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Long Noncoding RNAs of the Arterial Wall as Therapeutic Agents and Targets in Atherosclerosis

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Abstract

Long noncoding ribonucleic acids (lncRNAs) have been defined as transcripts which are > 200 ribonucleotides in size and are not translated into protein. Recent work has shown that many lncRNAs do have specific molecular functions and biological effects, and are involved in a growing number of diseases, including atherosclerosis. As a consequence, lncRNAs are also becoming interesting targets for therapeutic intervention. Here, we focus on lncRNAs which are expressed in the arterial wall, and describe potential RNA therapeutic approaches of atherosclerosis by manipulating lncRNAs without affecting genome deoxyribonucleic acid content: Starting out with an overview of all lncRNAs that have so far been implicated in atherosclerosis by *in vivo* studies, we describe methodologies for their activation, inactivation, and RNA sequence manipulation. We continue by addressing how artificial (nonnative) therapeutic lncRNAs may be designed, and which molecular functions these designer lncRNAs may exploit. We conclude with an outlook on approaches for chemical lncRNA modification, RNA mass production, and site-specific therapeutic delivery.

Keywords
- ► transcription
- ► splicing
- ► lncRNAs
- ► circRNAs
- ► gene expression

Introduction

It is well known today that a large portion of the human genome (~70%) is transcribed and that the majority of the produced transcripts are noncoding (ENCODE or FANTOM consortia).[1–4] Among the 200,000 known transcripts, around 28,000 stem from long noncoding ribonucleic acid (lncRNA) loci (GENCODE).[5] Genome-wide association studies (GWAS) recurrently find disease-linked genetic variation in the non-protein-coding sequence space,[6] often overlapping gene regulatory elements like enhancers, which are actively transcribed and give rise to noncoding RNAs.[7,8] Concurrently, transcriptomic analyses in patient cohorts reveal numerous lncRNAs which are differentially expressed in diseased tissues. Recent work has shown that many lncRNAs are functional, leading to the notion that lncRNAs may represent a large class of potential therapeutic agents and targets. In this minireview, we describe lncRNAs linked to atherosclerosis in humans which are expressed in cells of the arterial wall (wall endothelial cells [ECs], vascular smooth muscle cells [VSMCs], and circulating and resident immune cells). We do not cover the roles of lncRNAs regulating atherosclerosis risk factors, such as lipid metabolism, diabetes, or hypertension, or adaptation to ischemic stress. We describe molecular roles of relevant disease-linked lncRNAs, and techniques to therapeutically manipulate them at the RNA level, referred to as RNA therapeutics, an approach that focuses on controlling RNA form and sequence without affecting the deoxyribonucleic acid (DNA) in our genomes.

LncRNA Classes and their Suggested Therapeutic Potential

lncRNAs come in two major flavors, with tens of thousands of cases in each class: (1) linear lncRNAs from dedicated lncRNA genes with their own promoter and terminator and (2) covalently closed circular lncRNAs. The latter are produced through splicing from existing primary transcripts of any type of gene (►**Fig. 1A**, ►**Table 1**) (see Refs. [9] and [10] for review). Linear and circular lncRNA production and

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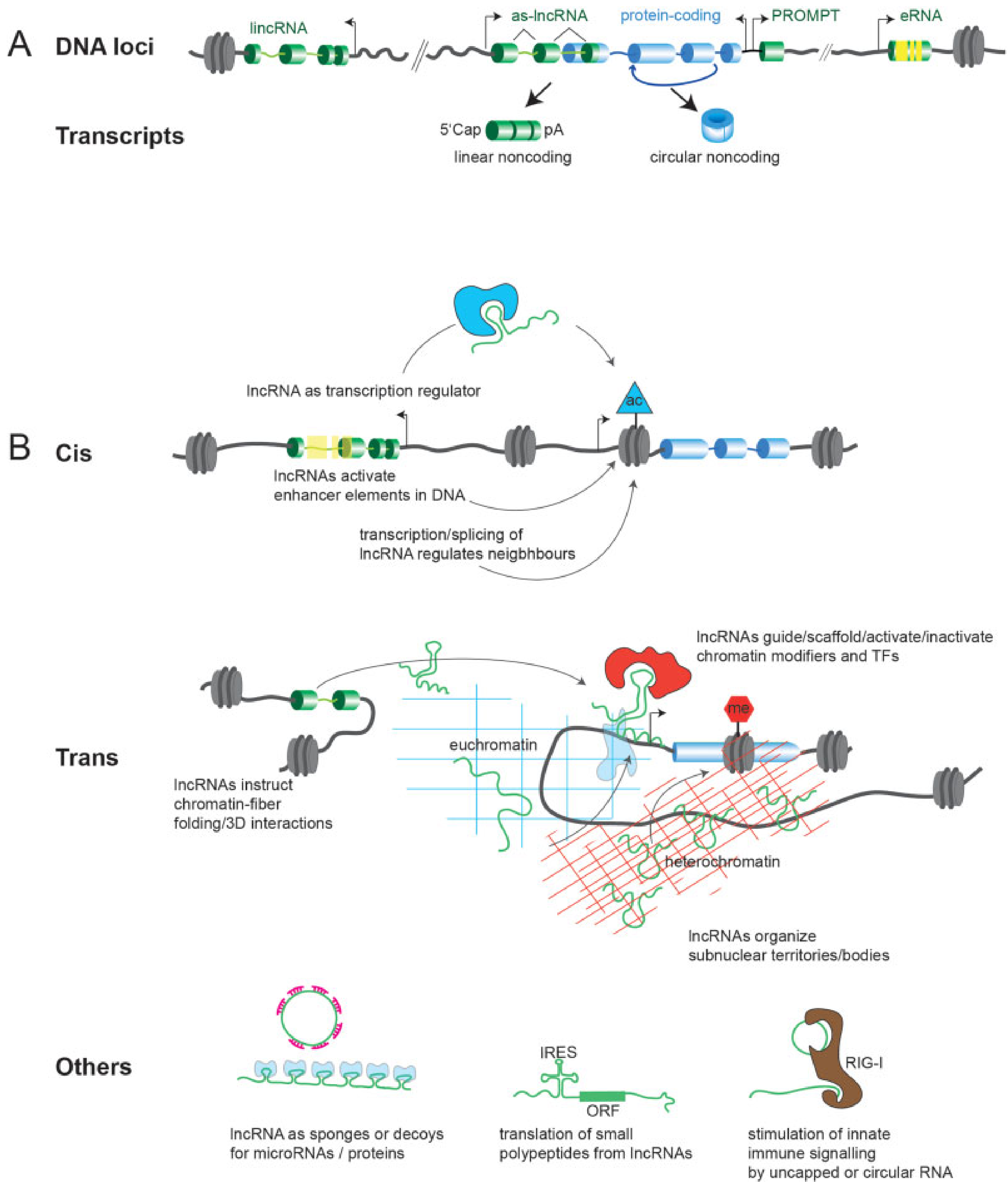

