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Perspectives:

Noncoding RNAs as drug targets

Masayuki Matsui and David R. Corey

UT Southwestern, Departments of Pharmacology and Biochemistry, Dallas, TX 75390-9041, USA

Abstract

Most of the human genome encodes RNA that does not code for protein. These noncoding RNAs (ncRNAs) may affect normal gene expression and disease progression, making them a new class of targets for drug discovery. Because their mechanisms of action are often novel, developing drugs targeting ncRNAs will involve equally novel challenges. However, many potential problems may already have been solved during development of technologies to target mRNA. Here, we will discuss the growing field of ncRNA – including microRNA, intronic RNA, repetitive RNA and long non-coding RNA - and assess the potential and challenges in their therapeutic exploitation.

The world of RNA inside mammalian cells has been expanding for decades¹. Each new discovery adds a new, and often surprising, layer to biological regulation and function. Experience has taught us not to dismiss a novel, unexpected, RNA-mediated activity. RNA species beyond mRNA include intronic RNAs, microRNAs (miRNAs)², long noncoding RNAs (lncRNA)³, circular RNAs (circRNA)⁴, and extracellular RNAs⁵. Collectively these are known as noncoding RNAs (ncRNAs) because they lack clear potential to encode proteins or peptides.

While ncRNAs lack potential to encode proteins, they can affect the expression of other genes through a variety of mechanisms. In some cases, their mechanisms of action are well known and strategies for controlling activity are well-established. In other cases, however, mechanisms are obscure or controversial. For example, the mechanism of oligonucleotides that bind to miRNAs to block their action and inhibit gene expression is proven⁶, while the many possible mechanisms for using lncRNAs to manipulate expression are only beginning to be understood³.

The ability of ncRNAs to control gene expression makes them targets for drug development. The drug discovery process is never easy. Uncertainty about how ncRNAs function (and even whether they have a function) makes lead identification and development even more challenging. This Perspective will first consider lessons learned from nucleic acid-based targeting of mRNA - including issues associated with target specificity and toxicity - which are applicable to ncRNA-based drug discovery. Recent advances involving the targeting of miRNAs, intronic RNA/exon skipping, and repetitive RNAs will be highlighted, while lncRNAs, a novel and challenging class of potential drug targets will be focused upon.

*Correspondence to David R. Corey; david.corey@utsouthwestern.edu.

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Targeting mRNA: principles and lessons learned

Basic Principles

mRNA has been experimentally pursued as a potential therapeutic target since the late 1970's⁸. The concept is simple: Identify an mRNA involved in disease, use the mRNA sequence to design a complementary oligonucleotide, introduce the oligonucleotide into cells so that upon binding to the mRNA the expression of a protein is disrupted and symptoms of the disease are alleviated^{9,10}.

One advantage of this approach is that the synthesis of complementary oligonucleotides is straightforward and it will almost always be much easier to design an oligonucleotide to bind an mRNA and inhibit protein production, than to identify a small molecule that can inhibit the protein's function directly. Furthermore, rather than requiring months or years, lead compound identification can usually be accomplished in just a few weeks.

Any RNA can be targeted by complementary base-pairing recognition and inhibitory oligonucleotides are chemically similar to one another. Oligonucleotides may vary in sequence, but have similar lengths and similar chemical modifications that are necessary to improve stability, efficacy, and pharmacokinetic properties (Damha and Dunleavy). Due to these similarities, preclinical or clinical experiences with compounds now approved or in the pipeline are likely to have substantial predictive value for planned trials of oligonucleotides that target other mRNAs, which will likely reduce risk relative to programs that are testing novel drug scaffolds.

Another advantage of synthetic nucleic acids is that they may be used to modulate activity of targets that appear to be “undruggable” by small molecules^{11,12}. Examples include protein:protein interactions, complex proteins for which it might be difficult to block all key functions by a single molecule, or proteins that are so closely related to others that it is impossible to achieve adequate selectivity^{11,12}.

However, while the therapeutic potential of oligonucleotides has been recognized for more than forty years, few drugs have received approval (*Vitravene, ocular/CMV retinitis, approved 1998; Macugen, ocular/macular degeneration, approved 2004; Kynamro, systemic/familial hypercholesterolemia approved*). Vitravene and Kynamro are antisense oligonucleotides that target mRNA, while Macugen is an aptamer that binds vascular endothelial growth factor.

This small number of approved agents reflects the obstacles that have been faced when attempting to target mRNA using synthetic oligonucleotides. Most drugs are hydrophobic small molecules, under 500 daltons in molecular weight with fewer than five hydrogen bond donors or acceptors¹³. Oligonucleotides by contrast, have masses in the thousands of daltons, containing many hydrogen bond donors and acceptors, and have multiple negative charges⁷. Unlike antibodies, which represent another class of large and chemically complex therapeutics, oligonucleotides that target cellular RNA must enter cells to be active.

Because they are so unlike traditional small molecule drugs, three decades of intense effort have been invested in developing them as plausible therapeutic candidates. Landmark

achievements in the therapeutic development of oligonucleotides have included 1) the ability to synthesize kilogram quantities of oligonucleotide at costs that are compatible with clinical application¹⁴, 2) the demonstration that chemical modifications can reduce off-target effects (see below), lower toxicity, and improve pharmacokinetic properties^{7,15}, 3) the demonstration that synthetic nucleic acids can be administered systemically and knock down their intended targets *in vivo*^{7,15}; and 4) the FDA approval of two synthetic oligonucleotides designed to target mRNA and numerous ongoing trials¹⁶⁻¹⁸.

Despite these achievements, it is not yet clear whether synthetic nucleic acids can successfully compete with small molecule drugs and follow the path of therapeutic antibodies by forming a large class of widely used compounds. Given the number and relative sophistication of current clinical trials that use nucleic acids, a much better understanding of the potential of agents that target mRNA is likely within the next five years.

Notably, small molecules have also been proposed to target disease-causing nucleic acid targets and modulate their function¹⁹. The advantage of this approach is that it would apply the advantages of small molecules to DNA and RNA “receptors”. In practice, due to the similarities between nucleic acids present in cells, it is difficult to identify small molecules that can both potentially bind nucleic acid targets and be selective for a disease target.

Classes of Lead Compounds

The most common strategies for controlling mRNA expression use antisense oligonucleotides (ASOs) and duplex RNAs (Figure 1).

A typical contemporary design for an ASO consists of a central DNA “gap” region flanked by chemically modified nucleotides that boost binding to a complementary target and increase nuclease resistance (Figure 1a)^{20,21}. These “gapmer” ASOs form a DNA:RNA hybrid with target mRNA that recruits RNase H and promotes degradation of mRNA.

