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Alzheimer's disease amyloid-β pathology in the lens of the eye

Juliet A. Moncaster[a,b], **Robert D. Moir**[c], **Mark A. Burton**[a], **Oliver Chadwick**[c], **Olga Minaeva**[a,b], **Victor E. Alvarez**[b,d], **Maria Ericsson**[e], **John I. Clark**[f], **Ann C. McKee**[b,d], **Rudolph E. Tanzi**[c], **Lee E. Goldstein**[a,b,*]

[a]Molecular Aging and Development Laboratory, Boston University School of Medicine, Boston, MA, 02118, USA

[b]Boston University Alzheimer's Disease Research Center, Boston University School of Medicine, 72 East Concord Street, B-7800 Boston, MA, 02118, USA

[c]Genetics and Aging Research Unit, Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, 02129, USA

[d]Edith Nourse Rogers Memorial Veterans' Hospital, Bedford, MA, 01730, USA

[e]Electron Microscopy Facility, Harvard Medical School, Boston, MA, 02115, USA

[f]Departments of Biological Structure and Ophthalmology, University of Washington, Seattle, WA, 98195, USA

Abstract

Neuropathological hallmarks of Alzheimer's disease (AD) include pathogenic accumulation of amyloid-β (Aβ) peptides and age-dependent formation of amyloid plaques in the brain. AD-associated Aβ neuropathology begins decades before onset of cognitive symptoms and slowly progresses over the course of the disease. We previously reported discovery of Aβ deposition, β-amyloidopathy, and co-localizing supranuclear cataracts (SNC) in lenses from people with AD, but not other neurodegenerative disorders or normal aging. We confirmed AD-associated Aβ molecular pathology in the lens by immunohistopathology, amyloid histochemistry, immunoblot analysis, epitope mapping, immunogold electron microscopy, quantitative immunoassays, and

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*Corresponding author. Molecular Aging & Development Laboratory, Boston University, School of Medicine, 670 Albany Street, Boston, MA, 02118, USA. lgold@bu.edu (L.E. Goldstein).

Authors' contributions

Juliet A. Moncaster designed and performed the experiments, analyzed the data, prepared figures and tables, wrote and reviewed drafts of the paper. Robert D. Moir designed and performed the experiments, analyzed the data, prepared figures and tables. Mark A. Burton performed the experiments. Maria Ericsson performed the experiments and reviewed drafts of the paper. Oliver Chadwick performed the experiments. Olga Minaeva analyzed the data, prepared figures and tables, and reviewed drafts of the paper. Victor E. Alvarez contributed reagents, materials, analysis tools, and reviewed drafts of the paper. John I. Clark contributed reagents, materials, analysis tools, and reviewed drafts of the paper. Ann C. McKee contributed reagents, materials, and analysis tools. Rudolph E. Tanzi contributed reagents, materials, analysis tools, and reviewed drafts of the paper. Lee E. Goldstein conceived and designed and performed the experiments, analyzed the data, prepared figures and tables, wrote and reviewed drafts of the paper.

Declaration of competing interest

Lee E. Goldstein declares the following competing interest: Cognoptix, Inc., Marlborough, MA 01752 USA. All other authors declare that they have no known competing financial or personal interests that could influence or appear to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.108974.

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tryptic digest mass spectrometry peptide sequencing. Ultrastructural analysis revealed that AD-associated Aβ deposits in AD lenses localize as electron-dense microaggregates in the cytoplasm of supranuclear (deep cortex) fiber cells. These Aβ microaggregates also contain αB-crystallin and scatter light, thus linking Aβ pathology and SNC phenotype expression in the lenses of people with AD. Subsequent research identified Aβ lens pathology as the molecular origin of the distinctive cataracts associated with Down syndrome (DS, trisomy 21), a chromosomal disorder invariantly associated with early-onset Aβ accumulation and Aβ amyloidopathy in the brain. Investigation of 1249 participants in the Framingham Eye Study found that AD-associated quantitative traits in brain and lens are co-heritable. Moreover, AD-associated lens traits preceded MRI brain traits and cognitive deficits by a decade or more and predicted future AD. A genome-wide association study of bivariate outcomes in the same subjects identified a new AD risk factor locus in the *CTNND2* gene encoding δ-catenin, a protein that modulates Aβ production in brain and lens. Here we report identification of AD-related human Aβ (hAβ) lens pathology and age-dependent SNC phenotype expression in the Tg2576 transgenic mouse model of AD. Tg2576 mice express Swedish mutant human amyloid precursor protein (APP-Swe), accumulate hAβ peptides and amyloid pathology in the brain, and exhibit cognitive deficits that slowly progress with increasing age. We found that Tg2576 trangenic (Tg^+) mice, but not non-transgenic (Tg^-) control mice, also express human APP, accumulate hAβ peptides, and develop hAβ molecular and ultrastructural pathologies in the lens. Tg2576 Tg^+ mice exhibit age- dependent Aβ supranuclear lens opacification that recapitulates lens pathology and SNC phenotype expression in human AD. In addition, we detected hAβ in conditioned medium from lens explant cultures prepared from Tg^+ mice, but not Tg^- control mice, a finding consistent with constitutive hAβ generation in the lens. *In vitro* studies showed that hAβ promoted mouse lens protein aggregation detected by quasi-elastic light scattering (QLS) spectroscopy. These results support mechanistic (genotype-phenotype) linkage between Aβ pathology and AD-related phenotypes in lens and brain. Collectively, our findings identify Aβ pathology as the shared molecular etiology of two age-dependent AD-related cataracts associated with two human diseases (AD, DS) and homologous murine cataracts in the Tg2576 transgenic mouse model of AD. These results represent the first evidence of AD-related Aβ pathology outside the brain and point to lens Aβ as an optically-accessible AD biomarker for early detection and longitudinal monitoring of this devastating neurodegenerative disease.

Keywords

Aβ; Alzheimer's disease; Amyloid-β; Amyloid precursor protein; Biomarker; Cataract; Down syndrome; Lens; Protein aggregation; Quasi-elastic light scattering; Transgenic mouse model

1. Introduction

1.1. Alzheimer's disease amyloid-β (Aβ) molecular pathology in the brain

Alzheimer's disease (AD) is a leading cause of morbidity and mortality and the most common cause of dementia worldwide (Prince et al., 2015; Patterson, 2018; Gao et al., 2019; Nichols et al., 2019; Guerchet et al., 2020; Livingston et al., 2020; Ahmad et al., 2021; Alzheimer's Association, 2021; Murphy et al., 2021). AD neuropathology (Fig. 1) is characterized by extracellular amyloid plaques (composed primarily of aggregated Aβ

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peptides) and intracellular neurofibrillary tangles (composed of hyperphosphorylated tau protein) in the brain of people affected by the disease (Glenner and Wong, 1984; Price and Morris, 1999; Duyckaerts et al., 2009; Selkoe and Hardy, 2016). Recent evidence indicates that other cellular and subcellular mechanisms also contribute to AD pathogenesis and progression (De Strooper and Karran, 2016; Long and Holtzman, 2019; Rayaprolu et al., 2021).

AD molecular pathology begins with accumulation of neurotoxic Aβ peptides in the brain years to decades before onset of cognitive deficits (Price and Morris, 1999; Sperling et al., 2011; Bateman et al., 2012; Dubois et al., 2016; Jack et al., 2019; Vermunt et al., 2019). Recognition of this protracted presymptomatic period, dubbed preclinical AD (Sperling et al., 2013; Dubois et al., 2016; Jack et al., 2018), and slow clinical progression of the disease has important implications for risk assessment, diagnosis, and treatment with emerging disease-modifying therapies (Gauthier et al., 2020; Cummings et al., 2021). The role of Aβ as an early driver of AD pathobiology is strongly supported by human genetics, clinical pathology, and experimental research (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Tanzi and Bertram, 2005; Haass and Selkoe, 2007; Choi et al., 2014; Bertram and Tanzi, 2019; Long and Holtzman, 2019; Kwak et al., 2020; Walsh and Selkoe, 2020). Aβ peptides (Fig. 2) comprise a family of amyloidogenic cleavage products derived from the amyloid precursor protein (APP) that is widely expressed in neuronal and non-neuronal cell types throughout the body (Leiden Open Variation Database, 2021; National Center for Biotechnology Information Database, 2021). Abnormal Aβ deposition in the brain is associated with pathogenic protein aggregation, synaptic dysfunction, and neurotoxicity. Aβ amyloidopathy also triggers other pathogenic cascades—including tau proteinopathy, neuroinflammation, and neurodegeneration—that are closely associated with clinical signs, symptoms, and progression of the disease (Selkoe and Hardy, 2016; Walsh and Selkoe, 2020).

1.2. Lens and brain share developmental, cellular, and molecular features relevant to AD

The lens is a continuously-growing, optically-transparent tissue in the anterior segment of the eye. The primary function of the lens is to focus visible light on the retina. The bulk of the lens is composed of long-lived lens fiber cells. The lens and brain share a common embryological origin in germinal ectoderm (Lovicu and Robinson, 2004; Stiles and Jernigan, 2010; Wormstone and Wride, 2011). The lens develops from surface ectoderm and the brain from neuroectoderm. The lens placode derives from a narrow strip of progenitor cells that reside on the rostral border of the neural plate from which the brain develops. Primordial lens cells are responsive to inductive signals from the underlying optic cup that promote placode differentiation and lens morphogenesis (Gunhaga, 2011; Cvekl and Zhang, 2017; Hintze et al., 2017). The lens and brain are composed of large numbers of long-lived, functionally--specialized cell types that differentiate from partially-committed epithelioid stem cells. In the lens, fiber cells terminally differentiate from a reservoir of epithelial cells that reside as a monolayer on the anterior surface of this tissue. Post-mitotic fiber cells are organized into tightly packed concentric growth shells and radial columns that surround a central core of organelle-free primary fibers in the embryonic lens nucleus (Kuszak and Costello, 1992; Augusteyn, 2010). The lens expresses the full complement of precursor

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protein (amyloid precursor protein, APP), proteolytic cleavage enzymes (presenilin 1 and 2), and modulators (e.g., δ-catenin) required to generate Aβ and express AD-related Aβ pathologies and phenotypes (Frederikse and Zigler, 1998; Goldstein et al., 2003; Li et al., 2003; Moncaster et al., 2010; Jun et al., 2012).

1.3. AD-associated Aβ molecular pathology and supranuclear cataract phenotype in the lens

We discovered Aβ molecular pathology and associated cataract phenotype in the lenses of people with AD (Goldstein et al., 2003). Specifically, we identified AD-specific subequatorial supranuclear cataracts (SNC) in lenses of autopsy donors with neuropathologically-confirmed AD, but not in lenses from donors with non-AD neurodegenerative diseases or normal aging (Fig. 3A and B). In AD lenses, Aβ peptides accumulate as electron-dense microaggregates that distribute in the cytoplasm of supranuclear lens fiber cells. AD-linked cytosolic Aβ microaggregates are initially expressed as molecular pathology in clear lenses. Age-dependent Aβ pathology in the AD lenses progresses over many years and ultimately manifests as the distinctive SNC phenotype associated with the disease (Goldstein et al., 2003). Mature SNC observed in advanced AD is typically expressed bilaterally with variable polymorphic phenotypic penetrance (Supplementary Fig. 1).

We deployed a wide range of histopathological, biochemical, and analytical techniques to establish that Aβ pathology is the molecular origin of the SNC phenotype associated with AD (Goldstein et al., 2003; Moncaster et al., 2010). Evidence of Aβ molecular pathology in AD lenses was obtained by anti-Aβ immunohistochemistry, immunogold electron microscopy, amyloid histochemistry, molecular epitope mapping, quantitative Aβ enzyme-linked immunosorbent assay (Aβ ELISA), and tryptic digest tandem mass spectrometry peptide sequencing (Table 1). We detected unique human Aβ tryptic fragments that unequivocally identified the two major Aβ proteoforms (i.e., $A\beta_{1-40}$, $A\beta_{1-42}$) in AD lenses (Fig. 2). Immunogold electron microscopic analysis revealed Aβ-containing microaggregates that localized in the cytosol of lens fibers in the subequatorial supranuclear (deep cortex) region of AD lenses. It is notable that Aβ microaggregates in AD lenses also contain αB-crystallin (HspB5), an abundant structural protein, small heat shock protein, and molecular chaperone present in mature lens fiber cells (Horwitz, 2000, 2003; Augusteyn, 2004). Intiguingly, αB-crystallin is also expressed in brain and associated with AD neuropathology (Iwaki et al., 1992; Lowe et al., 1992a, 1992b; Shinohara et al., 1993; Renkawek et al., 1994; Wilhelmus et al., 2006, 2009; Bjorkdahl et al., 2008; Bruinsma et al., 2011). Our finding that the cytosolic microaggregates detected in AD lenses contain both Aβ and αB-crystallin indicate that these ultrastructural lesions are hetero-oligomeric supramolecular complexes. Electron microscopic analysis also revealed that cytosolic Aβ microaggregates are electron dense. This finding is consistent with metal incorporation, a known feature of Aβ aggregates and amyloid plaque in brain from humans with AD (Bush et al., 1994; Lovell et al., 1998; Cherny et al., 2001; Viles, 2012) and transgene-expressing Tg2576 APP-Swe mice (Craddock et al., 2012). In this context, it is notable that both Aβ (Bush et al., 1994; Huang et al., 2000; Cuajungco et al., 2000; Curtain et al., 2001; Miller et al., 2010) and αB-crystallin (Ganadu et al., 2004; Biswas and Das,

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2008; Karmakar and Das, 2011; Biswas et al., 2016; Serebryany et al., 2021) bind zinc and redox-active transition metals (i.e., copper and iron) present in human lens (Cekic, 1998; Dawczynski et al., 2002; González-Iglesias et al., 2017). Ultrastructural analysis showed that the amorphously shaped Aβ microaggregates in AD lenses are variable in size (~10–100 nm), consistent with Rayleigh light scatterers. We also observed protofibrillar structures, but not mature amyloid fibrils, within the matrix of some Aβ microaggregates. It is notable that Aβ microaggregates were observed in the same supranuclear subregion of AD lenses that showed Aβ immunoreactivity, Congo red amyloid staining with apple-green birefringence under cross-polarized illumination, and supranuclear opacification (Goldstein et al., 2003).

