

The role of A β circRNA in Alzheimer's disease

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Abstract:

Circular RNAs are universally expressed and evolutionarily conserved; however, the functions of most circRNAs are still unknown. Previous studies have shown that circRNAs are enriched in neurons and accumulate during brain ageing, indicating possible roles in ageing-associated neurodegenerative disorders. Alzheimer's disease (AD) is the most common detrimental dementia, closely linked with advancing age. Amyloid- β (A β) peptide accumulation due to APP proteolytic processing is long believed to be the key step in AD neuropathology. Mutations in factors involved in A β peptide biogenesis have been shown to contribute to the mechanism of familial AD (FAD). However, this accounts for less than 1-5% of total AD patients, and mechanisms for the most common type of sporadic AD (SAD), remain largely unknown. Here, I demonstrate that a circRNA is expressed from the wild-type APP gene in human brain, encompassing the A β peptide sequence. Using my previously established circRNA expression strategy, I found A β circRNA-encoded expression of an A β related peptide, called A β circRNA-derived peptide, which up-regulates GSK3 β levels and tau phosphorylation, both hallmarks of AD progression. Thus, A β circRNA and its translated peptide may not only play a causative role in AD, but represent promising therapeutic targets in AD diagnosis and treatment.

Key words: Neurodegeneration, Alzheimer's disease (AD), Circular RNA (circRNA), intron-mediated enhancement (IME), amyloid beta (A β) circRNA, A β circRNA derived peptide (A β -circRNA-DP), GSK3 β , tau phosphorylation/hyper-phosphorylation.

Introduction:

Alzheimer's disease (AD) is the most common form of dementia associated with progressive loss of memory, thinking and behaviour capabilities¹⁻⁴. As an ageing-associated neurodegenerative disorder, the greatest known risk factor for AD is increasing age itself^{3,5}. Both the leading hypothesis and extensive studies have focused on the causative roles of amyloid- β (A β) peptide accumulation and tau hyperphosphorylation^{1-4,6,7}. It has been shown that mutations in genes (APP, Presenilin) participating in APP protein proteolytic processing accelerate the accumulation of A β peptides (A β 36-43). These polymerize into toxic forms, aggregate into insoluble amyloid plaques, and lead to tau protein hyperphosphorylation through GSK3 β activation, and subsequently neurofibrillary tangles forming inside neurons, triggering a cascade of events that ultimately cause neuron death^{1,2,8-12}. This type of Alzheimer's disease is normally called familial Alzheimer's disease (FAD). Since it develops at an earlier age, it is also described as early-onset familial Alzheimer's disease (eFAD).

Despite well-demonstrated roles of genetic mutations in FAD, this type of AD accounts for less than 1-5% of all AD patients^{13,14}. The most common form of AD is late-onset Alzheimer's (LOAD), which normally arises in people aged 65 and older. This type of AD is sporadic, correspondingly it is also called sporadic AD (SAD); the direct cause or gene(s) responsible for LOAD/SAD are largely unknown.

Although with different age of onset and genetic causes, both FAD and SAD are believed to share a similar overall sequence of symptoms and gradually increasing impairments. The

pathologic hallmarks of both FAD and SAD are plaques and tangles arising from A β accumulation, GSK3 β activation and tau hyperphosphorylation.

The sporadic incidence of LOAD poses the question of what is the exact mechanism of AD^{15,16}. Moreover, the recent extensive failures of clinical trials targeting A β have challenged the classic view that A β accumulation from APP proteolytic processing (the Amyloid- β hypothesis) is the key the detrimental factor in AD, especially SAD^{2,16}.

Circular RNAs (circRNAs) are a form of evolutionary conserved transcripts with a covalently jointed 5' and 3' structure derived from pre-mRNA back-splicing¹⁷. Previous studies have found that they are especially enriched in neurons¹⁸. Interestingly, brain circRNAs are regulated during ageing in both the fly and mouse, highlighting potential roles of circRNA in brain ageing and ageing-associated neurodegenerative diseases^{19,20}.

In this study, I found that a circRNA from the human APP gene is well expressed in the human brain frontal lobe and hippocampus. Since this circRNA contains the A β coding sequence, here I call it A β circRNA. Using my previously established method of intron-mediated enhancement (IME) of circRNA expression and translation²¹, this A β circRNA clearly expressed an A β related peptide, which induced GSK3 β up-regulation and tau protein phosphorylation, thus indicating a new direction in the mechanism of Alzheimer's disease.

Material and methods:

Plasmid construction

The genetic information about circRNAs from the human APP gene were reported by several groups^{18,22-24}. One isoform of circRNA from the APP gene, hsa_circ_0007556 (circBase), was called human A β circRNA for the purpose of this study. The cDNA of human APP circRNA (GRCh37/hg19, chr21:27264033-27284274) was inserted into pCircRNA-BE or pCircRNA-DMo vectors to generate pCircRNA-BE-A β or pCircRNA-DMo-A β as described previously²¹. Plasmid DNAs were purified with EndoFree Plasmid Maxi Kit (QIAGEN). Details of oligonucleotides are provided in Supplementary Table 1.