Fig. 1 Classes and functions of long noncoding ribonucleic acids (lncRNAs). (**A**) The positioning of lncRNA genes relative to protein-coding genes is a classification criterion. (1) Large intergenic noncoding RNAs (lincRNAs). (2) Natural antisense transcripts (as-lncRNAs). (3) Promoter upstream antisense transcripts (PROMPTs). (4) Enhancer RNAs (eRNAs). lncRNAs transcripts can be linear or circular. Circular lncRNAs are produced by backsplicing of a downstream exon to an upstream exon and do not carry 5′Cap or 3′polyA tail. (**B**) Functions of lncRNA with atherosclerotic relevance. Functions can be classified in *cis*- and *trans*-acting roles in transcriptional regulation in the nucleus, and in standalone effects (others) in the cytoplasm (bottom). During transcription control, either the lncRNA transcript is functional, or the progression of the RNA polymerase over the lncRNA gene body is the functional determinant (coinciding with chromatin-decompaction of deoxyribonucleic acid [DNA] elements at the lncRNA locus, or with splicing of nascent lncRNA still attached to its DNA template). Abbreviations: IRES, internal ribosome entry site; ORF, open reading frame; RIG-I, retinoic acid inducible gene I; TF, transcription factor.

Table 1 Dichotomy of linear and circular long noncoding RNAs

Linear lncRNAs	circRNAs (3′-5′-linked exon-containing circular RNAs)
> 200 nucleotides (nts) in length	
Non-protein-coding (lacking open reading frames > 100 codons)	
Tens of thousands of cases	
Many isoforms per case	
Produced with cell type- and context-specificity	
Mostly lower abundance per cell than average mRNAs	
Considered to be mostly functional	
Average length ∼1,000 nts	Average length ∼550 nts
Nuclear enrichment (not all)	Cytoplasmic enrichment (not all)
Mostly carrying 5′Cap and 3′ polyA tails (depending on lncRNA class)	Generically lacking 5′Cap and 3′ polyA tails due to covalent 3′-5′ backbone linkage after backsplicing
Produced by transcription from unique transcriptional units with their own promoter	Produced by spliceosome from primary transcripts (mRNAs or lncRNAs)

Abbreviation: lncRNA, long noncoding ribonucleic acid.

abundance are regulated and are cell-type specific. Both linear and circular lncRNAs have been shown to be overall functional in one pathway or another. lncRNAs effector mechanisms are diverse and complex (►**Fig. 1B**), making it nontrivial to decide which one to interfere with for therapy:

(1) lncRNAs guide, scaffold, and control transcription-regulating protein complexes:

Many lncRNAs are known to affect transcription of RNAP I and II target genes, often by impacting chromatin readers and writers in gene promoter control.[11] Examples with relevance for atherosclerosis are *ANRIL*, *H19*, *lincRNA-p21*, *MALAT1*, *MEG3*, *NEAT1*, or *TUG1* (►**Table 2**). lncRNAs affect transcription *in cis*, or also *in trans*, especially if their steady-state abundance is large and allows diffusion of the lncRNA throughout the nucleoplasm. Mechanistically, lncRNAs can recruit chromatin remodelers to target genes via hybridization to DNA, or control their enzymatic activity, or function as negative decoys. lncRNAs can also regulate the transcription of microRNA and other noncoding RNAs (a process distinct from RNA sponging, see 5).

(2) Enhancer RNAs/eRNAs:

Active enhancers for gene promoters have recently been found to be transcribed and to give rise to enhancer lncRNAs (eRNAs). These can confer enhancer activity by capturing the promoter-contacting Mediator protein complex.[12] Examples of eRNAs important for atherosclerosis are *HOTTIP*, *LEENE*, and *SMILR* (►**Table 2**). Depending on enhancer, both, the eRNA and the chromatin-opening during transcription of the eRNA locus can be therapeutically relevant.[11]

(3) Antisense lncRNAs/asRNAs:

Some lncRNA genes reside within protein-coding gene units, even overlapping coding exons in antisense. Effects on host genes can be positive and negative. Examples with relevance for atherosclerosis are *ANRIL*, *HOXC-AS1*, *MALAT1*, and *SENCR* (►**Table 2**). Globally, antisense transcription dampens transcriptional noise and is not part of signal-dependent expression control.[13] Therapeutic programming through asRNAs is complex, because it may require engineering the genomic locus.

(4) LncRNAs in subnuclear bodies:

Some lncRNAs can affect other genes through their architectural role in assembling eu- and heterochromatin subnuclear territories. Examples are *MALAT1* in Polycomb bodies,[14] or *NEAT1* in paraspeckles.[15] These indirectly affect gene expression and pre-messenger RNA (mRNA) processing, respectively, depending on the vicinity of genes to these subnuclear bodies. The broadness of the effect and the complexity of the process make it difficult to achieve specificity in therapy.

(5) lncRNAs as microRNA sponge:

Although many publications implicate endogenous lncRNAs as microRNA sponges (and as inhibitors of microRNA availability and function, therein), many of these reports are met with criticism because evidence often bases on uncontrolled lncRNA overexpression. Few lncRNAs pass the stoichiometric requirements for sponging, though, as most are endogenously not sufficiently highly expressed compared with the number of corresponding microRNA targets and copy numbers of microRNAs per cell (see Ref. [16] for overview).

