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Review

RNA duplexes in transcriptional regulation

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Abstract

Transcriptional regulation by small RNA molecules, including small interfering RNA and microRNA, has emerged as an important gene expression modulator. The regulatory pathways controlling gene expression, post-transcriptional gene silencing and transcriptional gene silencing (TGS) have been demonstrated in yeast, plants and more recently in human cells. In this review, we discuss the currents models of transcriptional regulation and the main components of the RNA-induced silencing complex and RNA-induced transcriptional silencing complex machinery, as well as confounding off-target effects and gene activation. We also discuss RNA-mediated TGS within the NF-κB motif of the human immunodeficiency virus type 1 5′ long tandem repeat promoter region and the associated epigenetic modifications. Finally, we outline the current RNA interference (RNAi) delivery methods and describe the current status of human trials investigating potential RNAi therapeutics for several human diseases.

Keywords: gene expression regulation; gene silencing; microRNAs; RNA interference; small interfering RNA.

Introduction

Small RNA duplexes play a pivotal role in transcriptional regulation via one of two pathways, post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). Small interfering RNAs (siRNAs), which are capable of inducing gene regulation via both pathways, have heralded a new age of gene manipulation and potential therapeutics against a range of diseases. These siRNAs were first shown to work in a PTGS manner, but more recently, siRNAs have been shown to induce transcriptional regulation via the TGS pathway (1, 2). Endogenous small RNA molecules, called microRNAs (miRNAs), have also recently been shown to play a critical role in protein translation within the cell (3). Recent evidence in lower organisms also point to a role of miRNAs in transcriptional regulation (4). We know now that miRNAs play a vital role in modulating the translation of mRNAs into protein and it is estimated that they control up to 50% of mRNA transcripts within the cell at any one point (5). This has also changed our understanding of the function of non-protein coding DNA (6). The purpose of this review is to look at our current state of understanding of transcriptional regulation by small RNA duplexes at the transcriptional and post-transcriptional level and to understand their role in future therapeutics.

PTGS mediated by small RNAs

The PTGS pathway, occurring mainly in the cytoplasm, involves recruitment of RNA duplex and Argonaute protein/s to form the RNA-induced silencing complex (RISC) and degrade mRNA via specific homologous target sequence cleavage (as illustrated in Figure 1). The process of PTGS was first described in plants when two groups reported overexpression of transgenes involved in flower pigmentation resulted in an unexpected phenotype – lighter coloured flowers (7, 8). This was because the introduced transgenes (which were used to increase pigmentation) were targeting sense transcripts and resulted in a 50-fold reduction in both the endogenous gene and transgene expression. This process was dubbed 'cosuppression'. In 1993, Lee and colleagues described the first miRNA, lin-4, in *Caenorhabditis elegans* (9). It was found that lin-4 produced two transcripts – the smaller transcript targeted the 3′ untranslated region (3′UTR) of lin-14 mRNA. They postulated that the lin-4 transcript was a negative regulator of lin-14 translation. In 1998, Fire and colleagues reported gene specific knockdown following introduction of double-stranded RNA into *C. elegans* (10). This phenomenon of gene knockdown by sequence specific RNA was later termed RNA interference (RNAi). Here, we describe the process of PTGS using endogenous miRNAs, but the same process occurs with siRNA-mediated PTGS.

PTGS induced by small RNAs

Most miRNAs are transcribed from non-coding regions of the DNA or alternatively from intronic DNA (approximately 25% of miRNAs) (11). Genes encoding miRNAs are found in all chromosomes and many are clusters of 2–7 genes (12). Numerous miRNAs are expressed in concert with protein coding genes, indicating a common transcriptional control mechanism (13). However, there are miRNA genes that are independently transcribed from their own unique promoter sequences (14). The biogenesis and processing of miRNA is outlined in Figure 2. The RNA polymerase II enzyme pro-

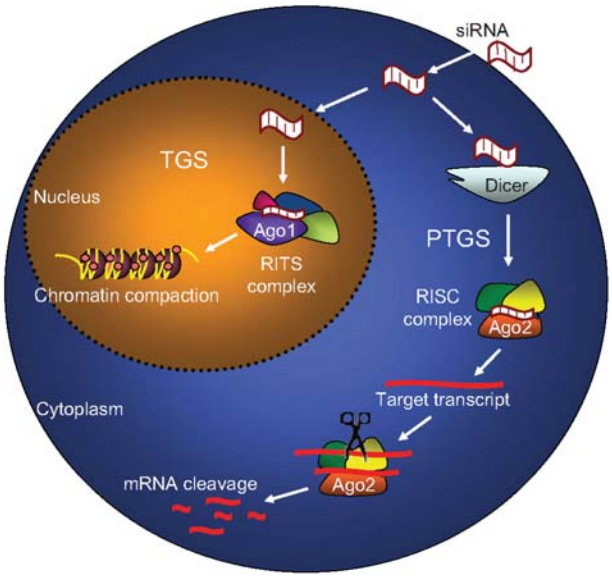

Figure 1 Mechanisms of siRNA-induced gene silencing.
RNA duplexes induce transcriptional gene silencing (TGS) in the nucleus via the RITS complex initiating epigenetic modifications, and post-transcriptional gene silencing (PTGS) via the RISC machinery initiating specific mRNA cleavage in the cytoplasm. (Ago1: Argonaute 1, Ago2: Argonaute 2, RISC: RNA-induced silencing complex, RITS: RNA-induced transcriptional silencing complex).

duces an initial transcript containing a 5′ cap and 3′ poly(A) tail, called a primary miRNA transcript (pri-miRNA) (15, 16). The pri-miRNA is processed by a microprocessor complex of Drosha and DGCR8 (DiGeorge syndrome critical region 8), which is responsible for cleaving the primary transcript to a stem-loop structure within the nucleus (17, 18). The pri-miRNA transcript is processed into a ∼70 nucleotide long precursor miRNA (pre-miRNA), containing a stem-loop structure, a 5′ phosphate and a 3′ two nucleotide overhang. DGCR8 plays a role in accurate Drosha processing by determining the Drosha cleavage site (19), which typically occurs 11 nucleotides from the base of the stem structure. The pre-miRNA molecule is then transported to the cytoplasm by Exportin V and Ran-GTP (20). Some intron-derived pri-miRNAs do not contain the 11-bp segment that is required for Drosha/DGCR8 processing. The first step in processing these 'mirtrons' is mediated by the spliceosome, instead of Drosha/DGCR8 (21, 22). After spliceosome processing, mirtrons are released as Lariat structures. These lariat mirtrons are linearised, folded into hairpin structures resembling pre-miRNAs, and then exported into the cytoplasm by Exportin 5/Ran-GTP (23).

