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Therapeutic Targeting of Long Non-Coding RNAs in Cancer

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Abstract

Long non-coding RNAs (lncRNAs) represent a significant population of the human transcriptome. Many lncRNAs exhibit cell and/or tissue/tumor specific expression making them excellent candidates for therapeutic applications. In this review we discuss examples of lncRNAs that demonstrate the diversity of their function in various cancer types. We also discuss recent advances in nucleic acid drug development with a focus on oligonucleotide-based therapies as a novel approach to inhibit tumor progression. The increased success rates of nucleic acid therapeutics provides an outstanding opportunity to explore lncRNAs as viable therapeutic targets to impact various aspects of cancer progression.

Long non-coding RNAs as novel players in tumorigenesis

Large-scale cancer genomics projects such as The Cancer Genome Atlas (TCGA) revealed that many of the mutations and copy number changes found in cancer do not overlap with protein-coding genes [1,2], but are frequently located in non-coding DNA; including both regulatory DNA elements such as enhancers as well as non-coding RNA genes [3]. MicroRNAs (miRNAs) were among the first non-coding RNAs to be investigated in the context of cancer, and their role as therapeutic targets or biomarkers in cancer has been previously reviewed [4]. Here, we focus on the clinical relevance of long non-coding RNAs (lncRNAs), representing the largest and most diverse class of non-coding transcripts, with up to 60,000 lncRNA genes present in the human genome [5]. lncRNAs are defined by length (>200 nt), are transcribed by RNA polymerase II, and commonly originate from intergenic regions [5,6]. lncRNAs can be capped, spliced, and polyadenylated, but lack a significant open reading frame. Members of this class of non-coding transcripts have been implicated as molecular scaffolds, architectural RNAs, or as regulatory molecules in a variety of cellular functions: including epigenetic gene regulation, splicing, mRNA stability and translation, as well as acting as decoys or “sponges” for miRNAs or transcription factors [7,8] (Figure 1).

While the first cancer-related lncRNAs were identified based on their altered expression in cancer cells or tumor tissue (see examples in Table 1), the function of a number of lncRNAs is beginning to be revealed. Many lncRNAs play a critical role in one or several hallmarks of

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cancer such as uncontrollable proliferation, evasion of cell death or metastasis [9,10], and can directly act as oncogenes or tumor suppressors, or indirectly by interacting with well-known oncogenes or tumor suppressors such as MYC or p53, on both a transcriptional or post-transcriptional level [11,12]. Here, we highlight several well-studied examples of lncRNAs involved in the acquired capabilities of cancer cells, and selected based on clinical data as well as *in vitro* and *in vivo* evidence. The majority of lncRNAs are expressed in a highly tissue- and cell-type specific manner [13,14], making them potential highly efficacious targets for systemic cancer treatment. We discuss examples of lncRNAs that highlight diversity of function in various types of cancer. We also outline recent advances in drug development aimed at targeting cancer-associated lncRNAs, with a focus on oligonucleotide-based therapies as a novel approach to inhibit tumor progression.

LncRNAs as Oncogenes

H19

H19, one of the first lncRNAs described to be overexpressed in a wide range of cancer types including hepatocellular carcinoma, colorectal cancer and breast cancer, is a paternally **imprinted gene**, initially found to be expressed in embryonic tissues during mouse development and silenced in most tissues at birth [15,16]. Loss of imprinting leads to *H19* re-expression which correlates with many steps of tumorigenesis as shown using mouse models and human cell lines [17,18]. Transcription of *H19* is controlled in part by the tumor suppressor and master cell cycle regulator p53, as well as by the ubiquitous oncogene MYC. Loss of functional p53 or up-regulation of MYC in various cancers correlates with increased *H19* expression [11,19]. In the absence of wild type p53, *H19* can also be up-regulated by hypoxic stress through hypoxia-induced factor 1 α (HIF1- α) [20]. Analysis of TCGA data has revealed increased levels of *H19* in colorectal and stomach cancer, but not in other types of cancer [21].

However, overexpression of *H19* cDNA has also been shown to lead to decreased tumorigenicity of human rhabdoid tumor cell lines *in vivo* [22]. In addition, in an **Apc mouse model of colorectal cancer**, *H19* knockout mice were reported to develop more polyps and a faster onset of tumorigenesis than wild type mice, revealing a tumor suppressive role [23,24]. Such divergent outcomes based on whether *H19* can act as an oncogene or as a tumor suppressor might be explained by the heterogeneity in the genetic dependencies of tumors, the use of different model systems, (i.e. transgenic mouse models compared to human cancer cell lines), or alternatively, might potentially reflect a dual, context-dependent role of *H19* in tumorigenesis, although this remains to be tested.

Several different pathways have been proposed to explain how *H19* can impact tumor progression. *H19* is a precursor for microRNA (miR)-675 [24], shown to target the tumor suppressor retinoblastoma (RB) protein in colorectal cancer. In this model, elevated levels of *H19* lead to increased expression of miR-675 and decreased expression of RB, in turn promoting cell proliferation in colorectal cancer cell lines [25]. There is mounting evidence that *H19*, in addition to being a precursor for miR-675, can function as a **competitive endogenous RNA (ceRNA)** for several different miRNAs [26]. In general, ceRNAs are lncRNAs that can compete with mRNAs for binding of common miRNAs, sequestering

miRNAs from the cellular pool [26]. *H19* has been proposed to act as a “sponge” for many different miRNAs, such as let-7 family members, miR-200 family members (including miR-141), as well as miR-138, miR-630, miR-138 and miR200a [25,27]. This model of action is consistent with the predominantly cytoplasmic localization of *H19* [28].

It is reasonable to speculate that elevated levels of *H19*/miR-675 in various cancers likely contribute to increased cancer cell proliferation and successful tumor metastasis. Hence, targeting *H19* may be an interesting therapeutic approach in a distinct set of cancer types where this lncRNA may act as an oncogene.

HOTAIR

HOX antisense intergenic RNA (HOTAIR) is transcribed from the *HOXC* locus during normal development. Early studies showed that *HOTAIR* acts in *trans* by recruiting the Polycomb repressive complex 2 (PRC2), the LSD1 and the CoREST/REST H3K4 demethylase complex to their target genes [29]. However, recent studies raise controversy with regard to its function and target genes during this time period [30]. *HOTAIR* can function as an oncogene in multiple tumor types including breast, lung, liver, pancreas, and colorectal promoting tumor invasion and metastasis, but can also act as an independent predictor of patient survival rates [31–33]. Therefore, *HOTAIR* may prove to be a valuable therapeutic cancer target, but this remains to be tested.

LUNAR1

Another example of a potential oncogenic lncRNA is the *Leukemia-induced Non-coding Activating RNA 1 (LUNAR1)*, originally identified in ten T-ALL (T cell acute lymphoblastic leukemia) patients harboring a mutation in NOTCH1 [34]. The *LUNAR1* gene is located adjacent to the insulin-like growth factor receptor 1 (*IGF1R*) gene, previously implicated in T-ALL and is a NOTCH1 target itself [35]. Knockdown of the nuclear-retained *LUNAR1* using either shRNAs or antisense oligonucleotides (ASOs) has been found to lead to repression of *IGF1R* as well as reduced cell growth of human T-ALL cells, both *in vitro* and in a mouse xenograft model [34]. Ectopic expression of *IGF1R* rescued the growth defect, indicating that *LUNAR1* could regulate T-ALL cell growth by activating *IGF1R* expression *in cis* [34]. LUNAR1 can bind the intronic *IGF1R* enhancer element, recruiting the Mediator complex, and resulting in full activation of the *IGF1R* promoter [34]. Thus, targeting *LUNAR1* therapeutically may be a viable alternative to targeting *IGF1R*, when aiming to attenuate the downstream effects of aberrant NOTCH signaling in T-ALL cells [34], but further testing will be required.

