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Review

The potential therapeutic applications of long non-coding RNAs

Phoebe Tamblin-Hopper[1], Endre Kiss-Toth[1], Ian Sudbery[2], David Young[3], J. Mark Wilkinson[1] [iD]

[1]Division of Clinical Medicine, School of Medicine and Population Health, University of Sheffield, Sheffield S10 2RX, UK.
[2]School of Biosciences, University of Sheffield, Sheffield S10 2TN, UK.
[3]Biosciences Institute, Newcastle University, Newcastle-Upon-Tyne NE1 3BZ, UK.

Correspondence to: Prof. J. Mark Wilkinson, Division of Clinical Medicine, School of Medicine and Population Health, University of Sheffield, The Medical School, Beech Hill Road, Sheffield S10 2RX, UK. E-mail: j.m.wilkinson@sheffield.ac.uk

Abstract

The field of RNA-based therapeutics is rapidly evolving and targeting non-coding RNAs (ncRNAs) associated with disease is becoming increasingly feasible. MicroRNAs (miRNAs) are a class of small ncRNAs (sncRNAs) and the first anti-miRNA drugs, e.g., Miravirsen and Cobomarsen, have successfully completed phase II clinical trials. Long ncRNAs (lncRNAs) are another class of ncRNAs that are commonly dysregulated in disease. Thus, they hold potential as putative therapeutic targets or agents. LncRNAs can function through a variety of mechanisms, including as guide, scaffold or decoy molecules, and understanding of these actions is critical to devising effective targeting strategies. LncRNA expression can be modulated with small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), CRISPR-Cas9, or small molecule inhibitors. These approaches have been employed to target a number of lncRNAs and tested in animal models of disease, including targeting ANRIL for non-small cell lung cancer and H19 for pancreatitis. However, there are currently no clinical trials registered in the ClinicalTrials.gov database that target lncRNAs as a therapeutic intervention. In order to translate lncRNA targeting into clinical use, several limitations must be overcome, such as potential toxicity and off-target effects. Overall, while significant progress has been made in the field, further development is required before the clinical application of the first therapeutics targeting lncRNAs. In this review, we discuss recent advances in our understanding of the mechanisms of action of lncRNAs that present avenues for clinical therapeutic targeting and consider off-target effects as a limiting factor in their application.

Keywords: lncRNAs, mechanism of action, therapeutics, ASO, siRNA, CRISPR-Cas9

INTRODUCTION

Non-coding RNAs (ncRNAs) are RNAs that do not encode protein but may possess important regulatory functions[1,2]. In this review, we discuss the potential application of long non-coding RNAs (lncRNAs) as therapeutic targets. Examples of lncRNAs that are dysregulated and causative in disease pathologies will be discussed in relation to their mechanism of action, together with how their roles in disease can make them suitable therapeutic targets, as well as examples of successfully targeting them. Targeting lncRNAs therapeutically has been hailed as an exciting field for some time and is rapidly expanding, but to date, we have found no registered clinical trials for this application. In this review, we explore the real potential of lncRNA targeting and where they provide a unique therapeutic opportunity. This will be achieved by looking at some of the best examples where there is genuine evidence for modulation of lncRNA expression in treating disease. Furthermore, while studies have linked many lncRNAs to a variety of diseases, not all of them are robust and many of their applications have limitations that are also considered in the review.
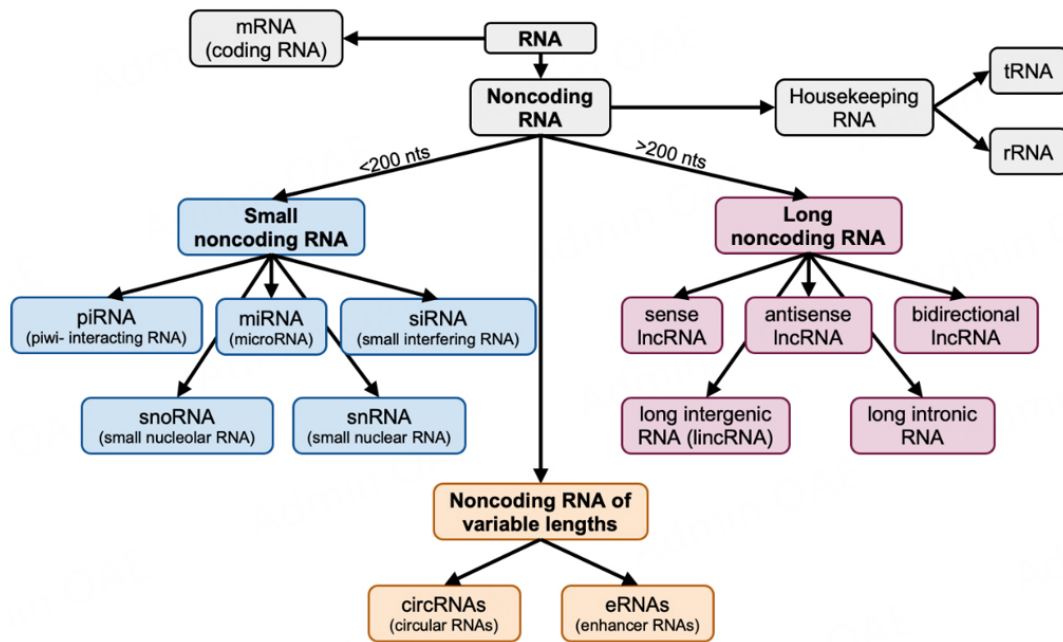
Non-coding RNAs

Regulatory ncRNAs are largely split into two classes: small non-coding (sncRNAs) RNAs of < 200 nucleotides in length and lncRNAs of > 200 nucleotides [Figure 1][3,4]. Some classes of ncRNAs are of variable length, so they can belong to either classification. These include enhancer RNAs (eRNAs)[5] and circular RNAs (circRNAs)[6].

There are numerous lncRNAs, with the GENCODE database annotating over 20,000 to date in humans[7], but other resources estimate over 90,000[8,9]. lncRNAs are found throughout the cell, but are most commonly in the nucleus[10] or cytoplasm[11,12]. The subcellular localisation of lncRNAs is highly regulated and is important in deciphering their functions[13]. Nuclear lncRNAs are generally less stable[14], but only a minority of lncRNAs are deemed unstable enough to make them unsuitable for therapeutic targeting[14]. In the nucleus, lncRNAs function in chromatin remodeling, transcriptional regulation, and as scaffolds for spatial organisation, whereas cytoplasmic lncRNAs are involved in translational control, post-transcriptional control of gene expression, and protein localisation[13,15]. The factors controlling their localisation have been reviewed in depth elsewhere[13].

