The role of Aβ circRNA in Alzheimer's disease: alternative mechanism of Aβ biogenesis from Aβ circRNA translation

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Abstract:
CircRNAs are ubiquitously expressed in brain and accumulated at higher levels within brain ageing, suggesting that they may play ageing-associated neurological roles.

Alzheimer's disease (AD) is the most prevalent form of detrimental dementia (brain disorder). Today it is known that the amyloid-β (Aβ) peptide plays a key role in the development of AD. In particular, in case of familial AD, Aβ peptides are generated from full-length APP by dysregulated proteolytic processing. However, the actual mechanism of Aβ biogenesis in sporadic AD remains largely unknown.

Here we reported the identification of 17 circRNAs that are derived from the APP gene and encompass the Aβ coding region. In accordance, they are named Aβ circRNAs. Using a well-established circRNA expression strategy, which is based on intron mediated enhancement (IME), we discovered an Aβ-related peptide from Aβ circRNA translation. Importantly, this peptide is further processed to form Aβ and the generation of amyloid-β plaques in primary neuron culture, significantly recapitulating the key hallmark of AD pathology and representing an alternative mechanism of Aβ biogenesis.

Furthermore, Aβ circRNA up-regulates GSK3β levels and correspondingly causes tau phosphorylation, another hallmark of AD. Thus, Aβ circRNA and translated peptides may not only play a causative role in AD but might represent therapeutic targets for the development of AD treatment.

Key words: Neurodegeneration, Alzheimer's disease (AD), sporadic AD, Circular RNA (circRNA), intron-mediated enhancement (IME), amyloid beta (Aβ) peptide, amyloid beta (Aβ) plaque, amyloid beta (Aβ) circRNAs, Aβ circRNA derived peptides (Aβ-circRNA-DP), GSK3β, tau 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[1-4,6-9]. It has been demonstrated that mutations within genes (APP, Presenilin) that participate in APP protein proteolytic processing accelerate the accumulation of Aβ peptides. These peptides polymerize to toxic oligomers and aggregate to insoluble amyloid plaques, and cause tau protein hyperphosphorylation *via* GSK3β activation; the subsequent formation of neurofibrillary tangles inside neurons is triggering a complex cascade of events that ultimately cause neuron death[1,2,10-14]. This variant of Alzheimer's disease is known as familial Alzheimer's disease (fAD). Since it develops at an earlier age, it is also described as early-onset familial

Alzheimer's disease (EFAD)[1,2,10-14]. Roles of various mutations in fAD are well-established, however this type of AD accounts for less than 1-5% of all AD patients[1,2].

The most common form of AD is late-onset Alzheimer's (LOAD), which normally arises in people aged 65 and older. As the genetic cause for this type of AD is unknown, correspondingly it is as often called sporadic AD (sAD).
Despite the differences between fAD and sAD, they share common symptoms leading to a gradually increasing impairment. The pathologic hallmarks of both fAD and sAD are plaques and tangles arising from Aβ accumulation and tau hyperphosphorylation, respectively. The sporadic incidence of LOAD poses the question of exact mechanism of AD development and progression[15-17]. Moreover, the process of Aβ accumulation is not understood for LOAD/sAD[2,16-18].

Circular RNAs (circRNAs) are evolutionary conserved transcripts with covalently joint 5' and 3' ends derived from pre-mRNA *via* back-splicing[19]. Previous studies revealed that circRNA expression is especially prominent in neurons[20]. Interestingly, brain circRNAs are regulated during ageing in multiple organisms, suggesting potential roles of circRNA in brain ageing and possibly ageing-associated neurodegenerative diseases[21-23].

Here we found 14 circRNAs derived from the human APP gene are expressed in the human brain. Since these circRNAs contain the Aβ coding sequence, we refer them as Aβ circRNAs. With our previously established method of intron-mediated enhancement (IME) for circRNA expression and translation[24], we demonstrate that at least one Aβ circRNA serves as template for Aβ-related peptide synthesis. Notably, the resulting product is further processed leading to active Aβ peptide in human HEK293 cell line; and it forms plaques in mouse primary neuron culture. These findings revealed novel aspects of Aβ biogenesis in Alzheimer's disease.

Material and methods:

Aβ circRNAs identification by RT-PCR and sequencing

All Aβ containing circRNAs from the APP gene were amplified *via* RT-PCR with specific 'divergent' primers targeting exon 17 of the APP gene with human brain total RNA as template. cDNA synthesis was performed with SuperScript™ III First-Strand Synthesis SuperMix (18080400, Invitrogen) with random hexamers. PCR was performed with PrimeSTAR® GXL DNA Polymerase (R050A, TaKaRa) with extension at 68 °C for 40 cycles.

For RNase R treatment, 15 µg total human brain RNAs were digested with 10 units of RNase R (RNR07250, Epicentre) for 1 hour at 37 °C and purified by phenol-chloroform extraction. Treated RNAs were used for cDNA synthesis and PCR amplification. Resulting PCR products were purified with E.Z.N.A. Gel Extraction Kit, (D2501-02, Omega Bio-tek), then digested by BamHI and XhoI and ligated into the pCMV-MIR vector (Origene). Positive clones were sequenced by Sanger sequencing.
PCR primers: AB-cRNAvBF2, atataggatccGTGATCGTCATCACCTTGGTGATGC
AB-cRNAvXR2, tatatctcgagCACCATGAGTCCAATGATTGCACC
For deep sequencing, RT-PCR products of Aβ circRNAs were used to prepare DNA library by TruSeq DNA Nano Kit (FC-121-4003, Illumina, Inc) and they were sequenced in HiSeq4000 (Illumina, Inc) at the Cologne Center for Genomics, University of Cologne. About one million reads were obtained for each samples and they were firstly mapped with STAR, then by DCC for circRNA detection at the bioinformatics core facility of Max Planck Institute for Biology of Ageing[25,26].

Plasmid construction
CircRNAs derived from the human APP gene were reported by several groups[20,27-29]. For the purpose of this analysis, one isoform of these circRNAs, i.e., human hsa_circ_0007556 (circBase), is named as Aβ circRNA-a. The cDNA of human APP circRNA-a (GRCh37/hg19, chr21:27264033-27284274) was inserted into pCircRNA-BE or pCircRNA-DMo vectors to generate pCircRNA-BE-Aβ-a or pCircRNA-DMo-Aβ-a as described previously[24]. Recombinant plasmids were purified with EndoFree Plasmid Maxi Kit (QIAGEN). Oligonucleotide sequences and further details are provided in Supplementary Table 1. All plasmids were verified by restriction endonuclease digestions and Sanger sequencing.

Cell culture and plasmid DNA transfection
Human HEK293 cell line culture and plasmid DNA transfection were performed as previously described[24].

Total RNA isolation and qRT-PCR
Total RNA from HEK293 cells was isolated with the TRIzol reagent (Ambion) according to the manufactures' recommendations. Total RNA of human brain frontal lobe and hippocampus was purchased from BioCat GmbH. cDNA synthesis and qRT-PCR was performed as previously described[24]. Details of qRT-PCR oligonucleotides are provided in Supplementary Table 1.

