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DNA Aptamers Targeting BACE1 Reduce Amyloid Levels and Rescue Neuronal Deficiency in Cultured Cells

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β-amyloid (Aβ) plays an essential role in the pathogenesis of Alzheimer's disease (AD). Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is indispensable for Aβ production, and knockout of BACE1 has no overt phenotypes in mouse. Thus, fine modulation of BACE1 may be a safe and effective treatment for AD patients. However, the large active site of BACE1 makes it challenging to target BACE1 with classical small-molecule inhibitors. DNA aptamer can have high affinity and specificity against diverse targets, and it provides an alternative strategy to target BACE1. In this study, we used a novel cell-systematic evolution of ligands by exponential enrichment (SELEX) strategy to select specific DNA aptamers optimized to target BACE1 under physiological status. After 17 rounds of selection, we identified two DNA aptamers against BACE1: BI1 and BI2. The identified aptamers interacted with BACE1 in pull-down assay, inhibited BACE1 activity in *in vitro* fluorescence resonance energy transfer (FRET) assay and HEK293-APP stable cell line, reduced Aβ in the culture medium of HEK293-amyloid protein precursor (APP) stable cell line and APP-PS1 primary cultured neurons, and rescued Aβ-induced neuronal deficiency in APP-PS1 primary cultured neurons. In contrast, the identified aptamers had no effect on α- or γ-secretase. In addition, cholesteryl tetraetylene glycol (TEG) modification further improved the potency of the identified aptamers. Our study suggests that it is feasible and effective to target BACE1 with DNA aptamers, and the therapeutic potential of the identified aptamers deserves further investigation.

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disorder with about 40 million patients worldwide. The autopsy of AD patients shows that extracellular amyloid plaques and intracellular neurofibrillary tangles are the pathological hallmarks of AD brains.[1] The genetic studies in familial AD patients identify numerous pathogenic mutations in genes encoding amyloid protein precursor (APP) and presenilins (PS). The relevance behind these findings is that β-amyloid (Aβ), the major component of plaques, is produced through sequential cleavages of APP by β- and γ-secretases. Based on the above evidence, the amyloid hypothesis believes that over-accumulation of amyloid in the brain is neurotoxic as it impairs synapse, neuron, and cognitive function.[2] Thus, targeting Aβ appears to be a plausible strategy to treat AD.

Unfortunately, nearly all efforts to target Aβ have failed so far.[3] Several clinical trials show that antibodies against Aβ bring no benefit to the cognition of AD patients.[4,5] Clinical trials also repeatedly demonstrate that targeting γ-secretase with potent inhibitor causes unwanted side effects without cognitive improvement.[6–8] Consistently, knockout of PS1/2 leads to neonatal lethal phenotype in mouse.[9] In contrast, knockout of BACE1, the β-secretase, produces no overt phenotype in mouse,[10–12] and subtle phenotypes are correlated with the gene dose of BACE1.[13,14] In addition, BACE1 expression and activity are upregulated in the brains of AD patients[15–17] and in cerebrospinal fluid (CSF) of patients with mild cognitive impairment.[18] Thus, BACE1 may play an essential role in the pathogenesis of AD, and fine modulation of BACE1 may be an effective treatment that is tolerable by human body.[19] However, the large active site of BACE1 makes it challenging to design classical small-molecule inhibitors with high affinity and ideal pharmacokinetics.[20] Thus, identification of novel modulators of BACE1 activity may open a safe and effective avenue to treat AD.

DNA aptamers are single-stranded DNA (ssDNA) molecules with a length typically less than 100 mer.[21] DNA aptamers can have high affinity and specificity against diverse targets after a selection

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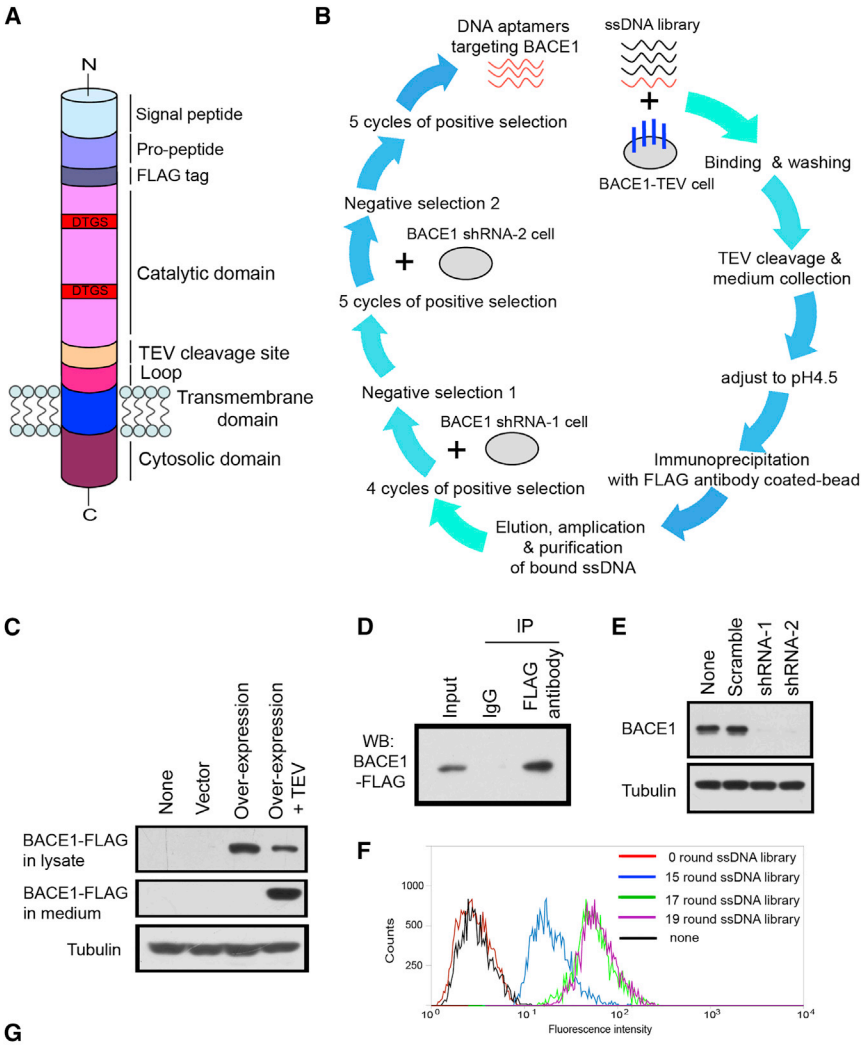

Figure 1. Selection of DNA Aptamers Targeting BACE1 Using Cell-SELEX

(A) Diagram showing the structure of BACE-TEV fusion. (B) Diagram showing the process of cell-SELEX. (C) Western blot with FLAG antibody showing the over-expression of BACE-TEV fusion in HEK293 stable cell line. (D) Immunoprecipitation with FLAG antibody showing the BACE-TEV domain in the culture medium after TEV proteinase treatment. (E) Western blot with BACE1 antibody showing its expression level in BACE-shRNA cell lines. (F) Flow cytometry showing the fluorescence intensity of HEK293-BACE1-TEV stable cell line alone (black curve) or after incubation with 0th (red curve), 15th (blue curve), 17th (green curve), and 19th (purple curve) round. (G) The sequences and frequencies of DNA aptamers enriched by the cell-SELEX strategy.

process called systematic evolution of ligands by exponential enrichment (SELEX).[22] Aptamers have shown great potential in targeted therapy, detection, and diagnostics.[23] Previous studies have developed aptamers against BACE1 in *in vitro* assays with recombinant BACE1 domains from *E. coli*. However, it is unclear whether or not these aptamers could bind BACE1 under the native state with proper modifications and subcellular localization. In this study, we used cell-SELEX strategy[24] to select specific DNA aptamers targeting BACE1. The identified aptamers were able to interact with BACE1, inhibit BACE1 activity, reduce Aβ, and rescue neuronal deficiency in cultured cells without effect on α- or γ-secretase. In addition, cholesteryl tetraetylene glycol (TEG) modification further improved the potency of the identified aptamers. Our study suggests that targeting BACE1 with DNA aptamers may be effective for AD treatment.

