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Challenges and Opportunities in Linking Long Noncoding RNAs to Cardiovascular, Lung, and Blood Diseases

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Abstract

The new millennium heralds an unanticipated surge of genomic information, most notably an expansive class of long noncoding RNAs. These transcripts, which now outnumber all protein-coding genes, often exhibit the same characteristics as mRNAs (RNA polymerase II-dependent, 5' methyl capped, multi-exonic, polyadenylated), yet they do not encode for stable, well-conserved proteins. Elucidating the function of all relevant lncRNAs in heart, vasculature, lung, and blood is essential for generating a complete interactome in these tissues. This is particularly evident as an increasing number of investigators perform RNA-sequencing experiments where, typically, annotated lncRNAs exhibit impressive changes in gene expression. How does one go about evaluating an lncRNA when the sequence of the transcript lends no insight into how it may function within a cell type? Here, we provide a brief overview for the rational study of long noncoding RNAs.

Keywords

noncoding RNA; genomics; CRISPR; disease; method

Our understanding of the human genome has changed considerably over the years, from a landscape of junk DNA to one replete with functional information. Of notable interest are the tens of thousands of long noncoding RNAs (lncRNAs) that, by strict definition, are processed transcripts >200 nucleotides that do not encode for a protein. We are only beginning to understand how lncRNAs work, mainly because of their weak expression, low sequence conservation, and capricious functions. Despite these challenging attributes, two facts underscore an urgent need to intensify our study of lncRNAs in the heart, vasculature, lung, and blood: (i) the vast majority of disease-associated sequence variants occur in

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Disclosure

None.

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noncoding sequence space where lncRNAs reside[1–3] and (ii) emerging evidence points to diverse roles for lncRNAs in maintaining or perturbing cellular homeostasis.[2, 4, 5] This mini-review offers some basic directions and resources for investigators to consider for the rational study of this large class of noncoding genes.

Identifying lncRNAs

LncRNAs are subdivided into several classes including enhancer RNAs, long intervening RNAs, natural antisense RNAs, sense or antisense RNAs, pseudogenes, and circular RNAs. One means of uncovering lncRNAs is through deep sequencing of polyadenylated or non-polyadenylated RNA from cell types in bulk or at the single-cell level either under normal or stimulus-provoking conditions. An important consideration prior to screening for lncRNAs is whether the initial RNA originates from human or experimental animal model. Since most lncRNAs exhibit low sequence conservation, finding homologous genes in other species is challenging and hence limits the scope of investigation. For example, loss-of- function studies in mice are not possible if the lncRNA is only present in humans. Nevertheless, short blocks of sequence homology combined with synteny offer opportunities to define lncRNA orthologs.[6] Of note, one need not even lift a pipetman to find an lncRNA of interest as they are increasingly annotated in the Ensembl (www.ensembl.org) and UCSC genome (www.genome.ucsc.edu) browsers. The trove of experimental and computational data at these and other websites (*e.g.*, http://www.lncipedia.org) provide some insight into which lncRNAs to evaluate.[7] For example, an increasing number of genome wide association study hits fall near or within an lncRNA, suggesting many of the cardiovascular, lung, and blood disease-associated variants will affect lncRNA expression or function.[1, 8]

Genome browsers also display a large number of lncRNAs closely juxtaposed to or overlapping protein-coding genes. Investigators would therefore be wise to examine the genomic landscape around their favorite protein-coding gene for neighboring lncRNAs. Analyzing these lncRNAs could reveal information about their regulation and potential function in the transcriptional or post-transcriptional control of the adjacent protein-coding gene. Such protein-coding/lncRNA gene pairs present new challenges and opportunities in genetic loss-of-function experiments where thoughtful planning is essential. In this context, the revolutionary clustered regularly interspaced short palindromic repeats (CRISPR) system affords precision-guided genome editing that can uncouple the role of an lncRNA from that of an overlapping protein-coding gene or another functional noncoding sequence.[9] Alternatively, it is now possible to target lncRNAs directly with CRISPR innovations that circumvent functional DNA elements or the role of active transcription of the lncRNA.[10, 11] One additional challenge in identifying lncRNAs relates to their annotation as authored names (*e.g., MALAT1, Braveheart, SENCR*) versus less informative labels (*e.g.*, LOC#, FLJ#, KIAA#, #Rik, Gm#). How is one to sort through a large list of such vague designations in an RNA-sequencing experiment? One way is to query any number of lncRNA websites[7] for an array of information that will help inform the investigator as to whether an lncRNA exhibiting stimulus-induced changes in gene expression should be pursued for further study.

