The role of Aβ 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 (Aβ) peptide accumulation due to APP proteolytic processing is long believed to be the key step in AD neuropathology. Mutations in factors involved in Aβ 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 Aβ peptide sequence. Using my previously established circRNA expression strategy, I found Aβ circRNA-encoded expression of an Aβ related peptide, called Aβ circRNA-derived peptide, which up-regulates GSK3β levels and tau phosphorylation, both hallmarks of AD progression. Thus, Aβ 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 (Aβ) circRNA, Aβ circRNA derived peptide (Aβ-circRNA-DP), GSK3β, 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 capabilities[1-4]. 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 (Aβ) 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 Aβ peptides (Aβ 36-43). These polymerize into toxic forms, aggregate into insoluble amyloid plaques, and lead to tau protein hyperphosphorylation through GSK3β 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 as 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 Aβ accumulation, GSK3β 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 Aβ have challenged the classic view that Aβ 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-splicing[17]. Previous studies have found that they are especially enriched in neurons[18]. 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 Aβ coding sequence, here I call it Aβ circRNA. Using my previously established method of intron-mediated enhancement (IME) of circRNA expression and translation[21], this Aβ circRNA clearly expressed an Aβ related peptide, which induced GSK3β 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 Aβ 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-Aβ or pCircRNA-DMo-Aβ as described previously[21]. 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 described[21]. Briefly, 2.5 μg 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 described[21]. Details of qRT-PCR oligonucleotides are provided in Supplementary Table 1.

Western blot assays

Total proteins were prepared in RIPA buffer as previously described[21]. For detecting Aβ circRNA-derived peptide in N2a cells, the PIPA buffer-insoluble proteins were extracted by 70% formic acid as described previously[25]. 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), GSK3β (#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

Aβ circRNA expression from the human APP gene in brain; IME promotes Aβ 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 Aβ peptide, in this study it is called Aβ 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 Aβ circRNA (Fig. 1A, C). Aβ circRNA was clearly well expressed in human frontal lobe and hippocampus. RT-PCR of Aβ 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 expression[21]. Here, I applied this method to boost Aβ 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-Aβ and pCircRNA-DMo-Aβ generated 2185- and 3268-fold more Aβ circRNA than was endogenously produced in HEK293 cells transfected with empty vector (Fig. 1C, D). RT-PCR confirmed that the overexpressed Aβ circRNA was spliced exactly as the wildtype Aβ circRNA (Supplementary Fig. 1). Sequencing the RT-PCR products further confirmed that their sequences were identical to wild type Aβ 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 Aβ circRNA in HEK293 cells provides a good model for studying Aβ 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.

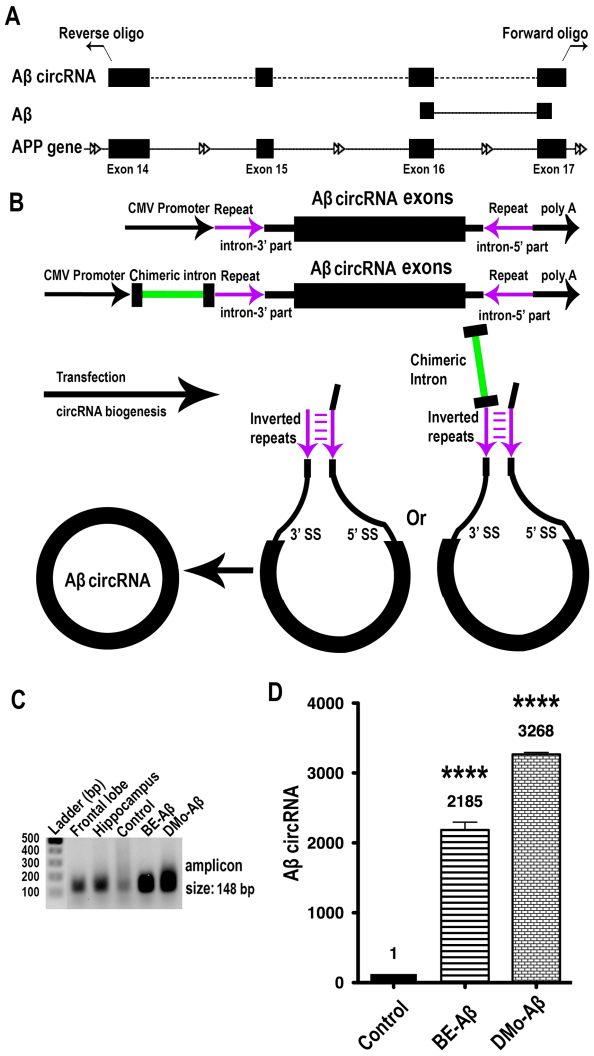

Fig. 1: Aβ circRNA expression from the APP gene in human brain and Aβ circRNA overexpressing HEK293 cells.

