The time has come,” the Walrus said, “to talk of many things.”1 With apologies to Lewis Carroll, understanding the role of myocardial long noncoding RNAs (lncRNAs) can be reminiscent of poor Alice’s adventures through the looking glass; one of the few certainties is that not much is (yet) certain. LncRNAs are broadly defined as RNA transcripts that are not expected to code for proteins and that are >200 nucleotides in length to distinguish them from other noncoding RNA families, such as microRNAs and small nucleolar RNAs.2 Nevertheless, empirical data trump predictions, as demonstrated by the use of ribosome RNA profiling techniques to document nonproductive translation of lncRNAs3 and recent discoveries of short open reading frames (microORFs) and resultant micropeptides embedded within some transcripts previously defined as lncRNAs.4,5 With prevalent usage of high read-depth RNA sequencing for RNA quantification and discovery in mammalian tissues and cells, the number of noncoding RNAs continues to grow. Current estimates of genomic loci harboring lncRNAs are as high as ≈79,000 in mouse and ≈90,000 in human, according to NONCODE2016.6 Previous efforts using RNA sequencing have established which of at least some lncRNAs recognized at the time of study are expressed in human and mouse hearts,7–9 and depending on the analytic platform used, ab initio reconstruction of previously unknown lncRNA transcripts from deep RNA sequencing data has been possible.7,9 LncRNAs have also been comprehensively catalogued in the embryonic, developing heart.8,10,11 Two lncRNAs shown to mediate critical transitions in shaping cardiomyocyte and cardiac fate in the mouse are Fendrr12 and Bvht (Braveheart),11 via association with and recruitment of histone-modifying complexes to effect chromatin modification. In both of these cases, gene targeting and a range of sophisticated molecular and cellular investigations were required to define lncRNA function, just as such methods are needed to attribute functions to other novel RNAs. Although not all of the >130,000 recognized lncRNA transcripts6 are necessarily expressed in the heart, a large number without mechanistic characterization still remain (and indeed, it would be premature to conclude that the function of every mRNA expressed in the heart is well understood, without even considering microRNAs and others). Several questions arise from these observations: Are at least some lncRNAs transcribed because the cellular machinery that produces really useful RNAs is not fully able to distinguish between their transcription start sites and others that are not optimal, that is, are such lncRNAs the result of transcriptional noise? Are some lncRNAs, such as enhancer RNAs, only produced to assist with modulation of local chromosome conformation and, thus, enjoy only a transient existence? Are all lncRNAs truly useful, including among their functions either cis or trans activity on genomic DNA together with as yet unrecognized cytosolic roles?11 Importantly, do higher-throughput methods currently exist that can be used to provide functional insight?

The manuscript by Touma et al14 uses a relatively high-throughput analytical method to focus on a key period in cardiac maturation, that of the immediate postnatal period in which the heart must successfully adapt to altered nutrient availability and maintenance of an independently oxygenated circulation via appropriate pulmonary blood flow. The authors have chosen to investigate primarily lncRNA abundance and alterations, although there is also a need to better understand coding mRNA changes during this period, and the rRNA-depleted, strand-specific RNA sequencing data that underlie this study do in fact permit full investigation of both coding and noncoding RNA species. Transcriptomes were studied in the left and right ventricles separately during postnatal days 0, 3, and 7 in the mouse, permitting chamber-specific analysis over a time course of early cardiac maturation. To identify and characterize differentially expressed mRNAs, it is a common procedure to select mRNAs that vary between conditions based on the magnitude of differences together with a statistical measure of confidence, such as the false discovery rate, and to then allocate such mRNAs to signaling networks. Although lncRNAs could well be selected in a similar manner, the paucity of empirically established functional data would compromise further characterization. Another valid but less commonly used approach to mRNA selection is to perform extensive correlation analyses of mRNAs across samples and conditions and to subsequently group mRNAs into modules, solely on the basis of their shared variance behavior. This procedure is referred to as weighted gene coexpression network analysis,15 and Touma et al14 use it here to independently group mRNAs and lncRNAs. By recognizing mRNA and lncRNA modules with similar correlation behavior across samples and conditions, clues can be gained to the function of clusters of lncRNAs based on the function of the covarying mRNA modules. At this stage of what are likely to be ongoing investigations, the conclusions drawn by the authors are fairly broad. Having studied postnatal cardiac transcriptomes in both a time- and chamber-dependent manner, the
highest number of gene expression changes are observed early in maturation (between P0 and P3), and differences between the left and right ventricles are dwarfed by the effect of the maturation process. Continued examination of key hub RNAs (those with the highest levels of connectivity to others within the same module, as assessed by correlation parameters) in both the mRNA and the lncRNA module sets should lead to further mechanistic insights. A total of 155 mRNAs and 17 lncRNAs were reported to act as hub RNAs, which is a large but not unreasonable number from which to select RNAs for detailed studies.

