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Pericyte Implantation in the Brain Enhances Cerebral Blood Flow and Reduces Amyloid- β Pathology in Amyloid Model Mice

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Abstract

Pericytes are a major component of cerebrovasculature playing a key role in maintaining cerebrovascular homeostasis. These cells have also been suggested to regulate brain metabolism of amyloid- β ($A\beta$), disturbances of which are believed to contribute to the pathogenesis of Alzheimer's disease (AD). To examine the effects of pericytes on brain $A\beta$ metabolism, C3H/10T1/2 mouse mesenchymal stem cells were differentiated into pericytes and stereotactically injected into the brains of amyloid AD model APP/PS1 mice at the age of 18 to 20 months. Consistent with a role of pericytes in modulating cerebrovascular function, brain microcirculation in the pericyte-injected hemisphere of the mice was increased 3 weeks after implantation compared to the contralateral hemisphere when measured by laser speckle contrast analysis technology. Importantly, enzyme-linked immunosorbent assay revealed that the levels of insoluble $A\beta$ 40 and $A\beta$ 42 were significantly lower in the hippocampus of the pericyte-injected hemisphere of the APP/PS1 mice than that of the contralateral side. Consistently, immunohistochemical analysis demonstrated that the pericyte implantation reduced $A\beta$ deposition in the hippocampus. When brain slices from the APP/PS1 mice were incubated with C3H/10T1/2 cell-derived pericytes, $A\beta$ 42 levels were significantly reduced in a manner that depends on the expression of a major $A\beta$ endocytic receptor, the low-density lipoprotein receptor-related protein 1 (LRP1). While LRP1 mediated the cellular uptake of $A\beta$ in the pericytes, the amounts of major $A\beta$ -degrading enzymes were not affected by LRP1 knockdown. Together, our findings indicate that mesenchymal stem cell-derived pericytes have the capacity to reduce brain $A\beta$ and related pathology, and suggest that cell-based therapy through transplantation of pericytes may be a promising approach to prevent and/or treat AD.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the content of this article.

AUTHOR CONTRIBUTIONS

M.T. and T.K. conceived the study. M.T., Y.Y., C.-C.L., G.B., and T.K. collected data. M.T., Y.Y., C.-C.L., G.B., and T.K. analyzed data. M.T. and T.K. wrote the first draft. All authors contributed in writing the final manuscript. All authors have approved the final version of the manuscript.

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Keywords

amyloid- β ; Alzheimer's disease; C3H/10T1/2; LRP1; pericytes; stem cell therapy

1. INTRODUCTION

Pericytes are vascular mural cells originally discovered as capillary adventitial cells (Sims, 1986). Pericytes are likely composed of heterogeneous populations, and sometimes display mesenchymal stem cell (MSC)-like properties (Crisan et al., 2008; Guimaraes-Camboa et al., 2017). In brain capillaries, pericytes helically surround the microvessels with their long processes, while other pericyte subtypes reside on pre-capillary arterioles (hybrid smooth muscle–pericyte cells and mesh pericytes) and post-capillary venules (mesh pericytes) (Hartmann et al., 2015; Kisler et al., 2017). As pericytes are more abundant in the central nervous system compared to peripheral tissues/organs (Armulik et al., 2011), which cover approximately 37% of the abluminal surface of endothelial tubes in rat brains (Mathiisen et al., 2010), pericytes play a critical role in maintaining cerebrovascular homeostasis. By interacting with endothelial cells, pericytes regulate blood-brain barrier integrity, angiogenesis, and hemodynamic responses in brain microvessels. Furthermore, pericytes have immune cell-like properties, such as responding to inflammatory stimuli, presenting antigen, and regulating fibrosis (Kisler et al., 2017; Rustenhoven et al., 2017; Sweeney et al., 2016; Thomas et al., 2017). Pericytes also have the phagocytic ability to eliminate toxic molecules, including amyloid- β (A β) peptides, in perivascular regions (Rustenhoven et al., 2017; Sweeney et al., 2016). Indeed, in an amyloid mouse model, the pericyte deficit caused by *Pdgfra* haploinsufficiency suppresses A β clearance, resulting in exacerbated brain A β deposition (Sagare et al., 2013).

Importantly, brain accumulation of A β likely triggers the pathogenic cascade of Alzheimer's disease (AD) (Musiek and Holtzman, 2015; Selkoe and Hardy, 2016), which is the most common cause of dementia in the elderly (Alzheimer's Association, 2017). A β deposition is predominantly detected as senile plaques in brain parenchyma and as cerebral amyloid angiopathy (CAA) in cerebrovasculature (Kapasi et al., 2017; Thal et al., 2015), where A β is less efficiently eliminated from the brain in AD patients compared to healthy individuals (Mawuenyega et al., 2010). While several proteases, including neprilysin (NEP) and insulin-degrading enzyme (IDE), can efficiently degrade A β in extracellular space (Saido and Leissring, 2012), most brain cells have sufficient capacity to endocytose A β for subsequent lysosomal degradation (Kanekiyo et al., 2014; LaFerla et al., 2007; Querfurth and LaFerla, 2010). In addition, A β is also cleared through the interstitial fluid drainage pathway and glymphatic system along the cerebrovasculature (Morris et al., 2016). Increasing evidence demonstrates that disturbances of cerebrovascular functions are involved in brain A β accumulation and AD development (Kapasi et al., 2017; Kisler et al., 2017). Consistently, diminished pericyte coverages on microvessels have also been demonstrated in AD patients (Sengillo et al., 2013). Because pericytes are critical components with diverse functions in the neurovascular unit, pericytes are predicted to directly or indirectly contribute to A β clearance through multiple pathways.

