REVIEW

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Exploiting Long Noncoding RNAs as Pharmacological Targets to Modulate Epigenetic Diseases

Bindu Prabhakar[a], Xiao-bo Zhong[a,b], & Theodore P. Rasmussen[a,b,*]

[a]Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, CT; [b]Institute for Systems Genomics, University of Connecticut, Storrs/Farmington, CT

Long non-coding RNAs (lncRNAs†) constitute the largest class of non-coding transcripts in the human genome. Results from next-generation sequencing and bioinformatics advances indicate that the human genome contains more non-coding RNA genes than protein-coding genes. Validated functions of lncRNAs suggest that they are master regulators of gene expression and often exert their influences via epigenetic mechanisms by modulating chromatin structure. Specific lncRNAs can regulate transcription in gene clusters. Since the functions of protein-coding genes in clusters are often tied to specific pathways, lncRNAs constitute attractive pharmacological targets. Here we review the current knowledge of lncRNA functions in human cells and their roles in disease processes. We also present forward-looking perspectives on how they might be manipulated pharmacologically for the treatment of a variety of human diseases, in which regulation of gene expression by epigenetic mechanisms plays a major role.

EPIGENETICS: CHROMATIN-MEDIATED EFFECTS ON GENE REGULATION

In 1942, Conrad Waddington coined the term 'epigenetics' and defined it as "the study of the causal interactions between genes and their products, which leads to phenotype changes during development" [1,2]. Today, epigenetics is a rapidly growing field of research and is now broadly defined as mechanisms leading to changes in gene expression that do not involve changes in DNA sequences *per se*. At a molecular level, epigenetic mechanisms are primarily mediated by alterations of chromatin structures and changes (especially in DNA methylation and post-translational modifications (PTMs) placed upon nucleosomal histones) can lead to alterations of the expression of genes presented in or near epigenetically modified nucleosomes. Epigenetic regulation is rooted in chromatin structures, which can be divided into two major classes: euchromatin and heterochromatin. Euchromatin is transcriptionally active and consists of DNA that is loosely associated with nucleosomes and thus is accessible to RNA polymerases. Heterochromatin, in contrast, is highly condensed and not readily transcribed, resulting in silenced gene states.

*To whom all correspondence should be addressed: Theodore P. Rasmussen, Ph.D., Department of Pharmaceutical Sciences, University of Connecticut, 69 North Eagleville Road, Unit 3092, Storrs, CT 06269; Tel: (860) 486-8339; Fax: (860) 486-5792; Email: theodore.rasmussen@uconn.edu.

†Abbreviations: ASO, Antisense oligonucleotide; BWS, Beckwith-Wiedemann syndrome; DMR, differentially methylated region; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; lincRNA, long intergenic noncoding RNA; lncRNA, long non-coding RNA; MBP, methyl-CpG-binding protein; NAT, natural antisense lncRNA; ORF, open reading frame; PTM, post-translational modification; PRC, Polycomb repressive complex; RPKM, reads per kilobase per million base pairs; SINE, short interspersed nuclear element SINEUP, to up-regulate translation; siRNA, small interfering RNA; TrxG, trithorax group; T-UCR, transcribed ultraconserved regions; UTR, untranslated region.

Long Non-coding RNAs

One of the major types of epigenetic regulation utilizes functionally untranslated RNA species. Long non-coding RNAs (lncRNAs) are non-coding RNAs with a length greater than 200 nucleotides. lncRNAs play versatile roles in many aspects of gene regulation, including transcription, mRNA splicing, translation, epigenetic silencing, genomic imprinting, X-chromosome inactivation, and the processing of small ncRNAs [3,4]. Many lncRNAs regulate gene expression by recruiting chromatin complexes by means of DNA methylation and histone PTMs, which are described below.

Roles of lncRNAs in the Regulation of DNA Methylation

The interaction between lncRNAs and DNA methylation enzymes plays a key role in epigenetic regulation. DNA methylation at position 5 within cytosine bases present in CpG dinucleotides plays a critical role in key biological functions, including embryonic development, genomic imprinting, X-chromosome inactivation, the silencing of transposable elements, and many others. Genomic imprinting is an epigenetic mechanism where certain genes are expressed from only one of the two parental chromosomes. Some imprinted genes are maternally expressed (e.g. *IGF2R* and *H19*), and others paternally expressed (e.g *IGF2*) [5]. Xist is transcribed from the X-inactivation center (Xic) and coats the inactive X-chromosome in females *in cis,* leading to silencing of hundreds of X-linked genes [6]. The Air lncRNA (Antisense Igf2r RNA) has been shown to silence imprinted genes [7]. H19 functions as a lncRNA but it has also been reported by Cai et al. that "H19 functions as a primary microRNA precursor decreasing the post-transcriptional down-regulation of mRNAs during development" [8].

Some lncRNAs are known to regulate DNA methylation through physical interactions with DNA methyltransferases (DNMTs). Ruscio et al. have reported that a lncRNA called Extra-coding CEBPA (ecCEBPA) physically interacts with DNMT1 (the primary maintenance cytosine methyltransferase) and prevents methylation of the *CEBPA* gene locus *in cis* [9]. lncRNA Dali physically interacts with DNMT1 protein and modulates DNA methylation of CpG island-associated promoters *in trans* [10]. The lncRNA Dum causes promoter methylation of developmental pluripotency associated 2 (*Dppa2)* gene through interactions with DNMT1, and the *de novo* methyltransferases DNMT3A, and DNMT3B [11]. lncRNAs can thus modulate DNA methylation *in cis* and *trans* through interactions with all three DNMTs. Hence, it is possible that dysregulated lncRNAs may be involved in epigenetic changes leading to human diseases.

