Decoding the Long Noncoding RNA During Cardiac Maturation
A Roadmap for Functional Discovery

Marlin Touma, MD, PhD; Xuedong Kang, PhD*; Yan Zhao, PhD*; Ashley A. Cass, BS; Fuying Gao, PhD; Reshma Biniwale, MD; Giovanni Coppola, MD; Xinshu Xiao, PhD; Brian Reemtsen, MD; Yibin Wang, PhD

Background—Cardiac maturation during perinatal transition of heart is critical for functional adaptation to hemodynamic load and nutrient environment. Perturbation in this process has major implications in congenital heart defects. Transcriptome programming during perinatal stages is an important information but incomplete in current literature, particularly, the expression profiles of the long noncoding RNAs (lncRNAs) are not fully elucidated.

Methods and Results—From comprehensive analysis of transcriptomes derived from neonatal mouse heart left and right ventricles, a total of 45 167 unique transcripts were identified, including 21 916 known and 2033 novel lncRNAs. Among these lncRNAs, 196 exhibited significant dynamic regulation along maturation process. By implementing parallel weighted gene co-expression network analysis of mRNA and lncRNA data sets, several lncRNA modules coordinately expressed in a developmental manner similar to protein coding genes, while few lncRNAs revealed chamber-specific patterns. Out of 2262 lncRNAs located within 50 kb of protein coding genes, 5% significantly correlate with the expression of their neighboring genes. The impact of Ppp1r1b-lncRNA on the corresponding partner gene Tcap was validated in cultured myoblasts. This concordant regulation was also conserved in human infantile hearts. Furthermore, the Ppp1r1b-lncRNA/Tcap expression ratio was identified as a molecular signature that differentiated congenital heart defect phenotypes.

Conclusions—The study provides the first high-resolution landscape on neonatal cardiac lncRNAs and reveals their potential interaction with mRNA transcriptome during cardiac maturation. Ppp1r1b-lncRNA was identified as a regulator of Tcap expression, with dynamic interaction in postnatal cardiac development and congenital heart defects. (Circ Cardiovasc Genet. 2016;9:395-407. DOI: 10.1161/CIRCGENETICS.115.001363.)

Key Words: congenital cardiac defect ▶ gene regulation ▶ lncRNA ▶ neonatal heart maturation ▶ neonatal mouse cardiomyocyte ▶ transcriptome

Perinatal cardiac growth and maturation is critical for functional adaptation of the heart to changes in hemodynamic load, respiration, and nutrient environment. At birth the adaptation of the cardiovascular system to extrauterine life, also referred to as perinatal circulatory transition, involves dramatic hemodynamic changes that lead to closure of the fetal shunts and separation of the pulmonary and the systemic blood flow. Concomitantly, the left ventricle (LV) and the right ventricle (RV) undergo functional modifications and structural remodeling to operate the systemic and the pulmonary circuits, respectively. Perturbation in this process has major implications in congenital heart defects (CHDs). Remarkably, during fetal to neonatal transition period, the vast majority of cardiomyocytes undergo dramatic changes in morphology, function, metabolism, gene expression, and proliferative capacity. These tightly regulated processes become significantly disrupted in the setting of a preterm birth or a CHD. The abnormal physiology of the premature myocardium and the altered anatomy of the impaired heart are further complicated by the persistence of fetal shunt pathways and pathological flow patterns. As such, the growing heart is particularly vulnerable during this critical period to multifactorial perinatal stresses (sepsis, respiratory failure, surgery) that can...
consequently lead to significant ventricular dysfunction and circulatory failure.

Transcriptome programming is the driving force for cardiac maturation and functional adaptation during perinatal circulatory transition in normal and pathological conditions. However, although changes in cardiac gene expression have been extensively studied in the developing fetal heart, in the postnatal heart, and in the failing adult heart, the molecular mechanisms of transcriptome programming in neonatal heart chambers during the critical window of fetal to neonatal maturation remain to be fully revealed. In addition, current stem-cell therapies share a common challenge of cellular immaturity upon differentiation. Therefore, understanding the transcriptome landscape during the perinatal window will fill an important gap in cardiac maturation and would have significant implications in CHD and cardiac regeneration.

Recently, the discovery of the long noncoding RNA transcripts (lncRNAs) has expanded the total functional complexity of transcriptome. Featuring temporal regulation, species and tissue specificities, and functional diversity, it is increasingly evident that the lncRNAs play important roles in gene regulation at different levels, including transcription, chromatin modification, and post-transcriptional editing. The importance of lncRNAs is increasingly recognized in heart development and pathogenesis. Several reports have identified hundreds of lncRNAs enriched in heart, dynamically transcribed during development, and differentially regulated in disease.