RESULTS

Selection of DNA Aptamers Targeting BACE1 Using Cell-SELEX

To select DNA aptamers targeting BACE1, we performed cell-based aptamer selection, the cell-SELEX, with minor modification from previous report.[24] BACE1-tobacco etch virus (TEV) fusion was constructed by inserting FLAG tag between the pro-peptide domain and catalytic domain and inserting TEV cleavage site between the catalytic domain and loop (Figure 1A). When BACE1-TEV fusion is expressed, matured, and displayed on cell surface, TEV protease could cleave TEV site to release the extracellular domain of BACE1 with N-terminal FLAG tag into culture medium. The cell-SELEX process was summarized in Figure 1B. First, HEK293 stable cell line overexpressing BACE1-TEV fusion was established using lentivirus for positive selection. Western blot with FLAG antibody confirmed that, in BACE1-TEV stable cell line, BACE1-TEV fusion was expressed at high level and TEV protease treatment released a large amount of extracellular domain of BACE1 with N-terminal FLAG tag into culture medium (Figure 1C). When BACE1-TEV stable cell was incubated with random ssDNA library and then digested with TEV protease, the bound ssDNA-BACE1 complex was released into culture medium. As β-secretase cleavage of APP takes place in endosome, which has an acidic environment, and BACE1 activity shows an optimum at pH 4.5,[25] the optimal aptamers targeting BACE1 should bind BACE1 both on the cell surface and within the acidic endosomes. Thus, we adjusted the medium containing the bound ssDNA-BACE1 complex to pH ≈ 4.5 to mimic the acidic environment of endosomes. Then, the bound ssDNA-BACE1 complex was immunoprecipitated by FLAG-antibody-coated bead (Figure 1D) and the bound ssDNAs were eluted and amplified using conventional methods. In this way, the DNA aptamers, which could

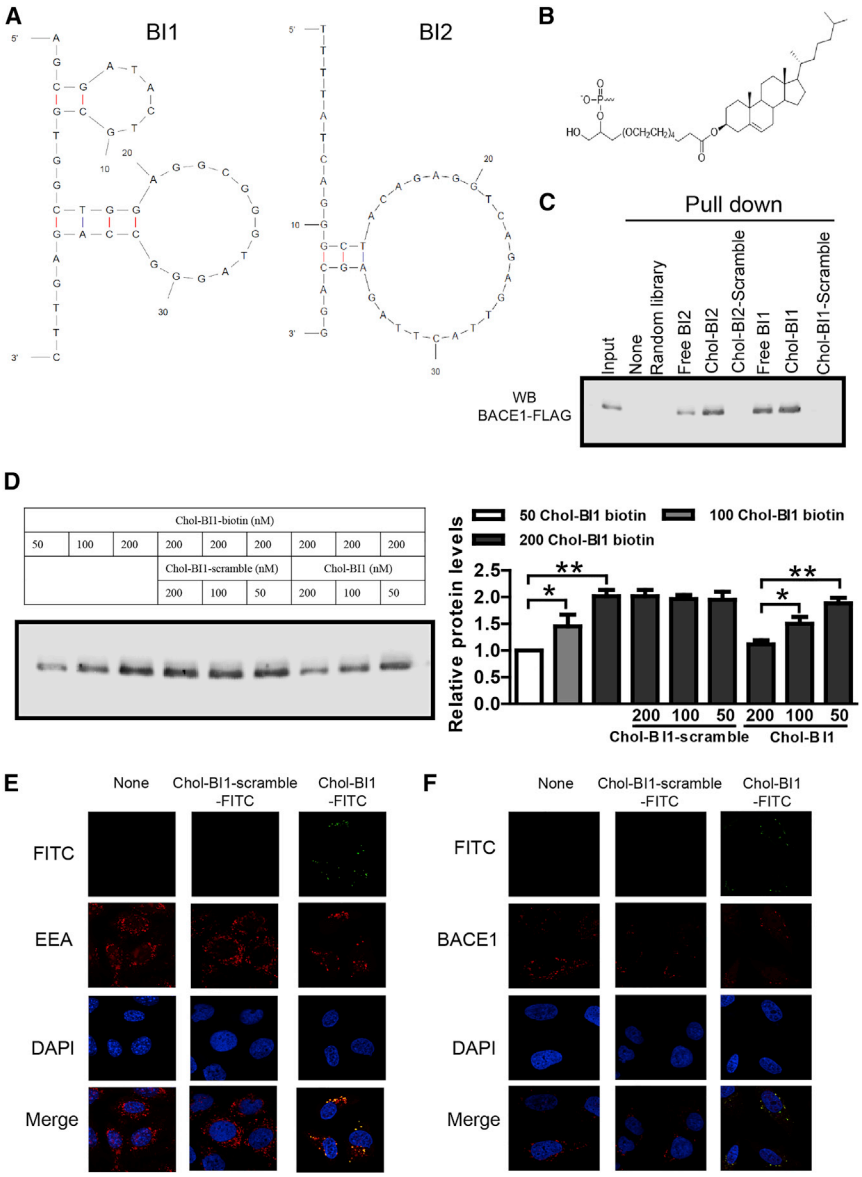

Figure 2. DNA Aptamers Interact with BACE1

(A) Diagram showing the predicted structure of DNA aptamers BI1 and BI2 by Mfold. (B) Diagram showing the structure of cholesteryl TEG moiety. (C) Western blot with FLAG antibody showing the precipitated WT-BACE1-FLAG in the pull-down assay. (D) Representative western blot with FLAG antibody showing the precipitated WT-BACE1-FLAG in the competition assay and its quantification (n = 3 biological replicates). (E) Representative immunofluorescence images showing localization of Chol-BI1-FITC, Chol-BI1-scramble-FITC, and endosome marker EEA1 in HEK293 cell. (F) Representative immunofluorescence images showing localization of Chol-BI1-FITC, Chol-BI1-scramble-FITC, and BACE1 in HEK293 cell. *p < 0.05; **p < 0.01.

bind BACE1 on the cell surface and within endosome-like environment, were enriched.

To eliminate DNA aptamers with non-specific binding to cell surface, HEK293-BACE1 knockdown stable cell lines were established for negative selection. Two different short hairpin RNAs (shRNAs) targeting human BACE1 mRNA sequence were used to avoid off-target effect. Western blot with BACE1 antibody confirmed that, in BACE1 shRNA-1 and BACE1 shRNA-2 stable cell lines, BACE1 expression was almost completely inhibited (Figure 1E). BACE1 shRNA cells were incubated with the bound ssDNA pool. Non-specific aptamers were absorbed by BACE1 shRNA cells, and specific aptamers remained in the medium and were used for the next round of positive selection.