MALAT1

The highly conserved *Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1)* lncRNA was originally identified as being over-expressed in human lung tumors exhibiting a higher propensity for metastasis [36] and loss of *MALAT1* in a lung cancer xenograft mouse model resulted in reduced metastasis [37], establishing *MALAT1* as an oncogene. Subsequently, increased *MALAT1* expression has been shown to be associated with tumorigenesis in different types of cancer (reviewed in [38]) including breast cancer. Human *MALAT1* expression has been found to be up-regulated 3–4 fold in breast tumors, as well as

in different human breast tumor cell lines, compared to normal tissues/cells, respectively [39]. In addition, *MALAT1* gene mutations frequently occur in luminal type breast tumors [1,40].

In normal tissues, *MALAT1* is an abundant ubiquitously expressed lncRNA, post-transcriptionally processed into a long ~6.7kb nuclear retained transcript and a tRNA-like small RNA, and which translocates into the cytoplasm [41]. Multiple mechanisms of action have been proposed for *MALAT1* including regulation of pre-mRNA splicing, and its knockdown in human cells can result in cell cycle arrest [42,43] or activation of E2F target genes by relocating them from **polycomb bodies** to transcriptionally active nuclear sites in a serum-dependent manner [44].

Malat1 knockout mice exhibit a normal phenotype: they are fertile and no major changes in gene expression or pre-mRNA splicing have been noted, indicating that *Malat1* is dispensible for normal mouse development and viability [45–47]. However, genetic knockout or ASO knockdown of *Malat1* in the MMTV-PyMT mouse model of breast cancer, has resulted in highly differentiated primary tumors and nearly 80% reduction in lung metastasis [48]. Therefore, *Malat1* likely functions in a context-dependent manner to play a critical role in regulating gene expression at both the transcriptional and post-transcriptional levels in various cancers. The lack of a phenotype upon loss or knockdown of *Malat1* in “normal” tissues/cells is likely due to its redundancy, which may be absent or “weak” in a cancer context, although this has not been directly demonstrated. Nevertheless, together, these studies indicate that *MALAT1* may constitute a critical player in tumor progression, and its therapeutic knockdown may represent an excellent option to impact metastatic disease [48].

NEAT1

The *Nuclear enriched abundant transcript 1 (NEAT1)* is another lncRNA that has been implicated in tumorigenesis [49–51]. *NEAT1* is required for **paraspeckle** formation in the nucleus and has been shown to mediate nuclear retention of Interleukin 8 (*IL8*) mRNA in virus infected mouse cells [47,52–54]. Several groups have now shown that *NEAT1* is a p53 target gene and enables tumorigenesis *in vivo* by promoting the survival of oncogene expressing cells such as activated *Kras* in genetically engineered mouse models [51]. Furthermore, high *NEAT1* expression has been reported to correlate with poor prognosis in different types of human cancers including glioma, ovarian cancer, hepatocellular carcinoma, melanoma and prostate cancer [50,51,54]. *NEAT1* upregulation in human prostate cancer cells also confers resistance to androgen receptor (AR) antagonists, leading to a poorer disease outcome [50]. However, a recent report suggested that *NEAT1* may also suppress transformation in p53 mutant cells [49]. Specifically, loss of *NEAT1* increased tumorigenicity in a p53 null/mutant *Kras* mouse model of pancreatic ductal adenocarcinoma [49], suggesting that similar to *H19*, *NEAT1* can elicit a context-specific function in either promoting or suppressing tumor formation; thus, future assessment is warranted.

MaTARs

Our laboratory recently performed an **RNA-seq** screen comparing mammary tumor cells to normal mammary epithelial cells from mouse models of mammary cancer. We identified and

characterized 30 lncRNAs dubbed *Mammary Tumor Associated RNAs (MaTARs)* [55]. 21 *MaTARs* were significantly overexpressed in human breast cancer based on TCGA data analysis, often dependent on hormone receptor status [55]. Independent ASO-mediated knockdown of 20 *MaTARs* resulted in reduced mouse mammary tumor cell proliferation, invasion and/or **tumor organoid branching** [55]. The process of organoid branching is driven by both collective cell migration and cell proliferation -- two essential characteristics of tumor progression; thus, organoids represent a powerful 3D *ex vivo* model system to study potential oncogenes [56]. In addition, some *MaTARs* seem to play important roles in other cancer types: *MaTAR1* (aka *LINC00461* or *ECONEXIN*) has been found to act as an oncogene in glioma [57]. Human *EXONEXIN/MaTAR1* was proposed to act as a sponge for miR-411-5p, in turn regulating topoisomerase 2 alpha in human glioma cell lines [57]. Inhibition of the putative oncogene using siRNAs resulted in decreased cell proliferation [57]. Ongoing studies aim to reveal the molecular mechanism(s) of action of this and additional *MaTARs*, investigating their potential as candidate therapeutic targets for various cancers.

LncRNAs regulating oncogenes

The most frequent **somatic copy-number amplification** in cancer is the gain of the oncogenic transcription factor *MYC* [58]. Several lncRNAs have been implicated in the regulation of *MYC* expression, such as *PVT1*, *PCAT1*, *CCAT1* and *CCAT2*. As attempts to target *MYC* directly in tumors have not been successful in the past, these regulatory lncRNAs may provide exciting new targets to modulate *MYC* expression or *MYC* activity indirectly.

PVT1

The *Plasmacytoma Variant Translocation 1 (PVT1)* gene is located adjacent to *MYC* on human chromosome 8q24 and is co-amplified with *MYC* in 98% of cancers [59]. *PVT1* lncRNA stabilizes *MYC* protein in a post-transcriptional manner, resulting in increased cell proliferation and tumorigenicity in cells with *MYC* amplifications [60]. Recent studies suggest that the generation of *PVT1* fusion proteins resulting from DNA rearrangements that can contribute to tumorigenesis, as in the case of the chimeric gene *PVT1-WWOX* in multiple myeloma [61]. *WWOX* (WW Domain Containing Oxidoreductase) has been described as a tumor suppressor implicated in mediating apoptosis in several cell lines [62]. *PVT1* fusion transcripts are frequently found in other cancer types, such as medulloblastoma, where the fusion genes result from a **chromothripsis-like mechanism** [63]. The *PVT1* locus also harbors a cluster of six miRNAs [64,65]; reduced expression of one of them, miR-1204 in a medulloblastoma cell line, decreased cell proliferation to similar levels to those achieved via siRNA-mediated knockdown of *MYC*. Of note, this anti-proliferative effect was only observed in cell lines with *PVT1-MYC* fusions [63]. The exact relationship between *PVT1* and *c-MYC* remains to be further investigated for devising optimal therapeutic strategies targeting PVT1.

PCAT-1

The *Prostate Cancer Associated Transcript 1 (PCAT-1)* was identified as an activator of cell proliferation in prostate cancer [66] and subsequently detected in several other types of cancer. For example, high levels of *PCAT-1* have been found to correlate with distant metastasis and lower patient survival rates in colorectal cancer [67]. *PCAT-1* in human prostate cancer cell lines modulates the transcription of cell cycle related genes, potentially acting as a transcriptional repressor in a complex with the polycomb repressive complex 2 (PRC2) [66]. *PCAT-1* has also been shown to function in the cytoplasm, where, it acts as a ceRNA, abrogating the binding of miR34-1 to *MYC* transcripts, which results in increased levels of *MYC* protein in human prostate cancer cells [68]. In this regard, *PCAT-1* may act similarly to *PVT1* by stabilizing *MYC* protein post-transcriptionally, albeit presumably, via a different mechanism. *PCAT-1* has also been implicated in DNA damage repair by reducing the RNA levels of *BRCA2*-- an important tumor suppressor-- in a post-transcriptional manner [69]. The same study also demonstrated increased sensitivity of *PCAT-1* over-expressing cells to Poly ADP Ribosyl Phosphatase (PARP) inhibitors, commonly used to treat tumors harboring mutations in *BRCA1* or *BRCA2* [69]. Thus, *PCAT-1* expression might serve not only as prognostic marker but also potentially, as an indicator of PARP sensitivity. These possibilities merit further investigation.