Histone Modifications

Histone modifications are also known to epigenetically regulate gene transcription as well as DNA repair and replication, chromosome condensation, and alternative splicing [12]. A vertebrate nucleosome consists of an octamer of core nucleosomal histones (two each of histones H2A, H2B, H3, and H4) and a linker histone H1, which binds short stretches of DNA between nucleosomes leading to chromatin compaction. All of the core histones undergo covalent PTMs, which include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation at specific amino acid residues, mostly within their N-terminal histone tails [13]. Transcriptional activation is mediated by histone acetyltransferases (HATs) and transcriptional repression is mediated by the action of histone deacetylases (HDACs). The polycomb repressive complexes (PRC1 and PRC2) contain both histone methyltransferases leading to H3K27Me3 and histone monoubiquitination, which results in gene silencing, respectively [14]. In contrast, trithorax group (TrxG) complexes methylate H3K4Me3, which facilitates gene transcription [15]. Therefore, histone PTMs are centrally involved in epigenetic regulation at the level of chromatin structure, and this notion is revisited frequently in the remainder of this review.

EPIGENETIC MECHANISMS OF DISEASE

Together, histone PTMs, CpG methylation, and lncRNAs regulate gene expression and are exquisitely involved in a wide range of biological processes, including development and the epigenetic regulation of genes. However, when errors in the maintenance of epigenetic states occur in somatic cells, a variety of human diseases and conditions can occur.

Beckwith-Wiedemann syndrome (BWS), an imprinting disorder of the *KCNQ1OT* gene, is mediated epigenetically, and at least 50 percent of patients have loss of DNA methylation and loss of histone H3K9Me2 on the maternal *KCNQ1OT* differentially methylated region (DMR) [16]. He at al. reported that a microdeletion in the human *H19* DMR can result in loss of insulin-like growth factor 2 (*IGF2*) imprinting and BWS [16]. lncRNAs can thus lead to the development of complex diseases through epigenetic mechanisms.

Perturbations in DNA methylation are common in many cancers and global alterations involving DNA hypomethylation are common in cancer, often typified by a 20 to 60 percent reduction in 5-methyl-cytosine content [12]. DNA hypomethylation at the promoter regions of oncogenes can activate their expression, and DNA hypermethylation at the promoters of tumor suppressor genes can lead to their silencing. Loss of imprinting of *IGF2*

and *H19* genes by demethylation can lead to carcinogenesis and tumor progression [17]. The lncRNA LET is repressed by histone deacetylase 3 (HDAC3), which is reported to contribute to hypoxia-mediated cancer cell invasion [18]. The lncRNA ANRASSF1 reduces the expression of Ras association domain family 1 isoform A (RASSF1A) protein by recruiting PRC2 to the *RASSF1A* promoter region, thereby increasing cellular proliferation and inhibiting cell death [19]. These and other examples too numerous to cite firmly establish altered DNA methylation and histone modification as being intimately involved in carcinogenesis, and often in conjunction with lncRNA actions.

lncRNAs in Detail

Recent advances in next generation sequencing and bioinformatics show that much of the mammalian genome is transcribed into RNA, and that much of this RNA is functional. This new view constitutes a paradigm shift in our view of the genome as a whole and the regulation of protein-coding genes. The ENCODE project consortium reported that at least 93 percent of human genomic DNA is transcribed [20]. After completion and analysis of the human genome, it was found that there are only 20,000 to 25,000 protein coding genes in the genome, corresponding to less than 2 percent of the genome [21]. The remaining 98 percent was originally thought to be "junk DNA" [22], but it is now known that much of this DNA encodes functional non-coding RNAs (ncRNAs) [23] and hence, cannot be interpreted simply as 'transcriptional noise' [24].

lncRNAs make up the majority of the ncRNA transcripts in the genome [25], and yet the functions of most lncNRAs are unknown. lncRNA lengths can range from 200 nucleotides to over 50 kilobases [26]. Various genomic consortiums and databases including GENCODE [27] have assisted in the task of computational identification, annotation, and interpretation of expansive ncRNA datasets. lncRNAs, such as XIST and H19, were initially identified from cDNA libraries [28]. Iyer et al. curated 7,256 RNA sequencing libraries and identified 58,648 lncRNA genes from the human transcriptome totaling 91,013 expressed genes [29]. lncRNAs have been discovered by a variety of methods including RNA immunoprecipitation (RIP), microarray and tiling array screens, and RNA-sequencing. All these approaches have limitations. For instance, isolating lncRNAs though RIP methods relies upon the specificity of antibodies. Although these studies have provisionally identified a large number of lncRNAs, their functions cannot be fully evaluated until each of these lncRNAs is experimentally validated. Nonetheless, these studies show that the human genome likely contains more lncRNA genes than protein-coding genes.

