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Article

Probing the therapeutic potential of TRPC6 for Alzheimer's disease in live neurons from patient-specific iPSCs

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The induced pluripotent stem cells (iPSCs) offer an unprecedented opportunity to model and study Alzheimer's disease (AD) under patient-specific genetic background. The lower expression of transient receptor potential canonical 6 (TRPC6) was associated with AD patients, which might be involved in AD pathogenesis. However, the role of TRPC6 that played in AD process still needs more investigation in patient-relevant neurons. In this study, the iPSCs were generated from peripheral blood cells of sporadic AD patients and efficiently differentiated into mature cortical neurons. These sporadic AD-bearing neurons displayed higher levels of AD pathological markers A and phospho-tau, but lower levels of TRPC6, than those of control neurons. Treatment of AD neurons with TRPC6 protein fragment or agonist inhibited the elevation of A and phospho-tau. Our results in live AD neurons manifest that the compromised expression of TRPC6 substantially contributed to A pathology of sporadic AD, suggesting that targeting TRPC6 could help to develop novel therapeutic strategies for the treatments of AD.

Keywords: transient receptor potential canonical 6 (TRPC6), Alzheimer's disease, patient-specific induced pluripotent stem cells (iPSCs), cellular models, amyloid-beta (Aβ)

Introduction

Alzheimer's disease (AD) is a progressive debilitating neurodegenerative disorder pathologically characterized by accumulation of amyloid-β peptide (Aβ), aggregation of hyperphosphorylated tau, and neuronal loss in the brain. The molecular mechanisms underlying pathogenesis of AD are complicated and needed more comprehensive investigation. In the past, animal models expressing human genes containing mutations associated with familial AD have been widely used in elucidating the etiology of AD. The majority of AD cases are sporadic and lack of an appropriate model system. Recently, the derivation of induced pluripotent stem cells (iPSCs) from somatic cells of AD patients and neural differentiation of iPSCs offer a new strategy to model AD-relevant phenotypes in live neurons with genetically identical background of individual patient (Marchetto et al., 2011). The patient iPSC-derived neurons were demonstrated to display canonical neuropathological features of AD (Israel et al., 2012; Kondo et al., 2013; Young et al., 2015). Then, the key challenges in the field are to explore the possible application of the resulting iPSC-based cellular models in uncovering the molecular mechanisms of AD pathogenesis and in subsequently

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developing new treatment strategies for AD, such as identifying or validating novel therapeutic target genes or drugs that are safe and effective for AD patients.

Transient receptor potential canonical 6 (TRPC6) is an important Ca^{2+}-permeable non-selective cation channel protein (Montell et al., 2002) and plays critical roles in brain development (Tai et al., 2009), neuronal survival (Jia et al., 2007), and synaptic formation (Zhou et al., 2008). Disruption of TRPC6 expression was reported to affect the development and function of neurons and may be involved in the etiology of autism spectrum disorders (Griesi-Oliveira et al., 2015). Previously, we found that TRPC6 specifically interacted with amyloid precursor protein (APP) via the second transmembrane domain (TM2), modulated the γ-secretase cleavage of APP, and reduced Aβ production (Wang et al., 2015). Moreover, the overexpression of TRPC6 led to the reduction of Aβ burden in the AD mice brain and improved the learning abilities of AD mice (Wang et al., 2015). The following study revealed the reduction of TRPC6 expression in peripheral blood of sporadic AD patients at different clinical stages (Lu et al., 2018). Based on these observations from AD model mice and cohort studies among AD patients, we have hypothesized that TRPC6 might be involved in the progress of AD by interfering with Aβ generation. Due to the fundamental species-specific differences between rodents and human, the mouse models are usually not faithfully reflective of the clinic conditions of AD patients, thus compromising the fidelity of assessing the therapeutic potential of TRPC6 for AD diagnosis and treatment. Thus, it is required to investigate the role of TRPC6 in AD pathogenesis under the genetic background of patients. However, the expression of TRPC6 in live neurons of AD patients and its correlativity with Aβ production remain largely unexplored. Hyperforin is a drug derived from plant (Singer et al., 1999), which can induce the expression of TRPC6 in short term cultured human skin explants (Muller et al., 2008) and mouse cortex (Gibon et al., 2013). However, it is still unknown whether hyperforin treatment can increase the expression of TRPC6 and then interfere with Aβ generation under genetic background of AD patients.

In this study, we generated multiple lines of sporadic AD patient-specific iPSCs that efficiently differentiated into mature AD neurons. Relative to control neurons, sporadic AD neurons exhibited higher levels of Aβ and phospho-tau but lower TRPC6, while increasing TRPC6 level by TRPC6 peptides or agonist hyperforin caused marked reductions of Aβ and tau in sporadic AD neurons, providing evidences that TRPC6 inhibits Aβ elevation in human AD neurons.

Results

The patient iPSC-derived neurons exhibit AD-related phenotypes

Twelve diagnosed sporadic AD patients and 17 age-matched healthy controls who participated in our previous study (Lu et al., 2018) were recruited and the detailed information was summarized in Supplementary Table S1. Altogether, 145 iPSC lines were generated from the peripheral blood mononuclear cells of these 29 participants using an episomal vector-mediated integration-free approach as previously reported (Chou et al., 2011; Dowey et al., 2012; Su et al., 2013). Immunostaining analysis showed that the iPSC colonies homogeneously expressed pluripotency markers OCT4, TRA-1-60, TRA-1-81 as well as SSEA-4 and acquired pluripotency (Supplementary Figure S1A). PCR analysis on DNAs from multiple iPSCs at early passages did not detect exogenous pluripotent genes, confirming that the iPSCs were integration-free (Supplementary Figure S1B). In addition, we observed that the patient-specific iPSCs displayed normal karyotypes as control iPSCs (Supplementary Figure S1C) and possessed pluripotent capacities to form three germ layers in teratoma assay (Supplementary Figure S1D).