A more immediate application of these data involved the mapping of those lncRNAs and mRNAs thought to exist in genomically proximal regions, with the aim of identifying lncRNAs capable of modulating mRNA transcription in cis. Here, the authors borrow a previous strategy for identifying cis-acting lncRNAs, although the co-localization distance is extended to from 10 to 50 kb of linear genomic DNA. Naturally, both in this study and the previous one, the lack of knowledge of open chromatin conformation and topologically associating domains made it impossible to account for genomic regions that may be proximal in 3-dimensional space, yet further apart on a linear basis; such data remain to be gathered using cutting-edge chromosome conformation assays for cardiac myocytes at various developmental stages. Nonetheless, from 2262 lncRNA/mRNA pairs with detectable expression in neonatal heart, 114 pairs demonstrated significantly correlated expression. The vast majority of these (=90%) exhibited positive correlation, suggesting concordant transcription from a region of open chromatin. Although precise functional characteristics of all these lncRNAs remain to be examined, it is perhaps more likely that lncRNAs that exhibit positive correlation with neighboring mRNAs represent transcriptional noise than those lncRNAs whose abundance is negatively correlated with mRNAs. Among these, 3 previously described lncRNA/mRNA pairs exhibiting negative correlation between embryonic and adult mouse hearts were validated, while several new pairs were described, including four pairs for which the lncRNA has a conserved ortholog in the human transcriptome. Although strong sequence conservation of lncRNAs across species has proven to be the exception rather than the rule, the identification of these 4 new pairs (together with the previously described n420212/KCNB1) permits studies of potential lincRNA/mRNA coregulation to be performed in human cardiac disease.

Validation of the newly discovered lincRNA/mRNA pairs requires moving beyond the correlative relationships obtained from the RNA sequencing data to independent experiments. Here, the authors again borrow a previous strategy, that of manipulating lncRNA levels in the more readily transfectable mouse skeletal myoblast C2C12 cell line using GapmeRs, a proprietary lncRNA-interference technology from Exiqon. During postnatal heart maturation, as well as during differentiation of C2C12 cells, Ppp1r1b-lncRNA levels decrease, while those of Tcap mRNA increase; Tcap encodes a key sarcomeric protein that anchors titin to the Z-disk. GapmeR-mediated interference with Ppp1r1b-lncRNA resulted in an increase of Tcap mRNA in C2C12 cells and in neonatal mouse cardiomyocytes. Interestingly, Ppp1r1b-lncRNA inhibition also interfered with C2C12 myotube fusion, perhaps indicating that adoption of a fully differentiated phenotype (as assessed by Tcap expression) requires cessation of fusion. More relevant to the heart, however, and particularly relevant to the focus of these studies on the early postnatal heart, is the finding that the negative correlation between Ppp1r1b-lncRNA and Tcap mRNA abundance is a feature of human infantile tetralogy of Fallot and of the presence of ventricular septal defects. Although not validated in C2C12 cells, the FUS-lncRNA/TRIM72 inversely correlated pair also exhibited an association with tetralogy of Fallot and ventricular septal defects. It may be of interest in future studies to increase expression of either Ppp1r1b- or FUS-lncRNA in the embryonic mouse heart to investigate whether dysregulation of these lncRNAs is sufficient to recapitulate these human congenital defects, or whether additional events resulting from dysregulated open chromatin are required.

As mentioned previously, lncRNAs generally exhibit little conservation at the nucleotide sequence level across species, and here Touma et al. made the most of the opportunity to link mouse and human studies using some of those few lncRNAs that are well conserved. It has been suggested previously that lncRNAs may exhibit much greater conservation at a structural rather than a sequence level, yet the experimental tools available to evaluate RNA structure are still limited in their reach. Recent studies of a key lncRNA for mouse cardiac development, Bvht, which is not obviously conserved at the sequence level in human, both indicate the likely path to overcoming difficulties in cross-species investigations while highlighting the degree of molecular understanding that will be necessary. Chemical probing of the 590-nt Bvht RNA was able to define important elements of its structure, and targeting of an 11 nt G-rich motif in Bvht eliminated its interaction with a zinc-finger protein CNBP/ZFPP; in the absence of this interaction, cardiomyocyte differentiation was impaired. This study adds evidence that the active regions of an lncRNA may represent a relatively small proportion of its total nucleotide count and suggest that as long as overall conformation is preserved, the exact composition of the remaining nucleotides may not be important. Although it represents an arduous road of investigation, our eventual ability to understand essential lncRNA roles and conservation may well depend on the degree to which structure-function relationships can be assessed.

The studies of Touma et al. thus, represent a vital effort in categorizing which lncRNAs are likely to be worthy of study with advanced resources. The comprehensive RNA sequencing data over the early postnatal transition period nearly fill a gap between previous studies comparing embryonic and adult hearts and studies examining postnatal hearts at P1, 3-week-old, and 5-week-old stages. The experimental toolkit for evaluating lncRNA function, especially with gene-targeting approaches, is subject to the same demands as for mRNAs, and the degree to which RNA structural assessment techniques to better predict lncRNA function can be accelerated is still uncertain. While awaiting these developments, the temptation exists to infer lncRNA function on the basis of neighboring mRNAs in linear genomic space, even to the extent of attempting to assign lncRNAs to Gene Ontology
categories for coding mRNAs and proteins. However, in the absence of at least coexpression data on lncRNAs and mRNAs under specific conditions of interest, such attempts at deriving functional insights risk merely talking about, as the Walrus nonsensically does, “why the sea is boiling hot—and whether pigs have wings.”

Disclosures

None.

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