FORUM REVIEW ARTICLE

Short and Long Noncoding RNAs Regulate the Epigenetic Status of Cells

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Abstract

Significance: The concepts of junk DNA and transcriptional noise are long gone as the existence of noncoding RNAs (ncRNAs) has been tested extensively in recent years. Given that the epigenetic status of cells affects many biological processes, how ncRNAs mechanistically contribute to these processes is of great interest.

Recent Advances: Recent studies show that various ncRNAs interact with epigenetic and/or transcription factors to modulate the epigenetic status of cells directly and/or indirectly. There exists growing interest in the field of cardiovascular research to understand the roles of ncRNAs. Due to the large number of ncRNAs in the mammalian genome, only a handful of ncRNAs have been functionally elucidated, which makes it difficult to understand how ncRNAs interact with protein-coding genes and their encoded proteins.

Critical Issues: Although the canonical function of microRNAs (miRNAs) to inhibit the translation of protein-coding genes is well established, the number of functionally annotated long noncoding RNAs (lncRNAs) is still small, which is especially true in the heart.

Future Directions: Future studies must connect the epigenetic controls of various cellular phenomena by incorporating both miRNAs and lncRNAs. *Antioxid. Redox Signal.* 29, 832–845.

Keywords: epigenetics, long noncoding RNAs, microRNAs

Introduction

BY DEFINITION, EPIGENETICS is the study of chromosomal changes that are not due to a change in DNA sequence (126). Epigenetics can alter gene expressions and can be inherited to the progeny of cells or of organisms. As such, epigenetic changes are important, and various techniques have been used to uncover their presence and contributions to biological processes, including those in the heart. Although many epigenetic studies have uncovered the contribution of proteins to such processes, a growing number of studies report that molecules other than proteins are important as well. Increasing evidence suggests that most of the mammalian genomes are transcribed as RNAs; however, not all RNAs are translated into proteins. The current estimate is that most of the mammalian genomes become RNAs (64), although only a very small part corresponds to the exons of protein-coding genes. Until recently, many of the remaining RNAs were considered as functionless transcriptional noise and experimental errors; however, recent studies show that noncoding

RNAs (ncRNAs) are functional, as in the case of microRNAs (miRNAs). Besides miRNAs, longer ncRNAs have been identified. If their lengths are longer than 200 nucleotides (nt), these ncRNAs are collectively called “long noncoding RNAs (lncRNAs).” Generally speaking, lncRNAs are expressed at low levels compared to protein-coding genes but are more cell- and/or tissue-specific (15). Although lncRNAs are of great interest in the research community, their functions are largely unknown. This is especially true in the cardiovascular field, which we recently reviewed (117). Since our review, more lncRNAs have been studied in the heart. As it is estimated that the number of lncRNAs surpasses that of protein-coding genes (81) and hundreds of lncRNAs have been detected in the heart (63, 91–93), the number of functionally studied lncRNAs is still too small at the moment.

In this review, we will examine the current status of miRNAs and lncRNAs in the heart. Based on our expertise in bioinformatics, current problems in the annotation of lncRNAs will be discussed.

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miRNAs and Epigenetics

As more and more miRNAs are functionally studied in the heart, it is not hard to imagine that there are numerous miRNAs that target epigenetic and/or transcriptional factors to control transcription in general. From the standpoint of translational inhibition, it is not hard to connect such relationship in the larger context. In reality, one miRNA targets more than one mRNA. Bioinformatic predictions list hundreds, even thousands, of potential targets of one miRNA, which complicates the whole study and the understanding of the relationship between miRNAs and epigenetic control. Rather than listing all the known (*i.e.*, experimentally validated) miRNAs and their targets in the heart, we will briefly introduce the relationship between miRNAs and epigenetic controls aside from canonical mechanism of miRNAs.

