

Transgenic *C. elegans* as a Model in Alzheimer's Research

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Abstract: Alzheimer's disease (AD) has been associated with aggregation of β -amyloid peptide ($A\beta$) and cell death in the brain. Using various models, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus*, investigators have attempted to imitate the pathology process of AD for better understanding of the cellular mechanisms and for possible therapeutic intervention. Among many *in vitro* and *in vivo* models of AD, transgenic *C. elegans* expressing human $A\beta$ has shown its own advantages. The transgenic *C. elegans* model have been used in studying AD due to its short life span, facility to maintain, ability to develop muscle-associated deposits reactive to amyloid-specific dyes and the concomitant progressive paralysis phenotype. Moreover, the transgenic *C. elegans* exhibits increased levels of reactive oxygen species (ROS) and protein carbonyls, similar to those observed in AD patients, supporting the current theory on $A\beta$ -induced oxidative stress and subsequent neurodegeneration in AD. DNA microarray assays of the worm demonstrated several stress-related genes being upregulated, particularly two genes homologous to human β -crystallin and tumor necrosis factor-related protein, which were also upregulated in postmortem AD brain. Studies in our laboratory along with others suggest that the transgenic *C. elegans* model is a suitable *in vivo* model to relate $A\beta$ -expression with its toxicity, which may underlie AD pathology. It may also be used as a tool for pharmacological evaluation of novel therapeutic agents.

Key Words: *Caenorhabditis elegans*, Amyloid β , Alzheimer's disease.

INTRODUCTION

During the last century, there has been a dramatic increase of life expectancy in the American people, from nearly 49 years to more than 76 years. Given that age is one of the most significant risk factors for developing neurodegenerative diseases, more individuals achieve an age at which neurodegenerative disorders are common. They suffer from decreased cognition and memory, and eventually develop severe dysfunction in all mental functions [1]. They progressively exhibit personality changes, difficulties in judgment, language, calculation and become demented. They increasingly lose functions of daily activities, and in the final stage, lose their life. Among all age-associated neurodegenerative disorders, Alzheimer's disease is the most devastating one [2].

Although the clinical symptoms of the syndrome were first described in patient August D. in 1906, little progress had been made in characterizing AD for many years. With the help of electron microscopy in the 1960's, neuroscientists described the ultrastructural alterations of AD: senile (neuritic) plaques and neurofibrillary tangles [3]. Senile plaques, one of the hallmarks of AD, are composed of an extracellular deposition of 40 to 43 amino acid polypeptides in which the 42-residue amyloid peptides ($A\beta_{1-42}$) is the major form [4]. Increasing number of reports revealed that $A\beta_{1-42}$ can also accumulate within the neurons and plays an important role in AD pathology [5-7].

Since the original identification of $A\beta$ [8], and of mutations in the amyloid precursor protein (APP) gene in some cases of familial AD (FAD) [9], a well known "amyloid hypothesis" [10] has been developed. Despite its wide acceptance, this hypothesis still remains controversial: it is not even clear whether the neurotoxic molecular species is the fibrillar $A\beta$ [11, 12], or the soluble $A\beta$ oligomers [13-17]. The leading theory is that $A\beta$ associated with oxidative stress induces neurodegeneration observed in AD [18-21]. The evidence for or against these hypotheses is critical for determining the mechanism of $A\beta$ toxicity and the specific therapeutic strategies.

Neurofibrillary tangles, on the other hand, are intraneuronal aggregates of paired helical filaments, which are assembled from the hyperphosphorylated microtubule-associated protein tau [22]. Also, there is evidence that the ϵ 4 allele of apolipoprotein E (ApoE) is associated with increased AD risk in many human populations [23]. During the mid 1970s, acetylcholine was found to play an important role in AD. The quantity and activity of the synthetic and degradative enzymes, choline acetyltransferase and acetylcholinesterase decrease significantly in the damaged regions of AD brain. This however, may be a consequence, rather than a cause of the disease. Although the whole pathogenesis of AD is still not well understood, damages in brain regions including neural circuits and neurons in the neocortex, hippocampus, amygdala, and basal forebrain cholinergic system have been observed.

