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5.42 RNA Interference Technology

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Glossary

Dicer Dicer is an endoribonuclease member of RNase III family encoded by *dicer* gene, which has the function of cleaving long double-stranded RNA or pre-miRNA into siRNA or miRNA that are in 20–25 base pairs length with 2 nt overhangs at 3′ ends.

MicroRNA (miRNA) miRNA is a kind of 22 nt small non-coding RNA fragments discovered in viruses, plants, animals and human, which have the functions of post-transcriptional regulation in gene expression.

p19 protein Plant tombusvirus 19 kDa protein (p19) is an RNA silencing suppressor protein. p19 selectively binds to exactly matched, double-stranded antiviral siRNAs of 21 nt, but not to imperfectly paired miRNAs, ssRNAs or other dsRNAs.

Prokaryotic siRNA (pro-siRNA) Pro-siRNA are bacterial RNase III products that have chemical and functional properties similar to eukaryotic siRNAs.

Ribonucleic acid (RNA) RNA is a linear molecule composed of four types of smaller molecules called ribonucleotide bases: adenine (A), cytosine (C), guanine (G), and uracil (U).

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RNA dependent RNA polymerase (RdRP) RdRP is an enzyme which can use an RNA template to catalyze RNA synthesis and replication.

RNA induced silencing complex (RISC) RISC is a multi-ribonucleoprotein complex that the whole structure is unsolved but associated with TRBP (protein with three dsRNA binding domains), and Argonaute protein as catalytic center. RISC can employ one single-strand RNA from small dsRNA fragments (siRNA or miRNA) as template to recognize complementary mRNA, then the Argonaute protein will induce the cleavage or suppression of the mRNA transcript and result in RNA interference.

RNA interference Ribonucleic acid interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules.

Short hairpin RNA (shRNA) shRNA is an artificial molecule, which consists of two complementary 19–22 nt RNA sequences linked by a 4–11 nt short loop and 2 nt overhangs at 3′ end that is similar to pre-miRNA so-called stem-loop structure.

Small interfering RNA (siRNA) siRNA, sometimes known as short interfering RNA, is a class of double-stranded RNA molecules, 20–25 base pairs in length, similar to miRNA, and operating within the RNAi pathway.

5.42.1 The Discovery of RNAi

The original discovery of RNAi relevant phenomenon can be retrospect to 1990. Napoli and Jorgensen claimed a transgene-induced "cosuppression" of endogenous chalcone synthase gene during generation of hybridized violet petunias.[1] Later in 1992, a similar phenomenon was observed by Romano and Macino in *Neurospora crassa*.[2] They described a "quelling" of the endogenous gene caused by the introduction of homologous RNA sequences. However, RNAi event in animal was first reported in *Caenorhabditis elegans* by Guo and Kemphues until 1995, noting introducing sense or antisense RNA can lead to the degradation of *par-1* mRNA. At the time, homologous antisense RNA was regarded as the tool of silencing to hybridize with endogenous mRNAs then form double-stranded RNA to stop translation. Guo and Kemphues used only sense *par-1* RNA has no hybridization capacity with the endogenous *par-1* transcript in their validation. Interestingly, the degradation of *par-1* mRNA was still observed. The results raised questions and uncertainty of potential regulation mechanism remaining undiscovered.[3]

Followed the investigation of these paradoxical findings, Fire and Mello published a paper stating possible explanations of "cosuppression, quelling and sense mRNA" against endogenous genes in 1998.[4] In their *C. elegans* models, they had employed both purified single-stranded RNAs (ssRNAs) and double-stranded RNAs (dsRNAs) targeted mRNA of *unc-22* gene. The dsRNAs consistently demonstrated higher efficacy than either ssRNAs in gene silencing results of individual and whole population. They proposed dsRNA could be the trigger of stoichiometric implicating integrated against endogenous mRNA in vivo, and possible catalytic or amplification components played a highlighting role during the interference process. These regional developing into systemic silencing effects required a stable intermediate system. Then, Hamilton and Baulcombe first demonstrated the existence of stable intermediate system in plants to support this hypothesis. They suggested dsRNAs need to unwind and the antisense RNA strand served as a guide to bind to the mRNA. Though full length antisense strand were never found, an estimated 25 nt antisense RNA is required for the specific binding based on their observation.[5] Besides, another two different teams found 21–23 nt RNAs could always be purified from *Drosophila* cells with RNAi, proposing a shorten convert of dsRNA mediated cleavage of targeted transcript.[6] Researchers further used 21–22 nt chemical synthesized dsRNA to determine this system in various species, finding short dsRNA mediated gene silencing also exist in mammalian cells.[7] Ambros's group found both *lin-4* and *let-7* genes can encode short temporal mature RNAs in the length of 22 nt in *C. elegans*. These short RNAs were further identified as miRNA.[8] Following investigation showed that genome sequences are tended to form hairpin loops, and these stem-loop sequences as precursor RNAs are the source of miRNAs. miRNA was demonstrated to have gene regulation effects at post-transcriptional level in various species.[9]

It has been determined that the substance that directly leads to gene silencing is small dsRNA. So, how is small dsRNA formed? Bernstein's team discovered RISC responsible to mRNA cleavage could be collected by high-speed centrifugation, whereas short converted dsRNA fragments still remained in the supernatant, which indicated the initiation phase of dsRNA cleavage into siRNA and the effect phase of mRNA cleavage destruction are the result of two different enzymatic activities.[10] Hannon's group hypothesized the initiate phase enzyme should be a dsRNA ribonuclease and then chose different RNase III family enzymes for investigation.[8] By using T7 epitope tag labeling and immunoprecipitation to quantify 21–23 nt siRNAs yields of different RNase III enzymes, they found only the enzyme encoded by gene CG4792 had the regulation activities and named it "Dicer". Sontheimer's group then confirmed depletion of Dicer would cause a loss of siRNAs production and fail to induce further silencing on different target genes.[11]

On the other hand, initial identification of RISC was carried out by Tuschl's team in human cervical cancer Hela cells.[9] They used biotin to conjugate with 3′ terminal of siRNAs in order to coimmunoprecipitate out whole associated protein complexes. Argonaute 1 and Argonaute 2 (Ago1 and Ago2) were isolated after purification. In 2004, Joshua-Tor's group purified an Argonaute protein from the archebacterium *Pyrococcus furiosus* (PfAgo).[10] PfAgo has a PIWI domain sharing high similarity with RNase H enzymes and have two identical DDE (aspartate-aspartate-glutamate) motif catalytic carboxylates which are also required for the cleavage activities in RNase H. Based on these results, Joshua-Tor then collaborating with Hannon's group used immunoprecipitation to investigate Ago1-4 complexes activities in 293T cells.[12] The results showed though siRNAs bound to all Ago proteins, but only Ago2 complex had cleavage activity. Hannon tried to mutate key amino acids of the PIWI domain in Ago2. The mutated Ago2

can only bind to siRNAs but has no cleavage capacity. Besides cleavage activities, how did siRNAs bind to RISC had raised a great research interest as well. Evidence showed only single-stranded siRNAs were found within RISC, which indicated a potential unwind and bind mechanism. Both Gregory's team and Matranga's team found Ago2 has the RNA helicase function to unwind duplex siRNAs, employ one guide strand to find complementary mRNA, remaining anti-guide strand will be cleaved and removed.[13,14] This mechanism is essential for the RISC activation. Although Ago2 hold function of cleaving both siRNA and target mRNA, only Ago2 protein could not recruit any siRNA. For RNAi event, researchers found RISC composed of Dicer, Ago2, and TRBP (the HIV-1 TAR RNA binding protein) is required. TRBP helps recruiting the Dicer complex to Ago2 which form a ternary complex induce RNAi activities.

The effect RNAi can be triggered by very little induction and systematically transmit to whole organism. Researchers observed and recognized that RdRP can employ aberrant accumulated RNAs as templates to generate secondary siRNAs against same mRNA and then cause sequences specific degradation, and this process was named "transitivity".[15] RdRPs play an important role in various forms of RNAi amplification. In *Arabidopsis*, unprimed RdRPs employed both 5′ and 3′ mRNA cleaved fragments as substrates to produce long dsRNAs. These dsRNAs were then cut by Dicer to generate secondary siRNAs and induced mRNA degradation. But in *C. elegans*, a different mechanism was described. Unprimed RdRPs can directly use primary siRNAs as template substrates. Through transitivity process, Dicer-independent secondary siRNAs were directly synthesized and presented same properties of sequences specific degradation on same mRNA.[16]

Upon constantly enriched knowledge of RISC formation and functional activities, the understanding of entire RNAi conceptual system had been greatly improved. Investigation aimed other crucial components as mRNA translation regulation[17] and mRNA decay center[18] were quickly carried out in next few years.