Bioinformatics of lncRNAs

De novo assembly of novel lncRNAs can be achieved with the assistance of a bioinformatician possessing a working knowledge of programs such as Scripture[12] that will assemble transcription units in a reference genome according to such parameters as splice donor/acceptor sequences and the relative expression of each exon. Basic properties of annotated lncRNAs can be gleaned through bioinformatics data archived in many lncRNA databases (*e.g.*, http://www.lncipedia.org; http://www.noncode.org; http://www.lncrnadb.org).[7] Whether an lncRNA is novel or already annotated, chromatin signatures and transcription factor binding site data through chromatin immunoprecipitation-sequencing experiments in the Ensembl or UCSC Genome Browser offer some insight into potential modes of lncRNA expression control. Further, ribosome profiling shows most cytoplasmic lncRNAs are engaged with the ribosomal machinery for the generation of small peptides.[13, 14] These peptides may have local functions related to translational control processes and then rapidly degraded. In some cases, a conserved open reading frame within an lncRNA, initially missed in early annotations, may encode for a stable protein carrying out an essential function within a cell.[15] An investigator should therefore, at a minimum, interrogate an lncRNA for open reading frames using programs such as ExPASy (http://www.expasy.org). It would also be instructive to either mine ribosomal profiling data for open reading frame "footprints" corresponding to the lncRNA-ribosome association or perform an *in vitro* transcription/translation assay to demonstrate the presence or absence of a stable peptide. Further evidence for a stable micro-peptide encoded within an lncRNA may be obtained through mass spectrometry analyses or integration of an in-frame epitope tag (such as FLAG or Hemagglutinin) using CRISPR genome editing. If an lncRNA encodes for a peptide, then it would be of interest to perform site-directed mutagenesis of the initiating start codon to assess whether the lncRNA has both coding and noncoding functions in a cell.

LncRNAs, with our without coding potential, may also harbor microRNA response elements and de-repress target genes by acting as competing endogenous (or 'sponge') RNAs through sequestration of microRNAs.[16] However, this competing endogenous RNA hypothesis is controversial and its physiological relevance in an *in vivo* setting may not be borne out.[17] Nevertheless, defining microRNA response elements within an lncRNA is easily accomplished (*e.g.*, http://starbase.sysu.edu.cn/) and could provide a fruitful research direction, provided the stoichiometry of microRNA-binding lncRNA to target sites is sufficient to have meaningful impact; this may be challenging given the low abundance of most lncRNAs. LncRNAs can also base-pair with mRNAs so as to trigger mRNA decay, and this can be determined with a simple basic local alignment search tool query (http://blast.ncbi.nlm.nih.gov/). Finally, secondary structure prediction programs such as RNAfold (http://rna.tbi.univie.ac.at/) or Mfold (http://unafold.rna.albany.edu/) can facilitate the optimal design of PCR primers or siRNA oligonucleotides to lncRNAs and may serve as a foundation for future classification of lncRNAs based on structural attributes.

Expression of lncRNAs

The main challenge here is prioritizing those lncRNAs, from among hundreds in an RNA-sequencing screen, for further workup. With a modest depth of sequencing of ~10 million