As neuroscientists attempted to identify the molecular mechanism involved in the formation of plaques, tangles, and toxicity leading to or induced by these pathological changes, they faced some difficulties. All the patients

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diagnosed were in the late stages of AD and were thus unlikely to provide the useful clues in early pathogenesis. Researchers thus have sought for the identification and validation of potential mutant genes associated with FAD. The discovery of mutant genes producing A⁺ aggregation in AD implies that these genes and the proteins they encoded are connected with AD pathology. A⁺ is produced by γ -secretase, a type-1 transmembrane glycosylated aspartyl protease which cleaves APP, and β -secretase, a large protein complex that includes presenilin (PS1 or PS2), Nicastrin, Aph-1 and Pen-2 [24]. The heterogeneity of β -secretase gives rise to a series of A⁺ species, mainly A₁₋₄₀ and a smaller amount of A₁₋₄₂. Most of FAD cases with the APP mutation, together with mutations in PS exhibit extensive accumulation of A₁₋₄₂ [24-27], providing strong evidence for the "amyloid hypothesis" [28].

Several models have been established for testing the amyloid hypothesis. Among various models of the disease, the whole organism models have an essential role in identification the pathological processes and may provide insight for pharmacological intervention. In order to comprehend the function of the candidate genes, scientists use transgenic technology to analyze the phenotypic consequence. Cell culture models allow one to transfect genes of interest into the cells, to manipulate the cells pharmacologically and obtain results quickly. However, a simple monolayer of cells cannot accurately reflect the activities in the whole organism. Also, the cellular models do not include all factors of pathogenesis, some of which may be crucial to understanding the mechanism of the disease. Therefore, gene-targeted and transgenic mice were developed for modeling various aspects of AD pathology. Several mouse models that express APP, dual PS and APP, or even triple transgenes, as well as A⁺ and apolipoprotein E (apoE) have successfully duplicated some features of the pathological process [29-33]. Moreover, tau gene mutations in fronto-temporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) facilitated the development of tauopathies in transgenic mice [34-36]. Although mice transgenic model for mutant forms of APP and Tau exhibits amyloid as well as tangles, the relationship between the two pathways has not been defined. Taken together, these models show some characteristics of AD, such as an age-dependent generation of amyloid plaques, they do not display all the neurodegeneration signs observed in AD.

Besides, genetic modulations in the mouse model need a longer time, and it is thus not suitable for pharmacological screening of potential new drugs.

Modeling human disease in simple invertebrate systems is attractive because genetic screens can be performed in a relatively short period of time to identify mutations that lead to age-dependent neurodegeneration. Both *C. elegans* and *Drosophila* are in the leading positions of invertebrate models of diseases. They have short reproduction time, informed genomics, variety of phenotypes, and are able to express human genes of interest. Theoretically, genetic screens in these models could lead to identify AD-relevant genes and processes, such screens are technically difficult due to their dependence on post-reproductive phenotypes, which are difficult to assay. Phylogenetic comparisons suggest that the worm PS-homologous genes (sel-12 and hop-1) are

quite divergent members of the PS family [37, 38]. Another member of the APP family, *apl-1*, has also been characterized in the worms [39], which lacks a recognizable A⁺ sequence [40]. cRNAi knockdown of the worm APP homologue, *apl-1*, results in a severe uncoordinated phenotype [41], and genetic deletion results in embryonic lethality. This organism's phenotype was traced to a possible defect in microtubule-based trafficking, suggesting that APP can act to link vesicles to kinesin I and participate in fast axonal transport [42]. A tau gene mutation model of *Drosophila* has replicated several features of the disease [43, 44]. From *in vitro* and *in vivo* study, Fossgreen *et al.* found that a modified fragment of human APP could produce A⁺ in *Drosophila* [45]. Also, it was reported that APP might act as a vesicular kinesin I receptor in *Drosophila* [46]. Using a *Drosophila* model for AD, Iijima *et al.* recently [47] dissected the pathological effects of A₁₋₄₀ and A₁₋₄₂, and demonstrated that accumulation of A₁₋₄₂ in the brain is sufficient to cause behavior deficits and neurodegeneration. Mutation in *sel-12* suppresses the egg-laying defect in *Drosophila* and *C. elegans* [37, 48, 49], implicated PS in a signaling pathway homologous to Notch pathway. Proteolysis of Notch by PS caused the intracellular domain of Notch to reach the nucleus and activate transcription [50].