In the cytoplasm, Dicer, another RNase III endonuclease, processes pre-miRNAs, or introduced siRNAs, into ∼22 nucleotide long mature miRNA or siRNA duplexes (24), containing a guide strand (antisense) and a passenger or sense strand. Dicer is a highly conserved cytoplasmic enzyme, containing a helicase domain, a PAZ (Piwi-Argonaute-Zwille) domain, a double-stranded RNA binding domain and two RNase III domains. It associates with TRBP (Tar RNA binding protein) (25, 26) and PACT (protein activator of the interferon-induced protein kinase) (27), which aid in the maturation of miRNAs. Dicer cleavage of pre-miRNAs leads to a two nucleotide overhang in the 3′ end of both strands, and following this the guide strand is incorporated into the RISC, whereas the passenger strand is degraded.

The RISC is formed by several proteins, including Dicer, Argonaute protein/s (typically Ago2), TRBP (28), and KSRP (KH-type splicing regulatory protein) (29). It has been recently been demonstrated that KSRP, a component of both Drosha and Dicer, binds to the terminal loop of miRNA precursors and promotes their maturation. There are four different Ago proteins in mammalian cells and all of these can bind to endogenous miRNAs; however, only Ago2 possesses endonuclease activity (30). The key region of the guide

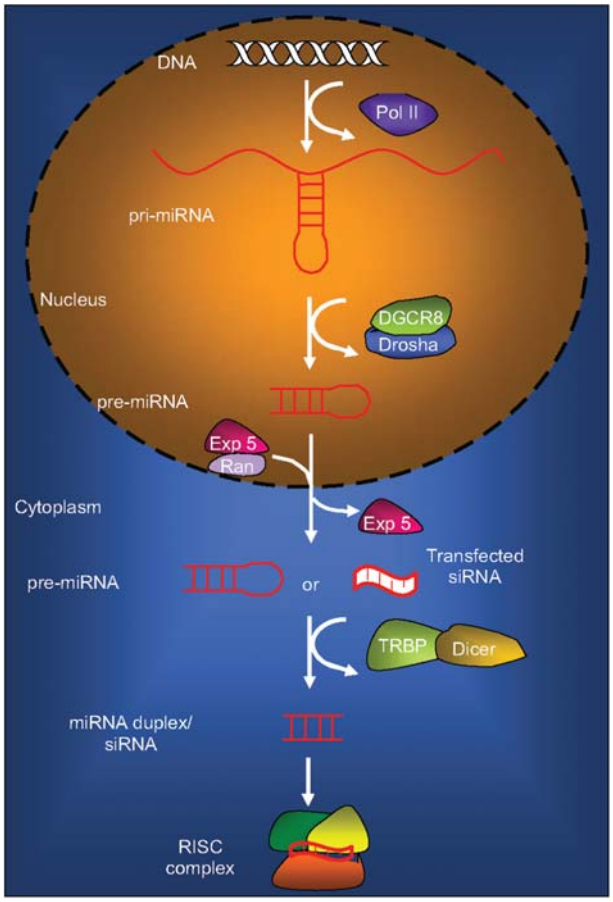

Figure 2 Small RNA processing and activity.
Processing of miRNA starts with pri-miRNA being transcribed by RNA polymerase II and forming a hairpin. The double-stranded RNA-specific ribonuclease Drosha, along with DGCR8, digests the pri-miRNA to generate pre-miRNA, which is exported from the nucleus to the cytoplasm via Exportin 5 (Exp 5) and Ran. Dicer cleavage and processing of the pre-miRNA, or siRNA, then occurs in the cytoplasm, producing a mature miRNA duplex or siRNA, which can act to control translation of target mRNAs via association with the RISC. (TRBP: Tar RNA binding protein, DGCR8: DiGeorge syndrome critical region 8).

strand is the seed region, which comprises nucleotides 2–8 from the 5′ end of the miRNA. Binding of this eight nucleotide seed to complementary nucleotides found in 3′UTR mRNA transcripts leads to RISC effector function (5). When perfect complementarity exists between siRNA and mRNA (i.e., a 22 nucleotide match), degradation occurs within the Ago2 catalytic region of RISC, whereas matches between the miRNA seed region and complementary mRNA sequences can lead to either mRNA degradation or translational repression. Prior to translation, mRNA exists in a circular form. The 5′ cap which binds eukaryotic initiation factors (eIFs) and the poly (A) tail which binds PABP [poly(A) binding protein] act in concert to stimulate cap-dependent initiation of translation. Following binding of the seed sequence to matching 3′UTR mRNA sequences, the Ago protein of RISC disrupts the PABP/eIF complex, thus disrupting initiation of translation (31, 32). There is also some evidence to suggest that deadenylation at the 3′ end of the mRNA by RISC also disrupts translation (33).

Cytoplasmic P-bodies (processing bodies) were initially thought to be the location of mRNA degradation (34) occurring after interaction with RISC. P-bodies contain various enzymes involved in mRNA degradation and RNAi-mediated gene silencing and the silencing complex contains at least an Argonaute protein and GW182 (35). However, it is not clear whether P-bodies are the site of RNAi silencing or the consequence of RNAi-medicated silencing (36, 37). It could also be possible that mRNA found in P-bodies can leave this compartment and undergo translation through the ribosome.

TGS induced by small RNAs

RNA duplexes also have the potential to act via the alternate TGS pathway, which takes place in the nucleus of the cell and involves the RNA-induced transcriptional silencing (RITS) complex, resulting in inhibition of transcription via chromatin modification (as shown in Figure 1). This process is distinct from PTGS, which can also occur in the nucleus (38); however, unlike PTGS, TGS does not involve mRNA cleavage. The first study coining the term TGS was based on experiments in tobacco plants, whereby small RNA duplexes were reported to induce DNA methylation within regions of the plant genome that was complementary to the small RNA sequence (39). The TGS field then slowly developed with further observations reported in another plant species (*Arabidopsis*) (40, 41) and fission yeast (*Schizosaccharomyces pombe*) (42), although the mechanism of transcriptional regulation in *S. pombe* was discovered to involve histone methylation and heterochromatin formation mediated by the Argonaute 1 protein in association with the RITS complex. The process appears to be critical for the regulation of centromeric chromatin in these organisms. Reports of TGS occurring in flies (*Drosophila*) and nematode worms (*Caenorhabditis elegans*) hinted towards the possibility of TGS in higher eukaryotes and indeed several groups, as well as our own, have more recently published observations of TGS in human cells (2, 43–50).

Several groups have suggested that siRNAs (and even endogenous miRNAs) can act in a functional way to direct epigenetic changes to coding regions of the genome (51). siRNAs appear to be able to recruit chromatin remodelling complexes to the transcribed loci that they contain homology to (44, 52). Evidence is accruing to suggest that single-stranded RNAs play an important role in controlling chromatin structures.

