Citation: Molecular Therapy—Nucleic Acids (2014) 3, e196; doi:10.1038/mtna.2014.45

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De-repressing LncRNA-Targeted Genes to Upregulate Gene Expression: Focus on Small Molecule Therapeutics

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Non-protein coding RNAs (ncRNAs) make up the overwhelming majority of transcripts in the genome and have recently gained attention for their complex regulatory role in cells, including the regulation of protein-coding genes. Furthermore, ncRNAs play an important role in normal development and their expression levels are dysregulated in several diseases. Recently, several long noncoding RNAs (lncRNAs) have been shown to alter the epigenetic status of genomic loci and suppress the expression of target genes. This review will present examples of such a mechanism and focus on the potential to target lncRNAs for achieving therapeutic gene upregulation by de-repressing genes that are epigenetically silenced in various diseases. Finally, the potential to target lncRNAs, through their interactions with epigenetic enzymes, using various tools, such as small molecules, viral vectors and antisense oligonucleotides, will be discussed. We suggest that small molecule modulators of a novel class of drug targets, lncRNA-protein interactions, have great potential to treat some cancers, cardiovascular disease, and neurological disorders.
Molecular Therapy—Nucleic Acids (2014) **3**, e196; doi:10.1038/mtna.2014.45; published online 18 November 2014
Subject Category: Non-coding RNAs

Insights Into The Complexity of The Human Genome

The Human Genome Project and subsequent large-scale transcriptomics efforts, notably: Functional Annotation of the Mouse and Encyclopedia of DNA Elements have overturned the central dogma of molecular biology and helped to make sense of the "transcriptional noise" that was thought to comprise the majority of the human genome.[1–5] These studies have shown that the genome is pervasively transcribed and that many genes are transcribed from both sense and antisense strands of DNA, resulting in protein products, non-protein coding transcripts, or combinations of the two depending on the gene locus.[1,2,5,6] Recently, we have come to appreciate that these non-coding RNAs (ncRNAs) are functionally important[3,7–9] and that unlike the size of the genome coding for proteins, the size of non-protein coding genome scales with organismal complexity.[3,10–13] Non-coding RNAs are divided into two broad classes based on length: small ncRNAs (<200 nucleotides) and long non-coding RNAs (>200 nucleotides) that can span up to hundreds of kilobases in length.[7,14] Although much is known about the mechanisms and functions of small noncoding RNAs (*e.g.*, microRNAs and small interfering RNAs (siRNAs)),[15–20] the biological functions of long noncoding RNAs (lncRNAs) are still being elucidated.

Although the field of lncRNA research has gained increasing momentum in the past decade, many questions about the *in vivo* function of lncRNAs remain unanswered. This is in part because of the challenge posed by the primate- or human-specific nature of a majority of these transcripts. Some lncRNAs are conserved in mice and have been studied *in vivo* in knockout models, with variable results ranging from changes in the expression of a limited repertoire of genes lacking phenotypic defects[21] to severe developmental abnormalities[22,23] and embryonic lethality.[23] For example, the lncRNA *MALAT1* has been shown *in vitro* to regulate splicing,[24,25] but interestingly, *Malat1* knockout mice are viable and do not have any reported defects *in vivo*.[21,26,27] However, targeted deletion of the lncRNA *Hotair* results in developmental defects and mimics findings from *in vitro* studies. Furthermore, a recent study of 18 lncRNA knockout strains revealed embryonic lethality in 3 mutant strains (*Fendrr*$^{-/-}$, *Peril*$^{-/-}$, and *Mdgt*$^{-/-}$) and distinct growth defects for two other strains (*linc-Brn1b*$^{-/-}$ and *linc-Pint*$^{-/-}$).[23] This evidence suggests that lncRNAs are a heterogeneous class of transcripts, many of which have important biological functions ranging from fine-tuning the expression of a limited number of genes to acting as master regulators of important cellular processes.

Emergence of Long Non-Coding RNAS as Important Regulatory Units Within the Cell

LncRNAs have been separated into several broad classes in terms of their mechanisms of regulation of mRNA transcription and translation: decoys, regulators of translation, enhancers and modular scaffolds that guide chromatin modifying enzymes to specific genomic loci (**Figure 1**). LncRNAs can regulate apoptosis and cell cycle; the lncRNA Growth arrest-specific 5 (*GAS5*) is induced during growth factor starvation and binds the glucocorticoid receptor through its hairpin domain, acting as a decoy to physically block the transcription of metabolic genes.[28,29] The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT-1*) localizes to nuclear speckles to associate with the serine/arginine-rich (SR) proteins involved in splicing.[24,25] Another important function of lncRNAs is to regulate translation, *e.g.*,

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Keywords: cardiovascular disease; cancer; epigenetics; gene upregulation; long intervening RNAs; long Noncoding RNA; natural antisense transcript; neurological disorders; protein-RNA interactions; RNA structure
Received 18 June 2014; accepted 8 August 2014; published online 18 November 2014. doi:10.1038/mtna.2014.45