Comparing the two models, the nematode exhibits its own advantages for studying the functions of mutant genes. The genome of *C. elegans* was sequenced in 1998, thus making the nematode the first multicellular organism [51]. Surprisingly, about 65% of the human disease genes have a counterpart in the nematode's genes [52]. A total of 302 neurons in *C. elegans* greatly facilitate studying the morphology and physiology, in parallel with the behavior of the mutant phenotype. Regardless of its obvious simplicity, the worm model allows us to identify protein interactions, which is rendered useful in understanding entire regulatory pathways. Despite many advantages and extensive research in the transgenic vertebrate models of mice, *Drosophila* and *C. elegans* offer models for mechanistic examination of the transgene products as well as tools for pharmacological analysis [53] within short time. In addition, the absence of endogenous A⁺ production in the worms, presents an opportunity to find roles of the multiple A⁺-related processes proposed to be involved in AD (Link, personal communication).

In this review article, we focus on development of the transgenic *C. elegans* model of AD, and on the application of this model in AD research and the potential use for drug intervention.

DEVELOPMENT AND CHARACTERIZATION OF THE TRANSGENIC *C. ELEGANS* MODEL

At a first attempt to develop a mutant strain by targeting endogenous APP gene, researchers found that the *C. elegans* genome does include genes that encode proteins related to human APP- *apl-1* [39]. Analogous to human APP, the invertebrate APP-family members are composed of single-pass transmembrane proteins with a large extracellular domain and a short intracellular domain, which can be cleaved to release intracellular and extracellular proteolytic fragments. However, APP-like genes in this nematode do not possess the region encoding the neurotoxic A₁₋₄₂. So, the

actual model of AD generated by mutation of endogenous APP cleavage seemed irrelevant. Thus, Link *et al.*, took an alternative approach and developed a transgenic *C. elegans* model, which can express human A₁₋₄₂ fragment intracellularly in the nematode body wall muscle with transgene-induced paralysis phenotype [54-56]. Although the *C. elegans* model obviously lacks the neuronal cognitive complexity of mammals, it turns out to be a valid model to replicate cellular processes that may underlie AD.

In developing the transgenic *C. elegans* model of AD, the minigene construct pCL12 containing the chimeric gene *unc-54/A₁₋₄₂* was introduced into the nematode by gonad microinjection [54] to produce A₁₋₄₂ constitutively expressed in the CL2006 strain of *C. elegans*. The pCL12 gene, along with the coding regions, was derived from human cDNA clones and regulatory sequences from a specific expression vector, on which the 42 amino acid version of the A peptide preceded by a synthetic signal was incorporated. Furthermore CL2006 has the pPD30.38 vector containing the *unc-54* promoter/enhancer sequence that produces high-level muscle-specific gene expression. This causes the expression of human β -amyloid deposits in the muscle cells of the animals [54]. To discriminate transgenic nematodes, these transgenes were co-injected with plasmids expressing the dominant morphological marker *rol-6* (pRF4), which causes the animal to rotate around its longitudinal axis. The resulting movement, due to this roller marker, is a distinctive non-sinusoidal one used to identify those animals maintaining the injected transgenes [55].

To characterize the location of intracellular deposit and the ultrastructure of amyloid fibrils, Link *et al.* employed immuno-electron microscopy and the amyloid-specific dye X-34 [56]. A newly developed and intensely fluorescent Congo red derivative X-34 can sensitively detect amyloid in senile and soluble A in post mortem AD brain tissue [57]. Figure 1A shows an example of A deposits stained with X-34 (blue) and a specific anti-A antibody (green). From the results of immuno-EM and fluorescence staining, Link *et al.* found that the amyloid deposits are located intracellularly and that the increased amyloid load in individual worms from mid-larva to adult stages was caused by an increase in the deposit size rather than the emergence of new deposits [56]. The finding that both endoplasmic reticulum (ER) and cytoplasmic heat-shock protein 70 (HSP70s) could be co-immunoprecipitated with A in the worm model [58] suggests that A was actively re-routed from the secretory pathway by the ER protein. This proposed retrograde transport of A from the ER is analogous to the cellular metabolism of prion protein (Link, personal communication), which is also subject to retrograde transport [59] and can be highly toxic when accumulated in the cytoplasm [60].