A second design for ASOs has more uniform chemical modifications and lacks a central DNA gap. These “steric block” ASOs function by binding to target RNA sequences and blocking access of key proteins. The most common use of steric block ASOs is to obstruct binding of regulatory proteins near critical splice junctions to redirect alternative splicing^{20,21}.

Duplex RNAs, by contrast, function through RNA interference (RNAi) (Figure 1b)²². Because RNAi is a natural mechanism for gene silencing, synthetic RNA duplexes benefit from interactions with proteins that have been optimized through evolution to promote recognition of complementary RNA. The potential for efficient recognition and silencing to be achieved in patients drives development duplex RNAs as a therapeutic capable of competing with gapmer ASOs.

It is currently unclear whether duplex RNAs or ASOs will prove superior in the clinic. The relative value of the approaches will differ depending on the nature of the target. However, experience using both approaches provide a basis for the discovery of agents targeting noncoding RNAs.

Lessons learned from targeting mRNA

By the mid 1990's it became clear that many published descriptions of the use of ASOs inside cells were unconvincing or inaccurate²³⁻²⁵. Similar concerns have been expressed for studies involving duplex RNAs²⁶. A key challenge in targeting mRNA is that duplex RNAs and antisense oligonucleotides, like any exogenous molecule, -have the potential to produce off-target effects when added to cells.

Several factors have combined to make off-target effects a recurring problem for research using nucleic acids to modulate gene expression (Box 1). Due to the simplicity of the concept, researchers have often assumed that any effect observed is due to recognition of the expected target, especially when the effect fits the hypothesis. Duplex RNAs or ASOs are complex molecules that can cause off-target effects through multiple mechanisms (Box 1, Box 2)^{27,28}. They are often delivered into cultured cells using cationic lipid²⁹, which while an excellent delivery tool, can compound the observation of off-target effects. Nucleic acids can also exert effects that are sequence-dependent, but independent of interactions with the intended RNA target.

How can an “on-target” mechanism be established? Fortunately, it is relatively simple to build a strong case for an on-target mechanism if diligent control experiments are designed and performed^{30,31}. When screening for ASOs or duplex RNAs, the goal is to identify at least two or three potent compounds that are capable of knocking down gene expression and are complementary to the target RNA. By identifying multiple compounds that share complementarity to the target and produce a common phenotype, the possibility that the phenotype is due to non-specific binding of the compounds to unintended targets is reduced. Nucleotide BLAST using databases of genomic DNA and transcript sequences would help remove candidates having significant potential of off-target interactions.

After identifying these lead compounds, one then designs multiple controls that are either “scrambled” (groups of bases swapped within the control compounds relative to the active parent compound) or “mismatched” (mismatched bases introduced relative to target, for example within a putative seed sequence of a duplex RNA). It is important to include control compounds that maintain groups of bases in the same order, in case small motifs are responsible for phenotypes independent of overall complementarity to target. For example, the CpG dinucleotide has the potential to exert potent immunostimulatory effects independent of the surrounding sequence or intended target³².

Experiments using positive or negative control nucleic acids are relatively rapid and inexpensive, involving lipid-mediated transfection of nucleic acids into cultured cells²⁹. The concentration of nucleic acid needed to produce an effect depends on the identity of the target and the type of cell culture dish used. For example, an experiment in a 96-well plate will produce different values relative to experiments performed in 6-well plates because the overall amount of nucleic acid used per cell are likely to differ. Cell growth and distributions sometimes change depending on plate formats, affecting transfection efficiency. Experimental conditions affect relative efficiencies, therefore comparisons become problematic when experiments are not run in parallel using similar protocols.

The combination of lipid and ASO or duplex RNA will invariably cause toxicity and cell death when used at higher concentrations, which can confound experimental interpretation,. This problem is especially troublesome when an anti-proliferative phenotype is being examined. “Gymnotic” delivery - in which the oligonucleotide is delivered directly in saline solution – may minimize toxicity and therefore be useful in studies examining proliferation or cell cycle changes, or analysis that require cells to be repeatedly treated over time^{33,34}. However, gymnotic delivery requires 10-50 times as much ASO and cannot be used for delivery of duplex RNA.

Emerging classes of RNA targets

Beyond mRNA, emerging classes of RNA targets include miRNAs, intron/exon junctions, repetitive RNA, and long noncoding RNAs. Each class of RNA performs different endogenous functions, providing a variety of opportunities and challenges for drug discovery.

When considering emerging classes of RNA, it is important not to be dogmatic with regard to terminology, as this may limit data interpretation and mechanistic understanding. For example, an intronic RNA may also contain a repetitive RNA target and therefore qualify for inclusion in both categories. Furthermore, the term lncRNA itself can be problematic, as long “noncoding” RNAs may encode miRNAs or peptides.

The experience gained with using oligonucleotides to target mRNA has important implications for targeting other RNA species. Indeed, designed nucleic acids may bind to any RNA species to modulate activity. Lessons learned regarding control oligomers and phenotype interpretation may therefore be applicable for emerging classes of RNA. Because ASOs and duplex RNAs that target mRNA act by reducing gene expression, targeting ncRNA RNA might be useful if the outcome was activation of gene expression. In addition, miRNAs typically regulate many genes and blocking a single miRNA might allow manipulation of a signalling pathway at multiple points.

miRNA

miRNAs form a major class of functional noncoding RNAs^{35,36} and their potential to be therapeutically targeted has been well-reviewed elsewhere^{2,37}. Oligonucleotides that are complementary to an miRNA can block its activity, while duplex or chemically-modified single-stranded RNAs that mimic a miRNA can trigger enhanced activity (Figure 1cd)^{2,6}.

Both miRNA inhibitors and mimics are currently being developed against a variety of targets^{38–41} and tested in clinical trials (Table 1). Examples include: mimics of miR-34 designed to repress oncogene expression and block tumor growth³⁹, single-stranded oligonucleotides complementary to miR-122 being developed to treat Hepatitis C virus⁴⁰ and single-stranded oligonucleotides complementary to miR-21 being applied to treat Alport nephropathy, a chronic kidney disease⁴¹.

Intronic RNA

Approximately thirty percent of the genome encodes intronic RNA, making it one of the most prevalent species of noncoding RNA⁴². Because steric blocking ASOs can be designed to be complementary to key regulatory sequences, they have the potential to redirect alternative splicing, leading to the biosynthesis of different protein isoforms (Figure 1e). The potential to “switch” from one splice variant to another suggests that targeting key splicing control sequences within introns or exons can offer opportunities for therapeutic development⁴³⁻⁴⁵. Because compounds that target exons and introns can affect splicing, both will be considered here.

Kole *et al.* first noted oligonucleotide-directed alternative splicing in 1993, using an ASO complementary to intronic RNA within B-globin RNA that corrected splice defects found in B-thalassemia in a cell free system⁴⁶.