Synthetic small interfering RNAs (siRNAs) are an indispensable tool to investigate gene function in eukaryotic cells and may be used for therapeutic purposes to knockdown genes implicated in disease. Thus far, most synthetic siRNAs have been produced by chemical synthesis.

5.42.2 Mechanism of RNA Interference

The elucidation of RNAi machinery is emerging as a two-phase mechanistic model based on the discoveries and studies of key components in the regulation. This combination includes an initiation phase, long dsRNAs cleaved into ~21–25 nt discrete small RNA fragments of siRNA; and an effector phase of siRNAs incorporating with multinuclease complex RISC to induce further cleavage and degradation of homologous mRNA.

In the initiation phase, siRNAs were generated from the cleavage of long dsRNA by type III endonuclease Dicer (**Fig. 1**). Dicer was first identified as a multi-domain RNase enzyme in *Drosophila*. It has two existence forms: Dicer-1/Loquacious (R3D1-L) which produces miRNA and Dicer-2/R2D2 which produces siRNA. Dicer-1 and Dicer-2 share structural homology features but display different properties like ATP dependency and substrate specifications.[19]

Dicer-1 is an ATP independent enzyme working as precursor related to miRNA biogenesis.[20] Previous research found that Dicer-1 needs to work together with a double-stranded RNA binding protein (dsRBDP). In *Drosophila*, dsRBDP Loquacious (Loqs) was identified to have interaction with Dicer-1.[20] Co-immunoprecipitation experiments have purified a functional pre-miRNA processing complex containing both Loqs and Dicer-1. This complex directly activated and participated in pre-miRNA processing activity. Structural analysis showed only Loqs-PA and Loqs-PB among Loqs protein species have three dsRNA binding domains (dsRBD) and Loqs-PB can increase the affinity of Dicer-1 toward pre-miRNA.[20]

Dicer-2 is an ATP-dependent enzyme that showed substrate specificity to dsRNA.[19] Sharing structurally homology with Dicer-1, Dicer-2 also needs to work together with a dsRBDP-R2D2. Dicer-2 associated with R2D2 can form a functional heterodimeric complex.[19,20] Unlike Loqs, R2D2 has only two dsRNA-binding domains which can interact with long dsRNA but does not regulate siRNA generation activities. R2D2 plays an important role in development and stability of Dicer-2. It has demonstrated that both dsRNA binding domains of R2D2 and Dicer-2 are essential to the process of Dcr-2/R2D2 complex binding with siRNAs then loading them into RISC.[21] Structural analysis showed Dicer-2 has an RNA helicase domain, a DUF283 domain, an N terminus' PAZ domain, tandem RNase III motifs and a C terminus' dsRBD motif. Since Dicer-2 or R2D2 alone does not bind with siRNAs, which indicated siRNA might bind with the interface between R2D2 and Dicer-2 then triggered a conformational change on complex allows them to coordinately bind siRNA to promote assembly of the siRISC complexes.[21]

In the effector phase, single strands siRNA or miRNA work as guide strands are incorporated into RNAi effector such as the RNA-induced silencing complex (RISC) which cleaves mRNA and represses translation, or the RNA-induced transcriptional silencing (RITS) complex which regulates heterochromatin assembly (**Fig. 1**).

Structural analysis showed RISC is composed of PAZ PIWI domain (PPD) proteins. PPD proteins have 100 amino acids PAZ at central and 300 amino acids PIWI at C-Terminal. Researchers found PPD proteins in RISC are Ago proteins. PPD proteins perform different functions in different systems. For example, PPD, RDE-1 and PPW-1 in *C. elegans* are responsible for the efficient siRNA mediated mRNA cleavage; Ago-2 in *Drosophila* mediates the incorporation of siRNA into RISC; and hAgo-2 in *Homo sapiens* is for the mRNA cleavage required catalytic activity.

During preliminary investigation, researchers found it was difficult to purify complete eukaryotic Ago protein.[12] Thus, the studies on prokaryotic homolog RNase H ribonuclease was carried out to mimic Ago protein. Structural analyses revealed Ago proteins are composed of N-terminal, PAZ domain, Middle domain and PIWI domain. Functional analyses revealed Ago protein is the catalytic

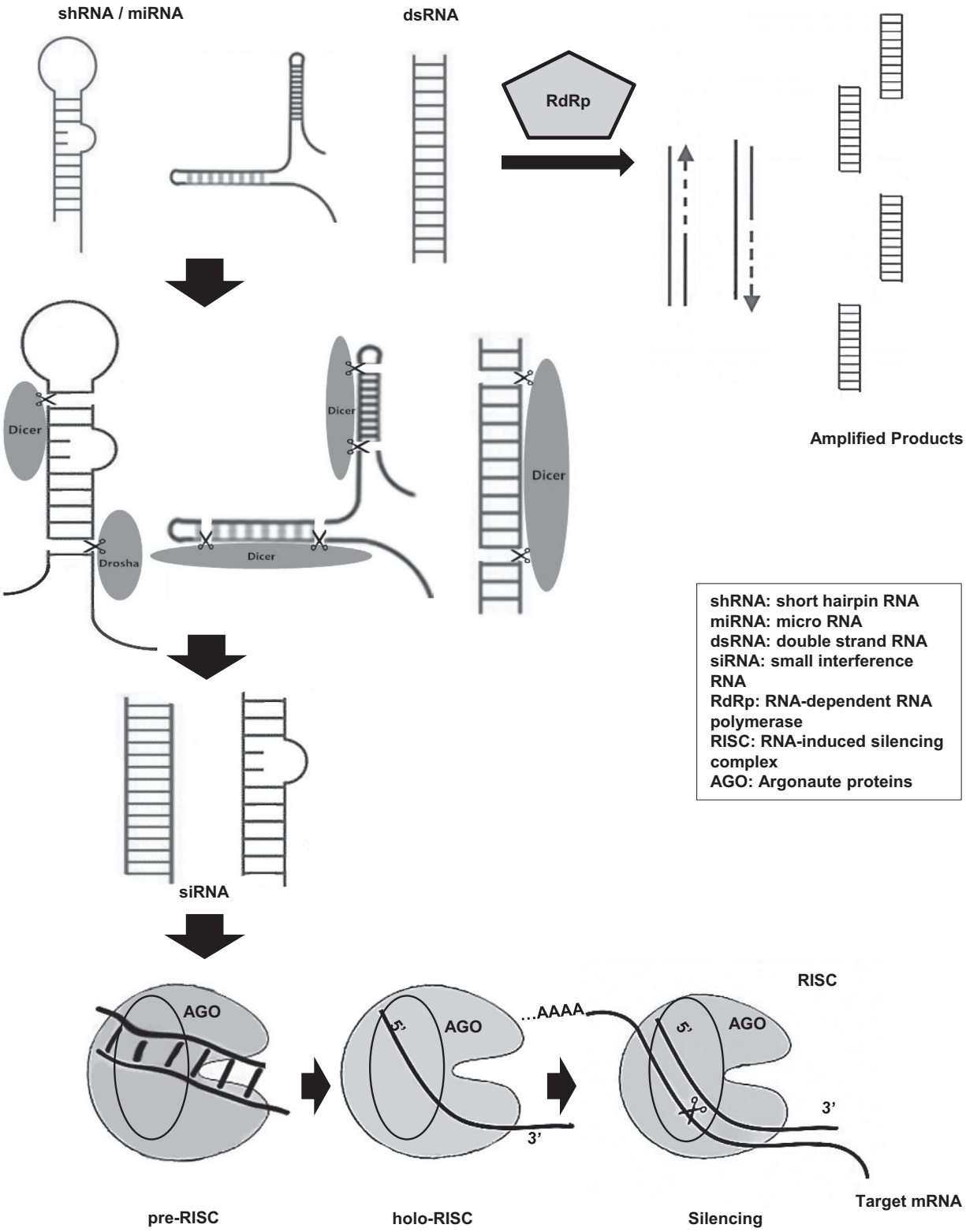

Figure 1 dsRNAs, shRNAs or miRNA precursors are cleaved by the Dicer or Drosha to produce siRNA/miRNA duplexes. Amplification process is induced due to the presence of RNA-dependent RNA polymerase (RdRP). These short dsRNAs bind to effector RNA-induced silencing complexes (RISCs) to form pre-RISC. Holo-RISC is the activated effector after effective removal of passenger strand, which can recruit mRNA and directly induce cleavage activities, then mediate translational repression or chromatin modification.

