Current Status for Application of RNA Interference Technology as Nucleic Acid Drug

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Abstract

RNA interference (RNAi) is a convenient and useful gene suppression technology induced by small interfering RNA (siRNA) composed of 21-nucleotide long double-stranded RNA. The successful application of RNAi for clinical use is expected for a long time. Although siRNA drug is categorized into a nucleic acid drug, it has a prominent advantage that genetic function can be suppressed by destroying mRNA at the posttranscriptional level without wounding genomic DNA. Nevertheless, unfortunately there are no siRNA certified as pharmaceuticals passing through clinical trials, since there are several problems, such as gene suppression efficiency, stability in blood stream, or other undesirable effects. Here, we describe the current status and future prospects for clinical application of the siRNA nucleic acid drug.

Keywords: RNA interference, siRNA, off-target effect, thermodynamic property, chemical modification

1. Introduction

In recent years, nucleic acid drugs have attracted attention as a next-generation medicine following low molecular weight drugs and antibody drugs. Research and development of these drugs for clinical application is advanced in major pharmaceutical companies, bio-ventures, or research institutions including universities. Nucleic acid drugs, such as DNA/RNA or their modified molecules, act directly on molecules causing diseases and regulate their functions by administering chemically synthesized nucleic acid to the body by local administration or subcutaneous injection. Unlike the hitherto known gene therapy, the nucleic acid drugs directly act on the target molecules and relieve symptoms of the diseases without manipulating the genomes. Although the effects of various nucleic acid drugs, including antisense RNA, small
interfering RNA (siRNA), aptamer, or decoy, are investigated in the clinical trials, only five examples including four antisense oligos and an aptamer are already approved. However, no siRNA drug is certified so far. In this manuscript, we outline the advantages, current status, and problems to be solved in the development of nucleic acid drugs, in particular, focusing on the development of siRNA drug.

2. RNA interference

RNA interference (RNAi) is a highly regulated, evolutionarily conserved mechanism of post-transcriptional gene regulation. siRNA, consists of double-stranded RNA with 19 nucleotides in length with 2 nucleotides overhangs, is the intermediate utilized in this mechanism [1].

![Figure 1. Mechanism of RNA interference. siRNA is the approximately 21-nt double-stranded RNA composed of the guide strand RNA and the passenger strand RNA. siRNA is incorporated into a protein complex called RISC and unwound into single-stranded RNAs. After unwinding, the guide strand is remained on the AGO protein, while the passenger strand is degraded. The mRNAs only with partial complementarities with the seed region of siRNA guide strand are off-target genes and are repressed by siRNA as a mechanism known to be “off-target effect.” the mRNA, which has a complete complementary sequence with the guide strand RNA, is cleaved by “RNAi” as an actual target.](image-url)
When siRNA is introduced into the cells, it is loaded onto the Argonaute (AGO) protein, which is a component of a protein complex called RNA-induced silencing complex (RISC) [2, 3]. In the RISC, siRNA is unwound into single-stranded RNAs, and a functional RNA strand called the guide strand is remained in the RISC and the opposite passenger strand is degraded [4–8]. The activated guide strand RNA-containing RISC binds to the target transcript in a sequence-specific manner. The perfectly complementary target transcript is then cleaved between the 10th and 11th nucleotide relative to the 5’ end of the guide strand [2]. Thus, gene functions of mRNAs which have the complementary nucleotide sequences of the guide strand are suppressed (Figure 1) [9]. This elegant, endogenous process has been extensively utilized in functional genomics studies and shows potential as a therapeutic platform [10]. However, although the clinical application of siRNA is expected for a long time, it has not been put into practical use due to some essential problems. For example, the difficult delivery system of siRNA to the target tissues: siRNA is easily degraded by RNA degradation enzymes when it is introduced into the blood, and it is extremely difficult to deliver siRNA to specific tissues. The other severe problem is off-target effects on messenger RNAs (mRNAs) other than the target gene (Figure 1). The siRNA often suppresses unintended mRNAs with partial complementarities in nucleotide sequences of the guide strand. The procedures to overcome such problems have been really expected. There is a new candidate method that has overcome the readily degradable property of RNA by enclosing them in lipid nanoparticles (LNP). Although its clinical trial has reached to phase III, there is no example approved as a formal pharmaceutical so far.