TGS-induced epigenetic changes caused by small RNAs targeting promoters

Epigenetics refers to heritable traits that are not carried in the DNA sequence. TGS occurring by siRNAs targeting the promoters of genes is thought to occur through epigenetic mechanisms. These epigenetic mechanisms encompass two major modifications, DNA methylation or post-translational histone modifications, which generate silent heterochromatin, also known as chromatin compaction (53). DNA methylation occurs almost exclusively in the context of CpG dinucleotides, with the ‘p’ referring to 3′ to 5′ phosphodiester bond between adjacent nucleotides. There are several active DNA cytosine methyltransferases (DNMT1, 3a and 3b) that add methyl groups to the 5′ position of cytosines, altering the appearance of the major groove of DNA to which the DNA binding proteins bind. Methylation of promoters, which are often CpG rich regions, is used to prevent transcriptional initiation and silencing of genes, such as X-linked inactivation.

There are over 60 different residues on histones where modifications have been detected, although these modifications are not uniformly distributed. Histone modifications are dynamic and occur rapidly, with a striking feature being the large number and type of histone modifications, particularly in the histone tail region (54). Modifications that can occur include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerisation (54). Acetylation of lysine residues generally correlates with chromatin accessibility and transcriptional activity, whereas reduced levels of acetylation are associated with silent heterochromatin. Methylation of lysine residues can have variable effects, depending on which lysine residue is methylated. Methylation of histone H3 lysine 4 (H3K4) and H3K36 is associated with transcribed chromatin, whereas methylation of H3K9, H3K27 and H4K20 generally correlates with translational repression, as shown in Figure 3, which depicts a model of siRNA targeting a promoter region to induce changes in epigenetic markers.

Histone methylation acts as a molecular ‘anchor’ which recruits proteins to either directly or indirectly modify chromatin. Current models for siRNA-mediated heterochromatin formation in fission yeast involve siRNAs guiding the Ago1 containing RITS effector complex to regions of sequence homology, often in the centromere, where the RITS complex (containing Ago1, Tas3 and Chp1) associates with the chro-

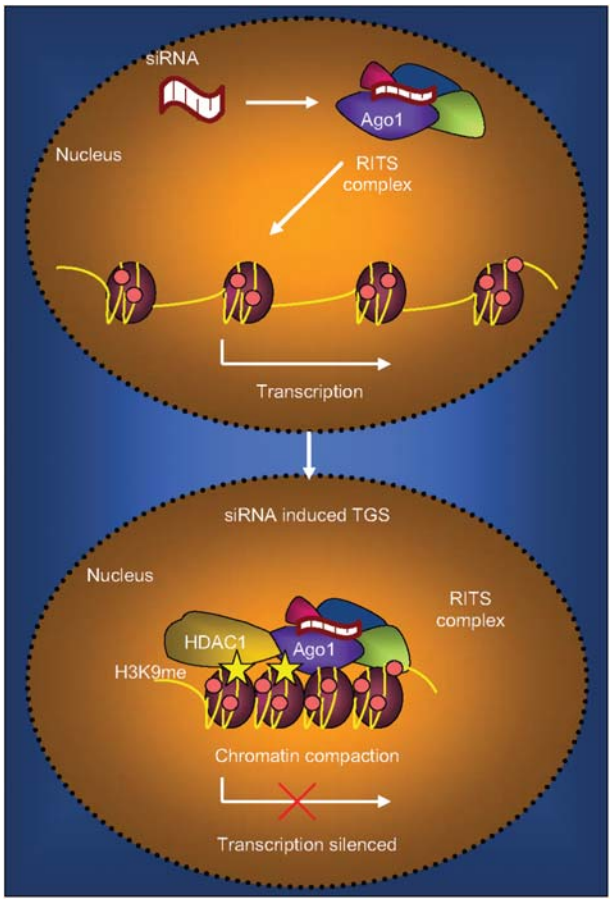

Figure 3 Model of induced heterochromatin formation by siRNA targeting a specific promoter region of HIV-1.
siRNA binds to Ago1 and initiates recruitment of the RITS complex. siRNA then binds to the target promoter sequence where HDAC, complexed to RITS, induces histone tail methylation and deacetylation [shown as H3K9me (yellow star)], resulting in chromatin compaction. (Ago1: Argonaute 1, HDAC1: histone deacetylase, RITS: RNA-induced transcriptional silencing). Adapted from Ref. (55).

matin through the chromodomain protein, Chp1. Transcript bound RITS then recruits RNA-directed RNA polymerase complex (RDRC) (comprising Rdp1, Cid12 and Hrr1) and Dicer, generating more siRNA transcripts, which through mechanisms unknown, recruit the histone methyltransferase Clr4, in association with the CLCR (Clr4-Rik1-Cul4) multisubunit complex, to initiate H3K9 methylation and create a binding site for HP1 proteins, Swi6 and Chp1. Recently, the LIM domain protein Stc1 was shown to be the key recruiting protein for CLCR and associated with both CLCR and Ago1, thereby mediating the association between CLCR and RITS, which is critical for the formation of heterochromatin (56). However, although the main components of TGS in yeast are known, the same mechanism cannot be directly applied to siRNA-mediated TGS in human cells, because several of the essential yeast RDRC components have no homologous human proteins. miRNAs have also been reported to induce TGS in yeast and plants, resulting in repressive chromatin structures from both DNA methylation and histone modification (57). Whether there are commonalities in the siRNA and miRNA pathways inducing these changes require further investigation.

There is growing evidence about the mechanism of TGS in human cells (58, 59). Weinberg and colleagues first demonstrated that the antisense strand alone of the siRNA duplex can direct sequence specific TGS in human cells using an siRNA which targeted elongation factor 1 α promoter (EF1α) (50). Han and colleagues demonstrated that the antisense strand of an siRNA, directed against EIFα, bound to the RNA Pol II expressed mRNA containing an extended 5′UTR that overlapped the EIFα gene promoter (52). The coupling between mRNA and siRNA functions as a recognition motif to direct further epigenetic changes to the promoter region.

There also appears to be a role for Ago2 when duplex RNAs are targeted directly at RNA Pol II binding sites (45, 60). Previously published research has shown a role for both Ago1 and Ago2 in siRNAs targeting genomic sequences. Chromatin immunoprecipitation (ChIP) assays with human cells treated with promoter targeted siRNAs (or a mismatch RNA duplex) demonstrated that both Ago1 and Ago2 associate with the promoter region and this did not occur with the mismatch RNA duplex. Looking at an alternate promoter (the GADPH promoter) showed no enrichment for Ago1 or Ago2, suggesting that these proteins are not normally associated with promoter elements in the absence of promoter specific siRNAs (45). A recent study has highlighted the importance of Ago2, in particular, in promoter targeted siRNA induced transcriptional silencing using an siRNA targeting the c-myc gene (60).

The question of whether DNA or RNA is the preferred target of siRNAs targeting promoters in the nucleus has been investigated by several groups. Gonzalez et al. demonstrated that chromatin remodelling required overlapping transcription and that siRNA targets the transcribed strand but not the template strand, which favoured siRNA interacting with the nascent RNA transcript. In addition, they showed that miRNAs might also be able to induce heterochromatin changes where there is sequence complementarity to the miRNA seed region with that of the targeted promoter region (61).