lncRNAs have been classified into different categories and have been extensively reviewed by Laurent et al. [30]. Briefly, lncRNAs are classified based on their locations in the genome, their lengths, proximity to protein-coding genes, association with DNA elements, mechanisms of action, and sub-cellular localization (nucleus or cytoplasm). lncRNA genes can regulate adjacent protein-coding genes near their sites of synthesis, and these are known as *cis*-acting lncRNAs. lncRNAs can also regulate genes in distant genomic locations (on other chromosomes), and these are called *trans*-acting lncRNAs. lncRNA genes often reside near protein-coding genes (often presented in gene clusters) and they can be divided into sense lncRNAs, natural antisense lncRNAs (NATs), and long intronic ncRNAs (linRNAs). Sense lncRNAs are transcribed from the sense strand with respect to regulated protein-coding genes, while NAT lncRNAs are transcribed from the antisense strand with respect to adjacent protein-coding genes (e.g. APOA1-AS) [31]. linRNAs reside within introns of protein-coding genes [32]. Genes for intergenic lncRNAs (located between protein-coding genes) are called long intergenic noncoding RNAs (lincRNAs), and these include the well-studied lncRNAs XIST, H19, and an antisense non-coding RNA in the *INK4* locus (ANRIL) [33]. Some lncRNAs are of extraordinary length; these are called very long intergenic noncoding RNAs (vlincRNA) and they consist of transcripts over 50 kilobases (e.g. HELLP) [34]. Based on subcellular localization, lncRNAs can be divided into nuclear or cytoplasmic classes. Most lncRNAs are nuclear (eg. XIST and maternally expressed gene 3; MEG3) but some, like H19, are located in the cytoplasm [23]. Genome-wide physical interactions of lncRNAs with DNA have been determined by biochemical approaches, including Chromatin Isolation by RNA Purification (ChIRP) [35] and Capture Hybridization Analysis of RNA Targets (CHART) [36]. Through these methods, some lncRNAs associate with enhancers (enhancer-associated lncRNA, or elncRNAs) [37], promoters (promoter-associated lncRNAs, or PLARS) [38], or telomeres (telomeric repeat-containing RNA, or TERRA) [39]. Other lncRNAs include transcribed ultraconserved regions (T-UCR) [40], which are so called due to their sequence conservation across species (e.g. PTENP1) [41]. The existing systems of classification have limitations, since individual lncRNAs can fit into multiple classifications. Annotation and classification nomenclature of lncRNAs are still coalescing; hence, new classification strategies that can consider lncRNA properties, functions, and relationships are desirable.

lncRNAs are generally transcribed by RNA polymerase II and undergo post-transcriptional modifications including 5'-capping, polyadenylation, and splicing [23].

RNA polymerase III, which is primarily involved in the transcription of tRNAs and 5S rRNA, also transcribes a neuronal lncRNA, BC200 [42]. More than 25 percent of lncRNAs are alternatively spliced to produce two or more related isoforms [23]. Some lncRNAs, however, are non-polyadenylated [23]. lncRNA promoters exhibit specific histone marks, including methylated H3K4, H3K27, H3K36, and acetylated H3K9 and H3K27, suggesting that they too undergo epigenetic regulation similar to protein-coding genes [23].

The presence of well-defined open reading frames (ORFs) distinguishes protein-coding genes from lncRNAs. The FANTOM consortium assumes that genes containing long ORFs are likely protein-coding genes in the mouse transcriptome [43]. However, some lncRNAs have ORFs consisting of over 100 codons, including Xist, H19, and KCNQ1OT, yet these are not translated into proteins [44]. In a surprising ribosome profiling study by Guttman et al, it was found that the ribosome occupancy on many lincRNAs is detectable, but was similar to small ncRNAs (including small nuclear RNAs, small nucleolar RNAs, microRNA precursors, and lncRNAs) and other non-coding regions, including 5'-untranslated regions (UTRs). This demonstrates that the presence of ribosome occupancy alone is insufficient to determine the coding potential of lncRNAs. However, they defined a method to accurately distinguish protein-coding transcripts from all classes of non-coding transcripts based on the release of translating ribosomes from translated RNAs upon encountering a *bona fide* stop codon [45].

lncRNAs sequences are generally less conserved across species than protein-coding genes, implying that they undergo evolutionary changes much more rapidly. Even though some lncRNAs are poorly conserved, they are functional, which suggests that recently evolved lncRNAs can still be of functional consequence. Human accelerated regions (HARs) are regions where increased rates of nucleotide substitution occur between the human and chimpanzee. The 118-nucleotide HAR1 region is a part of the HAR1F lncRNA, which is highly expressed in developing human brain. The HAR1 region is folded into an organized RNA secondary structure and mutations in this region in human were shown to stabilize the secondary structure as compared to the chimpanzee [46]. Lack of conservation is a limiting factor for the use of animal models of human diseases, and it is possible that some of the species-specific differences might be due to divergence in lncRNA conservation. However, some lncRNA sequences are conserved across species. lncRNA promoter regions are more conserved than the exonic sequences and exhibit levels of sequence conservation comparable to protein-coding genes in the mouse genome [23,29,47]. lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is highly conserved in mammals

but is not found in non-mammals [48]. Polyadenylated RNA (PAN RNA) is produced and retained in the nucleus during Kaposi's sarcoma-associated herpesvirus (KSHV) lytic infection. PAN RNA has an expression and nuclear retention element (ENE), which enables the formation of a triple helical structure. The triple helix renders lncRNA stability and also its nuclear retention [49]. The lncRNA thyroid cancer-associated transcript 126 (THCAT126) is presented in nearly all vertebrates [29]. Thus, not all lncRNAs are poorly conserved across species, and Iyer et al. identified 597 intergenic lncRNAs, which contained regions of ultraconserved elements (UCEs) (regions greater than 200 nucleotides that are highly conserved across species) [29].