3. Advantages for using RNAi technology for nucleic acid medicine

siRNA with complementary nucleotide sequence to the mRNA of target gene can be designed conveniently, if the nucleotide sequence of the target gene is known. In addition, since siRNA can be chemically synthesized, it is not necessary to undergo complicated manufacturing processes, such as immunization of animals or cell culture like synthesis of antibody drugs. Furthermore, due to the complementary binding of siRNA to the target mRNA, its specificity has been considered to be very high, and siRNA can target molecules, such as mRNAs or other noncoding RNAs at the posttranscriptional level that could not be regulated by traditional drugs. These excellent characteristics are reasons to be expected as the future medicine.

4. Problems to be solved for application of siRNA to nucleic acid medicine

Although clinical trials of siRNA application are performed for a long time, there are no authorized siRNA as a clinical drug until now. The current status of the development in RNAi technology and the major problems for its clinical application are discussed in the following section.
Figure 2. Sequence design algorithms for siRNAs with high RNAi effects. Three widely used siRNA sequence design rules; the Ui-Tei method (A), the Reynolds method (B), and the Amarzguioui method (C). Upper RNA strand indicates the passenger strand, and lower strand indicates the guide strand. The number under the guide strand indicates the nucleotide position measured from the 5′ end of the guide strand. Detailed algorithms are shown in main text.
4.1. Sequence design of highly functional siRNA

The siRNA design is known to define the RNAi efficiency essentially because its effectiveness is dependent on the base-pairing between siRNA and target mRNA. Basic studies so far revealed that the highly efficient RNAi can be induced by almost all siRNAs with any sequences in flies or nematodes, whereas RNAi efficiencies varied greatly depending on the sequences of siRNAs in mammals including human [11]. Among nucleic acid medicines, the nucleotide sequence of aptamer, which specifically acts on a target molecule successfully, is selected by an enormous screening experiment using an artificial nucleic acid library called systematic evolution of ligands by the exponential enrichment (SELEX) method. Such screening requires a great deal of labor and cost. However, the nucleotide sequences of functional siRNAs are designed systematically according to a few reliable algorithms, such as the Ui-Tei rule [11], Reynolds rule [12], or Amarzguioui rule [13] (Figure 2). The relationship between siRNA sequence and RNAi was determined by experimental analyses using 62 targets for 4 exogenous and 2 endogenous genes in mammalian cells [11], or 180 siRNAs targeting mRNAs of 2 genes [12], or by the statistical analysis of 49 siRNAs verified by 34 siRNAs [13]. The algorithm of each strategy for the selection of functional siRNA is as follows:

1. Ui-Tei rule
   i. Nucleotide at the 5′ end of the guide strand is A or U.
   ii. Nucleotide at the 5′ end of the passenger strand is preferably G or C.
   iii. A and U are abundant in the region corresponding to the 5′ terminal one-third of the guide strand.
   iv. It is better not to include a long GC stretch over the entire region.

2. Reynolds rule
   i. The content of G and C is 30–5% over the entire region.
   ii. At least three out of five nucleotides at the 3′ end of the passenger strand are preferably A or U.
   iii. Possible inverted repeats that form hairpin structures are absent.
   iv. The third nucleotide from the 5′ end of the passenger strand is A.
   v. The 10th nucleotide from the 5′ end of the passenger strand is U.
   vi. The 13th nucleotide from the 5′ end of the passenger strand is other than G.
   vii. The 19th nucleotide from the 5′ end of the passenger strand is preferably A (other than G or C).

3. Amarzguioui rule
   i. The nucleotide at the 5′ end of the passenger strand is G or C (other than U).
   ii. A or U is preferable at the 5′ end of the guide strand.
iii. The sixth nucleotide from the 5′ end of the passenger strand is A.

iv. The AU content of three nucleotides at the 5′ end of the passenger strand is relatively lower than that of the passenger strand.

The siRNAs with high RNai effects can be conveniently designed using these three siRNA design algorithms. The application of these algorithms incorporating each of all criteria is shown to improve potent siRNA selection. However, among them, siRNAs designed by the Ui-Tei rule can suppress the target genes with the highest probability of 95% or more. About 15% of all siRNAs complementary for human transcripts satisfy this algorithm, and such siRNA can be designed using siDirect 2.0 (Figure 3), which is an open access website [14, 15].

