Chapter 8

# RNAi Therapeutic Potentials and Prospects in CNS Disease

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62896

**Abstract**

Over the past 20 years, RNA interference (RNAi) technology has provided a new regulatory paradigm in biology. This technique can efficiently suppress target genes of interest in mammalian cells. Small non-coding RNAs play important roles in gene regulation, including both in post-transcriptional and in translational regulation. For *in vivo* experiments, continuous development has resulted in successful new ways of designing, identifying, and delivering small interfering RNAs (siRNAs). Proof-of-principle studies *in vivo* have clearly demonstrated that both viral and non-viral delivery methods can provide selective and potent target gene suppression without any clear toxic effects. There are also the persistent problems with off-target effects (OTEs), competition with cellular RNAi components, and effective delivery *in vivo*. Although recent researches and trials from a large number of animal model studies have confirmed that most OTEs are not dangerous, other important issues need to be addressed before RNAi-based drugs are ready for clinical use. Currently, RNAi may be harnessed as a new therapeutic modality for brain diseases. Finally, there are already several RNAi-based human clinical trials in progress. It is hoped that this technology will have also effective applications in human central nervous system (CNS)-related disease.

**Keywords:** RNAi therapy, brain, neurodegenerative disease, allele-specific, neurovascular

## 1. Introduction

During developmental stage and in response to internal and external cellular stresses, small RNA molecules regulate gene expression [1]. Specialized ribonucleases and RNA-binding proteins govern the production and action of small regulatory RNAs [2]. In most eukaryotic cells, RNA interference (RNAi) is a regulatory mechanism using small double-stranded RNA

(dsRNA) molecules to direct homology-dependent control of gene activity [3, 4]. Small size [20–30 nucleotide (nt)] non-coding RNAs and associated proteins regulate the expression of genetic information [5]. The discovery of RNAi phenomenon widened our understanding of gene regulation and revealed related pathways in small RNAs [6]. As it processes, RNAi has been finding widespread in plants [7] and animals [8]. Each small RNA associates with an Argonaute (AGO) family protein to form a sequence-specific complex. After then, gene-silencing ribonucleo-protein complex with specificity conferred by base pairing between the small RNA (guide RNA) and its target mRNA [5]. The pathway is well known as the RNA-induced silencing complex (RISC), which gives a target mRNA silencing by degradation or transcriptional regression [2]. Small interfering RNAs (siRNAs) loaded into RISC are double-stranded, and AGO-2, which having an active catalytic domain in human, cleaves and releases the "passenger" strand. RISC is activated with a single-stranded "guide RNA" molecule to impose the specificity recognizing the target by intermolecular base pairing [9].

MicroRNAs (miRNAs) are other endogenous substrates for the RNAi machinery, but the cellular origins of miRNA and siRNA are distinct. miRNAs are derived from the genome, whereas siRNAs may be endogenous or arise through viral infection or other exogenous sources [2]. Typically, miRNAs are initially expressed in the nucleus with a transcript as long as primary miRNA (pri-miRNA), and the transcripts are at least over 1000 nt. Pri-miRNAs are processed by the microprocessor complex (histone deacetylase proteins) consisting in Drosha-DGCR8 [DiGeorge critical region 8 (a double cysteine-ligated Fe (III) heme protein)—DGCR8] in the nucleus [10, 11]. They are cleaved in the nucleus into 60–70 base pair (bp) hairpins, which are consisted in single-stranded 5′- and 3′-terminal overhangs and about 10-nt distal loops [12]. In cytoplasm, the loop is further processed by the RNAse III Dicer, and one strand is loaded onto RISC. The mature miRNAs bind to the 3′ UTR of target mRNAs and then degrade the target [13]. Despite their differing origins, these RNA processing pathways converge once either type of RNA assembles into the RISC.

With development of an efficient delivery system in various diseases, RNAi has been an emerging therapeutic approach for *in vivo* studies with specific synthetic siRNAs against each disease. It should be considered as novel and interesting therapeutic challenge with the major concern how to administer the siRNAs with specific, efficient, and targeted way. Despite some hurdles for applying to clinical challenges such as anatomical barriers, drug stability and availability, various delivery routes, and different genetic backgrounds, an application of siRNAs has become extremely attractive in development of new drugs. Currently, one of the important challenges in siRNA bioinformatics is target prediction, when there is still no proper tool with certain drug design grade. Besides specific challenges in siRNA therapeutics, an efficient delivery method, targeting a specific tissue or cell, is another fundamental challenge.