CCAT1

The expression of *Colon Cancer Associated Transcript 1 (CCAT1)* [70], also known as *CARLo-5* [71] or *onco-lncRNA-40* [72]) correlates with the cancer-associated variant rs6983267, located within the human 8q24 *MYC* super-enhancer region, and associated with increased cancer susceptibility [71]. *CCAT1* knockdown *in vitro* using siRNAs resulted in decreased colon cancer cell proliferation due to G1 cell cycle arrest, caused by up-regulation of *CDKN1A/p21*. Injection of these siRNA-transfected cells resulted in prolonged tumor-free survival in mouse xenografts, implicating *CCAT1* in tumorigenesis [71]. The exact mechanism of action is not well understood for this lncRNA. Of note, *CCAT1* bears potential as a colorectal cancer biomarker as it can be detected in all stages of tumorigenesis from pre-malignant lesions to metastasis and is detectable in the peripheral blood of 40% of patients with colorectal cancer [70,73]. In addition to colon cancer, *CCAT1* is also up-regulated in prostate and lung cancer [71,72]. Recently, *CCAT1* has been described to be highly sensitive to **BET (bromodomain and extraterminal) protein inhibitors** such as JQ1, and was proposed to be a clinically relevant biomarker for patients who might benefit from treatment with BET inhibitors in colorectal cancer [74].

CCAT2

Like *CCAT1*, the *Colon Cancer Associated Transcript 2 (CCAT2)* is overexpressed in colorectal cancer, located upstream of *MYC* and is associated with the single nucleotide polymorphism (SNP) rs6983267 [75]. *CCAT2* overexpression has been found to be growth promoting in xenografted tumors, leading to a higher number of liver metastases in mice relative to controls [75]. *CCAT2*, predominantly located in the nucleus, has also been shown to induce chromosomal instability [75]. Furthermore, this lncRNA has been reported to increase *MYC* expression by enhancing Wnt signaling through the transcription factor

TCF7L2, possibly due to direct interactions between *CCAT2* and TCF7L2 [75]. Lastly, up-regulation of *CCAT2* has also been detected in other types of cancer such as human breast, lung, gastric and esophageal tumors [76] suggesting that this lncRNA might have pro-tumorigenic properties in several cancers.

LncRNAs as tumor suppressors

Not all cancer-associated lncRNAs are genomically amplified or over-expressed. Loss-of-function models have identified an increasing number of lncRNAs that can act as tumor suppressors, whereby inactivation contributes to tumor onset and/or drives tumor progression. While these lncRNAs may not be immediate targets for currently available therapeutic interventions, it is important to understand their function in the context of major cancer signaling pathways, as well as their value as putative prognostic markers. Future therapeutic advances may enable re-activation of tumor suppressors including lncRNAs.

GAS5

The *Growth Arrest-Specific Transcript 5 (GAS5)* was first identified as one of six genes preferentially expressed in growth-arrested mammalian cells [77]. *GAS5* is one of the most highly expressed lncRNAs, present in all human tissues and, similarly to *H19*, is involved in embryogenesis [78]. Its expression is significantly reduced in a variety of cancer types such as breast, prostate, bladder, gastric, colorectal, pancreatic and cervical cancer [79]. In addition, *GAS5* expression is inversely correlated with clinico-pathological characteristics such as tumor size, staging or metastasis [79]. Furthermore, overexpression of *GAS5* in xenografted breast cancer cell lines in nude mice has been reported to inhibit breast tumor growth *in vivo* by inducing cell cycle arrest and apoptosis, further supporting its role as a tumor suppressor [79]. On a molecular level, *GAS5* acts as a decoy for the glucocorticoid receptor (GR), thereby suppressing GR-dependent gene regulation in HeLa and HepG2 cell lines [80]. *GAS5* can bind other steroid hormone receptors such as androgen and progesterone receptors which play important roles in hormone-dependent cancers [81].

NORAD

The *Non-coding RNA Activated by DNA damage (NORAD)* transcript is induced in response to DNA damage, and was originally identified in a **doxorubicin** treatment screen in colon cancer cell lines [82,83]. This lncRNA is expressed in a p53-dependent manner upon DNA damage in colon cancer cells [82,83]. *NORAD* is ubiquitously expressed, very abundant and highly conserved [82]. As a cytoplasmic lncRNA it functions as a decoy for the RNA-binding proteins PUMILIO 2 (PUM2) and, to a lesser extent, PUMILIO 1 (PUM1) in colorectal carcinoma cells [82,83]. These proteins stimulate deadenylation and decapping of target mRNAs, leading to post-transcriptional gene repression [84] and loss of *NORAD* releases PUMILIO proteins, thus causing repression of their target mRNAs, many of them encoding proteins involved in mitosis, DNA replication and DNA repair [84]. Hence, hyperactivity of PUM1/2 in *NORAD* knockout cells leads to chromosomal instability, a phenotype frequently observed in the course of tumor progression [82,85]. Furthermore, the increased **aneuploidy** observed upon *NORAD* loss suggested that this lncRNA could act as a tumor suppressor [82].

LncRNAs that regulate tumor suppressors

Several lncRNAs can impact important regulators of the cell cycle, such as p21 or p53. These lncRNAs can thus act as oncogenes by regulating essential tumor suppressors in the cell.

ANRIL

The *INK4B-ARF-INK4A* locus on human chromosome 9p21 is a cluster of well-known tumor suppressor genes, frequently deleted in cancer [86,87]. This locus encodes p15^{INK4B} and p16^{INK54A}, two cyclin-dependent kinase inhibitors, as well as ARF, a regulator of the p53 pathway. This genomic region also harbors the Antisense Noncoding RNA in the INK4 locus” (*ANRIL*, also known as *p15-AS* or *CDKN2B-AS1*), originally identified in familial melanoma, and has since been reported as an oncogene in gastric, breast, lung, bladder and hepatocellular cancer, among others [88,89]. Expression of the *INK4B-ARF-INK4A* locus is tightly regulated by Polycomb group protein complexes (PcG) (reviewed in [89]). *ANRIL* recruits chromobox 7 (CBX7), a component of the Polycomb repressive complex 1 (PRC1), to the *INK4B-ARF-INK4A* locus, contributing to its epigenetic repression in prostate cancer cells [90]. Thus, the oncogenic activity of *ANRIL* might be explained by its transcriptional repression of tumor suppressor genes in *cis*. Knockdown of *ANRIL* resulted in reduced cell proliferation, migration and/or invasion and promoted apoptosis *in vitro* in many epithelial cancers. But in addition, therapeutic inhibition of *ANRIL* released repression of the *INK4B-ARF-INK4A* locus, thus re-activating the expression of the tumor suppressor [89].

LincRNA-p21

Similarly to *NORAD*, expression of *lincRNA-p21* can be induced upon DNA damage in a p53-dependent manner [91]. *LincRNA-p21*, located 16.7 kb upstream of the tumor suppressor gene p21 (*CDKN1A*), is transcribed in an antisense direction [12]. *LincRNA-p21* expression has been associated with tumor stage and invasion status in colorectal cancer [92]. Moreover, it can act as a transcriptional repressor *in trans* by binding to hnRNP-K [12], and it can also enhance p53 transcriptional activity by binding to **Mouse Double Minute (MDM2)** in cancer cells [93]. Early studies of *lincRNA-p21* did not detect an effect of *lincRNA-p21* expression on its neighboring gene p21, or on the cell cycle in general [12]. However, a recent study using *LincRNA-P21 LoxP* conditional knock-out mice revealed that it could predominantly function by activating p21 in *cis* and compromising the G1/S cell cycle checkpoint; however, it did not seem to affect apoptosis or to regulate target genes in *trans* [91]. This difference might be based on the use of RNAi-based knockdown methods in earlier studies compared to a genetic deletion, highlighting the importance of complementary approaches to alter and assess lncRNA function. In addition, *lincRNA-p21* has been reported to interact with **HuR** and repress the translation of β -catenin (*CTNNB1*) and JunB in breast cancer cells [94]. Regulation at both transcriptional and post-transcriptional levels is consistent with the localization of *lincRNA-p21*, found in the nucleus as well as the cytoplasm [94]. Furthermore, *lincRNA-p21* has been shown to be induced by hypoxia and involved in hypoxia-induced glycolysis in mouse embryonic fibroblasts, suggesting a role of this lncRNA in the regulation of the **Warburg effect** in cell metabolism [95]. In summary, current data suggests that *lincRNA-p21*, among various roles,

is involved in fine-tuning the transcriptional regulatory network of p21 and/or p53, two well-studied tumor suppressors.

