The Function and Therapeutic Potential of Long Non-coding RNAs in Cardiovascular Development and Disease

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The popularization of genome-wide analyses and RNA sequencing led to the discovery that a large part of the human genome, while effectively transcribed, does not encode proteins. Long non-coding RNAs have emerged as critical regulators of gene expression in both normal and disease states. Studies of long non-coding RNAs expressed in the heart, in combination with gene association studies, revealed that these molecules are regulated during cardiovascular development and disease. Some long non-coding RNAs have been functionally implicated in cardiac pathophysiology and constitute potential therapeutic targets. Here, we review the current knowledge of the function of long non-coding RNAs in the cardiovascular system, with an emphasis on cardiovascular development and biology, focusing on hypertension, coronary artery disease, myocardial infarction, ischemia, and heart failure. We discuss potential therapeutic implications and the challenges of long non-coding RNA research, with directions for future research and translational focus.

New sequencing technologies, combined with bioinformatics and computational tools, have allowed the scientific community to appreciate the great complexity of the transcriptome.[1] In particular, the discovery of various types of non-protein coding RNAs (ncRNAs) and their different functions in regulating developmental and disease processes is expanding our knowledge of molecular biology and could significantly advance therapeutic options for many patients, including those suffering from cardiovascular disease. Thousands of ncRNAs have been described and classified into two large groups: small ncRNAs, which are up to 200 nucleotides long, and long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides.

lncRNAs are a heterogeneous group of transcripts exerting major regulatory roles in gene expression, and their importance in cardiovascular disease has been reinforced.[2,3] The dynamic expression and specific profiles of lncRNAs in different pathophysiological states suggest their functional relevance and potential to be used as non-invasive markers of disease and therapeutic targets.[4,5] However, establishing the biological actions of each lncRNA is proving more complex than investigating

microRNAs (miRNAs, the most popular class of small ncRNAs within the biomedical community). This is due to lncRNAs' multiple modalities of action and their low conservation among vertebrates.[6] Therefore, a large gap remains between the number of lncRNAs identified, and then listed in databases, and their functional characterization and implications in pathophysiological situations. A list of databases and their major characteristics, such as number of lncRNAs, species, and association with function and other genes, is given in Table 1.

So far, it has been demonstrated that lncRNAs can regulate gene expression through functional mechanisms including epigenetic, transcriptional, and post-transcriptional, either activating or suppressing gene expression. lncRNAs can also mediate signaling, such as phosphorylation, and trafficking of proteins.[7,8] One way to classify lncRNAs is according to their mechanism of action: signal, decoy, guide, scaffold,[9] enhancer, or sponge lncRNAs (particularly circular lncRNAs [circRNAs]) (Figure 1).[10,11] In this review, we do not address in detail the lncRNAs mechanisms of action but instead refer to reviews on the subject.[2,12–14]

A growing number of lncRNAs are implicated in cardiovascular development and disease, although it is not clearly understood how they participate in pathological processes. Their potential as therapeutic targets has often been raised, and there are a few examples of in vivo modulation of lncRNAs. However, modulating lncRNAs has been a challenging task to date.[15–19] Here, we summarize the current understanding of lncRNA function in cardiovascular pathophysiology and discuss its potential for therapy.

Function of lncRNAs in the Cardiovascular System
Cardiac Development and Biology
Transcriptomics profiling and loss-of-function approaches in progenitor and embryonic stem cells (ESCs) have demonstrated the

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Table 1. List of Available Databases on lncRNAs

Database	What	Species	No. of lncRNAs	Last Update	Association with Function	Association with Protein-Coding RNAs	Association with miRNAs	Reference
ANGIOGENES	in silico screening of protein-coding genes and lncRNAs in ECs	human, mouse, zebrafish	24,382 (15,149 in human)	2016	X			164
ChIPBase	transcriptional regulation of lncRNA from ChIP-seq data	10	10,200 ChIP-seq datasets	2016		X		165,166
deepBase	identification, annotation, and function prediction of lncRNAs from RNA-seq data	14	191,547	2016		X		167,168
GENCODE	manually curated human and mouse lncRNA reference based on ENCODE project	human, mouse	42,302	2016				169,170
LincSNP	annotated disease-associated SNPs in human lncRNAs	human	244,545	2016	X			171
LNCipedia	annotated lncRNA sequences, structures, protein coding potential, and miRNA binding sites	human	118,777	2016		X	X	172,173
lncRNAdb	curated reference database of functionally annotated eukaryotic lncRNAs	71	295 (183 in human)	2015	X			174,175
LncRNADisease	experimentally supported and predicted associations between lncRNAs and diseases	human	1,564	2015	X			176
lncRNome	annotated human lncRNAs	human	17,547	2013	X	X	X	177
NONCODE	integrated annotation of ncRNAs, especially lncRNAs	16	487,164 (167,150 in human)	2016	X	X		178

ChIP-seq, chromatin immunoprecipitation sequencing.

importance of lncRNAs for cardiac development and cell differentiation. More than 1,000 lncRNAs were reported as being dynamically regulated during differentiation,[20,21] and further transcriptome analyses of embryonic and adult-stage murine hearts identified several lncRNAs specific to tissue and developmental stage.[22,23]

Among biologically validated lncRNAs, several have been associated with cardiac development (Table 2). For example, Braveheart (Bvht) has a critical role in cardiac lineage commitment in mouse. It is abundantly expressed in embryonic stem cells and regulates the transition from nascent mesoderm to cardiac progenitor.[24] Bvht, by modulating the core cardiovascular gene network and mediating the epigenetic regulation of cardiac fate, is necessary to maintain cardiac commitment.[25] Conversely, the lateral mesoderm-specific lncRNA Fendrr (fetal-lethal non-coding developmental regulatory RNA) controls mesodermal differentiation, as well as heart and body wall development, by binding to the histone-remodeling polycomb repressive complex PRC2 and TrxG/MLL to modulate chromatin status.[26]