Current therapeutics targeting ncRNAs

Due to their ability to regulate gene expression, ncRNA targeting offers exciting opportunities for disease treatment. To date, there are 85 trials listed on the Clinical Trials database (ClinicalTrials.gov) that contain "lncRNA" when searched in other terms[16]. Of these, the majority are testing or have tested the use of lncRNAs as biomarkers for monitoring disease progression/severity or as diagnostic tools. The few that are not looking at lncRNAs as biomarkers are concerned with identifying their mechanisms of action and role in a particular disease, for example, by investigating their relationship with other elements of the functional pathway (examples include www.clinicaltrials.gov NCT04937855, NCT06213493, and NCT04767750). None of these trials test drugs/treatments to target lncRNAs and all are listed as observational studies. This lack of interventional trials suggests an underexploited area or, alternatively, that there are significant barriers to overcome before treatments targeting lncRNAs can successfully reach the clinical trial stage.

However, although the field of lncRNA targeting in clinical trials is undeveloped, the therapeutic targeting of microRNAs (miRNAs) is more advanced. Several anti-miRNA drugs are currently undergoing clinical trials, including Miravirsen, which has successfully completed phase II clinical trials for hepatitis C[17,18]. Miravirsen is a locked nucleic acid (LNA)-modified antisense oligonucleotide (ASO) that binds miR-122, inhibiting its action in stabilising hepatitis C RNA[17]. This was one of the first drugs developed to specifically target an ncRNA, but it was discontinued due to the availability of other effective treatments.

Figure 1. Classification of RNAs. RNA can be classified as coding or non-coding RNA. Non-coding RNA can be separated into housekeeping ncRNAs and regulatory ncRNAs, which are the subject of this review. Regulatory ncRNAs can be separated according to their length, with small non-coding RNAs being less than 200 nucleotides and long non-coding RNAs greater than 200 nucleotides. snoRNAs and snRNAs are generally classified as sncRNAs but possess housekeeping functions. lncRNAs can be classified according to their relative location to protein-coding genes. The main types of small non-coding RNAs are also further classified here according to their function. Additionally, circRNAs and eRNAs are ncRNAs that can be of variable length, either greater or less than 200 nucleotides, so they do not fit into either length classification.

However, its development demonstrates the potential of this therapeutic approach. Other methods have also been developed and miRNA mimics, synthetic copies of endogenous miRNAs, such as MRX34 to augment *in vivo* miR-34a levels, are being tested clinically[19]. Despite immune-related severe adverse events causing early termination of this study, it demonstrates a method of exploration for ncRNA therapeutics[19]. Remlarsen, another miRNA mimic, is undergoing clinical trials for restricting fibrous scar tissue formation[20]. For a comprehensive overview of therapeutics that have completed or are currently undergoing clinical trials for targeting miRNAs and other ncRNAs, see Winkle *et al.* (2021)[21].

ncRNA-targeted therapeutics offer significant potential, but they do have several limitations, many of which are also relevant to the development of therapeutics targeting lncRNAs. One problem is successfully delivering RNA-based therapeutics to target tissues other than the liver. To this end, chemical modification of various parts of the nucleotide has enabled the successful delivery of ASOs to multiple tissues without a delivery agent[22]. Furthermore, novel polymer and peptide-based nanoparticle delivery systems have reduced issues with charge and electrostatic interactions to improve miRNA delivery[23,24]. The addition of surface peptides also improves cellular uptake[23]. These methods are safe and biodegradable and, thus, could be applicable to developing other ncRNA therapeutics[25]. However, other significant issues include sequence and tissue specificity, leading to off-target binding[26], as well as tolerability, leading to toxicity, particularly hepatotoxicity[27]. The Phase I MRX34 study encountered immunity-related toxicity, despite no problems being observed in animal studies[19], indicating that preclinical models do not consistently predict human responses. These issues must be thoroughly assessed before further clinical studies can be carried out safely and successfully.

The rationale for targeting lncRNAs

lncRNAs are present in a wide range of animals, plants, prokaryotes, yeast, and viruses, but their sequence is not well conserved between species[15]. They face less selection pressure than mRNAs, since they do not need to maintain a specific open reading frame[28]. Some lncRNAs, for example, X-inactive Specific Transcript (XIST), possess short regions with high conservation, suggesting a lack of evolutionary selection pressure on other regions, such that only the target binding sequences are conserved[29,30]. Thus, lncRNAs can evolve rapidly. lncRNAs have specific spatio-temporal expression, i.e. they are activated in specific tissues at specific times during development, and this expression often affects the expression of nearby protein-coding genes (PCGs) and contributes to the lineage-specific expression of PCGs[31]. Furthermore, even conserved lncRNAs often function differently between species due to alternate processing and localisation[32]. This initially led to questions over their importance and functionality, but subsequent research has uncovered diverse functions in the regulation of transcription, splicing, translation, differentiation, the cell cycle, nuclear bodies, and chromatin[13,15]. In fact, lncRNA promoters are conserved at a similar rate to PCG promoters[2], suggesting lncRNA expression is important for fitness, and even those with rapidly changing sequences often have orthologous functions between species and are expressed from syntenic locations[33,34].

The ubiquity of ncRNAs and their ability to target multiple genes within a pathway makes them excellent therapeutic targets[21]. A transcriptome-wide association study (TWAS) found that of 14,100 lncRNA genes, expression of 800 was associated with genetic traits of disease where the association was not due to any effects of neighbouring PCGs, making it less likely that the effects were due to alterations of cis-regulatory sequences overlapping the lncRNA[35]. This represents a large number of potential therapeutic targeting opportunities by modulating lncRNA expression. However, their ability to target multiple genes does raise the issue of off-target effects in other genes, as has been seen in therapeutics targeting other ncRNAs. lncRNAs, in particular, have high organ, tissue, and cell type specificity[2,36]. This spatial and temporal expression makes them excellent targets for lineage-specific gene therapy[37]. lncRNA dysregulation has been linked to cancer[38], e.g., MEG3 downregulation in multiple cancers[39], cardiovascular diseases[40], e.g., MALAT1 in diabetic retinopathy and angiogenesis[41], neurological disorders[42], e.g., BASE1-AS in Alzheimer's Disease[43], musculoskeletal disorders[44], e.g., ANRIL in osteoarthritis[45], and many other diseases. In cancer, lncRNAs have been identified as oncogenes, e.g., HOTAIR[46] and MALAT1[47] or tumour suppressors, e.g., MEG3[48]; therefore, they are ideal targets for new cancer therapeutics[38]. lncRNAs also play key roles in tumour microenvironments. For example, in influencing immune cell function, LNMAT1 recruits macrophages into tumour cells to enhance lymphatic metastasis[49] and LINC00301 increases levels of regulatory T cells while decreasing CD8+ T cells, in non-small cell lung cancer (NSCLC)[50].