Northern blot analysis
Northern blot hybridizations were performed with NorthernMax™ Kit (AM1940, Ambion). In brief, 15 µg total RNAs from HEK293 cells were separated on 5% native polyacrylamide gel (Bio-Rad) and transferred to positively charged nylon membrane. The hybridization was performed with 5' p^{32}-labeled DNA oligo for overnight at 42 °C (Aβ-NBR1: CCCACCAT GAGTCCAATGATTGCACCTTTGTTTGAACCCACATCTTCTGCAAAGAACACC).
Blot membrane was washed as the suggested method by the kit. For RNase R treatment, 15 µg total RNAs were digested with 10 units of RNase R (RNR07250, Epicentre) for 1 hour at 37 °C; treated RNAs were subsequently separated on gel and analysed by northern blot similarly.

Western blot assays
Total proteins were prepared in RIPA Buffer (50mM Tris-HCl pH 8.0, 150 mM NaCl, 1% (v/v) NP40, 0.1% (w/v) SDS, 0.5% (w/v) Na-Deoxycholate, 1* Roche cOmplete Protease Inhibitor and 1* PhosSTOP Phosphatase Inhibitor). 40 µg total proteins were separated on 18% or 4-20% Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad) and transferred to 0.2 µm nitrocellulose membranes. Immunoblotting was performed with Anti-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). Aβ42 (A9810, Sigma) was prepared in DMSO.

Immunoprecipitation/western blotting (IP-WB) of Aβ peptide
Aβ peptide detection was performed through immunoprecipitation of conditioned medium (CM), followed with western blot analysis as previously described[30]. In brief, HEK293 cells transfected with pCircRNA-BE-Aβ-a, pCircRNA-DMo-Aβ-a or empty vector (pCircRNA-DMo) were cultured in serum free medium overnight. Then CM was prepared with protease and phosphatase inhibitors (Roche) and pre-cleaned with Protein A/G (Dynabeads™ Protein A, 10002D, Dynabeads™ Protein G, 10004D, Invitrogen). Immunoprecipitation was

conducted with a mixture of Aβ antibodies (6E10, 6G8, BioLegend Inc.). Precipitated peptides were subsequently resolved in SDS loading buffer and analysed by western blot with an antibody derived against Aβ (D54D2, Cell signalling Technology).

Primary neuron culture and Immunocytochemistry (ICC)

C57BL6N mice were housed under the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Primary neurons were isolated from 13 days of embryos of mice, using a neural tissue dissociation kit (Miltenyi Biotec). Nucleofection of pCircRNA-DMo-Aβ-a vector or the empty vector control (pCircRNA-DMo) to the isolated neuron were performed with P3 Primary Cell 4D-Nucleofector™ X Kit (V4XP-3024, Lonza Cologne GmbH). The transfected neurons were seeded onto coated coverslips with 5 $x10^5$ cells per well and cultured in MACS Neuro Medium (#130-093-570, Miltenyi Biotec) with 1x MACS NeuroBrew-21 (130-093-566, Miltenyi Biotec),1x Glutamaine (25030081, Gibco) and 1x Penicillin-Streptomycin (15140122, Gibco). Of note, transfection efficiency of primary neuron with the Nucleofector technology was very high, although it caused a considerable number of neuron dying.

ICC was performed at the 10th day after nucleotransfection with antibodies against GFP (GFP-1020, Aveslab) and Aβ (6E10, BioLegend Inc.). Alexa Fluor® 488 Goat Anti-Chicken IgG (A-11039, thermo scientific) and Goat anti-Mouse Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody (M30010, thermo scientific) were used as secondary antibody in correspondingly. Nuclei were indicated by DAPI (D9542, Sigma) staining for 2 hours at room temperature. Images were taken by Leica SP8 X confocal Microscope.

Results

Aβ circRNA isoforms expression from the APP gene in human brain

Previous deep-sequencing analysis discovered several circRNAs from human APP gene and one circRNA (hsa_circ_0007556, circBase) contains the open reading frame for Aβ peptide[20,27-29]. To identify all circRNAs encoding the Aβ related sequences, we performed RT-PCR with divergent primers with the total RNA from human brain samples (Fig. 1). Then we used 5% native polyacrylamide gel electrophosis to analyze the RT-PCR products. As there was couple of bands in the gel, it indicated multiple of circRNAs with different lengths were amplified (Fig. 1A). So we performed deep-sequencing of the PCR product. After computational analysis of the obtained reads, we discovered 16 circRNAs isoforms (Table 1). hsa_circ_0007556 was contained within this list. Since all of these circRNAs include coding sequence for the Aβ peptide, we refer these RNAs as Aβ circRNAs. The main target of our analysis is hsa_circ_0007556, it is henceforth called Aβ circRNA-a (Fig. 1B, Table 1). Similarly, the other three related copies are named as Aβ circRNA-b, c, d (Fig. 1, Table 1).

Furthermore, we repeated the Aβ circRNAs identification with RNase R pre-treated human brain total RNA. Our data demonstrated that 15 of 16 Aβ circRNAs within our analysis were resistant to RNase R treatment and one additional Aβ circRNA copy was discovered (Table 1). Importantly, Aβ circRNA-a was the most abundant copy and it was enriched in RNase R treated brain RNA samples (Table 1). Aβ circRNA-a was therefore selected for the analysis of Aβ circRNA associated functions.

Moreover, to obtained the full sequence of Aβ circRNA-a, we ligated the amplified RT-PCR products of RNase R treated human brain RNA to the pCMV-MIR vector; few clones of the resulting constructs were analyzed by Sanger sequencing. Result showed that Aβ circRNA-a contained exon 14, 15, 16, 17 of APP gene without any intron retaining (data not shown).

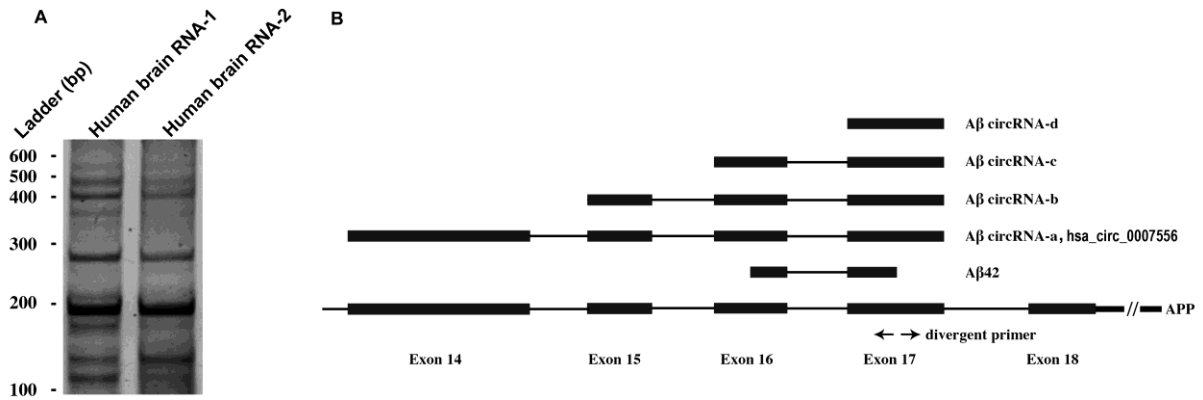

Fig. 1: Aβ circRNA isoforms identification

A) 5% native polyacrylamide gel electrophoresis of Aβ circRNA RT-PCR products with divergent primer located in the exon 17 of human APP gene in human brain samples. Two human brain RNA samples were used. B) the localizations of Aβ circRNA-a, b, c, d in APP gene. Aβ42 sequence was used as location reference. The short arrow indicates the location of divergent primer. has_circ_0007556 is named as Aβ circRNA-a in this study.

