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Strategies to target long non-coding RNAs in cancer treatment: progress and challenges

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Abstract

Background: Long non-coding RNAs are important regulators of gene expression and diverse biological processes. Their aberrant expression contributes to a verity of diseases including cancer development and progression, providing them with great potential to be diagnostic and prognostic biomarkers and therapeutic targets. Therefore, they can have a key role in personalized cancer medicine.
This review aims at introducing possible strategies to target long ncRNAs therapeutically in cancer. Also, chemical modification of nucleic acid-based therapeutics to improve their pharmacological properties is explained. Then, approaches for the systematic delivery of reagents into the tumor cells or organs are briefly discussed, followed by describing obstacles to the expansion of the therapeutics.

Main text: Long ncRNAs function as oncogenes or tumor suppressors, whose activity can modulate all hallmarks of cancer. They are expressed in a very restricted spatial and temporal pattern and can be easily detected in the cells or biological fluids of patients. These properties make them excellent targets for the development of anticancer drugs. Targeting methods aim to attenuate oncogenic lncRNAs or interfere with lncRNA functions to prevent carcinogenesis. Numerous strategies including suppression of oncogenic long ncRNAs, alternation of their epigenetic effects, interfering with their function, restoration of downregulated or lost long ncRNAs, and recruitment of long ncRNAs regulatory elements and expression patterns are recommended for targeting long ncRNAs therapeutically in cancer. These approaches have shown inhibitory effects on malignancy. In this regard, proliferation, migration, and invasion of tumor cells have been inhibited and apoptosis has been induced in different cancer cells in vitro and in vivo. Downregulation of oncogenic long ncRNAs and upregulation of some growth factors (e.g., neurotrophic factor) have been achieved.

Conclusions: Targeting long non-coding RNAs therapeutically in cancer and efficient and safe delivery of the reagents have been rarely addressed. Only one clinical trial involving lncRNAs has been reported. Among different technologies, RNAi is the most commonly used and effective tool to target lncRNAs. However, other technologies need to be examined and further research is essential to put lncRNAs into clinical practice.

Keywords: Long non-coding RNA, Therapeutic target, Systematic delivery, Cancer treatment

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Background

Long non-coding RNAs (lncRNAs) are a major group of non-coding RNAs. Similar to mRNA, they are transcribed by RNA polymerase II, 5′ capped, and polyadenylated at 3′ end. However, they do not have protein-coding capacity. They have exon/intron and dynamic secondary or tertiary structures [1, 2]. LncRNAs act widely in numerous aspects of gene regulation, including X chromosome inactivation, genomic imprinting, epigenetic regulation, transcription, mRNA splicing, and nuclear and cytoplasmic trafficking [2, 3]. They are crucial regulators of biological processes such as cell cycle, proliferation, differentiation, metabolism, apoptosis, and maintenance of pluripotency [4, 5]. LncRNAs function in different ways. Scaffold lncRNAs have domains that recruit various effectors [6]. Guide lncRNAs guide the ribonucleoprotein complexes to specific locations [7]. Decoying lncRNAs bind to their targets and inhibit their functions [8]. Signaling lncRNAs act as molecular signals in cellular processes such as activation of gene transcription by enhancer RNAs (eRNAs) or X chromosome inactivation [9, 10].

Comparative transcriptomic and genome-wide association studies (GWAS) indicated that single nucleotide polymorphisms (SNPs) in lncRNAs are associated with cancer and several other diseases [11, 12]. Engagement of lncRNAs in all of ten cancer hallmarks has been documented [13–16]. LncRNAs show low expression in normal conditions. However, they are upregulated or downregulated during cancers [17]. Deregulated lncRNAs are involved in the cancer-associated alterations at transcriptional and translational levels [18]. For example, some lncRNAs are associated with transcription factors (TF). Studies on glioblastoma revealed that dysregulation of these lncRNAs leads to tumorigenesis. They form specific lncRNA-TF-gene triplets such as HOX transcript antisense RNA-max-interacting protein1 CD58 antigen, lymphocyte function-associated antigen 3, and protein kinase C epsilon (*HOTAIR*-MXI1-CD58/PRKCE) and HOX transcript antisense RNA-activating transcription factor 5 and neural cell adhesion molecule1 (*HOTAIR*-ATF5-NCAM1). This enhances their target gene expression and, in turn, contribute glioblastoma prognosis [19]. The metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is known to regulate alternate splicing and modulate the activity of spliceosome complex, which is essential for correct splicing and activity of a transcription factor, Myb-related protein B (*B-Myb*), that involves in second growth phase G2 phase/mitosis M phase (G2/M) transition. Thus, elevated expression of *MALAT1* in cancer tissues leads to hyper-proliferation [20].

LncRNAs function as oncogenes and tumor suppressors in cancers. Oncogenic lncRNAs such as nuclear enriched abundant transcript1 (*NEAT1*), antisense non-coding RNA in the INK4 locus (*ANRIL*), *HOTAIR*, and *MALAT1* fulfill the definitions of oncogenes. *HOTAIR* is a transcript from the antisense strand of the homeobox gene (*HOXC*) cluster. It is overexpressed in solid tumors and promotes tumor progression, invasion, metastasis, and poor prognosis [21]. *HOTAIR* recruits histone methylase polycomb repressive complex 2 (PRC2) and lysine-specific histone demethylase 1A (LSD1) to the target gene promoters. Tri-methylation at the 27th lysine residue of the histone H3 protein (H3K27me3) and demethylation at lysine 4 (H3K4) occurs which can result in gene silencing of some tumor suppressor genes [22]. LncRNA *HOTAIR* is deregulated in hepatocellular and colorectal carcinomas, pancreatic tumors, ovarian cancer, and sarcomas [23–27]. In esophageal cancer, *HOTAIR* enhances cell invasion and metastasis and promotes the epithelial-mesenchymal transition (EMT), since it functions as a miR-148a sponge and positively regulates a transcription factor, zinc finger protein SNAI2 (Snail2) expression [28]. *MALAT1* localizes the nucleus and participates in RNA splicing and gene expression at transcriptional and posttranscriptional levels [29]. It involves proliferation, migration, invasion, metastasis, or apoptosis of tumor cells. The upregulation of *MALAT1* is associated with various types of cancers including breast, lung, bladder cancers, esophageal squamous cell carcinoma, and glioma [30–33].