subunit of RISC.[22] A crystal structure of a full-length Ago protein from the archebacterium *P. furiosus* (PfAgo) was determined the relationship. PAZ and PIWI were identified as functional domains among four major domains.[12]

siRNA binds to Dicer-2/R2D2 to form R2D2 Dicer initiator complex (RDIC). In an RNAi model, RDIC was found to have orientation binding preference: 5′ ends with lower melting temperature in siRNA duplex tends to interact with Dicer-2 the less stable end, while more stable end of siRNA tends to bind to R2D2. These results hypothesized thermodynamic asymmetry is cause of separation of guide strand and passenger strand.[23] Further investigation found enzymes responsible for the RISC assembly have selection function of incorporated siRNA strand on the basis of structure. Which indicates siRNA structure determines the orientation preference of Dicer-2/R2D2 complex then results in the select of guide strand incorporated into RISC.

Ago2-dependent complex was identified as pre-RISC containing duplex siRNA. Holo-RISC was characterized as RISC after effective removal of passenger strand. Studies found Ago2 was responsible for the conversion of pre-RISC to holo-RISC.[23] Efficient RNAi required 5′ ends of siRNA acting as a target recognition guide. Then Ago-2 as catalytic subunit of holo-RISC, with RNase H like activities, will induce the cleavage of target m-RNA backbone.

Researchers also found particular gene silencing can be induced by just a small induction in whole organism, which indicates potential amplification mechanism. RNA-dependent RNA polymerase (RdRP) could help synthesize new homology sequences that will be recruited for the destruction of same target.[24] During the amplification process, RdRP can synthesize new siRNA directly, or use introduced siRNA as a template to generate long dsRNA, which are then cleaved into siRNAs. Research found that the newly synthesized siRNA fragments were 24–26 nt instead of 20–22 nt, then these fragments will rejoin the production cycle of long dsRNA and sustains the amplification process.[24]

5.42.3 Technologies of RNAi

On account of the sequence specificity of siRNA, RNAi is generally considered as a negative switch that regulates the expression of genes. Therefore, it is able to introduce vectors that express suitable RNAs to induce RNAi, including synthetic siRNAs, short hairpin RNAs (shRNAs), long dsRNAs, endoribonuclease-prepared short interfering RNAs, pro-siRNAs, etc. The first two only have single sequence, while the others have multiple sequences. We compare the features of these methods simply in **Table 1**.

5.42.3.1 Synthetic siRNAs

Synthetically exogenous siRNA is completely base-paired 21–22 nucleotide (nt) with a 2-nt-long 3′ overhang. It is generally a chemical synthetic polynucleotide.

The sequence design of effective and specific siRNA is the key to successful gene silencing by RNAi. To date, various siRNA design algorithms and programs have been developed and widely used by biologists for siRNA design. These algorithms have certain rules called Tuschl's rules that are based on some parameters, including length, 3′ overhang, absence of intron regions, GC contents, absence of repeats, absence of single nucleotide polymorphism (SNP) sites, homology to other genes or sequences, RNA secondary structure, etc.[7,25–27] Two years later, Khvorova et al. suggested that the thermodynamic properties of siRNA are also critical parts in determining the function and longevity by involving in siRNA duplex unwinding and strand selection.[28]

After years of development, now there are various web-based programs that design siRNA candidates. The faculties of the University of Hong Kong combined 11 free online siRNA design tools together and developed their own filtering algorithm to filter ineffective siRNAs (http://i.cs.hku.hk/~sirna/software/sirna.php).[29] It has been shown that many siRNA design programs tend to select different siRNA candidates with a range of siRNA efficacies even though we run the same algorithms with the same target

Table 1 Compare different types of siRNAs

	Synthetic siRNA	*shRNA*	*Dicer-produced siRNA*	*esiRNA*	*RNAi feeding for* C. elegans	*miRNA inhibitor and mimic*	*Pro-siRNA*
Number of target sequences	Single	Single	Multiple	Multiple	Multiple	Single	Multiple
Potency	Variable	Higher than synthetic siRNAs	Higher than synthetic siRNAs	Higher than Dicer produced siRNAs	Variable	Variable	High
Off-target effects	Variable	Variable	Low	Low	Not studied	Variable	Low
Cost of production	High	Lower than synthetic siRNA	Lower than synthetic siRNA	Lower than synthetic siRNA	Low	High	Low
Scale-up production	Difficult	Easy	Difficult	Easy	Easy	Difficult	Easy
Renewable with minimum resource	No	Yes	No	No	Yes	No	Yes
Natural produce	No	Yes	No	No	Yes	No	Yes

sequences.[30] So, it is recommended to use two or more siRNA design programs and compare the multiple sets of candidates to search for the optimal siRNA sequences.

Once the siRNA that against the specific gene has been obtained, it has to be effectively delivered into cells. There are three efficient and inexpensive delivery methods of siRNA: transfection, electroporation and viral vector. The first two are non-viral delivery, the rest one is, as the name implies, viral delivery. Transfection generally has three modes: cationic liposomes, polymer nanoparticles, and lipid conjugation. It is relatively easy to transfect siRNAs into most of cell lines with high efficiency and reproducibility, and there are many commercial reagents offered. However, it is not suitable for all kinds of cells such as primary cells and non-dividing cells and has low efficiency in vivo.[31] For hard-transfected primary cells, stem cells and neuronal cells, electroporation, another intracellular delivery method, could be a good choice. The cell membrane is consisted of phospholipid bilayer which is susceptible to electric shock. When there is a powerful voltage pulse, the lipid molecules will reorient to form hydrophilic pores to let nucleic acids in via electrical current. But different cell types need different optimal parameters, and this method is easier to make cell die. Viral-mediated delivery, similar as transduction, deliver siRNA through some recombinant viral vectors based on retrovirus, adeno-associated virus (AAVs), adenovirus and lentivirus. It can be used for primary and non-dividing cells, compatible for in vivo experiments and stable expression. But problems arise because it can lead to possible mutagenic and immunogenic effects and need to generate and titrate viral particles.[32]

Dr. Mark Helm and his groupmates used the fluorescent-labelled siRNA to trace the fate and their subcellular location of synthetic siRNA. From the results of the fluorescence resonance energy transfer (FRET)-based visualization, once siRNA gets into cell, it translocates into the nucleus and reach high concentrations at an early time point, then relocates to the cytoplasm in next 4 h. Up to 48 h, nearly 99% siRNA have been degraded. That is in accordance with RNAi activity, which reaches the peak at about 24 h and decreases within 48 h.[33] In this process, siRNA assembles with RNA-induced silencing complex (RISC) with no need to continue processing with Tat-RNA-binding protein (TRBP)/PKR activating protein (PACT)/Dicer complex, which is needed for shRNAs or long dsRNAs.[34]

5.42.3.2 Short Hairpin RNA

The use of synthetic siRNA to strongly downregulate specific gene expression is a promising method. However, this reduction is basically transient, since the concentration of siRNA gradually reduce to so low level by cell division that leads to inefficient suppression of gene expression, especially in long-lived cells. For this reason, a vector-based method has been developed for gene silencing by the expression of a shRNA which mediates persistent suppression of gene expression.[35]

shRNA is an artificial molecule, too. Typically, it consists of two complementary 19–22 nt RNA sequences linked by a 4–11 nt short loop and 2 nt overhangs at 3′ ends that similar as pre-miRNA so-called stem-loop structure.[36] And it also can be designed by web-based programs. The shRNA is usually delivered as a gene construct that consists of a promotor, shRNA and a transcription termination sequence.[37]