The two major AD-associated Aβ species ($A\beta_{1-40}$, $A\beta_{1-42}$) specifically bind to αB-crystallin with high affinity (Kapp ~20 nmol/L) and exhibit substrate saturation and competitive inhibition binding characteristics (Goldstein et al., 2003). The binding of Aβ to αB-crystallin has been confirmed by other investigators (Liang, 2000; Fonte et al., 2002; Narayanan et al., 2006; Ghosh et al., 2007, 2008; Dehle et al., 2010; Shammas et al., 2011; Hochberg et al., 2014; Mainz et al., 2015). Research conducted by John Clark and colleagues at the University of Washington showed that specific domains in αB-crystallin bind not only to Aβ but also to other pathogenic aggregating proteins (e.g., α-synuclein) implicated in neurodegenerative disease (Ghosh et al., 2007, 2008). Molecular interaction between Aβ and αB-crystallin is not limited to binding. We showed that incubation of synthetic human Aβ with water-soluble human lens protein extract (containing αB-crystallin and other water-soluble lens proteins) generated hetero-oligomeric microaggregates that were identical in composition, ultrastructure, and amyloid histochemical properties to the cytosolic microaggregates detected in AD lenses. Importantly, we found that synthetic Aβ microaggregates dose-dependently scatter light detected by quasi-elastic light scattering (QLS) spectroscopy (Moncaster et al., 2010). This finding links AD-associated Aβ molecular pathology with increased light scattering and SNC phenotype expression in AD and DS lenses (Fig. 4). *Collectively, these results provide the first evidence of AD-associated Aβ pathology and tissue-specific phenotype outside the brain* (Goldstein et al., 2003).

1.4. AD-associated Aβ lens pathology and supranuclear phenotype in Down syndrome

Down syndrome (DS), the most common chromosomal disorder compatible with human survival, is present in ~1 in 700 live births (Mai et al., 2019) and is invariantly associated with early-onset, age-related Aβ brain pathology (Lott and Head, 2019). In the majority of cases (~95%), DS arises from sporadic nondisjunction of chromosome 21 (HSA21) and triplication of the entire chromosome in all somatic cells (trisomy 21). Far less common causes of DS include partial aneuploidy due to unbalanced chromosomal translocation or mosaicism (Shin et al., 2010). The triplicated chromosome or chromosomal subregion in nearly all DS cases includes the *APP* gene locus (21q21.3) that encodes the amyloid precursor protein (APP). Sequential endoproteolysis of APP by the amyloidogenic pathway generates a family of Aβ peptides and other cleavage products (Fig. 2). With the exception of a single reported case of an individual with a microdeletion in the *APP* gene locus (Prasher et al., 1998), all individuals with DS (including rare cases involving Robertsonian translocation, partial duplications, or trisomic mosaicism) are trisomic for the *APP* locus

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(Doran et al., 2017). Gene-dosage disequilibrium resulting from the aneuploidy leads to APP overexpression, increased Aβ production, age-dependent Aβ accumulation, and early-onset AD-related neuropathology with age-dependent progression (Wisniewski et al., 1985; Beyreuther et al., 1993; Lott and Head, 2005). Age-dependent Aβ accumulation and AD-related amyloidopathy in the brain are strongly associated with DS (Wisniewski et al., 1985; Mann, 1988; Lemere et al., 1996; Wiseman et al., 2015; Head et al., 2016). Early Aβ neuropathology has been reported in children with DS (Leverenz and Raskind, 1998). By the third decade of life, people with DS have abnormally high levels of Aβ and early AD-related amyloid pathology (diffuse plaques) in the brain. By the fifth decade of life, the majority of people with DS exhibit significant Aβ burden, amyloidopathy (diffuse, neuritic, and dense core plaques), and tau proteinopathy (neurofibrillary tangles) in the brain similar to that in older individuals with advanced late-onset sporadic AD. People with DS in the sixth decade of life and beyond are at elevated risk of further progression of AD-related neuropathology, neurodegeneration, and cognitive decline leading to clinical dementia (McCarron et al., 2014).

Lens abnormalities in people with clinical features of DS were first reported over a century ago (Oliver, 1891; Pearce et al., 1910) and have been confirmed in numerous reports since (Lowe, 1949; Igersheimer, 1951; Robb and Marchevsky, 1978; Moncaster et al., 2010; Little et al., 2020). The distinctive lens phenotype associated with DS is characterized by cerulean "blue dot" opacities with onset in the first or second decade of life, and in some cases, may be evident at birth (da Cunha and Moreira, 1996; Kallen et al., 1996; Creavin and Brown, 2009). This distinctive phenotype initially localizes to the supranuclear (deep cortex) region of the lens and bears strikingly resemblance to the SNC phenotype associated with AD (Fig. 3). While DS lens pathology has long been known to include deposits of granular material of uncertain composition (Pearce et al., 1910; Robb and Marchevsky, 1978; da Cunha and Moreira, 1996), the molecular origin and pathogenic mechanisms underpinning the distinctive DS cataract phenotype were not known until recently (Moncaster et al., 2010). Given our discovery of Aβ pathology in AD lenses, we hypothesized that the pathogenesis of the distinctive lens phenotype associated with DS reflects the same underlying Aβ lens pathology in AD (Fig. 4). Specifically, we hypothesized that the SNC phenotype associated with both disorders results from downstream effects of the same underlying lens pathobiology, namely, age-dependent Aβ accumulation, co-localizing Aβ amyloidopathy, and increased light scattering due to Aβ-containing microaggregates in the cytoplasm of lens fiber cells in the supranuclear (deep cortex) region of lenses of people affected by AD or DS (Fig. 4). As in our AD study (Goldstein et al., 2003), we used a wide range of techniques (stereomicroscopy, immunohistopathology, amyloid histochemistry, immunogold electron microscopy, quantitative immunoassays, and tryptic digest mass spectrometry peptide sequencing) to identify pathogenic Aβ accumulation as the molecular etiology underpinning early-onset lens pathology and age-dependent SNC phenotype expression in DS (Moncaster et al., 2010).

Thus, pathogenic Aβ accumulation represents the shared molecular origin of two defining features of AD and DS, namely, Aβ amyloidopathy in the brain and Aβ-linked molecular pathology and supranuclear cataract formation in the lens (Fig. 4). In DS, the pathogenic driver of Aβ amyloidopathy in lens and brain derives from the primary chromosomal

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disorder and triplication of all or part of chromosome 21 (HSA21). The resulting *APP* gene-dosage imbalance fosters increased Aβ production in the brain and lens. In DS brain, increased Aβ generation leads to early-onset Aβ amyloidopathy and progression of AD-related neuropathology. In the lens, increased Aβ accumulation leads to formation of Aβ-containing microaggregates that scatter visible light and clinically manifest as the distinctive SNC phenotype associated with DS. By contrast, Aβ amyloidopathy in late-onset AD, the most prevalent form of the sporadic disease, is driven by multiple factors (e.g., age, sex, genetic factors, environmental exposures, vascular disease, diabetes, metabolic derangements, etc.) that collectively contribute to decreased Aβ clearance and increased Aβ accumulation in brain and lens. In AD, the downstream effects of Aβ amyloidopathy in both compartments follow mechanistic pathways analogous to those in DS (Fig. 4). While Aβ tissue concentrations ($A\beta_{1-40}$, $A\beta_{1-42}$, total Aβ) are far greater in brain than lens, Aβ pathology and AD-associated phenotypes are expressed earlier in lens than brain (Moncaster et al., 2010). Moreover, Aβ accumulation occurs earlier and with more aggressive pathology in both compartments (brain and lens) in DS compared to AD. Indeed, we reported exceedingly high levels of Aβ in lens from an 18-month-old infant with DS that were comparable to Aβ in lenses from adults with end-stage sporadic AD or dominantly inherited familial AD (Moncaster et al., 2010). Finally, we detected substantial Aβ immunoreactivity in the epithelial monolayer on the anterior surface of the lens, a finding that suggests these metabolically-active cells may contribute to Aβ in aqueous humor (Goldstein et al., 2003).

1.5. Additional evidence of AD-related Aβ pathology in the lens and other ocular tissues

We investigated 1249 participants in the Framingham Eye Study and found that AD-associated quantitative traits in lens and brain are coheritable (Jun et al., 2012). This finding is notable given that all subjects in this longitudinal study were dementia-free at the time of the ophthalmic examination and ten years following the initial MRI examination. Co-heritability and AD association of brain and lens traits were detected only for phenotypes affecting the lens cortex (e.g., cortical cataract), but not the lens nucleus (e.g., common age-related nuclear cataract). These results are consistent with the SNC phenotype identified in AD and DS lenses. In addition, this study also found that AD-associated traits are expressed far earlier (by a decade or more) in lens than brain. Moreover, AD-associated lens traits predicted future MRI abnormalities in the brain (decreased temporal horn volume) and cognitive deficits indicative of AD. Importantly, a genome-wide association study (GWAS) of 187,657 single nucleotide polymorphisms (SNPs) for bivariate trait outcomes in the same subjects identified a new GWAS-significant AD risk locus in the *CTNND2* gene that encodes δ-catenin (Jun et al., 2012). This unexpected finding is significant since δ-catenin, a component of the cadherin-catenin complex, binds a critical hydrophilic loop domain in presenilin 1 (PS1) that regulates γ-secretase interactions with APP and modulates Aβ production (Zhou et al., 1997; Levesque et al., 1999; Stahl et al., 1999; Kouchi et al., 2009; Dai et al., 2019). Moreover, we identified a rare *CTNND2* missense mutation (G810R) that altered δ-catenin localization and increased secreted Aβ in neuronal cell culture. Finally, we detected elevated expression of δ-catenin in lenses from donors with autopsy-confirmed AD that localized to the same lens subregion that demonstrates Aβ accumulation, β-amyloidopathy, and SNC lens pathology in individuals with AD or DS.

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Taken together, these findings provide strong support for linkage between AD-associated molecular pathology in the lens and brain (Table 1).

Additional evidence supports this linkage (Table 1). We identified $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides in human primary aqueous humor (AH) obtained from living patients by anterior chamber paracentesis (Goldstein et al., 2003). As in cerebrospinal fluid, Aβ in primary AH is predominated by the $A\beta_{1-40}$ proteoform. The presence of Aβ in primary AH is consistent with findings of soluble Aβ in conditioned media from lens epithelial cell cultures prepared from human capsulorrhexis specimens (Xu et al., 2017). Together with new results reported below, these findings point to the lens epithelium as a likely source of Aβ in the anterior chamber of the eye. A recent study identified Aβ in human vitreous humor in the posterior chamber and found that decreased Aβ levels in this compartment are associated with AD cognitive deficits (Wright et al., 2019). The origin of Aβ in vitreous humor is unknown, but likely derives from retina rather than lens. Multiple studies have reported Aβ in the human retina (Koronyo et al., 2012; Hill et al., 2014; Krantic and Torriglia, 2014; Gupta et al., 2016; Hart et al., 2016; Chiquita et al., 2019; Dumitrascu et al., 2020; Lee et al., 2020; Schultz et al., 2020) and in retinae from murine and canine models of AD (Ning et al., 2008; Perez et al., 2009; Tan and Ge, 2010; Emptage et al., 2013, 2015; Barton et al., 2021). AD association, disease specificity, and temporal dynamics of retinal Aβ expression relative to clinical AD and age-related retinal diseases remain to be determined.

1.6. Lens Aβ as a molecular biomarker for early detection of AD

Early detection of AD is recognized as a critical prerequisite for effective and enduring treatment with emerging disease-modifying therapies (DeKosky and Marek, 2003; Frank et al., 2003; Morris et al., 2005; Fagan et al., 2007; Shaw et al., 2007; Jack et al., 2010; Niculescu et al., 2019). Clinical AD is preceded by a protracted “silent” period (known as preclinical AD) marked by Aβ accumulation in the brain that begins years to decades before onset of clinical symptoms and cognitive deficits that characterize the disease (Jack et al., 2018). Screening for and detection of preclinical AD is widely recognized as the sine *qua non* for effective intervention early in the course of disease when emerging disease-modifying anti-Aβ treatments are expected to have maximal impact (Pogacic Kramp, 2012; Langbaum et al., 2013; Cummings et al., 2021). Thus, the urgent need for reliable AD diagnostics for: (i) risk assessment, (ii) early detection, (iii) differential diagnosis, (iv) disease staging and prognosis; (v) longitudinal tracking, and (vi) therapeutic monitoring. Enabling technology is also needed to confirm therapeutic target engagement, assess therapeutic efficacy, and stratify patient cohorts to increase power and efficiency in clinical trials of new anti-Aβ treatments. The recent approval of the first anti-Aβ immunotherapy for AD (Lalli et al., 2021) has greatly increased the need for new diagnostic platforms for early detection of AD-associated Aβ amyloidopathy.

Various techniques have been developed to measure Aβ brain pathology and functional sequelae, including positron emission tomography (Shoghi-Jadid et al., 2002; Klunk et al., 2004; Nordberg, 2004; Mintun et al., 2006; Small et al., 2006; Rowe et al., 2007; Michael et al., 2017; Canu et al., 2018; Fantoni et al., 2018; Camarda et al., 2020), single photon-emission computed tomography (Kung et al., 2004; Wang et al., 2004; Alagille et

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al., 2011; Okumura et al., 2018), multiphoton imaging (Bacskai et al., 2001, 2003; Kwan et al., 2009) and magnetic resonance imaging (Benveniste et al., 1999; Poduslo et al., 2002; Sperling, 2007, 2011; Chhatwal and Sperling, 2012). Several of these techniques utilize systemically-administered radioactive tracers to detect Aβ in the brain (Herholz and Ebmeier, 2011; Johnson et al., 2013). Parallel research has targeted development of fluid bioassays to measure Aβ in cerebrospinal fluid and blood (Frank et al., 2003; Shaw et al., 2007; Janelidze et al., 2018; Ashton et al., 2020; Bernath et al., 2020; Fyfe, 2020; Soldan et al., 2020). Promising applications of these and other techniques have shown promise for detecting and differentiating AD in patient cohorts (Mintun et al., 2006; Fagan et al., 2007; Shaw et al., 2007; Jack et al., 2016; Fantoni et al., 2018), butnone are currently approved and available for early detection of AD in individual patients.

