Chapter

The Natural Antisense Transcript-Targeted Regulation Technology Using Sense Oligonucleotides and Its Application

Mikio Nishizawa, Tetsuya Okuyama and Richi Nakatake

Abstract

Natural antisense transcripts (NATs or AS transcripts) are frequently transcribed from many eukaryotic genes and post-transcriptionally regulate gene expression. The AS transcript is classified as noncoding RNA and acts as a regulatory RNA in concert with RNA-binding proteins that bind to cis-controlling elements on the mRNA, microRNAs, and drugs. The AS transcript that overlaps with mRNA regulates mRNA stability by interacting with mRNA, and the network of mRNAs, AS transcripts, microRNAs, and RNA-binding proteins finely tunes the output of gene regulation, i.e., mRNA levels. We found that single-stranded 'sense' oligonucleotides corresponding to an mRNA sequence decreased the mRNA levels by interfering with the mRNA-AS transcript interactions of several genes, such as inducible nitric oxide synthase (*iNOS*) and interferon-alpha1 (*IFN-A1*) genes. In contrast, AntagoNAT oligonucleotides, which are complementary to AS transcripts, are sense oligonucleotides when they overlap with mRNA, but they increase the levels of specific mRNAs. Collectively, the sense oligonucleotide is a powerful tool for decreasing or increasing mRNA levels. The natural antisense transcript-targeted regulation (NATRE) technology using sense oligonucleotides is a method with a unique modality for modulating cytosolic mRNA levels and may be used to treat human diseases in which AS transcripts are involved.

Keywords: antisense transcript, noncoding RNA, microRNA, mRNA stability, sense oligonucleotide, locked nucleic acid

1. Introduction

The transcripts whose sequences are complementary to those of mRNA have been reported in many genes regardless of species, from bacteria to mammals. Because protein is encoded by mRNA, whose sequence is the same as the sense strand of a gene, i.e., double-stranded DNA, the transcript has been called a natural antisense transcript (NAT or AS transcript) [1, 2]. The AS transcripts do not code for proteins or only short peptides and are classified as one class of noncoding RNA (ncRNA). Accumulating genome-wide transcriptome analyses have demonstrated that natural

antisense transcripts are transcribed from many eukaryotic genes [3]. HUGO proposed the nomenclature of human gene symbols for natural antisense transcripts *AS* (suffix) [4]. In this chapter, we use 'AS transcript' as a natural antisense transcript.

In contrast, classical types of ncRNA species, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA), are well known and have definite functions in gene expression. These classical ncRNAs do not overlap mRNAs. Among other ncRNA species, microRNA (miRNA or miR), which is 20–23 nucleotides (nt) in the length, was found in nematodes and mammals. This very short ncRNA species, which is complementary to the 3′-untranslated region (3′UTR) of several mRNAs, inhibits translation and induces mRNA degradation [5]. Therefore, microRNA hybridizes with mRNA to regulate its functions.

Furthermore, long ncRNAs (lncRNAs), which are more than 200 nt long [6], were found. At first, their functions were unclear, but it has gradually been revealed that these lncRNAs are involved in gene expression [7]. To date, huge number of lncRNA sequences have been reported by RNA-seq analysis and deposited in public databases, such as LNCipedia 5 (human lncRNA transcripts) [8]. Nowadays, ncRNA species, including miRNA, and lncRNAs, are known as *regulatory RNAs* [9].

Many studies have demonstrated that the AS transcript, one class of lncRNA, is involved in various steps during gene expression [7]. When focusing on the AS transcript that overlaps with an mRNA, this type of AS transcript interacts with mRNA and plays an important role in gene expression, especially at post-transcriptional levels [1, 2]. Interestingly, most AS transcripts are transcribed at low levels [1, 2]. The analyses and application of AS transcripts are summarized by the reviews, for example, see [1, 2, 10].

During our functional analyses of AS transcripts, we found the mRNA-AS transcript interactions that regulate mRNA stability (described later). Although conventional methods, i.e., antisense and short interference RNA (siRNA) technologies [11, 12] were available for the analyses of AS transcript functions [13], we first used synthetic sense oligonucleotides that are complementary to the AS transcript. We found that sense oligonucleotides resulted in decreases in cytoplasmic mRNA levels, which may be applied to 'knockdown of mRNA.'

Here, our method to regulate mRNA levels based on the mRNA-AS transcript interactions is described, and the application of this technology to treat disease is discussed.

2. Natural antisense transcripts that overlap with mRNAs

2.1 Structures of natural antisense transcripts

The AS transcript is frequently transcribed from inducible genes [14]. Our previous studies showed that an AS transcript harbors an overlapping sequence with 3′UTR of an mRNA [14]. Such a 3′UTR possesses a few AU-rich elements (AREs), which may be involved in mRNA stability because AREs may be the targets of miRNAs and RNA-binding proteins [15, 16]. Interestingly, the location of AREs in the 3′UTR of *iNOS* mRNA is conserved among species (rat, mouse, and human) [17, 18].

The sizes of AS transcripts are variable and show a smear pattern or discrete bands in Northern blot analysis. The former example is AS transcripts that are transcribed from rat inducible nitric oxide synthase (*iNOS*, *NOS2*) gene, and the size is ranging from 600 to 1000 nt (**Figure 1**) [19]. iNOS catalyzes the production of the inflammatory mediator nitric oxide (NO). iNOS is induced by various inflammatory stimuli; interleukin (IL)-1beta to hepatocytes and bacterial lipopolysaccharide (LPS) to macrophages [17, 18].

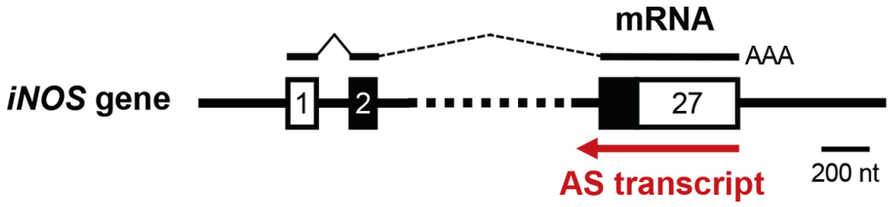

Figure 1.
mRNA and AS transcripts transcribed from the inducible nitric oxide synthase (iNOS) gene. The rat iNOS gene is schematically depicted. The iNOS gene consists of 27 exons, and the AS transcript overlaps with the exon 27, which includes the 3′UTR (white box) of the mRNA. The iNOS mRNA harbors AREs in its 3′UTR. The iNOS AS transcript starts at the end of exon 27 and stops at various sites, resulting in various sizes of the transcripts, which do not harbor a poly(A) tail. Figure reproduced with modification from [19] with permission.

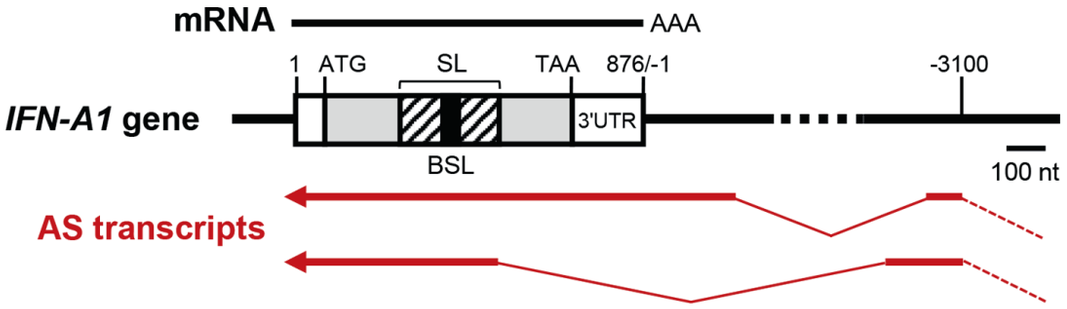

Figure 2.
mRNA and AS transcripts transcribed from interferon alpha1 (IFN-A1) gene. The human IFN-A1 gene consists of a single exon, which encodes IFN-alpha1 [20]. The coding sequence (from ATG to TAA) gives conserved stem-loop structures in the IFN-A1 mRNA, i.e., stem-loops (SL) and bulged-stem loop (BSL) [21]. These structures are responsible for chromosome region maintenance 1 (CRM1)-dependent nuclear export of IFN-A1 mRNA [21]. The IFN-A1 mRNA harbors AREs in its 3′UTR. Two AS transcripts are shown, both of which are spliced and harbor poly(A) tails. Nucleotides are numbered from the transcription initiation site. Figure reproduced with modification from [20] with permission.

