The Encyclopedia of DNA Elements (ENCODE) and the Functional Annotation Of Mouse (FANTOM) consortiums reported that there are far more transcripts than initially anticipated at the time of human genome sequencing in 1990s. Surprisingly, it turned out that coding transcripts represent only <3% of the total genome, whereas the majority of other transcripts have no coding potential (Figure 1). The existence of the large number of noncoding transcripts has been disregard as junk DNA and transcript noises for some time, but increasing evidence suggests that a significant part of such noncoding transcripts are functionally active as RNA molecules. Noncoding RNAs can be divided into small (<200 nt) noncoding RNAs, which are microRNAs (miRs), transfer RNAs, and small nuclear RNAs, and longer RNAs (>200 nt) that include ribosomal RNAs, natural antisense transcripts and other long noncoding RNAs (lncRNAs). The present article will introduce noncoding RNA species and will summarize the current understanding of lncRNAs in cardiovascular diseases.

Abstract: In recent year, increasing evidence suggests that noncoding RNAs play important roles in the regulation of tissue homeostasis and pathophysiological conditions. Besides small noncoding RNAs (eg, microRNAs), >200-nucleotide long transcripts, namely long noncoding RNAs (lncRNAs), can interfere with gene expressions and signaling pathways at various stages. In the cardiovascular system, studies have detected and characterized the expression of lncRNAs under normal physiological condition and in disease states. Several lncRNAs are regulated during acute myocardial infarction (eg, Novlnc6) and heart failure (eg, Mhrt), whereas others control hypertrophy, mitochondrial function and apoptosis of cardiomyocytes. In the vascular system, the endothelial-expressed lncRNAs (eg, MALAT1 and Tie-1-AS) can regulate vessel growth and function, whereas the smooth-muscle–expressed lncRNA smooth muscle and endothelial cell–enriched migration/differentiation-associated long noncoding RNA was recently shown to control the contractile phenotype of smooth muscle cells. This review article summarizes the data on lncRNA expressions in mouse and human and highlights identified cardiovascular lncRNAs that might play a role in cardiovascular diseases. Although our understanding of lncRNAs is still in its infancy, these examples may provide helpful insights how lncRNAs interfere with cardiovascular diseases. (Circ Res. 2015;116:737-750. DOI: 10.1161/CIRCRESAHA.116.302521.)

Key Words: angiogenesis effect • atherosclerosis • heart failure • hypertrophy • RNA, long noncoding
Description of Noncoding RNA Species

MicroRNAs

miRs are short noncoding RNAs that are transcribed from introns or intergenic regions as primary-miR transcripts and are subsequently processed by endonucleases into mature RNAs, which are ≈22-nucleotide (nt) long. Mature miRs are incorporated into the RNA-induced silencing complex to induce translational repression or degradation of targeted mRNAs. miRs bind to the target mRNA by the miR seed region and sequences outside of the seed, which serve to augment the binding. miRs thereby can affect the expression of hundreds of targets and control gene expression patterns. Among the noncoding RNAs, miRs have been studied extensively in the cardiovascular system, and the reader is referred to reviews summarizing the effects of miRs on cardiovascular differentiation, cardiovascular aging, vascular function, and cardiac biology. Particularly, the availability of tools to block miR functions in vivo by so-called antimiRs has allowed the identification of putative therapeutic strategies to treat cardiovascular diseases.

Long Noncoding RNAs

Although the biosynthesis and biological activities of miRs are well explored, the understanding of IncRNAs is limited. IncRNAs are defined as transcripts that are longer than 200 nt and do not code for proteins to separate them from miRs and from protein-coding genes. Owing greatly to the development of next generation sequencing techniques (especially RNA sequencing [RNA-seq]), a new class of IncRNAs is discovered and annotated each year. The actual number of IncRNAs in human genome is currently known, but it may vary as it is the case for protein-coding genes, whose number fluctuates whenever a new set of annotations is made. According to NONCODE database (version 4.0) (http://www.noncode.org), which includes the most comprehensive number of transcripts to date, there are 56 018 and 46 475 IncRNA genes for human and mouse, respectively, which are high compared with the current number of human protein-coding genes, which is 20 345 (based on the GENCODE, version 19, Ensembl 74 http://www.gencodegenes.org/stats.html).

Because the number of IncRNAs is more than twice the number of protein-coding genes in human, we are still in the infancy of naming, categorizing, and validating IncRNAs. It is unclear whether all of these RNA sequences that are found by RNA-seq indeed have biological functions because only few have been characterized to date. Given such background, the followings are generally accepted categorization of IncRNAs, which is in part based on association to nearby protein-coding genes (Figure 2):

- Sense: overlapping a protein-coding gene by usually sharing the same promoter.
- Antisense: located in antisense orientation (in an opposite strand) to a protein-coding gene.
- Intronic: arising from an intron of a protein-coding gene.
- Intergenic, also known as long intergenic noncoding RNAs (lincRNAs): located in between 2 protein-coding genes.
- Enhancer, also known as enhancer RNA (eRNA): arising from the enhancer region of a protein-coding gene.
- Circular: forms a covalently enclosed circular RNA, which usually arises from splicing of a protein-coding gene.

The above rough categorization does not take into account functions of IncRNAs; simply because of the fact that only a handful of IncRNAs have been studied in detail. In below, currently uncovered functions of IncRNAs are discussed in reference to the above classifications.

