Therapeutic targeting of non-coding RNAs

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Abstract

ncRNAs (non-coding RNAs) are implicated in a wide variety of cellular processes, including the regulation of gene expression. In the present chapter we consider two classes of ncRNA: miRNAs (microRNAs) which are post-transcriptional regulators of gene expression and IncRNAs (long ncRNAs) which mediate interactions between epigenetic remodelling complexes and chromatin. Mutation and misexpression of ncRNAs have been implicated in many disease conditions and, as such, pharmacological modulation of ncRNAs is a promising therapeutic approach. miRNA activity can be antagonized with antisense oligonucleotides which sequester or degrade mature miRNAs, and expressed miRNA sponges which compete with target transcripts for miRNA binding. Conversely, synthetic or expressed miRNA mimics can be used to treat a deficiency in miRNA expression. Similarly, conventional antisense technologies can be used to silence IncRNAs. Targeting promoter-associated RNAs with siRNAs (small interfering RNAs) results in recruitment of chromatin-modifying activities and induces transcriptional gene silencing. Alternatively, targeting natural antisense transcripts with siRNAs or antisense oligonucleotides can abrogate endogenous epigenetic silencing leading to transcriptional gene activation. The ability to modulate gene expression at the epigenetic level presents exciting new opportunities for the treatment of human disease.

Keywords: long non-coding RNA, microRNA, natural antisense transcript, transcriptional activation, transcriptional gene silencing.

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Introduction

Transcription of the mammalian genome is ubiquitous [1,2] and occurs in both sense and antisense orientations [3–5]. Only a small fraction (~1%) of the genome codes for protein and so the vast majority of cellular transcriptional output is therefore ncRNA (non-coding RNA). Although there are many classes of ncRNAs with wide-ranging functionalities (e.g. RNA editing, mediation of mRNA splicing, ribosomal function), in the present chapter we will primarily be concerned with the role of ncRNAs in the regulation of gene expression. As modulators of gene expression, ncRNAs are promising therapeutic targets. In the present chapter we will consider two general classes of ncRNA molecules: miRNAs (microRNAs) and lncRNAs (long ncRNAs).

miRNAs

miRNAs are small RNA sequences (21–23 nt) that are endogenous RNAi (RNA interference) effectors [6]. Typically, miRNAs are derived from longer pri-miRNA (primary-miRNA) transcripts that are transcribed by RNA Pol II (RNA polymerase II). Pri-miRNAs can either be intergenic transcripts or the mRNAs of protein-coding genes (with the miRNA sequences contained within one or more introns). Pri-miRNA transcripts are progressively processed by the enzyme Drosha (in the nucleus) and then Dicer (in the cytoplasm) to generate the pre-miRNA (precursor-miRNA) hairpin and mature miRNA species respectively. Following Dicer cleavage, one strand of the miRNA hairpin is loaded into the RNA-binding protein AGO2 (Argonaute 2), the so called ‘catalytic engine of RNAi’, and a component of the RISC (RNA-induced silencing complex) [7]. The mature miRNA sequence guides the RISC to its mRNA targets in the cytoplasm where it binds, typically in the 3′ UTR (untranslated) region, to form an imperfect duplex and induce translational repression and/or mRNA decay [8–10] (Figure 1). As miRNAs can induce silencing with only partial complementarity to a target transcript (i.e. multiple mRNA–miRNA mismatches are tolerated), each miRNA can bind to multiple mRNA targets. Similarly, each mRNA 3′ UTR contains multiple potential miRNA-binding sites. Consequently miRNAs can act as master regulators of gene expression by regulating families of transcripts with related functions [11]. Individual miRNAs have been implicated in a wide variety of physiological and pathophysiological processes and, as such, are potential pharmacological targets [12].

miRNA therapeutics

In instances where the activity of an miRNA is a causitive factor in pathology, strategies which antagonize miRNA activity are desirable. These fall into two broad categories: (i) small oligonucleotide miRNA inhibitors and (ii) expressed miRNA sponges. Conversely, miRNA replacement therapy can be utilized to correct an miRNA deficiency, or to modulate an endogenous protective pathway (Figure 1).

