SURVEY AND SUMMARY
Transcriptional gene silencing in humans

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ABSTRACT
It has been over a decade since the first observation that small non-coding RNAs can functionally modulate epigenetic states in human cells to achieve functional transcriptional gene silencing (TGS). TGS is mechanistically distinct from the RNA interference (RNAi) gene-silencing pathway. TGS can result in long-term stable epigenetic modifications to gene expression that can be passed on to daughter cells during cell division, whereas RNAi does not. Early studies of TGS have been largely overlooked, overshadowed by subsequent discoveries of small RNA-directed post-TGS and RNAi. A reappraisal of early work has been brought about by recent findings in human cells where endogenous long non-coding RNAs function to regulate the epigenome. There are distinct and common overlaps between the proteins involved in small and long non-coding RNA transcriptional regulatory mechanisms, suggesting that the early studies using small non-coding RNAs to modulate transcription were making use of a previously unrecognized endogenous mechanism of RNA-directed regulation. Here we review how non-coding RNA plays a role in regulation of transcription and epigenetic gene silencing in human cells by revisiting these earlier studies and the mechanistic insights gained to date. We also provide a list of mammalian genes that have been shown to be transcriptionally regulated by non-coding RNAs. Lastly, we explore how TGS may serve as the basis for development of future therapeutic agents.

INTRODUCTION
The history of RNA-directed transcriptional gene silencing (TGS)

Almost three decades ago, Marjorie Matzke et al. observed that over-expression of a transgene led to DNA hypermethylation and transcriptional silencing in doubly transformed tobacco plants (1) (Figure 1). Mechanistically, this type of silencing in plants was found to be the result of small non-coding RNAs directing epigenetic changes, specifically DNA methylation, to those loci containing sequences homologous to the small RNA. The phenomenon was termed small RNA-directed transcriptional gene silencing (TGS). TGS was later shown in Arabidopsis to require the action of RNA-dependent DNA methylation (2,3) and members of the Argonaut protein family (4). A few years later RNA interference (RNAi), mediated by double-stranded RNAs, was discovered as a powerful post-TGS (PTGS) system against messenger RNAs (mRNAs) in plants (5), and a few months later in Caenorhabditis elegans (6).

Transcriptional gene silencing in humans

The study of small non-coding RNA-directed TGS has been carried out in various model organisms such as plants (Arabidopsis thaliana), yeast (Saccharomyces pombe), flies (Drosophila melanogaster) and worms (C. elegans) (reviewed extensively in (7,8)). A decade ago, the first report of RNA-directed TGS in human cells was observed when exogenous siRNAs were used to silence a transgenic elongation factor 1α promoter driving a Green Fluorescent Protein (GFP) reporter gene (9) (Figure 1). Importantly, the observed silencing was clearly at the transcriptional level, as indicated by nuclear run-on analysis. Moreover, silencing was also epigenetic: inhibition was abrogated by 5′ Aza-cytadine (5′ AzaC) and Trichostatin A (TSA), com-
Figure 1. Regulatory non-coding RNA timeline. A timeline of some important observations in RNA biology are shown leading up to our collective understanding of non-coding RNA-directed transcriptional gene silencing (TGS). (5,71,170–177).

Mechanisms of small non-coding RNA-directed TGS

TGS is mechanistically distinct from the abundantly studied PTGS pathway of RNAi. One notable difference is that TGS results in long-term stable epigenetic modifications to gene expression that can be passed on to daughter cells during cellular division (reviewed in (12)). Early observations postulated that siRNA-directed TGS functioned through an epigenetic nuclear mechanism distinct from RNAi-mediated PTGS in the cytoplasm (13). For instance 5′ AzaC and TSA were functional in reverting the siRNA targeted TGS, indicating epigenetic modes of gene regulation were at play in siRNA-directed TGS, and not via a PTGS-based mechanism (9). Indeed, recent studies have observed that two different siRNAs, one targeted to the promoter and one targeted to exon 1 of the coding transcript, can functionally repress the targeted gene in a TGS or PTGS based manner (14). A lot has been gleaned over the last decade regarding the mechanism of action for RNA-directed TGS in human cells. Studies carried out to determine the underlying mechanism of siRNA-directed TGS revealed that RNA-directed TGS is operative through RNA-directed methylation of histone 3 lysine’s 9 and 27 (H3K9 and H3K27, respectively) and DNA methylation at the targeted promoter (9,11,15–21) (Figure 2). These promoter-directed siRNAs interact with a low level expressed (~1–2%) promoter associated RNA, which is essentially the 5′ UTR of the protein coding gene (16,22) (Figure 2). It is worth noting that most genes and gene promoters appear to be transcribed to some extent (23,24) and experimental observations suggest that non-coding RNAs interact with target loci via Watson–Crick-based RNA:RNA hybridization (16,22) and not by double-stranded DNA invasion. Temporal studies have determined that exogenously introduced siRNAs targeted to a promoter region interact first with Argonautes 1 and 2 (AGO1 and AGO2) (17,25,26). SiRNA and AGO interactions is found within the first 24 h, at the siRNA targeted promoter and is followed shortly thereafter with the recruitment of the H3K9me2 and H3K27me3 silent state epigenetic marks (17), and later by the recruitment of DNA methyltransferase and DNA methylation at 72–96 h for some genes (14). It should be noted, however, that the role of DNA methylation in TGS in human cells is not as clearly understood as in plants; DNA methylation at the targeted promoter is not always observed in human TGS applications (Table 1). These effects may be explained by the duration of RNA targeting to the promoter, the presence of robust siRNA targeting (e.g. delivery to the nucleus), the presence and abundance of promoter-occupied RNAs and/or the dynamic interplay of proteins interacting with the promoter. Despite differences in the various experimental observations, a key consistent feature has been the observations that promoter-directed small RNAs can modulate gene transcription and that some level of epigenetic based silencing is ongoing in the observed silenced genes.