Function 1: Imprinting

Imprinting is an epigenetic phenomenon in which only 1 allele of a gene (either paternal or maternal) is expressed. XIST is the
first lncRNA to be functionally described, which literally covers the whole X chromosome. Other well-studied imprinting lncRNAs are Airn, H19, and KCNQ1OT1. The complete list of imprinting genes, including protein-coding genes, can be found on the Genomic Imprinting Web site (http://www.geneimprint.com/site/genes-by-species). In general, such imprinting acts to inhibit the expression of the targeted loci, which are located near the imprinting lncRNA; thus, its mode of action can be considered as cis. Because imprinting is an important developmental process (as in the case of mouse Xist), the above studies suggest the functional importance of lncRNAs to development of an organism and a mode of actions.

**Function 2: Scaffold/Guide for Epigenetic and Transcription Factors**

Probably the most popular area of functional study of lncRNAs is uncovering their role as guiding molecules to direct various biological phenomena by recruiting functional proteins. One of the most influential studies to date in this context is the one by Mitchell Guttman and Eric Lander. In this study, the authors used functional genomics screening of 226 lincRNAs in mouse embryonic stem cells using lentiviral-based short hairpin RNAs followed by microarrays as readout. In this screening, silencing of >90% of the lincRNAs showed significant changes in gene expressions, which were in part as much as the expression changes caused by knocking down regulatory protein genes, including bona fide stem cell marker genes Oct4 and Nanog. When these high-throughput data were analyzed in detail, the authors found that most of such gene expression changes occur not for nearby genes of the knockdown lincRNA, which suggests trans (affecting protein-coding genes that are not genomic locationally near the target lincRNA) regulatory control of lincRNAs rather than cis (directly or indirectly resulting in the differential regulation of nearby (not necessary being overlapped) protein-coding genes of the target lincRNA). On the basis of this analysis, the authors perform RNA immunoprecipitation (RIP) assay, which is similar to chromatin immunoprecipitation (ChIP) that pulls down bound genomic DNA. In the case of RIP, the pulled-down RNA has been analyzed, using antibodies against 12 chromatin complexes (eg, Suz12, Suv39h1, and Hdac1), which resulted in the identification of 74 lincRNAs that were bound to ≥1 chromatin complex. From above results, authors proposed a model in which lincRNAs function as cell-type-specific flexible scaffolds to bring protein complexes (eg, epigenetic factors) to initiate transcriptional programs, which was previously proposed by Howard Chang’s group for HOTAIR. Of note, even low-expressed lincRNAs may exhibit such functions because activation or repression of a specific locus induces an amplification cascade that controls the biological response and as such a moderate to high expression may be not as important for epigenetic regulatory lincRNAs in comparison to IncRNAs that act as sponges (Discussed in Function 4: Molecular Sponges).

As the above-described molecular scaffold model is attractive for the growing field of epigenetics, several studies have been published using RIP assay followed by next-generation sequencing (therefore, it is called RIP-seq) to conduct genome-wide screening of bound RNAs. Although technically easier compared with other RNA-based techniques (eg, CLIP-seq), the generated data can be difficult to analyze because of the usage of an appropriate control, variation from one experimental assay to another, and so on. Moreover, unlike ChIP-seq experiment that relies on the idea of binding motif equals the genomic region, the identification of bound regions in RIP-seq data is difficult because the expressions of whole transcripts and their isoforms must be considered for the binding between the target protein and bound RNA molecules. To resolve this problem, 2 programs are freely available: RIPSeeker and ASPeak. Nevertheless, cross-linking of RNAs with proteins using either chemical (eg, formaldehyde) or UV will result in nonphysically relevant bindings, which might lead to unspecific binding of RNAs to the target protein.

Although the information that can be obtained from RIP-seq is valuable, there are some cautions that must be taken to interpret the results. Due simply to the availability of ChIP/RIP-grade antibodies, polycomb repressive complex is more often used for RIP assays than any other epigenetic factors. However, one needs to keep in mind that recent studies that reported ubiquitous RNA-binding ability of one of the component of polycomb repressive complex 2 Ezh2. Therefore, more rigorous evaluations are needed to identify proteins that specifically bind to the target lncRNA. Although one could argue that by performing the opposite experiment, which is called RNA pulldown by labeling the target lncRNA with biotin and mix it with cellular lysates followed by pulldown using streptavidin beads, the promiscuous binding of Ezh2 can be avoided; in reality, RNA pulldown suffers from its in vitro setting, where biotinylated lncRNA is folded into its shape either by heating it followed by slow cool down or by exposing it to a mixture of chemicals. Therefore, artificial folding of the biotinylated lncRNA may ultimately affect its capability to bind proteins. Furthermore, mere abundance of biotinylated lncRNA in the experimental condition (typically 50 pmol of RNA per condition) compared with the endogenous level of the target lncRNA will create pseudobinding events. To solve the above problems, Howard Chang’s group proposed a method, which is called domain-specific chromatin isolation by RNA purification (dChIRP). This method is an improvement to the previously published method called chromatin isolation by RNA purification (ChIRP). In principle, dChIRP works by mixing biotinylated antisense 20-mer oligonucleotides targeting several RNA domains (eg, species-conserved regions, devoid of low complexity) with nuclear extracts that are cross-linked chemically followed by a protocol similar to ChIP/RIP assays. The beauty of this assay is that pulled down materials can be divided for DNA, RNA, and proteins to comprehensively cover nuclear contexts that bind to the target lncRNA. There are other similar methods currently available, which include capture hybridization analysis of RNA targets (CHART) and RNA Antisense Purification coupled with DNA sequencing (RAP). As usual with a new technique, an optimization of the above assays is necessary for each lncRNA.

