RNA Interference as a Tool for Alzheimer's Disease Therapy

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Abstract: RNA interference is a biological process that controls gene silencing in all living cells. Targeting the RNA interference system represents a novel therapeutic strategy able to intercede with multiple disease-related genes and to target many neurodegenerative diseases. Recently, the design of small interfering RNA-selective compounds has become more straightforward because of the significant progress made in predictive modeling for new therapeutic approaches. Although *in vivo* delivery of RNA interference remains a significant obstacle, new data show that RNAi blocks gene function *in vivo*, suggesting a potential therapeutic approach for humans.

Some groups have demonstrated the efficacy of RNAi therapy in Alzheimer's disease. Results, based on animal models, show a down-regulation of the amyloid precursor protein and a consequent reduction of the amyloid-β peptide accumulation in the brain or the inactivation of β-secretase (BACE1). Indeed, lentiviral vectors expressing siRNAs targeting BACE1 reduce amyloid production and the neurodegenerative and behavioural deficit in APP transgenic mice.

This review highlights recent advances in RNA research and focuses on strengths and weaknesses of RNAi compounds in Alzheimer's disease.

GENERAL BACKGROUND

RNA INTERFERENCE

RNA interference (RNAi) is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing) or by activating a sequence-specific RNA degradation process (post-transcriptional gene silencing).

Several recent reviews [1-5] show that RNAi natural functions and the related processes seem to act as a protective mechanism of the genome against transposons, mobile genetic elements and more complex events in eukaryotic organisms. A simplified model of the RNAi pathway consists of two steps with three critical common elements: (i) inducers dsRNA, (ii) RNA target degradation in a homology-dependent manner, (iii) presence of proteins which are specifically involved in the degradative machinery. Remarkably, inducer dsRNA molecules do not act stoichiometrically. It has been estimated that only two dsRNA molecules per cell are able to induce RNAi of an abundantly expressed *C. elegans* gene such as *unc22* [6-7].

The Mechanism of RNA Interference

Recent studies have defined the molecular events occurring during the two step mechanism of RNAi.

The first step involves the binding of RNA nucleases to a large dsRNA, and its cleavage into -21- to -25 nucleotide RNA fragments (siRNA). Bernstein *et al*. [8] showed that RNase III is involved in the initiation of RNAi. RNAIII, named Dicer, digests dsRNA into homogeneously siRNA fragments, with 3-overhangs of 2 to 3 nucleotides, 5-phosphate and 3-hydroxyl termini [9]. Dicer has four distinct domains: (1) an amino-terminal helicase domain, (2) dual RNase III motifs, (3) a dsRNA binding domain, and (4) a PAZ domain. The last domain consists of 110-amino-acids: it is present in proteins like those of the RDE1/QDE2/Argonaute family that has been genetically linked to RNAi by independent studies [10-12]. Some Dicer proteins contain an ATP-binding motif along with the DEAD box RNA helicase domain [5, 10-16]. Each monomer of the Dicer possesses two catalytic domains, but only one has catalytic sequences. Consequently, Dicer acts as a dimer and digests dsRNA with the help of two compound catalytic centers. The crystal structure of the RNase III catalytic domain was recently solved and a model for the generation of 23-to 28-mer siRNA products was defined [5, 13-19]. In this model, the dimeric Dicer folds on the dsRNA substrate producing four compound catalytic sites. The two terminal sites, having the maximum homology with the consensus RNase III catalytic sequence, remain active; the two internal sites with partial homology, lose functional significance.

In the second step, the siRNAs bond a multinuclease complex, the RISC, that degrades the homologous single stranded mRNAs [1-2, 5, 20]. During RISC assembly, the siRNA is unwound in a strand specific manner. Only one strand of the original double stranded molecules, a guide strand, is incorporated into the RISC while the other, a passenger strand, is discarded. This was first observed with microRNAs, but also occurs with siRNAs and long dsRNAs. The incorporated strand is generally the one whose 5' termi-

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nus is at the thermodynamically less stable end of the duplex [3, 21-22]. Within the RISC, mRNA cleavage occurs between residues that are base paired to nucleotides -10 and -11 of the siRNA, and the cleavage itself does not require ATP. The guide siRNA remains associated with the complex, allowing it to carry out multiple rounds of RNA cleavage. The enzyme turnover depends on ATP [23]. This step involves an RNA helicase and several proteins implicated in RNAi. The Dcr-2-R2D2-siRNA ternary complex is the best characterized assembly in the RISC formation, in the absence of ATP. This step, in which the siRNA duplex undergoes unwinding and the Dcr-2/R2D2 heterodimer is gradually displaced by the Ago2 protein, requires additional proteins and ATP. Functional RISCs were isolated in other forms. "Minimal" active RISCs of 150 kDa may contain only Argonaute proteins associated with the siRNA guide strand and catalyze the mRNA cleavage. The largest complex, a holo-RISC, was identified in *Drosophila* [24], and represents smaller RISC forms associated with ribosomes and Argonaute proteins. This complex is localized in cytoplasmic foci known as P/GW bodies. Silencing of the GW-182 subunit, delocalizes resident P/GW-body proteins. Mutations that prevent Argonaute proteins from localizing in P/GW-bodies, prevent translational repression of mRNAs even when Argonaute proteins interact with their target in a siRNA-independent fashion. Armitage, identified in *Drosophila*, is required for the conversion of RLC into an active RISC. It is still unclear whether Armitage is involved in siRNA unwinding, since it is also needed when RISC assembly is programmed with the single-stranded siRNA [23]. However, proteins involved in specific steps of RNAi have not yet been established [25-27].

