Title: Pathological bases and clinical Application of long noncoding RNAs in cardiovascular diseases

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Running title: Long non-coding RNAs in cardiovascular disease

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Abstract

Increasing evidence has suggested that noncoding RNAs (ncRNAs) have vital roles in cardiovascular tissue homeostasis and diseases. As a main subgroup of ncRNAs, long noncoding RNAs (lncRNAs) have been reported to play important roles in lipid metabolism, inflammation, vascular injury, and angiogenesis. They have also been implicated in many human diseases including atherosclerosis, arterial remodeling, hypertension, myocardial injury, cardiac remodeling and heart failure. Importantly, it was reported that lncRNAs were dysregulated in the development and progression of cardiovascular diseases (CVDs). A variety of studies have demonstrated that lncRNAs could influence gene expression at transcription, post-transcription, translation and post-translation level. Particularly, emerging evidence has confirmed that the crosstalk amongst lncRNAs, mRNA and miRNAs is an important underlying regulatory mechanism of lncRNAs. Nevertheless, the biological functions and molecular mechanisms of lncRNAs in CVDs have not been fully explored yet. In this review, we will comprehensively summarize the main findings about lncRNAs and CVDs, highlighting the most recent discoveries in the field of lncRNAs and their pathophysiological functions in CVDs, with the aim of dissecting the intrinsic association between lncRNAs and common risk factors of CVDs including hypertension, high glucose and high fat. Finally, the potential of lncRNAs functioning as the biomarkers, therapeutic targets, as well as specific diagnostic and prognostic indicators of CVDs will be discussed in this review.

Keywords: Long non-coding RNAs, Cardiovascular diseases (CVDs), Biomarker, Pathogenesis.

Introduction

Cardiovascular diseases (CVDs) are major causes of morbidity and mortality in the world. Approximately 100 million American adults (>1 in 3) have ≥1 types of CVDs[1]. A total of 11.5% of American adults (27.6 million) have been diagnosed with heart disease, exerting a dramatic burden on the public health in the USA. As rare diseases in China during 1950s[2], CVDs have become a major threat to national health and socioeconomic development, undergoing a significantly increased mortality rate from 1990 to 2015, by 21.4% and 70% in urban and rural area, respectively[3]. Therefore, it is vital to uncover the molecular pathophysiology of CVDs and to find novel biomarkers for early prevention and diagnosis of CVDs[4]. In this respect, non-coding RNAs (ncRNAs), particularly long ncRNAs (lncRNAs) caught our attention due to their significant implications in pathogenesis, diagnosis and prognosis of CVDs[5].

Based on the Encyclopedia of DNA Elements (ENCODE) and the Functional Annotation of Mouse (FANTOM), human genome contains approximately 20,000 protein-coding genes, which only accounts for less than 2% of the entire genome, indicating that the vast majority of RNA transcripts belong to ncRNAs without any protein-coding potential, such as lncRNAs. It has been well-known that lncRNAs (>200 nt) with limited or no protein-coding capacity serve primarily as the regulatory components in cells[6]. Data from the NONCODE database (http://www.noncode.org)

which includes all the annotated ncRNAs to date, shows that there are 102,783, 87,553 and 27,793 lncRNA genes for human, mouse, and cow, respectively (**Table 1**). It has been shown that lncRNAs exert comprehensive effects on biological processes and are associated with numerous diseases including CVDs[7].

A variety of lncRNAs have been implicated in cardiac development and disease[8-10]. In this review, we will summarize current understanding of lncRNAs in CVDs including hypertension, myocardial infarction, cardiac fibrosis, cardiac hypertrophy, and heart failure (**Table 2**). Moreover, from a genetic and epigenetic perspective, we will also discuss the complex interactions between different risk factors (hypertension, hyperglucose and hyperlipemia) and lncRNAs. Finally, the potential applications of lncRNAs as a biomarker for CVDs diagnosis (**Table 3**) will be explored.

Overview of lncRNAs

LncRNAs are currently defined as a large and heterogeneous class of transcribed RNA molecules whose length is greater than 200 nucleotides, and lack an open reading frame of significant length. The most common categorization of the highly heterogeneous lncRNAs is based on the relative location of lncRNAs to protein-encoding genes in the genome: intergenic lncRNAs (lincRNAs), sense lncRNA, intronic lncRNAs, antisense lncRNAs (aslncRNAs) and bidirectional lncRNAs (**Figure 1**)[11, 12]. LncRNAs are usually identified from deep RNA-sequencing, and annotated using characteristic histone modification signatures that are associated with Pol II transcription, such as enrichment of histone H3 lysine 4 tri-methylation (H3K4me3) at the promoter and

H3K36me3 along the gene body[13]. LincRNAs, located between two protein coding-genes, are further classified into two broad categories: enhancer-(elncRNAs) and promoter-associated lncRNAs (plncRNAs). They are transcribed from enhancer regions or promoter-like lncRNA loci, and marked by high levels of H3K4me1 compared to H3K4me3 (elncRNAs) and high levels of H3K4me3 relative to H3K4me1, respectively[14]. While elncRNAs have been shown to regulate the expression of neighbouring protein coding genes on the same chromosome, plncRNAs mainly regulate chromatin states and epigenetic inheritance[7]. Sense lncRNAs, transcribed from the sense strand, overlaps with one or more exons of another protein-coding gene in the same strand[14]. Intronic lncRNAs are normally initiated and terminated within an intron of a protein-coding gene in either direction without overlapping exon[15]. AslncRNAs are transcribed from the complementary strand of protein-coding genes. They are located either on the same strand or the opposite strand of the nearest protein-coding genes, and overlap with at least one protein-coding exon[15, 16]. Bidirectional LncRNAs are transcribed from the same promoter as a protein-coding gene, but in the opposite direction[16]. No matter whether the lncRNAs originate from their own promoters or enhancers, their transcriptional start sites, in most cases, are marked by the H3K4me3 histone modification, and their transcribed regions are marked with the H3K36me3 mark, respectively[17].

Histone modifications including methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation play critical roles in gene regulation through two main mechanisms: by altering chromatin structure or recruiting histone modifiers[18].

Unsurprisingly, the majority of lncRNA genes are transcriptionally regulated by histone modification[19]. Wu et al.[20] found that the expression levels of numerous lncRNAs were positively and negatively associated with H3K4me3 (activating histone modification) and H3K27me3 (inhibiting histone modification) at promoter sites, respectively, in embryonic stem cells and terminally differentiated fibroblasts. Therefore, similar to protein-coding gene regulation, lncRNA expression was spatiotemporally regulated through multiple histone modifications.