Cell culture and plasmid DNA transfection

Human HEK293 and mouse neuro 2a (N2a) cell line cultures and plasmid DNA transfections were performed as previously described²¹. Briefly, 2.5 μ g of plasmid DNA was transfected into 0.5 million cells for three days (N2a) or six days (HEK293).

Total RNA isolation and qRT-PCR

Total RNA from HEK293 or N2a cells was isolated with TRIzol reagent (Ambion). Human brain frontal lobe and hippocampus total RNAs were purchased from BioCat GmbH. cDNA synthesis and qRT-PCR were performed as previously described²¹. Details of qRT-PCR oligonucleotides are provided in Supplementary Table 1.

Western blot assays

Total proteins were prepared in RIPA buffer as previously described²¹. For detecting A β circRNA-derived peptide in N2a cells, the PIPA buffer-insoluble proteins were extracted by 70% formic acid as described previously²⁵. Human frontal lobe protein samples were purchased from BioCat GmbH and BIOZOL Diagnostica Vertrieb GmbH. Protein extractions were separated on 18% Criterion™ TGX Stain-Free™ Protein Gel or AnykD™ Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad) and transferred to nitrocellulose membranes, then immunoblotted against β -amyloid (β -Amyloid (D54D2) XP® Rabbit mAb #8243, Cell

signalling Technology, which also recognizes APP full-length protein), GSK3 β (#12456, Cell signalling Technology), tau (#MN1000, Thermo Scientific), phospho-tau (AT8, #MN1020; AT100, #MN1060; ThermoFisher Scientific) and β -Actin (#A5441, Sigma). Quantitative analyses were performed with ImageJ (NIH).

Results

A β circRNA expression from the human APP gene in brain; IME promotes A β circRNA overexpression in cell lines

Expression of a candidate circRNA (hsa_circ_0007556) from the human APP gene was reported by several groups^{18,22-24}. Since this circRNA includes the sequence of the A β peptide, in this study it is called A β circRNA (Fig. 1A). To confirm its existence in human brain, I performed RT-PCR on human frontal lobe and hippocampus total RNA using oligonucleotides specifically designed to detect A β circRNA (Fig. 1A, C). A β circRNA was clearly well expressed in human frontal lobe and hippocampus. RT-PCR of A β circRNA with another set of two oligonucleotides showed that it contained exons 14, 15, 16 and 17 of the human APP gene without intron sequences (Supplementary Fig 1). Further sequencing of the RT-PCR product confirmed its sequence (data not shown).

To facilitate circRNA functional studies, I recently developed an intron-mediated enhancement (IME) strategy for robust circRNA expression²¹. Here, I applied this method to boost A β circRNA expression from vectors constructed in a similar manner to the described strategy (Fig. 1A, B)²¹. Transient expression in HEK293 cells showed that pCircRNA-BE-A β and pCircRNA-DMo-A β generated 2185- and 3268-fold more A β circRNA than was endogenously produced in HEK293 cells transfected with empty vector (Fig. 1C, D). RT-PCR confirmed that the overexpressed A β circRNA was spliced exactly as the wildtype A β circRNA (Supplementary Fig. 1). Sequencing the RT-PCR products further confirmed that their sequences were identical to wild type A β circRNA found in the human brain (data not shown).

Since HEK293 is a well-established cell line for Alzheimer's disease research^{26,27}, the robust IME strategy for transient expression of A β circRNA in HEK293 cells provides a good model for studying A β circRNA function. In the endogenous control, qRT-PCR showed that the level of human full-length APP mRNA was not changed (Supplementary Fig. 2), indicating that endogenous APP gene expression is not affected.