Another *C. elegans* strain, CL4176, contains the expression vector pPD118.60. This vector has the myo-3 body-wall specific myosin promoter and an abnormally long 3'untranslated region, which makes the transgenes expression dependent on *smg-1* function (mRNA surveillance system). The *smg-1* in *C. elegans* becomes inactive at the non-permissive temperature of 23°C, which allows the translation of the stabilized transgenes mRNA for human A₁₋₄₂ and makes the nematode lose the ability to

move (paralysis). Figure 1B represents the no-paralysis phenotype in CL4176 without transgene expression (top panel) and the paralysis phenotype with A expressed by temperature upshift (bottom panel). Although A expression is limited to the muscle cells, this specific strain allows us to establish a relationship between A expression and A toxicity [61].

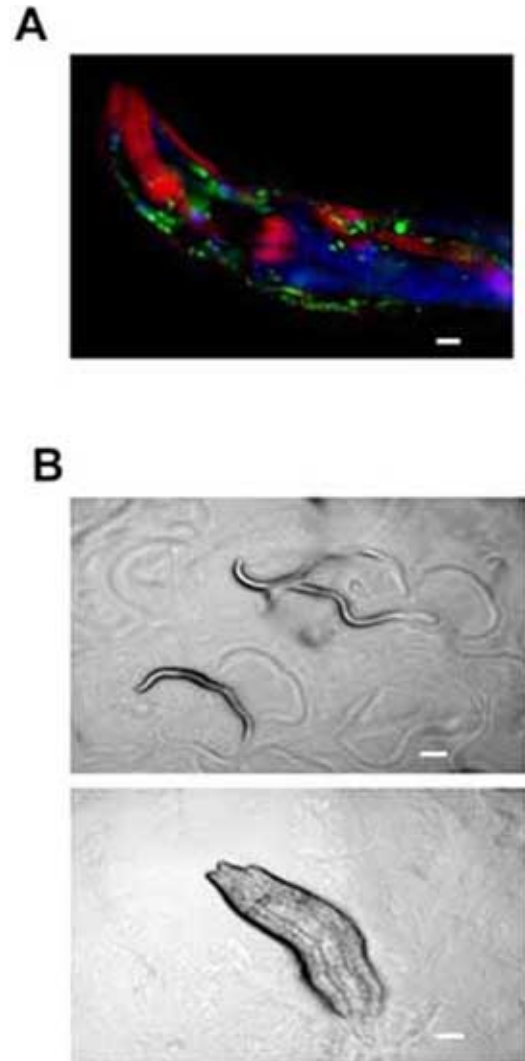


Fig. (1). Characterization of transgenic *C. elegans* model of Alzheimer's disease. A: Fluorescence staining of A deposition in the transgenic *C. elegans* model of AD (CL2006). Adult CL2006 animal were first stained (while alive) with X-34 (blue). The worms were then fixed, permeabilized and probed with anti-A peptide monoclonal antibody 4G8 and fluorescence-conjugated goat anti-mouse Ig secondary antibody (green). As a control, polymerized actin in muscle bundles was labeled with phalloidin (red) (Scale bar 20 μ m) **B:** Muscle-specific A expression leads to a progressive paralysis (CL4176). **Top panel:** transgenic animal CL4176 at L3 stage without temperature up-shift. Photographed was made one hour after transfer of the worms to a lawn of *E. coli* on agar. Note the obvious tracks and eggs laid. **Bottom panel:** The transgenic animal CL4176 undergone temperature up shift for 24 h to express A peptide (Scale bar 50 μ m). Note the absence of movement tracks and eggs (generously provided by Dr. C. Link).

INVESTIGATIONS OF INTRACELLULAR A TOXICITY USING THE TRANSGENIC *C. ELEGANS* MODEL

1. Sequence-dependence of A Aggregation in the Transgenic *C. elegans*

Some studies have indicated that A aggregation is strongly related with neurotoxicity both *in vivo* and *in vitro* [62]. The major components of neuritic plaque in AD brain are A₁₋₄₂ and A₁₋₄₃ peptides [63]. These forms were not detected in the soluble A from the cerebrospinal fluid of AD [64] [65]. Several groups found mutations in APP, PS1 and PS2 increased production of longer forms of A [66] [67] [68]. The structure of soluble and aggregated A is an important issue regarding to its toxicity. *In vitro* studies demonstrated that the state of A aggregation was crucial to neurotoxicity [69] [70] [71]. The process of conversion from soluble A to aggregated A was accompanied by an alteration from a α -helix / random coil state to a β -sheet structure, given the β -sheet conformation in amyloid fibrils [72] [71] [73] [74]. In order to understand the effect of full length A peptide, Link et al developed a few transgenic *C.elegans* lines for analyzing the A aggregation with a single-residue variant of A₁₋₄₂ [55]. The substitution of Leu¹⁷Pro and Met³⁵Cys did not produce observable deposits when stained with thioflavin S, an amyloid specific dye. These results suggested the importance of these amino acid residues in amyloid formation. In their experiment, they created a novel variant single-chain dimer, built by two tandem copies of A₁₋₄₂ joined by a synthetic 15-residue glycine-rich flexible linker. The *C.elegans* strain could express high levels of single-chain dimer, but it was unable to form thioflavin S-reactive deposits [55].

