Review

Long Noncoding RNAs in Cardiac Development and Pathophysiology

Nicole Schonrock, Richard P. Harvey, John S. Mattick

Abstract: **Heart function requires sophisticated regulatory networks to orchestrate organ development, physiological responses, and environmental adaptation. Until recently, it was thought that these regulatory networks are composed solely of protein-mediated transcriptional control and signaling systems; consequently, it was thought that cardiac disease involves perturbation of these systems. However, it is becoming evident that RNA, long considered to function primarily as the platform for protein production, may in fact play a major role in most, if not all, aspects of gene regulation, especially the epigenetic processes that underpin organogenesis. These include not only well-validated classes of regulatory RNAs, such as microRNAs, but also tens of thousands of long noncoding RNAs that are differentially expressed across the entire genome of humans and other animals. Here, we review this emerging landscape, summarizing what is known about their functions and their role in cardiac biology, and provide a toolkit to assist in exploring this previously hidden layer of gene regulation that may underpin heart adaptation and complex heart diseases. (*Circ Res*. 2012;111:1349-1362.)**

Key Words: cardiovascular disease ■ epigenetics ■ gene regulation ■ heart development ■ long noncoding RNAs

The heart is extraordinary, not only in its complex ontogeny, architecture and uninterrupted contractility, but also in its ability to respond acutely to changing physiological and neuropsychological circumstances. Significant perturbation to cardiac developmental pathways, whether genetic or environmental, can be catastrophic for the embryo or give rise to a spectrum of congenital heart defects, which place enormous burdens on patients and families alike and often stretch the limits of ethics, critical care medicine, and health budgets. The regulatory networks that control the development and adaptations of the heart therefore have been under intense investigation.[1,2] Emphasis is now being placed on elucidating the complex interplay between the many hierarchical levels of gene regulation that give a network its dynamic properties and ultimately arranges cells into a myriad of precisely sculpted 3-dimensional tissues and interacting organ systems. The 3 billion nucleotides of the human haploid genome encode the blueprint for development and function, perhaps best thought of as a zip file that is unfolded into countless different transcriptional programs. The epigenome overlays this information with several context-specific regulatory codes by way of secondary chemical modifications to the DNA itself and to the histones that fold DNA into organized structures and allow it to be read by the transcriptional machinery at specific times and places. Protein–protein interactions, catalysis, and second messengers are also fundamental network components, as are the site-specific DNA-binding transcription factors and their tissue-restricted codes that have executive control over cell fate decisions, a notion that has been reinforced in the era of cellular reprogramming.[3] Thus, regulation of the transcriptome, epigenome, and proteome requires a vastly expanded regulatory network governing the coordinated outputs of otherwise disparate molecular machineries.

The interpretation of genetic data and systems-level analysis of gene regulatory networks traditionally have been protein-centric.[4] However, gene cloning and more recent high-throughput genomic technologies have revealed many unexpected features of the human genome, including that its protein-coding genes are mosaics of introns and exons, that only ≈1.2% of the genome codes for amino acids in proteins, and that the 20 000 recognized human proteins are similar in number with largely orthologous functional overlaps to those found in other animals, including the simple nematode worm with only 959 somatic cells.[5] This has raised questions of what might be the function of the vast tracts of intronic and intergenic DNA, what additional information is required for higher organismal complexity and cognitive ability, and whether these are related. Alternative splicing, as well as posttranslational modifications, certainly contributes toward increasing the diversity and functionality of the proteome.

Interestingly, however, it is the proportion of non protein-coding DNA that has increased with developmental complexity, with <25% in prokaryotes, >60% in plants and metazoans, and 98.5% in humans.[5] Furthermore, the majority of the human

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From the Victor Chang Cardiac Research Institute, Darlinghurst, New South Wales, Australia (N.S., R.R.H.); St. Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Kensington, New South Wales, Australia (N.S., R.P.H., J.S.M.); and Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia (J.S.M.).

Correspondence to John S. Mattick, Garvan Institute of Medical Research, Darlinghurst, 2010 NSW, Australia. E-mail j.mattick@garvan.org.au

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Non-standard Abbreviations and Acronyms

eRNA	enhancer RNA
lincRNA	long intergenic noncoding RNA
MHC	myosin heavy chain
MIAT	myocardial infarction–associated transcript
miRNA	microRNA
NAT	natural antisense transcript
ncRNA	noncoding RNA
NFAT	nuclear factor of activated T-cells
PRC2	polycomb repressive complex 2
SRA	steroid receptor RNA activator
uaRNA	3′UTR-associated transcript
UCR	ultraconserved region
UTR	untranslated region

genome, and indeed of all animals, is dynamically transcribed in different cells and developmental stages, well beyond the boundaries of known protein-coding genes, producing a vast pool of RNA molecules. The majority of these transcripts have little or no protein-coding capacity and may hold the key to understanding the regulatory complexity inherent in advanced biological networks.[6] Although traditionally the central dogma has relegated RNA to the role of middle messengers of genetic information in the overall scheme of producing proteins as the effectors and regulators of biological processes,[7] it is now clear that many noncoding RNAs (ncRNAs) have intrinsic indices of functionality.[8] These include regulation by tissue-restricted transcription factors, dynamic developmental and cell type–specific expression patterns, localization to specific subcellular compartments, association with chromatin signatures indicative of active transcription, conservation of promoters, structure and genomic location, and association with human disease.[4] Thus, it seems that ncRNAs form a previously unrecognized but likely vital component of gene regulatory networks, although the field in terms of heart development and cardiovascular disease is clearly in its infancy.

A more sophisticated understanding of network structure and logic incorporating ncRNAs would impact profoundly on cardiovascular science and therapies and may provide unprecedented opportunities for intervention in disease progression. One described function of many long ncRNAs (lncRNA) is to direct chromatin modification complexes to their sites of action, indicating that they are central to the epigenetic control of development.[9] Elucidating the logic of ncRNA networks and their control of epigenetic modifications to DNA and histones that underpin genome integrity and function[10] are the new frontiers. The aim of this review is to provide insight into the world of lncRNAs as integral members of regulatory networks. We focus on their species, abundance, biogenesis, and known functions (including cardiac functions) and provide a toolbox of resources to facilitate their study in cardiac biology.

RNA as an Important Orchestrator of Biological Networks

The potential of RNA as a regulatory molecule was first recognized by Jacob and Monod >50 years ago.[11] RNAs not only encode primary sequence information, allowing them to directly interact with both DNA and other RNAs, but also contain great structural complexity and plasticity. lncRNAs are proposed to function as modular scaffolds, assembling diverse combinations of regulatory proteins through specific secondary structures of discrete domains, thus enhancing the repertoire of protein–protein interactions.[12] Many ncRNAs are alternatively spliced, and hence differential inclusion of exons in these transcripts can change the protein cargo and subsequent function of the resulting protein complex (Figure 1A). RNAs also can be guide molecules, functioning in *cis* on their neighboring genes or in *trans* on distally located genes to direct and tether protein complexes to specific loci through RNA–DNA, RNA–RNA, and RNA–protein interactions (Figure 1B). A single RNA molecule therefore can couple the primary sequence of nucleic acids to protein machinery, thus enhancing information flows and functional capabilities of biological networks.[13]