Regulation of the target genes by lncRNAs are closely related to chromosome silencing, genomic imprinting, chromatin modifications and structure, transcriptional activation, transcriptional interference, and nuclear transport (**Figure 2**). Simultaneously, lncRNAs have also been reported to influence exon splicing/skipping, RNA translation or degradation by binding to mRNAs or protein components of ribonucleoprotein complexes. Moreover, lncRNAs can specifically bind to other proteins and chromatin, and act as co-activators or co-enhancers. Furthermore, microRNA transcription is controlled by lncRNAs through inducing methylation of the miRNA promoter. Studies also showed that stability of mRNA is influenced by lncRNA[6]. Notably, lncRNA molecules have the potential to form highly structured macromolecules by folding into double-stranded stems, single-stranded loops and bulges, which again can fold further into three-dimensional structures using *in cis* and *in trans* dogma[21]. In sum, lncRNA can work as decoys to bind and titrate away proteins or RNAs to impart specificity to genomic locations through either RNA-protein or RNA-miRNA recognition rules, thereby exerting biological functions in multiple kingdoms of life.

Genetic and Epigenetic Studies of LncRNA

Evidence for a putative role of lncRNAs in vascular diseases came from genome-wide association studies that independently identified a susceptibility locus of coronary artery disease (CADs) on the human Chr9p21. This locus is adjacent to the last exon of an lncRNA, named antisense ncRNA in the INK4 locus (ANRIL). Interestingly, the exons of multiexonic lncRNAs were found to be GC rich, tending to have higher fractions of CpG dinucleotides and higher densities of H3K36me3 marks, compared with intronic or monoexonic lncRNAs[18]. The higher GC content in exons than that in introns is a hallmark of coding sequences and has been interpreted with more efficient transcription, splicing, or translating. The major epigenetic regulation in CVDs are DNA methylation, posttranslational histone modifications and ncRNAs including lncRNAs[22].

It has been well-known that evolutionary conservation of lncRNAs consists of four dimensions: the sequence, structure, function, and expression from syntenic genetic loci[23]. The splice sites of lncRNAs have been found to be more stable during evolution. In general, lncRNAs can act locally (*in cis)* to regulate neighboring genes or target genes by involving in DNA looping or act *in trans* as recruiters or decoys for chromatin modifiers and transcription factor[24].

LncRNA H19, located on 11p15.5, is an imprinted gene and is highly conserved throughout evolution, and it has been identified as an important regulator in mammalian development and diseases. Study showed that lncRNA H19 is mainly expressed in

skeletal muscle and heart in adults, playing a role in myoblast differentiation and CVDs[25]. Depletion of H19 influenced endothelia cell (EC) transcriptome, increased EC apoptosis and growth arrest, and impaired the formation of capillary-like structure[26]. Hadji F et al. found that epigenetic-related dysregulation of DNA methylation was occurred in the promoter of H19, which was inversely associated with H19 expression in calcific aortic valve disease (CAVD)[26]. Interestingly, exons of H19 carry a miRNA containing hairpin which has been reported to act as the template for miR-675, and it has been shown that miR-675 can confer functionality of H19. Further study found that miR-675 regulated cyclin-dependent kinase 6 (CDK6) expression, affecting cell proliferation and migration in glioma[27]. CDK6 siRNA could efficiently inhibit phenylephrine-induced hypertrophy in rat cardiomyocytes by inactivating CDK6-Rb pathway[28]. Furthermore, increased plasma levels of H19 was associated with increased risk of CAD in Chinese population, and multivariate logistic regression analysis indicated that plasma H19 level was an independent predictor for CAD, with an area under the curve of 0.631 for H19[29].

MANTIS, namely lncRNA n342419, reported as a disease-relevant lncRNA, is associated with chromatin-remodeling complexes in the regulation of transcriptional events, and enhances endothelial angiogenic function[30]. A recent study showed that MANTIS was downregulated in patients with pulmonary arterial hypertension and in rats treated with monocrotaline, whereas it was upregulated in carotid arteries and in ECs from human glioblastoma patients. Depletion of MANTIS reduced EC angiogenic function *ex vivo* and *in vivo*. Mechanically, the histone demethylase JARID1B was

found to bind to H3K4me3-rich regions near the transcriptional start site (TSS) of MANTIS, and the expression of MANTIS was controlled by JARID1B at the post-transcriptional level, suggesting that MANTIS expression is dependent on the changes of histone modification rather than direct transcriptional regulation. Chromatin immunoprecipitation assay indicated that MANTIS could directly interact with ATPase subunit BRG, leading to a higher activity of BRG1 by binding with BAF155. Thus, activation of BRG1 facilitated the binding of BAF155 and RNA Pol II to endothelial gene promoters. Furthermore, MANTIS depletion decreased the interaction of BRG1 with BAF155 and the ability of BRG1 to bind its target gene promoters. Taken together, MANTIS interacts with BRG1, leading to a higher ATPase activity of BRG1 and chromatin remodeling, thereby facilitating EC gene expression. These findings suggest that MANTIS plays a significant and unique role for EC function by acting as a scaffolding lncRNA within a chromatin-remodeling complex, and mediating efficient transcription of key endothelial gene.

The Cardiac Hypertrophy Associated Epigenetic Regulator (Chaer), which is necessary and sufficient for cardiac hypertrophy and hypertrophic gene induction, acts as an early epigenetic checkpoint in cardiac hypertrophic reprogramming[31]. It was demonstrated that down-regulation of Chaer significantly blunted cardiac hypertrophy and pathological progression, but had no effect on the development of post-stressed heart[31]. From a translational perspective, Wang Z et al. observed a dynamic change in global histone methylations at the H3K9 and the H3K27 sites but not at the H3K4 site. Chaer deficiency specifically increased di- and tri-methylation at H3K27, which was

catalyzed by PRC2, a well-known molecular target of lncRNAs. These data indicated a specific but negative regulation of H3K27 methylation by Chaer in cardiomyocytes[31]. In addition, methylation at H3K4 by trithorax group (TrxG)/mixed-lineage leukemia (MLL) caused gene activation, while methylation at H3K27 by Chaer-PRC2 resulted in gene silence. Notably, the decrease of H3K27me3 was significantly reversed by mTOR inhibition, suggesting that Chaer/PRC2-mediated H3K27 methylation in cardiomyocytes via a mTOR-dependent mechanism[32]. Therefore, Chaer was defined as an epigenetic checkpoint in cardiac hypertrophy[33].

One of the well-studied functions of lncRNAs is their regulatory roles in regulating gene and genome activity. Xist is one of the best-studied lncRNAs to date in this aspect. It is required for the silencing of hundreds of genes on the inactive X chromosome by favoring the formation of a chromatin structure with an epigenetic profile linked to transcription repression, a process known as X chromosome inactivation (XCI). Xist specifically captures PRC2 through a conserved repeat motif and takes it to the inactive X chromosome[34]. Xist also coats and inactivates the distal X-chromosome by exploiting the three-dimensional conformation of the X-chromosome, modifying chromosome structure, and spreading to newly accessible locations[35].