lncRNAs typically have lower expression levels in tissues as compared to protein-coding genes. Computational analysis of RNA sequencing data from 16 human tissues obtained from the Illumina Human Body Map Project revealed that lncRNAs in general had lower expression in all tissues than protein-coding genes, except in the testis [50]. lncRNAs also have higher tissue-specificity as compared to coding genes. Custom microarray studies on 9,747 lncRNA transcripts from GENCODE version 3c annotation, which assayed lncRNA content from human tissues and cell lines, also suggest that lncRNAs have far lower levels of expression relative to mRNAs [23,50]. lncRNA expression profiles are not identical for polyadenylated and non-polyadenylated RNAs. Djebali et al. reported that lncRNA gene expression ranged over six orders of magnitude (10^{-2} to 10^4 reads per kilobase per million reads (RPKM)) for polyadenylated lncRNAs and five orders of magnitude (10^{-2} to 10^3 (RPKM) for non-polyadenylated lncRNAs) [51]. It has thus been shown by different studies that most lncRNAs have lower expression and higher tissue specificity of expression than protein-coding genes.

lncRNAs are often more unstable than mRNAs. Two independent studies determined the range of lncRNA stability and reported that their half-lives varied from unstable to highly stable [52,53]. Clark et al. used custom microarray studies in a mouse cell line to identify the half-lives of approximately 800 lncRNAs and 1,200 mRNAs in the mouse Neuro-2a. Most of the lncRNAs were stable, with half-lives greater than 16 hours, and approximately 240 lncRNAs were unstable, with half-lives less than 2 hours. The mean half-life of lncRNAs was found to be 4.8 hours and this was less than mRNAs, which had a mean half-life of 7.7 hours [53]. It has been observed that the nuclear paraspeckle assembly transcript 1 (Neat1) is highly unstable, but is functional and required for the nuclear localization and dynamic regulation of paraspeckles at the Neat1 locus [53]. Several pathways are known to be involved in the degradation of lncRNAs, including nucleolytic degradation by nuclear exosomes and cytoplasmic

nonsense-mediated decay [54]. lncRNAs thus regulate key cellular functions irrespective of their stability within the cellular compartment (nucleus or cytoplasm).

Mechanisms of lncRNA Action

lncRNAs play a critical role in many cellular and biological processes, including early embryonic development, embryonic stem cell (ESC) pluripotency and differentiation, cell cycle regulation, proliferation, apoptosis, and senescence [3,4,55]. The molecular mechanisms whereby lncRNAs exert their regulatory impacts on protein-coding genes have been extensively reviewed [56-59]. Individual lncRNAs can operate as a signal, decoy, scaffold, and guide lncRNAs [56,57]. These classes of lncRNAs are discussed below:

Signal lncRNAs: lncRNAs can act as molecular signals to regulate transcription in response to different stimuli. Transcription of some lncRNAs is both tissue-specific and temporally specific. It has been noted by Wang et al. that some signal lncNRAs have regulatory functions while others are triggered by transcriptional events [57]. Wang et al. have reported that "lncRNAs act as signals marking space, time, developmental stage, and expression for gene regulation" [57]. lncRNA *KCNQ1OT1* acts as a signal lncRNA and induces transcriptional repression by recruiting G9a histone methyltransferases and PRC2 to the genes both *in cis* and *in trans* [60]. Thus, they serve as a marker of transcriptional activity. lncRNAs that are transcribed from the four human homeobox transcription factors (*HOX*) clusters exhibit specific spatial and temporal patterns of gene expression during development. For example, HOTAIR regulates sequential *Hox* gene expression during mouse embryogenesis, thus serving as a signal of anatomic position during development [57,61]. lncRNAs also respond to environmental stimuli, such as cold, which triggers their action (e.g., COLDAIR and COOLAIR) [62]. Signal lncRNAs thus serve as sensors that regulate important biological functions.

Decoy (sponge) lncRNAs: Some lncRNAs serve to sequester key cellular components. lncRNAs can bind to transcription factors and microRNAs, where they sequester these factors, thus preventing their action. lncRNA GAS5 acts as a decoy glucocorticoid response element (GRE) and binds to the DNA-binding domains of the glucocorticoid receptor, thereby preventing its association with DNA, downstream effects on the cell cycle and apoptosis [3,63]. lncRNAs also act as decoys (sponges) for miRNAs and splicing factors [4,57]. Pseudogene lncRNA PTENP1 acts as a sponge and sequesters microRNAs that bind the 3'-UTR of the tumor-suppressor gene *PTEN*, thus indirectly influencing translation of *PTEN* mRNA [64]. The lncRNA hepatocellular carcinoma upregulated long non-coding RNA (HULC) acts as an endogenous miRNA sponge for miR-372 and down-

regulates its target gene protein kinase cAMP-activated catalytic subunit beta *(PRKACB)* [4,65]. Decoy lncRNAs thus inhibit the function of the effector transcription factors and microRNAs by sequestration, and thereby negatively regulate transcription.