2. Gene Expression Profile in the A -expressing *C. elegans* Strain

With the transgenic A -expressing *C.elegans* model established and characterized, Link *et al.* employed the DNA microarray technique by cDNA hybridization to glass slide microarray containing probes for almost all known or predicted *C.elegans* genes, to reveals global gene expression changes in the A -expressing strain CL4176 [75]. Among the top induced genes, they found that the heat shock protein-16 (HSP-16) gene was up regulated, corresponding with their previous observations that a GFP reporter transgene driven by the *hsp-16-2* promoter could be induced by constitutive A expression [76] (Table 1). HSP-16 protein

was co-localized with A in the A expression strain CL2006 [58]. Two genes, probably involved in A toxicity-induced apoptosis, were up-regulated as well: F37C12.2 (assumed homology of apoptosis-inducing protein ei24/PIG8 [77]) and Y50E8.N (putative homology of phospholipid scrambles linked with the process of apoptosis [78]). There were two other closely related genes, F22E5.6 and ZC239.12 (82% protein sequence similar as F22E5.6) on the top induced gene list. These two genes demonstrated surprising resemblance to the TNFAIP1 gene family [79]. TNFAIP1 was considered to be a tumor necrosis factor -induced protein [80]. Barger et al showed tumor necrosis factor treatment could protect hippocampal neurons from A toxicity [81]. To verify their experiment results, Link *et al.* used quantitative RT-PCR to measure the expression of TNFAIP1 and B-crystallin (CRYAB), which is homologous to the HSP-16 gene, in post mortem AD brain. RNA of the two genes increased considerably in parts of the superior frontal gyrus and the cerebellum of AD patients compared with the controls. Taken together, the *C.elegans* model permits us to view the gene expression profile alteration after expression of A in an organism.

In another experiment, Fonte and Link et al found some chaperone proteins interacting with intracellular A aggregates, which may play an early role in A metabolism [58]. From early studies it was shown that increased expression of HSP-70 class and B-crystallin-related proteins might have effects on the formation of plaques in AD brain [52] [82]. In the *C.elegans* model, one of these chaperone proteins is HSP-16, which was closely colocalized with intracellular A. HSP-16 protein was found to be related with anti-A antibody (4G8) immunoreactive deposits, but not with the A aggregation stained by X-34. Therefore, HSP-16 may interrelate with A monomer or some prefibrillar A oligomers. Another major chaperone protein in A coimmunoprecipitates is F26D10.3, a cytoplasmic HSP70 that can interact with A₁₋₄₂ specifically. Based on these results, Fonte et al presumed chaperon proteins might work directly in early stage of AD [58].

3. Oxidative Stress Induced by A Expression in the Transgenic *C. elegans*

Numerous evidence supports the view that A plays a central role in the controversial topic in this field. Several lines of experimental evidence suggests that oxidative stress might be on the top list of the cellular damage induced by

Table 1. Some Highly Up-Regulated Genes in a Temperature-Induced A -Expression Strain CL4176, Compared with Control Strain CL4175 which do not Express A [75]

Gene	Description	Fold increase (induced/control)	Human homolog
F22E5.6	TNF-induced protein1 homolog, contains K ⁺ channel tetramerization domain	32	MSTP028
ZC239.12	TNF-induced protein1 homolog, contains K ⁺ channel tetramerization domain	11.5	MSTP028
Y46H3A.D	Small heat shock protein HSP-16-2	9.86	CRYAB
Y46H3A.E	Small heat shock protein HSP-16-41	9.19	CRYAB