Regulatory RNA sequences also are more malleable than protein-coding sequences and freer to evolve, especially under positive selection for phenotypic innovation. This higher tolerance to mutation explains their lower sequence conservation and probably has allowed many ncRNAs to go unrecognized in genetic screens (because of phenotypic, expectational, technical, and interpretive biases).[4] Furthermore, by reversibly changing their secondary structure in response to changes in their microenvironment (such as temperature or ligand concentration), RNAs can function as biological riboswitches that rapidly change their information content available for interaction with the molecular circuitry (Figure 1C), an allosteric feature well-established in bacteria.[14] Furthermore, there is now good evidence to suggest that regulatory RNA information can be modulated by RNA editing, allowing context-dependent alteration of regulatory circuitry underpinning environment–epigenome interactions in learning and physiological adaptation.[15]

Diverse World of ncRNAs

ncRNA species are diverse in structure, biogenesis, and function, and can be broadly classified according to their size (Figure 2A). Small ncRNAs are generally defined as those that are <200 nucleotides, whereas lncRNAs can range up to tens or even hundreds of thousands of nucleotides in length and therefore have vastly more complex secondary structures. Many previously characterized ncRNAs, such as ribosomal RNA and transfer RNAs, have well-established roles as components of the translational machinery. In addition, small nuclear RNAs and small nucleolar RNAs play prominent roles in splicing and RNA modifications.

Recently, much interest has arisen in a heterogeneous class of small regulatory ncRNAs that directly affect the expression or function of protein-coding genes. These include microRNAs (miRNAs), endogenous small interfering RNAs, and PIWI-interacting RNAs, which interact with the RNA interference machinery.[16] PIWI-interacting RNA ensures genome stability in the germline by silencing repetitive and transposable elements,[17] whereas a recently described class of neuronal PIWI-interacting RNA functions in the epigenetic control of synaptic plasticity and long-term

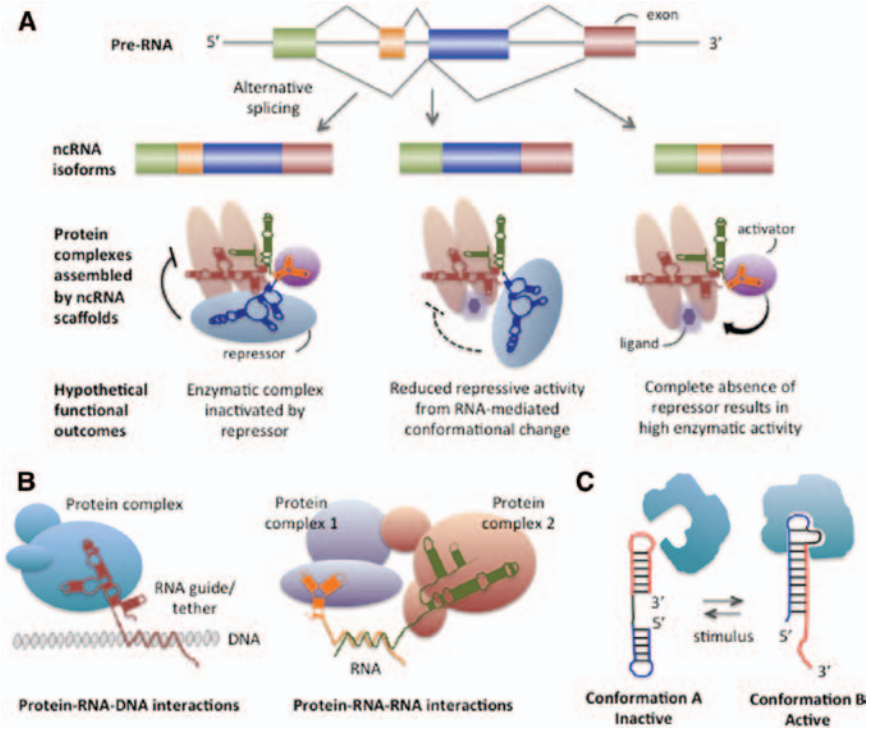


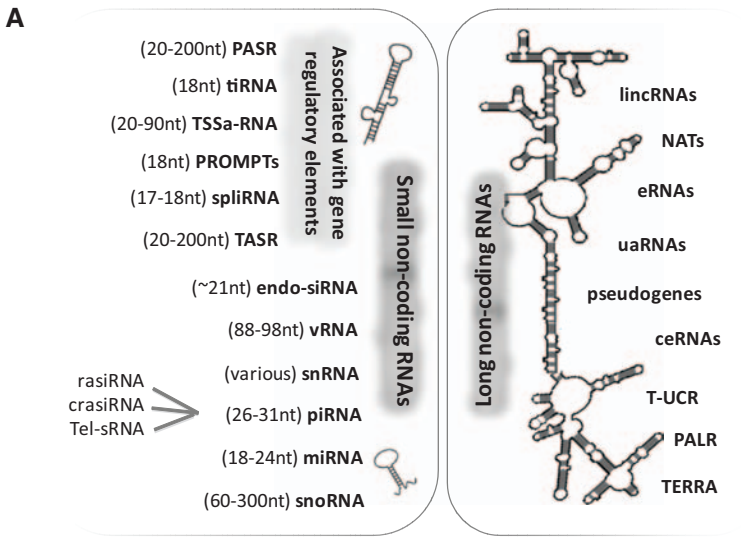
Figure 1. Conceptual overview of long noncoding (lncRNA) regulatory diversity and versatility. **A**, Noncoding RNAs (ncRNAs) can function as modular scaffolds through folding into complex protein interaction interfaces and assembling diverse protein complexes. Alternative ncRNA splicing can change the modular domains of the RNA molecule, effectively altering the protein cargo and resulting function of the protein complex. **B**, ncRNAs can increase the repertoire of protein–DNA or protein–protein interactions by functioning as a guide or tether to deliver a protein complex to specific genomic loci. Through RNA–RNA interactions, ncRNA scaffolds could effectively link 2 otherwise noninteracting protein complexes. **C**, Dynamic conformational transitions allow RNA to function as a biological switch in response to cellular stimuli, such as protein binding, metabolites, pH, or thermal triggers.

memory formation.[18] Endogenous small interfering RNAs, derived from long double-stranded RNA precursors, seem to function mainly in host defenses against exogenous double-stranded RNAs and also target retrotransposons.[19] miRNAs represent the most extensively studied class of ncRNA since their discovery in the 1990s. These highly conserved ≈22 nucleotide single-stranded RNAs function as guides for RNA-induced silencing complex–mediated repression of target mRNAs with partial sequence complementarity. miRNAs are predicted to exert fine-tuning of posttranscriptional regulation to >60% of mammalian protein-coding mRNAs and play key roles in heart development and cardiovascular disorders.[20] There are also other classes of small RNAs, including transcription-associated and splice site–associated tiny RNAs, which are ≈18 nucleotides in length and whose function is unknown but may reflect and regulate nucleosome position.[21,22]

lncRNAs, however, comprise the bulk of the human noncoding transcriptome. They can be nuclear or cytoplasmic and may or may not be subject to alternative splicing or polyadenylation.[23] Many serve vital molecular functions, including structural or trafficking roles, controlling cell cycle, differentiation, and apoptosis, and serving as precursors for smaller RNAs. To illustrate diversity among the lncRNAs, here we discuss their classification, focusing on 4 classes (Figure 2B) with potential broader regulatory impact on gene networks, and then highlight specific examples of their breadth of function in biology before discussing important functional roles in heart development and cardiovascular disease. It is important to note that with pervasive transcription producing complex webs of overlapping sense and antisense transcripts, the definitions of genes and intergenic regions are quickly evolving.[24] Thus, the boundaries between lncRNA classes remain rather vague, and parsing them according to their position relative to protein-coding genes is likely to be artificial.