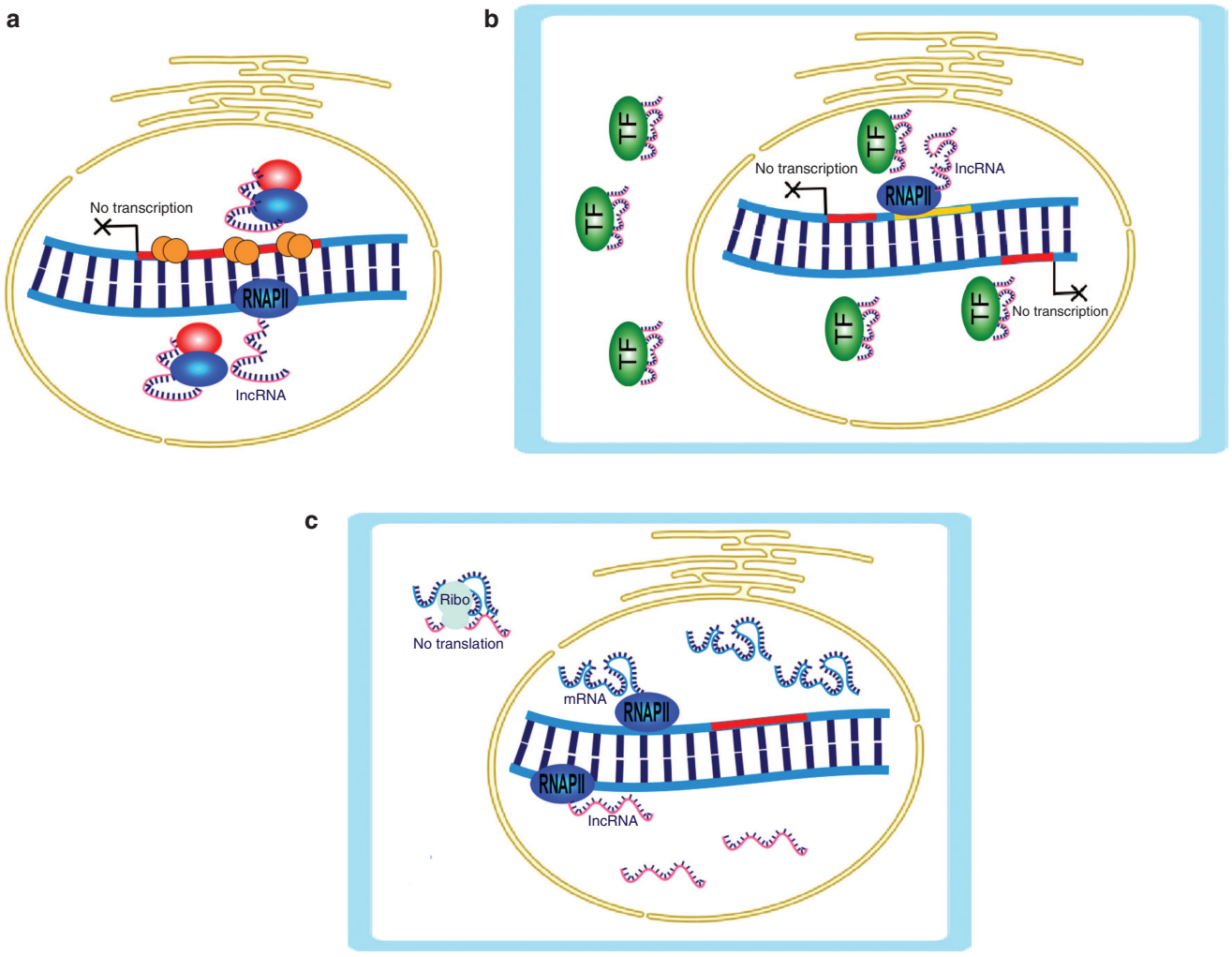

Figure 1 Possible mechanisms of gene repression by long noncoding RNAs. (**a**) Long noncoding RNAs can interact with chromatin-modifying enzymes (red and blue) to guide them to promoters of target genes (red) to repress chromatin structure and downregulate gene expression. (**b**) LncRNAs act as decoys, binding to transcription factors in the nucleus or the cytoplasm to prevent transcription at the promoters of target genes (red). (**c**) LncRNAs bind to target mRNA in the cytoplasm to stall the ribosome and block protein translation.

beta-site APP cleaving enzyme 1-antisense (*BACE1*-AS) masks miRNA-binding sites on its cognate mRNA *BACE1* to form a stable duplex and prevent mRNA degradation.[30,31] LncRNAs can also enhance the expression of neighboring genes, acting as positive regulators of gene expression[32] as is the case with ncRNAs arising from the *SCL/TAL1*, *SNAI1*, and *SNAI2* loci.[33] Finally, lncRNAs play an important role in epigenetic regulation, functioning as modular scaffolds to bring protein complexes into proximity to position epigenetic enzymes to specific genomic loci,[34–37] resulting in changes in gene expression *in cis*[38] (at the same gene locus) or *in trans*[39] (at different loci). The most extensively studied lncRNA is *Xist*, a roughly 17 kb mammalian-specific lncRNA that regulates X-chromosome inactivation (through an epigenetic mechanism that involves interactions with polycomb proteins), by silencing one of the two X-chromosomes in females.[40,41] LncRNAs are also involved in imprinting; *Kcnq1ot1* and *Air* act as scaffolds to recruit chromatin-modifying enzymes. The 91-kb lncRNA *Kcnq1ot1* interacts with two chromatin modifying enzymes: G9a and Polycomb repressive complex-2 (PRC2) during imprinting to produce lineage-specific transcriptional silencing patterns.[42–44] The *Air* lncRNA silences gene expression at the *Igf2r/Slc22a2/Slc22a3* locus of paternal alleles by targeting G9a to chromatin.[45,46]