Typically, the mechanism of splice modulation involves blocking the binding site for splice factors⁴⁵. One alternative mechanism and an example of an unexpected pathway for oligonucleotide-directed splice modulation involves recruitment of interleukin enhancer binding factor 2 and 3 (*ILF2/3*) complex to target sites through interaction with ASOs containing 2'-fluoro modifications⁵².

Perhaps because of the relative simplicity of the predominant mechanism for splice correction, substantial progress has been made in both pre-clinical and clinical studies⁴⁷⁻⁵⁸ (Table 1). Successful splice correction has now been achieved for dystrophin (*DMD*, Duchenne muscular dystrophy)^{49-51,55,56}, *USH1* (Usher syndrome)⁵³, survival motor neurons 2 (*SMN2*; spinal muscular atrophy (SMA))⁵⁴ among others. Most clinical attention has focused on drisapersen⁴⁹⁻⁵¹ and eteplirsen⁵⁶ for treating Duchenne muscular dystrophy and nusinersen⁵⁷ for spinal muscular atrophy.

Case study: Dystrophin

Clinical studies of two compounds, drisapersen (BioMarin Pharmaceutical) and eteplirsen (Sarepta Therapeutics), have been pursued for dystrophin restoration. Drisapersen is a 2'-O-methyl phosphorothioate oligonucleotide, while eteplirsen is a phosphorodiamidate morpholino oligomer. Both compounds are designed to target a sequence within exon 51, induce exon skipping, and generate a shorter mRNA that will produce a partially active dystrophin. This “exon-skipped” dystrophin is produced by patients with Becker's muscular dystrophy, a condition with a better prognosis⁴³.

As of early 2016, Phase III clinical trials had not demonstrated drisapersen to be sufficiently effective to win FDA approval⁵⁸. However, BioMarin Pharmaceutical is still pursuing the development of other exon skipping ASOs such as BMN 044, BMN 045, and BMN 053, which are currently in phase I/II clinical trials (Table 1). It is now suspected that an increased and wider distribution of the drug will be required to lead to better efficacy⁵⁹. As the dose of drisapersen was limited to 6 mg/kg because of its relatively high kidney toxicity and the use of modified oligonucleotide chemistries may improve the therapeutic window. Preclinical and clinical studies have suggested that another exon skipping morpholino-based

ASO, eteplirsen, may have safer biological properties for *in vivo* application, enabling the application of much higher doses (30, 50 mg/kg) in the clinical trials⁵⁶.

Case study: USHI

Usher syndrome is a hereditary hearing impairment with a frequency as high as 1 in 6000⁶⁰, caused by mutations in the *USH1C* gene encoding harmonin protein. For the Acadian population, all cases involve a mutation that creates a cryptic splice site that is used in place of the normal location⁶¹. Treatment of mice with an ASO capable of blocking the cryptic splice site increased expression of wild-type protein and partially restored hearing⁵³.

Although there have been no efforts as yet to develop this clinically, this finding demonstrates the therapeutic potential of this approach and warrants further investigation.

Case study: SMN2

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by a loss of function mutation within the survival motor neuron 1 (SMN1) gene⁶². Humans possess a paralogous gene, SMN2, the splicing of which differs slightly from SMN1, resulting in more rapid degradation which prevents it from substituting for defective SMN1. Nusinersen (Ionis/Biogen), a 2'-O-methoxyethyl modified phosphorothioate ASO that targets intron 7 within *SMN2* pre-mRNA, restored splicing and facilitated production of full length SMN2 in mouse models of SMA⁵⁴.

Phase I clinical trials of nusinersen have been completed in patients with type II and type III SMA. Patients ranged in age from two to fourteen years and were treated with a single intrathecal injection of nusinersen between 1 and 9 mg/kg⁵⁷. The drug was well tolerated in all patients and significant improvements in motor function were observed at the highest dose one month after administration.

Recently released results from a Phase II trial involving 15 infants with type I SMA reported no new adverse events and achievement of developmental milestones such as unsupported sitting in eight subjects, standing with or without support in five subjects, and walking in 2 subjects⁶³. Such milestones are rarely achieved by SMA patients, therefore this interim data is encouraging. Further trials are ongoing and are likely to have important implications for targeting splice junctions and for neurological applications of antisense oligonucleotides in general.

Repetitive RNA

Over forty neurological diseases are caused by an expansion of repetitive sequences within DNA^{64,65}. These expanded repeats, and nearby sequences if they are within introns, are attractive targets for ASOs and duplex RNAs⁶⁶⁻⁶⁸. In contrast to single nucleotide polymorphisms, these mutations are shared by all patients, therefore any drug would be applicable to an entire patient population.

Because the repeat mutation causes the RNA to differ from wild-type, it is possible to achieve allele-selective inhibition of mutant expression if preservation of wild-type expression is desired⁶⁸. For some diseases, the expanded repeats are within exons that lead

to production of toxic proteins (e.g. Huntington's disease, Machado-Joseph disease)^{64,65}. Expanded repeats within introns or 5'/3' untranslated regions can produce toxic mutant RNAs (e.g. myotonic dystrophy) or affect production of protein (e.g. Friedreich's ataxia, Fragile X syndrome)^{64,65}.

Case study: Myotonic dystrophy

Myotonic dystrophy type 1 (DM1), the most common form of muscular dystrophy, is caused by expansion of a CUG repeat within the 3' untranslated (3' UTR) region of the dystrophin myotonia protein kinase (*DMPK*) gene⁶⁹. This expanded repeat binds muscleblind like splicing regulator 1 (*MBNL1*) protein, a splicing regulator, and perturbs alternative splicing. The expanded repeat also causes aberrant activation of protein kinase C (PKC), contributing to hyperphosphorylation of CUG-binding protein 1 (CUGBP1). The hyperphosphorylation increases the half-life of CUGBP1. The resultant increase in CUGBP1 levels plays pathogenic roles in DM1 by disrupting alternative splicing and translation⁷⁰.

For therapy of DM1, two approaches targeting different regions of the *DMPK* transcript have been tested so far (Figure 2a). One approach is to use a CUG repeat-targeting ASO. *In vivo*, non-RNase H dependent 2'-O-methyl phosphorothioate oligonucleotides⁷¹ and morpholino oligomers⁷² complementary to the CUG repeat, successfully released MBNL1 protein from the repeat and mitigated RNA toxicity of the repeat RNAs *in vivo*.

Another therapeutic approach is to use a gapmer ASO targeting outside of the CUG repeats to silence nuclear *DMPK* transcripts^{73,74}. Systemic administration of a 2',4'-constrained ethyl (cEt)-modified gapmer (ISIS 486178) induced rapid knockdown of CUG repeat RNAs and corrected pathogenic features of the disease⁷³. A Phase I/II clinical study for a similar gapmer ASO containing cEt and 2'-O-MOE groups (IONIS-DMPK-2.5Rx (ISIS 598769)) was initiated in 2014 to evaluate its safety and tolerability⁷⁵.