Here, we performed comprehensive analysis of the lncRNAs in neonatal mouse hearts. We found a significant number of these lncRNAs dynamically expressed along postnatal maturation. Further, we identified several lncRNA co-expression modules coordinately regulated during postnatal development similar to protein coding genes. Furthermore, we find 5% of the highly regulated lncRNAs significantly correlate with the expression of their corresponding neighboring genes. Finally, we demonstrate that the Ppp1r1b-lncRNA can potently modulate its neighboring partner gene Tcap, and the expression ratio of Ppp1r1b-lncRNA/Tcap segregates different types of CHDs. These insights would advance our current understanding of the gene regulatory network and potential disease mechanisms in perinatal heart.

Methods

All animal-related experimental protocols were approved by the University of California Los Angeles Animal Care and Use Committee. All human studies were conducted in accordance with regulation of the University of California Los Angeles Institutional Review Board.

Gene expression data have been deposited within the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo) under Neonatal Heart Maturation SuperSeries GSE85728 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85728).

Detailed methods are provided in the Data Supplement, including description of experiments, study design, bioinformatics analysis, weighted gene co-expression network analysis (WGCNA), network analysis and statistical methods, in vitro functional studies, human specimens, molecular validation, primers, and GapmeR designs.

RNA Sequencing and Bioinformatics Analysis

Deep RNA sequencing was performed using total RNA isolated from male C57B/6 mouse LV and RV at postnatal day 0 (P0; before the ductal closure), day 3 (P3; transition), and day 7 (P7; terminal differentiation of the vast majority of cardiomyocytes; Figure IA in the Data Supplement). Strand-specific cDNA libraries were constructed from 3 LV and RV total RNA samples (biological replicates) at each time point, with the exception of only 2 RV samples for day 7. Paired end sequencing reads were aligned to reference mouse genome (UCSCmm9) using TopHat. Reads mapped to reference genome by TopHat were used for assembly. Reference annotation–based assembly method was implemented to reconstruct the transcripts using Cufflinks to predict novel lncRNAs and using Cuffmerge to merge them.

Identifying Known and Novel lncRNAs

The constructed transcripts were compared with reference annotation database (NONCODE v4.0) by using Cuffcompare, and the known lncRNAs were filtered. The transcripts that do not exist in NONCODE v4.0 database were regarded as candidate novel lncRNAs (Figure IA; Figure IB and IC in the Data Supplement). To separate potential protein coding transcripts from putative novel lncRNAs, isceRNA software, an open reading frame–based analyzer capable of defining short open reading frames, was used. Finally, further analysis filters that are based on lncRNA definition (transcript length ≥200 bp) and features (contains at least one splice junction, given ≥98% of lncRNAs undergo alternative splicing) were performed to identify the novel lncRNAs. Expression levels of the novel and known lncRNAs, as well as the mRNAs, were estimated using the reads per kilobase per million of mapped reads (RPKM) measure. Cutoff values of 3 RPKM and 0.3 RPKM were used to define significantly expressed mRNA and lncRNA, respectively.

Differential Expression Analysis

Genes with mean ≥3 RPKM and lncRNAs with mean ≥1 RPKM in at least one sample (3 biological replicates) of each category and variation (V) ≥0.2 across samples were included. Unless otherwise specified, significantly differentially expressed genes/lncRNAs are defined as those with fold change (FC) ≥2, at a false discovery rate FDR ≤0.05.

Weighted Gene Co-Expression Network Analysis

Genes with mean RPKM ≥3 and lncRNAs with mean RPKM ≥0.3 in at least one sample (3 replicates) of each category and variation ≥0.2 across samples were included to construct signed network modules using the R package. The module–trait correlation relationships were calculated (Figure II in the Data Supplement). Unique stage-specific modules were defined as those with correlation coefficient r ≥0.7 and P value ≤0.005 between the module Eigengene and the maturation stage (P0, P3, or P7).

Genomic Position

Known and novel lncRNAs were evaluated if they can be located within ±50 kb upstream or downstream of a protein coding gene by comparing to reference genome. For each lncRNA, all Ensembl genes within the ±50 kb region that do not overlap with the lncRNA were identified. Requiring no sequence overlaps between the neighboring gene and the lncRNA of interest eliminates the host genes that overlap with a putative lncRNA sequence. The correlation of gene expression for each lncRNA/mRNA pair was calculated using Pearson’s correlation and Benjamini–Hochberg correction methods. A Benjamini–Hochberg adjusted correlation P value ≤0.05 was considered significant.

Human Studies

Pediatric patients with clinical diagnosis of teratology of fallot or ventricular septum defect were enrolled in accordance with the regulation of the University of California Los Angeles Institutional Review Board–approved protocol. Informed consents were obtained from all participants. Human heart specimens from the RV outflow tract were collected during clinically indicated cardiac operations. The specimens were immediately snap frozen using liquid nitrogen. Total RNAs were isolated using standard methods.
Statistics
Quantified results are presented as mean±SEM. Comparisons between groups were evaluated using analysis of variance or the Student’s t test; \( P \leq 0.05 \) was considered significant, unless specified otherwise in Methods in the Data Supplement.