Expression of shRNA in cells can be achieved by delivery of plasmids, viral vectors or bacterial vectors. Transfection can be used to introduce plasmids into cells which is similar as the transfection of siRNA. It can be accomplished by using commercial transfection reagents in vitro but cannot be used in vivo. Viral-mediated delivery also can be used for shRNA through some recombinant viral vectors based on adeno-associated virus, adenovirus and lentivirus. The gene construct is simple and small so that they can be easily inserted into vectors. The genomes of AAVs and adenoviruses are episomal so that they cannot be integrated into the genome of the host cells. In addition, AAVs have diminished packing capacity to limit the induction of immune responses in host cells. But with cell dividing, the virus will be lost quickly. Different from AAVs and adenoviruses, lentiviruses can integrate its own genome into sections of transcriptionally active chromatin and be passed on to progenies so that increase the risk of insertional mutagenesis. The other kind of vectors that can be used for delivery of shRNA is bacterial vector. Dr. Shuanglin Xiang and his co-workers engineered the *Escherichia coli* to produce plasmids of shRNA to knock down gene expression in vitro and in vivo.[38]

Different with siRNA, shRNA is synthesized in the nucleus of cell. Once the vector into the cells, the gene will integrate into the host genome and is then transcribed by Pol III or Pol II depending on the type of promoters. That's why shRNA can induce sustainable RNAi. Then, shRNA further transports to the cytoplasm and assimilates into the miRNA pathway.[34] The common used one is Pol III based on U6, H1 or tRNA promotors.[35,39,40] These promoters direct high levels of shRNA expression and afterward mediate highly efficient gene knockdown. However, the high concentration of shRNA also has side effects: increase the off-target possibility and lead to non-specific effects such as interferon response and cellular toxicity.[41] And they lack cell specificity. Compared with Pol III, Pol II may be used to direct cell- or tissue-specific gene silencing.[37]

shRNA is 10-fold more efficient than siRNA. For most of siRNAs, the effective concentrations for knocking down target genes are usually on nanomolar level, while less than five copies of shRNA are sufficient for being integrated in host genome.[34] Even though, many researchers still study on the improvement of efficacy. Replaced polymerase III by highly efficient polymerase II,[37] overexpressing exportin 5,[42] and expressing multiple shRNA in a single plasmid vector.[43]

shRNA also has some limitations. Due to the advantage of stable expression, it can be used to generate stable knockdown cell lines. But this project is a time-consuming task since the design of gene construct and the selection of shRNA-positive cells.[36]

5.42.3.3 In Vitro Produced Long dsRNA

It can be seen from the mechanisms of RNAi that long dsRNA is an effective inducer of RNAi. So, it is also a good idea to synthesize long dsRNAs in vitro. For instance, in *Drosophila*, dsRNAs of more than 150 bp or even longer were shown to trigger gene silencing

effectively.[25] But they are not fit for mammals. This is because long dsRNAs activate the expression of genes in the interferon pathway, including the gene encoding dsRNA-activated protein kinase PKR, which inhibits translation by phosphorylating eukaryotic initiation factor 2 α-subunit (eIf2α) on Ser51 and induces transcription of inflammatory genes[44] and 2′,5′-oligoadenylate synthetase (OAS) which can cause nonspecific effect by activation of RNase L,[45] resulting in termination of these two protein syntheses and finally causing cell death. Since the activities of siRNAs that target to different sequences have profound differences and the expensive cost for synthesizing siRNAs, although, they can suppress selective gene expression without triggering interferon responses, an alternative method that using bacteriophage promoters to mediate transcription from linearized DNA templates to obtain long dsRNA has been developed. The most popular one is using T7 RNA polymerase.[46,47]

5.42.3.3.1 Dicer Produced siRNA

With the deepening of research, many biochemical research results show that RNAi is completed by a multicomponent nuclease. And the function of this complex is guided by a small guide strand (21–22 nt siRNA). As a result of biochemical fractionation and genetic approaches, a nuclease called Dicer from RNase III family has been identified. It can cleave dsRNA into siRNAs in vitro. The Dicer kit has already been commercialized (Genlantis). This method is cheaper and can obtain a large number of siRNAs.[10,48] In 2005, Dr. Dong-Ho Kim with his co-workers found that some dsRNAs have greater potency than synthetic 21 nt siRNAs directed to the same target site, and that this property seemed to correlate with length. With the increasing of the length of the siRNA, synthetic RNA duplexes 25–30 nt in length is 100-fold more potent. Especially, the duplex length of 27 nt has the maximal inhibitory and it doesn't induced interferon or activate protein kinase R (PKR). That means that synthetic RNAs that are substrates for Dicer are more potent than conventional RNAs. It is possibly because that increasing the length of an siRNA duplex with an unprocessed end is thermodynamically more stable and prompting the incorporation with Dicer/TRBP/PACT complex for more efficient RISC assembly.[49]

5.42.3.3.2 Endoribonuclease-Prepared siRNA

Although Dicer is an enzyme that also involved in dsRNA cleavage in vivo, using it in vitro is very inefficient, especially for short dsRNAs. Because of this, Dr. Dun Yang and his groupmates reported a similar method that using *E. coli* RNase III to process dsRNA into esiRNA and said this method is more efficient than the former.[50] *Escherichia coli* RNase III is a dsRNA-specific endoribonuclease that can be easily produced in a large scale. It can digest dsRNAs into small pieces with 12–15 bp so that unable to trigger immunology responses in mammalian cells. Although the length of these fragments is different from that of traditional siRNAs, their end structures are the same, 5′ phosphate, 3′ hydroxyl termini and 2 to 3 nt 3′ overhangs.[51] And these end structures are reported to be important for RNAi activity.[26] There is now a commercial *E. coli* RNase III (Invitrogen).

5.42.3.4 RNAi Feeding for *Caenorhabditis elegans*

RNAi was first discovered in *C. elegans* and have been used as a tool for several years before it was named RNAi. Initially, RNAis were performed by microinjecting corresponding dsRNAs into worms to observe the phenotypic changes of the parents and progeny.[4] In the same year, Lisa Timmons and Andrew Fire found that *C. elegans* show gene-specific manners depending on the dsRNAs in the exposure environment. *C. elegans* normally feed on bacteria, sucking in the pharynx and subsequently digesting in gut. Thus, the bacteria that expressing dsRNAs can result in specific gene silencing on nematodes that feed on bacteria.[52] Hirlaki Tabara, Craig C. Mello and their groupmate also showed that feeding and soaking worms in dsRNA are effective ways in gene silencing.[53] And these methods hold promise for high-throughput RNAi.[54–56]

This method begins with the creation of feeding strain. First select a target region of approximately 500 bp that corresponding to the mRNA and link into plasmids that having specific structure. Then, the plasmid should be transformed into an *E. coli* RNase III mutant strain to express dsRNAs. One point that needs special mention here is that the effect of gene silencing on liquid culture medium or plates feeding nematodes is similar, depending on penetration and expression efficiency. However, the experimental processes and the restrictions are still different.[57]

5.42.3.5 microRNA Inhibitor and Mimic

Similar to siRNA, miRNA is also a kind of small non-coding RNAs containing about 22 nt that regulate gene expression through mRNA degradation and translational inhibition. They are associated with differentiation, homeostasis and disease. miRNAs have already been found in plants, animals and some viruses and they are conserved in both plants and animals.

miRNA can regulate mRNA expression while miRNA also can be regulated by other regulators. One kind of the regulators is miRNA inhibitor which can be used to study the functions and mechanisms of endogenous miRNAs. miRNA inhibitors are mainly divided into two categories, natural miRNA inhibitors and artificial miRNA inhibitors. Natural miRNA inhibitors are competing endogenous RNAs (ceRNAs) (such as viral RNAs, mRNAs, pseudogene RNAs, lncRNAs, and circRNAs), which competitively recognize miRNA recognition elements (MREs) to reduce the inhibitory effect of miRNA and release target mRNA.[58–60] ceRNA model describes potential communication networks among all transcripts and provides an opportunity to discover the novel therapeutic targets for human cancer.