Long Non-Coding RNAs as Epigenetic Regulators

LncRNAs are expressed at lower levels than protein-coding genes and are mostly localized in the chromatin fraction of the nucleus, where they interact with epigenetic enzymes. LncRNAs exhibit tissue-specific expression patterns (with a large proportion expressed in the testes and brain)[5,6,47–50] because of the tight regulation of promoters that regulate their transcription.[51,52] The tight transcriptional regulation and tissue-specificity of lncRNAs, their presence in low-copy number and localization to chromatin, support the notion that lncRNAs are potent regulators of gene accessibility by modulating chromatin structure at developmental-specific timepoints in the cell. Chromatin-associated lncRNAs exert their regulatory effects by acting as modular

scaffolds, conferring specificity to ubiquitously expressed epigenetic enzymes, by guiding these enzymes to specific genomic loci.[33–35] Therefore, lncRNAs play a pivotal role in fine-tuning gene expression at the chromatin level via their interactions with chromatin-modifying enzymes. It is becoming increasingly evident that these transcripts play an important role in development as well as disease through their interactions with epigenetic effectors[39,53–55] (**Table 1**).

Long ncRNAs and Cancer: De-Repressing Tumor Suppressor Genes

Hox antisense intergenic RNA, *HOTAIR*, is a 2.2 kb lncRNA transcribed from the *HOXC* locus that negatively regulates the expression of *HOXD* genes *in trans*.[39] *HOTAIR* interacts with the PRC2 complex to silence genes by depositing repressive histone 3 lysine 27 trimethyl marks (H3K27me3) on the *HOXD* locus.[39] Importantly, *HOTAIR* is overexpressed in metastatic tumors, in particular, breast,[56,57] ovarian,[58] esophageal,[59] gastric[60,61] as well as a number of other cancers.[62–68] The current understanding, at least in breast cancer, is that *HOTAIR* overexpression promotes tumor metastasis by re-targeting PRC2 to metastasis suppressor genes. Furthermore, knockdown of *HOTAIR* inhibits cancer invasiveness, highlighting its potential as a target for cancer therapeutics.

The lncRNA *ANRIL* was first identified as an antisense transcript overlapping the *INK4b/ARF/INK4a* tumor suppressor locus that emerges from the 403 kb germ-line deletion observed in patients with melanoma- neural system tumor syndrome.[69] *ANRIL* negatively regulates its cognate transcript, *INK4b*, *in cis*, by an epigenetic mechanism that induces heterochromatin formation.[70] Prostate cancer tissues have significantly elevated levels of *ANRIL* and CBX7 (a chromobox protein (CBX) that is part of the polycomb repressive complex 1 (PRC1)) that correlates with a significant reduction in *INK4a*. *ANRIL* associates with CBX7 and other members of the PRC1 protein complex that recognizes H3K27me3 via the chromobox protein. Point mutations of the CBX7 chromodomain disrupts lncRNA binding, H3K27met3 and de-represses the *INK4b/ARF/INK4a* locus.[53]

Some lncRNAs are unique to specific diseases, *e.g.*, prostate,[71] colorectal cancers,[72] and hematopoietic tumors[73] while others are overexpressed in several cancers.[57,66,67] The prostate cancer-associated lncRNA transcript-1 (*PCAT-1*) is overexpressed solely in metastatic prostate cancer and knockdown of this transcript reduces proliferation in prostate cancer cells.[71] Suppressor of zeste 12 homolog (SUZ12), a component of the repressive PRC2 complex, binds to the *PCAT-1* promoter to suppress transcription of metastatic genes. Therefore, targeting *PCAT-1* could de-repress genes that help prevent prostate cancer progression.[71]

The important tumor suppressor gene phosphatase and tensin homolog gene (*PTEN*) is silenced in a number of cancers and is regulated in part by antisense lncRNA transcripts that arise from the PTEN pseudogene (*PTENpg1*) locus. The α isoform of *PTENpg1* as RNA functions *in trans* and interacts with EZH2 and DNA methyltransferase 3a (DNMT3a) to negatively regulate the expression of *PTEN*, making the α isoform of *PTENpg1* a potential target in several cancers.[74]

The lncRNA *H19* positively regulates tumor growth[75] via a PRC-2 dependent mechanism. *H19* physically interacts with EZH2 to reduce E-cadherin and promote metastasis in oral tongue squamous cell carcinoma[76] and bladder cancer.[77]

During cancer, several lncRNAs are dysregulated and act to repress important tumor suppressor genes. However, some lncRNAs promote tumor suppression by regulating p53 signaling.[78,79] The 7.1 kb lncRNA *TUG1* is induced by p53 and interacts with PRC2 to repress 120 genes, most of which are involved in cell cycle regulation.[37] Interestingly, a recent report identifies one of the genes repressed by *TUG1* to be *HOXB7*, a known oncogene.[79] Similarly, the *PINT* lncRNA acts as a tumor suppressor that reduces cell proliferation by regulating the expression of genes involved in p53 signaling via a PRC2-dependent mechanism.[78] These p53-associated lncRNAs silence oncogenic and cell proliferation-promoting genes. Therefore, these lncRNAs have the potential to be exploited for their potential therapeutic function in regulating p53 signaling.