Long Intergenic ncRNAs

Thousands of long intergenic noncoding RNA (lincRNAs) transcripts have been identified in mammalian genomes, including ≈1600 and ≈3300 from 4 mouse[25] and 6 human cell types, respectively.[26] lincRNAs are predominately transcribed by RNA polymerase II, producing polyadenylated RNAs ranging in length from 2000 to 20 000 nucleotides, and a substantial portion are multiexonic, producing alternatively spliced isoforms.[27] Their genes are marked with known chromatin signatures, such as trimethyation of lysine 36 of histone 3 (H3K36me3) along their transcribed region and trimethyation of lysine 4 of histone 3 (H3K4me3) at promoters, which also are associated with regulatory transcription factors.[25] lincRNAs are readily detectable, although their expression levels are somewhat lower than those of protein-coding genes, which is not surprising if their function is regulatory and is consistent with the observation that their expression patterns tend to be more tissue-specific than protein-coding genes. lincRNA sequences display greater conservation than intronic or random genomic regions and contain patches of higher conservation that may represent specific network-interaction domains that could provide insight into the lincRNA function. Many are associated with chromatin-modifying complexes,[26] as are other classes of lncRNAs,[28,29] and it is likely that lincRNAs will be subdivided into several classes, each with distinct roles; however, the current challenge lies in understanding how primary sequence translates into lncRNA function.



PASRs : promoter-associated short RNAs [138]; **tiRNAs** : transcription initiation RNAs [21]; **TSSa-RNA** : transcription start site-associated RNAs [139]; **PROMPTs** : promoter upstream transcripts [140]; **spliRNAs** : splice junction-associated RNAs [22]; **TASRs** : termini-associated short RNAs [141]; **endo-siRNAs** : endogenous small interfering RNAs [19]; **vRNAs** : vault RNAs [142]; **snRNAs** : small nuclear RNAs; **piRNAs** : piwi-interacting RNAs [17]; **rasiRNAs** : repeat associated small interfering RNA; **crasiRNAs** : centromere repeat associated short interacting RNAs [143]; **Tel-sRNA** : telomere specific small RNAs [144]; **miRNAs** : microRNAs [20]; **snoRNAs** : small nucleolar RNAs [145]; **lincRNAs** : long intergenic non-coding RNAs [27]; **NATs** : natural antisense transcripts [31]; **eRNAs** : enhancer RNAs [40]; **uaRNAs** : 3′UTR-derived RNAs [36]; **ceRNAs** : competing endogenous RNAs [146]; **T-UCR** : transcribed ultraconserved region [48]; **PALRs** : promoter-associated long RNAs; **TERRA** : telomeric repeat-containing RNA [147]

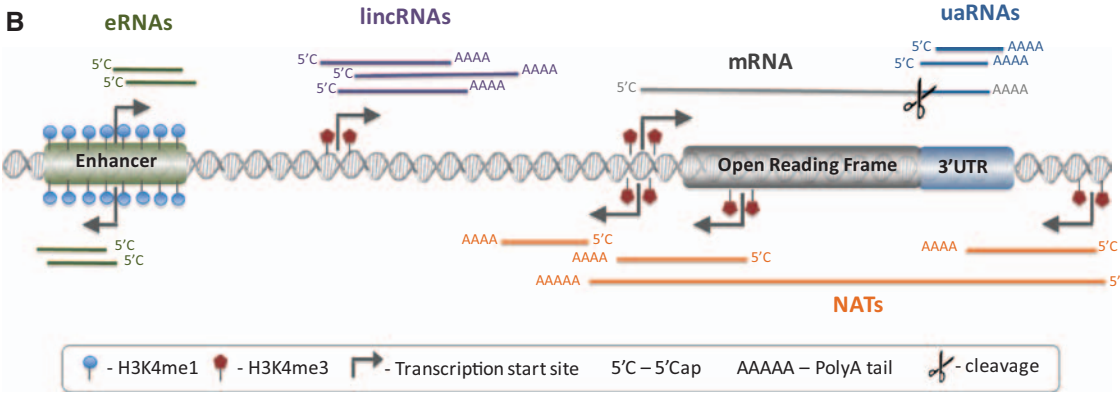


Figure 2. Overview of the current landscape of non coding RNAs (ncRNAs). **A**, ncRNAs can be superficially divided into 2 classes based on their size, long (>200 nt) and short (<200 nt), of which there are many types and many names given in different studies. **B**, Characteristics of 4 long ncRNA classes. Enhancer RNAs (eRNAs) derived from bidirectional transcription of enhancer domains marked by histone 3 lysine 4 monomethylation (H3K4me1) are 5′ capped but generally are not polyadenylated. Long intergenic ncRNAs (lincRNAs) are generated from independent promoters, characterized by the presence of histone 3 lysine 4 trimethylation (H3K4me3), distal to and not overlapping any protein-coding gene. In contrast, natural antisense transcripts (NATs) arise from independent transcription of the opposite DNA strand and may be intronic, intragenic, or bidirectional to protein-coding genes, and may share some sequence complementarity with their sense transcript. Despite having a 5′ cap, 3′ untranslated region (UTR)–derived RNAs (uaRNAs) are unlikely to be independently transcribed and may be a cleavage product of a longer messenger RNA (mRNA) precursor.

Natural Antisense Transcripts

Natural antisense transcripts (NATs) are derived from the opposite strand of either protein or nonprotein coding genes, with the most prominent form being a non protein-coding antisense RNA partner of an annotated protein-coding gene.[30] They form an abundant class of lncRNAs, with an estimated 70% of mouse genes undergoing antisense transcription to form sense/antisense pairs of coding and ncRNAs. Both the 5′ and 3′ ends of protein-coding genes have a tendency for antisense transcription, with particular enrichment observed 250 nucleotides upstream of the transcription start site and 1.5 kb downstream of sense mRNAs.[31] Those that have been identified exhibit typical mRNA properties, including a 5′ cap and 3′ polyadenylation; however, unlike lincRNAs, NATs display very little sequence conservation.[27] Many antisense transcripts are alternatively spliced[27,32] and contain H3K4me3

marks at their 5′ end, indicative of independent transcription and regulation, although they tend to exhibit lower expression than their corresponding sense transcripts. Antisense RNAs display a wide range of biological functions, including regulation of transcription as well as mRNA stability, splicing, and translation.[31] NATs often partially overlap with their corresponding sense mRNAs and therefore share some exonic sequence complementarity. Through formation of sense–antisense RNA duplexes either in the nucleus or in the cytoplasm, NATs may effectively mask miRNA-binding sites, splice sites, or even may serve as templates for generating endogenous siRNAs, with all of these mechanisms affecting sense RNA or encoded protein expression in some way.[33] Because these ncRNAs can remain tethered to the site of transcription, they can cotranscriptionally direct enzymatic activities to specific regions generally affecting their neighboring genes.[34] Altering the levels of antisense transcripts often has a positive or negative effect on sense RNA expression.[35] Recently, however, they have received much attention for their ability to direct epigenetic transcriptional regulation by binding chromatin-modifying proteins, associating with particular forms of activated histones, and affecting chromatin modifications, DNA methylation, or monoallelic expression such as genomic imprinting and X-chromosome inactivation.