4.2. Avoid the adverse suppression effects on genes other than the target

The siRNA exerts its RNAi effect by binding to the target mRNA via complete complementarity of nucleotide sequence (Figure 1). However, its suppression effects are often observed on the genes, other than the target gene, with nucleotide sequences partially complementary to the siRNA. Such phenomenon is known to be “off-target effects,” which is an unintended adverse effect of RNAi [16–19]. Especially, the region called “seed” positioned at nucleotides 2–8 from the 5′ terminus of the guide strand contributed to the off-target effects [18, 19] (Figure 4), since the seed region loaded on the Argonaute protein in quasi-helical structure and stably form base-pairings with the off-target mRNAs. To avoid such adverse effects, it is desirable to design siRNAs that do not interact with off-target mRNAs other than the target mRNA as much as possible. However, since the off-target effect by siRNA is induced by sequence complementarity of only seven nucleotides sequence in the seed region, it is impossible to select siRNA sequence with no seven nucleotides complementarities with off-target genes. However, such off-target effect was not always observed. It was revealed that the seed-dependent off-target effect is induced only when the base-pairing stability between the guide strand seed region and the off-target mRNA is strong (Figure 5). It means that the off-target effect is avoidable when the stability in the seed-target duplex is weak [19, 20]. The melting temperature of RNA–RNA duplex calculated by the nearest-neighbor procedure may be a useful parameter for evaluating the RNA–RNA duplex stability (Figure 5).

Thus, the sequence design of siRNA with high specificity for a target gene without off-target effect on the untargeted genes comes to be possible. First, such siRNA satisfies the functional siRNA selection rules, such as Ui-Tei rules. Second, it is preferable to have a lower thermodynamic stability of base-pairing between the siRNA seed region and the target mRNA. The siRNAs simultaneously satisfying these two conditions are considered to show high RNai effect and reduced off-target effect. However, only 3% of siRNAs, satisfying both conditions simultaneously, is selectable in human. This is relevant to the fact that it is impossible to select any siRNA sequences for about 5000 genes, one quarter of all human genes. Thus, this limitation is inevitable for sequence design of human siRNA [15].

4.3. Reduction of off-target effects using chemical modifications

The off-target effect has been found to depend on the thermodynamic stability of the seed-target duplex, and such thermodynamic property is basically determined according to the nucleotide
sequence. However, the base-pairing stability can be modified by introducing chemical modifications into siRNA. The chemical modifications, including DNA and unlocked nucleic acid (UNA), are known to decrease thermodynamic stabilities. In fact, introduction of DNA molecules into siRNA seed region successfully reduced off-target effects [21, 22], since the DNA-RNA duplex in the seed region shows weak base-pairing stability compared to RNA-RNA duplex.

Figure 3. Highly efficient and target-specific siRNA design website: siDirect. (A) the first screen shot. “1” indicates the box to enter the accession number of target gene. When click “2,” you can get nucleotide sequence from GenBank. However, you can directly paste the nucleotide sequence (<10 kbp) in “3.” for design siRNA, click “4.” (B) the second screen “a” indicates the target positions. “B” indicates siRNA target sequences with links to the off-target lists. “C” indicates the functional siRNA design algorithms used for selection of the indicated siRNA. “D” shows homology search results against mRNAs. Numbers of hits with complete match (0), one mismatch, two mismatches, or three mismatches are shown. The number 1 in the 0(+) column usually indicates a complete match against intended target mRNA. “E” shows calculated tm to the siRNA seed region. An siRNA with lower seed tm value reduces off-target effects. “F” is a graphical view of designed siRNA. Off-target lists can be seen by click each siRNA. “G” indicates tab-delimited siRNA list. You can copy and paste the result into excel or text editors, etc. (C) List of off-target candidates for individual siRNA. “6” is siRNA information. “7” shows the alignment between each off-target candidate and each siRNA sequence, clarifying the mismatch positions. (D) Design strategy of guide and passenger strand RNA oligonucleotides based on siDirect result.
In contract, the thermodynamic stability of RNA duplex with 2′-O-methyl (2′-OMe) or LNA is known to increase in the protein-free condition. However, we showed that these modifications also reduced the off-target effects [23]. The chemical modification often changes the nucleotide conformation in addition to thermodynamic stability. In RNAi, the guide strand RNA is preloaded on the AGO protein via seed region and form duplex with target mRNA. The 2′-OMe modification in all of the seven nucleotides of siRNA seed region does not disturb the preloading on the AGO protein, but the base-pairing formation with complementary RNA on the AGO protein is apparently disturbed by steric hindrance. As a result, siRNA with 2′-OMe in the guide strand seed region shows weak off-target effect without reduction of RNAi effect [23]. LNA showed more strong effect in the interaction between siRNA and AGO protein or off-target mRNAs. The siRNA modified with LNAs in all of the seven nucleotides in the seed region cannot preload on AGO protein and it cannot base-pair with the complementary RNA. However, siRNA with LNA modifications in three nucleotides among seven