The lncRNAs *HOTAIR*, *ANRIL, PCAT-1, PTENpg-1*α isoform, and *H19* all form complexes with epigenetic enzymes to silence cancer-promoting genes. Therefore, targeting the

Table 1 The various mechanisms through which lncRNAs function during disease

Transcript	Target	Mechanism	Disease Relevance	Reference
GAS5	*Glucocorticoid receptor (GR)*	Decoy	Cancer, Autoimmune disease	28,29
APOA1-AS	*APOA1*	Scaffold, Guide	Cardiovascular disease	83
BDNF-AS	*BDNF*	Scaffold, Guide	Neurodegenerative disorders	38
DLEU1, DLEU2	*13q14.3 gene locus*	Scaffold, Guide	Leukemia	73
H19	*E-cadherin*	Scaffold, Guide	Cancers	75–77
HOTAIR	*HOXD 8-11, 13*	Scaffold, Guide	Cancers	56–68
KCNQ1OT1	*KCNQ1*	Scaffold, Guide	Beckwith-Wiedemann Syndrome, adrenal neuroblastoma	43,44
ANRIL	*Ink4b/ARF/INK4a*	Scaffold, Guide	Cancers	69,70
PCAT-1	*BRCA2, CENPF, CENPE*	Scaffold, Guide	Prostate cancer	71
PINT	p53 pathway genes	Scaffold, Guide	Cancers	78
PTENpg1-α isoform	*PTEN*	Scaffold, Guide	Cancers	74
TUG1	*HOXB7*	Scaffold, Guide	Cholangiocarcinoma	79

LncRNAs are involved in the epigenetic regulation of genes and have been implicated in a number of diseases.

lncRNAs that bring chromatin modifying enzymes into contact with specific loci, could be key in developing anticancer therapies. More specifically, targeting the lncRNA or disrupting the lncRNA-protein (*HOTAIR*-PRC2, *ANRIL*-CBX7, *PCAT-1*-PRC2, *PTENpg-1*α isoform-PRC2, *H19*-EZH2) interactions, could de-repress cancer-suppressing genes, in a highly specific fashion by altering chromatin structure to promote senescence and slow cancer progression. Furthermore, upregulating *TUG1* and *PINT* lncRNAs that interact with PRC2 to silence oncogenic genes can also be therapeutically valuable.

Although the examples of lncRNAs presented have been shown to exert their effects primarily by interacting with histone modifying complexes, it is important to note that lncRNAs can also interact with DNA methyltransferases directly to regulate DNA methylation. In doing so, lncRNAs have another, perhaps more significant role, that of regulating long-term epigenetic inheritance via their interactions with *de novo* (DNMT3a and DNMT3b)[74,80] and maintenance (DNMT1)[81] DNA methyltransferases.

LncRNAs and Cardiovascular Disease: Upregulating Therapeutic Genes

Apolipoprotein A-1 (APOA-1) is the major protein component of high-density lipoprotein (HDL) in plasma where it plays an important role in cholesterol efflux. Elevated HDL levels have been shown to protect against coronary heart disease,[82] making APOA1 a strong candidate for pharmacological intervention for patients at risk for heart disease. Analysis of the *APOA1* gene locus resulted in the identification of along noncoding antisense transcript, *APOA1*-AS. The *APOA1*-AS transcript regulates *APOA1 in cis*, purportedly through an epigenetic mechanism.[83] Knockdown of *APOA1*-AS upregulates *APOA1*, increases activating histone 3 lysine 4 trimethylation (H3K4met3) marks and reduces suppressive H3K27 trimethylation spanning the promoter and enhancer regions of the *APOA1* gene. The reduction in H3K27met3 suppressive marks correlates with a reduction in the SUZ12 protein, a core component of the PRC2 complex. Importantly, knockdown of *APOA1*-AS *in vivo* results in a significant increase in endogenous APOA1 protein. Therefore, *APOA1*-AS can be used to directly modulate APOA1 protein, making it a potential therapeutic target for cardiovascular disease.[83]

The emerging use of stem cell therapy for the treatment of patients with cardiovascular disease seems to hold great promise. However, in order for this regenerative therapy to be successful, it is critical to understand and re-create the processes that result in the differentiation of mature cardiomyocytes from pluripotent stem cells. A novel lncRNA, *Braveheart* (*Bvht*), was recently identified to be important in specifying lineage commitment in mice via a PRC2-dependent mechanism. *Bvht* interacts with SUZ12 during multiple stages of cardiomyocyte differentiation.[84] This work suggests that mediating the interaction between *Bvht* and PRC2 might be a means to promote differentiation of stem cells into cardiomyocytes. Understanding the function and mechanisms of lncRNAs in stem cell differentiation might aid in the production of more efficacious stem cell therapies. Furthermore, strengthening the interaction of *Bvht* with PRC2 might be a means by which to promote cardiomyocyte differentiation in stem cells.

LncRNAs *APOA1*-AS and human mimics of *Bvht* are both potential therapeutic targets for the specific upregulation of therapeutic genes in the heart. Blocking interactions between *APOA1*-AS and epigenetic enzymes might de-repress the *APOA1* gene to upregulate apololipoprotein A1. In the case of *Bvht*, strengthening the interaction of this lncRNA with PRC2 could increase cardiomyocyte formation by de-repressing differentiation in pluripotent stem cells.