The latter examples are about 4-kilobase (kb) AS transcript that is transcribed from the human interferon-alpha1 (*IFN-A1*) gene (**Figure 2**) [20]. *IFN-A1* gene encodes the cytokine IFN-alpha1 and is one subset of human *IFN-A* genes, which consist of 13 subsets [22]. Viral infection induces IFN-alpha1, a member of the type I interferon family, which is a main innate immunity response. AS transcripts are also transcribed from other subtypes of *IFN-A* genes [23].

Another example is the AS transcript from the rat tumor necrosis factor-alpha (*Tnf*) gene, which is about 2.5-kb long [24]. AS transcripts are transcribed from many genes that are involved in inflammation, such as mRNAs that encode alpha subunit (p19) of IL-23 (IL-23A), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X_3-C) motif ligand 1 (CX3CL1), p65 and p50 subunits of nuclear factor kappaB (NF-kappaB) [14]. Additionally, AS transcript is transcribed from the human gene encoding ephrin type A receptor 2 (EPHA2), which is a receptor tyrosine kinase whose over-expression is observed in various cancers [25, 26].

2.2 mRNA-AS transcript interactions and mRNA stability

2.2.1 iNOS mRNA-AS transcript interaction

When the AS transcript overlaps with the relevant mRNA, the interaction between the AS transcript and mRNA is expected. Indeed, the AS transcript is transcribed from the rat *iNOS* gene and interacts with and stabilizes the *iNOS* mRNA [19].

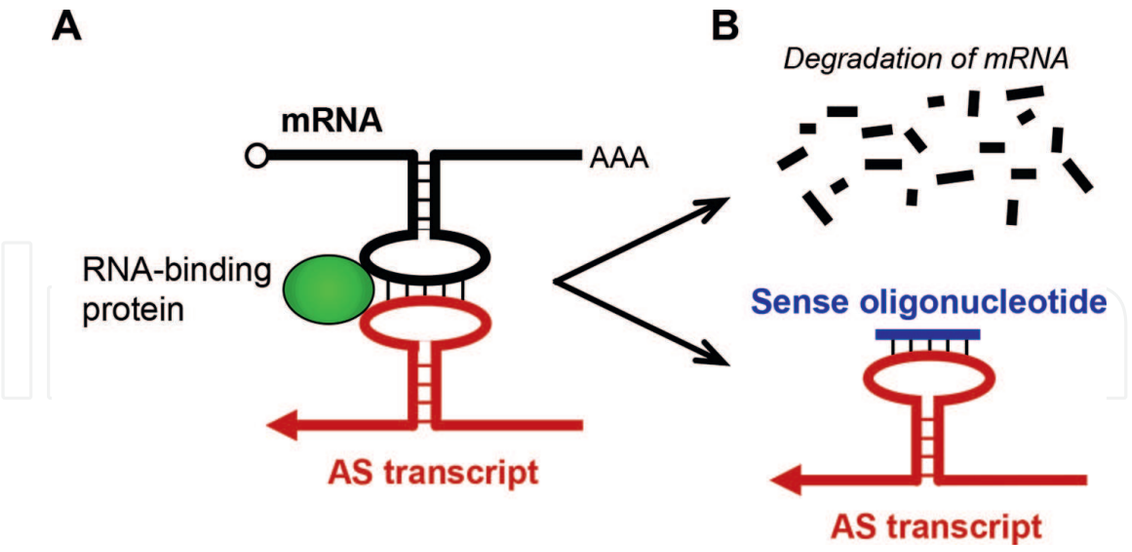

Figure 3.
A putative mechanism of the mRNA-AS transcript interaction and mRNA degradation by the introduction of a sense oligonucleotide (iNOS gene). (A) Stable complex on the iNOS mRNA. When the iNOS AS transcript is expressed, it partially hybridizes with a single-stranded loop in the iNOS mRNA that harbors a cap structure (open circle) and a poly(A) tail. By recruiting an RNA-binding protein, it forms an mRNA-AS transcript-protein complex to stabilize the iNOS mRNA in the cytoplasm. (B) Interference with the mRNA-AS transcript interaction. Because a sense oligonucleotide to the iNOS mRNA harbors the same sequence as the mRNA, it competitively inhibits the hybridization of iNOS mRNA with the iNOS AS transcript, leading to interference with the mRNA-AS transcript interaction and then the mRNA degradation. This mechanism confers to the basis of the NATRE technology.

Further analyses demonstrated that the *iNOS* AS transcript interacts with the *iNOS* mRNA at the single-stranded loop or bulge of the overlapping region between the mRNA and AS transcript (**Figure 3**) [19].

Because the orientation of RNA is 5′-to-3′, base complementarity indicates that the secondary structure of AS transcript is a mirror image of that of mRNA. This means that stem-loop structures in the AS transcript are formed at the complementary sites in the corresponding mRNA, leading to loop-loop hybridization between the mRNA and AS transcript [1, 2]. This loop-loop hybridization forms a short RNA:RNA duplex (usually <10 base pairs), which is thermodynamically unstable due to the low melting temperature of the duplex. Then, RNA-binding proteins (e.g., HuR) bind to the *iNOS* mRNA and/or AS transcript to stabilize the mRNA-AS transcript-protein complex, which protects from the degradation of RNAs and facilitates translation in the cell [19].

2.2.2 INF-A1 mRNA-AS transcript interaction

As another putative mechanism, the human *IFN-A1* AS transcript interacts with and stabilizes *IFN-A1* mRNA by blocking the microRNA binding to *IFN-A1* mRNA [20]. *IFN-A1* AS transcript is expressed at a low level. After the AS transcript transiently interacts with *IFN-A1* mRNA, it moves on and targets the next mRNA molecule in a 'hit-and-run' fashion [1, 20].

Sense oligonucleotides to BSL of *IFN-A1* mRNA (see **Figure 2**) decreased *IFN-A1* mRNA levels. Because a potent binding site of a microRNA (miR-1270) is present in BSL, miR-1270 may bind to BSL of *IFN-A1* mRNA. A microRNA-binding site is also called a microRNA-responsive element (MRE). Next, a short AS oligoribonucleotide (asORN), which is the part of *IFN-A1* AS transcript corresponding to BSL, was