This chapter introduced two of main themes. The first is the possibilities of therapeutics using RNAi principles and technique. The second is the challenges with siRNAs or miRNAs specifically in the area of brain disease. In addition, this chapter provided some prospects of siRNAs or miRNAs on disease prognosis, progress, and therapeutics in the present and future.

http://dx.doi.org/10.5772/62896

## 2. Principles of RNAi therapy

As far as it is true that siRNA has promising benefits, and, concomitantly, siRNA has still some of technological barriers to be widely used in clinical therapy, which generally due to the lack of efficient delivery tools. To success with siRNA therapies, an effective and safe carrier system is required that would overcome the inherent defects of siRNA and achieve maximum gene-silencing effect. There are many approaches that are being developed to achieve the efficient delivery of siRNA. In that, non-viral vectors have advantages of reproducibility, low immunogenicity, and relatively low production cost [14]; therefore, non-viral vectors made siRNA to be a potential therapeutic and nucleic acid–based drugs, such as plasmid DNAs or antisense oligonucleotides (ASOs) [15].

### 2.1. Advantages of RNAi

Theoretically, all disease-associated genes could be amenable to antisense-mediated RNAi suppression. RNAi can be a strategy for silencing of virtually all annotated protein-encoding genes in the human genome in large scale. The high specificity of siRNA lets targeting of disease-specific alleles that differ from the normal allele by only one or few nucleotide substitutions. This high fidelity and specificity of siRNAs are useful for targeting for some oncogenes, too.

The first advantage is the powerfulness of RNAi when compared with other antisense strategies, such as antisense DNA oligonucleotides and ribozymes [16]. It is important fact that the effector molecules work at much lower concentration than any other antisense oligomers or ribozymes, suggesting that RNAi has higher potency. This is a critical point to set therapeutics.

The second is efficacy. The efficacy is generally presented by the half level of maximal inhibition or the value of IC50 against target site. The efficacy level is crucial for determining thermodynamic stability [17], targeted gene accessibility [18], or structure [18] of designed siRNA. For designing siRNA, the most important thing is end stability that is different from each end and is also meaning asymmetry and consistent with selected miRNA [19]. However, to date, our knowledge of siRNA and the selection of targets are incomplete and being explored. The identification of "hyperfunctional" siRNAs, functioning at sub-nanomolar concentration, remains an elusive task.

### 2.2. Basic strategies for targeting-specific molecules

RNAi can be triggered by two different pathways: (1) a RNA-based approach, where the 21-nt long duplexed siRNA effectors are delivered to target cells, and (2) a DNA-based strategy, where the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts [3]. DNA-based strategy is based on short hairpin RNA (shRNA) synthesis in nucleus and transportation to the cytoplasm through miRNA machinery, which subsequently is processed by Dicer. Although the direct use of siRNA effectors is simple and effective way for gene silencing, the effect is transient. Therefore, it is costly for clinical usage due to the need

of multiple large-scale application. In contrast, DNA-based RNAi drugs have the potential and stably introduced for application in a gene therapy. In principle, DNA-based RNAi allows a single treatment of viral vector that delivers shRNA genes to the targeted cells/or tissues.

### 2.3. Delivery routes for targeting

The effective delivery of siRNAs acts to be significant step in accelerating RNAi-based treatments. The instability of RNA and the relatively inefficient encapsulation process of siRNA remain critical issues toward the clinical translation of RNAi as a therapeutic tool. There are several obstacles for extracellular introduction of siRNA to deliver the target. Under normal physiological condition, the introduced molecules ought to have a positive charge to diffuse to cell membrane [20]. It is the simplest way of naked nucleotides or transfecting siRNAs to deliver into cells [21]. Another technique is microinjection and electroporation for direct delivery, but it has higher level of cellular toxicity [22]. The delivery routes can be intra-peritoneal, intra-vascular, intra-muscular, intra-splenic, intra-cranial, and intra-tumoral injection. In addition, siRNAs can be delivered through subretinal, subcutaneous, mucosal, topical application, and oral ingestion to improve delivery [22]. However, these transfection processes should be optimized for siRNA concentration, cell density, and ratio of transfection reagent to siRNA [23]. Carriers for delivery of siRNA with cationic environment surrounding of siRNAs can be liposomes and dendrimers. These carriers reduce the nuclease activity and improve siRNA delivery into cells [24].

