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REVIEW

Long Non-Coding RNAs as Key Regulators of Cardiovascular Diseases

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Protein-coding genes account for less than 2% of the whole genome. However, the advances in RNA sequencing and genome-wide analysis have demonstrated that most of the genome is capable of being transcribed. Moreover, recent studies have suggested that long non-coding RNAs (lncRNAs) are critical regulators of gene expression and epigenesis in both physiological and disease states. Several lncRNAs are functionally involved in cardiovascular diseases and may be potential therapeutic targets. Here, we review the current strategies for the discovery of functional lncRNAs and recently discovered lncRNAs in the cardiovascular field, focusing on cardiac development, hypertrophy, heart failure, and atherosclerosis. We also discuss the therapeutic potentials of synthetic RNAs to modulate these lncRNAs and future directions in this research field.

Key Words: Atherosclerosis; Cardiac hypertrophy; Heart failure; Non-coding RNAs

Recent developments in RNA-seq methods in conjunction with bioinformatics have enabled characterization of all RNA transcripts.[1] The discovery of various types of non-protein-coding RNAs (ncRNAs) has expanded our knowledge of molecular biology. Thousands of ncRNAs have been classified into 2 groups depending on their length: small ncRNAs up to 200 nucleotides long, and long non-coding RNAs (lncRNAs) longer than 200 nucleotides. The functions of microRNAs (miRNAs), which are the most numerous class of small ncRNAs, are well documented.[2–4] However, establishing the biological actions of each lncRNA is still challenging because of their low conservation among vertebrates, variety of structures, and multiple modalities of action. Therefore, there still remain a large number of lncRNAs with unknown functions.

Recently, the FANTOM5 project identified 27,919 human lncRNA genes and their expression profiles across 1,829 samples from major human primary cells and tissues.[5] Moreover, they suggested that almost 70% of these lncRNAs are potentially functional. A selected subset of lncRNAs will be functionally characterized in more detail using other complementary technologies in the FANTOM6 project (http://fantom.gsc.riken.jp/6/). A list of ncRNA databases currently available is shown in **Table 1**.

Strategies for the Identification of Functional lncRNAs

Genome-wide transcriptomic approaches are commonly used for screening for lncRNAs. Recently, computational approaches have also been utilized for the identification of lncRNAs from RNA-seq data because of improvements in lncRNA annotations. Although RNA-seq is the most commonly used technique, multiple microarrays that contain probes for coding- and non-coding transcripts have been produced. The use of arrays has an advantage of providing rapid and efficient analysis; however, RNA-seq is superior for detecting low-abundance transcripts.

Validation of the transcripts is the next step for functional investigations. Quantitative PCR is utilized for validation of the expression levels of candidate ncRNAs. Consequently, the rapid amplification of cDNA ends (RACE) technique is commonly used to identify the full sequences of lncRNAs. In addition, determination of the localization of lncRNAs of interest is important to estimate their potential biological actions. RNA fluorescent in situ hybridization (RNA FISH) may provide precise information on the subcellular localization of lncRNAs.

It is also important to test the coding potential of lncRNAs, because some transcripts currently annotated as lncRNAs may encode small proteins despite the annotation efforts. For example, the *Dworf* RNA transcript was annotated as lncRNA; however, it was later proven to encode a peptide of 34 amino acids.[6] The use of bioinformatics tools or in vitro transcription and translation assays enables the identification of possible peptide production from lncRNAs.[7–9]

The functions of lncRNAs range broadly from guiding chromatin-modifying factors to genomic targets to providing ribonuclear protein complexes with a scaffold for support. Therefore, several techniques have been developed to identify lncRNA interactions with the genome, proteins, and RNAs.