Numerous enhancer-associated lncRNAs have been implicated in cardiogenic differentiation,[21,27] among which the enhancer lncRNA Novlnc6 modulates expression of MKX2.5, a transcription factor critical for cardiac differentiation and maturation.[27] CARMEN (cardiac mesoderm enhancer-associated non-coding RNA) is also responsible

for cardiogenic specification and differentiation in precursor cells, possibly by regulating PRC2.[28] Furthermore, several lncRNAs regulate specific mRNA abundance during heart development, although as yet there is no clear understanding of their role during cardiogenic differentiation. For example, n411949 regulates Mccc1 mRNA, which metabolizes leucine, and n413445 modulates ReIb, which is involved in the nuclear factor κB (NF-κB) pathway.[22] Overall, fetal gene program reactivation constitutes a hallmark in multiple cardiovascular diseases. Although a moderate number of dynamically regulated lncRNAs in embryonic cells are equally regulated in the hypertrophic heart,[22] some lncRNAs associated with cardiac pathologies may also be implicated in cardiac development.[27] As for cell proliferation, a study identified eight lncRNAs putatively implicated in the proliferative capacity of cardiac cells in fetal heart[29] that require further investigation.

Cardiomyocyte repolarization during the final stage of the action potential needs potassium fluxes mediated by the $K_v7.1$ channels. In late embryogenesis, lncRNA Kcnq1ot1 (potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1) regulates the expression of the transcript Kcnq1, which encodes the potassium channel $K_v7.1$.[30] Such regulation fulfills the requirement for increased cardiac contractile activity in this late developmental stage. Dysregulation of KCNQ1OT1 expression has
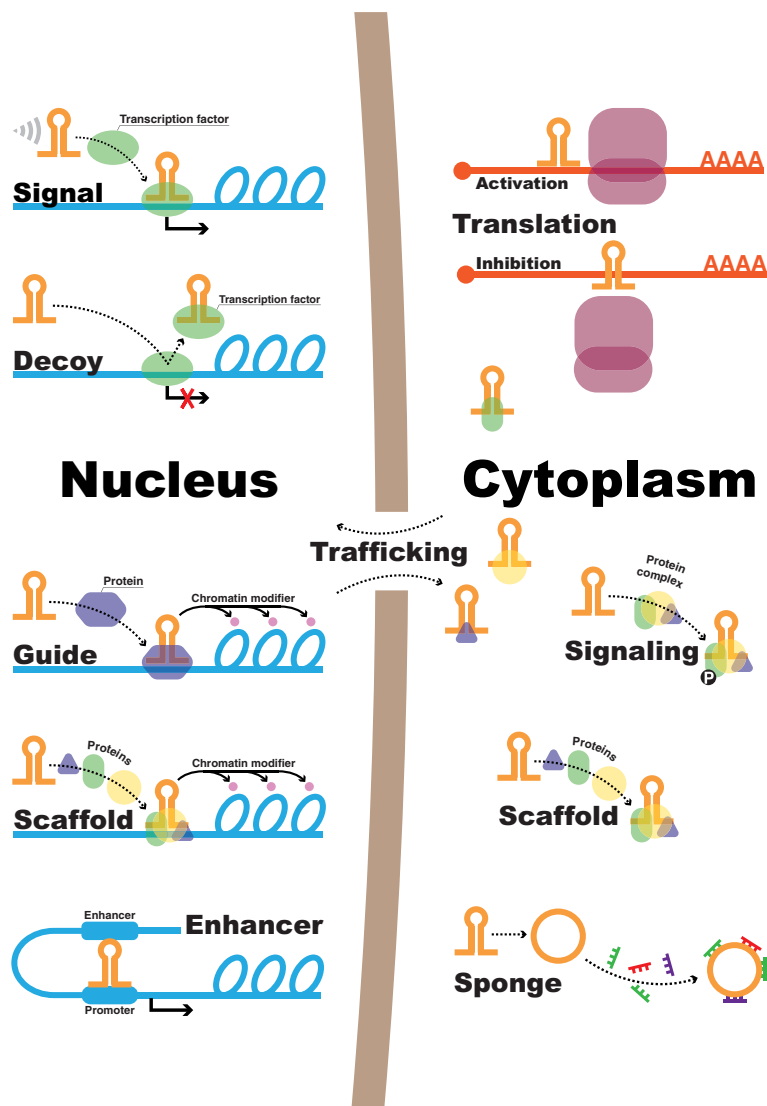
Figure 1. Classification of lncRNAs by Mechanism of Action

Signal lncRNAs respond to specific stimuli and thus show expression specific to cell type. Decoys bind transcription factors and other proteins away from their target site, repressing transcription. Guides interact with regulatory proteins, forming ribonucleoprotein complexes, and direct them to their target sites in subcellular locations. Scaffolds serve as platforms to bring different proteins together, both in the cytoplasm and in the nucleus, activating or repressing transcription. Enhancers are regulatory sequences in which transcription factors bind to initiate transcription; these regions of the genome produce several transcripts, enhancer lncRNAs, which act in *cis* to regulate expression of target genes. In the cytoplasm, lncRNAs can activate or inhibit translation by binding to target mRNAs. They can also regulate protein trafficking and signaling, such as phosphorylation. Sponging miRNAs is another way lncRNAs (including circular RNAs) regulate gene expression post-transcriptionally.