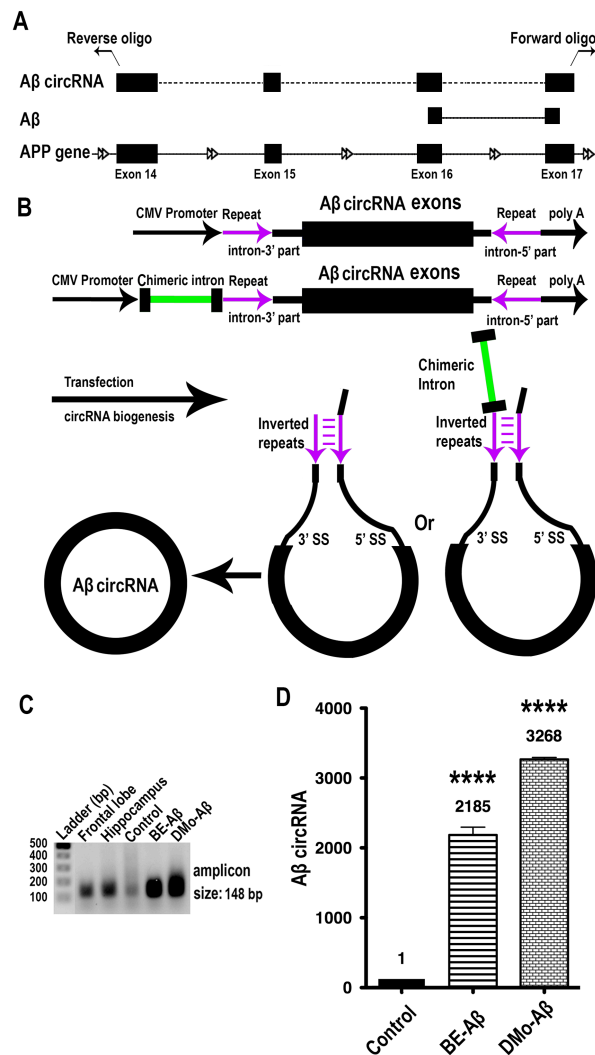


Fig. 1: Aβ circRNA expression from the APP gene in human brain and Aβ circRNA overexpressing HEK293 cells.

A) Aβ circRNA is encoded by exons 14-17 of the human APP gene, without the introns. The amyloid-β (Aβ) sequence is located in exons 16 and 17. Reverse and forward oligonucleotides were used to amplify Aβ circRNA by RT-PCR. B) Aβ circRNA over-expression constructs. C) RT-PCR verification of Aβ circRNA expression in human brain samples (frontal lobe and hippocampus) and HEK293 cells overexpressing Aβ circRNA; Control, pCircRNA-DMo empty vector transfection into HEK293; BE-Aβ, pCircRNA-BE-Aβ transfection into HEK293; DMO-Aβ, pCircRNA-DMo-Aβ transfection into HEK293; RT-PCR verification of Aβ circRNA by another set of oligonucleotides is shown in Supplementary Fig. 1. D) Aβ circRNA expression in HEK293 cells mediated by pCircRNA-BE and pCircRNA-DMo vectors. Control, empty vector (pCircRNA-DMo); BE-Aβ, pCircRNA-BE-Aβ; DMO-Aβ, pCircRNA-DMo-Aβ. All statistical T tests were performed in comparison to the control sample, ****, $P \leq 0.0001$, $n = 4$.

Aβ circRNA can be translated into an Aβ circRNA-derived peptide

Previous studies have shown that certain circRNAs are translatable^{21,28-31}. Examination of the Aβ circRNA revealed that it contained an open reading frame (ORF) (Fig. 2A) that would translate into a putative protein with a calculated molecular weight of 19.2 kDa (Supplementary Fig. 3). Western blots to detect whether an Aβ peptide is translated in HEK293 cells overexpressing Aβ circRNA used an antibody (β-Amyloid (D54D2) XP® Rabbit mAb) that detected Aβ peptides as well as APP full-length protein. Specifically, I detected an obvious Aβ-related peptide signal with a size around 15 to 20 kDa, confirming the translation of Aβ

circRNA. Products of A β circRNA translation were called A β circRNA-derived peptide (A β circRNA-DP).

Interestingly, control HEK293 cells transfected with empty vector contained detectable amounts of A β circRNA-DP. As I showed above (Fig. 1), HEK293 cells expressed low levels of endogenous A β circRNA, thus detectable A β circRNA-DP in control HEK293 cells represents its endogenous translation. Importantly, such detectable A β circRNA-DP in HEK293 cells confirmed its authenticity, highlighting its biological relevance.

Clearly, as in my previous study²¹, intron-mediated enhancement (IME) increased A β circRNA translation. pCircRNA-DMo-A β expressed about 3.3-fold more A β circRNA-DP than the endogenous expression in HEK293 cells, while pCircRNA-BE-A β only moderately enhanced A β circRNA-DP levels (1.4-fold; Fig 2B, C). Interestingly, the enrichment of protein product (A β circRNA-DP) was much less significant than the elevated expression of A β circRNA, indicating that the translation of A β circRNA was regulated by other unknown mechanisms. Comparing expression of endogenous full-length APP protein levels, I found these to be similar in control and A β circRNA overexpressing cells, indicating that proteolytic degradation of APP does not generate A β circRNA-derived peptide.