To determine the lncRNA binding sites in the genome, chromatin isolation by RNA purification (ChIRP) was first developed by Chu et al.[10] This method uses antisense

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Table 1. List of Available Databases of lncRNAs

Database	Description	No. of lncRNAs in humans	No. of lncRNAs in mice	Last update
LNCipedia	Integrated database of human lncRNAs Secondary structure information, protein-coding potential and microRNA binding sites are also available	120,353	0	2017
LncRNAdb	Database providing annotations of eukaryotic lncRNAs	65	184	2015
GENCODE	High-quality reference gene annotations and experimental validation for human and mouse genomes	15,778	12,374	2017
NONCODE	Integrated knowledge database dedicated to ncRNAs in 17 species	172,216	131,697	2017
FANTOM CAT	Comprehensive atlas of 27,919 human lncRNA genes with high-confidence 5'-ends	27,919	0	2017

lncRNAs, long non-coding RNAs.

Table 2. Summary of lncRNA Analysis

Stage	Technique	Reference
Identification of existence	RNA-seq, microarray, Cap-assisted gene expression sequencing, and nuclear run-on assay	5
Validation	Quantitative PCR, RACE, RNA FISH, and databases	21, 23
Assessment of the coding potential	Bioinformatic tool, in vitro transcription assays and mass spectrometry	6–9
Mapping of binding sites	ChIRP, Chart, and RAP	10–12
Identification of lncRNA-bound proteome	ChIRP-MS and Chart-MS	13, 14
RNA-RNA interaction	CLASH	15
Analysis of RNA structure	SHAPE	16
Functional assay in vivo	Genetic knockout, promoter insertion, PolyA insertion, RNAi, CRISPR repressor, promoter knockin, and transgenic	17–19

Chart-MS, capture hybridization analysis of RNA target-mass spectrometry; ChIRP, chromatin isolation by RNA purification; CLASH, cross-linking, ligation, and sequencing of hybrids; CRISPR, clustered regularly interspaced short palindromic repeat; FISH, fluorescent in situ hybridization; lncRNAs, long non-coding RNAs; PCR, polymerase chain reaction; RAP, RNA antisense purification; RNA, ribonucleic acid; SHAPE, 2'-hydroxyl acylation and primer extension.

DNA oligonucleotides to capture and purify the lncRNA-chromatin complexes. Two other similar approaches have also been reported: capture hybridization analysis of RNA target (Chart)[11] and RNA antisense purification (RAP).[12]

To identify the lncRNA-bound proteome, ChIRP-like methods such as ChIRP-mass spectrometry (MS) and Chart-MS are being utilized.[13,14] RNA-RNA interactions can also be assessed by ChIRP; however, it does not differentiate direct RNA-RNA hybridization from interactions with intermediate proteins. In contrast, cross-linking, ligation, and sequencing of hybrids (CLASH) is used to detect only direct base-pairing between RNA molecules.[15]

The unique secondary and tertiary structures of each lncRNA contributes to its biological function. Thus, several techniques have been developed to elucidate RNA structures. RNA-selective 2'-hydroxyl acylation and primer extension (SHAPE) can identify bases that are in a flexible and probably single-stranded conformation.[16]

Finally, gene knockout or modification strategies are required to determine the physiological functions and the contribution to diseases of lncRNAs in vivo. The recent development of clustered regularly interspaced short palindromic repeat (CRISPR) technology has created a versatile platform for the generation of loss-of-function models.[17] A modified catalytically inactive Cas9 can inhibit gene transcription without modifying genome elements.[18] A similar approach can be used to activate transcription by fusing multiple copies of a transcriptional activator domain to Cas9.[19] A summary of lncRNA analysis methods is shown in **Table 2**.