Then, we analyzed the neural differentiation capacity of the generated iPSC lines. We found that differentiation from human iPSCs to neurons was hardly initiated relying on the embryoid body (EB) formation in serum-free culture conditions, which is suitable for human embryonic stem cell (hESC) neural differentiation (Yue et al., 2015; Li et al., 2017). To promote the neural differentiation of human iPSCs, an approach combining the EB-based method with dual-SMAD inhibitors (TGF-β inhibitor SB431542 and BMP inhibitor dorsomorphin) (Chambers et al., 2009) was established (Figure 1A). With the treatment of two inhibitors at the first 6 days, the expression of *OCT4* quickly decreased, while the neuroepithelia markers *OCT6* and *ZIC2* increased and the neural differentiation of iPSCs initiated (Figure 1B). Then the differentiating cells acquired the forebrain identity by expressing anterior forebrain neuroectoderm marker *OTX2* and anterior forebrain progenitor marker *FOXG1* (Figure 1B). Finally, the expression of neural progenitor markers *PAX6* and *SOX1* as well as subsequent neuronal markers *TUJ1* and *MAP2* was indicative of the progressive fate commitment from human iPSCs to neuronal progenitors, then to neurons (Figure 1B). At Day 50, ~90% of cells in iPSC-neural differentiated cultures were TUJ1- or MAP2-positive neurons, showing the high efficiency of our approach (Figure 1C and D). Majority of iPSC-derived $TUJ1^+$ neurons were mature cortical neurons and expressed mature neuronal marker NEUN (75.5%) and cortical marker TBR1 (83.6%) (Figure 1E and F). In addition, $VGLUT^+$ glutamatergic neurons (89.3%) among iPSC-derived cortical neurons were dominant compared with other subtypes, such as $GAD67^+$ GABAergic neurons (6.9%), TH^+ dopaminergic neurons (5.1%) and $ChAT^+$ cholinergic neuron (not detected) (Figure 1E and F). Whole-cell patch-clamp recording revealed that most of iPSC-derived neurons fired action potentials (APs) (15/17) at Days 55–65, either repetitive (9/17) or one APs (Figure 1G and H) and some neurons generated spontaneous APs (Figure 1I) or expressed post-synaptic currents (Figure 1J), indicating that iPSC-derived cortical neurons possessed functional membrane properties.

Next, the reprogramming efficiency and neural differentiation capacity between control and AD group were evaluated. First, the efficiency to generate iPSCs from peripheral blood mononuclear cells (PB MNCs) of AD patients vs. controls was comparable (Supplementary Figure S2A). The similar expression pattern