**Function 3: Enhancer Activation**

For transcription to occur, the chromatin must open to allow for transcriptional machinery (eg, RNA polymerase II) to bind at the site of transcription. This process is initiated by the binding
of transcription factors at the site called enhancer, where the transcriptional coactivators (eg, p300, CREB [cAMP response element-binding protein]) bind to in a tissue-specific manner. In 2010, a survey was made in primary murine neuronal culture using ChIP- and RNA-seq experiments, which identified the presence of thousands of transcripts that arise from enhancer regions. These RNAs are termed eRNAs, whose lengths are <2000 nt and a majority of them are nonpolyadenylated. Furthermore, their expression levels correlate with nearby protein-coding genes suggesting the existence of an interaction between an enhancer and a promoter of the protein-coding gene. A subsequent study in murine T cells identified that 60% of eRNAs are polyadenylated and nonpolyadenylated, respectively, which is similar to distribution as other types of lncRNAs. On the basis of this study, the following 2 classes of eRNAs are defined: (1) 1d-eRNA, which is polyadenylated and unidirectionally transcribed to the enhancer region and (2) 2d-eRNA, which is nonpolyadenylated and bidirectionally transcribed. Like other lncRNAs, the functional studies of eRNAs are scarce.

Recently, a series of articles was published from Richard Young’s laboratory suggesting the presence of so-called super enhancers. These enhancers are composed of clusters of enhancers that are marked by the presences of tissue/cell type–specific transcription factors and mediators. For example, the enhancer CCAT1-L has a length of 200 nt, is located at 515 kb upstream of oncogene MYC, and is expressed specifically in human colorectal cancers. By interacting with CCCTC-binding factor, which binds to a transcriptional insulator element, CCAT1-L participates in the chromatin looping formation in the MYC promoter. Because the median size of super enhancer is 19.4 kb, there will be many more eRNAs of super enhancer regions to be studied in the near future.

**Function 4: Molecular Sponges**

It was a surprise to many researchers that the number of human protein-coding genes is similar to that of *C. elegans*. There are several possible reasons being proposed that make ourselves different from worms: One is alternative splicing events to generate protein isoforms from 1 gene; and another is the presence of increasing number of lncRNAs during the evolution. A particular class of lncRNA called circular RNAs combines these 2 possible reasons, which is well reviewed by Jeck and Sharpless recently. As the name suggests, a circular RNA forms a covalent link between its 3′- and 5′-ends, which can arise from introns (sometimes, such circular RNAs are called circular intronic RNAs) or from exons of protein-coding genes; where it is found that former is more abundant in the nucleus, whereas latter is predominantly in the cytoplasm. Jeck et al proposed a term called backsplice to describe abundant (>25 000 in human foreskin fibroblast cell line) circular RNAs that joins exons of protein-coding genes in a reverse order that is annotated.

As in the case of other types of lncRNAs, the functions of circular RNAs are slowly being elucidated. One of such functions is that circular RNAs sequester miRs, which is exemplified by antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as) (also known as circular RNA sponge for miR-7 [ciRS-7]) that binds miR-7. As a matter of fact, such function is not exclusive to circular RNAs; there are growing lists of linear lncRNAs that also function as miR sponges: highly upregulated in liver cancer (HULC), linc-MD1 and imprinting lncRNA H19, which bind miR-372, miR-133, and let-7, respectively. More intensive researches are needed to uncover functional roles of circular RNAs.

To function as a molecular sponge, an appropriate localization and a certain concentration of the lncRNA are required. Particularly, for the many low expressed lncRNAs, their function as a sponge may be less likely unless the respective lncRNA or circRNA is upregulated during disease stages. A recent study by David Bartel’s group argues against the abundance of most circRNAs at baseline conditions: Considering that in homogeneous cell types 1 molecule per cell usually corresponds to an FPKM (fragments per kilobase of transcript per million mapped reads) of 1 to 4, most circRNAs only accumulated to a few molecules per cell. Even if a low concentration may be compensated by a high number of binding sites, a critical assessment of the stoichiometry seems essential for both lncRNAs and circRNAs.

**Long Noncoding RNAs in Cardiovascular Biology and Disease**

**lncRNAs in Vascular Biology and Disease**

*Insights From Genetic Studies*

First evidence for a putative role of lncRNAs in vascular disease came from genome-wide association studies that independently identified a susceptibility locus of coronary artery disease (CAD) on the human chromosome 9p21. This locus is adjacent to the last exon of a lncRNA named antisense noncoding RNA in the INK4a locus (ANRIL, also known as CDKN2BAS), whereas the other 2 protein-coding genes (cyclin-dependent kinase inhibitors 2A and 2B; CDKN2A and CDKN2B, respectively) lie >100 kb from associated single nucleotide polymorphisms (SNPs), suggesting that SNPs on ANRIL are more likely to contribute to the susceptibility of CAD. Indeed, subsequent studies showed that ANRIL expression is associated with the risk for coronary atherosclerosis, peripheral artery disease, and other vascular disease (for review, see reference 59).