circRNA number	circRNA name	Aβ circRNA genomic positions	junction reads of brain RNA-1 without RNase R treatment	junction reads of brain RNA-2 without RNase R treatment	junction reads of brain RNA-1 with RNase R treatment	junction reads of brain RNA-1 with RNase R treatment	spliced exon size
1	n.n	chr21:25881397-25891834	498	445	218	84	488 nt
2	n.n	chr21:25881459 -25891868	0	3	25	11	450 nt
3	n.n	chr21:25881489 -25897673	21	3	37	1	531 nt
4	n.n	chr21:25881523 -25897626	69	108	39	25	466 nt
5	n.n	chr21:25881523 -25911815	12	13	0	0	626 nt
6	n.n	chr21:25881537- 25891834	0	6	10	4	348 nt
7	n.n	chr21:25881611 -25891834	17	20	27	19	274 nt
8	n.n	chr2125881611 -25891868	59	37	53	15	300 nt
9	n.n	chr21:25881695 -25891855	0	0	20	8	211 nt
10	n.n	chr21:25881695 -25897673	35	22	13	17	325 nt
11	**Aβ circRNA-d**	**chr21:25891722 -25891868**	**1519**	**643**	**755**	**571**	**147 nt**
12	**Aβ circRNA-c**	**chr21:25891722 -25897673**	**114**	**100**	**339**	**159**	**248 nt**
13	**Aβ circRNA-b**	**chr21:25891722 -25905077**	**104**	**88**	**218**	**139**	**302 nt**
14	**Aβ circRNA-a**	**chr21:25891722 -25911962**	**1464**	**1113**	**6965**	**3503**	**524 nt**
15	n.n	chr21:25891733 -25897650	0	11	0	25	214 nt
16	n.n	chr21:25891753 -25891834	0	51	0	23	82 nt
17	n.n	chr21:25891753 -25897626	347	33	73	4	170 nt

Table 1 Aβ circRNA isoforms from the APP gene in human brain.

Aβ circRNA genomic position and reads number from deep sequencing of RT-PCR products. The most enriched circRNA 14, 13, 12, 11 are named as Aβ circRNA-a, b, c, d respectively (bold). n.n., not named yet.

IME promotes Aβ circRNA-a overexpression in cell lines.

To further confirm Aβ circRNA-a's existence in human brain, we performed RT-PCR on human frontal lobe and hippocampus total RNA using oligonucleotides specifically designed

to detect Aβ circRNA-a (Fig. 2A, C). Indeed, Aβ circRNA-a was clearly expressed both in human frontal lobe and hippocampus (Fig. 2A, C).
To facilitate circRNA functional studies, we recently developed an intron-mediated enhancement (IME) strategy for robust circRNA expression[24]. Here, we applied this method to boost Aβ circRNA-a expression from vectors constructed in a similar manner to the described strategy (Fig. 2A, B)[24]. Transient expression in HEK293 cells showed that pCircRNA-BE-Aβ-a and pCircRNA-DMo-Aβ-a generated 2185- and 3268-fold more Aβ circRNA-a than that was endogenously produced in HEK293 cells transfected with empty vector (Fig. 2C, D). RT-PCR confirmed that the overexpressed Aβ circRNA-a was spliced exactly as the wild type Aβ circRNA-a (Supplementary Fig. 1). Sequencing the RT-PCR products further confirmed that their sequences were identical to wild type Aβ circRNA-a found in the human brain (Supplementary Fig. 1C, D). Moreover, we used northern blot to detect Aβ circRNA-a expression. As shown in Supplementary Fig. 1E, the detected Aβ circRNA-a migrated faster than the linear counterpart in native 5% native polyacrylamide gel. RNase R treatment abolished the linear counterpart but the suggested Aβ circRNA-a bands were not affected, demonstrating the circularity of the expressed Aβ circRNA-a in HEK293 cell (Supplementary Fig. 1E). Importantly, being similar as described in previous study[24], northern blot also shown that both pCircRNA-BE-Aβ-a and pCircRNA-DMo-Aβ-a did not produce linear counterpart mature mRNA, thus excluding the possibility of linear contamination of followed function study.

Since HEK293 is a well-established cell line for Alzheimer's disease research[31,32], the robust IME strategy for expression of Aβ circRNA-a in HEK293 cells provides a good model for studying Aβ circRNA-a 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 was not affected.

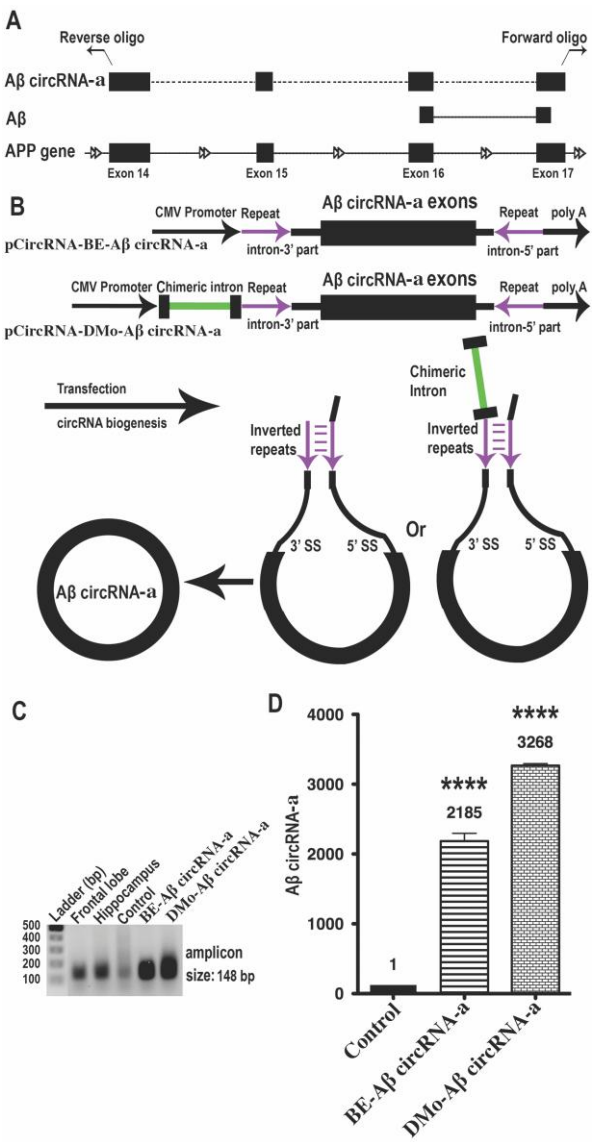

Fig. 2: Aβ circRNA-a overexpressing HEK293 cells.
A) Aβ circRNA-a is encoded by exons 14-17 of the human APP gene, without the introns. The amyloid-β (Aβ) sequence is located in exons 16 and 17. Reverse and forward oligonucleotides were used to amplify Aβ circRNA-a by RT-PCR. B) Aβ circRNA-a over-expression constructs. C) RT-PCR verification of Aβ circRNA-a expression in human brain samples (frontal lobe and hippocampus) and HEK293 cells overexpressing Aβ circRNA-a; Control, pCircRNA-DMo empty vector transfection into HEK293; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a transfection into HEK293; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a transfection into HEK293; RT-PCR verification of Aβ circRNA-a by another set of oligonucleotides is shown in Supplementary Fig. 1. A, B, C, D) Aβ circRNA-a expression in HEK293 cells mediated by pCircRNA-BE circRNA-a and pCircRNA-DMo circRNA-a vectors. Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a. All statistical T tests were performed in comparison to the control sample, ****, $P \leq 0.0001$, $n = 4$.