miRNAs are highly expressed in many diseases. Inhibition of miRNAs can alleviate their over-repression of target mRNAs and restore normal cell phenotype, so it is considered as a promising therapeutic strategy. Different from natural ones, artificial miRNA

inhibitors are exogenous RNAs, including anti-miRNA oligonucleotides (AMOs) and miRNA sponges. AMOs are single-stranded short RNA oligonucleotides that are fully complementary to the miRNA. Due to their single-stranded structure, they are easily degraded by nucleases. Chemical modification can be a good way to increase affinity for target miRNA, stabilize miRNA inhibitor to avoid nuclease degradation, prevent cleavage by RISC and promote tissue uptake for in vivo delivery.[61] Barbara Robertson and her colleagues showed that the function of the inhibitors varies with the position of the mismatch in the inhibitors. The "seed region" (3rd–8th nucleotides) and the "3′ region" (13th–18th nucleotides) are equally important in determining recognition of the inhibitor target by endogenous miRNAs.[62] With the deep research on AMOs, they are widely used in miRNA loss-of-function study and miRNA suppression therapy. However, there are still many shortcomings that inhibit the development of this discovery. The inhibition still could not be sustainable and permanent; AMOs are toxic and can trigger innate immune response and finally cause cell death; the concentration of AMOs that used in transfection cannot exert satisfactory inhibition, whereas the higher concentration may cause cell death.[63] The miRNA sponge is a designed RNA transcript that expressed from strong promotor and bind to target miRNA competitively via multiple and tandem miRNA binding sites.[64] Different from AMOs, once miRNA sponges can be transfected and expressed stably in cells, they can play a prolonged even permanent role in suppressing individual miRNA or the whole family.[63] miRNA sponges are also widely used in miRNA loss-of-function study and miRNA suppression therapy.

The other kind of inhibitor is chemical synthesized miRNA mimic. Because miRNAs are double-stranded RNAs in cells, miRNA mimics typically retain both strands. And chemical modifications are often introduced to miRNA mimics to improve stability, selectively exclude the passenger strand and facilitate guide miRNA loading into RISC.[65] miRNA mimic has the opposite function as miRNA inhibitor. miRNA inhibitors suppress the miRNA, whereas miRNA mimic can mimic the high-level expression of endogenous mature miRNAs in cells to enhance the regulation of endogenous miRNAs. It is a powerful tool for miRNA function research. miRNA mimic is simple and efficient. It can be transfected into cells only by encapsulation with transfection reagents, without the need to construct vectors and without the fear of virus protection.

Although the application of double-stranded miRNA mimics is promising, they still have some drawbacks for their use in therapy. The presence of the passenger strand adds complexity that not only increases the need for synthesis of the double strand but also may result in potential off-targeting effects that caused by the passenger strand.[66] Double-stranded RNAs are more difficult to enter cells or tissues than single-stranded RNAs and often require the use of appropriate transport vectors.[67,68]

5.42.3.6 Pro-siRNA

Pro-siRNA is a recent developed approach that produces a pool of highly efficacious siRNAs in *E. coli*. Like chemically synthesized siRNAs, the mediated gene silencing is also temporary.

How do siRNAs produce in *E. coli*? The key point here is that a plasmid that consists of *p19*, T7 promotors and a pair of inverted repeat sequences that encoding the gene of interest with a linker is designed and transformed into bacteria. The p19 protein found in the plant RNA virus *tombusvirus* can stabilize siRNAs by binding with them in a sequence-independent way.

Once the plasmid transformed into *E. coli*, the inverted repeat sequences will be transcribed and form a long hairpin RNA with the linker, being co-express with p19 protein. After the processing by RNase III of *E. coli*, hairpin RNA is digested into a numerous of siRNAs and combined with p19 proteins to form complexes. Then one can capture the complexes with Ni beads by affinity interaction, elute siRNAs and purify by HPLC.

Plant tombusvirus 19 k Da protein (p19) is an RNA silencing suppressor protein. p19 selectively binds to exactly matched, double-stranded antiviral siRNAs of 21 nt, but not to imperfectly paired miRNAs, ssRNAs or other dsRNAs. When p19 was expressed in *E. coli*, a small RNA species accumulates that is double-stranded and ~21 nt in length, like eukaryotic siRNAs. We named those bacterial siRNA-like RNAs pro-siRNAs for "prokaryotic siRNA". *Escherichia coli* RNase III, which belongs to the same RNase III endoribonuclease family as eukaryotic Dicer, is responsible for the generating pro-siRNAs. RNase III is also known to generate ~21 nt dsRNAs in vitro.[69]

This method relies on ectopic expression of p19, a siRNA-binding protein found in a plant RNA virus. When expressed in *E. coli*, p19 stabilizes ~21 nt siRNA-like species produced by bacterial RNase III. Transfection of mammalian cells with siRNAs, generated in bacteria expressing p19 and a hairpin RNA encoding 200 or more nucleotides of a target gene, at low nanomolar concentrations reproducibly knocks down gene expression by ~90% without immunogenicity or off-target effects. Because bacterially produced siRNAs contain multiple sequences against a target gene, they are especially useful for suppressing polymorphic cellular or viral genes.[70]

5.42.4 RNAi Screening

Sequence specific RNAi can lead to silence of target gene expression and associated with phenotypic changes, which can be applied on the large-scale mechanism or functional interrogation on various organism models from different species. RNAi screen provide new approaches for the understanding of unknown biological pathways. On one aspect, it can be performed as high-throughput genome-wide screen to help to identify all possible regulators in a general process. On the other hand, it can also serve as limited screen to help to narrow down crucial factors in a specific pathway.[71]

In RNAi screen, sequence specific RNAi reagents were introduced into model organisms to cause degradation of targeted transcripts. RNAi process employed the ~21–25 nt double-stranded siRNA fragments with a characterized 2-nt 3′ overhang, which are produced from the cleavage of long dsRNA by type III endoRNase Dicer. Different cell types, approaches and delivery methods of

cell-based RNAi screening has determined the setup of appropriate RNAi reagent libraries. In models that lack the defensive of type I interferon response, such as *C. elegans* and *Drosophila*, long dsRNA can be taken up for the intracellular cleavage activities of Dicer to produce a highly specific overlapping siRNA pool against target genes.[72] But in mammalian cells, introduction of exogenous long dsRNA can activate antiviral type I interferon response and dsRNA-binding protein kinase R, which leads to the disruption of phenotypic screening. In order to minimize interferon response, reagent pools prepared with recombinant bacterial RNase III or Dicer, in the form of small interfering RNAs (siRNAs), endoribonuclease-prepared siRNAs (esiRNAs) or small hairpin RNAs (shRNAs) are used for mammalian cell screens. These RNAi reagents can escape interferon response and defense system at low concentrations.[73]

Chemical synthesized siRNA is designed to mimic the final products of Dicer cleavage. Chemical modification offered superior duplex stability and high target mRNA knockdown efficiency for synthetic siRNA. Current commercial siRNA libraries are available to cover complete genome for several species. Most libraries provide three or more siRNA sequences against each target gene can reach estimated 95% silencing efficiency after transfection.[71] The extended protein depletion associated phenotypic changes are varied due to different protein lifetimes.

But the siRNA delivery by transfection is inefficient on primary cells. Research showed successful gene silencing in primary T cells required tenfold higher siRNA dosage than human cervical cancer HeLa cell line. Similar results were observed in the experiment aimed to efficient knockdown NF-kappa B p50 in primary human dendritic cells.[74] The critical requirement of 500 nM siRNA was significantly higher than usual effective dosage of 10–50 nM in most established cell models. Retroviral and lentiviral approaches are more suitable for efficient knockdown in primary cells. Short hairpin RNA (shRNA) delivered by viral transduction can continuously express in cells and mediate stable gene silencing regardless with transfection efficiency. Several shRNA libraries are also available for primary cells RNAi screen.