miRNA antagonism

There are numerous examples of miRNAs that promote pathology in human disease and are consequently therapeutic targets. For example, many viruses express specific miRNA genes...
Figure 1. The miRNA pathway and targets for therapeutic intervention

Schematic diagram showing the miRNA pathway whereby pri-miRNA transcripts are progressively processed to generate the mature miRNA species. First, a complex of Drosha and DGCR8 (Di George Syndrome critical region gene 8) cleaves the pri-miRNA transcript to produce the pre-miRNA in the nucleus. Export of the pre-miRNA hairpin from the nucleus is facilitated by exportin-5. In the cytoplasm, the pre-miRNA is cleaved by Dicer generating a ~21 bp RNA duplex. Dicer is part of the RISC along with AGO2 and TAR RNA-binding protein 2 (TRBP). One strand of the duplex is loaded into AGO2 and the other strand (labelled miRNA*) is subsequently degraded. RISC is then guided to complementary target mRNAs and induces translational repression or mRNA decay. Approaches for miRNA replacement therapy and miRNA antagonism are indicated. miRNA mimics enter the miRNA pathway at various stages. Expressed pri-miRNA and pre-miRNA mimics enter at the Drosha and Dicer cleavage steps respectively. Conversely, mature miRNA mimics (either bulged duplexes or completely complementary siRNAs) enter RISC directly without prior processing. Anti-miRNA oligonucleotides or antagomirs bind to mature miRNA species in the cytoplasm and induce degradation or sequestration of the target miRNA. Similarly, oligonucleotides can bind to target mRNAs and mask an miRNA-binding site. Expressed miRNA sponges are transcripts containing multiple miRNA-binding sites which compete with endogenous mRNAs for miRNA binding.
[13], or are dependent upon host cell miRNAs for viral replication [14]. One promising application of miRNA inhibition is in the treatment of cancer, as miRNA-mediated gene regulation has been implicated in tumorigenesis and metastasis [15]. For example, inhibition of miR-10b in a mouse mammary tumour model resulted in a reduction in lung metastasis [16]. Anti-miRNA technologies are currently the most advanced miRNA-based therapeutic strategies with the most commonly used approach being AMOs (anti-miRNA oligonucleotides). These are single-stranded oligonucleotides consisting of the reverse complement sequence of a target miRNA that function by either degrading or sequestering the target miRNA. Alternatively, oligonucleotides complementary to target mRNAs block miRNA binding at individual recognition sites [17]. This target masking strategy allows for the inhibition of specific miRNA–mRNA interactions.

The in vivo delivery of AMOs is a substantial obstacle to their effective use as therapeutics. Typically, AMOs contain extensive chemical modification to both the oligonucleotide backbone and the ribose sugar (e.g. substitution at the 2′-hydroxy with O-methyl or O-methoxyethyl groups) in order to increase nuclease stability, reduce clearance and improve pharmacokinetic properties. For example, the commonly used phosphorothioate backbone modification has the dual effect of improving oligonucleotide nuclease stability and conferring non-specific protein binding with a concomitant increase in serum half-life. The incorporation of LNA (locked nucleic acid) bases results in an increase in the T_m (melting temperature) of AMOs and favours binding to RNA over DNA [18]. Similarly, the nucleic acid analogue PNA (peptide nucleic acid) has also been utilized to antagonize miRNA activity [19,20]. Alternatively, the conjugation of AMOs with lipophilic moieties, such as cholesterol in the case of antagonimirs, results in improved cellular uptake [21,22] (Figure 2). AMOs have successfully been delivered systemically to liver, kidney, bone marrow and adipose tissue, and locally delivered to lung, gut, brain and eye in model organisms (reviewed in [23]). It has also recently been shown that naturally derived lipid microvesicles called exosomes can deliver exogenous RNA cargos to brain following systemic administration [24]. It will be interesting to see whether AMOs can be delivered via a similar strategy.