Microsponge is one of the mediators for siRNA delivery. Carrier and cargo combine and self-assemble into nanoscale pleated sheets of hairpin RNA. Subsequently, this complex forms sponge-like microspheres [25]. The complex of siRNA and microsponges consists in cleavable RNA strands, and the stable hairpin RNA converts into working siRNA once cells uptake the complex. Therefore, it can provide a protection for siRNA during delivery and transport it to the cytoplasm. Single microsponge complex can deliver more than half a million copies of siRNA when uptaken into a cell [25].

### 2.4. Stabilizing the siRNA delivery

The stability of the siRNA complexes, penetrating into target cells without stimulating immune responses, is one of the limiting factors and the major bottleneck for developing siRNA therapeutic tools. It restricts the delivery of siRNA macromolecular complexes to the desired cell types, tissues, or organs. Usually, siRNAs do not easily penetrate the cellular membrane because of their negative charge and macromolecular size. Manipulation of nucleotide bases is needed to increase stability and protein interactions, which can harness to increase the structural improvement of siRNAs [26]. The delivery systems for siRNA consist of four main methods, namely naked, lipid-based, peptide-based, and polymer-based delivery [27]. Basically, polymer-based methods are similar to lipid-based methods in targeting, except some special triggers, such as temperature, pH, or pulse release [28].

Initial efforts to improve stability addressed above were focused on incorporating chemical modifications into the sugar backbone or bases of siRNA duplexes [29]. The modified siRNA

molecules increased stability, which effectively lowered the dose to achieve measurable and reproducible gene silencing [30]. Several modifications were introduced. The thio (−SH), hydroxyl (−OH), or iodo (−I) can modify bases in specific sites or utilize the pseudouracil base in siRNA, which would augment potency of naked siRNA [31]. There are three most popular chemical modification sites on siRNA structure containing the phosphodiester backbone, ribose 2′-hydroxyl group (R-2′-OH), and ribose ring. Endogenous cellular endonucleases can easily digest phosphodiester bond in RNA backbone [32]. Alternative modification is oxygen bridges of RNA backbone that can be replaced with phosphorothioate, although it would increase toxicity and reduce silencing activity [33, 34]. Another alternative is boranophosphate linkages. These are more nuclease resistant and less toxic compared to phosphorothioate [35]. Phosphonoacetate linkages are other candidates [36]. The linkage is completely resistant to nuclease and is electrochemically neutral when they are esterified [36, 37]. Another modification is 2′-*O*-methoxyethyl (2′-*O*-MOE), 2′-*O*-alkyl, and other bulky groups. These modifications can improve anti-nuclease shield of siRNA that simultaneously makes them less tolerable when they are positioned on 3′ overhangs [38]. Despite disturbing thermodynamic asymmetry of siRNA by addition of 2′-aminoethyl at 3′ end of passenger strand, this modification improves efficiency of target silencing [39].

On the other hand, alterations in sugar compartment of nucleotides reduced flexibility and nuclease sensitivity of siRNA structure [39, 40]. Binding of ribose 2′O into 1′C with methylene bridges, which finally produces oxetane, forms a locked conformation nucleic acid (locked nucleic acid—LNA) [41]. *In vivo* nuclease resistance of this structure is enhanced [42]. In contrast to LNA, derivatives of RNA without C2′–C3′ sugar bonds (unlocked nucleic acid—UNA) destabilize a sequence structure [43]. Substitution of pentose with hexose monosaccharides, such as cyclohexenyl, anitrol, and arabinose, was applied to develop CeNA, ANA, and 2′-F-ANA [44], subsequently resulting in enhanced stability of siRNA *in vivo* [45]. During systemic delivery, however, internal modifications failed to improve central nervous system (CNS) entry and uptake. Researchers put new efforts to move toward using liposomes, nanoparticles, and cell-penetrating peptides (CPPs), among others, to stabilize and navigate siRNAs into and throughout the brain [46].

### *2.4.1. Liposomes*

Generally, liposomes are classified into three classes: multilmellar vesicle (0.5~20 μm), small unilamellar vesicles (25~100 nm), and large unilamellar vesicles (100~500 nm) [47]. Liposomes are developed for passive or active targeting mechanisms in different complexes of liposome and other interacting molecules, namely lipoplex (cationic liposome-pDNA complex), liposome polycationic DNA, mannose liposome, and so on [48, 49]. The siRNA with mannose (Man)-coated liposomes would be useful for treatment of some cancers, especially liver and brain cancers [50].