Recently it has been elucidated that microRNA (miRNA) is a 21-nucleotide with a regulatory RNAs activity. It is likely that miRNA is responsible for the inhibition of mRNAs traduction. This mechanism was discovered in plants and shows similarities between siRNA and miRNA pathways. Indeed, maturation of both RNA classes involves Dicer proteins and like siRNAs, miRNAs function as RNP particles, miRNPs or miRISCs, whose composition and probably also their assembly are related to those of the RISC [28-29].

ALZHEIMER'S DISEASE

Alzheimer's Disease (AD), originally described in a 51-year-old woman by Alois Alzheimer, is the most common cause of dementia in humans [30-34]. External examination of the brains of AD patients reveals significant cortical atrophy which is particularly evident in the limbic and association cortices, together with the enlargement of the lateral ventricles. The hallmarks include: neuronal loss, intraneuronal neurofibrillary tangles, extracellular deposits of amyloid filaments of a 40- to 42-residue amyloid β protein (Aβ), and the Aβ surrounded by altered neuritic processes and glia. These are called senile plaques and are not AD specific. After a period of time, Aβ deposits become increasingly fibrillar and gradually acquire the classical features of amyloid plaques. Generally, mature amyloid plaques are associated with numerous dystrophic axons and dendritic processes that lie within or immediately around the fibrous amyloid deposit accompanied by neurofibrillary tangles [35-36].

Ten to 15% of cases of AD occur in an autosomal dominant Mendelian pattern, but a much higher percentage of patients has a clinical history with a highly similar dementing syndrome. Due to the late onset of most AD cases, it is difficult to ascertain whether members of previous generations really suffered from the disease. However, growing evidence suggests that a high percentage of subjects has inherited some type of genetic predisposition to the disease, as demonstrated by the identification of specific DNA mutations in a large number of families [30-33]. It is likely that, despite this etiological heterogeneity, there is a common AD pathogenetic cascade which can result from distinct gene defects and/or unknown environmental factors.

Alzheimer's Disease Pathway

Despite the fact that molecular events leading to the generation of amyloid plaque deposition have been explored in great detail, a clear molecular etiology of AD is still unclear.

In 1987 the amino-terminal sequence of the Aβ protein was defined, the gene encoded for its precursor polypeptide APP was cloned and the localization of its gene to the long arm of chromosome 21 was established [37-38]. This finding provided an explanation for the long-standing neuropathological observation that patients with trisomy 21 develop amyloid-bearing plaques and other AD lesions [38]. Subsequently, cloning of APP cDNAs from other mammals demonstrated that this gene is totally conserved in the cynomolgus monkey and humans. The full-length cDNA encoding APP is a 695-residue protein that contains a single domain with a hydrophobic putative transmembrane sequence next to its carboxy terminus [39-40]. APP belongs to a protein family with two mammalian paralogues, the amyloid precursor-like proteins (APLP) 1 and 2 [41-42]. APP/APLPs share highly conserved protein domain organization, are able to form homo- and heterotypic interactions and are proteolytically processed in a similar manner. APP exists as a heterogeneous group of polypeptides ranging from 105 to 140 kDa [41] and, during its post-translational maturation in the secretory pathway, it undergoes *N*- and *O*-glycosylation as well as tyrosine sulfation [40]. It has been demonstrated that APP polypeptides arise from alternative splicing that generate transcripts of 751 and 770 amino acids. Both transcripts have an inserted exon encoding a Kunitz-type serine protease inhibitor (KPI motif). Further examination of the exon/intron structure of the APP gene revealed that also the 40- to 42-amino-acid Aβ fragments contain portions of two adjacent exons and, for this reason, must arise from proteolytic processing rather than alternative splicing [43-46].

Normal APP cellular processing includes a pathway that involves protein maturation in the Golgi apparatus, trafficking to the plasma membrane and cleavage at residue 16 within the Aβ domain (residue 687 of APP770). In this α-secretory processing, the large amino-terminal hydrophilic portion of the precursor is released into the medium [45] whereas the membrane-associated carboxy-terminal fragment is retained inside the cell [45-48]. APP intracellular domain (ICD) contains a YENPTY motif that interacts with the adaptor protein Fe65 [49-50], Fe65L1 [42], or X11α and X11β [42,49]. Since Fe65 is also a nuclear protein, it has been suggested that APP could function as an extracellular

anchor, thus preventing Fe65 nuclear translocation [42,49]. The APP cytoplasmic tail also contains a similar consensus sequence for the internalization of cell-surface receptors *via* clathrin-coated vesicles. In fact, an alternative processing route involving the re-internalization of holo-APP from the cell surface and its trafficking to endosomes and lysosomes has been demonstrated [45-47]. During the above mentioned process, APP is first cleaved by α-secretase(s), and then the resultant membrane attached fragments are cut by γ-secretase into p3/p3-like fragments (Fig. **1A**). The products of this α-cleavage-γ-cleavage are highly soluble and non-amyloidogenic [51-53]. However, the normal functions of APP metabolism are yet to be determined.