Scaffold lncRNAs: Scaffold lncRNAs are structural in nature and provide a framework upon which one or more proteins can simultaneously assemble within different lncRNA domains and regulate chromatin modifications [66]. HOTAIR acts as a scaffold and simultaneously binds to two different protein complexes. The 5' domain of HOTAIR binds to the PRC2 complex (involved in H3K27 methylation and gene silencing) and the 3' domain of HOTAIR binds to LSD1-CoREST complex to mediate H3K4Me2 demethylation [67]. ANRIL binds to both PRC1 and PRC2, leading to repression of the *INK4b/ARF/INK4a* gene locus [68]. KCNQ1OT1 serves as a scaffold by binding both PRC2 and G9a in order to mediate H3K27 and H3K9 trimethylation and consequent gene silencing [69]. lincRNA functional intergenic repeating RNA element (Firre) acts as a scaffold through its 156-bp repeating RNA domain (RRD). Firre interacts with the nuclear-matrix factor hnRNPU through the Firre RRD for nuclear localization of Firre transcripts and for binding to different chromosomal locations [70]. Thus, scaffold lncRNAs form a binding platform for tethering one or more chromatin-modifying complexes and enzymes to mediate RNA-protein interactions.

Guide lncRNAs: Guide lncRNAs act a guide for the localization of regulatory protein complexes, such as trithorax group proteins, polycomb group proteins, and transcription factors to their target DNA sites *in cis* or *in trans* [57]. Xist [71,72] and Air [73] act *in cis* to regulate syntenic genes that are either subject to dosage compensation or imprinting, respectively. The lncRNA HOXA transcript at the distal tip (HOTTIP) acts functionally as both a signal and guide lncRNA to regulate the *HOXA* locus, by recruiting the adaptor protein WD repeat domain 5 (WDR5) and mixed lineage in leukemia-1 (MLL-1) protein, which in turn mediate histone methylation and gene transcription in the *HOXA* locus [74]. *Trans*-acting lincRNAs like HOTAIR [75] and Jpx also regulate gene expression and chromatin modification upon other chromosomes [76]. Guide lncRNAs can thus regulate gene expression through complex epigenetic mechanisms.

lncRNAs can regulate mRNA stability both positively and negatively [77]. Antisense lncRNA BACE1-AS is known to increase the stability of BACE1 mRNA. BACE1-AS forms a RNA-RNA duplex with BACE1 mRNA. BACE1-AS competes with miR 485-5p for the same binding site in the BACE1 mRNA. This abrogates miRNA-induced repression and thus stabilizes the BACE1 mRNA. BACE1-AS expression is increased in the brains of Alzheimer's patients [78]. Alu elements are

primate-specific repeat elements. lncRNAs containing Alu repeats can destabilize mRNA through Staufen mediated decay (SMD). SMD is a process by which mRNA degradation is mediated by the binding of Staufen 1 (STAU1) (a protein that binds to double-stranded RNA) to STAU1-binding sites (SBS) within the 3'-UTR of the target mRNA. Alu elements within the 3'-UTR of lncRNAs imperfectly base-pair with the mRNA 3'-UTR Alu elements, creating a double stranded SBS. STAU-1 binds to this site and destabilizes the target mRNA. Gong et al. have shown that lncRNA-AF087999 base-pairs with the *SERPINE1* mRNA at its 3'-UTR Alu element sequence and facilitates the binding of STAU1 protein to mRNAs leading to SMD [79]. In summary, lncRNAs are involved in various aspects of mRNA stability.

lncRNAs in Human Disease

Dysregulated or mutated lncRNAs play a critical role in the etiology and pathogenesis of many diseases (Table 1). Many different types of cancers and syndromes are associated with mutations in lncRNA genes [3]. Genome-wide array studies show differential lncRNA expression patterns in comparisons between normal and tumor cells [80-82]. Transcribed ultraconserved regions (T-UCRs) are highly conserved sequences between orthologous regions of human, rat, and mouse genomes located in intra- and intergenic regions. Genome-wide microarray profiling studies of T-UCRs indicate that they are differentially expressed in human leukemias and carcinomas and are regulated by miRNAs both *in vitro* and *in vivo* [83]. HOTAIR is associated with multiple types of cancer (reviewed by Hajjari et al.), [84] where it interacts with PRC2 and LSD1 to repress target gene transcription [85]. ANRIL binds to chromobox 7 (CBX7), which is a component of the PRC1 complex, to induce gene silencing, and both ANRIL and CBX7 are upregulated in prostate cancer [86]. lincRNA-p21 is a p53 repressor and is associated with the development and progression of prostate cancer, chronic lymphocytic leukemia, atherosclerosis, and rheumatoid arthritis [87,88]. A chromosomal translocation involving the lncRNA gene *DISC2* on chromosome 1 (1;11) (q42.1;q14.3) is associated with schizophrenia and psychiatric disorders in a large Scottish family [89]. Genome-wide association studies (GWAS) have shown that germline deletion (403 kb) of *INK4b/ARF* locus including the *ANRIL* gene is associated with hereditary cutaneous malignant melanoma (CMM) and neural system tumors (NST) syndrome [90]. Expansion of CTG trinucleotides in the primary sequence of lncRNA gene *ATXN8OS* produces a toxic RNA that alters RNA splicing of spinocerebellar ataxia type 8 (*SCA8*) mRNA [91]. BWS is an imprinting disorder associated with abnormal imprinting of the *KCNQ1OT* and *IGF2* genes resulting in congenital malformation and tumor predisposition [16].