Several other lncRNAs, such as *PANDA* (Table 1) and *PINT* are induced by p53 and can either enhance the p53 transcriptional response in the cell or antagonize it [96,97].

Consequently, therapeutic targeting of lncRNAs within these transcriptional networks rather than the cell cycle regulators themselves may provide a viable alternative to overcome the limitations of targeting those proteins.

Therapeutic targeting of lncRNAs

Noncoding RNAs play a significant role in cancer pathogenesis [98]. Given the diversity in their potential modes of action, as described earlier, lncRNAs can be targeted by multiple approaches (Key Figure, Figure 2): i) Post-transcriptional RNA degradation pathways can knockdown pathogenic RNAs. This can be achieved by using siRNAs that will invoke a **Dicer** and **Argonaute** dependent cleavage pathway. Alternatively, antisense oligonucleotides (ASOs) with chemical modifications can be used to target the RNA of interest for degradation via an RNase H dependent mechanism.; ii) Modulation of lncRNA genes by steric blockade of the promoter or by using genome-editing techniques; iii) Finally, one can also achieve loss of function by creating steric inhibition of RNA-protein interactions or preventing **secondary structure formation**. RNA binding small molecules, or ASOs, can be used in this case. Here, we describe examples of current advances in each of the above-mentioned strategies to target lncRNAs, as well as their potential therapeutic applications.

Post-transcriptional targeting of lncRNAs

Post-transcriptional targeting provides a straightforward means of impacting any RNA of interest. The highly selective nature of RNA-RNA or RNA-DNA duplex formation has enabled investigators to explore the potential of oligonucleotide-based therapeutics [99,100]. These nucleic acid based drugs can target any unique region of the human transcriptome and importantly, provide the ability to impact the ‘undruggable’ portions of the genome. This is currently impossible with small molecules or antibody based drugs, which have a much smaller repertoire of molecules and targets [101,102]. Currently there are two major approaches employing nucleic acid therapeutics; double stranded RNA-mediated interference (RNAi) and single stranded antisense oligonucleotides (ASOs) (see below). Newer generation nucleic acid therapeutics have demonstrated improved stability, higher efficacy and result in significantly reduced off-target effects, leading to the generation of drugs at various stages of clinical development for a number of diseases, including malignancies [103–105]

RNAi—The discovery of the RNA interference (RNAi) pathway in *Cenorhabditis elegans* followed by the demonstration of specific knockdown of target RNAs using exogenous double-stranded RNA ignited the field of RNAi [106–108]. Addition of a double stranded small interfering RNA engages a degradation pathway that involves Dicer, an RNaseIII enzyme and a multiprotein complex RISC (RNA induced silencing complex) along with the endonuclease Argonaute 2 (Ago2) [109]. RNAi is also active in human cells and this has led to investigating the use of synthetic siRNAs to target RNAs in human cells and mouse

models [110,111]. The first report of a successful *in vivo* study used unmodified siRNAs to knockdown FAS mRNA in a mouse model of fulminant hepatitis [112]. Since then, numerous studies have demonstrated the successful use of siRNAs against target mRNAs for different pathological conditions such as cancer, neurological diseases, and metabolic disorders [113]. Several pharmaceutical companies including Alnylam Pharmaceuticals, siRNA Therapeutics, and miRNA Therapeutics have pioneered the development of siRNA and miRNA based therapies [114]. Since double stranded RNAs are inherently susceptible to nucleases, they require additional chemical modification to prevent them from serving as substrates for subsequent enzymatic degradation pathways [115]. Advances in chemical modifications such as 2'-O methyl (2'-O-Me) sugar residues and phosphothioate linkages in the 3'-end of the RNA have improved the pharmacological properties of siRNA-based drugs [116].

Several lncRNAs have been knocked down using traditional siRNAs in cell lines [115,116]. However, *in vivo* experiments using siRNAs have been quite challenging. This is in part due to the lack of efficient delivery methods and limited bioavailability of siRNAs in animals [117,118]. MicroRNAs were the first non-coding RNAs that were pharmacologically targeted using RNA based therapeutics (reviewed in [119]). However, pre-clinical studies using siRNAs/shRNAs to target lncRNAs are very limited. siRNAs directed against *MALAT1* in human prostate cancer cell lines resulted in inhibition of cell growth, invasion, migration, and induced cell cycle arrest [120]. siRNA-mediated knockdown of *HOTAIR* inhibited matrix invasion in human breast cancer cell lines [32]. Furthermore, subcutaneous injection of human gastric cancer cell lines transfected with *HOTAIR* shRNA, inhibited engraftment efficiency in nude mice [31]. In a recent shRNA-based screen in a mouse model of leukemia, several lncRNA species were identified as essential for leukemia maintenance, and revealed that a number of lncRNAs can act by promoting leukemia stem cell signatures [121].

Antisense oligonucleotides (ASOs)—ASOs bind to RNA via standard Watson-Crick base pairing. Zamecnik and Stephenson first demonstrated that a short 13mer single-stranded DNA targeting Rous sarcoma virus RNA could inhibit viral replication in cell lines [122,123]. Currently, ASOs targeting different mRNAs have entered clinical trials for several diseases including cancer [124]. They are also emerging as a promising therapeutic approach for targeting lncRNAs [48]. Upon binding to their target RNA, ASOs can inhibit or alter gene expression via steric hindrance, splicing alterations, initiation of target degradation, and/or other events. The evolution of ASO chemistry has clearly contributed to their clinical success in various scenarios. The newer generation of ASOs consist of 15–20 nucleotides with a backbone modification containing phosphothioate linkages [125]. The phosphothioate increases the resistance to degradation by cellular nucleases [126,127]. However, upon binding to their target, these ASOs are able to invoke an RNase H dependent cleavage mechanism resulting in an endo-nucleolytic cleavage of the target RNA [127]. In addition to the phosphothioate modification, chemical modification of the sugar backbone especially in the 2' position (2'-O-Methoxyethyl) has conferred drug-like properties to ASOs [128]. These second generation ASOs show improved binding affinity and sustained pharmacokinetics [129]. While all the sugars in the backbone can be modified resulting in

‘**uniformly modified ASOs**’, these ASOs demonstrate very low or no RNase H activity and cannot be utilized for RNA knockdown as the modified bases are resistant to RNase H cleavage [130]. However, these are effective as splice switching ASOs and can be used to modulate splicing patterns of target RNAs by blocking splicing enhancers or repressor binding sites [103]. The first clinically approved splice switching ASO drug Spinraza™ (nusinersen, Biogen Inc) is based on this technology and is used for correcting a splice switch in the *SMN2* (*survival of motor neuron 2*) gene in the CNS of patients suffering from **Spinal Muscular Atrophy (SMA)** [103,131].

In order to achieve RNase H-mediated knockdown of target RNAs, chimeric ASOs referred to as ‘gapmers’ are used. Gapmer ASOs are RNA-DNA-RNA hybrids, where RNA residues contain a 2′-O-MOE modified sugar backbone [130]. Kynamro® (mipomersen sodium) is the first FDA approved drug used to knockdown APOB-100 mRNA to treat familial hypercholesterolemia [132,133]. However, there have been reports of liver toxicity associated with Kynamro® usage and it is not clear if this is sequence dependent [132]. The gapmer chemistry coupled with various sugar modifications have given rise to much improved drug-like attributes to ASOs [134]. One of the major advances in ASO chemistry is the development of **locked nucleic acids (LNA)** and **S-constrained ethyl (cEt) modifications**, which have demonstrated improved potency and promising pharmacokinetic profiles that have advanced to clinical trials for various cancer related and neurological diseases [135–137]. cEt gapmers for STAT3 and Androgen receptor developed by Ionis Pharmaceuticals have entered phase II clinical trials [104,138]. While ASO-mediated knockdown works for cytoplasmic RNAs, ASOs also function effectively in the cell nucleus. This is due to the fact that the major effector nuclease RNase H is enriched in the nucleus [127]. A large number of lncRNAs are enriched in the nucleus [8], hence, ASOs represent an ideal approach for achieving significant lncRNA knockdown.