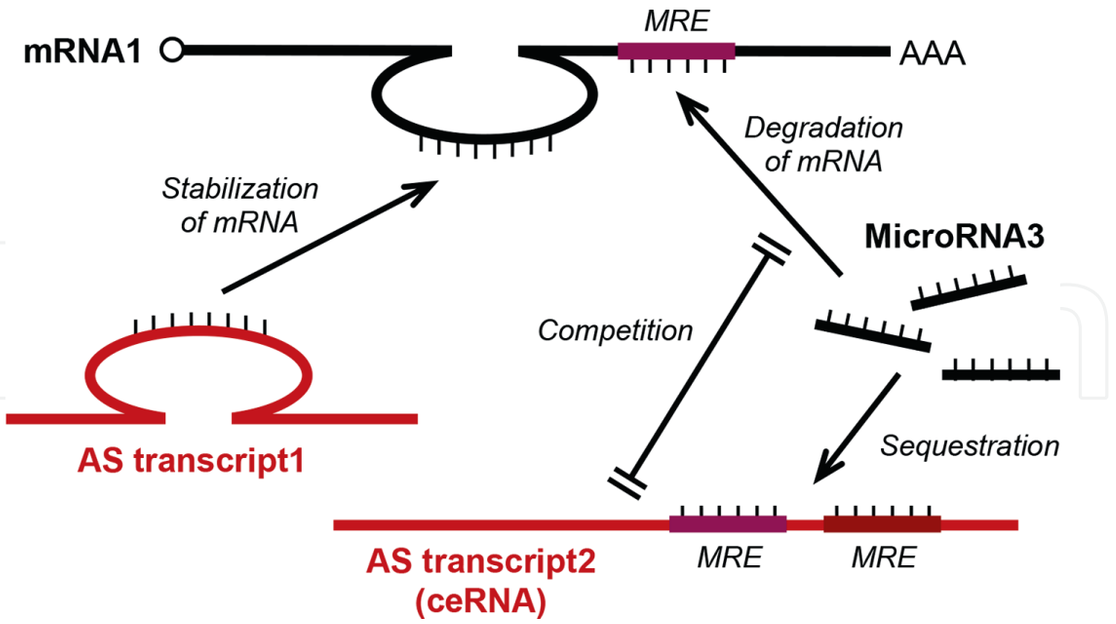

Figure 4.
A model of interactions among transcripts to regulate mRNA levels. mRNA1 has a site of interaction (loop) with AS transcript1 and an MRE for microRNA3. AS transcript1 stabilizes mRNA1, whereas microRNA destroys mRNA1 through its MRE. AS transcript2 transcribed from another gene has common MREs. AS transcript2 sponges microRNA3, resulting in the sequestration of microRNA3. Therefore, AS transcript2 competes with microRNA3 and functions as a ceRNA. A typical example is seen among IFN-A1 mRNA and AS transcripts from the specific subsets of IFN-A gene family. See details in the text.

introduced into the cells (This is not NATRE technology.). *IFN-A1* asORN increased *IFN-A1* mRNA levels, but it did not alter *IFN-A1* AS transcript levels [20]. These data imply that *IFN-A1* asORN stabilizes *IFN-A1* mRNA by simulating the AS transcript.

When the levels of transcripts were measured, miR-1270 was much more excess to *IFN-A1* AS transcript [23]. Although *IFN-A1* AS transcripts include several MREs, they are stoichiometrically unable to sponge all miR-1270 molecules. Further study indicated another mechanism.

When microRNAs are shared by mRNAs and AS transcripts, the transcripts function as competing endogenous RNAs (ceRNAs). Several AS transcripts are transcribed from the specific subsets of *IFN-A* gene family (*IFN-A1*, *A7*, *A8*, *A10*, and *A14* genes) and harbor common MREs for miR-1270 [23]. MiR-1270 can bind to both mRNA and AS transcript from *IFN-A* gene, as well as mRNAs from the specific subsets of *IFN-A* gene family (*IFN-A8*, *A10*, *A14*, and *A17* genes). Because the mRNAs and AS transcripts share MREs for miR-1270, they sponge and sequester the miR-1270 molecules. Collectively, *IFN-A* mRNAs and AS transcripts from *IFN-A* gene family form a ceRNA network to antagonize miR-1270 [23, 27]. This network finely tunes *IFN-A1* mRNA levels by common MREs that are present in the mRNAs and AS transcripts at the post-transcriptional level. Possible interactions among transcripts are depicted in **Figure 4**. See detailed discussion in [28].

2.2.3 Tnf mRNA-AS transcript interaction

Different from the *iNOS* and *IFN-A1* mRNA cases, AS transcripts sometimes downregulate gene expression. The stability of *Tnf* mRNA is modulated by RNA-binding proteins that bind to the AREs in its 3′UTR: human homolog R of embryonic lethal-abnormal visual protein (HuR), which stabilizes the mRNA; and tristetraprolin

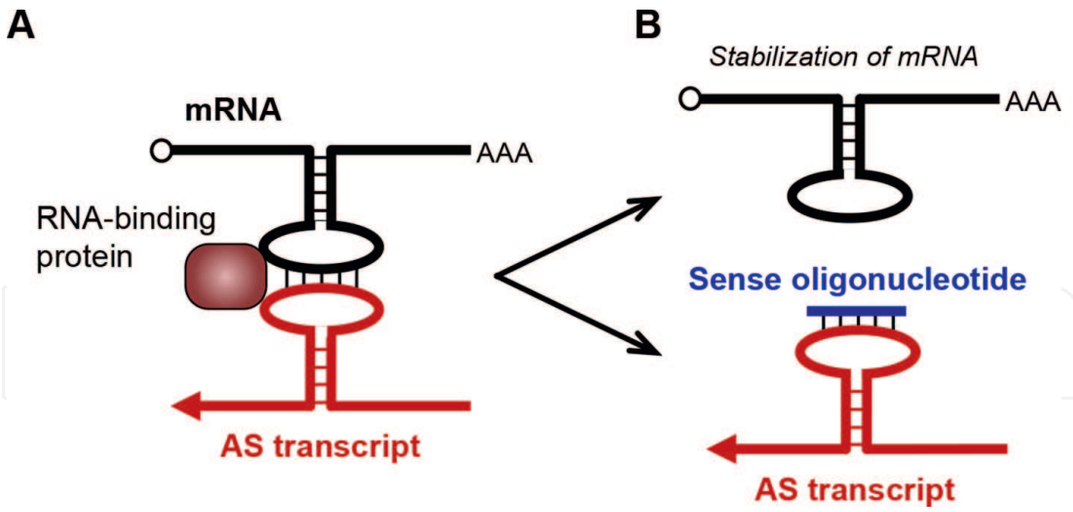

Figure 5.
A putative mechanism of the increase in mRNA levels by the introduction of a sense oligonucleotide (Tnf gene). (A) The Tnf mRNA that forms with a destabilizing RNA-binding protein. The Tnf AS transcript partially hybridizes with a single-stranded loop in the Tnf mRNA. An RNA-binding protein forms an mRNA-AS transcript-protein complex. (B) Interference with the mRNA-AS transcript interaction. A sense oligonucleotide to the Tnf mRNA competitively inhibits the hybridization of Tnf mRNA with the Tnf AS transcript, releasing a destabilizing RNA-binding protein. Finally, the Tnf mRNA becomes stable in the cytoplasm.

(TTP), which destabilizes the mRNA [24]. A putative mechanism of mRNA destabilization is schematically shown in **Figure 5** and discussed in [1, 2]. Possible involvement of microRNA in the *Tnf* mRNA-AS transcript interaction is also mentioned [24].

Other than these mechanisms, there are several AS transcript-mediated mechanisms that regulate gene expression [7]. For example, AS transcripts may epigenetically repress transcription at the chromatin level.

3. Natural antisense transcript-targeted regulation technology

3.1 Natural antisense transcript-targeted regulation technology using sense oligonucleotides

If the mRNA-AS transcript interactions are inhibited, it is speculated that an mRNA-AS transcript-protein complex is not formed and that the mRNA becomes unstable [1, 2]. According to this hypothesis, we used single-stranded *sense* oligonucleotides to block the mRNA-AS transcript interactions at a post-transcriptional level. The sense oligonucleotide harbors the same sequence as that of the relevant mRNA.

We first applied the sense oligonucleotide corresponding to the *iNOS* mRNA sequence to decrease the *iNOS* mRNA levels in hepatocytes [19]. In a search of the literature to date, there are not any other reports on applying sense oligonucleotides to knockdown AS transcript(s) and finally mRNA. After refinement of this method and confirmation of its versatility, we established this method as a natural antisense transcript-targeted regulation (NATRE) technology using sense oligonucleotides [14, 18]. The introduction of sense oligonucleotides may regulate the mRNA levels of AS transcript-expressing genes. Similarly, the sense oligonucleotides to other mRNAs reduced the levels of *IFN-A1* and other mRNAs [14, 20]. Therefore, the introduction of sense oligonucleotides may be used as loss-of-function experiments of AS transcripts.