The endogenous pathway of TGS in human cells; rise of long non-coding RNAs

While small RNAs were observed early on to regulate gene transcription in human cells by the targeting of epigenetic silencing complexes to those loci containing complementarity to the small RNAs (Figure 1), the endogenous mechanism(s) driving this form of gene regulation in the context of human cells remained largely unknown. MicroRNAs (miRNAs) have been shown to be endogenous drivers of TGS with some genes in human cells (27–30) (Table 1). In 2005, through the efforts of the FANTOM and ENCODE consortia, it started to become apparent that a large fraction of the human genome was generating long non-coding RNAs (lncRNAs) and that many of these transcripts were antisense to protein-coding counterparts (31,32). Several of these sense/antisense or bidirectionally-transcribed genes are evolutionarily conserved, suggesting some functional cues for retention of these elements (33,34). Indeed, studies with imprinted genes and X-inactivation found that cis acting long non-coding RNAs (lncRNAs) were actively in-
| Gene(s)                                                                 | Gene symbol       | Effector RNA          | Cell line                  | Therapeutic relevance                                                                 | References |
|------------------------------------------------------------------------|-------------------|-----------------------|----------------------------|----------------------------------------------------------------------------------------|------------|
| Eukaryotic translation elongation factor 1 |
| HIV-1/SIV                                                               | EEF1A1            | siRNA                 | HEK293T                    |                                                                                       | (9,16,18)  |
| nitric-oxide synthase                                                  | NOS               | siRNA                 | HeLa, 293                  | Regulate HIV-1 (18–21, 26, 79, 99, 100, 104, 140–144)                                   | (18–21, 26, 79, 99, 100, 104, 140–144) |
| E-cadherin                                                            | CDH1              | siRNA                 | HCT116, MCF7               | Cancer, tumor suppressor (10)                                                          | (10)       |
| BCL-2 (oncogene)                                                      | BCL-2             | sasRNA                | HeLa                       | Cancer, oncogene (146)                                                                 | (146)      |
| Fibronectin                                                           | FN1               | siRNA                 | HeLa                       | Cardiac disease (129,147)                                                               | (129,147)  |
| Huntingtin gene                                                       | HTT               | siRNA                 | Glioblastoma               | Monogenetic diseases (148)                                                             | (148)      |
| Non-sense codon-containing immunoglobulin minigenes                   | (Ig)mu and Ig-gamma | siRNA               | HeLa                       | Immunologic diseases (149)                                                             | (149)      |
| INK4A/Cyclin-dependent kinase inhibitor 2B/p15 + ARF + INK4A/ Cyclin-dependen kinase inhibitor 2A isoform 3/p16 | CDKN2B+            | siRNA                 | HEK293T                    | Cancer, tumor suppressor (150)                                                          | (150)      |
| Plasminogen activator, urokinase                                      | PLAUR             | siRNA                 | PC3 and invivo             | Cancer (152)                                                                           | (152)      |
| Chemokine receptor 5                                                  | CCR5              | siRNA                 | HEK293T                    | HIV-1 co-receptor (16,17)                                                               | (16,17)    |
| Breast cancer-associated gene 1                                       | BRCA1             | siRNA                 | T47D                       | Cancer, oncogene (153)                                                                 | (153)      |
| Progesterone receptor                                                 | PGR               | siRNA                 | T47D                       | Cancer (11,25,119,153,154)                                                             | (11,25,119,153,154) |
| Huntingtin                                                            | HD                | siRNA                 | T47D                       | Monogenetic diseases (23)                                                              | (23)       |
| Androgen receptor                                                     | AR                | siRNA                 | T47D                       | Cancer, spinal bulbar muscular atrophy (25,155)                                        | (25,155)   |
| v-myc avian myelocytomatosis viral oncogene homolog                   | c-MYC             | siRNA/sasRNA          | PC3, HCT113, 293, Hela, MCF7 | Cancer, oncogene (22,97,156,157)                                                       | (22,97,156,157) |
| Papillomavirus-16 oncogenes                                           | HPV-16            | siRNA                 | HeLa                       | HPV (158)                                                                             | (158)      |
| v-akt murine thymoma viral oncogene homolog 1                         | AKT-1             | siRNA                 | 293HEK                     | Cancer, oncogene (156)                                                                 | (156)      |
| Kirsten rat sarcoma viral oncogene                                     | KRAS              | siRNA                 | 293HEK                     | Cancer, oncogene (156)                                                                 | (156)      |
| Dual specificity phosphatase 6                                        | DUSP6             | siRNA                 | CFPAC                      | Cancer, tumor suppressor (156)                                                         | (156)      |
| Myostatin                                                             | MSTN              | siRNA                 | Cyclin-dependen kinase inhibitor 2A isoform 3/p16 |
| Runt-related transcription factor 3                                    | RUNX3             | siRNA                 | Cyclin-dependen kinase inhibitor 2A isoform 3/p16 |
| Small nuclear 7k (RNAi functional in the nucleus of human cells)       | 7K                | siRNA                 | Cyclin-dependen kinase inhibitor 2A isoform 3/p16 |
| met proto-oncogene (hepatecty growth factor receptor)                  | c-Met             | siRNA/sasRNA          | SKHep1C3 cells             | Cancer, oncogene (161)                                                                 | (161)      |
| Periostin                                                             | POSTN             | siRNA/sasRNA          | PC3                        | Cancer and metastasis (101)                                                            | (101)      |
| Heparanase (endo-l- D-glucuronidase)                                   | HPA               | sasRNA                | PC3, EJ and SGC-7901 cells | Cancer, angiogenesis (162)                                                             | (162)      |
| Interleukin 2                                                          | IL2               | siRNA                 | Jurkat                     | Immunologic (163)                                                                     | (163)      |
| Transforming growth factor-α receptor II                               | UBC               | sasRNA, sRNA          | HEK293GT                   | Immunologic (14)                                                                      | (14)       |
| Vascular endothelial growth factor                                     | VEGF-A            | sRNA                  | mouse C166 and invivo      | Cancer, angiogenesis (107,122)                                                         | (107,122)  |
| Ras association domain family 1                                        | RASSF1A           | sRNA                  | HeLa                       | Cancer, oncogene (15,17)                                                               | (15,17)    |
| Tubulin folding cofactor E-like                                        | TBCEL/ LRRFC35    | miRNA                 | HCT116                     | Kenny-Caffey syndrome (KCS)                                                            | (150)      |
| Ras p21 protein activator 2                                            | RASA2             | miRNA                 | HCT116                     | Cancer, tumor suppressor (150)                                                         | (150)      |
| Rhophilin, Rho GT Pase binding protein 2                               | RPHN2             | miRNA                 | HCT116                     | Cancer, tumor suppressor (150)                                                         | (150)      |
| Wolf-Hirschhorn syndrome candidate 1                                   | WHSC1             | miRNA                 | HCT116                     | Wolf-Hirschhorn syndrome (150)                                                         | (150)      |
| Homeobox D4                                                            | HOXD4             | miRNA                 | MCF7, MDA-MB-231            | HIV-1 infection (164)                                                                 | (164)      |
| HIV-1                                                                 | LTR               | miRNA                 | Jurkat, T-cells             | HIV-1 infection (28)                                                                  | (28)       |
| OCT4 and Nanog (pluripotent factor)                                    | OCT4 and Nanog    | mRNA                  | MCF7                        | Cancer, pluripotency (54)                                                              | (54)       |
| PTER (antisense, pseudogene)                                           | PTEN              | miRNA                 | (Trans-antisense,          | Cancer, tumor suppressor (53)                                                         | (53)       |
|                                                                                               |                  |issyRNA               | 293, Hela, Jurkat            |                                                                                       |            |
| P21 tumor suppressor                                                   | P21               | lncRNA (antisense)    | MCF7                        | Cancer, tumor suppressor (39)                                                          | (39)       |
volved in epigenetic regulation of these, dosage-dependent, regulated loci (35).

In 2008, a number of important studies confirmed the role of lncRNAs as endogenous drivers of TGS in human cells, in particular those, which were antisense to their protein-coding counterparts (reviewed in (36,37)). Antisense lncRNAs were shown to regulate the p15 (38) and p21 (39) tumor suppressor genes (Table 1). The over-expression of these antisense lncRNAs resulted in TGS of their protein-coding counterpart while their repression resulted in the derepression/transcriptional activation (38,39). Support for the role of antisense lncRNAs as active endogenous regulators of gene expression was evident in an earlier understated study, which indicated that antisense transcripts might also be involved in CpG methylation in thalassemia (40). Antisense lncRNAs are now known to affect TGS for genes such as BDNF (41,42), MYCN (43), DHRS4 (44), KCNQ1 (45–47), NBAT (48) and HIV-1 (49–52) (Table 1). Interestingly, non-coding transcripts derived from pseudogenes of Phosphatase and tensin homolog (PTEN) (53) and OCT4 (54), which contain significant homology to their protein-coding counterparts, have also been observed to be involved in directing TGS and subsequent PTEN and OCT4 suppression (Table 1). Also, the PTEN antisense pseudogene-directed TGS of PTEN is one of the first bona fide examples of a trans-functional IncRNA (53). LncRNAs directly regulate ribosomal genes (56) as well as p21 (57) and MYCN (43), genes involved in cell regulation and cancer. The plethora of lncRNA functions are extensive and the range includes: protein modifiers (58,59), scaffolds for tethering proteins (43,60,61), miRNAs (62–64), splicing modifiers (65), cellular body transformation (66–69), enhancer function and gene activation (70–73), and epigenetic modifiers (53,74,75), (reviewed in (76)). Collectively, the observations to date suggest that we are only now just beginning to realize the complexity and pervasiveness of lncRNA functional regulation in epigenetic and transcriptional states.