Aβ circRNA-a can be translated into an Aβ related peptide.
Previous studies have shown that certain circRNAs are translatable[24,33-36]. Examination of the Aβ circRNA-a revealed that it contained an open reading frame (ORF) (Fig. 3A) 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 related peptide was translated

in HEK293 cells overexpressing Aβ circRNA-a used an antibody (β-Amyloid (D54D2) XP® Rabbit mAb) that detected all Aβ containing peptides. Specifically, we detected an obvious Aβ-related peptide signal with a size around 15 to 20 kDa, confirming the translation of Aβ circRNA-a (Fig. 3B). Products of Aβ circRNA-a translation were called Aβ circRNA-a-derived peptide (Aβ circRNA-a-DP).

Interestingly, control HEK293 cells transfected with empty vector contained detectable amounts of Aβ circRNA-a-DP. As we showed above (Fig. 2), HEK293 cells expressed low levels of endogenous Aβ circRNA-a, thus detectable Aβ circRNA-a-DP in control HEK293 cells represents its endogenous translation. Importantly, such detectable Aβ circRNA-a-DP in HEK293 cells confirmed its authenticity, highlighting its biological relevance.

Clearly, as in our previous study[24], intron-mediated enhancement (IME) increased Aβ circRNA-a translation. pCircRNA-DMo-Aβ-a expressed about 3.3-fold more Aβ circRNA-a-DP than the endogenous expression in HEK293 cells, while pCircRNA-BE-Aβ-a only moderately enhanced Aβ circRNA-a-DP levels (1.4-fold; Fig 3B, C). Interestingly, the enrichment of protein product (Aβ circRNA-a-DP) was much less significant than the elevated expression of Aβ circRNA-a, indicating that the translation of Aβ circRNA-a was regulated by other unknown mechanisms beyond the circRNA expression level.

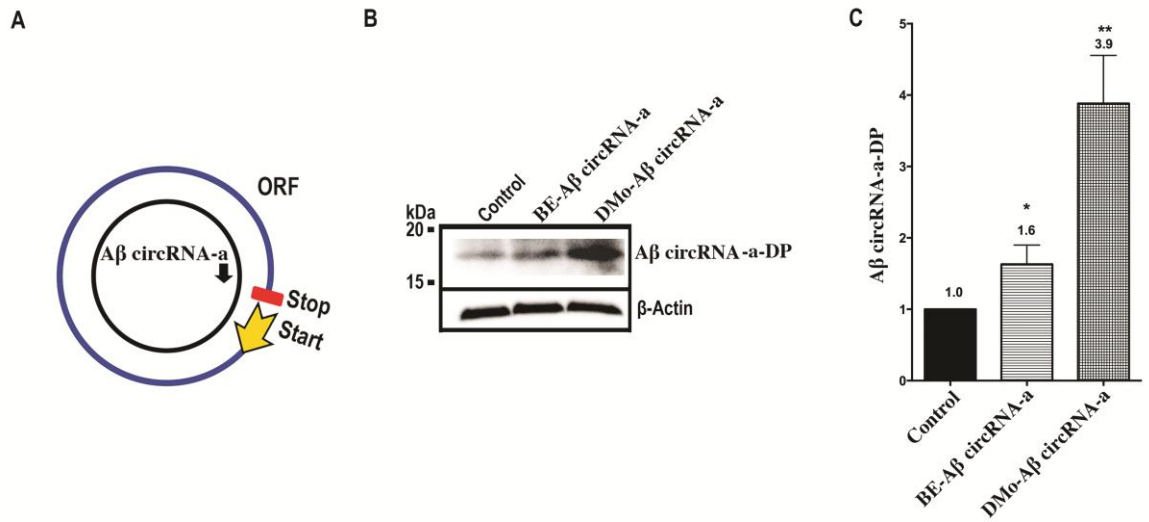

Fig. 3: Expression of Aβ related peptide from Aβ circRNA-a translation in HEK293
A) The open reading frame (ORF) of Aβ circRNA-a is represented by a blue circle; yellow arrow, translation start codon; red square, stop codon; the inner black arrow shows the start of the Aβ circRNA-a. B) Western blot of Aβ-related peptides in HEK293 cells. Control, empty vector (pCircRNA-DMo); BE-Aβ circRNA-a, pCircRNA-BE-Aβ circRNA-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ circRNA-a. The detected peptides indicated on the right: Aβ circRNA-a-DP, Aβ circRNA-a-derived protein; β-Actin used as a loading control. C) quantification of Aβ circRNA-a-DP levels. All statistical T tests were performed with respect to the control sample; *, $P \leq 0.05$; **, $P \leq 0.01$; $n \geq 3$.

Aβ circRNA-a derived peptide is further processed to form Aβ.
The production of Aβ related peptide from Aβ circRNA-a translation highlights the potential of Aβ biogenesis. Moreover, the predicted protein sequence of Aβ circRNA-a-DP contain β and γ-secretase cleavage site, indicating that Aβ can be produced from Aβ circRNA-a-DP by β and γ-secretase proteolytic processing (Supplementary Fig. 3). So, we used immunoprecipitation/western blotting (IP-WB) by specific Aβ antibodies (6E10, 4G8) to analyse the possible Aβ expression in the conditioned medium (CM) of Aβ circRNA-a

overexpressed HKE293 cells. Strikingly, we detected a peptide band corresponding to Aβ in western blot with another Aβ antibody (D54D2), thus confirming the biogenesis of Aβ from Aβ circRNA-a translation (Fig. 4A, B). Compared to empty vector transfection, pCircRNA-BE-Aβ-a caused 2.6-fold increase of Aβ peptide expression in the condition medium (Fig. 4A, B). Strikingly, pCircRNA-DMo-Aβ-a caused as more as 6-fold increase of Aβ peptide expression (Fig. 4A, B). Of note, the difference trend of Aβ expression level between three samples (control, pCircRNA-BE-Aβ-a, pCircRNA-DMo-Aβ-a) was consistent with the difference trend of Aβ circRNA-a-DP, representing the up-regulated Aβ was processed from circRNA-a-DP.