At the time of writing the most advanced anti-miRNA therapy is currently in Phase IIa clinical trials (http://www.clinicaltrials.gov). Miravirsen, developed by Santaris Pharma A/S to treat chronic HCV (hepatitis C virus) infection, is a 15-mer LNA-modified phosphorothioate antisense oligonucleotide inhibitor of miR-122. Endogenous miR-122 is required for HCV viral replication [14] and antagonism of this miRNA was shown to reduce viraemia in a chronically infected chimpanzee model with no evidence of toxicity [25].

The effects of AMOs are transient as they are dependent on the presence of the effector molecule. Consequently, expressed miRNA decoys or sponges have been developed in order to elicit longer-term miRNA inhibition [26]. These virus or plasmid-encoded transcripts contain multiple miRNA target sites and compete with endogenous target mRNAs for miRNA binding. miRNA sponges have been used successfully to inhibit miR-9 in highly malignant 4T1 cells, leading to suppression of metastasis [27]. Expressed miRNA inhibitors are an alternative therapeutic modality to the use of AMOs although, as they will probably require viral vector-mediated delivery in vivo, they are subject to the limitations and risks associated with classical gene therapy [28].

miRNA inhibition strategies are not limited to diseases in which aberrant miRNA expression is a causative factor in the pathology. In the muscle wasting condition DMD
(Duchenne muscular dystrophy), loss-of-function mutations in the gene which encodes the dystrophin protein lead to progressive muscle weakness and are ultimately fatal. miR-31 is highly up-regulated in dystrophic muscle and acts to suppress translation of dystrophin mRNA. This interaction is clinically relevant as the activity of miR-31 limits the efficacy of efforts to restore dystrophin protein expression by exon skipping therapy. Inhibition of miR-31 using an miRNA sponge in combination with exon skipping was shown to be more effective at restoring dystrophin than exon skipping alone [29]. This study demonstrates that modulating miRNA activity can be an effective means of boosting the expression of therapeutically relevant genes.
miRNA replacement therapy

miRNA mimics are synthetic or expressed oligonucleotides that mimic the function of endogenous miRNAs. As miRNA expression is frequently dysregulated in tumours [30] and some miRNAs have been shown to have tumour-suppressive functionality [31], miRNA mimics are potential anti-cancer therapeutics. For example, adenovirus-mediated delivery of an expressed miR-26a mimic resulted in inhibition of tumour progression in a murine hepatocellular carcinoma model [32]. Conversely, miRNA mimics can be used to modulate pathophysiological processes. miR-29 is known to regulate fibrosis by suppressing the expression of collagens, fibrillins and elastin [33]. miR-29 expression is reduced in dystrophic muscle, leading to fibrogenesis in DMD. Consequently, treatment with a synthetic miR-29 mimic was capable of reducing fibrosis and improving pathology in the mdx mouse model of DMD [34].

Considering that AMOs are required only to bind mature single-stranded miRNAs with high affinity and specificity there are relatively few constraints on what chemical modifications can be incorporated in oligonucleotide design. Conversely, synthetic miRNA mimics have much more stringent requirements on chemical composition as RISC incorporation is essential for function. Synthetic miRNA mimics must primarily consist of RNA nucleotides with relatively few chemical modifications tolerated. As such, the development of therapeutic miRNA mimics has lagged behind that of anti-miRNA technology. Additionally, the delivery of synthetic miRNA mimics is subject to the same delivery obstacles as siRNAs (small interfering RNAs) (reviewed in [35]).