Vascular Development and Biology

Growing evidence describes lncRNAs as key molecular players of vascular and endothelial cell (EC) biology. *SENCR* (smooth muscle and EC-enriched migration/differentiation-associated lncRNA) was among the first lncRNAs to be identified in human vascular smooth muscle cells (VSMCs) and ECs, being involved in their differentiation.[37,38] The lncRNA *SMILR* (smooth muscle-induced lncRNA) appears to promote VSMC proliferation and may achieve this by regulating expression of adjacent transcripts, *HAS2* (hyaluronan synthase 2).[39] *HAS2* plays a role in proliferation in saphenous vein-derived VSMCs, in which small interfering RNA (siRNA) targeting of *HAS2* resulted in reduced proliferation ability.[40] *SMILR* could be a target for therapy of atherosclerosis because VSMC aberrant function is one of the defining features of atherosclerotic plaques (see Coronary Artery Disease and Atherosclerosis). *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) is one of the most abundant lncRNAs in mammalian cells, and it was observed in ECs. Due to its increased expression in hypoxia, *MALAT1* is proposed to have a role in vascularization, although knockout mice did not present severe developmental abnormalities.[17] *MALAT1* is involved in alternative splicing[41] and possibly regulates gene expression during vascular development and disease.[17]

In vitro differentiation of embryonic stem cells can recapitulate the development process, with transcriptome studies being employed to identify novel lncRNAs whose role is important for either commitment of cardiovascular progenitors or endothelial commitment.[38,42] A study that identified hundreds of novel ncRNA transcripts in the vascular setting functionally characterized the lncRNA *PUNISHER*,

been associated with left ventricular (LV) dysfunction after myocardial infarction (MI),[31] thereby potentially linking this lncRNA to cardiac contractility and arrhythmia in a clinical setting.

A switch in myosin heavy chain (MHC) isoforms accompanies the acquisition of the adult cardiac contractile phenotype. The expression of α-MHC in adult left and right ventricles is associated with higher filament sliding velocity, while the slower β-MHC confers higher force at lower energy cost.[32] In rodent models (which mainly express the α-MHC isoform in the normal adult stage), the intergenic region between the two genes has been shown to regulate the transition from β- to α-MHC during cardiac development through co-transcription of an antisense RNA, called *β-RNA*, targeting and inhibiting the myosin heavy chain 7 (*MYH7*) transcript (encoding the β-MHC isoform).[33,34] This mechanism is responsive to thyroid status and further implicated in the response to pressure overload.[35,36]

Table 2. lcnRNAs Associated with Cardiovascular Biology

lncRNA	Expression	Biological Context	Action	Genomic Localization	Organism	Reference
Differentiation and Cardiac Development						
Bvht	embryonic stem cells	cardiomyocyte differentiation	signal	intergenic	mouse	24,25
Fendrr	lateral plate mesoderm	development of heart and body wall	signal	intergenic	human, mouse, rat	26
Novlnc6	embryonic stem cells (particularly left ventricle), cardiomyocytes	cardiac differentiation and maturation	decoy		human, mouse	27
CARMEN	cardiac precursor cell	cardiomyocyte differentiation of cardiac precursor cells	enhancer	intergenic	human, mouse, rat	28
n411949	embryonic heart	cardiac development	unknown	antisense	mouse	22
n413445	embryonic heart	cardiac development	unknown	intronic	mouse	22
Contractile Function						
KCNQ1OT1		cardiac development	signal	antisense	human, mouse	30
β-RNA	adult ventricles	contractile phenotype, pressure overload	unknown	antisense	rat	33
Vascular Development						
SENCR	smooth muscle cells	maintenance of smooth muscle cells' differentiated state	decoy	antisense	human	37,38
SMILR	VSMCs	proliferation of smooth muscle cells	proposed scaffold or enhancer	intergenic	human	39
MALAT1	ECs	proliferation of ECs and vascularization	decoy	intergenic	human, mouse	17
PUNISHER	ECs	identity of ECs	guide	antisense	human, mouse, zebrafish	42

which is expressed in mature ECs.[42] Morpholino targeting of *PUNISHER* in zebrafish culminated in extensive vascular defects, including abnormal branching and vessel formation. *PUNISHER* silencing in human umbilical vein ECs (HUVECs) using short hairpin RNA (shRNA) revealed similar defects. Overall, *PUNISHER* compromises EC function, but its mechanism of action or its involvement in vascular pathologies have not yet been elucidated.[42]

lncRNAs in Cardiovascular Disease

The association between lncRNAs and cardiovascular disease is just coming to light with several reports about their specific expression in different cardiac diseases. Dysregulation of certain lncRNAs has been shown in both human and rodent models (Figure 2), in which some studies present encouraging results for disease prognosis and therapy. The biological context of lncRNAs discussed in this section is illustrated in Figure 3. However, because of their poor conservation across species, translation of animal findings to human applications should be approached with caution. Here we overview the role of lncRNAs in hypertension, coronary artery disease (CAD), MI, ischemia, and heart failure.

Some lncRNAs differentially expressed during cardiovascular development also participate in a pathological setting, while others are involved in more than one cardiovascular disease. *SENCR* and *H19*, for example, are widely implicated in cardiovascular disease. *SENCR*, besides playing a role in VSMC and EC differentiation during devel-

opment, has also been suggested to affect CAD. This lncRNA was found to be downregulated in VSMCs from a type 2 diabetes mellitus murine model, promoting proliferation and migration.[43] In addition, *SENCR* overexpression protected against the effects of high glucose stress on mouse VSMCs,[43] and its reduced expression has been associated with premature CAD in humans.[38]