reads, basal expression of the majority of lncRNAs will be less than 5 fragments per kilobase of transcript per million mapped reads (JMM, unpublished). This is a reasonable cutoff for the determination of known attributes of an lncRNA using the above bioinformatics analyses. Initial wet-lab studies will typically validate the level of expression of an lncRNA by quantitative RT-PCR in the original cells or tissue used in the RNA-sequencing experiment. Additional expression studies should include a spectrum of cell types (± agonist or other stimulus) and tissue types. The main challenge with analyzing expression of lncRNAs is their weak expression as compared to mRNAs; cycle threshold values are generally ≥35 for most lncRNAs. One hypothesis for such low level expression of an lncRNA is transcriptional noise with no intrinsic biological information.[18] On the other hand, low level lncRNAs could have functions in *cis* through direct or indirect transcriptional control processes involving sequestration of key transcription factors, scaffolding of chromatin remodeling factors, or the formation of RNA-DNA triplex structures.[19] Alternatively, the simple act of lncRNA transcription could influence local gene expression.[20] Distinguishing between these modes of regulation is possible with carefully designed CRISPR strategies (below). It is also important to define the full length of an lncRNA with 5' and 3' rapid amplification of cDNA ends since many lncRNAs are incompletely annotated with respect to transcribed sequences.[21] Defining the fully processed transcript of an lncRNA is critical if promoterology or gain-of-function studies are to be carried out. Assessing transcript half-life with timed actinomycin D experiments will also inform an investigator as to the stability of an lncRNA. This parameter may find utility with lncRNA/protein-coding gene pairs, which may be co-regulated by common regulatory regions; variable RNA decay rates in an lncRNA/protein-coding gene pair could imply the regulation of one over the other. A database of mouse embryonic stem cell mRNA decay rates is available for comparison to lncRNA half-lives.[22]

Another challenge with low abundant lncRNAs is their accurate detection and quantitation in individual cells or tissues. Most lncRNAs are expressed below the level of detection by *in situ* hybridization of a tissue section. However, opportunities exist to define where in isolated cells an lncRNA resides with cell fractionation followed by quantitative RT-PCR. The latter assay provides a reasonable first approximation, but quantitative RNA-fluorescence in situ hybridization and digital PCR are more powerful measures of intracellular locale and absolute levels of an lncRNA. Defining lncRNA localization is a key step towards elucidating lncRNA function. For example, nuclear lncRNAs tend to have functions related to gene transcription, splicing, or nuclear architecture[23, 24] whereas cytoplasmic lncRNAs have myriad and unpredictable functions including the control of signal transduction pathways,[25] post-transcriptional control of other processed transcripts,[26] regulation of translation,[27] and facilitative control of cytoplasmic-nuclear trafficking of proteins.[28] Of note, lncRNAs, particularly those expressed abundantly (*i.e.*, cycle threshold values ≤30), may be detected in plasma or other body fluids suggesting their transport may reflect (patho)physiological processes of clinical interest.[29] Indeed, many lncRNAs have been described as biomarkers of disease (*e.g.*, cancer).[2] An important challenge here is the standardization of detection methods for reliable reporting of circulating lncRNAs in disorders of the heart, vasculature, lung, and blood.

Function of lncRNAs

Illuminating the function of an lncRNA is a formidable challenge. Unlike microRNAs, with well-defined sequence features (seed sequence and limited size range) and mechanisms of action (mRNA destabilization and/or translational repression), the function of lncRNAs is rarely revealed through defined sequence rules; a notable exception is the presence of *Arthrobacter luteus* elements in human lncRNAs that mediate Staufen-mediated mRNA decay.[30] The importance of defining sub-cellular localization of an lncRNA for functional studies cannot be over-emphasized. For example, while lncRNAs can form numerous types of interactions including RNA–RNA, RNA–DNA, and RNA–protein, the establishment of a cytoplasmic localization profile would direct efforts to understand RNA-RNA or RNA-protein interactions rather than an RNA-DNA interaction. In some cases, there may be localization both in the nucleus and cytoplasm. In this instance, one could fractionate compartments for further study. A common approach to ascertain biological function of an lncRNA is RNA-sequencing in cells following loss-of-function (*e.g.*, siRNA knockdown) of the lncRNA. Gain-of-function transcriptome analysis may be difficult to interpret due to non-physiological levels of an lncRNA, although controlled expression of an lncRNA in genetic rescue experiments is important in accurately interpreting a loss-of-function phenotype. Further, since there may be structural motifs conserved between lncRNAs with otherwise dissimilar sequences, it may prove informative to examine the function of a human lncRNA in the mouse using a gain-of-function approach with the lncRNA expressed in a cell-restricted manner. In addition, human lncRNAs can be evaluated in embryonic stem cells[31] as well as inducible pluripotent stem cells that are differentiated to a cell type in which the lncRNA is suspected to have some function.