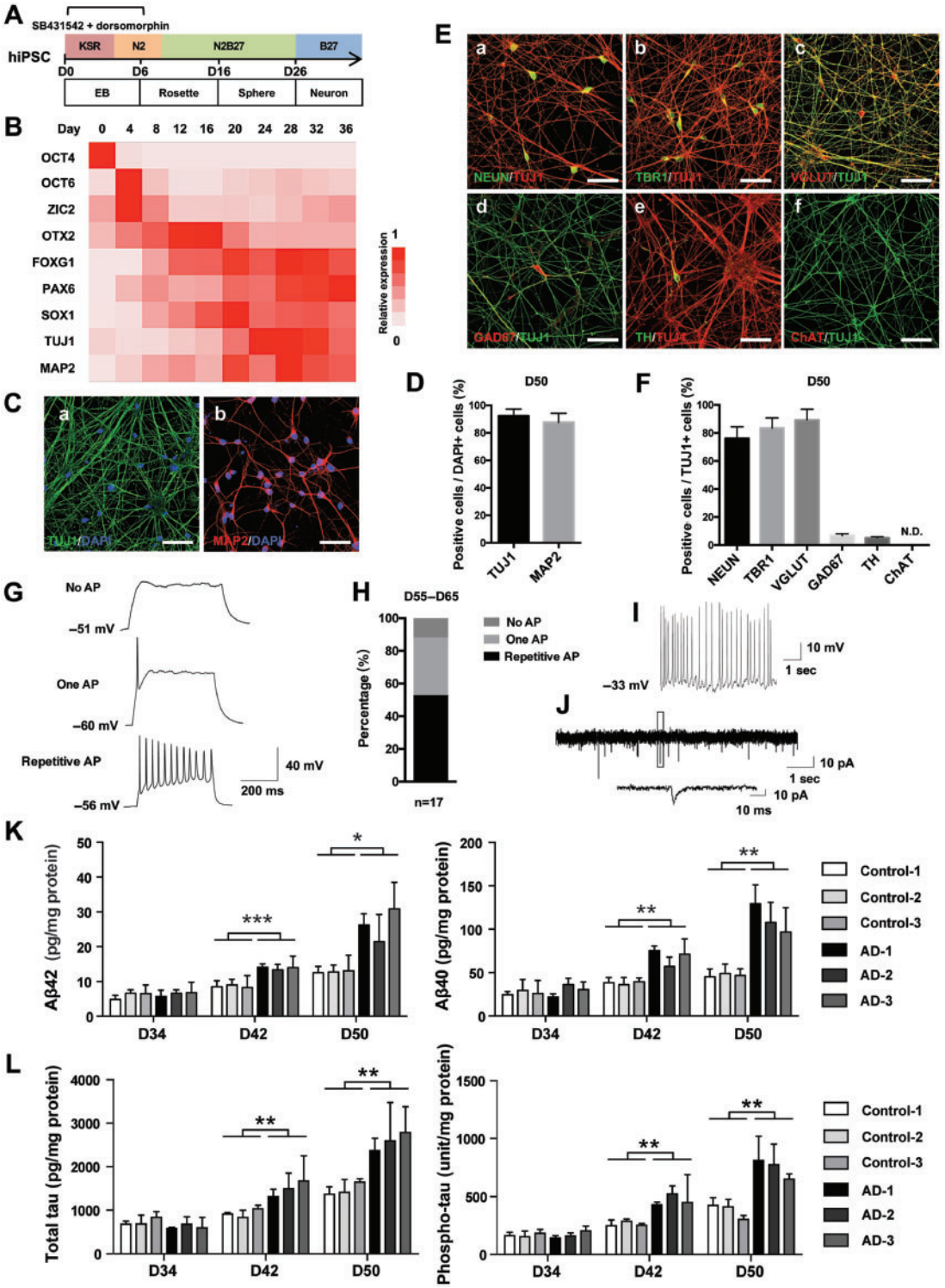

Figure 1 The efficient differentiation from patient-specific iPSCs into neurons displaying pathological features associated with AD. (**A**) Schematic representation of the approach used to direct the differentiation from iPSCs into neurons. (**B**) Gene expression heatmap for marker genes during the neural differentiation of human iPSCs. The value indicates the fold change that is relative to *GAPDH* and normalized to the highest value. Red and white colors represent higher and lower gene expression levels, respectively. (**C**) Immunocytochemistry analysis of the expression levels of neuronal markers TUJ1 (a) and MAP2 (b) in iPSC-derived neurons at Day 50. (**D**) Quantification of the results shown in **C**. $n = 3$. (**E**) Immunocytochemistry analysis of the expression levels of mature neuronal marker NEUN (a), cortical neuronal marker TBR1 (b), glutamatergic neuronal marker VGLUT (c), GABAergic neuronal marker GAD67 (d), dopaminergic neuronal marker TH (e), and

of representative marker genes during neural differentiation measured showed the similar neural differentiation process between AD and control iPSCs (Figure 1B; Supplementary Figure S2B). Consequently, both control and AD iPSC lines showed preference to give rise to cortical neurons with similar efficiency (Figure 1C–F; Supplementary Figure S2C and D). Total protein levels measured at Day 50 were slightly but not significantly lower in AD neurons than those in controls (Supplementary Figure S2E). The levels of apoptosis-associated markers, PARP (a substrate of activated Caspase-3), cleaved PARP, and Caspase-3, were similar between AD and control neurons, indicating that the genetic differences between AD and controls did not entail evident apoptosis in AD-bearing neurons up to Day 50 (Supplementary Figure S2F and G). Thus, from the PB MNCs of AD patients and controls, we generated faithfully reprogrammed iPSC lines with similar capacity and efficiency of neural differentiation, which allowed us to compare the Aβ secretion and tau level between control and AD iPSC-derived neurons in a reliable and consistent way.

To detect the AD-relevant phenotypes in sporadic AD neurons, iPSC lines of three AD patients and three controls were simultaneously subjected to neural differentiation. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the levels of Aβ in medium and tau in cell lysates of neurons derived from each iPSC line at Days 34, 42, and 50, respectively. Both the secreted Aβ (Aβ42 and Aβ40) and intracellular tau (total tau and phospho-tau) increased at a time-dependent manner in AD and control neurons (Figure 1K and L). The levels of Aβ42, Aβ40, total tau, and phospho-tau were similar to controls at Day 34, but became markedly higher than controls from Day 42 in AD neurons and maintained higher thereafter (Figure 1K and L).

Collectively, the sporadic AD patient-specific iPSCs efficiently differentiated into mature cortical glutamatergic neurons that display pathological features associated with human AD at later differentiation days, suggesting that the iPSC-derived sporadic AD neurons could serve as a cellular platform to study the pathogenesis of AD.

Compromised expression of TRPC6 in patient iPSC-derived sporadic AD neurons

Since we have observed that TRPC6 is protective against pathology of AD in mouse model, we next investigate this gene by assessing the expression of TRPC6 and the correlativity between TRPC6 and Aβ in the patient iPSC-based cellular platform. The quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed in the control and AD iPSC lines same as those used for the measurements of Aβ and tau. The mRNA level of TRPC6 in sporadic AD iPSCs was much lower than that in controls (Figure 2A). During the neural differentiation, the mRNA levels of TRPC6 kept elevating from Day 34 to Day 50 in a linear manner with the maturation of neurons in both control and sporadic AD neurons. However, the expression of TRPC6 in sporadic AD neurons was markedly lower from Day 34 and kept lower at all the following time points measured relative to controls, indicative of the compromised expression of TRPC6 in sporadic AD neurons (Figure 2B). In addition, western blotting analysis was performed to measure the protein levels of TRPC6, TRPC5 (another TRPC family member), APP, and PS1 in sporadic AD and control neurons at Days 34, 42, and 50, respectively (Figure 2C). The quantitative analysis revealed that the levels of these proteins in both sporadic AD and control neurons increased in a time-dependent manner (Figure 2D). However, TRPC6, but not other proteins, in sporadic AD neurons was markedly lower than in controls, displaying similar expression pattern as TRPC6 mRNAs (Figure 2D). Furthermore, the mRNA levels of key factors involved in APP processing, including α-secretase ADAM10 and TACE, β-secretase BACE1, as well as γ-secretase subunits PS1, PEN2, nicastrin, and APH-1 in AD neurons, were comparable with those in control neurons (Supplementary Figure S3), indicating that sporadic AD neurons with lower TRPC6 displayed relatively normal transcriptional levels of key factors involved in APP processing. Thus, we confirmed that the expression of TRPC6 at both mRNA and protein levels in sporadic AD neurons was compromised. In addition, we observed that during the neural differentiation, the sporadic AD neurons exhibiting compromised expression of TRPC6 displayed evidently higher Aβ and tau (Figure 1K and L). It is interesting to note that the detectable reduced expression of TRPC6 in AD neurons occurred at Day 34, which is earlier than the elevation of Aβ and tau in AD neurons, which begins from Day 42. Thus, the consistent compromised expression of TRPC6 might be an early molecular event during AD progression, which was even prior to abnormal Aβ accumulation or tauopathy, suggesting that TRPC6 might be a potentially therapeutic target gene of AD.