**Mechanisms of small and long non-coding RNA-directed TGS**

To date there are ~55 reports of small RNA-directed TGS and ~10 of antisense IncRNA-directed TGS (Table 1). Mechanistically, much of what we know about how small non-coding RNAs, such as siRNAs, miRNAs and small antisense RNAs (sasRNAs)-directed TGS, have been determined from cell culture studies. The promoter-targeted small RNAs interact with various proteins to guide TGS, beginning in the first 24 h, with direct interactions with AGO1 and AGO2 (17,18,25) followed shortly thereafter by interactions at the targeted promoter with DNMT3a (14,18,77,78), HDAC1 (14,20) and resulting ultimately in histone 3 lysine 9 di-methylation and histone 3 lysine 27 tri-methylation (H3K9me2 and H3K27me3, respectively)(14,16–18,20,79,80) (Figure 3). SiRNA-directed
Figure 2. Small non-coding RNA pathways in human cells. Small non-coding RNAs can be generated as priRNAs where they are (A) processed by Drosha and DGCR8 into miRNAs which are (B) exported from the nucleus and (C) loaded into RISC where they can affect mRNA expression by (D) binding and blocking mRNA translation or (E) cutting the target mRNA. Some miRNAs may also be retained in the nucleus (F) where they can interact with epigenetic remodeling proteins and (G) recruit the complexes to target loci in the genome resulting in (H) localized chromatin compaction and epigenetic silencing.

TGS has also been observed to occur in the absence of DNA methylation, suggesting that alternative routes may be present for RNA-mediated transcriptional and epigenetic silencing (10). Small RNA-directed TGS appears to require a template or target transcript at the corresponding targeted promoter (16,22), similar to the method by which plants utilize RNA Polymerase V transcribed and processed siRNAs to regulate DNA methylation and TGS (reviewed in (81)). Notably, in plants there is a requirement for RNA-dependent RNA polymerase (RdRP) activity to amplify RNA polymerase V transcript-directed TGS (81), whereas humans lack such a polymerase, which opens up a methodology for specific RNA-directed epigenetic modes of regulation. Curiously, this is exactly what lncRNAs appear to be doing in human cells via cis and trans-specific targeting of epigenetic complexes to particular loci (Figure 3 and Table 1), similar to what is also observed in Saccharomyces cerevisiae, which also lacks RdRP activity (82,83).

Early studies carried out with S. cerevisiae indicated that antisense non-coding RNAs function endogenously to direct epigenetic gene silencing in place of RdRP-mediated mechanisms (82,83). The parallels between S. cerevisiae and previous observations of small antisense RNA-directed TGS in human cells (18) have emerged, suggesting that antisense transcripts also function to direct TGS (Figure 3). Most notable are the observations that particular antisense
Figure 3. Antisense RNA-directed TGS. Small antisense non-coding RNAs can be (A) introduced into the nucleus and (B) interact with and recruit epigenetic silencing complexes consisting of DNMT3a, Ago1, EZH2 and HDAC1 to homology containing targeted loci by interactions with low copy promoter-associated transcripts resulting in (C) epigenetic silencing consisting of histone and DNA methylation and ultimately chromatin compaction of the targeted locus. (D) Long antisense non-coding RNAs have also been observed to interact with similar epigenetic silencing complexes (53,54) and (E) localize with these complexes at targeted loci resulting in (C) epigenetic silencing of the lncRNA targeted locus.

lncRNAs, first observed in tumor suppressor genes, p15 (38) and p21(39), function to epigenetically modulate their protein-coding counterparts (Figure 1, Table 1). One interesting, and surprisingly overlooked, early study found antisense transcription was involved in DNA methylation in Thalassemia (40), and even early work linked antisense transcripts and DNA methylation in regulating HIV (51) and MYC (84,85).

Mechanistically, far less is known about how antisense lncRNAs direct epigenetic silencing in human cells. Studies carried out with the lncRNA, HOTAIR, indicate that bimodal chromatin modifying complexes can be localized to the HOX locus via the action of this lncRNA (86). A common theme is also evident with Kcnq1ot1 (45) and the p53 regulatory lncRNA p21, which indicates that the entire p53 expressed pathway is controlled by the action of this lncRNA at the p53 locus (57). Indeed, many lncRNAs have been observed to be associated with chromatin (87), but mechanistic insights into the process of lncRNA-directed gene regulation remain less clear. Interesting insights into the mechanism of action of lncRNA-directed TGS came from a recent study looking at the PTEN pseudogene. It had been reported previously that the PTEN pseudogene functions as a miRNA ‘sponge’ (64), similar to the CEBPA lncRNA that acts to sponge DNMT1 away from the CEBPA promoter (88). Studies to interrogate the PTEN pseudogene in greater detailed determined that this pseudogene also expressed an antisense lncRNA in trans which functions to direct TGS to the PTEN promoter and control PTEN expression epigenetically (53). Mechanistically, the PTEN pseudogene expressed antisense lncRNA modulated PTEN transcription by recruiting DNMT3a and EZH2 to the PTEN promoter. The parallels between the functions of the PTEN pseudogene and previous observations with small antisense ncRNA-directed TGS are notable, as both involved the action of DNMT3a (Figure 3). It is noteworthy that DNMT3a is the only known de novo DNA methyltransferase in human cells (89) and has been observed previously to be the only DNA methyltransferase to bind non-coding RNAs including small ncRNAs, both antisense and double stranded RNAs (18,77,78,90), and lncRNAs (53,91). There is an interesting connection between DNMT3a and epigenetic silencing, which includes studies indicating DNTM3a co-immunoprecipitates with HDAC1 (92,93) and EZH2 (94), as well as early predictions that DNA methylation is an active participant in X-inactivation (95), one of the first bona fide lncRNA regulatory pathways described. Collectively, a paradigm is emerging in human cells, which proposes that non-coding RNAs, both small and long forms (Figure 3), function through the action of
DNMT3a to modulate chromatin and epigenetic states of gene expression. While there are several other mechanisms of action described for lncRNAs in human cells, the interactions with DNMT3a and targeting of transcriptional and epigenetic states is of particular interest, as this mode of gene regulation has the potential to be long-lasting, heritable and may be of significant relevance to the development of targeted therapeutics (reviewed in (96)).