Results
LncRNAs Are Dynamically Regulated in Neonatal Heart Chambers
We implemented deep RNA sequencing to establish mRNA/lncRNA landscape in neonatal LV and RV. We obtained tissues from P0 (before the ductal closure), P3 (transition), and P7 (terminal differentiation of the vast majority of cardiomyocytes; Figure 1A; Figure I in the Data Supplement). Out of the average 30 million paired sequencing reads obtained from each sample, 77% were uniquely mapped to mouse genome and classified according to their categories in reference to UCSDmm9 and NONCODE v4.0. Using cutoff values of 0.3 RPKM and 3 RPKM in at least one sample to define the expressed lncRNAs or mRNAs, respectively, a total of 45,167 unique transcripts were detected, including 21,218 mRNAs and 23,949 lncRNAs. Among the lncRNAs, 2033 are novel.
Wide range of lncRNA expression levels in neonatal heart was observed, involving known and novel lncRNAs varying from <0.1 RPKM for Braveheart and Fendrr, both of which are important for maintaining cardiac fate and differentiation of cardiac progenitors, to >30 RPKM for the well-known H19 (NONMMUT064276). This wide range of expression is consistent with previous observations in fetal and adult heart.\(^1\) Among a total of 23,949 lncRNAs expressed in neonatal heart, 1,286 exhibited significant variation exceeding 0.2 across samples. Among them, 1099 (863 known and 236 novel) lncRNAs are abundantly expressed (RPKM ≥1), including 242 (214 known and 28 novel) lncRNAs with average RPKM exceeding 3 (Figure 1B; Table I in the Data Supplement).

Principal component analysis of the top-ranked 500 protein-coding mRNAs (based on magnitude of changes) across all samples revealed developmental stage as a major contributor to the underlying expression changes (84%), while chamber specificity has a relatively modest role (12%; Figure 1C, upper panel). Consistent with principal component analysis, unsupervised hierarchical clustering analysis at whole transcriptome level revealed that samples from the same developmental stage clustered together, regardless of their chamber identity. Interestingly, transcripts from P3 and P7 clustered relatively close but further away from P0, indicating more robust transcriptome changes during the P0 to P3 window. Furthermore, chamber-specific gene expression clusters were identified in P3 and more clearly in P7 hearts, but not in P0, suggesting an increasing level of transcriptome divergence between LV and RV as the heart matures (Figure 1D, upper panel).

Similar to mRNAs, the principal component analysis of the top 500 varying lncRNAs (ranked based on magnitude of changes) also revealed that developmental time point is a major source of variation (PC1≈85%; Figure 1C, lower panel). However, chamber-based separation was much lower, mainly at P7. Likewise, unsupervised hierarchical clustering on all detected lncRNAs revealed a developmental stage-specific lncRNA signature in the neonatal heart without discernable chamber-specific differences (Figure 1D, lower panel). Similar expression patterns were also revealed by differential expression analysis that was structured along 2 schemes: development time points (P0 versus P3, P3 versus P7, and P0 versus P7) and ventricular chamber identity (LV versus RV; Figure 2A). A total of 196 lncRNAs are significantly expressed in at least one maturation window at cutoff values of RPKM≥2, V≥0.2, FDR ≤0.05, and FC≥2 (i.e., log FC ≥1), with concordant regulation patterns in both LV and RV (Figure 2B–2D; Table II in the Data Supplement). In contrast, NONMMUT054852 was the only differentially expressed lncRNA between LV and RV at FC>2. Given that the chamber-specific genes may generally change <2 fold, we relaxed the FC criterion while tightening the FDR P value to ≤0.01. Using these alternate criteria, 16 lncRNAs were identified to be differentially expressed in chamber-specific manner, including 5 lncRNAs upregulated in LV and 11 lncRNAs upregulated in RV from at least one time point (Figure 2E and 2F; Table III in the Data Supplement).

### Concordant mRNA/LncRNA Network Modules During Neonatal Heart Maturation

To establish functional relevance of mRNAs and lncRNAs associated with postnatal heart maturation, we performed an unsupervised WGCNA\(^3\) across the 3 time points. WGCNA on mRNA transcripts (RPKM ≥3, V≥0.2) revealed 18 gene co-expression modules (Figure 3A). Among them, 8 modules were identified to exhibit significant stage-specific expression correlation in both LV and RV (r≥0.7 and P≤0.005; Figure 3B). In total, 2826 member genes were found in the P0-specific modules (brown, blue, and magenta), comparing to 1234 genes in the P3 (light cyan, salmon, and magenta) and 955 genes in the P7 (green and red) modules. Only 2 of the 8 stage-specific modules also exhibit chamber specificity. This observation is consistent with the notion that the greatest changes in gene expression in neonatal heart are between P0 and P3, whereas relatively more gradual changes occur between P3 and P7, and variations in neonatal heart gene expression are predominantly driven by developmental process rather than LV versus RV differentiation.