In the absence of an AS transcript, a sense oligonucleotide cannot hybridize with the relevant mRNA and does not affect mRNA stability. Therefore, the presence of AS transcript and mRNA-AS transcript interactions are essential for the NATRE technology using sense oligonucleotides.

3.2 Design of sense oligonucleotides

3.2.1 Prediction of secondary structure

A sense oligonucleotide should harbor an overlapping sequence of an mRNA-AS transcript interaction, i.e., a single-stranded loop or bulge [18, 20]. The single-stranded loops are the potential sites of mRNA-AS transcript interactions. To seek the single-stranded regions of an mRNA where the relevant AS transcript interacts with, secondary structures of mRNA (especially, 3′UTR) were predicted using the mfold program [29]. Other prediction programs can be used. Regions conserved among predicted mRNA (especially 3′UTR) structures are selected, and candidates of several sense oligonucleotides are designed from the stem-loop regions.

Generally, it is unnecessary to predict the secondary structures of AS transcripts. As mentioned above, the overlapping sequence of an AS transcript is complementary to that of the corresponding mRNA. Therefore, the secondary structures of the AS transcript are a mirror image of the mRNA. The stems and loops of the AS transcript are formed at the same positions as the mRNA.

3.2.2 Design of sense oligonucleotides

The sense oligonucleotide consists of either synthetic oligodeoxyribonucleotide (DNA) or synthetic oligoribonucleotides (RNA) with modifications of the oligonucleotide backbone, sugars, bases, and the 5′-phosphate (described later). The sequences of sense oligonucleotides (about 20 nt long in our cases) designed according to the mRNA sequence included at least one single-stranded loop in the conserved region [14, 19]. From the sequences of sense oligonucleotides, the elements that may provoke innate immunity responses through Toll-like receptors (TLR3, 7, 8, and 9) should be eliminated, such as GU-rich motifs (e.g., 5′-GUGU-3′), CG, GGG, GGGG, and CCCC [14, 19, 30]. It is possible that some oligonucleotides show off-target effects, even after the exclusion of these motifs. To attain the specificity of a target mRNA and avoid off-target effects, homology search in the DDBJ/EMBL/GenBank databases should be performed. Trials and errors are necessary to select effective sense oligonucleotides among several candidates.

Note that not all the candidate sense oligonucleotides reduce the levels of specific mRNA species, whereas some oligonucleotides increase the mRNA levels. As above-mentioned, AS transcripts modulate the expression of each gene either positively or negatively; the AS transcript stabilizes mRNA (**Figures 3** and **4**) or destabilizes mRNA (**Figure 5**).

Changes in mRNA levels also depend on the region of the mRNA-AS transcript interactions [14, 24]. Six sense oligonucleotides to the 3′UTR of the rat *Tnf* mRNA were designed to the regions whose secondary structures were conserved [24]. Among them, only one sense oligonucleotide decreased *Tnf* mRNA levels, four increased, and one did not alter. RNA-binding proteins, e.g., HuR and TTP, may change the effect of each sense oligonucleotide.

Additionally, both several RNA-binding proteins and microRNAs control the mRNA stability (see Section 2.2). Therefore, it is difficult to predict whether knockdown of AS transcript using a sense oligonucleotide causes either an increase or decrease in mRNA levels (see also Section 3.3).

3.2.3 Negative controls of sense oligonucleotides

Several types of oligonucleotides are frequently used as negative controls. A negative control that is suitable for your experiments should be selected because not all the negative controls work well in the experiments.

3.2.3.1 Sense oligonucleotides to stems

A sense oligonucleotide to stem regions is used as a negative control. A stem region consists of double-stranded RNA:RNA hybrid and is not involved in the interactions with an AS transcript [20].

3.2.3.2 Mismatch oligonucleotides or mutated oligonucleotides

Mismatch oligonucleotides are designed by introducing mutated bases at the site of mRNA-AS transcript interactions [20, 31].

3.2.3.3 Random oligonucleotides

Random oligonucleotides (20 nt) harbor random sequences, i.e., 5′-N_{20}-3′ (N = A, C, G, or T) with phosphorothioate bonds [18].

3.2.3.4 Scrambled oligonucleotides and mock transfection

A scrambled oligonucleotide is designed by base shuffling without changing the base composition of the relevant sense oligonucleotide [31]. Homology search screened by the BLAST program must eliminate candidates harboring unexpected homology to other mRNAs in the DDBJ/EMBL/GenBank databases.

When a sense oligonucleotide is introduced into cells using a transfection reagent, mock transfection is also necessary as a negative control of transfection. The mock transfection requires a transfection reagent alone, and an oligonucleotide is not introduced to the cells [18, 20].

3.2.4 Modification of sense oligonucleotides

A variety of nucleases are present in the cells and blood, such as exonuclease, endonuclease, and ribonuclease (RNase) H1. RNase activity is very high in various cell lines, as well as blood and cells in many organs, including the liver. To protect sense oligonucleotides from these nucleases, phosphorothioate bonds and modified nucleic acids are commonly introduced to replace the phosphodiester bonds and (deoxy)ribose rings of native nucleotides, respectively [32, 33]. Indeed, *iNOS* sense oligonucleotides without modification did not reduce *iNOS* mRNA levels [18]. Locked nucleic acid (LNA) [34] and 2′-*O*-methyl nucleic acid (OmeNA) are frequently used as modified nucleic acids. In our cases, *iNOS* sense oligonucleotides were substituted with partial phosphorothioate bonds and LNAs or OmeNAs

reduced the levels of *iNOS* mRNA and iNOS protein in hepatocytes [18, 19]. This is a critical point at which modifications are included to obtain effective sense oligonucleotides.

3.2.5 Conjugation of sense oligonucleotides

When modified, but non-conjugated oligonucleotides are introduced in the body, they are transferred to the liver and kidney. To improve *in vivo* delivery to an organ or a tissue, sense oligonucleotides are often conjugated at their ends with cell-penetrating arginine-rich peptides and cell-permeable hydrophobic molecules [32, 33]. Cell-penetrating arginine-rich peptides are derived from the Tat protein of human immunodeficiency virus (HIV)-1 [35] and synthetic arginine oligomer peptides (Arg_6); and cell-permeable hydrophobic molecules are cholesterol [36] and C_{12} spacer.

The conjugation does not affect the potency of *iNOS* oligonucleotides to decrease *iNOS* mRNA expression [18]. When an *iNOS* sense oligonucleotide conjugated to these molecules was introduced into hepatocytes, all conjugated sense oligonucleotides were as effectively decreasing *iNOS* mRNA levels as the non-conjugated sense oligonucleotide [18]. Appropriate conjugation may facilitate the delivery of sense oligonucleotides to target tissues or organs (see Section 3.7).

3.3 Regulation of mRNA levels by sense oligonucleotides in culture cells

Because the modification of sense oligonucleotides is essential, modified sense oligonucleotides were used for the introduction to cells. The *in vitro* effects of NATRE technology (including AntagoNAT technology) on mRNA levels in the cells are summarized in **Table 1**.

mRNA levels*	Gene from which AS transcript transcribed (product)	Technology	Reference
Decrease	*iNOS* (iNOS/NOS2)	NATRE	[18, 19]
	Il23A (IL-23, alpha subunit)	NATRE	[14]
	IFN-A1 (IFN-alpha1)	NATRE	[20]
	EPHA2 (EPHA2)	NATRE	[26]
Increase	*Ccl2* (CCL2)	NATRE	[14]
	Ccl20 (CCL20)	NATRE	[14]
	Cx3xl1 (CX3CL1)	NATRE	[14]
	Cd69 (CD69)	NATRE	[14]
	RelA (NF-kappaB, p65 subunit)	NATRE	[14]
	Tnf (TNF-alpha)	NATRE	[24]
	Bdnf (BDNF)	AntagoNAT	[37]
	Gdnf (GDNF)	AntagoNAT	[37]

** Genes were classified by the main effect of sense oligonucleotides on the mRNA levels.*

Table 1.
Examples of the in vitro effects of NATRE technology on mRNA levels.