LncRNAs and Neurological Disorders: Upregulating Neuroprotective Proteins

Neurotrophins have been shown to play an important role in the maintenance of neuronal survival, outgrowth, and differentiation.[85] Brain-derived neurotrophic factor (*BDNF*), one of the most abundant growth factors in the brain, is a protein that is critical for the development, survival and maintenance of neurons in the nervous system. Furthermore, BDNF is a therapeutic target for several neurodegenerative and neurodevelopmental disorders in which BDNF is severely downregulated.[86–88] The *BDNF* locus gives rise to a 2.2 kb antisense transcript, called *BDNF*-AS, that discordantly regulates *BDNF* mRNA and protein levels *in cis*.[38,89] Blocking or knocking down *BDNF*-AS correlates with a reduction in H3K27me3 at the *BDNF* promoter region and a reduction of enhancer of zeste 2 (EZH2, catalytic subunit of the PRC2 complex) occupancy, suggesting that *BDNF*-AS exerts its repressive effect on the *BDNF* locus by interacting with PRC2 to silence *BDNF* expression *in cis*. Therefore, *BDNF*-AS or its site of interaction with PRC2 could be a viable therapeutic target to upregulate BDNF protein expression. This mechanism of specific gene upregulation might be extended to other neuroprotective genes, *e.g.*, glial-derived neurotrophic factor (*GDNF*), and ephrin B2 receptor (*EPHB2*), which were also shown to be discordantly regulated by their cognate antisense transcripts, opening the door for further study of the epigenetic regulation of these potentially therapeutic CNS genes.[38]

An analysis of genes both enriched in the brain and implicated in Autism revealed that 40% of "disease" genes had overlapping antisense transcription that exhibited region-specificity in the brain of Autism spectrum disorder (ASD) patients.[90] An antisense transcript to *SYNGAP1*, called *SYNGAP1*-AS, was differentially expressed in the brain regions of patients with ASD compared to non-ASD control subjects. SYNGAP1 is a protein that is important during the maturation of learning, memory and cognition and its dysregulation has been implicated in ASD.[91] Furthermore, *SYNGAP1*-AS is found primarily in the chromatin compartment of the cell and is discordantly expressed with respect to *SYNGAP1* in autism patients. Although a mechanism for *SYNGAP1*-AS has not been explored, the data suggest that *SYNGAP1*-AS, due to its localization to chromatin, may exert its effects on *SYNGAP1* through an epigenetic mechanism that could be of therapeutic value to ASD patients.[90]

Gene repression by lncRNAs is prevalent in the brain and occurs at several loci that encode for proteins involved in nervous system development and maintenance as well as synapse formation and maturation. Since these lncRNAs are potent regulators of transcription, inhibition of these repressive transcripts could result in locus specific upregulation of therapeutic genes. Importantly, one of the first examples of *in vivo* endogenous gene upregulation was described by blocking mouse *Bdnf*-AS.[38] The potential to target lncRNAs and produce changes in neurotrophin and synaptic proteins has major implications in treating neurodegenerative and neurodevelopmental diseases where BDNF protein is dysregulated, *e.g.*, Alzheimer's Disease, Rett's Syndrome, and Parkinson's Disease.[92] These disorders all exhibit marked reductions in BDNF and can potentially be protected against or in part rescued by the de-repression and upregulation of endogenous BDNF protein.[92]

Designing LncRNA-Directed Therapeutics

Despite the multiple ways that genes can be silenced, methods for upregulating the expression of therapeutic genes remains a challenge.[93] LncRNAs represent attractive therapeutic targets due to their presence in a development and cell-type specific manner as well as their ability to target a specific subset of genes. However, it must be noted that although *cis* or *trans* regulatory mechanisms have been identified for certain lncRNAs, effects on global gene expression changes are in most cases unknown and should also be carefully considered. LncRNAs have mainly been targeted using antisense oligonucleotides (ASOs), siRNAs and viral vectors that contain shRNAs (**Figure 2**). Although lncRNAs represent appealing pharmacological and therapeutic targets, inhibiting lncRNAs *in vivo* remains a challenge.[94]