Next, I used a mouse neuroblastoma cell line to further validate the transcription and translation of A β circRNA. These N2a cells apparently do not express any detectable A β circRNA or translation product (Fig 2E, G). However, pCircRNA-DMo-A β expressed 4.6-fold more A β circRNA than pCircRNA-BE-A β , whereas the internal control, App mRNA, showed no change (Fig 2E, F). In both A β circRNA overexpressing N2a cells, A β circRNA-DP was well expressed, and consistent with HEK293 cells, IME dramatically enhanced A β circRNA-DP expression (Fig 2E, G). Since N2a cells do not express A β circRNA-DP or related peptides endogenously, the detection of A β related peptide at 15-20 kDa further clearly confirmed that A β circRNA was the template for translating A β circRNA-DP. Interestingly, overexpression of A β circRNA in N2a cells generated another band migrating at around 15 kDa (Fig 2 G), suggesting that A β circRNA-DP may also undergo proteolytic processing.

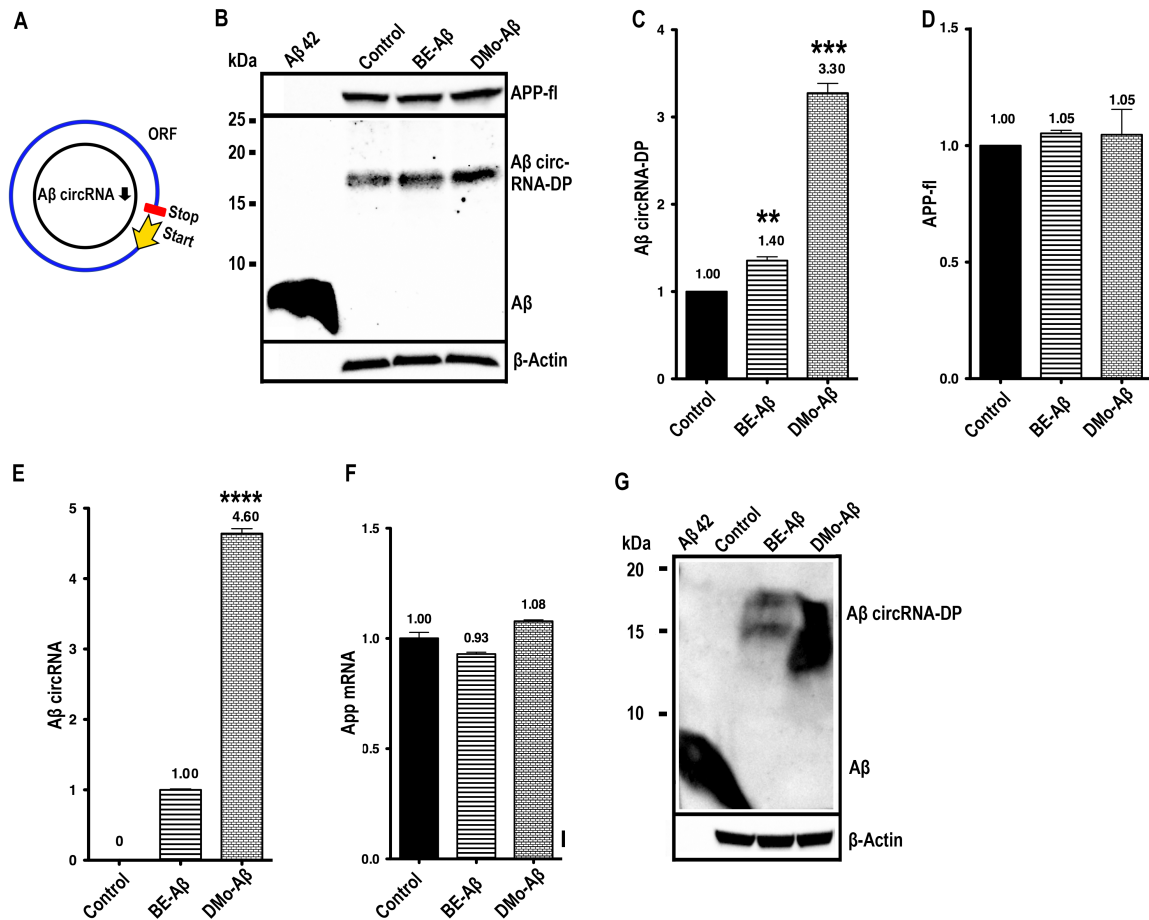


Fig. 2: Expression of A β circRNA-DP from A β circRNA translation in HEK293 and N2a cells.

A) The open reading frame (ORF) of A β circRNA is represented by a blue circle; yellow arrow, translation start codon; red square, stop codon; the inner black arrow shows the start of the A β circRNA. B) Western blot of A β -related peptides in HEK293 cells. 5 ng of *in vitro* synthesized A β 42 was used as a positive control peptide and migration indicator. Control, empty vector (pCircRNA-DMo); BE-A β , pCircRNA-BE-A β ; DMO-A β , pCircRNA-DMo-A β . The detected peptides indicated on the right: APP-fl, APP full-length protein; A β circRNA-DP, A β circRNA-derived protein; A β , synthetic peptide A β 42; β -Actin used as a loading control. C) quantification of A β circRNA-DP levels. D) Quantification of APP full-length protein (APP-fl) levels.