The expression of TRPC6 inhibits the elevation of Aβ and tau in live AD neurons

To assess the therapeutic potential of TRPC6, we next explored whether the rescue of TRPC6 expression could alleviate

cholinergic neuronal marker ChAT (f) in human iPSC-derived neurons at Day 50. (**F**) Quantification of the results shown in **E**. $n = 3$. (**G**) Representative traces of no AP (upper), one AP (middle), and repetitive AP (lower) from human iPSC-derived neurons at Days 55–65. (**H**) Percentages of human iPSC-derived neurons firing different types of AP presented in **G**. (**I**) Representative traces of spontaneous AP recorded in human iPSC-derived neurons at Days 55–65. (**J**) Representative traces showing spontaneous PSCs received by human iPSC-derived neurons at Days 55–65. (**K** and **L**) The levels of Aβ42 and Aβ40 (**K**) as well as total tau and phosphorylated tau (**L**) in control or AD iPSC-derived neurons at Days 34, 42, and 50. Data are normalized to total protein of whole-cell lysates. $n = 4$. Scale bar, 50 μm. Data are represented as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

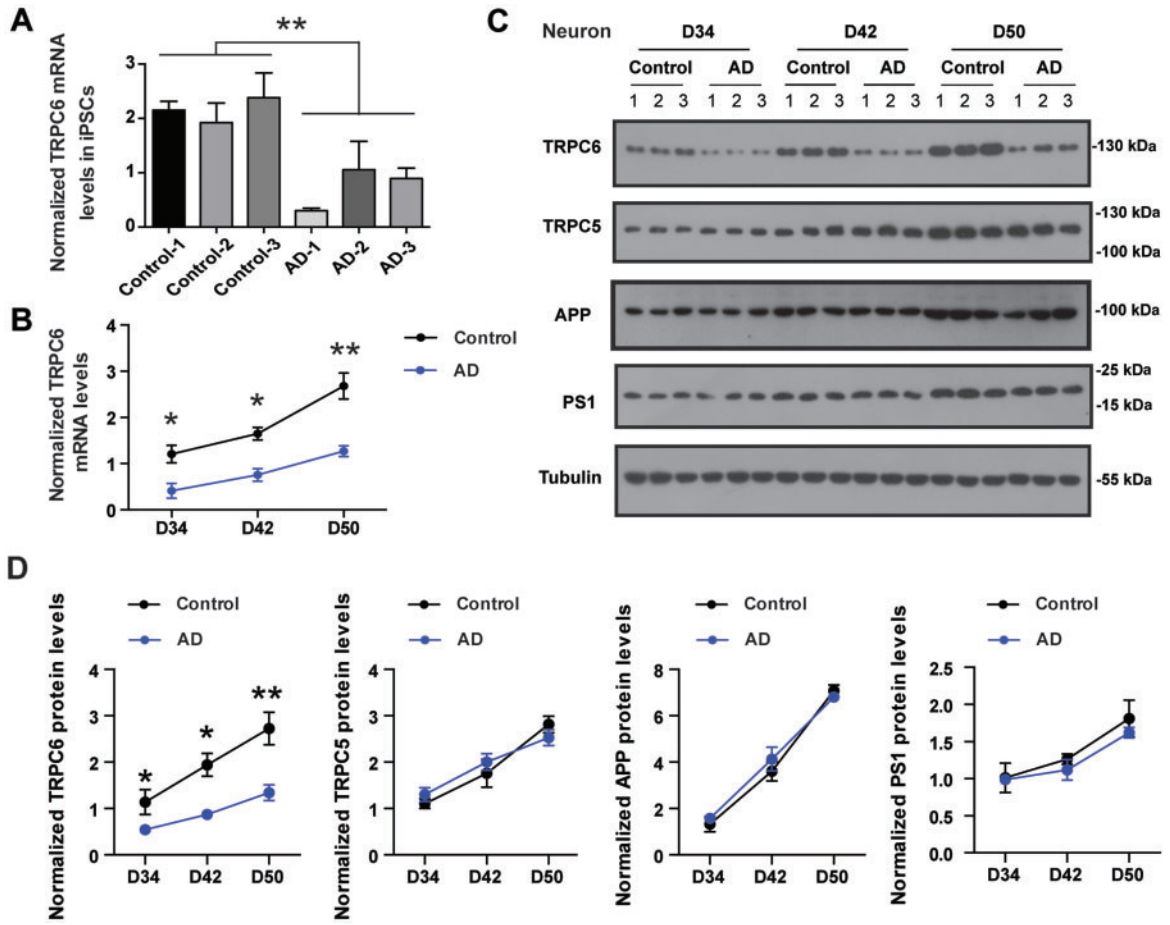

Figure 2 The TRPC6 expression in sporadic AD neurons derived from patient-specific iPSCs. (**A**) The mRNA levels of TRPC6 in AD and control iPSCs derived from PB MNCs. $n = 3$. (**B**) The mRNA levels of TRPC6 in iPSC-derived AD or control neurons at differentiation Days 34, 42, and 50. $n = 8$. (**C**) The protein levels of TRPC6, TRPC5, APP, and PS1 in iPSC-derived AD or control neurons at Days 34, 42, and 50. (**D**) Quantification of the results shown in **C**. $n = 6$. Data are represented as mean ± SD. *$P < 0.05$ and **$P < 0.01$.