Alternatively, APP should be first cleaved by β-secretase and then, the different resultant membrane attached fragments processed by γ-secretase [41-42, 44, 46]. The product generated by the β-cleavage-γ–cleavage pathway is the 99-residue C-terminal fragment of APP (CTF fragment). This fragment may be cleaved by distinct γ-secretases at either residue 40- or residue 42- of the Aβ region (Fig. **1B**). Thereafter, Aβ is secreted from the presynaptic terminals into the extracellular matrix, and thus fibrillary Aβ deposits in AD are formed outside of the neurons. Indeed, Aβ40 and Aβ42 are insoluble [34-35, 46-47]. This was also confirmed by electrophysiological examinations of AD mouse models, suggesting an essential role of dimeric Aβ as inducers of synaptic plasticity disruption [53]. It is likely that Aβ dimers can influence calcium homeostasis and other physiological processes in the synapses, resulting in synaptic degeneration that may involve a marked reduction of presynaptic neprilysin [53]. Remarkably, small amounts of Aβ are continuously released from a variety of cultured cells under normal metabolic conditions [54-56], indicating that the Aβ peptide is a normal metabolic product of APP throughout life [57].

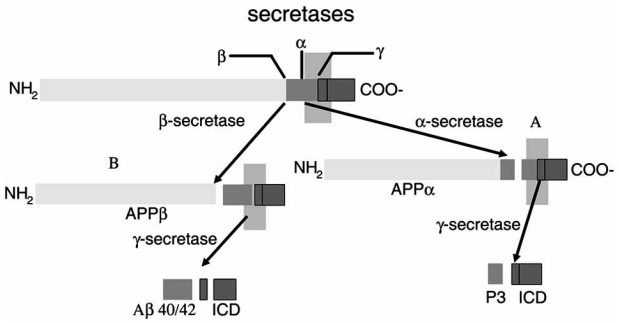

Fig. (1). APP processing. **A)** α-secretase-γ-secretase cleavage generate APPα, P3 and ICD peptides. **B)** β-secretase-γ-secretase cleavage generate APPβ, Aβ40/Aβ42 and ICD peptides.

Interestingly, both processing pathways liberate the APP corresponding intracellular domains (ICDs) [54]. On the basis of the recent observations indicating a function in nuclear signaling for the APP/APLP ICDs, APP/APLPs processing is supposed to be a crucial step in the pathology of AD as well as an important factor in the physiological function of APP/APLPs [54].

Familial AD mutations on the APP gene either enhance β-cleavage or alter the activity of γ-secretase to increase the ratio of amyloidogenic Aβ42 to Aβ40 [58-60]. Linkage analyses and positional cloning led to the identification of the presenilin 1 (PS1) gene on chromosome 14, encoding for a membrane protein of 467 amino acids, as one of the genes responsible for AD [61-62, 65]. After the cloning of PS1, a highly homologous gene called presenilin 2 (PS2) was identified on chromosome 1 [66]. Generally, PS1 and PS2 mutations linked to familial AD seem to generate a disregulation of γ-secretase, selectively enhancing APP proteolysis at Aβ42 [67-71]. Investigations are underway to see whether presenilin mutations increase the Aβ42 generation. Other studies have indicated the involvement of additional polypeptides. Apolipoprotein E has been suggested as one of such proteins [72]. Genetic analyses confirm these data, showing that ε4 polymorphism of the ApoE gene was substantially over-represented in sporadic AD subjects compared to age-matched controls, representing a major risk factor for the development of the disease [73-74]. A major indication is based on the observation that AD subjects with two ε4 alleles have a significantly higher number of Aβ deposits in the brain than subjects with no ε4 alleles, while subjects with one ε4 allele generally fewer deposits are present [75-78].

Senile plaques are mainly composed of Aβ40 and/or Aβ42, and neurofibrillary tangles consist of twisted filaments of hyperphosphorylated microtubule-associated protein (MAP) Tau [79,80]. Tau has six isoforms in the adult human brain, which vary in length from 352 to 441 residues that result from alternative splicing of the *tau* gene (human chr 17 at 17q21) [81]. Tau N-terminal domain, composed of an acidic region at the N-terminus and a proline-rich region in the interior, has been suggested to be involved in several functions including determining axonal microtubule spacing [82] and interaction or interconnection with cytoskeletal components [82-84], mitochondria [85], and the plasma membrane [86]. The C-terminal side of Tau contains the microtubule-binding domain with either three or four microtubule-binding repeat domains, depending on the isoform. This microtubule-binding domain is ubiquitinated by at least three different types of polyubiquitin conjugations when Tau is in PHFs (Paired Helical Filaments) [87]. Hyperphosphorylation of Tau (P-tau) induces the protein to dissociate from microtubules and form PHFs (16). However, the relation between hyperphosphorylation and PHF formation is not yet clear since native Tau is already phosphorylated and it is likely that Tau need not be phosphorylated to assemble into PHFs. Similarly, the relation between Tau hyperphosphorylation and neuronal toxicity is unclear [88-91]. It seems that AD P-tau sequesters normal tau (N-tau), and it disassembles microtubules and self-assembles into PHFs [92-96]. AD P-tau is able to destroy the microtubules formed with all of the tau isoforms [97]. Dephosphorylation of AD P-tau as well as PHF converts them into normal-like protein, which promotes assembly and stabilizes microtubules [93, 98-99]. Moreover, as P-tau is present in different aggregation states, it is not known whether the toxic entity is in the misfolded modified protein, the oligomer, or the filaments. [100]. Recently, it has been demonstrated that the temporal relationship between Aβ monomers, oligomers, and fibril formation. appears to commence intraneuronally suggesting that the action of Aβ oligomers may be a key event in the initiation of the tau pathology [100]. Yet, new evidence indicates that tau may mediate neurotoxicity by altering the organization and the dy-