3.4 Administration of sense oligonucleotides to animals

When NO is excessively produced by iNOS in hepatocytes and Kupffer cells (resident macrophages) of the liver, it leads to multiple organ failure [38]. Endotoxemia model rats with hepatic failure are often used to evaluate drugs. These model rats are prepared either by intravenous injection of D-galactosamine (GalN) and LPS [38–40], or by LPS injection after partial hepatectomy [38, 41]. These rats resemble the animals suffering from sepsis or septic shock.

After optimization of the sequence and modification of *iNOS* sense oligonucleotides in hepatocytes, the best sense oligonucleotide was administered into the endotoxemia model rats [38]. When the sense oligonucleotide was intravenously injected with GalN and LPS to rats, the survival rate was markedly increased, and apoptosis in the hepatocytes markedly decreased in the sense oligonucleotide-treated rats [38].

Because LNA is efficiently accumulated in the liver [34], the LNA-modified *iNOS* sense oligonucleotide may function in the liver where the *iNOS* gene is highly expressed. Taken together, NATRE technology using *iNOS* sense oligonucleotides may be applicable to treat sepsis and septic shock.

3.5 AntagoNAT technology

To increase mRNA levels by modulating the mRNA-AS transcript interactions, an *AntagoNAT* oligonucleotide has been used, which is an antagonist to an AS transcript (NAT) and defined as a single-stranded antisense oligonucleotide to a specific AS transcript [37]. Each AntagoNAT oligonucleotide contained a mixture of OmeNAs and LNAs.

When an AS transcript overlaps with its corresponding mRNA, the AntagoNAT is identical to a sense oligonucleotide. Therefore, AntagoNAT technique is very close to NATRE technology. Both technologies use sense oligonucleotides to knockdown AS transcript. However, NATRE technology has been applied to decrease mRNA levels, whereas AntagoNAT technology has been applied to increase mRNA levels.

It has been reported that AntagoNAT-mediated knockdown of brain-derived neurotrophic factor (*Bdnf*) and glial-derived neurotrophic factor (*Gdnf*) AS transcripts resulted in increased levels of *Bdnf* and *Gdnf* mRNAs in HEK293T cells [37]. The underlying mechanism may be similar to those indicated in **Figure 5**, or to other mechanisms that were previously mentioned [7].

AntagoNAT oligonucleotides can be administered to animals. When *Bdnf*-AntagoNAT was intracerebroventricularly delivered, the *Bdnf* mRNA levels increased in the mouse brain [37]. Collectively, the *Bdnf*-AntagoNATs functioned *in vitro* and *in vivo*, although it increased the *Bdnf* mRNA levels.

AntagomiR (antagomir), which is a synthetic oligonucleotide complementary to a microRNA, is used to sequester endogenous microRNA [42]. Each antagomir sequence is identical to a specific mRNA and similar to several mRNAs that share microRNA-binding sites (seed sequences). Therefore, antagomirs are another type of sense oligonucleotides. When microRNA is involved in the mRNA-AS transcript interactions, the antagomir technology may be applied to analyze these interactions. See an example in [23].

3.6 Comparison with other methods

The mechanisms of two conventional technologies, i.e., antisense and siRNA technologies [11], are schematically shown (**Figure 6**).

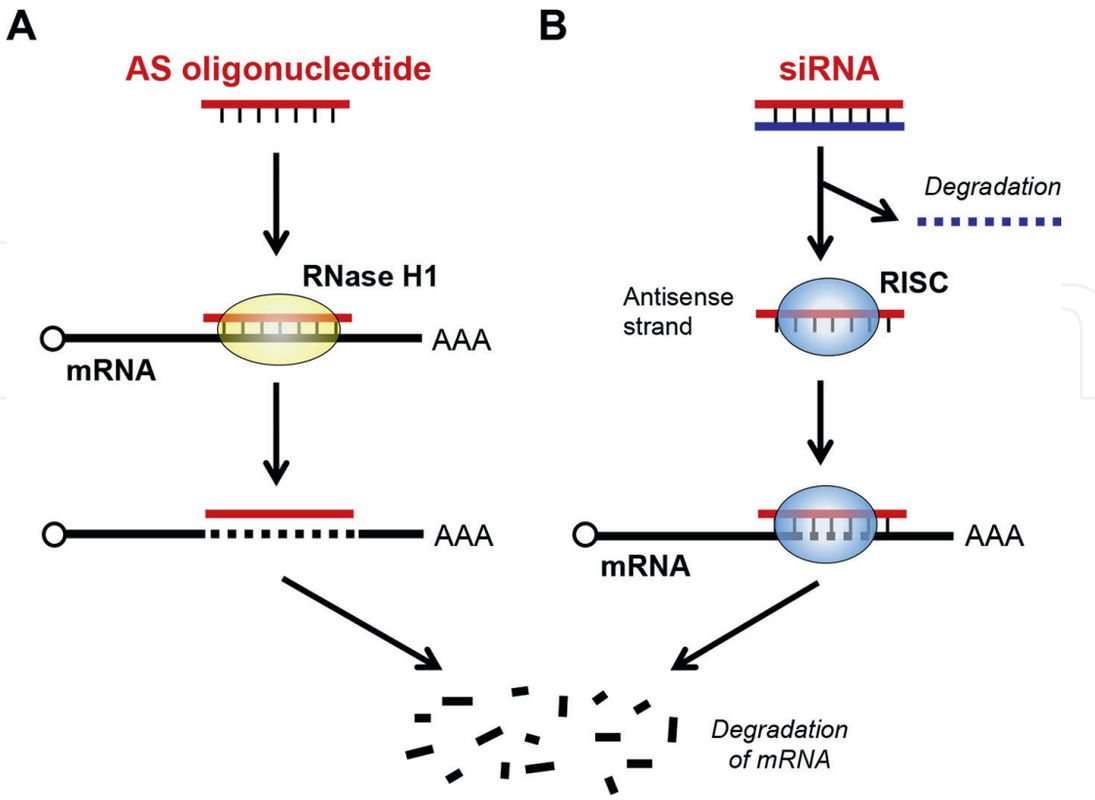

Figure 6.
Mechanisms of conventional mRNA knockdown technologies. (A) Antisense technology. The mRNA that hybridizes with an antisense oligonucleotide is digested by RNase H1. (B) siRNA technology. siRNA recruits Argonaut (Ago) proteins to form RISC, which destroys mRNA. See details in the text.

3.6.1 Antisense technology

A single-stranded antisense oligonucleotide hybridizes with an mRNA and forms a local DNA:RNA hybrid. RNase H1 recognizes DNA:RNA hybrids and selectively digests the RNA strand, leading to the degradation of the mRNA. Therefore, the antisense oligonucleotides should be DNA. The presence of an AS transcript is not essential for this method.

3.6.2 siRNA technology

siRNA is a synthetic double-stranded RNA, and one strand of the siRNA (i.e., guide strand) is complementary to a target mRNA. Typical siRNA consists of 19 base pairs and 2-nt 3′ overhangs. siRNA interacts with Argonaut (Ago) proteins to form RNA-induced silencing complex (RISC) and then binds to the target mRNA. The guide strand of siRNA hybridizes with the mRNA (especially, 3′UTR) in the RISC, resulting in degradation of the target mRNA. The other RNA strand (i.e., passenger strand) is destroyed during the RISC formation. This mechanism mimics mRNA degradation by microRNA.

As mRNA knockdown methods, NATRE technology using sense oligonucleotides is compared with conventional methods, i.e., antisense technology and siRNA technology (**Table 2**). Other than these technologies, there are various oligonucleotide technologies that are applied to therapies of disease.