From these modules, we identified 155 hub genes based on their intramodular gene connectivity (≥0.9 and P≤10\(^{-10}\)). Many of these genes are also identified independently as hub genes in publicly available data sets. For example, Sox4, a hub gene for the light cyan mRNA module (Figure 3B), is also consistently identified and verified as a hub gene in separate data sets regulating memory CD8\(^+\) T cell development.\(^3\) Notably, Sox4 has also been implicated in ventricular septation and outflow tract development.\(^3\) We also identified several hub genes that have not been established in other public data sets, providing novel candidates for future mechanistic studies. By carrying out functional enrichment analysis, we found glucose metabolism, cell cycle, chromatin organization, and RNA processing enriched in the P0 gene modules; mitochondria, fat metabolism, and intracellular organelle formation enriched in P3 gene modules; and signaling, protein synthesis, and multicellular organ development enriched in P7 gene modules (Figure 3C). Together, these changes reflect stepwise progression in cardiacmyocyte differentiation, metabolic maturation, and cellular growth during postnatal transition.

When significantly expressed, lncRNAs (RPKM≥0.3, V≥0.2) were subjected to parallel gene network analysis using WGCNA; 9 modules were identified (Figure 3D). Among them, 6 modules are developmental stage specific (Figure 3E). Similar to the observations made from mRNAs, the developmental stage-specific lncRNA modules were largely shared between LV and RV. In contrast, only one lncRNA module (lncRNA-yellow) exhibits both stage- and chamber-specific association. In total, 710 lncRNAs were identified in developmental stage-specific modules at P0 (brown, turquoise, and blue), 152 lncRNAs at P3 (magenta and red), and 143 lncRNAs at P7 (yellow), parallel to the same trends observed for mRNAs in terms of number of changes observed during P0 to P3 window in contrast to P3 to P7 window (Figure 3E). Remarkably, these stage-specific lncRNA modules exhibited reciprocal transition between P0 and P7 during maturation (Figure 3F).

Notably, 17 lncRNAs were identified to exhibit hub gene like properties with significant intramedullary connectivity. Of these hub-lncRNAs, 11 are members of stage-specific
Figure 2. Long noncoding RNAs (LncRNAs) are dynamically regulated in neonatal heart along maturation stages. A, Schematic representation of pair-wise comparative (differential expression) analysis along 2 schemes: stage specific and chamber specific. B, Numbers of significantly differentially expressed LncRNAs (cutoff values: \( \text{RPKM} \geq 1, \text{V} \geq 0.2, \text{Fold Change} (\text{FC}) \geq 2, \text{FDR} \text{ P value} \leq 0.05 \)) in stage-specific and (Continued)
modules (Figure 3E). H19 (NONMUT064276) was identified as a potential hub gene in the P0–brown–lncRNA module (Figure IVA and IVB in the Data Supplement). We further analyzed potential interaction between lncRNA and mRNA modules by implementing the hypergeometric test for individual modules. A total of 33 lncRNAs (11 known and 22 novel) were identified to be significantly concordant with their corresponding mRNA modules. Notably, more than half of them are members of the P0 lncRNA modules, while 7 of them are from the P7 lncRNA module. Based on their local genomic structure, 11 were identified as natural antisense transcripts, 19 as sense, and 3 as intronic lncRNAs (Table IV in the Data Supplement). Together, these analyses suggest that the overall architectures of the mRNA and the

Figure 3. Weighted gene co-expression network analysis (WGCNA) revealed stage-specific mRNA and long noncoding RNAs (lncRNA) module gene network in neonatal heart. A and D, WGCNA dendrograms of protein coding mRNAs (A) and lncRNAs (D) expression reveal different expression modules. Branches in the hierarchical clustering dendrograms correspond to modules. Color-coded module membership is displayed in the color bars below the dendrograms. y Axes (height) represent module significance (correlation with external trait). B, Heat map depicting expression profiles of stage-specific mRNA modules member genes. Eight stage-specific modules overlapping in LV and RV and numbers of genes corresponding to each module are shown (color bars). Eigengene expression of a given module is presented (bar graphs). Color code of the modules is preserved. C, Top gene ontology (GO) terms enriched in corresponding stage-specific modules are listed with their P values. D, Expression heat maps (Z score) of chamber-specific differentially expressed lncRNAs in LV and RV. Columns represent lncRNAs, and rows represent expression ratio between developmental stages being compared (red, upregulated; green, downregulated). FC indicates fold change; and RPKM, reads per kilobase per million.
lncRNA transcriptome are largely shared between LV and RV and are likely the product of developmentally regulated transcription programs in neonatal heart.