Therefore, to further explore the potential mechanism of pericyte-mediated A β clearance and to develop pericyte-targeted therapy for AD, we implanted mouse mesenchymal C3H/10T1/2 cell-derived pericytes (Bose et al., 2013; Darland et al., 2003; Hirschi et al., 1998; Reznikoff et al., 1973) into the brains of amyloid model APP/PS1 mice and investigated their effect on A β pathology. In addition, we assessed the contribution of low-density lipoprotein receptor-related protein 1 (LRP1) (Kanekiyo and Bu, 2014), a major A β clearance receptor, to the pericyte-mediated A β metabolism using *ex vivo* and *in vitro* approaches. Our study demonstrates that C3H/10T1/2 cell-derived pericytes are capable of eliminating brain A β deposition with competence.

2. MATERIAL AND METHODS

2.1. Cell culture

The C3H/10T1/2 cell line was purchased from ATCC (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS) in normal culture condition, and differentiated into pericytes by culturing with mouse transforming growth factor- β (TGF- β ; 1 ng/ml, Invitrogen [Carlsbad, CA]) for at least 3 days before beginning experiments (Bose et al., 2013). In some experiments, LRP1 knockdown and green fluorescent protein (GFP)-transfection were performed in the cells. Single-stranded sense and antisense RNA oligonucleotides for mouse LRP1 were synthesized by Dharmacon (GE Healthcare; Lafayette, CO), and double-stranded RNA molecules were generated according to the manufacturer's instruction. The sequences of the single-stranded RNAs were 5'-GGA GUC ACU UAC AUC AAU AUU-3' (sense) and 5'-UAU UGA UGU AAG UGA CUC CUU-3' (antisense). Non-targeting siRNA was also purchased from Dharmacon (GE Healthcare) and used as the control. For GFP transfection, the pmax GFP vector provided in Amaxa Basic Nucleofector Kit (Lonza; Cologne, Germany) was used. LRP1-siRNA (300 nM) or GFP plasmid vector (2 μ g) was transfected into C3H/10T1/2 cell-derived pericytes by nucleofection using the Amaxa Basic Nucleofector Kit (Lonza) according to the manufacturer's instructions (program A-033). Cells were cultured for an additional 48 hours before being used for experiments.

2.2. Animals and cell injection

All animal procedures were approved by the Institutional Animal Care and Use Committee at Mayo Clinic and in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. APPswe/PSEN1 E9 (APP/PS1) mice were purchased from Jackson Laboratory (Jankowsky et al., 2004). Both male and female APP/PS1 mice were subjected to intracranial cell implantation at the age of 18 to 20 months. The mice were subjected to anesthesia, and the skull was exposed by skin incision. Stereotactically, holes were bored above both hemispheres (lateral 2.0 mm and posterior 2.0 mm from the bregma). C3H/10T1/2 cell-derived pericytes in 2 μ l of phosphate-buffered saline (PBS; 1×10^5 cells/ μ l) were injected into the right hemisphere (depth 2.0 mm from surface of the skull) using a Hamilton Neuros Syringe (Reno, NV) with 33-gauge needle at a rate of 1 μ l/min. After injection, the needle was held steady for 2 minutes, and then gradually withdrawn for 1 minute. In the same manner, PBS (2 μ l) was injected in the left hemisphere as a control.

2.3. Monitoring of brain microcirculation

To monitor cerebral microcirculation in real time, laser speckle contrast analysis (LASCA) technology was used through PeriCam PSI HR (Perimed AB, Sweden). The mouse was anesthetized with ketamine and xylazine, and placed on a small animal stereotaxic device equipped with a heating blanket to maintain body temperature. The surgical site was prepared by shaving, following transection of the skin along the midline to expose the skull to the anterior-posterior coordinates in the brain, bregma, and lambda. The cerebral microcirculation of the mouse was monitored for 5 to 10 minutes to ensure that stable perfusion was obtained. The region of interest was selected around the injection site in both hemispheres, and the average cerebral microcirculation rate was quantified.

2.4. A β enzyme-linked immunosorbent assay (ELISA)

Mouse hippocampus samples were homogenized using a Polytron homogenizer (KINEMATICA; Lucerne, Switzerland) in 10 times volumes (w/v) of ice cold tris-buffered-saline with 1% triton-X (TBSX) containing cOmplete protease inhibitor cocktail (Roche; Mannheim, Germany) and PhosSTOP phosphatase inhibitor (Roche). After incubation with mild agitation at 4°C for 1 hour, samples were centrifuged at 100,000 g at 4°C for 60 minutes. The residual pellet was further homogenized in 5 M of guanidine hydrochloride (GDN-HCl; pH 7.6) and incubated with mild agitation at room temperature for 12 to 16 hours, followed by centrifugation at 16,000 g for 1 hour. Levels of A β 40 and A β 42 were determined by ELISA as previously described (Kanekiyo et al., 2013; Liu et al., 2017). Samples were incubated with horseradish peroxidase-linked streptavidin (Vector Laboratories; Burlingame, CA) and developed with 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich; St Louis, MO) by a Synergy HT plate reader (BioTek Instruments; Winooski, VT) followed by colorimetric measurement. All measured values were normalized against protein concentration as determined by BCA protein assay kit (Pierce; Rockford, IL).