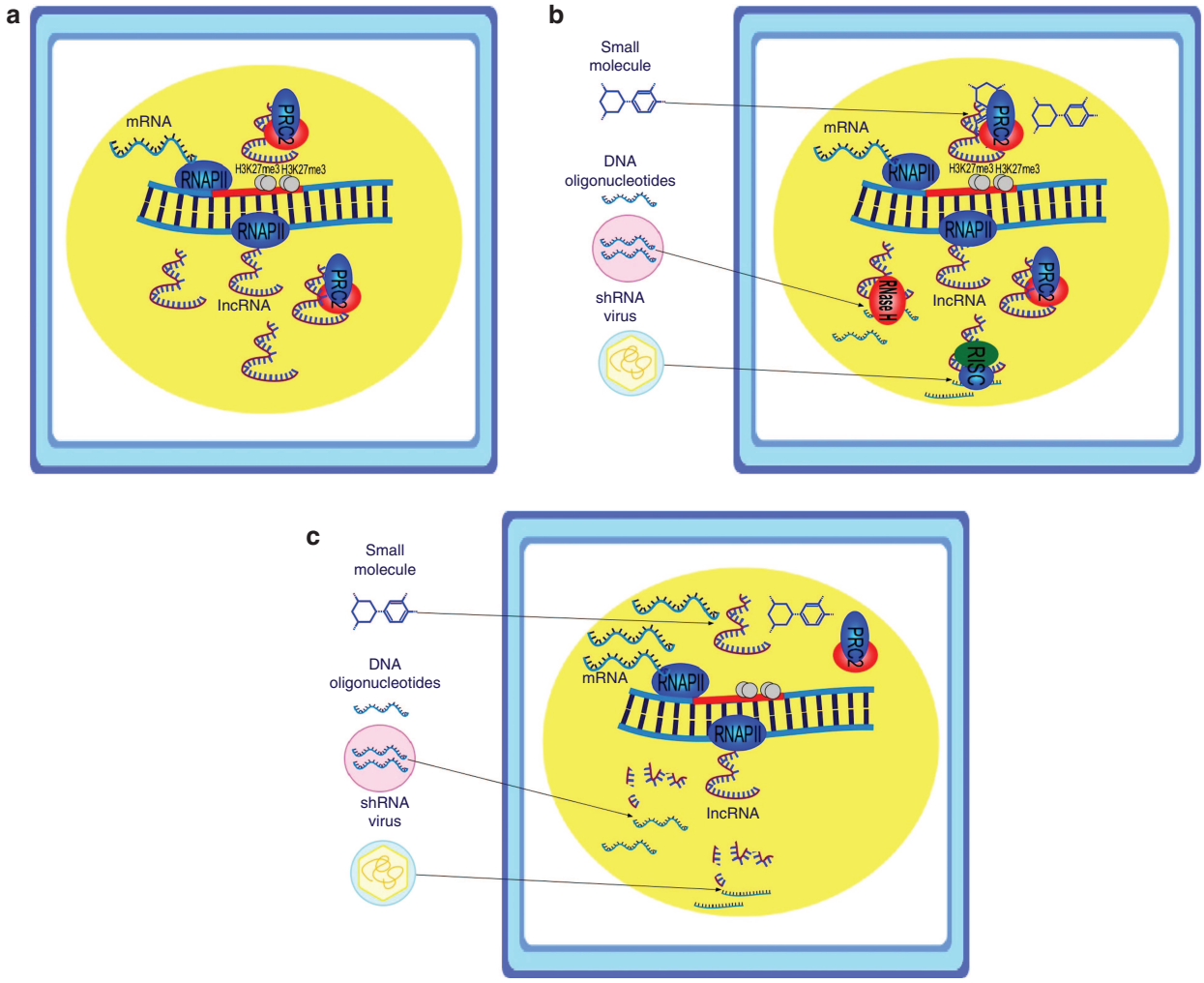

Figure 2 Inhibition of lncRNAs to upregulate gene expression by blocking interactions with epigenetic complexes. (**a**) LncRNAs bind to the PRC2 complex and guide the complex to target gene promoters (red) to induce H3 lysine 27 trimethylation (H3K27met3) and repress transcription. (**b**) Potential approaches to inhibit the repressive action of lncRNAs. Top: Small molecules can inhibit interaction of the PRC2 complex at the lncRNA binding site to de-repress transcription. Middle: Naked or liposome-encapsulated DNA oligonucleotides can target lncRNAs to form DNA-lncRNA duplexes that recruit RNase H. Bottom: Viral particles containing lncRNA-targeting shRNAs can deliver shRNAs that generate siRNAs (targeting the RISC complex to the lncRNA) in the cell. (**c**) Top: Small molecule drugs prevent the formation of a functional PRC2-lncRNA complex. Middle: RNase H results in lncRNA cleavage and decay. Bottom: The RISC complex cleaves and degrades lncRNAs. These lncRNA-targeting approaches result in the dissociation of the PRC2 complex from the target gene promoter, de-repressing genomic loci to upregulate the expression of therapeutic genes.

Knockdown of disease genes, *in vitro*, usually involves the use of siRNAs that are unstable and non-penetrating to target cells *in vivo.* Modified antisense oligonucleotides are stable, nuclease-resistant, short (13–25 nucleotides), single-stranded DNA oligonucleotides that are complementary to RNAs of interest, have been used to induce RNase H-dependent cleavage of lncRNA targets. Various modified nucleotides have been tested both *in vitro* and *in vivo* and these modifications are continually undergoing enhancements in chemistry. Modified oligonucleotides targeting antisense lncRNAs, also called AntagoNATs, usually contain a mixture of 2′-O-methyl RNA and Locked Nucleic Acid (LNA) modifications at their 5′ and 3′ ends to protect against nuclease cleavage and phosphothioate backbone to enhance their cellular uptake.[95]

Knockdown of lncRNAs *in vivo* has led to successful upregulation of therapeutic target genes and proteins resulting in measurable phenotypic responses.[38] Continuous *in vivo* infusion of AntagoNATs targeting mouse *Bdnf-AS* produced a locus- specific upregulation in Bdnf mRNA and protein. Mice treated with BDNF AntagoNATs display a marked increase in proliferating cells and neuronal survival in the brain as compared to controls, indicating that an increase in functional Bdnf protein was achieved. This effect is made all the more significant because of the difficulty in upregulating neurotrophins *in vivo*, even through small molecule modulators of neurotrophin receptors.[96] Repeated administration of AntagoNATs targeting monkey *ApoA1*-AS resulted in the upregulation of HDL levels. Nuclear-localized lncRNA *Malat1* was successfully targeted for downregulation in skeletal muscle with the systemic administration of a gapmer, that might have therapeutic applications in cancer.[97] Subcutaneous administration of antisense oligonucleotides targeting *MALAT1* also effectively inhibited human lung cancer cell proliferation in a mouse xenograft model.[98] In the past couple of years, around 100 ASOs and 40 RNAi-based therapies are put forward for clinical trials, including over 20 in advanced clinical trials (phase 2 or 3).[99] Two oligonucleotide drugs, Fomivirsen[100] and Mipomersen,[101] have received FDA-approval to treat Cytomegalovirus retinitis and high blood cholesterol, respectively. Additionally, several ASO-based therapeutics are in stage III clinical trials, including ASOs that induce exon skipping in Duchenne muscular dystrophy.[102] Although the use of ASOs for gene upregulation seems promising, poor intracellular uptake and chemistry-dependent toxicity are still major concerns when developing therapeutics.[103]