E) A β circRNA expression in N2a cells. Control, empty vector (pCircRNA-DMo); BE-A β , pCircRNA-BE-A β ; DMO-A β , pCircRNA-DMo-A β . F) Mouse App mRNA expression in A β circRNA overexpressing N2a cells. G) Western blot of A β related peptides in A β circRNA overexpressing N2a cells. The synthetic A β 42 peptide (Fig. 2) was used as a positive detection and migration control; Control, empty vector (pCircRNA-DMo); BE-A β , pCircRNA-BE-A β ; DMO-A β , pCircRNA-DMo-A β . Panels C, D, all statistical T tests were performed with respect to the control sample; **, $P \leq 0.01$; ***, $P \leq 0.001$; $n \geq 3$. Panel E, F, all statistical T tests were performed in comparison to the control sample (in panel E this was BE-A β set at 1); ****, $P \leq 0.0001$; $n = 4$.

A β circRNA-DP significantly up-regulates GSK3 β and tau phosphorylation

Previously studies have shown that A β peptides are toxic to neural cells and cause GSK3 β activation, which promotes the phosphorylation of tau proteins. To evaluate the potential roles

of A β circRNA-DP, I analysed GSK3 β levels and the phosphorylation of tau proteins in HEK293 cells overexpressing A β circRNA using Western blots. A β circRNA overexpression significantly increased GSK3 β levels, by 2.3-fold following pCircRNA-BE-A β transfection and 3.5-fold after pCircRNA-DMo-A β transfection (Fig. 3A, B). Interestingly, tau protein levels were also upregulated when A β circRNA was overexpressed (HT7 antibody; Fig. 3A, C). Using AT8 (Ser202, Thr205) and AT100 (Thr212, Ser214) antibodies to analyse tau phosphorylation, pCircRNA-BE-A β transfection resulted in a 2.6-fold increase in phosphorylation detected by AT8 and a 2.9-fold increase detected by AT100. Correlating with the IME enhanced higher expression of A β circRNA-DP, pCircRNA-DMo-A β transfected cells contained 3.7-fold or 5.0-fold more phosphorylated tau detected with AT8 or AT100 antibodies (Fig. 3A, C, D, E).

In summary, A β circRNA-DP seems to significantly up-regulate GSK3 β and consequently promote tau phosphorylation, as depicted in Figure 3F.