### *2.4.2. Dendrimers*

Dendrimers are hyper-branched, tree-shaped, and 3-D structures [51]. Dendrimer can utilize broad spectrum, and the broad range of functional groups makes it possible to introduce

dendrimers with extensive applications. There are different classes of cationic and anionic dendrimers, such as polyamidoamine (PAMAM), polypropylene imine (PPI), and polyethylene glycol (PEG)-grafted carbosilane [52]. Specific dendritic polymers like PAMAM have been widely utilized in *in vivo* drug delivery [53]. Conjugation of Tat peptide (GRKKRRQRRRPQ) with PAMAM-G5 can efficiently inhibit multi-drug resistance-1 (MDR-1) gene expression *in vitro* [51]. Capping poly-l-lysine (PLL) dendrimers with methotrexate enhances stability and decreases toxicity [54].

#### *2.4.3. Cationic polymers*

Cationic polymers include chitosan, gelatin, cationic dextran, cationic cellulose, and cationic cyclodextrin and some synthetic biocompatible polyethyleneimine (PEI), PLL, poly(amidoamine)s (PAAs), poly(amino-co-ester), and poly(2-*N*,*N*-dimethylaminoethylmethacrylate). Moreover, they are less immunogenic response because these polymers are natural biodegradable [55].

#### *2.4.4. Cationic peptides*

CPPs are cationic peptides. CPPs interact covalently or non-covalently through disulfide or electrostatic–hydrogen interactions with siRNAs [56]. Viral protein (VP22) [57], MPG (a peptide vector) [58], amphipathic peptide [59], and poly-arginine [60] were reported the same abilities. In addition, small cationic polypeptides (poly His, Lys, and Arg) coat and neutralize siRNA helping to pass through membrane [61].

#### *2.4.5. Nanoparticles*

For systemic delivery, a targeted nanocarrier-siRNA complex has been used. There are some studies that have experimentally condensed DNA or RNA into cancer-targeted nanoparticles with PEI, PLL, and cyclodextrin-containing polymers [62]. PEI–PEG–arginine–glycine–aspartic acid (RGD) fusion was used to inhibit vascular endothelial growth factor receptor-2 (VEGFR-2) expression [63]. Angiogenesis can be inhibited by downregulation or silencing of VEGFR-2 expression [64]. PEGylation of nanoparticles causes "muco-inert" properties, which enhances diffusion process through mucus and peptidoglycan barriers [65].

#### *2.4.6. Aptamer*

siRNAs can be coupled with aptamers or oligodeoxynucleotide through a disulfide bond. This releases actively into targeted cells siRNAs before cytosolic uptake. Conjugate of aptamer siRNA has suggested a novel therapeutics with widespread applications in medicine [66].

### 2.5. Limitations

#### *2.5.1. Competition with endogenous RNAs*

In human brain diseases and normal brain development, RNAi potentiates the important role in normal neuronal function, although it is underestimated. When exogenous shRNA is

http://dx.doi.org/10.5772/62896

introduced into the neuron, it might be considered whether the RNAi machinery perturbs normal physiologic condition of the system. Bioactive drugs that rely on cellular processing to exert their action face the risk of saturating such pathways and hence perturb the natural system. Sometimes, ectopically introduced RNAi does not trigger the silencing process because siRNA/shRNA activity may depend on the endogenous miRNA to achieve efficient target silencing. Mice that received liver-directed associate adeovirus (AAV)-encoded shRNAs were damaged in liver with dose-dependent manner. Within 2 months, the mice were killed by introducing high doses of AAV-encoded shRNAs. It was interpreted that the liver-specific miRNA was unexpectedly down-regulated by introducing shRNA [67]. The enhanced expression of Exportin 5, the nuclear export component, increased RNAi efficacy, which was shown by competition assay [68].

#### *2.5.2. Stimulation of innate immune responses*

RNAi therapy is importantly considered because of its potential for generating an adverse immune response, particularly in neurodegenerative diseases with affected brain. It has been already known as "heightened state of alert" to start chronic pro-inflammatory signaling cascades [30]. All evolutionary conserved mechanisms aimed at combating against invading viral pathogens [69]. In general, innate immune responses to non-virally delivered siRNAs are mediated by members of the toll-like receptor (TLR) family or by the two different dsRNA-sensing proteins: retinoic acid-inducible gene-1 and dsRNA-binding protein kinase [70]. Certain siRNA sequence motifs invoked TLR7-dependent immune stimulation [71]. The particular sequence motif (5′-GUCCUUCAA-3′) seems to be recognized by TLR7 in plasmacytoid dendritic cells and activates immune responses. The GU-rich regions, so-called "danger motifs," stimulated innate immune responses and lead to secretion of inflammatory cytokines in a cell type and sequence-specific manner. As siRNA-mediated immune induction seems to rely on endosome-located TLR receptors (TLR7 and TLR8) [72], the delivery and compartmentalization of the siRNA significantly influence the cellular responses [3]. These interactions can occur during endosomal or lysosomal compartments' internalization or intracellular release of the siRNA molecule. It has a manner of dose and sequence dependence. Importantly, the chemically modified or nanoparticle-encased siRNA complexes avoid stimulation of immune response.