(6) lncRNAs which bind and regulate proteins:

Mass spectrometric analyses showed that a single lncRNA can bind dozens of different proteins in the nucleus and in the cytoplasm, and thereby affect multiple molecular mechanisms at once[17]: This concept is best seen for well-studied lncRNAs like *XIST*, which was found to bind > 80 proteins, indicating that it participated in DNA and histone modification and RNA remodeling machineries.[17] With relevance to atherosclerosis, circular *ANRIL* (*circANRIL*) binds to the rRNA processing PeBoW complex for protein translation

control,[18] whereas linear *ANRIL* interacts with members of the PRC1[19] as well as with the PRC2 Polycomb-repressive complexes[19–21] during presumptive transcription control of target genes (►**Table 2**). In another case, *TUG1* can serve as competing endogenous RNA, and also promote gene activity by chromatin fiber positioning,[14] and was even suggested to regulate cytoskeletal contractility by enhancing the cytoplasmic activity of Ezh2 toward methylating α-actin[22,23] (►**Table 2**). Conceptually, a lncRNA may also hierarchically regulate a single transcriptional regulator, thereby influencing, in one step, a range of downstream genes and cellular processes. For example, *NRON* participates in scaffolding and restraining the nuclear factor of activated T cell (NFAT) transcription factor in a latently active form in the cytoplasm in unstimulated resting T cells.[24,25] Transcription factor control is recurrently ascribed to many lncRNAs (►**Table 2**). Together, there is potential for lncRNAs in tuning protein complex activity as "RNA-drugs," akin to small-molecule drugs, but lncRNA-dependent activity changes in lncRNA:protein complexes are difficult to study, and so far not understood in any case in mechanistic and structural detail.

(7) Protein translation from noncoding RNA:

Despite being nonprotein-coding by definition, some lncRNAs do contain small open reading frames (sORFs), some of which can be translated. Atherosclerosis-specific functions of sORFs are so far unknown. Still, protein expression is, in principle, possible from, both, linear and circular lncRNAs, if the required translation-initiating signals are artificially incorporated into synthetic constructs[26] (see chapter on "Disease Therapy by Artificial (Nonnative) Designer lncRNAs" below).

(8) Bifunctional noncoding mRNAs:

The clear separation between coding and noncoding RNA is blurring and some mRNAs carry functions also as untranslated RNAs. For example, the *steroid receptor RNA*, which is in principle protein-coding, also functions as lncRNA-in chromatin regulation at specific target genes in the nucleus.[27,28] And p53, as noncoding RNA, binds and affects the activity of the MDM2 enzyme through structural RNA motifs.[29] In another more indirect case, under stress cohorts of several hundred mRNAs become transcribed from alternative upstream transcription start sites, a process that blocks their transcription from the actual promoters. This shift leads to a novel longer RNA isoforms that include a short upstream ORFs with a new stop codon, whose translation blocks the translation of the actual functional protein encoded in a given locus.[30] Thereby, functionality lies less in an active role as *cis/trans*-acting noncoding RNA, but more in indirect effects of translation control on the proteome.[31,32] These findings expand the operational space for noncoding RNA therapeutics (►**Table 1**).

Known Roles of lncRNAs in Atherosclerosis

Limiting our review to those lncRNAs expressed in the vascular wall and functioning in atherosclerosis, 31 lncRNAs have so far been implicated in vascular cell types (►**Table 2**). Most were initially found because of being differentially expressed in patient cohorts. Only a few (*ANRIL*, *circANRIL*, *MIAT*, *H19*, *LINC00305*), were identified by unbiased GWAS. Fifteen of the listed lncRNAs were studied in immune cell types (such as peripheral blood monocytes, circulating and vascular wall macrophages, or foam cells), 17 in ECs, and 13 in VSMCs.

In the following, we highlight lncRNAs from ►**Table 2** where *in vivo* evidence for therapeutic potential exists. Four groups may be distinguished: (1) lncRNAs with a documented therapeutic benefit for atherosclerosis, (2) lncRNAs with a benefit for other vascular diseases, (3) lncRNAs essential for normal vascular biology, and (4) lncRNAs generally involved in inflammatory signaling (with expected relevance for atherosclerosis).

1. *In vivo* evidence for therapeutic a potential in atherosclerosis has been determined only for one lncRNA: *Neat1*, a well-known lncRNA,[76] is upregulated in plaques, and knocking-out *Neat1* in mice decreased neointimal lesions in an atherosclerosis model.[64] Since a full-body mouse knockout was analyzed, it remained unclear in which cell type *Neat1* functioned.[64] A function in VSMCs was tested *in vitro*: During carotid artery injury, VSMCs usually dedifferentiate from a quiescent to a proliferative/synthetic phenotype, and *Neat1* promoted this atherogenic switch by repressing the function of the chromatin activator WDR5/MLL on serum response factor (SRF) target genes.[64] Consequently, therapeutically reducing *NEAT1* in VSMCs in lesions might be useful for antagonizing the proatherogenic myocardin-SRF-dependent phenotypic switching of VSMCs,[64,77] or the proatherogenic oxidized low-density lipoprotein (ox-LDL)-dependent inflammatory signaling in macrophages.[78] However, given that VSMC proliferation and matrix synthesis are in other contexts also considered beneficial (for example, for plaque repair or for fibrous cap stability) (see Ref. [79] for review), more work is needed before *NEAT1* can be considered for cell-type-specific therapy.
2. Three lncRNAs showed therapeutic potential at least regarding other vascular diseases: Downregulation of *H19* ameliorates aneurysms,[38] and downregulation of *Miat*,[62] *circHipk3*,[33] or *cZNF609*[34] ameliorates diabetic retinopathy.
3. Two lncRNAs, *MeXis*[61] and *Malat1*,[57,80,81] have been studied by knockouts in mice. Nevertheless, the therapeutic potential of these lncRNAs remains untested: In the first case, *MeXis* levels were found to increase by ox-LDL stimulation of macrophages, upon which this lncRNA induced the *Abca1* transporter and cholesterol efflux.[61] Since knockout of *MeXis* led to increased plaque growth in bone marrow reconstitution experiments of $ldlr^{-/-}$ mice, therapeutically increasing *MeXis* (human *TCONS00016111*) expression, especially in patients with single-nucleotide polymorphisms (SNPs) in this gene,[61] might potentially be therapeutically relevant. Care is advised, however, when interpreting data for *Malat1*, which has opposing roles in different cardiovascular conditions: On the one hand, *MALAT1* was found to be downregulated in the plaque,[55] and knocking-