namics of the actin cytoskeleton [81-82]. Fulga and collaborators, using *Drosophila* and mouse models of tauopaties, have shown that tau-induced neurodegeneration is associated with the accumulation of filamentous actin (F-actin). In fact, modulating F-actin levels genetically leads to a dramatic modification of this protein. In the same study the authors demonstrated that human Aβ synergistically enhances the ability of wild-type tau to promote alterations in the actin cytoskeleton and neurodegeneration. These findings raise the possibility that a direct interaction between tau and actin may be a critical mediator of tau-induced neurotoxicity in Alzheimer's disease and related disorders [101-102].

Efforts aiming to elucidate the mechanisms of abnormal tau hyperphosphorylation have led to the identification of several protein kinases that may catalyze tau phosphorylation in the brain [103]. Among these tau kinase candidates, glycogen synthase kinase 3 (GSK-3β and GSK-3α) [104], cyclin-dependent protein kinase 5 (cdk5) and cAMP-dependent protein kinase (PKA) have been most implicated. The former two were actually described as tau kinase I and tau kinase II, respectively [9,10]. These two kinases can phosphorylate tau at multiple sites. It has been observed that prephosphorylation at certain sites primes tau to be a better substrate *in vitro* as well as *in vivo* for GSK-3β. PKA-induced tau phosphorylation promotes its subsequent phosphorylation at most sites catalyzed by GSK-3β, whereas it differentially affects its subsequent phosphorylation by cdk5 [103].

Aβ over-production has not been observed in sporadic AD. On the basis of the observation that the steady-state level of Aβ is presumed to be maintained by the balance of synthesis and clearance of Aβ, it is likely that the onset of sporadic AD may be attributed to an impaired clearance of Aβ. Major processes involved in Aβ clearance include Aβ proteolytic degradation and Aβ transfer from the brain tissue to the cerebrospinal fluid and plasma. However, there is evidence that Aβ42 can be generated early during the secretory trafficking of APP, namely, in the endoplasmic reticulum and Golgi [105-109]. Therefore, the proteases referred to as β- and γ-secretases are apparently distributed to several subcellular compartments.

Neprilysin is the key enzyme responsible for Aβ degradation in the brain [110-112]. Neprilysin is a type II membrane metalloendopeptidase composed of 750 residues, with an active site containing a zinc-binding motif (HEXXH) at the extracellular carboxyl terminal domain. It is placed on plasma membrane as a non-covalently associated homodimer [110-111]. On the basis of the size of known substrate peptides and cleaves a hydrophobic residue in the P1V position of substrate peptides, neprilysin mainly acts on peptides smaller than 5 kDa (5–40 amino acid). The Aβ 1–42 polypeptide is the longest known neprilysin substrate [112]. Interestingly, the active site of neprilysin faces the extracellular side, where Aβ should be released [112].

Other potential Aβ breakers are likely to act in different locations from the neprilysin-rich presynaptic surface. It should also be noted that Insulin Dependent Enzyme (IDE) can attenuate Aβ-related neurotoxicity by proteolysis of ICD, as well as Aβ [34, 113-114]. However, IDE cannot degrade oligomers [114]. Since a variety of cytoskeletal components (tau, neurofilaments, microtubule-associated protein 2 and actin) are substrates for two major families of cysteine proteases, (calpains and caspases) [115-119], it can be hypothesized that these enzymes are activated by Aβ [97]. This phenomenon is presumed to be activated by calcium influx, as calcium homeostasis is disturbed by the accumulation of Aβ [120-121].

Recent studies have identified a disturbance of the endocytic pathway as one of the earliest known manifestations in sporadic AD. Lysosomal dysfunction has been linked to neurodegeneration and directly implicated in cell death programs in certain pathologic states [122-124]. Cathepsin D levels in AD brain increase [124] but, unlike in early AD, several other cathepsins remain constant or decrease [124-125]. Within this context, we have demonstrated that the CTSD gene is down-regulated at both transcriptional and translational level in skin fibroblasts of patients affected either by sporadic or familial forms of AD [126]. Moreover, in a previous study, we provided evidence that an up-regulation of lysosomal glycohydrolases (α-D-mannosidase, β-D-hexosaminidase, and β-D-galactosidase) takes place in skin fibroblasts from patients affected either by AD sporadic or familial forms. This event is also detectable in presymptomatic subjects carrying the above mentioned mutations but healthy at the time of skin biopsy. Yet, enzyme activity increase was a result of a transcriptional up-regulation and the mechanism seemed to be controlled by the Ras oncogene [127].