Functional Implications of lncRNAs

To identify potential regulatory lncRNAs, we focused next on identifying lncRNAs that were located upstream or downstream of an annotated protein coding gene to examine their potential impact on paired neighboring gene expression. The lncRNA/gene pairs were defined as being co-located within 50 kilobases of genomic distance, but with no sequence overlap between the neighboring gene and the lncRNA of interest, thus, eliminating the potential lncRNA/host gene pairs. Based on these criteria, a total of 6996 lncRNA/mRNA pairs were identified on mouse genome. Among them, 2262 unique lncRNA/gene pairs were detected in neonatal heart (defined as lncRNA-RPKM ≥ 1, mRNA-RPKM ≥ 3, V ≥ 0.2). Of these, 114 lncRNAs, including 12 novel lncRNAs, showed significantly correlated expression pattern with a neighboring mRNA (Pearson’s correlation Benjamini–Hochberg–corrected P value ≤ 0.05) during at least one developmental period (Table V in the Data Supplement). 90.4% of these pairs are positively correlated (eg, UCP2-lncRNA/UCP3) and 9.6% are negatively correlated (eg, Ppp1r1b-lncRNA/Tcap; Figure 4A and 4B). By gene ontology analysis of the lncRNA-correlated mRNAs, RNA process (eg, Hnrnpa1, Sf3b6), cardiac development (eg, Nkx2-5, Hand2), and metabolism (eg, Lc7is, UCP3) are found to be the top enriched functional ontology terms. Remarkably, the majority of significantly correlated lncRNA/mRNA pairs are also members of developmental stage–specific lncRNA or mRNA modules with significant correlation with their corresponding module Eigengenes.

LncRNAs are generally poorly conserved across species. However, we found that 5 lncRNAs have conserved ortholog in human, including UCP2-lncRNA (NONMMUT 062940), n420212 (NONMMUT041263), Fus-lncRNA (NONMMUT063779), Ppp1r1b-lncRNA (NONMMUT011874), and H19 (NONMMUT064276). Their expressions were detected in infantile heart and altered in different CHDs (Figure 5a–5d; Figure IVC in the Data Supplement). Importantly, the genomic position and the inverse expression relationship between Fus-lncRNA and Ppp1r1b-lncRNA with their corresponding neighboring genes Trim72 and...
Tcap (Figure 5C and 5D), respectively, were also conserved in human infantile hearts (Figure 5c and 5d). Similarly, the genomic position relationships and concordant expression patterns of n420212/KCNB1 and UCP2-lncRNA/UCP3 pairs (Figure 5A and 5B) were also conserved in human postnatal heart (Figure 5a and 5b). Together, these data suggest that some cardiac lncRNA/mRNA pairs discovered in mouse are also preserved in human.

To validate regulatory relationship of the identified lncRNA/mRNA pairs, we investigated the Ppp1r1b-lncRNA/Tcap pair further. As shown in Figure 5D, the Ppp1r1b-lncRNA/Tcap mRNA pair exhibit tightly correlated dynamic expression during neonatal heart maturation. In addition, they both are members of the inversely related lncRNA/mRNA modules. While Ppp1r1b-lncRNA significantly correlates with P0–lncRNA–brown module Eigengene, Tcap is a member of the P7–mRNA–green module in neonatal mouse heart (Figure VB and VC in the Data Supplement). Tcap encodes Titin Cap, a muscle-specific protein involved in cardiomyocyte sarcomere organization and myogenesis by anchoring Titin filaments at the disk.35,36

As illustrated in Figure 6A and Figure 8A, the Ppp1r1b-lncRNA is positioned within 28 kb upstream of the Tcap gene on mouse chromosome 11 and similarly positioned on human chromosome 17. The Ppp1r1b-lncRNA consists of partial exonic and intronic segments from its host gene Ppp1r1b, as well as a nonoverlapping exon fragment that we targeted using GapmR (Figure 6A; Figure VI in the Data Supplement). A short open reading frame is identified in the mouse Ppp1r1b-lncRNA, but is predicted to be nonfunctional. Consistent with quantitative reverse transcriptase polymerase chain reaction–validated expression time course in neonatal mouse heart (Figure 6B I and II), the expression time course of Ppp1r1b-lncRNA and Tcap in differentiating C2C12 myoblast cells revealed reciprocal relationship (Figure 6C I and II).