MiRNAs in the nucleus

Given that translation takes place in ribosomes, it was originally thought that miRNAs loaded onto the RNA-induced silencing complex (RISC) bind to their targets outside of the nucleus. However, increasing evidence indicates the presence of RISC (90) and mature miRNAs in the nucleus (47, 54, 58, 60, 68, 99, 101, 115), which challenges the canonical mechanism of miRNAs in general. For example, both pre- and mature *miR-373* bind to the promoters of *E-cadherin* and *CSDC2* to induce their gene expressions (99). Instead of gene activation, *miR-320* directs the association of AGO1 and EZH2 with the promoter of the nearby protein-coding gene *POLR3D* by silencing its promoter (60). At the level of RNA, *miR-709* directly binds to the pri-*miR-15a/16-1* to prevent maturation, which results in the suppression of *miR-15a/16-1* to target the antiapoptotic gene *Bcl-2* (115). A more detailed mechanism was elucidated recently in zebrafish, in which *miR-9* and AGO are transported to the nucleus of quiescent adult neural stem cells *via* the RISC protein TNRC6, activating the expression of *her4* with Notch3 for quiescence of adult neural stem cells, although the exact mechanism (direct or indirect) of the activation of Notch signaling is currently unknown (58). These examples suggest more epigenetic controls of miRNAs inside of the nucleus (Fig. 1).

FIG. 1. Nuclear miRNAs. (A) Both pre- and mature *miR-373* bind to the promoters of *E-cadherin* and *CSDC2* in a human prostate cancer cell line “PC-3.” (B) *miR-320* binds to the promoter of *POLR3D* to silence its expression in a human kidney cell line “HEK-293.” (C) Mouse *miR-709* directly binds to the pri-*miR-15a/16-1* to prevent their maturations. (D) *miR-9* controls the Notch signaling pathway in the zebrafish adult neural stem cells for their quiescence status. miRNA, microRNA

miRNAs and super-enhancers

In 2013, a new enhancer domain was reported, which is called “super-enhancers” (45, 138). By definition, a super-enhancer encompasses a cluster of enhancers and is bound by several transcriptional regulators, which control the cell-type-specific expressions of a number of genes (138). Recent studies show that super-enhancers are involved in the regulation of miRNAs at the transcriptomic level (31, 109). Furthermore, a member of the bromodomain and extra-terminal domain family of epigenetic readers BRD4, which marks super-enhancers (29), is suppressed by *miR-9* through its binding to the 3'-UTR of *BRD4* gene in cardiomyocytes (107). These studies suggest regulation of epigenetic remodeling by miRNAs.

LncRNAs and Epigenetics

Definition, identification, and functional characterization of lncRNAs

As we recently surveyed (134), the number of protein-coding genes is steadily decreasing in the annotation provided by the Ensembl database, while the number of lncRNAs is increasing (Fig. 2). This is mostly due to the fact that previously thought protein-coding genes, which are categorized as such based on the computational predictions, are not anymore protein coding as more evidence (again based mostly on the computational predictions) suggests that they are noncoding. Thus, Ensembl frequently updates its annotations. This is problematic as RNA sequencing (RNA-seq) data analysis changes depending on annotations, such as the biotype and length of a particular transcript. Such changes cause problems in the downstream analysis, especially in the quantification of transcripts (*e.g.*, FPKM values) (134).

Although the abovementioned problems of RNA-seq exist, there is no other technology currently available to perform unbiased, genome-wide screening of lncRNAs. As the cost of next-generation sequencing (NGS) has been decreasing, RNA-seq is increasingly being used to analyze transcriptomes of various conditions, including those in the heart. Currently, there are numerous RNA-seq data sets available from the sequence read archive, most of which are already

FIG. 2. Numbers of human protein-coding genes and ncRNAs. The statistics is based on the gene counts in the primary assembly provided by the Ensembl database (www.ensembl.org/Homo_sapiens/Info/Annotation). Of note, the category for other ncRNAs ("miscellaneous other ncRNAs") became available from the GRCh38.p2 assembly. In GRCh37.p7 and NCBI 36, only "RNA genes" category exists for all kinds of ncRNAs. lncRNA, long noncoding RNA; ncRNA, noncoding RNA.

published in the literature. However, only few data sets have been analyzed for lncRNAs (63, 91–93). More importantly, many data sets were analyzed with the older version of genome assembly (*i.e.*, hg19) rather than the newest version (*i.e.*, GRCh38/hg38). As we have recently shown (134), the mapping rate is better using GRCh38/hg38 than hg19, which means that more sequencing reads can be utilized for further analysis. In other words, secondary analysis of RNA-seq is necessary to maximize the power of RNA-seq to identify lncRNAs and quantify them using the latest genome assembly and annotation.