Case study: Friedreich's ataxia

Friedreich's ataxia (FA) is caused by expansion of a GAA repeat within intronic RNA of the frataxin (*FXN*) gene⁷⁶. Normal frataxin protein is made by cells, but transcription is reduced and the level of protein is lowered. Evidence suggests that the mechanism of action involves binding of the expanded intronic repeat RNA directly to chromosomal DNA through R-loop formation, putting the break on transcription by inducing suppressive histone modifications⁷⁷.

Our laboratory has targeted steric block locked nucleic acid (LNA) ASOs and duplex RNAs to the expanded GAA repeat in FA patient-derived cells (Figure 2b)⁷⁸. These compounds reduce R-loop formation and increase *FXN* mRNA and protein levels to those found in wild-type cells. The *FXN* locus is a good example of the unexpected mechanisms employed by repeat RNA and the ability of designed duplex RNAs and ASOs to manipulate mechanism and alter gene expression towards a therapeutically valuable endpoint.

Repeat-targeting dsRNA or ASOs may have some advantages when allele-specific gene silencing or activation is required to avoid adverse effects that might be caused by knocking down gene expression from normal alleles. However, targeting simple repeat sequence

motifs increases the potential risk of off-target effects. Using gapmer ASOs that target sequences within the mutant gene that are outside of the expanded repeat, may be more effective for diseases like DM1 that are caused by nuclear RNA gain-of-function toxicity.⁷⁴

Long noncoding RNA (lncRNAs)

lncRNAs are a diverse group of transcripts whose natural functions and potential as drug targets remain largely undefined. These RNA species does not encode protein and they are greater than 200 nucleotides in length. Transcriptome analysis over the past decade had identified many lncRNAs⁷⁹⁻⁸² that fall into various subclasses depending on length, association with protein-encoding genes, sense or antisense orientation relative to protein-encoding genes, association with repeats, and other criteria^{3,83,84}. Some lncRNAs are encoded between genes and are known as long intergenic noncoding RNAs (lincRNAs)⁸⁵. Intergenic noncoding RNAs that are greater than 50 kB in length are known as very long intergenic RNAs (vlincRNAs)⁸⁶.

Although transcriptome studies have revealed that approximately 80% of the genome is transcribed into RNA⁷⁹⁻⁸², it remains unclear, how many lncRNAs are functional, how many are expressed at significant levels inside cells, or even how to define how much RNA is necessary to achieve a biological effect⁸⁷⁻⁸⁹. It is likely that many lncRNAs have no function, complicating predictions and analysis. For lncRNAs that might play a role in controlling gene expression, mechanisms will often be unknown and require substantial effort to establish.

The first step towards understanding the existence or potential function of a lncRNA, is to examine databases such as FANTOM5 (<http://fantom.gsc.riken.jp/5/>), ENCODE (<https://www.encodeproject.org>), and NONCODE (<http://www.noncode.org>). However, the information in these databases does not provide quantitative measures of lncRNA expression and is derived from selected cell lines or tissues.

Confirming the existence of an RNA transcript and determining the number of RNA transcripts per cell (using a combination of quantitative reverse transcription PCR (RT-qPCR), 5'/3' RACE (rapid amplification of cDNA ends) analysis and droplet digital PCR (ddPCR)) is critical to understanding potential mechanisms and functions. A transcript present at a just a few copies per cell would be sufficient to act *in cis* relative to the locus that encodes it. A transcript present at dozens, hundreds, or thousands of copies per cell might act *in trans* because sufficient numbers are available to diffuse to distant sites and exert an effect. A transcript present at much less than one per cell would be less likely to have a significant function, although it should not be dismissed.

lncRNAs can act in either the nucleus or cytoplasm, therefore determining the subcellular localization is also important for understanding mechanism and designing strategies for manipulating lncRNA expression and function. This can be done by stringent isolation of nuclei followed by demonstration of purity^{92,93}. Fluorescence *in situ* hybridization (FISH) assay is also useful for semi-quantitative evaluation of subcellular localization of lncRNAs. Localization in the nucleus would be consistent with an effect on transcription or splicing,

whereas predominant localization in the cytoplasm would suggest the possibility of an effect on translation and imply action *in trans*. Another important step in identifying potential functionality of a non-coding RNA and its relevance to disease is to search for potential correlations between lncRNA expression and disease outcomes or symptoms.

Lack of conservation between lncRNA sequences in humans and experimental animal models may complicate development of related therapeutics. The divergence of mRNA sequences is constrained by the need to maintain critical protein functions and structures, making it more likely that some sequences will be shared and that a single ASO or duplex RNA will be effective in more than one species. lncRNAs, by contrast, are expressed from intronic or intergenic regions which are often less conserved between species (e.g. human vs. mouse/rat) [This is also a well known observation, no need to cite its source]. Drug candidates obtained from screening using human cells may not be applicable to *in vivo* evaluation using rodent or other disease models because the functions of lncRNAs may differ in humans relative to other animals. As a result, it may be necessary to engineer animal models to express the lncRNA. Engineering the complex interplay of lncRNA and target gene is likely to be challenging and will require a detailed understanding of mechanism.

Gene silencing can be used to investigate the function of a lncRNA, using either ASOs or duplex RNAs. However, the use of gene silencing for lncRNAs is less straightforward than for mRNA because, as indicated above, cellular localization differs among lncRNAs, as does the efficacy of different gene silencing strategies⁹⁴. Duplex RNAs are a reliable approach for targeting mRNAs or lncRNAs in the cytoplasm and inhibiting gene expression [The usefulness of standard cytoplasmic RNAi is well known]. In the nucleus, RNAi factors are present and can promote cleavage of target sequences⁹², suggesting that RNAi can also be used to silence nuclear lncRNAs. Recently, it has been shown that ASOs are more reliable gene silencing agents than duplex RNAs for RNAs that are localized to cell nuclei⁹⁴.

ASOs may, therefore, be the silencing method of choice when a target RNA is thought to function in the nucleus, while duplex RNAs may be a better choice when a target is thought to function in the cytoplasm.

Finally, as lncRNAs are novel targets with often undefined mechanisms, it is critical that proper controls be used during gene silencing studies in order to ensure that observed phenotypes are due to on-target interactions.

Regulation of gene expression

A central question for drug discovery is whether a lncRNA modulates expression at a specific locus. Linkage of a specific lncRNA to a specific gene suggests that the lncRNA might provide an alternative to small molecule or antibody approaches for controlling the activity of the gene product⁹⁵.