We monitored the enrichment of the selection pools by flow cytometry in binding assay. The binding of ssDNA pool at the 17th round improved compared to that at the 15th round, but no improvement was seen at the 19th round (Figure 1F). Thus, we chose the ssDNA pool at the 17th round for clone sequencing. After 17 rounds of selection, bound ssDNAs were amplified by PCR, cloned into plasmid, and transformed into *E. coli*. A total of 70 colonies of transformed cells were sequenced. Two sequences designed as BI1 and BI2 (which stand for BACE1 inhibitor) were highly enriched, and their sequences and frequencies were shown in Figure 1G. In the following experiments, BI1 and BI2 were used as DNA aptamers targeting BACE1 and their scrambles were used as controls (BI1 scramble: 5′-GTGGCTCGGACGGG TTCGCATGGGAGGCAGACGGGTACAT-3′; BI2 scramble: 5′-GCAAGATTGGAGGAATT TATCAGGGACTCTGGCGTCTATA-3′).

DNA Aptamers Interact with BACE1

The structures of aptamers BI1 and BI2 were predicted by Mfold (Figure 2A).[26] Previous study demonstrates that linking a sterol moiety to a small-molecule β-secretase inhibitor greatly improves its inhibitory effect by anchoring the inhibitor to cell membrane and promoting its co-localization with BACE1 in lipid rafts.[27] Thus, BI1 and BI2 were also modified by 5′-cholesteryl TEG moiety to improve their potential potency (Figure 2B).

To demonstrate that BI1 and BI2 interact with BACE1, we labeled random library, free BI1, cholesteryl-BI1 (Chol-BI1) and cholesteryl-BI1-scramble (Chol-BI1-scramble), free BI2, cholesteryl-BI2 (Chol-BI2), and cholesteryl-BI2-scramble (Chol-BI2-scramble) with 3′-biotin moiety. They were used to incubate cell lysate of HEK293-wild-type BACE1 stable cell at the final concentration of 200 nM for 4 h. Then, the lysates were precipitated by streptavidin bead,

and BACE1 level was detected by western blot using FLAG antibody (Figure 2C). In this pull-down assay, free BI1 precipitated more BACE1 than free BI2 did, and cholesteryl modification of BI1 and BI2 increased the amount of precipitated BACE1. In contrast, random library or scramble controls of Chol-BI1 and Chol-BI2 could not precipitate BACE1. In addition, in a competition assay, biotinylated Chol-BI1 precipitated BACE1 in cell lysate of wild-type BACE1 stable cell in a dose-dependent manner, although Chol-BI1 without biotin label competed to bind BACE1 and reduced the amount of BACE1 precipitated by biotinylated Chol-BI1 dose dependently (Figure 2D).

To support the interaction between Chol-BI1 and BACE1, we performed immunofluorescence to evaluate the localization of Chol-BI1 with 3′-fluorescein isothiocyanate (FITC) label. HEK293 cells were incubated with Chol-BI1-FITC (500 nM; 12 h) in DMEM and then washed, fixed, permeabilized, and stained with antibody against endosome marker EEA1. The results show a co-localization between Chol-BI1 and endosome marker EEA1 (Figure 2E). In contrast, Chol-BI1-scramble-FITC shows no visible signal. These results support that Chol-BI1 is efficiently internalized into endosomes. We also stained endogenous BACE1 to check the co-localization of Chol-BI1 with BACE1. The results show that the distribution of Chol-BI1 overlaps with BACE1, and Chol-BI1-scramble with 3′-FITC shows no visible signal (Figure 2F).

Taken together, these results suggest that aptamers BI1 and BI2 interact with BACE1 and modification with 5′-cholesteryl TEG moiety further increases their interaction with BACE1. In addition, the co-localizations of Chol-BI1 with endosome marker and BACE1 support that DNA aptamers attached to BCAE1 on the cell surface could be efficiently internalized into endosomes.

DNA Aptamers Inhibit BACE1 Activity

As BI1 and BI2 interact with BACE1, we try to explore whether they have any effect on BACE1 activity. In the *in vitro* FRET assay for β-secretase, the substrate is an internally quenched fluorogenic peptide and the hydrolysis of the substrate by β-secretase activity results in fluorescence enhancement. HEK293 cell lysate was incubated with fluorogenic substrate alone or in the presence of random library (200 nM), free BI1 (200 nM), Chol-BI1 (200 nM), Chol-BI1-scramble (200 nM), free BI2 (200 nM), Chol-BI2 (200 nM), Chol-BI2-scramble (200 nM), or β-secretase inhibitor IV (200 nM) for 4 h. The results showed that free BI1 and BI2 inhibited β-secretase activity and the inhibitory effects of Chol-BI1 and Chol-BI2 were more potent (Figure 3A). In contrast, random library, Chol-BI1-scramble, or Chol-BI2-scramble had no effect on β-secretase activity. In the same assay, Chol-BI1 inhibited β-secretase activity in a dose-dependent manner and Chol-BI1-scramble had no such effect (Figure 3B).

When APP is cleaved by β-secretase, the extracellular domain of APP, sAPP-β, is released into culture medium. Thus, sAPP-β level in culture medium is a marker for β-secretase activity. HEK293-APPsw stable cell was incubated with random library (500 nM), free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), free BI2 (500 nM), Chol-BI2 (500 nM), Chol-BI2-scramble (500 nM), or β-secretase inhibitor IV (1 μM) in DMEM for 12 h. Western blot showed that free BI1 and BI2 reduced sAPP-β level in culture medium and the inhibitory effects of Chol-BI1 and Chol-BI2 were more potent (Figure 3C). In contrast, random library, Chol-BI1-scramble, or Chol-BI2-scramble had no effect on β-secretase activity. In the same assay, Chol-BI1 reduced sAPP-β level in culture medium dose dependently and Chol-BI1-scramble had no such effect (Figure 3D).

Taken together, these results suggest that aptamers BI1 and BI2 inhibit BACE1 activity, and modification with 5′-cholesteryl TEG moiety further increases their inhibitory potency on BACE1 activity.

DNA Aptamers Have No Effect on of α- or γ-Secretase Activities

As drug safety remains a great challenge for the development of AD therapy and potential off target of DNA aptamers may result in serious side effects, we measured the potential effects of aptamers BI1 and BI2 on α- or γ-secretase. In the fluorogenic substrate assay for α-secretase, HEK293 cell lysate was incubated with fluorogenic substrate alone or in the presence of random library (200 nM), free BI1 (200 nM), Chol-BI1 (200 nM), Chol-BI1-scramble (200 nM), free BI2 (200 nM), Chol-BI2 (200 nM), Chol-BI2-scramble (200 nM), or α-secretase inhibitor GI254023X (100 nM) for 4 h. The results showed that none of them had effect on α-secretase activity except for the potent inhibitor GI254023X (Figure 4A).

When APP is cleaved by α-secretase, the extracellular domain of APP, sAPP-α, is released into culture medium. Thus, sAPP-α level in culture medium is also a marker for α-secretase activity. HEK293-APPsw stable cell was incubated with random library (500 nM), free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), free BI2 (500 nM), Chol-BI2 (500 nM), Chol-BI2-scramble (500 nM), or α-secretase inhibitor GI254023X (1 μM) in DMEM for 12 h. Western blot showed that none of them had effect on sAPP-α except for the potent inhibitor GI254023X (Figure 4B).

