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$A\beta_{1-42}$ disrupts the expression and function of KLF2 in Alzheimer's disease mediated by p53

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ABSTRACT

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. Blood brain barrier (BBB) dysfunction and impaired permeability are implicated in the pathological process of AD, but the underlying mechanisms are poorly understood. Kruppel-like factor 2 (KLF2) plays a critical role in regulating vascular functions, including vascular barrier permeability. The expression patterns and functions of KLF2 in AD progress are still unknown. In the current study, we investigated whether alterations in KLF2 contribute to cerebrovascular dysfunction in AD. Our results demonstrated that decreased expression level of KLF2 in the brains of Tg2576 transgenic mice was due to accumulation of Aβ. Importantly, overexpression of KLF2 could completely rescue impaired expression of tight junction protein Occludin induced by $A\beta_{1-42}$ in primary human brain endothelial cells (HBMECs). At last, p53 was verified to mediate $A\beta_{1-42}$ induced reduction of KLF2. Overall, this is the first time to report that KLF2 is involved in cerebrovascular dysfunction in Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD), characterized by the presence of extracellular senile plaques (SP) comprised of the amyloid-beta (Aβ) peptide and intracellular neurofibrillary tangles (NFT) comprised of the tau protein, is the leading cause of dementia in the elderly [1,2]. Cerebrovascular pathology and AD are strongly linked in the current studies. Cerebral amyloid angiopathy (CAA), defined as cerebrovascular amyloid deposition, was proved to play a critical role in the progress of AD [3]. Moreover, breakdown of the BBB associated with CAA was implicated in the pathogenesis of AD [4]. BBB plays a critical role in regulating the transport of various molecules and restricting permeability across brain endothelium [5]. The tight junction protein Occludin was described to mainly express in brain endothelium and to be responsible for a recent study demonstrated that $A\beta_{1-42}$ triggered an intracellular signaling cascade and disrupted tight junctions, leading to the breakdown of the integrity of BBB [6].

Kruppel-like factor 2 (KLF2), a member of the mammalian Kruppel-like factors (KLF) family, is a DNA-binding transcription factor. KLF2 has been reported to be extensively expressed in endothelial cells and to control vascular functions [7], including regulating endothelial barrier function [8]. The present study was designed to explore mechanisms through which altered expression of KLF2, a key regulator of vascular functions, may influence AD amyloid neuropathology and to test the hypothesis that promotion of KLF2 expression in brains might be developed as a novel therapeutic strategy for impaired BBB function in AD.

2. Materials and methods

2.1. Animals

Tg2576 transgenic (Tg/+) mice, with the age of 18–24 months, were used in this study. The mice were purchased from Taconic Farms (Germantown, USA), and were maintained on mixed C57Bl6/SJL background by mating heterozygous Tg2576 males to C57Bl6/SJL F1 females. All experimental procedures were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Weifang Medical University and were in accordance with Weifang Medical University guidelines for the care and use of laboratory animals.

2.2. Cell culture and transfection

Primary HBMECs were from Promocell, USA. Cells were cultured in EBM-2 media with supplemental growth factors according to the instructions. $A\beta_{1-42}$ peptide (American Peptide, USA) was dissolved in hexafluoroisopropanol (Sigma, USA) for 2 d at room

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temperature (RT), and the lyophilized peptide was dissolved in dimethylsulfoxide (DMSO) [9].

An upstream 2000 bp fragment of KLF2 proximal promoter was generated by PCR and subsequently cloned in pGL3 firefly luciferase reporter (Promega, USA) according to manufacturer's instructions. Human p53 and a non-specific control siRNA were acquired from Invitrogen, USA and were transfected into HBMECs using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Human Lentiviral KLF2 was purchased from ABM, INC, CA. Briefly, HEK 293T cells were used to generate lenti-viruses and virus-containing supernatants were titrated on HBMECs to determine the titers needed to transduce >95% of the cells [10].

2.3. Real time PCR

Total RNA was extracted from cultured cells and brain tissues with Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. Real-time PCR was performed with LightCycler® 480 Probes Master on Applied Biosystems Step One Real-Time PCR system with gene specific primers and probes. The following primers were used: Human KLF2: forward 5′-tgcggcaagacctacaccaagagt-3′, reverse 5′-agccgcagccgtcccagtt-3′; Human GAPDH: forward 5′-ccacatcgctcagacaccat-3′, reverse 5′-ccaggcgcccaatacg-3′; Human Occludin: forward 5′-cactatgagacagactacacaactgg-3′; reverse 5′-ttgatctgaagtgataggtggatatt-3′; mKLF2: forward 5′-accaagagctcgcacctaaa-3′; reverse 5′-gtggcactgaaagggtctgt-3′; Mouse GAPDH: forward 5′-tgtgtccgtcgtggatctga-3′; reverse 5′-cctgcttcaccaccttcttga-3′.