Polycomb repressive complex-2 (PRC2) presents a model for how lncRNAs might contribute to the regulation of gene expression^{96,97}. PRC2 is a multi-protein complex including EZH2, RBBP4, AEBP2, SUZ12, and EED, involved in modifying chromatin with

H3K27me3 mark and epigenetic silencing⁹⁸. The noncoding X-inactive specific transcript (*XIST*) has been implicated in PRC2 recruitment during X chromosome inactivation^{99,100} and thousands of other lncRNAs have been reported to bind PRC2 by studies using RNA-seq¹⁰¹.

The hypothesis that selective binding of PRC2 affects gene silencing has obvious implications for drug development, as the use of ASOs to block the binding of PRC2 to specific lncRNAs by ASOs would be expected to activate gene expression. Supporting this hypothesis, ASOs were reported to be able to displace PRC2 and disrupt interactions between lncRNA and chromatin¹⁰². The ASOs used in this study were not designed to induce cleavage of the lncRNA and were thought to act by competing with PRC2 for association with the target lncRNA.

To successfully cooperate during gene silencing, PRC2 must be selective for target RNA sequences. However, PRC2 is a promiscuous RNA binding protein that, presumably, would have little preference on its own for one sequence versus another¹⁰³. Similar promiscuous binding has also been shown for FUS protein, another protein factor that had been thought to associate with specific RNAs¹⁰⁴. A subsequent study reported that different sequences embedded within RNAs of similar lengths could confer differences in FUS binding (3–8-fold), but also that results could vary depending on experimental conditions and the nature of the RNAs used for comparisons¹⁰⁵. Although a 3–8 fold difference cannot explain selective recognition by PRC2 acting alone, it remains possible that specificity is achieved through combinatorial binding of several protein factors. These studies of PRC2 are significant because they reveal that the mechanism for lncRNA action, even at a relatively well-studied model target, remains incompletely understood.

Recognition of lncRNAs by small RNAs and the RNA interference (RNAi) machinery is another potential mechanism for selective control of gene expression^{106,107}. Both miRNAs and RNAi protein factors exist in the nuclei of human cells, providing the components necessary for recognition⁹². RNAi mediated recognition by synthetic duplex RNAs and miRNAs has been demonstrated to alter transcription through recognition of RNAs complementary to transcripts that overlap gene promoters and mRNA transcription start sites (described below)¹⁰⁸ and splicing through recognition of sequences at intron/exon junctions¹⁰⁹.

Since lncRNAs often overlap genes, they might be able to affect expression *in cis* without relying on sequence-specific binding by proteins. In that case, inactivation of a lncRNA by an ASO would be sufficient to affect expression of the nearby gene. LNA gapmers targeting an antisense transcript at the brain-derived neurotrophic factor (*BDNF*), glial derived neurotrophic factor (*GDNF*), and ephrin receptor B2 (*EPHB2*) loci were also reported to lead to increased expression of the parent gene¹¹⁰.

Disease-associated lncRNAs and therapeutic potential

Case Study: MALAT1—Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a nuclear localized RNA that is upregulated in cancer cells¹¹¹. It is an unusually abundant lncRNA, present at concentrations of a few thousand per cell, which is

well above that of essential house-keeping genes^{91,111}. *MALAT1*'s abundance makes it a representative for candidate lncRNAs that might possess activity *in trans*.

MALAT1-mediated regulation of alternative splicing has been implicated in human HeLa cells¹¹². RNA immunoprecipitation revealed an interaction of *MALAT1* with the serine/arginine-rich (SR) family of nuclear phosphoproteins (SR proteins) that have been well characterized as alternative splicing regulators. Depletion of *MALAT1* using ASOs increased cellular levels of SR proteins and changed the ratio and the cellular distribution of phosphorylated/dephosphorylated SR proteins, leading to a change in alternative splicing of pre-mRNAs.

However, another study investigating the involvement of *MALAT1* in splicing regulation by using *MALAT1* knockdown/knockout mice or cultured cells, found that elimination of *MALAT1* does not significantly change global pre-mRNA splicing and phosphorylation status of SR proteins¹¹³. Other groups have also reported that *MALAT1* knockout in mouse does not lead to obvious defects in development, fertility, or normal adult functions^{114,115}. These inconsistent observations between different groups emphasize the unsettled state of insight into the physiological functions of *MALAT1* for splicing regulation.

In contrast to the lack of effects on normal development, depletion of *MALAT1* by genetic knock-out or treatment with ASOs can reduce metastasis in mouse mammary or lung carcinoma models^{116,117}. Reduced cancer metastasis by knockdown of *MALAT1* by siRNA has also been shown in human bladder cancer cells¹¹⁸. The molecular mechanism proposed in this study is that *MALAT1* functions as a scaffold for recruiting SUZ12 (one of the PRC2 components) and mediates downregulation of the E-cadherin (*CDH1*) gene. Depletion of *MALAT1* decreases H3K27me3 levels at the E-cadherin promoter, leading to upregulation of E-cadherin and concurrent downregulation of N-cadherin and fibronectin as EMT (epithelial–mesenchymal transition) markers. The mechanism by which *MALAT1* can effect the expression of other genes remains uncertain. Studies have suggested that *MALAT1* may bind to active chromatin directly or to nascent RNAs^{119,120}, although the molecular interactions that might mediate binding remain unknown.

Although *MALAT1* is highly abundant and well-studied, fundamental gaps remain in our knowledge of its mechanism and function¹²¹. ASOs targeting *MALAT1* have been shown to reduce tumor growth and metastasis, but converting these observations into a viable drug development program will require reliable methods for delivering ASOs to tumor cells and be facilitated by a better understanding of *MALAT1*'s role in normal human cells and tumors.

Case Study: Angelman syndrome antisense transcript—Angelman's syndrome is a severe neurodevelopmental disorder with a prevalence of 1 in 10,000–20,000 individuals and no known curative treatments¹²². Angelman's syndrome is caused by a defect affecting expression of the maternal gene *UBE3A* encoding an E3 ubiquitin-protein ligase. An antisense transcript is expressed at the paternal *UBE3A* locus and represses gene expression of that allele^{123–125} (Figure 3a), raising the possibility that inactivating the noncoding

transcript might increase transcription of the paternal allele to compensate for the lack of maternal expression.

Reducing murine *Ube3a* antisense transcript (*Ube3a-ATS*) levels was achieved using phosphorothioate 2'-methoxyethyl gapmer ASOs in mice¹²⁶. Reducing transcript levels by two different ASOs led to increased expression of the paternal allele of *Ube3a*.