RNAi screen aimed to high-throughput detect desired phenotype, is defined by different setup of assay. Arrayed screening assays use multi-well plate to contain single RNAi reagent separately for the knockdown experiment. Readouts for arrayed screening can be the measurement of specific luciferase or fluorescence indicators. Moreover, high-content image-based readout can report quantitative parameters on morphology change, proliferation, viability, apoptosis and other cellular functions of individual targets or whole populations, by employing specific fluorescence imaging and labeling of samples in a high-throughput measurable format. Other than arrayed screening assays, pooled screen is to introduce a whole set of RNAi libraries into bulk populations of cells. The readout of pooled screen is required to present a selectable change in cell phenotype. It allows rapid analyses on multiple cell types.[71]

Off-target effect of RNAi-based screen has stated as the major issue responsible to false discovery of candidates. Different types of off-target effects were distinguished from various studies.[75] For example, if a siRNA shares an identical sequence with an unrelated mRNA, the undesired silencing would produce a confused phenotype then result in false positive event. In addition, if the seed region of a siRNA duplex has a weakly complementary sequence in the 3′ untranslated region of an unrelated mRNA, this siRNA can act like microRNA (miRNA) to induce undesired mRNA degradation or translational block, then cause non-target proteins depletion. In mammalian cells, introduction of short siRNA fragments can sometime sequence-independently activate the antiviral type I interferon response, then cause unpredicted false disturbance. To minimize off-target effects, non-target or scrambled RNAi sequences should be setup for the control. Different delivery method of siRNA should be monitored and evaluated. Multiple siRNA species should be used against each target gene.[75]

Many complex phenotypes displayed on in vivo level cannot be analyzed through cell-based *in vitro* assay. With the availability of genome or transcriptome sequences information, in vivo RNAi makes it possible for researchers to perform functional genomics studies directly in model organism. RNAi phenomenon was first identified in *C. elegans*. Studies found that RNAi in *C. elegans* is systematic and heritable. Thus, various in vivo RNAi screens were conducted in *C. elegans* and provided numerous new understandings for diverse biological process such as aging and obesity.[76] *Drosophila* is another popular in vivo RNAi screen model. With a well-annotated genome and multi-specific genetic modify tools, tissue and stage specific studies were performed on *Drosophila*. With the ability of tissue or stage specific RNAi, *Drosophila* allows screens not only on embryo or larval, but on adults as well.[6] With the idea of pooled RNAi screen for the comparison of different cell populations, ex vivo screens by introducing pooled shRNA transfected cells in mice were performed. For example, by injecting transduced lymphoma cells into in vivo context, their ability and contribution for tumorigenesis can be directly assessed.[77] In vivo RNAi screen provides a straightforward and quick approach to screen an interest phenotype in a tissue and stage specific manner.

5.42.5 Technical Considerations for Using RNAi Technology

Due to the ability of specific gene silencing and the generality of therapeutic targets, many scientists have paid much attention to RNAi technology not only for the functional analysis of specific genes by observing the variation of physiological-biochemical characteristics before and after gene silencing but also for discovering genes involved in various diseases that cannot be cured with traditional pharmaceutical approaches and treating diseases as medicinal targets.

5.42.5.1 Advantages

Compared with other antisense-based methods such as antisense DNA oligonucleotide and ribozymes, the use of RNAi for therapeutic applications has great advantages.

5.42.5.1.1 Specificity

The main advantage of RNAi is specificity. In theory, the sequence-specific siRNAs designed by Watson-Crick base pairing interactions can be used to silence any gene without restriction, whether we know the function of the gene or not, and regardless of what protein the gene is expressed in, and the structure and function of the protein. This high specificity even allows siRNAs to be mapped to a mutation site that is different from the normal allele, even if there is only one or a few nucleotide substitutions.

5.42.5.1.2 Potency

Due to the differences in sequence design algorithm and target sequence selection, it is difficult to directly compare the gene silencing efficacy among several technologies. But what is certain is that RNAi-mediated inhibition is more efficient than other antisense oligonucleotides. Antisense oligonucleotides have been developed and widely used for gene regulation in vitro and in vivo in the past decades. Although the result is consistent with RNAi, the mechanism is indeed completely different. Based on mechanisms of action, ODN can be classified into two groups: one is the RNaseH-dependent oligonucleotides which result in the degradation of mRNA; the other is the steric-blocker oligonucleotides, which structurally suppress the progression of splicing or translation. RNaseH is an endoribonuclease which hydrolyzes the phosphodiester bonds of RNA in RNA/DNA duplex, so it can target to any regions of the mRNA for inhibition. However, the most steric-blocker oligonucleotides are efficient only at 5′- or AUG initiation codon region, phosphorothioate oligonucleotides, it may not always be an optimal site for targeting.[78] Furthermore, the siRNA appears to be more potent at very low concentrations (<50 nM) and more resistant in cell culture and in mice.[79]

5.42.5.2 Challenges

Although siRNA is commonly used in nowadays and seems promising in therapeutic applications due to its specificity and potency, it is still fraught with challenges. To take full advantage of the potential of this technology, many intracellular and extracellular barriers need to be overcome, such as stability, off-target effects and immune responses.

5.42.5.2.1 Stability

Since siRNA is an exogenous molecule, how to successfully transport siRNAs to specific genes, cells or tissues and exert their gene silencing activities is the first problem to be solved. Although siRNA is double-stranded and more stable than single-stranded RNA, it can still be quickly degraded by nucleases in plasma and tissues. For this purpose, efforts should be made for chemical modification of siRNAs to reduce their susceptibility to RNases and alter the pharmacokinetic properties to increase half-life in vivo. Several studies have shown that the half-life period of unmodified siRNAs in vivo is about several minutes, but it can be significantly improved by protective vehicles and chemical modification (**Table 2**), even sometimes at the cost of reducing activity of suppression.[80–84]

Table 2 Chemical modification for siRNA stability

Classification	*Modification*	*Function*	*References*
Modifications to overhangs and termini	RNA-3′-PNA chimers on both strands	Enhances nuclease resistance in serum	85
Modifications to conjugate groups	Cholesterol	Long duration	86
	Polyethylene glycol (PEG)	Greater stability	87, 88
	Carbon-nanotubes	Chemical stability	89, 90
Base modifications	Propynyl and methyl substitutions at C-5 positions	Increase the thermal stability	91
	2,4-Difluorotoluyl ribonucleoside (rF) modification	Improves nuclease resistance in serum	92
Sugar modifications	2′-Deoxy-2′-fluoro-β-D arabinonucleotide units/ 2′-deoxy-2′-fluoro-5-methyl-4′-thioarabinonucleotide units	Greater serum stability and longer half-life	93, 94
	Locked nucleic acid (LNA)	Nuclease resistance and reduces off-target effects	95
	4′-Thio-RNA	Increases thermal and plasma stability	96–98
	2′-O-methyl ribosyl substitution at position 2 in the guide strand	Reduces silencing of most off-target transcripts with complementarity to the seed region of the siRNA guide strand	99
	DNA-RNA chimeras	Reduces off-target effects	100
Backbone modifications	Phosphodiester or phosphorothioate linkages	More stable during prolonged incubations in serum	101
Architecture modifications	Synthetic small hairpin RNAs	High stability	102
	Asymmetric interfering RNA (aiRNA)	Reduces off-target effects	103, 104

5.42.5.2.2 Off-Target Effects

After optimization of the stability of siRNA before entering the cell and reaching the target sites, how to make gene silencing more effective becomes another major problem. Off-target effect is that siRNA inhibits not only the expression of desired genes but also undesired genes. It may lead to unpredictable genetic changes. Several studies show that off-target gene regulation is a result of degradation of mRNA with partial identity to the "seed region" of the siRNA sequence, the position 2–8 nucleotides from 5′ end of the guide strand.[66,105] Bioinformatic studies have shown that the "seed region" is usually found in 3′-UTR, which is suggested as a miRNA-like mechanism.[106]

There are two strategies to solve the off-target effects mentioned above due to the "seed region". The most straightforward way is to ensure that the sequence complementary to the "seed region" is unique to the target gene. But this possibility exists only at the theoretical level. As the other option, many studies have shown that off-target effect can be weakened by chemical modification of the "seed region" (Table 1).

5.42.5.2.3 Immune Responses

Because of the double-stranded structure, siRNAs are recognized as "foreign" by the innate immune system. Although siRNAs are more tolerated than long dsRNAs, in some cases it can also trigger immune responses.