#### *2.5.3. Suppression of off targets*

Harmfulness of RNAi is "OTE." Genome-wide sequencing analyses have clearly demonstrated that siRNA-treated cells show off-target silencing of a large number of genes [73]. The research result suggests that siRNAs with a 2′-*O*-MOE modification at the second base can significantly reduce off target without compromising the degree of silencing target [74]. Experimentally, it has been verified that off targets have 6~7-nt long matching to the siRNA, and it is called "seed" region [75]. When the siRNA guide strand contains seed-sequence matching to mRNA 3′-UTR regions, the siRNA guide strand functions as a miRNA, which might lead to harmful OTEs by translational repression [76]. To avoid siRNA seed matching with mRNA 3′ UTRs, the use of online 3′-UTR search algorithms would potentially reduce the detrimental OTEs [75].

The OTEs can also derive from non-specific changes in gene expression due to the activation of the interferon response (IR) [77]. The OTEs can change another gene by binding either strand of the shRNA to partially complementary sequences rather than binding to the intended target gene [77]. In case of dsRNA, it can result in a signaling cascade that culminates with the activation of interferon responsive genes and global translational repression [78]. Nevertheless, IR activation was variable among the siRNAs used for each of these studies, and one recent report did not detect IR activation by siRNAs [79]. In mice, injection of naked siRNA did not show detectable induction of an IR in one study while another study showed sequence-dependent induction of innate immunity [79, 80].

## 3. Applications of RNAi

RNAi has been used to generate model systems to identify novel molecular targets [81], to study gene function [82], and to create a new niche for clinical therapeutics [83]. Many researchers reported that siRNAs have successfully been tested in various disease animal models. Recent reports reviewed the therapeutic potential of synthetic siRNAs in various human diseases and disorders [84].

### 3.1. Application for therapy with RNAi *in vivo*

Applications, such as gene function analysis, target identification and validation, and therapeutic agents, are the main spots of this new technology [26]. Although RNAi is an efficient technique for *in vitro* studies, there are some challenges for *in vivo* applications. siRNAs have undesired characteristics, such as non-specific silencing of non-targeted genes and dose-dependent immunogenic response [85]. In addition, it is extremely complicated to avoid the OTEs due to spatiotemporal gene expression pattern of these molecules [73]. Furthermore, age, sex, tissue, organ, tumor, and individual-specific specificity should be also considered as other variables [86]. Prediction of susceptible off-target domains that can influence silencing efficiency is the first step for applying *in vivo* therapy [73, 87]. Some studies recommend utilization of more sensitive alignment algorithms or siDirect instead of BLAST database [85, 88] to predict a target for siRNA matching without cross-reactivity [89].

The administration route for siRNA, such as oral or intravenous, is not feasible and not efficiently delivered the siRNA into target cells. A single injection of naked siRNA into the brain parenchyma failed to good efficacy [90]. A study reported that continuous infusion of siRNA into the ventricular CSF success with very high concentration [91]. To penetrate the blood–brain barrier (BBB) and reach the target cells in the interesting site, receptor-specific pegylated immunoliposome (PIG) is used. PIGs encapsulate the plasmid vector–encoding siRNA or shRNA and are administered with peripheral route to the brain. This tool has been tried in brain cancer animal model and successfully worked [92]. Another study showed effective and long-term knock down of endogenous tyrosine hydroxylase (TH) in rodent brain using shRNA-expressing adeno-associated virus (AAV) [93]. There have been many successful *in vivo* studies with using viral vector. They are included two models of autoimmune hepatitis

[94], hepatitis B virus [95], respiratory viruses such as influenza virus [96], respiratory syncytial virus [97], parainfluenza virus, and sexually transmitted disease such as herpes simplex virus-2 [98]. Both non-viral and viral shRNA delivery systems have been trailed.

### 3.2. Application for therapy with RNAi in brain diseases

Many works using RNAi to suppress dominant disease genes have occurred primarily in cell culture models [99, 100]. Allele-specific silencing aims to suppress the disease gene without affecting any other normal genes. The possible therapeutic applications of RNAi for neurological diseases are broad, ranging from acquired diseases, such as viral infections, to purely genetic disorders.