the AD-relevant phenotypes in sporadic AD neurons. We previously found that the TRPC6 inhibits APP processing via its TM2 domain and mutations in TM2 (TM2-mut) abolished the inhibitory effect of TRPC6 in HEK293 cells (Wang et al., 2015). Then, the TM2 fused with transmembrane peptide TAT was added to neurons from sporadic AD and control iPSCs at Day 48. Two days later, the secretion of both Aβ42 and Aβ40 decreased in a dose-dependent manner in sporadic AD or control neurons (Figure 3A). In addition, TM2 induced marked decrease of total tau and phospho-tau (Figure 3B). The treatment with TAT only or TM2-mut had no effect on Aβ secretion or tau expression in either AD or control neurons (Figure 3C and D). These results indicate that TRPC6 can inhibit the elevation of Aβ and phospho-tau via TM2 in sporadic AD neurons.

Hyperforin is a drug, which was reported to increase TRPC6 expression (Muller et al., 2008; Gibon et al., 2013). We confirmed that treatment with hyperforin increased the expression of TRPC6 at both mRNA and protein levels in HEK293 cells (Supplementary Figure S4A and B). Exposure of sporadic AD or control neurons to hyperforin resulted in an increase of TRPC6 protein in a dose-dependent manner and 1 μM or higher hyperforin markedly induced the protein expression level of TRPC6 (Figure 4A). More importantly, co-immunoprecipitation assay showed that more TRPC6 proteins bound to APP due to hyperforin treatment, which might further modulate the APP processing (Figure 4B). We also consistently observed that hyperforin treatment resulted in a reduction of secreted Aβ42 or Aβ40 in a dose-dependent manner and 1 μM or higher hyperforin caused evident decrease of Aβ in both AD and control neurons, and the sporadic AD neurons were more sensitive to hyperforin than controls (Figure 4C; Supplementary Table S2). Similar inhibitory effects on phospho-tau and total tau by hyperforin were observed in both AD and control neurons (Figure 4D; Supplementary Table S2).

Together, these results reveal that increasing TRPC6 by TM2 peptide and hyperforin inhibits the elevation of Aβ and tau in sporadic AD neurons, suggesting that targeting TRPC6 might help to develop novel therapeutic strategies for the treatment of AD.