Figure 4. Quantitative analysis of off-target mRNAs by microarray. (A) all of the expressed mRNAs were divided into 15 groups by the region of complementarities with siRNA. (B) Cumulative frequency curve of changes in the expression level of each gene group. When curve shifts to the left, the expression levels of these genes are suppressed. A gene group having a sequence complementary to the siRNA seed region (positions 2–8) in their 3′ UTRs was suppressed.
can preload on the AGO protein, but it cannot base-pair with the complementary RNA. Thus, siRNA with seven LNA modifications in the seed region has neither RNAi effect nor off-target effect, but siRNA with three LNA modifications shows weak off-target effect without reduction of RNAi effect [23]. Thus, since the chemical modifications can regulate the binding capability of siRNA to the target mRNA depending on the thermodynamic and structural characteristics, the proper application of chemical modifications may be a useful strategy for selection of highly effective and off-target effect-reduced RNAi.

4.4. Increasing stability in blood and efficient transport to the target tissues or cells of siRNA

Nucleic acids are degraded by nucleolytic enzymes when they are released into blood. Fomivirsen is an oligonucleotide used as an antisense antiviral drug that was applied to the treatment of cytomegalovirus retinitis in patients with acquired immunodeficiency syndrome (AIDS). Pegaptanib is a pegylated antivascular endothelial growth factor (VEGF) aptamer and used as an antiangiogenic medicine for the treatment of neovascular age-related macular degeneration (AMD). These nucleic acid drugs are treated by local administration into vitreous bodies. Mipomersen is the second antisense drug used for the treatment of homozygous familial hypercholesterolemia and is administered through subcutaneous injection. The various chemical modifications were introduced into these nucleic acid drugs to increase the stability in the blood. Fomivirsen is involved in the first generation of antisense therapeutics.

Figure 5. Correlation between thermodynamic stability (Tm value) in the seed-target duplex and off-target effect. siRNAs with low seed tm values in the seed region show low off-target effects, while siRNAs with high seed tm values showed high off-target effects.
containing phosphorothioate linkages between bases to prevent nuclease digestion. Five 2′-OMe modifications were introduced into mipomersen to allow the resistance to degradation by endonucleases and exonucleases maintaining high affinity and specificity to the target mRNA. Pegaptanib has both modifications of phosphorothioate and 2′-OMe. Incorporation of them greatly improved the aptamer half-life in urine and also improved binding affinity for VEGF. Since it has also reported that siRNAs with chemical modifications, such as 2′-OMe and 2′-fluoro (2′-F), are resistant to degradation by RNA degrading enzymes [24, 25], it is possible to use proper chemical modifications for each siRNA to enhance its stability in blood.

Furthermore, since the nucleic acid has anionic charge, the permeability of the cell membrane is low. Then, the development of drug delivery system (DDS) to transport nucleic acid drugs to target tissues or cells stably using lipid or collagen are also on going. Many trials are performed using lipid nanoparticle (LNP) technology in which pharmacokinetics are indicated to be much better than a naked RNAi approach. Several types of nanoparticles, including LNP, N-acetylgalactosamine (GalNAc) conjugates and dynamic polyconjugated (DPCs), are used clinically. The most successful DDS so far is lipid nanoparticle (LNP) developed by Tekmira. LNP, which forms a lipid bilayer membrane similar to the cell membrane, protects siRNA by encapsulation and assists the transport of siRNA to the target tissues. Alnylam has developed a therapeutic agent (ALN-TTR 02) for familial amyloid polyneuropathy by using this LNP, which is currently conducting phase III of clinical trials. In addition, the method for conjugation of atelocollagen and siRNA has also been developed. Atelocollagen makes siRNA less susceptible to degradation by RNase enzymes, which results in a long-lasting RNA silencing effect.