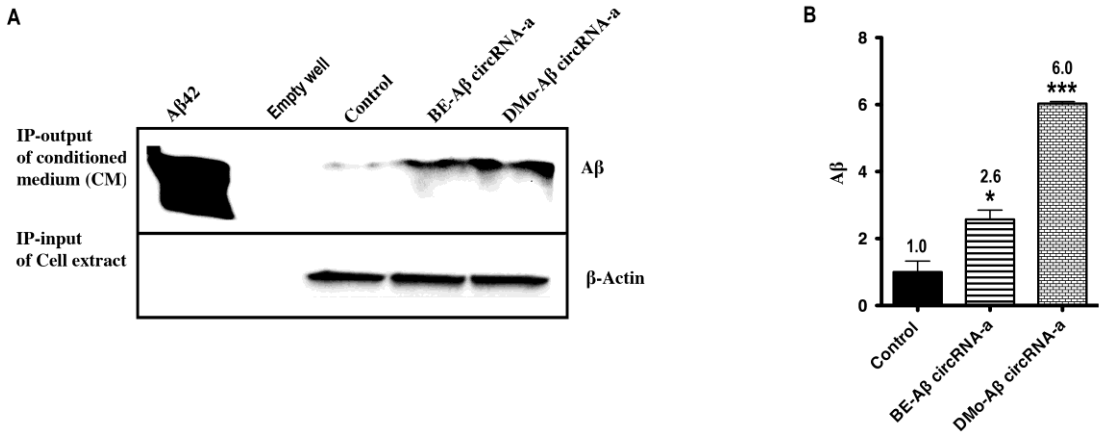

Fig. 4: IP-WB of Aβ peptides from in the conditioned medium of Aβ circRNA-a overexpressed cells.
The conditioned medium of HKE293 with transfection of Aβ circRNA-a overexpression vector was immunoprecipitated with antibody against Aβ (6E10, 4G8). Control, pCircRNA-DMo; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a; Aβ antibody (D54D2) was used in western blot; β-Actin was used as loading control. 5 ng in vitro synthesised Aβ42 was used as Aβ migration maker in western blot. All statistical T tests were performed in comparison to the control sample; *, $P \leq 0.05$; ***, $P \leq 0.001$; n = 3.

Aβ circRNA-a overexpression develops Aβ plaques in primary neuron culture.
As we have shown that Aβ peptides are produced from Aβ circRNA-a translation, then we want to know that whether these Aβ peptides could develop Aβ plaques outside of neuron, which is the key hallmark of AD. So, we transfected the pCircDNA-DMo-Aβ-a plasmid into mouse embryonic neuron by electroporation and cultured them for 10 days. Then we used the specific antibody 6E10 against Aβ to detect any plaques in the neuron culture. Strikingly, we observed clearly Aβ plaque signals in the neuron culture when Aβ-a circRNA was expressed (red signals in Fig. 5B). As negative control, the neuron culture with empty vector transfection did not develop Aβ plaque signal (Fig. 5A). Of note, as both the empty vector and the circRNA expression vector contained GFP expression cassette[24], immunofluorence against GFP served as good control for neuron cell location. As shown in Fig.5 B, the merged image of GFP and Aβ plaque signal clearly demonstrated that Aβ plaque was located outside of neuron, which was consistent with previous studies. In summary, we concluded that Aβ plaques were formed in primary neuron culture with Aβ circRNA-a overexpression.

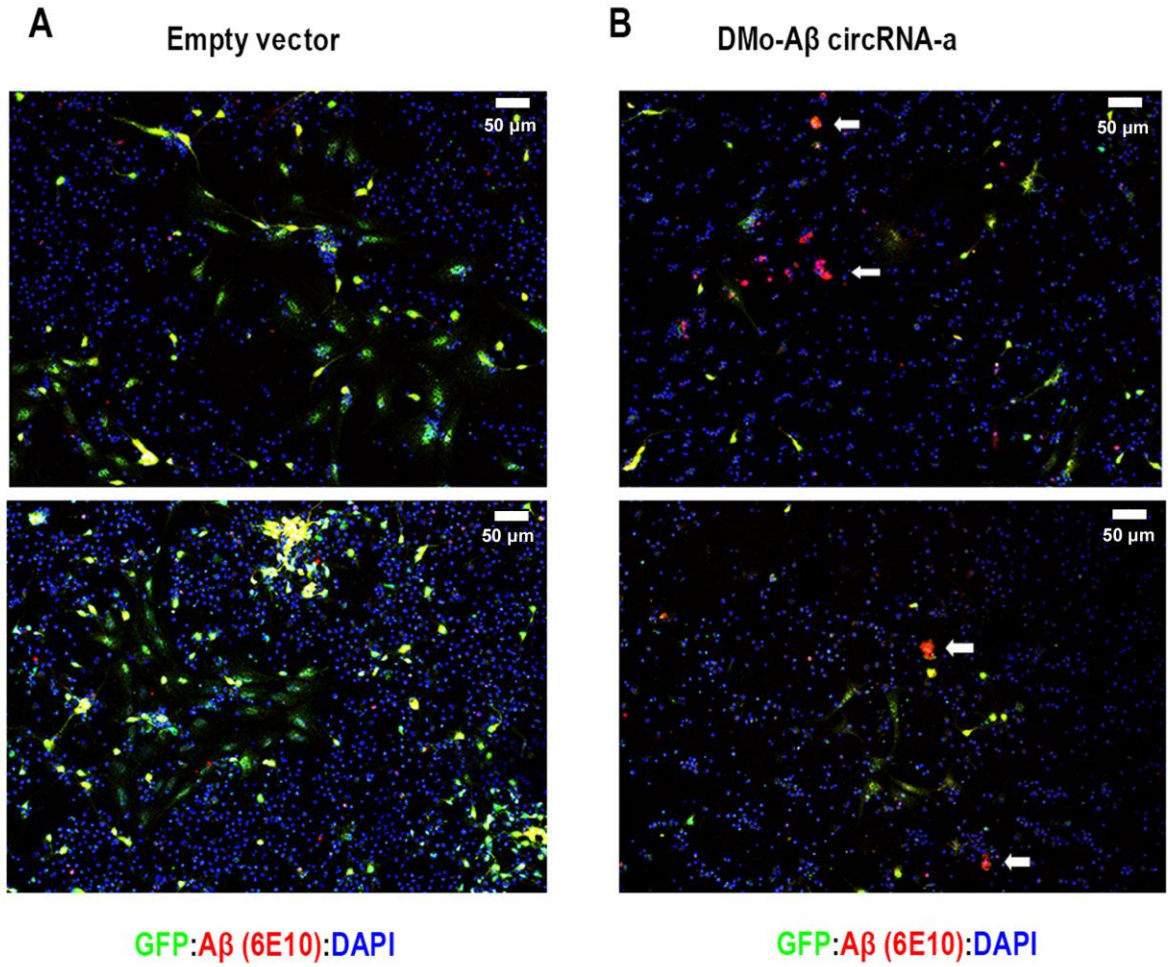

Fig. 5: Aβ circRNA-a overexpression generates Aβ plaques in mouse primary neuron culture.
A, B) pCircRNA-DMo was used as empty vector control. DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a; GFP was shown in green; Aβ (6E10) was shown in red; white arrows indicate the Aβ plaques positions; DAPI (nuclei staining) was shown in blue. Of note, same amount of starting neurons were used in transfections. Different density of neurons (green) observed in images between A and B most likely was caused by the toxicity of Aβ peptides. In both A and B, two individual images were taken from the primary neuron culture after 10 days of transfection.

Aβ circRNA-a overexpression significantly up-regulates GSK3β and tau phosphorylation

Previous 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-a translated peptides, especially these Aβ peptides, we analysed GSK3β levels and the phosphorylation of tau proteins in HEK293 cells overexpressing Aβ circRNA-a using western blots. Indeed, Aβ circRNA-a overexpression significantly increased GSK3β levels, by 2.3-fold following pCircRNA-BE-Aβ transfection and 3.5-fold after pCircRNA-DMo-Aβ circRNA-a transfection (Fig. 6A, B). Interestingly, tau protein levels were also upregulated when Aβ circRNA-a was overexpressed (HT7 antibody; Fig. 6A, C). Using AT8 (Ser202, Thr205) and AT100 (Thr212, Ser214) antibodies to analyse tau phosphorylation, pCircRNA-BE-Aβ circRNA-a 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-a-DP, pCircRNA-DMo-Aβ circRNA-a

transfected cells contained 3.7-fold or 5.0-fold more phosphorylated tau detected with AT8 or AT100 antibodies (Fig. 6A, C, D, E).

