Advances with RNA interference in Alzheimer’s disease research

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Abstract: Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized clinically by memory and cognitive dysfunction. Unfortunately, there is no effective therapeutic method for AD treatment or ways to halt disease progression. Many mechanisms are involved in the disease, including genes mutation and protein dysfunction. RNA interference (RNAi) technology may potentially be able to control AD. It can inhibit the protein expression of specific genes by activating a sequence-specific RNA degradation process. This is a powerful tool with which to study gene function, investigate the mechanism of the disease, and validate drug targets. In this review, we highlight the advances in RNAi technology in the investigation and treatment of AD.

Keywords: RNAi, β-amyloid, tau, amyloid precursor protein

Introduction
Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized clinically by memory and cognitive dysfunction. It was first reported in 1907 by the German neurologist Alois Alzheimer. The disease is generally classified into two types: sporadic AD (SAD) and familial AD (FAD). The former accounts for more than 90% of all cases and occurs in patients aged 65 years or older. The latter is rare and the age of onset is earlier than that of SAD, with symptoms appearing when patients are in their 40s or 50s. Three genes lead to FAD – amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin (PS2) – while the ε4 allele of the apolipoprotein E gene has been identified as the major risk factor for SAD.

The neuropathology of AD is characterized by two types of lesions, extracellular senile plaques and intracellular neurofibrillary tangles (NFTs), which are composed of, respectively, β-amyloid (Aβ), a cleavage product of APP, and aberrantly phosphorylated tau, a microtubule-associated protein. The detailed mechanism of the disease is still not clear and there are several hypotheses accounting for it, the most influential of which is the amyloid cascade hypothesis. According to this hypothesis, the accumulation of Aβ aggregates in the brain triggers a complex neurodegenerative cascade, which results in progressive cognitive impairment and dementia. A variety of factors may induce AD and it is difficult to clarify what the functions of these factors are and how they work together to result in AD; however, RNA interference (RNAi) may make it easy to determine these.

RNAi was discovered in 1998 by Andrew Fire and Craig Mello, who were awarded the Nobel Prize in Physiology and Medicine for their discovery 8 years later. RNAi regulates the expression of genes by controlling the synthesis of protein with a posttranscriptional...
gene-silencing mechanism. RNAi is triggered by the presence of long pieces of double-strand RNA, which cleave into the fragment known as small interfering RNA (siRNA) (21–23 nucleotides long) by the Dicer enzyme in the cytoplasm. Thereafter, siRNA is incorporated into a protein complex called the “RNA-induced silencing complex” and the sense strand of the

Figure 1 A normal neuron (left) and an Alzheimer’s disease-affected neuron (right).

Note: Extracellular amyloid plaques and intracellular neurofibrillary tangles are the main pathological characteristics of the latter. Copyright © 1988. Proceedings of the National Academy of Sciences of the United States of America. Adapted with permission from Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A. Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. Proc Natl Acad Sci U S A. 1988;85(11):4051–4055. Copyright © 1989. Elsevier. Adapted with permission from Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA. Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer’s disease. Neuron. 1989;3(4):519–526.

Amyloid cascade hypothesis

Missense mutation in APP, PS1, or PS2 genes

Increased Aβ42 production and accumulation

Aβ42 oligomerization and deposition as diffuse plaques

Subtle effect of Aβ oligomers on synapses

Microglial and astrocytic activation (complement factors, cytokines, etc)

Progressive synaptic and neuritic injury

Altered neuronal ionic homeostasis; oxidative injury

Altered kinase/phosphatase activities

Widespread neuronal/neuritic dysfunction and cell death with transmitter deficits

Dementia

Figure 2 Hypothetical pathogenetic steps of the amyloid cascade hypothesis.

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siRNA is cleaved. The antisense strand guides the RNA-induced silencing complex to bind with messenger RNA (mRNA), which is complementary to the antisense strand and degrades it (Figure 3).