STRATEGIES TO TARGET LNCRNAS

There have been several well-documented methods used to target lncRNAs and modulate their expression, including siRNAs, ASOs, and CRISPR-Cas9. ASOs and siRNAs have been used in many studies of lncRNA knockdown. Typically, siRNA silencing is most effective for cytoplasmic lncRNAs, whereas ASOs are considered most effective for nuclear lncRNAs, but can also act in the cytoplasm[51,52].

siRNAs are short oligonucleotides complementary to target ncRNAs and work by recruiting the RNA-induced silencing complex (RISC) to degrade lncRNAs [Figure 2A][53]. To date, six siRNA-based drugs are approved by the Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA), and all target mRNA in the liver [Table 1][54,55], demonstrating the efficacy of this approach for targeting RNA. This method has also been successful in several lncRNA preclinical models, but concerns remain over the potential adverse effects of targeting molecules other than the intended lncRNA[51].

Table 1. Currently approved siRNA/ASO-based drugs (as of May 1, 2024). This shows the type of sequence targeted by these drugs and which condition they are approved to treat. Two ASO drugs have recently been discontinued by the FDA and are highlighted in grey

	Active substance (drug name)	Brand name	Target sequence	Condition treated	Licensed by (EMA and/or FDA)
siRNA	Givosiran	Givlaari	mRNA	Acute hepatic porphyria	EMA, FDA
	Inclisiran	Leqvio	mRNA	High cholesterol(primary hypercholesterolaemia, mixed dyslipidaemia)	EMA, FDA
	Lumasiran	Oxlumo	mRNA	Primary hyperoxaluria type 1	EMA, FDA
	Nedosiran	Rivfloza	mRNA	Primary hyperoxaluriatype 1	EMA, FDA
	Patisiran	Onpattro	mRNA	Polyneuropathy in hereditary tranthyretin-mediated amyloidosis	EMA, FDA
	Vutrisiran	Amvuttra	mRNA	Polyneuropathy in hereditary tranthyretin-mediated amyloidosis	EMA, FDA
ASO	Aganirsen	Olisens	mRNA	Ocular neovascularisation	EMA
	Casimersen	Amondys 45	exon	Duchenne muscular dystrophy	FDA
	Eteplirsen	Exondys 51	exon	Duchenne muscular dystrophy	FDA
	Fomivirsen	Vitravene	mRNA	CMV infection	FDA (now discontinued)
	Golodirsen	Vyondys 53	pre-mRNA	Duchenne muscular dystrophy	FDA
	Inotersen	Tegsedi	mRNA	Homozygous familial hypercholesterolemia	EMA, FDA
	Mipomersen	Kynamro	mRNA	Homozygous familial hypercholesterolemia	FDA (now discontinued)
	Nusinersen	Spinraza	pre-mRNA	Spinal muscular atrophy	EMA, FDA
	Vitolarsen	Viltepso	exon	Duchenne muscular dystrophy	FDA
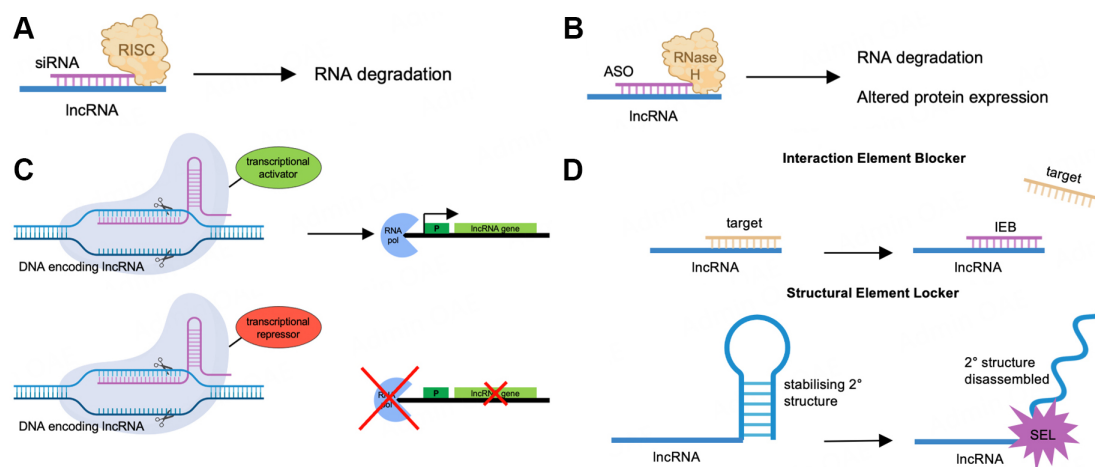
Figure 2. Schematic illustration of strategies used to target lncRNA. (A) siRNAs: siRNAs bind to lncRNA and recruit RISC, resulting in degradation of the lncRNA. (B) ASOs: ASOs bind to lncRNA and recruit RNaseH, resulting in degradation of the lncRNA and altered downstream protein expression. (C) CRISPR-Cas9: The inactive Cas9 domain is bound to a transcriptional activator domain or a transcriptional stop signal, so that when it binds to the complementary DNA that encodes the lncRNA gene, it results in either transcriptional activation at the promoter, or repression through blocking RNA polymerase, respectively. (D) Small molecules: the first small molecules designed for modulating lncRNA expression can be classified as interaction element blockers (IEBs) or structural element lockers (SELs). IEBs block the binding of lncRNA to its target, which in some cases can be used to increase expression levels of lncRNAs that would normally undergo nonsense-mediated decay due to their normal binding. SELs work by binding to lncRNAs and disrupting secondary (2°) structures which stabilise the lncRNA, thus resulting in destabilisation and reduced expression.

ASOs are 15-25 bp oligonucleotides that can bind complementary lncRNA, and commonly recruit RNase H to promote RNA degradation and alter downstream protein expression when coding elements are targeted [Figure 2B][51]. ASOs can also act by binding mRNA to alter splicing that results in exon inclusion where

mutations have led to exon skipping[56], or they can be used to cause exon skipping in diseases such as Duchenne muscular dystrophy where deletion mutations shift the reading frame and generate premature stop codons[57]. Additionally, ASOs can alter the site of polyadenylation to destabilise RNA[58]. To date, seven ASO-based drugs currently have marketing authorisation, with many more currently undergoing clinical trials [Table 1][54,55,59]. However, some ASO-based drugs have been discontinued from the market after their authorisation due to hepatotoxicity problems[60]. The ASO drug Inotersen also contains an FDA warning for hepatotoxicity on its label[60]. These mechanisms, however, are less applicable to lncRNA targeting. Although these ASOs do not target lncRNAs, their success in reaching the market demonstrates that they can be effective RNA-targeted drugs. Through their ongoing development, some initial problems have been overcome - the latest ASOs designed have high affinity and stability[51]. However, toxicity issues remain, including hepatotoxicity[51] and renal toxicity that can lead to potentially fatal glomerulonephritis[61]. Furthermore, the fact that they cannot be administered orally is a limitation[51]. Both ASOs and siRNAs can be used in conjunction with LNAs to increase potency, but this can also increase hepatotoxicity[62].