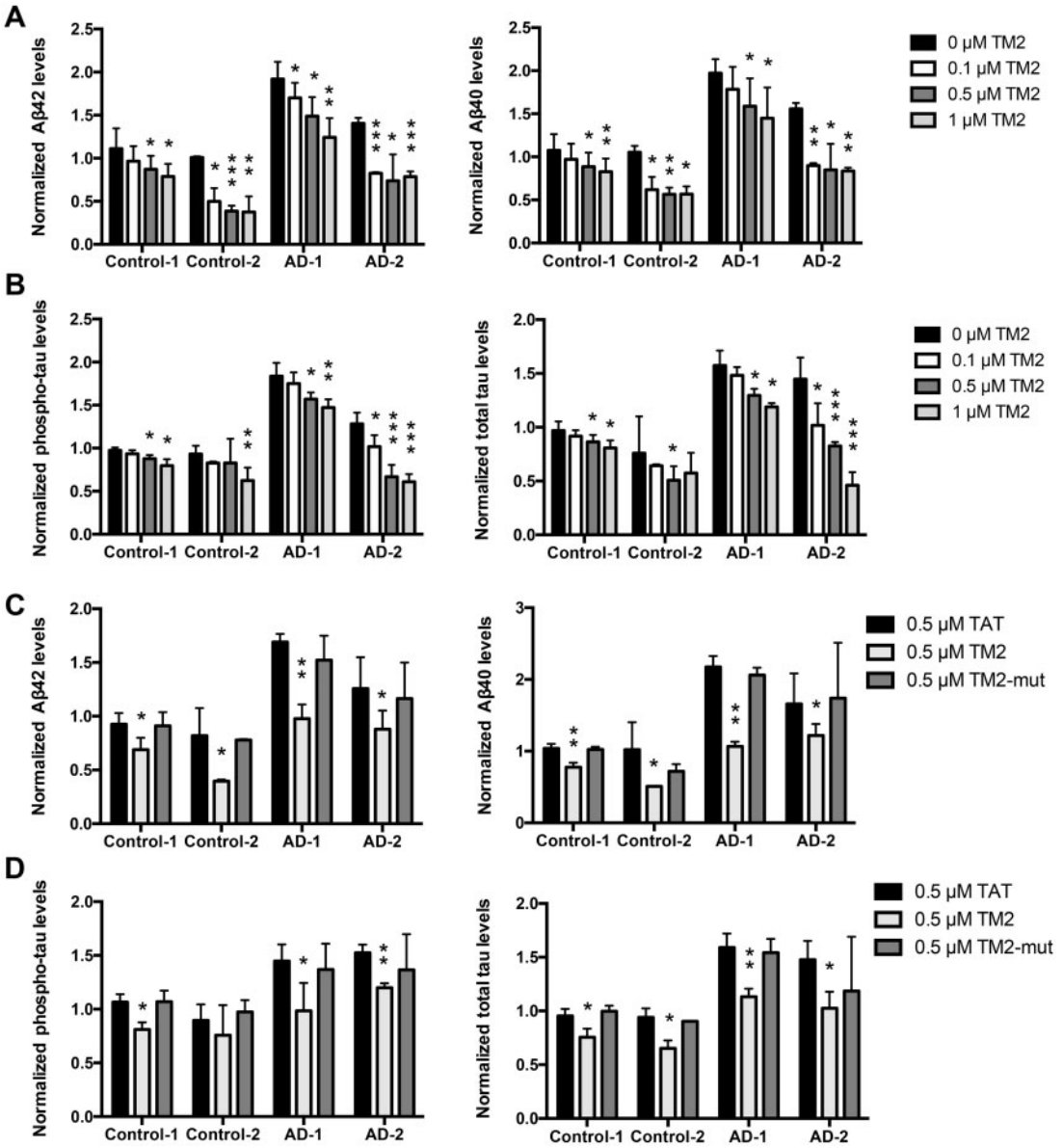

Figure 3 The decrease of Aβ and tau due to the treatment of TRPC6 peptide fragments in sporadic AD neurons. (**A** and **B**) The secretion of Aβ42 and Aβ40 (**A**) as well as the intracellular tau and phospho-tau (**B**) in AD and control neurons exposed to second transmembrane of TRPC6 fused with transmembrane peptide TAT (TM2) at different concentrations. (**C** and **D**) The secretion of Aβ42 and Aβ40 (**C**) as well as the intracellular tau and phospho-tau (**D**) in AD and control neurons exposed to TAT, TM2, and mutated TM2 (TM2-MUT). Data are normalized to total protein of whole-cell lysates and represented as mean ± SD. $n = 3$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Discussion

In this study, we report the derivation of patient-specific sporadic AD iPSCs and the efficient differentiation of iPSCs into AD neurons. Relative to controls, the sporadic AD neurons exhibited severer neuropathological phenotypes associated with AD and compromised expression of TRPC6. More importantly, we observed that the increase of TRPC6 inhibits the elevation of both Aβ and tau in sporadic AD neurons. Together, these results indicated that TRPC6 is involved in the pathogenesis of AD by interfering with the generation of Aβ, even tau. Our data in iPSC-derived live AD neurons are consistent with our previous findings in mouse models and AD patients, suggesting that TRPC6 might be a potentially therapeutic target for AD.

The differentiation process from AD iPSCs to neurons recapitulates aspects of neural developmental and degenerative processes. The efficient neural differentiation of iPSCs is critical to generate the patient iPSC-based AD cellular models. However, the previous studies reported that human iPSCs showed poor efficiency in neural commitment, delayed differentiation and line variations during neural differentiation (Hu et al., 2010;