Cardiovascular physiopathology and lncRNAs

Hypertension

Hypertension is a well-known and common risk factor for CVDs. The core characteristics of hypertensive patients were endothelial dysfunction and activation of

inflammatory system. The increases in reactive oxygen species (ROS) production and decreases in nitric oxide (NO) bioavailability were also present in subjects with hypertension. Moreover, sustained hypertension was a predisposing factor for stroke, myocardial infarction, coronary artery disease, and heart failure[36].

GAS5, namely growth-arrest specific transcript 5, located in 1q25, consists of 12 exons and encodes 10 box C/D snoRNAs within the introns. A recent study demonstrated that lncRNA GAS5 was predominantly expressed in ECs and vascular smooth muscle cells (VSMCs) of hypertensive patients and played a vital role in vascular remodeling induced by high blood pressure[37]. Specifically, GAS5 was responsible for controlling blood pressure, regulating endothelial cell activation, VSMC phenotypic conversion and arterial remodeling. Interestingly, the expression of GAS5 was significantly downregulated in the plasma of hypertensive patients and other arteries of hypertensive mice, including caudal artery, carotid artery, renal artery, and thoracic artery. GAS5 knockdown caused an increase of systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure. In response to hypoxic stress, GAS5 increased the viability of VSMCs and inhibited the apoptosis of VSMCs. Moreover, GAS5 inhibitor could increase the number of new blood vessels, capillary degeneration and capillary leakage, while incubation of VSMCs with the medium from GAS5 overexpressed ECs significantly reduced the proliferation and migration of VSMCs. β-catenin signaling pathway plays a critical role in cell proliferation, cell polarity, and cell fate determination, and is characterized by cytoplasmatic β-catenin stabilization, β-catenin

nuclear translocation, and increased expression of β-catenin target genes. GAS5 interacts with β-catenin and prevents β-catenin nuclear translocation, thereby altering β-catenin signaling[37]. Li et al. [38]reported that overexpression of GAS5 inhibited abnormal activation of the Wnt/β-catenin signaling pathway in myocardial tissues of CAD rats, thus alleviated myocardial injury. β-catenin nuclear translocation is controlled by GAS5, suggesting that GAS5 regulates EC and VSMC function through β-catenin signaling[38]. Therefore, GAS5 controls EC and VSMC function, or EC-VSMC crosstalk via exosomes in β-catenin dependent manner, ultimately affecting the development of hypertension.

AK098656 (1 isoform of LINC01227), located on human Chr16 (the minus strand) and containing 3 exons, was predominantly expressed in VSMCs and strongly upregulated in the plasma of hypertensive patients. Additionally, increased blood pressure, elevated VSMCs synthetic phenotype and narrowed resistant arteries were observed in AK098656 transgenic rats[39]. Overexpression of AK098656 increased VSMC proliferation and migration, with elevated levels of extracellular matrix proteins but decreased expressions of VSMC contractile proteins. Moreover, RNA immunoprecipitation assay and fluorescence in situ hybridization assay confirmed the colocalization and direct binding of AK098656 with myosin heavy chain-11 (MYH11, a VSMCs-specific contractile protein) and fibronectin-1 (an essential component of extracellular matrix), and such interactions resulted in a decreased protein level by promoting their protein degradation[39]. Collectively, all the data indicate that AK098656, an lncRNA is predominantly expressed in VSMCs, is a novel regulator in hypertension.

High glucose (HG) and diabetes

It is widely recognized that hyperglycemia is a key determinant of endothelial dysfunction. The advanced glycation and products from hyperglycemia could contribute to endothelial dysfunction. High glucose stimulated free radical production and ROS formation. Excess glucose stress could cause serious oxidative stress during diabetic complications. High glucose significantly increased endogenous ROS production in a time-dependent and concentration-dependent manner[40].

Whole transcriptome sequencing showed that cardiomyocyte hypertrophy induced by hyperglycemia could be attributed to many differentially expressed genes and ncRNAs including lncRNAs[41]. High glucose increased myocardial infarction-associated transcript (MIAT) expression in HEK293 cells (a cell line derived from human embryonic kidney) and EA.hy926 cells (a human cell line derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549), whereas MIAT knockdown reduced expression of proapoptotic proteins, thus suppressing high glucose-mediated apoptosis of cardiomyocytes. Targeted inhibition of lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) decreased cellular inflammatory response and reduced EC pyroptosis[42]. Mechanistic studies showed that MALAT1-targeting antisense oligonucleotide (ASO) transfection could significantly suppress the expression of NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), and knockdown of NLRP3 significantly increased the levels of total cholesterol and triglycerides, causing hypercholesterolemia and high levels of inflammatory factors. Additionally, MALAT1 could act as a ceRNA to bind and sequester miR-22-3p. MiR-

22-3p overexpression abrogated the effect of MALAT1 on high glucose-induced EA.hy926 cell pyroptosis, and negatively regulated NLRP3 expression and inflammation, which consequently controlled the progression of diabetes-associated CVDs.

Atherosclerosis

Atherosclerosis occurred under different risk conditions. Previous studies have shown that atherosclerosis is a chronic inflammatory disease caused by a combination of pathological conditions including lipid disorder, endothelial cell dysfunction, oxidative stress, hemodynamic changes, and biochemical stimuli[6]. Handful of lncRNAs have been reported to play a role in atherogenesis. Specifically, high expression levels of lncRNAs ANRIL, MIAT and RNCR3[43-46], but low expression levels of MALAT1 were observed in the atherosclerotic arteries[47].

ANRIL, also known as CDKN2B-AS1, is a 3.8kb lncRNA consisted of 19 exons and is highly expressed in ECs. Genome-wide association studies (GWAS) have identified genetic variants at the Chr9p21 locus contributing to the risk of CVDs[48]. The expression levels of ANRIL encoded from Chr9p21, but not the other neighboring genes in the Chr9p21 region, were associated with atherosclerosis severity[49]. Mechanistically, ANRIL acts as a sponge for multiple microRNAs including miR-199a[50], miR-125a[51], miR-186[52], and miR-323[53], and mediates their activity in various diseases. ANRIL also plays a regulatory role in various signaling pathways, including the ataxia telangiectasia mutated (ATM)/E2F1 pathway[54], the vascular endothelial growth factor (VEGF)

pathway[55], and the nuclear factor kappa B (NF-κB) pathway[56]. A bioinformatic analysis of ANRIL and its promoter regions highlighted a common presence of Alu elements, suggesting functional involvement of Alu motifs in target gene regulation and modulating atherogenic cell functions[49]. Further experiments found that ANRIL might bind to chromatin through an Alu-mediated interaction, and guide the PRC proteins to ANRIL-regulated genes[57]. The expression of ANRIL is affected by CVD-associated single nucleotide polymorphisms in the 9p21 locus, which in turn modulates atherogenic cell functions via regulating CDKN2A/B[48].