CRISPR/Cas9 is another tool that can be used to target nuclear or cytoplasmic lncRNAs, but in one study, it was only effective in 38% of ~16,000 lncRNA loci tested[63]. CRISPR/Cas9 may upregulate lncRNA expression by activating the promoter with a fusion protein of inactive Cas9 with a transcriptional activator domain[64], or disable the lncRNA gene with a transcriptional stop signal to block RNA polymerase [Figure 2C][40,65]. Some loci are not readily targeted with CRISPR, due to their bidirectional, internal, or proximal promoter, or due to off-target effects in neighbouring genes[63].

More recently, strategies have been developed to target ncRNAs with small molecules specifically targeted to their secondary structures [Figure 2D][21]. The ability to predict structures and virtually screen compounds accelerates this process[21,66]. This method has been used for lncRNAs; AC1NOD4Q, a compound targeting the lncRNA homeobox antisense intergenic RNA (HOTAIR), has been developed to selectively interfere with HOTAIR-EZH2 binding, thus blocking its activity[67]. This was achieved by developing 3D models to predict hairpin loop structures that could be targeted with small molecules, followed by virtual screening of potential molecules[67]. This methodology can be applied to the design of small molecules to target other lncRNAs, but requires the high-resolution 3D structure of the respective lncRNAs[66].

TARGETING LNCRNAS THERAPEUTICALLY ACCORDING TO THEIR MECHANISM OF ACTION

The mechanisms of action for lncRNAs can be broadly separated into scaffold, guide, or decoy RNAs[15,68,69]. These mechanisms are distinct, but many lncRNAs can act via multiple mechanisms[69] and thus there may be multiple ways to exploit their actions therapeutically.

lncRNAs as scaffold molecules

When functioning as scaffolds, lncRNAs act as a platform to assemble different regulatory proteins together to perform a specific function[70]. This is possible through the presence of different domains that simultaneously bind various effector molecules, such as transcriptional activators or repressors, which have specific effects when brought together both spatially and temporally [Figure 3][69]. lncRNAs can act in a cis manner on neighbouring genes, or a trans manner on distant genes[71]. In transcription, scaffold lncRNAs can activate or silence specific genes by binding different subunits of chromatin-modifying complexes to facilitate their assembly, such as the polycomb repressive complex (PRC) 1 and PRC2[68]. Therefore, knockdown of scaffold lncRNAs would inhibit the effector molecules from interacting with their target and double knockdown of lncRNAs with the effectors should exacerbate these effects[69]. Understanding how lncRNAs assemble and regulate these effector molecules is thus crucial to targeting them effectively.
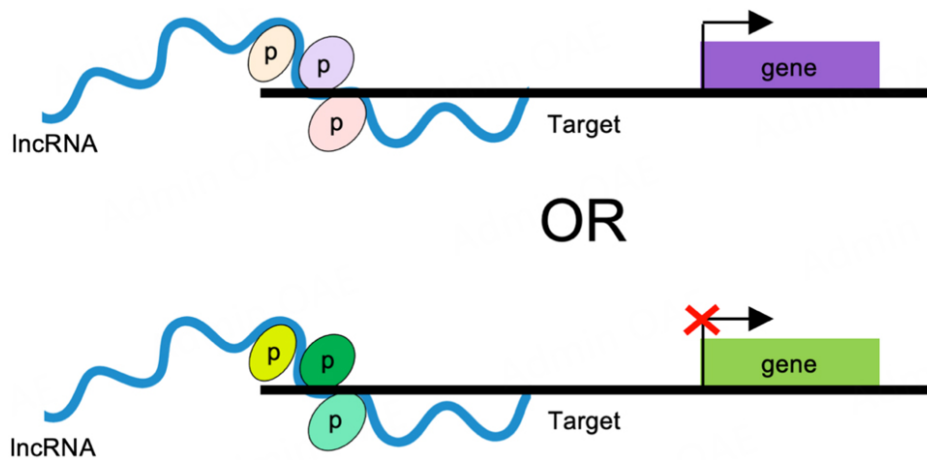
Figure 3. Schematic illustration of the functional mechanism of scaffold lncRNAs. Scaffold lncRNAs act by using different modules to bring together different proteins (labelled "p"), such as transcriptional activators and repressors, in time and space to cause specific effects on target molecules.

The lncRNA HOTAIR can act through a scaffold mechanism. HOTAIR binds PCR2 and LSD1 chromatin remodelling complexes and acts as a platform to target these to HOX loci, to influence cell epigenetic states[72,73]. HOTAIR has four independently folded domains, with two of them recruiting and interacting with various transcription factors, nine of which are involved in pan-cancer processes[74]. Increased HOTAIR expression in primary tumours strongly predicts metastases and death, for example, in breast cancer, where it is commonly highly expressed[46,75]. Consequently, enforced expression in multiple breast cancer cell lines led to increased cancer invasiveness and PRC2-dependent metastasis, while knockdown by siRNAs inhibited cancer invasiveness, with a bias for cells with high PRC2 activity[46]. Furthermore, grafting HOTAIR-expressing cells into murine fat pads accelerated primary tumour growth and promoted lung metastasis[46]. HOTAIR led to selective retargeting of PRC2 across the genome by aiding the localisation of its subunits to 854 genes, which gain PRC2 occupancy and are consequently downregulated in the most aggressive breast cancer tumours[46]. This identifies HOTAIR as a relevant target for new cancer therapeutics, particularly by exploiting its interactions with PRC2. However, as HOTAIR acts in trans, validating its role would be more robust if HOTAIR could be knocked out and then the phenotype rescued by expressing it from an independent transgene[65]. Furthermore, the study of human HOTAIR *in vivo* is challenging, as there is poor sequence conservation between human and murine HOTAIR, but the orthologs do have similar functions and conserved RNA structures[76,77]. Ma *et al.* (2022) created a transgenic murine model with inducible expression of human HOTAIR to study the role of HOTAIR in breast cancer progression[78]. Mice overexpressing HOTAIR were crossed with MMTV-PyMT mice, a commonly used model of breast cancer. Overexpression of HOTAIR for several months increased the invasiveness of breast cancer cells, promoting their migration and metastasis to the lungs[78]. Removal of this overexpression abrogated these effects. Mechanistically, HOTAIR alters chromatin states and the transcriptome to cause changes that result in the promotion of metastatic pathways, by influencing both repressive and activatory modifications[78]. This demonstrates that HOTAIR could be a target for downregulation in breast cancer treatment. Although this model did not test potential therapeutics to knock down HOTAIR, it does represent an important tool that could be used to test siRNAs, ASOs, or other therapeutics targeting HOTAIR.