Similarly, in the fluorogenic substrate assay for γ-secretase, HEK293 cell lysate was incubated with fluorogenic substrate alone or in the presence of random library (200 nM), free BI1 (200 nM), Chol-BI1 (200 nM), Chol-BI1-scramble (200 nM), free BI2 (200 nM), Chol-BI2 (200 nM), Chol-BI2-scramble (200 nM), or γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)-S-phenylglycine t-butyl ester (DAPT; 1 μM) for 4 h. The results showed that none of them had effect on γ-secretase activity except for the potent inhibitor DAPT (Figure 4C).

C99 is derived from β-secretase cleavage of APP and therefore is the direct substrate for γ-secretase. We established HEK293 stable cell line overexpressing C99, and γ-secretase cleavage of C99 would release Aβ into culture medium. HEK293-C99 stable cell was incubated with random library (500 nM), free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), free BI2 (500 nM), Chol-BI2 (500 nM), Chol-BI2-scramble (500 nM), or γ-secretase inhibitor

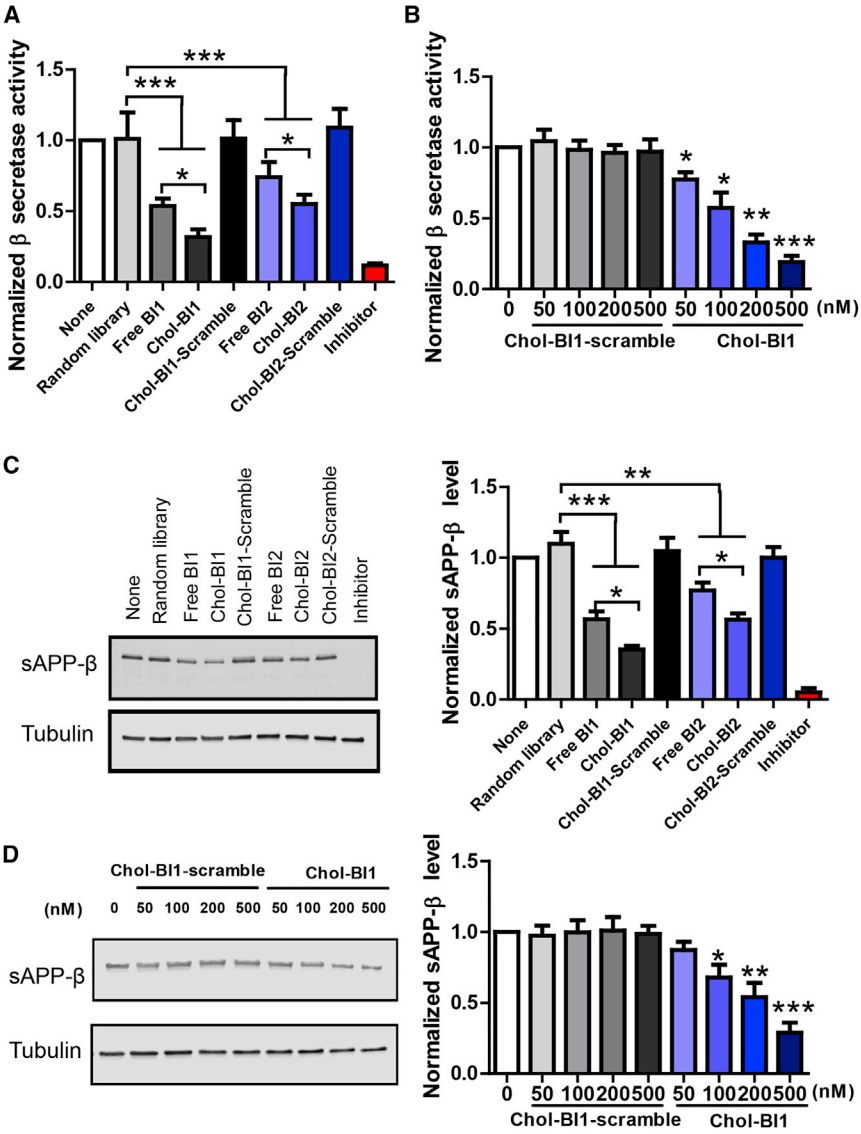

Figure 3. DNA Aptamers Inhibit BACE1 Activity

(A) Normalized β-secretase activity under indicated treatments in the *in vitro* FRET assay (n = 5 biological replicates). (B) Normalized β-secretase activity in the presence of Chol-BI1 or Chol-BI1-scramble at indicated concentrations in the *in vitro* FRET assay (n = 5 biological replicates). (C) Representative western blot and its quantification showing the sAPP-β levels in the culture medium of HEK293-APP stable cell under indicated treatments (n = 5 biological replicates). (D) Representative western blot and its quantification showing the sAPP-β levels in the culture medium of HEK293-APP stable cell in the presence of Chol-BI1 or Chol-BI1-scramble at indicated concentrations (n = 5 biological replicates). For all, difference was analyzed by one-way ANOVA with Newman-Keuls post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DAPT (2 μM) in DMEM for 12 h. ELISA quantification of Aβ40 and Aβ42 in culture medium showed that none of them had effect on Aβ except for the potent inhibitor DAPT (Figure 4D).

Taken together, these results suggest that aptamers BI1 and BI2 have no effect on α- or γ-secretase. It supports that aptamers BI1 and BI2 are specific inhibitors for BACE1, the β-secretase.

DNA Aptamers Reduce Aβ Levels in Cultured Cells

To measure the effects of aptamers BI1 and BI2 on Aβ, HEK293-APPsw stable cell was incubated with random library (500 nM), free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), free BI2 (500 nM), Chol-BI2 (500 nM), Chol-BI2-scramble (500 nM), or γ-secretase inhibitor DAPT (2 μM) in DMEM for 12 h. ELISA quantification of Aβ in culture medium showed that free BI1 and BI2 reduced Aβ40 and Aβ42 in culture medium, and the inhibitory effects of Chol-BI1 and Chol-BI2 were more potent (Figure 5A). In contrast, random library, Chol-BI1-scramble, or Chol-BI2-scramble had no effect on Aβ. In the same assay, Chol-BI1 reduced Aβ40 and Aβ42 in culture medium dose dependently and Chol-BI1-scramble had no such effect (Figure 5B).

Moreover, primary cortical neurons from APP-PS1 mouse were also used to test the effects of aptamers BI1 and BI2 on Aβ40 and Aβ42 in culture medium. APP-PS1 primary neurons were incubated with random library (500 nM), free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), free BI2 (500 nM), Chol-BI2 (500 nM), Chol-BI2-scramble (500 nM), or γ-secretase inhibitor DAPT (2 μM) in Neurobasal A for 12 h. Consistently, ELISA quantification of Aβ showed that free BI1 and BI2 reduced Aβ40 and Aβ42 in culture medium and the inhibitory effects of Chol-BI1 and Chol-BI2 were more potent (Figure 5C). In contrast, random library or Chol-BI1-scramble or Chol-BI2-scramble had no effect on Aβ. In the same assay, Chol-BI1 reduced Aβ40 and Aβ42 in culture medium dose dependently, and Chol-BI1-scramble had no such effect (Figure 5D).