2.5. Immunohistochemical analysis

Frozen sections (30- μ m thickness) were permeabilized in PBS with 0.3% triton X-100, blocked with 3% bovine serum albumin, and incubated with anti-platelet-derived growth factor receptor β (PDGFR β) antibody (1:50, R&D Systems; Minneapolis, MN) or anti-collagen IV antibody (1:100, EMD Millipore) at 4°C overnight followed by incubation with Alexa Fluor 568 secondary antibody (1:200, Thermo Fisher Scientific; Waltham, MA). Subsequently, sections were incubated with anti-GFP antibody (1:500, Aves Labs; Tigard, OR) at 4°C overnight, followed by a secondary Alexa Fluor 488 antibody (1:200). Stained sections were captured using a Zeiss LSM 510 META confocal microscope. For the quantification of A β plaque burden, frozen brain sections were stained by mouse monoclonal anti pan-A β 33.1.1 (human A β 1–16 specific) antibody, visualized with 3,3'-diaminobenzidine (DAB), and captured with an Aperio AT2 scanner (Aperio Technologies Inc; Vista, CA) (Kanekiyo et al., 2013; Kanekiyo et al., 2012; Liu et al., 2017). The immunoreactivity in the hippocampus was measured by ImageJ software in a blinded manner.

2.6. Ex vivo cellular A β clearance assay

Using APP/PS1 mouse brain slices, *ex vivo* cellular A β clearance assay was performed as previously described (Koistinaho et al., 2004; Wyss-Coray et al., 2003; Xiang et al., 2016), with some modifications. Briefly, brains from 12-month-old APP/PS1 mice were coronally cryostat-sectioned (10- μ m thickness), mounted on poly-L-lysine-coated coverslips (Neuvitro; Vancouver, WA), and transferred to 12 well plates. Sections were coated with gelatin (100 μ g/cm²; Sigma) at 37°C for 2 hours, and incubated with or without C3H/10T1/2 cell-derived pericytes (6 $\times$ 10⁵ cells/well) in DMEM with 10% FBS at 37°C for 24 hours. For immunofluorescent staining, pericytes were stained with CM-DiI (Molecular Probes; Eugene, OR) according to the manufacturer's protocol and cultured on the brain slices. After being fixed in 4% paraformaldehyde, brain slices were immunostained with pan-A β 33.1.1 antibody at 4°C overnight, followed by incubation with Alexa Fluor 488 secondary antibody for 2 hours. For A β 42 measurement, cells were carefully removed by trypsin treatment at 37°C for 15 minutes. Brain sections were lysed in 5 M GDN-HCl (pH 7.6), incubated at room temperature for 12 to 16 hours using mild agitation, and centrifuged at 16,000 g for 1 hour. The supernatant was collected and subjected to A β 42 ELISA.

2.7. Fluorescence-activated cell sorter (FACS)-based internalization assays

5-carboxyfluorescein (FAM)-A β 40 and FAM-A β 42 were purchased from AnaSpec Inc (Fremont, CA). The peptides were dissolved in trifluoroacetic acid (Sigma), distilled with nitrogen, washed with 1,1,1,3,3,3-hexafluoro-2-propanol, and further distilled with nitrogen. FAM-A β peptides were freshly dissolved in dimethyl sulfoxide at 200 μ M before each experiment. Cells were plated onto 12-well plates and allowed to grow to 90% confluency. Cells were then incubated with FAM-A β 40 (500 nM), FAM-A β 42 (500 nM), or Fluoresbrite YG Carboxylate Microspheres (1.1 $\times$ 10⁷ particles/ml; Cat# 15702-10, Polysciences Inc; Warrington, PA) in DMEM with 10% FBS. Following incubation, cells were harvested using trypsin (Gibco) and resuspended in PBS containing 1.5% FBS, 1% sodium azide, and 1% paraformaldehyde. In each sample, 1 $\times$ 10⁴ cells were analyzed for the shift of fluorescence intensity using a FACSCalibur machine (BD Biosciences) (Kanekiyo et al., 2013; Kanekiyo et al., 2012; Liu et al., 2017).

2.8. Western blotting

Samples were lysed in Radioimmunoprecipitation Assay buffer with protease inhibitor cocktail (Roche). The same concentration of protein for each condition was subjected to SDS-PAGE. Immunoreactivity was detected and quantified using the Odyssey CLx Imaging System (LI-COR Biotechnology; Lincoln, NE). In-house polyclonal anti-LRP1, anti-matrix metalloproteinase (MMP) 2 and MMP9 (EMD Millipore; Billerica, MA), anti-IDE and anti-CD10 (Neprilysin; Abcam; Cambridge, United Kingdom), and anti- β -actin antibodies (Sigma) were used.

2.9. Statistical analysis

Statistical analysis was performed using the 2-tailed Student's *t* test (non-paired/paired) or ANOVA followed by post-hoc Tukey's test. A value of *P*<0.05 was considered statistically significant.

3. RESULTS

3.1. Implanted C3H/10T1/2 cell-derived pericytes survive in mouse brains and increase cerebral microcirculation

First, C3H/10T1/2 cells were differentiated into pericytes by culturing with TGF- β . Consistent with the previous report (Bose et al., 2013), we confirmed that the cells abundantly expressed PDGFR β , a pericyte marker, through immunocytochemistry (Fig. 1A). To examine potential effects of pericyte implantation on A β pathology, C3H/10T1/2 cell-derived pericytes were injected into the cortex of APP/PS1 mice at the age of 18 to 20 months (Fig. 1B). When GFP-expressing C3H/10T1/2 cell-derived pericytes were implanted into the mouse cortex, immunostaining revealed that PDGFR β immunoreactivity was stronger in the ipsilateral pericyte-implanted site than the contralateral site, where PBS was injected as a vehicle control 2 weeks after implantation. Although the localization of GFP-positive cells was limited only around the injected region, most of the cells were immunostained for PDGFR β (Fig. 1C) and closely associated with collagen IV-positive microvessels (Fig. 1D). Since C3H/10T1/2 cells have been reported to form blood vessels when implanted with endothelial cells into mice (Koike et al., 2004), we measured the brain microcirculation in the APP/PS1 mice by LASCA after 3 weeks of cell implantation (Fig. 2A). We found that microcirculation in the specific region of pericyte-implanted hemisphere was considerably increased compared to that of the contralateral hemisphere (Fig. 2B), suggesting that the implantation of pericytes may facilitate microvessel formation. These results indicate that the implanted C3H/10T1/2 cell-derived cells survive in mouse brains and sufficiently preserve the properties of pericytes.