RNAi is well suited to probe the biological function of individual genes, genes in pathways, genes known to be associated with diseases (including inherited genetic diseases) and viral pathogens. Further, RNAi may be used to discover novel genes critical to pathogenic processes. Therefore, RNAi has major implications for basic and biomedical research that may lead to a number of clinical applications. Compared with other therapeutics, the principal advantage of RNAi is that all targets – including those that are usually unable to be targeted with drugs – are able to be drugged using RNAi. This is because, in theory, any transcript that encodes a protein that causes or contributes to a disease can be targeted by RNAi (see Table 1). To date, RNAi has been widely used in basic bioscience – including in the study of AD, which may
be caused by different genes and proteins – and such usage may lead to novel therapies in the future. Here, we review the recent advances in the use of RNAi in AD research, investigation, and treatment.

**RNAi and Aβ**

Aβ is a peptide of 40–42 amino acids that is processed from APP, which is a transmembrane protein expressed in many tissues and concentrated in neuron synapses. The primary function of APP is unknown, although it has been suggested that it is a regulator of synapse formation, neural plasticity, and iron export. The metabolism of APP is well characterized, mediated by a series of enzymes termed “secretases” (α, β, and γ). Cleavage of APP by α-secretase occurs in the middle of the peptide, generating non-amyloidogenic Aβ; the common isoforms are fragments of 40 or 42 amino acids called Aβ40 and Aβ42, respectively (Figure 4). The Aβ40 form is the more common of the two, but Aβ42 is the more fibrillogenic and is considered to be primarily responsible for neuronal damage. Aggregated

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Table 1 Comparison of RNAi with traditional pharmaceutical drugs

| Small molecules | Biologics (proteins and antibodies) | RNAi |
|-----------------|------------------------------------|------|
| Antagonist or agonist of targets | Antagonist or agonist of targets | Antagonist only |
| Extracellular and intracellular targets | Extracellular targets | All targets, including non-druggable targets |
| Not all targets can be modulated selectively and potently | High selectivity and potency | High selectivity and potency |
| No allelic specificity | Low allelic specificity | High allelic specificity |
| Lead ID and optimization slow | Lead ID and optimization slow | Lead ID and optimization rapid |
| Low cross species reactivity | Low cross species reactivity | High cross species reactivity |
| Easy to manufacture | Difficult to manufacture | Easy to synthesize and manufacture |
| Easy to deliver | Difficult to deliver | Difficult to deliver |

**Notes:** The principal advantages of RNAi over small-molecule and protein therapeutics are that all targets, including “druggable” and “non-druggable” targets, extracellular and intracellular targets, and mutant alleles can be targeted with RNAi. One of the major advantages of sequence-based targeting technologies is the ability to design precisely targeted therapeutics for almost any target sequence, both coding and non-coding, regardless of the function of the gene product, whether that function is known, and in the absence of any target protein structure information. Copyright © 2006. Nature Publishing Group. Reproduced with permission from Bumcrot D, Manoharan M, Koteltansky V, Suh DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol. 2006;2(12):711–719.

**Abbreviations:** RNAi, RNA interference; ID, identification.

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Figure 4 Proteolytic processing of APP by α-, β-, and γ-secretase.

**Notes:** Sequential APP cleavage by β- and γ-secretase is referred to as the “amyloidogenic pathway” and generates Aβ. Beta-secretase cleavage occurs within the ectodomain of APP close to the transmembrane domain, resulting in the shedding of the membrane-bound C-terminal fragment C99 (C-terminal 99 amino acid of APP). Gamma-secretase cleavage of C99 leads to Aβ secretion and the formation of the APP intracellular domain (AICD). In the alternative, non-amyloidogenic pathway, APP is first cleaved by the metalloprotease α-secretase. This cleavage yields the soluble APP ectodomain sAPP α and a C-termina fragment (CB3), which is further processed by γ-secretase, leading to the secreted p3-peptide and AICD. Copyright © 2006, BioMed Central. Reproduced with permission from Zheng H, Koo EH. The amyloid precursor protein: beyond amyloid. Mol Neurodegener. 2006;1:5.