As the cost of RNA-seq decreases and the protocol to perform it is standardized, the screening of transcripts using RNA-seq is no longer a challenge for laboratories around the world. Thus, various transcripts, including lncRNAs, can be identified and associated with a particular pathophysiological condition by analyzing the data as such (mostly guilt-by-association). The challenge now is to functionally characterize the identified lncRNAs. Despite the fact that lncRNAs interact with DNA, RNA, and proteins, the current consensus in the field of lncRNAs is that lncRNAs carry out their functions largely *via* interaction with proteins (36). Thus, it is essential that binding proteins be identified using biochemical assays, such as RNA pull-down (104) or chromatin isolation by RNA purification (22). Once the binding proteins are identified, research continues to find out how the interaction with the target lncRNAs and their associated proteins is affected on loss of the target lncRNA, which can be achieved with siRNAs and/or LNA GapmeRs. LNA GapmeRs is an especially important technology as it can be used *in vivo* (82, 121), which opens avenues for clinical use. Given that many lncRNAs are cell-type specifically expressed, LNA-GapmeR-based knockdown of lncRNAs might avoid off-target effects in other cell types. Given the power and accessibility of the CRISPR/Cas9 system, it is becoming more important to completely ablate the target lncRNA instead of transiently silencing it. However, there exists a challenge in such genetic tools as the complete deletion of the target lncRNA is required as interference of translation is not possible for lncRNAs as in the case of protein-coding gene. Thus, in the case of the CRISPR/Cas9 system, two guide RNAs designed to flank the target lncRNA are required to

achieve complete ablation. Alternatively, poly-A signal can be inserted to truncate an lncRNA as in the case of *Lockd* (94). As more experimental tools are available, more functional studies are necessary to uncover the functions of lncRNAs and their associated proteins, which are much more challenging tasks to be achieved than simply screening by RNA-seq experiments.

lncRNAs and genomic imprinting

Classically, lncRNAs are known for their activity of epigenetic regulation *via* their action as genomic imprinting, which is an epigenetic phenomenon through which genes are expressed in a parent-of-origin dependent manner (98). Such imprinting lncRNAs include *Airn* (65), *H19* (6, 103), *MEG3* (83), and *MEG8* (19) (the complete list of imprinting genes can be found in the Geneimprint database [www.geneimprint.com]). Besides the classic imprinting lncRNAs, the lncRNA *XIST* is

FIG. 3. Functionally characterized lncRNAs in relation to interacting partners in the cardiomyocytes. APF, autophagy promoting factor; CARL, cardiac apoptosis-related lncRNA; CARMEN, CARDiac mesoderm enhancer-associated noncoding RNA; Chaer, cardiac hypertrophy-associated epigenetic regulator; CHRF, cardiac hypertrophy-related factor; Mhrt, myosin heavy chain-associated RNATranscripts; PRC2, polycomb repressive complex 2.

known to silence one X chromosome during the development of female mammals (11–14). Recent studies show that *XIST* interacts with several proteins [e.g., a member of the SWI/SNF family of chromatin remodeling proteins ATRX (106), a member of the SPEN family of transcriptional repressors SHARP (79)] to directly regulate the exclusion of RNA polymerase II on the X chromosome, thereby suppressing the X chromosome [reviewed extensively in Ref. (25)]. Furthermore, other lncRNAs [i.e., *XACT* (119), *TSIX* (66, 67)] are also involved in controlling X chromosome activity *via* interacting or

competing with *XIST*. As is the case for imprinting lncRNAs and *XIST*, many imprinting lncRNAs interact with epigenetic factors and other proteins to control the epigenetic status of the loci that they target, highlighting the importance of lncRNAs in the context of epigenetic control of the development of an organism.

LncRNAs in the heart

In the heart, only a handful lncRNAs have been elucidated with respect to their functions, which we have recently