Evidence supporting linkage of AD-related Aβ amyloidopathy in lens and brain is summarized in Table 1. The *clinical significance and diagnostic implications of this linkage derive from the unique properties of the lens as an optically-accessible transparent tissue that exhibitsAβ molecular pathology and clinical phenotypes with early-onset expression, age-dependent progression, region-specific localization, and disease-specific association linked to AD.* These collective findings (Table 1) spurred development and clinical testing of a novel eye scanner for early detection of AD-associated Aβ lens pathology in living patients (Kerbage et al., 2013, 2015). This drug-device lens scanner detects Aβ in the lens by combining two components: (i) a topically administered small-molecule fluorescent tracer that specifically binds Aβ in the lens (Sutharsan et al., 2010), and (ii) a purpose-designed confocal scanning laser ophthalmoscope with an integrated fluorescent lifetime decay spectroscopy analyzer (Kerbage et al., 2013, 2015). The platform features use of a well-characterized Aβ-binding fluorescent tracer, Aftobetin-HCl (2-propenoic acid 2-cyano-3-[6-(1-piperidinyl)-2-napthalenyl]-,2-[2-(2-methoxyethoxy) ethoxy] ethyl ester HCl (PubChem, 2011). The fluorescent tracer is compounded in a sterile ophthalmic ointment for topical application to the lower eyelid. Aftobetin in the ointment contacts and penetrates the cornea, traverses the anterior chamber, and enters the lens where it binds Aβ microaggregates in people with AD. Tracer-bound Aβ complexes exhibit a characteristic Stokes shift relative to unbound tracer that is detected by the eye scanner and quantitatively analyzed by fluorescent lifetime decay spectroscopy (Kerbage et al., 2013, 2015). Early clinical testing has shown that this investigational drug-device combination eye scanner and tracer are safe for clinical use and reliably detect lens Aβ in living patients with AD (Kerbage et al., 2013, 2015). Clinical trials are underway to evaluate the utility of lens Aβ scanning to discriminate patients with minimal cognitive impairment (MCI, an early AD prodrome) or early-stage AD compared to age-matched normal controls.

1.7. AD-related Aβ lens pathology in the Tg2576 transgenic mouse model of AD

Here, we report findings in the Tg2576 mouse model of AD (Hsiao et al., 1996) that link AD-related Aβ expression, molecular pathology, and tissue-specific phenotypes in the lens and brain. Tg2576 transgenic (Tg^{+}) mice express mutant human APP (APP-Swe, isoform 695) containing the Swedish double substitution mutation (K670N, M671L) identified in two kindreds affected by dominantly-inherited familial AD (FAD). The Swedish mutation is adjacent to the β-secretase site, the only known mutation affecting this region of APP (Fig.

2). Affected individuals present with memory loss and all individuals carrying the mutation develop early-onset AD neuropathology resulting from increased Aβ production (Mullan et al., 1992; Citron et al., 1994). Tg2576 transgenic (Tg^+) mice, but not non-transgenic ($Tg^−$) control mice, constitutively express mutant human APP-Swe, produce abundant quantities of human Aβ (hAβ) peptides in the brain, and age-dependently develop AD-related Aβ neuropathology (including amyloid plaques) and neurobehavioral deficits (Hsiao et al., 1996; Kawarabayashi et al., 2001). The findings reported here shed light on the molecular etiology and ultrastructural compartmentalization of AD-related Aβ pathology in the lens and temporal relationship to corresponding Aβ pathology in the brain.

2. Methods

2.1. Animal subjects

Tg2576 transgenic (Tg^+) mice and aged-matched non-transgenic ($Tg^−$) littermate control mice were group housed and maintained in accordance with an approved animal use protocol at Boston University School of Medicine. The Tg2576 transgene contains the human gene for an AD-linked variant APP with the Swedish double mutation (Hsiao et al., 1996) driven by the promoter for prion protein (PrP), a cytosolic protein expressed in mouse lens (Frederikse et al., 2000).

2.2. Ex vivo lens stereomicroscopy

Lenses were dissected from freshly enucleated eyes and illuminated with a slit lamp attached to a modified Zeiss OpMi-1 surgical stereomicroscope (Carl Zeiss, Thornwood, NY) with a Zeiss-Urban stereoscopic beamsplitter (Urban Engineering, Burbank, CA). Lenses were imaged with a Nikon D70 digital camera and Nikon Capture Control software (Nikon USA, Melville, NY).

2.3. Aβ immunohistochemistry and amyloid histopathology

Lenses and brains were fixed in freshly prepared 4% paraformaldehyde, embedded in paraffin, and sectioned by microtomy at 8 μm thickness. Tissue sections were stained with alkaline Congo red (Prophet et al., 1992) and examined by brightfield and polarized light photomicroscopy, or treated with 90% formic acid, immunostained with anti-human Aβ monoclonal antibodies 6E10 or 4G8 (Signet Laboratories, Covance, Dedham, MA) directed against epitopes in the human Aβ ectodomain (4G8: Aβ aa17–24; 6E10: Aβ aa3–8) and processed by conventional immunohistochemistry (Vectastain, Vector Laboratories, Burlingame, CA). Fibrillar Aβ X-34 staining was adapted from a modified protocol (Styren et al., 2000). Briefly, slide sections were placed in 100 μM solution of X-34 in 40% ethanol, 60% Tris buffer 10 mM (adjusted to pH 10) for 10 min. Slides were then washed in water, differentiated in 0.2% NaOH, 80% ethanol for 2 min, and rinsed five times in water before mounting. Bielschowsky silver staining was adapted from a modified protocol (Sevier and Munger, 1965). Briefly, slide sections were incubated in 20% silver nitrate for 15 min, washed in ammonia water, and developed using ammoniacal silver formalin solution. Congo red amyloid staining was performed by Bennhold's modified method (Armed Forces Institute of Pathology, 1992). Congo red-stained slides were examined under brightfield and cross-polarized illumination and visualized by photomicroscopy (Nikon Eclipse E600

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microscope, Nikon USA, Melville, NY; Spot Slider RT digital CCD camera and software, Diagnostic Instruments, Sterling Heights, MI).

2.4. Anti-Aβ immunogold electron microscopy

Anti-Aβ immunogold electron microscopy was conducted as previously described (Goldstein et al., 2003; Moncaster et al., 2010) on ultrathin cryosections without plastic embedding. Specimens were adsorbed to a carbon-coated grid, blocked for 10 min in 1% BSA, immunolabeled with anti-Aβ monoclonal antibody 4G8 (Signet Laboratories, Covance, Dedham, MA), incubated with rabbit anti-mouse bridging antibody followed by incubation with immunogold-conjugated Protein A (University Medical Center, Utrecht, Netherlands). Specimens were negatively stained with uranyl acetate in 2% methyl cellulose (Sigma Aldrich) and examined on a JEOL 1200EX transmission electron microscope (Electron Microscope Facility, Harvard Medical School). All analyses included specificity controls in which identically processed specimens were incubated with immunodepleted detection antibody prepared by pre-absorption with synthetic human Aβ peptide (Keck Laboratory, Yale University, New Haven, CT) as previously described (Goldstein et al., 2003).

2.5. Whole lens explant culture and measurement of human Aβ in conditioned media

Lenses (n = 4) were dissected from 10-week-old Tg2576 transgenic (Tg^+) mice and age-matched non-transgenic (Tg^-) littermate control mice (n = 4). One lens from each mouse was placed in a separate well containing tissue culture media (M199; Sigma-Aldrich, Burlington, MA) supplemented with 1% penicillin/streptomycin (Invitrogen/Thermo-Fisher Scientific, Waltham, MA) and 10% heat-inactivated normal mouse serum (Innovative Research, Southfield, MI). Cultures were incubated with humidification, 5% carbon dioxide, at 37 °C (Sanderson et al., 2000). Samples of conditioned media from cultures containing explanted intact lenses were collected after 2 days and 6 days of incubation. Media collected from cultures incubated without dissected lenses served as a negative control. Quantitative measurement of human Aβ species was performed on conditioned and control media samples using an anti-human Aβ fluorescent enzyme-linked immunosorbent assay (BioSource, Invitrogen, Carlsbad, CA).

2.6. Identification of human Aβ by tryptic digest tandem mass spectrometry peptide sequencing

Five pooled Tg2576 transgenic (Tg^+) mouse lenses were homogenized in 70% formic acid and centrifuged at 106,000×*g* for 1 h. Supernatant was concentrated under a nitrogen stream before neutralization with sodium hydroxide. Samples were diluted 50-fold in Tris buffered saline containing 10 μg of anti-human Aβ monoclonal antibody 6E10 (Signet Laboratories, Covance, Dedham, MA). Antibody-antigen complexes were precipitated by incubation with magnetic beads covalently coupled to sheep anti-mouse antibody (Invitrogen, Carlsbad, CA) followed by exposure of the suspension to a magnetic field. Immunoprecipitated peptides and protein were released from the beads under acidic conditions (100 mM glycine, pH 3), loaded onto a reverse-phase liquid chromatography column (ZORBAX 300 SB-C8 column; Agilent, Santa Clara, CA), and eluted under isocratic conditions (80 °C, 1 ml/min, with an aqueous mobile phase containing 0.1% TFA, 30.5% acetonitrile). Peak Aβ eluate

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fractions were collected at elution times determined from analysis of chromatographic profiles of synthetic human Aβ standards. The HPLC-isolated eluate fractions were dried by centrifugation in a vacuum and resolubilized in buffer containing trypsin. Tryptic digests were fractionated by reverse-phase liquid chromatography and subjected to electrospray ionization mass spectrometry and LCQ-DECA ion-trap mass spectrometry (ThermoFinnigan, San Jose, CA). Eluting peptides were isolated and fragmented by tandem mass spectrometry. We identified peptide sequences by a computer search program (Sequest, ThermoFinnigan) that matches the acquired fragmentation pattern to all known proteins. Analytical blank controls were prepared from eluates collected by immunoprecipitation and reverse-phase liquid chromatography of non-tissue samples using the same protocol and sequencing conditions used for the mouse lens samples.

2.7. Biochemical analysis of human Aβ in Tg2576 mouse lens and brain

Subcellular fractions were prepared from whole mouse lenses and brain hemispheres according to the methods of Moir et al. (1992). Briefly, tissue homogenates were centrifuged (106,000×*g* for 1 h). The supernatant (soluble subcellular fraction) was removed and the pellet extracted with 1% sodium dodecyl sulfate (SDS). Insoluble material in the SDS-extract was pelleted by a second ultracentrifugation step. Human $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were measured by high-sensitivity anti-human Aβ fluorescent enzyme-linked immunosorbent assay (BioSource, Invitrogen, Carlsbad, CA) in subcellular fractions of homogenized lenses and brains dissected from Tg2576 Tg^{+} and non-transgenic Tg^{-} mice. Samples were diluted before assay in sample buffer supplied by the manufacturer. Final detergent concentration was <0.1%. Test samples and Aβ standards were prepared in the same buffer and assayed according to the manufacturer's instructions. Briefly, Aβ was captured to solid phase by overnight incubation in wells coated with anti-human $A\beta_{1-40}$ or anti-human $A\beta_{1-42}$ antibody. Wells were then incubated with an anti-Aβ antibody conjugated to horse radish peroxidase and developed with fluorescent substrate. Lens and brain subcellular fractions were also analyzed by anti-human Aβ immunoblotting as previously described (Goldstein et al., 2003; Moncaster et al., 2010). Samples in each lane were normalized for total protein concentration and probed with anti-human Aβ monoclonal antibody W02 (hAβ, aa4–10; The Genetics Company, Zurich, Switzerland) or 6E10 (hAβ, aa3–8; Signet Laboratories, Covance, Dedham, MA).

2.8. Mouse lens protein aggregation assayed by quasi-elastic light scattering (QLS) spectroscopy

Water-soluble mouse lens protein extract (mTLP) was prepared from freshly dissected mouse lenses by homogenization in 0.22 μm filter-sterilized Millipore water and ultracentrifuged at 100,000×*g* for 1 h at 4 °C. Supernatant was collected and diluted in sterile phosphate-buffered saline (PBS) without calcium or magnesium, pH 7.4, to a final concentration of 1 mg/mL. Human $A\beta_{1-40}$ (hAβ40) and inactive reverse sequence human $A\beta_{40-1}$ ($hA\beta40_{rev}$) were synthesized and purified at the Keck Laboratory, Yale University, New Haven, CT and stored at −80 °C in gas-tight containers purged with argon. Protein concentrations were determined by bicinchoninic acid assay (ThermoFisher, Waltham, MA). Lens protein extract preparations were aliquoted into sealed glass cylindrical cuvettes and incubated under sterile conditions in the dark (to minimize photodynamic effects) at 37.4 °C.

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Four groups of samples (n = 5 per group) were prepared: mTLP (1 mg/ml) with synthetic human $A\beta_{1-40}$ (hAβ40, 1 μM); mTLP (1 mg/ml) with inactive reverse sequence human $A\beta_{40-1}$ ($hA\beta40_{rev}$, 1 μM); mTLP (1 mg/ml) without added hAβ; phosphate-buffered saline without mTLP or hAβ peptides. Quasi-elastic light scattering (QLS) measurements were acquired during incubation using a custom-built QLS instrument and analytical procedures as previously reported (Minaeva et al., 2020).