The findings that ANRIL is expressed in endothelial, smooth muscle, and inflammatory cells and can be alternatively spliced giving rise to various isoforms including a circular forms of ANRIL challenged the identification of the mechanism by which it may regulate CAD. Particularly, first evidence for a differential regulation and biological properties of transcript variants suggests a more complex picture of ANRIL’s functions. Carriers of the risk allele showed increased whole blood RNA levels of the short variants DQ485454 and EU741058, whereas the long variant DQ485453 was decreased. An additional study confirmed the upregulation of the transcript EU741058, which was significantly increased in peripheral blood mononuclear cells and atherosclerotic plaques in carriers of the risk haplotype, but the transcript variant DQ485454 remains unaffected in this study. The expressions of the transcripts EU741058 and NR_003529 were further correlated with the severity of atherosclerosis. Despite ample correlative studies, the biological function of...
strategies. Inhibitions of several ANRIL isoforms containing exon 19 reduced cell viability, but changes of gene expression were different between the 2 targeting variants by targeting exon 13 also reduced cell proliferation, whereas its overexpression promoted cell growth, metabolic activity, and cell adhesion of tumor cell lines. In addition, some studies suggested a role of the 9p21 locus in the inflammatory response of endothelial cells. Harismendi et al showed that the CAD locus physically interacts with the CKN2A/B locus, the MTAP gene and an interval down-stream of the type 1 interferon gene IFNA21 in endothelial cells. The authors suggest that the risk allele interferes with the binding of STAT1 and that the interactions between enhancer structures at the CAD locus with other genes is remodeled during inflammation (eg, by treatment with interferon-γ). However, subsequent studies did not confirm a correlation of type 1 interferon expression in plasma or peripheral mononuclear cells with the risk variant, however, meanwhile the endothelial nitric oxide-synthase antisense transcript hAS-2 but not the other isoforms suppressed Tie-1 expression and inhibited tube formation in human endothelial cells in vitro. Together, this study represents a typical example of how natural antisense constructs may interfere with the sense mRNA and thereby regulate cellular functions.

Another antisense RNAs that might play a role in vascular biology are the natural antisense transcripts of hypoxia-inducible factor 1α (HIF-1AS), which are expressed in various tissues. Bioinformatics prediction suggests that the antisense RNA can bind to HIF-1 mRNA and might change its hairpin structure; leading to a new conformation allowing easier protein binding and possibly an increased mRNA decay at the translational step. However, these computational predictions have not been validated by experiments, and biological functions of the HIF-1AS RNAs in the cardiovascular system are unknown. Recent studies however report the regulation of HIF-1AS in patients with myocardial infarction (see Regulation of IncRNAs During Myocardial Infarction and Heart Failure).

Antisense Transcripts and Endothelial Cell Functions
Antisense transcripts were among the first studied lncRNAs in vessel growth. A conserved natural noncoding antisense RNA in the tie-1 locus (tie-1AS) was identified in zebrafish, mouse, and human. During zebrafish development, tie-1AS was expressed in the axial vessels, dorsal aorta, and posterior cardinal vein. Because antisense transcripts may work via binding to the sense RNA, the authors further explored a direct binding and showed by bioinformatics prediction and RNAs protection assays that tie-1AS indeed binds to the tie-1 mRNA both in the cytoplasmic and nuclear fractions. Injection of tie-1AS in zebrafish not only reduced tie-1 mRNA expression but phenocopied the tie-1 morpholino induced impairment of vascular cell contact junctions (Figure 3). In addition, 3 different forms of the human transcripts were studied in endothelial cells, and the transcript hAS-2 but not the other isoforms suppressed Tie-1 expression and inhibited tube formation in human endothelial cells in vitro. Together, this study represents a typical example of how natural antisense constructs may interfere with the sense mRNA and thereby regulate cellular functions.

Deep sequencing identified several other IncRNAs that are highly expressed in endothelial cells. Among them, not only known lncRNAs MALAT1, MEG3, and TUG1 but also unknown additional lncRNAs were identified. MALAT1, MEG3, and to some extent TUG1 were increased by hypoxia in vitro, and MALAT1 expression also was augmented in ischemic limbs. The study further characterized the function of MALAT1, which was first described as Metastasis Associated in Lung Adenocarcinoma Transcript. In endothelial cells, inhibition of MALAT1 by siRNA or LNA GapmeR, which induces RNA degradation by an RNase H-dependent mechanism, promoted migration of tip cells but blocked proliferation of subsequent stalk cells leading to a disturbed formation of vessel-like structures in vitro. In vivo studies confirmed that genetic deletion of MALAT1 or pharmacological inhibition of MALAT1 with LNA GapmeRs impairs vascularization of the retina and the ischemic hindlimb, respectively. Bioinformatics analysis of MALAT1-regulated genes in endothelial cells showed that MALAT1 supports the proliferation of endothelial cells (Figure 3), which is a finding that is consistent with the known cell cycle regulatory effect of MALAT1 in other cell types. However, the cell cycle regulatory mechanism(s) appear distinct from other cell. For example, regulation of splicing as it occurs in fibroblasts did
not change in MALAT1-silenced endothelial cells. The dysregulation of cyclins and cell cycle inhibitors in endothelial cells is likely mediated by another mechanism. MALAT1 is expressed not only in endothelial cells but also in muscle cells. A recent report showed that MALAT1 is induced and promotes skeletal muscle differentiation. Therefore, a function of MALAT1 in other cardiovascular cells (eg, myocytes or SMCs) may be possible and needs to be further investigated.