RNA interference approaches have been successful in downregulating the expression of lncRNAs *in vitro* to promote upregulation of their target genes. *In vivo*, RNAi-based approaches require a delivery vehicle such as liposomes, nanoparticles or viruses to protect siRNA or shRNA vectors from nuclease degradation, prevent their accumulation in the liver and enhance cellular uptake.[104] There have been multiple successful attempts to target an mRNA with RNAi *in vivo*,[105] and currently, 40 RNAi therapies are in clinical trials.[105] Development of new RNAi delivery vehicles for the inhibition of lncRNAs and locus-specific gene upregulation holds promise. Viral vectors are effective in delivering shRNAs *ex vivo* and *in vivo* to produce stable and specific knockdown of target RNAs and have been used multiple times *in vitro* and *in vivo* to knockdown lncRNAs to upregulate target genes. Lentiviral vectors for gene therapy have successfully been used for transducing hematopoietic stem cells in the course of clinical trials for treating two rare genetic disorders: metachromatic leukodystrophy (MLD)[106] and Wiskott–Aldrich syndrome (WAS).[107] Immune cells genetically modified with lentiviral vectors have been used in multiple paradigms of cancer immunotherapy.[108] Moreover, *ex vivo* gene therapy with lentiviral transduction of shRNA to stably downregulate target disease mRNA has been used in transplantation of CD34(+) cells to treat HIV.[109] Overall, antisense oligonucleotides and RNAi therapies targeting repressive lncRNAs are promising but require additional developments in oligonucleotide chemistry and use of vehicles for safe and effective delivery.

Small Molecule Modulators of LncRNA-Protein Interactions

Many chromatin-modifying enzymes have a flexible backbone and it is believed that they fold and form distinct structures only when bound to other proteins, RNA or DNA. This flexibility is believed to allow proteins to interact with several partners and form stronger, more stable interactions.[110–113] Chromatin-modifying enzymes are mostly ubiquitously expressed proteins that lack rigidity in protein structure, which makes them undesirable or difficult therapeutic targets using a small molecule approach.[114]

Chromatin-modifying enzymes that interact with lncRNAs are being studied for their ability to specifically bind RNA. Although some reports suggest that in the case of PRC2, the histone methyltransferase EZH2 is a promiscuous RNA binding protein,[115,116] other studies[117,118] have shown that the EZH2 component of the PRC2 complex does exhibit RNA-binding specificity when studying well-known PRC2-interacting lncRNAs *Xist*[117] and *HOTAIR*[117,118] as compared to control transcripts. It is important to mention that novel methods need to be developed in order to reliably identify RNA-protein interactions. Current methodologies have serious limitations, for example, in immunoprecipitation experiments, a protein of interest might interact with many RNAs, however due to methods of crosslinking, might grossly over or underestimate RNA protein interactions.[119] Furthermore, RNA-centered approaches, using a labeled RNA of interest to pull down interacting proteins followed by mass spectrometry can detect abundant RNA binding proteins which might be experimental artifacts as only a few of these RNAs have been purified.[119] Additionally, the reported non-specific RNA binding to EZH2 might be an artifact due to technical limitations in studying RNA-protein interactions (particularly using EMSA alone) or could be a general feature of RNA-binding proteins. We suggest that better *in vitro* assays are needed to test lncRNA-protein interactions and that these studies should incorporate valid RNA and protein controls, while taking the RNA-binding affinity of proteins of interest in the assay design.

Chromatin-modifying enzymes usually need lncRNAs to form complexes with other effector proteins and to be targeted to specific genomic loci. Therefore, one viable approach would be to target interactions between lncRNAs

and proteins with small molecules, in order to increase specificity and to overcome problems with tissue and cell penetration. We suggest the use of small molecules as an alternative therapeutic approach and are actively pursuing this by designing measurable pharmacological assays to quantitate lncRNA-protein interactions (Fatemi RP, Salah-Uddin H, Faghihi MA, manuscript in preparation). Targeting lncRNA-protein interactions, instead of ubiquitously expressed effector proteins, is a superior approach due to cell, tissue and developmental time point specificity of lncRNA expression. To design small molecules targeting RNA, it is necessary to understand higher order RNA structures. Although lncRNAs lack sequence conservation, they are structurally conserved, making them more stable and predictable physical targets, with their promoters conserved in a manner similar to that of protein-coding genes.[120,121] Although the "druggability" of RNAs with small molecules has been studied with some success, a more detailed understanding of RNA-ligand interactions, additional RNA targets and active compounds that target RNAs are needed.[122,123] The most widely studied RNA targets for small molecule therapy include the bacterial 16S rRNA A site, HIV-1 Rev Response element (RRE) and HIV-1 trans-activating region (TAR) RNAs. The most successful RNA-targeting approach to date has been the use of aminoglycosides to target the bacterial 16S rRNA, however these compounds are limited to *in vitro* use.[123] Netilmicin, an aminoglycoside derivative, has been used to target TAR and has been effective *in vitro*; however, due to poor absorption, can only be injected for use as an antibiotic in extreme cases.[124,125]

Therefore, targeting lncRNAs at their site of interaction with chromatin modifying enzymes is a well-defined alternative approach as compared to targeting either proteins or lncRNAs alone. Targeting lncRNA-protein interactions would increase the specificity of compounds, reduce off-target effects, and provide reversible inhibition of chromatin modifying enzymes at non-catalytic domains of the protein.