2.4. Immunoblotting

After boiled in SDS–PAGE gel loading buffer, protein lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were electrotransferred to Immobilon-P (Millipore, Billerica, MA, USA). After blocking with 10% non-fat dry milk, sequentially incubated with primary and secondary antibodies, blots were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) or the immunoCruz (Santa Cruz Biotechnology, USA) [11].

2.5. Immunofluorescence microscopy

HBMECs were washed in PBS, pH 7.4, and then fixed in 4% paraformaldehyde (PFA) for 10 min at RT. After permeabilization with 0.4% Triton X-100 followed by blocking with 5% BSA and 2.5% FBS in PBST. Cells were incubated with anti-KLF2 (Invitrogen, USA) or anti-Occludin (Invitrogen, USA) in PBS with 5% BSA for 2 h at RT and followed by incubating with FITC or TRITC conjugated secondary antibodies for 1 h at RT (Invitrogen, USA). The cells were counterstained with DAPI (4′, 6′-diamidino-2-phenylindole dihydrochloride) and analyzed on a fluorescence microscope (Olympus DP50) [12].

3. Results

Firstly, we measured the expression levels of KLF2 in the brains of Tg 2576 mice. As shown in Fig. 1A, real-time PCR studies of transgenic mice and age-matched controls suggested a 50% decrease in KLF2 mRNA levels in Tg2576 mice. Immunoblotting analysis also revealed that the protein level of KLF2 was significantly reduced in the brains of Tg2576 mice (Fig. 1B).

The pathological mechanism of AD is very complicated. And Aβ was considered to play a critical role in the process of AD. To evaluate whether the reduction of KLF2 in Tg2576 mice was induced by over burden of Aβ or other factors, primary HBMECs were incubated with 5 μM $A\beta_{1-42}$ for 48 h. After incubated with $A\beta_{1-42}$, the expression level of KLF2 was reduced at both mRNA and protein levels (Fig. 2A and B). Accordingly, KLF2 immunoreactivity declined after exposure to $A\beta_{1-42}$, shown in Fig. 2C. We next assessed the effect of $A\beta_{1-42}$ on KLF2 promoter activity. A 2000 bp region upstream of the transcription start site (−2000) in the KLF2 gene was used in promoter-reporter assays. And the results in Fig. 2D showed that $A\beta_{1-42}$ inhibited KLF2 promoter activity, implying that Aβ could inhibit the expression of KLF2 at the transcriptional level.

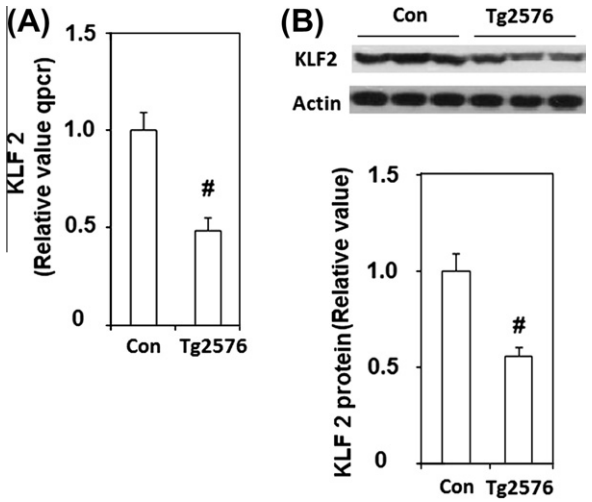

Fig. 1. The expression level of KLF2 was reduced in Tg2576 mice. (A) Real time PCR analysis revealed that the mRNA level of KLF2 was significantly reduced in Tg2576 brains compared with controls (#, $p < 0.01$ vs Controls, Student's *t*-test); (B) representative immunoblot and quantification analysis revealed that the protein level of KLF2 was significantly reduced in Tg2576 brains compared with age-matched controls (#, $p < 0.01$ vs Control, Student's *t*-test); actin was used as an internal loading control.