miRNA-mediated transgene inactivation

Classical gene therapy typically involves the delivery of DNA encoding a therapeutic transgene to treat or manage disease. Transgene expression in professional APCs (antigen-presenting cells) can lead to immune-related vector clearance and consequently limit therapeutic efficacy. To address this problem, Brown et al. [36] engineered a lentiviral vector expressing a GFP (green fluorescent protein) reporter to contain miRNA target sites complementary to miR-142-3p (which is highly expressed in immune cells) in its 3′ UTR. This strategy restricted GFP expression to non-haemopoietic cells and resulted in stable transgene expression in the desired target tissues [36]. A similar approach has been used with respect to adenovirus-mediated oncolytic virotherapy for cancer. In this case, high expression levels of the adenoviral E1A protein in hepatocytes results in acute liver toxicity. To abrogate this toxicity, miRNA target sites for a liver-specific miRNA, miR-122, were inserted into the 3′ UTR of the viral transgene. Mice treated with these miRNA-restricted adenoviral vectors showed reduced viral replication in the liver and almost no liver toxicity [37]. Thus, by taking advantage of endogenous miRNA regulation, the expression of therapeutic transgenes can be fine-tuned to minimize toxic off-target effects.

IncRNAs

IncRNAs are a heterogeneous group of RNA transcripts >200 nt in length with low protein-coding potential that are processed in similar ways to mRNAs (i.e. the majority are spliced and
many are polyadenylated). IncRNAs can be sense or antisense transcripts with respect to a neighbouring protein-coding gene locus, intron-derived, the products of divergent bidirectional transcription or reside in the space between genes [i.e. lincRNAs (long intergenic ncRNAs)] (Figure 3a). Many IncRNAs show distinct spatial, temporal, cell-type specific and subcellular-specific expression patterns. It has been argued that this is indicative of tightly regulated transcription and therefore unlikely to be transcriptional ‘noise’ [38,39]. In addition, many IncRNAs show a high degree of evolutionary conservation consistent with biological function [40]. Given that there are thousands of IncRNAs, many of which have been implicated in cellular processes, these transcripts represent a multitude of potential drug targets.

**IncRNA function**

IncRNAs primarily act as adaptors that mediate interactions between chromatin, proteins and other RNAs. Two properties of IncRNA molecules enable them to function in this manner. First, they can bind to DNA or other RNA molecules by (i) complementary Watson–Crick base pairing to form hetero- or homo-duplexes, (ii) formation of DNA–DNA–RNA triplexes by Hoogstein and reverse Hoogstein base pairing, or (iii) by direct RNA recognition of chromatin surface features [41]. Secondly, the inherent flexibility of RNA permits the formation of complex secondary structures that can function as binding domains for proteins or small molecules (Figure 3b). The combination of these properties enables a much wider range of functions than is possible with miRNAs. Additionally, IncRNAs may also contain multiple binding modules allowing for complex multi-functional interactions.

Arguably the most exciting role of IncRNAs is as epigenetic regulators of gene expression. IncRNAs form riboprotein complexes where the ncRNA acts as a guide that targets

**Figure 3. Genomic organization and function of IncRNAs**

(a) Genomic organization of IncRNAs (blue) with respect to protein coding genes (black). Arrows indicate the direction of transcription. IncRNAs can be partially or completely overlapping with protein coding genes, reside within introns (intrinsic) or the space between genes (intergenic), or result from bidirectional transcription at promoters. (b) IncRNAs can interact with other RNA molecules (RNA–RNA homoduplex), transient single-stranded genomic DNA (gDNA) regions (RNA–DNA heteroduplex), form RNA–DNA–DNA triplexes with gDNA, or fold into secondary structures which can directly bind to chromatin.
chromatin-modifying activities or transcription factors to specific genomic loci. For example, RepA ncRNA [derived from the 5′ region of the Xist (X-inactive specific transcript) transcript] binds PRC2 (Polycomb Repressive Complex 2) and guides it to induce H3K27 (histone H3 Lys27) tri-methylation and heterochromatin formation at the Xist promoter. This event initiates the process of XCI (X-chromosome inactivation) [42]. Similarly, in the case of rRNA genes, a promoter-associated RNA transcript forms a triplex structure at the rRNA promoter and recruits DNMT3B [DNA (cytosine-5-)-methyltransferase 3 β] in order to induce promoter DNA methylation and transcriptional gene silencing [43,44]. Thus RepA and the rDNA promoter transcript act as guides for epigenetic modifiers in cis (i.e. influencing neighbouring genes). Conversely, IncRNAs can also function as guides in trans (i.e. affecting distal genes). For example, lincRNA-p21 is able to guide epigenetic remodelling at multiple genomic loci via recruitment of hnRNP (heterogeneous nuclear ribonucleoprotein)-k [45].