**Abbreviation:** APP, amyloid precursor protein.
Aβ forms insoluble filaments and these are deposited both intra- and extraneuronally. These filaments constitute one of the main components of senile plaque, the primary hallmark of AD. In many cases of AD, the senile plaques are also found deposited in the walls of cerebral blood vessels.

Aβ is produced in a variety of subcellular locations, including the endoplasmic reticulum/intermediate compartment, the trans-Golgi network (TGN), and the endosomal/lysosomal system. The intracellular trafficking of Aβ production remains uncertain. The β-site APP-cleaving enzyme (BACE) is the main β-secretase synthesized as an immature and inactive form in the endoplasmic reticulum (ER) and this undergoes maturation during its transport to the cell surface along the secretory pathway. The mature BACE is internalized from cell surface to endosomes, followed by sorting to the TGN for recycling or to lysosomes for degradation. Using an RNAi approach, it has been found that the majority of APP is processed in the TGN to produce Aβ40 and that the retromer is key in mediating APP recycling from early endosomes to the TGN. This results in the accumulation of endocytosed APP in early endosomes and a reduction in APP processing.

In addition, increasing levels of sortilin in post-mortem brain tissue of AD patients leads to increased BACE1-mediated cleavage of APP in cultured cells. RNAi suppression of sortilin results in decreased BACE1-mediated cleavage of APP. Further, sortilin expression redistributes BACE1 from the TGN to the endosomes and substantially reduces the retrograde trafficking of BACE1. It has been suggested that sortilin may be a modulator of BACE1 retrograde trafficking and subsequent generation of Aβ.

Receptor tyrosine kinases (RTKs) comprise a large family of cell surface receptors, which transduce various extracellular signals to the interior of cells. BACE and subsequent Aβ production can be enhanced after stimulation of RTKs in cultured cells as well as in mouse hippocampus. Stimulation of RTKs also induces BACE internalization into endosomes and Golgi apparatus. The enhancement of BACE activity and Aβ production on RTK activation could be specifically inhibited by Scr family kinase inhibitors and by depletion of endogenous c-Ser with RNAi. GGA3, an adaptor protein which is recruited to the TGN by the Arf1-GTPase, is involved in BACE trafficking. If the levels of GGA3 are reduced, BACE levels are increased. RNAi silencing of GGA3 can elevate BACE and Aβ levels.

“Autophagy” is a conserved membrane trafficking pathway that mediates the delivery of cytoplasmic substrates to the lysosomes for degradation. Impaired autophagic function is implicated in the pathology of various neurodegenerative diseases. It has been found that Aβ expression causes autophagosome accumulation. Moreover, decreased insulin-receptor signaling promotes the maturation of autophagosomes into degradative autolysosomes, whereas Aβ impairs this process. RNAi-mediated knockdown of lysosomal components results in enhanced Aβ-toxicity and autophagosome accumulation. So, insulin-receptor signaling promotes the autophagic degradation of Aβ. Amyloid precursor-like protein (apl-1) is a member of APP family, and loss of apl-1 leads to a severe molting defect and early larva lethality. RNAi knockdown of apl-1 followed by drug testing on the acetylcholinesterase inhibitor aldicarb showed that loss of apl-1 leads to aldicarb hypersensitivity, indicating a defect in synaptic function. Rab5, a small GTPase, regulates endosomal trafficking of vesicles from the plasma membrane to the early endosome. Knockdown of the small GTPase rab-5 also leads to a dramatic decrease in the amount of apl-1 expression in neurons, suggesting that trafficking from the plasma membrane to the early endosome is important for apl-1 function.