**IncRNAs and SMCs**

Deep sequencing studies were conducted to identify IncRNAs in human coronary artery SMCs by comparing their profiles to those of HUVEC. By screening 31 candidates, Bell et al further studied 1 IncRNA in detail, which is highly expressed in endothelial cells, SMCs, and aortic tissue and is transcribed in antisense orientation to the Fli1 gene. This IncRNA was named smooth muscle and endothelial cell–enriched migration/differentiation-associated long noncoding RNA (SENCR) and expression of 2 variants was confirmed by real-time polymerase chain reaction experiment. The function in endothelial cell is not reported, but SENCN knockdown led to a significant increase in SMC migration and morphological evidence for a hypermigratory phenotype. Silencing of SENCN downregulated expressions of several SMC contractile proteins and increased 2 promyogenic genes (namely MDK and PTN; Figure 3). Inhibition of 2 upregulated genes prevented the SENCN silencing induced promigratory effect, suggesting the causal involvement of these 2 genes in mediating the SENCN phenotype. The mechanism by which SENCN regulates targets is unclear. The authors demonstrate that SENCN is preferentially localized in the cytoplasm and does not affect the expression of the overlapping Fli1 gene. Moreover, SENCN has a low predicted coding potential; however, the absence of coding potential has not been validated by experimental studies. On the basis of the currently available information, one may speculate that SENCN plays a role as a miR sponge or may exhibit other functions in the cytoplasm (eg, RNA–mRNA interactions and RNA–protein interactions).

**LincRNA-p21** is a p53-induced IncRNA, which recently was shown to control SMC proliferation and atherosclerosis. Inhibition of lincRNA-p21 increased proliferation and reduced apoptosis by interfering with p53. LincRNA-p21 enhances p53 transcriptional activity by binding to 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 patients and its inhibition enhanced neointima formation in mice models. Angiotsensin-induced IncRNAs in SMCs were studied by Leung et al, and the highly upregulated Linc-Ang362 was identified. This IncRNA seems to represent the host transcript from which miR-221 and miR-222 are excised, which are 2 miRNAs with well-known function in SMC proliferation.

Another study evaluated the regulation and function of IncRNAs in human aortic valve cells to identify IncRNAs that may control aortic valve calcification. The authors showed that cyclic stretch reduced the expression of the IncRNA HOTAIR. Silencing of HOTAIR elevated expressions of calcification-related genes, such as ALPL and BMP2. Activation of canonical Wnt signaling repressed HOTAIR and increased calcification related genes; however, the causal involvement of the proposed signaling pathway has not been determined.

**IncRNAs in Cardiac Biology and Diseases**

Given that IncRNAs are more tissue/cell-type specifically expressed and potentially regulated than protein-coding genes, it is assumed that they should have functions during the development of an organism, including the heart. *Fendrr* (Fetal-lethal nondcoding developmental regulatory RNA) is 1 excellent example. Werber et al searched for differentially expressed IncRNAs in 6 different tissues (mesoderm caudal to the somites, somites, heart, head, presumptive spinal cord, and remainder [carcass]) dissected from early somite-stage mouse embryos (TS12, E8.25, and 3–6 somites) using RNA-seq and ChIP-seq experiments. On the basis of this screening, authors focused on *Fendrr*, which is highly expressed in the posterior mesoderm, which give rise to heart. After expression analysis, authors created a knockout mouse strain by replacing the first exon of *Fendrr* with a transcriptional stop signal, which was embryonically lethal around E13.75 possibly because of impaired proliferation of cardiomyocytes. Further molecular analysis showed that *Fendrr* interacts with polycomb repression complex 2 and TrxG/MLL complexes to bring polycomb repression complex 2 to its target promoter, which results in H3K27 trimethylation to reduce the expressions of the target genes. Recently, John Rinn’s group created 18 IncRNA knockout mouse strains, which is the largest null allele screening of IncRNAs to date. In this collection of knockout mice, *Fendrr* is included. In this study, the authors used a different knockout strategy (replacement of the target IncRNA with a LacZ reporter cassette) than the one by Grote et al. In their *Fendrr* mice, although the expression of LacZ gene was not observed in the heart of embryos (at E14.5, E18.5, and postnatal stage), intraventricular septal heart defects were observed before E18.5, which confirm the importance of *Fendrr* in the development of the heart.

As written above, *Fendrr* was initially screened from the posterior mesoderm of embryos. Because the heart arises from the posterior mesoderm, it is interesting to note that another important IncRNA *Braveheart* is in the heart (or specifically in the cardiomyocytes) is involved in the upstream regulation of *Mesp1* (Mesoderm posterior protein 1). However, up until now, no ortholog of *Braveheart* could be identified in human, whereas human ortholog of *Fendrr* exists. Further researches in these IncRNAs are needed to understand the functional importance of IncRNAs in the development of the human heart.