In response to Ppp1r1b-lncRNA inhibition in myoblasts, myocyte maturation as measured by myotubes fusion index was blocked by Ppp1r1b-lncRNA inactivation (Figure 7A and 7B). Furthermore, significant upregulation of Tcap-mRNA and protein abundance was observed in differentiating myotubes, supporting negative regulatory relationship (Figure 7C and 7D) along with suppressed expression of other myogenesis genes, including Myom2 and Myoz2 (Figure 7E). Notably, Ppp1r1b-lncRNA inhibition did not impact the host gene expression, nor other nearby genes (eg, Stard3-mRNA was not affected). Moreover, the inverse correlation relationship was preserved in GapmR-targeted neonatal cardiomyocytes (Figure 7F). These data support that the putative Ppp1r1b-lncRNA transcript is not another splice product of the host gene.

More interestingly, both PPP1R1B-lncRNA and the corresponding partner gene TCAP were found to be tightly and inversely coregulated in human infantile hearts with tetralogy of fallot, with age ranging from 2 to 12 months (Figure 8B). Importantly, the negatively correlated relationship between PPP1R1B-lncRNA and TCAP expression was also observed in infant hearts with tetralogy of fallot or ventricular septal defect (Figure 8C). Moreover, the ratio of PPP1R1B-lncRNA and TCAP expression significantly segregated the 2 types of CHDs (Figure 8D).

Discussion

Here we performed in-depth characterization of the expression landscape of lncRNAs relative to mRNAs and revealed interactions among lncRNAs, as well as between lncRNA and mRNA gene networks. We find that the majority of lncRNAs exhibit developmental stage–specific regulation, parallel with mRNA expression patterns. We also observed concordant dynamic regulation of lncRNAs more evident during postnatal heart development than in postnatal chamber specification. Furthermore, we identified novel lncRNA/mRNA pairs implying potentially important roles of lncRNAs in neonatal cardiomyocyte maturation.

The RNA profiling reported in this study complements 2 recent reports where RNA sequencing analyses were performed in postnatal hearts at specific time points, focusing on data from P2 and P13 hearts and from P1 and P21 hearts, respectively. However, our current analysis focuses on lncRNA profile and correlation with mRNA in neonatal hearts during the critical window of perinatal circulatory transition (before and after the ducal closure), which was not included in these 2 data sets. We include more description on the different times during the critical window of perinatal circulatory transition (before and after the ducal closure). We also use methods different from these 2 studies. To our knowledge, this is the first time the neonatal heart gene and lncRNA expression profiles have been systematically analyzed in spatial–temporal manner, providing a high-resolution profile of their signature and characteristics during critical fetal to neonatal transition, all of which may help identify important lncRNAs of potential regulatory function impacting neonatal heart maturation and disease.

Our data revealed 1099 lncRNAs abundantly expressed and varied in neonatal heart chambers. Gene network analysis identified coordinated lncRNA and mRNA modules that were shared in a developmental stage–specific fashion. Remarkably, sharp changes of the transcriptome complex, including mRNA and lncRNA networks, occurred during P0 to P3 maturation window, likely reflecting the rapid adaptation of cardiovascular system to dramatic changes in circulation, nutrient environment, and cellular respiration. These changes impact cardiomyocyte energy demands and metabolism, as well as regeneration and functional properties.

A recent report investigated cardiac-specific lncRNAs in contrast to liver and skin lncRNAs and identified 117 heart-enriched and 104 adult cardiomyocyte–enriched lncRNAs (RPKM≥2.3) in mice. Out of these previously reported cardiac lncRNAs, we identified 27 cardiac-enriched and 35 adult cardiomyocyte–enriched lncRNAs that were expressed in neonatal heart exhibiting dynamic regulation during maturation (Table VI in the Data Supplement). Remarkably, our findings agree with their report that genomic position of the lncRNAs and their correlated expression with neighboring genes is an important predictor of putative functionality. Therefore, we further compared their differential expression results with our results revealed by combined differential expression and WGCNA analyses. Particularly, all cardiomyocyte and cardiac-enriched lncRNAs that are found to be abundantly expressed in our samples (RPKM≥2) revealed stage–specific module membership in neonatal heart (Figure 7 in the Data Supplement). In addition to the validated cardiac-enriched lncRNA/mRNA pair n420212 (NONMMUT041263)/
Figure 5. Conserved expression correlation relationship between lncRNA/mRNA partners in human infantile heart. A–D, Expression time courses (RNA sequencing) of lncRNAs (Ucp2-lncRNA, n420212, FUS-lncRNA, and Ppp1r1b-lncRNA) in LV (light pink) and RV (light blue) and partner mRNAs (Ucp3, Kcnb1, Trim72, and Tcap, respectively) in LV (dark pink) and RV (dark blue). a–d, Correlation graphs depicting expression correlation relationship between lncRNAs (UCP2, NONMMUT041263, FUS-lncRNA, and PPP1R1B-lncRNA; x axis) and (Continued)
Kcnb1 mRNA, we found other previously validated pairs, such as n411949 (NONMMUT042600)/Mccc1 and n413445 (NONMMUT042600)/Relb, displaying similar correlated expression in our study. These observations suggest that cardiac-specific and highly abundant lncRNAs are also highly correlated and connected to their developmental module network, implicating potential functionality in coordinated gene regulation.

