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Novel human neuronal tau model exhibiting neurofibrillary tangles and transcellular propagation

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A B S T R A C T

Taupathies are a class of neurodegenerative diseases, including Alzheimer's disease, frontotemporal dementia and progressive supranuclear palsy, which are associated with the pathological aggregation of tau protein into neurofibrillary tangles (NFT). Studies have characterized tau as a "prion-like" protein given its ability to form distinct, stable amyloid conformations capable of transcellular and multigenerational propagation in clonal fashion. It has been proposed that progression of tauopathy could be due to the prion-like propagation of tau, suggesting the possibility that end-stage pathologies, like NFT formation, may require an instigating event such as tau seeding. To investigate this, we applied a novel human induced pluripotent stem cell (hiPSC) system we have developed to serve as a human neuronal model. We introduced the tau repeat domain (tau-RD) with P301L and V337M (tau-RD-LM) mutations into hiPSC-derived neurons and observed expression of tau-RD at levels similar to total tau in postmortem AD brains. Tau aggregation occurred without the addition of recombinant tau fibrils. The conditioned media from tau-RD cultures contained tau-RD seeds, which were capable of inducing aggregate formation in homotypic mode in non-transduced recipient neuronal cultures. The resultant NFTs were thioflavin-positive, silver stain-positive, and assumed fibrillary appearance on transmission electron microscopy (TEM) with immunogold, which revealed paired helical filament 1 (PHF1)-positive NFTs, representing possible recruitment of endogenous tau in the aggregates. Functionally, expression of tau-RD caused neurotoxicity that manifested as axon retraction, synaptic density reduction, and enlargement of lysosomes. The results of our hiPSC study were reinforced by the observation that Tau-RD-LM is excreted in exosomes, which mediated the transfer of human tau to wild-type mouse neurons *in vivo*. Our hiPSC human neuronal system provides a model for further studies of tau aggregation and pathology as well as a means to study transcellular propagation and related neurodegenerative mechanisms.

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1. Introduction

Tau pathology is characterized by the presence of abnormally hyperphosphorylated tau protein that has misfolded into insoluble aggregates known as neurofibrillary tangles (NFT). Tau protein is abundant in neurons, acting to stabilize microtubules and facilitate axonal transport. When hyperphosphorylated, tau has a heightened tendency to misfold, losing its ability to interact with microtubules (Iqbal et al., 2009). An accumulation of pathologically aggregated tau protein is often observed in postmortem brains from patients suffering from tauopathies. In Alzheimer's disease (AD), there is build-up of both amyloid plaques and neurofibrillary tangles, with the appearance of NFTs

Abbreviations: AD, Alzheimer's disease; NFT, neurofibrillary tangles; hiPSC, human induced pluripotent stem cell; PHF, paired helical filament; FTD, frontotemporal dementia; NDC, non-demented control; PS-1, presenilin 1; YFP, yellow fluorescent protein.

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correlated with clinical expression of dementia (Alafuzoff et al., 1987; Arriagada et al., 1992; Knopman et al., 2013).

The processing, misfolding and aggregation of tau are all implicated in the progression of neurodegeneration. Abnormal hyperphosphorylation is an early pathological event that precedes successive truncations at both the N- and C-termini, leaving the tau repeat domain (tau-RD) at the core of the cleaved protein (Garcia-Sierra et al., 2003; Mondragon-Rodriguez et al., 2008). The repeat domain of tau, which interacts with microtubules, aggregates more readily, likely due to tau-RD's propensity to form β-pleated sheets and assume amyloid-patterned aggregates (Mandelkow et al., 2007). Tau-RD has been found to be a primary component of NFTs (Endoh et al., 1993; Novak et al., 1991).

Patient (human) derived induced pluripotent stem cells (hiPSC) have emerged as a powerful new tool to model human neurodegenerative diseases (Yuan and Shaner, 2013). Human neuronal cultures are grown from hiPSC derived from cells obtained through skin biopsies of carriers of tau mutations associated with frontotemporal dementia (FTD). Previous human models of tauopathy have demonstrated early pathological phenotypes, such as hyperphosphorylated tau and pre-tangle AT-8 positive puncta, yet failed to manifest NFT formation (Fong et al., 2013; Iovino et al., 2015). NFTs were also absent following overexpression of full-length 4-repeat (4R) tau (Mertens et al., 2013). Tau aggregation, as demonstrated on Western blot, can be induced with exogenous tau fibrils in hiPSCs overexpressing full-length tau; albeit, the resultant NFTs do not exhibit thioflavin staining, a confirmation of β-pleated sheet formation (Medda et al., 2016; Verheyen et al., 2015). However, overexpression of both the presenilin-1 (PS-1) familial mutations and the amyloid precursor protein (APP) have been shown to initiate hyperphosphorylation of tau as well as formation of SDS-insoluble, silver stain-positive NFTs in 3-dimensional (3-D) cultures (Choi et al., 2014).

Tau-RD has been shown to spread from cell-to-cell (Frost et al., 2009), and has been demonstrated to form distinct aggregate species, which can be transmitted to other cells, like prions (Sanders et al., 2014). It has further been demonstrated that aggregates found in human brain tissue from patients with tauopathies, can induce new aggregates in a naïve mouse brain (Clavaguera et al., 2013; Sanders et al., 2014). Recently, we demonstrated that neuronally-derived exosomes (NDEs) from AD patients seeded tau aggregation and induced AD-like neuropathology in normal mouse brains (Winston et al., 2016). Exosomes represent a subclass of secreted membrane vesicles that have been shown to shuttle protein cargo and mRNAs (Valadi et al., 2007) for intracellular communication between cells, to eliminate damaged or excess protein cargo (Raposo and Stoorvogel, 2013), and to mediate the propagation of Aβ peptides (Rajendran et al., 2006) and prion proteins (Fevrier et al., 2004).