3.2 Implantation of C3H/10T1/2 cell-derived pericytes reduces A β deposition in the hippocampus of APP/PS1 mice

To examine whether pericytes have the ability to eliminate brain A β , we implanted the C3H/10T1/2 cell-derived pericytes or vehicle control (PBS) into brain hemispheres at the lateral area of cortex on external capsule in APP/PS1 mice at 18 to 20 months of age. To avoid the potential effects of scar formation, we investigated A β amounts in the entire hippocampus from each brain hemisphere 3 weeks after the implantation. Both A β 40 and A β 42 levels in detergent-soluble TBSX fractions and detergent-insoluble GDN-HCl soluble fractions were measured by ELISA (Fig. 3). When ipsilateral site and contralateral site were compared, there were no substantial differences in soluble A β 40 (Fig. 3A) and A β 42 (Fig. 3B) levels. On the other hand, we found that amounts of insoluble A β 40 (Fig. 3C) and A β 42 (Fig. 3D) were significantly less in the hippocampus from the pericyte-implanted hemispheres than those from contralateral hemispheres in the APP/PS1 mice. Consistent with the results from ELISA, immunohistochemical analysis also revealed the reduced A β plaque burdens in the hippocampus of the ipsilateral site compared to the contralateral site in the pericyte-implanted APP/PS1 mice (Fig. 4). These results indicate that implantation of C3H/10T1/2 cell-derived pericytes can facilitate the elimination of insoluble A β deposition rather than soluble A β in aged APP/PS1 mice.

3.3. LRP1 mediates the A β clearance in C3H/10T1/2 cell-derived pericytes

To further validate the properties of C3H/10T1/2 cell-derived pericytes in A β metabolism, the pericytes were cultured on brain slices from APP/PS1 mice for 24 hours. While some pericytes co-localized with A β plaques, such co-localization was not specific because the pericytes attached on entire brain slices. (Fig. 5A). Next, the cells were removed using trypsin, and the brain slices were lysed in 5 M GDN-HCl and subjected to A β measurement by ELISA. Since LRP1 is known as a major A β clearance receptor in pericytes (Sagare et al., 2013), we knocked down LRP1 in C3H/10T1/2 cell-derived pericytes through specific siRNA (Fig. 5B). Our results demonstrated that A β 42 levels in the mouse brain slices were considerably reduced in the presence of intact C3H/10T1/2 cell-derived pericytes (Fig. 5B), consistent with results from the *in vivo* pericyte implantation model. While the incubation with LRP1-suppressed pericytes also decreased the A β 42 amounts, the effect was inferior to that with control pericytes (Fig. 5B). In addition, when C3H/10T1/2 cell-derived pericytes were incubated with FAM-labeled soluble A β 40 (Fig. 6A) or A β 42 (Fig. 6B) for 24 hours, FACS analysis showed that the pericytes possessed a sufficient ability to endocytose those A β species, which were partially mediated by LRP1. Amounts of cell-associated FAM-A β 40 (Fig. 6A) and A β 42 (Fig. 6B) were considerably less in LRP1-suppressed C3H/10T1/2 cell-derived pericytes compared to control pericytes, although there was no difference in the phagocytic ability for fluorescently-labeled microsphere beads (Fig. 6C). In addition, knockdown of LRP1 in C3H/10T1/2 cell-derived pericytes did not have substantial effects on A β degrading enzymes including NEP, IDE, MMP2, and MMP9 examined by Western blotting (Fig. 7). Taken together, these results suggest that C3H/10T1/2 cell-derived pericytes can eliminate A β aggregates through endocytic pathways in a manner regulated by LRP1.

4. DISCUSSION

AD is a progressive and irreversible neurodegenerative disease. In the United States, approximately 5.5 million individuals are suffering from Alzheimer's dementia, and this number will likely reach 13.8 million by 2050 (Alzheimer's Association, 2017). In addition, AD is recognized as one of the leading causes of death in the United States; deaths due to AD increased 89% between 2000 and 2014, whereas other causes such as cancer, stroke, and heart disease decreased (Alzheimer's Association, 2017). However, because of the complexity and heterogeneity of AD pathogenesis, no effective treatments are currently available to prevent and/or cure the disease. Given that vascular risk factors, including diabetes mellitus, hypertension, and hypercholesterolemia are considerably correlated with AD development (Santos et al., 2017), better understanding the contributions of cerebrovascular cell types to the disease pathogenesis might be critical to the development of novel therapeutic strategies. Thus, we investigated A β metabolism in C3H/10T1/2 cell-derived pericytes by implanting them into the brains of aged APP/PS1 mice. Microarray analysis has confirmed that C3H/10T1/2 cells can be differentiated into pericytes expressing pericyte specific markers by culturing with TGF- β (Kale et al., 2005). While loss or dysfunction of pericytes could be involved in AD pathogenesis (Halliday et al., 2016; Miners et al., 2017; Sengillo et al., 2013) and compromise A β clearance *in vivo* (Sagare et al., 2013), how restoration of pericyte functions affects AD pathology remains to be

elucidated. To our knowledge, our study for the first time demonstrated that implantation of pericytes into mouse brains induces the beneficial consequence of eliminating A β deposition.