It is assumed that α- and β-secretase compete for APP as a substrate, but have opposite effects on Aβ generation, as mentioned earlier. Increasing the APP α-secretase cleavage is considered a therapeutic approach for AD, as it is assumed to reduce Aβ generation. Alpha-secretase activities have been identified, all belonging to a metalloprotease of the A disintegrin and metalloproteinase (ADAM) family. RNAi-mediated knockdown of ADAM10 completely suppressed APP α-secretase cleavage in different cell lines and in primary murine neurons. In different cell lines, the reduction of α-secretase cleavage is not paralleled by a corresponding increase in the Aβ generating β-secretase cleavage, revealing that both alpha-secretase and beta-secretase do not always compete for APP as a substrate.

Thimet oligopeptidase (THOP1) is a thiol-sensitive metalloprotease with Zn2+, Mn2+, and Co2+ as cofactors, which cleaves peptide substrates of less than 18 amino acids. It is primarily present in tissues rich in neuropeptides and hormones, suggesting a role in the processing of bioactive peptides. The enzyme has been associated with the processing of APP at a β-secretase site and with degradation of Aβ peptide. Its overexpression is neuroprotective against Aβ toxicity, while RNAi knockdown made neurons more vulnerable to amyloid peptide, which suggests that THOP1 operates against the toxic effects of Aβ in the early stages of AD pathology and an increase in THOP1 expression might
be part of a compensatory defense mechanism of the brain against an increased Aβ load.\(^{57}\)

Some other enzymes may also be involved with Aβ, such as acyl-coenzyme A cholesterol acyltransferase (ACAT-1), an enzyme that controls cellular equilibrium between free cholesterol and cholesteryl ester and modulates the proteolytic processing of APP in cell-based and animal models of AD.\(^{58,59}\) ACAT-1 RNAi, through a single transfection of ACAT-1 siRNA oligonucleotides, reduces cellular ACAT-1 protein by \(\sim 50\%\), cholesteryl ester levels by 22%, and causes a slight increase in the free cholesterol content of ER membranes, which correlates with reduced proteolytic processing of APP and a 40% decrease in Aβ secretion.\(^{60}\) In addition, endogenous ferritin knockdown by RNAi can decrease iron in mammal cell cultures and promote the formation of well-ordered aggregates of Aβ while decreasing its toxicity.\(^{61}\)

**RNAi and tau**

Tau proteins interact with tubulin, which makes up microtubules, to stabilize microtubules and promote the assembly of tubulin into microtubules. They are the product of alternative splicing from a single gene that in humans is designated “MAPT” (microtubule-associated protein tau). These proteins are mostly found in neurons rather than non-neuronal cells. When tau proteins are defective, they no longer stabilize microtubules properly.\(^{62}\) They are capable of aggregating and fibrillating to form NFTs, another pathological hallmark of AD\(^{5,6}\) (Figure 5).

Tau proteins play a critical role in AD pathogenesis; however, their molecular basis is unknown. Hyperphosphorylation of tau proteins can result in the self-assembly of paired helical filament and straight filament tangles, which are involved in the pathogenesis of AD.\(^{63}\) SUT-2 is a gene required for tau neurotoxicity in a transgenic *Caenorhabditis elegans* model of tauopathy.\(^{64}\) RNAi knockdown of *MSUT2* (mammalian SUT-2) in cultured human cells overexpressing tau causes a marked decrease in tau aggregation.\(^{65}\) Cyclin-dependent kinase 5 (CDK5) is a relevant kinase that has been hypothesized to contribute to the tau pathology.\(^{66,67}\) Silencing of CDK5 reduces the phosphorylation of tau in primary neuronal cultures and in the brain of wild-type C57BL/6 mice. Further, in one study, the knockdown of CDK5 strongly decreased the number of NFTs in the hippocampus of triple transgenic mice (3xTg-AD mice).\(^{68}\)

A recent concept that has emerged suggests that, rather than the amyloid plaques, small Aβ non-fibrillar oligomers

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**Figure 5 (A)** Tau facilitates microtubule stabilization within cells and is particularly abundant in neurons. **(B)** It is thought that tau function is compromised in Alzheimer’s disease and other tauopathies.