Therapeutic applications of RNA-directed epigenetic regulation of gene expression

The utility of small RNA-induced TGS as a therapeutic has been largely ignored, mainly due to the pervasiveness of using RNAi targeted approaches to degrade mRNAs. The main concern with RNAi and post-transcriptional mechanisms of gene silencing (Figure 2) is the duration of their therapeutic effect. The effector siRNAs required to drive RNAi must be administered continuously to repress a therapeutic target gene. This is not the case with RNA-induced TGS, where stable, long-term, silencing can be achieved following a relatively short duration of promoter targeting with the siRNAs (19,20,97–100) or small antisense RNA (14,101). This is because the mode of action for the observed gene silencing is transcriptional and driven ultimately by epigenetic silencing (79,102) and not ‘slicing’ of the genes messenger RNA as is the case with RNAi. One universal hurdle that both RNAi and RNA-induced TGS face with is the targeted delivery of the effector RNAs to those cells requiring treatment. One approach is to utilize synthetic antisense oligonucleotides targeted to promoters of interest. This approach has worked with regards to blocking transcription (103) but was not found to induce robust epigenetic silencing, unless the particular oligonucleotides were RNA based (104). However, it may be that better interrogation of each non-coding RNA targeted promoter is required to delineate the best promoter-associated transcripts to target and that many of the earlier studies may have neglected this notion. Indeed, establishing TGS in the absence of a target promoter RNA has not been reported and attempts by some groups, including ours, have proven fruitless. Another approach might be to deliver the effector RNAs using receptor targeted aptamers, which has shown promise for targeting HIV infected cells (105,106). While delivery remains an important concern, the notion that one needs to only target a particular gene for 2–4 days to instill stable epigenetic silencing is promising with regards to minimizing the need for sustained delivery. Recent studies suggest that small RNA-directed TGS is feasible and that stable epigenetic marks can be imposed at small RNA target loci in vivo (99,107).

Another area of therapeutic utility can be found in the plethora of lncRNAs that are appearing to be involved in various diseases. Emerging evidence suggest that non-coding RNAs play a wide role (108) in various disease states in humans. Genome-wide observations of diseased states, such as heart failure (109), indicate significant differential and discordant expression between protein-coding and non-coding antisense and pseudogenes is prevalent (110). To date the list of those lncRNAs involved in human diseases is expanding at an unprecedented rate. LncRNAs have been observed in disease ranging from Cancer (57,86,105,106), to HIV (111,112), to autism (113), to pluripotency and differentiation (114–116). It is worth underscoring that many of the disease relevant lncRNAs have been observed to be antisense to particular protein coding genes. A significant obstacle to using RNAi and other post-transcriptional effectors for targeting antisense lncRNAs is the fact that double stranded siRNAs have an ability to target both sense and antisense transcripts (117). The use of RNA-directed TGS avoids this issue by targeting the lncRNA promoter with single stranded antisense transcripts (52). The targeting of endogenous effector antisense lncRNAs can result in the de-repression and subsequent transcriptional activation of the lncRNA targeted locus (Figure 4). Using this mode of action, it becomes feasible to activate gene expression to affect those protein-coding genes under sustained lncRNA-directed TGS (Figure 4). This has proven an effective approach to inducing genes both in vitro (39,52,118–121) and in vivo (42,107,122), but presupposes that there are known antisense lncRNAs regulating the therapeutic target gene. Collectively, the advantages to using RNA-directed TGS as a therapeutic are many and include: (i) strand specific targeting of a gene, (ii) stable long-term epigenetic based silencing can be established to particular genes of therapeutic interest and (iii) antisense RNA-based approaches work as well, if not better than double stranded RNAs, as the endogenous pathway of RNA-directed TGS appears to contain significant overlap with small antisense RNAs and antisense lncRNAs (Figure 3).

CONCLUSION

It has been roughly 10 years since the first observation that promoter-directed RNAs can affect gene transcription (Figure 1 and Table 1). This seminal observation in 2004 (9) was indicative of a role for RNA in regulating gene expression, a notion proposed~5 decades ago but largely overlooked (123,124). Possible reasons for the poor early adoption of RNA-directed TGS (Table 1) are varied but may include (i) the unfortunate retraction of a similar paper published in Nature (125), and/or (ii) the overwhelmingly positive response to PTGS and the rejection of any RNAi-related phenomena occurring in the nucleus, despite the fact that RNAi was shown to be functional in the human nucleus in 2005 (126) and confirmed in many subsequent studies (25–27,127–131).

The notion that RNA may function as the master gene regulator in the cell was something proposed by Britten and Davidson in 1969 (123), which at the time was largely neglected by the broad scientific community. With the advent of high-throughput technologies and the findings from ENCODE, that most of the human genome is transcribed and likely plays a functional role (132–139), it is becoming apparent that Britten and Davidson’s theory should be reappraised. Certainly, lncRNAs are abundantly active in the nucleus, and many of them are active modulators of transcriptional and epigenetic modes of gene expression (reviewed in (37,76) and appear to share many of the mechanistic characteristics observed in small RNA-directed TGS (Figure 3). Collectively, the mounting observations that an-
Figure 4. LncRNA pathways of transcriptional silencing and de-repression. LncRNAs can be expressed in (A) *cis* or *trans* and can (B) interact with those proteins involved in epigenetic silencing. The LncRNAs act to (C) target and tether the epigenetic silencing complexes to homology containing loci resulting in (D) chromatin compaction and transcriptional gene silencing of the targeted locus. These endogenous regulatory LncRNAs can be targeted with (E) antisense oligonucleotides or (F) siRNAs, which results in the loss of the LncRNA and activation/de-repression of those loci actively under LncRNA regulation.

tisense non-coding RNAs, both small and long RNAs, directed to gene promoters can affect transcription by the recruitment of silent state epigenetic complexes suggests that a pervasive and underappreciated role for non-coding RNAs is part of the basic fabric of life. Knowledge of this molecular pathway may prove incredibly insightful with regards to the development of disease, including epigenetic silencing of gene expression and the development of new-targeted therapeutics aimed at specifically affecting gene expression. The next decade could prove an exciting time for our understanding of non-coding RNAs in the transcriptional gene expression and their application as novel therapeutics.

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Conflict of interest statement
None declared.