Suppression of human immunodeficiency virus type 1 (HIV-1) infection

We have also previously demonstrated that RNA duplexes targeting the promoter region of integrated HIV-1 can induce TGS, resulting in sustained suppression of viral replication (48, 62, 63). The RNA duplex targeted to the tandem repeat of NF-κB (nuclear factor kappa beta) motif within the U3 promoter of HIV-1 5′ long tandem repeat (5′LTR), designated κB-siRNA, had the most profound effect on virus inhibition. Indeed, when we measured the reverse transcriptase levels of culture supernatants to assess new HIV-1 virions being released from infected cells, the reduction was over 1000-fold in HIV-1 infected cultures treated with κB-siRNA, compared to mock transfected cultures and the suppression lasted for over 30 days after a single siRNA transfection. More

recently, we have also shown a T cell line stably expressing a short hairpin RNA (shRNA) targeted to the same NF-κB motif can also suppress HIV-1 gene expression for prolonged periods of greater than a year (63). We have also reported both siRNA and shRNA targeting the NF-κB region suppressed viral mRNA expression and *de novo* virion formation in culture supernatants, whereas proviral HIV-1 DNA was detected at all time points, indicating that virus suppression occurs at the post-integration stage. Nuclear run-on assays, capable of distinguishing transcriptional from post-transcriptional effects, confirmed the suppression of HIV-1 transcription in infected nuclei cultures by both siRNA- and shRNA-mediated TGS (62, 63), and silencing of a 5′LTR HIV-1 driven luciferase reporter construct by the same shRNA was also observed (63). Our findings are consistent with viral transcriptional regulation via heterochromatin formation as modelled in Figure 3. Silencing was associated with increased H3K9me2 (dimethylation of lysine 9 of histone 3), H3K27me3 (trimethylation of lysine 27 of histone 3), decreased H3 acetylation and recruitment of histone deacetylase HDAC1 in the promoter region of the virus (47, 48, 62, 63). Interestingly, these markers of heterochromatin formation are consistent with those reported in *in vitro* studies of the mechanisms underlying HIV-1 latency (64–70). Two particular single-stranded RNAs, LTR-247 antisense and LTR-362 antisense, targeting approximately 100 bases upstream and 7 bases downstream of our targeted NF-κB region, respectively, have also been reported to mediate TGS of HIV-1. Consistent with our observations, this effect was associated with elevated H3K27me3 levels and recruitment of histone methyltransferase, Enhancer of zeste 2 and the histone deacetylases HDAC1 and HDAC3a in the 5′LTR region (50, 71–73). In addition, the DNA metyltransferase3a was also shown to co-immunoprecipitate with the small RNAs. This suggests the involvement of *de novo* CpG methylation in this process in addition to histone modification (74). A recent study has demonstrated that RNA directed transcriptional silencing of HIV-1 can be delivered by a mobilisation competent vector to cause long-term (1 month) suppression of virus without the emergence of resistance mutations (73).

Suppression of SIV infection

Our group has also observed siRNA-induced TGS in simian immunodeficiency virus (SIV) infection. Using a human lymphoid cell line, CEMx174, we targeted a specific promoter sequence approximately 200 base pairs upstream of the transcription start site in the 5′LTR of SIVmac251. We reported a strong TGS-mediated effect with greater than 1000-fold reduction in virus replication at day 12 post-transfection (47). Our heterochromatin analyses using ChIP also revealed enrichment of the methylation markers H3K9me2 and H3K27me3 in the SIV LTR of silenced cultures. Furthermore, the HDAC inhibitor, Trichostatin-A, partially blocks heterochromatin enrichment in SIV-infected cultures, which further supports the role of epigenetic changes in RNA-mediated TGS. Our reports of RNA-mediated TGS in SIV infection reiterate our findings in HIV-1 and suggest that *in vivo* testing of this approach to retroviral gene silencing is warranted.

Small RNA-induced gene activation

Although the main focus of research efforts have highlighted the role of non-coding RNAs in translational repression, there is some evidence to suggest that they can have the opposite effect and activate transcription via targeting of the gene transcription start site (75–79). One such example is induced E-cadherin expression via siRNA targeting the promoter region. The resulting prolonged transcriptional activation appears to be related to the induction of histone modifications, such as decreased H3K9me2 and H3K9me3 levels (76). However, the regulation of this process is complex and our understanding of the underlying biology is far from complete, as illustrated by studies involving siRNA effects on the regulation of the progesterone receptor (PR). In this study, small RNAs targeted to the transcription start site of the PR inhibited transcription in T47D breast cancer cells, which constitutively highly express PR (45). However, the same siRNAs activated PR transcription in MCF7 breast cancer cells, which constitutively express low PR levels (75). Interestingly, the same RNA duplex was shown to activate PR expression in T47D cells under modified growth conditions leading to low PR expression, while repressing PR expression in T47D cells expressing high levels of PR (78). These changes were associated with histone modifications in the promoter of the PR. Thus, in this system the basal PR expression levels are important in determining PR specific gene regulation. The antisense RNA strand was later shown to have a central role in PR promoter recognition and suggested initial regulation via basal PR expression levels (78), although it remains unclear how siRNA regulate PR based on endogenous antisense transcript levels.

Mechanistic insight into the role of gene activation by siRNAs has recently emerged. An endogenous gene regulatory mechanism exists which benefits from bidirectional transcription (80). The antisense transcript in the bidirectionally transcribed gene was demonstrated to function in directing silent state epigenetic marks to the sense gene promoter. siRNAs can act by targeting the long non-coding antisense transcript, thus leading to suppression of the non-coding antisense transcript resulting in gene activation (81).

This phenomenon of small RNA-mediated gene activation has also been reported for miRNAs. AU-rich elements (AREs), present at the 3′UTR, are important post-transcriptional regulatory signals, especially in cytokines, and this has been demonstrated for TNF-α (82). Two micro-RNP proteins, fragile-X-retardation relation protein 1 (FXR1) and Ago2, associate with the ARE during translational activation. The endogenous miRNA, miR-369-3 directs the association of FXR1 and Ago2 with the ARE, leading to translational activation (83). Other miRNAs that have also demonstrated translational activation include the let-7 family of miRNAs. These studies demonstrate that the fundamental transcriptional regulation of small RNAs is not restricted or limited

to inhibitory effects, but clearly shows the potential for a complex system of both inhibitory and activatory regulatory effects at the levels of transcription and translation.