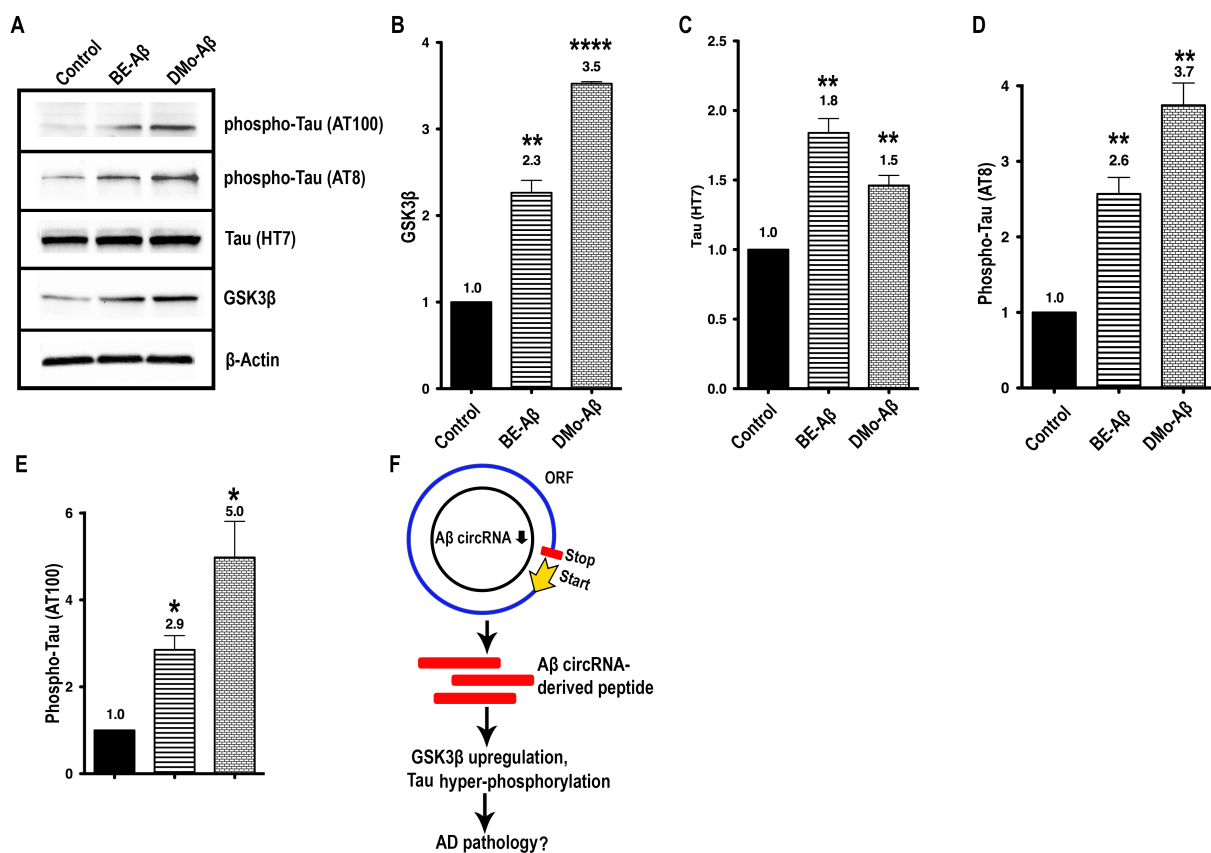


Fig. 3: A β circRNA up-regulates GSK3 β expression and promotes tau protein phosphorylation.

A) GSK3 β and tau protein (HT7 antibody) levels, and tau phosphorylation detected in HEK293 cells overexpressing A β circRNA. β -actin was used as a loading control. Control, pCircRNA-DMo empty vector; BE-A β , pCircRNA-BE-A β ; DMo-A β , pCircRNA-DMo-A β . B) Quantification of GSK3 β levels; C) quantification of tau levels; D) quantification of tau phosphorylation levels detected by antibody AT8; E) quantification of tau phosphorylation detected by antibody AT100. F) Model of how A β circRNA could function in Alzheimer's disease. All statistical T tests were performed with respect to the control sample; **, $P \leq 0.01$; ****, $P \leq 0.0001$; $n \geq 3$.

Translation of A β circRNA in human brain

The results above indicate that translation of A β circRNA into A β -related peptide (A β circRNA-DP) can dramatically cause GSK3 β up-regulation and tau phosphorylation in a human cell line. However, although I detected A β circRNA-DP in the well-established Alzheimer's disease cell line HEK293, it is still unknown whether A β circRNAs are translated into this peptide in the human brain. Using Western blot analysis of human brain samples, a strong band around 15 to 20 kDa, similar to A β circRNA-DP detected in cell lines, along with smaller A β peptides, were detected in human brain frontal lobes from two donors (Fig. 4), demonstrating the existence of A β circRNA-DP in human brain. Interestingly, in human frontal lobe-1, although A β circRNA-DP was detected, no A β peptides were observed, demonstrating that the detected A β circRNA-DP had not resulted from oligomerization of A β . Notably, no A β circRNA-DP peptides were detected in mouse brain frontal lobe (Fig. 4).

In summary, translation of A β circRNA apparently generates A β -related peptide, A β circRNA-DP, in human brain, highlighting a potential role of A β circRNA and A β circRNA-DP in the pathology of Alzheimer's disease.

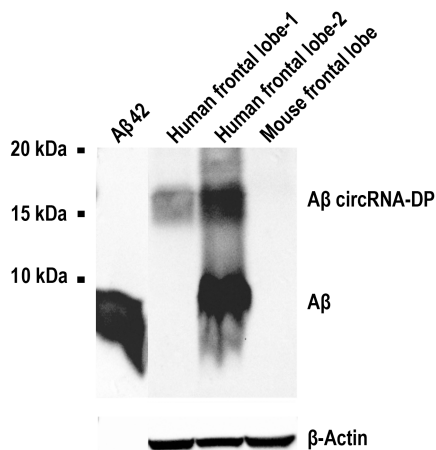


Fig. 4: Expression of A β circRNA-DP in human brain.

Human and mouse brain samples (frontal lobe) were immunoblotted with the antibody against β -amyloid (β -Amyloid (D54D2) XP® Rabbit mAb). Synthesized A β 42 peptide was used as a positive control and indicator of A β -related peptide migration.

Discussion

My discovery of biological roles for A β circRNA and its translated peptide, A β circRNA-DP not only represents a milestone for the function exploration of circRNA, but also reveals a ground-breaking potential mechanism for causing Alzheimer's disease.

With the help of intron-mediated enhancement of circRNA expression, I could verify that A β circRNA can be translated into A β related peptide, subsequently inducing GSK3 β up-regulation and tau phosphorylation, thus indicating a new direction in the search for molecular mechanisms of Alzheimer's disease (Fig. 5). Such a mechanism is dramatically different from A β accumulation through dysregulation of APP prototypic processing. Unlike factor mutations in familial Alzheimer's disease, no genetic mutation is required for A β circRNA biogenesis. Correspondingly, the entire human population may express A β circRNA and A β circRNA-DP, which may play a key role in sporadic Alzheimer's disease.