In the past two decades, many studies on siRNA-induced interferon response have been reported. Although the induction of IFN is usually beneficial to the body, uncontrolled induction of innate defense mechanism can be harmful. It has been demonstrated that some but not all siRNA duplexes can induce potent IFN-mediated activations by upregulating Toll-like receptors in a cell type-specific manner and even make cells die.[107–111] Some studies have systematically analyzed these siRNAs capable of inducing interferon or not. For example, Hornung and his groupmates have identified that a specific sequence, that the nine bases at the 3′ end of the sense strand (5′-GUCCUUCAA-3′), seemed to be more related to the immunological activity.[108]

Therefore, avoiding the RNAi-induced immune responses is a necessary condition for using RNAi for therapeutics. Some strategies can be used to avoid immune stimulation, including avoiding offending sequences during siRNA design, inactivating offending sequences by chemical modification and using siRNA delivery strategies. The first strategy is still not feasible because not all stimulatory motifs have been identified. However, the next one has been confirmed by many studies. In mammalian cells, more than 25% nucleotides are modified, such as 2-thiouracil (s2U), 5-methylcytidine (m5C), N^6-methyladenosine (m6A), N^7-methylguanosine (m7G), inosine, pseudouridine and many 2′-O-methylated nucleosides, thus the capacity of activating dendritic cells and TLR-expressing cells are suppressed.[112–114]

5.42.5.3 Resolutions

5.42.5.3.1 Delivery

A good small RNA can only play a silent role if it can be accurately transported to the "destination". Therefore, the ideal delivery vector should be stable, effective, safe and specific. A stable and effective carrier can ensure that small RNAs are transported in cells or tissues for a long period of time without being degraded before reaching the target site. The safe vector will not bring about other side effects to the organism while exerting gene silencing. And an ideal vector needs to be able to distinguish target genes from normal genes to prevent other abnormalities in the body during treatment.

From the animal studies so far, viral and nonviral vectors, local and systemic administrations have all been used to achieve efficacy. They all have advantages and disadvantages.

Viral approaches vs. nonviral approaches: Viral approaches provide excellent tissue-specific tropism and transduction efficiency that only single administration could lead to durable down-modulation of targets and suitable for both dividing cells and non-dividing cells. Nonviral vectors are also efficient and lower doses needed than naked small RNAs. They can help to form complexes with polyanionic nucleic acids and interact with negatively charged cell membranes due to the cationic nature, safer than viral vectors, simplicity of use and possibility of large-scale production.

Viral approaches can induce mutations and trigger immunogenic and inflammatory responses, cost highly for production, are impossible to fully estimate drug exposure with regarding to both amount and timing, and are limited in loading capacities. Whereas nonviral approaches are less efficient in gene silencing than viral vectors because of the limited ability to reach and cross the nuclear membrane. Lipids and polymers can be cytotoxic so that limit the use for delivery. Some proteins and antibodies may influence normal functions and produce side effects by their own biological activity.[115–119]

Local administration vs. systemic administration: There are no clear advantages and disadvantages of these two administrations. Which one to be chosen is entirely based on the requirements. The principle of choosing these two methods is the dosage needed for the target sites and the possible effects of drugs on non-target sites. Usually, the doses of small RNAs needed for local administration is lower than that for systemic administration. Since local administration only applies small RNAs into or near the target tissues, while systemic one circulates small RNAs around the whole body. Besides, the possible effects of exposure drugs on nonspecific tissues also should be considered. That is to say, local administration might prevent undesirable effects from systemic administration.[118]

5.42.5.3.2 Chemical Modification

There are many kinds of modification for RNA, and usually they can be divided into three major categories: modifications to overhangs and termini and conjugate groups (also can be considered as a kind of delivery method), modifications within the oligonucleotides, and architecture modification. A specialized database (http://crdd.osdd.net/servers/sirnamod/) is developed for chemical

modification of siRNAs.[120] Chemical modification can optimize a variety of therapeutics problems, such as increasing silencing potency, improving nuclease resistance, enhancing half-life, inhibiting immunostimulation, reducing off-target effects, etc. However, when optimizing one of these problems, it can also add to other problems. So, you should consider more when you decide which kind of chemical modification you want to use.

5.42.6 RNAi Therapeutics and Agriculture

5.42.6.1 RNAi Therapeutics

After more than 10 years of clinical development, RNAi therapeutics begins to realize its promises in multiple disease areas including genetic mutations, viral infections, cancers and many other. siRNA drugs fill the need for therapies to manipulate genes implicated in diseases, including previously undruggable gene products. However, the bottleneck of clinical application of RNAi still exist that is the lack of effective siRNA delivery method in vivo, though effective approaches are available for delivering siRNAs into cell lines. In the early days of RNAi development, it has been shown that siRNAs can be systemically administrated into experimental animals by hydrodynamic intravascular injection, cholesterol conjugation, liposome, cell-penetrating peptide and various types of nanoparticles. However, some of the approaches are not practical or not safe for applications in human. And the efficiencies of those early approaches were typically low. Thus, a large number of siRNAs (typically tens of milligrams per kg) are required, which could make siRNA medicines prohibitively expensive. Large doses of siRNAs are more likely to cause undesired side-effects including immune activations. Enabling efficient delivery of siRNAs into specific tissues and across the cell membrane is a hotly pursued area of research. siRNA delivery technologies are also rapidly evolving. Many siRNA drugs are already in advanced clinical trials. Recent advances in the manufacture of lipid nanoparticles (LNPs) and the use of specific chemical conjugations have revitalized the clinical development of siRNA drugs.

5.42.6.1.1 Lipid Nanoparticle siRNA Drugs

A lipid nanoparticle is typically spherical with the size of around 80 nm in diameter which is much smaller than the commercial cationic liposome-based transfection reagent (several hundred nm). Besides the advantage of size, the feature of containing cationic lipid which is to bind to negatively charged siRNA also makes lipid nanoparticles better than other delivery vectors. In particular, Alnylam and Tekmira, two leading RNAi therapeutic companies, have developed new generations of LNPs that are exceedingly efficient for delivering siRNAs into liver cells by intravenous administration.

The most advanced clinical program of siRNA drug is patisiran (ALN-TTR02) from Alnylam. Patisiran is designed to treat Transthyretin (TTR)-mediated amyloidosis which is an inherited disease caused by genetic mutations in the TTR gene. Patients carrying the mutated TTR gene produce misfolded TTR proteins in the liver, which can aggregate to form amyloid fibrils in tissues such as the peripheral nerves and heart. The resulting disease is polyneuropathy or cardiomyopathy which is progressively debilitating, and often fatal. Patisiran, composed of synthetic siRNAs targeting TTR gene and delivered by LNPs, led to knockdown of serum TTR protein levels of up to 96% in a Phase II clinical trial. Patisiran is the first FDA approved siRNA drug in the market in 2018.

Another important area for siRNA therapeutics is viral infection which traditionally lacks effective treatment options and has huge impact on public health. The current pandemic of Ebola virus in West Africa and newly discovered coronavirus in Middle East highlighted the urgent needs for effective anti-viral treatment for highly infectious and emerging viruses. For Ebola virus, a siRNA drug, TKM-Ebola, is the most advanced Ebola therapy in clinical development (in Phase I trial). TKM-Ebola is a mixture of three siRNAs targeting three Ebola genes (delivered by LNPs) and has shown 100% protection of monkeys from deadly Ebola infections. siRNA drugs are also in development for treating chronic hepatitis B virus (HBV) infection, a disease prevalent in Chinese populations. An siRNA drug, ARC-520, developed by Arrowhead Research, which contains hepatocyte-tropic cholesterol-conjugated siRNAs targeting HBV coagulation factor VII and is delivered by Dynamic Poly Conjugate technology, has shown promising result in a Phase I clinical trial and has just entered a Phase II clinical trial.

For cancer therapy, siRNAs offer a quick way to design gene targeted and personalized therapy based on the rich knowledge in cancer biology and oncogenes. One of the key clinical targets is polo-like kinase 1 (PLK1), a protein involved in cell cycle regulation and often overexpressed in tumor cells. *PLK1* is a well-validated pro-oncogene in multiple cancers including colon and lung cancer cells. TKM-PLK1, an LNP formulated siRNA therapy from Tekmira, is in early clinical trials for adrenocortical carcinoma, gastrointestinal neuroendocrine tumors and hepatocellular carcinoma. Another notable clinical program is ALN-VSP from Alnylam which is composed of LNPs containing two different siRNAs targeting vascular endothelial growth factor A (VEGF-A; for inhibiting tumor angiogenesis) and kinesin spindle protein (KSP; for inhibiting tumor mitosis). In a Phase I clinical trial for ALN-VSP, promising results showed that siRNAs were delivered into the tumor and siRNA-mediated mRNA cleavage of VEGF-A and regression of liver metastases were observed in some patients.