Functional lncRNAs in Cardiac Development

Loss-of-function studies have been applied to progenitors and embryonic stem (ES) cells to reveal the functions of lncRNAs in cardiac development and differentiation. It has been proven that knockdown of lncRNAs has major effects on gene expression patterns, and causes either exit from the pluripotent state or upregulation of lineage commitment programs.[20]

Through the use of multiple ES cell differentiation techniques, an lncRNA named Braveheart (*Bvht*) was found to be necessary for the activation of a core gene-regulatory system that included key cardiac transcription factors (e.g., MesP1, Gata4, Hand1, Hand2, Nkx2.5, and Tbx5) and epithelial-to-mesenchymal transition genes (e.g., Snai and Twist).[21] There was a significant overlap of the genes regulated by MESP1 and *Bvht*. It was also revealed that *Bvht* interacts with SUZ12, a core component of the polycomb-repressive complex 2 (PRC2), suggesting that this interaction may be critical for the epigenetic regulation of the cardiac gene-regulatory network. Of note, *Bvht*-deficient cells showed similar morphology on neuronal differentiation by treatment with retinoic acid. Further experiments determined the secondary structure of *Bvht* using chemical probing methods and showed that the

Table 3. Features of eRNA, lncRNA, and mRNA

Feature	eRNA	lncRNA	mRNA
Number	~40,000–65,000	Several to tens of thousands	~23,000
RNA polymerase II	Yes	Yes	Yes
Splicing	Rare	Common	Yes
Polyadenylation	Some	Mostly	Mostly
Stability	Low	Low to medium	High
Conservation	Low	Medium to high	High
Tissue specificity	Extremely high	High	Low
Subcellular enrichment	Nuclear and chromatin bound	Nuclear and chromatin bound and cytoplasmic	Mostly cytoplasmic
H3K4 me1	High	Medium	Low
H3K4 me3	Low	Medium	High
H3K36 me3	No	Yes	Yes/high
H3K27ac	High	High	High

eRNA, enhancer RNA; lncRNA, long non-coding RNA.

~590-nucleotide transcript of *Bvht* has the potential to interact with cellular nucleic acid binding protein (CNBP/ZNF9), a zinc-finger protein known to bind single-stranded G-rich sequences. This work first determined the role of RNA structure in regulating cardiovascular lineage commitment.[22]

In a similar way, the lateral mesoderm-specific lncRNA *Fendrr* was found to be essential for proper heart and body wall development in mice. Embryos without *Fendrr* displayed upregulation of several transcription factors controlling lateral plate or cardiac mesoderm differentiation. This was accompanied by a drastic reduction in PRC2 occupancy at their promoters. *Fendrr* binds to both the PRC2 and Trithorax group/MLL protein complexes (TrxG/MLL), suggesting that it acts as a chromatin signatures modulator that defines cardiac gene activity.[23]

Enhancers are a specific class of regulatory modules, which are located far from the transcriptional start sites of their target genes. Their function involves both direct and indirect promotion of transcription at target gene promoters. Enhancers strengthen transcriptional initiation and elongation by direct interaction with the basal transcriptional machinery and indirect interaction with the local chromatin environment at target gene promoters.[24] Recent studies have indicated that most of the active enhancers consistently generate ncRNAs and that they are functionally required for enhancer activity.[25–27] Thus, enhancer RNAs (eRNAs) are known to be implicated in specific gene expression. The features of and differences between eRNAs, lncRNAs, and mRNAs are summarized in **Table 3**. Through a systematic bioinformatic analysis, a catalog of eRNAs with active cardiac enhancer sequences that are expressed during ES cell differentiation into cardiomyocytes has been generated.[28] In this experiment, knockdown of 2 eRNAs resulted in the specific downregulation of their predicted target genes. Another lncRNA associated with active cardiac enhancers and super-enhancers, cardiac mesoderm enhancer-associated non-coding RNA (*CARMEN*), is also responsible for cardiac development and differentiation.[29]

Functional lncRNAs in Cardiac Hypertrophy

Cardiac hypertrophy is an initially adaptive response to various stresses, including pressure or volume overload, and reduces the increased wall tension and helps to maintain cardiac output. Thus, the adaptive process is beneficial and can initially improve cardiac function; however, persistent exposure of the heart to increased work load can lead to the impaired blood flow, resulting in relative hypoxia and a subsequent loss of cardiomyocytes, ultimately resulting in heart failure (HF). With the utilization of high-throughput RNA sequencing, many studies have already been carried out to investigate the role of lncRNAs in cardiac hypertrophy.