Our laboratory has pioneered the use of cEt gapmers to target *Malat1* lncRNA in various models of breast cancer. Subcutaneous delivery of *Malat1* ASO in the MMTV-PyMT mouse model of luminal B breast cancer resulted in differentiation of primary tumors and a nearly 80% reduction in metastasis relative to nonspecific ASO treated control mice [48]. Further, *Malat1* knockdown using ASOs also reduced **branching morphogenesis** in a 3D organoid model derived from MMTV-PyMT tumors and a Her2 amplified mouse mammary tumor model [48]. Apart from breast tumors, *MALAT1* ASOs have also been shown to elicit a potent anti-metastatic response in a lung cancer xenograft model. Specifically, systemic knockdown of *MALAT1* in nude mice intravenously injected with human lung cancer cells reduced homing of the cells to the lungs by more than 70% relative to control ASO injected mice [37]. These results suggest that *MALAT1* ASO may emerge as a potential therapeutic for metastatic disease in several cancer types, but further assessment will be needed [139]. In addition, as discussed earlier, numerous *MaTARs*, are also susceptible to ASO mediated knockdown in *ex vivo* organoid models and show a significant anti-tumor response [55,56]. Ongoing pre-clinical studies in patient-derived xenograft models as well as in patient-derived tumor organoids are focusing on moving these studies towards possible clinical trials (Box 1).

Box 1**Useful Pre-clinical Models for LncRNA Research**

The biggest challenge in lncRNA research is the lack of conservation for many lncRNA species. It can occur at different levels: primary sequence conservation, small blocks of sequence conservation, syntenic conservation, and in some instances, structural conservation. Some cancer-associated lncRNAs such as *MALAT1* [45], *NEATI* [49], *H19* [18] are well conserved. However, most human cancer associated lncRNAs do not exhibit sequence conservation across mammalian species. This lack of conservation impedes the translation of lncRNAs that are identified in human cancer to pre-clinical mouse studies for therapeutic targeting. Thus, models can be used for pre-clinical studies assessing lncRNA targeting.

Genetically Engineered Mouse Models (GEMMs)

GEMMs can be used to study all aspects of tumor progression including transformation and tumor initiation in a genetically defined context (e.g. activation of an oncogene or knockout of a tumor suppressor). The lncRNA of interest is required to be evolutionarily conserved in mouse. Genetic knockouts of lncRNAs such as *H19* [22], *MALAT1* [48], and *NEATI* [49,51] have been used in combination with other oncogene targeted GEMMs. RNA therapeutics targeting lncRNAs can also be used in combination with GEMM models [48].

Patient-Derived Xenograft Models (PDX)

For lncRNAs that are restricted to humans or for which human orthologs have been identified, PDX models are a valuable resource for pre-clinical evaluation. PDX tumors transplanted into nude mice provide a means to evaluate tumor growth rate and therapeutic response upon targeting lncRNAs. PDXs have been used to translate lncRNA basic research to pre-clinical studies: *HOTAIR* [31,32], *SAMMSON* [203] etc. have been targeted using siRNAs or ASOs in breast and melanoma models, respectively. The caveat of this method is the inability to study tumor initiation and earlier events leading to transformation. In addition, most PDX models do not metastasize limiting the insight they provide.

Xenografted Human Cell Lines

Human cancer cell lines can be xenografted into nude mice in pre-clinical experiments. Cancer cell lines are readily accessible and can be simultaneously tested in a high throughput fashion. The potential caveat is that cell lines remain in culture for prolonged periods of time, bearing distinct genomic and transcriptomic profiles that can potentially introduce artifacts in readouts. lncRNAs such as *HOTAIR* [32], *LUNAR* [34], *MALAT1* [37] etc., have been knocked down in xenografted human cell lines in breast cancer, T-ALL and lung cancer, respectively.

Patient-Derived Tumor Organoids

Derived from surgically resected tumors, they can serve as a powerful tool to manipulate lncRNA levels in a patient-specific manner. These organoids maintain tumor

heterogeneity and grow in 3D culture on an extracellular matrix scaffold that preserves tumor-extracellular matrix characteristics. They can be used for both *ex-vivo* knockdown studies and can also be orthotopically transplanted into nude mice for *in vivo* therapeutic validation. An advantage is that they can be developed from corresponding normal tissue from the same patients for patient-specific comparisons. A caveat of this method is that the culture conditions for patient-derived tumor organoids is rather complex and not all tumor types have thus far been efficiently established into 3D organoids. Tumor organoids derived from mouse mammary tumor models have been used to knockdown *Malat1* and *MATARs* using ASOs to study branching morphogenesis [48, 55].

Zebrafish Models

Recently, the use of zebrafish to study cancer progression has gained interest. The role of the lncRNA *THOR* in melanoma progression has been evaluated in a Zebrafish model [208]. It remains to be determined whether other lncRNAs can be similarly targeted.

Morpholinos—Morpholino oligonucleotides are nonionic DNA analogs originally used in developmental biology for loss of function studies in zebrafish [140,141]. They have also been used to modulate miRNA activity in a variety of organisms [142]. Morpholinos are generally 25 nucleotides long and are used to prevent translation or promote splice switching upon binding to target mRNAs/pre-mRNAs [141,143]. Morpholino ASOs can be chemically modified with methylenemorpholine rings replacing sugar moieties and contain non-ionic phosphorodiamidate linkages that substitute for the anionic phosphates of DNA or RNA [144]. The first clinically approved morpholino, Exondys 51 (eteplirsen, Sarepta Therapeutics, Inc) has been used to achieve splicing modulation of the dystrophin mRNA (*DMD*) in patients with **Duchenne Muscular Dystrophy** [105,145,146]. However, pre-clinical studies targeting lncRNAs using morpholinos are very limited. Nevertheless, given the clinical success and antisense binding to targets, morpholinos may prove useful for steric interference between lncRNAs and their protein or DNA targets in other pathological conditions.

Limitations of nucleic acid based therapies—While there is immense enthusiasm surrounding nucleic acid based therapies for various disorders including cancers, there are some caveats associated with such strategies. Crossing the cellular plasma membrane is the first and foremost issue. Additionally, the presence of cellular nucleases and the innate immune response to foreign RNAs, such as via **Toll-like receptor (TLR)** and retinoic acid inducible gene-I like (RIG-I) RNA helicase pathways, may pose a substantial block in effective cellular uptake of these molecules [147,148]. Additionally, entrapment of synthetic ASOs in the endosomal compartment can significantly reduce the bioavailability of these molecules [117,149,150]. Finally, it is critical to ensure that the oligonucleotides possess minimal to no off-target effects or toxicity. Due to the aforementioned issues it has taken more than 20 years from the initial discovery of antisense therapeutics [122, 123] to the initial therapeutic successes [103, 132]. The pharmacological evolution of newer generation chemistries detailed above has played a critical role in overcoming many of these barriers including the ability to achieve “free-uptake”, increased stability, nuclease resistance, and extended pharmacokinetics. To overcome innate immune responses triggered by TLRs,

ASOs are extensively screened to identify sequences that are well tolerated, and also by avoiding CpG motifs that might provoke an immune response [151,152]. A major advance in targeting has been the development of N-Acetylgalactosamide (GalNAc) conjugated siRNAs to achieve liver specific targeting (Alnylam Pharmaceuticals) [153]. Asialoglycoproteins such as GalNAc bind to the asialoglycoprotein receptor (ASGPR), and because hepatocytes express numerous copies of ASGPR on their cell membrane, effective uptake of such conjugated siRNAs can occur in the hepatic tissue at a very low dose [153]. Other conjugation methods include lipids such as cholesterol, **Peptide Nucleic Acids (PNAs)** and antibodies, among others [154,155]. Oligonucleotide conjugation is emerging as an exciting area of research aiming to extend the specific targeting obtained in hepatic tissues to other tissue types. However, it may require identification of tissue- or tumor-specific receptors or cell surface markers and substrates amenable for conjugation. Off target effects remain a potential issue that also needs to be thoroughly addressed. One must employ bioinformatics analyses to eliminate sequences with potential off-target matches. Additionally, with advancements in gene editing methods, one can score for off target effects by performing knockdowns in cells that are null for the target gene and evaluate any gene expression changes induced in the absence of the target RNA.