TABLE 1. LIST OF LONG NONCODING RNAs KNOWN TO BE FUNCTIONAL IN THE HEART

<i>lncRNA</i>	<i>Organism(s)</i>	<i>Disease/condition</i>	<i>Functions in the heart</i>	<i>References</i>
<i>APF</i>	Mouse	Myocardial infarction	Binds <i>miR-188-3p</i> , which targets <i>Atg7</i> to control cardiac autophagy.	(127)
<i>Braveheart</i>	Mouse	ES-derived cardiomyocytes	Binds PRC2 and CNBP/ZNF9 to regulate cardiovascular lineage commitment.	(61, 141)
<i>CARL</i>	Mouse	Myocardial infarction	Binds <i>miR-539</i> , which targets <i>Phb2</i> for mitochondrial fission and apoptosis.	(131)
<i>CARMEN/MIR143HG</i>	Human, mouse	Aortic stenosis, idiopathic dilated cardiomyopathy, myocardial infarction	Arises from the super-enhancer region and binds PRC2 to control the expression of cardiac genes; and controls MIR-143/145 expression and the smooth muscle cell fate in adult cardiac progenitor cells.	(91, 100)
<i>Chaer</i>	Human, Mouse	Hypertrophy	Binds PRC2 to inhibit H3K27 methylation at the promoter regions of genes involved in cardiac hypertrophy.	(132)
<i>Chast</i>	Human, mouse	Aortic stenosis, hypertrophy	Regulates neighboring <i>Plekha1</i> gene (<i>cis</i> -regulation), which is a regulator of autophagy.	(121)
<i>CHRF</i>	Human, mouse	Heart failure, hypertrophy	Binds <i>miR-489</i> , which targets prohypertrophic factor <i>Myd88</i>	(129)
<i>DWOLF</i>	Human, mouse	Ischemic heart failure	Encodes a 34aa peptide and enhances SERCA activity for myocyte contractility.	(86)
<i>H19</i>	Human, rat, mouse	Calcific aortic valve disease, diabetic cardiomyopathy, hypertrophy,	Encodes <i>miR-675</i> , which targets <i>CaMKIIδ</i> for cardiac hypertrophy, <i>VDAC1</i> for cardiomyocyte apoptosis, and <i>UPSP10</i> in c-kit+ cardiac progenitor cells; prevents the recruitment of p53 to the promoter of <i>NOTCH1</i> .	(16, 39, 72, 74)
<i>LINC00323/lnc-DSCAM-1</i>	Human	EHTs	Binds eIF4A3 to control the translation of <i>GATA2</i> mRNA.	(33)
<i>lncRNA-ROR</i>	Mouse	Hypertrophy	Binds <i>miR-133</i> to attenuate the effects of cardiac hypertrophy.	(55)
<i>Mhrt</i>	Human, mouse	Hypertrophy	Binds Brg1 to interfere its genomic DNA binding capability that causes aberrant gene expression and cardiac myopathy.	(40)
<i>MIAT</i>	Rat, mouse	Angiotensin II-induced H9c2 cells	Binds <i>miR-150</i> , which is possibly linked to the enhancement of cardiac hypertrophy.	(149)
<i>MIR503HG/lnc-PLAC1-1</i>	Human	EHTs	Regulates neighboring <i>miR-424</i> to control angiogenesis.	(33)
<i>NRF</i>	Mouse	Hydrogen peroxide-treated neonatal cardiomyocytes	Binds <i>miR-873</i> , which targets procardiomyocyte necrosis factors RIPK1 and RIPK3.	(128)
<i>SMILR</i>	Human	Aortic stenosis	Regulates neighboring <i>HAS2</i> gene (<i>cis</i> -regulation).	(5)
<i>Upperhand</i>	Mouse	Knockout mice	Its transcription (but not its RNA) is required in <i>cis</i> to promote <i>Hand2</i> expression <i>via</i> GATA4 binding and super-enhancer activity.	(4)

“EHTs” stands for “human-induced pluripotent stem cell-derived engineered heart tissues.”

aa, amino acids; APF, autophagy promoting factor; CARL, cardiac apoptosis-related lncRNA; CARMEN, CARDiac mesoderm enhancer-associated noncoding RNA; Chaer, cardiac hypertrophy-associated epigenetic regulator; Chast, cardiac hypertrophy-associated transcript; CHRF, cardiac hypertrophy-related factor; DWOLF, dwarf open reading frame; ES, embryonic stem cells; Mhrt, myosin heavy chain-associated RNATranscripts; PRC2, polycomb repressive complex 2.