REFERENCES
1. Matzke,M.A., Primig,M., Trnovsky,J. and Matzke,A.J.M. (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J., 8, 643–649.
2. Mette,M.F., Aufsatz,W., Van der Winden,J., Matzke,A.J.M. and Matzke,M.A. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J., 19, 5194–5201.
3. Wassenegger,M., Heimes,S., Riedel,L. and Sanger,H.L. (1994) RNA-directed de novo methylation of genomic sequences in plants. Cell, 76, 567–576.
4. Lippman,Z., May,B., Yordan,C., Singer,T. and Martienssen,R. (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol., 1, e67.
5. Waterhouse,P.M., Graham,M.W. and Wang,M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. U.S.A., 95, 13959–13964.
6. Fire,A., Xu,S., Montgomery,M.K., Costas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by small interfering RNAs in Caenorhabditis elegans. Nature, 391, 806–811.
7. Green,V.A. and Weinberg,M.S. (2011) Small RNA-induced transcriptional gene regulation in mammals, therapeutic applications, and scope within the genome. Prog. Mol. Biol. Transl. Sci., 102, 11–46.
8. Morris,K.V. (2009) RNA-directed transcriptional gene silencing and activation in human cells. Oligonucleotides, 19, 299–306.
9. Morris,K.V., Chan,S.W., Jacobsen,S.E. and Looney,D.J. (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. Science, 305, 1289–1292.
10. Morris,K.V. (2008) RNA-mediated transcriptional gene silencing in human cells. J. Cell. Biochem., 105, 296–301.
11. Carninci,P., Kasukawa,T., Katayama,S., Gough,J., Frith,M.C., et al. (2005) The transcriptional landscape of the mammalian genome. Nature, 435, 84–90.
12. Carninci,P., Kasukawa,T., Katayama,S., Gough,J., Frith,M.C., et al. (2005) The transcriptional landscape of the mammalian genome. Science, 310, 1268–1272.
13. Carninci,P., Kasukawa,T., Katayama,S., Gough,J., Frith,M.C., et al. (2005) The transcriptional landscape of the mammalian genome. Nature, 435, 84–90.
14. Hawkins,P.G., Santoso,S., Adams,C., Anest,V. and Morris,K.V. (2009) PROMOTER-MARKER small RNAs induce long-term transcriptional gene silencing in human cells. Nucleic Acids Res., 37, 2894–2905.
15. Castanotto,D., Tommasi,S., Li,M., Li,H., Yanow,S., Pfeifer,G.P. and Rossi,J.J. (2005) Short hairpin RNA-directed cytotoxic (CpG) methylation of the RASSF1A gene promoter in HeLa cells. Mol. Ther., 12, 179–183.
16. Han,J., Kim,D. and Morris,K.V. (2007) Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc. Natl. Acad. Sci. U.S.A., 104, 12422–12427.
17. Kim,D.H., Villeneuve,L.M., Morris,K.V. and Rossi,J.J. (2006) Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. Nat. Struct. Mol. Biol., 13, 793–797.
18. Weinberg,M.S., Villeneuve,L.M., Ehsani,A., Amargurouli,M., Aagaard,L., Chen,Z.X., Riggs,A.D., Rossi,J.J. and Morris,K.V. (2006) The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. RNA, 12, 256–262.
19. Suzuki,K., Ishida,T., Yamagishi,M., Aihenstiel,C., Swaminathan,S., Marks,K., Murray,D., McCartney,E.M., Beard,M.R., Alexander,M. et al. (2011) Transcriptional gene silencing of HIV-1 through promoter targeted RNA is highly specific. RNA Biol., 8, 1035–1046.
20. Suzuki,K., Juelich,T., Lim,H., Ishida,T., Watanabe,T., Cooper,D.A., Rao,S. and Kelleher,A.D. (2008) Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter region. J. Biol. Chem., 283, 23533–23536.
21. Yamagishi,M., Ishida,T., Miyake,A., Cooper,D.A., Kelleher,A.D., Suzuki,K. and Watanabe,T. (2009) Retroviral delivery of promoter-targeted shRNA induces long-term silencing of HIV-1 transcription. Microbes Infect., 11, 500–508.
22. Napoli,S., Pastori,C., Magistri,M., Carbone,G.M. and Catapano,C.V. (2009) Promoter-specific transcriptional interference and e-myc gene silencing by siRNAs in human cells. EMBO J., 28, 1708–1719.
23. Seila,A.C., Calabrese,I.M., Levine,S.S., Yeo,G.W., Rahl,P.B., Flynn,R.A., Young,R.A. and Sharp,P.A. (2008) Divergent transcription from active promoters. Science, 322, 1849–1851.
24. Core,L.J., Waterfall,J.J. and Lis,J.T. (2008) Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science, 322, 1852–1857.
25. Janowski,B.A., Huffman,K.E., Schwartz,J.C., Ram,R., Nordsell,R., Shames,D.S., Minna,J.D. and Corey,D.R. (2006) Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. Nat. Struct. Mol. Biol., 13, 787–792.
26. Ahlenstiel,C.L., Lim,H.G., Cooper,D.A., Ishida,T., Kelleher,A.D. and Suzuki,K. (2012) Direct evidence of nuclear Argonauta distribution during transcriptional silencing links the actin cytoskeleton to nuclear RNAi machinery in human cells. Nucleic Acids Res., 40, 1579–1595.
27. Kim,D.H., Saetrom,P., Snoe,O.Jr and Rossi,J.J. (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc. Natl. Acad. Sci. U.S.A., 105, 16230–16235.
28. Omo,T.S. and Fuji,Y.R. (2005) Regulation of human immunodeficiency virus 1 transcription by nfil microRNA. J. Gen. Virol., 86, 751–755.
29. Klase,Z., Kale,P., Winograd,R., Gupta,M.V., Heydarian,M., Berro,R., McCaffrey,T. and Kashanchi,F. (2007) HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. BMC Mol. Biol., 8, 63.
30. Tan,Y., Zhang,B., Wu,T., Skogerbo,G., Zhu,X., Guo,X., He,S. and Chen,R. (2009) Transcriptional inhibition of Hoxd4 expression by noncoding RNAs in human breast cancer cells. BMC Biol., 10, 12.
31. Carninci,P., Kasukawa,T., Katayama,S., Gough,J., Frith,M.C., Maeda,N., Oyama,R., Ravasi,T., Lennard,B., Wells,C.E. et al. (2005) The transcriptional landscape of the mammalian genome. Science, 309, 1559–1563.
32. Katayama,S., Tomaru,Y., Kasukawa,T., Waki,K., Nakamishii,M., Nakamura,M., Nishida,H., Yap,C.C., Suzuki,M., Kawai,J. et al. (2005) Antisense transcription in the mammalian transcriptome. Science, 309, 1564–1566.
33. Dahary,D., Elroy-Stein,O. and Sorek,R. (2005) Naturally occurring antisense: transcriptional leakage or real overlap? Genome Res., 15, 364–368.
34. Lavorgna,G., Dahary,D., Lehner,B., Sorek,R., Sanderson,C.M. and Casari,G. (2004) In search of antisense. Trends Biochem. Sci., 29, 88–94.
35. Latos,P.A. and Barlow,D.P. (2009) Regulation of imprinted expression by macro non-coding RNAs. RNA Biol., 6, 100–106.
36. Malecova,B. and Morris,K.V. (2010) Transcriptional gene silencing through epigenetic changes mediated by non-coding RNAs. Curr. Opin. Mol. Ther., 12, 214–222.
37. Morris,K.V. (2009) Long antisense non-coding RNAs function to direct epigenetic complexes that regulate transcription in human cells. Epigenetics, 4, 296–301.
38. Yu,W., Gius,D., Onyango,P., Muldoon-Jacobs,K., Karp,J., Feinberg,A.P. and Cui,H. (2008) Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. Nature, 451, 202–206.
39. Morris, K.V., Santos, S., Turner, A.M., Pastori, C. and Hawkins, P.G. (2008) Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. PLoS Genet., 4, e1000258.

40. Tufarelli, C., Stanley, J.A., Garrick, D., Sharpe, J.A., Ayyub, H., Wood, W.G. and Higgs, D.R. (2003) Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat. Genet., 34, 157–165.

41. Lipovich, L., Dachet, F., Cai, J., Bagla, S., Balan, K., Jia, H. and Loeb, J.A. (2012) Activity-dependent human brain coding/noncoding gene regulatory networks. Genetics, 192, 1133–1448.

42. Mocharelli, F., Faghimi, M.A., Lopez-Toledano, M.A., Fatemi, R.P., Magistri, M., Brothers, S.P., van der Brug, M.P. and Wahlestedt, C. (2012) Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. Nat. Biotechnol., 30, 453–459.

43. Wade, N., Saayman, S., Lenox, A., Ackley, A., Clemson, M., Burdach, J., Hart, J., Vogt, P.K. and Morris, K.V. (2015) MYCNOS functions as an antisense RNA regulating MYCN. RNA Biol., 12, 893–899.

44. Li, Q., Xu, Z., Liu, G., Song, X., Liu, T., Chang, X. and Huang, D. (2012) AS1DHRS4, a head-to-head natural antisense transcript, silences the DHR54 gene cluster in cis and trans. Proc. Natl. Acad. Sci. U.S.A., 109, 14110–14115.