IncRNAs can also act as RNA scaffolds that remain associated with a chromatin locus and recruit multiple epigenetic modifiers. As a result, complex changes in chromatin states can be dynamically co-ordinated in response to external signals. The IncRNA HOTAIR, which is transcribed from the HOXC cluster, binds to the HOXD cluster. HOTAIR associates with PRC2 at its 5′ region [46] and a complex containing LSD1, CoREST and REST at its 3′ region [47]. Consequently, the activities of these two complexes [i.e. tri-methylation of H3K27 and demethylation of H3K4 (histone H3 Lys4) respectively] are co-ordinated in order to induce transcriptional silencing. Similarly, scaffold IncRNAs transcribed from pericentromeric satellite regions have been shown to associate with SUMOylated-HP1 (heterochromatin protein 1) which induces further recruitment of additional HP1 molecules and transcriptional silencing [48]. The epigenetic silencing complexes SMCX and PRC1, and the epigenetic activating complex WDR5/MLL have also been shown to associate with IncRNAs [49,50].

Recently, it was shown that IncRNAs can influence the subnuclear localization of genomic loci. The IncRNA TUG1 was shown to bind methylated Pc2 (a polycomb component) and thereby direct the accompanying genomic DNA to polycomb bodies where it is epigenetically silenced. Conversely, unmethylated Pc2 was bound by another IncRNA, MALAT1 (metastasis associated in lung adenocarcinoma transcript-1; also known as NEAT2), which resulted in localization to ICGs (interchromatin granules) which are associated with active transcription [51]. Thus post-translational modification of a non-histone protein is capable of influencing the subnuclear localization of chromatin through interactions with IncRNAs.

IncRNAs also act as ‘riboregulator’ decoys by binding and sequestering proteins (e.g. transcription factors and chromatin modifiers) or other RNAs. For example, the IncRNA Gas-5 (growth arrest-specific 5) forms an RNA secondary structure that binds to the DNA binding domain of GR (glucocorticoid receptor) and prevents it from interacting with its DNA target sites, thus repressing GR activity [52]. Additionally, IncRNAs have been shown to act as endogenous miRNA sponges [53,54] or to mask miRNA-binding sites on target mRNAs [55]. Similarly, MALAT1 alters the subnuclear localization of the splicing factor SR through sequestration in nuclear speckles [56,57].

IncRNAs also exert non-epigenetic effects on gene expression. Transcription of upstream IncRNA genes is sufficient, in some cases, to inhibit transcription of downstream genes, a phenomenon known as transcriptional interference [58]. In the case of the DHFR (dihydrofolate reductase) locus, a 5′ sense promoter RNA forms a direct association with DHFR promoter.
DNA in order to inhibit transcription (in addition to acting as a decoy for the basal transcription factor TFIIB) [59].

Where overlapping bidirectional transcription occurs, there is potential for hybridization between the pair of sense and antisense transcripts. In the case of BACE1 (β-secretase 1), a gene involved in the pathophysiology of Alzheimer’s disease, a 3′-overlapping antisense RNA (BACE1-AS) forms an RNA duplex with the BACE1 mRNA leading to stabilization and increased β-secretase expression [60,61]. Additionally, the formation of dsRNA (double-stranded RNA) as a result of overlapping transcription can form substrates for Dicer leading to the production of endo-siRNAs [endogenous-siRNAs (small interfering RNAs)] capable of silencing complementary target mRNAs [62,63]. Mechanisms of lncRNA action are summarized in Figure 4.