reviewed (117). Since our review, the functions of additional lncRNAs have been elucidated to a certain extent in the heart (Fig. 3 and Table 1). With regard to epigenetic regulation, some lncRNAs [e.g., *Braveheart* (61, 141), *CARMEN* (91), *Chaer* (132)] can directly bind to epigenetic factors (namely, PRC2) to exert gene regulations. However, caution should be used, as although PRC2 is known to specifically bind RNAs, the number of PRC2-bound RNAs is very high (23, 26, 27). Although such direct interaction of lncRNAs with epigenetic factors is easy to understand, some lncRNAs function as miRNA sponges to sequester otherwise available miRNAs (127, 129, 131, 149). For example, the lncRNA *CARL* binds *miR-539*, which targets one of the two subunits of prohibitin complex *Phb2* for mitochondrial fission and apoptosis (131). Although this study investigated their effects in mitochondria, *PHB2* is an estrogen receptor-selective coregulator and acts as a mediator of transcriptional repression (84), suggesting a potential mechanism in which lncRNA controls the epigenetic status *via* miRNA binding. More recently, a promoter-associated lncRNA called “*Upperhand (Uph)*” was reported to be important for its transcription (but not its RNA) in *cis* to promote cardiac transcription factor *Hand2* expression *via* GATA4 binding and super-enhancer activity (4). In line with this study, recent bioinformatic analysis of long intergenic ncRNAs (lincRNAs) shows that many lincRNAs are associated with enhancers/promoters and control the expression of nearby protein-coding genes (especially, *cis*-regulation of disease-associated/suspected genes) (46, 113), suggesting that many lncRNAs may alter the epigenetic status of nearby protein-coding genes. In line with this, experimental evidence suggests that super-enhancers are also controlled by lncRNAs (97, 139) as in the case of the lncRNA *CARMEN* in the heart (91).

As it is estimated that the number of lncRNAs exceeds that of protein-coding genes (81) and hundreds of lncRNAs have

been detected in the heart (63, 91–93), the number of functionally studied lncRNAs is too low at the moment. Although the usage of knockdown technologies (e.g., siRNAs, LNA GapmeRs) is convenient, all phenotypes must be confirmed genetically using knockout (KO) mice (7). In the cardiovascular field, only a handful of lncRNAs [e.g., *Chaer* (132), *DWOLF* (86), and *Uph* (4)] have been validated *via* KO mice. Thus, extensive research is needed to firmly establish the importance of lncRNAs in cardiovascular biology; in particular, their potential connections to cardiovascular disease are still poorly understood (Figs. 4 and 5).

Circular RNAs

Aside from lncRNAs, an emerging field is that of circular RNAs (circRNAs), which are by-products of splicing events (more specifically, “backsplicing”) of mostly protein-coding genes (10, 52, 53). As early as the 1990s, it was known that circRNAs are stable and localized predominantly in the cytoplasm (24, 88). With the increased usage of NGS, bioinformatic tools have been built to detect circRNAs from RNA-seq data [reviewed recently in Refs. (42, 110)], including those in the heart (50, 59, 114, 130, 137). When some circRNAs are knocked down *via* siRNAs, it has been reported that there are phenotypes observed, which may not be observed when the parental transcripts of circRNAs (e.g., protein-coding genes whose exons [or introns] give rise to circRNAs) are knocked down (10, 35). Some studies suggest that circRNAs function as miRNA sponges (34, 41, 76, 80, 148). However, recent comprehensive bioinformatic analysis (38) and our biological validation experiments (10, 136) indicate that circRNAs acting as miRNA sponges are extremely rare. A recent study suggests yet another mechanism in which circRNAs might function; that is, they encode for proteins

FIG. 4. LncRNAs involved in myocardial infarction. (A) The lncRNA *APF* sequesters *miR-188-3p* to impede its binding to the target *Atg7*, which results in upregulation of *Atg7* to control cardiac autophagy in cardiomyocytes. (B) *CARL* binds *miR-539*, which targets *Phb2* for mitochondrial fission and apoptosis in cardiomyocytes. (C) *CARMEN* (also known as “*MIR143HG*”) is transcribed from the super-enhancer region and binds PRC2 to control expressions of cardiac genes in cardiomyocytes. Of note, although located on the same locus, *CARMEN* gives rise to its transcripts independently from the *MIR-143/145* precursor. (D) *DWOLF* encodes a 34aa peptide and enhances SERCA activity for myocyte contractility in cardiomyocytes. aa, amino acid; DWOLF, dwarf open reading frame.

FIG. 5. LncRNAs involved in cardiac hypertrophy. (A) *Chaer* binds PRC2 to inhibit H3K27 methylation at the promoter regions of hypertrophy-related genes in cardiomyocytes. (B) *Chast* regulates neighboring *Plekhm1* gene (*cis*-regulation), which is a regulator of autophagy in cardiomyocytes. (C) *CHRF* binds *miR-489*, which targets prohypertrophic factor *Myd88* in cardiomyocytes. (D) *H19* harbors *miR-675*, which targets *CaMKIId* for cardiac hypertrophy and *VDAC1* for cardiomyocyte apoptosis. (E) *Mhrt* binds Brg1 to interfere its genomic DNA binding capability that causes aberrant gene expression and cardiac myopathy in cardiomyocytes.