Taken together, these results suggest that aptamers BI1 and BI2 reduced Aβ in the medium of cultured cells and modification with 5′-cholesteryl TEG moiety further increases their inhibitory potency on Aβ.

DNA Aptamer Rescues Neuronal Deficiency in APP-PS1 Neurons

As Aβ impairs synapse, BACE1 targeting aptamers may rescue the Aβ-induced neuronal deficiency. To test the potential protective

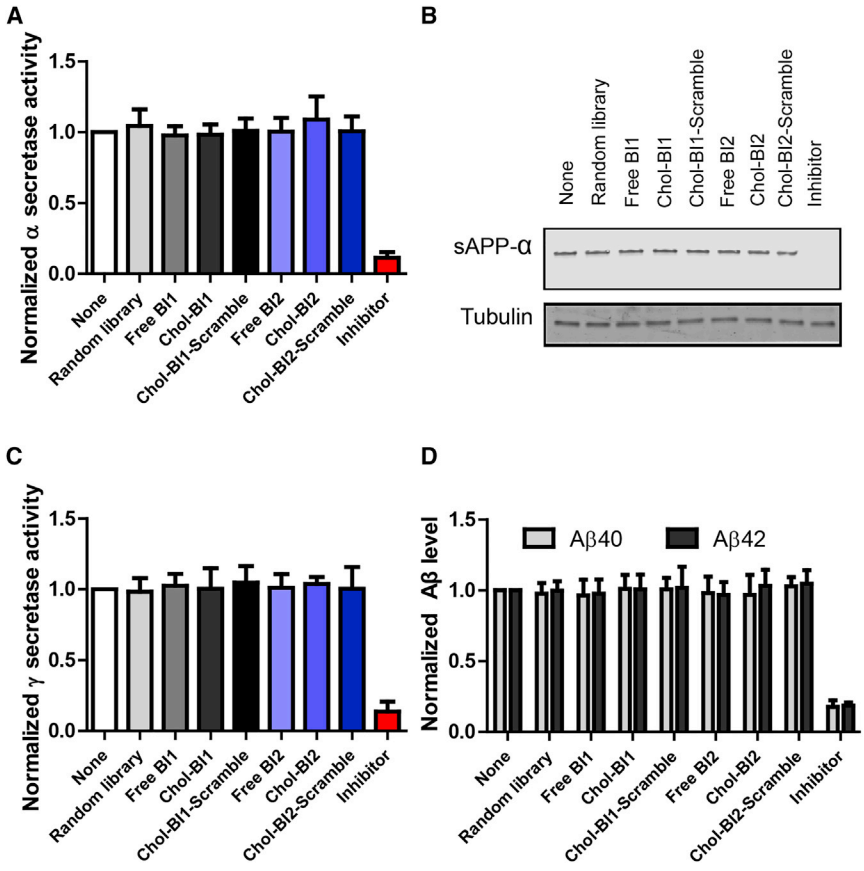

Figure 4. DNA Aptamers Have No Effect on the Activities of α- or γ-Secretase

(A) Normalized α-secretase activity under indicated treatments in the *in vitro* FRET assay (n = 5 biological replicates). (B) Representative western blot showing the sAPP-α levels in the culture medium of HEK293-APP stable cell under indicated treatments. (C) Normalized γ-secretase activity under indicated treatments in the *in vitro* FRET assay (n = 5 biological replicates). (D) ELISA quantification of Aβ in the culture medium of HEK293-C99 stable cell under indicated treatments (n = 5 biological replicates). For all, difference was analyzed by one-way ANOVA with Newman-Keuls post hoc test.

effects of aptamer BI1, APP-PS1 primary neurons were incubated with free BI1 (500 nM), Chol-BI1 (500 nM), Chol-BI1-scramble (500 nM), or γ-secretase inhibitor DAPT (2 μM) in Neurobasal A for 12 h. Their effects on the total length of dendrites and the total number of dendritic tips were evaluated (Figure 6A). The results showed that the total length of dendrites and the total number of dendritic tips of APP-PS1 neurons were dramatically reduced compared to wild-type neurons. Treatment with γ-secretase inhibitor DAPT could rescue the dendritic deficiency, suggesting that the dendritic deficiency was induced by high Aβ. Similarly, free BI1 could partially rescue the dendritic deficiency of APP-PS1 neurons, and the protective effect of Chol-BI1 was more potent (Figures 6B and 6C). In contrast, Chol-BI1-scramble had no such effect.

In addition, the effects of aptamer BI1 on synaptic proteins, including PSD-95, Synapsin-1, GluR1, and synaptophysin, were measured by western blot (Figure 6D). The results showed that PSD-95, synaptophysin, and GluR1 in APP-PS1 neurons were dramatically reduced compared to wild-type neurons. Treatment with γ-secretase inhibitor DAPT could rescue the levels of these synaptic proteins, suggesting that the compromised expression of synaptic proteins resulted from high Aβ. Similarly, free BI1 increased the protein levels of PSD-95, synaptophysin, and GluR1 in APP-PS1 neurons, and the protective effect of Chol-BI1 was more potent. In contrast, Chol-BI1-scramble had no such effect.

Taken together, these results suggest that aptamer BI1 is protective against Aβ-induced neuronal deficiency in APP-PS1 neurons and modification with 5′-cholesteryl TEG moiety further increases its protective effects.

DISCUSSION

BACE1 has been proposed as an important therapeutic target for AD since the discovery that BACE1 is indispensable for Aβ production.[10] BACE1 also appears to be one of the few remaining targets for AD treatment after the continuous failures of clinical trials for γ-secretase inhibitors and Aβ immunotherapy. Thus, great efforts have been made to target BACE1 through the strategy of small-molecule inhibitor[28] or antibody.[29] Here, our study represents an alternative strategy to target BACE1 to modulate Aβ for the treatment of AD. We performed cell-SELEX to identify specific DNA aptamer targeting BACE1 and confirmed its inhibitory effect on Aβ and its protective effect on Aβ-induced neuronal deficiency. Compared to conventional small-molecule inhibitor or antibody, DNA aptamers have numerous unique features. First, aptamers could achieve comparable binding affinity and specificity to antibody within a short time. Second, aptamers could be produced by cost-effective chemical synthesis and modification. Third, DNA aptamers could be stored and transported under room temperature. The great potential of aptamers has been translated into U.S. Food and Drug Administration (FDA)-approved drugs like Macugen for patients with age-related macular degeneration, and numerous clinical trials for aptamers are ongoing.[30] Encouraged by these clinical translations, aptamers against potential therapeutic targets, such as α-synuclein in Parkinson's disease[31] and amyloid[32] and Tau[33] in AD, are also developed. These efforts suggest that DNA aptamer is an attractive therapeutic strategy alternative to conventional small-molecule compound or antibody.