H19 is an important regulator of mammalian development and disease in that it inhibits cell proliferation.[44] It is normally highly expressed during in utero development and downregulated at birth; however, studies reveal a re-expression of lncRNA *H19* in cardiovascular disease settings, although not all mechanisms and involved players have been described.[45–53] Human genome-wide association studies (GWASs) have demonstrated significant associations between H19 locus and systolic or mean arterial blood pressure.[54] High *H19* expression has been linked to hyperhomocysteinemia, a known risk factor for CAD,[45,51] and polymorphisms correlate with CAD risk.[47] Methylation at the IGF2/H19 locus have been implicated in regulation of glucose metabolism and development of diabetes,[46,53,55] renal development,[56] pre-eclampsia,[57] and aortic stenosis,[58] indicating possible links. Furthermore, *H19* was reported to sponge let-7 family miRNAs,[59] which are believed to have atheroprotective[60–63] or proatherosclerotic[64] roles and are downregulated in CAD patients.[65] In addition, *H19* was identified as differentially expressed in normoxic versus hypoxic ECs.[66] Finally, it is a precursor of miR-675, which inhibits cardiomyocyte hypertrophy[52] and contributes to cardiac
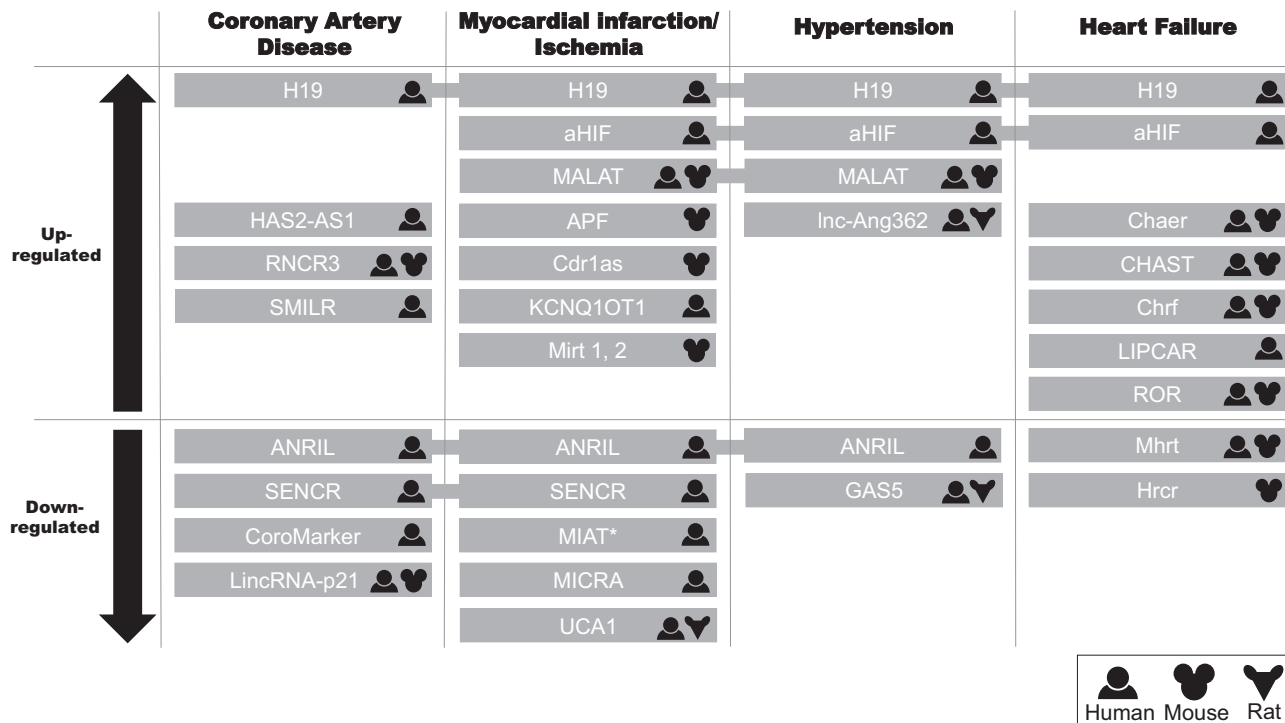
Figure 2. lncRNAs that Are Up- or Downregulated in Cardiovascular Diseases

The human, mouse, or rat symbol indicates in which organism the lncRNA has been described. *MIAT is downregulated in ST-elevation myocardial infarction (STEMI) patients compared to non-ST-elevation myocardial infarction (NSTEMI) patients; it correlates with hypertension, but there is no change in regulation.

fibroblast proliferation and fibrosis, acting through repression of *DUSP5/ERK1/2* (Figure 4).[67]

Hypertension

Hypertension has a complex etiology, involving an interplay of environmental and genetic components, and is a major risk factor for other cardiovascular diseases. Although many miRNAs have been shown to act in the pathogenesis of hypertension,[68] reports on lncRNAs that relate to hypertension are scarce. However, studies based on animal and cell models in this area are emerging. One lncRNA involved in hypertension and vascular remodeling is *GAS5* (growth arrest-specific 5).[69] *GAS5* was found to be downregulated in the plasma of human hypertensive patients and in the arteries and retina of a rat model, the spontaneously hypertensive rat. In these rats, *GAS5* knockdown exacerbated the hypertensive phenotype, arterial remodeling, and microvascular dysfunction.[69] In HUVECs and VSMCs, proliferation, migration, and resistance to oxidative stress were altered by *GAS5* siRNAs. Co-culture experiments suggested that *GAS5* participates in extracellular vesicle-mediated cross-talk between ECs and VSMCs.

A screening approach using angiotensin II (Ang II)-treated rat VSMCs identified differentially expressed lncRNAs, one of which, *lnc-Ang362*, is proximal to miR-221 and miR-222.[70] These two miRNAs, which regulate the proliferation of rat VSMCs[71] and the

migration of HUVECs,[72] were also upregulated in response to Ang II and appeared to be co-transcribed with the lncRNA. Consistently, *lnc-Ang362* siRNA knockdown decreased miR-221/222 expression and reduced VSMCs proliferation.[70] Thus, together with miR-221/222, *lnc-Ang362* may represent an interesting therapeutic target that deserves further investigation, because it is conserved in human.

Given the poor evolutionary conservation of many lncRNAs, any approach based solely on animal models may limit discovery. In a cohort of patients with acute MI, expression of four lncRNAs—*ANRIL* (antisense non-coding RNA in the INK4 locus), *aHIF* (hypoxia-inducible factor 1A antisense RNA 2), *MIAT* (MI-associated transcript), and *MALAT1*—was found to be significantly associated with hypertension.[31] Although their mechanisms of action in hypertension are not yet described, *ANRIL* affects cell adhesion, proliferation, and apoptosis,[73] and *MALAT1* modulates EC migration, sprouting, and proliferation.[17] A novel bioinformatics tool, LncDisease, has been used to predict four human lncRNAs associated with hypertension, three of which were validated as dysregulated in Ang II-treated human VSMCs.[74] Further investigation is required to firmly establish their validity in human hypertension.