Another example of a scaffold lncRNA is the antisense non-coding RNA in the INK4 Locus (ANRIL) that also recruits and binds to PRC1/2 to modify transcription[79]. High levels of ANRIL expression are associated

with increased metastases and tumour size, leading to poor prognosis in NSCLC patients[80]. Knockdown of ANRIL through RNAi in six human NSCLC cell lines resulted in impaired cell proliferation in five of the cell lines and induced apoptosis, as well as inhibiting tumour growth *in vivo* when one cell line was transfected into mice[80]. The mechanism for this involves ANRIL binding to EZH2, a core subunit of PRC2, to silence KLF2 and P21[80]. Both KLF2 and P21 play significant roles in cancer, where KLF2 expression is typically reduced and is also associated with NSCLC cell apoptosis[80]. Thus, investigating methods to downregulate ANRIL in NSCLC, through its scaffolding role in binding PRC2, could provide potential therapeutic options for its treatment.

lncRNAs as guide molecules

lncRNAs can act as guides by binding proteins and guiding their localisation to specific targets, causing alterations in gene expression [Figure 4][81]. This can affect transcription by guiding the recruitment of transcriptional activators, e.g., Trithorax group proteins, or suppressors, e.g., Polycomb group proteins, in a site-specific manner[69]. Guide lncRNAs can act in cis or trans to their protein-coding targets[71]. Similar to scaffold lncRNAs, the knockdown of guide lncRNAs inhibits the localisation of the effector molecule to its target, resembling a loss of function phenotype, while a double knockdown with the effector molecule should augment this effect[69].

XIST is one of the first lncRNAs to be characterised and acts via a cis guide mechanism to initiate X-chromosome inactivation (XCI). XIST assists in recruiting the inactive X chromosome to the nuclear lamina and binds across it, with greater affinity for the most gene-dense regions[82,83]. XIST recruits PRC2 and promotes repressive chromatin modifications that cause transcriptional silencing[82,84]. XIST is thus differentially expressed in X-linked diseases, and plays a critical role in many sex-biased diseases including autoimmune diseases such as rheumatoid arthritis[85,86], neurological disorders such as Alzheimer's Disease (AD)[87,88], pulmonary arterial hypertension[89,90], and sex-biased cancers, as reviewed in[91]. In cancer, the role of XIST is complex and can have conflicting effects in protecting against or promoting cancer progression[91]. In breast cancer, which predominantly affects females, XIST is abnormally downregulated relative to normal female breast tissue[92]. Additionally, studies *in vitro* and in mice have successfully altered XIST expression to slow bladder[93], colorectal[94], and lung cancer progression[95]. In AD, higher levels of XIST are observed, alongside increased inflammatory cytokines[88]. In murine models of AD and *in vitro*, XIST promotes Aβ protein accumulation and neuroinflammation by epigenetically silencing neprilysin (NEP), an Aβ degrading enzyme, through recruitment of EZH2[88]. Knockdown of XIST in the same murine AD model reduced cell injury and neuronal inflammation, as NEP levels were increased, suggesting a potential route for therapeutics could be to downregulate XIST[88]. However, it is important to consider that attempts to target XIST for specific diseases could have wider consequences for other diseases influenced by XCI. Thus, XIST targeting must be approached cautiously and will require a thorough understanding of its mechanisms. Furthermore, attempts have also been made to utilise XIST's chromosome silencing function to target Down's Syndrome, by inserting an inducible XIST transgene into chromosome 21 of pluripotent stem cells with trisomy 21[96,97]. This successfully led to chromosome-wide silencing and methylation to create a Barr body with the additional chromosome 21[96]. This approach to chromosome silencing could also be investigated for the treatment of other trisomy conditions.

Maternally expressed gene 3 (MEG3) is a lncRNA that also interacts with PRC2, guiding it to modulate the activity of TGF-β-regulated genes by binding to chromatin in a trans manner, and is recruited to loci by the formation of RNA-DNA triplex structures[98]. MEG3 is downregulated in many cancers[99-101], for example, in non-functioning pituitary adenomas (NFA), where MEG3 is silenced[102]. Restoring MEG3 expression in cells derived from human pituitary tumours significantly slowed tumour growth when grafted into mice *in vivo*, by inducing G1 cell cycle arrest. This tumour suppression required the presence of functional p53. This
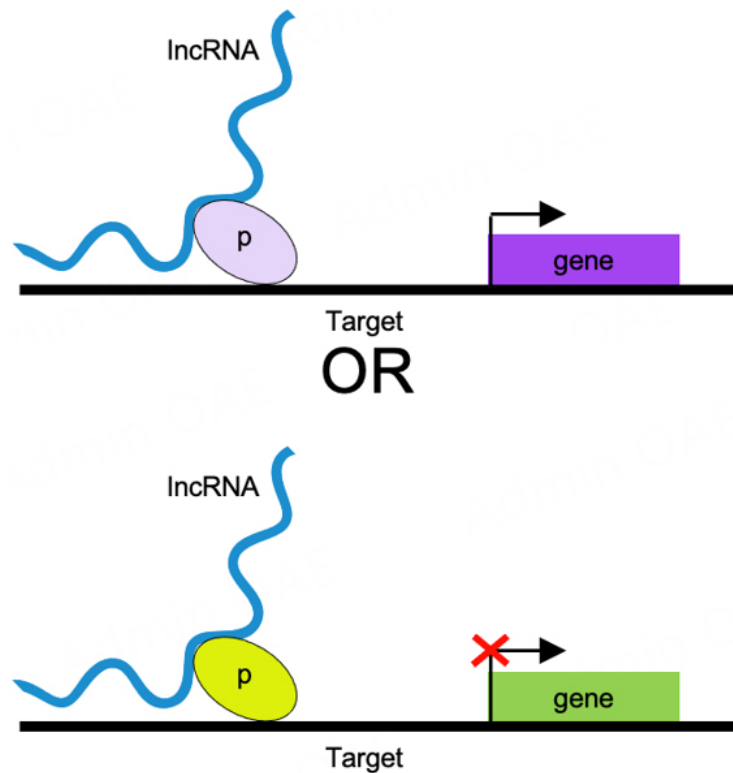
Figure 4. Schematic illustration of the functional mechanism of guide lncRNAs. Guide lncRNAs function by guiding proteins (labelled "p") to localise to specific targets in cis or trans, resulting in changes in gene expression. These effects can include activation or repression of genes depending on the specific protein.