Thus, as more and more lncRNAs are being functionally validated, there is increasing evidence for the role of dysregulated or mutated lncRNAs in the etiology and prognosis of various neurodegenerative, cardiovascular, metabolic diseases, and cancer of various organs.

lncRNAs as Drug Targets: Future Strategies

Existing pharmaceutical agents to treat diseases through epigenetic mechanisms are nonspecific. Epigenetic drugs, such as the DNA methyltransferase (DNMT) inhibitor 5-aza-2'-deoxycytidine, and histone deacetylase (HDAC) inhibitors, such as sodium valproate, depsipeptide, tetrapeptide suberoylanilide hydroxamic acid (SAHA), and CI-994 (N-acetyldinaline) [92], have all been used to treat various types of cancer; however, their modes of action alter chromatin structure throughout the genome. Histone- or DNA-modifying enzymes attached to gene-specific zinc finger proteins may in the future lead to targeted treatments that will specifically bind to targeted epimutation sites [93]. However, since these are proteins, they must be delivered by venous injection. Since lncRNAs normally regulate relatively small sets of genes, often with related functions, the ability to target lncRNAs pharmacologically should result in improved specificity and lowered incidence of side effects. Gene clusters often contain genes with similar functions (i.e. they function in a shared pathway), and these gene clusters are typically regulated by one or few specific lncRNAs. Therefore, lncRNAs constitute attractive and potentially highly specific drug targets.

A number of opportunities and challenges exist for the future pharmacological manipulation of lncRNAs. RNA therapeutics can take advantage of various lncRNA cellular functions and target those pathways through gene silencing and structure disruption mechanisms. lncRNAs are functional molecules that can be detected in the body fluids; hence, they can serve as diagnostic biomarkers for various diseases. Occasionally, a single lncRNA can target several mRNAs, and in such situations, manipulating the lncRNA can help to modulate multiple genes and their functions. Extensive secondary structures and long lncRNA size may hinder the design of effective small interfering RNAs (siRNAs) and small molecule inhibitors. Toxicity might be observed with siRNA or antisense oligonucleotide (ASO)-mediated knockdown strategies designed to disrupt lncRNA functions. Single-stranded ASOs are highly unstable in cells and subject to nucleases. Other difficulties encountered with ASOs include low target affinity and low potency, which may require the use of higher concentrations that in turn could lead to off-target effects. Chemical modifications to ASOs, such as phosphorothioate modifications, heterocyclic modifications, 2'-O-methyl modifications, and 5'-, 3'-end-locked nucleic acid (LNA) modifications, are known to increase

Table 1. lncRNAs associated with human diseases

Affected organ system	Disease	Associated lncRNAs	References
Autoimmune Diseases	Psoriasis	PRINS	[122]
	Rheumatoid arthritis	HOTAIR	[123]
Cardiovascular diseases	Cardiac hypertrophy	7SK; CHRF	[124,125]
	Myocardial infarction	MIAT; KCNQ1OT1; ANRIL	[126,127]
Digestive system disorders	Barrett's	AFAP1-AS1	[128]
	Crohn's	DQ786243	[129]
Endocrine & metabolic disorders	Pseudohypoparathyroidism type Ib	GNAS-AS1	[130]
Genetic disorder	Fragile X syndrome	FMR4; FMR5; FMR6	[131,132]
	HELLP syndrome	HELLPAR	[34]
Infectious diseases	Leishmania	7SL	[133]
Musculoskeletal system disorders	Duchenne muscular dystrophy	KUCG1; linc-MD1	[134,135]
	Facioscapulohumeral muscular dystrophy	D4Z4; DBE-T	[136]
Neurological diseases	Angelman syndrome	UBE3A-AS1	[137]
	Parkinson's	naPINK1	[138]
	West syndrome	BX118339	[139]
Reproductive system diseases	Mullerian aplasia	H19	[140]
Cancer	Adenocarcinoma	HNF1A-AS1; ZXF1	[141,142]
	Breast cancer	GAS5	[143,144]
	Colorectal cancer	MALAT1; H19; HOTAIR	[145-147]
	Esophageal squamous cell cancer	ANRIL; SPRY4-IT1	[148,149]
	Gastric cancer	GHET1	[150]
	Kaposi's	PAN	[151]
	Liver	HULC	[152,153]
	Lung	MALAT1; LincRNA-p21	[154,155]
	Testicular cancer	BOK-AS1	[156]

affinity and cellular uptake and decrease toxicity [94]. The low expression of lncRNAs may permit the use of lower doses, which may alleviate some toxicities. Off-target hybridization effects can be minimized by careful bioinformatics selection of ASO sequences. Some of the possible pharmacological approaches to target lncRNAs are discussed below and are summarized in Figure 1.

SINEUPs: Short interspersed nuclear elements (SINEs) are short (< 500 bp) non-coding repetitive sequences that can transpose into new parts of the genome by means of an RNA intermediate and reverse transcriptase. SINEUPs are a new class of natural antisense lncRNAs that contain an embedded SINEB2 (SINE of B2 family element), and these can Up-regulate translation of target mRNAs. SINEUPs are known to enhance protein synthesis of their target mRNA and function post-transcriptionally [95,96]. SINEUPs contain two functional domains: a Binding Domain (BD), which provides specificity (via base-pairing) to the targeted mRNA, and an Effector Domain (ED) that acts as an activator of translation. The BD consists of 72 nucleotides, which include sequences that are complimentary to the 5'-UTR, the translation initiation codon and initial codons. The ED contains repetitive SINEB2 sequences. The SINEUP lncRNA base-pairs with the mRNA, and the SINEB2 element facilitates association of the mRNA with polysomes, thus increasing the rate of translational initiation [96].