Modulation of lncRNA-expressing loci using CRISPR-Cas9—lncRNA genes, similar to protein-coding genes, are transcribed by RNA polymerase II. With recent advances in genome editing methods such as **CRISPR-Cas9**, it is possible to achieve transcriptional silencing of lncRNA expressing loci using CRISPR-interference (CRISPRi) [156,157]. In this approach dead-Cas9 is fused to transcriptional repressors and this fusion protein is targeted to a specific gene promoter by **guide RNAs** to achieve transcriptional silencing [158]. In a genome wide CRISPRi study, guide RNAs were developed to target the promoters of more than 16,000 lncRNAs in the human genome [159]. CRISPRi was used to selectively inactivate lncRNA genes in an array of seven human cell lines, including six cancer cell types and a line of induced pluripotent stem cells (iPSCs); approximately 500 lncRNAs were found to be essential for cancer cell growth. Many of these were essential in only one cell type, underscoring the fact that lncRNA function is highly specific for a given cell type [159]. These experiments indicate that transcriptional silencing of lncRNAs using CRISPR based approaches is feasible and will likely be used in the future for therapeutic targeting of these molecules at the transcriptional level [156,160]. Recently, the RNA targeting CRISPR-Cas13 system was identified [161], representing another promising approach to knockdown lncRNAs and which may prove to be useful for therapeutic development. Thus, lncRNAs implicated in blood related cancers such as *PVT1*, and *LUNARI*, may be targeted more easily if CRISPR based approaches are considered [34,60]. While several pre-clinical studies are taking advantage of CRISPR-editing methods, it remains to be seen how well they will translate into clinical scenarios for cancer related illness.

Transcriptional upregulation by targeting Natural Antisense RNAs—The majority of current therapeutic strategies operate via inhibition or knockdown of target molecules. Yet, there is a considerable lack of therapeutic strategies to upregulate gene expression. Such up-regulation is critical for activating tumor suppressors in cancers and

other critical growth factors or transcription factors whose expression confers a growth disadvantage to proliferating cells. **Natural Antisense RNAs (NATs)** are a subset of noncoding RNAs that overlap with protein coding genes and are transcribed in an antisense direction [162]. Overlapping NATs are found in a number of protein coding loci in the human genome [162]. The majority of such transcripts affect the transcription of their overlapping genes, thus acting *in cis* [163,164]. Inhibition of *cis* acting antisense transcripts can result in up-regulation of the neighboring/overlapping protein-coding genes, suggesting that a significant number of NATs can act as repressors of the sense coding transcripts [163,164]. NATs are present near many critical tumor suppressor genes such as *CDKN2B* (*ANRIL*) [165] and *CDKN1A* (*P21-AS*) [91]. In several cancers, elevated expression of NATs suppress the expression of the corresponding tumor suppressor genes [166]. Thus, therapeutic inhibition of NATs using ASOs present a potential to upregulate tumor suppressors. These ASOs are known as ‘**AntagoNATs**’ which are single-stranded, chemically modified LNAs or other ASOs [164]. Currently, OPKO-CURNA and RaNA Therapeutics hold several AntagoNATs in various stages of pre-clinical and clinical development. These examples provide exciting possibilities of modulating lncRNA expression to exploit multiple facets of gene regulation and therapeutic interference.

Steric inhibition of lncRNA function

Accumulating evidence suggests that many lncRNAs manifest their function by binding to proteins or to integral components of protein complexes [167,168]. Many nuclear lncRNAs are bound to chromatin-modifying complexes such as members of the PRC complex, as described above. In these cases, the RNA will dock onto the protein surface which can be sequence or structure dependent [169]. Morpholinos and uniformly modified ASOs that cannot stimulate RNase H activity can be used in such cases to bind specifically to the RNA, and block the RNA-protein interface resulting in loss of function [142].

Small molecules—lncRNAs, like other non-coding RNAs, can potentially form stable secondary and tertiary structures [170]. While computational predictions based on a covariance model suggest that very few lncRNAs have conserved structural features [171], *in vivo* chemical crosslinking studies have demonstrated secondary and/or tertiary structures for several lncRNAs [170,172,173]. With the development of RNA structure determination assays such as **SHAPE** and **PARIS** it is possible to precisely map the secondary and tertiary structure of lncRNAs [174–176]. Clinical trials are underway to target highly structured bacterial or viral riboswitches using small molecule inhibitors for bacterial and viral infections respectively [177]. Several human genetic disorders including Huntington’s disease, fragile X syndrome and myotonic dystrophy are caused by trinucleotide repeat expansions in RNA transcripts, which fold into stable RNA hairpin structures and result in impaired splicing and/or translation [172,178,179]. These disease-causing RNA elements represent a completely novel class of drug targets. It is thus reasonable to speculate that similar structured RNA elements might also be present in numerous pathogenic lncRNAs [175]. *MALAT1* and *NEAT1* are classic examples where the 3’ end of the transcripts fold into a unique triple helical structure [172,173,180,181]. Small molecule inhibitors targeting these unique structural elements in lncRNAs could potentially destabilize the transcript or

allosterically interfere with protein binding to confer a therapeutic effect, although this remains to be tested.

Therapeutic manipulation of lncRNA promoters—Aside from the fact that lncRNAs themselves could serve as potential therapeutic targets, recent findings from clinical trials utilizing lncRNA promoters are providing encouraging insights. BC-819 (DTA-H19), is a double-stranded DNA plasmid carrying the gene for the A subunit of diphtheria toxin under the regulation of the *H19* gene promoter, administered to elicit an anti-tumor response in various solid tumors (BioCancell Therapeutics Inc) [182]. Originally tested in a mouse bladder cancer model, BC-819 is now also being tested in non-small cell lung carcinoma, colon, pancreatic and ovarian cancers [183,184]. A phase I/IIa clinical trial in patients with invasive bladder cancer receiving intravesical BC-819 has reported mild, local toxicity along with complete and partial response rates of 22 and 44%, respectively [183]. Highly selective tissue expression of several lncRNAs such as *H19*, combined with the therapeutic responses in early stage clinical trials with BC-819, have created significant interest among scientists and clinicians, prompting the investigation of using tissue or cell-type specific lncRNA promoters to drive the expression of toxins to stimulate cytotoxic effects in disease-related cells.

Concluding Remarks

lncRNAs are emerging as important players in tumorigenesis. A detailed understanding of their expression and mechanisms of action is critical in order to appreciate the importance of this unique class of molecules. The fact that lncRNAs are generally expressed in a cell- or tissue-specific manner makes them exceptional therapeutic targets. Further, sequence-based nucleic acid therapeutics are evolving at a rapid pace. Identification and evaluation of a lncRNA target in the context of cancer may be translated into clinical scenarios in a reasonable time window. Although many questions and challenges remain to be addressed (see Outstanding Questions and Box 2), the increased success rate of nucleic acid therapeutics provides an exciting opportunity to explore lncRNAs as viable candidate therapeutic targets in cancer and other pathologies.

Box 2

Clinician's Corner

- lncRNAs are emerging as critical players in cancer pathogenesis. Deep sequencing of patient tumor DNA and RNA have identified many lncRNAs that are up- or down-regulated in tumor tissues. Many mutations and recurrent CNVs in cancers map to non-coding regions of the genome.
- An understanding of the molecular functions of several lncRNAs in different cancers is just emerging. Many lncRNAs have been shown to play a direct role in cancer cell proliferation, cancer progression and/or metastasis.
- Tissue-specific expression of lncRNAs renders these exciting candidates for diagnostic marker development or as putative therapeutic targets for systemic treatment.