**IncRNA in disease**

Considering that lncRNAs are involved in a wide variety of gene regulation processes including epigenetic remodelling and memory, control of transcription and translation [60], cellular differentiation [64,65], XCI [66], mono-allelic expression of imprinted loci [67] and modulation of splicing [57] to name only a few, it is unsurprising that some lncRNAs are also implicated in human disease. Given that only a small portion of the genome (~1%) codes for protein, the majority of mutations occur in non-coding regions [68]. Consequently, lncRNA mutations and misexpression have been linked to disease [69]. For example, mutations have been identified in lncRNAs transcribed from ultra-conserved regions in patients with CLL (chronic lymphocytic leukaemia) and CRC (colorectal cancer) [70]. Similarly, expression of HOTAIR is increased in breast tumours and correlates well with metastasis [71]. Increased levels of HOTAIR result in retargeting of the PRC2 to novel genomic sites with consequent changes in gene expression and increased tumour invasiveness. Similarly, MALAT1 was initially identified due to its association with lung metastasis [72].

In a landmark study by Tufarelli et al. [73] an antisense RNA was shown to be the direct cause of α-thalassaemia in a patient with a rare chromosome rearrangement. In this instance, the LUC7L promoter was found to be translocated immediately downstream of the HBA2 (α-globin) gene resulting in transcription of a novel antisense RNA through the CpG island in the HBA2 promoter. This antisense RNA mediates hypermethylation of the CpG island leading to epigenetic silencing of HBA2 [73]. This study was the first to demonstrate how misexpression of a ncRNA could directly lead to human disease.

A study by Lewejohann et al. [74] revealed that mice in which the neuron-enriched ncRNA, BC1, was knocked-out showed no obvious physical or neurological defects relative to wild-type controls when under normal laboratory conditions. However, mutant mice exhibited reduced exploratory behaviour, increased anxiety and decreased survival upon reintroduction into a semi-natural outdoor environment [74]. This study raises the possibility that ncRNAs may also be involved in complex behavioural phenotypes in humans, specifically in disorders with cryptic aetiologies. Furthermore, the human homologue of BC1, BC200, is found to be up-regulated in the brains of Alzheimer’s disease patients and is mislocalized to neuronal cell bodies instead of dendritic spines, suggesting that it may be involved in disease pathophysiology [75].
Figure 4. Mechanisms of gene regulation by lncRNAs

lncRNAs can act as guides for chromatin-modifying activities or transcription factors in cis. (a) RepA guides PRC2 to the Xist locus resulting in H3K27 tri-methylation and heterochromatin formation. (b) An rRNA promoter-associated RNA guides Dnmt3b to the rRNA promoter resulting in hypermethylation of the rRNA promoter and gene silencing. In addition rRNA pRNA also forms a triplex structure with the rRNA promoter. lncRNAs can also act as guides in trans. (c) lincRNA-p21 guides hnRNP-K to multiple sites in the genome and initiates gene silencing events. (d) lncRNAs can act as scaffolds for epigenetic-modifying complexes. The lncRNA HOTAIR binds both PRC2 and a complex containing LSD1, CoREST and REST to facilitate co-ordinated H3K27 tri-methylation and H3K4 demethylation at the HOXD locus. (e) Differential binding of lncRNAs TUG1 and MALAT1 to methylated or unmethylated Pc2 targets chromatin to polycomb bodies or interchromatin granules respectively. (f) lncRNAs can also act as riboregulator decoys for transcription factors (e.g. Gas-5 sequesters the glucocorticoid receptor) or miRNAs (e.g. the pseudogene PTENP1 acts as an miRNA sponge to relieve miRNA repression of PTEN). (g) Overlapping lncRNAs can hybridize with complementary mRNAs and stabilize them. In the case of the sense–antisense pair BACE1–BACE1-AS this is, at least in part, achieved by masking a binding site for miR-485-5p in the BACE1 3′ UTR. (h) Bidirectional transcription of lncRNAs can generate substrates for Dicer which are processed into endo-siRNAs. These can induce post-transcriptional gene silencing of complementary target RNAs (e.g. endo-siRNAs generated from bidirectional transcription of L1 retrotransposon sites leads to inhibition of retrotransposition).
Cross-talk between short and long RNAs