We have noticed that two previous studies report aptamers targeting recombinant domains of BACE1.[34,35] The first study develops RNA

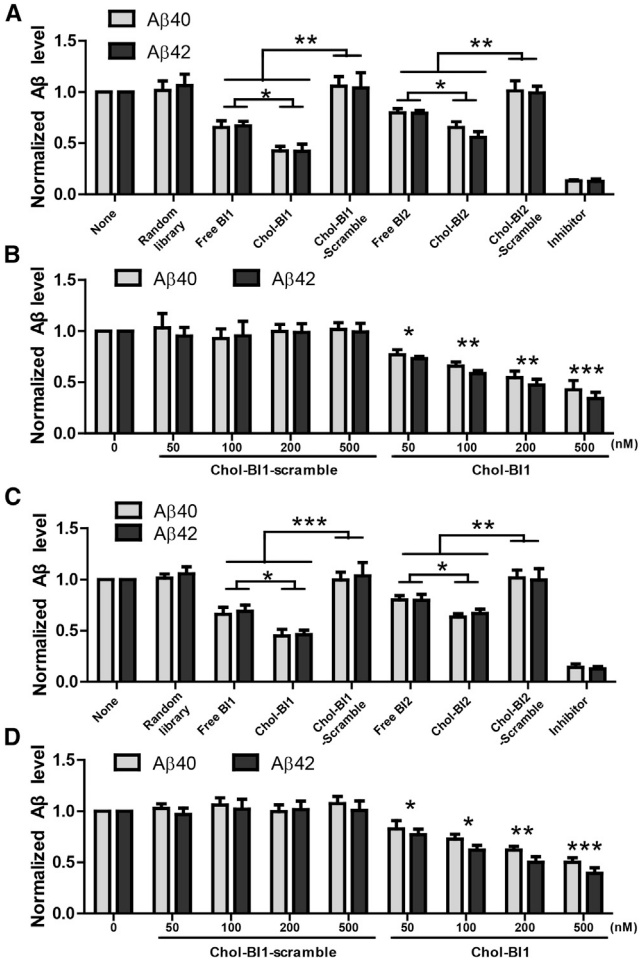

Figure 5. DNA Aptamers Reduced Aβ Levels in Cultured Cells

(A) ELISA quantification of Aβ in the culture medium of HEK293-APP stable cell under indicated treatments (n = 5 biological replicates). (B) ELISA quantification of Aβ in the culture medium of HEK293-APP in the presence of Chol-BI1 or Chol-BI1-scramble at indicated concentrations (n = 5 biological replicates). (C) ELISA quantification of Aβ in the culture medium of APP-PS1 primary cultured neurons under indicated treatments (n = 5 biological replicates). (D) ELISA quantification of Aβ in the culture medium of APP-PS1 primary cultured neurons in the presence of Chol-BI1 or Chol-BI1-scramble at indicated concentrations (n = 5 biological replicates). For all, difference was analyzed by one-way ANOVA with Newman-Keuls post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

aptamer targeting the recombinant intracellular domain of BACE1, but its potential effect on BACE1 activity or Aβ production is unknown.[34] The second study develops DNA aptamer targeting the recombinant extracellular domain of BACE1 and evaluates its effects on BACE1 activity and Aβ production.[35] Together with our study, these reports support that it is feasible and effective to target BACE1 using aptamers. However, our cell-SELEX-based study has significant advantages in the following aspects. First, BACE1 has diverse post-translational modifications, such as phosphorylation, glycosylation, and palmitoylation.[36] These modifications are essential for the structure and function of BACE1. In addition, it seems that BACE1 forms dimer to cleave APP.[37,38] Thus, screening aptamers against recombinant domains of BACE1 would certainly miss the critical modifications and dimerization of BACE1 in physiological status. Instead, our study uses a novel cell-SELEX procedure, in which a TEV site was inserted between the catalytic domain and loop of BACE1, so the ssDNAs captured by matured BACE1 on the cell surface could be enriched. Second, it seems that these two studies, as well as many other studies, do not take into account the acidic environment required for β-secretase activity, so the aptamers or inhibitors targeting BACE1 under normal pH may fail to engage their target under acidic environment, such as endosomes. In our cell-SELEX process, the medium containing cleaved BACE1-bound ssDNA complex was adjusted to acidic pH to mimic the acidic environment of endosome. Thus, the DNA aptamers derived from our cell-SELEX are supposed to bind BACE1 on the cell surface as well as in the endosome-like environment. Third, the identified aptamers are further improved by cholesteryl TEG modification.[39] The improvement in the potency of cholesteryl TEG-aptamer is fully consistent with previous study.[27] Through the above combinational strategies, we identified DNA aptamers optimized to target BACE1 under physiological status.

We also notice the relatively low diversity of identified aptamers that only two unique sequences were enriched after 17 round selections. However, the low diversity is unlikely due to excessive rounds of selection. In fact, it appears common to see that only 1 or 2 dominant aptamers are identified in previous studies.[33,35] Those two studies performed 7 and 12 rounds of selection, respectively. It looks like we performed more rounds of selection (15 rounds of positive selection and 2 rounds of negative selections), but it is important to note that those studies were based on *in vitro* SELEX with recombinant protein domains from *E. coli* so that the target proteins are homogeneous at high concentrations. Instead, in our cell-SELEX process, the target protein is heterogeneous (due to various modifications) at low concentration and at natural state. So our cell-SELEX takes more rounds of selection to identify aptamers. In addition, we monitored the enrichment of the selection pools by flow cytometry. Thus, we believe that these two aptamers were enriched because of their high affinity to the target protein.

There are some limitations in our study. First, our cell-SELEX may miss certain cell-internalizing aptamers, as we only captured the aptamers bound to BACE1 on the cell surface. But it would be very difficult to isolate the endosomes and the BACE1-binding aptamers therein. In addition, aptamers binding to BACE1 on the cell surface are not necessarily excluded from internalization. In fact, once synthesized in endoplasmic reticulum (ER), BACE1 will traffic to the cell surface along the secretory pathway and then internalize into endosomes. As there is a dynamic flow of BACE1 between cell surface and endosomes, it is very likely that the aptamers attached to BCAE1 on the cell surface will also be internalized into endosomes along with BACE1, but we can only capture them at the moment when they are on the cell surface. Indeed, co-localizations of aptamer BI1 with endosome marker and endogenous BACE1 support that