- The ability to target lncRNAs at various functional levels provides a wide range of therapeutic options. Targeting approaches may include nucleic acid based drugs, small molecule inhibitors, and gene editing methods.
- Nucleic acid based RNA targeting approaches are evolving at a rapid pace to potentially treat various pathologies. Recent clinical success and FDA approval of antisense drugs for Spinal Muscular Atrophy and Duchenne Muscular Dystrophy have led to various pre-clinical studies targeting lncRNAs using nucleic acid based therapies.
- Further functional studies using appropriate pre-clinical models will validate the importance of several lncRNA species in cancer pathogenesis leading to a broad exploration of this class of molecules as viable therapeutic targets in many cancer types.

Outstanding Questions

- How many lncRNAs are functionally and clinically relevant for specific cancers? Is there a functional interplay between these lncRNAs?
- How can we develop systematic genomic and functional approaches to better understand the role of lncRNAs in tumor initiation, progression and metastasis?
- How can we best address the off-target effects of systemic nucleic acid based therapies?
- What are the best preclinical models to study human lncRNAs?
- How can we best integrate patient genomic and transcriptomic data to establish a lncRNA discovery pipeline to drive preclinical studies in animal models?

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Glossary

Aneuploidy

the presence of an abnormal number of chromosomes in a cell; a common feature of tumor cells.

AntagoNATs

Short, chemically-modified, single-stranded oligonucleotides that interfere with the function of natural antisense transcripts (NATs).

Apc mouse model

Mouse model of colorectal cancer, where the *Apc* (Adenomatous Polyposis Cancer) gene is mutated.

Argonaute

Belongs to a family of RNaseH domain containing proteins. Is an essential component of the RNA-induced silencing complex (RISC), the key mediator of RNA interference.

BET Inhibitors

A class of drugs that reversibly bind the bromodomains of Bromodomain and Extra-Terminal motif (BET) proteins and prevent their interaction with acetylated histones.

Branching Morphogenesis

A developmental process that involves formation of tree-like structures directed by a gene expression program and tissue remodeling.

Chromothripsis

Large scale genomic rearrangements occurring in a single event in confined genomic regions in one or a few chromosomes.

Competing Endogenous RNA (CeRNA)

class of lncRNAs that regulate other RNAs by competing for shared miRNA binding sites and acting as a sponge to sequester miRNAs.

CRISPR/Cas9

A genome editing technology that uses the bacterial nuclease *Cas9* to direct specific editing events in mammalian genomes, guided by a short complementary RNA.

Doxorubicin

Chemotherapeutic agent used for treatment against various cancers.

Dicer

An RNase III enzyme involved in cleavage of double-stranded RNAs and pre-miRNAs into short double-stranded RNA fragments.

Duchenne Muscular Dystrophy

X- linked recessive disorder characterized by severe muscular weakness.

Guide RNA

short stretch of RNA that can direct Cas9 nucleases to cut at a specific genomic location.

Imprinted Gene

gene expressed from either the maternal or paternal allele

Locked Nucleic Acid (LNA)

modified RNA nucleotide. The ribose sugar moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon.

Mouse double minute 2 homolog (MDM2)

E3 ubiquitin-protein ligase; important negative regulator of the p53 tumor suppressor.

Natural Antisense RNA (NATs)

subclass of non-coding RNAs that are transcribed antisense to a protein coding gene.

Paraspeckle

dynamic sub-nuclear compartment thought to be involved in nuclear RNA retention.

PARIS (Psoralen Analysis of RNA Interactions and Structures)

method to determine RNA-RNA and RNA- protein interactions at high resolution.

Peptide Nucleic Acid (PNA)

synthetic chemically modified oligonucleotide analog where the nucleotides are linked to the backbone composed of repeating amino ethyl glycines.

Polycomb Bodies

sub-nuclear compartment formed by accumulation of polycomb proteins that remodel chromatin and cause epigenetic silencing of genes.

RNA-Seq (RNA sequencing)

Transcriptome-wide shotgun sequencing that uses a next-generation sequencing (NGS) approach to determine RNA complexity and abundance in cells.

S-Constrained Ethyl (cET)

A modified RNA nucleotide similar to LNA containing a constrained ethyl substitution in the 4' Carbon of the ribose sugar.

SHAPE (Selective 2'-hydroxyl acylation analyzed by primer extension)

chemical assay to determine RNA secondary structure

Somatic Copy Number Amplification

process by which clustered regions of the genome are repeated and the number of repeats in the genome varies between normal and diseased somatic cells.

Spinal muscular atrophy (SMA)

rare genetic disorder characterized by loss of motor neurons and progressive loss of neuromuscular function.

Toll-like receptors (TLRs)

class of proteins that play a critical role in the innate immune system, recognizing bacterial or viral RNAs.

Uniformly modified ASO

Phosphothioate oligonucleotides in which the sugar residues in all the nucleosides are chemically modified to resist nuclease cleavage.

Warburg effect

High rate of aerobic glycolysis accompanied by lactic acid fermentation in tumor cells.

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Highlights

- Approximately 27% of annotated human genes encode lncRNAs.
- Recent tumor genome sequencing efforts have identified several lncRNA loci that are deleted, amplified and/or mutated in various cancers.
- Many lncRNAs are up- or down-regulated in cancers compared to respective normal tissues. Several lncRNAs have been shown to play a critical role in various aspects of cancer progression.
- Tissue-specific expression of lncRNAs positions them as interesting potential therapeutic targets for a variety of pathologies.
- Nucleic acid based therapeutics are emerging as a promising approach to target pathogenic lncRNAs. RNA targeting therapies have been clinically approved for several diseases.
- Nucleic acid based therapeutics have shown success in several pre-clinical studies targeting lncRNAs in cancers.

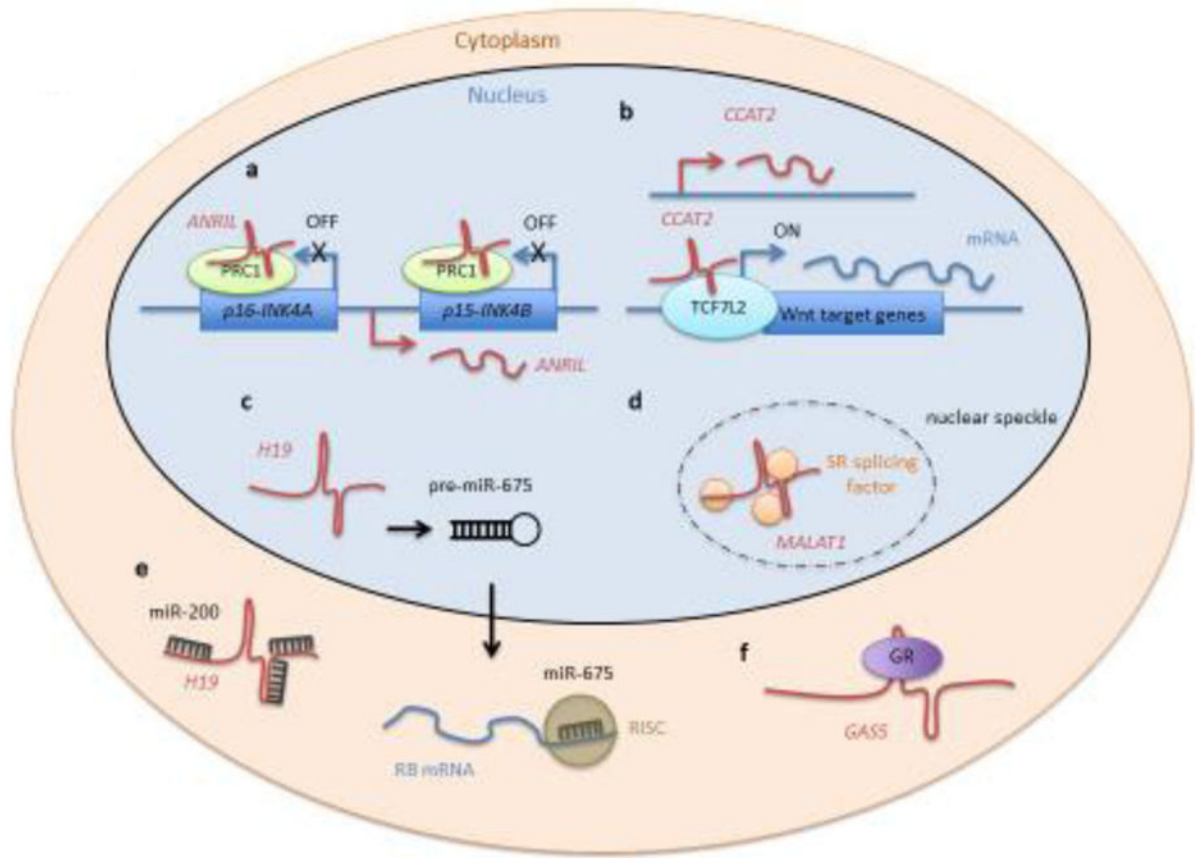
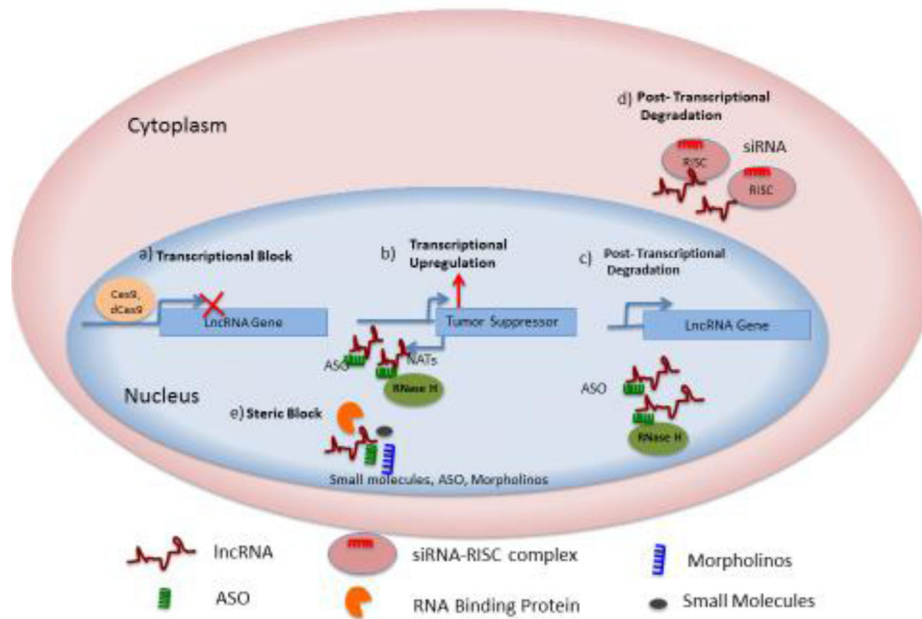