Whereas the targeting of mRNA sequences with antisense technologies (i.e. siRNAs and antisense oligonucleotides) for post-transcriptional gene silencing is now commonplace, in recent years a number of reports have demonstrated that targeting non-coding regions can also influence gene expression. Specifically, it is now apparent that transcription occurs at many promoters, enhancers and 3′ gene termini [76–78], and that these transcripts are targets for therapeutic modulation. Early studies targeting promoters with siRNAs reported both TGS (transcriptional gene silencing) [79–81] and TGA (transcriptional gene activation) [also known as RNAa (RNA activation)] [82–84]. Tentative mechanistic models have been proposed to explain these opposing phenomena [85].

Figure 5. Model of small-RNA-mediated transcriptional gene silencing

(a) pRNA is transcribed from the promoter region of a hypothetical gene locus. This transcript remains tethered to the locus through association with RNA Pol II. (b) Targeting the pRNA with an siRNA recruits the RNA-induced transcriptional silencing complex (RITS) which includes AGO1, a histone deacetylase (HDAC1), histone methyltransferases (EZH2 and EHMT2) and a DNA methyl transferase (DNMT3A) leading to local chromatin remodelling. (c) Heterochromatin formation and promoter DNA methylation (black lollipops) results in transcriptional gene silencing of the targeted promoter.
In the case of TGS, the effector siRNA, shRNA or expressed antisense RNA is bound by AGO1 and directs the RITS (RNA-induced transcriptional silencing complex) to low-copy pRNAs (promoter-associated RNAs). RITS recruitment at a target promoter triggers remodeling of the local chromatin such that there is enrichment of silent-state chromatin modifications [i.e. H3K9me2 (histone H3 Lys9 dimethylation) and H3K27me3 histone H3 Lys27 trimethylation]) and, in some cases, promoter DNA methylation. There is evidence that RITS is comprised of HDAC1 (histone deacetylase 1), the histone methyltransferases EZH2 and EHMT2 (formerly G9a), and DNMT3A [86,87] (Figure 5). pRNAs are sense orientation transcripts that are initiated upstream of the conventional transcription start site and are required for TGS [80]. They may act as cis-regulatory sequences that remain bound to the promoter chromatin or, alternatively, they may simply be tethered to the locus by association with RNA Pol II. There is evidence that unstable pRNAs may exist at the majority of gene loci [76].

NATs (natural antisense transcripts) are RNA transcripts which overlap a sense protein-coding gene and often act to regulate the associated loci through the recruitment of histone-modifying complexes and induction of transcriptional silencing. Targeting these NATs with siRNAs or antisense oligonucleotides (also known as antagoNATs [88]) results in loss of this epigenetic silencing and consequently, TGA of the sense gene [82,84,85,88] (Figure 6). It has since been demonstrated that miRNAs can also act to regulate transcription by TGS and TGA, suggesting that cross-talk between short and long ncRNA activities is an endogenous mechanism of gene regulation [89–91]. As a result, the use of anti-miRNA oligonucleotides may also be able to disrupt endogenous networks of epigenetic regulation.

Small RNA-mediated transcriptional regulation permits both silencing and activation of therapeutic target genes. Targeting promoter proximal transcripts or NATs allows for gene-specific epigenetic manipulation with numerous potential therapeutic applications. The majority of transcriptional modulation studies have focused on the silencing of the HIV-1 provirus [81,92–94], silencing of oncogenes [95–97] and the activation of tumour suppressors [82,85], although theoretically any gene can be targeted. Recent studies targeting VEGF (vascular endothelial growth factor) [98] and BDNF (brain-derived neurotrophic factor) [99] have reported TGS and TGA in vivo, suggesting that these are plausible therapeutic strategies. The ability to regulate genes at the epigenetic level has several advantages over conventional RNAi and antisense oligonucleotide approaches (which regulate gene expression at the post-transcriptional level). Epigenetic modifications are stable and heritable, meaning that long-term silencing can be achieved through a single treatment (or short course of treatments) [86,94,100]. Repeat administration is not required as the silencing or activation effects are not dependent on the presence of effector molecules. Consequently, off-target effects are minimized, saturation of endogenous RNA processing pathways is avoided and the material cost of treatment is greatly reduced.