3. Results

3.1. Human Aβ expression, AD-related lens pathology and SNC phenotype in Tg2576 mouse lenses

Based on our previous findings of Aβ pathology in human lenses from people with AD (Goldstein et al., 2003) or DS (Moncaster et al., 2010), we hypothesized that homologous AD-related Aβ pathology and SNC phenotype would be age-dependently expressed in lenses from Tg2576 transgenic (Tg^+) mice. Tg2576 Tg^+ mice express mutant human APP-Swe ($APP_{K670N,\ M671L}$), constitutively produce human Aβ (hAβ), and age-dependently develop amyloid plaque neuropathology and cognitive deficits (Hsiao et al., 1996; Kawarabayashi et al., 2001). Conversely, we hypothesized that age-matched non-transgenic (Tg^-) control mice would not show evidence of human Aβ expression, molecular pathology, or associated phenotypes in lens or brain. The Tg2576 transgenic mouse model was selected for this study because Tg^+ mice age-dependently express AD-related Aβ phenotypes and temporal progression of hAβ molecular pathology that recapitulate the slow progression of amyloidopathy and cognitive deficits in human AD (Hsiao et al., 1996; Kawarabayashi et al., 2001). Early amyloid brain lesions are detectable by ~7–10 months-of-age in Tg2576 Tg^+ mice. Dense-core amyloid plaques are not observed in the hippocampus and cerebral cortex until ~15 months-of-age (Kawarabayashi et al., 2001). The Tg2576 mutant human *APP-Swe* transgene is under control of the promoter for the prion protein (PrP), a protein with known expression in lens (Frederikse et al., 2000; Singh et al., 2020).

Given these features of the Tg2576 transgenic mouse model of AD, we reasoned that transgene-expressing Tg^+ mice, but not non-transgenic Tg^- control mice, would develop AD-related hAβ lens pathology and express an age-dependent SNC lens phenotype as in human AD and DS. To test this hypothesis, we used slit lamp stereomicroscopy to examine freshly dissected lenses from middle-aged (10-months) and advanced-aged (24-months) Tg2576 transgenic Tg^+ mice compared to same-age non-transgenic Tg^- control mice (Fig. 5A and B). We observed mature supranuclear cataracts (SNC) in the deep cortex of lenses harvested from aged (24-months) Tg^+ mice (Fig. 5B, *right panel*) but not from middle-aged (10-month) Tg^+ mice (Fig. 5A, *right panel*). Expression of the SNC phenotype in Tg^+ mice exhibited a characteristic pattern of age-dependent opacification localizing in the supranuclear (deep cortical) subregion of the lens (Supplementary Fig. 2). Lens pathology in aged Tg^+mice (Fig. 5B, *right panel*) showed phenotypic resemblance to SNC in human lenses from people with AD (Fig. 3). By contrast, lenses from non-transgenic Tg^- control mice did not exhibit lens opacification at either age and were grossly normal by *ex vivo* slit

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lamp examination (Fig. 5A and B; *left panels*). These results support mechanistic (genotype-phenotype) linkage of AD-related phenotypes in the lens and brain (Table 1).

We identified and localized human Aβ (hAβ) expression in Tg2576 Tg^+ mouse lenses by anti-hAβ immunohistopathology (Fig. 5C). We detected region-specific hAβ immunostaining in the supranuclear (deep cortex) region of lenses from Tg^+ mice (Fig. 5C, *middle panel*), but not lenses from age-matched Tg^- control mice (Fig. 5C, *left panel*). We also detected hAβ immunoreactivity in the epithelial layer on the anterior surface of lenses from Tg^+ mice. We did not detect immunoreactivity in any region of the lens when staining was conducted without detection antibody (procedural control) or with immunodepleted detection antibody prepared by pre-absorption with synthetic hAβ (specificity control; Fig. 5C, *right panel*). Next, we conducted anti-hAβ immunogold electron microscopy to identify suspected Aβ ultrastructural pathology in Tg2576 Tg^+ lenses (Fig. 5D). As in human AD or DS lenses, we observed hAβ-immunoreactive microaggregates in the cytoplasm of lens fibers from Tg^+ mice (Fig. 5D, *left panel*). We did not observe hAβ-immunoreactivity in lenses from Tg^- control mice (Fig. 5D, *right panel*). Transmission electron microscopic examination of ultrathin cryosectioned lenses from Tg2576 Tg^+ mice revealed hAβ immunostaining in amorphous electron-dense microaggregates in the cytoplasm of fiber cells in the supranuclear (deep cortex) subregion of Tg^+ lenses (Fig. 5D, *middle panel*). Pre-absorption of the anti-hAβ detection antibody with synthetic hAβ abolished immunogold staining in Tg^+ lenses, thus confirming hAβ detection specificity. These results indicate that hAβ-containing microaggregates in Tg2576 Tg^+ lenses are compositionally, morphologically, and ultrastructurally similar to the Aβ microaggregates observed in human lenses from people with neuropathologically-confirmed AD (Goldstein et al., 2003) or karyotype-confirmed DS (Moncaster et al., 2010).

3.2. Biochemical identification of human Aβ expression in Tg2576 mouse lenses

We deployed tryptic digest tandem mass spectrometry peptide sequencing to identify human Aβ (hAβ) expression in Tg2576 Tg^+mouse lenses (Fig. 6A). Pooled lenses were harvested from aged Tg2576 Tg^+ mice (18–24 months-of-age), mechanically homogenized, solubilized in SDS, immunoprecipitated, and fractioned by reverse-phase liquid chromatography. Collected ~4 kDa eluates corresponding to monomeric hAβ ($hA\beta_{1-42}$: 4514 Da; $hA\beta_{1-40}$: 4330 Da) were digested with trypsin and analyzed by tandem MS/MS LCQ-DECA ion-trap mass spectrometry. Peptide sequencing analysis yielded a 5-amino acid residue tryptic fragment (DAEFR, 636.662 Da) with +1 charge state that identified a unique human trypsin cleavage site in the N-terminus of hAβ that is not present in the murine Aβ (mAβ) sequence. The detected tryptic peptide definitively identified human Aβ peptides in Tg2576 Tg^+ mouse lens (Fig. 6A).

Next, we conducted anti-hAβ immunoblot analysis of fractionated lens protein extract prepared from freshly dissected lenses harvested from 24-month-old Tg2576 Tg^+ mice and Tg^- control mice (Fig. 6B). Immunoblots probed with detection antibodies for anti-hAβ (6E10: hAβ aa4–9, FRHDSG; WO2: hAβ aa4–10, FRHDSGY) identified a discrete ~4 kDa hAβ immunoreactive band that co-migrated with synthetic monomeric hAβ. This immunoreactive band was detected in SDS-soluble and pellet fractions of lens and brain

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homogenates from Tg2576 Tg^+ mice, but not from lens or brain from age-matched Tg^- control mice. Anti-hAβ immunoblot analysis of lens and brain pellet fractions contained several-fold more hAβ signal than corresponding soluble (supernatant) fractions. This result is consistent with the presence of substantial quantities of aggregated Aβ in Tg^+ lenses. Importantly, our results showed that Tg2576 Tg^+ mice express full-length human APP (hAPP) in lens and brain. Detection of hAPP and hAβ in Tg2576 Tg^+ mouse lenses is consistent with: (i) promoter and transgene activation, (ii) expression and proteolytic processing of human APP-Swe, and (iii) production and accumulation of hAβ in this tissue. Complementary analyses were conducted on lens and brain from 10-month-old Tg2576 Tg^+ and Tg^- mice (Supplementary Figs. 3 and 4).

Next, we used an hAβ-specific enzyme-linked immunosorbent assay (ELISA) to measure hAβ peptides in lens and brain from Tg2576 Tg^+ mice at 24 months-of-age (Figs. 6C) and 10 months-of-age (Supplementary Fig. 4). The majority of hAβ partitioned in SDS-insoluble pellet fractions in both tissue compartments (% hAlens: 96.5% at 10 months; 81.6% at 24 months; brain: 73.2% at 10 months; 98.8% at 24 months). The ratio of $hA\beta_{1-42}$ to $hA\beta_{1-40}$ in the pellet fraction was lower in lens (~1:2) than brain (~3:1). Average total hAβ concentration (soluble + pellet fractions) was ~3.7-fold greater in brain (13.8 μg/g) than lens (3.73 μg/g) at 10 months-of-age. As expected, the average total hAβ concentration increased with age in both brain (24 months: 1763.3 μg/g) and lens (24 months: 80.1 μg/g), representing ~22.0-fold difference in hAβ concentrations in brain compared to lens in aged Tg2576 Tg^+ mice. Absolute and relative hAβ tissue levels may reflect age-dependent effects on extraction efficiency. It is notable that Tg2576 Tg^+ mice at 10 months-of-age did not show evidence of AD-related Aβ amyloidopathy in hippocampus or cerebral cortex (evaluated by Bielschowsky's silver staining, Congo red histochemistry, and anti-hAβ immunohistochemistry), whereas Tg^+ mice at 24 months-of-age showed widespread Aβ amyloidopathy in these brain regions (Supplementary Fig. 5).

3.3. Tg2576 transgenic mouse lenses produce human Aβ (hAβ) peptides

Given that we detected hAPP expression and hAβ accumulation in Tg2576 Tg^+ mouse lenses, and also that we previously identified Aβ peptides in primary aqueous humor (Goldstein et al., 2003), we sought to determine whether hAβ would be liberated into conditioned medium from whole lens explant cultures prepared from 10-week-old Tg2576 Tg^+ mice but not from age-matched Tg^- control mice (Fig. 7). We sampled conditioned media from Tg^+ and Tg^- single lens explant cultures after 2-day and 6-day incubation and analyzed the collected media by hAβ-specific fluorescent enzyme-linked immunosorbent assay. Human Aβ was detected in conditioned media at both incubation time points in single intact lens cultures prepared from Tg2576 Tg^+ mice, but not from whole lens cultures prepared from Tg^- mice (genotype control) or from lens-free cultures containing unconditioned media (experimental control). These results are consistent with hAPP expression, endoproteolytic processing, and Aβ production in: (i) Tg2576 Tg^+ lenses (hAPP, hAβ; Results 3.1,2, above), (ii) immortalized B-3 human lens cell cultures (full-length hAPP, C-terminal fragments, CTF; Supplementary Fig. 6), and (iii) primary human lens cell cultures prepared from cataract capsulorhexis specimens (hAβ, hAPP; Xu et al., 2017).

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3.4. Human Aβ promotes mouse lens protein aggregation and light scattering

We used quasi-elastic light scattering (QLS) spectroscopy to investigate hAβ molecular interaction with mouse lens proteins in a cell-free *in vitro* system that models the protein-rich cytoplasm in Tg2576 Tg^+ lens fibers (Fig. 8). For these experiments, we used synthetic human $A\beta_{1-}40$ (hAβ40) and inactive reverse human $A\beta_{40-1}$ ($hA\beta40_{rev}$) for two reasons. First, the 40-mer proteoform (hAβ40) is the predominant soluble Aβ species in Tg2576 Tg^+ lens (~98.5% of total soluble hAβ in lenses from 10-month-old Tg2576 Tg^+ mice; Supplementary Fig. 4). Second, while hAβ40 is amyloidogenic, this proteoform can be solubilized in aqueous solution for experimental analysis. By contrast, the far lower abundance hAβ42 peptide rapidly auto-aggregates and precipitates from aqueous solution. We incubated synthetic hAβ40 (1 μM) or inactive reverse sequence control peptide $hA\beta40_{rev}$ (1 μM) with freshly prepared water-soluble mouse lens protein extract (mTLP, 1 mg/ml) prepared from non-transgenic mouse lens homogenate. Each incubated sample (n = 5 per group) was serially analyzed by QLS. Mean light scattering intensity values and autocorrelation functions (ACFs) were determined at regular time intervals (0 h, 24 h, 48 h, 72 h; Fig. 8). At the initial time point (0 h), QLS intensity readings from all samples were low (baseline) and statistically indistinguishable. However, mouse lens protein samples incubated with synthetic hAβ40 showed progressive time-dependent increases in light scattering intensity at all subsequent incubation time points (24 h, 48 h, 72 h). No changes in light scattering intensity were detected in mouse lens protein samples incubated with inactive reverse sequence $hA\beta40_{rev}$ control peptide (Fig. 8A). These results are consistent with hAβ-mediated lens protein aggregation. Corresponding autocorrelation functions (ACFs) obtained from samples of mouse lens protein incubated with hAβ40 showed a rightward shift relative to samples containing inactive $hA\beta40_{rev}$ control peptide (Fig. 8B). These results are consistent with increasing molecular size (mean hydrodynamic radii) and decreasing molecular mobility of mouse lens protein aggregates promoted by incubation with hAβ40 peptide. These changes were not detected at any time point in mouse lens protein samples incubated with inactive reverse sequence $hA\beta40_{rev}$ control peptide or without added Aβ peptide. Taken together, these findings indicate that amyloidogenic hAβ peptides interact with murine lens proteins to promote time-dependent protein aggregation and increased light scattering.

4. Discussion

We identified human Aβ peptides, AD-related Aβ lens pathology, and an age-dependent AD-related lens phenotype in the Tg2576 transgenic mouse model of AD. Tg2576 Tg^+ mice express Swedish double mutant human APP (APP-Swe; K670N, M671L), constitutively produce human Aβ (hAβ) in the brain, and age-dependently develop progressive Aβ amyloid neuropathology and cognitive deficits that recapitulate key features of the human disease (Hsiao et al., 1996; Kawarabayashi et al., 2001). We deployed multiple methods to identify and characterize corresponding AD-related human APP expression, hAβ generation and molecular pathology, and age-dependent supranuclear opacification (SNC) in lenses from Tg2576 transgenic (Tg^+) mice, but not age-matched non-transgenic (Tg^-) control mice. As in human AD and DS lenses, Tg2576 Tg^+ mouse lenses show evidence of Aβ-containing cytosolic microaggregates that accumulate in the cytoplasm of supranuclear (deep cortex)

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lens fiber cells. Moreover, we found that hAβ promotes mouse lens protein aggregation and increased light scattering *in vitro*, thereby linking AD-related Aβ molecular pathology and SNC phenotype expression in Tg2576 mouse lenses. Notably, age-dependent expression of Aβ pathology in lens paralleled that in brain. Collectively, these findings provide strong support for genotype-phenotype linkage driven by AD-related Aβ molecular pathology in lens and brain (Table 1, Fig. 4).