Recent studies have implicated circRNA as a regulator of cellular stress³². For example, the translation of Circ-ZNF609 circRNA could be induced by heat shock stress²⁸. In brain ageing, neurons are particularly vulnerable to various stresses³³. Presumably, expression of A β circRNA and its translated peptide could be activated by various (age-related) stresses, and

cause a detrimental downstream cascade, leading to neurodegeneration. My discovery of A β circRNA and its translated protein, A β circRNA-DP may open up new strategies for Alzheimer's disease prevention, diagnosis and treatment.

The rapidly emerging role of circRNA is apparently serving as a template for protein synthesis. However, it is unknown whether peptides produced from circRNA have any function in organisms. Here, for the first time I show that the protein produced from A β circRNA translation may play a critical role in Alzheimer's disease, thus endorsing further function exploration of circRNA, and reasoning that proteins translated from other circRNAs may also play essential, biologically significant roles.

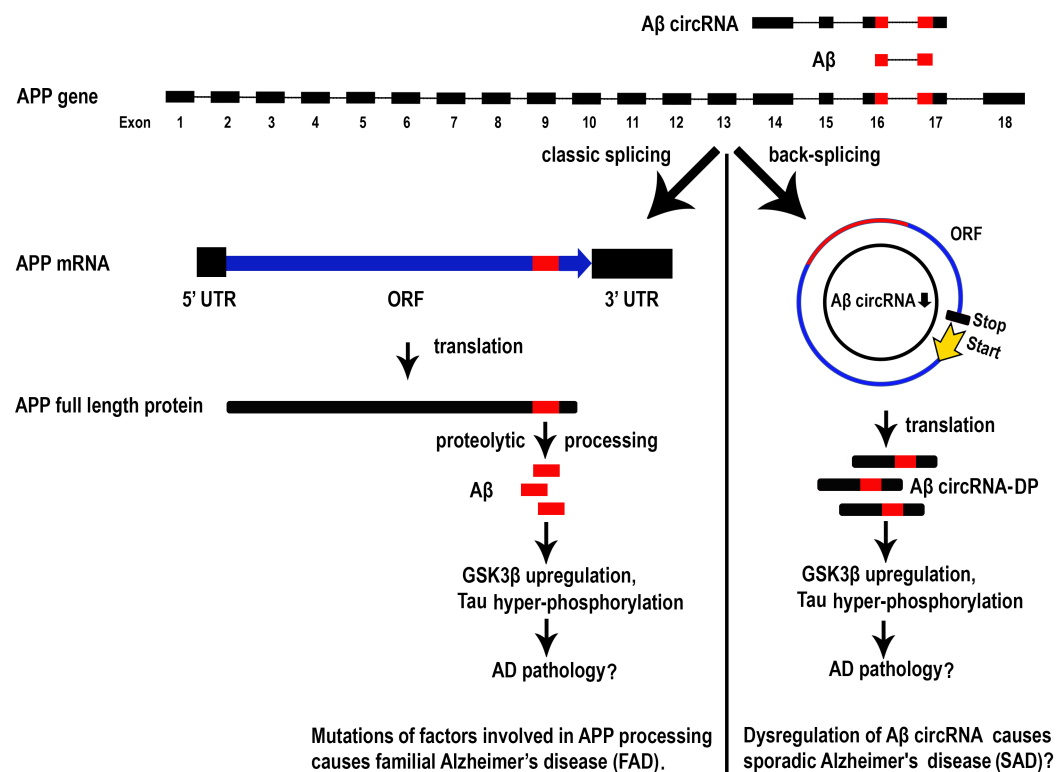


Fig. 5: Two possible APP gene processing pathways in Alzheimer's disease.

At the top, exon sequences contained in A β circRNA and A β peptides (in red) are aligned with the full-length APP gene. On the left, linear APP mRNA transcribed from the APP gene undergoes classic splicing before being translated into full-length APP protein. Proteolytic processing of APP protein generates A β peptides (A β 40, A β 42, in red), which play causative roles in AD pathology through GSK3 β activation and tau protein hyperphosphorylation. Mutations in factors involved in APP processing cause familial Alzheimer's disease. On the right, A β circRNA is synthesised by back-splicing of the APP gene. The open reading frame (ORF) is in blue, with the A β sequence in red, the start codon in yellow arrow and the stop codon in black. Translation of A β circRNA produces A β -related peptide (A β circRNA-DP), which could cause the sequential scenario of GSK3 β activation and tau protein hyperphosphorylation, leading to AD pathology. Since no mutations are required for A β circRNA biogenesis, this process may depend on intracellular environments rather than genetics. Changes in A β circRNA expression and translation may cause sporadic Alzheimer's disease.

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Author Contributions

DM designed and conceived the study. DM performed the experiments, analysed the results, prepared the figures, and wrote the manuscript.

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Conflict of interest.

None declared.