Although the antisense transcript that overlaps the *Ube3a* locus also encodes the genes *Snrpn*, *Snord116*, and *Snord115*, the expression of these genes was not affected¹²⁶. This was thought to be due to the fact that because the genes are far upstream of the target site for the ASOs, they are fully spliced before transcription reaches the ASO target site. This complex interplay between coding and noncoding RNA is a good example for why understanding mechanism in detail is necessary for informed interpretation of results. Importantly, administration of the ASOs to animals partially reversed some cognitive defects in a mouse model¹²⁶.

Case Study: Targeting Transcripts that Overlap Gene Promoters—

Cyclooxygenase-2 (*COX-2/PTGS2*) catalyzes the conversion of arachidonic acid into prostaglandins and is a critical regulator of gene expression. The *COX-2* gene is controlled by a TATA-box and almost all transcription begins at a single +1 transcription start site.

The *COX-2* locus encodes more than mRNA. There are additional transcripts that overlap the promoter in both the sense and antisense directions (Figure 3b)¹⁰⁸. miR-589 has two adjacent binding sites within the *COX-2* promoter transcripts and acts to up-regulate *COX-2* expression by eight fold¹⁰⁸. Designed duplex RNAs that are fully complementary to the promoter transcript enhanced expression by up to twenty fold. A gapmer targeting the promoter transcript reduced levels of the transcript and *COX-2* expression in A549 lung cancer cells, consistent with the hypothesis that the promoter RNA contains a binding site for an activating miRNA.

As little as one mismatch within the seed sequence was sufficient to disrupt activation by the promoter-targeted dsRNAs¹⁰⁸. Activation was dependent on expression of the RNAi factors AGO2 and TNRC6A and also required WDR5, a protein that stimulates histone methyltransferase activity. While AGO2 expression was required, cleavage of the promoter transcript by AGO2 was not necessary. Activity in the absence of cleavage suggests that the promoter transcripts act as a scaffold for assembly of factors near the promoter, rather than a trigger whose cleavage provokes a change in gene expression.

The gene adjacent to *COX-2* is *PLA2G4A* (phospholipase A2 group IVA, cPLA2). PLA2G4A protein produces the substrate for COX-2 enzyme, so the genes are both spatially and functionally linked. Analysis of *PLA2G4A* expression revealed that duplex RNAs targeting the *COX-2* promoter also increased *PLA2G4A* expression¹⁰⁸. As with *COX-2*, activation was dependent of expression of AGO2, TNRC6A, and WDR5. Analysis by chromosome conformation capture (3C) showed that the promoters for *COX-2* and *PLA2G4A* are physically linked, providing a means for RNA recognition to control two genes that are separated by almost 150,000 bases. For drug development, promoter-targeted

duplex RNAs might be an effective strategy for upregulating gene expression when additional amounts of a therapeutic protein are desired.

Case Study

NORAD, a noncoding RNA that sequesters PUMILIO proteins: The lncRNA *NORAD* was identified as being upregulated in response to DNA damage¹²⁷. In contrast to *COX-2* promoter RNA, but similar to *MALAT1*, *NORAD* is well expressed and present at up to 1000 copies per cell. Genetic inactivation of *NORAD* triggered chromosomal instability. *NORAD* possesses a repetitive 400 nucleotide domain and binds to PUMILIO protein 1 and 2 (PUM1, PUM2), which mediate chromosomal instability when overexpressed. These data implicate *NORAD* as a regulatory factor maintaining genomic stability.

With regard to drug discovery, these findings suggest that compounds that mimic *NORAD* might be useful for maintaining chromosome stability. Alternatively, reducing the level of *NORAD* might increase instability and make cancerous cells more susceptible to some anticancer agents.

The experimental strategy used in the study emphasizes the importance of quantitating RNA. Understanding mechanism depends on accurate evaluation of the relative amounts of PUMILIO protein and *NORAD* RNA, due to the possibility that PUMILIO function depends on *NORAD*:PUMILIO interactions. A 2-fold increase in *NORAD* expression is able to affect cellular processes profoundly, because increased *NORAD* provides ~7000 additional binding sites per molecule. It is worth considering that the pool of highly expressed and conserved lncRNAs might form a group of outstanding targets for studies exploring biological functions, with the *NORAD* study providing a useful roadmap.

Case Study: SAMMSON—The lncRNA *SAMMSON* (survival associated mitochondrial melanoma specific oncogenic non-coding RNA) has recently been linked to melanomagenesis and may represent a potential therapeutic target¹²⁸. Based on comprehensive qPCR and RNA-Seq analysis for 60 different cancer cell lines or more than 8000 tumor specimens, *SAMMSON* appears to be expressed exclusively in melanoma cells. Subcellular fractionation and FISH assay revealed that *SAMMSON* is mainly localized in the cytoplasm, especially in the mitochondria. *SAMMSON* was found to exhibit pro-oncogenic functions in melanomas *in trans* by interacting with p32, which is a known master regulator of mitochondrial homeostasis and metabolism. Intravenous injection of ASO gapmers targeting *SAMMSON* in patient-derived xenograft (PDX) melanoma models significantly suppressed tumor growth, decreased cell proliferation, and increased apoptosis, suggesting significant therapeutic potential.

Although these findings are promising additional confirmatory studies, as discussed above, would be useful prior to further development of this therapeutic approach. The studies described above, using gapmer ASOs, employed only one control oligonucleotide which was not based on the sequence of either active ASO. In addition, the animal studies did not demonstrate reduction in *SAMMSON* expression or provide evidence of ASO uptake in the target tissue. Furthermore, the number of *SAMMSON* molecules are not calculated, nor

related to the number of p32 proteins per cell. Finally the mass spectrometry data identifying p32 as an outstanding interacting partner is difficult to interpret.

Despite these drawbacks, the potential of targeting *SAMMSON* clearly warrants further investigation.

Conclusions

Cellular RNAs play critical roles during disease progression and comprise a diverse class of targets for drug discovery. Three decades of research have demonstrated that synthetic nucleic acids are a promising starting point for drug development, but that they have the potential to mislead the unwary. This valuable experience can be applied to noncoding RNAs. If used wisely, prior experience will speed the development process by avoiding past mistakes.

Noncoding RNAs act through mechanisms that are novel and often poorly understood. While it is tempting to believe that simply observing a physiologic effect is sufficient as the basis for a drug discovery platform, knowledge of mechanism will be necessary. Projects that build a strong case that a physiologic effect is due to interactions at noncoding RNA will be much more likely to succeed over the long term than those that do not.

Which class of noncoding RNAs have the most potential to be developed as therapeutic targets? In the near term, compounds that bind to miRNAs or that affect splicing function act through the best understood mechanisms and will be the focus of most clinical development. In the longer term, it is likely that our understanding of the mechanism of lncRNAs and other noncoding RNAs will grow. New classes of potential noncoding targets may emerge. As our understanding of mechanism improves, the design of effective development programs will be put on a firmer foundation and the likelihood of clinical success will increase.