LncRNAs also act as tumor suppressors: the maternally expressed gene 3 (*MEG3*), growth arrest specific 5 (*GAS5*), neuroblastoma-associated transcript-1 (*NBAT-1*), and long intergenic non-protein coding RNA, P53-induced transcript (*LINC-PINT*) have key roles in cellular processes. They are downregulated in cancers. *LINC-PINT* localizes downstream of p53 and acts as its regulatory effector and inhibits tumor invasion [34]. Its downregulation contributes to tumorigenesis in mouse models and *LINC-PINT* expression is lost in many tumors [35]. *MEG3* is a polyadenylated lncRNA. Its imprinted gene is on the delta-like non-canonical notch ligand1-the maternally expressed gene 3 (*DLK1-MEG3*) locus of chromosome 14q32.2 [36]. *MEG3* has high expression in normal human tissue, which is stimulated by cyclic adenosine monophosphate (cAMP). Highly expressed *MEG3* inhibits proliferation and promotes apoptosis of tumors through interactions with different microRNAs [37–39]. Besides, *MEG3* shows decreased or no expression in many cancers namely, brain, lung, colon, liver, and leukemia. *MEG3* expression is under epigenetic control, and aberrant CpG methylation has been demonstrated in several types of cancer [37, 40]. *MEG3* involves the modulation of transforming growth factor-b (TGF-b) pathway genes that affect cell invasion and immune regulation. In addition, it activates p53 [41, 42]. Some lncRNAs can act both as oncogenes and tumor suppressors such as V-Raf murine

sarcoma viral oncogene homolog B1 (BRAF)-activated noncoding RNA (*BANCR*), lncRNA of H19 gene (*H19*), X-inactive specific transcript (*XIST*), and *MALAT1* [43].

LncRNAs have the potential to be diagnostic or prognostic biomarkers and therapeutic targets, since they are expressed in a cell, tissue, developmental, or disease-specific manner. Several lncRNAs are only expressed in cancer cells [44–47]. The prostate cancer antigen 3 (PCA3) promotes the proliferation and invasion of prostate cancer. PCA3 has been approved as a urine biomarker for prostate cancer. This lncRNA exhibits better specificity and sensitivity compared to the prostate-specific antigen (PSA) test [48, 49]. It is over-expressed 60- to 100-fold in prostate tumors compared to benign prostatic tissue and is undetectable in other cancer types [50, 51].

The existing approaches for the treatment of cancer are suboptimal. Targeting lncRNAs can replace or supplement present strategies because they are minimally invasive and convenient therapeutic targets. This review illustrates the potential of lncRNAs as prospective therapeutic targets in cancer. Strategies of targeting cancer-associated lncRNAs therapeutically to modulate their level or function are discussed. Also, chemical modification of nucleic acid-based therapeutics to improve their pharmacological properties is explained. Finally, approaches for the systematic delivery of reagents into the tumor cells and challenges in targeting lncRNAs are described.

Main text

Targeting lncRNAs in cancer treatment

LncRNAs could be promising therapeutic targets in cancer. They are easily detectable in the saliva, serum, plasma, urine, and tissues of cancerous patients. Their flexible and complex structures can be targeted while they are participating in cellular complexes. Specific expression of lncRNAs provides the possibility of killing cancer cells selectively. Their low expression allows the use of lncRNA targeting drugs at lower doses, thereby avoiding toxicities relevant to oligonucleotide therapies. Unlike cellular signaling pathways that encompass signal amplification cascade, lncRNAs function at absolute expression levels that facilitate easier manipulation. In addition, strategies such as enzyme replacement therapy that have been designed to restore deficient or eliminated expression of genes have side effects. Targeting lncRNAs might be an alternative to upregulate such genes in a locus-specific pattern [52, 53]. In this regard, various strategies have been developed to suppress oncogenic lncRNAs or alter their epigenetic effects. Besides, technologies to interfere with their functions have been designed. LncRNAs regulatory elements and expression patterns have been recruited for cancer treatment. Moreover, downregulated or lost lncRNAs can be restored as natural drugs.

Suppression of oncogenic lncRNAs

Oncogenic lncRNAs are upregulated in cancers so they can be targeted via using different technologies to reduce their levels. Here, these techniques are described, which, among them, nucleic acid-based methods have dominated. Furthermore, these technologies allow the functional analysis of lncRNAs and targeting epigenetic modifications.

Antisense oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) are single-stranded antisense oligonucleotides with a DNA stretch (at least 6mer) at the central part, which is native, or phosphorothioated (chemically modified) and RNA nucleotides at flanking parts of the molecule. DNA forms RNA/DNA heteroduplex with target lncRNA that will be cleaved by endogenous RNaseH1 [54]. ASOs typically are used to alter mRNA expression and have succeeded in treating several diseases [55, 56]. They can be exploited to suppress highly expressed lncRNAs in cancers. Several designs of ASOs including locked nucleic acid GapmeRs (LNAGapmeRs), antagonist to NATs (antagoNAT), and mixmers are used with different modes of action.

Locked nucleic acid GapmeRs (LNA GapmeRs)

LNA GapmeRs are very similar to ASOs in structure and function (16 nucleotides), except that they have chemically modified LNA in flanking arms whereas the gap DNA segment lacks the LNA. LNA increases binding affinity and nuclease resistance. Phosphorothioated backbones have been designed to make GapmeRs resistant to enzymatic degradation [57].

Researchers designed two LNAs for targeting different regions of repeat C on lncRNA *XIST* through base pair formation. They were used to study the localization of *XIST* along the X chromosome. Repeat C consists of 14 tandem repeats with a c-rich sequence, situated 3 kb downstream of repeat A that is a silencing domain at the 5′ end [58]. A localization domain was detected, and displacement of polycomb repressive complex 2 (PRC2) and *XIST* coincided. This study suggested that PRC2 and *XIST* bind to different sites of the X chromosome at the same time and do not occupy all binding sites immediately, while the displacement of *XIST* from X takes place with fast kinetics. However, H3K27me3 marks and gene silencing were stable. As a result, the LNA technology allows high-throughput functional analysis of lncRNAs and may provide an opportunity to target epigenetic modifications in vivo for therapeutic applications [59].

Antagonist to NATs (antagoNATs)

Natural antisense transcripts (NATs) are coded from the opposite strand to the host gene locus. They are divided into *cis*-NATs that regulate the expression of the sense

transcripts of the same locus and into *trans*-NATs, which regulate the expression of a transcript from other genomic loci [12]. NATs mediate transcriptional silencing of the related loci via histone-modifying complexes. The antagonist to NATs is a single-stranded oligonucleotide that is designed to inhibit sense-antisense interactions. Therefore, it can be used for the elimination of the epigenetic silencing effect of NATs. Brain-derived neurotrophic factor (*BDNF*) transcription naturally is repressed by BDNF-AS. In a study to target, activate brain-derived neurotrophic factor antisense (BDNF-AS) in vivo, DNA-based, antagoNAT gapmer with three LNA substitutions at each end and phosphorothioate-modified backbones was built to activate *BDNF* expression. This resulted in *BDNF* mRNA upregulation, which led to increased protein levels and induced neuronal outgrowth and differentiation in vitro and in vivo [60]. Thus, antagoNAT strategy provides a useful tool for targeting lncRNAs that act as natural antisense NATs to genes of therapeutic interest.