Table 2 Mammalian lncRNAs implicated in atherogenesis by affecting cells of the vasculature

lncRNA	Regulation in atherosclerotic vascular wall	Immune cells	Endothelial cells	Smooth muscle cells	Molecular function	Effector mechanism	References
ANRIL	Up	x	x	x	Transcription Guiding chromatin regulators (cis/trans-regulation) Enhancer elements in locus	• PBMCs, VSMCs proproliferative, antiapoptotic • PBMCs, ECs proadhesive, proinflammatory • ECs proinflammatory, proapoptotic	19,21
circANRIL	Down	x		x	Protein regulation: Inhibiting rRNA processing PeBoW complex	VSMCs, MΦ antiproliferative, proapoptotic	18
circHIPK3	n.d.		x		ceRNA/microRNA sponge	EC proproliferative, promigrative, antiapoptotic	33
cZNF609	Down		x		ceRNA/microRNA sponge	ECs antiproliferative, proapoptotic, antimigrative	34
GAS5	Up	x	x	x	Parent molecule for small RNA SnoRNAs ceRNA/microRNA sponge Transcription Decoy for TFs	• EC, MΦ antiproliferative, proapoptotic • VSMC antidifferentiative	35,36
H19	Down (in plaques) Up (in aneurysm)	x	x	x	Transcription Antagonizing microRNAs Tethering chromatin modifiers Parent molecule for microRNAs Binding mRNAs mRNA decay	• MΦ proinflammatory • VSMC proapoptotic, invasive • ECs proproliferative, anti-inflammatory, proangiogenic	37–39
HAS2-AS1	Up			x	Transcription Activating sense transcript	VSMC proproliferative	40,41
HOTAIR	Down		x		Transcription	• MΦ proinflammatory, proapoptotic • EC proproliferative, promigrative, antiapoptotic	42,43
HOTTIP	Up		x		Transcription Tethering chromatin modifiers eRNA-like	EC proproliferative, promigrative	44
HOXC-AS1	Down	x			Transcription Activating sense transcript	MΦ anticholesterol-loading	45
LEENE	n.d.		x		Transcription eRNA	EC anti-inflammatory, antiadhesive	46
Lethe	n.d.	x			Transcription Decoy for TF	MΦ anti-inflammatory	47
LINC00305	Up	x	x		Protein regulation Scaffolding receptors ceRNA/microRNA sponge	• PBMC proinflammatory • EC antiproliferative; proapoptotic	48,49
LincRNA-Cox2	n.d.	x			Transcription Scaffolding chromatin modifier	MΦ immunomodulatory, proinvasive	50–52

Table 2 (*Continued*)

lncRNA	Regulation in atherosclerotic vascular wall	Immune cells	Endothelial cells	Smooth muscle cells	Molecular function	Effector mechanism	References
lincRNA-Dynlrb2–2	n.d.	x			n.d.	MΦ anticholesterol	53
lincRNA-p21	Down	x	x	x	Transcription Tether for hnRNPK, corepressor for p53 Protein regulation Inhibiting E3 ligase Binding to mRNAs Suppression of translation	• VSMC, MΦ antiproliferative, proapoptotic • EC proapoptotic	54
Malat1	Down	x	x	x	Transcription Binding chromatin remodelers 3D chromatin positioning Splicing control Parent molecule for small RNA Cytoplasmic mascRNAs	• PBMC, MΦ anti-inflammatory • EC antimigrative, proproliferative • SMC prodifferentiative	55–57
Mantis	n.d.		x		Transcription Scaffold of chromatin modifier	• EC proangiogenic	58
Meg3	Down	x	x	x	Transcription Tether of chromatin modifier Protein regulation Interacting with TF CeRNA/microRNA sponge	• VSMC antiproliferative, antimigrative, proapoptotic • EC antiproliferative	59,60
MeXis	Down	x			Transcription	MΦ cholesterol efflux	61
MIAT/Gomafu	Up		x		Splicing control ceRNA/microRNA sponge	• EC proproliferative, promigrative, proangiogenic	55,62
Myoslid	n.d.			x	n.d.	VSMC antiproliferative, antimigrative	63
Neat1	Up			x	Transcription Decoy for chromatin regulator	VSMC proproliferative, promigrative; antidifferentiative	64,65
NRON	n.d.	x			Protein regulation Inhibiting TF	T cells immunomodulatory	24
Pacer	n.d.	x			Transcription Inhibiting TF	MΦ anti-inflammatory	66
RNCR3/LINC00599	Up		x	x	ceRNA/microRNA sponge	• VMC proproliferative and promigrative ECs antiapoptotic	67
SENCR	Down		x	x	n.d.	• VSMC proproliferative, promigrative • EC proproliferative promigrative, proangiogenic	68,69
SMILR	Up			x	n.d.	• VSMC proproliferative;	70
STEEL/HOXD-AS1	n.d. (Up)		x		Transcription Binding chromatin modifier	EC proangiogenic, angiogenic patterning	71

(*Continued*)

Table 2 (*Continued*)

lncRNA	Regulation in atherosclerotic vascular wall	Immune cells	Endothelial cells	Smooth muscle cells	Molecular function	Effector mechanism	References
THRIL/Linc1992	n.d.	x			Transcription: Binding chromatin factor	MΦ immunomodulatory	72
Tug1	Up	x	x	x	Transcription: 3D chromatin positioning Protein regulation ceRNA/microRNA sponge	• VSMC, MΦ proproliferative, antiapoptotic, proinflammatory • VSMCs actin polymerization ECs proapoptotic, antiadhesive	22,73,74
Uca1	n.d.		x		Transcription	• EC proproliferative, promigrative, antiapoptotic	75

Abbreviations: 3D, three-dimensional; EC, endothelial cell; lncRNA, long noncoding ribonucleic acid; PBMC, peripheral blood mononuclear cell; TF, transcription factor; VSMC, vascular smooth muscle cell.

out *Malat1* in mice was recently found to trigger immune dysregulation and atherosclerosis in an *apoe*$^{-/-}$ mutant background, remarkably even without the challenge by a coronary artery disease-triggering fat-rich diet.[81] With a similar direction of effect, *Malat1* knockouts developed larger infarcts in brain ischemic mouse models.[82,83] These two studies suggested that it might be worthwhile to normalize *MALAT1* through overexpression during therapy. Yet, in other contexts, such as aortic thoracic aneurysms or vascular diseases of the Marfan syndrome, beneficial effects seemed to lie rather in *MALAT1* inhibition, and not in its induction.[57] Also, bluntly overexpressing *Malat1* may have limitations because it is known from other studies that increases in *MALAT1* would promote cancer by effects on cell migration, metastasis, and angiogenesis in hypoxic conditions.[84,85] Summarizing, different studies showed context- and cell type-dependent therapeutic requirements for *MALAT1*. Consequently, further studies and tools for achieving tissue tropism in delivering therapeutic lncRNA only to specific cells, or for conditional activation/inactivation of lncRNAs in specific conditions and cells, will be required.