A β , which is able to generate reactive oxidative radicals (ROS) [3] [83]. Protein oxidation and lipid peroxidation have been demonstrated to occur in AD brain [18] [84]. To support the hypothesis that neuronal protein oxidation is a result of A β -associated free radicals production [18], Yatin and Link et al measured protein carbonyl, a key marker of protein oxidation [85] [86] in cultured hippocampal neurons, which were exposed to exogenous synthetic A β_{1-42} , and in transgenic *C. elegans* model [87]. Their results showed free radicals formation occurs in A β_{1-42} solution, and the reverse peptide A β_{42-1} and Met³⁵Nle substituted peptide did not produce free radicals. In the *C. elegans* model, the content of protein carbonyl of the strain expressing A β_{1-42} is more than that of the strain with a Met³⁵Cys substitution in A β_{1-42} . This result suggested methionine is critical in free radical production by A β_{1-42} . As mentioned above, Met³⁵ is also critical for β -sheet formation observed in the transgenic *C. elegans* lines [55], β -sheet structure may thus implicated in ROS production.

In order to identify the effect of A β expression and toxicity, Jennifer and Link et al employed the temperature-inducible A β expression *C. elegans* CL4176 to scrutinize the temporal relationship between A β expression, oxidative stress and A β fibril formation [61]. The results sustain the hypothesis that free radicals were related to A β toxicity. They observed that A β_{1-42} expression in *C. elegans* increased protein carbonyl formation significantly at 24 and 32 hours after temperature up shift, and oxidative stress occurred in the absence of significant A β fibril formation, suggesting that the pre-fibrillar A β is the toxic species [61], which is in agreement with other observations that fibrils might not be necessary for its toxicity [88] [15] [89] [13]. From Link's experiment, oxidative stress induced by A β expression caused phenotypic paralysis in the transgenic *C. elegans* (CL4176). The result indicated a multimer or conformer that serves as an intermediate in fibril formation, which is assumed to relate with A β toxicity [61]. Growing evidence support the speculation that non-fibrillar intermediates (oligomers) instead of fibrils are toxic in AD and other neurodegenerative diseases [90] [91].

Several research groups [83, 92, 93] have conducted studies on possible relevance of free radicals induced oxidative damage to the development of neurodegenerative diseases. To determine whether endogenous A β -expression increases H₂O₂-associated ROS levels, we employed the *C. elegans* strains CL2006 and CL4176. Smith et al [94] and (Fig. 2A) show significantly higher ROS levels in both A β -expressing transgenic *C. elegans* models compared to wild type [94], or transgenic control counterparts (Fig. 2A). The A β -expressing strain CL2006 showed 2.5-fold higher endogenous ROS levels than the wild type *C. elegans* (N2) [94], and the temperature-inducible A β -expressing strain CL4176 exhibited 2.6 fold increase in the levels of ROS compared with the transgenic control strain CL4175 (Wu, unpublished).

4. Pharmacological Evaluation of Ginkgo Biloba Extract in the *C. elegans* Model

The transgenic *C. elegans* model of AD enabled investigators to study the basic mechanism of AD *in vivo*, especially to correlate A β -induced molecular changes with