Notes: This probably results from both tau hyperphosphorylation, which reduces the binding of tau to microtubules, and the sequestration of hyperphosphorylated tau into neurofibrillary tangles (NFTs), which reduces the amount of tau that is available to bind microtubules. The loss of tau function leads to microtubule instability and reduced axonal transport, which could contribute to neuropathology. Copyright © 2009, Nature Publishing Group. Reproduced with permission from Brunden KR, Trojanowski JQ, Lee VM. Advances in tau-focused drug discovery for Alzheimer’s disease and related tauopathies. Nat Rev Drug Discov. 2009;8(10):783–793.\(^{97}\)
could be the earliest mediators of neuronal dysfunction.\textsuperscript{69,70} Isolated Aβ dimers from the cortices of typical AD subjects first induce hyperphosphorylation of tau at AD-relevant epitopes in hippocampal neurons then disrupt the microtubule cytoskeleton and cause neuritic degeneration, all in the absence of amyloid fibrils. In one study, the researchers found that knocking down endogenous tau fully prevented the neurotic changes, whereas overexpressing human tau accelerated them. The results suggest natural Aβ dimers can induce AD-type tau phosphorylation then neuritic dystrophy.\textsuperscript{71}

**RNAi and other investigation**

AD is a neurodegenerative disorder characterized by deregulation of the neuronal cell cycle and differentiation control, eventually resulting in cell death.\textsuperscript{72} During brain development, neuronal differentiation is regulated by Smad proteins, which are elements of the canonical transforming growth factor β (TGF-β) signaling pathway, linking receptor activation to gene expression.\textsuperscript{73} In the normal adult brain, Smad proteins are constitutively phosphorylated and predominantly localized in neuronal nuclei.\textsuperscript{74} Using RNAi in cell cultures to mimic the neuronal deficiency of Smad proteins observed in AD results in elevation of cell cycle-dependent kinase 4 and retardation of neurite outgrowth, giving rise to neuronal dedifferentiation and cell death.\textsuperscript{75} Polo-like kinase 1 (Plk1) is an established regulator of many cell cycle-related events. Inhibition of Plk1 kinase activity or depletion of Plk1 by RNAi reduces Aβ-induced neuronal cell death.\textsuperscript{76}

While, as previously noted, three gene mutations lead to the development of FAD, SAD has only one clear genetic modifier: the ε4 allele of the apolipoprotein E gene.\textsuperscript{7} RNAi knockdown of apl-1, lipoprotein receptor-related protein 1, and cholesterol starvation leads to aldicarb hypersensitivity, indicating a defect in synaptic function.\textsuperscript{77} Mutations associated with FAD change the ratio of Aβ42 to Aβ40 by increasing the production of Aβ42, which is linked to amyloid plaque formation.\textsuperscript{78,79} To determine whether the two types of AD share a common underlying molecular cause, RNAi has been used to systematically silence 24 genes linked to SAD. The results show that SAD genes do not specifically alter the Aβ42 to Aβ40 ratio, thus suggest that these genes probably contribute to AD through distinct mechanisms.\textsuperscript{80}

Mitochondrial function – which relies heavily on its morphology and distribution – and alterations in mitochondria morphology and distribution have been increasingly implicated in neurodegenerative diseases such as AD.\textsuperscript{81} Dynamin-like protein 1 (DLP1), a regulator of mitochondrial fission and distribution,\textsuperscript{82} has been found at significantly lower levels in SAD fibroblasts.\textsuperscript{83} Expression of DLP1 by miR RNAi in human fibroblasts from normal subjects significantly increased mitochondrial abnormalities, which suggests that DLP1 reduction causes mitochondrial abnormalities in SAD fibroblasts.\textsuperscript{84} Experiments have also demonstrated that elevated oxidative stress and increased Aβ production are likely to be the pathogenic factors that cause DLP1 reduction and abnormal mitochondrial distribution in AD cells.\textsuperscript{84}