Considering that lncRNAs and their epigenetic effectors are implicated in gene regulation in a wide variety of diseases, it would stand to reason that targeting lncRNAs at their site of interaction with epigenetic modifying enzymes would alter gene expression. In targeting the interaction between lncRNAs and enzymes of interest, it is possible to modify gene expression without altering the protein's other effects in the cell, *e.g.*, the epigenetic enzymes JARID2[126] and EZH2[116] have distinct sites at which they bind lncRNAs. A great degree of specificity can be achieved when targeting lncRNAs that act *in cis* compared to more widespread effects in *trans*-acting lncRNAs, however one important caveat to this is that global changes in gene expression upon lncRNA modulation must first be characterized, since lncRNAs might act both *in cis* and *in trans* and might even have cytoplasmic effects. Furthermore, tissue and cell-type as well as developmental-specific lncRNA expression patterns add another level of specificity to this approach. However, in order to target this specific interaction, it is crucial to understand the biochemistry of how RNAs and proteins interact.

Small molecules are used to treat various diseases as these compounds have greater cellular uptake and less administration challenges than antisense oligonucleotides or viral vectors for the delivery of RNAi. In order to use small molecule drugs to target lncRNA-protein interactions, pharmacological rules for rational drug discovery must be applied. Applying the rules for rational drug design necessitates two main criteria: (i) to have a clearly defined target whose modulation is of therapeutic value and (ii) that the target is in fact druggable.[127] We have described above evidence to support the first criteria and find that the modulation of these lncRNAs is indeed of therapeutic value in several disease contexts.[38,56,69,83,90] It is also critical to understand the drug targets themselves: RNA higher order structures, protein tertiary structure and the unique conformational changes that occur when an RNA and protein interact, *i.e.*, a lncRNA interacts with a chromatin modifying enzyme.

The next task is to define whether these targets are receptive to small molecule modulation. To answer this question, there is a need for the development of target-based pharmacological assays that can quantify lncRNA-protein interactions for the purpose of high throughput compound screening. There are several widely used non-quantitative methods for measurement or RNA-protein interactions such as, RNA footprinting, various RNA pulldown methods (Crosslinking and immunoprecipitation (CLIP), RNA Immunoprecipitation (RIP), Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP)), co-staining by fluorescence *in situ* hybridization and immunofluorescence (FISH-IF), and RNA electrophoretic mobility shift assays (RNA EMSA). However, these methods are cumbersome and are not amenable to compound screening. Recently, two new methods for the quantitative analysis of RNA-protein interactions have been introduced, RNA on a massively parallel array (RNA-MaP)[128] and high throughput sequencing-RNA affinity profiling (HiTS-RAP).[129] These two methods are currently being used to characterize the RNA-protein interactome, and when targets are identified, have the potential to be modified for compound screening. By and large, a pharmacological assay that can quantitate the degree and affinity of lncRNA-protein interactions and is amenable to screening will be a tremendous asset to the field.

Because of the malleability of proteins and RNAs, the most efficient site to target lncRNAs with small molecules should ideally be at the binding cleft where the lncRNA and chromatin-modifying enzyme interact. In order to achieve this, modeling the conformation of proteins and RNAs and understanding the unique conformations these molecules undertake when they interact is key. Several studies have focused on high throughput approaches to understanding RNA structure and RNA-protein interactions on a large-scale.[128,130–135] However, methods to assay for small molecules must also be implemented in order to make use of this structural information. Furthermore, X-ray crystallography and NMR spectroscopy should be incorporated to produce models to better understand how small molecules would both fit and bind in the lncRNA-protein binding cleft. This approach has been successfully used in modeling a single protein or protein-protein interactions, but should also be extended large-scale to lncRNA-protein interactions. It should be noted that these approaches have important limitations, *e.g.*, the length of time and difficulty in producing crystal structures and the limitations in nucleic acid length and protein size in NMR, thereby making it difficult to produce a structure for most full-length

long noncoding RNAs.[136] Biochemical studies are necessary to accurately model the minimum regions required to allow lncRNAs to interact with specific domains of chromatin modifying enzymes. Furthermore, accurate methods to measure the strength of these lncRNA-protein interactions are lacking in the field but are necessary to develop assays to screen for small molecule modulators.[123] Therefore, quantitative assay methods should be employed in conjunction with biochemical approaches and structural modeling so that high throughput compound screening can be performed and hit compounds can be optimized using accurate structure models. The use of small molecules would overcome many of the challenges observed in the above-mentioned therapies and holds tremendous potential therapeutically.

Long noncoding RNA-protein interactions represent an important class of molecular effectors within the cell. As such, they also represent a new and unexplored set of druggable targets with the potential for developing highly specific targeted therapeutics that can surpass the efficacy of existing drugs used to treat diseases in various contexts. The use of small molecules that target specific lncRNA-protein interactions to finely tune gene expression and modify chromatin state can overcome many of the complications introduced by using other approaches. Furthermore, directing therapies to these specific interactions can help reduce off-target side effects that plague drugs currently on the market.

Conclusions

Although a wealth of transcriptomics data exists indicating the potential presence of thousands of lncRNAs, validation of the function of these ncRNAs still remains a challenge. To date, the function and mechanisms of only a few of these lncRNAs have been studied, yet their mechanisms and global effects on gene expression require further research.[30,41,56] However, in order to design effective therapeutics, we must further study the function of lncRNAs, elucidate their mechanisms, understand their structures, and develop approaches to quantitatively assay for drugs that modulate their interactions with proteins to ultimately de-repress genomic loci and to effectively upregulate therapeutic genes.

Acknowledgments. The authors thank Marco Magistri for critical reading of the manuscript. This work was done during the term of an Award from the American Heart Association Greater Southeast Affiliate to R.P.F. The US NIH NINDS R01NS081208-01A1 awarded to M.A.F supported this work. The authors declare no conflict of interest.

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