4. Twelve lncRNAs have been indirectly implicated in atherogenesis through *in vitro* studies in cultured vascular cell types, or through *in vivo* insight in their effectors. A major group in this class comprises 6 lncRNAs involved in modulating inflammatory signaling *in vivo* (*lincRNA-Cox2*, *PACER*, *Lethe*, *THRIL*, *NRON*, *STEEL*) (►**Table 2**). These have considerable therapeutic potential because of the intimate contribution of inflammation to atherogenesis (see following chapter on "Disease Therapy by Manipulating Endogenous lncRNAs".). The rest of lncRNAs in this group control ECs (*Miat*), VSMCs (*Myoslid*), or both (*Gas5*, *lincRNA-p21*, *Meg3*), through functioning in diverse processes.

Translating Molecular Function to Therapeutic Value

In the following, we summarize key therapeutic principles centered on lncRNAs (►**Fig. 2A**). Thereby, we distinguish (1) therapeutic approaches exploiting endogenous lncRNA functionality, and (2) approaches based on artificial (nonnative) designer lncRNAs.

Disease Therapy by Manipulating Endogenous lncRNAs

During the onset of disease, lncRNAs expression levels change in cell- and context-dependent modes. For therapy, lncRNAs that are overactive in disease can be normalized by knockdown approaches. Gain-of-function approaches or RNA sequence correction can be used to antagonize disease-linked changes at the RNA level in other lncRNAs.

(1) *Transcriptional control* is, so far, the major known function of noncoding RNAs. Twenty-one of 31 atherosclerosis-linked lncRNAs function as guides, scaffolds, and regulators of chromatin factors (►**Table 2**). In contrast to