Previous evidence implicated KLF2 as a key regulator of vascular barrier function through regulating the expression of tight junction protein Occludin [8]. And $A\beta_{1-42}$ was reported to disrupt tight junctions in endothelial cells [6]. Here, we examined the effect of KLF2 on $A\beta_{1-42}$ induced reduction of Occludin. After infected with lentiviral KLF2, HBMECs were treated with $A\beta_{1-42}$ for 48 h. Compared with control ones, lenti-viral overexpression of KLF2 protected against $A\beta_{1-42}$ induced reduction of Occludin at mRNA level (Fig. 3A). Also, the result was confirmed by immunocytochemistry (Fig. 3B).

Transcriptional regulation of KLF2 expression is complex. The previous study demonstrated that p53 could suppress the expression of KLF2 in endothelial cells at the transcriptional level [13]. And p53 was associated with AD pathogenesis in the previous study [14]. In order to examine whether p53 mediated $A\beta_{1-42}$-induced reduction of KLF2, the expression pattern of p53 was investigated in HBMECs after Aβ treatment. Since post-translational phosphorylation at serine 15 plays a critical role in the stability and transactivation of p53, we examined the impact of Aβ on the expression of phospho-p53 ser 15 expression. Exposure to $A\beta_{1-42}$ for 48 h significantly increased phosphor-p53 ser 15 expression (shown in Fig. 4A). To further assess whether p53 activation played a causal role in reduced expression of KLF2 induced by $A\beta_{1-42}$, the effect of $A\beta_{1-42}$ on KLF2 expression was investigated in HBMECs following siRNA mediated downregulation of p53. Indeed, the reduction of KLF2 induced by $A\beta_{1-42}$ could be rescued by down-regulation of p53 at both the mRNA and protein levels (Fig. 4B and C).