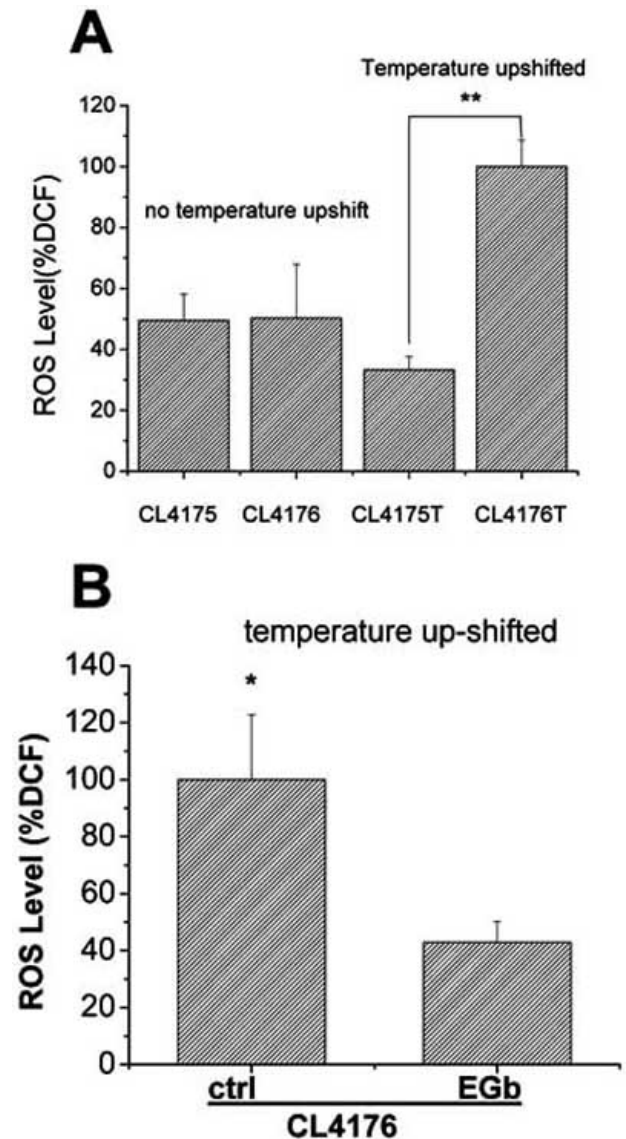


Fig. (2). H₂O₂ levels in the temperature inducible A β -expression *C. elegans* strain CL4176. **A.** Synchronized eggs of the control transgenic *C. elegans* strain (CL4175), or temperature-inducible A β -expressing transgenic *C. elegans* strain (CL4176) were maintained at 16°C on solid nematode growth medium (NGM) seeded with a 100 μ l spot of *Escherichia coli* (OP50) (~ 100 eggs/plate). To induce the transgene expression, the worms were maintained at 16°C for 38 h after hatching followed by up-shifting the temperature to 23 C for 36 h. Intracellular ROS were measured in the *C. elegans* nematodes using 2,7-dichlorofluorescein diacetate (DCF-DA; Molecular Probes).

B. The transgenic *C. elegans* strain (CL4176) grown on the culture plate containing either vehicle (Ctrl), or EGb 761 (100 μ g/ml) were temperature up-shifted to express A β . At least 60 animals from each group were analyzed for ROS assay, and results are expressed as percentage of fluorescence (%DCF) relative to Ctrl. * Statistically significant, $p < 0.05$; ** $p < 0.001$. (Wu, et al., 2004 unpublished)

A β -induced toxicity. It also allowed us to evaluate pharmacological effect of potential drugs for treatment of AD.

Our research involves a programmatic exploration for the mechanisms of neuroprotection provided by a ginkgo biloba extract EGb 761. Using neuronal cell culture, organism nematode *C. elegans* and animals behavioral test in mammals, our previous results shown that EGb 761 exhibits multiple cellular and molecular neuroprotective mechanisms, including modulating the cellular survival machinery [95], inhibition of A β aggregation [96], and augmentation of organism's stress-response [97, 98].

The standard *Ginkgo biloba* leaf extract EGb 761 is taken by the general population to enhance mental focus and by the elderly to delay the onset of age-related loss of cognitive function. EGb 761 was first on market in the 70's in France and Germany, and it becoming one of the most popular dietary supplements in the States. During the past decade, *in vivo* and *in vitro* experiments in mammalian systems and clinical studies in humans demonstrated that EGb 761 exhibits a range of biochemical and pharmacological effects that include cognition enhancement and stress alleviation [99]. In human studies, available data have confirmed the clinical efficacy of EGb 761 in primary degenerative dementia of Alzheimer's type [100-103]. Some data support the view that the extract enhances learning and longevity in rats [104] [105] and has neuromodulatory and neuroprotective properties in several species [106]. However, the evidence of an effect on memory in healthy humans is still inconclusive [107].

Smith *et al.* employed the transgenic *C. elegans* constitutively expressing human A β (CL2006) and observed a significantly lower level of H₂O₂-related ROS in AD-associated transgenic models fed with EGb 761 compared with the wild type controls [94]. Further, treatment with fractionated flavonoid components in EGb 761, kaempferol (Kaemp) or quercetin (Querc), or vitamin C (L-ascorbate) significantly attenuated ROS levels when compared to untreated transgenic control nematodes (attenuation with kaempferol by 69%) [94].

These result supports the well-known free radical theory of aging [108], and the role of A β in pathological processes of AD. It also explains another observation of ours in which EGb 761 extends life span in *C. elegans* [98]. Wu *et al* reported that the treatment of the wild-type worms with EGb 761 moderately extended median life span under physiological conditions, significantly increased their maximum life span under chronic oxidative challenge (Fig. 3A), and increased their resistance to oxidative stress and thermotolerance [98]. These results indicate that EGb 761 augments the natural anti-stress system of *C. elegans*, thus increasing stress resistance and life span.