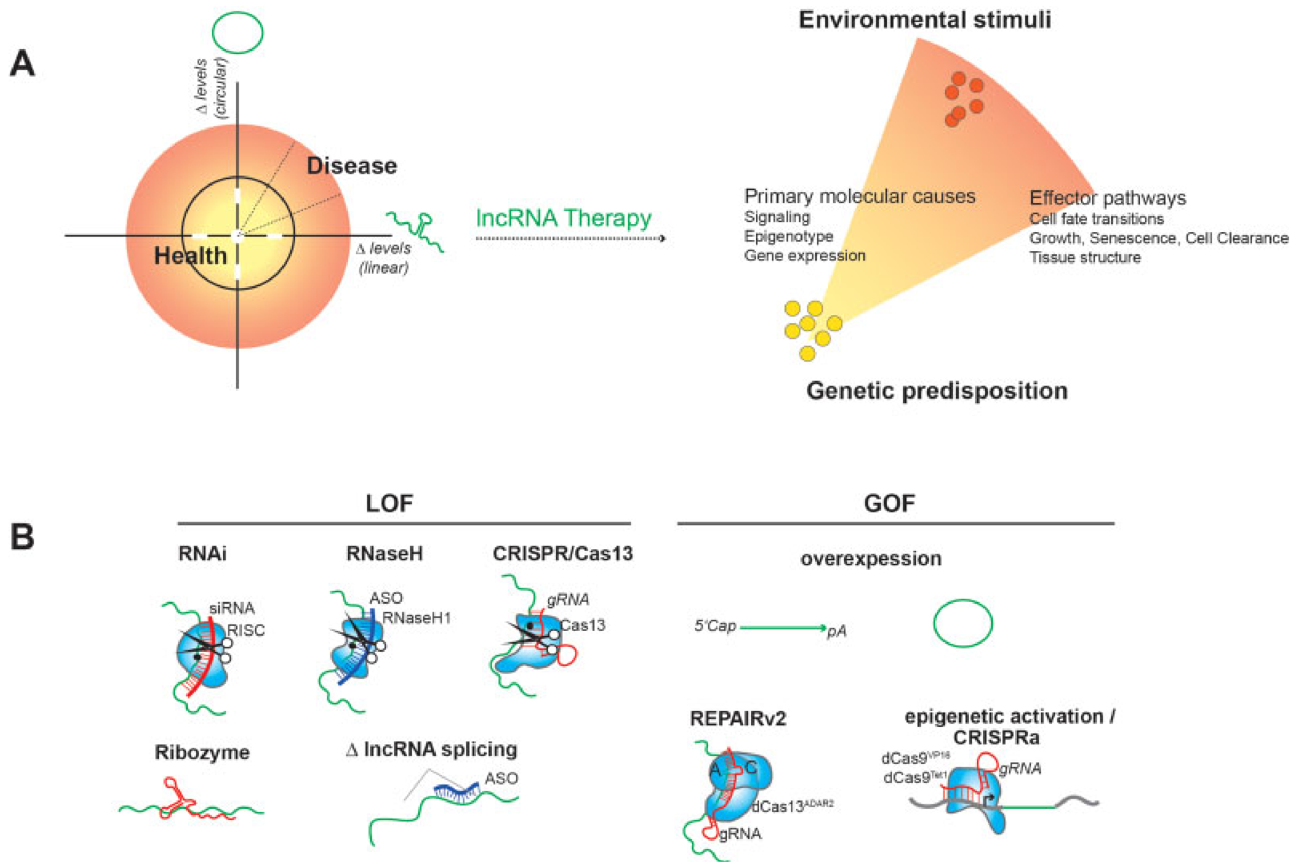

Fig. 2 Concepts and tools for using long noncoding ribonucleic acids (lncRNAs) of the arterial wall in antiatherogenic therapy. (**A**) Concepts for lncRNA therapy. lncRNA genotype and the ratio of linear and circular lncRNA transcripts must be considered during therapy (left panel). lncRNAs can be therapeutically used to correct the primary molecular causes of disease or to block the pathologically induced cellular effector pathways that confer plaque growth (right panel). Another option would be to, more generally, kill-off disease-causing cells in plaques (right panel). The healthy cell state is denoted in yellow, the diseased state in red. (**B**) Tools for manipulating lncRNA levels in cells, classified under LOF (loss-of-function) and GOF (gain-of-function) approaches. Target RNAs are labeled in green. Targeting RNAs or ribozymes are marked in red, targeting deoxyribonucleic acid (DNA)-like antisense oligonucleotides (ASOs) in blue. Scissors denote target RNA cleavage by nucleases. Abbreviations: CRISPRa, CRISPR activation for promoter activation; dCas9^{VP16}, inactive Cas9 fused to the generic herpes simplex viral protein 16 (VP16) activation domain; dCas9^{Tet1}, inactive Cas9 fusion with catalytic domain of Tet1 (DNA 5-methylcytosine demethylase); dCas13^{ADAR2}, catalytically inactive Cas13 RNA-guided RNase fused to the ADAR2 (adenosine deaminases acting on RNA 2); gRNA, CRISPR guide RNA; pA – poly (A) tail; REPAIR, RNA Editing for Programmable A to I Replacement; RNAi, RNA interference.

small-molecule drugs which inhibit broadly-acting chromatin factors (such as DNA/histone methyltransferases, demethylases, acetylases, and chromatin readers),[86] epigenetic therapy through manipulating lncRNA levels and sequence may have the advantage that it allows, in principle, to sequence-specifically control specific subsets of target genes. Separately, it becomes a therapeutic option to guide generic repressor proteins (KRAB- and Chromo-domains) or activators (VP16) to promoters of disease-linked genes via noncoding RNAs.[87,88]

(2) *Controlling splicing* is equally important for therapy as transcription control. Splice-regulating antisense oligonucleotides (ASOs) can be used to tune splicing in mRNAs and lncRNAs. Additionally, a new therapeutic front was opened when it was found that cotranscriptional circRNA biogenesis interfered with linear RNA biogenesis.[89–91] A well-studied case is *ANRIL*, transcribed from Chr9p21 in humans, the most prominent atherosclerosis risk loci known so far.[18,92] Linear *ANRIL* levels positively correlate with atherosclerosis severity, while *circANRIL* levels anticorrelate with atherosclerosis.[18,21] While linear *ANRIL* expression causes transitions reminiscent of atherogenesis (inflammation, overproliferation, adhesion in macrophages and VSMCs),[19–21,93–95] *circANRIL* mediates opposite effects by blocking proliferation and enhancing cell death rates.[18] Thus, one way to decrease atherosclerosis risk would be to coincidently decrease linear *ANRIL* and increase circ*ANRIL*, because linear splice forms are known to be increased and circular isoforms decreased in atherosclerosis. How to therapeutically re-engineer the wild-type splicing pattern in a locus is highly complex, as learned from diseases like muscular dystrophies that arise from splicing defects (see Ref. [96] for a recent review), and it has not yet been practically achieved to simultaneously correct levels of linear and circular *ANRIL*. Theoretically, and confined to RNA-centric approaches, it would be possible to deliver *in vitro* produced synthetic *circANRIL* together with ASOs that target exonic *ANRIL* sequences

that are not contained in the circular isoforms. Other options would be to use splice-changing ASOs, that inhibit forward splicing, and promote intron-backfolding for assisting backsplicing and circularization. Finally, deeper insight into how specific atherosclerosis-linked SNPs in *ANRIL* affect splice site choice[92] (e.g., through RNA-binding proteins), may allow to get a grip on deliberately affecting circularization.

(3) *Proinflammatory signaling* drives atherogenesis and various lncRNAs regulate inflammation-dependent gene expression in different cell types. For example, during nuclear factor kappa B signaling in macrophages, *LincRNA-Cox2* represses numerous genes by scaffolding RelA-p50 into SWI/SNF complexes.[51] In contrast, *PACER* upregulates *COX-2*, a proatherogenic cyclooxygenase, by interfering with the formation of repressive p50:p50 homodimers,[66] and *Lethe* inhibits RelA binding to DNA.[47] Other lncRNAs are active in T cells or ECs: *NRON* scaffolds the cytoplasmic IQGAP1/NFAT1 protein complex, thereby restraining calcineurin from activating the proinflammatory NFAT1 transcription factor in T cells.[24,97] In ECs, *STEEL* recruits the activating poly-adenosine diphosphate ribosylase PARP1 to *KLF2* and *eNOS*.[71] Depleting *PARP1* is known to limit atherosclerotic plaque growth,[98] suggesting that inhibiting *STEEL* could be interesting. Together, upregulating *Lethe* and downregulating *lincRNA-Cox2*, *PACER*, and *THRIL* may be therapeutically useful (see Refs. [99] and [100] for review on atherogenic inflammation).

Disease Therapy by Artificial (Nonnative) Designer lncRNAs

To achieve lncRNAs with novel functionality, endogenous RNA sequence can be altered, or synthetic constructs are overexpressed. These can contain sequence combinations not existing in endogenous RNA, or stem from *in vitro* evolution routines:

(1) Although *microRNA sponging* is not considered a common endogenous function for lncRNAs, therapeutically overexpressed lncRNAs are more abundant than endogenous RNAs and may very well become sponges. Such artificial sponges can be optimized by increasing microRNA-binding sites, and by optimally spacing them.[101] Furthermore, if target-matched sites are shortened from 8 to 6 nt, microRNA degradation happens instead of sponging, opening therapeutic possibilities even further.[16] circRNAs may become the superior type of sponges because circRNAs are more stable against cellular exoribonucleases than linear RNAs.[9,10]