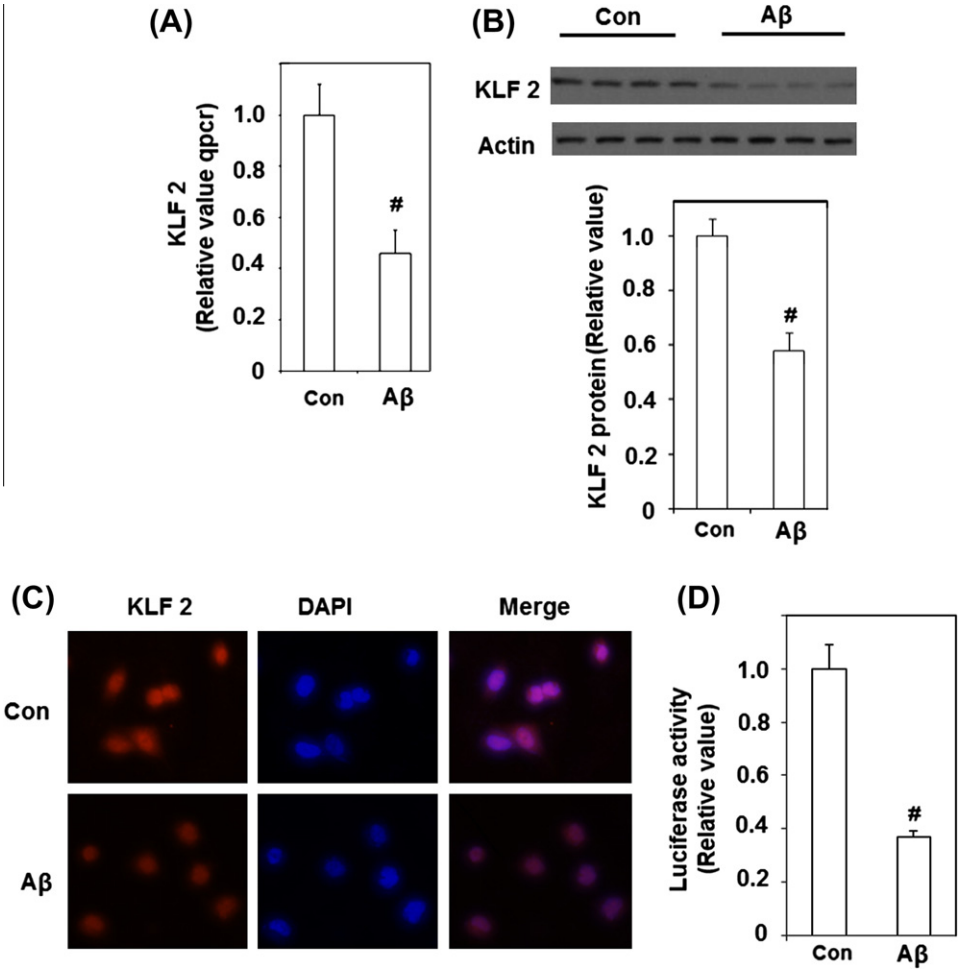

Fig. 2. $A\beta_{1-42}$ induced alterations of KLF2 in HBMECs. (A) HBMECs were treated with $A\beta_{1-42}$ (5 μM) for 48 h, real time PCR analysis revealed that the mRNA level of KLF2 was significantly reduced after $A\beta_{1-42}$ treatment (#, $p < 0.01$, Student's *t*-test); (B) HBMECs were treated with $A\beta_{1-42}$ (5 μM) for 48 h, representative immunoblot and quantification analysis revealed that the protein level of KLF2 was significantly reduced after $A\beta_{1-42}$ treatment (#, $p < 0.01$, Student's *t*-test); (C) HBMECs were treated with $A\beta_{1-42}$ (5 μM) for 48 h, immunoreactivity (red) of KLF2 in the presence of $A\beta_{1-42}$ was lower than the control group. (D) $A\beta_{1-42}$-induced inhibition of KLF2 promoter. Activity of −2000 bp KLF2 promoter was measured in HBMECs in presence of $A\beta_{1-42}$, normalized (firefly/protein concentration) promoter activity is expressed relative to DMSO control (#, $p < 0.01$; $n = 4$, Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

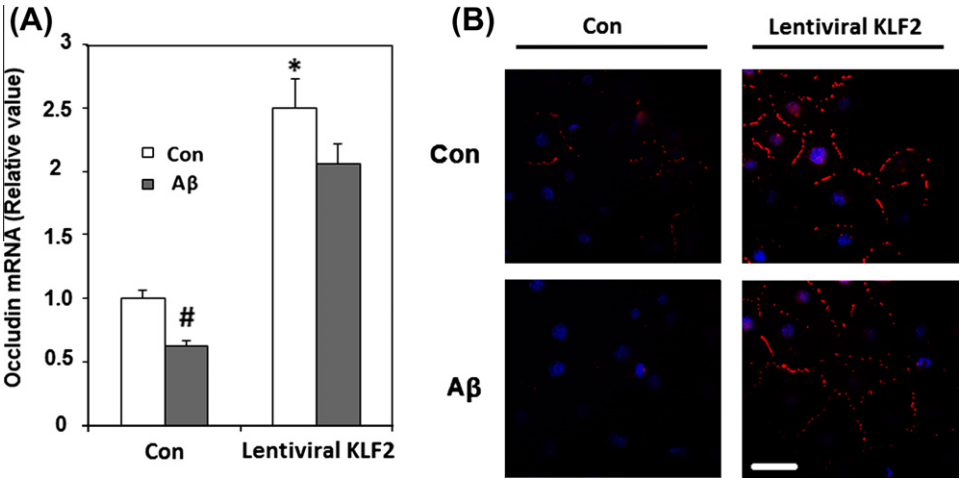

Fig. 3. $A\beta_{1-42}$-induced alterations of Occludin in HBMECs are attenuated by lenti-viral KLF2. (A) Transiently infected HBMECs with mock or full-length human KLF2 were incubated with 5 μM $A\beta_{1-42}$ for 48 h, real time PCR revealed that $A\beta_{1-42}$ induced reduction of Occludin was attenuated by lenti-viral overexpression of KLF2 (#, $p < 0.01$ vs Vector(Control), ∗, $p < 0.01$ vs Vector (Control)). (B) Transiently infected HBMECs with mock or full-length human KLF2 were incubated with 5 μM $A\beta_{1-42}$ for 48 h, immunocytofluorescence experiment revealed that $A\beta_{1-42}$ induced reduction of Occludin was attenuated by lenti-viral overexpression of KLF2, red signal is Occludin, Scale bar, 60 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