Accumulating evidence has shown that transplantation of neural stem cells or MSCs through intracranial or intravenous injection can restore cognitive functions in amyloid AD mouse models by supplying neurotrophic growth factors and modulating immune responses (Duncan and Valenzuela, 2017; Hunsberger et al., 2016). In addition, MSC transplantation increases A β degrading enzymes and activates microglia, thereby ameliorating A β pathology in the mouse models (Kim et al., 2013; Lee et al., 2010; Yang et al., 2013). Our results also suggest that MSC-derived pericytes have the ability to eliminate brain A β through phagocytosis or protease-mediated degradation. Thus, it is possible that transplanted MSCs facilitate A β clearance by differentiating into vascular mural cell lineage. Currently, several phase I and II clinical trials using MSCs are ongoing for AD therapy (Duncan and Valenzuela, 2017; Hunsberger et al., 2016), although a completed phase I study failed to detect significant positive effects on cognitive performances (Kim et al., 2015). Further studies should clarify whether or not implantation of MSCs differentiated into vascular mural cells has better effects on cognition and AD-related pathologies.

Of note, pericytes have been shown to contribute to tissue repair by regulating angiogenesis and fibrosis (Wong et al., 2015). Indeed, intramyocardial injection of pericytes or pericyte progenitor cells could improve ischemic heart repair through activation of the angiogenesis process in acute myocardial infarction model mice (Chen et al., 2013; Katare et al., 2011). In addition, intramuscular injection of blood-derived pericyte-like cells also sufficiently enhanced revascularization in a mouse hind limb ischemia model (Blocki et al., 2015). Consistent with those findings, our results showed that intracranial implantation of C3H/10T1/2 cell-derived pericytes considerably increased brain microcirculation in an amyloid mouse model, suggesting accelerated microvessel formation in their brains. Given that reduction of cerebral blood flow likely precedes the onset of evident symptoms in AD (Iturria-Medina et al., 2016; Kisler et al., 2017), transplantation of pericytes may have an advantage in cell-based AD therapy not only by accelerating A β clearance, but also by restoring cerebral circulation in the disease. Interestingly, there is a study showing that implantation of VEGF-secreting microencapsulated fibroblasts into the brain increases cerebrovascular density, decreases brain A β deposition, and ameliorates behavioral impairment in APP/PS1 mice (Spuch et al., 2010). Because C3H/10T1/2 cell-derived pericytes are known to produce VEGF in abundance (Darland et al., 2003), the pericyte implantation may induce therapeutic effects by providing VEGF, although further studies are needed.

While increased brain microcirculation may potentially impact A β metabolism in the mouse model, our *ex vivo* and *in vitro* experiments indicate that C3H/10T1/2 cell-derived pericytes possibly contribute to A β clearance through phagocytosis. C3H/10T1/2 cell-derived pericytes efficiently removed A β deposition from mouse brain slices, where the function was diminished in the absence of LRP1. We also found knockdown of LRP1 suppressed cellular A β uptake in the pericytes, although amounts of those A β -degrading enzymes were not affected. LRP1 is a large and efficient endocytic receptor expressed in diverse brain cell

types, and it mediates the endocytosis for over 40 ligands, including A β (Kanekiyo and Bu, 2014; Shinohara et al., 2017). Deficiency of LRP1 in neurons (Kanekiyo et al., 2013), astrocytes (Liu et al., 2017), endothelial cells (Storck et al., 2016), or vascular smooth muscle cells (Bell et al., 2009; Kanekiyo et al., 2012) has been shown to exacerbate A β pathology in mouse models. Consistent with our results from C3H/10T1/2 cell-derived pericytes, LRP1-specific antibody or LRP1 knockdown through siRNA also interfered with A β uptake in isolated mouse brain pericytes (Sagare et al., 2013). Thus, endocytosis and lysosomal degradation through LRP1 are predicted to substantially contribute to pericyte-mediated A β clearance. However, it remains unclear how the LRP1-mediated pathway is predominantly involved in the A β clearance through C3H/10T1/2 cell-derived pericyte implantation due to technical limitations, as long-term LRP1 deficiency will likely influence survival and proliferation of the pericytes.

In conclusion, we demonstrated that C3H/10T1/2 cell-derived pericytes have the capacity of eliminating A β through several mechanisms. As pericyte functions can be preserved when implanted into the brains, the transplantation of MSC-derived pericytes or pericyte-like cells could be a promising therapeutic approach for AD. Further studies should rule out their potential complications, in particular their possible contributions to tumorigenesis. Alternatively, restoring endogenous pericyte functions in AD brains may also be a potential strategy to prevent and/or treat the disease.

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ABBREVIATIONS

AD	Alzheimer's disease
Aβ	amyloid- β
CAA	cerebral amyloid angiopathy
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FAM	5-carboxyfluorescein
FBS	fetal bovine serum
GDN-HCl	guanidine hydrochloride
GFP	green fluorescent protein

IDE	insulin degrading enzyme
LASCA	laser speckle contrast analysis
LRP1	low-density lipoprotein receptor-related protein 1
MMP	metalloproteinase
MSC	mesenchymal stem cell
NEP	neprilysin
PBS	phosphate-buffered saline
PDGFRβ	platelet-derived growth factor receptor β
TBSX	tris-buffered-saline containing 1% triton X
TGF-β	transforming growth factor- β