Small RNA-directed off-target effects

The potential for off-target effects when employing small RNAs, both siRNA and miRNA, to regulate transcription has been highlighted (51, 79, 84) with the result being a call for more comprehensive controls and target validation. Both transcriptional regulation pathways, PTGS and TGS, have the potential for off-target effects, including RNA-induced interferon responses, unintended gene targeting, spread of epigenetic modifications to adjacent promoters and miRNA pathway saturation. These off-target effects can be the result of siRNAs acting like miRNAs, in the sense that although siRNAs are completely complementary to the target promoter DNA or mRNA sequence, the eight nucleotide 'seed' region has the potential to bind to complementary mRNAs and yield unintended gene silencing effects. The comprehensive controls required to confirm transcriptional regulation include: (a) multiple siRNAs targeting different regions of the same gene and showing the same effect; (b) mutant and scrambled siRNAs showing the effect is diminished or abrogated, respectively; (c) determining the small RNA minimum working concentration to eliminate side effects for high doses; and finally (d) mutating the target region to verify specific-sequence binding. Target validation can be performed using other assays, such as reporter plasmid assays, microarray and other global or extensive target gene assays. More reports of off-target effects emphasise the necessity for careful experimental controls when confirming the specificity of small RNA transcriptional gene regulation.

Delivery of RNA duplexes to target tissues

The process of RNAi delivery has recently been considered, with multiple forays into developing this approach for viable human therapeutics (85). There are, however, several key issues which require attention when applying RNAi to human disease treatment. Chief amongst these is finding appropriate RNAi delivery methods to specifically target the cells of interest. The other main concern with this approach is to limit off-target effects, which have the potential to derail the benefits of target gene regulation.

With regard to delivery, these can be divided into non-selective and specific delivery methods. Some of these delivery methods can overlap depending on modifications, such as coating nanoparticles with monoclonal antibodies. One option is direct delivery of 'naked' siRNAs either into the blood or by direct injection into target organs. In this case, the modification of nucleotides at 2′ position of the ribose with 2′-*O*-methylpurines and 2′-fluoropyrimidines (86) leads to an increase in siRNA stability by making the constructs relatively resistant to RNase activity in the serum or extracellular milieu. The other advantage of adding 2′-*O*-methylpurines to the siRNA of interest is that this appears to avoid induction of interferon responses. Other non-selective delivery methods include conjugation of siRNAs to a cholesterol group (87) or packaging siRNAs into a liposomal particle to improve cellular uptake (88). Polymers have also been successfully used to deliver siRNAs to target tissues (89). A recent advance for delivery is through the use of stable nucleic acid lipid particles (or SNALPs), which are coated with polyethylene glycol polymers. SNALPs have been used to deliver siRNAs directed against apolipoprotein B and peroxisome proliferator-activated receptor α (90). These non-selective methods are often suitable where there is direct injection into the target tissue. Viruses, particularly lentiviral vectors, can also be used to incorporate shRNA transgenes into cells of interest. There appears to be several advantages of using shRNA over siRNA to knockdown gene expression. Around 99% of siRNAs are degraded after being introduced into cells by 48 h, whereas only a small number of integrated shRNAs are required for continuous gene knockdown. Also, shRNA appears to be more efficiently loaded onto RISC compared to siRNA. Slightly longer nucleotide sequences (29–30 nucleotides) in siRNA/shRNA also appear to improve processing by Dicer/TRBP/PACT and subsequent loading onto RISC. Lentiviral vectors have been used in general gene therapy approaches over the past two decades with some safety issues noted particularly with regard to insertional mutagenesis leading to malignancy in patients treated with gene therapy for X-linked severe combined immunodeficiency (91). There are also concerns using other viral vectors, such as adenovirus, which can lead to strong immune responses, thereby reducing their effectiveness in the clinical setting. An example of issues linked to the adenoviral delivery platform is the failed STEP trial, which aimed to deliver HIV-1 antigens to HIV-1 negative hosts in the hope of developing an effective immune response against HIV-1. Analyses showed that those patients with high pre-existing adenoviral antibody titres had a higher rate of HIV-1 acquisition compared to patients with low titres – a finding which is currently being heavily investigated (92–94).

Selective delivery methods that have been utilised include aptamers, antibody fragments and nanoparticles. Aptamers are structured RNA ligands, which can bind to specific cell surface receptors and can be covalently linked to siRNAs (95). Binding to these surface receptors via the aptamer leads to release of the siRNA once the aptamer is internalised into the cell. Another specific delivery method utilises the unique specificity of antibody fragments. The fragment antigen binding (Fab) portion of an antibody contains the unique antigen binding site and this Fab fragment has been conjugated to positively charged protamines which, in turn, link to the negatively charged siRNA molecules (96). Nanoparticles have also been used with success to deliver siRNAs *in vivo* (97) and can be coupled with cell-type specific ligands, such as antibodies, and carry siRNAs to the tissue of interest (98). Pseudotyping of lentiviruses to contain proteins that target molecules has the potential to target specific tissues with an shRNA of interest (99).

Potential side effects of RNAi as a therapeutic

The next issue needing careful consideration is the potential for off-target effects of RNAi. As addressed above, although siRNAs are designed to have complete sequence homology with the target mRNA, the eight nucleotide 'seed' region (in particular nucleotides 2–7 from the 5′ end of the siRNA) can act similarly to endogenous miRNAs. The seed region can therefore bind to complementary mRNAs and lead to translational repression and unintended gene silencing effects. It would seem that careful consideration to sequence homology is essential, especially in this region when designing putative siRNA molecules. The other issue arising from RNAi therapeutics is induction of interferon responses. It has been long recognised that the introduction of exogenous single-stranded or double-stranded RNA can lead to the upregulation of type I interferons, which in general terms lead to an anti-viral phenotype. These innate sensing receptors include RIG-I, activation of the dsRNA protein kinase pathway and Toll-like receptors. Chemical modifications to siRNAs, mentioned above, and also the use of retroviral vectors to deliver shRNAs, appear to avoid interferon inducible gene expression in targeted cells.

As discussed above, siRNAs acting via the TGS pathway are capable of inducing heterochromatin changes. These heterochromatin changes can spread distally (in the direction of 5′ to the 3′ end) beyond the target sequence to be silenced (44). The possibility of epigenetic off-target effects from spreading heterochromatin should be considered when designing therapeutic siRNAs.

Despite the incredible specificity of gene knockdown guaranteed by RNAi therapeutics, adverse events have been noted in animal studies. One murine study showed that of 49 adeno-associated virus shRNAs (directed to six targets within the liver), 36 were associated with an adverse event due to the shRNA itself, including 23 shRNAs which eventually led to death mostly due to acute liver failure (100). The authors suggested there could have been oversaturation of endogenous small RNA pathways and that this can be reduced by keeping the shRNA dose to a minimum. The short duration of action of siRNA versus the longer acting shRNA offers some protection against prolonged triggering of small RNA pathways. This can be both advantageous in terms of reduced toxicity but might also have reduced efficacy against longer acting shRNAs.

Clinical applications of RNA duplexes

The first animal model testing the efficacy of siRNAs targeted to Fas was in a murine model of autoimmune hepatitis, where mice receiving siRNA treatment were protected against liver fibrosis. The first human trials began in 2004 with the phase I trial of an siRNA used to treat wet age-related macular degeneration. Today, the areas where RNAi duplexes have been deployed as therapy include cancer, pathogenic viruses, ocular disorders (such as macular degeneration) and several inflammatory conditions (Table 1 lists current RNAi clinical trials).