In summary, Aβ circRNA-a significantly up-regulate GSK3β and consequently promote tau phosphorylation.

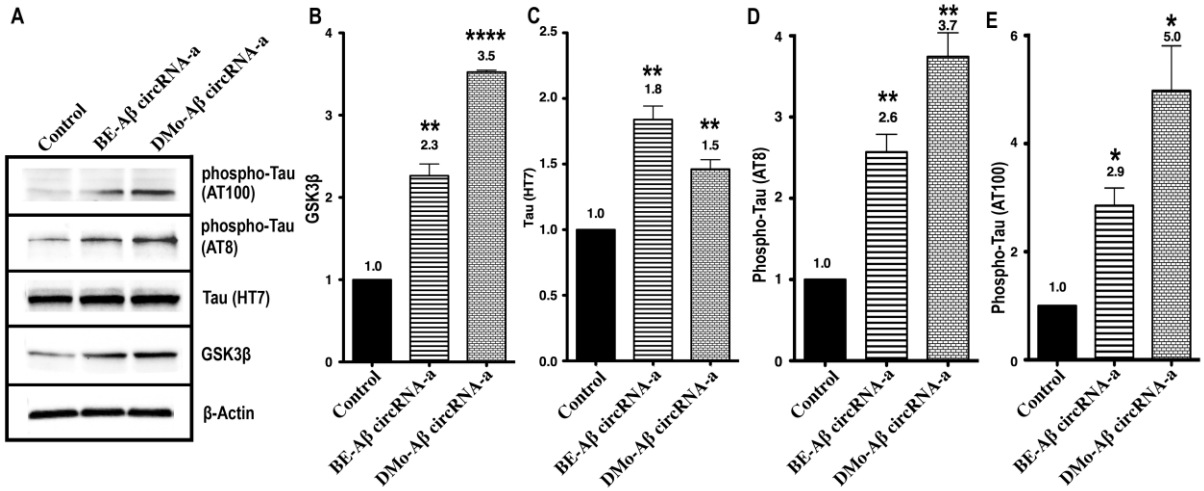

Fig. 6: Aβ circRNA-a up-regulates GSK3β expression and promotes tau protein phosphorylation.
A) GSK3β and tau protein (HT7 antibody) levels, and tau phosphorylation detected in HEK293 cells overexpressing Aβ circRNA-a. β-actin was used as a loading control. Control, pCircRNA-DMo empty vector; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a. 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. All statistical T tests were performed with respect to the control sample; **, $P \leq 0.01$; ****, $P \leq 0.0001$; $n \geq 3$.

Discussion
Although extensive studies have illustrated the biogenesis mechanism of Aβ peptide in fAD, the pathways of Aβ peptide production in sAD remain elusive.

In this study, we discovered 17 Aβ circRNAs from APP gene. With the help of intron-mediated enhancement of circRNA expression, we could verify that Aβ circRNA-a can be translated into Aβ related peptide and subsequently processed to form Aβ and develop Aβ plaques, correspondingly inducing GSK3β up-regulation and tau phosphorylation, thus indicating a new direction in the search for molecular mechanisms of Alzheimer' disease (Fig. 7). Such a mechanism is dramatically different from Aβ accumulation through dysregulation of full length APP prototypic processing (Fig. 7). Thus, it provides an alternative pathway of Aβ biogenesis (Fig. 7). Unlike factor mutations in familial Alzheimer's disease, no genetic mutation is required for Aβ circRNAs biogenesis. Correspondingly, the entire human population may express Aβ circRNA and Aβ circRNA-DPs, which may play a key role in sporadic Alzheimer's disease.

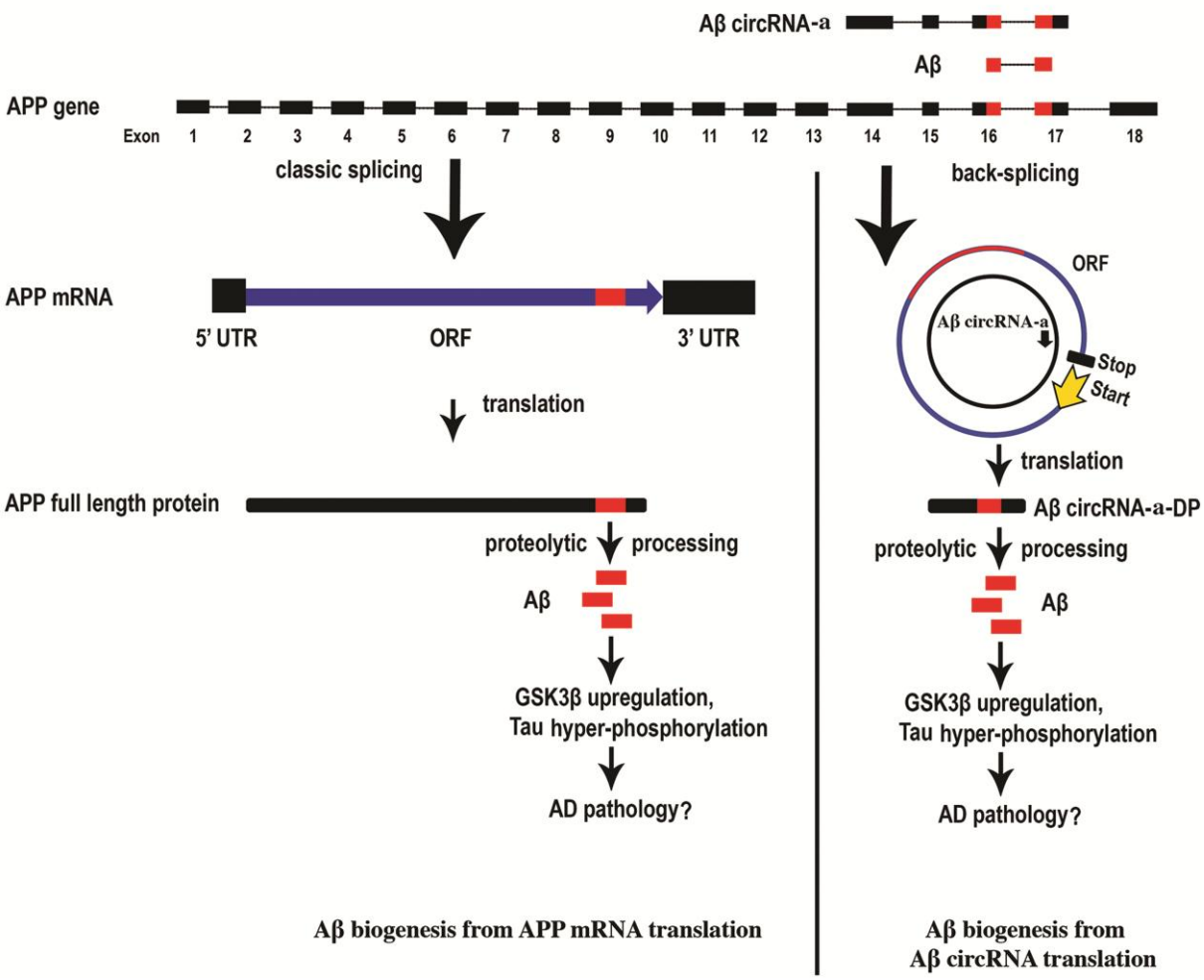

Fig. 7: Two Aβ biogenesis pathways in Alzheimer's disease.
At the top, exon sequences contained in Aβ circRNA-a and Aβ peptides (in red) are aligned with the full-length APP gene. On the left, liner APP mRNA transcribed from the APP gene undergoes classic splicing before being translated into full-length 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-a 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-a produces Aβ-related peptide (Aβ circRNA-a-DP), which is further processed to form Aβ. The accumulation of Aβ 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.