3′UTR-Associated Transcripts

The 3′ untranslated regions (UTRs) are conventionally thought to operate in *cis* by recruiting regulatory proteins and miRNAs that control mRNA stability, translation, and localization. However, certain 3′UTRs recently have been shown to exhibit dynamic expression, independent of their associated mRNA, in a developmental-specific, tissue-specific, stage-specific, and subcellular-specific manner.[36] Through mining of publically available capped analysis of gene expression and cDNA libraries, large numbers of 3′UTR-associated RNA transcripts (uaRNAs) have been identified in mouse and humans. Despite the presence of a 5′ cap normally associated with RNA polymerase II–dependent transcriptional start sites, uaRNAs do not seem to be independently transcribed. They lack specific chromatin marks (H3K4me1/2/3) normally indicative of transcriptional start sites and are not enriched for RNA polymerase II occupancy. Instead, uaRNAs seem to be derived from cleavage of the full-length transcript.

Considering the rapid expansion of 3′UTRs during eukaryotic evolution together with the distinct expression patterns of uaRNAs, 3′UTRs may contain far more sophisticated functionality than simply serving as *cis*-regulatory sites for their mRNAs. The 3′UTRs of muscle structural genes encoding troponin I, tropomyosin, and α-cardiac actin can function as distinct RNAs in *trans* to regulate cell division and differentiation.[37] This occurs independently of their associated coding regions, and expression of the uaRNAs is required for their function. Myocyte enhancer factor 2C is an important transcription factor in heart development, and the *Mef2c* transcript also produces a validated uaRNA derived from its 3′UTR (mm8-chr13:84144078-84144117), although its precise function is currently unknown.[36]

Enhancer RNAs and Ultraconserved Elements

Enhancers are traditionally regarded as long-range *cis*-acting elements that regulate regional gene expression during development, but there is increasing evidence that they are specifically transcribed in the cells in which they are active.[38–40] In general terms, enhancers have been defined as regulatory DNA elements that are located distally from known transcription start sites, contain unique histone modifications, and are associated with the transcriptional coactivator p300/CBP.[41,42] They are thought to function by recruiting and depositing transcription factors to target promoters through induced chromosomal looping.[43] RNA polymerase II also is recruited to enhancer regions in an activity-dependent manner, where it transcribes bidirectionally only within the enhancer domain marked by the presence of the histone mark H3K4me1 to produce a class of ≈2-kb short-lived lncRNAs termed enhancer RNAs (eRNAs).[40] eRNAs generally are not detected in RNA deep sequencing technology experiments using polyA+ RNA fractions, suggesting that most eRNAs are not polyadenylated. Their expression is also positively correlated with the level of transcription at the nearest promoter, suggesting that they are actively involved in promoting mRNA synthesis. eRNAs also are found in various species, with ≈2000 identified in mouse and 3000 in humans, suggesting a universal mechanism of gene regulation.[44] Furthermore, a significant subset of lncRNAs has been shown to emulate the functional properties of enhancers in cell-based assays.[29] Although the act of transcription per se at enhancer domains may be important for enhancer function, eRNA transcripts themselves also may serve other functions.[45]

Smemo et al[46] have recently discovered a homozygous single nucleotide polymorphism (SNP) in a patient with Holt-Oram (heart-hand) syndrome located in an enhancer ≈90 kb downstream of the gene for TBX5, a transcription factor essential for heart development. This SNP abolishes *Tbx5* expression only in heart and not in limbs, yet no myocardial transcription factors were predicted to bind to this region. This suggests an alternative possibility that an eRNA is produced from this locus and that the SNP could disrupt its expression or function in driving cardiac *Tbx5* expression.

Although extreme evolutionary sequence conservation has been used to identify enhancers, many, including those functional in heart, are in fact poorly conserved.[47] However, mammalian genomes share ≈480 ultraconserved regions (UCRs) longer than 200 bp and >10 000 UCRs longer than 100 bp that display 100% sequence identity, suggestive of important evolutionary function.[48,49] There is a dearth of SNPs in human UCRs, suggesting purifying selection and some association with SNPs and human disease traits.[50] Both exonic and intergenic UCRs can be transcribed, predominantly in a single orientation and often in a tissue-specific manner, with expression patterns altered in cancer,[51] suggesting that UCR transcription is important for its function. Although deletion of 4 nonexonic UCRs did not lead to observable phenotypes in mice,[52] many have been shown to function as enhancers in splicing or as coactivators within transcriptional complexes.[53]

Functions of lncRNAs

ncRNAs Are Central to Epigenetic Gene Regulation

A hallmark function of lncRNAs is their ability to mediate epigenetic regulation, and they represent a previously hidden layer of information necessary for the architectural control of development, function, and adaptation. Many of the identified protein partners of lncRNAs are chromatin modifiers,[54] and 30% of lincRNAs expressed in mouse embryonic stem cells were found to associate with at least 1 of 12 chromatin complexes involved in reading, writing, and erasing histone modifications.[55] Furthermore, inhibition of epigenetic modifications also alters the expression pattern of lncRNAs, indicating that they are regulated by chromatin state and feed-forward to control it.[56]

Many lncRNAs bind to protein complexes of the trithorax chromatin-activating or polycomb group chromatin-repressing families and guide them to their sites of action.[9,26,28,57] These complexes act antagonistically to lay down either activating or repressive histone marks on specific loci and likely represent a global mechanism for epigenetic memory. The polycomb repressive complex, PRC2, regulates chromatin structure through trimethylation of lysine 27 of histone 3 (H3K27me3), which is then recognized and bound by a distinct polycomb group complex, PRC1, which catalyzes monoubiquitylation of lysine 119 on histone 2A (H2AK119Ub).[58] Remarkably, 20% of human lincRNAs are associated with PRC2, and knockdown of several PRC2-associated lincRNAs caused altered expression of PRC2-regulated genes.[26]

An extreme example of lncRNA-mediated epigenetic regulation is that of the complete shutdown of the X chromosome by the lncRNA *Xist* during random X inactivation. *Xist* is an ≈17-kb lncRNA that is expressed only from the inactive and not the active X chromosome. It contains a conserved structural A-repeat sequence at its 5′ end that functions in silencing through interaction with PRC2, whereas other sequence elements associate specifically with chromatin to allow *cis*-spreading of *Xist* over the entire X chromosome.[59] The function and expression of *Xist* are controlled by 2 ncRNA switches, antisense Tsix, which represses *Xist* on the active X chromosome, and Jpx, which activates *Xist* on the inactive X chromosome.[60] Another example is homeobox (HOX) antisense intergenic RNA, expressed from within the HOXC cluster of homeotic patterning genes, which functions as a *trans*-acting repressor targeting PRC2 to the distally located HOXD gene cluster.[61] It simultaneously binds PRC2 and the LSD1–CoREST complex that reinforces PRC2 repression by catalyzing demethylation of the active H3K4me2 histone mark.[62] In contrast, HOTTIP and Mistral function in a *cis*-acting fashion on neighboring genes of the HOXD and HOXA clusters by recruiting the activating H3K4me3 methyltransferase MLL1, a component of the trithorax complex.[63,64]