(143). By using bioinformatic techniques and validation by biological experiments, the authors demonstrated that 25 out of 7700 circRNAs detected in human fibroblasts possess coding potential, although their functions are currently unknown.

One interesting feature of circRNAs is that they are more stable than noncircular RNAs (*e.g.*, lncRNAs) as RNases are not easily accessible due to absence of free ends of RNA. Because of their stability, they may exist in the blood; indeed, several studies reported the detection of circRNAs in the circulation, which points to possible use of circRNAs as biomarkers (62, 77). However, more functional studies are needed to uncover the potential functions of circRNAs and their possible relationship to cardiovascular disease.

LncRNAs and Bioinformatics

As already mentioned above, RNA-seq is the preferred technology to detect lncRNAs in various experimental contexts. Although there are several well-established bioinformatic tools available to analyze RNA-seq data to detect lncRNAs [reviewed extensively in Refs. (48, 120)], depending on the genome assembly/annotation and normalization method used, the results differ significantly in the identification of lncRNAs and their expression values (2, 71, 108). The problem arises from the reference genome to be used for mapping the sequencing reads and the annotation of transcripts/genes (*i.e.*, gene transfer format [GTF] file) (134). Furthermore, transcripts that are not included in the annotation for assembling RNA-seq alignments also cause an additional problem. Our previous study (135) shows

that 77,656 novel isoforms of annotated reference transcripts and 102,848 intergenic transcripts are identified, with 58,789 (75.70%) and 101,993 (99.17%) being predicted as noncoding, respectively, from 12 human tissues (87), while 181,434 annotated transcripts (87.13% out of 208,244 transcripts in Ensembl version 77) are expressed in at least 1 of 12 tissues analyzed. Although we could validate the presence of novel lncRNAs by reverse transcription polymerase chain reaction (RT-PCR) experiments, many novel lncRNAs contain repetitive elements, such as microsatellites (9) and short interspersed nuclear elements, including ALU elements (43), suggesting that novel lncRNAs detected from RNA-seq must be examined carefully before proceeding to biological experiments.

Since the presence of many annotations for a particular lncRNA constitutes a significant problem, we developed a bioinformatic tool called "UGAHash" web tool (134). UGAHash ("Universal Genomic Accession Hash") is an algorithm for creating consistent nonconflicting accessions for genomic features asynchronously. In our UGAHash web tool, annotations from the Ensembl (versions 60–84), LNCipedia (versions 1.2, 2.1, and 3.1) (122, 123), NCBI (versions 103–107), NONCODE (versions 4 and 2016) (140, 144, 145), and lincRNA catalog (www.broadinstitute.org/genome_bio/human_lincrnas/?q=lincRNA_catalog) are included so that when a researcher identifies a novel transcript, it is possible to check its existence in major databases above.

Although the identification of lncRNAs is important, it is only the start of a project, especially for those lncRNAs that are identified in humans. The emergence of induced

pluripotent stem cells (112) and the CRISPR/Cas9 system (44, 49, 51, 57) has led to advances in functional studies using human cells, but it is still not possible to understand the functions of lncRNAs *in vivo* without using model organisms. Given that the sequence conservation of lncRNAs from one species to another is lower than that of protein-coding genes (28), it is a challenge to identify a homolog of a human lncRNA. To this end, instead of searching for sequence conservation, genomic positional conservation has been used recently (118, 133), which is based on the presence of conserved protein pairs, defined as the set of all pairs of adjacent protein-coding genes within an organism's genome that are also adjacent when compared to orthologs of other species. Building on this conservation of lncRNAs, we introduced a series of knowledge databases to assist researchers interested in screening for lncRNAs in humans, mice, and zebrafish: "ANGIOGENES" (85) for expression profiles of transcripts in endothelial cells; "C-It-Loci" (133) for evolutionary-conserved, tissue-enriched, protein-coding genes and lncRNAs; and "RenalDB" (136) for enriched/specific transcripts with respect to nephrotic tissues and cells, developmental stages, and other metadata. Besides our knowledge