References

- Alzheimer's, Association. 2017 Alzheimer's disease facts and figures. *Alzheimers Dement.* 2017; 13:325–373.
- Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell.* 2011; 21:193–215. [PubMed: 21839917]
- Bell RD, Deane R, Chow N, Long X, Sagare A, Singh I, Streb JW, Guo H, Rubio A, Van Nostrand W, Miano JM, Zlokovic BV. SRF and myocardin regulate LRP-mediated amyloid-beta clearance in brain vascular cells. *Nat Cell Biol.* 2009; 11:143–153. [PubMed: 19098903]
- Blocki A, Wang Y, Koch M, Goralczyk A, Beyer S, Agarwal N, Lee M, Moonshi S, Dewavrin JY, Peh P, Schwarz H, Bhakoo K, Raghunath M. Sourcing of an alternative pericyte-like cell type from peripheral blood in clinically relevant numbers for therapeutic angiogenic applications. *Mol Ther.* 2015; 23:510–522. [PubMed: 25582709]
- Bose A, Barik S, Banerjee S, Ghosh T, Mallick A, Bhattacharyya Majumdar S, Goswami KK, Bhuniya A, Banerjee S, Baral R, Storkus WJ, Dasgupta PS, Majumdar S. Tumor-derived vascular pericytes anergize Th cells. *J Immunol.* 2013; 191:971–981. [PubMed: 23785117]
- Chen CW, Okada M, Proto JD, Gao X, Sekiya N, Beckman SA, Corselli M, Crisan M, Saparov A, Tobita K, Peault B, Huard J. Human pericytes for ischemic heart repair. *Stem Cells.* 2013; 31:305–316. [PubMed: 23165704]
- Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badyak S, Buhning HJ, Giacobino JP, Lazzari L, Huard J, Peault B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell.* 2008; 3:301–313. [PubMed: 18786417]
- Darland DC, Massingham LJ, Smith SR, Piek E, Saint-Geniez M, D'Amore PA. Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev Biol.* 2003; 264:275–288. [PubMed: 14623248]
- Duncan T, Valenzuela M. Alzheimer's disease, dementia, and stem cell therapy. *Stem Cell Res Ther.* 2017; 8:111. [PubMed: 28494803]
- Guimaraes-Camboa N, Cattaneo P, Sun Y, Moore-Morris T, Gu Y, Dalton ND, Rockenstein E, Masliah E, Peterson KL, Stallcup WB, Chen J, Evans SM. Pericytes of Multiple Organs Do Not Behave as Mesenchymal Stem Cells In Vivo. *Cell Stem Cell.* 2017; 20:345–359. e345. [PubMed: 28111199]
- Halliday MR, Rege SV, Ma Q, Zhao Z, Miller CA, Winkler EA, Zlokovic BV. Accelerated pericyte degeneration and blood-brain barrier breakdown in apolipoprotein E4 carriers with Alzheimer's disease. *J Cereb Blood Flow Metab.* 2016; 36:216–227. [PubMed: 25757756]

- Hartmann DA, Underly RG, Grant RI, Watson AN, Lindner V, Shih AY. Pericyte structure and distribution in the cerebral cortex revealed by high-resolution imaging of transgenic mice. *Neurophotonics*. 2015; 2:041402. [PubMed: 26158016]
- Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol*. 1998; 141:805–814. [PubMed: 9566978]
- Hunsberger JG, Rao M, Kurtzberg J, Bulte JWM, Atala A, LaFerla FM, Greely HT, Sawa A, Gandy S, Schneider LS, Doraiswamy PM. Accelerating stem cell trials for Alzheimer's disease. *Lancet Neurol*. 2016; 15:219–230. [PubMed: 26704439]
- Iturria-Medina Y, Sotero RC, Toussaint PJ, Mateos-Perez JM, Evans AC. Alzheimer's Disease Neuroimaging I. Early role of vascular dysregulation on late-onset Alzheimer's disease based on multifactorial data-driven analysis. *Nat Commun*. 2016; 7:11934. [PubMed: 27327500]
- Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL, Younkin SG, Borchelt DR. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet*. 2004; 13:159–170. [PubMed: 14645205]
- Kale S, Hanai J, Chan B, Karihaloo A, Grotendorst G, Cantley L, Sukhatme VP. Microarray analysis of in vitro pericyte differentiation reveals an angiogenic program of gene expression. *FASEB J*. 2005; 19:270–271. [PubMed: 15579670]
- Kanekiyo T, Bu G. The low-density lipoprotein receptor-related protein 1 and amyloid-beta clearance in Alzheimer's disease. *Front Aging Neurosci*. 2014; 6:93. [PubMed: 24904407]
- Kanekiyo T, Cirrito JR, Liu CC, Shinohara M, Li J, Schuler DR, Shinohara M, Holtzman DM, Bu G. Neuronal clearance of amyloid-beta by endocytic receptor LRP1. *J Neurosci*. 2013; 33:19276–19283. [PubMed: 24305823]
- Kanekiyo T, Liu CC, Shinohara M, Li J, Bu G. LRP1 in brain vascular smooth muscle cells mediates local clearance of Alzheimer's amyloid-beta. *J Neurosci*. 2012; 32:16458–16465. [PubMed: 23152628]
- Kanekiyo T, Xu H, Bu G. ApoE and Abeta in Alzheimer's disease: accidental encounters or partners? *Neuron*. 2014; 81:740–754. [PubMed: 24559670]
- Kapasi A, DeCarli C, Schneider JA. Impact of multiple pathologies on the threshold for clinically overt dementia. *Acta Neuropathol*. 2017; 134:171–186. [PubMed: 28488154]
- Katara R, Riu F, Mitchell K, Gubernator M, Campagnolo P, Cui Y, Fortunato O, Avolio E, Cesselli D, Beltrami AP, Angelini G, Emanuelli C, Madeddu P. Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. *Circ Res*. 2011; 109:894–906. [PubMed: 21868695]
- Kim HJ, Seo SW, Chang JW, Lee JI, Kim CH, Chin J, Choi SJ, Kwon H, Yun HJ, Lee JM, Kim ST, Choe YS, Lee KH, Na DL. Stereotactic brain injection of human umbilical cord blood mesenchymal stem cells in patients with Alzheimer's disease dementia: A phase 1 clinical trial. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*. 2015; 1:95–102.
- Kim KS, Kim HS, Park JM, Kim HW, Park MK, Lee HS, Lim DS, Lee TH, Chopp M, Moon J. Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging*. 2013; 34:2408–2420. [PubMed: 23623603]
- Kisler K, Nelson AR, Montagne A, Zlokovic BV. Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer disease. *Nat Rev Neurosci*. 2017; 18:419–434. [PubMed: 28515434]
- Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK. Tissue engineering: creation of long-lasting blood vessels. *Nature*. 2004; 428:138–139. [PubMed: 15014486]
- Koistinaho M, Lin S, Wu X, Esterman M, Koger D, Hanson J, Higgs R, Liu F, Malkani S, Bales KR, Paul SM. Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides. *Nat Med*. 2004; 10:719–726. [PubMed: 15195085]
- LaFerla FM, Green KN, Oddo S. Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci*. 2007; 8:499–509. [PubMed: 17551515]
- Lee H, Bae JS, Jin HK. Human umbilical cord blood-derived mesenchymal stem cells improve neurological abnormalities of Niemann-Pick type C mouse by modulation of neuroinflammatory condition. *J Vet Med Sci*. 2010; 72:709–717. [PubMed: 20124762]