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 Aβ circRNA translation were called Aβ circRNA-derived peptide (Aβ circRNA-DP).

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

Clearly, as in my previous study[21], intron-mediated enhancement (IME) increased Aβ circRNA translation. pCircRNA-DMo-Aβ expressed about 3.3-fold more Aβ circRNA-DP than the endogenous expression in HEK293 cells, while pCircRNA-BE-Aβ only moderately enhanced Aβ circRNA-DP levels (1.4-fold; Fig 2B, C). Interestingly, the enrichment of protein product (Aβ circRNA-DP) was much less significant than the elevated expression of Aβ circRNA, indicating that the translation of Aβ 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 Aβ circRNA overexpressing cells, indicating that proteolytic degradation of APP does not generate Aβ circRNA-derived peptide.

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

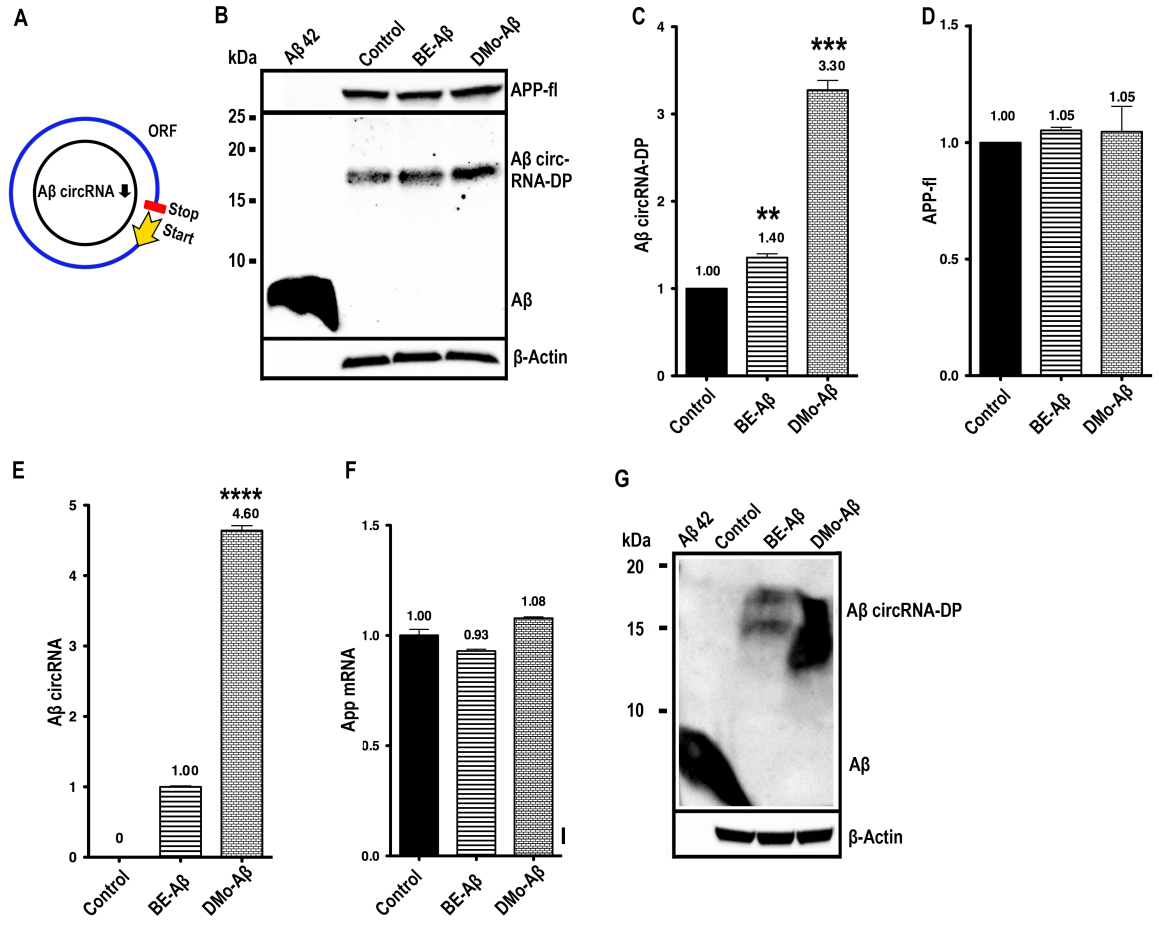

Fig. 2: Expression of Aβ circRNA-DP from Aβ circRNA translation in HEK293 and N2a cells.