So far, it is not known that how many percent of Aβ is derived from Aβ circRNAs in aged people and AD patients. The significance of this alternative Aβ biogenesis in AD pathology remains to be determined. As an analogy, although Aβ is endogenous expressed in rodent brain, but it is surprising that wild type mouse neither appears Aβ deposit in old brain nor develops Alzheimer's disease[37-39]. Compared to human Aβ, despite there are three amino acids variations in the N-terminal, endogenous murine Aβ peptides are neurotoxic and have quite similar ability to produce Aβ deposit provided that the murine APP gene harbours fAD mutations[40]. It sounds that there is very small amount of Aβ is derived from mouse APP

protein processing. Moreover, wild type human APP protein (hAPPwt) overexpression mice would not generate obvious Aβ, demonstrating that full length wild type APP protein would not be the major source of Aβ production in sporadic AD[41].

With the discovery of alternative Aβ biogenesis pathway from Aβ circRNA in human, it will be interesting to find out whether mouse express Aβ circRNA or not. Using RT-PCR with divergent primers located at Aβ sequence region, we did not detect any mouse Aβ circRNA copy (Supplementary Fig. 4). As positive control, a circRNA spanning exon 9 to exon 13 of mouse App gene was detected (Supplementary Fig. 4). Apparently, mouse does not express Aβ circRNA, thus lacking the alternative Aβ biogenesis pathway existed in human, possibly explaining the difference of Aβ deposition in elderly brain between human and mouse. At the other side, efficient generation of Aβ plaque from Aβ circRNA-a overexpression in mouse primary neuron strongly indicates that the Aβ biogenesis from Aβ circRNA translation may constitute the major part of Aβ population in human brain and play essential role in the sporadic AD development.

In this study, the proteolytic processing of Aβ circRNA-a derived peptide is not studied. It will be interesting to investigate how α, β and γ-secretase participate in the Aβ biogenesis mechanism from Aβ circRNA-a-DP.
Furthermore, other Aβ circRNA isoforms also trigger further investigations. For example, whether they can also produce Aβ related peptides which can be eventually processed to form Aβ.

In AD pathology, ageing itself is the most causative factor. Aβ accumulates in human brain in an ageing-related manner[17,42]. Although studies shown the defective clearance of Aβ in aging brain may partially contribute to this process, but mechanism precede clearance is largely unknown[17]. Recent studies have implicated circRNA as a regulator of cellular stress[43]. For example, the translation of Circ-ZNF609 circRNA could be induced by heat shock stress[33]. In brain ageing, neurons are particularly vulnerable to various stresses[44]. Presumably, expression of Aβ circRNAs and their translated peptides could be activated by various (age-related) stresses, and cause a detrimental downstream cascade, leading to neurodegeneration.

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, we show that the protein produced from Aβ circRNAs translation may play a critical role in Alzheimer's disease, thus endorsing further function exploration of circRNA, and indicating that proteins translated from other circRNAs may also play essential, biologically significant roles.

The discovery of biological roles for Aβ circRNAs and their translated peptides may not only represent a milestone for the function exploration of circRNA, but also reveals a potential ground-breaking mechanism for causing Alzheimer's disease and opens up new strategies for diagnosis, prevention and treatment of Alzheimer's disease.

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assistances during this study. C57BL6N mice were housed in the Transgenic Core Facility and ICC images were acquired in the FACS & Imaging Core Facility of the host institute.

Author Contributions

DM designed and conceived the study. DM performed the experiments, analysed the results, prepared the figures and wrote the manuscript. DC performed the primary neuron culture and ICC experiments. XL participated in some experiments and project discussion.

Conflict of interest.

None declared.

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The role of Aβ circRNA in Alzheimer's disease: alternative mechanism of Aβ biogenesis from Aβ circRNA translation

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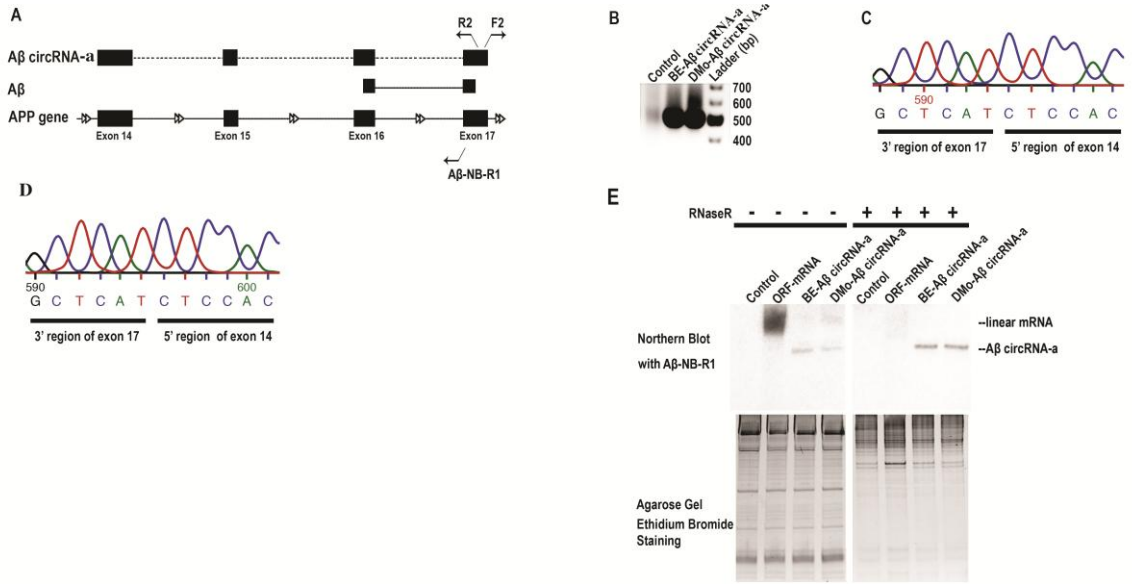
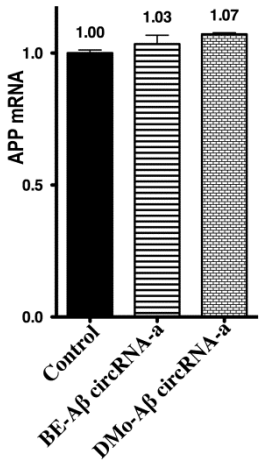
Aβ circRNA expression plasmid construction oligos	
Abeta-circF	gtttgtttttcagATGAGCTGCTTCAGAAAGAGCAAAACT
ABeta-circR	gcatggattattacCTCCACCACACCATGATGAATGG
circDMO-LF	GTCGACTGGATCcaacgttaaccc
DMo-Ab-LR	CTGAAGCAGCTCATctgaaaaacaaacagaatacaacctcagc
DMo-Ab-RF	GGTGTGGTGGAGgtaataatccatgcaccgtctcacc
circDMO-RR	cactttgCTCGAGctcatcaacatg
Aβ circRNA verification oligos	
AB-cRNAvF2	GTGATCGTCATCACCTTGGTGATGC
AB-cRNAvR2	CACCATGAGTCCAATGATTGCACC
human Aβ circRNA-a	
Abeta-cF	GTCATAGCGACAGTGATCGTC
Abeta-cR	CTTGGTTCACTAATCATGTTGGC
Human APP mRNA	
hAPP-mF	TTTGTGATTCCCTACCGCTG
hAPP-mR	TGCCAGTGAAGATGAGTTTCG
Human ACTB mRNA:	
hACTB-F	ACCTTCTACAATGAGCTGCG
hACTB-R	CCTGGATAGCAACGTACATGG
mouse App mRNA qRT-PCR,	
App-mF	ATCTTCACTGGCACACCG
App-mR	GCATACAAACTCTACCCCTCG
mouse putative Aβ circRNA amplification	
F3	Gctgtctctcattggctgcttcc
R3	agcttgtagagacacacatggccag
mouse App mRNA RT-PCR at Aβ peptide region	
F1	GGACATGATTCAGGATTTGAAGTCC
R1	CACCAGGGTGATGACAATCACG
mouse putative Aβ circRNA amplification	
R2	GGACTTCAAATCCTGAATCATGTCC
F2	CGTGATTGTCATCACCCTGGTG