For conserved lncRNAs of clinical significance, CRISPR genome editing in mice can provide some understanding of the regulation, function, or sequence variation of an lncRNA within a complex integrative system.[32] This becomes particularly relevant when the question arises as to whether the lncRNA functions in *cis* to control gene expression. Here, two-component CRISPR (defined as Cas9 endonuclease and sgRNAs)[32] could be used to delete the promoter and first exon of the lncRNA. A parallel approach using three-component CRISPR (defined as Cas9 endonuclease, sgRNA, plus a single-strand oligonucleotide repair template)[32] would involve the integration of a polyadenylation signal to interrupt transcription resulting in a truncated lncRNA product. These two approaches of editing an lncRNA could discriminate between the processed lncRNA versus lncRNA transcription *per se* in mediating a *cis* effect on gene expression. Of course, this type of approach would be reserved for a nuclear lncRNA. To date, only one lncRNA (*Rian*) has been inactivated in the mouse though the approach (a 23-kb deletion) is somewhat problematical.[33] Indeed, lncRNA inactivation by CRISPR-Cas9 genome editing demands careful deliberation to minimize deletion of other functional elements that could confound interpretation of the phenotype.

Definitive evidence for functionality requires biochemical studies that disclose the macromolecule with which the lncRNA interacts. Thankfully, there is a growing arsenal of methods designed to uncover lncRNA interactions including chromatin isolation by RNA purification, which, despite its name, can be used to reveal RNA-DNA, RNA-RNA, and

RNA-protein interactions; RNA antisense purification; capture hybridization analysis of RNA targets; cross-linking and immunoprecipitation; and ribonucleoprotein immunoprecipitation.[34] When coupled to mass spectrometry or next-generation sequencing, potential lncRNA binding partners will be revealed. For example, ribonucleoprotein immunoprecipitation using a biotinylated sense RNA probe followed by mass spectrometry demonstrated that the conserved dendritic cell-specific lncRNA, *WFDC21P*, interacts with STAT3 promoting its phosphorylation by physically inhibiting dephosphorylation of a critical tyrosine residue in STAT3. Knockdown of *WFDC21P* reduced dendritic cell differentiation in both human and mouse demonstrating the importance of this lncRNA in the normal function of these important immune cells.[25] A similar approach was used to show that the lncRNA, *NRON*, physically binds importin beta 1 subunit (*KPNB1*) and inhibits cytoplasmic-nuclear translocation of the NFAT family of transcription factors thus having an indirect effect on gene transcription programs.[28] It should be noted that to authenticate specificity of an lncRNA-macromolecular interaction, several biochemical assays should be utilized in parallel.

Currently, only ~50 lncRNAs have been characterized and studied in the heart, vasculature, and lung (Supplemental Table) and, of these, only a small handful have been shown to have effects in a pathological setting. The *Mhrt* lncRNA is cardiac-specific and is subject to repression upon cardiac hypertrophy via stimulation of a repressor complex that converges over the *Mhrt* promoter region; reinstating *Mhrt* expression could prevent a heart failure phenotype.[35] The conserved lncRNA, *lincRNA-p21*, is reduced in patients with coronary artery disease and a mouse model of atherosclerosis and was shown to reduce smooth muscle cell proliferation and promote apoptosis through a feed-forward mechanism of p53 activation.[36] These examples suggest important roles for lncRNAs in cardiovascular pathology. Given their massive number, increasing correlation with genome wide association study hits, and diverse mechanisms of action, an all-out assault in defining every lncRNA and its expression and function (*e.g.*, with directed genome-wide CRISPR-Cas9 screens[37]) is needed to optimally fuel basic and clinical research related to diseases of the heart, vasculature, lung, and blood. Integrating knowledge of lncRNAs with protein-coding and other noncoding genes is critical to paint a complete portrait of the underlying signaling and transcriptional events directing normal homeostatic processes and how such finely-tuned systems are perturbed in disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations used in text

CRISPR	clustered regularly interspaced short palindromic repeats
lncRNAs	long noncoding RNAs

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Highlights

- Long noncoding RNAs are a rapidly growing class of noncoding genes.
- Unlike microRNAs, long noncoding RNAs are spatially and functionally diverse in nature.
- There is a paucity of information about the function of long noncoding RNAs in heart, vasculature, lung, and blood.
- A battery of tools exists to accurately determine the localization and potential function of long noncoding RNAs.