Figure 1. Proposed molecular functions of mammalian lncRNAs

The schematic depicts examples of proposed lncRNA (red) molecular functions and their location in corresponding cellular compartments.

- a) *ANRIL* represses gene transcription of the *INK4* locus in *cis* by binding to polycomb repressive complex 1 (PRC1, green).
- b) *CCAT2* activates expression of WNT target genes including *MYC*, possibly by directly interacting with the transcription factor TCF7L2 (light blue).
- c) *H19* is a precursor for miR-675. The lncRNA is processed to pre-miR-675, which is exported into the cytoplasm and further processed to miR-675. The mature miRNA assembles with RNA-induced silencing complex (RISC, dark green). The tumor suppressor RB is one of the targets of miR-675.
- d) *MALAT1* associates with SR splicing factors (orange) and is located in nuclear speckles.
- e) *H19* also acts as a sponge for many different miRNAs, including members of the miR-200 family.
- f) *GAS5* functions as a decoy for glucocorticoid receptor (GR, purple), preventing GR-dependent gene activation.



Key Figure, Figure 2.

Therapeutic targeting of human lncRNAs

The schematic summarizes different approaches to target lncRNAs in the nucleus and cytoplasm.

a) Transcriptional inhibition can be attained through classical CRISPR/Cas9 to delete regions of interest in lncRNA loci. Alternatively, dead-Cas9 fused to a repressor complex can inhibit transcription of lncRNA genes.

b) Transcriptional upregulation of tumor suppressors can be attained through knockdown of the corresponding Natural Antisense Transcripts (NATs). NATs can be targeted post-transcriptionally using ASOs.

c) ASOs can also be used to post-transcriptionally knockdown lncRNAs that are overexpressed in cancers.

d) Post-transcriptional silencing can also be achieved by siRNAs targeting lncRNAs. SiRNAs stimulate Dicer activity in the cytoplasm and recruit RISC complex (RNA Induced Silencing Complex) to post-transcriptionally degrade target RNAs

e) Steric inhibition of lncRNA-protein interactions can be achieved using small molecules, morpholinos, or uniformly-modified ASOs that cannot stimulate an RNA degradation pathway.

Table 1

Selected human lncRNAs and their expression in tumorigenesis

<i>Name</i>	Function	Hallmarks	References
<i>aHIF</i>	Up-regulated	5	18
<i>ANCR</i>	Down-regulated	6	185
<i>ANRIL</i>	Up-regulated	1, 2, 3, 6, 9	86,89
<i>BCAR4</i>	Up-regulated	6	186
<i>CCAT1</i>	Up-regulated	1, 2, 6	73,74,75
<i>CCAT2</i>	Up-regulated	1, 6, 9	76
<i>ECONEXIN/MaTARI</i>	Up-regulated	1	57
<i>FILNC1</i>	Down-regulated	7	187
<i>GAS5</i>	Down-regulated	2	77,78,79
<i>H19</i>	Up-regulated/Down-regulated	1, 5, 6	11,12,19
<i>HOTAIR</i>	Up-regulated	6	29,32
<i>HOTTIP</i>	Up-regulated	1,6	188
<i>HULC</i>	Up-regulated	1, 5, 7	189
<i>Lilam</i>	Up-regulated	1	121
<i>Linc-PINT</i>	Down-regulated	6	97
<i>Linc-ROR</i>	Up-regulated	6	190
<i>lincRNA-EPS/MaTARI8</i>	Up-regulated	10	55
<i>lincRNA-p21</i>	Up-regulated	3, 5, 6, 7, 9	91,95
<i>lncRNA-ATB</i>	Up-regulated	6	191
<i>lncRNA-LET</i>	Down-regulated	6	192
<i>lncRNA-MIAT</i>	Up-regulated	5, 6	193
<i>LUNARI</i>	Up-regulated	1	34
<i>MALAT1</i>	Up-regulated	6	36,37,48
<i>MaTARs</i>	Up-regulated	1, 6	55
<i>MEG3</i>	Down-regulated	1, 3	194
<i>MVIH</i>	Up-regulated	1, 6	195
<i>NBAT1</i>	Down-regulated	1,6	196
<i>NBR2</i>	Down-regulated	7	197
<i>ncRAN</i>	Up-regulated	1, 6	198
<i>NEAT1</i>	Up-regulated/Down-regulated	3	49,50,51
<i>NORAD</i>	Down-regulated	9	82
<i>PANDA</i>	Up-regulated/Down-regulated	4, 9	199
<i>PCAT-1</i>	Up-regulated	1, 6	66,68
<i>PCAT-29</i>	Down-regulated	1,6	200
<i>PCGEMI</i>	Up-regulated	2, 3, 7	201
<i>PTENP1</i>	Down-regulated	2	202
<i>PVT1</i>	Up-regulated	1, 3, 6	60

<i>Name</i>	Function	Hallmarks	References
<i>SAMMSON</i>	Up-regulated	7	203
<i>SChLAPI</i>	Up-regulated	6	204
<i>TERRA</i>	Down-regulated	4	205
<i>TUG1</i>	Up-regulated	1, 6	206
<i>THOR</i>	Up-regulated	1, 7	207
<i>u-Eleanor</i>	Up-regulated	1	208
<i>UCAI</i>	Up-regulated	2	209

Hallmarks of cancer:

(1) sustained proliferative signaling, (2) insensitivity to growth suppressors, (3) evasion of apoptosis, (4) replicative immortality, (5) induced angiogenesis, (6) tissue invasion and metastasis, (7) abnormal metabolic pathways, (8) immune evasion, (9) genomic instability, (10) inflammation [16,17].