Mixmers

Mixmers comprise chemically modified nucleotides such as LNA and different types of monomers. They do not have ordinary sequential DNA nucleotides and are not a substrate for RNase H1. Therefore, they sterically inhibit the linkage between lncRNA, ribonucleoproteins, or nucleic acids. They can be used to prevent epigenetic remodeling complexes, alter gene expression, which is regulated by pseudogene transcript, redirect alternative splicing, repair defective RNA, and restore protein production [61, 62]. Researchers designed OMe/LNA (2′-*O*-methyl/LNA) mixmers that suppressed transactivation response element-transactivator of transactivation (TAT-TAR) interactions by the steric blockade in Hella cells. The replication of human immunodeficiency virus type 1 (HIV-1) depends on these interactions. Also, a tricyclo-DNA/oligonucleotides (16 nucleotides) mixmer inhibited expression of β-galactosidase in Hella cells [62].

Small interfering RNAs (siRNAs) and short hairpin RNA (shRNAs)

Small interfering RNA (siRNA) is a knockdown strategy. siRNAs are short double-stranded RNAs. They unwind into single strands, attach to RNA-induced silencing complex (RISC), and create a base pair with a lncRNA of interest, leading to argonaute degradation of the target transcript [63] (Fig. 1). RNAi has different forms, including transcriptional and posttranscriptional gene silencing. RNAi libraries consist of synthetic or enzymatic interfering RNAs. Treatment of double-strand RNA (dsRNAs) with Dicer or RNase III provides endoribonuclease-made siRNAs (esi-RNAs), which are directly delivered into the cytoplasm. Chemically synthesized siRNAs represent the conventional forms, which also are delivered into the cytoplasm directly. Although both siRNAs cause strong suppression of target transcripts, their effect is temporary. Conventional siRNAs indicate a more off-target effect compared to esi-RNAs. Another form of RNAi is short hairpin RNA (shRNA), which is expressed inside the cell. shRNAs yield silencing responses that may be transient or stable and show a much more off-target effect than esi-RNAs. Tumor cells are transfected by shRNA or siRNA plasmid vectors [64].

A siRNA mediated knockdown of second chromosome locus associated with prostate-1 (*SChLAP1*), resulting in reduced cell proliferation and invasiveness. LncRNA *SChLAP1* causes aggressive prostate cancer by preventing the tumor-suppressive activity of the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex [65].

MALAT1 expression levels are highly increased in cervical cancer (CC) cells and tissues. A plasmid vector with a DNA fragment, encoding hairpin RNA, was used to attenuate *MALAT1* levels. Also, an shRNA vector lacking hairpin oligonucleotides as a negative control was built. This strategy prevented metastasis and invasion in vitro and in vivo in CC cells. Downregulation of *MALAT1* increased E-cadherin and Zonula occludens-1 (ZO-1) while decreased b-catenin, vimentin, and transcription factor snail. Hence, *MALAT1* is a target for the inhibition and therapy of cervical cancers [66].

Researchers demonstrated that novel pyk-reg-90-containing *lncRNA* (*N-BLR*) is highly expressed in gastric cancer cell lines and tissues compared to normal gastric cells and adjacent normal tissues. Two siRNAs significantly reduced cell proliferation and suppressed migration and invasion of gastric cancer cells. LncRNA *N-BLR* expression was inversely associated with miR-200c, which is known to regulate EMT. Therefore, *NBLR* proves to be a regulator of the EMT process in gastric cancer [67].

Short hairpin RNAs to knockdown three molecules, secretory carrier membrane protein 1 lncRNA (*SCAMP1*), transcription factor LIM homeobox transcription factor 1 alpha (*LMX1A*), and NLR family, CARD domain containing5 (*NLRC5*) gene, were constructed in a vector. The oncogenic function of (*SCAMP1*) was repressed in glioma cells. Inhibition of *SCAMP1* prevented cell proliferation, migration, and invasion while induced apoptosis due to acting as a molecular sponge of miR-499a-5p. This microRNA acts as a tumor suppressor in glioma cells because it targets the 3′ untranslated (3′-UTR) region of *LMX1A*, which is upregulated in glioma cells and tissues. *LMX1A* activates the *NLRC5* expression that stimulates the Wnt/β-catenin signaling pathway, which promotes the malignancy of glioma cells. Therefore, targeting the *SCAMP1/miR-499a-5p/LMX1A/NLRC5* pathway can be a new therapeutic approach for glioma treatment [68].

Scientists built siRNAs to knockdown, *SOX2* overlapping transcript (*SOX2OT*), *MALAT1*, and *ANRIL*. Furthermore, a universal siRNA as a negative control without homology with mammalian gene sequence was designed. Down-regulation of polycomb group RING finger protein 1 (*NSPc1*) expression with *MALAT1*, *SOX2OT*, and *ANRIL* prevented the proliferation and raised apoptosis in primary glioblastoma cell line (U87) cells. This result indicated that *MALAT1*, *SOX2OT*, and *ANRIL* combine and crosstalk with *NSPc1* in U87 glioblastoma cells to modify proliferation and apoptosis [69].

siRNAs can also be used for NAT-related dysregulation, to target the antisense transcript in a region that does not directly overlap the sense transcript [60, 70].

Deoxyribozymes and ribozymes

Deoxyribozymes are enzymatic DNA molecules with one strand, synthesized to bind to the target RNA according to Watson-Crick base pairing rule. They catalyze RNA cleavage, resulting in fragments of 2′,3′-cyclic phosphate and 5′-hydroxyl ends. They also mediate bond formation via ligation between the 3′-hydroxyl and 5′-triphosphate terminal in RNA [71, 72] with the help of Ca2, Mg2, Pb2, andZn2 cofactors [73].

Scientists have designed site-specific cleaving deoxyribozymes, which are sensitive to the modified nucleotide N^6-methyladenosine (m6A) in cellular RNAs (Fig. 2). They were used to investigate the methylation state of DG (m6A/A) CH motifs (D = A, G, or U; H = A, C, or U). One type of these DNA enzymes offered faster cleavage of methylated RNA, whereas others were significantly prevented by the modified nucleotide. In humans, lncRNA *MALAT1* A 2577 contains m6A [74, 75]. Treatment of this lncRNA with one of these DNA enzymes (VMC10) confirmed the high methylation of the target site [76].

Engineered ribozymes with better catalytic activities and substrate recognition domains have been designed to gain new diagnostic and therapeutic applications. They cleave RNA independent of protein at specific sites in cis or trans [77].