Neurotransmitter deficit has been detected in neurons affected in the AD brain. These include marked decline in the activities of choline acetyltransferase and acetylcholinesterase and dysfunctions in noradrenergic and serotonergic cells in the brainstem, cells producing somatostatin or corticotropin-releasing factor in the neocortex and neurons releasing glutamate, GABA, substance P and/or neuropeptide Y [128].

DEVELOPMENT OF AD TREATMENTS: CURRENT STATUS

AD treatments aim at blocking the pathogenic Aβ peptide generation and at rescuing vulnerable neurons from degeneration (Fig. **2**).

Using an Aβ immunotherapy approach, it is possible to reduce amyloid burden in numerous cases. Initial clinical testing with AN1792, composed of Aβ42 and an adjuvant, has yielded important insights into both the clinical potential of the approach and the impact of Aβ peptide on the disease [129-132]. However, the application in human trials was accompanied by moderate brain inflammation in a subset of patients immunized with two vaccine vectors: the first expressing Aβ42 alone, and the second expressing Aβ42 fused with the tetanus toxin Fragment C as molecular adjuvant. It means that this approach needs to be clinically validated. Conversely, peripheral administration of these vaccines in Tg 2576 AD mice augmented humoral responses to Aβ and reduced CNS Aβ deposition [132-133].

Based on their crucial role in the disease, secretases are a centre of attraction for new drug research. Indeed, a large number of specific drug-like γ-secretase inhibitors have been discovered and tested [134-137].

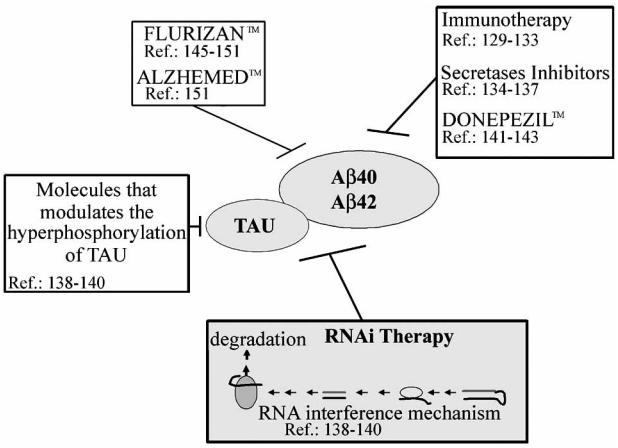

Fig. (2). Scheme of Alzheimer's Disease treatments. See text for details.

Similarly, pharmacological modulation of tau hyperphosphorylation as a downstream target for many kinases and signalling cascades, or inhibition of tau aggregation, might represent a valid and feasible therapeutic strategy for such disorders. Therefore, GSK-3β, CDK5 and ERK2 are being explored as drug candidates for AD treatment [138-140].

Other approaches investigate acetylcholinesterase inhibition [141-142]. Acetylcholinesterase inhibitors appear to cause selective muscarinic activation of α-secretase and to induce the translation of APP mRNA; they may also restrict amyloid fiber assembly. Activation of N-methyl-D-aspartate receptors is considered to be a probable cause of chronic neurodegeneration in AD, and memantine has been widely used in AD patients to block cerebral N-methyl-D-aspartate receptors that normally respond to glutamate [141]. Donepezil™ acts as an acetylcholinesterase inhibitor. Clinical studies have shown that using this drug at an early stage results in delayed progression of the disease, and increased longevity. Basic research suggests that Donepezil™ also has a neuroprotective effect due to the ability of this drug to inhibit the aggregation and toxicity of the Aβ peptide. In addition Donepezil™ is most effective in delaying disease progression in patients with the apolipoprotein E genotype, who have very high levels of senile plaque formation [143].

Further studies are needed to determine whether antioxidants such as vitamins C and E are effective, through various mechanisms, in patients with mild to moderate AD [144].

Although, additional data are required regarding the use of non-steroidal anti-inflammatory drugs, some of these molecules, such as nonaspirin NSAIDs, are associated with protection from the development of AD [145-150]. Indeed, trials of select NSAIDs in AD such as R-flurbiprofen are ongoing [151]. The R-enantiomer of racemic flurbiprofen, is undergoing development by Myriad Genetics Inc, under license from Encore Pharmaceuticals Inc, for the potential treatment of Alzheimer's disease. Up to date FLURIZAN™ has completed a Phase2 human clinical trial in 207 AD patients and is currently being studied in two Phase 3 clinical trials in patients with mild AD [151].

Similarly ALZHEMED™, a drug designed to prevent amyloid formation and deposition in the brain, are now in a trial by the FDA at Phase 3. ALZHEMED™ acts on two levels: preventing and stopping the formation and deposition of amyloid fibrils in the brain as well as binding to soluble Aβ, and inhibiting the inflammatory response associated with amyloid build-up in AD [151].

RNA INTERFERENCE FOR ALZHEIMER'S DISEASE THERAPY

RNAi potential in research and therapeutics has been highlighted by a large number of papers [152], investigating its effectiveness on the therapy of different types of diseases, including neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) [153], Spinocerebellar ataxia [154], and Huntington disease [155]. The efficacy of RNAi is particularly impressive in the ALS model, where it was reported to double the latency period and prolong the lifespan by approximately 80% after intramuscular injections of shRNAs [153].