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Supplementary table 1

Abeta circRNA expression plasmid construction oligos

Abeta-circF gtttgttttcagATGAGCTGCTTCAGAAAGAGCAAACCT

ABeta-circR gcatggattattacCTCCACCACCCATGATGAATGG

circDMO-LF GTCGACTGGATCcaacgттаacc

DMo-Ab-LR CTGAAGCAGCTCATctgaaaaacaacagaatacaacctcagc

DMo-Ab-RF GGTGTGGTGGAGgtaataatccatgcaccgtctcacc

circDMO-RR cactttgCTCGAGctcatcaacatg

Abeta circRNA verification oligos

AB-cRNAvF2 GTGATCGTCATCACCTTGGTGATGC

AB-cRNAvR2 CACCATGAGTCCAATGATTGCACC

qPCR oligos

human Abeta circRNA

Abeta-cF GTCATAGCGACAGTGATCGTC

Abeta-cR CTTGGTTCACCTAATCATGTTGGC

Human APP mRNA

hAPP-mF TTTGTGATTCCCTACCGCTG

hAPP-mR TGCCAGTGAAGATGAGTTTCG

mouse App mRNA

App-mF ATCTTCACTGGCACACCG

App-mR GCATACAAACTCTACCCCTCG

Mouse Actb mRNA:

mActbF1 ACCTTCTACAATGAGCTGCG,

mActbR1 CTGGATGGCTACGTACATGG

Human ACTB mRNA:

hACTB-F ACCTTCTACAATGAGCTGCG

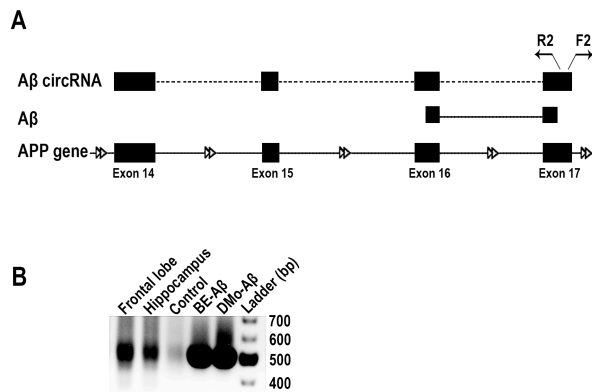
hACTB-R CCTGGATAGCAACGTACATGG

The role of A β circRNA in Alzheimer's disease

Dingding Mo^{*,#}

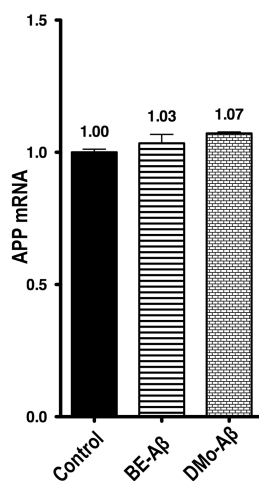
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Supplementary Fig. 1: RT-PCR verification of A β circRNA.

A) RT-PCR primers sites in A β circRNA. B) Agarose gel electrophoresis of RT-PCR products (503 bp) using primers A β -VR2 (R2) and A β -VF2 (F2); human frontal lobe; hippocampus; BE-A β , pCircRNA-BE-A β transfected HEK293 cells; DMO-A β , pCircRNA-DMo-A β transfected HEK293 cells. RT-PCR products were sequenced and aligned, confirming that all A β circRNAs were identical and comprised exons 14, 15, 16 and 17 of the APP gene without introns (data not shown).



Supplementary Fig. 2: Endogenous APP mRNA expression in HEK293 cells overexpressing A β circRNA.

qRT-PCR analysis of endogenous APP mRNA expression in A β circRNA overexpressing HEK293 cells. Primers targeted exons 3 and 4, which are not contained in A β circRNA: hAPP-m, TTTGTGATTCCCTA CCGCTG and hAPP-mR, TGCCAGTGAAGATGAGT TTCG. Control, pCircRNA-DMo empty vector; BE-A β , pCircRNA-BE-A β ; DMO-A β , pCircRNA-DMo-A β . No significant differences were observed.


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> Aβ circRNA-DP
1 50
miseprisygndalmpsltetkttvellpvngefslddlqpwhsfgadsv

51 100
pantenevepvdarpaadrglttrpgsgltnikteisevkmdaefrhds

101 150
gyevhhqklvffaedvgsnkgaiiglmvggvviatvivitlvmlkkkqyt

151 175
sihhgvveMSCFRKSKTIQMTSWPT
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Supplementary Fig. 3: The putative amino acid sequence of Aβ circRNA-DP.

Predicted from the identical sequences of all circRNA RT-PCR products: red, sequence of Aβ42; blue, the unique sequence of Aβ circRNA-DP resulting from the circular translation of Aβ circRNA, which is not present in the APP full-length protein. The calculated molecular weight of Aβ circRNA-DP is 19.2 kDa.