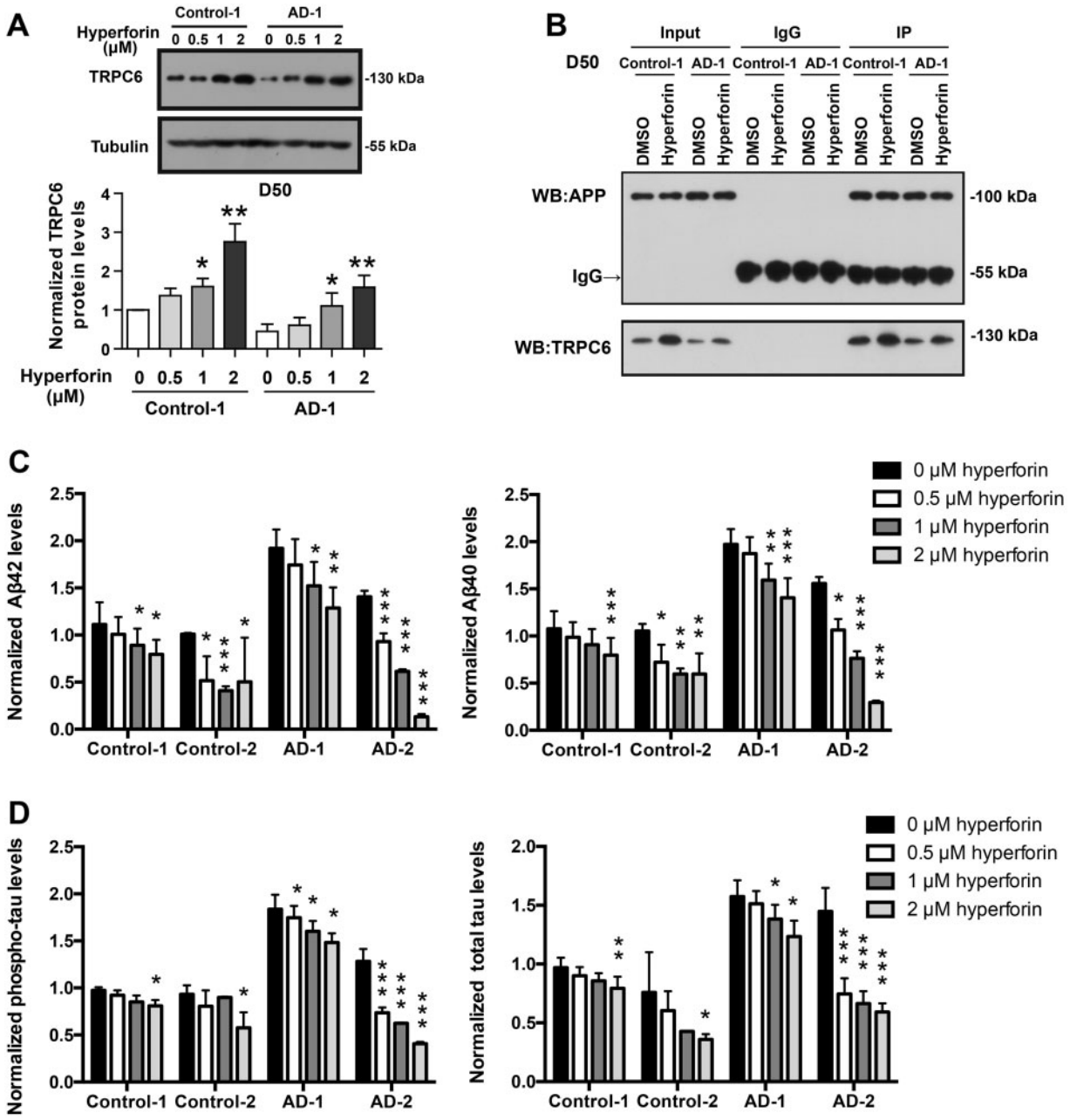

Figure 4 The decrease of Aβ and tau due to the treatment of TRPC6 agonist in sporadic AD neurons. (**A**) Western blotting analysis of TRPC6 expression in AD-1 and Control-1 neurons exposed to hyperforin at different concentrations (top) and quantification (bottom). (**B**) The immunoprecipitation analysis of whole-cell lysates from AD and control neurons treated with DMSO or hyperforin precipitated by anti-APP antibody and immunoblotted by anti-TRPC6 antibody. (**C** and **D**) The secretion of Aβ42 and Aβ40 (**C**) as well as the intracellular tau and phospho-tau (**D**) in AD and control neurons exposed to hyperforin at different concentrations. Data are normalized to total protein of whole-cell lysates and represented as mean ± SD. $n = 3$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Kim et al., 2010; Chambers et al., 2012). Consistently, we found that the serum free, floating EB approaches for human ESC neural differentiation cannot sufficiently induce neural differentiation of human iPSCs (data not shown). The dual-SMAD inhibition (TGF-β inhibitor SB431542 and BMP inhibitor dorsomorphin) strategy, which was widely used in monolayer neural differentiation of human ESCs (Chambers et al., 2009), was reported to be able to promote neural commitment (Kim et al., 2010). In this study, we combined the EB formation and dual-SMAD inhibitors to establish a neural differentiation method for human iPSCs. This approach efficiently directed both sporadic AD and control iPSCs into TUJ1$^+$ (92.3%) or MAP2$^+$

(87.7%) neurons (Figure 1C and D) and the high efficiency was consistent among independent iPSC lines (Supplementary Figure S2C). In addition, majority of iPSC-derived neurons was mature and functional cortex neurons (Figure 1E–J) and these AD neurons from sporadic AD iPSCs recapitulated canonical AD features including increased Aβ secretion and tau phosphorylation (Figure 1K and L) as previously reported in familial AD iPSCs (Israel et al., 2012; Kondo et al., 2013; Young et al., 2015). Collectively, these results suggested that our system based on efficient neural differentiation of sporadic AD-specific iPSCs could serve as a cellular platform to provide new insights into AD, especially sporadic AD, in live neurons identical to patient-genetic background.

Then, we detected a novel candidate target gene, TRPC6, for AD in this patient iPSC-based cellular platform. Our previous studies performed in AD mouse revealed that TRPC6 interfered with Aβ generation and in AD patients showed the reduction of TRPC6 mRNA levels in the peripheral blood, suggesting that TRPC6 might be involved in the progress of AD (Wang et al., 2015; Lu et al., 2018). Then, it is crucial to know whether reduced expression of TRPC6 could induce AD-associated phenotypes in the patient brain. To address this issue, it can take decades by longitudinal follow-up of TRPC6 expression in AD patients over disease progression. Then, the iPSC-derived neurons from patients provide an alternative opportunity to explore the correlativity between TRPC6 and AD-relevant phenotypes in short term. Here, we compared the expression patterns of TRPC6 vs. Aβ as well as tau in live sporadic AD neurons derived from patient-specific iPSCs and found that sporadic AD neurons with compromised expression of TRPC6 displayed evident increase of Aβ secretion and tauopathy (Figures 1K, L and 2B–D). This observation is of particular interesting and suggested that, at least in a subgroup of population, the individuals with lower TRPC6 might be predisposed to develop abnormal Aβ secretion or tau accumulation during AD progression. Thus, these results from live sporadic AD neurons provide evidences to further support our hypothesis that TRPC6 might be a potential target in early stage of AD pathogenesis.

Finally, we tested the therapeutic potential of TRPC6 by modulating its expression levels in iPSC-derived AD neurons. We demonstrated that treatment with TRCP6 peptide caused the decrease of Aβ and tau in AD neurons (Figure 3), which is consistent with the inhibitory effects of TRPC6 on Aβ in cell-free *in vitro* assay, HEK293-APP cell, rat primary neuron, and AD mouse in our previous studies (Wang et al., 2015; Lu et al., 2018). Hyperforin, the agonist of TRPC6 and a clinical drug, was reported to reduce the accumulation of Aβ and alleviate the cognitive symptoms associated with AD in mouse models (Dinamarca et al., 2006; Cerpa et al., 2010; Inestrosa et al., 2011) , but the mechanism underlying remained largely unclear. Our results indicated that the sporadic AD neurons were sensitive to hyperforin and hyperforin exposure resulted in a marked increase in the expression of TRPC6 and evident decreases in the production of Aβ and tau (Figure 4), which is consistent with the observations in AD mouse. It was reported that hyperforin regulated protein kinases and transcription factors via activation of TRPC6 channels, effect downstream targets like BDNF/TrkB pathway in neurons (Gibon et al., 2013; Thiel and Rossler, 2017). Hyperforin also has been proposed to functions as a protonophore and causes cytosolic acidification in a TRPC6-independent manner (Sell et al., 2014). In addition to the mechanism illustrated in our previous study (Wang et al., 2015; Lu et al., 2018), we deduced that hyperforin might also modulate Aβ and tau levels by activating TRPC6 channel-associated pathways. Thus, the hyperforin treatment illustrates the potential to increase TRPC6 expression by drugs in AD patients and the development of TRPC6-based therapies for the treatment of AD.