The hammerhead ribozyme (HHRz) has been found in all species. The secondary structure of the HHRz contains three variable stems (stem I/II/III). They are linked to a central catalytic core of 13 conserved nucleotides that is vital for self-cleaving. The cleavage occurs behind the nucleotides (GUC↓) (Fig. 3, hammerhead ribozyme). The catalytic activity of the HHRz is boosted by three-dimensional interaction between the loop in stem II and the bulge in loop I [78]. Engineered HHRz was created using two independent molecules. Splitting of loop III resulted in a transformation from intramolecular *cis*-cleavage to intermolecular *trans*-cleavage. NUX↓ group is essential for the cleaving site. Here, N is any nucleotide, U is uridine, and X stands for any nucleotide except guanosine [79] (Fig. 3, engineered ribozyme). Any RNA molecule containing inner NUX↓ that matches the HHRz binding arms can be cleaved in trans [80, 81]. Some researchers claimed this ribozyme could not cleave efficiently at GUG↓ site. They designed hammerhead ribozymes with a new cleaving site, "DWH" (D = A/U/G, W = A/U, and H = A/U/C) and an optimal binding arm length of (8–9 nucleotides) to achieve *trans*-cleavage of a single-stranded RNA molecule [82].

Genome engineering tools

Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system are genome-engineering approaches, which can be recruited to decrease lncRNA expression levels. The genome can be manipulated by ZFNs in a site-specific manner. RNA destabilizing elements (RDE) were integrated into the locus of *MALAT1* by utilizing ZFNs in human cancer cells. Its expression was decreased 1000

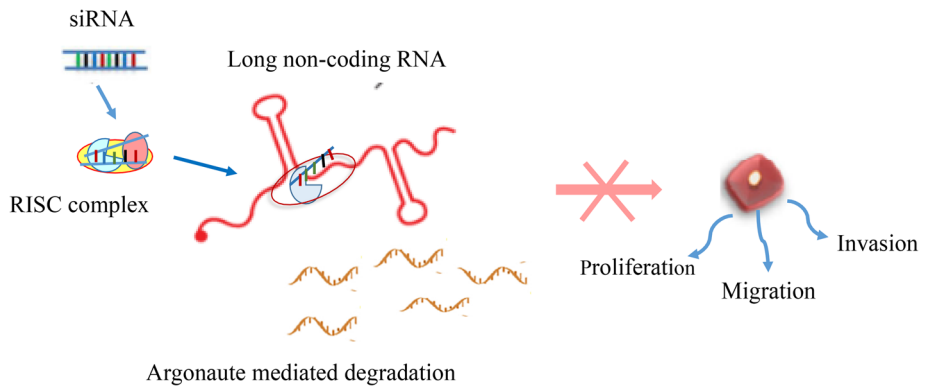

Fig. 1 siRNA technology to suppress oncogenic lncRNAs in cancer

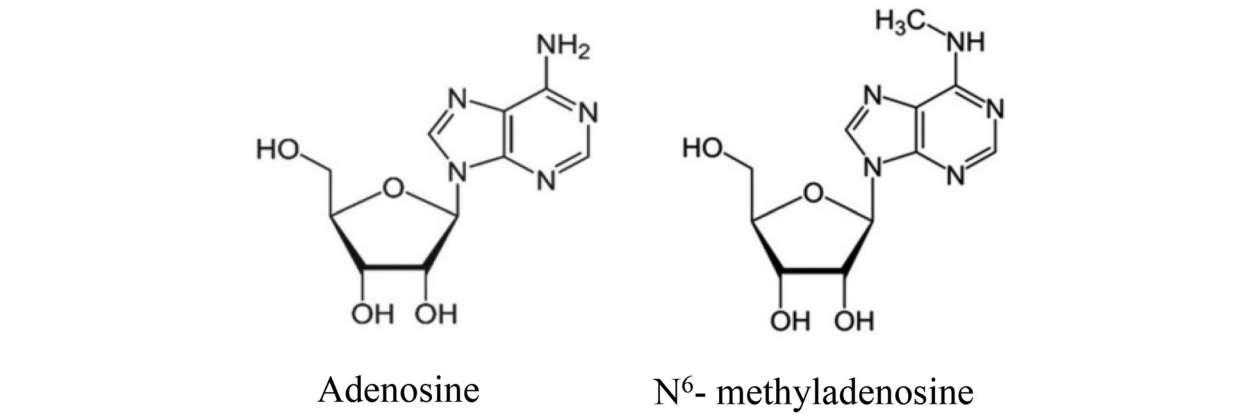

Fig. 2 Adenosine (left); N^6-methyladenosine (right)