LncRNA taurine-up-regulated gene 1 (TUG1), a 6.7-kb lncRNA, is located at Chr22q12, and contributed to the proper formation of photoreceptors in the developing rodent retina[58]. It has also been reported that tanshinol reduces atherosclerotic lesions in ApoE-/-mice by inhibiting TUG1, indicating the therapeutic potential of TUG1 in atherosclerosis[59]. TUG1 knockdown ameliorated atherosclerosis by inhibiting hyperlipidemia and decreasing inflammatory response. TUG1 overexpression accelerated cell growth, improved inflammatory factor expression, and inhibited apoptosis, while these effects were abated after co-transfection with miR-133 mimic. As an endogenous molecular sponge, TUG1 binds to miR-133a and de-suppresses FGF1 expression[59].

LncRNAs and Endothelial Cell Functions

One hundred sixteen EC-enriched lncRNAs were identified in ECs by lncRNA microarray analysis, including HHIP-AS1, FLI-AS1 (SENCR),

LOC100505812/CARD8-AS, AF161442/HSPC324, and STEEL[60]. RNA deep sequencing showed that MALAT1, MEG3, TUG1, linc00493 and linc00657 were highly expressed in HUVECs[61].

SENCR, a smooth muscle and endothelial cell-enriched migration/differentiation-associated lncRNA, is located on the Chr11 and transcribed from the first intron of FLI1 which is an earliest expressed transcription factor involved in EC specification and development[62]. It was reported that SENCR could be induced with laminar shear stress in aortic EC of humanized mice. SENCR binds to RNA-binding protein called cytoskeleton-associated protein 4 (CKAP4). Specifically, studies show that there are two potential SENCR-binding domains in CKAP4, RBD1 and RBD2, and the RBD1 domain is essential for SENCR and CKAP4 protein interaction. Functionally, SENCR inhibition enhances the association of CKAP4 with VE-cadherin at the EC membrane, triggering VE-cadherin internalization and causing perturbation of adherens junctions, which in turn disrupts membrane integrity and increases EC permeability[63].

Additionally, SENCR is dynamically regulated during differentiation of human embryonic stem cells, and plays a critical role in endothelial differentiation from stem cells and EC angiogenesis[64]. SENCR loss- and gain- of function experiments confirmed a role for SENCR in controlling proliferation, migration, and neo-vessel formation of ECs[65, 66]. SENCR was equally distributed in nucleus and cytoplasm in HUVEC[63], and SENCR overexpression increased the expression levels of EC-specific genes and miRNAs including CD144, CD31, FLT1, miR-126 and miR-27b. Conversely, SENCR knockdown decreased the expression levels of proangiogenic genes including

CCL5, CEACAM1, and CX3CL1, causing abnormal EC membrane integrity and permeability[64]. Collectively, SENCR can stabilize vascular endothelial cell adherens junctions and control cell lineage specification during embryonic development.

LncRNA maternally expressed gene 3 (MEG3), transcribed from Chr14q32, is aberrantly expressed in multiple cancers and its down-regulation can augment cisplatin resistance of lung cancer[67]. MEG3 is regulated by HIF-1α under hypoxia condition in primary HUVECs[68, 69]. MEG3 inhibition significantly suppressed VEGFR2 expression, but had no influence on gene expression of VEGFR1. MEG3 inhibition also suppressed VEGF-induced endothelial migration and angiogenesis. Furthermore, MEG3 silencing reduced the tube formation and the spheroid sprouting of primary HUVEC under normoxic and hypoxic conditions. Altogether, MEG3 regulated by HIF-1α is required to maintain VEGFR2 expression in ECs, suggesting that MEG3 is an important regulator of EC functions[36].

The expression of HOTTIP, long noncoding RNA (lncRNA) HOXA transcript at the distal tip, was relatively high in CAD tissues and proliferating endothelial cells (ECs). Ectopic expression of HOTTIP facilitated EC proliferation and migration via the activation of the Wnt/β-catenin signaling pathway. These results suggested that HOTTIP might manipulate the EC proliferation and migration by activation of the Wnt/β-catenin signaling pathway[70].

LncRNAs and Vascular smooth muscle cell (VSMCs) Functions

VSMCs phenotype switching from contractile to synthetic, VSMC proliferation and

migration are the well-known key pathological events for hypertension, atherosclerosis and restenosis[71]. It was reported that Angiotensin II (AngII) promoted hypertension and atherosclerosis by activating the enhancers and super-enhancers of the growth-promoting and pro-inflammatory genes in VSMCs[72].

LincRNA-p21, a p53-induced lncRNA, was recently reported to control VSMC proliferation and facilitate atherosclerosis progression. Inhibition of lincRNA-p21 increased VSMC proliferation and reduced VSMC apoptosis by interfering with p53. LincRNA-p21 enhanced p53 transcriptional activity by binding mouse double minute 2 (MDM2) and unleashing MDM2-mediated inhibition of p53. Importantly, lincRNA-p21 was significantly reduced in carotid artery tissue and peripheral blood mononuclear cells from CAD patients, and its inhibition enhanced neointima formation in mice models[73]. Many angiotensin-induced lncRNAs in VSMCs were identified by Leung et al.[74], and one of the highly upregulated lncRNAs in VSMCs by angiotensin was Lnc-Ang362. Interestingly, this lncRNA is the host transcript of miR-221 and miR-222, two miRNAs with renowned functions in VSMC proliferation and neointimal hyperplasia.

LncRNAs in cardiomyocytes

The lncRNA, small nucleolar RNA host gene 6 (SNHG6), was found to be upregulated in fetal cardiac tissues with ventricular septal defect (VSD), and down-regulated during cardiomyocytes differentiation from P19 cells. Additionally, SNHG6 controls P19 cell proliferation, apoptosis, and cardiomyocytes differentiation through a negative regulation of miR-101, and activation of Wnt/β-catenin signaling pathway. Finally, this

study also showed that SNHG6 promoted VSD formation via regulating miR-101 and Wnt/β-catenin signaling pathway[75].

LncRNAs NR_045363 is highly expressed in cardiomyocytes, but rarely in non-cardiomyocytes. NR_045363 overexpression reinforced DNA synthesis and cytokinesis in neonatal cardiomyocytes in vitro. Interestingly, NR_045363 silencer and miR-216a inhibitor had a synergistic effect on cardiomyocyte proliferation. Mechanistic analysis revealed that NR_045363 promoted cardiomyocyte proliferation through interaction with miR-216a, thereby modulating the JAK2-STAT3 signaling pathway[76].

LncRNAs in acute myocardial infarction

Acute myocardial infarction (AMI) is the most serious type of coronary atherosclerotic diseases. Many risk factors, and abnormal expression of genes, proteins, and ncRNAs including lncRNAs have been identified in the pathophysiology of AMI[77].