In this review we report *in vivo* and *in vitro* data that raise the hope for a RNAi-based therapy for AD (Figs. **2**,**3**).

RNA Interference for AD: *In Vivo* Studies

Significant evidence has arisen from the *in vivo* approach with viral vectors expressing siRNAs targeting BACE1, in APP transgenic mice (Fig. **3A**). These results demonstrate the potential utility for exploiting these vectors, both as novel gene therapeutics for AD and as tools to elucidate the role of APP and Aβ in the pathogenesis of AD [156-158].

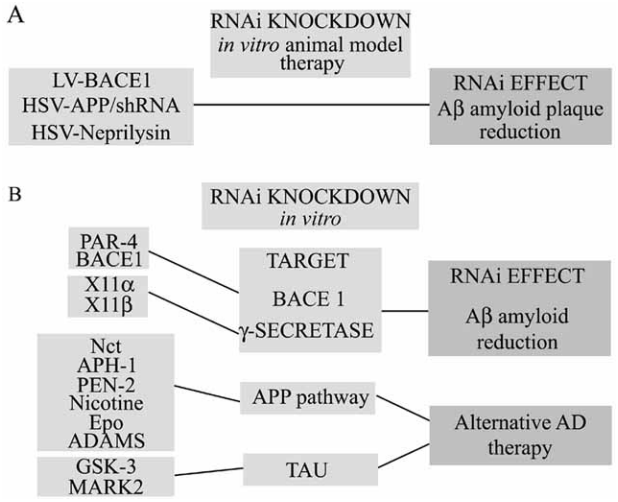

Fig. (3). Overview of Alzheimer's Disease and RNAi. **A**) *In vivo* RNAi for Alzheimer's Disease. **B**) *In vitro* RNAi for Alzheimer's Disease. See text for details.

Using lentiviral vectors expressing siRNAs targeting BACE1, Singer *et al*. obtained a reduced cleavage of APP as well as a reduction in amyloid burden and amelioration of the dendritic and synaptic pathology in the hippocampus (site of injection) [156]. These results were consistent with other *in vitro* studies demonstrating that delivery by lipofectamine of siBACE in APP-transfected primary neuronal cultures from APP tg mice resulted in reduced APP CTFs and Aβ production and was protective from the neurotoxic effects of peroxide [157].

Relevant results were also obtained by Hong and colleagues [158]. They generated HSV vectors expressing either (i) short RNA directed to the APP transcript (HSV-APP/ shRNA), or (ii) neprilysin, (HSV-neprilysin) [158] that were injected into the hippocampus of a novel mouse model generated using a lentiviral vector (LV-APPSw) to deliver a familial AD mutant form of the APP gene (the 'Swedish mutation'). The basic experimental paradigm of this study was also attractive. The hippocampus on one side of a mouse was stereotactically co-injected with LV-APPSw and either HSV-APP/shRNA or HSV-neprilysin; the contralateral hippocampus was co-injected with LVAPPSw and QOZHG, the parental HSV vector that is isogenic to HSV-APP/shRNA and HSV-neprilysin, except for the absence of Aβ-targeting genes. At 10 days or 4 weeks post-injection, brain sections were examined. Both the HSV-APP/ shRNA and HSV-neprilysin vectors efficiently reduced Aβ expression in the lentiviral mouse model of Aβ accumulation [158].

RNA Interference for AD: *In Vitro* Studies

Based on AD pathology pathway, it is widely believed that any mechanism causing a reduction in the toxic Aβ cleavage products, either by reducing its formation or by increasing its degradation, would provide benefits to the treatment of AD (Fig. **3B**).

To this end, recent advances using RNAi technology have opened a new window to testing the impacts of β- and γ-secretase inhibition.

PAR-4 (prostate apoptosis response-4) is a leucine zipper protein that forms a complex with the cytosolic tail of β-secretase BACE1 [159-163]. Silencing of PAR-4 expression by RNAi significantly decreased the β-secretase cleavage of APP, suggesting that PAR-4 may be directly involved in regulating APP cleavage activity of BACE1 [163]. Results identified PAR-4 as an endogenous regulator of BACE1 and indicated novel therapeutic strategies for AD.

APP adaptor proteins with phosphotyrosine-binding domains, including X11α and X11β, can bind to the conserved YENPTY motif in the APP C-terminus [164]. The overexpression of X11α and X11β alters APP processing and Aβ production. For the first time, Xie and collaborators described the effects of RNA silencing of X11α and X11β expression on APP processing and Aβ production. They demonstrated that RNAi silencing of X11α in H4 human neuroglioma cells, stably transfected to express either full-length APP or APP-C99, inhibited APP γ-secretase cleavage and reduced Aβ levels in both cell lines. On the contrary, X11β silencing reduced Aβ levels, but apparently not *via* the attenuation of APP γ-secretase cleavage. These results were of particular relevance since they suggested X11α as a therapeutic target for γ-secretase inhibition [164].