A major finding of this study is that lncRNAs exhibit tight temporal regulation during fetal to neonatal maturation and may have important regulatory function during this window. In particular, we identified potential novel regulatory interactions between correlated lncRNAs and neighboring mRNAs in neonatal heart. Tcap encodes a cardiomyocyte-specific protein involved in cardiac myogenesis. Trim72 is enriched in heart and regulates sarcolemma response to oxidative stress. The inverse relationship between Ppp1r1b-lncRNA and its neighboring partner gene Tcap and between Fus-lncRNA and Trim72 in neonatal heart suggests potential role of these lncRNAs in cardiomyocyte maturation and early adaptation to postnatal stress. Indeed, our functional studies have demonstrated significant functional impact of Ppp1r1b-lncRNA inactivation on myogenesis and sarcomere assembly by blocking differentiation of C2C12 cells. Additionally, the induction of UCP2-lncRNA and associated UCP3 mRNA may be important for metabolic switch during postnatal myocardium maturation. Together, our findings are of direct clinical value to further understand neonatal heart programming not only during normal maturation, but also during pathological maturation, especially in the context of prematurity.

Figure 6. Ppp1r1b-lncRNA (NONMMUT011874) and Tcap are inversely regulated in neonatal mouse heart and in C2C12 cell line.

A. Genomic position of mouse Ppp1r1b-lncRNA in relation to Tcap on mouse Chromosome 11, and schematic representation of GapmeR (antisense Oligo) targeting site.

B. Quantitative validation (qRT-PCR) of Ppp1r1b-lncRNA, Tcap, and Ppp1r1b-mRNA expression time course in neonatal mouse heart (I: RNA sequencing data, II. qRT-PCR data).

C. Quantitative validation of Ppp1r1b-lncRNA, Tcap, and Ppp1r1b-mRNA expression during C2C12 differentiation (n=3 replicates). lncRNA indicates long noncoding RNAs; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; and RPKM, reads per kilobase per million.
and CHDs when metabolic substrates are altered, fetal shunts are often persistent, and fetal to neonatal adaptation is delayed or disrupted, leading to cardiac and circulatory failure early after birth.

Importantly, some of the mouse neonatal heart lncRNAs have conserved counterparts in human infant hearts with CHDs, and some of the lncRNA/mRNA pairs observed in mouse are also preserved in human. Indeed, the emerging links between lncRNAs and heart development indicate that lncRNAs contribute to core transcriptional regulatory circuits involving key transcription factors that underlie human CHDs. Our findings showcase that specific lncRNA/mRNA pairs can not only have a significant impact on cellular morphology and function but also be valuable to segregate diseases. In particular, we identified Ppp1r1b-lncRNA/Tcap expression ratio as a molecular signature that differentiated tetralogy of fallot and ventricular septum defect in human infantile hearts. Therefore, comprehensive profiling of lncRNAs may yield novel biomarkers and therapeutic targets for CHDs and pediatric heart diseases.

Important limitations of our study remain. We used whole ventricle tissue for RNA sequencing analysis without first sorting or purifying different cell types. Therefore, the low overall expression levels of certain lncRNAs may mask the high level and important expression of these lncRNAs in specific subset of cardiac cells. In addition, future studies using RNA sequencing should include more replicates. Furthermore, there are significant deficiencies in the current algorithms for lncRNA identification, and some of these currently annotated lncRNAs may produce micropeptides as shown by Nelson et al.

Notably, we observed that vast majority of the lncRNAs that exhibited concordant regulation with protein coding genes are particularly localized within 2-kb distance of their neighboring genes, suggesting a potential cis regulatory mechanism of function. However, we recognize that the neighboring gene pair based analysis is limited to identify potentially functional lncRNAs because it will miss many long-range trans-acting lncRNAs with their targets, at either transcriptional or post-transcriptional levels. Further, some natural antisense transcripts exhibited significant overlap with concordant lncRNA–mRNA modules, suggesting that at least some lncRNAs also act in trans manner, potentially via recruitment of chromatin remodeling complexes. Importantly, we acknowledge that the finding obtained from our C2C12 model system may not fully recapitulate maturation

Figure 7. Ppp1r1b-lncRNA (NONMMUT011874) regulates Tcap expression and myogenesis in cultured C2C12 myoblast cell line. A, Light microscopy (upper) and confocal images (lower) of cultured C2C12 cells in response to GapmeR treatment compared with scrambled control. B, Semiquantitative analysis of myogenic differentiation using fusion index. C and E, Quantitative expression of Ppp1r1b-lncRNA, Tcap, Myoz2, Myom2, Ppp1r1b, and Stard3 in C2C12 cells in response to GapmeR compared with scrambled control 48 hours post treatment. D, Increased Tcap protein abundance in response to Ppp1r1b-lncRNA knockdown. F, Quantitative expression of Ppp1r1b-lncRNA, Tcap, and Ppp1r1b-mRNA in neonatal rat ventricular myocytes (NRVMs) in response to GapmeR compared with scrambled control. (n=3 replicates per condition). lncRNA indicates long noncoding RNAs.
process of cardiomyocytes. Using human cardiomyocytes derived from induced pluripotent stem cells will serve as an ideal approach of functional interrogation in future studies.