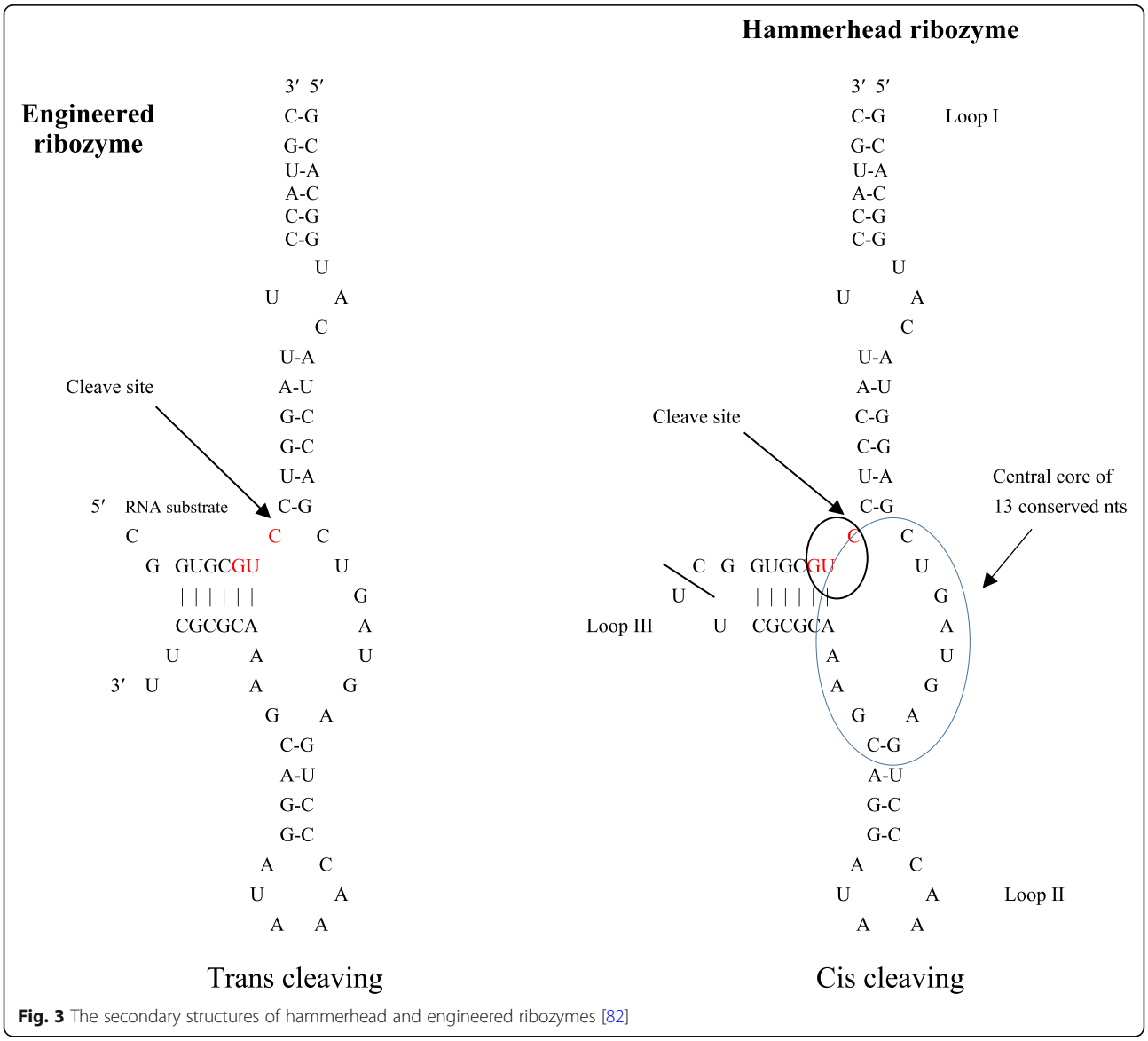

Fig. 3 The secondary structures of hammerhead and engineered ribozymes [82]

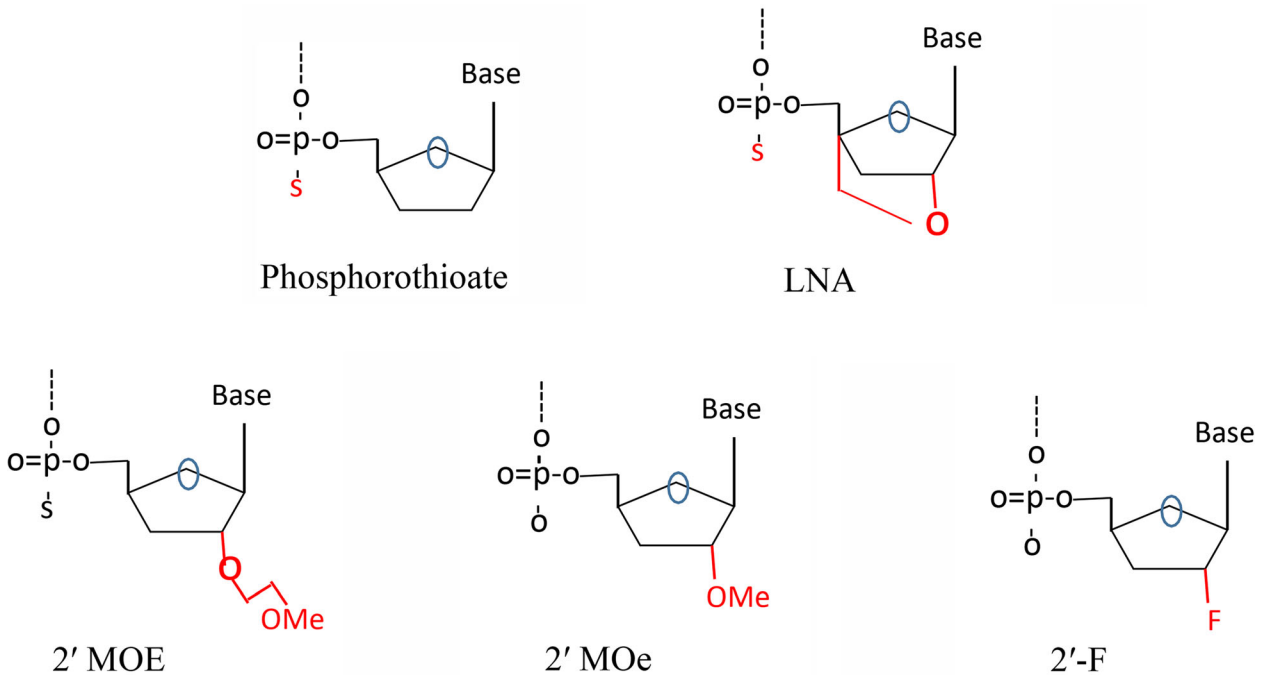

Fig. 4 Chemical modifications of nucleic acid-based therapeutics [52, 83]

fold in stable knockout colons, inferring a loss of function model [84, 85]. Although this inactivation did not affect alternative splicing, fewer tumor nodules and cell migration were observed in mouse xenograft revealing that *MALAT1* has the potential to be a therapeutic target to prevent metastasis in lung cancer [30]. RDEs are integrated into the genome loci such as poly-A signals leading to the silencing of downstream sequences by acting as termination elements. Also, pseudogenes can be silenced by RDE [84, 86].

Unlike protein-coding genes, long ncRNA genes are not vulnerable to a few base insertion, deletion, or frameshift mutations. Thus, they may act through general structures that should be manipulated on large scales. This is possible utilizing the CRISPR/Cas9 system and paired single guide RNAs (sgRNAs) whereby 23 kb of *Rian*, a maternally expressed lncRNA gene, was deleted in mice. Also, the deletion efficacy was enhanced by using numerous sgRNAs, and deletions were inherited as well [87].

Transcription of lncRNA genes can be suppressed sterically by CRISPR interference (CRISPRi). CRISPRi comprises a guide RNA (gRNA) to recognize the target gene and catalytically dead Cas9 (dCas9) protein without endonucleolytic activity [88]. gRNAs target template strand sequences, or regions that are 100 bp upstream of the promoter, or non-template DNA strand in the promoter, or -35 regions. This is more effective in eukaryotic cells compared to direct blockage of RNA polymerase [88]. Also, dCas9 can be integrated into a repressor domain, Kruppel-associated box (KRAB), that leads to the silencing of gene expression epigenetically [89].

Strategies to interfere with LncRNAs function

To target lncRNAs function several technologies namely, small molecules, nanobodies, aptamers, and RNA decoys have been proposed to disrupt interactions between lncRNA/protein via competition or steric blockade [53].

Small molecules

Small molecules bind to either lncRNA or RNA-binding proteins (RBP), change their secondary or tertiary structures, or directly mask protein-binding sequences of RNAs or lncRNA binding domain of the RBPs to disrupt interactions between them [90]. Thus, a profound understanding of LncRNA-protein interactions is essential to meet this goal. Various methods have been developed for the identification of the physical interactions between lncRNAs and proteins. For example, using methods like capture hybridization analysis of RNA targets (CHART) and RNA affinity purification (RAP), proteins that bind to functional intergenic repeating RNA element (FIRRE), *XIST*, *MALAT1*, and *NEAT1* with relevant gene sequences were identified. These methods are categorized as the RNA-centric study of RNA-protein interactions in vivo via cross-linking [91–93].