4.5. Suppression of excessive immune response induced by introduction of exogenous nucleic acid

In general, nucleic acid medicine introduces a large amount of artificially synthesized nucleic acids into the body. However, the excessive autoimmune response is often induced by the exogenous introduction of nucleic acids. In mammals, it is well-known that the activation of virus sensor proteins, like Toll-like receptors (TLRs) [26, 27] and RIG-I-like receptors (RLRs) [28], or Protein kinase R (PKR) [29] induces interferon response. Interferon response may upregulate the production of inflammatory cytokines and can possibly cause systemic inflammation. It has been reported that LNA- or 2′-OMe-modified siRNA suppresses the activation of TLR 7/8 and does not induce the excessive immune response without reducing RNAi activity [30, 31]. It was also reported that induction of interferon response was not observed even when a complex of atelocollagen and siRNA was introduced into the cells [32]. By revealing the detailed molecular mechanism of the immune response by introduction of the exogenous nucleic acids, more appropriate chemical modifications to avoid such immune response should be developed.

5. Conclusion

RNAi is a field in which its clinical application is strongly expected, but its first wave for clinical application failed due to the difficulties in the initial stage. However, due to the improvement
of its delivery system and the sequence selection method, the new wave has come in 2012. Current clinical trials are applied to targets, including the eye, liver, cancer, blood, gastrointestinal tract, dermis, and others, since it is relatively easy to deliver siRNA to these tissues with currently available nanoparticles [33].

Antibody drugs and low molecular weight drugs were widely used so far. They were synthesized through complicated manufacturing processes, so that mass production at low cost was impossible. However, the nucleic acid drugs can be artificially synthesized by a simple manufacturing process. Then, once a production line is established, it can be synthesized inexpensively in large quantities. In addition, for antibody drugs and low molecular weight drugs, basic data from scratch are required every time when the target changes, but nucleic acid drugs such as siRNA drugs can be designed relatively easily by identifying the nucleotide sequences of target genes. Thus, it is expected that the siRNA is a powerful candidate for nucleic acid therapeutics.

Genome editing techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins 9 (Cas9) system [34] and transcription activator-like effector nucleases (TALEN) [35] can directly modify the genomic DNA that causes disease. Although it is extremely attractive as a potential drug candidate in the future, there are many ethical issues to overcome when the edited genome is transmitted to the next generation. On the other hand, siRNA does not introduce the permanent modification in the genomic DNA and its action is transient, since siRNA knockdown the target mRNA. Then, CRISPR interfering (CRISPRi) system modified from the CRISPR/Cas9 system is developed. CRISPRi inhibit the gene expression without cleavage of genomic DNA by introducing mutations into DNA cleavage domains in Cas9 [36]. Such system may also promote the nucleic acid medicine.

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References

[1] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001;411:494-498

[2] Hutvagner G, Simard MJ. Argonaute proteins: Key players in RNA silencing. Nature Reviews. Molecular Cell Biology. 2008;9:22-32

[3] Doi N, Zenno S, Ueda R, Ohki-Hamazaki H, Ui-Tei K, Saigo K. Short-interfering-RNA-mediated gene silencing in mammalian cells requires dicer and eIF2C translation initiation factors. Current Biology. 2003;13:41-46

[4] Matranga C, Tomori Y, Shin C, Bartel DP, Zamore PS. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. 2005;123:607-620

[5] Miyoshi K, Tsukumo H, Nagami T, Siomi H, Siomi MC. Slicer function of Drosophila Argonautes and its involvement in RISC formation. Genes & Development. 2005;19:2837-2848

[6] Leuschner PJ, Ameres SL, Kueng S, Martinez J. Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Reports. 2006;7:314-320

[7] Kawamata T, Seitz H, Tomari Y. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nature Structural & Molecular Biology. 2009;16:953-960

[8] Yoda M, Kawamata T, Paroo Z, Ye X, Iwasaki S, Liu Q, Tomari Y. ATP-dependent human RISC assembly pathways. Nature Structural & Molecular Biology 2009;17:117-123

[9] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391:806-811

[10] Ryther RCC, Flynt AS, Phillips JA, Patton JG. siRNA therapeutics: Big potential from small RNAs. Gene Therapy. 2004;12:5-11

[11] Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Research. 2004;32:936-948

[12] Reynolds A, Leake D, Boese Q, Scringe S, Marchall WS, Khvorova A. Rational siRNA design for RNA interference. Nature Biotechnology. 2004;22:326-330