A) The open reading frame (ORF) of Aβ 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 Aβ circRNA. B) Western blot of Aβ-related peptides in HEK293 cells. 5 ng of *in vitro* synthesized Aβ42 was used as a positive control peptide and migration indicator. Control, empty vector (pCircRNA-DMo); BE-Aβ, pCircRNA-BE-Aβ; DMo-Aβ, pCircRNA-DMo-Aβ. The detected peptides indicated on the right: App-fl, APP full-length protein; Aβ circRNA-DP, Aβ circRNA-derived protein; Aβ, synthetic peptide Aβ42; β-Actin used as a loading control. C) quantification of Aβ circRNA-DP levels. D) Quantification of APP full-length protein (APP-fl) levels.

E) Aβ circRNA expression in N2a cells. Control, empty vector (pCircRNA-DMo); BE-Aβ, pCircRNA-BE-Aβ; DMo-Aβ, pCircRNA-DMo-Aβ. F) Mouse App mRNA expression in Aβ circRNA overexpressing N2a cells. G) Western blot of Aβ related peptides in Aβ circRNA overexpressing N2a cells. The synthetic Aβ42 peptide (Fig. 2) was used as a positive detection and migration control; Control, empty vector (pCircRNA-DMo); BE-Aβ, pCircRNA-BE-Aβ; DMo-Aβ, pCircRNA-DMo-Aβ. 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-Aβ set at 1); ****, $P \leq 0.0001$; $n = 4$.

Aβ circRNA-DP significantly up-regulates GSK3β and tau phosphorylation

Previously studies have shown that Aβ peptides are toxic to neural cells and cause GSK3β activation, which promotes the phosphorylation of tau proteins. To evaluate the potential roles

of Aβ circRNA-DP, I analysed GSK3β levels and the phosphorylation of tau proteins in HEK293 cells overexpressing Aβ circRNA using Western blots. Aβ circRNA overexpression significantly increased GSK3β levels, by 2.3-fold following pCircRNA-BE-Aβ transfection and 3.5-fold after pCircRNA-DMo-Aβ transfection (Fig. 3A, B). Interestingly, tau protein levels were also upregulated when Aβ circRNA was overexpressed (HT7 antibody; Fig. 3A, C). Using AT8 (Ser202, Thr205) and AT100 (Thr212, Ser214) antibodies to analyse tau phosphorylation, pCircRNA-BE-Aβ 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 Aβ circRNA-DP, pCircRNA-DMo-Aβ 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, Aβ circRNA-DP seems to significantly up-regulate GSK3β and consequently promote tau phosphorylation, as depicted in Figure 3F.