Quantitative analysis of these interactions at a large scale is vital to investigate small molecule modulators [94]. Technologies such as high-throughput sequencing-RNA affinity profiling (HiTS-RAP) assay and RNA on a

massively parallel array (RNA-MaP) can be adopted for this purpose [95]. Meanwhile, databases and libraries of small molecules that modulate ncRNAs can be explored [96, 97]. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy may help to create structural models to find out how small molecules are attached to and fit in the lncRNA-protein binding cleft [94].

PerkinElmer (Waltham, MA) technology was introduced to quantify the lncRNA-protein interaction, named AlphaScreen technology. Based on AlphaScreen technology, the interaction of *HOTAIR* and *BDNF*-AS, with enhancer of zeste homolog 2 (EZH2), was assessed. Besides, ellipticine was discovered, which upregulates the *BDNF* transcription [90].

Targeting lncRNA-protein interactions lead to reversible inhibition of chromatin-modifying enzymes in their non-catalytic domains since it is illustrated that these enzymes have different sites for long ncRNA binding [94].

In a breast cancer model, small molecules have been used to interfere with the interactions of *HOTAIR* and PRC2 or LSD1 complexes to limit the metastatic potential of tumors [98].

LncRNA *MALAT1* does not have a polyA tail; however, it has a very unusual 3′-terminal structural motif, known as the stability element for nuclear expression (ENE) [99]. A triple helix is formed through interactions between a U-rich hairpin and the transcript 3′ A-rich tail. This ENE motif protects *MALAT1* from degradation and high levels of transcript accumulate in the nucleus [100, 101]. Using a small molecule microarray strategy, two ligands were detected, which specifically bind to the mouse *Malat1* ENE triplex. They had ~ 90% homology with human *MALAT1* ENE triplex [101]. Both ligands decreased *Malat1* RNA levels in cell culture and branching morphogenesis in a mammary tumor model by inducing structural changes, (but in different ways). One of the ligands regulated *Malat1* downstream genes whereas it did not affect lncRNA *Neat1* that possesses a similar ENE triplex structure. This illustrates the specificity of this ligand for *Malat1* over another virus-coded ENEs and *Neat1* [102].

Nanobodies

Nanobodies are capable of disrupting cancer-related RNA-protein networks. They are a variable part of camelid heavy-chain antibodies (HcAbs) with high affinity and specificity. Besides, they are very stable and soluble antigen-binding proteins, with similarity to human Immunoglobulin heavy chain V gene (VH) sequences; thus, they are non-immunogenic. They interfere with protein-nucleic acid or protein-protein interactions and have the capacity to interrupt cancer-specific RNA-RBP networks [103].

Researchers designed a gene library of synthetic nanobodies, able to bind to nucleic acids. A nanobody (cAbB-C1rib3) was identified that specially binds to *ϕBC1*, a structured RNA (stRNA) in nanomolar concentrations. Also, the nanobody binds to various non-related stRNAs. However, it did not bind to single or double-stranded DNA/RNA or proteins with negative charges. The thermal unfolding/refolding processes were not affected by the presence or absence of nanobody. Therefore, nanobodies can be engineered to recognize stRNA epitopes. Nevertheless, their specificity should be improved in future works [104].

Aptamers

Aptamers are single-stranded nucleic acids (DNA/RNA) with high specificity and affinity to targets. In other words, they are nucleic acid analogs of antibodies but with better tissue penetration and transport and lower immunogenicity [105]. They act through three-dimensional structures, recognize secondary structures of lncRNAs, and interfere with RNA-protein interactions [105].

Systematic evolution of ligands by exponential enrichment (SELEX) is used to recognize and expand aptamers in vitro with the possibility of incorporating modified nucleic acids to produce nuclease resistant RNA aptamers [18].

RNA decoy

RNA decoys could be generated as imitators of lncRNAs and act through attachment to proteins and thus sequester proteins. They may function as an approach to disrupt the creation of functional lncRNA-RBP complexes. Scientists designed an anti-HIV decoy that targets the viral protein, Tat. It has (TAR) RNA hairpin and binds to Tat protein. This decoy localizes in the nucleolus whereas natural TAR RNA is located in the nucleus [106].

LncRNA regulatory elements or expression patterns

The gene of diphtheria toxin-A beside the H19 promoter was integrated into a double-stranded DNA plasmid BC-819 (DTA-H19) in overexpressed H19 tumor cells. It was injected to intratumoral regions of various cancer types where reduction of tumor size was reported [107].

Chemical modifications

RNA is a dramatically unstable molecule and has poor pharmacological properties due to the presence of various endogenous ribonucleases. RNA has a negative charge and is hydrophilic. Besides, the 2′-OH group of ribose sugar makes it catalytically active. Therefore, chemical modification of RNA-based therapeutics is necessary to increase their stability without affecting biological activity [52]. (2′-OH) group of the ribose can be replaced by 2′-methoxy (2′-OMe), 2′-methoxyethoxy (2′-MOE), 2′-4′-O-methylene Bridge, locked nucleic

acid (LNA), and 2′-fluoro (2′-F) to improve the pharmacological potential of siRNAs and ASOs [52] (Fig. 4).

In ASOs, the 2′-MOE-modified oligonucleotides are more stable in the serum and have higher RNA affinity compared with the 2′-OMe-modified analogs [108]. (2′-OMe)-modified ASOs show increased stability against nucleases and high affinity with RNA compared to unmodified oligonucleotides. However, they are still sensitive to serum nucleases [109]. To avoid this limitation, 2′-OMe was modified along the whole chain, and to reduce the degradation by exonucleases short phosphorothioate fragments replaced 3′- and 5′-ends. Also, the hydrophobicity of ASOs increased with cholesterol moiety at the 3′-end of the chain [110]. The 2′-F group increases nucleotide's affinity to the target RNA [111].

LNA is a class of bicyclic RNA analogs in which the 2′-O and 4′-C atoms are connected by a methylene linkage so that the furanose ring of the ribose sugar is chemically locked, resulting in higher thermal stability and the highest RNA affinity among typical ASOs [62, 112] (Fig. 4). The LNA strategy is also recruited to produce highly stable aptamers [113].

Phosphorothioate group forms when sulfur substitutes for non-binding oxygen atom of the phosphate group in a nucleotide, to enhance both their stability and hydrophobicity [114] (Fig. 4). It is used in the synthesis of ASOs and less often with aptamers and siRNAs [115].

Chemical modifications in the antisense strand of siRNAs are restricted to one or two internal nucleotides including 2′-OMe nucleotide substitutions and at the 3′-end. Phosphorothioate internucleotide linkages are exerted to improve the resistance of the siRNAs to nucleases. In the sense strand, more internal nucleotides can tolerate the 2′-OMe nucleotide substitutions so that siRNA remains functional within RISC. Such chemical modifications improve target cell penetration of siRNAs and assist their metabolic stability [61, 116]. Unmodified siRNAs poorly uptake into target cells and organs and are rapidly degraded by nucleases that circulate in the blood [117].