The expression levels of five lncRNAs, HIF1A antisense RNA 2, ANRIL, KCNQ1OT1, MIAT, and MALAT1, were measured by quantitative polymerase chain reaction in peripheral blood cells from patients with AMI. The result showed increased levels of HIF1A antisense RNA 2, KCNQ1OT1, and MALAT1, but decreased levels of ANRIL in patients with AMI. It was interesting to note that MIAT, also known as RNCR2, AK028326 or GOMAFU, was remarkably up-regulated in a mouse model of AMI. To confirm if MIAT can influence the pathological process but not merely a consequence of AMI, MIAT was up-regulated, and subsequently an elevated expression level of

transforming growth factor-β (TGF-β) was observed in mouse model of AMI. Additionally, knockdown of endogenous MIAT by its siRNA was found to result in improved cardiac function and reduced cardiac fibrosis in AMI. Furthermore, knockdown of MIAT substantially alleviated Ang II induced upregulation of ANP, BNP and β-MHC in AMI[78]. RNA-induced silencing complex (RISC) is responsible for the binding of miRNA to target mRNA transcripts. Notably, as a core component of RISC, Argonaute2 (Ago2) knockdown caused marked increase in MIAT level. Yan B et al. revealed that MIAT or miR-150-5p was found to be enriched in Ago2-containing miRNPs in the nuclear fraction using Ago2 antibody, suggesting that MIAT is targeted by miR-150-5p in the nucleus in an Ago2-dependent manner[44]. Therefore, increasing evidence supports a crucial role for MIAT in governing pro-fibrotic secretion and cardiac fibrosis after AMI[79].

It has been reported that overexpression of the lncRNA, component of mitochondrial RNA processing endoribonuclease (RMRP), aggravates hypoxia-induced injury in H9c2 cells (a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue), and activation of PI3K/Akt/mTOR signaling pathway is the key downstream pathway mediating the cardioprotection of lncRNA-RMPR/miR-206/ATG3 axis against myocardial I/R injury[80, 81]. Specifically, overexpression of RMRP activated PI3K/AKT/mTOR pathway in hypoxia-treated H9c2 cells, which was reversed by miR-206 overexpression. Another study using a rat model of myocardial I/R injury revealed a role for the MIAT/miR-150-5p/P300 signaling axis in cardiac hypertrophy[76].

The role of lncRNAs in cardiac fibrosis

Several dysregulated transcripts have been found in cardiac fibroblasts (CFs) isolated from mice after chronic pressure overload by Global lncRNA profiling. Among them, CF-enriched MEG3 was found to be down-regulated during late cardiac remodeling. MEG3 was found not only to be one of 22 lncRNAs expressed in the adult murine heart, but also presented in other tissues, particularly rich in the brain. In comparison with other cardiac cell types, MEG3 was the most abundant lncRNA both in freshly isolated and cultured CFs. MEG3 was significantly reduced in the CF fraction by MEG3 GapmeR, whereas a lower silencing efficiency was achieved in the cardiomyocytes or EC fractions. MMP-2 expression and activity in CFs was controlled by TGFβ1/MEG3/P53 axis. Interestingly, CF apoptosis and cell cycle progression were not affected by MEG3 silencing[9]. This study provides clear evidence to support a regulatory role for MEG3 in cardiac fibrosis.

The role of lncRNAs in cardiac hypertrophy

Cardiomyocyte hypertrophy is a physiological adaptation triggered by various physiological or pathological stimuli in an attempt to augment or preserve cardiac function for short periods. Long-term cardiomyocyte hypertrophy often progressed to heart failure. Several dysregulated lncRNAs have been identified in hypertrophic cardiomyocytes. One of such dysregulated lncRNAs is the highly abundant and conserved imprinted lncRNA H19. Interestingly, both H19 and its encoded miR-675

were up-regulated in pathological cardiac hypertrophy and heart failure. Further studies demonstrate the H19-miR-675 axis targets CaMKIIδ and acts as a novel negative regulator of cardiac hypertrophy[27]. The lncRNA cardiac hypertrophy related factor (CHRF) is another star lncRNA in pathological cardiac hypertrophy. CHRF was found to act as an endogenous sponge of miR-489 and suppress miR-489 expression in cardiomyocytes and hypertrophic hearts in response to Ang-II treatment. Importantly, myeloid differentiation primary response gene 88 (Myd88) was identified as a miR-489 target to mediate the function of miR-489 in cardiac hypertrophy, and CHRF could directly bind to miR-489 and regulate Myd88 expression and cardiac hypertrophy, suggesting that CHRF/miR-489/Myd88 is a powerful regulatory axis in cardiac hypertrophy[82]. Apart from miR-489/Myd88, miR-93/Akt3 has been identified as another downstream signaling axis to exert the pathological effects of CHRF on cardiac hypertrophy[83].

Further study found that in H9c2 cells treated with LPS, the silence of CHRF promoted cell viability, but inhibited apoptosis, as well as suppressed ROS generation and inflammatory genes expression. Interestingly, there was no protective effect on H9c2 cells against LPS when miR-221 was suppressed. Also, the inhibitory effects of lncRNA CHRF silencing on the activation of NF-κB and JNK pathways were flattened by miR-221 suppression. CHRF silencing protected H9c2 cells against LPS-induced injury via up-regulating miR-221, and modulating NF-κB and JNK signaling pathways which were significantly activated by LPS[84]. Therefore, silence of lncRNA CHRF exhibited cardioprotective effects through up-regulating miR-221 and thus blocking the

activation of NF-κB and JNK pathways.

The role of lncRNAs in heart failure (HF)

LncRNAs microarray analysis showed that 2066 mRNAs and 1197 lncRNAs were upregulated, while 2871 mRNAs and 1403 lncRNAs were downregulated in rat cardiac tissues with ischemic heart failure. Moreover, 331 pairs of differentially expressed lncRNAs and nearby coding genes, comprised of 291 lncRNAs and 296 mRNAs, were identified. Expression levels of four lncRNA-mRNA pairs (MRAK140148-KCND2, MRAK078262-CCRK, MRAK018538-CS, and MRAK053119-Corin) might have a role in the pathogenesis of ischemic heart failure[85]. Despite its reported role in vascularization, no critical role for MALAT-1 was found in pressure overload-induced heart failure in mice[86]. Study showed that the plasma levels of lncRNA LIPCAR were elevated in HF patients with reduced ejection fraction, and increased levels of lncRNA LIPCAR were closely associated with left ventricular remodeling and poor outcomes[86]. In a doxorubicin-induced HF model, both lncRNA CHRF and TGF-β1 were found to be up-regulated *in vivo* and *in vitro*. Valsartan supplementation alleviated the cardiac dysfunction and injury in HF, while overexpression of CHRF reversed the cardiac protective effect of Valsartan *in vivo*[87]. This study demonstrated that Valsartan might protect from doxorubicin-induced heart failure through CHRF/TGFβ1 signal pathway. Kcna2, a newly discovered 2.52-kb native lncRNA, was increased in the left ventricular myocardium of rats with congestive heart failure (CHF). Kcna2 silencing by Kcna2 antisense RNA (Kcna2 AS) resulted in reducing delayed rectifier potassium current (I_K),

prolonging action potential (AP), and decreasing occurrence of ventricular arrhythmias. Kcna2 knockdown in the heart decreased the I_{Ks} and prolonged APs in cardiomyocytes, which is consistent with the changes observed in heart failure. Conversely, Kcna2 overexpression in the heart significantly attenuated the CHF-induced decreases in the I_{Ks}. Thus, Kcna2 AS may be a new therapeutic for the prevention and treatment of ventricular arrhythmias in patients with HF[88].