Mouse Actb mRNA:	
mActbF1	ACCTTCTACAATGAGCTGCG
mActbR1	CTGGATGGCTACGTACATGG

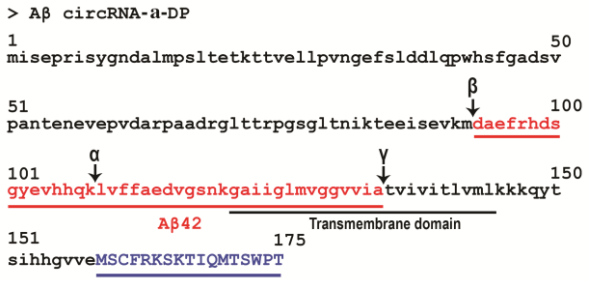
Supplementary Table 1
DNA oligos used in this study



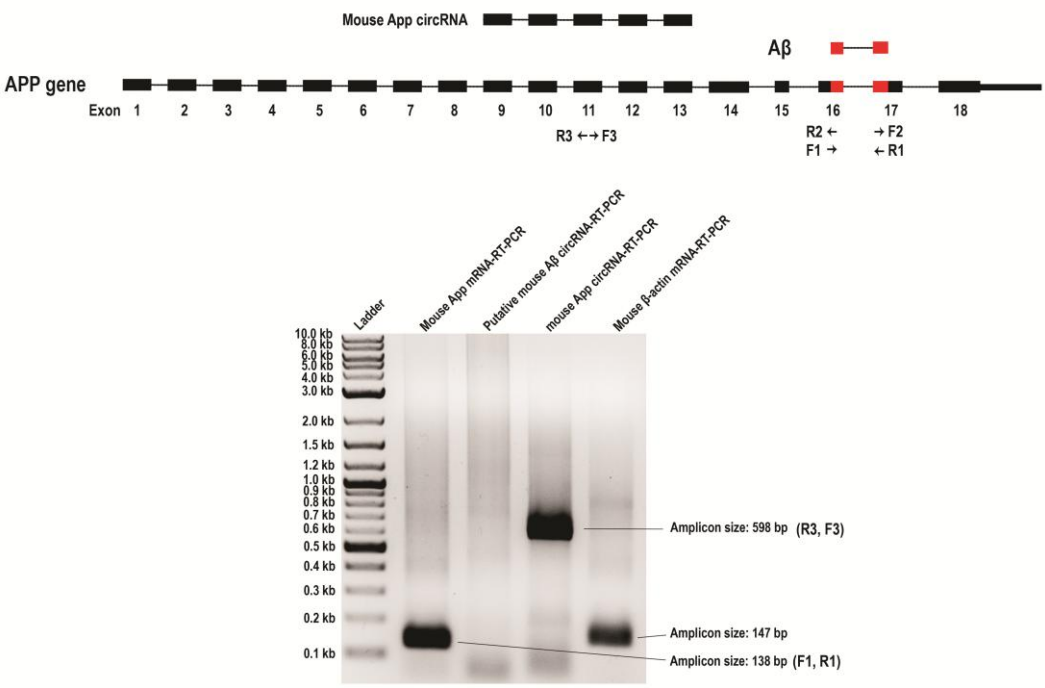
Supplementary Fig. 1. RT-PCR and northern blot verification of Aβ circRNA-a expression in HEK293 cells.
A) RT-PCR primers sites in Aβ circRNA. B) Agarose gel electrophoresis of RT-PCR products (503 bp) using primers Aβ-VR2 and Aβ-VF2; BE-Aβ circRNA-a, pCircRNA-BE-Aβ-a transfected HEK293 cells; DMo-Aβ circRNA-a, pCircRNA-DMo-Aβ-a transfected HEK293 cells. RT-PCR products were cloned, sequenced and aligned, confirming that Aβ circRNA-a were identical and comprised exons 14, 15, 16 and 17 of the APP gene without introns (data not shown); C, D) the sequencing of Aβ circRNA-a junction region of back-splicing from pCircRNA-BE-Aβ-a and pCircRNA-DMo-Aβ-a; E) Northern blot analysis of Aβ circRNA-a expression in HEK293 cells; ORF-mRNA, the linear cognate ORF mRNA of Aβ circRNA-a. For RNase R treatment, 15 µg total RNA was digested with 10 unites of RNase R for 1 hour at 37 °C; -, no treatment; +, with treatment; Oligo Aβ-NB-R1 (CCCACCATGAGTCCAATGATTGCACCTTTGTTTGAACCCACATCTTCTGCAAAGA ACACC) was used in northern blot; ethidium bromide staining of the agarose gel was used as loading control.



Supplementary Fig. 2. Endogenous APP mRNA expression in HEK293 cells overexpressing Aβ circRNA-a.
qRT-PCR analysis of endogenous APP mRNA expression in Aβ circRNA-a overexpressing HEK293 cells. Primers targeted exons 3 and 4, which are not contained in Aβ circRNA-a: hAPP-m, TTTGTGATTCCCTACCGCTG and hAPP-mR, TGCCAGTGAAGATGAGTTTCG. Control, pCircRNA-DMo empty vector; BE-Aβ CircRNA-a, pCircRNA-BE-Aβ-CircRNA-a; DMo-Aβ CircRNA-a, pCircRNA-DMo-Aβ CircRNA-a. No significant differences between different groups were observed. n = 4.



Supplementary Fig. 3. The putative amino acid sequence of Aβ circRNA-a–DP.
Predicted from the identical sequences of all circRNA RT-PCR products: red, sequence of Aβ42; blue, the unique sequence of Aβ circRNA-a-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-a-DP is 19.2 kDa; the arrow indicates the possible secretase sites.



Supplementary Fig. 4. Mouse brain does not express Aβ circRNA copies.
Primers F1 and R1 were used to amplify App mRNA; the divergent primers R2 and F2 located at the same position as F1 and R1were used to amplify putative Aβ circRNA copies; R3 and F3 were used to amplify the App circRNA (exon 9 to exon 13) as positive control circRNA RT-PCR; amplicon from mouse β-actin mRNA was also used as RT-PCR positive control.