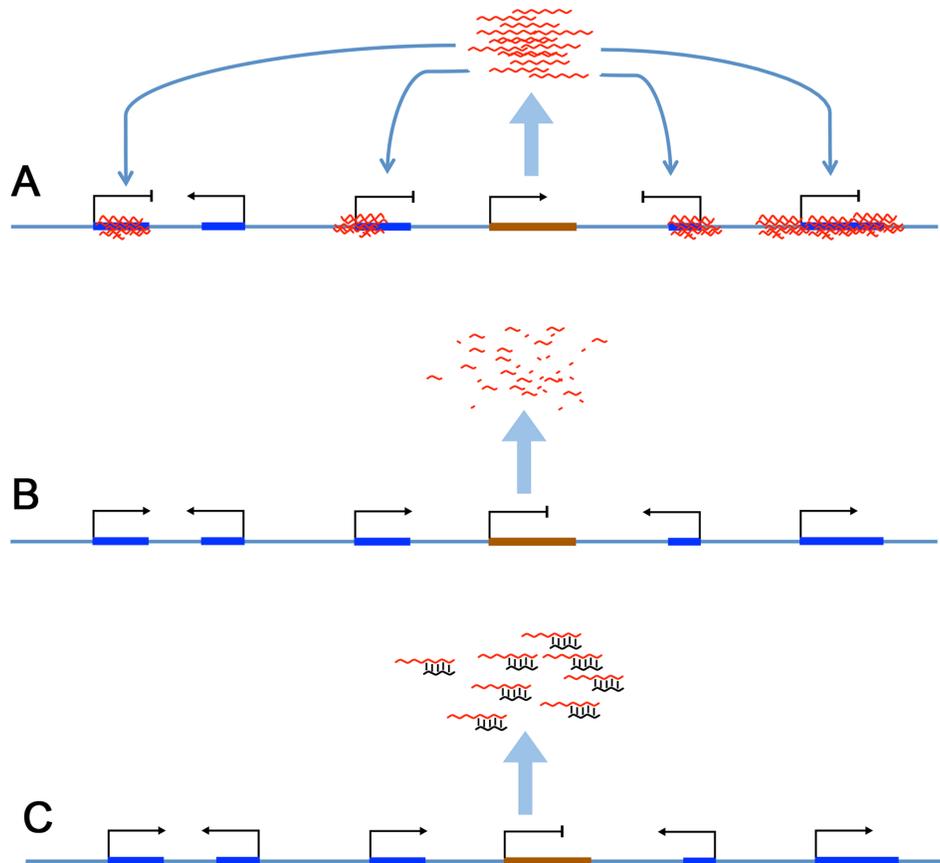The use of synthetic SINEUPs to increase protein

Figure 1. An Overview of pharmacological strategies to modulate lncRNA functions. A. A diagram of a typical gene cluster regulated by a lncRNA. lncRNA genes produce a pool of lncRNAs, which then interact with nearby genes. Since a gene cluster often contains genes that function in the same pathways, a single lncRNA can coordinately regulate the locus and consequently a pathway of interest. **B.** Pharmacological targeting strategies can be designed whose mode of action is the direct destruction of lncRNAs. Examples include antisense oligonucleotides, siRNA approaches, and ribozymes. **C.** Pharmacological strategies can also be devised whose mode of action is the competitive inhibition of interactions between lncRNAs and their target genes or interacting ribonucleoproteins. Examples include small molecule inhibitors and synthetic stabilized oligonucleotides.

synthesis has been demonstrated both by *in vitro* and *in vivo* studies. *Uchl1* (ubiquitin carboxyterminal hydrolase L1) is a gene associated with Parkinson's disease and other neurodegenerative diseases. AS Uchl1 is a natural anti-sense lncNRA that targets Uchl1 mRNA. Under conditions of cellular stress, AS Uchl1 shuttles to the cytoplasm, where it induces Uchl1 mRNA to become highly loaded with ribosomes, thereby increasing its translation [97]. Mutations in subunit 7B of cytochrome C oxidase (*cox7B*) result in microphthalmia with linear skin defects (MLS) syndrome. In an *in vivo* study conducted on medakafish (*Oryzias latipes*), MLS (microphthalmia and microcephaly) was induced by downregulating cox7B using morpholinos that targets *cox7B*. A synthetic (SINE-UP-cox7B) reversed the microphthalmia and microcephaly in MLS Cox7B morphants in a dose-dependent manner, without any increase in the Cox7B mRNA levels

[98]. More research into efficient *in vivo* delivery systems may enable the use of SINEUPs as a therapeutic agent to treat a wide variety of diseases caused by reduced mRNA translation.

RNA interference (RNAi)**:** RNA interference is a mechanism for inducing gene silencing by double stranded RNA. siRNAs are 21-23 nucleotide long RNAs with 3'-dinucleotide overhangs produced by cleavage of double-stranded RNAs by the enzyme Dicer. siRNAs are incorporated into a protein-RNA complex, the RNA-induced Silencing Complex (RISC). The siRNA then binds to the target mRNA and degrades it through perfect sequence complementarity, leading to the recruitment of ribonucleases. siRNAs are highly potent and generally do not require any chemical modifications for activity. siRNA drugs designed to disrupt mRNAs involved in cancer are currently in clinical trials [99]. Similar siRNA strate-

gies can be applied to target lncRNAs. Efficient delivery of siRNAs using nanoparticles and lipid-encapsulation will increase uptake and pharmacokinetic duration of drug delivery [100]. A siRNA directed against MALAT-1 lncRNA in prostate cancer cells resulted in down-regulation of MALAT-1, inhibited cell growth, invasion, migration, and induced cell cycle arrest [101]. siRNA-mediated knockdown of HOTAIR lncRNA inhibited matrix invasion in breast cancer cell lines [85], and injection of siRNA-transfected cells inhibited xenograft efficiency of gastric tumors and metastasis in peritoneal and non-small cell lung cancer [102,103]. Thus, RNAi is a robust and effective strategy to downregulate pathogenic lncRNAs.