Actually, transcriptomic analysis of pressure-overload-induced failing hearts in mice revealed almost 150 lncRNAs that are significantly dysregulated.[30] One of them was a heart-enriched lncRNA, named cardiac-hypertrophy-associated epigenetic regulator (*Chaer*), which was necessary for the development of cardiac hypertrophy. Mechanistically, *Chaer* directly binds with the catalytic subunit of PRC2. Thus, *Chaer* interferes with PRC2 targeting of genomic loci, which results in the inhibition of histone H3 lysine 27 methylation in the promoter regions of cardiac hypertrophy-related genes.[31]

Myosin heavy-chain-associated RNA transcripts (*Myheart* or *Mhrt*), are cardiac-specific, abundant ncRNAs in adult hearts that consist of a cluster of RNAs of 709–1,147 nucleotides in length (*Mhrt* RNAs).[32] *Mhrt* antagonizes the function of Brg1, which is a chromatin-remodeling factor activated by various stress to trigger abnormal gene expression and cardiomyopathies.[33] *Mhrt* prevents Brg1 from recognizing its genomic DNA targets by binding to the helicase domain of Brg1, a domain that is crucial for tethering Brg1 to chromatinized DNA targets. Thus, a *Mhrt*-Brg1 feedback circuit is crucial for cardiac function, and *Mhrt* is a cardioprotective lncRNA.

Cardiac hypertrophy-associated transcript (*Chast*) was identified by global lncRNA expression profiling during pressure overload-induced cardiac hypertrophy in mice.[34] Mechanistically, *Chast* negatively regulated Pleckstrin homology domain-containing protein family M member 1 (opposite strand to *Chast*), preventing autophagy of cardiac cells and enhancing cardiac hypertrophy. In addition, transcription of a *CHAST* homolog in humans was significantly enhanced in samples of hypertrophic hearts of patients with aortic stenosis.

In contrast to the chromatin- or gene-regulatory effect

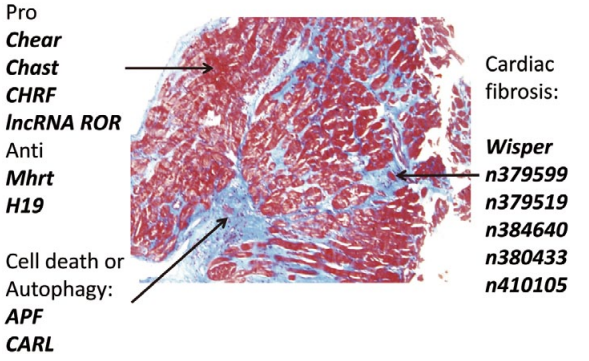

Figure 1. Long non-coding RNAs in the heart.

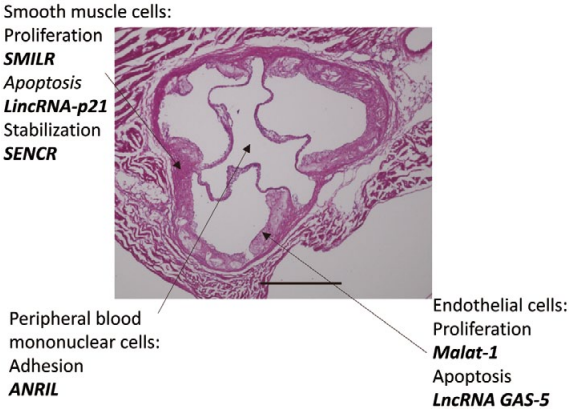

Figure 2. Long non-coding RNAs in atherosclerosis.

of other lncRNAs, there are several lncRNAs that inhibit the functions of the miRNAs that affect cardiac hypertrophy. Cardiac hypertrophy-related factor (*CHRF*) was found to serve as a sponge for miRNA-489.[35] *CHRF* was found to be upregulated in hypertrophic hearts in both mice and human HF samples. *CHRF* downregulates miR-489 expression levels, which in turn upregulates Myd88 expression and induces cardiac hypertrophy.