	NATRE technology	Antisense technology	siRNA technology
Targets	AS transcript (direct) and mRNA (indirect)	mRNAs	mRNAs
Oligonucleotides* (strand)	Single-stranded DNA or RNA (sense)	Single-stranded DNA (antisense)	Double-stranded RNA (both)
Underlying mechanism	mRNA-AS transcript interactions**	None	None
mRNA degradation	RNases and other nucleases	RNase H1	RISC
Disadvantage	Impossible when mRNA-AS transcript interactions are absent	Difficult to optimize the sequence to the relevant mRNA	Difficult when stable secondary structures are present
Examples of human application	Not yet (Successful results in animal experiments)	Mipomersen, casimersen, etc. [11]	Patisiran, givosiran, etc. [11]

**The backbone, sugars, bases, and the 5′-phosphate of the oligonucleotide are modified.*
***MicroRNAs may interfere with these interactions.*

Table 2.
Comparison of the methods to knockdown mRNAs.

3.6.3 RNA aptamers

Aptamers are oligoribonucleotides that form 3D structures and function like proteins, such as ligands and antibodies [42]. For example, pegaptanib, which is an aptamer drug developed for the treatment of macular degeneration, blocks vascular endothelial growth factor (VEGF) by preventing its binding to VEGF receptors [42]. Although there are no reports about the mRNA knockdown using aptamers up to date, the aptamers that simulate RNA-binding proteins may be utilized to modulate mRNA levels by affecting the mRNA-AS transcript interactions.

3.7 Drug delivery system (DDS)

The introduction of oligonucleotides to cells requires transfection reagents using liposomes, e.g., Lipofectamine (Thermo Fisher Scientific Inc., Waltham, MA, USA) and using iron nanoparticles, e.g., MATra A reagent (IBA, Göttingen, Germany) or PolyMag Magnetofection reagent (OZ Biosciences, Marseille, France).

To improve the *in vivo* delivery of sense oligonucleotides, several techniques have been developed [32, 33]. Because LNA is efficiently accumulated in the liver [34], *iNOS* sense oligonucleotides were modified with LNAs [38].

Recently, *N*-acetylgalactosamine (GalNAc) has been conjugated at the ends of the oligonucleotides to efficiently deliver the oligonucleotides to the liver [11, 43]. Because the liver and kidney receive high blood flow and permeability of their capillaries is high, sense oligonucleotides conjugated with GalNAc will improve the delivery and accumulation in these organs. To cross the blood-brain barrier, *Bdnf*-AntagoNATs in liposomes were delivered by nasal approaches [44, 45]. Additionally, aptamers capable of entering the cells may facilitate the delivery of oligonucleotides into the cells [42].

Because guinea pigs maintain a functional *MX1* gene for the IFN-alpha1 pathway, they were infected with influenza virus to verify that the AS transcript-mRNA

regulatory axis exerts *in vivo* control of innate immunity [46]. When an AS oligoribonucleotide (asORN) to guinea pig *IFN-A1* mRNA in poly (D,L-lactide-co-glycolide (PLGA) nanoparticles was pulmonary-administered, it inhibited *in vivo* viral proliferation by modulating *IFN-A1* mRNA levels. Although this experiment is not NATRE technology, PLGA nanoparticles can be used to deliver sense oligonucleotides to animals. Various lipid nanoparticles (LNPs) have been used to deliver nucleic acids, including oligonucleotides, in the body [47]. New-generation LNPs can deliver long RNA, such as LNP-based mRNA vaccines for COVID-19 [47].

4. Perspectives

Administration of oligonucleotides, including NATRE technology, is a unique therapeutic modality. Because the sequence of an oligonucleotide specifies the gene, one mRNA is selectively downregulated or upregulated (**Figure 7**). For example, administration of an *iNOS* sense oligonucleotide to endotoxemia/sepsis model rats showed little effects on endothelial NOS (eNOS) and neuronal NOS (nNOS) (unpublished data). Enzyme inhibitors (e.g., NOS inhibitors) generally have a broader specificity. Furthermore, it is rare that antibodies against oligonucleotides are raised.

NATRE technology is a powerful method to modulate *in vitro* and *in vivo* gene expression. Note that sense oligonucleotides to the mRNAs can be designed to inducible genes and many other genes. iNOS AS transcript was successfully administered to endotoxemia/sepsis model rats and improved their survival rate [38]. As shown in **Table 1**, many genes involved in inflammation are candidates suitable for clinical uses in the future. When a gene that is involved in diseases is selected, sense oligonucleotides to this gene can be easily designed and examined. Instead of sense oligonucleotides, antisense oligoribonucleotides (asORN) may be used to increase mRNA levels. Indeed, *IFN-A1* asORN inhibited the proliferation of Influenza virus in guinea pigs [46].

Sense oligonucleotides may apply to cancer, neurodegenerative disorders, and other diseases. For example, EPHA2 is over-expressed in various cancers, and the *EPHA2*

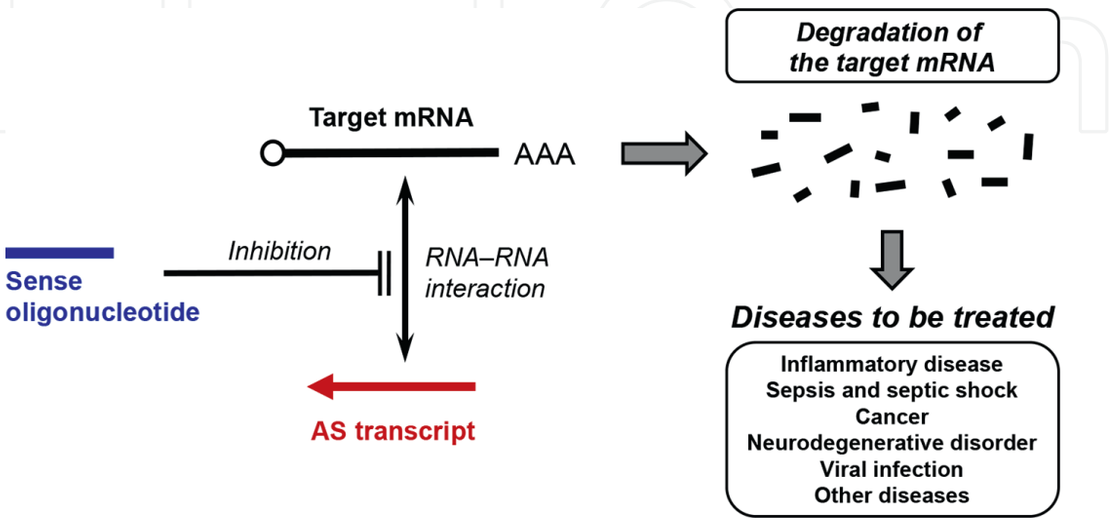

Figure 7.
The NATRE technology using sense oligonucleotides. The principle of the NATRE technology is schematically shown. Potential therapeutic application using this technology is also shown.

AS transcript may be involved in a subtype of breast cancer [26]. It is possible that sense oligonucleotides inhibit cancer progression and proliferation of cancer cells. Furthermore, the administration of *Bdnf*- and *Gdnf*-AntagoNATs to mice [44, 45] may facilitate the regeneration of neurons and glial cells in the central nervous system. When a gene that is involved in diseases is found, sense oligonucleotides to this gene can be designed. Furthermore, sense oligonucleotides can be designed in the genome of a pathogenic virus to inhibit viral multiplication.

Drugs and some constituents in functional foods and crude drugs of Japanese Kampo medicine mimic sense oligonucleotides by modulating mRNA stability [1]. When sodium salicylate reduced *iNOS* mRNA levels in hepatocytes, decreased mRNA stability was speculated [48]. It was reported that acetyl salicylate (aspirin) interacts with RNA by intercalating with the RNA duplex and destabilized the helix, resulting in a conformational change of the stem-loop structure of the RNA [49]. Because the drug-RNA interaction may affect mRNA stability, drugs and constituents may interfere with *iNOS* mRNA-AS transcript interactions like sense oligonucleotides. Indeed, several drugs or constituents in functional foods and crude drugs decreased the levels of both *iNOS* mRNA and AS transcript: dexamethasone (anti-inflammatory drug) [50], cucurbitacin B (triterpenoid in the fruit of *Momordica charantia*) [51], sakuranetin (flavonoid in the bark of *Prunus jamasakura*) [52], and standardized oligomerized-polyphenol from *Litchi chinensis* fruit extract (OPLFE) [53]. The investigation of the drugs and constituents may clarify the mechanism of action of the sense oligonucleotide in future.