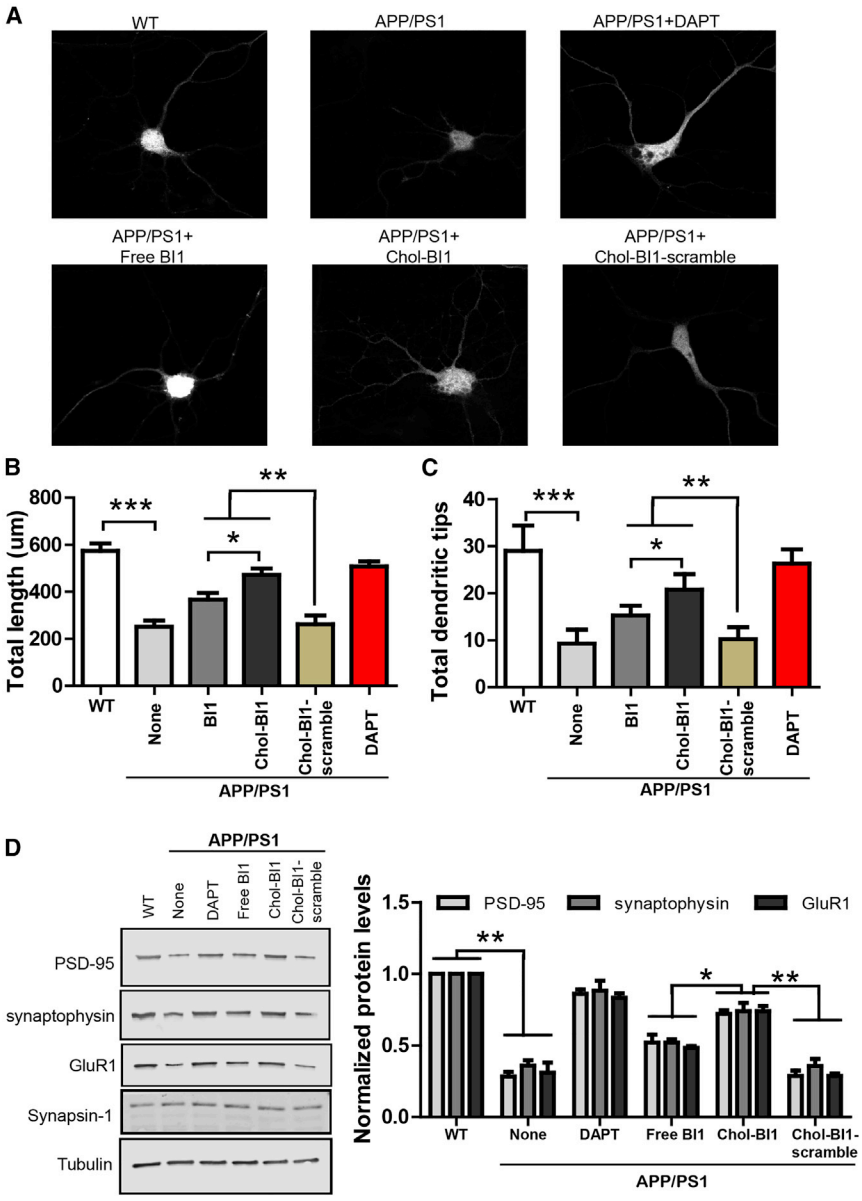

Figure 6. DNA Aptamer Rescues Neuronal Deficiency in APP-PS1 Neurons

(A) Representative images of APP-PS1 primary cultured neurons under indicated treatments. (B) Quantification of total length of APP-PS1 neurons in (A) (n = 20 biological replicates). (C) Quantification of total dendritic tips of APP-PS1 neurons in (A) (n = 20 biological replicates). (D) Representative western blot and its quantification showing the levels of indicated synaptic proteins in APP-PS1 neurons under indicated treatments (n = 5 biological replicates). For all, difference was analyzed by one-way ANOVA with Newman-Keuls post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DNA aptamers attached to BCAE1 on the cell surface could be efficiently internalized into endosomes. So our cell-SELEX strategy represents a significant advance compared to previous SELEX in *in vitro* assays with recombinant protein domains from *E. coli*.

The second limitation of our study is that the effects of BACE1 targeting aptamers remain to be tested in AD mouse model. The ssDNA may induce immune response, but it could be minimized by further modification and engineering.[40] In fact, several DNA-aptamer-based therapies have entered clinical trials and passed phase I trial.[41,42] It suggests that DNA-aptamer-based therapies could be as safe as conventional small-molecule drugs or antibodies. It would be a great challenge to deliver aptamers into brain, and some encouraging progresses have been made in the brain-targeting strategy. For example, systemic injection of exosomes could deliver small interfering RNA (siRNA) to mouse brain,[43] and intranasal delivery also could target therapeutic agents into the CNS.[44,45] Thus, we might have non-invasive technologies to deliver DNA aptamers to brain in the near future. In addition, extensive optimization and modification may be required to improve its pharmacokinetics, such as half-life, in future study to evaluate its *in vivo* therapeutic efficacy.

In summary, using a novel cell-SELEX strategy, we identify DNA aptamer optimized to target BACE1 under physiological status. Its inhibitory effect on BACE1 activity and Aβ production, and its protective effect on Aβ-induced neuronal deficiency, are validated in various *in vitro* and cellular assays. The potential of BACE1-targeting aptamer as an intervention for AD deserves further investigation.

MATERIALS AND METHODS

Cell Cultures

The coding sequence of human BACE1 (NM_012104.4) tagged with C-terminal FLAG was cloned into pLenti-CMV-GFP-Puro vector, and it was used to generate HEK293-wild-type BACE1 stable cell line. The coding sequences of human APP (NM_201414.2) with Swedish mutation and C99 tagged with C-terminal FLAG were cloned into pLenti-CMV-GFP-Puro vector, respectively, and they were used to generate HEK293-APPsw and HEK293-C99 stable cell lines, respectively.

To generate BACE1-TEV fusion from wild-type BACE1 coding sequence, FLAG tag was inserted between the pro-peptide domain and catalytic domain, and TEV cleavage site was inserted between the catalytic domain and loop. The BACE1-TEV fusion was cloned into pLenti-CMV-GFP-Puro vector, and it was used to generate HEK293-BACE1-TEV stable cell line.

To generate BACE1 knockdown cell lines, two different shRNAs targeting human BACE1 mRNA sequence and a scrambled control were constructed into the pLentiLox3.7 (pLL3.7) lentiviral vector, and their sequences were as follows: BACE1 shRNA-1: 5′-TG CAACGTCACTGTGCGTGCCAACATTTTCAAGAGAAATGTTG GCACGCACAGTGACGTTGCTTTTTTC-3′ (forward), 5′-TC GAGAAAAAAGCAACGTCACTGTGCGTGCCAACATTTC TC TTGAAAATGTTGGCACGCACAGTGACGTTGCA-3′ (reverse); BACE1 shRNA-2: 5′-TGCGGGCACTGTTATGGGAGCTGTT ATTTCAAGAGAATAACAGCTCCCATAACAGTGCCCGCTTTT TTC-3′ (forward), 5′-TCGAGAAAAAAGCGCCTGGAGATTAG CAAGACCATTA TCTCTTGAAATAACAGCTCCCATAACAGT GCCCGCA-3′ (reverse); scramble shRNA: 5′-TGCAAACTGTCC GTGTGACCCACGATTTTCAAGAGAAATCGTGGGTCACACG GACAGTTTGCTTTTTTC-3′ (forward), 5′-TCGAGAAAAAAG CAAACTGTCCGTGTGACCCACGATTTC TCTTGAAAATCGT GGGTCACACGGACAGTTTGCA-3′ (reverse). Target sequences were underlined.

All above lentiviruses were packaged and amplified in HEK293T cells. HEK293 cell line was infected at a MOI of 5 and maintained in DMEM with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO2 at 37°C.

APP-PS1 mice were from Jackson Laboratory (no. 004462), and all animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Zhongshan Hospital, Shanghai, China. Primary cortical neurons of APP-PS1 transgenic mouse were dissected from embryonic day 17 (E17) brains and cultured in Neurobasal A. Neurons at 12 days *in vitro* (DIV12) were used for experiments.

Cell-SELEX

The cell-SELEX strategy was modified from previous report.[24] In brief, random ssDNA library (1 nmol) in 1-mL binding buffer (4.5 g glucose, 100 mg tRNA, 50 mg of salmon sperm DNA, 1 g BSA, and 5 mL of 1 M MgCl21 in 1 L of Dulbecco's phosphate buffered saline [DPBS]) was heated at 95°C for 5 min, snap cooled on ice, pre-cleared by FLAG-coated bead, and subjected to 5 cycles of positive selection in HEK293-BACE1-TEV stable cell, 1 cycle of negative selection in BACE1 shRNA-1 stable cell, 5 cycles of positive selection in HEK293-BACE1-TEV stable cell, 1 cycle of negative selection in BACE1 shRNA-2 stable cell, and 5 cycles of positive selection in HEK293- BACE1-TEV stable cell.