LncRNA regulator of reprogramming (*ROR*) was first found as an lncRNA that regulates the pluripotency and differentiation of ES cells.[36] LncRNA-*ROR* promotes cardiac hypertrophy by acting as a sponge for miR-133, which is known as an antihypertrophic miRNA.[37] Because the expression level of miR-133 is high in cardiac myocytes, it is also possible that miR-133 serves as an lncRNA-*ROR* sponge and attenuates the prohypertrophic effect of lncRNA-*ROR*.

It is notable that the lncRNA *H19*, a highly abundant and conserved imprinted gene, encodes miR-675, which was shown to mediate the inhibitory effect of *H19* on cardiomyocyte hypertrophy by targeting CaMKIIδ.[38]

Functional lncRNAs in HF

HF is a complex condition involving declining cardiac function in response to various pathophysiological insults, which result in maladaptive cardiac hypertrophy and cell death. Therefore, dysregulation of all of the aforementioned *Chaer*, *Mhrt*, *Chast*, *CHRF*, and lincRNA *ROR* is associated with HF development (**Figure 1**).

There are several cell-death-related lncRNAs. An lncRNA named autophagy-promoting factor (*APF*) was found to regulate autophagy in the heart.[39] It regulates miR-188-3p, and thus affects ATG7 expression and autophagic cell death. *APF* knockdown by siRNA resulted in a significant reduction in infarct size and the amelioration of cardiac function in an ischemia-reperfusion model in mice.

Abnormal mitochondrial fission participates in the promotion of cell death. The lncRNA cardiac apoptosis-related lncRNA (*CARL*) was reported to suppress mitochondrial fission and apoptosis by impairing miR-539-dependent downregulation of prohibitin 2 (PHB2).[40] A detailed ultrastructural analysis in mouse embryonic fibroblasts revealed the defective morphogenesis of cristae in the absence of prohibitins,[41] and that study revealed a model of mitochondrial fission regulation that is composed of *CARL*, miR-539, and PHB2.

RNA deep-sequencing of cardiac samples from patients with ischemic cardiomyopathy and from controls identified 145 differentially expressed lncRNAs in hearts with ischemic cardiomyopathy.[42] Expression correlation coefficient analyses of differentially expressed lncRNAs revealed a strong association between lncRNAs and extracellular matrix protein-coding genes. Overexpression or knockdown experiments in cardiac fibroblasts suggested that selected lncRNAs such as *n379599*, *n379519*, *n384640*, *n380433*, and *n410105* are important regulators of fibrosis and the expression of extracellular matrix synthesis genes.

By using an integrated genome screen, Wisp2 super-enhancer-associated RNA (*Wisper*) was identified as a cardiac fibroblast-enriched lncRNA that regulates cardiac fibrosis after injury.[43] *Wisper* expression correlated with fibrosis levels in a mouse model of myocardial infarction and in samples from humans with aortic stenosis. Mechanistically, *Wisper* regulates cardiac fibroblast gene expression partly through its association with TIA1-related protein to control the expression of lysyl hydrosylase 2.

Functional lncRNAs in Atherosclerosis

Atherosclerosis is accompanied by a cascade of inflammatory responses, fatty plaque formation, migration of monocytes, and proliferation of smooth muscle cells in conjunction with abnormal lipid metabolism. All of these physiological and pathological and steps may be affected by lncRNAs (**Figure 2**).