Polycomb is essential for cardiac development because conditional deletion of polycomb group component proteins *Ezh2* or *Eed* in cardiomyocytes leads to inappropriate activation of transcription factors and cell cycle regulators, leading to a variable spectrum of congenital and postnatal abnormalities, including septal defects, ventricular hypoplasia and noncompaction, hypertrophic cardiomyopathy, and fibrosis.[65,66] Rae28/Phc1, a component of PRC1, is essential for early heart development and maintenance of expression of the cardiomyocyte transcription factor Nkx2-5, although its function is required principally in a noncardiomyocyte cell type.[67]

There is increasing evidence that DNA methylation is also RNA-directed. DNA methylation plays a key role not only in imprinting and X-chromosome inactivation but also in gene silencing, stabilizing chromosome structure, and altering retrotransposon mobility.[68] lncRNAs may influence DNA methylation as illustrated by overexpression of *Khps1a* ncRNA isoforms that cause demethylation of CG sites in the differentially expressed and methylated region of the sphingosine kinase-1 gene implicated in calcium mobilization.[69]

Transcriptional and Posttranscriptional Gene Regulation by lncRNAs

The diverse roles of regulatory RNAs extend well beyond the epigenome as they regulate transcription in diverse ways[70] (Figure 3). ncRNAs can basepair with DNA or RNA targets to mask splice junctions, miRNA-binding sites, and promoters, thus altering gene expression or protein function.[71] A well-studied example is the regulation of the metabolic gene encoding dihydrofolate reductase.[72] An lncRNA produced from a minor dihydrofolate reductase gene promoter binds to the general transcription factor IIB and to the DNA of the dihydrofolate reductase major promoter in a specific and stable tripartite complex. This results in dissociation of the preinitiation complex and transcriptional repression. lncRNAs also can function as transcriptional coactivators.[73] The 3.8-kb polyadenylated ncRNA Evf2 is transcribed from an UCR in the *Dlx5/6* locus, encoding developmental transcription factors. Evf-2 functions in *trans* to bind and regulate the transcriptional activity of the homeodomain-containing Dlx2 protein to activate transcription of adjacent protein-coding genes.

Although lncRNAs can be regulatory targets of miRNAs, they also can act as miRNA sponges.[74,75] One such example is the ncRNA PTENP1, which is transcribed from a pseudogene homolog of the tumor suppressor gene phosphatase and tensin homology (*PTEN*), an antagonist of the PI3K kinase pathway that has broad roles in cardiovascular growth, adaptation, and disease. PTEN and PTENP1 are highly homologous, sharing only 18 mismatches throughout the PTEN coding sequence (including a mutated start codon that precludes PTENP1 protein production) and a 3′UTR that contains similar miRNA-binding sites. miRNAs fine-tune PTEN expression levels,[76] which are critical in determining cancer susceptibility.[77] By functioning as a decoy of *PTEN*-targeting miRNAs, *PTENP1* can compete for miRNA binding and indirectly regulate *PTEN* expression levels, thus influencing disease pathogenesis. lncRNAs also can function as inhibitory decoys of regulatory proteins. The lncRNA Gas5, for example, through its secondary structure, can mimic the DNA-binding motif of the glucocorticoid receptor.[78] When induced on growth factor starvation, Gas5 interacts with and inhibits binding of the glucocorticoid receptor to its DNA targets, thus preventing proper regulation of metabolic genes.

Protein localization and therefore function also can be modulated by lncRNAs, such as noncoding repressor of

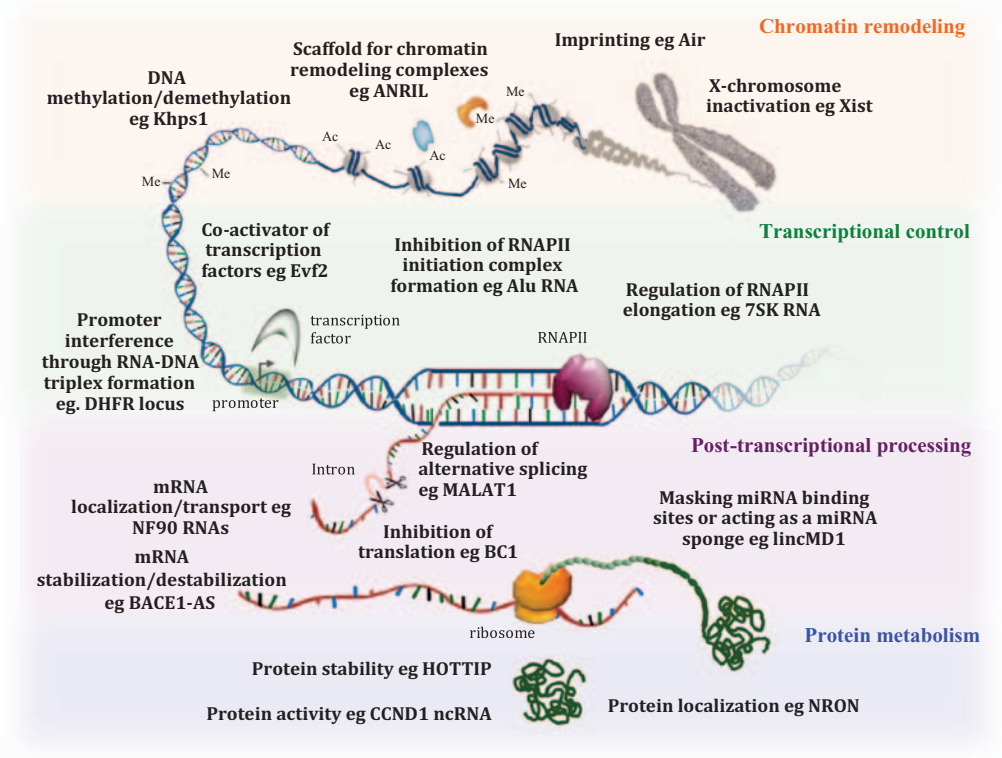


Figure 3. Functions of long noncoding RNAs (lncRNAs). lncRNAs can impact genetic output at almost every stage of a gene's life cycle—from epigenetic regulation and chromatin remodeling to transcriptional and post-transcriptional control to protein metabolism. Listed are a few examples of lncRNA gene regulation.

nuclear factor of activated T-cells (NFAT) (NRON). In response to extracellular signals, the nuclear factor of activated T-cells (NFAT) is dephosphorylated by the calcium-regulated phosphatase calcineurin and localizes to the nucleus, where it activates gene expression. The Ca^{2+}/calcineurin/NFAT pathway is central to pathological hypertrophy and is a potential therapeutic target,[79] but also functions in coronary angiogenesis[80] and cardiovascular development.[81] NRON has no influence on the transcription-activating properties of NFAT but rather interacts with nuclear import factors such as importin-β to specifically disrupt NFAT nuclear localization and to prevent its transcriptional activation.[82]

lncRNAs in Cardiovascular Function and Disease

Analysis of lncRNAs in the heart is still in its infancy, and the full impact of this field on cardiovascular biology is yet to be realized. We highlight some other examples of when lncRNAs are known or suggested to be involved in the cardiovascular system (Table). Most stories are incomplete and only hint at the rich layers of ncRNA function that are yet to be discovered and which may represent new therapeutic targets.

lncRNAs in Pluripotency and Cardiomyocyte Differentiation

During development, ncRNAs have reported functions in promoting developmental transitions and maintaining developmental states by ensuring a robust commitment to cell fate.[83] lncRNAs are integral components of embryonic stem cell gene regulatory networks, and altering their expression can interfere with embryonic stem cell maintenance and lineage-specific differentiation.[55,84] lncRNA expression is often tightly correlated with that of pluripotency markers,[28] and many lncRNAs are regulated by key transcription factors such as Nanog and Oct4.[84]

Detailed investigations have begun to reveal how lncRNAs function to regulate lineage commitment. Boyer et al at Massachusetts Institute of Technology have begun to study lncRNAs important for heart development and have identified a novel lncRNA (AK143260) required for specification of the cardiac lineage in vitro (Klattenhoff and Boyer, personal communication). Depletion of this lncRNA results in loss of beating cardiomyocytes during embryonic stem cell differentiation and a failure to activate a network of genes specifying key cardiac transcription factors and myofibril assembly components. Notably, these defects can be rescued by overexpression of the transcription factor MesP1, a key transcription factor that marks the earliest cardiac cell population in the developing embryo that ultimately gives rise to all cell types of the heart.[85] These data suggest that this lncRNA is required for mediating the transition from mesoderm to multipotent cardiac progenitors.