Using a similar approach the same authors investigate the effects of reduced expression of ShcA and ShcC on APP processing and Aβ production. ShcA (*SHC1* gene) and ShcC (*SHC3* gene), the phosphotyrosine-binding domain-containing adaptor proteins that signal cellular differentiation and survival pathways, are other types of APP adaptor proteins that also bind to and interact with the YENPTY motif of APP. Xie and collaborators established RNAi for ShcA and ShcC in H4 human neuroglioma cells overexpressing FL-APP (H4-FL-APP cells) and APP-C99 (H4-APP-C99 cells) and evaluated the effects of RNAi-mediated silencing of ShcA and ShcC on APP processing and Aβ production. RNAi silencing of ShcC led to reductions in the levels of APP-C-terminal fragments (APP-CTFs) and Aβ in H4 human neuroglioma cells stably overexpressing full-length APP (H4-FL-APP cells) but not in those expressing APP-C99 (H4-APP-C99 cells). RNAi silencing of ShcC also led to reductions in BACE1 levels in H4-FL-APP cells. In contrast, RNAi silencing of the homologue ShcA had no effect on APP processing or Aβ levels. RNAi silencing of Fe65 increased APP-CTF levels, although also decreased Aβ levels in H4-FL-APP cells. These findings suggest that pharmacologically blocking interaction of APP with ShcC and Fe65 may provide novel therapeutic strategies against AD [165].

RNAi technique has also been explored for human AD PS1 [166]. Findings showed that there was a time-dependent relationship between the transcript of PS1 gene and the production of Aβ42. Moreover it was shown that PS1 is essential for γ-secretase activity since its inhibition decreases Aβ42 levels. These studies indicate a new pathway towards which new strategies for AD treatment should be directed.

The core of the active γ-secretase complex is PS1, which contains the proteolytic active site, and three other membrane proteins: nicastrin (Nct), anterior pharynx defective-1 (APH-1), and presenilin enhancer protein-2 (PEN-2) [167]. Depletion of the CD147 γ-secretase subunit by RNAi was found to increase the production of Aβ peptides without changing the expression level of other γ–secretase components or APP substrates [167-168]. In this context, Xie *et al*., establishing RNAi for PEN-2, and demonstrated that the metabolism of wt-PS1 FAD-linked Delta9-PS1 was specifically and differentially affected by loss of function of PEN-2 [168]. Furthermore, investigation on the functions of nicastrin (Nct) within the PS1/γ-secretase complex demonstrated that the loss of Nct expression in the embryonic fibroblast cells (Nct knockdown cells) resulted in dramatically decreased levels of APH-1, PEN-2, and PS1 fragments, accompanied by a significant accumulation of full-length PS1 [169]. The relevance of this work is also the demonstration that, in the Nct knockdown cells, PEN-2 and full-length PS1 are subjected to proteasome-mediated degradation, whereas the degradation of APH-1 is mediated by both proteasomal and lysosomal pathways. Moreover, conversely to WT cells, the majority of residual PEN-2, APH-1, and the uncleaved full-length PS1, in Nct knockdown cells, reside in the endoplasmic reticulum with a significant amount of full-length PS1 and PEN-2, also on the plasma membrane. This study demonstrates a critical role of Nct in the stability and proper intracellular trafficking of other components of the PS1/ γ-secretase complex, but not in maintaining the association of PEN-2, APH-1, and full-length PS1 [169-170].

The importance of RNAi technology for AD also arose from growing research investigating the role of proteins that in a different way are involved in the AD pathway. Below some examples of these studies are reported.

Important findings were obtained by Hiltunen *et al*. [171]. They reported that DNA variants in Ubiquilin 1

(UBQLN1) increase the risk for AD. UBQLN1 is a ubiquitin-like protein, playing a key role in regulating the proteasomal degradation of various proteins, including presenilins [171-172]. Down-regulation of UBQLN1 by RNAi accelerates the maturation and intracellular trafficking of APP without interfering with α–, β-, or γ-secretase levels or their activities. UBQLN1 knockdown increased the ratio of mature/immature APP, increased levels of full-length APP on the cell surface, enhanced the secretion of APP (α- and β-) and increased the levels of secreted Aβ40 and Aβ42. Furthermore, using the knockdown cell model, it was observed that UBQLN1 and APP move into intact cells, in a presenilin independent manner, suggesting that UBQLN1 may normally serve as a cytoplasmic "gatekeeper" that controls APP trafficking from intracellular compartments to the cell surface [171].

Other groups employing RNA technology elucidated the nicotine pathway in order to improve its therapeutic benefit in treating AD**.** Liu Q. *et al*., showed that nicotine decreases Aβ accumulation in the cortex and hippocampus of APP (V717I) transgenic mice. Using RNAi experiments, they showed that the nicotine-mediated process requires α7 nAChR to be effective [173].

Protective capacity of EPO in AD remains unclear. Yet, understanding the mechanism is of relevance for therapy. Chong and colleagues showed that EPO, in a concentration dependent manner, is able to prevent the loss of both apoptotic genomic DNA integrity and cellular membrane asymmetry during Aβ exposure [139]. Using the RNAi system, they demonstrated that EPO is an effective entity at the neuronal cellular level against Aβ toxicity and requires the close modulation of the NF-kappaB p65 pathway [174].