[13] Amarzguioui M, Prydz H. An algorithm for selection of functional siRNA sequences. Biochemical and Biophysical Research Communications. 2004;316:1050-1058

[14] Naito Y, Yamada T, Ui-Tei K, Morishita S, Saigo K. siDirect: Highly effective, target-specific siRNA design software for mammalian RNA interference. Nucleic Acids Research. 2004;32:W124-W129
[15] Naito Y, Yoshimura J, Morishita S, Ui-Tei K. siDirect 2.0: Updated software for designing functional siRNA with reduced seed-dependent off-target effect. BMC Bioinformatics. 2009;10:392. DOI: 10.1186/1471-2105-10-392

[16] Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, Fesik SW, Shen Y. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Research. 2005;33:4527-4535

[17] Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E, Santoyo-Lopez J, Duncan DJ, Long A, Kelleher D, Smith Q, Beijersbergen RL, Ghazal P, Sharmu CE. 3'UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nature Methods. 2006;3:199-204

[18] Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. Widespread siRNA “off-target” transcript silencing by seed region sequence complementarity. RNA. 2006;12:1179-1187

[19] Ui-Tei K, Naito Y, Nishi K, Juni A, Saigo K. Thermodynamic stability and Watson-crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effects. Nucleic Acids Research. 2008;36:7100-7109. DOI: 10.1093/nar/gkn902

[20] Ui-Tei K, Nishi K, Takahashi T, Nagasawa T. Thermodynamic control of small RNA-mediated gene silencing. Frontiers in Genetics. 2012;3:101. DOI: 10.3389/fgene.2012.00101

[21] Ui-Tei K, Naito Y, Zennno S, Nishi K, Yamato K, Takahashi F, Juni A, Saigo K. Functional dissection of siRNA sequence by systematic DNA substitution: Modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. Nucleic Acids Research. 2008;36:2136-2151. DOI: 10.1093/nar/gkn042

[22] Takahashi T, Zennno S, Ishibashi O, Takizawa T, Saigo K, Ui-Tei K. Interaction between the non-seed region of siRNA and RNA-binding RLC/RISC proteins, ago and TRBP, in mammalian cells. Nucleic Acids Research. 2014;42:5256-5269. DOI: 10.1093/nar/gku153

[23] Iribe H, Miyamoto K, Takahashi T, Kobayashi Y, Leo J, Aida M, Ui-Tei K. Chemical modification of the siRNA seed region suppresses off-target effects by steric hindrance to base-pairing with targets. ACS Omega. 2017;2:2055-2064. DOI: 10.1021/acsomega.7b00291

[24] Sproat BS, Lamond AI, Beijer B, NEuner P, Ryder U. Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives: Novel probes that are resistant to degradation by RNA or DNA specific nucleases. Nucleic Acids Research. 1989;17:3373-3386

[25] Peiken WA, Olsen DB, BEnseler F, Aurup H, Eckstein F. Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. Science. 1991;253:314-317

[26] Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/toll/cactus controls the potent antifungal response in drosophila adults. Cell. 1996;86:973
[27] Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the drosophila toll protein signals activation of adaptive immunity. Nature 1997;388:394-197

[28] Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature Immunology. 2004;5:730-737

[29] Meurs E, Chong K, Galabru J, Thomas NS, Kerr IM, Williams BR, Hovanessian AG. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell. 1990;62:379-390

[30] Dahlgren C, Wahlestedt C, Thonberg H. No induction of anti-viral responses in human cell lines HeLa and MCF-7 when transfecting with siRNA or siLNA. Biochemical and Biophysical Research Communications. 2006;341:1211-1217

[31] Judge AD, Bola G, Lee AC, MacLachlan I. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vitro. Molecular Therapy. 2006;13:494-505

[32] Takeshita F, Minakuchi Y, Nagahara S, Honma K, Sasaki H, Hirai K, Teratani T, Namatame N, Yamamoto Y, Hanai K, Kato T, Sano A, Ochiya T. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2005;102:12177-12182

[33] Bobbin ML, Rossi JJ. RNA interference (RNAi)-based therapeutics: Delivering on the promise? Annual Review of Pharmacology and Toxicology. 2016;56:103-122

[34] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337:816-821. DOI: 10.1126/science.1225829

[35] Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics. 2010;186:757-761. DOI: 10.1534/genetics.110.120717

[36] Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stem-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013;154:442-451. DOI: 10.1016/j.cell.2013.06.044