CAD and Atherosclerosis

Atherosclerosis is typified by the formation of a fibro-fatty plaque in the arterial vessel wall and involves the molecular and functional
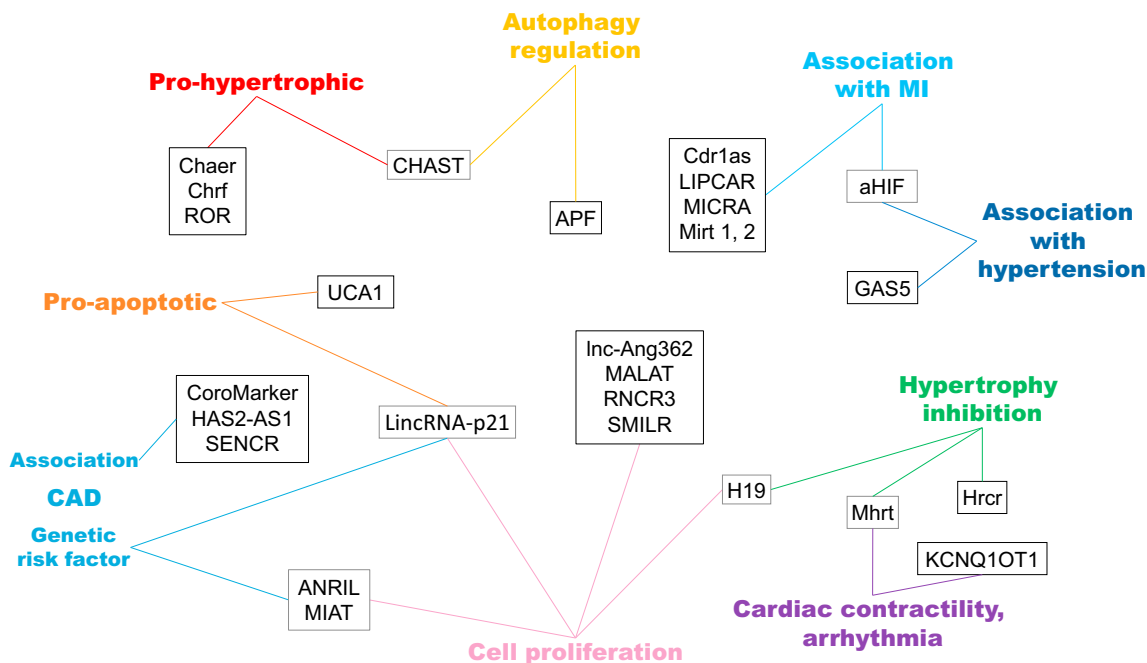
Figure 3. Biological Context of lncRNAs Associated with Cardiovascular Disease and Their Mechanism of Action
CAD, coronary artery disease; MI, myocardial infarction.

dysregulation of ECs, VSMCs, macrophages and other leukocytes, and platelets.[75] When atherosclerosis occurs in the epicardial vessels of the heart, it is referred to as CAD, which is a leading cause of death worldwide. Dysregulated expression of specific lncRNAs has been reported to contribute to CAD.

The lncRNA *RNCR3* (retinal non-coding RNA 3) expression is altered during atherosclerosis, being overexpressed in atherosclerotic VSMCs and ECs compared with non-atherosclerotic tissue in mouse and human.[76] Compared with control mice, downregulation of *RNCR3* with shRNA aggravated atherosclerosis in thoracic aorta tissue and increased inflammatory factors in plasma. In vitro treatment with oxidized low-density lipoprotein (ox-LDL) increased *RNCR3* levels in HUVECs and VSMCs, reducing proliferation and viability and increasing apoptosis. These data suggest that *RNCR3* is atheroprotective. Moreover, the same study demonstrated in vitro that exosomes derived from ECs are rich in *RNCR3*, which is transferred to VSMCs and induces their proliferation and migration. The proposed mechanism of action in ECs is that *RNCR3* regulates the transcription factor KLF2 by sponging miR-185-5p, which targets *KLF2*. Thus because of the atheroprotective role of *RNCR3* in atherosclerosis, its induced upregulation potentially represents a therapeutic intervention.[76]

LincRNA-p21 was identified as a transcriptional target of p53.[77] In VSMCs, this lncRNA disrupted the binding between p53 and its inhibitor, mouse double minute 2 (MDM2), with consequent effects on cell proliferation and apoptosis.[78] *LincRNA-p21* protected against

neointimal hyperplasia in the carotid artery injury mouse model and was downregulated in aortic atherosclerotic plaques and in coronary artery tissues from CAD patients.[78] Furthermore, polymorphisms in *lincRNA-p21* have been associated with CAD risk.[79] The therapeutic potential of *lncRNA-p21* in acute vascular injury is suggested by its regulatory role of cell proliferation and apoptosis in CAD.

Remodeling of the extracellular matrix and neointimal formation are additional key features of CAD. Deposition of hyaluronan, synthesized by *HAS2*, contributes to this process. Two lncRNAs independently regulate *HAS2*: *HAS2-AS1* (HAS2 antisense RNA 1) and *SMILR*. The antisense transcript *HAS2-AS1* was increased in atherectomy samples collected from severely diseased carotid arteries and appears to promote *HAS2* transcription in VSMCs in the presence of O-GlcNAcylation by altering chromatin configuration.[80] This may be particularly relevant to neointimal formation in diabetic patients. *SMILR*, which promotes VSMC proliferation, was significantly upregulated in VSMCs stimulated with interleukin 1α and platelet-derived growth factor compared to unstimulated cells. In addition, its levels in plasma correlated with the inflammatory marker C-reactive protein and were upregulated in human carotid artery atherosclerotic plaques compared with adjacent healthy tissue.[39]