Despite several trials taking place that employ siRNAs to target pathogenic molecules, to date there is scant information regarding their efficacy in human trials. An early study demonstrated that siRNAs (targeting respiratory syncytial virus) delivered intranasally to humans was associated with limited adverse effects with minimal systemic bioavailability (101). One recently published trial demonstrated targeted knockdown of ribonucleotide reductase (RRM2) in tumour cells by a systemically delivered siRNA, which used a nanoparticle delivery method. The nanoparticles targeted tumour cells by a using a transferrin protein targeting ligand (on the surface of nanoparticles), which bound to transferrin receptors overexpressed in tumours. This group demonstrated dose-dependent accumulation of systemically delivered siRNA nanoparticles in the solid tumours targeted (102). A recent study highlighted the use of the first siRNA used to treat a skin disorder, pachyonychia congenita. The siRNA was injected directly into the skin lesion and efficacy was noted in the siRNA treated arm of the study as opposed to the vehicle-treated arm (103).

Future outlook

The past decade has underlined the importance of small RNAs as master regulators of the transcriptome. Our group and others have provided evidence to suggest that RNA duplexes can target pathogenic processes in human cells. Minimisation of unintended off-target effects and greater precision in delivering these target specific molecules to tissues provide very powerful laboratory tools for gene manipulation, which can herald a new age of disease-specific therapy. Although our understanding of these processes is as yet incomplete, recent advances indicate that small RNA molecules will play an important role in deciphering disease processes, as well as providing exciting new therapeutics. With several phase II trials and one phase III trial currently taking place, we predict RNAi therapeutics have a promising future.

Highlights

- Small RNAs have been shown to mediate both PTGS and TGS regulation pathways in human cells.
- Components of the RISC machinery and mechanisms involved in small RNA-mediated PTGS are well defined in human cells.
- Current fission yeast models of TGS define the main components of the RITS complex, although homologous proteins are not present in human cells.
- Typical TGS-induced epigenetic modifications include elevated H3K9me2 and H3K27me3 methylation, reduced H3 acetylation and recruitment of HDAC.
- We and others have shown RNA duplexes targeted to the NF-κB motif within the U3 promoter of HIV-1 5′LTR can profoundly suppress HIV-1 replication.

Table 1 RNAi-based therapeutic trials.

siRNA	Company/sponsor	Condition	Stage of trial	Trial complete
siRNA targeting proteasome	Duke University	Malignant melanoma	Phase I	No
SV40 siRNAs targeting CML	Hadassah Medical Organisation	Chronic myeloid leukaemia (CML)	Phase I	Yes
CALAA-01	Calando Pharmaceuticals	Solid tumour	Phase I	No
Atu027	Silence Therapeutics AG	Advanced solid tumours	Phase I	No
AGN211745	Allergan	(a) Age-related macular degeneration (b) Choroidal neovascularisation	Phase I Phase II	Yes Yes
QPI-1007	Quark Pharmaceuticals	(a) Chronic optic nerve atrophy (b) Non-arteritic anterior ischaemic optic neuropathy	Phase I	No
Bevasiranib	Opko Health, Inc.	Diabetic macular oedema	Phase II	Yes
Bevasiranib	Opko Health, Inc.	Age-related macular degeneration	Phase III	No
Cand5	Opko Health, Inc.	Wet age-related macular degeneration	Phase II	Yes
I5NP	Quark Pharmaceuticals	Acute kidney injury	Phase I	No
I5NP	Quark Pharmaceuticals	(a) Delayed graft function (b) Kidney transplant	Phase I Phase II	No
TD101	Pachyonychia Congenita Project	Pachyonychia congenita	Phase I	Yes
RSV01	Alnylam Pharmaceuticals	Respiratory syncytial virus	Phase II	Yes
NUC B1000 anti-tat/rev shRNA	Nucleonics Benitec	Hepatitis B HIV	Phase I Phase I	No No

- Reports of small RNA-mediated gene activation demonstrate both inhibitory and activated transcription regulatory effects.
- Small RNA-mediated off-target effects have been reported for PTGS and TGS, emphasising the necessity for comprehensive controls and specific target validation.
- RNAi delivery has developed selective approaches targeting specific cells/tissues with aptamers, antibody fragments and nanoparticles, as well as non-selective approaches employing viral vectors.
- Current phase II and phase III trials indicate that the first licensed RNAi therapeutics is imminent.

Expert opinion

RNA duplexes have revolutionised the field of gene regulation and opened up a new therapeutic era of gene-specific silencing targeting pathogenic proteins. Small RNA duplexes, such as siRNA, can be used to target mRNA precisely but parallel research into the mechanism of action of endogenous miRNAs has allowed us to predict important off-target effects. Predicting off-target effects will be an important component when designing therapeutic siRNAs for the future. Silencing can occur via two main pathways: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). TGS offers longer lasting silencing effects compared to PTGS, as epigenetic phenomena are responsible for causing a silent chromatin state. However, it might not be applicable to all genes. The PTGS approach also allows for gene-specific silencing, but its mechanism of action is shorter in duration which could be beneficial in reducing cell toxicity. Therapeutics with these small RNA duplexes can be delivered via selective or non-selective means. Ultimately, delivery methods are needed which are able to target diseased tissues precisely. This will aid in maximising efficacy and reducing toxicity from these small RNA molecules. Current trials with siRNAs are utilising several delivery methods. Ideally, RNAi will be directed to the tissue of interest and knockdown specific gene targets with minimal off-target side effects. Currently, we are a long way from this ideal scenario. However, there are several trials taking place and we are starting to see results from these initial human trials. If issues regarding delivery and off-target effects can be countered, RNAi therapeutics has a very promising future.