databases, there are many excellent lncRNA databases currently available (Table 2). For example, besides the hallmark databases of lncRNAs NONCODE (145), LNCipedia (122), lncRNAdb (2a, 102), and NRED (30), ALDB (69) provides expression profiles of cow, pig, and chicken lncRNAs; lncRBase (17) for expression profiles of human and mouse lncRNAs from microarray data; and lncRNAtor (96) for flies, worms, and yeasts. In the case of disease-related lncRNAs, Catalogue of Cancer Genes/lncRNAs (CCG) (75) provides manually curated information for protein-coding genes and lncRNAs in various cancers; KTCNlncDB (111) for keratoconus (KTCN) and non-KTCN human corneas; lnc2Cancer (89) for manually curated information regarding 666 human lncRNAs from 97 human cancers; lncRNADisease (20) for both manually curated and predicted relationship to various diseases; and TRANRIC (http://ibl.mdanderson.org/tanric/_design/basic/index.html) for expression profiles from over 8000 tumor samples of ~20 The Cancer Genome Atlas cancer types. Although not necessary in the causal relationship to diseases in most cases, lncRNASNP (37) provides single-nucleotide polymorphisms in human/mouse lncRNAs; and lncVar (21) for lncRNA-associated genetic variations in

TABLE 2. LIST OF LONG NONCODING RNA DATABASES

Database	URL	Organisms	Expressions?	References
ALDB	http://res.xaut.edu.cn/aldb/index.jsp	Cow, pig, chicken	Yes	(69)
ANGIOGENES	http://angiogenes.uni-frankfurt.de	Human, mouse, zebrafish	Yes	(85)
C-It-Loci	http://c-it-loci.uni-frankfurt.de	Human, mouse, zebrafish	Yes	(133)
CCG	http://ccg.xingene.net	Human	Yes	(75)
ChIPBase	http://rna.sysu.edu.cn/chipbase/	Human, mouse, rat	No	(142)
Co-lncRNA	www.bio-bigdata.com/Co-lncRNA/	Human	Yes	(147)
DIANA-lncBase	http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex	Human, mouse	Yes	(95)
Ensembl	www.ensembl.org/	Many	No	(105)
Entrez Gene	https://www.ncbi.nlm.nih.gov/gene	Many	No	(1)
KTCNlncDB	http://rhesus.amu.edu.pl/KTCNlncDB/	Human	Yes	(111)
lnc2Cancer	www.bio-bigdata.com/lnc2cancer/	Human	No	(89)
lncATLAS	lncatlas.crg.eu/	Human	Yes	(78)
LNCipedia	www.lncipedia.org/	Human	No	(122)
lncRBase	http://bicresources.jcbose.ac.in/zhumur/lncbase/	Human, mouse	Yes	(17)
lncRNAdb	www.lncrnadb.org/	Many	Yes	(2a, 102)
lncRNADisease	www.cuilab.cn/lncrnadisease	Human	Yes	(20)
lncRNAMap	http://lncnamap.mbc.nctu.edu.tw/php/	Human	Yes	(18)
lncRNASNP	http://bioinfo.life.hust.edu.cn/lncRNASNP/	Human	No	(37)
lncRNAtor	lncrnator.ewha.ac.kr/	Human, mouse, zebrafish, fly, worm, yeast	Yes	(96)
lncRNome	http://genome.igib.res.in/lncRNome/	Human	No	(8)
lncVar	http://bioinfo.ibp.ac.cn/lncVar/	9 species	No	(21)
LongTarget	http://lncrna.smu.edu.cn	Human	No	N/A
ncFANs	www.bioinfo.org/ncfans/	Human, mouse	Yes	(73)
NONCODE	www.noncode.org	17 species	Yes	(145)
NRED	http://nred.matticklab.com/cgi-bin/ncrnadb.pl	Human, mouse	Yes	(30)
RenalDB	http://renaldb.uni-frankfurt.de	Human, mouse, zebrafish	Yes	(136)
starBase	http://starbase.sysu.edu.cn/index.php	Human, mouse, worm	Yes	(70)
TF2lncRNA	https://mlg.hit.edu.cn/tf2lncrna/analyze.jsp	Human	No	(56)
TRANRIC	http://ibl.mdanderson.org/tanric/_design/basic/index.html	Human	Yes	N/A
UCSC Genome Browser	http://genome.ucsc.edu	Many	Yes	(116)