LncRNAs as biomarkers in CVDs

Creatine kinase (CK), creatine kinase isoenzyme (CK-MB), and type B natriuretic peptide (BNP), are well-established biomarkers for AMI and are widely used in clinical practice. As recommended by the American Association for Clinical Chemistry, one perfect biomarker should able to precisely reflect the progression of diseases and meet following requirements: high stability, convenience to be detected with minimal or non-invasive procedures, high sensitivity, and excellent specificity. There is an increasing appreciation that altered expressions of lncRNAs are associated with stage-specific CVD pathologies in human patient cohorts. In comparison with those conventional biomarkers mentioned above, lncRNAs seem to have superior effect and better performance due to their relative stability and distinct expression levels in plasma.

Increased expression levels of aHIF, KCNQ1OT1 and LIPCAR, but decreased expression levels of HOTAIR, UCA1, MIAT, MALAT1, ANRIL, and CPNE3 were observed in ST-elevation myocardial infarction (STEMI) patients. Moreover, the diagnostic values of these lncRNAs for CVDs patients were further evaluated by

receiver operating characteristic curve (ROC) analyses. The ROC curve showed that LIPCAR (AUC=0.782, 95% CI: 0.707-0.0.894) had better diagnostic accuracy. Furthermore, correlation analysis indicated that LIPCAR was positively correlated with myocardial enzymes, but negatively correlated with left ventricular ejection fractions. More interestingly, the expression level of LIPCAR in STEMI patients was downregulated after coronary angioplasty (P<0.05), suggesting that LIPCAR can serves as a warning sign for the diagnosis of STEMI (HR=5.93; 95% CI, 1.46-9.77; P=0.001) [88]. Plasma level of LIPCAR was positively associated with age (R = 0.201, P < 0.001), but negatively associated with HDL-C (R = −0.203, P < 0.001). Stratification analysis showed that LIPCAR was more prominent in younger subjects (Adjusted OR = 1.306, 95% CI = 1.061–1.607, P = 0.012), non-diabetic subjects (Adjusted OR = 1.227, 95%CI = 1.090–1.382, P = 0.001), and non-smoking subjects (Adjusted OR = 1.682, 95% CI = 1.198–2.361, P = 0.003)[88]. In short, LIPCAR levels are associated with the risks of CAD and may be utilized as novel biomarkers for CAD. Plasma lncRNA ANRIL expression was significantly upregulated in patients with in-stent restenosis (ISR), and higher ANRIL expression was associated with ISR. Multiple logistic regression models showed that ANRIL (OR=2.21, 95% CI: 1.68-2.92, P<0.001), drinking (odds ratio [OR]=2.09, 95% CI: 1.08-4.04, P=0.028), hypertension (OR=2.01, 95% CI: 1.14-3.57, P=0.017), diabetes (OR=3.15, 95% CI: 1.63-3.57, *P*<0.001), and low-density lipoprotein (OR=3.14, 95% CI: 1.57-6.31, P=0.001) were the independent risk factors for ISR[89]. The ROC of plasma ANRIL was 0.745, suggesting that ANRIL is an optimal prognostic factor for ISR. Additionally, the specific mitochondrial

lncRNAs uc004cov.4 and uc022bqu.1 were reported to be upregulated in patients with hypertrophic obstructive cardiomyopathy, and could be used as biomarkers for cardiac remodeling in patients with hypertrophic cardiomyopathy[90].

Current technical limitations in the extraction and quantification procedures for circulating ncRNAs need to be circumvented in order to implement solid protocols for their consideration as clinically useful biomarkers. In fact, confronted with many functions of lncRNAs, investigation into which lncRNA is specifically and extensively expressed at different stages of CVDs, with an excellent diagnostic and prognostic outcome can be a hard nut to crack. Interestingly, Cheng et al. [91] reported that a new integrated microfluidic system equipped with highly sensitive field-effect transistors could be used to extract and isolate target miRNA from extracellular vesicles in the blood, which allowed early diagnosis of CVDs by detecting the level of the target miRNA biomarker. Mabbott et al.[92] also proposed a paper-based diagnostic test, which combine paper-based three-dimensional microfluidic technology, colorimetric detection and surface enhanced Raman scattering analysis, to rapidly detect miR-29a in AMI patients. These newly reported techniques can also be used to detect serum/plasma levels of lncRNA in the diagnosis of CVDs.

Future Perspectives and Conclusion

Accumulating evidences shows that lncRNAs are aberrantly expressed in CVD patients and these dysregulated lncRNAs play causal roles in the physiological and pathophysiological processes of CVDs by affecting cell differentiation, proliferation,

apoptosis, necrosis and autophagy. Although increasing genetic, experimental, and epidemiologic evidences has suggested critical roles for lncRNAs in chromatin alteration, RNA transcription/splicing/stability/translation, and miRNA sequestration, investigations into their cellular functions, implications in human diseases, and molecular mechanisms involved remain at its infancy stage.

Recently, the successful application of clustered regulatory interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) technology to plant models has given us new inspiration. The lncRNAs editing protocol based on CRISPR-Cas9 technology has been widely used in a variety of CVDs. The utilization of genome editing with CRISPR/Cas9 system represents a promising opportunity for designing effective therapeutics for CVDs. For example, targeting the lncRNA-UCA1 via specifically designed gRNAs of CRISPR/Cas9 system effectively inhibited the proliferation, migration and invasion of cells *in vitro*. Moreover, a tissue-specific promoter could be used to drive Cas9 expression, thus providing a relatively high specificity of CRISPR/Cas9 for specific cells. However, study showed that only 38% of 15929 lncRNAs loci are safely amenable for CRISPR/Cas9 applications, while the remaining lncRNA loci are risky to be edited via CRISPR/Cas9, suggesting that some but not all lncRNAs regions can be targeted by CRISPR/Cas9 system. Several new techniques, such as SpCas9-HF1[93], a high-fidelity variant harboring alterations designed to reduce non-specific DNA contacts, have been utilized to reduce the off-target effects of CRISPR/Cas9. Interestingly, a combination of both CRISPR/Cas9 based targeted disruption as well as CRISPR/Cas9 based targeted knock-in rescue

approaches could be applied for a mammalian positional cloning study to better define the functional and quantitative trait of lncRNAs. Using these 3 novel gene editing approaches, lncRNA Rffl-lnc1, transcribed from a region on HSA17 strongly linked to cardiac QT-interval, has been shown to be important for the pleiotropic regulation of both cardiac QT-intervals and blood pressure[94].