We hypothesized that end-stage pathologies, like NFT formation, require an instigating event such as prion-like seeding, which may be lacking in previous human neuronal cell culture systems. We observed that tau-RD is excreted from the neurons inside exosomes, which mediated the transfer of tau to naïve mouse neurons *in vivo*. We then demonstrated the intercellular transfer of tau in a novel model of human tauopathy. In our model, expression of tau-RD was sufficient to initiate NFT production, with continued spread of tau pathology propagating between neurons *via* the intracellular media. This hiPSC system can serve as a human neuronal model for studying tauopathy, neurodegeneration, and the mechanisms of pathological seed propagation both *in vitro* and *in vivo*.

2. Material and methods

2.1. Generation of neuronal cultures

Neuronal cultures derived from non-demented controls (NDC) and PS-1 A246E mutation carriers (causative of early onset familial AD) were generated per a previously published protocol (Yuan et al., 2011). Neural stem cells (NSC) were seeded at a density of 150,000 cells/cm^2 on either Matrigel-coated [70 μg/mL] plastic cell culture dishes or onto glass coverslips coated with both polyornithine [20 μg/mL] and Matrigel. NSCs were grown to 80% confluency, at which time neuronal differentiation was initiated through withdrawal of fibroblast growth factor (bFGF) from the NSC media (DMEM-12, 1% N-2, 2% B-27, Pen-Strep, 20 ng/mL bFGF).

2.2. Preparation of the soluble and insoluble portion of the protein

We followed the previously published protocol (Higuchi et al., 2002; Rissman et al., 2004) with adaptation for cell culture. NDC-derived cells were collected directly from confluent 6-well plates and subjected to sequential extraction with reassembly buffer (RAB), radioimmunoprecipitation assay buffer (RIPA), and formic acid. Collected cells were re-suspended in RAB [100 mM MES, 1 mM EGTA, 0.5 mM $MgSO_4$, 0.75 mM NaCl, protease inhibitor cocktail set 1 (Calbiochem), phosphatase inhibitor single-use cocktail (Thermo Fisher)] and incubated on ice for 30 min. Samples were then centrifuged at 40,000 ×*g* for 40 min at 4 °C. Supernatant was collected and the pellet was re-suspended with RIPA buffer [50 mM Tris pH 7.4, 150 mM NaCl, Triton-100×, 1 mM EDTA pH 7.5, 0.1% SDS, 0.25% Na-Deoxycholate, 1 mM EGTA, protease inhibitor cocktail set 1 (Calbiochem, #539131), phosphatase inhibitor single-use cocktail (Thermo Fisher, #78428)] and centrifuged at 40,000 ×*g* for 20 min at 4 °C. Supernatant was again collected and the cell pellet was re-suspended in 50 μL of 70% formic acid and sonicated 3 times (10 s/sonication), prior to centrifugation at 135,000 ×*g* for 1 h at 4 °C. The sample was diluted 1:20 in formic acid neutralization buffer [1 M Tris, 0.5 M NaH_2PO_4] and its concentration determined by bicinchoninic acid protein assay (BCA).

2.3. Western blot

10–20 μg of soluble or insoluble protein was collected as previously described, diluted with 4× Laemmli sample buffer (BioRad), and resolved on 12% SDS polyacrylamide gel. Protein was transferred to polyvinylidene difluoride (PVDF) membranes using Trans-Blot SD Semi-Dry transfer cell (BioRad). Blots were blocked in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) at room temperature for 45 min on a platform shaker. Primary antibodies were applied overnight at 4 °C in blocking solution. The primary antibodies used were paired helical filament 1 (PHF1) (1:2000; gift from Peter Davies), tau-RD rabbit anti-tau antibody (1:2000; Abcam ab64193), total tau-K9JA (1:30,000; DAKO), CP13 (1: 200; gift from Peter Davies), Alz50 (1:250; gift from Peter Davies), β-III-tubulin (1:4000; Covance), β-actin (1:8000; Sigma). Following a series of washes, blots were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:2000; Thermo-Fisher). Enhanced chemiluminescence (ECL) substrate was applied before visualization by GelDoc (BioRad). Blot analysis was performed on ImageLab 5.2.1 (BioRad). Graphs were created using Prism (GraphPad).

2.4. Immunofluorescence studies

NDC and PS-1-derived cultures were fixed at room temperature with 4% paraformaldehyde (Lamp2 samples were post-treated with cold 100% methanol for 5 min at −20 °C) 4 days post-transduction or transfection. Fixed cultures were blocked for 45 min at room temperature in blocking solution (3% bovine serum albumin (BSA), 0.3% Triton X-100, 1× phosphate buffered saline (PBS)), stained for 2 h at room temperature or overnight at 4 °C, and washed twice with PBS (5 min/wash). The primary antibodies used were Map2b (1:500; Sigma), β-III-tubulin (1:500; Covance), synapsin (1:2000; Abcam), lamp2 (1:500; DSHB). All secondary antibodies were diluted 1:1000 in blocking solution and applied for 45 min at room temperature. Antifade

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