- Liu CC, Hu J, Zhao N, Wang J, Wang N, Cirrito JR, Kanekiyo T, Holtzman DM, Bu G. Astrocytic LRP1 Mediates Brain Abeta Clearance and Impacts Amyloid Deposition. *J Neurosci.* 2017; 37:4023–4031. [PubMed: 28275161]
- Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP. The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. *Glia.* 2010; 58:1094–1103. [PubMed: 20468051]
- Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science.* 2010; 330:1774. [PubMed: 21148344]
- Miners JS, Schulz I, Love S. 2017 Differing associations between Abeta accumulation, hypoperfusion, blood-brain barrier dysfunction and loss of PDGFRB pericyte marker in the precuneus and parietal white matter in Alzheimer's disease. *J Cereb Blood Flow Metab.* 271678X17690761
- Morris AW, Sharp MM, Albargothy NJ, Fernandes R, Hawkes CA, Verma A, Weller RO, Carare RO. Vascular basement membranes as pathways for the passage of fluid into and out of the brain. *Acta neuropathologica.* 2016; 131:725–736. [PubMed: 26975356]
- Musiek ES, Holtzman DM. Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. *Nat Neurosci.* 2015; 18:800–806. [PubMed: 26007213]
- Querfurth HW, LaFerla FM. Alzheimer's disease. *N Engl J Med.* 2010; 362:329–344. [PubMed: 20107219]
- Reznikoff CA, Bertram JS, Brankow DW, Heidelberger C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* 1973; 33:3239–3249. [PubMed: 4796800]
- Rustenhoven J, Jansson D, Smyth LC, Dragunow M. Brain Pericytes As Mediators of Neuroinflammation. *Trends Pharmacol Sci.* 2017; 38:291–304. [PubMed: 28017362]
- Sagare AP, Bell RD, Zhao Z, Ma Q, Winkler EA, Ramanathan A, Zlokovic BV. Pericyte loss influences Alzheimer-like neurodegeneration in mice. *Nat Commun.* 2013; 4:2932. [PubMed: 24336108]
- Saido T, Leissring MA. Proteolytic degradation of amyloid beta-protein. *Cold Spring Harb Perspect Med.* 2012; 2:a006379. [PubMed: 22675659]
- Santos CY, Snyder PJ, Wu WC, Zhang M, Echeverria A, Alber J. Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular risk: A review and synthesis. *Alzheimers Dement (Amst).* 2017; 7:69–87. [PubMed: 28275702]
- Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med.* 2016; 8:595–608. [PubMed: 27025652]
- Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, Zlokovic BV. Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathol.* 2013; 23:303–310. [PubMed: 23126372]
- Shinohara M, Tachibana M, Kanekiyo T, Bu G. Role of LRP1 in the pathogenesis of Alzheimer's disease: evidence from clinical and preclinical studies. *J Lipid Res.* 2017; 58:1267–1281. [PubMed: 28381441]
- Sims DE. The pericyte--a review. *Tissue Cell.* 1986; 18:153–174. [PubMed: 3085281]
- Spuch C, Antequera D, Portero A, Orive G, Hernandez RM, Molina JA, Bermejo-Pareja F, Pedraz JL, Carro E. The effect of encapsulated VEGF-secreting cells on brain amyloid load and behavioral impairment in a mouse model of Alzheimer's disease. *Biomaterials.* 2010; 31:5608–5618. [PubMed: 20430437]
- Storck SE, Meister S, Nahrath J, Meissner JN, Schubert N, Di Spiezio A, Baches S, Vandenbroucke RE, Bouter Y, Prikulis I, Korth C, Weggen S, Heimann A, Schwaninger M, Bayer TA, Pietrzik CU. Endothelial LRP1 transports amyloid-beta(1–42) across the blood-brain barrier. *J Clin Invest.* 2016; 126:123–136. [PubMed: 26619118]
- Sweeney MD, Ayyadurai S, Zlokovic BV. Pericytes of the neurovascular unit: key functions and signaling pathways. *Nat Neurosci.* 2016; 19:771–783. [PubMed: 27227366]
- Thal DR, Walter J, Saido TC, Fandrich M. Neuropathology and biochemistry of Abeta and its aggregates in Alzheimer's disease. *Acta Neuropathol.* 2015; 129:167–182. [PubMed: 25534025]