A recent study characterized cardiac-enriched IncRNAs. By comparing RNA sequencing data from mouse hearts, livers, and skin cells, the study identified 321 cardiac-expressed IncRNAs (defined as >0.3 RPKM [reads per kilobase of transcript per million mapped reads]). Among them, 52 transcripts were abundant and highly cardiac enriched (defined as >1 RPKM) and included the above-mentioned *Braveheart*. 67% of the abundant and cardiac-enriched IncRNAs were also highly expressed in isolated cardiomyocytes, whereas only 2% were highly expressed in fibroblasts, suggesting that the majority of the highly expressed IncRNAs in the mouse hearts are expressed by cardiomyocytes. The study additionally reported that IncRNA expression is largely consistent among different mouse strains.
conserved between mouse strains, and only few statistically significant differences were observed between FVB/N and C57/BL6 hearts.

**Regulation of IncRNAs During Myocardial Infarction and Heart Failure**

First insights into a putative role of IncRNAs in heart diseases came from genetic studies showing that SNPs were enriched in a susceptible locus for myocardial infarction that encodes a noncoding RNA, which the authors named myocardial infarction associated transcript (MIAT). The cDNA of this novel noncoding transcript was cloned, and in vitro studies demonstrated that it indeed represents a noncoding RNA because no protein translation was detected. The authors characterized the impact of SNPs on this newly identified RNA and showed that a minor variant of 1 SNP in its exon 5 increased the expression levels of MIAT and changed its binding to an uncharacterized protein. MIAT expression in peripheral blood mononuclear cells was significantly reduced in patients with ST-segment-elevation myocardial infarction. Although the function of MIAT in the heart is still unknown, MIAT (which is also known as RCRZ2/AK028326/Gomafu) was shown to be Oca4-activated, highly expressed in the retina and controls the differentiation of pluripotent stem cells. Recent studies report that manipulation of MIAT triggers pleiotropic effects on brain development, which are at least in part mediated by aberrant splicing of Wnt7b. Given the important role of Wnt signaling in the cardiovascular system, it will be interesting to determine the role of MIAT in this context.

Expression profiling using microarrays or deep sequencing identified regulated IncRNAs in the heart, peripheral blood mononuclear cells or plasma (Table 1). Several IncRNAs were identified by microarray analysis and confirmed to be up-regulated (AK137898, AK049728, ENSMUST00000142855, AK044955, ENSMUST00000127230) or downregulated (ENSMUST00000143888, uc.115-, AK139454, NR_028277, NR_036631) in murine hearts after isoproterenol treatment. This study also explored the expressions of IncRNAs in the whole blood and plasma and confirmed the regulation of several distinct IncRNAs. Another mouse study reports the regulation of IncRNAs after ischemia/reperfusion injury and showed several highly upregulated (AK035396, ENSMUST00000156081, AK005401, ENSMUST00000118172) and down-regulated (uc.007.prv.1, AK080112, ENSMUST00000170410, AK156124) IncRNAs.

In human failing left ventricular samples of paired ischemic and nonischemic patients before left ventricular assist device implantation, 18 480 IncRNAs were detected; of which, 679 and 570 were differentially expressed in ischemic versus nonischemic heart failure, respectively. After left ventricular assist device, ≈ 10% of IncRNAs were improved or normalized. Interestingly, bioinformatics analysis showed that IncRNAs strongly correlate with cis-expressed mRNA, suggesting that IncRNAs may interfere with the expressions of protein coding genes through cis-regulation.

Recently, mice challenged with acute myocardial infarction (AMI) were comprehensively characterized at the level of transcriptomics using RNA-seq. Using bioinformatics analysis, authors identified 1521 novel IncRNAs that are not listed in the UCSC (University of California, Santa Cruz)-based annotations of mouse genome (mm9). Further bioinformatics characterization of these novel IncRNAs revealed that ≈ 73% of them were mapped to the human genome, which indicates possible conservation in human. Validated downregulated IncRNAs include Novlnc6, Novlnc13, Novlnc44, Novlnc76, Novlnc95, Novlnc96, and Novlnc103; whereas Novlnc35 and Novlnc174 were increased particularly in the border zone after AMI. Interestingly, the human ortholog of Novlnc6 (chr6:122316007–122333735 [–]) was also suppressed in patients with dilative cardiomyopathy when compared with that in controls. Further characterization revealed that Novlnc6 is a cardiomyocyte-enriched, cytoplasmic localized IncRNA, which is predicted to have a low coding potential. Novlnc6 inhibition by GapmeRs induced a downregulation of Bmp10 and Nkx2.5 mRNA expressions in cardiomyocytes suggesting that this IncRNA interferes with functionally important regulatory transcripts (Figure 4). The mechanism(s) underlying this regulation are unknown. Given that under the pathological conditions, fetal gene program is activated in cardiomyocytes, the same group led by Thierry Pedrazzini conducted further screening to identify eRNAs that are expressed during cardiomyocyte differentiation of murine embryonic stem cells; mm67 (flanking Myocardin), mm77 (Cux2-Myl2), mm85 (Myocardin-Map2k4), mm104 (Sla-Wisp1), mm130 (Tbx20-Herpud2), mm132 (Cap2), mm172 (Ednra-Tic29), and SMAD7-IncRNA (Smad7 [SMAD family member 7]). Through in vitro and in vivo experiments using transgenic mouse enhancer assay (the Vista Enhancer Browser [http://enhancer.lbl.gov/100,101]), the authors demonstrated the specific activities of the above identified cardiac eRNAs. Furthermore, knockdown of 2 eRNAs (mm85 and SMAD7-IncRNA) resulted in the specific down-regulation of target protein-coding genes (Myocardin and Smad7, respectively) to highlight cis-regulation of these eRNAs.