Further, we investigated the molecular mechanism of EGb 761 on alleviating effects of oxidative stress using transgenic *C. elegans* expressing a jellyfish green fluorescent protein (GFP)-tagged inducible small heat-shock protein gene (*hsp-16-2*) [109]. The expression of *hsp-16-2* induced by the pro-oxidant juglone was significantly suppressed by 86% in the transgenic nematode fed with EGb 761 (Fig. 3B). These effects of EGb 761 correlate with its ability to increase

mean survival rate of the nematode in response to acute oxidative and thermal stresses, as well as to attenuate the basal levels of hydrogen peroxide in the organism [109]. Post-administration effects of EGb 761 on suppressing *hsp-16-2* expression [109] suggest that the extract functioned not only as a scavenger for oxidative free radicals that prevent the propagation of free radical damage, but also as the enhancer of repair or turn-over of damaged macromolecules. Thus, Strayer *et al* interpret the suppression of *hsp-16-2/GFP*

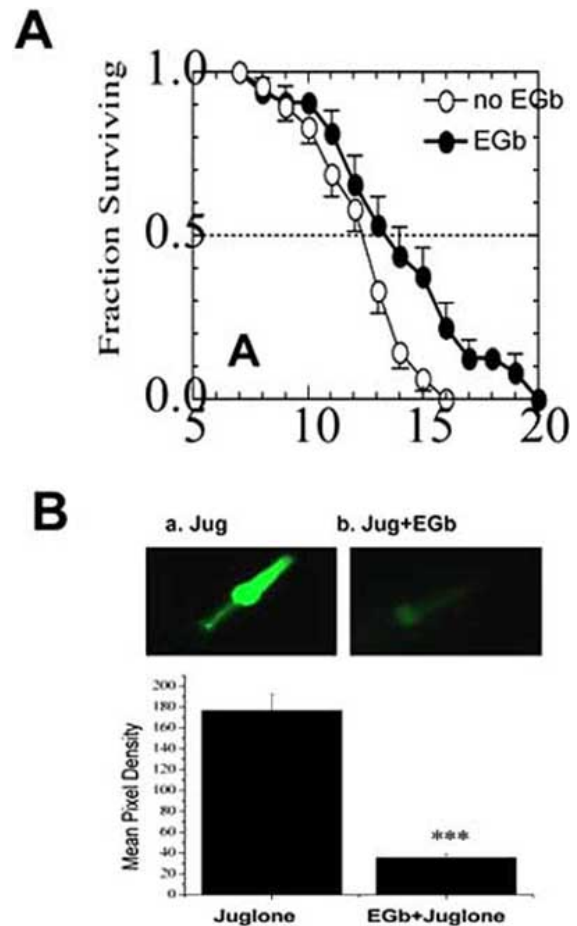


Fig. (3). EGb 761 extends life span and modulates the expression of the stress-response gene *hsp-16-2*. **A.** Effect of EGb 761 on life span of *C. elegans* pre-exposed to oxidative stress. The worms were oxidatively stressed by exposure to 40 μ M juglone on food for 24 h, after which they were transferred and fed with the food supplemented with or without 100 μ g/ml EGb761. Survival curves of the worms pre-exposed to juglone with (filled circles) or without EGb 761 treatment (open circles) [98]. **B.** GFP fluorescence images of the CL2070 worms fed with (b) or without (a) 100 μ g/ml EGb 761 for 48 h before 160 μ M juglone challenge for 24 h. The *C. elegans* were examined by epifluorescence microscopy, and the fluorescence images were captured with an Olympus fluorescence microscope attached to a digital camera. The photographs include the entire anterior part of the pharynx showing both nerve rings. For quantifying a population of GFP reporter animals, each 40x image was analyzed using ImageProPlus software. Data are expressed as GFP mean pixel density obtained from 4 independent experiments with at least 24 worms in each experimental group. ***Statistically significant, $p < 0.0001$ [109].

expression as an indication that EGb 761 decreases cellular stress resulting from exogenous treatments, therefore leading to a decreased transcriptional induction of the reporter transgene [109]. Since small heat shock proteins are remarkably expressed in A β -expressing *C.elegans* (CL4176, [75]), this result suggests a modulatory role of the extract in the function of a stress-response gene, and indicates that the effect of EGb 761 is beyond its known function as a scavenger for oxidative free radicals.

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