Further, RNAi know-how was crucial for the clarification of the implication of ADAMs proteins in AD. It was established that ADAM9, ADAM10, and ADAM17 catalyze α-secretory cleavage and therefore act as α-secretases, as well as that ADAM19 is tightly associated with constitutive Alzheimer's disease APP α-secretase in A172 cells [175-176].

On the basis that the phosphorylation of tau is an essential event in the pathogenesis of AD, the explicit role of GSK-3β in the pathogenesis and the activation mechanism of MARK are two enticing areas of study. It was found that GSK-3β inhibition resulted in the suppression of Ser-262 phosphorylation of tau in neurohybridoma F11 cells, indicating that GSK-3β is involved in the phosphorylation of tau at Ser-262. Because GSK-3β cannot phosphorylate this site directly, it was suggested that GSK-3β and MARK cooperated in inducing the phosphorylation of tau at Ser-262. Down-regulation of either GSK-3β or MARK2 by RNAi suppressed the level of phosphorylation on Ser-262 of tau. Consequently GSK-3β is responsible for Ser-262 phosphorylation and of the activation of MARK2 [177-180].

Similar RNAi studies were carried out using natural antisense transcripts of AD associated genes including PS1, PS2, BACE1, BACE2, APP, APOE, TAU, PRION, α-SYNUCLEIN (SNCA), NICASTRIN, PEN2, APH1A, APH1B as well as CD147. As already demonstrated for RNAi, the inhibition of one of the genes involved in AD pathway may provide important information about transcription regulation of AD associated genes [181-182].

Throughput Screens for Identifying Effective RNAi Probes for AD

Combinatorial gene inactivation using an RNAi library is a powerful approach to discover novel functional genes [183]. There are several methods of designing RNAi vector libraries with regard to their targets. The first approach is to construct individual siRNAs against defined sequences of each target. The second approach is to generate a combinatorial library by randomizing the entire siRNA sequence, thus covering every possible gene [184-186]. The third approach is to clone short double-stranded cDNA sequences of 21-27 bp directly into expression vectors. There are reports of successful examples using this strategy, but the efficiency of construction and the representativeness of these libraries are still not optimal [187-189].

Kumar *et al*. have developed a quantitative procedure for the rapid identification of an effective siRNA/shRNA for the inhibition of target gene expression [190]. Effective RNAi probes were identified on the basis of their ability to inactivate cognate sequences in an ectopically expressed target gene-reporter chimeric mRNA. Using either a fluorescent or enzymatic reporter, the siRNA effect is monitored quantitatively. Furthermore, using microarray-based cell transfections, it was demonstrated that this approach can be tailored to high-throughput screens for identifying effective siRNA probes in mammalian systems. Such a screen would have unlimited potential for analyzing AD gene function on a genome-wide scale. Using the siRNA screening technology**,** Majercak and collaborators assessed 15,200 genes for their role in Aβ42 secretion and identified two regulators of APP processing mapping to the region of chromosome 10 associated with LOAD and plasma Aβ42, *LRRTM3* and *RUFY2*. They focused on *LRRTM3* because of its proximity to genetic variability associated with LOAD in *APOE* ε*4*-positive individuals, its similarity to the Nogo receptor, its neuronal expression pattern, and its effect on BACE1[191].

Kreamer and collaborators, describe a genome-wide RNAi screen for genes that modify the tau-induced Unc phenotype. They tested RNAi sequences for 16,757 genes and found 75 that enhanced the transgene-induced Unc phenotype. Forty-six of these genes have sequence similarity to known human genes and fall into a number of broad classes including kinases, chaperones, proteases and phosphatases. This work uncovers novel candidate genes that prevent tau toxicity, as well as genes previously implicated in tau-mediated neurodegeneration [192].

CONCLUSIONS

Despite the recent efforts to understand AD molecular pathogenesis, two critical issues concerning the cascade hypothesis remain to be clarified. Firstly, there is no clear evidence of an elevated level of Aβ production in sporadic AD. Although genetic mutations in familial AD result in the overproduction of total Aβ and/or relatively amyloidogenic species of Aβ [34-35, 47], the mechanisms through which Aβ accumulates in the brains of sporadic AD patients are yet

to be elucidated. Secondly, the molecular processes linking Aβ and tau pathologies still remain unsettled [193].

We strongly believe that RNAi technology might be helpful to understand these unresolved questions using both *in vitro* and *in vivo* approaches. In this regard, the combination of RNAi and the gene transfer procedure should allow us to overcome, at least in part, technical problems arising when RNAi is delivered *in vivo*.

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ABBREVIATIONS

RNAi	=	RNA interference
dsRNA	=	double strand RNA
siRNA	=	small interfering RNA
RNP	=	Ribo-Nuclear Protein
AD	=	Alzheimer's disease
Aβ	=	β-Amyloid peptide
APP	=	Amyloid Precursor Protein
CTF	=	C-terminal fragment
PS1	=	Presenilin 1
PS2	=	Presenilin 2
CNS	=	Central Nervous System
PHFs	=	Paired Helical Filaments
GSK-3	=	Glicogen Syntase Kinase-3
NSAIDs	=	Non Steroid Antiflammatory Drugs
BACE1	=	beta-site APP cleaving enzyme 1
EPO	=	Erythropoietin
FDA	=	Food and Drug Administration

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