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References

1. Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 2002; 297: 1833–7.
2. Morris KV, Chan SW, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 2004; 305: 1289–92.
3. Ambros V. The functions of animal microRNAs. Nature 2004; 431: 350–5.
4. Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W. Transcriptional control of gene expression by microRNAs. Cell 2010; 140: 111–22.
5. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215–33.
6. Amaral PP, Dinger ME, Mercer TR, Mattick JS. The eukaryotic genome as an RNA machine. Science 2008; 319: 1787–9.
7. Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell 1990; 2: 279–89.
8. van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 1990; 2: 291–9.
9. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843–54.
10. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391: 806–11.
11. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281–97.
12. Tanzer A, Stadler PF. Evolution of microRNAs. Methods Mol Biol 2006; 342: 335–50.
13. Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, Proudfoot NJ. Primary microRNA transcripts are processed co-transcriptionally. Nat Struct Mol Biol 2008; 15: 902–9.
14. Corcoran DL, Pandit KV, Gordon B, Bhattacharjee A, Kaminski N, Benos PV. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. PLoS One 2009; 4: e5279.
15. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 2006; 13: 1097–101.
16. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004; 23: 4051–60.
17. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 2004; 18: 3016–27.
18. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature 2003; 425: 415–9.
19. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The microprocessor complex mediates the genesis of microRNAs. Nature 2004; 432: 235–40.
20. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science 2004; 303: 95–8.
21. Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell 2007; 130: 89–100.
22. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature 2007; 448: 83–6.
23. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 2004; 10: 185–91.
24. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409: 363–6.
25. Daniels SM, Melendez-Pena CE, Scarborough RJ, Daher A, Christensen HS, El Far M, Purcell DF, Laine S, Gatignol A. Characterization of the TRBP domain required for dicer interaction and function in RNA interference. BMC Mol Biol 2009; 10: 38.
26. Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gatignol A, Filipowicz W. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 2005; 6: 961–7.
27. Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. The role of PACT in the RNA silencing pathway. EMBO J 2006; 25: 522–32.
28. Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K, Doudna JA, Nogales E. Structural insights into RNA processing by the human RISC-loading complex. Nat Struct Mol Biol 2009; 16: 1148–53.
29. Trabucchi M, Briata P, Garcia-Mayoral M, Haase AD, Filipowicz W, Ramos A, Gherzi R, Rosenfeld MG. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature 2009; 459: 1010–4.
30. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell 2004; 15: 185–97.
31. Walters RW, Bradrick SS, Gromeier M. Poly(A)-binding protein modulates mRNA susceptibility to cap-dependent miRNA-mediated repression. RNA 2010; 16: 239–50.
32. Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates JR 3rd, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell 2009; 35: 868–80.
33. Beilharz TH, Humphreys DT, Clancy JL, Thermann R, Martin DI, Hentze MW, Preiss T. microRNA-mediated messenger RNA deadenylation contributes to translational repression in mammalian cells. PLoS One 2009; 4: e6783.
34. Sheth U, Parker R. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science 2003; 300: 805–8.
35. Liu J, Rivas FV, Wohlschlegel J, Yates JR 3rd, Parker R, Hannon GJ. A role for the P-body component GW182 in microRNA function. Nat Cell Biol 2005; 7: 1261–6.
36. Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol 2007; 27: 3970–81.

37. Omer AD, Janas MM, Novina CD. The chicken or the egg: microRNA-mediated regulation of mRNA translation or mRNA stability. Mol Cell 2009; 35: 739–40.
38. Robb GB, Brown KM, Khurana J, Rana TM. Specific and potent RNAi in the nucleus of human cells. Nat Struct Mol Biol 2005; 12: 133–7.
39. Matzke MA, Primig M, Trnovsky J, Matzke AJ. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J 1989; 8: 643–9.
40. Wassenegger M, Heimes S, Riedel L, Sanger HL. RNA-directed de novo methylation of genomic sequences in plants. Cell 1994; 76: 567–76.
41. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJ. Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 2000; 19: 5194–201.
42. Lippman Z, May B, Yordan C, Singer T, Martienssen R. Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol 2003; 1: E67.
43. Castanotto D, Tommasi S, Li M, Li H, Yanow S, Pfeifer GP, Rossi JJ. Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. Mol Ther 2005; 12: 179–83.
44. Hawkins PG, Santoso S, Adams C, Anest V, Morris KV. Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. Nucleic Acids Res 2009; 37: 2984–95.
45. Janowski BA, Huffman KE, Schwartz JC, Ram R, Nordsell R, Shames DS, Minna JD, Corey DR. Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. Nat Struct Mol Biol 2006; 13: 787–92.
46. Kim DH, Saetrom P, Snove O Jr, Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci USA 2008; 105: 16230–5.
47. Lim HG, Suzuki K, Cooper DA, Kelleher AD. Promoter-targeted siRNAs induce gene silencing of simian immunodeficiency virus (SIV) infection in vitro. Mol Ther 2008; 16: 565–70.
48. Suzuki K, Juelich T, Lim H, Ishida T, Watanebe T, Cooper DA, Rao S, Kelleher AD. Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter region. J Biol Chem 2008; 283: 23353–63.
49. Ting AH, Schuebel KE, Herman JG, Baylin SB. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. Nat Genet 2005; 37: 906–10.
50. Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, Riggs AD, Rossi JJ, Morris KV. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. RNA 2006; 12: 256–62.
51. Allo M, Buggiano V, Fededa JP, Petrillo E, Schor I, de la Mata M, Agirre E, Plass M, Eyras E, Elela SA, Klinck R, Chabot B, Kornblihtt AR. Control of alternative splicing through siRNA-mediated transcriptional gene silencing. Nat Struct Mol Biol 2009; 16: 717–24.
52. Han J, Kim D, Morris KV. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc Natl Acad Sci USA 2007; 104: 12422–7.
53. Callinan PA, Feinberg AP. The emerging science of epigenomics. Hum Mol Genet 2006; 15 (Spec No. 1): R95–101.
54. Kouzarides T. Chromatin modifications and their function. Cell 2007; 128: 693–705.
55. Suzuki K, Kelleher AD. Lessons from viral latency in T cells: manipulating HIV-1 transcription by siRNA. HIV Ther 2010; 4: 199–213.
56. Bayne EH, White SA, Kagansky A, Bijos DA, Sanchez-Pulido L, Hoe KL, Kim DU, Park HO, Ponting CP, Rappsilber J, Allshire RC. Stc1: a critical link between RNAi and chromatin modification required for heterochromatin integrity. Cell 2010; 140: 666–77.
57. Bayne EH, Allshire RC. RNA-directed transcriptional gene silencing in mammals. Trends Genet 2005; 21: 370–3.
58. Pulukuri SM, Rao JS. Small interfering RNA directed reversal of urokinase plasminogen activator demethylation inhibits prostate tumor growth and metastasis. Cancer Res 2007; 67: 6637–46.
59. Kim JW, Zhang YH, Zern MA, Rossi JJ, Wu J. Short hairpin RNA causes the methylation of transforming growth factor-beta receptor II promoter and silencing of the target gene in rat hepatic stellate cells. Biochem Biophys Res Commun 2007; 359: 292–7.
60. Napoli S, Pastori C, Magistri M, Carbone GM, Catapano CV. Promoter-specific transcriptional interference and c-myc gene silencing by siRNAs in human cells. EMBO J 2009; 28: 1708–19.
61. Gonzalez S, Pisano DG, Serrano M. Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. Cell Cycle 2008; 7: 2601–8.
62. Suzuki K, Shijuuku T, Fukamachi T, Zaunders J, Guillemin G, Cooper D, Kelleher A. Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. J RNAi Gene Silencing 2005; 1: 66–78.
63. Yamagishi M, Ishida T, Miyake A, Cooper DA, Kelleher AD, Suzuki K, Watanabe T. Retroviral delivery of promoter-targeted shRNA induces long-term silencing of HIV-1 transcription. Microbes Infect 2009; 11: 500–8.
64. du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, Mettling C, Baillat V, Reynes J, Corbeau P, Bertrand E, Marcello A, Emiliani S, Kiernan R, Benkirane M. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. EMBO J 2007; 26: 424–35.
65. Jordan A, Bisgrove D, Verdin E. HIV reproducibly establishes a latent infection after acute infection of T cells *in vitro*. EMBO J 2003; 22: 1868–77.
66. Pazin MJ, Sheridan PL, Cannon K, Cao Z, Keck JG, Kadonaga JT, Jones KA. NF-κB-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. Genes Dev 1996; 10: 37–49.
67. Van Lint C, Emiliani S, Ott M, Verdin E. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J 1996; 15: 1112–20.
68. Verdin E, Paras P Jr, Van Lint C. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. EMBO J 1993; 12: 3249–59.
69. Williams SA, Chen LF, Kwon H, Fenard D, Bisgrove D, Verdin E, Greene WC. Prostratin antagonizes HIV latency by activating NF-κB. J Biol Chem 2004; 279: 42008–17.
70. Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC. NF-κB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J 2006; 25: 139–49.
71. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F. The