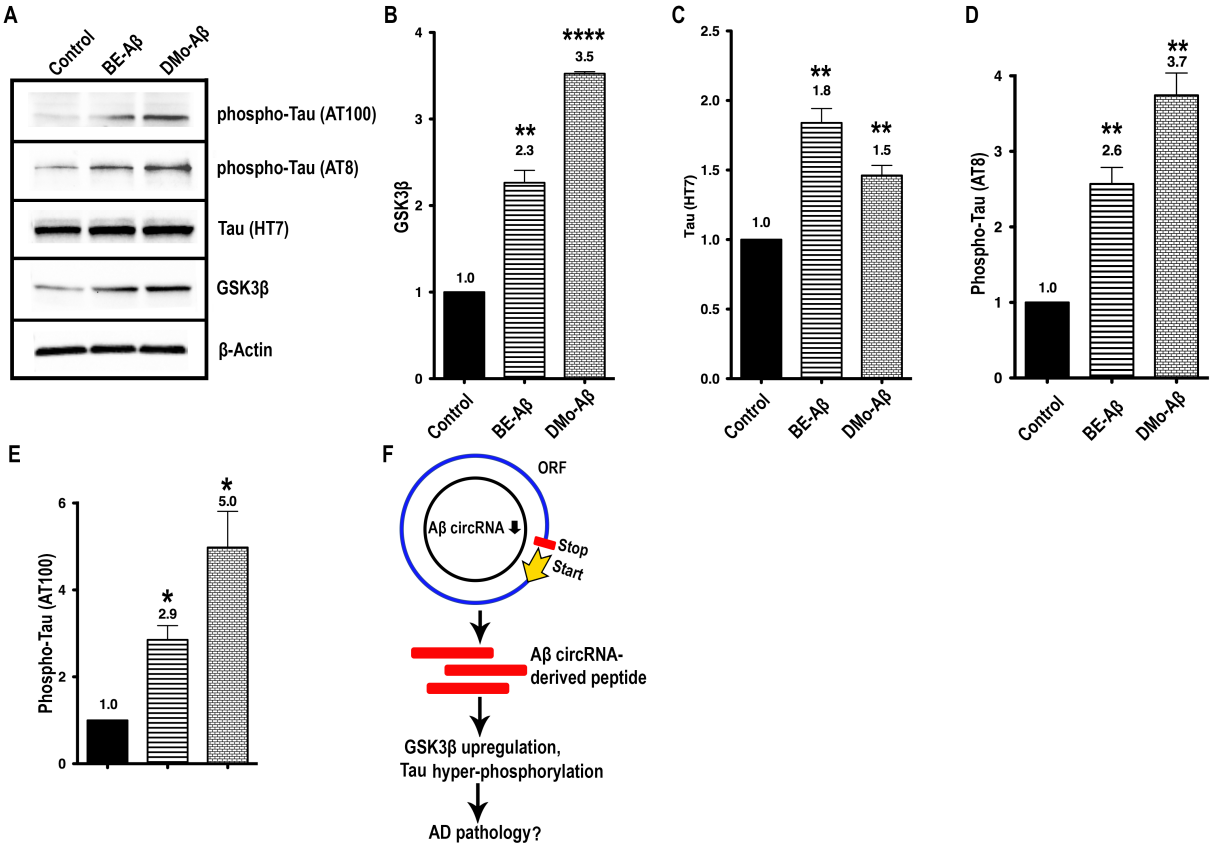

Fig. 3: Aβ circRNA up-regulates GSK3β expression and promotes tau protein phosphorylation.

A) GSK3β and tau protein (HT7 antibody) levels, and tau phosphorylation detected in HEK293 cells overexpressing Aβ circRNA. β-actin was used as a loading control. Control, pCircRNA-DMo empty vector; BE-Aβ, pCircRNA-BE-Aβ; DMo-Aβ, pCircRNA-DMo-Aβ. B) Quantification of GSK3β 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 Aβ 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 Aβ circRNA in human brain

The results above indicate that translation of Aβ circRNA into Aβ-related peptide (Aβ circRNA-DP) can dramatically cause GSK3β up-regulation and tau phosphorylation in a human cell line. However, although I detected Aβ circRNA-DP in the well-established Alzheimer's disease cell line HEK293, it is still unknown whether Aβ 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 Aβ circRNA-DP detected in cell lines, along with smaller Aβ peptides, were detected in human brain frontal lobes from two donors (Fig. 4), demonstrating the existence of Aβ circRNA-DP in human brain. Interestingly, in human frontal lobe-1, although Aβ circRNA-DP was detected, no Aβ peptides were observed, demonstrating that the detected Aβ circRNA-DP had not resulted from oligomerization of Aβ. Notably, no Aβ circRNA-DP peptides were detected in mouse brain frontal lobe (Fig. 4).

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

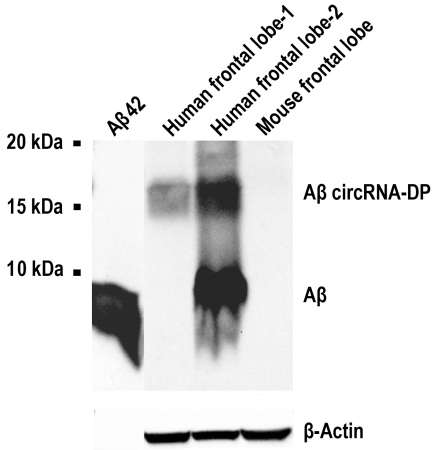

Fig. 4: Expression of Aβ circRNA-DP in human brain.
Human and mouse brain samples (frontal lobe) were immunoblotted with the antibody against β-amyloid (β-Amyloid (D54D2) XP® Rabbit mAb). Synthesized Aβ42 peptide was used as a positive control and indicator of Aβ-related peptide migration.