presents a potential opportunity for therapeutic targeting and could be relevant to many cancer types if similar mechanisms are found to be at play.

lncRNAs as decoy molecules

lncRNAs can act as molecular decoys by binding proteins, such as transcription factors, chromatin modifiers, or other regulatory factors, to up- or downregulate transcription[69]. lncRNAs can sequester these molecules to inhibit their target binding (frequently to chromatin), thus interfering with transcription [Figure 5A][68]. Knockdown or knockout of decoy lncRNAs may increase the expression of their targeted molecule, thus mimicking the gain of function for the protein[69].

Similarly, lncRNAs commonly contain miRNA binding sites and act as "molecular sponges" sequestering miRNAs away from their mRNA targets [Figure 5B][68]. miRNAs control the activity of PCGs by binding mRNA transcripts and recruiting protein complexes to repress translation and/or decrease mRNA stability[104]. As miRNAs are also dysregulated in many diseases, understanding these miRNA-lncRNA interactions could have far-reaching therapeutic potential by altering lncRNA expression.

One lncRNA that acts through a decoy mechanism is the maternally expressed H19, which plays a role in the imprinted gene network during embryonic growth[105]. H19 is upregulated in cardiac, pulmonary, hepatic, and renal fibrosis, and can act by sponging multiple miRNAs, as reviewed in[106]. In the pancreas, H19 acts as a competing endogenous RNA (ceRNA) and sponges miR-138-5p and miR-141-3p[107]. Knockdown of miR-138-5p and miR-141-3p suppresses autophagy by increasing the activity of the focal adhesion kinase (FAK) pathway and promoting cell proliferation by increasing β-catenin levels,
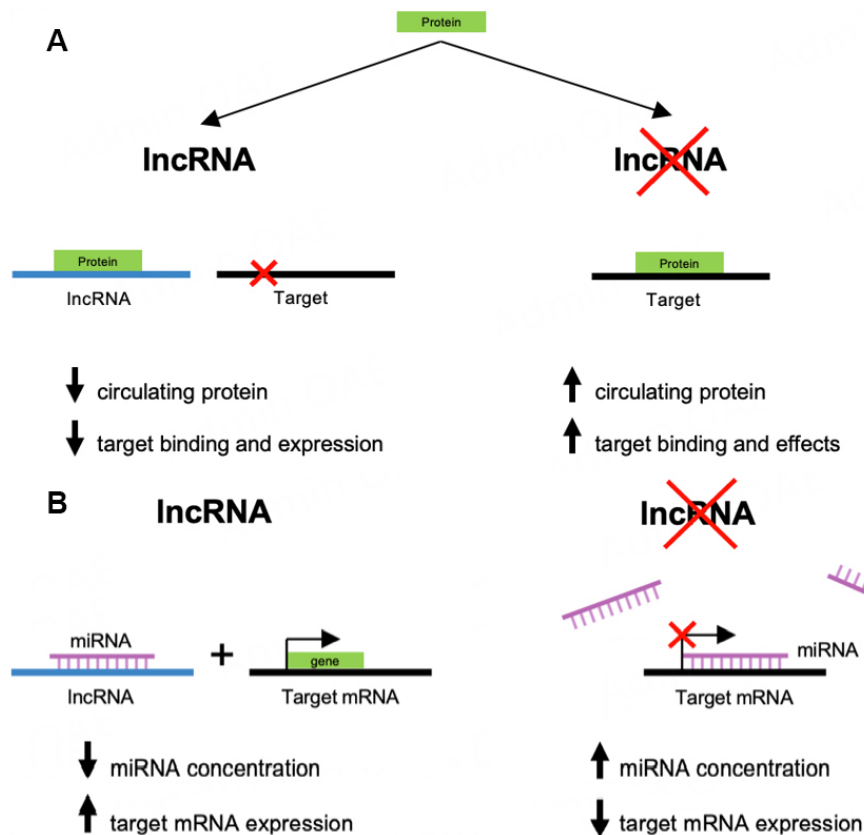
Figure 5. Schematic illustration of the functional mechanism of decoy lncRNAs. LncRNAs are shown in blue, target mRNA/molecules in black, miRNA in purple, and genes/proteins in green. (A) LncRNAs can act as molecular decoys for proteins, such as those involved in transcription, by binding the protein and stopping binding to their target molecule, e.g., chromatin. This stops any effects that are a result of the protein binding to its target. Without lncRNA presence, e.g., through knockout, there is an increase in circulating protein, so it can bind its target and produce the intended effects. (B) Here, lncRNAs act as competing endogenous RNAs to bind and sequester miRNAs, making them unable to bind to their target mRNA. Typically, miRNAs inhibit their target mRNA sequence, so this sequestering results in an increase in the expression of their target mRNA[103]. In the absence of lncRNAs, e.g., through knockout, miRNAs are free to bind to their target mRNAs, inhibiting their expression.

respectively[107]. However, in severe acute pancreatitis (SAP), H19 expression is suppressed, suggesting it could be a potential target for upregulation in SAP treatment. Rats with SAP were treated with mesenchymal stem cells (MSCs) transfected with a H19 overexpression plasmid, which increased the MSCs anti-inflammatory properties, promoted FAK-associated pathways, and increased cell proliferation[107]. Overall, this use of MSCs demonstrates an effective route to target and modulate H19 expression for SAP therapeutics. Furthermore, H19 plays a role in triple-negative breast cancer (TNBC), where it is upregulated and its expression levels are inversely correlated with lncRNA PTCSC3[108]. Overexpression of PTCSC3 inhibits TNBC cell proliferation by downregulating H19, while overexpressing H19 has no effect on PTCSC3 and promotes TNBC cell proliferation[108]. However, no evidence has been presented to indicate a physical interaction between H19 and PTCSC3, and further evidence is required to establish whether this interaction is causal in TNBC. If such evidence did come to light, then it is possible that downregulating H19 could provide therapeutic benefit, and this could be achieved through overexpression of PTCSC3[108].