In addition, we detected hAβ in conditioned media from whole lens cultures prepared from Tg2576 Tg^+ mice, but not in conditioned media from non-transgenic Tg^- lens cultures. This finding is consistent with expression and proteolytic processing of human APP in Tg2576 Tg^+ mouse lenses. These results support our hypothesis that lens Aβ is endogenously generated in the lens by sequential endoproteolytic processing of its precursor protein, APP. An alternative hypothesis that Aβ is exogenously generated in extra-lenticular tissues (e.g., brain, retina) and subsequently transported to the lens is not supported by our results. That the lens endogenously expresses APP and generates APP cleavage products is consistent with our finding that homogenates prepared from cultures of immortalized B-3 human lens cells (Andley et al., 1994; Fleming et al., 1998) express full-length APP as well as C-terminal fragments (CTFs) indicative of active APP endoproteolysis. This conclusion is further supported by an independent study that confirmed APP expression and Aβ generation in primary lens cells cultured from human capsulorrhexis specimens (Xu et al., 2017). We conclude that Aβ detected in the lens is endogenously generated from APP in this tissue.

We previously reported that Aβ in AD and DS lenses is present not only in supranuclear fiber cells, but also in the epithelial cell monolayer on the anterior surface this tissue (Goldstein et al., 2003; Moncaster et al., 2010). In AD lenses, the epithelium also shows strong immunoreactivity for δ-catenin, a protein that binds presenilin-1 (a key component of the γ-secretase complex that regulates APP cleavage) and modulates Aβ production in neurons (Jun et al., 2012). Results reported here indicate that Tg2576 Tg^+ mice also express hAβ in the lens epithelium. These observations indicate that the epithelium may be a source of soluble Aβ in aqueous humor (Goldstein et al., 2003). However, lenses from people with AD or DS and lenses from Tg2576 Tg^+ mice show prominent Aβ pathology and observable SNC phenotype that localize consistently in the supranuclear (deep cortex) subregion. How might these observations relate to one another? More importantly, do these findings provide clues regarding the regional distribution, cell-type specificity, subcellular localization, and molecular origin of AD-related Aβ pathology and SNC phenotype expression in the lens? Converging lines of evidence from our laboratory and others indicate that Aβ is endoproteolytically cleaved from APP that is constitutively expressed by the lens epithelium. We speculate that amyloidogenic Aβ peptides are released from membrane-bound processing compartments (e.g., endoplasmic reticulum, golgi apparatus, trans-golgi network, endosomes) into the cystosol of nascent lens fibers during organelle disintegration that accompanies epithelial-to-fiber cell terminal differentiation (Brennan et al., 2021). Given the fact that long-lived lens fibers have limited capacity to catabolize and clear aggregated protein, we speculate that AD-associated Aβ accumulation and molecular pathology in the lens temporally precede Aβ amyloidopathy in the brain. This hypothesis is supported by our findings of early-onset Aβ lens pathology and SNC phenotype expression

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in individuals with DS (Moncaster et al., 2010) as well as longitudinal comparison of AD-associated lens and brain traits and bivariate genetic analyses in Framingham Eye Study participants (Jun et al., 2012).

Research from our laboratory and others has shown that αB-crystallin (HspB5), an abundant cytosolic structural protein and molecular chaperone expressed in mature lens fibers (Horwitz, 2000, 2003; Augusteyn, 2004), avidly and specifically binds to Aβ and other amyloidogenic proteins linked to pathogenic protein aggregation (Liang, 2000; Fonte et al., 2002; Goldstein et al., 2003; Ghosh et al., 2006, 2007; Narayanan et al., 2006; Dehle et al., 2010; Shammas et al., 2011; Hochberg et al., 2014). Indeed, αB-crystallin is also expressed in the brain where this small heat shock protein is associated with neurodegenerative diseases including AD (Shinohara et al., 1993; Renkawek et al., 1994). While experimental studies have consistently shown that Aβ and αB-crystallin interact to form supramolecular complexes *in vitro*, we cannot empirically confirm whether these hetero-oligomeric complexes are stable, meta-stable, or transient *in vivo*. Our results indicate that Aβ-containing microaggregates in AD, DS, and Tg2576 lenses do *not* form mature amyloid fibrils. Rather, these light-scattering microaggregates are characterized by amorphous supramolecular organization that may contain localized areas with protofibrillar ultrastructure. Supranuclear Aβ pathology in AD and DS lenses co-localizes with intense congophilia that demonstrates classical apple-green birefringence under cross-polarized illumination. These properties fulfill protein and tinctorial criteria for designation as true amyloid (Sipe et al., 2016). Morover, *in vitro* incubation of synthetic Aβ with water-soluble lens protein extract generates Aβ-containing microaggregates that exhibit amyloid ultrastructure and congophilia identical to the Aβ molecular pathology in human lenses from people with AD or DS (Goldstein et al., 2003; Moncaster et al., 2010) and murine lenses from Tg2576 Tg^+ mice. Presumably, protofibrils and other intermediate amyloid-like structures serve as substrates for amyloid congophilia in AD, DS, and Tg2576 Tg + lenses. We speculate that formation of mature amyloid fibrils may be retarded (or prevented altogether) by molecular chaperoning afforded by interaction with αB-crystallin and αA-crystallin, both high-abundance chaperone proteins in the cytoplasm of mature lens fibers (Santhoshkumar and Sharma, 2004). Further research is needed to elucidate the protein chemistry underpinning these observations.

Experimental results reported here are consistent with our previous findings that Aβ promotes lens protein aggregation (Goldstein et al., 2003; Moncaster et al., 2010). Our results also indicate that the protein-rich milieu in the fiber cell cytoplasm attenuates Aβ auto-aggregation and suppresses formation of mature amyloid fibrils. These effects are dramatically demonstrated by comparing light scattering in aqueous solutions of Aβ peptides incubated with and without added lens protein extract. In the absence of added lens protein, Aβ peptides (especially $hA\beta_{1-42}$) auto-aggregate, fibrillize, and precipitate from solution. By contrast, Aβ peptides incubated with water-soluble lens protein extract (containing abundant quantities of αB-crystallin and αA-crystallin) results in slowly-progressive, time-dependent protein aggregation and increased light scatterering detectable by QLS. Synthetic hAβ-lens protein microaggregates generated *in vitro* are compositionally, morphologically, and ultrastructurally similar to Aβ-containing cytosolic microaggregates in human lenses from people with AD or DS (Goldstein et al., 2003;

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Moncaster et al., 2010) and murine lenses from Tg2576 mice. We conclude that light-scattering cytosolic Aβ microaggregates in supranuclear fiber cells represent the molecular pathology underpinning age-dependent expression and clinical progression of the distinctive SNC phenotype associated with AD and DS.

We report here that human Aβ also stimulates aggregation of mouse lens protein and formation of light-scattering Aβ-containing microaggregates that recapitulate molecular and ultrastructural pathology in supranuclear fiber cells in Tg2576 Tg^+ mouse lenses. Moreover, we found that AD-related Aβ lens pathology precedes SNC phenotype expression in Tg2576 Tg^+ mice. These results provide strong evidence supporting genotype-phenotype linkage of AD-related Aβ amyloid pathology and age-dependent phenotype expression in lens and brain (Table 1). With respect to the Tg2576 mouse model of AD, our results also support the hypothesis that age-dependent Aβ accumulation in Tg^+ mouse lenses represents a tissue-specific manifestation of the same underlying molecular pathology that drives Aβ amyloidopathy in the brain (Fig. 4). It is notable that Aβ pathologies and phenotypes in the lens and brain of Tg2576 mice bear striking resemblance to corresponding features of human AD and DS. In this context, it is also notable that Aβ neuropathology, congophilic amyloid angiopathy, and early-onset cataracts are characteristic features of familial Danish dementia (heredo-oto-ophthalmo-encephalopathy), a rare dominantly-inherited AD variant (Bek, 2000; Holton et al., 2002). Further research is needed to determine whether and to what extent Aβ lens pathology and SNC phenotype expression, progression, and penetrance are present in different dominantly-inherited familial AD kindreds.

It is important to note that AD-related Aβ lens pathology and distinctive SNC phenotype are readily differentiated from common age-related nuclear cataracts based on biochemical composition, molecular pathogenesis, anatomical (and subregional) localization, ultrastructural pathology, phenotypic expression, natural history, and ophthalmological sequelae. AD-related Aβ lens pathology is expressed initially in clear lenses and even late-stage disease is not visually disabling in AD patients. In late-onset sporadic AD, Aβ pathology remains largely restricted to the subequatorial periphery of the lens posterior to the iris such that central vision is not impacted by opacification. These lesions may be observed as clinically insignificant (incidental) findings during dilated ophthalmic examination of elderly patients with AD. By contrast, Aβ pathology in the lenses of people with DS is expressed with earlier onset and more fulminant natural history. Indeed, we reported substantial Aβ molecular pathology in a lens from an 18-month-old infant with DS that was comparable to that in lenses from older adults with end-stage sporadic AD or familial AD (Moncaster et al., 2010). By contrast, Aβ brain pathology in DS is not detectable by amyloid positron emission tomography brain imaging (amyloid-PET scans) until the third or fourth decade of life (Jennings et al., 2015; Annus et al., 2016), consistent with earlier expression of Aβ pathology in lens than brain. As we reported in 1249 Framingham Eye Study participants, AD-associated quantitative traits in lens and brain are not only co-heritable, but also expressed far earlier (by a decade or more) in lens compared to brain (Jun et al., 2012). We speculate that earlier onset of Aβ pathology in the lens may reflect the relative inefficiency of the lens in clearing pathogenic protein aggregates compared to brain.

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These considerations are important because AD-related Aβ molecular pathology in the brain precedes onset of clinical symptoms by many years (DeKosky and Marek, 2003; Frank et al., 2003; Morris et al., 2005). Indeed, recent evidence indicates that synaptic dysfunction and associated cognitive deficits correlate with accumulation of small neurotoxic Aβ oligomers as opposed to insoluble fibrillar amyloid associated with late-stage AD neuropathology (Walsh et al., 2002; Cleary et al., 2005; Glabe, 2006; Lesne et al., 2006; Townsend et al., 2006; Benilova et al., 2012; Long and Holtzman, 2019). Our results suggest that Aβ pathology in the lens represents an early, tissue-specific manifestation of the same underlying molecular disease that affects the brain in AD and DS (Fig. 4). Importantly, our findings underscore the potential of Aβ lens pathology as a viable, ophthalmically-accessible biomarker for early detection of AD before amyloid pathology is clinically detectable in the brain.

Questions regarding the supranuclear localization of Aβ pathology and SNC phenotype in AD, DS, and Tg2576 lenses remain unanswered. As in brain, APP and Aβ are generated in the lens, presumably throughout life. Homeostatic, metabolic, and clearance mechanisms contribute to controlling Aβ tissue levels, distribution, and compartmentalization. Inefficiencies in these and other factors (e.g., ApoE4 genotype, neuroinflammation, neurovascular dysfunction, metabolic deficits, etc.), as well as age-related changes in regional vulnerability (pathoklisis), modulate disease progression, pathology distribution, and clinical sequelae associated with sporadic AD. Specific mutations in genes coding for APP, PS1, or PS2 (in FAD) or chromosome 21 triplication (in DS) lead to increased Aβ generation and early-onset Aβ pathology in the brain of affected individuals. In the lens, age-dependent accumulation of Aβ in the fiber cell cytosol predominates in the supranuclear (deep cortex) subregion where cellular compaction, age-dependent vulnerability to Aβ-mediated oxidative stress, biometal dyshomeostasis, physiochemical properties (e.g., pH, ionic fluxes, hydration, reactive oxygen species), and other factors may contribute to increased microaggregate formation and progressive supranuclear opacification. The mechanisms underpinning initiation and progression of Aβ pathology in specific subregions of the lens warrant further investigation and may shed light on the pathobiology of AD-related amyloidopathy in the brain.

The findings reported here suggest a translational pathway for early detection and longitudinal monitoring of AD-related Aβ pathology in individual patients. Effective AD treatment with emerging anti-Aβ therapeutics critically depends on developing reliable quantitative means for early detection and disease tracking across the AD clinical spectrum. Furthermore, drug development and clinical trials require accurate, objective methods for patient selection, cohort stratification, and therapeutic monitoring. Collectively, our results suggest that lens Aβ may serve as a viable molecular biomarker for early detection and longitudinal monitoring of AD. Ongoing clinical testing will clarify the potential of assessing lens Aβ burden for AD risk assessment, early detection, clinical diagnosis, and longitudinal monitoring in individuals affected by this devastating neurodegenerative disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Aβ	amyloid-β
AD	Alzheimer's disease
AH	aqueous humor
APP	amyloid precursor protein; DS, Down syndrome (trisomy 21)
FAD	familial Alzheimer's disease
GWAS	genome-wide association study
MCI	mild cognitive impairment
QLS	quasi-elastic light scattering
SNC	supranuclear cataract
Tg2576	Swedish mutation (APP-Swe), transgenic mouse model of Alzheimer's disease

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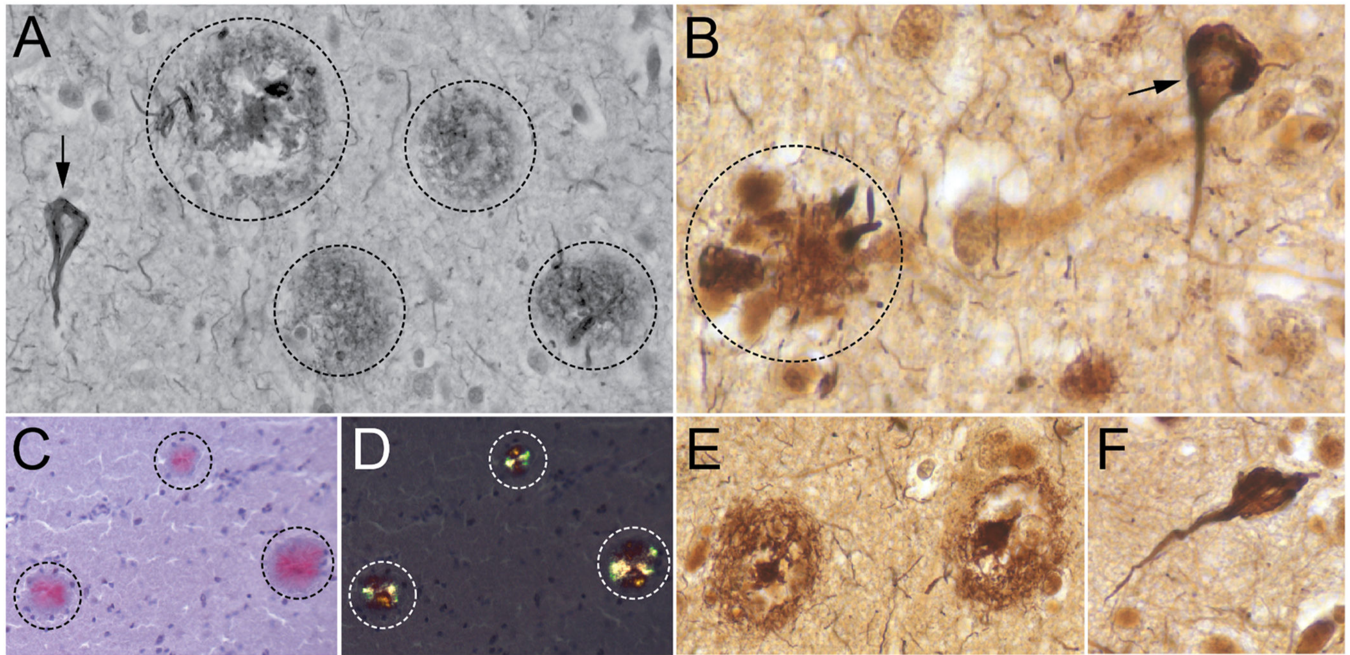

Fig. 1. Hallmarks of Alzheimer's disease neuropathology.