Antisense Oligonucleotides (ASO): Antisense oligonucleotides and antisense drugs have been explored as RNA inhibitors in the past to treat various diseases and are currently in different phases of clinical trials. The use of ASO technology to target lncRNAs is a logical next step [104]. ASOs are single-stranded DNA sequences and can be made complementary to the target lncRNAs [105]. In the nucleus, they hybridize with targeted lncRNAs to form RNA:DNA heteroduplexes, which trigger cleavage of the RNA moiety by endogenous RNase H1 activity [106]. ASO blocking of MALAT-1 was shown to prevent lung cancer metastasis [107] and ASO blocking of lncRNA APOA1-AS upregulated high-density lipoprotein particles (HDL) [31]. lncRNA lengths and complex secondary structures complicate ASO design, but systematic evolution of ligands by exponential enrichment (SELEX)-based approaches can be used to identify the best target RNA sequences [108,109]. However, lncRNAs must fold properly *in vitro* in order for SELEX-based approaches to correctly identify target sequences. ASOs can be delivered *in vivo* and chemical modifications such as phosphorothioate and LNA modifications enable its endocytotic uptake by cell surface receptors. Thus, ASOs serve as an important platform to modulate gene expression, but more research is required to optimize the synthesis of oligonucleotides to efficiently target lncRNAs, hopefully with high-potency and limited toxicity.

Ribozymes: Hammerhead ribozymes are approximately 30-nucleotide long, self-cleaving, and nuclease-resistant catalytic RNA oligonucleotides that can bind to and attack the 2′-OH that is 5′- to the scissile bond (a covalent bond, which can be broken by enzymes) in specific RNA targets, resulting in destabilization of the phosphodiester backbone of the targeted RNA molecule [110-112]. Once cleaved, the ribozymes dissociate from the products and may cleave other target RNAs. The cleavage is highly sequence-specific and is sensitive to single nucleotide mismatches; hence, toxicity due to off-target effects can be minimal [113]. Stability of short ribozyme sequences in the presence of endogenous ribonucleases poses a problem, but this has been addressed

by the chemical modification of the phosphate and sugar moieties. Incorporation of four phosphorothioate linkages at the 5′- end of the ribozymes and 2′-O-Me nucleotides in place of the 2′- hydroxyl group has been shown to stabilize ribozymes without altering their catalytic activity [114,115]. Targeted delivery of ribozymes to specific cellular compartments where lncRNAs are located using liposomes or peptide-based delivery systems provides an alternative to RNAi [116]. Synthetic ribozymes against VEGF mRNA administered *in vivo* decreased growth and metastasis of solid tumors, and the same methodology can be adapted to target specific lncRNAs [112,117]. Thus, ribozymes are an exciting future therapeutic tool that can be used to treat various lncRNA-associated diseases.

Small molecule inhibitors: Small molecule inhibitors can alter gene expression by perturbing the interactions of lncRNAs with chromatin modifying proteins. lncRNAs are folded into secondary structures that often change conformation upon interaction with ribonucleoproteins [118]. PAN RNAs produced by KSHV and MALAT-1 both have an ENE in their structure, which stabilizes these lncRNAs and retains them in the nucleus. ENEs are activated when the 3′-poly (A) tail hybridizes to the U-rich internal loop in the ENE to form a triple helical structure. This triple helix confers stability upon the lncRNA from rapid deadenylation-dependent decay [119]. Future small molecule inhibitors that can disrupt stable lncRNA secondary structure or inhibit lncRNA association with accessory proteins or target genes may provide other avenues to target dysregulated lncRNAs. Fatemi et al. used Amplified Luminescent Proximity Homogeneous Assay (ALPHA screen assay) to analyze the RNA-protein interactions and identified small molecules using high-throughput compound screening methods. They reported the specific and quantifiable binding of brain-derived neurotrophic factor antisense (BDNF-AS) lncRNA to protein EZH2 (component of PCR2) and also identified a small-molecule inhibitor Ellipticine that upregulated its downstream target genes [120,121]. In-depth studies involving lncRNA secondary structures are necessary in the future to allow the possibility of identifying new and efficient small molecule inhibitors that can specifically bind to the target lncRNAs.

CONCLUSIONS

An emerging paradigm in epigenetics research indicates that lncRNAs regulate many genes in clusters and this is an active area of research, which needs more focus in the future. lncRNA dysregulation has recently been observed as a common feature in a wide range of human diseases and disorders. lncRNAs function by a variety of mechanisms, but typically they function to regulate the

expression of protein-coding genes through epigenetic mechanisms, often by recruiting chromatin remodeling enzymes to gene clusters. Therefore, lncRNAs can be viewed as master regulators of gene expression and constitute attractive targets for specific epigenetic pharmacological therapy. Tractable pharmacological approaches are now available that can either degrade overexpressed lncRNAs or competitively inhibit their function. Now, for the first time, it may be possible to develop drugs that target specific lncRNAs, thus offering the ability to epigenetically modulate specific biochemical pathways. If so, lncRNA drugs will offer significant improvements to the very low specificity of existing drugs that function as epigenetic modifiers.

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