Particularly, one attractive group of candidate diseases for RNAi therapy is the dominantly inherited neurodegenerative diseases, including polyglutamine disorders such as Huntington's disease (HD) [101], amyotrophic lateral sclerosis (ALS) [102], familial Alzheimer's disease (AD) [103], and frontotemporal dementia caused by tau mutations [104]. HD has been approaching with animal model mimicking the human disease to provide some therapeutic clues with various ways. In the new preclinical study, single injection of a cholesterol conjugated-siRNA was targeting mutant Huntingtin (mhtt), and, subsequently, the pathologic symptoms containing behavioral dysfunction were improved [105].

The exciting recent works have taken place *in vivo* in mouse models of neurodegenerative brain disease. The best example of RNAi-mediated therapy to date is in spinocerebellar ataxia type-1 (SCA-1) [106]. As another case, RNAi-mediated therapy was tried on DYT1 dystonia with animal disease model. DYT1 dystonia is another inherited dystonia. DYT1 dystonia is caused by deletion of GAG that is coding TOR1A, which results in one of a pair of glutamic acid from the carboxyl terminal of the torsin A (TA) protein-coding region [107].

Prion disease is one of the brain diseases that is invariably fatal, and no therapy is available. Once serious damage to the brain has already occurred, clinical symptoms manifest after the untreatable brain damage. Causing this reason, prion disease treatments have aimed not to cure the disease but to slow disease progression [108]. Prion disease is caused by prions, in which a self-replicating, infectious protease resistant form of PrP (termed PrPSc), is the only essential component identified to date. PrPSc multiplies through conversion of the normal cellular PrP (PrPC) [109]. Some reviews are presenting that lentivector-mediated anti-PrPC shRNA expression effectively suppressed prion replication in a murine neuroblastoma cell line, and researchers created chimeric mice using embryonic stem cells, which were transfected with a lentiviral vector carrying an anti-PrPC shRNA. Results showed that the survival time after prion inoculation was markedly prolonged [110, 111].

## 4. Prospects of RNA therapeutics in CNS disease

The current phamaceuticals required more knowledge to decipher potentials of the RNAi in spite of flourishing future. It is crucial that each disease has not only a unique pattern but also

the understanding for pathogenesis relating pathways and activating or inhibiting factors [112]. To introduce the DNA therapeutics into the CNS is much more complicated due to the BBB, which can be only permeable to lipophilic molecules of less than 400 Da [113]. Using human viruses, DNA delivery system has been extensively trailed for over three decades. However, the results have been not satisfactory. Therefore, a critical goal for clinical neuroscience is to develop the efficient RNAi therapy to prevent the neuronal damage [77]. We categorized the neurological disease containing cancers in below sections.

### 4.1. Genetic neuronal disease-familial neurological disease

The application of siRNA has been advanced in development of various incurable disease therapies, apart from the widespread usage of RNAi in fundamental biological application. Particularly, dominant inherited disorders are major application field. Among familial neurobiological diseases, HD has been tried to lots of therapies based on RNAi and may be beneficial effect from the therapy using siRNA. In the N171-82Q transgenic HD mouse model, a study using shRNA showed a 50–55% decrease in the N171-82Q mRNA when injected to striatum and a complete elimination of mHtt protein inclusions from the neuronal cells [114]. There was also a rescue of motor dysfunctions. siRNAs against the "R6/2 huntingtin (htt) mRNA" reduced brain atrophy and neuronal inclusions in the R6/2 transgenic mouse model [115]. With using a rAAV5 vector and administrating to the striatum, long-term expression of a mHtt-siRNA partially reduced in neuropathology condition [116].

Besides AAV, there is lentiviral vector that can be applied after onset of symptoms [117]. Using lentivirus vector decreased htt protein expression by up to 35% and altered htt-related pathways but did not reduce cellular viability for at least 9 months after treatment. To enhance cellular uptake of siRNA, cholesterol-conjugated duplexes (cc-siRNA) have been applied to target htt mRNA [118]. Allele-specific targeting of mhtt helped to overcome the side effects of RNAi where ASO or single nucleotide polymorphisms (SNPs) in the mHtt allele have been used to specifically target only the mutant gene product [119]. Intra-cellular antibody fragments bind to abnormal aggregations, and allele-specific siRNA disrupts mhtt gene [120, 121]. Targeting of just three SNPs with five siRNAs covered most of the HD patients in the population studied [122].