45. Johnsson, P., Ackley, A., Vidarsdottir, L., Lui, W.O., Corcoran, M., Hawkins, P.G. and Morris, K.V. (2010) Transcriptional regulation of antisense RNA lnc-DC controls human dendritic cell differentiation. Science, 329, 689–693.

46. Wang, L., Guo, Z.Y., Zhang, R., Xin, B., Chen, R., Zhao, J., Wang, T., Wen, W.H., Jia, L.T., Yao, L.B. et al. (2013) Pseudogene OCT4-pg4 functions as a natural micro RNA sponge to regulate OCT4 expression by competing for miR-145 in hepatocellular carcinoma. Carcinogenesis, 34, 1773–1781.

47. Pandey, R.R., Mondal, T., Fraser, P. and Kanduri, C. (2008) Kcnq1ot1 antisense noncoding RNA mediates alternative splicing by local histone modifications: potential roles for enhancers. Mol. Cell, 32, 232–246.

48. Seyfried, T.N., Darnell, J.E., Jr., Kasten, J.M., Seashore-Ludlow, M., Murphy, L., Lipton, S.A., de St. Hilaire, S., Fratini, D. and Kajander, D. (2012) The association of pathogenic mutations in the human 5′ UTRs of Dicer and Argonaute proteins with neurodevelopmental disease. Nature Rev. Neurosci., 13, 399–409.

49. Landry, S., Halin, M., Lefort, S., Audet, B., Vaquero, C., Mesnard, J.M. and Verjovski-Almeida, S. (2013) The intronic long noncoding RNA LncMyoD, regulates skeletal muscle differentiation by blocking alternative splicing. J. Biol. Chem., 288, 1180–1189.

50. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wang, J.K., Salinas, R.D., Zarabi, H., Kriegstein, A.R. and Lim, D.A. (2015) The long noncoding RNA Prkzr regulates neuronal differentiation of embryonic and postnatal neural stem cells. Cell Stem Cell, 16, 439–447.

51. Orom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Nettles, K., Liu, Y. et al. (2010) Widespread transcriptional regulation at promoters of tissue-specific OCT4 target genes. Mol. Cell, 38, 79–91.

52. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wang, J.K., Salinas, R.D., Zarabi, H., Kriegstein, A.R. and Lim, D.A. (2015) The long noncoding RNA Prkzr regulates neuronal differentiation of embryonic and postnatal neural stem cells. Cell Stem Cell, 16, 439–447.

53. Orom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Nettles, K., Liu, Y. et al. (2010) Widespread transcriptional regulation at promoters of tissue-specific OCT4 target genes. Mol. Cell, 38, 79–91.

54. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wang, J.K., Salinas, R.D., Zarabi, H., Kriegstein, A.R. and Lim, D.A. (2015) The long noncoding RNA Prkzr regulates neuronal differentiation of embryonic and postnatal neural stem cells. Cell Stem Cell, 16, 439–447.

55. Orom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Nettles, K., Liu, Y. et al. (2010) Widespread transcriptional regulation at promoters of tissue-specific OCT4 target genes. Mol. Cell, 38, 79–91.
the expression of RASSF1A and increasing cell proliferation. *PLoS Genet.*, 9, e1003705.

75. Kotake, Y., Nakagawa, T., Kitagawa, K., Suzuki, S., Liu, N., Kitagawa, M. and Xiong, Y. (2011) Long non-coding RNA ANRIL is required for the PR2C recruitment to and silencing of p15(INK4B) tumor suppressor gene. *Oncogene*, 30, 1956–1962.

76. Morris, K.V. and Mattick, J.S. (2014) The rise of regulatory RNA. *Nat. Rev. Genet.*, 15, 423–437.

77. Holz-Schietinger, C. and Reich, N.O. (2012) RNA modulation of the human DNA methyltransferase 3A. *Nucleic Acids Res.*, 40, 8550–8557.

78. Ross, J.P., Suetake, I., Tajima, S. and Molloy, P.L. (2010) Recombinant mammalian DNA methyltransferase activity on model transcriptional gene silencing short RNA–DNA heteroduplex substrates. *Biochem. J.*, 432, 323–332.

79. Suzuki, K., Shijuku, T., Fukamachi, T., Zaunders, J., Guillemin, G., Cooper, D. and Kelleher, A. (2005) Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J. RNAi Gene Silencing*, 1, 66–78.

80. Turner, A.M., Ackley, A.M., Matrone, M.A. and Morris, K.V. (2012) Characterization of an HIV-targeted transcriptional gene-silencing RNA in primary cells. *Hum. Gene Ther.*, 23, 473–483.

81. Matzke, M.A. and Mosher, R.A. (2014) RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nat. Rev. Genet.*, 15, 394–408.

82. Camblong, J., Iglesias, N., Fickentscher, C., Dieppois, G. and Stutz, F. (2007) Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in *S. cerevisiae*. *Cell*, 131, 706–717.

83. Camblong, J., Beyrouty, N., Guffanti, E., Schlaepfer, G., Steinmetz, L.M. and Stutz, F. (2009) Trans-acting antisense RNAs mediate transcriptional gene co-suppression in *S. cerevisiae*. *Genes Dev.*, 23, 1534–1545.

84. Celano, P., Berchtold, C.M., Kizer, D.L., Weeraratna, A., Nelkin, B.D., Baylin, S.B. and Casero, R.A. Jr (1992) Characterization of an endogenous RNA transcript with homology to the antisense strand of the human c-myc gene. *J. Biol. Chem.*, 267, 15092–15096.

85. Siper, D.B. and Sonnenshein, G.E. (1992) An antisense promoter of the murine c-myc gene is localized within intron 2. *Mol. Cell. Biol.*, 12, 1324–1329.

86. Gupta, R.A., Shah, N., Wang, K.C., Kin, J., Horlings, H.M., Wong, D.J., Tsai, M.C., Hung, T., Argani, P., Rinn, J.L. et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature*, 464, 1071–1076.

87. Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Alberich-Jorda, M., Zhang, P. et al. (2013) DNM1-interacting RNAs block gene-specific DNA methylation. *Nature*, 503, 371–376.

88. Bestor, T.H. (2000) The DNA methyltransferases of mammals. *Hum. Mol. Genet.*, 9, 2395–2402.

91. Riggs, A.D. (1975) X inactivation, differentiation, and DNA methylation. *Cytogenet. Cell Genet.*, 14, 9–25.

92. Morris, K.V. (2009) Non-coding RNAs, epigenetic memory, and the passage of information to progeny. *RNA Biol.*, 6, 242–247.

93. Mehdiratta, M., Palamichamy, J.K., Pa,A., Bhagat, M., Singh, A., Sinha, S. and Chattopadhyay, P. (2011) CpG hypermethylation of the C-myc promoter by dsRNA results in growth suppression. *Mol. Pharm.*, 8, 2302–2309.

94. Esmaeili, F., Bamdad, T. and Ghaseem, S. (2010) Stable suppression of gene expression by short interfering RNAs targeted to promoter in a mouse embryonal carcinoma stem cell line. *In Vitro Cell. Dev. Biol. Anim.*, 46, 834–840.

95. Fuks, F., Burgers, W.A., Godin, N., Kasai, M. and Kouzarides, T. (2011) Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J.*, 30, 2536–2544.

96. Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vandervenne, J.M. et al. (2006) The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*, 439, 871–874.

97. Suzuki, K., Hattori, S., Marks, K., Ahlentiel, C., Maeda, Y., Ishida, T., Millington, M., Boyd, M., Symonds, G., Cooper, D.A. et al. (2013) Promoter targeting shRNA suppresses HIV-1 infection in vivo through transcriptional gene silencing. *Mol. Ther. Nucleic Acids*, 2, e17.