Discussion

My discovery of biological roles for Aβ circRNA and its translated peptide, Aβ 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 Aβ circRNA can be translated into Aβ related peptide, subsequently inducing GSK3β up-regulation and tau phosphorylation, thus indicating a new direction in the search for molecular mechanisms of Alzheimer' disease (Fig. 5). Such a mechanism is dramatically different from Aβ accumulation through dysregulation of APP prototypic processing. Unlike factor mutations in familial Alzheimer's disease, no genetic mutation is required for Aβ circRNA biogenesis. Correspondingly, the entire human population may express Aβ circRNA and Aβ circRNA-DP, which may play a key role in sporadic Alzheimer's disease.

Recent studies have implicated circRNA as a regulator of cellular stress[32]. For example, the translation of Circ-ZNF609 circRNA could be induced by heat shock stress[28]. In brain ageing, neurons are particularly vulnerable to various stresses[33]. Presumably, expression of Aβ 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 Aβ circRNA and its translated protein, Aβ 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 Aβ 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.

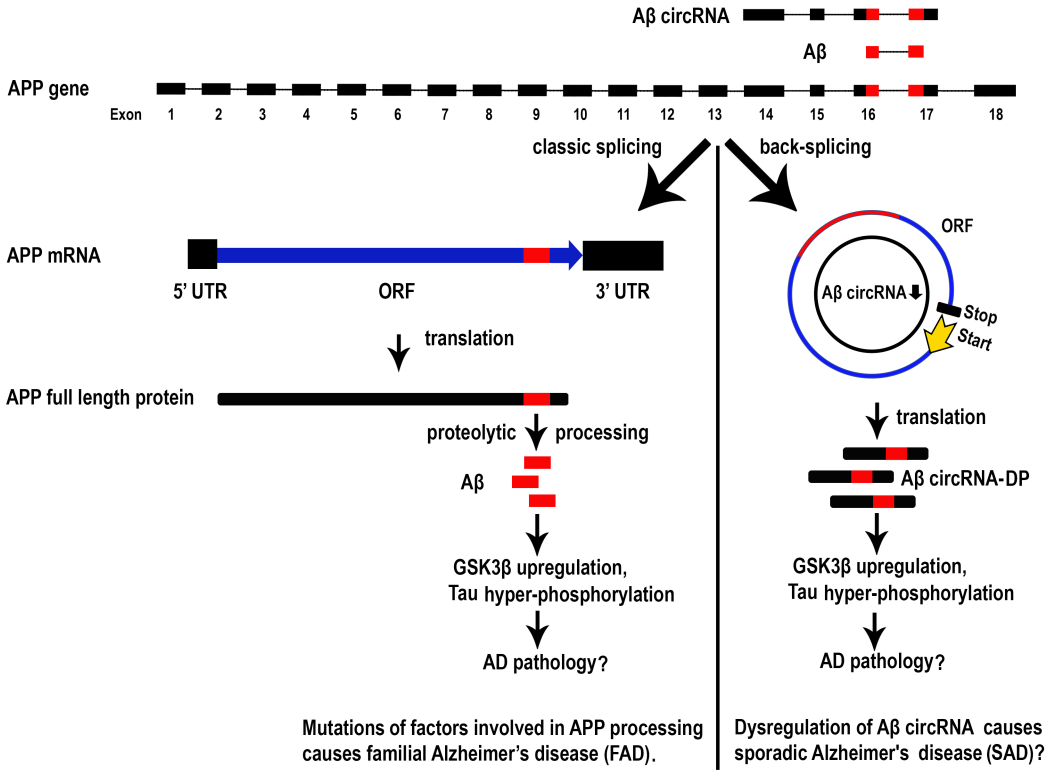

Fig. 5: Two possilbe APP gene processing pathways in Alzheimer's disease.
At the top, exon sequences contained in Aβ circRNA and Aβ peptides (in red) are aligned with the full-length APP gene. On the left, liner APP mRNA transcribed from the APP gene undergoes classic splicing before being translated into full-length APP protein. Proteolytic processing of APP protein generates Aβ peptides (Aβ40, Aβ42, in red), which play causative roles in AD pathology through GSK3β activation and tau protein hyperphosphorylation. Mutations in factors involved in APP processing cause familial Alzheimer's disease. On the right, Aβ circRNA is synthesised by back-splicing of the APP gene. The open reading frame (ORF) is in blue, with the Aβ sequence in red, the start codon in yellow arrow and the stop codon in black. Translation of Aβ circRNA produces Aβ-related peptide (Aβ circRNA-DP), which could cause the sequential scenario of GSK3β activation and tau protein hyperphosphorylation, leading to AD pathology. Since no mutations are required for Aβ circRNA biogenesis, this process may depend on intracellular environments rather than genetics. Changes in Aβ circRNA expression and translation may cause sporadic Alzheimer's disease.