Kumarswamy et al used IncRNA arrays to identify circulating long noncoding RNAs in plasma samples. Interestingly, highly expressed IncRNAs in plasma samples preferentially originated from the mitochondrial genome. This finding may be consistent with a recent observation that several known nuclear-encoded IncRNAs, which were highly expressed in peripheral blood mononuclear cells, were not detectable in serum or plasma. Circulating levels of one of these mitochondria-derived IncRNAs named long intergenic noncoding RNA predicting cardiac remodeling (LIPCAR; also known as iuc022qfs.1) were down-regulated after AMI but were increased during later stages of heart failure. LIPCAR expression was shown to be associated with future development of cardiac remodeling in patients who had an episode of AMI and independently predicted cardiovascular mortality in patients with chronic heart failure. This study is the first to test the use of IncRNAs as biomarkers for cardiovascular diseases and suggests that also IncRNAs (at least mitochondrial encoded IncRNAs) are detectable in plasma samples and may provide a prognostic tool. However, the mechanisms leading to the changes in circulating mitochondrial IncRNAs and the function of mitochondrial IncRNAs deserve further exploration. One may speculate that mitochondrial IncRNAs are...
simply higher expressed and therefore easier to detect. Indeed, in tissue samples from patients with ischemic and nonischemic heart failure, cardiac mitochondrial IncRNAs were highly expressed and negatively associated with nuclear-encoded mitochondrial regulatory genes.97 Alternatively, the release of mitochondrial IncRNAs may be regulated by (patho)physiological processes, such as cell death or mitophagy.

Others determined the expression of known IncRNAs in peripheral blood mononuclear cells of patients with AMI.70 This study reports that levels of HIF-1 AS, MALAT1, and KCNQ1OT1, which is an antisense ncRNA that is imprinted in early cardiac development,104 were higher in patients when compared with that in healthy controls, whereas the ANRIL transcript NR_003529 was lower in patients with myocardial infarction.70 The increase of MALAT1 level in ischemic patients is consistent with the hypoxia-induced up-regulation seen in cultured cells and ischemic limbs.73 With respect to the predictive value of the measured IncRNAs, the initial levels of ANRIL and KCNQ1OT1 in peripheral blood mononuclear cells were associated with left ventricular dysfunction at 4-month follow-up70; again suggesting that IncRNAs might be useful as indicators for left ventricular remodeling.

### Interference of IncRNAs With Cardiomyocyte Hypertrophy and Apoptosis

Bioinformatics analysis of RNA sequencing revealed that only a rather small number of 17 IncRNAs were regulated after early (1 week post operation) and late (4 weeks post operation) transverse aortic constriction with mild deterioration of heart function.90 Surprisingly, despite a markedly different IncRNA...
expression pattern between fetal and adult hearts, a minimal overlap of regulated lncRNAs in fetal and pressure-overload-ed hearts was observed, suggesting a limited role for lncRNAs in hemodynamically stressed adult hearts. A similar bioinformatics approach reported ≈15 differentially regulated lncRNAs in mouse hearts after transverse aortic constriction. Interestingly, the same study showed that 135 lncRNAs are regulated in heart failure, suggesting a more profound regulation in more severe disease states.

Despite the limited number of highly regulated lncRNAs during hypertrophy, several recent studies identified lncRNAs that control hypertrophy. Two studies suggest that lncRNAs can control hypertrophy and cardiomyocyte death by interfering with miRNAs (Figure 4). Wang et al determined the regulation of cardiomyocyte-enriched lncRNAs by anoxia and showed that among ≈100 moderately to highly expressed lncRNAs, 4 lncRNAs (namely, AK029547, AK041176, AK017121, and AK018416) were down-regulated by anoxia. AK017121, which the author named cardiac-apoptosis related lncRNA (CARL), suppressed apoptosis and mitochondrial fission in vitro.