Several other lncRNAs have been associated with inflammation,[81–88] including in the context of diabetes,[89–91] although direct links to CAD remain to be established. Downregulation of the lncRNA termed *CoroMarker*, originally identified as a biomarker of CAD,[92] has been shown to decrease pro-inflammatory cytokine secretion from
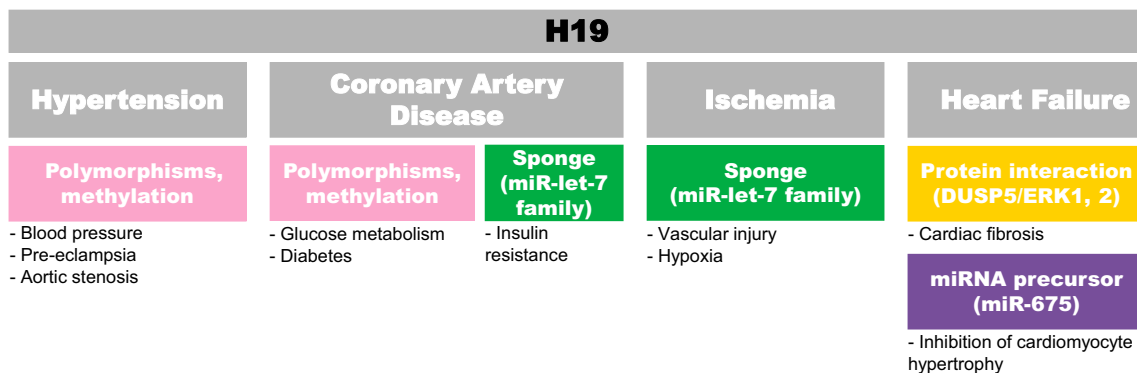
Figure 4. *H19* Is Associated with Hypertension, CAD, Atherosclerosis, Ischemia, and Heart Failure

Although mechanistic insights into the role of *H19* in cardiovascular disease are lacking, methylation regulation and sponging of miRNAs have been suggested and may overlap among diseases. Polymorphisms have been correlated with blood pressure and CAD. *H19* action as a sponge for the miRNA let-7 family has been linked to CAD and could be a possible mechanism in hypoxia. In heart failure, it acts by interacting with protein to regulate cardiac fibrosis and is a precursor of miR-675, which targets an inducer of hypertrophy.

THP-1 monocytic cells,[93] while *ANRIL* has been linked to the inflammatory response in ECs.[94] *CoroMarker*, for instance, is a prominent example of lncRNA as biomarker for CAD, because it correctly identified with high sensitivity most CAD patients in a large cohort.[92]

MI and Ischemia

Although advances in treatment and diagnosis of MI have increased patient survival and quality of life, MI is still a major cause of mortality and morbidity worldwide.[95] Several lncRNAs have been uncovered as being dysregulated in MI, and some may play roles in pathological angiogenesis and ischemic cardiac injury. Several GWASs have revealed an association between the INK4 locus and the risk of CAD, including MI. This locus is important for cell-cycle progression and cell growth and encodes tumor-suppressor genes *CDKN2A* and *CDKN2B* and the lncRNA *ANRIL*.[96–98] Several linear and circular isoforms of *ANRIL* have been identified, and their expression differs according to specific tissues and disease conditions.[99,100] Despite contradictions in the literature regarding its mechanisms of action,[73,101–105] there is a consensus that *ANRIL* affects cell proliferation.[73,101–103,106,107] Moreover, increased blood circulating *ANRIL* expression was associated with LV dysfunction.[31] The same study identified the lncRNAs *MIAT* and *MALAT1* as similarly associated.[31] A large-scale GWAS identified *MIAT* and six variants in its locus that confer susceptibility to MI. One variant was associated with upregulation of *MIAT* expression.[108] So far, the molecular mechanisms by which this lncRNA regulates MI remain largely unknown, although it may be involved in alternative splicing in the diseased heart.[109]

In a mouse model of MI induced by coronary ligation, several lncRNAs were dysregulated in the heart, among which the two most strongly upregulated were named *Mirt1* and *Mirt2* (myocardial infarction-associated transcript 1 and 2).[110] Their levels peaked 24 hr after MI and returned to baseline after 2 days, indicating that lncRNAs may be dynamically regulated in pathological processes. *Mirt1* and *Mirt2* levels correlated with expression of genes involved in reversing LV remodeling and preserved ejection fraction, suggesting a protective role in LV function. This study evidenced a therapeutic potential of these two lncRNAs. However, no human homologs of *Mirt1* and *Mirt2* have been described so far.

In addition to their potential as therapeutic targets, lncRNAs could serve as clinical biomarkers. One of the first studies to provide evidence of the feasibility of using lncRNAs as biomarkers for cardiovascular disease identified and validated *LIPCAR* (long intergenic non-coding RNA predicting cardiac remodeling) in a large number of patients.[111] *LIPCAR* levels were increased in plasma samples from patients with LV remodeling after acute MI[111] and in patients with type 2 diabetes mellitus.[112] Circulating *LIPCAR* may also have prognostic value because its levels correlated with higher mortality risk in patients with heart failure. *LIPCAR*'s therapeutic potential remains to be investigated.

Myocardial infarction-associated circular RNA (*MICRA*) was the first circRNA to be identified as a potential biomarker of LV dysfunction after MI, with its predictive value confirmed in two independent cohorts.[113] Therapeutic applications for this circRNA are yet to be discovered. A few circRNAs with relevance to cardiovascular disease have been unveiled and reviewed.[114–117] For example, *Cdr1as* (cerebellar degeneration-related protein 1 antisense transcript) was uncovered as a miR-7a sponge in cardiomyocytes, showing upregulation in MI mice and cardiomyocytes under hypoxia.[118]

The lncRNA *UCA1* (urothelial carcinoma-associated 1), proposed as a biomarker as well, presented altered expression in MI patients.[119] In rats with ischemia and reperfusion (I/R)-induced heart injury, another study found that *UCA1* contributes to cardiac injury by enhancing apoptosis of cardiomyocytes.[120] However, its application as a biomarker seems limited, because it performed worse than classical markers of MI (e.g., creatine kinase).[119] Nonetheless, because it has a role in I/R injury, future studies could reveal a therapeutic

potential to UCA1. Another lncRNA playing a role in the heart response to ischemia is *HIF1A-AS2*, also known as *aHIF*. It destabilizes the mRNA producing the hypoxia-inducible factor 1-α (HIF1α), which is considered the master transcriptional regulator of cellular response to hypoxia, including post-ischemic angiogenesis.[121] Besides being overexpressed in the failing heart,[121] *aHIF* was found to be dysregulated in the blood of patients after MI.[31]