5. Conclusion

When AS transcript is transcribed from a gene, NATRE technology can be applied to any gene to down- or up-regulate mRNA levels. NATRE technology using sense oligonucleotides may be useful to specifically inhibit mRNA-AS transcript interactions. Therefore, this method may be applied to many genes and contribute to the treatment of various human diseases in the future.

Acknowledgements

We thank Drs. Tominori Kimura, Tadayoshi Okumura, and Saki Shirako (Ritsumeikan University) for their valuable advice and discussion and Ms. Noriko Kanazawa for her secretarial assistance. This work was supported in part by the Japan Society for the Promotion of Science KAKENHI Grant Nos. 21K08697, 21K09032, and 19KK0413 and by the Asia-Japan Research Institute, Ritsumeikan University (Ibaraki, Osaka, Japan).

Conflict of interest

M.N. is an inventor of a patent describing the use of *iNOS* sense oligonucleotides to regulate *iNOS* mRNA levels. The other authors declare no conflict of interest.

DOI: http://dx.doi.org/10.5772/intechopen.108281

Author details

Mikio Nishizawa[1]*, Tetsuya Okuyama[2] and Richi Nakatake[2]

1 Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan

2 Department of Surgery, Kansai Medical University, Hirakata, Osaka, Japan

*Address all correspondence to: nishizaw@sk.ritsumei.ac.jp

References

[1] Nishizawa M, Okumura T, Ikeya Y, Kimura T. Regulation of inducible gene expression by natural antisense transcripts. Frontiers in Bioscience (Landmark Ed.). 2012;**17**:938-958. DOI: 10.2741/3965

[2] Nishizawa M, Ikeya Y, Okumura T, Kimura T. Post-transcriptional inducible gene regulation by natural antisense RNA. Frontiers in Bioscience (Landmark Ed.). 2015;**20**:1-36. DOI: 10.2741/4297

[3] Kiyosawa H, Mise N, Iwase S, Hayashizaki Y, Abe K. Disclosing hidden transcripts: Mouse natural sense-antisense transcripts tend to be poly(A) negative and nuclear localized. Genome Research. 2005;**15**:463-474. DOI: 10.1101/gr.3155905

[4] Wright MW. A short guide to long non-coding RNA gene nomenclature. Human Genomics. 2014;**8**:7. DOI: 10.1186/1479-7364-8-7

[5] Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;**75**:843-854. DOI: 10.1016/0092-8674(93)90529-y

[6] Wilusz JE, Sunwoo H, Spector DL. Long noncoding RNAs: Functional surprises from the RNA world. Genes & Development. 2009;**23**:1494-1504. DOI: 10.1101/gad.1800909

[7] Faghihi MA, Wahlestedt C. Regulatory roles of natural antisense transcripts. Nature Reviews Molecular Cell Biology. 2009;**10**:637-643. DOI: 10.1038/nrm2738

[8] Volders PJ, Anckaert J, Verheggen K, Nuytens J, Martens L, Mestdagh P, et al. LNCipedia 5: Towards a reference set of human long non-coding RNAs. Nucleic Acids Research. 2019;**47**:D135-D139. DOI: 10.1093/nar/gky1031

[9] Morris KV, Mattick JS. The rise of regulatory RNA. Nature Reviews Genetics. 2014;**15**:423-437. DOI: 10.1038/nrg3722

[10] Krappinger JC, Bonstingl L, Pansy K, Sallinger K, Wreglesworth NI, Grinninger L, et al. Non-coding natural antisense transcripts: Analysis and application. Journal of Biotechnology. 2021;**340**:75-101. DOI: 10.1016/j.jbiotec.2021.08.005

[11] Crook S, Liang XH, Baker BF, Crook RM. Antisense technology: A review. Journal of Biological Chemistry. 2021;**296**:100416. DOI: 10.1016/j.jbc.2021.100416

[12] Dykxhoorn DM, Lieberman J. The silent revolution: RNA interference as basic biology, research tool, and therapeutic. Annual Review of Medicine. 2005;**56**:401-423. DOI: 10.1146/annurev.med.56.082103.104606

[13] Wahlestedt C. Natural antisense and noncoding RNA transcripts as potential drug targets. Drug Discovery Today. 2006;**11**:503-508. DOI: 10.1016/j.drudis.2006.04.013

[14] Yoshigai E, Hara T, Okuyama T, Okumura T, Kaibori M, Kwon AH, et al. Characterization of natural antisense transcripts expressed from interleukin 1beta-inducible genes in rat hepatocytes. HOAJ Biology. 2012;**1**:10. DOI: 10.7243/2050-0874-1-10

[15] Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen, et al. Involvement of

microRNA in AU-rich element-mediated mRNA instability. Cell. 2005;**120**:623-634. DOI: 10.1016/j.cell.2004.12.038

[16] Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO. Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biology. 2008;**6**:e255. DOI: 10.1371/journal.pbio.0060255

[17] Yoshida H, Kwon AH, Habara K, Yamada M, Kaibori M, Kamiyama Y, et al. Edaravone inhibits the induction of iNOS gene expression at transcriptional and posttranscriptional steps in murine macrophages. Shock. 2008;**30**:734-739. DOI: 10.1097/SHK.0b013e318173ea0b

[18] Yoshigai E, Hara T, Araki Y, Tanaka Y, Oishi M, Tokuhara K, et al. Natural antisense transcript-targeted regulation of inducible nitric oxide synthase mRNA levels. Nitric Oxide. 2013;**30**:9-16. DOI: 10.1016/j.niox.2013.01.001

[19] Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, et al. Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes. Hepatology. 2008;**47**:686-697. DOI: 10.1002/hep.22036

[20] Kimura T, Jiang S, Nishizawa M, Yoshigai E, Hashimoto I, Nishikawa M, et al. Stabilization of human interferon-alpha1 mRNA by its antisense RNA. Cellular and Molecular Life Sciences. 2013;**70**:1451-1467. DOI: 10.1007/s00018-012-1216-x

[21] Kimura T, Hashimoto I, Nishizawa M, Ito S, Yamada H. Novel cis-active structures in the coding region mediate CRM1-dependent nuclear export of IFN-alpha1 mRNA. Medical Molecular Morphology. 2010;**43**:145-157. DOI: 10.1007/s00795-010-0492-5

[22] Génin P, Vaccaro A, Civas A. The role of differential expression of human interferon—A genes in antiviral immunity. Cytokine & Growth Factor Reviews. 2009;**20**:283-295. DOI: 10.1016/j.cytogfr.2009.07.005

[23] Kimura T, Jiang S, Yoshida N, Sakamoto R, Nishizawa M. Interferon-alpha1 competing endogenous RNA network antagonizes microRNA-1270. Cellular and Molecular Life Sciences. 2015;**72**:2749-2761. DOI: 10.1007/s00018-015-1875-5

[24] Yoshigai E, Hara T, Inaba H, Hashimoto I, Tanaka Y, Kaibori M, et al. Interleukin-1beta induces tumor necrosis factor-alpha secretion from rat hepatocytes. Hepatology Research. 2014;**44**:571-583. DOI: 10.1111/hepr.12157

[25] Sakamoto R, Kumagai K, Odaka T, Okuyama T, Nishizawa M, Kimura T. A standardized suppresses breast cancer cell proliferation by regulating the expression of EphA2 antisense RNA–mRNA axis independently of microRNA. Bioactive Compounds in Health and Disease. 2019;**2**:191-205. DOI: 10.31989/bchd.v2i9.642