98. Lin, H.G., Suzuki, K., Cooper, D.A. and Kelleher, A.D. (2008) Promoter-targeted siRNAs induce gene silencing of simian immunodeficiency virus (SIV) infection in vitro. *Mol. Ther.*, 16, 565–570.

99. Lister, N.C., Clemson, M. and Morris, K.V. (2015) RNA-directed epigenetic silencing of peristin inhibits cell motility. *R. Soc. Open Sci.*, 2, 140545.

100. Kawasaki, T., Zhang, Y.H., Zern, M.A., Rossi, J.J. and Wu, J. (2007) 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.*, 359, 292–297.

101. Watts, J.K., Yu, D., Charisse, K., Montaillier, C., Potier, P., Manoharan, M. and Corey, D.R. (2010) Effect of chemical modifications on modulation of gene expression by duplex antigen RNAs that are complementary to non-coding transcripts at gene promoters. *Nucleic Acids Res.*, 38, 5242–5259.

102. Knowling, S.S.K., Turner, A.M., Uhlmann, E., Lehmann, T., Vollmer, J. and Morris, K.V. (2012) Chemically modified oligonucleotides modulate an epigenetically varied and transient form of transcriptional silencing of HIV-1 in human cells. *Mol. Ther. Nucleic Acids*, 1, e16.

103. Zhou, J., Satheesan, S., Li, H., Weinberg, M.S., Morris, K.V., Burnett, J.C. and Rossi, J.J. (2015) Cell-specific RNA aptamer against human CCR5 specifically targets HIV-1 susceptible cells and inhibits HIV-1 infectivity. *Chem. Biol.*, 22, 379–390.

104. Neff, C.P., Zhou, J., Remling, L., Kuruvilla, J., Zhang, J., Li, H., Oudenaarden, A. et al. (2010) Human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc. Natl. Acad. Sci. U.S.A.*, 107, 11667–11672.

105. Zhou, J., Satheesan, S., Li, H., Weinberg, M.S., Morris, K.V. and Vogt, P.K. (2014) MYC regulates the non-coding transcriptome. *Oncotarget*, 5, 12543–12554.

106. Di Salvo, T.G., Guo, Y., Su, Y.R., Clark, T., Brittain, E., Abi, T., Maltais, S. and Hennes, A. (2015) Right ventricular long noncoding RNA expression in human heart failure. *Pulm. Circ.*, 5, 135–161.

107. Johnson, P., Lipovich, L., Grander, D. and Morris, K.V. (2013) Evolutionary conservation of long noncoding RNAs; sequence, structure, function. *Biochim. Biophys. Acta*, 1840, 1063–1071.

108. Saayman, S., Ali, S.A., Morris, K.V. and Weinberg, M.S. (2015) The therapeutic application of CRISPR/Cas9 technologies for HIV. *Expert Opin. Biol. Ther.*, 15, 819–830.

109. Kobayashi-Ishihara, M., Yamagishi, M., Harata, T., Matsuda, Y., Takahashi, R., Miyake, A., Nakano, K., Yamouchi, T., Ishida, T. and Watanabe, T. (2012) HIV-1-encoded antisense RNA suppresses viral replication for a prolonged period. *Retrovirology*, 9, 38.

110. Kerin, T., Ramanathan, A., Rivas, K., Grepo, N., Coetzee, G.A. and Sirtori, A.M. (2016) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Oncotarget*, 7, 2305–2312.

111. Nishikawa, T., Hattori, S., Marks, K., Ahlentiel, C., Maeda, Y., Ishida, T., Millington, M., Boyd, M., Symonds, G., Cooper, D.A. et al. (2013) Promoter targeting shRNA suppresses HIV-1 infection in vivo through transcriptional gene silencing. *Mol. Ther. Nucleic Acids*, 2, e17.
linRNAs act in the circuitry controlling pluripotency and differeniation. *Nature*, **477**, 295–300.

115. Loewer,S., Cabili,M.N., Guttman,M., Loh,Y.H., Thomas,K., Park,I.H., Garber,M., Curran,M., Onder,T., Agarwal,S. *et al.* (2010) Large intergenic non-coding RNA-Seq modulates reprogramming of human induced pluripotent stem cells. *Nat. Genet.*, **42**, 1113–1119.

116. Kretz,M., Siprashvili,Z., Chu,C., Webster,D.E., Zehnder,A., Qu,K., Harte,R., Balasubramanian,S., Tanzer,A., Diekhans,M. *et al.* (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature*, **489**, 57–74.

117. Consortium,E.P. (2004) The ENCODE (ENCyclopedia Of DNA Elements) Project. *Science*, **306**, 636–640.

118. Rin,J., and Chang,H. (2012) Genome regulation by long non-coding RNAs. *Annu. Rev. Biochem.*, **81**, 145–166.

119. Place,R.F., Li,L.C., Pookot,D., Noonan,E.J. and Dahiya,R. (2008) A multifunctional human Argonaute2-specific monoclonal antibody. *Antimicrob. Chemother.*, **52**, e89979.

120. Britten,R.J. and Davidson,E.H. (1969) Gene regulation for higher eukaryotes: a theory. *Cell. Mol. Biol.*, **13**, 165–172.

121. Britten,R.J. and Davidson,E.H. (1971) Repetitive and non-repetitive DNA sequences and a speculation on the origins of evolutionary novelty. *Q. Rev. Biol.*, **46**, 111–138.

122. Taira,K. (2006) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature*, **441**, 1176.

123. Britten,R.J. and Davidson,E.H. (1971) Repetitive and non-repetitive DNA sequences and a speculation on the origins of evolutionary novelty. *Q. Rev. Biol.*, **46**, 111–138.

124. Turner,A.M., De La Cruz,J. and Morris,K.V. (2009) Mobility-driven transcriptional silencing by short interfering RNA targeting NF-κB binding site within 5′ LTR. In: 10th Conference on Retroviruses and Opportunistic Infections, Boston.

125. Turner,A.M., De La Cruz,J. and Morris,K.V. (2009) Mobilization-competent Lentiviral Vector-mediated Sustained Transcriptional Modulation of HIV-1 Expression. *Mol. Ther.*, **17**, 360–368.

126. Turner,A.M. and Morris,K.V. (2010) Controlling transcription with non-coding RNAs in mammalian cells. *Biotechniques*, **48**, ix–xvi.

127. Singh,A., Palanichamy,J.K., Ramalingam,P., Kassab,M.A., Bhagat,M., Andrabi,R., Luthra,K., Sinha,S. and Chattopadhyay,P. (2013) Long-suppression of HIV-1 virus production in human peripheral blood mononuclear cells by LTR heterochromatinization with a short double-stranded RNA. *J. Antimicrob. Chemother.*, **69**, 404–415.

128. Gagnon,K.T., Li,L., Chu,Y., Janowski,B.A. and Corey,D.R. (2014) Antisense transcripts are involved in the circuitry controlling pluripotency and lincRNAs act in the circuitry controlling pluripotency and differentation. *Nature*, **489**, 231–235.

129. Rosenbloom,K.R., Dreszer,T.R., Long,J.C., Malladi,V.S., Bhagat,M., Andrabi,R., Luthra,K., Sinha,S. and Chattopadhyay,P. (2013) Long-suppression of HIV-1 virus production in human peripheral blood mononuclear cells by LTR heterochromatinization with a short double-stranded RNA. *J. Antimicrob. Chemother.*, **69**, 404–415.