NATs Regulate Cardiac Muscle Contraction

Antisense transcripts also have been implicated in regulating several genes essential for heart function, including those for the cardiac troponin I (cTNI) and myosin heavy chains (MHCs) and light chains.[86] cTNI is exclusively expressed in adult myocardium, and reduction of cTNI often is observed in heart disease with detrimental effects on the mechanical properties of the heart. Various polyadenylated cTNI NATs have been identified in humans and rat, which differ in size between species.[87] All have the ability to form sense–antisense RNA duplexes, and the ratio of antisense to sense cTNI RNA changes from birth to adulthood, suggesting a role in the regulation of cTNI expression or translation.

Table. lncRNAs in Cardiac Development and Disease

lncRNA	Class	Function	Disease Association	Reference
Braveheart (AK143260)	lincRNA	Functions upstream of MesP1, necessary for cardiac lineage specification	None described	PC
cTNI antisense	NAT	Regulation of cTNI expression or translation	None described	87
β-MHC antisense	NAT	Regulation of isoform switching between α- and β-MHC	None described	89
ALC-1 antisense	NAT	Regulation of ALC-1	Induced in hypertrophic ventricles	92
ANRIL (CDK2BAS/ P15AS)	NAT	Scaffold for polycomb group complexes. Regulation of CDKN2A/B as well as of genes involved in nuclear regulation and chromatin architecture	Strongest genetic susceptibility locus for coronary artery disease	95,97
DMPK 3′UTR	uaRNA	Induction of Nkx2-5	DM1	108
SRA transcripts	lncRNAs	Coactivators of nuclear receptor signaling, muscle differentiation, components of gene insulator complexes	DCM	110,113
MIAT (Gomafu/RNCR2)	lincRNA	Retinal cell fate specification, splicing, function in MI unknown	MI	116

ALC-1 indicates atrial myosin alkali light chain; ANRIL, antisense noncoding RNA in the INK4 locus; cTNI, cardiac troponin I; DM1, myotonic muscular dystrophy; DMPK, myotonin-protein kinase; DCM, dilated cardiomyopathy; lincRNA, long intergenic noncoding RNA; MIAT, myocardial infarction–associated transcript; lncRNAs indicates long noncoding RNAs; PC, personal communication; NAT, natural antisense transcript; β-MHC, β-myosin heavy chain; SRA, steroid receptor RNA activator; uaRNA, 3′UTR-associated RNA; MI, myocardial infarction.

The ratio of α-MHC to β-MHC is a major determinant of contractility and energy turnover in the heart.[88] Hearts with higher α-MHC display elevated contractility, whereas those enriched for β-MHC contract more slowly and are more energy-efficient. Isoform switching readily occurs under pathophysiological conditions, such as diabetes mellitus, hypothyroidism, pressure overload, and caloric deprivation, which enhances β-MHC expression while repressing α-MHC, highlighting the presence of a mechanism of opposing regulation whereby expression of one isoform leads to repression of the other. This isoform switching is potentially mediated through NATs derived from transcription within the 4.5-kb intergenic space between the α-MHC and β-MHC genes.[89] A bidirectional promoter within this intergenic space coordinately regulates transcription of α-MHC sense mRNA and β-MHC antisense RNA.[90] The β-MHC NAT overlaps the β-MHC gene and extends into its promoter. Thus, when intergenic transcription is stimulated, the β-MHC NAT negatively influences β-MHC sense transcription and only α-MHC is produced, thus allowing the rapid switch from α-MHC to β-MHC under pathophysiological situations. However, the exact mechanism of repression used by MHC NATs is unclear.[91]

The human heart uses 2 myosin light chain isoforms: ventricular-myosin light chain (VLC-1) and atrial myosin light chain (ALC-1). ALC-1 expression disappears from the ventricles soon after birth but remains in the atria for life. However, ventricular reexpression is seen during pressure overload, and in patients with tetralogy of Fallot there are lower ALC-1 levels, despite higher *ALC-1* mRNA expression.[92] An ALC-1 antisense RNA is induced in hypertrophic ventricles, and higher antisense and sense ACL-1 ratios result in low ALC-1 protein levels. This suggests an important role for this NAT in post-transcriptional gene regulation of heart function.

ANRIL Variants and Coronary Atherosclerosis

lncRNA transcript levels are often dynamically altered in various human diseases, and changes in lncRNA expression or structure as well as their associated RNA-binding proteins are central to several disorders ranging from cancer to neurodegeneration.[93] Results from genome-wide association studies also suggest that a significant proportion of genetic variation associated with complex human diseases lies in non protein-coding regions of the genome.[94] Because most of the genome is transcribed, these mutations are transmitted to the transcriptome, where they can potentially affect many regulatory RNAs.

The strongest genetic susceptibility locus for coronary artery disease is located in the chromosome 9p21 gene desert, and 8 SNPs in this region map to the 3′ end of an lncRNA called antisense noncoding RNA in the INK4 locus (ANRIL).[95] ≈21% of the population is homozygous for a risk haplotype, giving them ≈2-fold greater chance of suffering myocardial infarction. This locus since has been linked to other human disorders, including cancer, type 2 diabetes mellitus, intracranial aneurism, periodontitis, Alzheimer disease, endometriosis, frailty, and glaucoma.[96] ANRIL, also known as CDKN2B antisense RNA (CDK2BAS) and P15 antisense RNA (P15AS), spans a 126.3-kb genomic region that is overlapping and antisense to the tumor suppressor gene CDKN2B (encoding p15) at its 5′ end.[97] The full ANRIL transcript (≈3.8 kb) consists of 20 exons, which are subject to alternative splicing to produce variants that show tissue-specific expression.[98]

ANRIL transcripts are detected in smooth muscle, endothelial, and immune cells, and patients with the risk haplotype exhibit elevated ANRIL expression in peripheral blood mononuclear cells and atherosclerotic plaque,[99] with transcript levels directly correlated with the severity of atherosclerosis.[100] ANRIL functions as a scaffold for polycomb group complexes by associating with both PRC1 and PRC2 and can function in *cis* to epigenetically repress its neighbors CDKN2A/B, whose expression levels are reciprocally related.[101,102] However, overexpression of 1 ANRIL variant altered the expression of many genes involved in nuclear regulation and chromatin architecture, indicating diverse *trans*-regulatory effects that go beyond the *cis*-effects seen at 9p21.[103] Thirty-three enhancers recently

have been discovered in this 9p21 gene desert, making it the second-densest gene desert for predicted enhancers.[104] One of the 10 SNP variants in this region most consistently associated with coronary artery disease (the G allele at rs10757278) is located within 1 of the predicted enhancers and disrupts the binding of the STAT1 transcription factor, resulting in altered ANRIL expression.[104] STAT1 is an effector of the interferon-γ inflammatory pathway, which in endothelial tissue is associated with the pathogenesis of atherosclerosis.[105]