Although growing number of studies have reported that lncRNAs are excellent biomarkers for CVDs, identifying the altered expressions of lncRNAs which are closely and specifically associated with stage-specific CVD in patients remains a major obstacle to surmount. Specifically, many technical and analytical factors should be carefully considered by investigators in designing and implementing biomarker studies of lncRNAs to ensure their accuracy and reproducibility[94, 95]. Particularly, the cellular sources of lncRNAs (cardiac cells, VSMCs, ECs, macrophages, or other inflammatory cells), the cell or tissues specificity of target lncRNAs, sample types (whole blood, serum, or plasma), sample preparation and storage conditions, the quality of blood samples (e.g., hemolysis), the methods for RNA extraction and detection (quantitative RT-PCR, lncRNA microarrays, and next-generation RNA-seq), methods for data normalization (using the intrinsic/housekeeping RNAs, or the spike-in lncRNAs), and medications used by individual (heparin and antiplatelet drugs), are several critical factors among many others. Therefore, more investigations into the diagnostic and prognostic applications of the dysregulated lncRNAs in CVDs are urgently needed before lncRNAs can be reliably used as biomarkers for CVDs.

AUTHOR'S CONTRIBUTIONS

Each author made substantial contributions to the manuscript in writing and editing. All authors read and approved the final manuscript.

FUNDING

This project was supported by the Natural Science Foundation of Anhui Province (KJ2019A0246), the Natural Science Foundation of Anhui Province (1808085MH235), the China Postdoctoral Science Foundation (NO: 2019M662207). This work was also partially supported by British Heart Foundation (PG/15/11/31279, PG/15/86/31723, PG/16/1/31892, and PG/20/10458 to QX). This work forms part of the research portfolio for the National Institute for Health Research Biomedical Research Centre at Barts.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPLIANCE WITH ETHICS GUIDELINES

Cheng-xin Zhang, Kai-yuan Niu, Pan-pan Lian, Ying Hu, Tao Xu, Sheng-lin Ge, Qing-zhong Xiao and Zhao-lin Chen declare that they have no conflict of interest.

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Figure legend

Figure 1.

The major classifications of lncRNAs. A: LincRNAs, positioned between two protein coding-genes. B: Intronic lncRNAs, transcribed from an intron of a protein-coding gene in either direction. C: Sense lncRNA, transcribed from sense strands of protein-coding gene. D: aslncRNAs, transcribed from complementary strands of protein-coding gene. E: Bidirectional lncRNAs, transcribed from the same promoter as a protein-coding gene, but in the opposite direction.

Figure 2. Mechanisms of action of lncRNAs. (1) Induce chromosome conformational changes by modulating histone modifications. (2) Function as transcriptional activator. (3) Act as transcriptional repressor. (4) Serve as scaffold to bring together multiple functional proteins including transcriptional factors, mRNAs, and other ncRNAs. (5) Regulate mRNA stability. (6) Control mRNA alternative splicing. (7) Decay miRNAs or other ncRNAs/mRNAs.

Table 1. LncRNAs in different species.

Table 2. Expression and functions of LncRNAs in CVDs.

Table 3. LncRNAs as biomarkers in CVDs

Figure 1

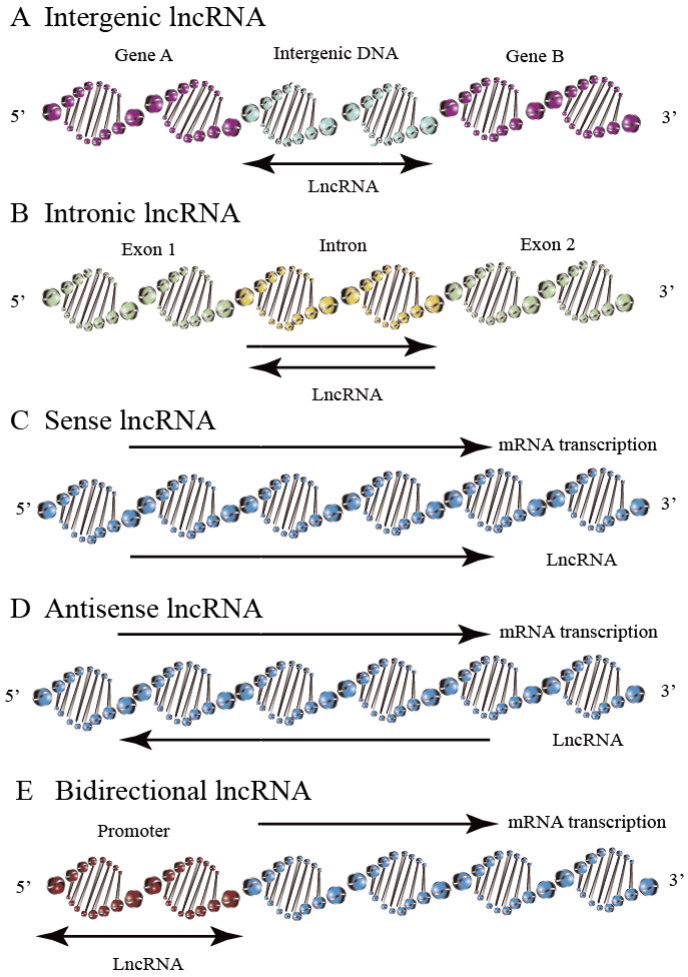
A Intergenic lncRNA
Gene A
Intergenic DNA
Gene B
5'
3'
LncRNA
B Intronic lncRNA
Exon 1
Intron
Exon 2
5'
3'
LncRNA
C Sense lncRNA
mRNA transcription
5'
3'
LncRNA
D Antisense lncRNA
mRNA transcription
5'
3'
LncRNA
E Bidirectional lncRNA
Promoter
mRNA transcription
5'
3'
LncRNA