For positive selection, HEK293-BACE1-TEV fusion stable cell was plated in a 10-cm dish and cultured for 48 h. Cells were washed with PBS and incubated with random library in 1-mL binding buffer with gentle rotation for 1 h at 37°C. After washing with PBS, cells were incubated with 50 μg/mL TEV nuclear inclusion endopeptidase (TEV protease) in PBS for 30 min at 30°C. The medium was collected, centrifuged, and adjusted to pH ≈ 4.5. The acidified medium was incubated for 30 min at 37°C and then immunoprecipitated with FLAG-antibody-coated bead. The bound ssDNA pool was eluted and amplified.

For the negative selection, BACE1 shRNA stable cell was plated in 10-cm dish and cultured for 48 h. Cells were washed with PBS and incubated with bound ssDNA pool in binding buffer with gentle rotation for 1 h at 37°C. The medium was collected, centrifuged, and used for the next positive selection.

In each round of selection, the concentration of ssDNA pool was maintained at 1 μM. After PCR amplification, the sense ssDNA was separated from biotinylated antisense ssDNA by alkaline denaturation and affinity purification with streptavidin beads. The enrichment of the selection pools is monitored by flow cytometry in binding assay. Phycoerythrin (PE)-labeled ssDNA pools at 15^{th}, 17^{th}, and 19^{th} rounds in 200 μL of binding buffer were incubated with 3×10^5 target cells at 37°C for 2 h. The PE-labeled initial library (0 rounds) was used as a negative control. After incubation, cells were washed, resuspended in 500 μL of binding buffer, and analyzed with a FACSVerse flow cytometer (BD Biosciences, USA). After 17 cycles of selection, the PCR product was cloned into pGEM-T Vector (Promega). A total of 70 colonies of transformed cells were sequenced.

DNA Aptamer Synthesis and Modification

The synthetic ssDNA library consists of a random sequence of 40 nt in the middle ($\sim 10^{14}$ DNA molecules) and two flanked primer hybridization sites (5′-ATCCAGAGTGACGCAGCA-N40- TGGA CACGGTGGCTTAGT-3′). The random library, aptamers BI1 and BI2, their scramble controls, and labeled aptamers with 3′-biotin, 3′-FITC, or 5′-cholesteryl TEG modification were synthesized by Shanghai Sangon biotech and purified by reverse phase high-performance liquid chromatography (HPLC).

Pull-Down Assay with DNA Aptamer

Wild-type (WT)-BACE1 stable cell line was lyzed with radio-immunoprecipitation assay (RIPA) buffer containing 1% (v/v) complete protease inhibitor cocktail, and lysates were centrifuged. The supernatant was collected, and protein concentration was determined by BCA method. Dynabeads M-280 streptavidin was incubated with biotinylated aptamers for 30 min at 37°C and washed with phosphate buffered saline Tween-20 (PBST). The bead-aptamer mixtures were incubated with cell lysates for 4 h at 4°C. The mixtures were centrifuged, washed, and heated for 15 min at 100°C in loading buffer. The samples were analyzed via western blot with FLAG antibody.

In Vitro FRET Assays for Secretase Activity

The secretase substrates are short peptides with a fluorescent donor on one end and a quenching acceptor group on the other end. As a result of intramolecular energy transfer to the quenching acceptor, fluorescence from the donor is quenched. Once substrate is cleaved by corresponding secretase, the energy transfer is disturbed and fluorescent signal is enhanced. Cells were homogenized and centrifuged. Secretases in the pellet were re-solubilized, and 20 μg fraction was used to incubate fluorogenic substrate in each reaction. The

α-secretase activity was measured in 100 mM sodium acetate (pH 7.0) with 2 mg fluorogenic substrate (Calbiochem; 565767). The β-secretase activity was measured in 100 mM sodium acetate (pH 4.5) with 2 mg fluorogenic substrate (Calbiochem; 565758). The γ-secretase activity was measured in 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, and 0.25% CHAPSO (w/v) with 2 mg fluorogenic substrate (Calbiochem; 565764). After incubation at 37°C for 4 h, the fluorescence intensities were measured with an excitation wavelength at 340 nm and an emission wavelength at 490 nm for α-secretase, 350 and 490 nm for β-secretase, and 355 and 440 nm for γ-secretase, respectively.

Western Blot

Proteins were extracted from cultured cells or culture medium using SDS lysis buffer (2% SDS, 10% glycerol, 0.1 mM DTT, and 0.2 M Tris-HCl [pH 6.8]). Protein samples were resolved by SDS-PAGE and detected with indicated antibodies. The protein bands were quantified by densitometry analysis using ImageJ software, and the intensity of each target protein was normalized by tubulin intensity.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 1 h at room temperature, and fixed cells were permeabilized and blocked with PBS containing 5% BSA and 0.3% Triton X-100 for 1 h at room temperature. Then, cells were incubated overnight at 4°C with indicated antibodies. The secondary antibody was applied for 1 h at room temperature followed by three washes with PBS. Images were obtained using Nikon confocal microscope (Nikon, Japan).

Quantification of Neuronal Morphology

To visualize the neuronal morphology, GFP construct transfection was performed using the Nucleofector Device II and the Rat Neuron Nucleofector Kit (Amaxa). Briefly, dissociated cortical neurons from E17 mouse brain were mixed with 100 μL Neuron Nucleofector Solution containing 2 μg of GFP construct. The mixture was transferred to the cuvette, and program O-003 was used. Neuronal tracing was performed on neurons for which the shortest dendrite was at least three times longer than the cell soma diameter using ImageJ. The total dendritic length includes the summed length of all dendrites per neuron.

Antibodies and Drugs

The following antibodies were used: BACE1 (MAB5308; Chemicon); FLAG (clone M2; Sigma); sAPP-α (SIG-39139; Covance); sAPP-β (SIG-39138; Covance); PSD-95 (MABN68; Merck Millipore); Synapsin-1 (S193; Sigma); GluR1 (04-855; Merck Millipore); and synaptophysin (MAB5258; Merck Millipore). The α-secretase inhibitor GI254023X was from Sigma (SML0789). The β-secretase inhibitor IV was from Calbiochem (565788). The γ-secretase inhibitor DAPT was from Calbiochem (565770). TEV was from Invitrogen (10127-017).

Aβ ELISA

For the measurement of Aβ40 and Aβ42, the culture medium were collected in polypropylene tubes and stored at −80°C. Aβ40 and Aβ42 were quantified by Aβ40 Human ELISA Kit (Invitrogen; KHB3482) and Aβ42 Human Ultrasensitive ELISA Kit (Invitrogen; KHB3544) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. All data were presented as mean ± SD, and statistical analysis was performed by two-tailed Student's t test for two groups and one-way ANOVA with Newman-Keuls post hoc test for more than two groups. Statistically significant differences were defined as $p < 0.05$. For all, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

AUTHOR CONTRIBUTIONS

J.X., W. Zhang, and X.-F.C. performed the experiments, carried out the statistical analysis, and prepared the manuscript; they contributed equally to this work. M.C., Z.-H.Y., F.Y., W. Zhu, X.-T.L., and T.W. were involved in experiment performance and data collection. J.X., J.-S.Z., and D.-F.C. were responsible for the supervision of the entire project and were involved in the study design, data interpretation, manuscript preparation, and funding. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that they have no competing interests.

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