DMPK 3′UTR and Nkx2-5 Regulation

Expression of 3′UTR variants also can contribute to heart dysfunction. Myotonic muscular dystrophy is a common autosomal-dominant neuromuscular disorder showing cardiac abnormalities involving sinoatrial and atrioventricular conduction block, accompanied by fibrosis, fatty infiltration, and cellular atrophy, and often leading to sudden death. Myotonic muscular dystrophy is a toxic RNA disease in which a CTG triplet repeat in the 3′UTR of the myotin protein kinase (DMPK) gene becomes expanded 50 to >2000 times.[106] Part of the disease mechanism involves accumulation of the mutant RNA in the nucleus, where it sequesters RNA-binding proteins involved in splicing and other RNA-mediated nuclear functions.[107] The disease can be replicated in mice carrying inducible transgenes overexpressing the normal *DMPK* 3′UTR harboring 5 CUG repeats.[108,109] In this mouse model, *DMPK* 3′UTR overexpression resulted in downregulation of connexins 40 and 43, gap junction proteins essential for propagation of action potentials, as well as upregulation of the homeodomain transcription factor Nkx2-5, which normally regulates connexin gene expression. Nkx2-5 expression also was induced in skeletal muscle, and genetic studies showed that Nkx2-5 upregulation in heart was responsible for the progressive heart block. Although the exact mechanism of how this 3′UTR induces Nkx2-5 expression remains elusive, it is clear that Nkx2-5 is a genetic modifier of myotonic muscular dystrophy RNA toxicity and indicates important functionality of this 3′UTR, independent of its mRNA, in heart dysfunction.

SRA and Human Dilated Cardiomyopathy

Steroid receptor RNA activator (SRA) is a multifunctional gene that gives rise to both steroid receptor RNA activator protein (SRAP) coding and noncoding transcripts.[110,111] ≈20 SRA noncoding transcripts have been identified that show tissue-specific expression patterns with the highest levels in liver, heart, and skeletal muscle. Differences in 5′ and 3′ extensions, point mutations, or presence of either a full intron or a portion of the intron determine the coding v noncoding potential of SRA transcripts. SRA RNAs show ≤11 structural elements and function as a coactivator of nuclear receptor signaling in a ligand-dependent manner. The SRA proteins also bind their own RNAs, and SRA/SRA coding transcripts also act in muscle myogenic differentiation as cofactors of myogenic differentiation (MyoD),[112] are associated with nuclear receptor–dependent cancers, and are a component of gene insulator complexes.[110] SRA also is present in a 600-kb linkage disequilibrium block associated with human dilated cardiomyopathy in 3 independent populations.[113] Knockdown of the zebrafish SRA homolog caused myocardial contractile dysfunction predominantly in ventricular heart chambers,[113] perhaps consistent with the role of nuclear receptors in cardiomyocyte maturation and function.[114]

MIAT lincRNA Variants Confer Susceptibility to Myocardial Infarction

Myocardial infarction–associated transcript (MIAT), also known as Gomafu or RNCR2, is an ≈9-kb lincRNA predominately expressed in the nervous system and has a known role in retinal cell fate specification in development.[115] Genome-wide SNP analysis has identified genetic variants in MIAT (rs2301523) that confer susceptibility to myocardial infarction.[116] One SNP (A11741G) caused a 1.3-fold increase in MIAT transcription in vitro, with the normal A allele exhibiting tighter binding to an unidentified nuclear factor. Despite its mRNA-like characteristics, being a long polyadenylated transcript arising from multiple exons, MIAT escapes nuclear export and is associated with an enigmatic subnuclear domain in a subset of neurons.[117] Chicken MIAT binds to splicing factor 1 through its tandem UACUAAC RNA repeats and may affect splicing by altering the nuclear concentration of splicing factors.[118] How it confers susceptibility to myocardial infarction is unclear.

lncRNA Toolkit for Functional Studies

Identification and Annotation of lncRNAs

There is need for accurate annotation of lncRNAs in specific cell and tissue types in various species. Several recent databases have been established, such as RNAdb,[119] which provides information on a wide range of ncRNAs, and lncRNAdb,[23] which is more specific for eukaryotic lncRNAs backed by experimental data. Each manually curated entry in lncRNAdb contains referenced information on RNA sequence, genomic location, structure, expression patterns, subcellular localization, conservation, and function when known. Entries are linked to the University of California Santa Cruz (UCSC) genome browser for ease of visualization and ncRNA expression database, which provides gene expression information for thousands of human and mouse lncRNAs.[120] Large-scale transcriptomic efforts to annotate human and mouse ncRNAs are underway by several large international consortia, such as Functional Annotation of the Mammalian Genome (FANTOM) and Encyclopedia of DNA elements (ENCODE),[94] which use RNA deep sequencing technology data, chromatin-state maps, and computer prediction algorithms to identify lncRNAs. Many lncRNAs are now included on microarrays, such as NCode ncRNA expression arrays, commercially available from Life Technologies, which can simultaneously profile 10 800 unique noncoding transcripts and 25 179 protein-coding transcripts. TaqMan quantitative polymerase chain reaction assays also are available to precisely interrogate individual lncRNA expression patterns.

Novel lncRNAs can be identified using an RNA deep sequencing technology, and several pipelines for data analysis have been suggested.[121] Because nonpolyadenylated transcripts comprise the major proportion (44%) of the transcriptional output of the human genome,[122] lncRNA studies should include both polyA+ and polyA− RNA fractions. In addition, 50% of all transcripts are found only in the nucleus, most

of which are ncRNAs,[122] and therefore additional separation into nuclear and cytoplasmic fractions could give insight into potential biological function. Importantly, a new technology called RNA-Capture-Seq recently has revealed the transcriptomic complexity at specific genomic loci, which indicates that the human transcriptome is far from fully characterized.[123] This technique first uses tiling arrays to capture specific portions of the transcriptome before sequencing them, giving a remarkable depth of coverage that has already revealed unannotated exons, splicing patterns, and noncoding intergenic transcription in the exceptionally well-studied p53 and HOX loci. It may be equally applied to fully characterizing the transcriptional landscape around important loci in heart development and function.