130. Ahlenstiel,C., Mendez,C., Lim,S.T., Marks,K., Turville,S., Cooper,D.A., Kelleher,A.D. and Suzuki,K. (2015) Novel RNA duplex locks HIV-1 in a latent state via chromatin-mediated transcriptional silencing. *Mol. Ther. Nucleic Acids*, **4**, e261.

131. Jiang,M.X., Zhang,C., Shen,Y.H., Wang,J., Li,X.N., Chen,L., Zhang,Y., Coselli,J.S. and Wang,X.L. (2008) Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. *Mol. Biol. Cell.*, **19**, 3997–4005.

132. Gonzalez,S., Pisano,D.G. and Serrano,M. (2008) Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. *Cell.*, **132**, 275–280.

133. Bulbule,M., Mohn,F., Stalder,L. and Muller-Hermelink,H.O. (2005) Transcriptional silencing of nonsense codon-containing immunoglobulin minigenes. *Nat. Genet.*, **37**, 307–317.

134. Gonzalez,S., Pisano,D.G. and Serrano,M. (2008) Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. *Cell Cycle.*, **7**, 2601–2608.

135. Wah,S., Fung,Y., Pan,L., Wang,Y., Xu,X., Liu,J. and Huang,B. (2007) The proximal GC-rich region of p16(INK4a) gene promoter plays a role in its transcriptional regulation. *Mol. Cell. Biochem.*, **301**, 259–266.

136. Pulukuri,S.M. and Rao,J.S. (2007) Small interfering RNA directed reversal of urokinase plasminogen activator demethylation inhibits prostate tumor growth and metastasis. *Cancer Res.*, **67**, 6657–6666.

137. Yue,X., Schwartz,J.C., Chu,Y., Younger,S.T., Gagnon,K.T., Yau,J., Andrau,J.C., Young,R., Morozova,N., Fenouil,R., Descostes,N., and Corey,D.R. (2012) ENCODE whole-genome data in the UCSC Genome Browser: update 2012. *Nucleic Acids Res.*, **40**, D912–D917.

138. Beautiful,M., Flockhart,R.J., Groff,A.F., Chow,J. *et al.* (2013) ENCODE whole-genome data in the UCSC Genome Browser: update 2012. *Nucleic Acids Res.*, **40**, D912–D917.

139. Beautiful,M., Flockhart,R.J., Groff,A.F., Chow,J. *et al.* (2013) ENCODE whole-genome data in the UCSC Genome Browser: update 2012. *Nucleic Acids Res.*, **40**, D912–D917.

140. Suzuki,K., Suter,S., Ward,R., Cooper,D.A. and Kelleher,A.D. (2003) Silencing gene expression by short interfering RNA targeting NF-κB binding site within 5′ LTR. In: 10th Conference on Retroviruses and Opportunistic Infections, Boston.
RNAs capable of epigenetically modulating transcriptional gene silencing and activation in human cells. Mol. Ther. Nucleic Acids, 2, e104.

157. Zakaria, M. K., Khan, I., Mani, P., Chattopadhyay, P., Sarkar, D. P. and Sinha, S. (2014) Combination of hepatocyte specific delivery and transformation dependent expression of shRNA inducing transcriptional gene silencing of c-Myc promoter in hepatocellular carcinoma cells. BMC Cancer, 14, 582.

158. Palanichamy, J. K., Mehdiratta, M., Bhagat, M., Ramalingam, P., Das, B., Das, P., Sinha, S. and Chattopadhyay, P. (2010) Silencing of integrated human papillomavirus-16 oncogenes by small interfering RNA-mediated heterochromatization. Mol. Cancer Ther., 9, 2114–2122.

159. Roberts, T. C., Andaloussi, S. E., Morris, K. V., McClurey, G. and Wood, M. J. (2012) Small RNA-mediated epigenetic myostatin silencing. Mol. Ther. Nucleic Acids, 1, e23.

160. Peng, X. Z., He, X. S., Zhuang, Y. Z., Luo, Q., Jiang, J. H., Yang, S., Tang, X. F., Liu, J. L. and Chen, T. (2008) Investigation of transcriptional gene silencing and mechanism induced by shRNAs targeted to RUNX3 in vitro. World J. Gastroenterol., 14, 3006–3014.

161. Salvi, A., Arci, B., Portolani, N., Giuliani, S. M., De Petro, G. and Barlati, S. (2007) In vitro c-met inhibition by antisense RNA and plasmid-based RNAi down-modulates migration and invasion of hepatocellular carcinoma cells. Int. J. Oncol., 31, 451–460.

162. Jiang, G., Zheng, L., Pu, J., Mei, H., Zhao, J., Huang, K., Zeng, F. and Tong, Q. (2012) Small RNAs targeting transcription start site induce hepatarsis silencing through interference with transcription initiation in human cancer cells. PLoS One, 7, e31379.

163. Murayama, A., Sakura, K., Nakama, M., Yasuzawa-Tanaka, K., Fujita, E., Tateishi, Y., Wang, Y., Ushijima, T., Baba, T., Shibuya, K. et al. (2006) A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. EMBO J., 25, 1081–1092.

164. Tan, Y., Zhang, B., Wu, T., Skogerbo, G., Zhu, X., Guo, X., He, S. and Chen, R. (2009) Transcriptional inhibition of Hoxd4 expression by miRNA-10a in human breast cancer cells. BMC Mol. Biol., 10, 12.

165. Ouellet, D. L., Plante, I., Landry, P., Barat, C., Janelle, M. E., Flamand, L., Tremblay, M. J. and Provost, P. (2008) Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element. Nucleic Acids Res., 36, 2353–2365.

166. Cho, D. H., Thienes, C. P., Mahoney, S. E., Analau, E., Filippova, G. N. and Tapscoott, S. J. (2005) Antisense transcription and heterochromatin at the DM1 CTG repeats are constrained by CTCF. Mol. Cell, 20, 483–489.

167. Wang, Q. Y., Zhou, C., Johnson, K. E., Colgrove, R. C., Coen, D. M. and Knipe, D. M. (2005) Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc. Natl. Acad. Sci. U.S.A., 102, 16055–16059.

168. Mohammad, F., Mondal, T., Guseva, N., Pandey, G. K. and Kanduri, C. (2010) Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1. Development, 137, 2493–2499.

169. Takayama, K., Horie-Inoue, K., Katayama, S., Suzuki, T., Tsutsuishi, S., Ikeda, K., Urano, T., Fujimura, T., Takagi, K., Takahashi, S. et al. (2013) Androgen-responsive long noncoding RNA CTBP1-AS promotes prostate cancer. EMBO J., 32, 1665–1680.

170. Brenner, S., Jacob, P. and Meselson, M. (1961) An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature, 190, 576–581.

171. Berget, S. M., Moore, C. and Sharp, P. A. (1977) Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. U.S.A., 74, 3171–3175.

172. Chow, L. T., Gelinas, R. E., Broker, T. R. and Roberts, R. J. (1977) An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. Cell, 12, 1–8.

173. Brannan, C. I., Dees, E. C., Ingram, R. S. and Tilghman, S. M. (1990) The product of the H19 gene may function as an RNA. Mol. Cell. Biol., 10, 28–36.

174. Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S. A. and Rastan, S. (1992) The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell, 71, 515–526.

175. Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) Posttranscriptional gene silencing in Caenorhabditis elegans. Cell, 75, 855–862.

176. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double stranded RNA in Caenorhabditis elegans. Nature, 391, 806–811.