Tuberous sclerosis is a common, dominantly inherited disorder caused by mutations in the tumor suppressor complex-1 (TSC1) or tumor suppressor complex-2 (TSC2) genes [123]. The proteins hamartin (encoded by TSC1) and tuberin (encoded by TSC2) form a complex. This protein complex represses mTOR-S6K-4E-BP signaling pathway [123]. Mutated TSC1 and TSC2 lead to loss of activity resulting in unchecked cell growth and hamartoma formation in the CNS. Recent studies propose that the target may be the GTPase Rheb [124]. RNAi suppression of Rheb might respond the dysregulated cell proliferation in tuberous sclerosis.

Particularly, allele-specific silencing is apt for inherited neurological diseases. DYT1 is the most commonly inherited dystonia [125]. Although the pathogenesis of DYT1 is unclear, several facts make DYT1 a good candidate to explore the therapeutic potential of RNAi [77]. The three nucleotide difference between the wild type and the mutated gene has been enough to allow

allele-specific silencing against mutant TA (the mutated protein in DYT1) in cultured cells using *in vitro* synthesized siRNA [107].

Allelic discrimination has also been demonstrated for superoxide dismutase (SOD) mutations responsible for familial ALS [100], and also a mutation in an acetylcholine receptor subunit causes congenital myasthenia [126]. In a tau mutation responsible for fronto-temporal dementia, siRNAs can act by discriminating between sequences differing by a single nucleotide [99].

An important role for RNAi in the brain is also presented for Fragile X syndrome (FXS) in human [127]. FXS is the one of the most common forms of inherited mental retardation caused by mutations in Fragile X Mental Retardation Protein (FMRP), a protein influencing synaptic plasticity [127]. FXS is stemmed from mutations in FMRP and is supported by the involvement of the RNAi process in human neurological disease [127]. Increasing evidences from different studies support the view that FMRP regulates protein translation by regulating RNAi in neurons [128, 129].

### 4.2. Sporadic neurodegenerative diseases

Neurodegenerative diseases are age dependent, and many of them are inherited. However, non-genetic neurological diseases, such as sporadic AD or migraine, are much more common than diseases due to single-gene mutations.

The most common sporadic neurodegenerative disease, AD, is also the best studied with siRNA therapy. Many studies of AD pathogenesis investigate an essential role for β-amyloid (Aβ) in familial and sporadic forms of AD [130]. Different RNAi strategies have been applied to regulate this pathogenic cascade. Researchers tried by directly silencing of amyloid precursor protein (APP) [131], by silencing of β-secretase (BACE1) that is one of two proteases required for Aβ production but not essential gene in mice [132], or by silencing of tau expression that is a component of the neurofibrillary tangles of AD neurons. Therapeutic use of RNAi is now being tested in animal models of AD targeting these proteins.

Migraine, one of the most common neurological disorders, is caused by diminished production of calcitonin gene-related peptide (CGPR) in the trigeminal system. CGPR can protect from migraine attacks [133]. The CGPR-limited animals are normal, but the paroxysmal nature of this disorder necessitates to use promoters for CGPR. From the beginning of the pathogenic cascade, expression of the shRNA targeting CGPR can terminate the growing pain of this disease. This pain alleviating therapy for migraine is limited because of high threshold dose needed for RNAi [133].

### 4.3. Motor dysfunction disease

A viral delivery of shRNA was used to achieve a long-term RNAi in the CNS. In some reports, the delivery of shRNA-expressing lentivirus showed a rescue of spinal motor neurons with behavioral and histopathological phenotypes in a mouse model having dominant familial ALS [134].

Parkinson's disease (PD) is the second most common neurodegenerative disease. Patient brain of PD is often littered with Lewy body, which is abnormal protein aggregate primarily of alpha-synuclein ($\alpha$-syn) [135, 136]. parkinsonism is linked to hereditary to a single-point mutation in the $\alpha$-syn as well as genetic duplication or triplication of the $\alpha$-syn (SCNA) [104]. The studies targeting the $\alpha$-syn expression revealed RNAi as a therapeutic approach to PD [30, 137]. To date, conflicting results were reported. Regarding the effectiveness and tolerability, there is a report that nigrostriatal degeneration was detected after depleting the $\alpha$-syn level in the brain [138]. It can be inferred that RNAi approaches can be used to validate them in genetic and sporadic models of PD.