Identification of Genomic lncRNA Targets

Deciphering lncRNA function requires knowledge of where they act. Two similar techniques capture hybridization analysis of RNA targets (CHART) and chromatin isolation by RNA purification (ChIRP), analogous to chromatin immunoprecipitation for proteins, are used to map the genomic binding sites of endogenous lncRNAs.[124,125] CHART is a hybridization-based technique that, instead of antibodies, uses cocktails of several short, complementary, affinity-tagged oligonucleotides designed against accessible RNase-H–sensitive regions of the lncRNA (not occluded by secondary structure or protein binding).[124] These complementary, affinity-tagged oligonucleotides allow specific enrichment for endogenous lncRNAs, along with their DNA targets and protein cargo from reversibly cross-linked chromatin extracts. CHART–enriched material can then be analyzed by sequencing the DNA to identify the associated genomic loci or even by Western blot to analyze the interacting proteins. Similarly, ChIRP also hybridizes biotinylated complementary oligonucleotides to cross-linked chromatin.[125] However, tiling oligonucleotides are used to retrieve specific lncRNAs, which requires no previous knowledge of RNA secondary structure or functional domains. Other techniques, such as chromatin precipitation using antibodies to RNA–DNA hybrids or triplexes, also may potentially be used to identify RNA/DNA-binding sites genome-wide.[126]

Determining lncRNA-Interacting Proteins

Genome-wide techniques for identifying which lncRNAs are bound to particular proteins already have been used successfully for several chromatin modifiers.[55,127] RNA immunoprecipitation using antibodies to specific proteins can pull-down associated transcripts that can then be identified by sequencing (RNA immunoprecipitation-Seq). There are a range of commercial RNA immunoprecipitation kits available. Alternatively, NanoString nCounter technology can be used for identifying transcripts after RNA immunoprecipitation.[128] This system captures and counts individual RNA molecules using color-coded probe pairs and eliminates the need for enzymatic reactions and amplification cycles often used during RNA deep sequencing technology library preparation. It is, however, biased toward interrogating expression of a known custom-selected set of ≈500 lncRNAs. Because this technology uses ≈300 ng of total RNA or less as starting material, it is well-suited for analysis in which RNA amounts are limiting, such as from microdissected samples, tissue biopsies, cells sorted by flow cytometry, or RNA immunoprecipitation samples.[55] Some studies have identified protein partners for specific lncRNAs through use of human protein microarrays. For example, Cy5-labeled Six3OS RNA probed to human protein microarrays identified 5 proteins that specifically interacted with both mouse and human Six3OS. This included histone modification enzymes and known transcriptional coregulators of *Six3*, suggesting that Six3OS functions as an RNA-based transcriptional scaffold.[129]

Effects of lncRNAs on Gene Expression and Phenotype

Identifying the functional role of an lncRNA and whether it is *cis*- or *trans*-acting generally requires direct perturbation experiments, including loss-of-function or gain-of-function analysis. lncRNA knockdown has been performed using RNA interference techniques, such as short hairpin RNAs, followed by sequencing or microarray hybridization to determine their global effects on transcription.[55] lncRNA function is then inferred on the basis of the genes that are affected by loss of function of the RNA. The RNA Interference Consortium contains a useful reference set of RNA sequences for knocking down lncRNAs. Although questions have been raised about the efficacy of these techniques because many lncRNAs are located in the nucleus and the machinery used for knocking down RNA is thought to lie predominantly in the cytoplasm, experience has not supported these concerns, with increasing numbers of successful knockdowns of lncRNAs being reported.[4] The majority of these knockdowns exhibit discernable phenotypes in cell culture. An alternative site-directed method uses zinc finger nucleases to integrate RNA-destabilizing elements into lncRNAs (such as polyA signals, AU elements, self-cleaving ribozymes, or RNaseP substrates) that function as termination elements and destabilize downstream sequences,[130] which may be more specific and efficient than using RNA interference techniques.

Regulation of lncRNAs

Regulation of ncRNA transcription is evidently highly complex, with many annotated transcripts potentially arising from posttranscriptional processing of primary transcripts of protein-coding genes. However, many ncRNAs are independent transcriptional units, whether overlapping or distal to protein-coding genes, controlled by their own promoter elements and influenced by conserved local enhancers. Chromatin immunoprecipitation experiments followed by deep sequencing (chromatin immunoprecipitation-Seq) have revealed that many lncRNAs show specific binding at their promoters by key developmental transcription factors. The genomic targets of GATA-binding protein 4 (GATA4), NK2 transcription factor–related, locus 5 (NKX2-5), T-box 5 (TBX5), serum response factor (SRF), and myocyte-enhancer factor 2A (MEF2A) in the HL-1 cardiomyocyte cell line[131] show the importance of combinatorial transcription factor regulation and that ncRNAs form an important component of the cardiac gene regulatory network. In addition, RNA editing or RNA modifications may have a profound impact on RNA function. There are currently >100 RNA modifications described, of which >90% affect ncRNA species.[132] Although the function of most

modifications remains unknown, they may have the ability to alter conformational plasticity or metabolic stability of RNA molecules, thus impacting their function and interaction with cellular components. It recently was shown using RNA bisulfite sequencing that 5-methylcytosine, a landmark epigenetic DNA modification, also affects >10 000 sites in both coding and ncRNAs.[133] Therefore, it remains important to decipher the role of RNA modifications in the posttranscriptional control of cellular RNA function.

Deciphering lncRNA Structure

The primary sequence of lncRNAs generally is not well-conserved between species, although there is a wide variation from ultraconserved to lineage-specific species. However, RNA secondary structure may show higher evolutionary conservation and could provide a meaningful way of classifying lncRNAs. Characterization of the SRA secondary structure shows that even RNA nucleotide sequences with poor conservation do not exist as disordered regions but show defined structural organization.[134] In addition, the RNA structural core of SRA has been evolutionary conserved, often at the expense of the protein product, indicating importance of this gene as a functional lncRNA. Traditionally, RNA structure has been determined for 1 molecule at a time using chemical agents or nucleases that can distinguish between single-stranded or base-paired regions. Recent advances have developed efficient computational structural prediction programs that can interrogate many RNA sequences simultaneously (for a list of secondary structure prediction programs and uses).[135] One technique termed parallel analysis of RNA structure uses high-throughput sequencing of RNA fragments digested with structure-specific enzymes to determine whether an RNA nucleotide is in a double- or single-stranded conformation on a genome-wide scale.[136] A similar technique, fragmentation sequencing, digests only single-stranded RNA using nuclease P1 before sequencing and mapping these regions to the genome to yield information on RNA structure (PARS). Such analyses will be essential to building a compendium of recognizable RNA domains that interact with different types of effector proteins, which will enable a richer informatic parsing of the lncRNA functional landscape, such as is possible using Pfam domain recognition in proteins themselves.

Conclusion

We are currently at the beginning of our understanding of the complex world of lncRNAs. It is clear that ncRNA structures and functions are highly diverse and that their repertoire of functions are likely to rival those of the proteome. This regulatory layer of RNA exhibits a dynamic interplay with other network components to increase network performance and robustness. The full extent of the cardiac noncoding transcriptome needs to be determined and a comprehensive list of annotated lncRNAs and short ncRNAs expressed in specific cardiac regions at different stages of development and disease needs to be documented. High-throughput genomic approaches that interrogate both polyA+ and polyA− RNA fractions will open new windows into genome biology, revealing not only transcriptional complexity but also potentially new players in the regulatory network governing cardiac development and congenital heart disease.

The language used by lncRNAs to interact with network components is still largely elusive. Unlike the well-studied miRNAs, lncRNAs do not seem to function via a common pathway; therefore, no predictions can be made about their function based on their primary sequence or secondary structure. A major challenge lies in decoding the functional elements and modules in the primary sequence of noncoding genes, including structural motifs and regulatory elements that define their roles. By understanding the function of this sea of *cis*- and *trans*-acting regulatory sequences engulfing islands of protein-coding information, we hope to gain new insight into developmental processes, disease mechanisms, and new targets for pharmacological intervention.[137] The field of lncRNAs in cardiac development and disease has vast potential for discovery and will remain a rich field of investigation for the foreseeable future.

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