In addition to its more well-known roles in transcriptional regulation, HOTAIR also has sponging functions. In diabetic cardiomyopathy (DCM), HOTAIR is downregulated, while its target, miR-34a, is upregulated[109]. Observation of HOTAIR knockdown in a mouse model of DCM showed increased

inflammation, oxidative stress, and cell death in the heart, which represent the hallmarks of DCM[109]. Increasing HOTAIR levels in DCM mice alleviated cardiac dysfunction and inhibited cardiac fibrosis[109]. The putative mechanism for this is HOTAIR acting as ceRNA and sponging miR-34a, stopping its inhibition of SIRT1, an important gene involved in DCM regulation[109,110]. This was evidenced through the knockout of SIRT1 in mice, which removed the benefits of HOTAIR expression and resembled the effects of HOTAIR knockout[109]. Furthermore, only mature miR-34a (and not the primary or precursor transcripts) was upregulated in HOTAIR knockout cells, suggesting HOTAIR downregulates miR-34a post-transcriptionally, rather than regulating its transcription[109]. Alongside evidence from RNA pull-down assays and luciferase reporter assays, this strongly supports HOTAIR directly targeting miR-34a[109]. This evidence suggests HOTAIR could be targeted for DCM through methods to increase its expression.

Aberrant expression of ANRIL has been identified in osteoarthritis (OA) tissue, as it is significantly upregulated in synoviocytes of the OA-affected joint[45]. Downregulation of ANRIL halts cell cycle progression and promotes apoptosis in synoviocytes, possibly by sponging miR-122-5p, which regulates *DUSP4*[45]. As synoviocyte proliferation is a common component of OA pathology, this suggests ANRIL downregulation could be a potential therapeutic pathway for OA by influencing the miR-122-5p/DUSP4 axis. However, this research is limited to an *in vitro* study of patient OA tissue and has not examined ANRIL mechanisms *in vivo*. There may also be potential issues with reducing DUSP4 expression, as it can have roles as a tumour suppressor, for example, in breast[111] and colorectal cancer[112].

The lncRNA dishevelled binding antagonist of beta catenin3 antisense1 (DACT3-AS1) is downregulated in gastric cancer (GC) and plays a role in its chemoresistance[113]. DACT3-AS1 aids suppression of cell proliferation, migration, and invasion, as identified through *in vitro* and *in vivo* experiments in a xenograft tumour mouse model[113]. This is achieved by targeting the miR-181a-5p/sirtuin 1 axis, which may operate through a sponging mechanism[113]. miR-181a-5p levels are increased in GC and negatively correlated with DACT3-AS1[113]. DACT3-AS1 directly targets miR-181a-5p and inhibits its levels in GC cell lines, whereas DACT3-AS1 silencing enhances miR-181a-5p levels[113]. This negative regulation suggests that DACT3-AS1 may play a role in the transcription of miR-181a-5p, thereby affecting its expression levels, but the location of DACT3-AS1 in the cytoplasm does support a sponging mechanism[113]. miR-181a-50 negatively regulates sirtuin 1 and this DACT3-AS1/ miR-181a-5p/ sirtuin 1 axis was demonstrated to suppress malignant characteristics of GC cells[113].

Other mechanisms

Acting as signalling molecules has been suggested as an additional mechanism of action, where lncRNAs act as molecular signals through their spatial and temporal expression[69]. The initiation, elongation, or termination of these lncRNAs is in itself regulatory, or they can also harbour additional regulatory functions[69]. One example is lincRNA-p21, activated by p53, which acts as a transcriptional repressor and promotes apoptosis[114]. Its expression is downregulated in coronary artery disease patients and in murine atherosclerotic plaques *in vitro*, as it represses proliferation and is pro-apoptotic in vascular smooth muscle cells and murine mononuclear macrophages *in vitro*[115]. Furthermore, *in vivo* silencing of lincRNA-p21 caused neointimal hyperplasia after endothelial injury[115]. lincRNA-p21 acts by binding MDM2, to enhance p53 activity[115]. This presents an opportunity for therapeutic targeting. However, lincRNA-p21 knockdown caused upregulation of 331 genes and downregulation of 274 genes, many of which are p53 target genes[115]. Thus, altering lincRNA-p21 expression could have serious problems if off-target effects are produced by targeting it in unintended locations.

MALAT1 can act via a decoy mechanism as previously discussed but has additional functions in cancer and other diseases. There are several suggested mechanisms, including acting as a scaffold for nuclear speckles and chromatin, as a guide RNA in signalling pathways, or as a sponge for miR-200, but the exact mechanism remains unclear[116]. MALAT1 plays a role in diabetic retinopathy and is upregulated in animal diabetes models[41]. Its knockdown improves retinal function in diabetic rats by alleviating retinal vessel impairment and inflammation[41]. Elevated MALAT1 levels have also been detected in human retinal endothelial cells[117], as well as in association with other diabetic complications[118-120]. MALAT1 acts by reducing levels of phosphorylated p38, part of the MAPK signalling pathway, to regulate endothelial cell function[41]. Inhibiting MALAT1 presents a potential therapeutic target for diabetic retinopathy, although p38 MAPK signalling pathways act in many physiological processes, with the risk of off-target effects in other locations. Specific knockdown of MALAT1 in retinal tissues would be required by any potential therapeutics to avoid impacting MALAT1 throughout the body and, importantly, in nuclear speckles. MALAT1 is also implicated in angiogenesis, where siRNA silencing of MALAT1 in mice causes endothelial cell migration instead of proliferation[121]. This implicates MALAT1 in angiogenesis regulation and in controlling the expression of cell cycle regulators. Thus, inhibiting MALAT1 could be of therapeutic benefit to induce antiangiogenic effects within tumour environments[121]. MALAT1 is frequently upregulated in cancers and has been successfully knocked down in mice using ASOs, resulting in differentiation of the primary tumour and a reduction in lung metastasis[122]. Knockdown in breast cancer organoids also inhibited branching morphogenesis and altered expression of pro-tumourigenic and differentiation-related genes[122]. This demonstrates the potential of ASO-based therapeutics in breast cancer, and, as MALAT1 plays a role in a large number of cancers, the potential for therapeutic targeting, although further studies with physiologically relevant *in vivo* models will be necessary.