**RNAi and AD therapy**

Over the past decade, at least 21 siRNA therapeutics have been developed for more than a dozen diseases, including various cancers, viruses, and genetic disorders.\textsuperscript{84,85} Many endeavors have also been made to improve AD treatment. The most advanced of these has focused on Aβ peptide production and clearance.\textsuperscript{3} The therapeutic potential of RNAi in AD has been demonstrated through allele-specific gene silencing by short-hairpin RNA (shRNA) to selectively suppress mutant APP.\textsuperscript{86} An anti-APPsw shRNA was delivered by the recombinant adeno-associated virus to the hippocampus of AD transgenic mice (APP/PS1). No neuronal toxicity was detected in short- and long-term transduction experiments. Over the long-term, bilateral hippocampal expression of anti-APPsw shRNA mitigated abnormal behaviors in this mouse model of AD. The difference in phenotype progression was associated with reduced levels of soluble Aβ but not with a reduced number of amyloid plaques.\textsuperscript{87} Intravenously injected rabies viral glycoprotein-targeted exosome delivered siRNA specifically to neurons in mouse brain, resulting in strong mRNA (60%) and protein (62%) knockdown of BACE1 without the corresponding level of immune stimulation.\textsuperscript{88} In addition, CBP-1 has been inhibited by RNAi to assess the age-dependent acceleration of the mortality rate of 30 drugs that reliably protected mice (APP/PS1). No neuronal toxicity was detected in short- and long-term transduction experiments. Over the long-term, bilateral hippocampal expression of anti-APPsw shRNA mitigated abnormal behaviors in this mouse model of AD. The difference in phenotype progression was associated with reduced levels of soluble Aβ but not with a reduced number of amyloid plaques.\textsuperscript{87} Intravenously injected rabies viral glycoprotein-targeted exosome delivered siRNA specifically to neurons in mouse brain, resulting in strong mRNA (60%) and protein (62%) knockdown of BACE1 without the corresponding level of immune stimulation.\textsuperscript{88} In addition, CBP-1 has been inhibited by RNAi to assess the age-dependent acceleration of the mortality rate of 30 drugs that reliably protected mammalian neurons.\textsuperscript{89} Further, when a high-throughput RNAi approach is used to screen 572 kinases in the human genome for effects on tau hyperphosphorylation, EIF2AK2 effects may result from effects on tau protein expression, whereas DYRK1A and AKAP13 are likely to be more specifically involved in tau phosphorylation pathways.\textsuperscript{90}

However, several obstacles remain in the clinical development of RNAi-based therapeutics, the greatest of which is delivery. Suitable delivery methods are needed to transfer siRNA to the specified targets and control of potential off-target effects also needs to be considered.\textsuperscript{85,86} AD is genetically heterogeneous and multifactorial.\textsuperscript{95} It may not be treated with a single siRNA. Thus, how to formulate the
different RNAi components and deliver them into the target is a significant problem. Further, there have been few ideal vectors for clinical application.92

Conclusion
AD has the greatest unmet medical needs in neurology.93 Although a century has elapsed since the identification of AD, there are still no ideal therapeutic approaches for treating the disease. Current drugs improve symptoms but do not have profound disease-modifying effects.94 A much deeper insight into this disease is required. RNAi has become a valuable research tool to help us understand this disease and to provide an efficient method for clinical applications.95,96 Due to its advantages over other current therapeutics, we strongly believe that RNAi technology will be helpful in addressing unresolved questions concerning AD via in vitro and in vivo approaches. Given the pace of new findings and discovery of applications, RNAi is likely to remain a major new therapy for the foreseeable future.

Acknowledgment
This research was supported in part by the Projects of National Science Foundation of China (No.81071025 and 81171203), and Projects of the Shanghai Committee of Science and Technology, China (No.11nm0503300, 11410708900 and 12XD1403800). The authors thank the Analytical Center of Shanghai JiaoTong University for technical support.

Disclosure
The authors declare no conflicts of interest in this work.

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