A, Postmortem brain from a 78-year-old male demonstrating the classical hallmarks of Alzheimer's disease (AD) neuropathology: (i) amyloid plaques (hashed circles) composed primarily of fibrillar amyloid-β (Aβ) surrounded by dystrophic neurites, (ii) neurofibrillary tangles (arrow) composed of abnormally phosphorylated tau protein. Dorsolateral frontal cortex, silver stain. **B**, AD neuritic plaque (hashed circle) and neurofibrillary tangle (arrow). Dorsolateral frontal cortex, Bielschowsky's silver stain. **C, D**, Amyloid deposits (hashed circles) stained with Congo red reveals classical congophilia (red staining) with brightfield illumination (C) and colocalizing birefringence with cross-polarized illumination (D). These histochemical features fulfill tinctorial criteria for designation as amyloid. Hippocampus, Tg2576 mouse model of AD. Tg2576 mice constitutively express mutant human APP and Aβ and age-dependently develop Aβ plaques in the brain. **E, F**, Amyloid plaques (E) and neurofibrillary tangle (F). Bielschowsky's silver stain, same human brain as in A, B, above.

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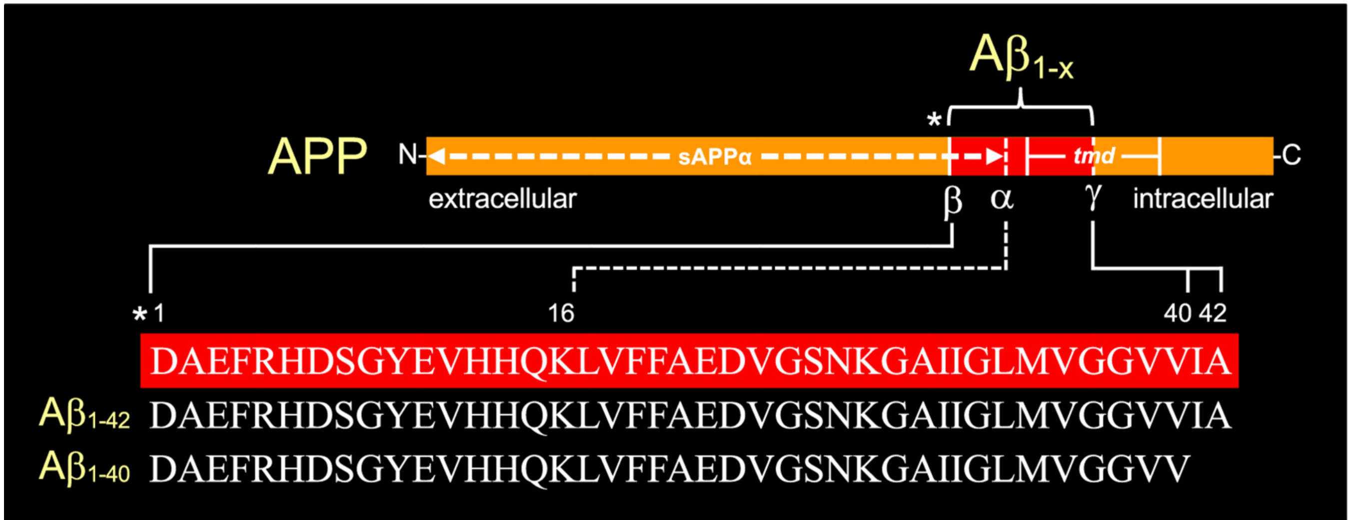

Fig. 2.

Generation of human Aβ (hAβ) peptides by endoproteolytic processing of human amyloid precursor protein (hAPP). Serial endoproteolytic cleavage of hAPP generates hAβ peptides that accumulate in AD brain as neurotoxic oligomers and amyloid plaque. The amyloidogenic processing pathway involves two endoproteolytical cleavages: (i) β-secretase cleavage (β) in the APP ectodomain, and (ii) variable cleavage by the γ-secretase complex (γ) in the APP transmembrane domain (tmd) that generates Aβ 42-mers ($A\beta_{1-42}$) and 40-mers ($A\beta_{1-40}$), the two major Aβ species in AD brain, cerebrospinal fluid, and lens. The non-amyloidogenic processing pathway involves alternative cleavage by α-secretase (α) in the APP ectodomain that generates soluble APP (sAPPα) and other proteolytic products but precludes Aβ production. Asterisk (*) marks the position of the Swedish double mutation (K670N, M671L) on the N-terminal side of the β-secretase site. The Swedish double mutation is one cause of familial AD (FAD) that shifts APP processing towards the amyloidogenic pathway and increases production of Aβ. The Tg2576 transgenic mouse model of AD incorporates a transgene that encodes Swedish mutant human APP (APP-Swe). Hemizygous overexpression of the transgene results in tissue- specific expression of human APP-Swe, increased generation of human Aβ peptides, progression of human Aβ amyloidopathy, and age-dependent phenotype expression in the brain and lens.

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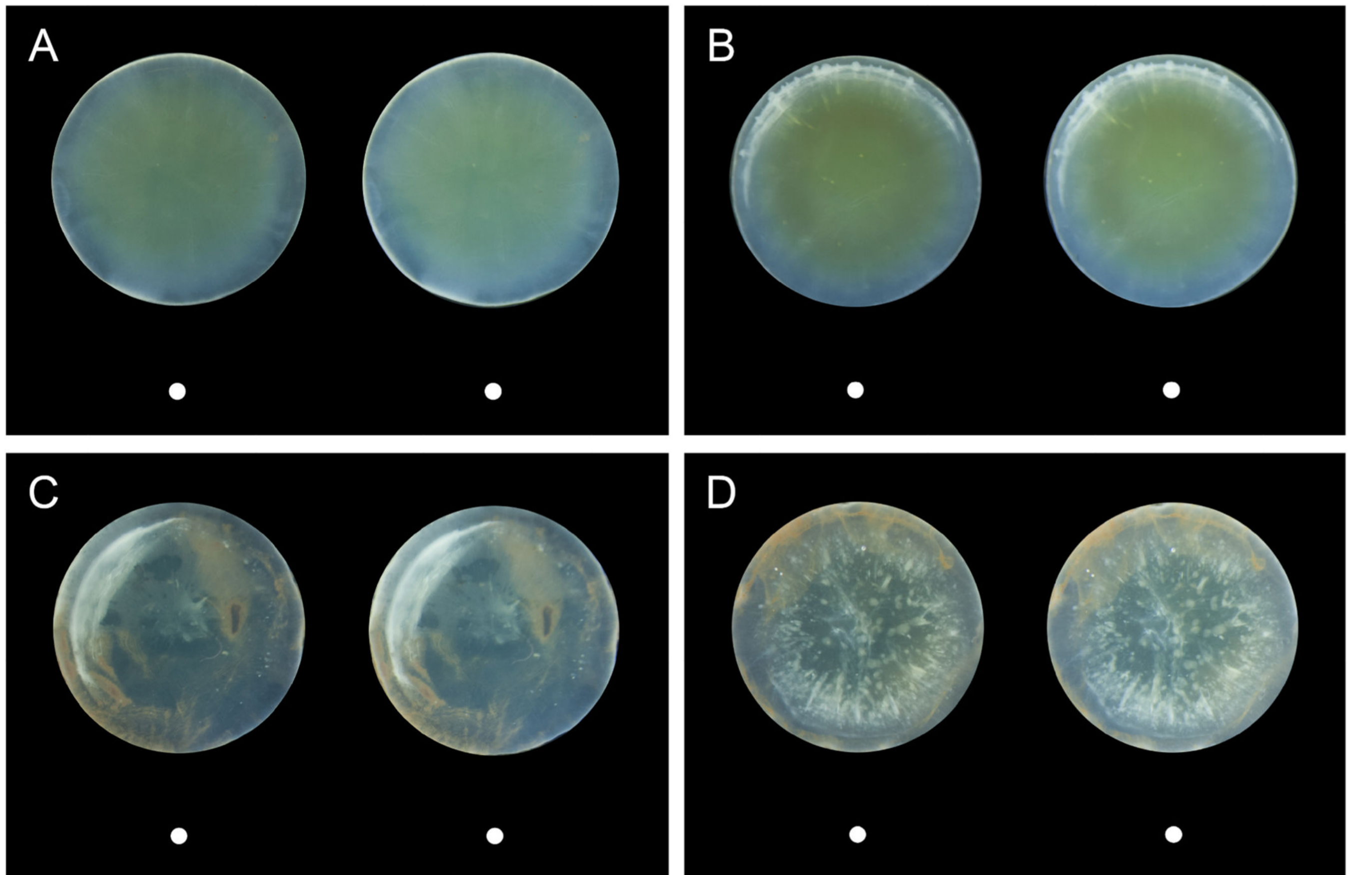

Fig. 3.

Alzheimer's disease-related Aβ supranuclear cataract (SNC) phenotype expressed in sporadic late-onset Alzheimer's disease (AD) and Down syndrome (DS). Stereomicroscopy image pairs of single postmortem lenses from patients with neuropathologically-confirmed AD or karyotype-confirmed DS (trisomy 21) exhibit the distinctive AD-related Aβ supranuclear cataract (SNC) phenotype that is not observed in postmortem lenses from individuals with non-AD neurodegenerative diseases or normal aging. A, Clear postmortem lens from a 79-year-old Caucasian male normal control. This lens shows minimal brunescence and no evidence of age-related nuclear or supranuclear cataract. B, Postmortem lens from an 85-year-old Caucasian male with neuropathologically-confirmed AD exhibiting the distinctive AD-associated supranuclear opacification at the equatorial periphery of the lens (~150° arc, left and right upper quadrants). This lens shows moderate brunescence and no evidence of comorbid age-related nuclear cataract. C, Postmortem lens from a 39-year-old Caucasian male with karyotype-confirmed DS. Note the characteristic DS-associated supranuclear opacification at the equatorial periphery of the lens (~120° arc, left quadrants, top and bottom). This lens shows minimal brunescence and no evidence of comorbid age-related nuclear cataract. Brown material is adherent zonular tissue on the surface of the lens capsule. D, Postmortem lens from a 47-year-old Caucasian female with karyotype-confirmed DS exhibiting advanced circumferential DS-associated supranuclear opacification with characteristic cerulean "blue dot" appearance and anterior-posterior radiating extensions (spokes). This lens shows minimal brunescence and no evidence of

comorbid age-related nuclear cataract. Brown material is adherent zonular tissue on the capsular surface of the lens.

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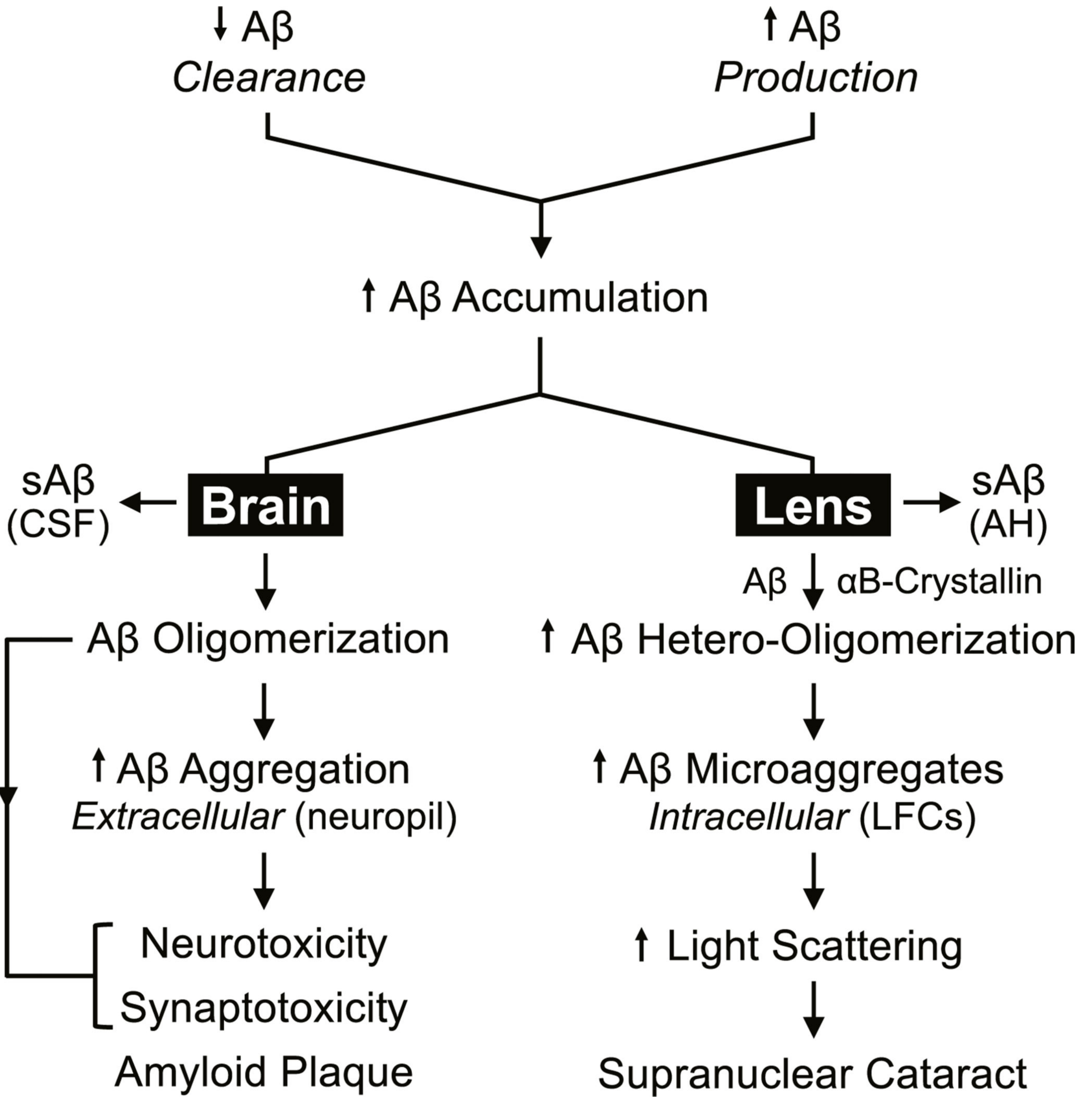

Fig. 4.