(2) *Artificial RNA aptamers*, as high-affinity binders of biomolecules, constitute a large therapeutic class. Endogenous lncRNAs fold into secondary structures, and more so than mRNAs.[102] Notable are conserved stem-loop structures in protein-interaction interfaces. With a novel *in vitro* sequence evolution methods in development (e.g., SELEX with Pol θ CS13 ribonucleotidyl transferases that deliver random RNA libraries and tolerate 2′-functionalized ribonucleotides[103]), it becomes tangible to engineer protein- or metabolite-binding RNAs, much like small molecules are classically used as protein-targeting "drugs." An alternative application of RNA aptamers is to bind surface receptors in diseased cells and confer cell entry of drugs and effectors fused to RNA.[104]

(3) *Therapeutically relevant peptides* can be expressed from designer lncRNA: Although the vast majority of linear and circular lncRNAs are endogenously not translated to polypeptides by ribosomes, there are exceptions: small ORFs encoding micropeptides are known in lncRNAs, and some have cardiovascular relevance (*LINC0094 8*→myoregulin; *SMIM6* →endoregulin; *LOC100507537*→DWORF).[105] Also, a tiny fraction of native circRNAs can be translated if noncoding RNA segments in the circle fold into internal ribosome entry sites (IRES) to drive translation initiation (*circZNF609*).[26] Therefore, one future therapeutic option would be to circularize mRNA to obtain stable expression of therapeutic proteins from a designer circRNA containing an artificial IRES sequence.

(4) *Immunotherapy of atherosclerosis by boosting innate immune signaling*: One future option for atherosclerosis therapy is to boost specific branches in innate-adaptive immune system cross-talk, a concept stemming from research on antitumor strategies (see Ref. [106] for overview). This may potentially supplement the more classical anti-inflammatory strategies to fight atherosclerosis.[107] Recent insight shows that also noncoding RNA may have a place in immunotherapy: First, externally provided synthetic noncoding RNA or RNA analogs are already used as adjuvants to increase the immunogenicity of peptide-based vaccination, by virtue of their ability to stimulate cytoplasmic innate immunity receptors RIG-I and MDA5 as "nonself."[108–110] Vaccinations, such as with the tolerizing apoB100 epitope,[111] might in the future benefit. Controlling RIG-I signaling, as far as known from the cancer field, may well also be therapeutically useful for atherosclerosis therapy. The aim here may be to induce programmed cell death by natural killer cells, to enhance phagocytosis by dendritic cell subtypes, to leverage the contribution of specific T cell subtypes in resolving lesions, and to promote neoantigen presentation to lymphocytes.[112] A broad MDA-5 and RIG-I activation by RNA is, however, certainly not the goal, because this is known to promote proinflammatory signaling in ECs and macrophages[113] or osteogenic calcification in aortic VSMCs.[114] As of yet, too little is known about the cross-talk between innate[115] and adaptive immunity[116] in atherosclerosis. Therefore, it is open whether it would be beneficial to transfect synthetic noncoding (uncapped or circular) RNAs as therapeutic triggers of RIG-I [117,118] into specific protective immune cell types, or whether, oppositely, it is the reduction of endogenous RIG-I signaling that may bear protective effects.

Molecular Techniques for lncRNA-Based Therapy

In the following, we briefly describe different modern technologies to knockdown, overexpress, and study aberrantly

expressed or spliced lncRNAs or to change their sequence content for the purpose of therapy (►**Fig. 2**). On a general note, drug development in atherosclerosis often starts out with disease modeling in animals because not all relevant disease-initiating cell–cell interactions can be recapitulated in cell culture models.

Therapeutic Modulation of lncRNA Levels In Vivo

In the first place, identifying lncRNAs with potential therapeutic relevance necessitates finding animal orthologs of human disease-linked lncRNAs. In atherosclerosis, this can be straightforward (*Malat1*, *Miat*, *Rncr3*, *H19*), or more complicated (*ANRIL*[119]), because evolutionary selective constraint for the majority of lncRNA sequences is modest at most, and exon–intron structure changes accordingly faster than for protein-coding genes.[120] If the genomic structure of an atherosclerosis-relevant locus is overall conserved but yields noncoding RNA with only limited conservation, humanizing synthetic disease-linked regions in mice with relevant human lncRNAs through knock-ins may be a viable approach.

If mouse lncRNA orthologs exist, knockdown techniques can be directly executed, involving lncRNA depletion via the RNAi machinery (mostly in the cytoplasm), RNase-H1-type enzymes (also in the cell nucleus), or RNA-cutting ribozymes (►**Fig. 2B**). Conversely, lncRNA overexpression occurs from plasmids or viral vectors. For circRNA biogenesis from DNA vectors, in many studies, reverse complementary intronic repeats are routinely placed adjacent to circularizing exons, which support backsplicing through backfolding (see Refs. [9] and [10] for review). An interesting new concept are self-amplifying RNA "replicons," derived from disarmed, cytoplasmic, self-replicating RNA-alphaviruses, which can make RNA therapy permanent without the need for genome integration of RNA-generating vectors.[121] Another novel approach employs guide RNAs to target heterologous activator or repressor domains to lncRNA promoters, which causes up-/downregulation of transcription efficiency.[122]

Apart from expression via vectors, lncRNAs can also be locally provided to the target tissue, which is the arterial lesion-containing vessel wall, by transfection of *in vitro*-produced RNA molecules: One problem of this approach is that routine solid-phase chemical RNA synthesis is still size-limited (currently 100 nts). Thus, lncRNAs, due to their size, are often transcribed by T7 RNA polymerase *in vitro*. Also, RNA circularization is possible *in vitro*. Thereby, linear T7 transcripts are circularized, either chemically through artificial linkers (e.g., via phosphotriester or click chemistry) or enzymatically through 2′-5′ or 3′-5 backbone linkage (via T4 DNA/RNA ligases, tRNA ligases, or ribozymes such as group II intron derivatives) (see Ref. [123] for review). Recombinant expression in heterologous hosts (*Escherichia coli*, yeast) would allow higher linear or circular lncRNAs yield than achieved by *in vitro* transcription, but so far suffered from stability issues and heterogeneity of RNA ends. Novel unconventional bacterial hosts, such as the marine *Rhodovulum sulfidophilum*, circumvent some problems, as they secrete nucleic acids but do not contain RNases in their extracellular space.[124] Together, novel expression hosts, RNA affinity tags, and methods like exponential *in vitro* synthesis of RNA through polymerase chain transcription[125] allow producing sufficient amounts of high-quality RNA for therapy (see Ref. [126] for an overview).