### 4.4. Neurovascular disease

RNAi can be applied to cardiovascular and cerebrovascular diseases. Cardiovascular disease results from the progressive occlusion of arteries, and it is most common in a process called atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke [139]. It may be a trigger for the death of cardiac muscle cells or neurons [139]. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis in such cardiac myocytes and brain neurons [140, 141]. RNAi technology may be used to intervene in atherosclerosis or to reduce the damage of heart tissue and brain cells following a myocardial infarction or stroke [142].

Another vascular disease is an ocular disease. Representatively, there were two RNAi clinical trials. The trials performed direct intra-vitreal injection of siRNAs that are targeting VEGF or the VEGFR to test for the safety and efficacy in ocular diseases [143]. siRNAs, targeting VEGF and VEGFR1, are currently in the early stages of clinical trials. The direct injection approach can also prove its usefulness for the other ocular diseases.

### 4.5. Cancer

A chemo-resistance or radio resistance is a major obstacle in cancer treatment. Targeted therapies that enhance cancer cell sensitivity have the potential to increase drug efficacy while reducing toxic effects on untargeted cells (144). Actually, oncogenes expressed at abnormally high levels are attractive targets for RNAi-based therapies against cancers [145], and such approaches have effectively inhibited tumor growth *in vivo* in mouse models.

In nasopharyngeal carcinoma, hyaluronan receptor (CD44) gene silencing resulted in profound reduction of malignant potential of the cells: tumorigenesis and metastasis of tumors in nude mice [105, 146]. It is also suggested a possible therapeutic effect of direct introduction of siRNA to CD44 into some human solid tumors with high expression of the CD44 gene [146]. Although the role of epidermal growth factor receptor (EGFR) in altering tumor chemosensitivity has not yet been fully elucidated, selectively targeting EGFR supplies the reversal possibility of chemoresistance in many tumor types [147]. Reduction of EGFR expression and increased chemosensitivity to docetaxel are emerging an effective strategy for the sensitization of cancer cells to taxane chemotherapy [147]. siRNA-PG-Amine polyplexes can be systemically

http://dx.doi.org/10.5772/62896

delivered to tumors in mice [148], and siRNA-nanocarrier system can efficiently inhibit expression of a specific gene in tumor cells. Once the intact siRNA molecule moves to the target, the gene of interest gets silenced. The PG-amine-based delivery system actually combines both tumor passive targeting with the sequence selectivity of siRNA [148].

The limiting point of targeted therapy is alternative pathway compensation by gene amplification. The "synthetic lethality" is proposed idea to overcome the above problem [149]. This concept suggests that two genes may be considered to have a synthetically lethal relationship [150]. When a mutation is existed either of the two genes alone has no effect on cell survival, but when mutations in both genes cell death is triggered at the same time. By genome-wide RNAi library screening, some synthetic lethal molecules have been discovered. Anaphase-promoting complex/cyclosome (APC/C) and polo-like kinase (PLK) are synthetically lethal with the RAS oncogene in colorectal cancer [151]. The STK33 gene is also synthetic lethal interacting with a RAS mutation in multiple cancer cells from different tumor types [152]. Modified EGFR (amplification or truncation) and hyperactivation of AKT play a major role in the development of glioblastoma, one of the extreme malignancies [153]. There are approaches to develop the siRNA delivery efficiency such as the use of dsRNA-binding domain (DRBD) with a TAT peptide transduction domain (PTD) delivery peptide [154]. These facilities are stable and efficient delivery of siRNAs into cells [155].

## 5. Conclusions

Small RNAs and non-coding small RNAs were important discovery for molecular cell biology; these small RNAs have a vital role in gene regulation that can be controlled by RNA interfering technology. Presently, attempts to integrate gene expression profiling and protein interaction mapping are the main research objectives. The proof-of-principle studies *in vivo* have clearly demonstrated that both viral and non-viral delivery methods can provide selective and efficient target gene suppression without any clear toxic effects. Initial results have been very promising, and many pharmaceutical companies are already focusing on commercialization of various disease-specific RNAi drugs. Despite successful trials in a large number of animal model studies including brain diseases, to develop an efficient therapeutic application, there are numerous hurdles and concerns regarding targeted delivery of siRNAs into brain subregions that must be overcome before wide clinical application of RNAi as a new therapeutic solution. The OTEs, competition with endogenous cellular RNAi components, and effective delivery *in vivo* remain to be optimized. Although recent research has improved the safety and toxicity from the OTEs, it still remains a crucial issue and needs to be addressed before RNAi-based drugs are ready for clinical use. Translational research using RNAi has taken place with an unprecedented speed, and already there are several RNAi-based human clinical trials in progress that will provide breakthrough therapeutic tools for effective treatment human CNS-related disease.

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