Pathogenic pathways in Alzheimer's disease-related Aβ amyloidopathy in the brain and lens. Decreased Aβ clearance is a primary driver of Aβ amyloidopathy in the brain in sporadic late-onset Alzheimer's disease (AD), whereas increased production of Aβ peptides promotes AD-related Aβ amyloidopathy in Down syndrome (DS) and familial AD (FAD). In DS (trisomy 21), increased Aβ production is caused by triplication of the *APP* gene on chromosome 21 (HSA21). This gene codes for the amyloid precursor protein (APP), the substrate for endoproteolytic cleavages that generate Aβ peptides. FAD is a rare autosomal

dominantly-inherited familial (genetic) form of AD that is linked to specific mutations in one of three genes coding for proteins involved in Aβ generation and processing (APP; presenilin-1, PS1; presenilin-2, PS2). In the brain of people with AD, DS, or FAD, extracellular accumulation of amyloidogenic Aβ peptides leads to oligomerization, aggregation, formation of higher-order supramolecular complexes (Aβ56mer*, annular aggregates, globulomers, Aβ-derived diffusible ligands, protofibrils), and eventually amyloid plaques that characterize progressive stages of molecular pathology associated with these diseases. In the lenses of people with AD or DS, Aβ binds αB-crystallin, an abundant lens structural protein and molecular chaperone, to form hetero-oligomeric complexes that progressively organize into amorphous light-scattering microaggregates in the cytosol of supranuclear lens fiber cells. AD-related Aβ molecular pathology in the lens age-dependently progresses to clinically observable supranuclear cataract (SNC). Abbreviations: Aβ, amyloid-β. sAβ, soluble Aβ. AH, aqueous humor. CSF, cerebrospinal fluid. LEC, lens epitheial cells. LFC, lens fiber cells.

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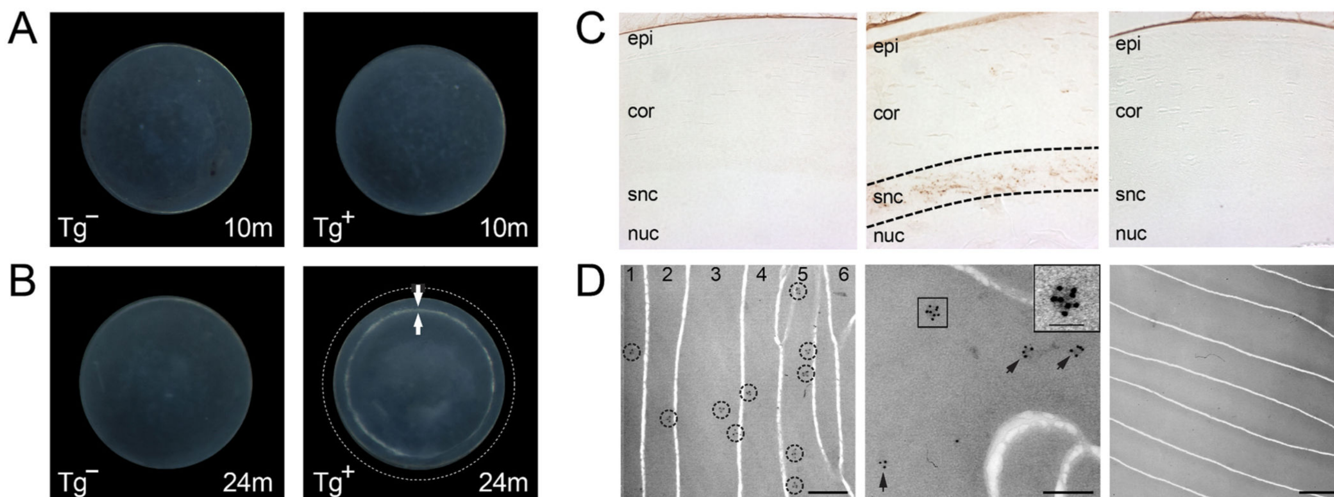

Fig. 5.

Human Aβ (hAβ) amyloid lens pathology and supranuclear cataracts in the Tg2576 transgenic mouse model of Alzheimer's disease. Tg2567 transgenic (Tg$^+$) mice express mutant human APP (APP-Swe, isoform 695) with the Swedish familial AD mutation (KM670/671NL). Tg2576 Tg$^+$ mice constitutively generate human Aβ peptides and age-dependently develop amyloid plaque in the brain. Non-transgenic (Tg$^-$) mice do not express human Aβ and do not develop AD-related Aβ brain pathology or phenotypes. A, B, Representative *ex vivo* slit lamp photomicrographs of dissected lenses from non-transgenic Tg$^-$ control mice at 10 and 24 months-of-age (A, *left panel*: 10-month-old Tg$^-$; B, *left panel*: 24-month-old Tg$^-$) compared to transgenic Tg$^+$ mice (A, *right panel*: 10-month-old Tg$^+$; B, *right panel*: 24-month-old Tg$^+$). Hashed circle and arrowheads denote circumferential subequatorial supranuclear opacification in the lens of a 24-month-old Tg$^+$ mouse (B, *right panel*). The central nuclear region of this Tg$^+$ mouse lens is clear. The SNC phenotype detected in aged Tg$^+$ mice recapitulates the distinctive SNC phenotype observed in human lenses from patients with Alzheimer's disease (AD) or Down syndrome (DS). C, Human Aβ (hAβ) molecular pathology detected in lenses from Tg2576 transgenic Tg$^+$ mice (*middle panel*; magnification: 40X) but not non-transgenic Tg$^-$ control mice (*left panel*; magnification: 40X). Anti-Aβ immunohistochemical staining with the anti-hAβ monoclonal detection antibody 6E10 (epitope: human Aβ amino acids 3–8, EFRHDS). A control section of Tg$^+$ lens is devoid of hAβ immunostaining when the detection antibody was immunodepleted by pre-absorption with synthetic human Aβ (*right panel*; magnification: 40X). Lens subregions are designated by abbreviations: *epi,* epithelium; *cor,* cortex; *snc,* supranucleus (hashed lines demarcate approximate boundaries); *nuc,* nucleus. Localization of anti-Aβ immunostaining in the supranuclear (deep cortex) subregion recapitulates AD-related Aβ lens pathology in human AD and DS. D, Human Aβ ultrastructural pathology detected in ultrathin cryosections of lenses from Tg2576 transgenic Tg$^+$ mice by anti-hAβ immunogold electron microscopy. *Left panel*, Aβ-containing microaggregates (hashed circles) localize in the cytoplasm of fiber cells (numbered 1–6) in the supranuclear region of Tg$^+$ lenses. Round (10 nm) black particles identify cytosolic hAβ microaggregates. Bar, 500 nm. *Middle panel*, higher magnification micrograph showing electron-dense Aβ-containing microaggregates (arrows) in the cytoplasm of supranuclear fiber cells in

Tg^+ lenses. Round (10 nm) black particles identify Aβ immunostaining. Box, single cytosolic Aβ microaggregate, Tg^+ lens fiber cell. Bar, 200 nm. Inset, the same cytosolic Aβ microaggregate at higher magnification. Bar, 50 nm. *Right panel,* absence of anti-Aβ immunogold staining in Tg^+ lens incubated with detection antibody immunodepleted by pre-absorption with synthetic hAβ peptide confirms molecular specificity of hAβ detected in Tg^+ mouse lens. Bar, 500 nm.

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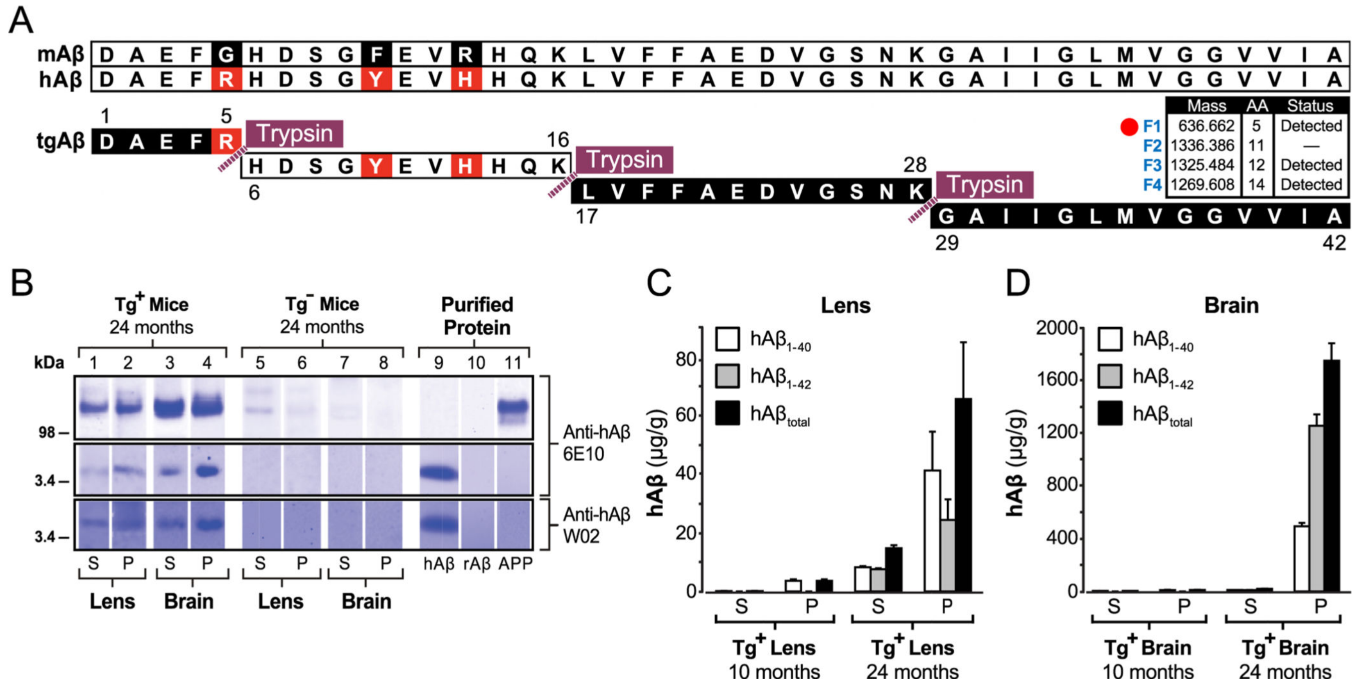

Fig. 6.

Identification and characterization of human Aβ (hAβ) in lenses from the Tg2576 transgenic mouse model of Alzheimer's disease. A, Tryptic digest mass spectrometry peptide sequencing identified three human Aβ (hAβ) tryptic fragments (F1, F3, F4) obtained from peptides in an excised ~4 kDa band following electrophoretic gel separation of homogenized lenses from 24-month-old Tg2576 transgenic (Tg$^+$) mice. Amino acid sequences for human Aβ (hAβ) and murine Aβ (mAβ) are shown at the top of the panel and aligned for comparison. Note that the human and murine amino acid sequences differ at three positions (R5G, Y10F, H13R). A 5-amino acid tryptic fragment (F1; DAEFR; large red dot) uniquely identified human Aβ (hAβ) expression in lenses from aged Tg2576 Tg$^+$ mice. B, Anti-Aβ immunoblot analysis of homogenized lens and brain from 24-month-old Tg2576 transgenic Tg$^+$ mice (lens: lanes 1, 2; brain: lanes 3, 4) and non-transgenic Tg$^-$ control mice (lens: lanes 5, 6; brain: lanes 7, 8). Detection with anti-hAβ monoclonal antibodies 6E10 (epitope: hAβ amino acids 3–8, EFRHDS) and W02 (epitope: hAβ amino acids 4–10, FRHDSGY). Purified synthetic human Aβ (hAβ; lane 8) and synthetic rodent Aβ (rAβ; lane 9) were assayed as positive and negative controls, respectively. SDS-soluble (S) and SDS-insoluble pellet (P) fractions of tissue homogenate were analyzed separately as indicated. Human APP (hAPP) was detected in homogenate fractions prepared from Tg$^+$ lens (lanes 1, 2) and brain (lanes 3, 4), but not Tg$^-$ lens (lanes 5, 6) and brain (lanes 7, 8). The detected hAPP showed expected full-length APP isoforms (~110 kDa) that co-migrated with hAPP purified from human AD brain (lane 11). C, D, Quantitative enzyme-linked immunosorbent assays detected hAβ peptides (hAβ_{1-40}, hAβ_{1-42}) and total hAβ (hAβ_{total}) in SDS-soluble (S) and SDS-insoluble pellet (P) fractions of lens (C) and brain (D) homogenates prepared from Tg2576 Tg$^+$ mice at 10-months and 24-months-of-age.