5.42.6.1.2 N-Acetylgalactosamine Conjugation siRNA Drugs

It was also shown that a version of three galactose molecules (GalNAc) linked to the 3′ end of siRNAs, which enables the binding of siRNAs to ASGPR receptors on the surface of hepatocytes, can direct siRNAs go into livers by subcutaneous administration. GalNAc is an amino sugar derivative of galactose. The affinity between ASGPR and GalNAc is greater than galactose. With subcutaneous administration, siRNA-GalNAc conjugates inhibit expression of target mRNA in liver. The ligands derived from GalNAc are compatible with solid-phase oligonucleotide synthesis and deprotection conditions, with synthesis yields comparable to those of standard

oligonucleotides. The optimally modified siRNA-GalNAc conjugates are hepatotropic and long-acting and have the potential to treat a wide range of diseases involving liver-expressed genes.[121]

Both LNP and GalNAc approaches were shown to be safe for human use in clinical trials. In summary, siRNA drugs can now be used to target diseases with liver associations. Once the delivery barriers to tissues outside the liver are removed, siRNAs will have a broad range of therapeutic applications. This makes the future of RNAi therapeutics much brighter.

5.42.6.2 RNAi in Agriculture

RNAi in the agricultural industry has become an exciting area of commercial development. RNAi was mainly used as a tool for creating genetically modified (GM) plants.[122] The modifications can offer either improvement of agronomic traits; increase of survivability against biotic or abiotic stress; or development of resistance to certain damage caused by pathogens, insects or parasites.

5.42.6.2.1 RNAi Improve Agronomic Traits

How to create GM crops with superior agronomic traits is the trending topic in agriculture development. Using RNAi approach to improve nutritional values and grain yield in different crops is promising. Cui's team showed hairpin RNA-mediated gene silencing of oleate desaturase can generate a high oleic peanut line. Nutrition level of peanut oil is conventionally qualified by the proportion of oleic and linoleic acids. Oleate desaturase (FAD2) gene is the negative regulator to high oleic acid production. Using Agrobacterium-mediated transformation, FAD2 gene was successfully suppressed and a significant increase of oleic acid content was observed in transgenic peanut line.[123] Similarly in wheat, Morell's group found starch-branching enzyme encoding genes SBEIIa and SBEIIb are related to the production of amylose content. They had successfully created a transgenic wheat line by using RNAi technique to regulate the expression of SBEIIa and SBEIIb. The transgenic wheat obtains a high level of amylose production and various health benefits.[124] For the grain yield improvement, researchers had shown that RNAi-mediated transgenic rice line for qSW5 has an increased seed weight.[125] Moreover, a semi-dwarf transgenic rice line generated by RNAi suppression of target GA 20-oxidase (OsGA20ox2) gene from original taller rice, has shown increased number of seeds per panicle and larger panicle size.[126]

5.42.6.2.2 RNAi in Drought Tolerance

Drought has a crucial impact on the yield and quality of agriculture production. Combined with the physiological understanding of plants, RNAi technique was applied to help develop tolerance against drought stress in different studies. Huang's group found using AtHPR1 promoter driving a RNAi construct can effectively down regulate farnesyltransferase in canola. This modification offered a decent yield protection of canola under drought stress.[127] In addition, similar effect was obtained in rice. Receptor of C-kinase 1 is the negative regulator of stomatal closure related to water loss. Liang's team has successfully created a transgenic rice line whose RACK1 gene was suppressed by RNAi. Their transgenic rice line showed a great tolerance against drought stress.[128] Another team used virus-induce gene silence method to suppress the expression of proteinase APRO2 gene and transcription factor JMJC gene in peanuts. The GM peanut was proven to has a secured production yield and better-quality under water deficit stress.[129]

5.42.6.2.3 RNAi in Insect Pests Resistance

RNAi is helpful for the control of insect pests. Various studies showed that different insects could take up exogenous dsRNA. Wong's team designed an oral delivered sequence specific dsRNA experiment which can induce RNAi activity and selectively kill target species.[130] Oral administration of dsRNAs targeted 3′UTR of the gamma-tubulin gene can selectively kill *Drosophila*. Price and Gatehouse used *in-planta* produced dsRNAs as feeding component and successfully silenced different target genes in several pest species, Lepidoptera and Coleoptera.[131] The experiment has demonstrated the potential of developing insect-resistant plant and crop lines. Nevertheless, different studies had identified western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte is a special coleopteran specie which can ingest dsRNA diets and trigger robust RNAi activity within their bodies. Robert's group showed transgenic corn plants engineered to produce WCR dsRNAs could induce a significant reduction in WCR feeding damage in a growth chamber assay.[132] *In-planta* expressed WCR dsRNA can successfully inhibit the growth of WCR and prevent the damage on transgenic corns. Another study has identified a cytochrome P450 gene (CYP6AE14) is highly correlated with the growth of cotton bollworm (*Helicoverpa armigera*). Larvae fed with plant diet modified to express specific dsRNA against CYP6AE14, showed a retarded growth.[133] Followed the lead of these promising findings, further studies on how to control dsRNA transmission between host plant and insects, how to make feeding silencing effects inheritable etc. are emerging.

5.42.6.2.4 RNAi in Parasites Control

Parasitic weed is a major cause of poor crop production in agriculture. RNAi signals transmit through host crop to parasites was examined as a potential method to control parasitic weeds. Yoder's group transformed an interfering hairpin RNA-targeted GUS gene into lettuce. Then they used hemi-parasitic plant *Triphysaria versicolor* which expressed GUS reporter gene to parasitize the root of either transgenic or non-transgenic lettuce. After GUS staining, *Triphysaria* roots attached to transgenic lettuce showed respectfully lower GUS activity and transcript quantification confirmed a significant reduction in GUS mRNA level.[134] This result suggested sequence specific silencing signal can be successfully transmitted through host plant to parasitic plant and induce further suppression of target genes within parasites. Following this idea, Radi and his colleagues investigated RNAi transitions between tomato and parasitic *Orobanche*.[135] *Orobanche* spp. *(broomrape)* are parasitic plants subsisted through the attachment to the root of host plant. They can cause severe damage to various crops, including tomato. During the growth of *Orobanche*, strong mannitol

accumulation was observed and mannose 6-phosphate reductase (M6PR) is the essential enzyme related to mannitol synthesize activities. Radi's team produced a transgenic tomato line stable bearing M6PR-siRNAs against *Orobanche aegyptiaca's* M6PR-mRNA. The RT-PCR quantification showed the level of endogenous M6PR mRNA in *Orobanche aegyptiaca* grown on transgenic tomato was significantly reduced by 60%–80%.

5.42.6.2.5 RNAi Improving Disease Resistance

Current studies showed that RNAi modification targeted either genes involved in pathogenicity, genes essential for pathogen's survival and metabolism, or genes associated to pathogen's resistance to plant immune and toxicity response, can help develop resistance against pathogens and diseases in plants. During the past decade, researchers have demonstrated that fatty acids metabolism plays important roles in pathogenicity and related immune response. Takatsuji's group used RNAi method to silence OsSSI2, an essential gene responsible for desaturase activity of fatty acid.[136] The genetically modified Arabidopsis and soybean they produced showed resistance to multiple pathogens infection. Yet a following study focused on the fatty acid gene family members, OsFAD7 and OsFAD8 was conducted. With the suppression of OsFAD7 and OsFAD8 genes, an enhanced resistance against *Magnaporthe grisea* was observed in the GM rice line.[137] Other than metabolism related investigations, Fritz's team found that in Arabidopsis, flagellin can trigger specific miRNA expression and downregulate the Toll and immune deficiency pathways, which resulted in a significantly enhance of innate immune defense then further develop into resistance to the infection of different bacterial and fungal.[138] In tobaccos, studies have demonstrated RNAi mediated silencing of glutathione S-transferase can enhance immune response and develop the resistance to black shank disease.[139]

5.42.6.2.6 siRNA Sprays in Agriculture

Very recently agribiotech companies begin to test the feasibility of applying synthetic siRNAs directly to crops to achieve desired agricultural traits without having to make genetic modifications. The idea is to create "siRNA sprays", similar to chemical sprays, which can be directly applied on the surface of plants. The siRNA spray approach has huge advantages over the GM approach because it is very easy to choose different siRNA sequences to target various crop genes or pathogens, eliminating the lengthy process of breeding GM crops.[140] In addition, siRNAs work temporally and do not alter the crop genomes, which help to alleviate public sentiments toward GM crops for causing permanent environmental impacts.[141]

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