Table 2. Annotations and Allocations of lncRNAs

<table>
<thead>
<tr>
<th>Name in the Main Text</th>
<th>Alias (Separated by a Comma)</th>
<th>Human Genomic Coordinates (hg19)</th>
<th>Mouse Genomic Coordinates (mm10)</th>
<th>Zebrafish Genomic Coordinates (danRer7)</th>
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</thead>
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<tr>
<td>AINR</td>
<td>2810051F02Rik, 281043M15Rik, Alr, B930018I07Rik, and D17Ertd669e</td>
<td>chr6:160424323-160428696</td>
<td>chr17:12741311–12830123</td>
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<td>AK018416</td>
<td>RIKEN clone 8430415L13</td>
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<td>chr8:123092877–123093998</td>
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<td>AK035396</td>
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<td>chr14:32115643–32117499</td>
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<td>AK041176</td>
<td>RIKEN clone A530007P14</td>
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<td>chr8:105225187–105246560</td>
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<td>AK049728</td>
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<td>AK080112</td>
<td>Gmn4151</td>
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<td>Braveheart</td>
<td>Bhnv and Gm20748</td>
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<td>CARL</td>
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<td>CCAT1-L</td>
<td>CCAT1 and CARL-5</td>
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<td>chr8:128219629–12823133</td>
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<td>CDR1as</td>
<td>CDR1-AS, CDR1AT, and CIRS-7</td>
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<td>ENSMUST0000011817</td>
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<td>Fendr</td>
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<td>chr8:121059119–121083032</td>
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<td>chr7:14257532–142578146</td>
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<td>HIF-1AS</td>
<td>HIF1A-AS2, aHIF, and 3’aHIF-1A</td>
<td>chr14:62213757–62215807</td>
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<th>Zebrafish Genomic Coordinates (danRer7)</th>
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<td>HCCAT1, LINC00078, and NCRNA00078</td>
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<td>KCNQ10T1</td>
<td>KCNQ1-AS2, KvDMR1, KvLQT1-AS, LIT1, and NCRNA00012</td>
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<td>chr2:20669882–20682958</td>
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<td>lincRNA-p21</td>
<td>Trp53Cr1, Aki44811, Gm16197, linc-p21, and OTTMUSG00000031656</td>
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<td>chr7:29194419–29215906</td>
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<td>MALAT1</td>
<td>HCN, LINC00047, MALAT-1, mascRNA, NCRNA00047, NEAT2, PRO1073, 2210401K01Rik, and 9430072K23Rik</td>
<td>chr11:65265233–65273939</td>
<td>chr19:5795690–5802671</td>
<td>chr14:48566202–48573730</td>
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<td>MEG3</td>
<td>GTL2, LINC00023, NCRNA00023, 2900016C05Rik, 3110050O07Rik, 6330408G06Rik, and D12Bwg1266e</td>
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<td>MIAT</td>
<td>Gomafu, C22orf35, FLJ25967, LINC00066, NCRNA00066, Rncr2, and A230057G18Rik</td>
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<td>mm77</td>
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<td>mm104</td>
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<td>n411949</td>
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<td>NONMMUTO60164, ENSMUST00000180681, TCONS_00010925, and TCONS_00029269</td>
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<td>NR_036631</td>
<td>Zm3, 1700128I23Rik, and ZNF657</td>
<td>chr19:57646546–57696570</td>
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<td>chrX:73040486–73072588</td>
<td>chr10:53460373–53482323</td>
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n.d. indicates no data available.
reduced ischemia/reperfusion injury in vivo. Mechanistically, the authors showed that CARL binds to miR-539 and acts as a sponge to block miR-539 functions in mitochondrial fission. CARL thereby derepressed the miR-539 target PHB2 and prevented anoxia-induced mitochondrial fission.

A similar strategy was used to determine angiotensin II-regulated lncRNAs.90 Here, AK048451, which was subsequently named cardiac hypertrophy related factor (CHRF), turned out to be substantially elevated by angiotensin II treatment in vitro. In vivo studies confirmed the upregulation in murine hearts after transverse aortic constriction and in human heart failure samples. CHRF is widely expressed in cardiovascular cells and other tissues but has a specific function in cardiomyocytes. CHRF induced cardiomyocyte hypertrophy in vitro and apoptosis in vivo. The mechanism of action was attributed to a binding of miR-489, which derepresses the miR-489 target Myd88 to regulate cardiomyocyte hypertrophy. A cluster of antisense transcripts from the Myh7 locus (named myosin heavy-chain-associated RNA transcripts [MyHEART or Mhrt]) was recently shown to interfere with cardiac hypertrophy and subsequent heart failure.108 Mhrt is highly enriched in the nuclear fraction of cardiomyocytes and is down-regulated by pressure overload. Over-expression of Mhrt protected the heart and reduced the pathological switch of Myh6/7 expressions. The inhibition of the Myh switch did not involve direct RNA-RNA sequence interferences between Mhrt and Myh but was mediated by the Brg1/Baf chromatin remodeling complex. Mhrt directly binds to Brg1 thereby blocking its chromatin binding and helicase function. It is interesting to note that the lncRNA Mhrt and miR-208a/b are encoded by the same locus but exhibit distinct functions; namely, providing a protective ([miR-208]) effect on heart function after pressure overload.109

The opposing biological function coincides with an inverse regulation in vitro and apoptosis in vivo. The mechanism of action has only been identified for a few lncRNAs. Despite these challenges, first genetic studies using gain- or loss-of-function approaches suggest that lncRNAs can indeed contribute to cardiac or vascular dysfunction. Importantly, first pharmacological inhibition studies suggest that targeting of lncRNAs might be possible not only in tumors but also in hearts or vessels. This may open up the opportunity for the development of new therapeutic strategies.

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Disclosures

None.

References

15. Thakur N, Tiwari VK, Thomassin H, Panedy RR, Kanduri M, Gondor A, Grange T, Ohlsson R, Kanduri C. An antisense RNA should be given (eg, chromosomal localization, deposition of the identified transcript into publicly-available databases [eg, GenBank, ENSEMBL]). Furthermore, the mechanism of action has only been identified for a few lncRNAs. Despite these challenges, first genetic studies using gain- or loss-of-function approaches suggest that lncRNAs can indeed contribute to cardiac or vascular dysfunction. Importantly, first pharmacological inhibition studies suggest that targeting of lncRNAs might be possible not only in tumors but also in hearts or vessels. This may open up the opportunity for the development of new therapeutic strategies.


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