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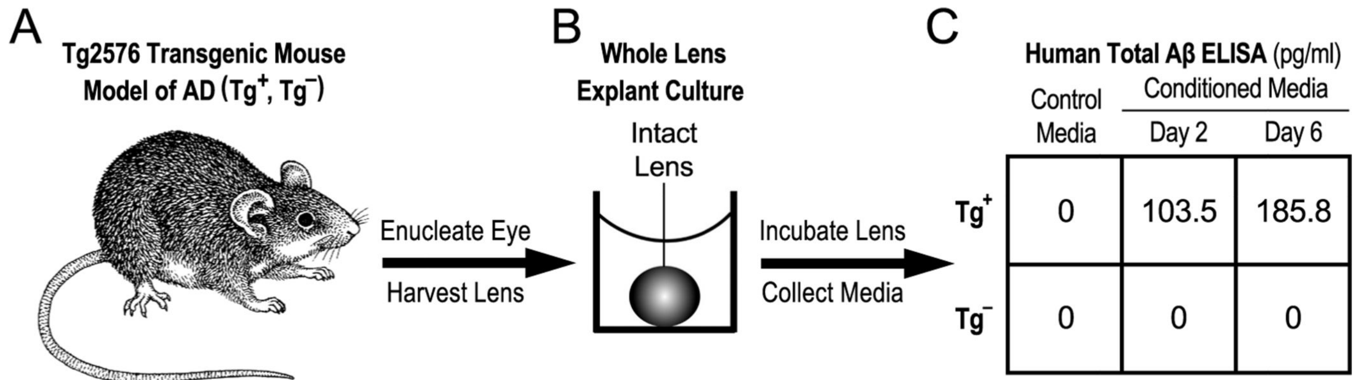

Fig. 7.

Identification of human Aβ (hAβ) in conditioned medium from intact whole lens explant cultures prepared from Tg^{+} mice, Tg2576 transgenic mouse model of Alzheimer's disease. A, B, Freshly dissected lenses from Tg2576 transgenic mice (A; Tg^{+}, transgene carrier mice; Tg^{-}, age-matched non-transgenic control mice) were maintained in single lens explant culture (B). Conditioned medium from single lens explant cultures prepared with Tg^{+} or Tg^{-} lenses, and unconditioned control medium from culture wells without lenses, were collected at day 0, day 2, and day 6. Media specimens were analyzed for hAβ by enzyme-linked immunosorbent assay. C, Human Aβ was detected in 2-day and 6-day conditioned media from Tg^{+} lens cultures, but not from Tg^{-} lens cultures.

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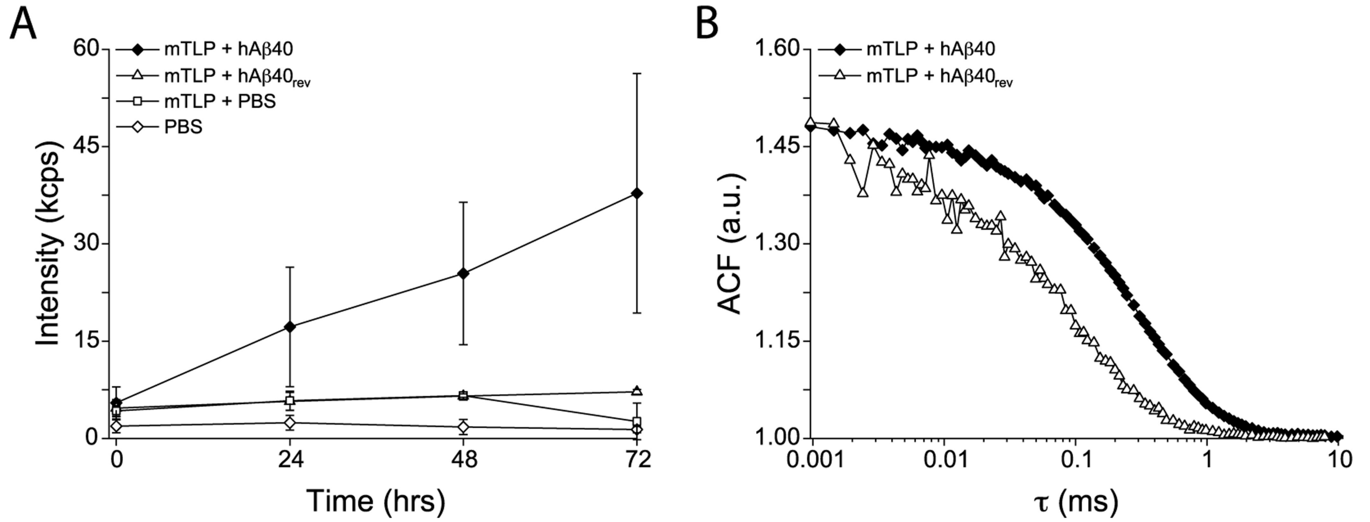

Fig. 8.

Human Aβ (hAβ) promotes time-dependent aggregation of mouse lens protein (mTLP) *in vitro.* A, Quasi-elastic light scattering (QLS) spectroscopic analysis of water-soluble mouse total lens protein extract (mTLP, 1 mg/ml) incubated with purified synthetic human $A\beta_{1-40}$ (hAβ40, 1 μM), but not inactive reverse sequence human $A\beta_{40-1}$ ($hA\beta40_{rev}$), showed time-dependent increase in light scattering intensity consistent with Aβ-mediated lens protein aggregation. Values are plotted as means ± standard deviation. B, Representative autocorrelation functions (ACF, 72 h) from mTLP extract incubated with synthetic human hAβ40 and inactive reverse sequence $hA\beta40_{rev}$. Note the rightward ACF shift in mTLP incubated with hAβ40 active peptide relative to inactive reverse $hA\beta40_{rev}$ control peptide. This finding is consistent with increased mean hydrodynamic radii (particle size) of light-scattering aggregates induced by incubation with amyloidogenic hAβ40 peptide.

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Table 1

Clinical and scientific evidence supporting Alzheimer’s disease (AD)-related amyloid-β (Aβ) pathology and phenotypes in the lens of the eye (Goldstein et al. (2003); Moncaster et al. (2010); Kerbage et al. (2013); Kerbage et al. (2015); Jun et al., 2012; Xu et al. (2017); Ghosh et al. (2008)).

Evidence Category	Disease	Detection Methods (Bioanalytical, Clinical)	Major Findings	References
1. AD-Related Aβ Molecular Pathology	Alzheimer’s Disease	Aβ immunohistopathology, human lenses (AD) Aβ immunohistopathology, human lenses (non-AD, NC) Amyloid pathology (Congo red staining + birefringence) Anti-Aβ immunogold electron microscopy Double immunogold electron microscopy Tryptic digest mass spectrometry peptide sequencing Aβ immunoblot analysis, epitope mapping Aβ immunoblot analysis, lens homogenate Quantitative Aβ ELISA, lens homogenate Lens Aβ fluorescent ligand eye scanning Lens Aβ fluorescent ligand eye scanning	Identification of AD-associated Aβ amyloid lens pathology Aβ amyloidopathy not detected in non-AD, NC lenses Aβ and amyloidopathy co-localize in SNC, AD lenses Cytosolic Aβ aggregates identified in AD lens fibers Aβ and aB-crystallin[1] co-localize in AD lens aggregates ***Definitive identification of Aβ in AD lenses*** Aβ peptides detected in AD lens homogenate Increased Aβ peptides in AD *vs* NC lenses Increased Aβ peptides in AD vs NC lenses Increased lens Aβ in living AD patients Increased lens Aβ in living AD patients	Goldstein et al. (2003) " " " " " " Moncaster et aL (2010) " Kerbage et al., (2013) Kerbage et al. (2015)
	Down Syndrome	Aβ immunohistopathology, human lenses (AD, DS, NC) Amyloid pathology (Congo red staining + birefringence) Anti-Aβ immunogold electron microscopy Tryptic digest mass spectrometry peptide sequencing Aβ immunoblot analysis, epitope mapping Aβ immunoblot analysis, lens homogenate Aβ immunoblot analysis, lens homogenate Quantitative Aβ ELISA, lens homogenate	Identification of DS-associated Aβ amyloid lens pathology Aβ and amyloidopathy co-localize in SNC, DS lenses Cytosolic Aβ aggregates identified in DS lens fibers ***Definitive identification of Aβ in DS lenses*** Aβ peptides detected in DS lens homogenate Increased Aβ peptides in DS (≈AD) vs NC lenses Elevated Iras Aβ, DS infant ≈ advanced AD, FAD Increased Aβ peptides in DS (≈AD) vs NC lenses	Moncaster et aL (2010) " " " " " " "
2. Lens Phenotype Characterization	Alzheimer’s Disease	Slit lamp stereomicroscopy	AD-associated supranuclear cataract (SNC)	Goldstein et al. (2003)
	Down Syndrome	Slit lamp stereomicroscopy	DS-associated supranuclear cataract (SNC)	Moncaster et aL (2010)
3. Clinicopathological Correlation	Alzheimer's Disease	Disease phenotype association Disease phenotype association Clinicopathological correlation *Framingham Eye Study* (n=l,249) Phenotype co-heritability analysis Phenotype co-heritability analysis Bivariate genome-wide association study (GWAS) Bivariate genome-wide association study (GWAS) Aβ immunohistopathology Neuronal cell phenotyping	AD-associated, age-related SNC phenotype SNC not detected in non-AD or NC lenses Aβ amyloidopathy localizes to SNC in AD lenses Earlier onset of AD traits in lens vs brain (~10 yrs) AD lens and brain phenotypes are co-heritable AD lens traits predict future AD (MRI, cognitive ↓) GWAS-significant risk locus, *CTNND2* (δ-catenin) Identified AD-linked *CTNND2* missense mutation Abnormal subregional δ-catenin expression, AD lenses	Goldstein et al. (2003) " " " Jun et al. (2012) " " " " "

Evidence Category	Disease	Detection Methods (Bioanalytical, Clinical)	Major Findings	References
			δ-Catenin binds PS1; G810R mutant ↑Aβ in neurons	
	Down Syndrome	Disease phenotype association Disease phenotype association Clinicopathological correlation	DS-associated, age-related SNC phenotype Earlier-onset SNC phenotype in DS *vs* AD Aβ amyloidopathy localizes to SNC in DS lenses	Moncaster et aL (2010) " "
4. Additional Supporting Evidence	Molecular Biology	SELDI mass spectrometry Human lens cell culture (B-3 lens cells) Human lens cell culture (primary LECs)	Aβ1–42/40 detected in primary aqueous humor[2] Human LECs express and process fl-APP, CTFs Human LECs express and process fl-APP, Aβ	Goldstein et al. (2003) Results 3.3 (q.v.) Xuetal. (2017)
	Protein Chemistry	Protein binding assay Protein biochemistry Protein biochemistry Quasi-elastic tight scattering (QLS) spectroscopy	Aβ binds the cytosolic lens protein, αB-crystallin[3] Synthetic Aβ-lens protein amyloid created *in vitro* Aβ promotes lens protein aggregation *in vitro* Aβ-lens protein microaggegates scatter light	Goldstein et al. (2003) Ghosh et ai (2008) Moncaster et aL (2010) Results 3.4 (q.v.)
	Tg2576 Mouse Model of AD[4]	Tissue, cellular, molecular phenotyping Tryptic digest mass spectrometry peptide sequencing Tissue, cellular, molecular phenotyping Tissue, cellular, molecular phenotyping	Human APP, Aβ expressed in Tg+ mouse lenses ***Identification of human Aβ in Tg2576 mouse lenses*** Human Aβ lens pathology and SNC in Tg+ mice Human Aβ in Tg+ lens explant conditioned media	Results 3.1,3.2 (q.v.) Results 3.2 (q.v.) Results 3.1,3.2 (q.v.) Results 3.3 (q.v.)

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; CTFs, C-terminal fragments of ABP; DS, Down syndrome (trisomy 21); ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer's disease; fl-APPs, full-length amyloid precursor protein; GWAS, genome-wide association study; LECs, lens epilhelial cells; NC, normal control; PS1, presenilin-1; QLS, quasi-elastic light scattering; SELDI, surface-enhanced laser desorption ionization mass spectrometry; SNC, supranuclear (deep cortical) cataract; Tg+, Tg2576 transgenic mice (transgene positive).

[1] These findings represent the first evidence of AD-related Aβ pathology outside the brain and point to lens Aβ as an optically-acces sible AD biomarker for early detection and longitudinal monitoring of AD.

[2] Human primary aqueous humor (obtained by anterior chamber paracentesis in living patients): [Aβ1–40] > [Aβ1–42]; Aβ42/40 ratio in primary aqueous humor ≈ AB42/40 ratio in cerebrospinal fluid (Goldstein et al., 2003).

[3] αB-crystallin (HspB5), a highly-conserved small heat shock protein, is expressed in mature lens fiber cells where this abundant cytosolic structural protein functions as a molecular chaperone. In AD lenses, αB-crystallin binds Aβ with high affinity (KApp ~20 nmol/L) to form pathogenic microaggregates (~10–100 nm) in the cytoplasm of supranuclear fiber cells. Cytosolic Aβ microaggregates scatter visible light, thus linking AB lens pathology and age-related SNC phenotype expression in humans with AD or DS (Goldstein et al., 2003; Moncaster et aL, 2010; Jun et aL, 2012) and in Tg2576 Tg+ mice. See text for details.

[4] Tg2576 transgenic mouse model of AD: Tg+ mice constituitively express Swedish mutant human amyloid precursor protein (ABP-Swe), age-dependently accumulate hAβ peptides and amyloid pathology in the brain, and develop cognitive deficits that progressivly worsen with increasing age (Hsiao etaL, 1996; Kawarabayashi et al., 2001).

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