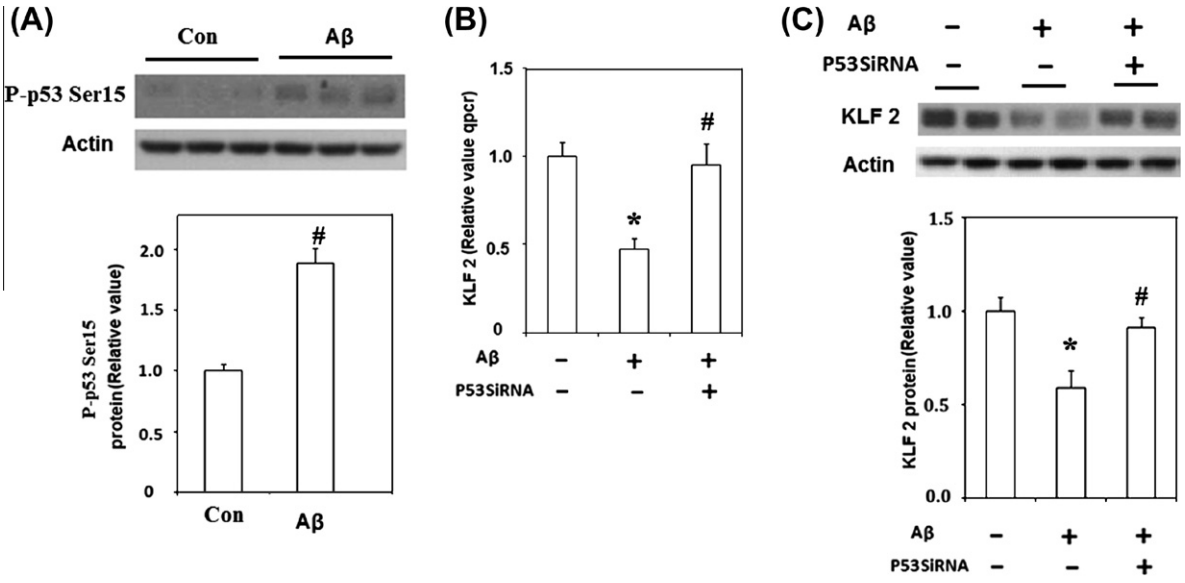

Fig. 4. p53 mediates $A\beta_{1-42}$-induced reduction of KLF2. (A) HBMECs were treated with $A\beta_{1-42}$ for 48 h, immunoblot and quantification analysis revealed that the level of p-p53 ser15 was increased by $A\beta_{1-42}$ (#, $p < 0.01$). (B) After transfected with p53 siRNA, cells were incubated with $A\beta_{1-42}$ for 48 h, real time PCR results revealed that $A\beta_{1-42}$-induced reduction of KLF2 could be attenuated by knockdown of p53 (∗, $p < 0.01$ vs Non-treatment; #, $p < 0.01$ vs $A\beta_{1-42}$ treatment). (C) After transfected with p53 siRNA, cells were incubated with $A\beta_{1-42}$ for 48 h, immunoblot and quantification analysis revealed that $A\beta_{1-42}$-induced reduction of KLF2 could be attenuated by knockdown of p53 (∗, $p < 0.01$ vs Non-treatment; #, $p < 0.01$ vs $A\beta_{1-42}$ treatment).

4. Discussion

We report for the first time, to our knowledge, that KLF2 expression is decreased in the brains of AD animal model Tg2576 mice. Moreover, we found that $A\beta_{1-42}$ could suppress the expression of KLF2 in HBMECs at transcription level. KLF2 was found to be highly expressed in vascular endothelium, and to regulate a range of endothelial functions. It was also reported to protect endothelial cells from oxidative stress mediated injury and apoptosis [15]. Thus, reduced level of KLF2 induced by accumulation of Aβ implies impaired endothelial functions in AD. CAA was defined as accumulation of Aβ in small arteries, arterioles and capillaries. Up to 90% of patients with Alzheimer disease have changes of CAA at autopsy [16].Vascular accumulation of Aβ in AD has been proved to be associated with cerebral hemorrhage, atherosclerosis and arteriosclerosis, and impaired BBB [17]. BBB dysfunction was identified in both animal models of AD [18] and AD patients [19]. Moreover, compared to age matched wild-type littermates, a significant increase in the incidence of disrupted TJs was found in aged Tg2576 AD mice [20]. These evidences suggest that the accumulation of Aβ in the CNS and brain vessels leads to cerebral microvascular dysfunction, including impaired BBB and changes in permeability. Here, we found that lenti-viral overexpression of KLF2 could attenuate $A\beta_{1-42}$-induced reduction of Occludin, but control lenti-virus did not. It is suggested that KLF2 might play an important role in the alterations of Occludin by $A\beta_{1-42}$.

p53 has been intensively associated with the pathogenesis of AD. It was reported to mediate $A\beta_{1-42}$ induced neurotoxicity through activating the expression of Bax [21]. Cytosolically expressed $A\beta_{1-42}$ in transgenic neurons significantly increased the level of p53 [22]. Here, we found that the activated p53 by $A\beta_{1-42}$ treatment participated in decreasing the expression of KLF2, suggesting a novel role of p53 in AD. Overall, our results suggest the accumulation of Aβ in AD might result in the activation of p53, leading to alteration of KLF2 that ultimately results in impaired expression of tight junction Occludin, implying a potential pathological mechanism of AD.

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