**Conclusions**

The use of oligonucleotides to influence miRNA and lncRNA activity is an exciting prospect for future gene therapies. By inhibiting or mimicking miRNA function whole gene networks can be exogenously regulated, providing the potential to attenuate pathological processes. There are numerous instances of miRNA involvement in human pathologies and many of these are attractive therapeutic targets. Given that the target sequence for an anti-miRNA
oligonucleotide is highly limited, the pool of potential lead compounds to screen is small, in stark contrast with other small molecule drugs. Consequently, it is also practical to screen these compounds in vivo. Ongoing progress in the development of chemically modified oligonucleotides will probably lead to nucleic acid drugs with improved pharmacokinetic and pharmacodynamic properties. Indeed, antisense oligonucleotide therapies are steadily advancing towards the clinic and an anti-miRNA therapy for HCV infection is just over the horizon. Additionally, an understanding of miRNA biology is enabling new approaches to enhance existing therapeutic strategies by restricting expression of exogenous transgene expression to

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Figure 6. Model of small-RNA-mediated transcriptional gene activation
(a) A hypothetical gene locus in which a NAT suppresses transcription of its corresponding sense mRNA by recruiting PRC2. This complex includes the histone methyltransferase EZH2 which tri-methylates Lys9 on histone H3 in order to induce epigenetic silencing. (b) Inhibition of the NAT by siRNAs or antisense oligonucleotides (also known as antagoNATs) leads to abrogation of endogenous epigenetic silencing and loss of repressive histone modifications. (c) Sense transcription increases.
specific cell types or by maximizing expression of an endogenous therapeutic gene through abrogation of miRNA regulation.

Although less well understood than miRNAs, it is clear that IncRNAs are highly important in the epigenetic control of gene expression and are potential therapeutic targets for conventional antisense technologies. Specifically, in cases where a IncRNA is directly linked to disease pathogenesis, conventional RNAi or antisense oligonucleotides can be used to modulate its expression. At present, the number of such cases is relatively small, although further investigation into the role that IncRNAs play in disease is likely to identify a plethora of novel therapeutic targets. Given the infancy of this field, the limitations of such approaches are currently unknown. For example, as many IncRNAs exhibit high degrees of secondary structure or are exclusively nuclear-localized, they may be relatively inaccessible to siRNA or antisense oligonucleotide approaches. The use of RNA aptamers targeting IncRNAs could potentially circumvent this problem [101]. Similarly, the complex tissue-specific patterns of expression of some IncRNAs may be an additional obstacle to their therapeutic modulation.

Exogenous small RNAs can regulate gene expression at the transcriptional level by either recruiting chromatin-modifying activities to specific gene promoters and inducing transcriptional silencing, or by disrupting endogenous ncRNA-mediated epigenetic regulation so as to activate transcription. Reciprocal transcriptional silencing and activation, of potentially any gene, can be achieved by targeting a sense pRNA (promoter-associated RNA) or NAT respectively. Initial reports demonstrating in vivo epigenetic modulation are highly encouraging [98,99], although future work must address the universal applicability of these phenomena and demonstrate that they are feasible therapeutic strategies.

### Summary

- Non-coding RNAs function to regulate gene expression in mammalian cells and are implicated in physiological and pathophysiological processes.
- Oligonucleotides that inhibit or mimic miRNA or long non-coding RNA activity are potential pharmaceutical agents.
- Delivery of oligonucleotides to target tissues remains a substantial obstacle to effective therapy.
- Epigenetic control of gene expression (both silencing and activation) may be possible through the targeting of long non-coding RNAs.

### References