The application of these strategies depends on the nature of the target. siRNAs effectively target lncRNAs in the cytoplasm [118]; however, successful silencing of lncRNAs by siRNAs has been reported irrespective of their intracellular location [119]. siRNAs are not as effective as ASOs and ribozymes in targeting secondary structures of lncRNAs [30, 120–122]. ASOs are more reliable for silencing highly expressed lncRNAs, which localize in the cell nuclei [123]. ASOs are less immunogenic and their small sizes let them enter the nucleus easier compared to double-stranded siRNAs [124, 125]. Besides, ASOs have higher specificity and fewer off-target effects [52]. However, ASOs have shown off-target effects and difficulties in cellular uptake [30, 120–122]. Ribozymes exhibit less off-target effects since they are sensitive to single nucleotide mismatches [126]. In some cases, ASOs are preferable to small molecules owing to their specificity and ability to impair correct folding of lncRNAs [43].

Approaches for systemic delivery of therapeutics

The targeting strategies will be successful if they can be delivered to the right target organs or cells with adequate efficacy and safety. ASOs can be taken up freely by cells in vivo. However, efficient delivery to the target tissue is a major limitation of their use. Also, they have very long half-lives when entering cells, i.e., from 2–4 weeks in the liver [127] to 4–6 months in the central nervous system (CNS) [128]. Systemically delivered ASOs exhibit rapid clearance from the blood and accumulate in the liver and kidney [129]. Other materials exploit delivery vehicles [30].

Liposomes are mostly used for nucleic acid delivery among lipid-based vectors. In a mouse model of ovarian cancer, the upregulated lncRNA ceruloplasmin (*NRCP*) was silenced using a phosphocholine derived, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) nanoliposome containing siRNA. Substantial reduction of tumor growth and increased sensitivity to cisplatin was observed [130]. Polymeric vectors have low immunogenicity or toxicity, whose surfaces are manipulated to increase stability, tissue specificity, and cellular uptake [52]. As an example, dendrimers are utilized to deliver siRNAs. In lung cancer, a delivery platform comprising modified poly amidoamine (PAMAM) with polyethylene glycol (10C PEG) and 10 bromodecanoic acids to improve transfection efficiency, an aptamer for targeting nucleolin ligand on target cancer cells and shRNA plasmid for knockdown of B-cell lymphoma-extra large protein (Bcl-xL) were constructed. Modified vector markedly improved the transfection efficiency via covalent or non-covalent aptamer binding compared to the non-targeted vector [131]. Genetically engineered adenovirus, adeno-associated virus (AAV), retrovirus, or herpes simplex virus have been used for targeted delivery of RNA [132, 133]. Viral vectors show efficacy in delivering shRNAs in vivo and ex vivo. They suppress targeted RNAs stably and specifically. Using lentiviral frame plasmids as vectors against *HOTAIR* prevented proliferation and invasion of endometrial carcinoma cells in vitro and in vivo [134]. Efficient gene knockdown has been achieved via the fusion of aptamer to siRNA (aptamer-guided RNAi). This strategy allows the delivery of siRNAs through receptor-mediated endocytosis for cell-specific targeting. The production on large scales with high purity and unlimited targeting of any gene in any cell type is the advantage of this approach. Linking aptamers with nanoparticles have also been exploited to

increased cellular uptake and retention of drugs in cancer cells and target cells selectively [135]. In a mouse model of pancreatic cancer, engineered exosomes from normal fibroblast-like mesenchymal cells were utilized to deliver siRNA or shRNA against oncogenic *K-RasG12D* (K-Ras genes substitution-missense, position 12, G➡D). This suppression led to increased overall survival and inhibited tumor formation [136]. LncRNAs within exosomes remained functional when presented to target cells. Thus, they are appropriate to retrieve the expression of tumor-suppressive lncRNAs in tumor cells [137].

Challenges in therapeutic targeting of lncRNAs

Functional assessment and in vivo validation of therapeutics are challenging. The expression of human lncRNA should be evaluated in model animals which needs recognizing complex interactions between lncRNA and target genes and proteins [138]. However, lncRNAs are poorly conserved across the species. Many human lncRNAs could not be found in mice [139, 140] and a few orthologous lncRNAs were identified among humans and mouse [53]. Producing engineered mouse models with larger human genome segments or entire chromosomes or the exchange of mouse genome proteins can be beneficial [141].

Sometimes, it is difficult to gain consistent results while studying lncRNAs. Researchers demonstrated that *MALAT1* involved in the regulation of alternative splicing in human Hella cells [142]. Although, concerning another study repression of *MALAT1* in cultured cells or mice did not alter total splicing and phosphorylation of serine and arginine-rich (SR) proteins [143]. Furthermore, the normal phenotype was observed in defective *Neat-1*, *H19*, and *MALAT1* mice [140]. However, in some cell lines, knockdown of *MALAT1* led to apoptosis or cell cycle arrest [144]. Thus, high-throughput functional analysis is required for precise determination of molecular mechanisms of lncRNAs actions. CRISPR-Cas9 genome editing technology might be a strong tool for functional screens and the determination of onco-lncRNAs, therapeutic targets, and drug resistance [145].

Long ncRNAs have tumor-specific expression patterns although differential lncRNA expression patterns were reported in some cases. Cancer heterogeneity may be causative so that a detailed analysis of cancer tissue may be more accurate compared to bulk tissue examination. Since lncRNAs are highly subjected to alternative splicing, we may lose a transcript isoform of lncRNAs by a general assessment of tumor tissue [53]. In situ hybridization of fluorescent RNA (FISH) to fresh-frozen or fixed tumor specimens [146] as well as single-cell RNA-seq [147, 148] might be a solution to this problem [53].

Toxicity and off-target effects are other limitations. Sugar modifications to give a high affinity to nucleic acids cause increased off-target cleavage of ASOs and siRNAs [148–150]. This is because in this case, 1–2 mismatches are tolerable and hybridization can take place in shorter regions of homology [151]. Phosphorothioated oligonucleotides demonstrated pro-inflammatory properties [152]. The transfection of cultured HeLa cells with 5–10–5 gapmer phosphorothioate-antisense oligonucleotides (PS-ASO) which undergo 2′-F nucleoside modifications (2′-F PS-ASO) caused DNA damage and cell death [152, 153]. It randomly bound to cellular proteins with greater affinity than that of PS-ASOs containing 2′-MOE or constrained-ethyl-bicyclic-nucleic-acid (cEt) modifications [153, 154]. Remarkable loss of RNase H1 activity will occur even with a single nucleotide mismatch in the cleavage site, three or more mismatches result in complete loss of activity [155, 156]. Bioinformatics can help to predict some nonspecific hybridization to reduce off-target oligonucleotides base pairing. However, only 10–50% of the designed ASOs for gene silencing decreases the expression of the target [157]. RNA deep sequencing approaches (RNAseq) may contribute to eliminating off-target effects of oligonucleotides; however, they could not prepare quantitative information [158].