The lncRNA nuclear enriched abundant transcript 1 (NEAT1) also has multiple suggested mechanisms of action. Importantly, it plays an essential role in the structure of nuclear paraspeckles by interacting with EZH2 and acting as a scaffold[123]. However, there is also evidence that it could act through sponging mechanisms to contribute to fibrosis development[124]. Knockdown of NEAT1 successfully reduces fibrosis *in vitro* and in various *in vivo* mouse models[124]. This identifies NEAT1 as a potential therapeutic target to prevent fibrosis of the liver[125], kidney[126], heart[127], and lung[128] that are implicated in the progression of the diseases[124]. Evidence suggests that the mechanisms may differ somewhat between tissues. In the heart, NEAT1 recruits the PRC2 subunit EZH2 to Smad7, appearing to act as a scaffold, resulting in Smad7 inhibition and accelerating cardiac fibrosis[127]. NEAT1 knockdown reduced cardiac fibrosis and dysfunction in mice[127]. In the liver, NEAT1 upregulation with subsequent downregulation of miR-506 is associated with nonalcoholic fatty liver disease (NAFLD)[129]. NEAT1 knockdown increases miR-506 expression, and inhibits GLI3, a miR-506 target, resulting in reduced fibrosis and inflammatory response[129]. As luciferase assays identified miR-506 binding NEAT1 and GLI3, it is possible that this interaction is a sponging mechanism, but further evidence would be required to rule out NEAT1 regulating miR-506 transcription as a mechanism. As NEAT1 is known to act via interactions with PRC2 in other tissues, the latter may be more likely. Regardless of the exact mechanism, it is possible that NEAT1 knockdown could be used therapeutically to treat fibrosis in various tissues.

LINC00301 is upregulated in NSCLC tumours and promotes cell proliferation, invasion, and tumourigenesis, while suppressing cell cycle arrest and apoptosis[50]. This was demonstrated both *in vitro* and *in vivo* in a mouse model of human disease[50]. LINC00301 is regulated by the transcription factor FOXC1, but there is evidence of it displaying multiple mechanisms of action. In the nucleus, it was found to interact with the PRC2 component, EZH2, suggesting either a guide or scaffold mechanism to affect transcription[50]. However, LINC00301 is also present in the cytoplasm, and here, it may act as a ceRNA to

sponge miR-1276[50]. Regardless of which mechanism is most significant to the role of LINC00301 in NSCLC pathogenesis, this represents an opportunity for therapeutic targeting.

CHALLENGES AND FUTURE DIRECTIONS

Several unresolved issues limit the clinical introduction of lncRNA therapeutic targeting. The low conservation of lncRNAs between species is a barrier for many research models. Humanised models or organoid cultures may be required to produce clinically translatable findings[40]. Many studies discussed here have used mouse or rat models for knockout, knockdown, or overexpression studies. Although many have yielded promising results, it will be a new challenge to replicate these findings in human disease. Furthermore, human embryonic stem cells (ESCs) and mouse ESCs have different subcellular localisations of their lncRNAs, and as localisation is closely linked to function, this may affect how findings in other organisms relate to humans[32]. lncRNA stability also varies between mice and humans; for example, MALAT1 and NEAT1 are both highly stable in humans, but unstable in mice[14]. Again, this can mean that findings in other organisms are not clinically relevant in humans. Another consideration is that lncRNAs are commonly expressed in multiple isoforms, as they undergo extensive alternative splicing[13]. These variants may have different functions, making mechanistic studies more challenging and further complicated by the fact that splicing varies between species[40]. Additionally, orthologs for human lncRNAs are only found for 38% and 35% of transcripts in mice and rats, respectively[130]. Many lncRNAs can also be modified, for example, through methylation, which may further affect their functions[40]. Furthermore, the ability of lncRNAs to target multiple genes means attempts to target them could produce off-target effects and this risk has been highlighted in several of the examples of lncRNAs provided here. Careful consideration must be given to the likelihood and extent of these effects so that they can be most safely mitigated. The method of targeting lncRNAs is also a challenge and potential issues must be noted, particularly the ongoing problems with toxicity, which may not always be fully understood through animal studies.

Although much of the lncRNA research is promising, many assumptions are made regarding their association with particular diseases, as discussed in[131]. This includes assuming that differential expression of a lncRNA in disease is causal and that interactions between a lncRNA and a protein implicated in disease indicate the lncRNA is responsible for modulating the disease risk[131]. Moreover, the presence of disease-associated single nucleotide polymorphisms (SNPs) within a lncRNA locus does not inherently imply a causal relationship with the disease[131]. It is important to remember that although some lncRNAs have essential functions or play significant roles in disease phenotypes, not all of them present opportunities for therapeutic targeting. Going forward, a useful strategy to employ could be TWAS, which was developed in recent years to complement genome-wide association studies, to detect genes associated with traits (such as disease) and determine the regulatory relationship between them. TWAS offers improved gene interpretability, particularly for non-coding regions, and enables investigation of diseases on a tissue-specific basis[132]. For lncRNAs, the genetic association signals for transcript abundance in a specific tissue can be compared with signals for a particular disease, and if colocalisation is seen, then there is evidence of a causal role in the disease[131]. Through such methods, the lncRNAs most relevant for experimental study can be identified and then tested, ideally with humanised models, to better understand their mechanism of action.

Overall, it can be challenging to characterise the functionally-relevant mechanisms of lncRNAs, particularly in discerning the difference between lncRNAs that sponge miRNAs and those that affect the transcription of their miRNA targets. However, if there is good evidence supporting the causal role of lncRNAs in a disease, precise characterisation of the mechanism may be of less importance and therapeutics targeting the lncRNA

can still be developed. This makes it easier to develop therapeutics, but there are still other problems to overcome, including toxicity and off-target effects. Off-target effects can be a problem in several ways: either by affecting the intended target in an unintended location and causing undesirable effects, by targeting the correct molecule but producing undesired effects in its other downstream effectors that are unrelated to the disease, or by affecting the expression of molecules other than the intended target. The small molecule method of targeting lncRNAs offers an exciting avenue, as this reduces toxicity problems. The development of RNA-based therapeutics and particularly RNA vaccines in recent years has resulted in much research into the safety and efficacy of RNA-based therapeutics. There is also a trend for improvement in the development of ASOs (and other ncRNA-targeting therapeutics) for targeting PCGs and other ncRNAs. This knowledge is transferable to targeting lncRNAs, and ideally should make the journey to successful therapeutics somewhat easier.

CONCLUSION

There is excellent potential for developing therapeutics targeting lncRNAs for a wide range of diseases, but these are only currently in early-stage development. There is a long journey ahead to successfully target these lncRNAs therapeutically, overcome problems such as delivery efficiency, toxicity, and off-target effects through preclinical testing, then perform clinical trials, and eventually get approval to bring these treatments to market.

DECLARATIONS

Authors' contributions
Review design: Tamblin-Hopper P, Kiss-Toth E, Sudbery I, Young D, Wilkinson JM
Literature research, manuscript drafting: Tamblin-Hopper P
Manuscript editing and revisions: Tamblin-Hopper P, Kiss-Toth E, Sudbery I, Young D, Wilkinson JM

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All authors declare that there are no conflicts of interest.

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