Oxidized low-density lipoprotein (oxLDL) is known to be associated with the onset of atherogenesis involving macrophages and endothelial cells. It was shown that expression of lncRNA-growth arrest-specific 5 (lncRNA *GAS5*) was significantly increased in atherosclerotic plaque.[44] Knockdown of lncRNA *GAS5* reduced apoptosis of THP-1 cells induced by oxLDL. In contrast, overexpression of lncRNA *GAS5* enhanced the apoptosis of THP-1 cells treated with oxLDL.[45] Of note, exosomes derived from lncRNA *GAS5*-overexpressing THP-1 cells enhanced the apoptosis of vascular endothelial cells. Thus, lncRNA

GAS5 may regulate the apoptosis of both macrophages and endothelial cells via exosomes in atherosclerotic plaque.

Long intergenic non-coding RNA (LincRNA)-*p21* was shown to act as a key regulator of apoptosis or cell proliferation during the progression of atherosclerosis.[46] The levels of lincRNA-*p21* were significantly reduced in atherosclerotic plaques of ApoE(−/−) mice. Loss- and gain-of-function approaches indicated that lincRNA-*p21* induces apoptosis and represses cell proliferation in vascular smooth muscle cells and mouse macrophage cells in vitro. Moreover, inhibition of lincRNA-*p21* resulted in neointimal hyperplasia in a carotid artery injury model in vivo. It was also shown that lincRNA-*p21*, which is a transcriptional target of p53, enhances p53 transcriptional activity by binding to mouse double minute 2 (MDM2) in a feedback manner. The association of lincRNA-*p21* and MDM2 releases MDM2 repression of p53, which enables p53 to interact with p300 and to bind to the promoters/enhancers of its target genes.

Smooth muscle-induced lncRNA enhances replication (*SMILR*) expression was detected in human saphenous vein vascular smooth muscle cells after stimulation with interleukin-1α and platelet-derived growth factor.[47] *SMILR* increased cell proliferation, by regulation of the proximal gene *HAS2*. Increased expression of *SMILR* was observed in unstable atherosclerotic plaques and the plasma of patients with high levels of C-reactive protein, suggesting an association with inflammation and vascular smooth muscle cell proliferation.

The chromosome 9p21 (Chr9p21) locus involved in coronary artery disease was identified in a genome-wide association study.[48] It is known that Chr9p21 encodes an lncRNA, antisense non-coding RNA in the INK4 locus (*ANRIL*). *ANRIL* expression was reported to be associated with the Chr9p21 genotype and correlated with atherosclerosis severity.[49] Overexpression of *ANRIL* in peripheral blood mononuclear cells leads to increased cell adhesion. Mechanistically, *ANRIL* can bind to and recruit polycomb group proteins to Alu motifs marking the promoters of *ANRIL* target genes.[50]

The lncRNAs that regulate vascular and endothelial cell biology have also been identified, and dysregulation of them may be associated with atherosclerosis. Smooth muscle and endothelial cell-enriched migration/differentiation-associated lncRNA (*SENCR*) was among the first lncRNAs to be identified that seem to stabilize the smooth muscle cell contractile phenotype.[51] Endothelial-enriched lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*Malat1*) is one of the most abundant lncRNAs in mammalian cells, and is reported to sustain endothelial cell proliferation.[52]

Future Directions of lncRNAs Studies

The lncRNAs have been shown to be important regulators in physiological and pathological states. Recent studies provide considerable evidence of the effect of lncRNAs on various stages of cardiovascular diseases. Because each lncRNA has a distinct mode of action, further investigations are required to understand the complexity of lncRNA biology. In the case of some lncRNAs, inhibition or activation leads to beneficial effects on a disease condition; therefore, the development of techniques that enable the spatiotemporal regulation of lncRNAs may hold the promise of clinical therapeutic applications.

Conclusions

There are many functional lncRNAs in cardiovascular diseases, including cardiac hypertrophy, HF, and atherosclerosis. The therapeutic potential of synthetic RNAs to modulate these lncRNAs is being tested and may be utilized in the future.

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Disclosures

None.

Conflict of Interest

None.

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