Conclusions

Long non-coding RNAs play key roles in cellular physiology, development, and disease states including cancer. Thus, they have an appealing potential to be therapeutic targets and drugs in cancer treatment. Nevertheless, the experience with therapeutic targeting of lncRNAs is limited. Most of the mentioned targeting strategies and delivery systems have been examined on mRNA and microRNAs. Only one clinical trial (www.clinicaltrials.gov, NCT02641847) [18] involving lncRNAs has been reported. The obstacles to the development of lncRNAs targeting therapeutics should be precisely explored. Bioinformatics, comprehensive databases, and high-throughput technologies might help establish a deep understanding of lncRNAs localization, structures, functional motifs, mechanisms of action, and interrelations with other biological molecules. The extensive functional screen is required to identify appropriate lncRNAs as therapeutic targets. Also, it is essential to study the exact features of modified oligonucleotides to avoid toxicity and produce efficient and safe drugs.

Abbreviations

(CC) cells: Cervical cancer cells; (CRISPR)/Cas9 system: Clustered regularly interspaced short palindromic repeats; "DWH": (D = A/U/G, W = A/U, and H = A/U/C) sequence; 2′-F: 2′-Fluoro; 2′-MOE: 2′-Methoxyethoxy; 2′-OMe: 2′-Methoxy; 3′-UTR: 3′ Untranslated region; AAV: Adeno-associated virus; *ANRIL*: Antisense non-coding RNA in the *INK4* locus; antagoNAT: Antagonist to NATs; ASOs: Antisense oligonucleotides; ATF5: Activating transcription

factor 5; *BANCR*: BRAF-activated noncoding RNA; Bcl-xL protein: B cell lymphoma-extra large protein; *BDNF*: Brain-derived neurotrophic factor; *BDNF*-AS: Brain-derived neurotrophic factor antisense; B-Myb: Myb-related protein B; *BRAF*: V-Raf murine sarcoma viral oncogene homolog B1; cAMP: Cyclic adenosine monophosphate; CD58: Lymphocyte function-associated antigen 3; cEt: Constrained-ethyl-bicyclic-nucleic-acid; CHART: Capture hybridization analysis of RNA targets; CNS: Central nervous system; CRISPRi: CRISPR interference; dCas9: Catalytically dead Cas9; DG (m6A/A) CH motifs: (D = A, G, or U; H = A, C, or U) sequence of RNAs; DLK1: Delta like non-canonical notch ligand 1; DOPC: 1,2-Dioleoyl-sn-glycero-3-phosphocholine; dsRNA: Double-stranded RNA; EMT: Epithelial-to-mesenchymal transition; ENE: Stability element for nuclear expression; eRNAs: Enhancer RNAs; esi-RNA: Endoribonuclease-made siRNA; EZH2: Enhancer of zeste homolog 2; *FIRRE*: Functional intergenic repeating RNA element; FISH: Fluorescence in situ hybridization; G2/M: Second growth phase (G2 phase)/mitosis (M phase); *GAS5*: Growth arrest specific 5; gRNA: Guide RNA; GWA: Genome-wide association; *H19*: Long noncoding RNA of *H19 gene*; H3K27me3: Tri-methylation at the 27th lysine residue of the histone H3 protein; H3K4: Histone H3 lysine 4 demethylation; HcAbs: Camelid heavy-chain antibodies; HHRz: The hammerhead ribozyme; HiTS-RAP: High-throughput sequencing–RNA affinity profiling; HIV: The human immunodeficiency viruses; *HOTAIR*: *HOX* transcript antisense RNA; *HOX* genes: Homeobox genes; KRAB: Kruppel-associated box; K-Ras G12D: K-Ras genes substitution - missense, position 12, G➡D; *LINC-PINT*: Long intergenic non-protein coding RNA, P53 induced transcript; *LMX1A*: Transcription factor *LIM* homeobox transcription factor 1 alpha; LNAGapmeRs: Locked nucleic acid GapmeRs; LncRNA: Long non-coding RNA; LSD1: Lysine-specific histone demethylase 1A; m6A: N^6-methyladenosine; *MALAT1*: Metastasis-associated lung adenocarcinoma transcript 1; *MEG3*: Maternally expressed, imprinted long non-coding RNA; miRNAs: microRNA; mRNA: Messenger RNA; MXI1: Max-interacting protein 1; NATs: Natural antisense transcripts; *NBAT-1*: Neuroblastoma-associated transcript-1; *N-BLR*: Novel pyk-reg-90-containing *lncRNA*; NCAM1: Neural cell adhesion molecule1; *NEAT1*: Nuclear-enriched abundant transcript 1; *NLRC5*: NLR family, CARD domain containing 5; NRCP: lncRNA ceruloplasmin; *NSPc1*: Polycomb group RING finger protein 1; OMe/LNA: 2′-*O*-methyl/locked nucleic acid; p53: Tumor suppressor p53; PAMAM: Poly amidoamine; PEG: Polyethylene glycol; PRC2: Polycomb repressive complex 2; PRKCE: Protein kinase C epsilon; PS-ASOs: Phosphorothioate-antisense oligonucleotides; RAP: RNA affinity purification; RBP: RNA-binding proteins; RDE: RNA destabilizing elements; RISC: RNA-induced silencing complex; RNA-seq: RNA sequencing; RNAi: RNA interference; RNA-MaP: RNA on a massively parallel array; *SCAMP1*: Secretory carrier membrane protein 1; *SChLAP1*: Second chromosome locus associated with prostate-1; SELEX: Systematic evolution of ligands by exponential enrichment; sgRNAs: Single guide RNA; siRNAs: Small interfering RNA; shRNA: Short hairpin RNA; Snail2: Zinc finger protein SNAI2; SNALP: Stable nucleic acid lipid particle; snRNAs: Small nuclear RNA; *SOX2OT*: SOX2 overlapping transcript; SR proteins: Serine and arginine-rich proteins; stRNAs: Structured RNAs; SWI/SNF: SWItch/Sucrose Non-Fermentable, also known as BRG1/BRM-associated factor (BAF) chromatin modifier family complex; TALENs: Transcription activator-like effector nucleases; *TAR*: Trans-activation responsive element; Tat: Trans-activator of transcription; TF: Transcription factor; TGF-b: Transforming growth factor-b; tRNAs: Transfer ribonucleic acid RNA; U87: Primary glioblastoma cell line; VH: Human immunoglobulin heavy-chain-variable region gene; VMC10: A deoxyribozyme; Wnt: Wingless signaling transduction pathway; *XIST*: X-inactive specific transcript; ZFNs: Zinc finger nucleases; ZO-1: Zonula occludens-1 ZO-1 or Tight junction protein-1

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