Drug discovery involving noncoding RNA will need to walk a fine line. On one hand, RNA has a proven repeatedly capable of surprising biological activities and daring hypotheses and remarkable results should be taken seriously. On the other hand, RNA has been prone to irreproducible research. Drug discovery and development should combine an eagerness to test the limits of the possible with a skepticism that demands a deep focus on the link between molecular interactions and biological outcomes.

Masayuki Matsui earned his Ph.D. from Kobe University in 2007 for his research on construction of DNA-based glycoclusters. Dr. Matsui then joined the Corey lab at University of Texas Southwestern Medical Center as a postdoctoral fellow. His research interests are gene expression regulation and its therapeutic applications by oligonucleotides. He has been involved in several different research projects including allele-specific silencing of triplet repeat disease genes by antisense oligonucleotides, gene activation by promoter-targeted dsRNAs, and development of chemically-modified single-stranded siRNAs/miRNAs.

David Corey earned a Ph.D. in chemistry from the University of California at Berkeley. After postdoctoral work at the University of California San Francisco he joined the faculty of the Department of Pharmacology of UT Southwestern Medical Center. Dr. Corey's

research has focused on the intersection of nucleic acid chemistry and biology. Research interests have included inhibition of human telomerase, allele-selective inhibition of genes containing expanded trinucleotide repeats, RNA-mediated activation of gene expression, and RNAi in mammalian cell nuclei.

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Glossary

Noncoding RNA

(ncRNA). RNA transcripts that do not code for protein

MicroRNA

(miRNA). Small (~22 nucleotides) noncoding transcripts generally thought to silence gene translation through RNA interference

Long noncoding RNA

(lncRNA). Relatively long (>200 nucleotides) noncoding transcripts

Antisense oligonucleotide

(ASO). A synthetic single-stranded oligonucleotide that is designed to bind a complementary cellular RNA sequence by Watson-Crick base-pairing

Off-target effect

Phenotypic effects in a cell or animal upon addition of a synthetic compound that are not caused by interactions with the intended cellular target

Droplet digital PCR

(ddPCR). A PCR technology that uses nanoliter-sized oil-water emulsion droplets as PCR reaction vessels and quantifies concentrations of target DNA templates based on counting the number of PCR-positive droplets by a flow cytometer

Box 1**Sources for off-target effects**

Irreproducible research and misguided interpretation of results are an important roadblock to biomedical science^{129,130}. Irreproducible research is especially troubling for gene silencing because it is easy to assume that agent X silences gene Y through interactions at the intended target site because agent X and gene Y have predictable Watson-Crick complementarity. Unfortunately, the situation is not so simple. ASOs and duplex RNAs are large, polar, and highly charged. Because of their size and complexity, nucleic acid-based drugs can provoke off-target effects by multiple mechanisms:

- Protein binding - Nucleic acids can bind to proteins on the surface of cells or inside cells. Such binding can cause many changes including induction of the interferon response. This outcome is so potent that oligonucleotides designed to provoke the immune response have been developed as antiproliferatives and vaccine adjuvants³².
- Partial complementarity to unintended targets - Even if designed to be fully complementary to only their intended target, nucleic acids will also have partial complementarity to many other targets. The potential magnitude of this problem is emphasized by the realization that as few as seven base complementarity (seed match) between miRNA and its target is adequate for mediating expression.
- Toxicity - Any synthetic organic molecule has the potential to affect cell proliferation if the concentration is high enough. In our hands, with synthetic nucleic acids in cultured cells delivered by cationic lipid, the window for efficacy is between 1-50 nM for cells cultured in six well dishes (efficacy varies as media volume to well surface area changes).

Suggestions for increasing research reliability include: 1) Identify alternate hypotheses to explain interesting results and vigorously test them; 2) Identify multiple active ASOs or duplex RNAs; 3) Test multiple negative controls (scrambled or mismatched oligomers); 4) Determine the copy number of the RNA target per cell; 4) Validate key conclusions by multiple independent experimental approaches; 5) Perform key experiments multiple times in independent trials; and 6) Investigate mechanism sufficiently to establish a working hypothesis to explain activity.

Box 2**Genasense - A lesson why mechanism matters**

The development of genasense (Oblimersen sodium; Genta)¹³¹, a “first generation” antisense oligonucleotide composed of phosphorothioate DNA (PS-DNA) that targets the mRNA encoding Bcl-2, provides an instructive example of the need to understand the mechanism of action of nucleic-acid based drugs. Bcl-2 is overexpressed in some cancer cells and blocking its expression was thought to make cells more susceptible to treatment with established anticancer agents and apoptosis^{132,133}.

PS-DNA lacks the affinity for target sequences that characterizes “second generation” gapmer designs that contain various ribose modifications on the 3′ and 5′ flanks in addition to PS linkages²¹. In retrospect, initial experiments showed no more than modest potential. Nevertheless, Genasense moved through clinical trials that went on for more than a decade. In spite of promising initial clinical results, repeated phase III trials failed to show sufficient benefit^{134,135}.

While these trials went on, the Stein laboratory showed that the pro-apoptotic effects of Genasense were independent of its sequence¹³⁵⁻¹³⁷. Inhibiting Bcl-2 expression by duplex RNA that act through RNAi did not induce apoptosis, further suggesting that observed anti-proliferative effects could not be due to recognition and silencing of the intended Bcl-2 target. Oligonucleotides that contain phosphorothioate modifications are known to be susceptible to off-target effects¹³⁸ and interactions with unpredicted targets probably explains Genasense' action.

Up to one billion dollars was spent on the development of Genasense, before bankruptcy ended trials in 2012. Had mechanism been thoroughly investigated early in the drug discovery process, it is likely that these trials would never have been initiated, patients would have been spared a treatment that yielded no clear benefits, and resources directed towards more fruitful efforts.

During the years of Genasense' development and sometimes promising clinical trial results, the question was raised regarding whether understanding mechanism mattered^{139,140}. A patient may not care about the mechanism of a successful drug, but knowing the mechanism of a drug development candidate will make it much more likely that the drug will be successful and applied in a manner most likely to be beneficial.

Key points

- Noncoding RNAs (ncRNAs) - which include microRNAs, repetitive RNAs, intronic RNAs, and long ncRNAs - are a diverse group of biomolecules with broad potential to control gene expression.
- Compounds that target ncRNAs have the potential to control expression of disease-related genes
- Development of compounds to target ncRNAs can benefit from understanding the lessons learnt from decades of research using antisense oligonucleotides (ASOs) and duplex RNAs to control expression of mRNA.
- ASOs that affect splicing or that are complementary to miRNAs are already being tested in multiple clinical trials in a variety of diseases, including cancer and muscular dystrophy
- Long noncoding RNAs can affect transcription or splicing and are emerging as a promising class of novel drug targets.