Together, our study supports putative regulatory role of lncRNAs in transcriptome programming during a critical window of neonatal heart maturation, adaptation, and disease. Our observations reinforce the notion that lncRNAs may use diverse molecular mechanisms to regulate their target genes. Additional functional studies will be needed to clarify their diverse mechanisms and biological function during myocardium maturation providing molecular basis for future investigations on congenital heart disease and stem cell therapy.

Acknowledgments

We acknowledge Dr. Thomas Vondriska for the critical review of the article. We acknowledge the support of the National Institute of Neurological Disorders and Stroke (NINDS) Informatics Center at University of California Los Angeles (UCLA).

Sources of Funding

This work was supported by grants from National Institutes of Health (NIH)/Child Health Research Center (5K12HD034610/ K12) and the UCLA-Children’s Discovery and Today and Tomorrow Children’s Fund for Dr Touma; NIH/Predoctoral training grant (T90DE022734) for A.A. Cass; R01HG006264 for Dr Xiao; HL070079, HL103205, HL108186, and HL110667 and UCLA-CTSI-Cardiovascular Pilot Team Research Grant UL1TR000124 for Dr Wang.

The NINDS Informatics Center for Neurogenetics and Neurogenomics (P50 NS062691) for Drs. Coppola and Gao.

Disclosures

None.

References

1. Finnemore A, Groves A. Physiology of the fetal and transitional circulation. *Semin Fetal Neonatal Med.* 2015;20:210–216. doi: 10.1016/j.siny.2015.04.003.
2. Donn MS. Fetal-to-neonatal maladaptation. *Semin Fetal Neonatal Med.* 2006;11:166–173.
3. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, et al. Transient regenerative potential of the neonatal mouse heart. *Science.* 2011;331:1078–1080. doi: 10.1126/science.1200708.
4. Harvey RP. Patterning the vertebrate heart. *Nat Rev Genet.* 2002;3:544–556. doi: 10.1038/nrg843.
5. Srivastava D. Making or breaking the heart: from lineage determination to morphogenesis. *Cell.* 2006;126:1037–1048. doi: 10.1016/j.cell.2006.09.003.
6. Vincent SD, Buckingham ME. How to make a heart: the origin and regulation of cardiac progenitor cells. *Curr Top Dev Biol.* 2010;90:1–41. doi: 10.1016/S0070-2153(10)90001-X.
7. Olson EN. Gene regulatory networks in the evolution and development of the heart. *Science.* 2006;313:1922–1927. doi: 10.1126/science.1132292.
8. McCulley DJ, Black BL. Transcription factor pathways and congenital heart disease. *Curr Top Dev Biol.* 2012;100:253–277. doi: 10.1016/B978-0-12-387786-4.00008-7.
9. Bruneau BG. The developmental genetics of congenital heart disease. *Nature.* 2008;451:943–948. doi: 10.1038/nature06801.
10. Laffamme MA, Murry CE. Heart regeneration. *Nature.* 2011;473:326–335. doi: 10.1038/nature10147.
Collectively phrased as perinatal circulatory transition, fetal to neonatal transition of the heart is a complex but tightly regulated process. Its perturbation may have major implications in congenital heart defects and failure of the premature heart. Transcription programme is the driving force for functional adaptation and maturation of the heart. However, our current understanding of transcription programme changes in neonatal heart chambers, in particular involving long non-coding RNA species, is limited. Here we undertook a multilayered and integrated systems analysis of neonatal heart transcriptome at three time points of perinatal circulatory transition. We find that transcription programme changes, including both protein coding RNAs and long non-coding RNAs, are more significantly driven by developmental stage than ventricular chamber specificity. Chamber-specific and developmental stage–specific long non-coding RNA/mRNA gene networks are identified to be associated with dynamic changes in myocyte proliferation and maturation. Novel interactions between mRNA and long non-coding RNAs species are revealed with potentially significant impact on myocyte function. The result of this study will help dissect the molecular mechanisms involved in perinatal circulatory transition in heart and facilitate the discovery of potential diagnostic and therapeutic targets for congenital heart defects and failure of the premature heart.