[26] Okuyama T, Sakamoto R, Kumagai K, Nishizawa M, Kimura T, Sugie T, et al. EPHA2 antisense RNA modulates EPHA2 mRNA levels in basal-like/triple-negative breast cancer cells. Biochimie. 2020;**179**:169-180. DOI: 10.1016/j.biochi.2020.10.002

[27] Nishizawa M, Kimura T. RNA networks that regulate mRNA expression and their potential as drug targets. RNA & DISEASE. 2016;**3**:e864. DOI: 10.14800/rd.864

[28] Kimura T. Non-coding natural antisense RNA: Mechanisms of action in the regulation of target gene expression

and its clinical implications. Yakugaku Zasshi. 2020;**140**:687-700. DOI: 10.1248/yakushi.20-00002

[29] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Research. 2003;**31**:3406-3415. DOI: 10.1093/nar/gkg595

[30] Forsbach A, Nemorin JG, Montino C, Muller C, Samulowitz U, Vicari AP, et al. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. Journal of Immunology. 2008;**180**:3729-3738. DOI: 10.4049/jimmunol.180.6.3729

[31] Muratani T, Nishizawa M, Matsumura S, Mabuchi T, Abe K, Shimamoto K, et al. Functional characterization of prostaglandin F2alpha receptor in the spinal cord for tactile pain (allodynia). Journal of Neurochemistry. 2003;**86**:374-382. DOI: 10.1046/j.1471-4159.2003.01840.x

[32] Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. Nature Biotechnology. 2017;**35**:238-248. DOI: 10.1038/nbt.3765

[33] Johannes L, Lucchino M. Current challenges in delivery and cytosolic translocation of therapeutic RNAs. Nucleic Acid Therapeutics. 2018;**28**:178-193. DOI: 10.1089/nat.2017.0716

[34] Straarup EM, Fisker N, Hedtjärn M, Lindholm MW, Rosenbohm C, Aarup V, et al. Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in mice and non-human primates. Nucleic Acids Research. 2010;**38**:7100-7111. DOI: 10.1093/nar/gkq457

[35] Abes S, Moulton H, Turne J, Clair P, Richard JP, Iversen P, et al. Peptide-based delivery of nucleic acids: Design, mechanism of uptake and applications to splice-correcting oligonucleotides. Biochemical Society Transactions. 2007;**35**:53-55. DOI: 10.1042/BST0350053

[36] Holasová S, Mojzísek M, Buncek M, Vokurková D, Radilová H, Safárová M, et al. Cholesterol conjugated oligonucleotide and LNA: A comparison of cellular and nuclear uptake by Hep2 cells enhanced by Streptolysin-O. Molecular and Cellular Biochemistry. 2005;**276**:61-69. DOI: 10.1007/s11010-005-2912-8

[37] Modarresi F, Faghihi MA, Lopez-Toledano MA, Fatemi RP, Magistri M, Brothers SP, et al. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. Nature Biotechnology. 2012;**30**:453-459. DOI: 10.1038/nbt.2158

[38] Okuyama T, Nakatake R, Kaibori M, Okumura T, Kon M, Nishizawa M. A sense oligonucleotide to inducible nitric oxide synthase mRNA increases the survival rate of rats in septic shock. Nitric Oxide. 2018;**72**:32-40. DOI: 10.1016/j.niox.2017.11.003

[39] Tanaka H, Uchida Y, Kaibori M, Hijikawa T, Ishizaki M, Yamada M, et al. Na^+/H^+ exchanger inhibitor, FR183998, has protective effect in lethal acute liver failure and prevents iNOS induction in rats. Journal of Hepatology. 2008;**48**:289-299. DOI: 10.1016/j.jhep.2007.09.017

[40] Hijikawa T, Kaibori M, Uchida Y, Yamada M, Matsui K, Ozaki T, et al. Insulin-like growth factor 1 prevents liver injury through the inhibition of TNF-alpha and iNOS induction in D-galactosamine and LPS-treated rats. Shock. 2008;**29**:740-747. DOI: 10.1097/SHK.0b013e31815d0780

[41] Nakatake R, Tanaka Y, Ueyama Y, Miki H, Ishizaki M, Matsui K,

et al. Protective effects of active hexose correlated compound in a rat model of liver injury after hepatectomy. Functional Foods in Health and Disease. 2016;**6**:702-717. DOI: 10.31989/ffhd.v6i11.305

[42] Lundin KE, Gissberg O, Smith CIE. Oligonucleotide therapies: The past and the present. Human Gene Therapy. 2015;**26**:475-485. DOI: 10.1089/hum.2015.070

[43] Debacker AJ, Voutila J, Catley M, Blakey D, Habib N. Delivery of oligonucleotides to the liver with GalNAc: From research to registered therapeutic drug. Molecular Therapy. 2020;**28**:1759-1771. DOI: 10.1016/j.ymthe.2020.06.015

[44] Padmakumar S, Jones G, Khorkova O, Hsiao J, Kim J, Bleier BS, et al. Osmotic core-shell polymeric implant for sustained BDNF AntagoNAT delivery in CNS using minimally invasive nasal depot (MIND) approach. Biomaterials. 2021;**276**:120989. DOI: 10.1016/j.biomaterials.2021.120989

[45] Pawar G, Parayath NN, Sharma AA, Coito C, Khorkova O, Hsiao J, et al. Endonasal CNS delivery system for blood-brain barrier Impermeant therapeutic oligonucleotides using heterotopic mucosal engrafting. Frontiers in Pharmacology. 2021;**12**:660841. DOI: 10.3389/fphar.2021.660841

[46] Sakamoto R, Jiang S, Tsukada Y, Tsujimoto H, Kimura T. IFN-Alpha1 antisense RNA represses human influenza A virus growth in a Guinea pig system. Frontiers in Bioscience (Landmark Ed.). 2019;**24**:798-818. DOI: 10.2741/4752

[47] Tenchov R, Bird R, Curtze AE, Zhou Q. Lipid nanoparticles—From liposomes to mRNA vaccine delivery, a landscape of research diversity and advancement. ACS Nano. 2021;**15**:16982-17015. DOI: 10.1021/acsnano.1c04996

[48] Sakitani K, Kitade H, Lnoue K, Kamiyama Y, Nishizawa M, Okumura T, et al. The anti-inflammatory drug sodium salicylate inhibits nitric oxide formation induced by interleukin-1beta at a translational step, but not at a transcriptional step, in hepatocytes. Hepatology. 1997;**25**:416-420. DOI: 10.1053/jhep.1997.v25.pm0009021956

[49] Neault JF, Tajmir-Riahi HA. RNA-aspirin interaction studied by FTIR difference spectroscopy. Journal of Physical Chemistry B. 1997;**101**:114-116. DOI: 10.1021/jp9619292

[50] Ozaki T, Habara K, Matsui K, Kaibori M, Kwon AH, Ito S, et al. Dexamethasone inhibits the induction or iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. Shock. 2010;**33**:64-69. DOI: 10.1097/SHK.0b013e3181a7fd74

[51] Dwijayanti DR, Okuyama T, Okumura T, Ikeya Y, Nishizawa M. The anti-inflammatory effects of Indonesian and Japanese bitter melon (*Momordica charantia* L.) fruit extracts on interleukin-1beta-treated hepatocytes. Functional Foods in Health and Disease. 2019;**9**:16-33. DOI: 10.31989/ffhd.v9i1.560

[52] Yamauchi Y, Okuyama T, Ishii T, Okumura T, Ikeya Y, Nishizawa M. Sakuranetin downregulates inducible nitric oxide synthase expression by affecting interleukin-1 receptor and CCAAT/enhancer-binding protein beta. Journal of Natural Medicines. 2019;**73**:353-368. DOI: 10.1007/s11418-018-1267-x

[53] Yamanishi R, Yoshigai E, Okuyama T, Mori M, Murase H, Machida T, et al. The anti-inflammatory effects of Flavanol-rich lychee fruit extract in rat hepatocytes. PLoS One. 2014;**9**:e93818. DOI: 10.1371/journal.pone.0093818