Figure 2

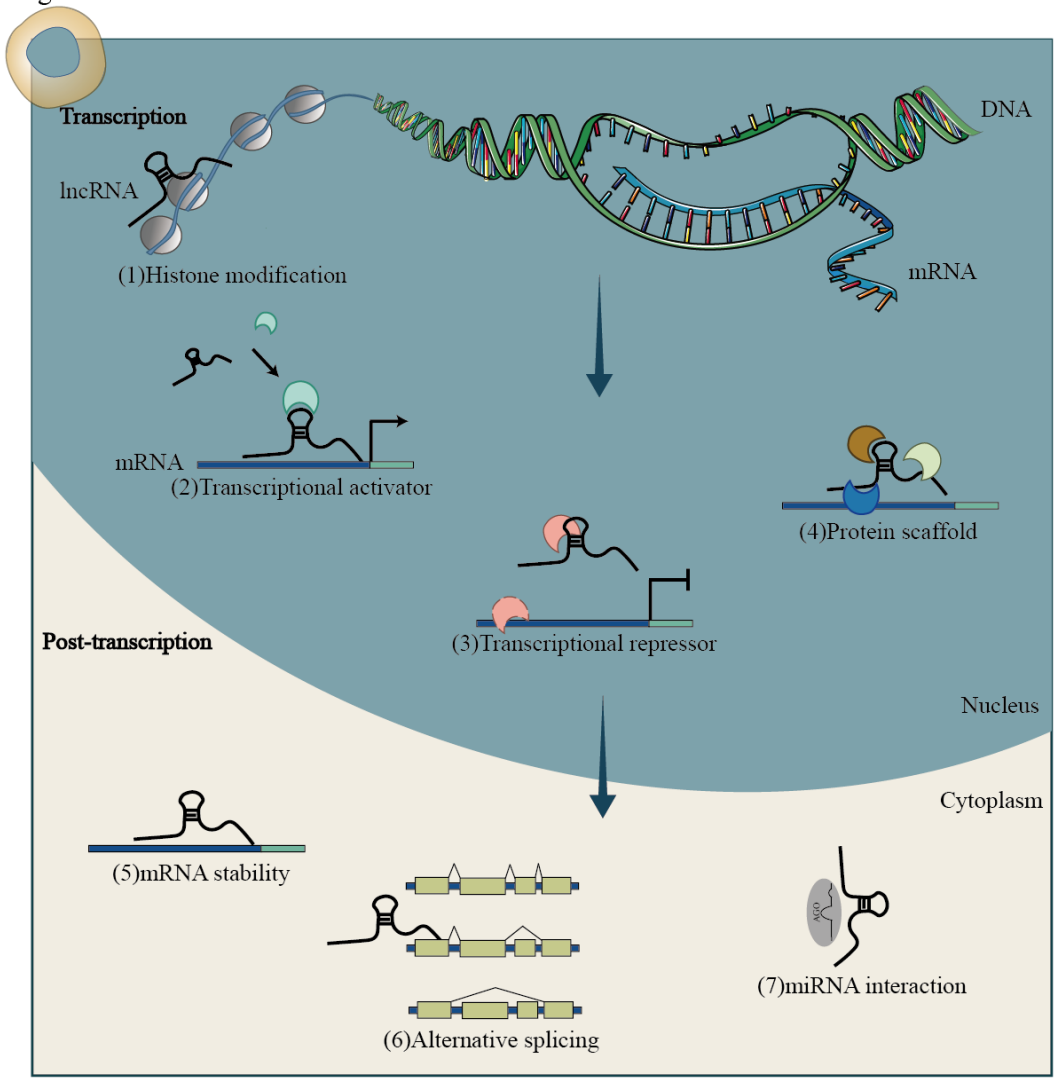
Transcription
lncRNA
(1)Histone modification
DNA
mRNA
mRNA
(2)Transcriptional activator
(4)Protein scaffold
(3)Transcriptional repressor
Post-transcription
Nucleus
Cytoplasm
(5)mRNA stability
(6)Alternative splicing
(7)miRNA interaction

Table 1. LncRNA in different species

Species	Number of lncRNA transcripts	Number of lncRNA genes
Human	172,216	96,308
Mouse	131,697	87,774
Cow	23,515	22,227
Rat	24,879	22,127
Chimp	18,004	12,790

Table 2. Expression and functions of LncRNAs in CVDs

LncRNA	Genomic Category	Neigh-boring Gene	Location	Cytology	Expression	Function	Reference
UIHTC/ NONHSAT094064	N.A	N.A	N.A	MI	+	Protect myocardial cells from apoptosis	PMID: 29887450
HOTAIR	LincRNA	HOXC11/ HOXC12	Nucleus	MI Cardiomyocytes,	-	Hypoxia-induced apoptosis	PMID: 29258067 PMID: 17604716
Meg3	LincRNA	RTL1	Nucleus	MI Cytoplasmic and nuclear (predominated in the nuclear)	+	Hypoxia-induced apoptosis	PMID: 30287867
MALAT1	LincRNA	LTBP3	Nucleus	Myocardial Tissue	+	Myocardial apoptosis Diagnosis and Therapy	PMID: 29990866
MALAT1	LincRNA	SCVL1	Nucleus	Endothelial cells (EA.hy926 cells)	N.A	N.A	N.A

UCA1	LincRNA		Cytoplasm	H9C2 cells Ischaemia/Reperfusion (I/R)	-	Biomarker I/R-induced ER stress	PMID: 26949706 PMID: 30877641
APF	LincRNA			Male mice MI	+	Autophagy and Cell death	PMID: 25858075
CAIF	LincRNA			Myocardial tissue	-	Increased autophagy	PMID: 29295976
CHRF	LincRNA			Cardiac hypertrophy	-	Reduced hypertrophic	PMID: 24557880
ANRIL	Antisense	CDKN2B	Nucleus	N.A	N.A	N.A	N.A
DEANR1	LincRNA	FOXA2	Cytoplasm and Nucleus	N.A	N.A	N.A	N.A
H19	LincRNA	IGF2/NCTC1	Nucleus	Endothelial cell	+	Adhesion Migration	PMID: 7536897 PMID: 24532688
MiAT	LincRNA	CRYBA4	Nucleus	Cardiomyocytes	+		PMID: 29157986
DANCR	LincRNA	ERVMER34	Nucleus	N.A	N.A	N.A	PMID: 22302877

Kcna2	Antisense	N.A	Nucleus	N.A	N.A	N.A	PMID: 29263036

MI, Myocardial infarction; N.A, Not Available.

Table 3. LncRNAs as biomarkers in CVDs

LncRNA	Specimen	Predicted diseases	Biomarker	AUC	Sensitivity	Specificity	Reference
H19	Plasma	CAD	Diagnosis	0.631	53.6%	73.0%	PMID: 28790415
ANRIL	Plasma	In-Stent Restenosis	Prognosis	0.745	68.4%	75.0%	PMID: 28970468
HOTAIR	Plasma	AMI	Diagnosis	N.A	N.A	N.A	PMID: 29258067
UCA1	Plasma	AMI	Diagnosis	0.757	N.A	N.A	PMID: 26949706
	Plasma	CHF	Prognosis	0.89	100%	76.12%	PMID: 28490726
	Plasma	HF	Prognosis	OR>1	NONE	NONE	PMID: 24663402
LIPCAR	Plasma	STEMI	Diagnosis	0.782	82%	75%	PMID: 30030914
	Plasma	diastolic function and remodeling	Predictors	0.745			PMID: 27874027
	Plasma	CAD	Diagnosis	0.722	72.2%	62.3%	PMID: 28790415

CAD, Coronary artery disease; AMI, Acute myocardial infarction; CHF, Congestive heart failure; HF, High FFAs; STEMI, ST-elevation myocardial infarction; N.A,

Not Available.

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