Although a certain level of autophagy has been shown to be cardioprotective in ischemia,[122] reports suggest that the accumulation of autophagosomes can trigger cardiomyocyte death, particularly during post-ischemic reperfusion.[123,124] The lncRNA *APF* (autophagy-promoting factor) participates in the regulation of autophagy and MI in mice.[125] *APF* is increased during I/R injury and sequesters miR-188-3p, resulting in an upregulation of the miR-188-3p target gene *ATG7*, a promoter of autophagy. *APF* is important in determining myocardial I/R injury. Because inhibition of autophagy can be protective in the setting of MI, both *APF* and miR-188-3p represent potential targets for therapy.[125]

Several studies have identified lncRNAs involved in limb ischemia. Inhibition of *MALAT1* using GapmeRs led to worse outcomes following experimental hindlimb ischemia. This suggested the possibility that *MALAT1* plays a reparative, proangiogenic role.[17] Moreover, *SENCR* was found to be reduced in human critical limb ischemia muscles.[38] Several additional lncRNAs, including *H19*, were identified as differentially expressed in normoxic versus hypoxic ECs, followed by expressional and functional validation in the mouse limb ischemia model.[66]

Heart Failure

Heart failure is a complex condition of declined cardiac function in response to various pathophysiological stresses that cause cardiac remodeling, characterized by maladaptive hypertrophy. Maladaptive remodeling in the failing heart is considered an important target for therapy, and several lncRNAs are implicated in this process.[126,127]

CHRF (cardiac hypertrophy-related factor), which is conserved between humans and mice, stimulates cardiac hypertrophy and was the first lncRNA reported to have implications in heart failure.[126] It acts as a sponge to miR-489, hence upregulating its downstream target, *MYD88,* which is a key gene in activating cardiac hypertrophy.[126] This was observed in cardiomyocytes of mice with pressure overload-induced cardiac hypertrophy, but the authors also reported a natural overexpression of *CHRF* in human heart failure tissue, emphasizing that this transcript may have a similar function in human.[126]

The heart-enriched lncRNA *Chaer* (cardiac hypertrophy-associated epigenetic regulator) is also required for cardiac hypertrophy and is functionally conserved between mouse and human.[128] By interacting with PRC2, *Chaer* inhibits histone lysine methylation at the promoter regions of pro-hypertrophic genes, thus allowing their expression.[128]

Similarly, *CHAST* (cardiac hypertrophy-associated transcript) plays a role in promoting hypertrophy and is functionally conserved.[15] *CHAST* levels were endogenously increased during cardiac hypertrophy in mice and in hypertrophic heart tissue from patients with aortic stenosis, a cause of cardiac hypertrophy and fibrosis. Induced overexpression of *Chast* in mice led to cardiomyocyte hypertrophy, while its suppression attenuated remodeling and hypertrophy without signs of toxicity.[15] This is one of the most prominent examples of the strong therapeutic potential of lncRNAs for cardiac remodeling, showing that manipulation of a specific lncRNA can improve cardiac function.

A study identified in mice a cluster of cardiac-specific lncRNAs termed *Mhrt* (myosin heavy-chain-associated RNA transcripts) that are transcribed from the Myh7 locus, which is critical for cardiac contraction.[129] Besides being transcribed from the same locus as *β-RNA* (antisense lncRNA involved in cardiac development), the transcripts have different sequences. Although highly expressed in adult hearts, *Mhrt* transcripts were suppressed during pathological stress. Induced restoration of *Mhrt* levels prevented cardiac hypertrophy and failure, revealing their cardio-protective role. *Mhrt* acts as a decoy to inhibit the aberrant expression of pathogenic genes involved in cardiac contractility, thus maintaining cardiac function. Finally, the authors found that the human version of *MHRT* was repressed in different cardiomyopathies (hypertrophic, ischemic, or idiopathic), indicating a conserved mechanism.[129] This study provides further evidence in support of lncRNAs as potential therapeutic targets, for which development of a related therapy is facilitated by the conserved epigenetic regulation in human and mouse.

It has been uncovered that the lncRNA *ROR* (regulator of reprogramming) plays a role in cardiac hypertrophy. This transcript was naturally overexpressed in murine hypertrophic heart and cardiomyocytes, and its knockdown with siRNA attenuated hypertrophy. *ROR* enhanced cardiac hypertrophy by interacting with miR-133, a muscle-enriched miRNA that plays a role in hypertrophy.[130] Both RNA molecules could be investigated as anti-hypertrophic therapeutic targets.

The circRNA *Hrcr* (heart-related circular RNA) has a protective role in cardiac hypertrophy and heart failure in mice. It acts as an endogenous sponge for miR-223, thus upregulating the expression of ARC (apoptosis repressor with caspase recruitment domain).[117] ARC protein is normally highly expressed in the heart and is involved in cardiomyocyte hypertrophy and apoptosis.[131,132] Overexpression of miR-223 in mice using adenovirus-induced cardiac hypertrophy, and levels of *Hrcr* were downregulated in failing mouse hearts, indicating that *Hrcr* might constitute another target to treat heart failure if the mechanism is conserved in humans.[117]

Therapeutic Applications of lncRNAs

Although lncRNAs offer a multitude of prospective targets due to the diversity of actions and cellular processes implicated, few practical examples of therapeutic applications of lncRNAs have been reported so far. The up- or downregulation of specific lncRNA abundance have

been the most thoroughly investigated approaches. Strategies for up-regulation of lncRNAs include the use of recombinant adeno- or lentiviruses. Adeno-associated viral (AAV) vectors may represent a more promising approach due to their low pathogenicity.[133–136] This strategy used for targeting miRNAs in preclinical models showed promising results[137–139] and reached successful clinical trials to deliver protein coding genes,[133,140,141] but its use to deliver lncRNAs remains to be determined.