- Thomas H, Cowin AJ, Mills SJ. The Importance of Pericytes in Healing: Wounds and other Pathologies. *Int J Mol Sci.* 2017;18.
- Wong SP, Rowley JE, Redpath AN, Tilman JD, Fellous TG, Johnson JR. Pericytes, mesenchymal stem cells and their contributions to tissue repair. *Pharmacol Ther.* 2015; 151:107–120. [PubMed: 25827580]
- Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, Husemann J. Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. *Nat Med.* 2003; 9:453–457. [PubMed: 12612547]
- Xiang X, Werner G, Bohrmann B, Liesz A, Mazaheri F, Capell A, Feederle R, Knuesel I, Kleinberger G, Haass C. TREM2 deficiency reduces the efficacy of immunotherapeutic amyloid clearance. *EMBO Mol Med.* 2016; 8:992–1004. [PubMed: 27402340]
- Yang H, Xie Z, Wei L, Yang H, Yang S, Zhu Z, Wang P, Zhao C, Bi J. Human umbilical cord mesenchymal stem cell-derived neuron-like cells rescue memory deficits and reduce amyloid-beta deposition in an AbetaPP/PS1 transgenic mouse model. *Stem Cell Res Ther.* 2013; 4:76. [PubMed: 23826983]

Fig. 1. Implantation of C3H/10T1/2 cell-derived pericytes into the mouse cortex

A, C3H/10T1/2 cell-derived pericytes were immunostained for a pericytic marker PDGFR β with the counterstaining for F-actin (Alexa Fluor 568 phalloidin) and nucleus (DAPI). B, The pericytes (2×10^5 cells) and PBS were stereotactically injected into the right and left cortex (2.0 mm lateral and 2.0 mm posterior from the bregma, depth 2.0 mm from surface of the skull) of APP/PS1 mice at the age of 18 to 20 months, respectively. C and D, The pericytes expressing GFP were implanted, and the brain sections were immunostained for PDGFR β (C) and a vascular basement membrane marker collagen IV (D) 2 weeks after the injection. Scale bar = 50 μ m.

Fig. 2. Implantation of C3H/10T1/2 cell-derived pericytes increases brain microcirculation
 A, Brain microcirculation was measured by laser speckle contrast analysis (LASCA) technology in the brains of APP/PS1 mice (18–20 months old) 3 weeks after stereotactic implantation of the pericytes. B, The region of interest with a consistent area size was selected around the injection site in the ipsilateral and contralateral hemisphere, and the average cerebral microcirculation rate was compared. ** $P < 0.01$ by paired Student t test (n=8).

Fig. 3. Implantation of C3H/10T1/2 cell-derived pericytes reduces insoluble Aβ levels in the hippocampus of APP/PS1 mice

The pericytes and PBS were injected into the brains of APP/PS1 mice (18–20 months old), and Aβ levels in the hippocampus were quantified by ELISA 3 weeks after the injection. Levels of Aβ40 and Aβ42 in TBSX fraction (A and B) and GDN-HCl fraction (C and D) were compared between the pericyte-implanted ipsilateral side and PBS-injected contralateral side (n=9). * $P < 0.05$ by paired Student t test; n.s., not significant.

Fig. 4. Implantation of C3H/10T1/2 cell-derived pericytes ameliorates A β plaque burden in the hippocampus of APP/PS1 mice

A, Brain sections from APP/PS1 mice (18 months old) 3 weeks after stereotactic implantation of the pericytes were immunostained for A β deposition. Scale bar = 200 μ m. B, The A β plaque burden in the hippocampus was compared between ipsilateral and contralateral sides (n=3). ** P <0.01 by paired Student t test.

Fig. 5. C3H/10T1/2 cell-derived pericytes eliminate Aβ from mouse brain slices through LRP1
 A, The CM-Dil-labeled pericytes (6×10^5 cells) were cultured on brain slices from APP/PS1 mice (12 months old) for 24 hours, and the Aβ in the slice was immunostained with pan-Aβ antibody. Scale bar = 200 μm. B, The pericytes treated with control or LRP1-specific siRNA were cultured on the continuous brain sections from APP/PS1 mice for 24 hours. The amounts of Aβ42 in the brain slices were measured by ELISA after removing the pericytes by trypsin treatment. Data are plotted as mean $\pm$ S.E.M. (n=4). ** $P < 0.01$; *** $P < 0.001$ by ANOVA followed by Tukey's post-hoc test.

Fig. 6. LRP1 mediates cellular uptake of A β in C3H10T1/2 cell-derived pericytes

The pericytes treated with control or LRP1-siRNA were incubated with FAM-A β 40 (A), FAM-A β 42 (B), or fluorescent microspheres (C) for 24 hours. The amounts of cell-associated A β or microspheres were analyzed by FACS. Data are plotted as mean $\pm$ S.E.M. (n=3). * $P < 0.05$ by Student t test; n.s., not significant.

Fig. 7. LRP1 knockdown has no impact on the level of Aβ-degrading enzymes in C3H10T1/2 cell-derived pericytes

The protein levels of Aβ-degrading enzymes (MMP2, MMP9, NEP, and IDE) in the pericytes treated with control or LRP1-siRNA were analyzed by Western blotting. Data are plotted as mean ± S.E.M. (n=3). n.s., not significant.