six species (human, mouse, zebrafish, worm, fruit fly, and arabidopsis). Possibly related to functional characterization of lncRNAs, Co-LncRNA (147) provides Gene Ontology annotations and KEGG pathways inferred from the co-expressed protein-coding genes; DIANA-LncBase (95) for both experimentally validated and predicted binding between lncRNAs and miRNAs; LncAtlas (78) for subcellular localization information about lncRNAs; LongTarget for the predictions of DNA binding domains and their binding sites of lncRNAs (<http://lncrna.smu.edu.cn>); starBase (70) for binding sites of RNA-binding proteins and miRNAs; and TF2LncRNA for the possible relationship between transcription factors and lncRNAs (56). On top of these valuable tools, UCSC Genome Browser (116) provides almost one-stop-shop for much of the information mentioned above through a genome viewer, which users could explore for their favorite lncRNAs by entering the genomic coordinates. Readers are strongly encouraged to explore these resources when starting an lncRNA project. We anticipate that these bioinformatic tools will facilitate further research into lncRNAs as many lncRNAs are expressed in the heart, while others are specifically expressed in a certain cell type and/or disease condition. With the option to identify homologs in other species, it is possible to utilize model organisms to understand the functions of human lncRNAs, which are provided in our databases for mice and zebrafish.

Conclusion and Outlook

Given the potential (but yet to be proven) diverse functions of lncRNAs, the most optimistic speculation is that lncRNAs might be the missing link to understand the yet unknown pathogenesis of cardiovascular disease. However, since the number of lncRNAs with known functions is very limited, considerable additional work will be needed to fully understand the impact of lncRNAs on the pathophysiology of the heart. More importantly, a paradigm shift is necessary for lncRNA research because most of the published studies are focused on their binding protein partners or miRNAs, as some lncRNAs function as miRNA sponges, which target proteins. In other words, most of the published studies are in fact analyses of proteins and/or miRNAs that consider lncRNAs as scaffolds or components of protein-centered research, based on the concept that proteins are the ultimate products controlling the pathophysiology of our body. If this concept stands, then there is a concern that lncRNAs may not be as important as one wishes, which is still early to determine because this field is only emerging. This is a concern particularly because most lncRNAs are expressed at much lower levels than protein-coding genes and proteins. However, recent reports from Eric Olson's laboratory have demonstrated that some lncRNAs are not truly noncoding, since they may code for small peptides as in the case of *DWOF* (86) and *myoregulin* (3). Since it is very hard to detect peptides less than 38 amino acids by mass spectrometry (32), mass spectrometry-based screening may not reveal such small peptides encoded by lncRNAs. Further studies are needed to understand how such small peptides impact heart functions and physiology.

As research into lncRNAs intensifies, in near future, it will yield various aspects of lncRNAs by technologically advancing the field by introducing experimental techniques that

better characterize lncRNAs. Furthermore, functions of lncRNAs other than molecular scaffolds will be elucidated, thereby enhancing our understanding of the pathophysiology of organs, including the heart. Further advances in NGS and bioinformatic tools will make it possible to identify links between additional lncRNAs, including circRNAs, and certain cardiovascular pathological conditions, which may lead to the use of lncRNAs as diagnostic biomarkers in clinical settings. Given that lncRNAs are generally more cell-type specific than mRNAs and miRNAs, by carefully selecting lncRNAs enriched in a certain cell type, it might be possible to identify a better therapeutic target for a disease as in the form of "RNA therapeutics." Finally, building on the existing knowledge, systems biology will take center stage, enabling us to predict the cause and outcome of cardiovascular disease by incorporating genes, proteins, miRNAs, and lncRNAs into one large network.

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Abbreviations Used

aa = amino acid
APF = autophagy promoting factor
CARL = cardiac apoptosis-related lncRNA
CARMEN = CARDiac mesoderm enhancer-associated noncoding RNA
Chaer = cardiac hypertrophy-associated epigenetic regulator
Chast = cardiac hypertrophy-associated transcript
CHRF = cardiac hypertrophy-related factor
circRNA = circular RNA
DWORF = dwarf open reading frame
KO = knockout
KTCN = keratoconus
lincRNA = long intergenic noncoding RNA
lncRNA = long noncoding RNA
Mhrt = myosin heavy chain-associated RNA transcripts
miRNA = microRNA
ncRNA = noncoding RNA
NGS = next-generation sequencing
PRC2 = polycomb repressive complex 2
RISC = RNA-induced silencing complex
RNA-seq = RNA sequencing
Uph = upperhand