lncRNA downregulation can be obtained using shRNA or siRNA, more suitable for cytoplasmic lncRNAs,[142] antisense oligonucleotides (ASOs)-mediated knockdown using aptamers,[143] or GapmeRs forming heteroduplexes with their target lncRNAs that are then recognized and cleaved by the RNase H.[144] The latter application, more suitable for nuclear lncRNAs and with fewer off-target effects than shRNA,[145] is already undergoing testing in cell and animal models,[15,17,18] while ASO and siRNA have been used to deplete *MALAT1* in human cancer cells and animal models, reducing metastasis.[146–148] Hopefully, it will be possible to transfer some findings in other diseases to help accelerate the development of therapies for cardiovascular disease. Ribozymes or deoxyribozymes, catalyzing the cleavage of the flanked region of the RNA target, represent an additional tool to knock down lncRNAs.[149,150] Finally, small molecules that compete with ligands to bind lncRNAs or induce conformational change in lncRNAs are being identified through large screening efforts.[151]

The most promising lncRNA targets for therapeutic applications in cardiovascular disease are those for which mechanism of action and effect are well described and preferably cell specific. One example is *CHAST*, for which the GapmeR-mediated silencing attenuated transverse aortic constriction-induced cardiac remodeling in mice.[15] No apparent side effects were observed due to treatment with GapmeRs. Future experiments will determine whether cardiac hypertrophic remodeling may similarly be targeted through the downregulation of *CHRF*, which regulates Myd88, a factor associated with hypertrophy development,[126] or by restoring expression levels of *MHRT*, thus preventing cardiomyopathy by restricting stress-associated aberrant gene expression mediated by the chromatin-remodeling factor, Brg1.[129] Other approaches to decrease cardiac cell death following MI could target apoptotic or autophagic processes by downregulating *APF*.[125] In vascular disease, prevention of *MALAT1* upregulation could be used as anti-angiogenic therapy to prevent diabetes-associated microvascular complications.[17,90,91]

Challenges and Next Steps

As interest in the role of lncRNAs increases and technology to detect them becomes more sophisticated, widespread use of RNA sequencing (RNA-seq) screens has identified promising candidates for therapy. lncRNAs potentially represent a powerful tool for personalized medicine due to their specific expression patterns associated with distinct pathologies. The detection of lncRNAs in circulating exosomes[152] opened interesting perspectives both scientifically, in terms of signaling regulation and intercellular communication, and for further translational applications to diagnostics.

Several limitations and challenges remain to be resolved before lncRNAs can reach clinical application. Foremost is target specificity, given the pleiotropic implications of a single lncRNA in pathophysiological processes throughout the human body. Although lncRNAs may show dysregulation specific to certain diseases, they exhibit various functions in the organism and some lncRNAs may act through more than one mechanism. As an example, modulation of *ANRIL*, for which SNPs are associated with CAD,[106,153] is probably hazardous given its implication in cancer development and progression.[154,155]

Second, the low conservation of lncRNAs across evolution[156] makes both the identification of human lncRNAs and their clinical testing real challenges, because rodents may not be an adequate model. The hurdles to translate animal findings to human are illustrated by *Mirt1* and *Mirt2*, which may have a protective role in LV function, but no homologs in human have been described so far.[110] However, it has been suggested that it is the secondary structure of lncRNAs that is conserved and functional, rather than the primary sequence. For example, *GAS5* acting as decoy or signal may depend on its secondary structures and their affinities for different ligands.[157,158] This may explain why some lncRNAs with important mechanistic roles have not been observed in other species. If structure is more critical to function than sequence, then lncRNAs previously considered non-conserved may have structural homologs in other species, which would enable the use of existing animal models.

Third, before therapeutic application, the structure-function relationship of each lncRNA must be further elucidated, using newly developed methods to resolve secondary and tertiary structures.[24,159,160] Finally, treatments must ensure the tissue-specific delivery of the ASOs or pharmacological agents, as well as their penetration into the intracellular compartment of interest. The translation of lncRNA-based therapy into clinical applications should also consider challenges such as route of delivery, low immunogenicity of the delivery system, stability of RNA drug in both circulation and cells, duration of treatment, dosage adjustment, and off-target effects.

Future work is required for a thorough functional characterization of lncRNAs in cardiovascular pathology, both at the molecular and at the cellular level. The role of lncRNAs as epigenetic regulators is critical for gene regulation and disease pathogenesis, yet the fine molecular mechanisms involved remain to be fully elucidated. Although several RNA-seq experiments have been conducted and many potential candidates have been identified,[27,66,83,152,161] few have been sufficiently characterized, either in terms of regulation of the disease or in the ability to be externally regulated. Therefore, much remains to be done to retrieve the most promising candidates for therapeutic development from the huge amount of sequencing data available.

The involvement of circRNAs in cardiovascular pathologies has emerged.[113,117,162] Although appealing due to their resistance to degradation by exoribonucleases, their use as therapeutic targets requires further investigation. Considering the number of previously

characterized circRNAs and the plethora of circRNAs that remain to be characterized, this new branch of the ncRNA family constitutes an invaluable reservoir of therapeutic targets and may be useful to move theranostics a step forward, because they may be used for both diagnostics (biomarkers) and therapeutic purposes. Finally, gene editing with the CRISPR system appeared as an appealing approach for therapy, and a study reported an efficient downregulation of *MALAT1* with this system,[163] representing a potential tool for therapeutic applications in cardiac disease through modulation of the expression of lncRNAs.

CONSORTIA

The authors Clarissa P.C. Gomes, Helen Spencer, Kerrie L. Ford, Y.M. Michel, Andrew H. Baker, Constanza Emanueli, Jean-Luc Balligrand, and Yvan Devaux are writing on behalf of the Cardiolinc network.

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