Polycomb group protein EZH2 directly controls DNA methylation. Nature 2006; 439: 871–4.
72. Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. EMBO J 2001; 20: 2536–44.
73. Turner AM, De La Cruz J, Morris KV. Mobilization-competent lentiviral vector-mediated sustained transcriptional modulation of HIV-1 expression. Mol Ther 2009; 17: 360–8.
74. Datta J, Ghoshal K, Sharma SM, Tajima S, Jacob ST. Biochemical fractionation reveals association of DNA methyltransferase (Dnmt) 3b with Dnmt1 and that of Dnmt 3a with a histone H3 methyltransferase and Hdac1. J Cell Biochem 2003; 88: 855–64.
75. Janowski BA, Younger ST, Hardy DB, Ram R, Huffman KE, Corey DR. Activating gene expression in mammalian cells with promoter-targeted duplex RNAs. Nat Chem Biol 2007; 3: 166–73.
76. Li LC, Okino ST, Zhao H, Pookot D, Place RF, Urakami S, Enokida H, Dahiya R. Small dsRNAs induce transcriptional activation in human cells. Proc Natl Acad Sci USA 2006; 103: 17337–42.
77. Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci USA 2008; 105: 1608–13.
78. Schwartz JC, Younger ST, Nguyen NB, Hardy DB, Monia BP, Corey DR, Janowski BA. Antisense transcripts are targets for activating small RNAs. Nat Struct Mol Biol 2008; 15: 842–8.
79. Weinberg MS, Barichievy S, Schaffer L, Han J, Morris KV. An RNA targeted to the HIV-1 LTR promoter modulates indiscriminate off-target gene activation. Nucleic Acids Res 2007; 35: 7303–12.
80. Morris KV, Santoso S, Turner AM, Pastori C, Hawkins PG. Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. PLoS Genet 2008; 4: e1000258.
81. Morris KV. RNA-directed transcriptional gene silencing and activation in human cells. Oligonucleotides 2009; 19: 299–306.
82. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science 2007; 318: 1931–4.
83. Vasudevan S, Steitz JA. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 2007; 128: 1105–18.
84. Hawkins PG, Morris KV. RNA and transcriptional modulation of gene expression. Cell Cycle 2008; 7: 602–7.
85. Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. Nature 2009; 457: 426–33.
86. Harborth J, Elbashir SM, Vandenburgh K, Manninga H, Scaringe SA, Weber K, Tuschl T. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev 2003; 13: 83–105.
87. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, Stoffel M. Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol 2007; 25: 1149–57.
88. Hughes J, Yadava P, Mesaros R. Liposomal siRNA delivery. Methods Mol Biol 2010; 605: 445–59.
89. Howard KA, Rahbek UL, Liu X, Damgaard CK, Glud SZ, Andersen MO, Hovgaard MB, Schmitz A, Nyengaard JR, Besenbacher F, Kjems J. RNA interference *in vitro* and *in vivo* using a novel chitosan/siRNA nanoparticle system. Mol Ther 2006; 14: 476–84.
90. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, Bertin SL, Reppen TW, Chu Q, Blokhin AV, Hagstrom JE, Wolff JA. Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. Proc Natl Acad Sci USA 2007; 104: 12982–7.
91. Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, de Ridder D, Gilmour KC, Adams S, Thornhill SI, Parsley KL, Stall FJ, Gale RE, Linch DC, Bayford J, Brown L, Quaye M, Kinnon C, Ancliff P, Webb DK, Schmidt M, von Kalle C, Gaspar HB, Thrasher AJ. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 2008; 118: 3143–50.
92. Benlahrech A, Harris J, Meiser A, Papagatsias T, Hornig J, Hayes P, Lieber A, Athanasopoulos T, Bachy V, Csomor E, Daniels R, Fisher K, Gotch F, Seymour L, Logan K, Barbagallo R, Klavinskis L, Dickson G, Patterson S. Adenovirus vector vaccination induces expansion of memory CD4 T cells with a mucosal homing phenotype that are readily susceptible to HIV-1. Proc Natl Acad Sci USA 2009; 106: 19940–5.
93. Hutnick NA, Carnathan DG, Dubey SA, Makedonas G, Cox KS, Kierstead L, Ratcliffe SJ, Robertson MN, Casimiro DR, Ertl HC, Betts MR. Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific $CD4^+$ T cells. Nat Med 2009; 15: 876–8.
94. O'Brien KL, Liu J, King SL, Sun YH, Schmitz JE, Lifton MA, Hutnick NA, Betts MR, Dubey SA, Goudsmit J, Shiver JW, Robertson MN, Casimiro DR, Barouch DH. Adenovirus-specific immunity after immunization with an Ad5 HIV-1 vaccine candidate in humans. Nat Med 2009; 15: 873–5.
95. McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 2006; 24: 1005–15.
96. Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, Lieberman J. Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol 2005; 23: 709–17.
97. Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hellermann G, Behera S, Singam R, Lockey RF, Mohapatra SS. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat Med 2005; 11: 56–62.
98. Wang Y, Li Z, Han Y, Liang LH, Ji A. Nanoparticle-based delivery system for application of siRNA *in vivo*. Curr Drug Metab 2010; 11: 182–96.
99. Maurice M, Verhoeyen E, Salmon P, Trono D, Russell SJ, Cosset FL. Efficient gene transfer into human primary blood lymphocytes by surface-engineered lentiviral vectors that display a T cell-activating polypeptide. Blood 2002; 99: 2342–50.
100. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 2006; 441: 537–41.
101. DeVincenzo J, Cehelsky JE, Alvarez R, Elbashir S, Harborth J, Toudjarska I, Nechev L, Murugaiah V, Van Vliet A, Vaishnaw AK, Meyers R. Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Res 2008; 77: 225–31.

102. Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 2010; 464: 1067–70.
103. Leachman SA, Hickerson RP, Schwartz ME, Bullough EE, Hutcherson SL, Boucher KM, Hansen CD, Eliason MJ, Srivatsa GS, Kornbrust DJ, Smith FJ, McLean WI, Milstone LM, Kaspar RL. First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. Mol Ther 2010; 18: 442–6.