Figure 1. Regulating RNA levels or splicing with ASOs and duplex RNAs

a) Reduction of cellular RNAs by ASO gapmers. RNase H recognizes the DNA-RNA duplex and cleaves the target. **b)** Reduction of cellular RNAs by small silencing RNAs (siRNAs). One strand (guide strand) is loaded into argonaute 2 (AGO2) protein and an active RISC complex is formed. The complex binds to the complementary sequences on a target RNA and cleaves it. **c)** miRNA inhibitors complementary to specific endogenous miRNAs bind to specific miRNAs and inactivate them. **d)** Gene expression regulation by miRNA mimics. Double-stranded or chemically modified single-stranded miRNA mimics reduce target gene expression by destabilizing target RNAs and/or inhibiting translation. **e)** Regulation of alternative splicing by ASOs targeting regions close to intron-exon junctions. **f)** Modulation of transcription and epigenetic status by ASOs or dsRNAs targeting promoter-associated RNAs.

Figure 2. ASOs and duplex RNAs targeting repetitive RNAs

a) Antisense oligonucleotides used for therapy of myotonic dystrophy type 1 (DM1). DM1 is caused by gain-of-function toxicity from CUG-repeat containing DMPK transcripts. ASOs targeting the CUG repeats can be used to prevent binding of muscleblind like splicing regulator 1 (*MBNL1*) to the repeats. Gapmer ASOs targeting outside the repeat are also used for depleting toxic nuclear DMPK transcripts. **b)** Activating *FXN* expression using GAA repeat-targeting oligonucleotides. *FXN* encoding frataxin is a causative gene for FA and its expression is suppressed at least partly because of the R-loop formation between the expanded GAA repeats within the intron1 of *FXN* pre-mRNA and the genomic DNA in patients (upper). Double-stranded RNAs or single-stranded locked nucleic acid (LNA) ASOs targeting the GAA repeats can inhibit the R-loop formation in the repeat region and change histone modifications surrounding the repeats, leading to activation of *FXN* gene expression (lower)⁷⁸. Image 2b was modified, with permission, from REF. 77.

Figure 3. Modulating gene expression by targeting cis-acting ncRNAs or promoter RNAs

a) Targeting *UBE3a-ATS* ncRNAs using ASOs. *UBE3a* encoding an E3 ubiquitin ligase is the imprinted gene. In the brain, paternal *UBE3a* is silenced by antisense transcripts *UBE3a-ATS* and maternal deficiency of *UBE3a* causes Angelman syndrome. Depletion of nuclear *UBE3a-ATS* using specific gapmers activates paternal *UBE3a* expression¹²⁶. **b)** *COX-2* expression regulation mediated by promoter RNAs produced from the upstream of the *COX-2* promoter. The transcript could function as a scaffold for interaction of complementary small RNAs in complex with AGO and TNRC6A. Binding of the protein-small RNA complex to the promoter RNAs could further trigger recruitment of some histone modifiers (e.g. WDR5) and transcription factors (e.g. NFκB, CREB1) to the *COX-2* promoter, causing transcriptional upregulation of *COX-2*. These molecular recognitions also affect expression of the adjacent *PLA2G4A* gene¹⁰⁸. Image 3b was modified, with permission, from REF. 108.

Table 1
Representative oligonucleotide-based drugs targeting or mimicking ncRNAs currently in clinical trials*

Company	Drug name	Chemistry**	Target	Disease	Clinical Status	Refs
Mima	MRX-34	dsRNA (liposomal nanoparticle formulation)	miR-34 targets	Solid tumors and hematological malignancies	I	39,141
Regulus	RG-101	GalNAc-conjugated	miR-122	Hepatitis C virus (HCV)	II	142,143
	RG-012	n/a	miR-21	Alport syndrome	I	144
	RG-125 (AZD4076)	GalNAc-conjugated	miR-103/107	Non alcoholic steatohepatitis	I	145
Roche	miravirsen (SPC3649)	LNA anti-miR-122	miR-122	Hepatitis C Virus (HCV)	II	146–149
BioMarin	drisapersen/ GSK-2402968/ PRO051	2'-OMe, PS,	Exon 51 of dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	III (completed)	50,51,58
	BMN 044/ PRO044	2'-OMe, PS	Dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	I/II, II	–
	BMN 045/ PRO045	2'-OMe, PS	Dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	IIb	–
	BMN 053/ PRO053	2'-OMe, PS	Dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	I/IIa	–
Sarepta	eteplirsen/ AVI-4658	PMO	Exon 51 of dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	III	56
	SRP-4053	PMO	Dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	I/II	–
	SRP-4045	PMO	Dystrophin pre-mRNA	Duchenne muscular dystrophy (DMD)	I	–
Ionis/ Biogen	nusinersen (IONIS-SMN _{Rx})	2'-O-MOE, PS, gapmer	Intron 7 of <i>SMN2</i> pre-mRNA	Spinal muscular atrophy (SMA)	II, III	57, 150
	IONIS-DMPK-2.5 _{Rx}	2'-O-MOE, cEt, PS, gapmer	<i>DMPK</i> mRNA	Myotonic dystrophy type 1 (DM1)	I/IIa	–

* This table focuses on oligonucleotide-based drugs targeting or mimicking ncRNAs (e.g. microRNAs, repetitive RNAs, intronic RNAs). Clinical status presented in this table is based on the information from each company's website or the databases (ClinicalTrials.gov (<https://clinicaltrials.gov/ct2/home>), Adis Insight (<http://adisinsight.springer.com>)) as of March 2016. Mirna Therapeutics (<http://www.mirna Therapeutics.com>); Regulus Therapeutics (<http://www.regulusrx.com>); Roche (<http://www.roche.com/index.htm>); BioMarin Pharmaceutical Inc. (<http://www.biopharm.com>); Sarepta Therapeutics Therapeutics (<https://www.sarepta.com>); Ionis Pharmaceuticals (<http://www.ionispharma.com>); Biogen (https://www.biogen.com/en_us/home.html);

GalNAc, N-acetylgalactosamine; PS, phosphorothioate; LNA, locked nucleic acid; 2'-OMe, 2'-O-methyl; PMO, phosphorodiamidate morpholino oligomer; 2'-O-MOE, 2'-O-methoxyethyl; cEt, 6'-(5)-CH₃ bicyclic nucleoside; HCV, hepatitis C virus; DMD, Duchenne muscular dystrophy; *DMPK*, dystrophin myotonia protein kinase; *SMN2*, survival of motor neuron 2, centromeric SMA, spinal muscular atrophy; DM1, myotonic dystrophy type 1. n/a, information not available.