The live AD neurons derived from patient-specific iPSCs provide a platform to reveal the potential of TRPC6 as a target gene of AD, supporting our previous conclusions in AD mouse models and AD patients and suggesting an alternative functional assay for the identification and validation of candidate targets for the therapeutic intervention and treatment of AD.

Materials and methods

Participants

This use of human adult peripheral blood was approved by the Research Ethics Committees of all the participating hospitals and institutes and conducted according to the principles of the Declaration of Helsinki. The iPSCs were generated from 12 AD patients and 17 age-matched healthy controls recruited in Shanghai, China (Supplementary Table S1). Written informed consent was obtained from all participants or their primary caregivers. Diagnosis of AD was based on the criteria of International Classification of Diseases (ICD-10) and National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and performed in our previous study (Lu et al., 2018).

The generation of iPSCs from human peripheral blood mononuclear cells

The generation of integration-free iPSCs from PB MNCs was performed according to a previously reported protocol (Dowey et al., 2012). PB MNCs were isolated from 3–8 ml peripheral blood of AD patients or controls by density-based centrifugal separation using Ficoll-Paque Premium (GE healthcare). The proliferative erythroblasts from PB MNCs were specifically enriched after an 8–10-day expansion with defined MNC medium as previously described (Chou et al., 2011). Cell numbers were counted every 2 days. Two million of PB MNCs in exponential phase were nucleofected with a mixture of 4 μg EBNA1/OriP-based episomal vectors EV SFFV-OCT4-2A-SOX2 (pEV-OS), 4 μg EV SFFV-MYC-2A-KLF4 (pEV-MK), and 2 μg EV SFFV-BCL-XL (kind gifts from Xiao-bing Zhang, Loma Linda University, USA) using Amaxa Nucleofector II (Lonza human $CD34^+$ cell nucleofector Kit, program 'T-016'). Two days later, transfected MNCs were plated onto mouse embryonic

fibroblasts (MEFs) and maintained with hESC medium supplemented with 10 ng/ml bFGF (Pufei) and 0.25 mM NaB (Sigma). The iPSC colonies were picked up 2–3 weeks after transduction and maintained on MEF with hESC medium. For each individual, six iPSC lines from different mono-colonies were established. The iPSC lines from three sporadic AD patients (designated as AD-1, AD-2, AD-3) and three controls (designated as Control-1, Control-2, Control-3) were used for the subsequent neural differentiation and analysis, respectively (Supplementary Table S1). Two or three iPSC lines of each individual were used. All reagents were purchased from Life Technology if not otherwise specified.

The Neural differentiation of human iPSCs

To direct the differentiation of human iPSCs into neurons, the feeder cells were removed from iPSC cultures. Then, the iPSCs were dissociated into clumps to form EBs. The EBs were cultured in hESC medium without bFGF for 4 days and then with N2 medium for another 2 days. During EB stage, both media were supplemented with 10 μM SB431542 (Selleck) and 2 μM dorsomorphin (Sigma). At Day 6, EBs were transferred into matrigel-coated culture plates and attached to form rosettes maintaining in N2 medium and then N2B27 medium from Day 8. At Day 16, the rosettes were digested and cultured in Petri-dish for another 10 days to allow the formation of neural spheres in N2B27 medium. At Day 26, neural spheres were digested into single cells and reseeded on matrigel-coated dishes in B27 medium (Sigma) for neuronal differentiation and maturation. Neuronal culture medium was refreshed every 2 days. All reagents were purchased from Life Technology if not otherwise specified.

ELISA assays for the measurement of Aβ40, Aβ42, total tau, and phospho-tau

To measure Aβ40 and Aβ42, the culture medium of iPSC-derived neurons was collected in polypropylene tubes at differentiation Day 34, 42, or 50 and stored at −80°C, while the neuronal lysates were collected for protein concentration analysis. Levels of Aβ40 and Aβ42 were assayed by Aβ40 Human ELISA Kit (Invitrogen, KHB3482) and Aβ42 Human Ultrasensitive ELISA Kit (Invitrogen, KHB3544), following the manufacturer's instructions, respectively. To measure the intracellular total tau and phospho-tau, whole-cell lysates of iPSC-derived neurons were extracted using cell extraction buffer were assayed by Total Tau Human ELISA Kit (Invitrogen, KHB0042) and TAU [pT231] Phospho-ELISA Kit, Human (Invitrogen, KHB8051). All experiments were repeated twice with duplicate samples for each iPSC line.

Drugs and peptides

Hyperforin was purchased from Abcam (ab144276). The following peptides were synthesized by ChinaPeptides and resolved in DMSO. TAT: GRKKRRQRRRC; TM2: TSCFSWMEM LIISWVIGMIWAGRKKRRQRRRC; TM2-MUT: TSCFSWMEMLIISDDIG MDDAGRKKRRQRRRC. Hyperforin and peptides were supplemented into neuronal cultures at differentiation Day 48 and treated for 48 h.

Statistics

For qRT-PCR analysis, *GAPDH* was used as a reference gene and the relative levels of target genes were calculated by the comparative Ct method (Schmittgen and Livak, 2008) . For ELISA analysis, data sets are normalized to total protein of whole-cell lysates and are relative to the mean of control. For western blotting analysis, data sets are normalized to the expression of tubulin and are relative to the mean of control. All statistical analyses were performed using GraphPad Prism. Sample size (n) values were provided in the relevant text, figures, and figure legends. Data of each sample were calculated from 2−3 technical replications. The statistical analyses were obtained from three independent experiments. All data were presented as mean ± SD. Two-tailed Student's t-test was used for two groups and one-way ANOVA with Newman–Keuls *post hoc* test for more than two groups. The $P < 0.05$ was considered as statistically significant. For all, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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