Acknowledgements

The author acknowledges the department of biological mechanisms of ageing led by Linda Partridge for sharing chemicals and instruments. The mouse sample used in this study was from the transgenic core facility of the host institute. The author also appreciates Gabriella B Lundkvist for reading the manuscript.

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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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The role of Aβ circRNA in Alzheimer's disease

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Supplementary table 1

Abeta circRNA expression plasmid construction oligos
Abeta-circF gtttgtttttcagATGAGCTGCTTCAGAAAGAGCAAAACT
ABeta-circR gcatggattattacCTCCACCACACCATGATGAATGG
circDMO-LF GTCGACTGGATCcaacgttaaccc
DMo-Ab-LR CTGAAGCAGCTCATctgaaaaacaaacagaatacaacctcagc
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 CTTGGTTCACTAATCATGTTGGC

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 Aβ circRNA in Alzheimer's disease

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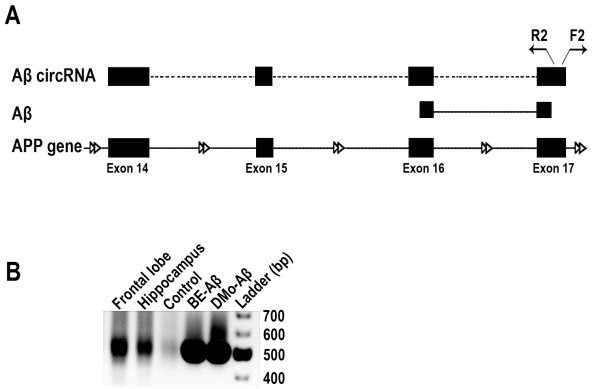
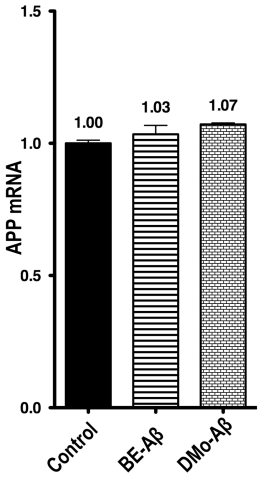
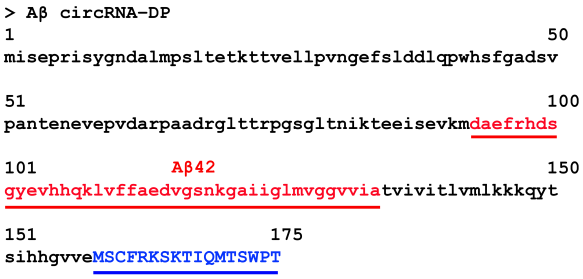


Supplementary Fig. 1: RT-PCR verification of Aβ circRNA.
A) RT-PCR primers sites in Aβ circRNA. B) Agarose gel electrophoresis of RT-PCR products (503 bp) using primers Aβ-VR2 (R2) and Aβ-VF2 (F2); human frontal lobe; hippocampus; BE-Aβ, pCircRNA-BE-Aβ transfected HEK293 cells; DMo-Aβ, pCircRNA-DMo-Aβ transfected HEK293 cells. RT-PCR products were sequenced and aligned, confirming that all Aβ 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 Aβ circRNA.
qRT-PCR analysis of endogenous APP mRNA expression in Aβ circRNA overexpressing HEK293 cells. Primers targeted exons 3 and 4, which are not contained in Aβ circRNA: hAPP-m, TTTGTGATTCCCTA CCGCTG and hAPP-mR, TGCCAGTGAAGATGAGT TTCG. Control, pCircRNA-DMo empty vector; BE-Aβ, pCircRNA-BE-Aβ; DMo-Aβ, pCircRNA-DMo-Aβ. No significant differences were observed.



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.