Designing lncRNA Function by Modulating RNA Sequence

In addition to modifying lncRNA levels, a second therapeutic approach is to create designer lncRNAs, whose RNA sequence, structural motifs, or posttranscriptional modifications are purposely engineered. Enabling sequence modifications *in vivo*, the technical evolution of Cas enzymes has recently made a significant step forward. Instead of modifying DNA via the classical Cas9 enzymes, Cas13 nucleases were found to target RNA. In one application, Cas13 derivatives allow to purposefully destruct RNAs.[127,128] But Cas13 derivatives also serve to modify RNA sequence when fusing Cas13 to the ADAR2 enzyme: The latter confers adenosine deamination to inosine in a target RNA, with inosine being functionally equivalent to guanosine in translation and splicing (termed REPAIR tool in ►**Fig. 2B**).[128] Linking other RNA-modifying enzymes to Cas13 has potential to modify target RNAs in different ways.

A range of artificial chemical RNA modifications (both at bases and in the phosphodiester backbone) have been chemically introduced in synthetic therapeutic nucleic acids, and benefits for therapy have been determined, mostly from experience with ASOs. Some modifications improve resistance against nucleases, increase potency, or improve pharmacokinetic properties and cellular uptake (see Ref. [129] for review). Covalent modifications, as used in ASOs, can theoretically be applied equally to *in vitro* synthesized lncRNAs, such as links to the ribose 2′ position (2′-fluoro, 2′-O-methoxyethyl, or cEt-constrained 2′-O-Ethyl). But to date, chemically modified lncRNAs have not yet been used for therapeutic purposes, in part because any modifications may negatively affect interactions with proteins or client RNAs. On the other hand, insight into the roles of some endogenously occurring posttranscriptional chemical modifications of RNAs (both coding and noncoding) are emerging, paving the field of "epitranscriptomics," as allusion to the so important concept of epigenomic control. For example, methylation (m^5C, m^6A), pseudouridylation (Ψ), and editing (deamination of A-to-I), known since more than 50 years, have more recently been functionally related to stability (also) of noncoding RNA,[130] to the formation of higher-order structure necessary for contact with proteins,[131,132] to splicing,[133,134] to RNA backbone rigidity[135] and base-pairing features,[136] and functional recognition of microRNA binding sites.[137,138] But this knowledge has not yet been exploited for engineering lncRNA therapy.

Structure–Function Studies in Designer lncRNAs

In comparison to modulating RNA sequence and covalent modifications on RNA, designing RNA function through engineering secondary and tertiary RNA folds is even more

complex. Although several native RNAs have evolved to regulate proteins, engineering a protein-activity-regulating lncRNA by bioinformatically designing RNA structure *de novo* is not yet possible. However, exploring native RNA: protein complexes with new structural methods (e.g. cryoEM), and through chemical probing, now even possibly inside living cells (PARIS, SHAPE-MaP),[139] will help to control how lncRNAs bind, scaffold, and regulate their molecular targets.

Hand-in-hand goes the development of novel technologies for profiling the molecular detail of lncRNA interactions with chromatin factors in DNA complexes, a major role of lncRNAs. Techniques for this structure–function analysis at a higher level include ChIRP, CHART, RAP, and ChOP (see Ref. [140] for an overview). Insight from these technologies is central because knowing the chromosomal lncRNA targets in disease-relevant cell types is necessary to achieve specificity in lncRNA therapy.[127,128]

Potential Side Effects during Systemic lncRNA Therapy and Therapeutic Implications

If thoroughly studied, virtually every lncRNA becomes known to engage multiple effector mechanisms in cell type-specific manners. Consequently, side effects are likely in systemic and long-lasting therapy, as applying to atherosclerosis. For example, *MALAT1* levels drop in plaques,[55] and *Malat1* knockouts developed atherosclerosis,[81] suggesting that therapeutic upregulation of *MALAT1* might ameliorate disease. On the other hand, *MALAT1* is upregulated in cancer cells and is cancer-promoting.[84] Such dichotomy necessitates tools for conditional lncRNA delivery. Similar disastrous side effects and dangers regarding cancer development and metabolic syndrome apply to *circHIPK3* and *Gas5*.

To avoid these side effects, and also to counteract off-targeting, conditional delivery schemes are necessary: For guiding their expression in atherosclerotic lesions within the vascular wall, synthetic lncRNAs can be conjugated to plaque-homing peptides (e.g., Ac2–26/LyP-1),[141] antibodies or lipid/lipoprotein carriers (high-density lipoprotein),[142] or be packaged in lipid vesicles with targeting cues on their surface (e.g., CCR receptors). The most modern relevant approach for conditional expression in biomedicine is localized delivery through photo- and optoacoustic approaches.[143] An alternative is to elute RNA from coated stents or from perivascular hydrogels. Not last, lncRNA activity can, in principle, be controlled by laser light, when lncRNA are synthesized with optogenetically regulatable backbones or caging groups,[144] but optogenetic control of lncRNAs has not yet been performed in therapy *in vivo*, so far.

Conclusion

To date, around 70 clinical trials are known to center on RNA therapeutics, and these exclusively involve small interfering ASOs/siRNAs and therapeutic mRNAs, but not yet lncRNAs.[145] Despite the relatively slow translation of RNA-centered therapy into the clinics,[145] RNA therapeutics is gaining renewed interest, not last through novel insights into lncRNA biology. Further, the ease by which candidate lncRNA can be screened and optimized in their interaction with disease targets surpasses the work with classical small molecule drugs whose targeting to proteins is complex to predict, control, and modify. Any future therapy with lncRNAs will benefit from insight into RNA mass production, chemical modifications, and cellular delivery schemes developed for ASOs/siRNAs over the last decades. As many of the previously unknown cell subtypes that contribute to plaque growth currently become molecularly characterized by novel methods like mass spectrometric cytometry,[146–148] and as methods for RNA chromatin profiling at single-cell resolution from limited tissue sources emerge,[149] the vision of a highly specific lncRNA-centered therapy in atherosclerosis is materializing, possibly sooner than expected.

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Conflict of Interest
None declared.

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