

Noncoding RNA Expression in Myocardium From Infants With Tetralogy of Fallot

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Background—The importance of noncoding RNAs (ncRNA), especially microRNAs (miRNAs), for maintaining stability in the developing vertebrate heart has recently become apparent; however, there is little known about the expression pattern of ncRNA in the human heart with developmental anomalies.

Methods and Results—We examined the expression of miRNAs and small nucleolar RNAs (snoRNAs) in right ventricular myocardium from 16 infants with nonsyndromic tetralogy of Fallot (TOF) without a 22q11.2 deletion, 3 fetal heart samples, and 8 normally developing infants. We found 61 miRNAs and 135 snoRNAs to be significantly changed in expression in myocardium from children with TOF compared with normally developing comparison subjects. The pattern of ncRNA expression in TOF myocardium had a surprising resemblance to expression patterns in fetal myocardium, especially for the snoRNAs. Potential targets of miRNAs with altered expression were enriched for gene networks of importance to cardiac development. We derived a list of 229 genes known to be critical to heart development and found 44 had significantly changed expression in TOF myocardium relative to normally developing myocardium. These 44 genes had significant negative correlation with 33 miRNAs, each of which also had significantly changed expression. The primary function of snoRNAs is targeting specific nucleotides of ribosomal RNAs and spliceosomal RNAs for biochemical modification. The targeted nucleotides of the differentially expressed snoRNAs were concentrated in the 28S and 18S ribosomal RNAs and 2 spliceosomal RNAs, U2 and U6. In addition, in myocardium from children with TOF, we observed splicing variants in 51% of genes that are critical for cardiac development. Taken together, these observations suggest a link between levels of snoRNA that target spliceosomal RNAs, spliceosomal function, and heart development.

Conclusions—This is the first report characterizing ncRNA expression in a congenital heart defect. The striking shift in expression of ncRNAs reflects a fundamental change in cell biology, likely impacting expression, transcript splicing, and translation of developmentally important genes and possibly contributing to the cardiac defect. (*Circ Cardiovasc Genet.* 2012;5:279-286.)

Key Words: tetralogy of Fallot ■ cardiac development ■ microRNA ■ miRNA ■ small nucleolar RNA ■ snoRNA

The heart is the first major internal organ to form during embryogenesis, and it is critical for the viability of the embryo. A multitude of genes and genetic networks contribute to the spatial and temporal specification of cell lineage required for proper embryological heart formation.¹ Failure of proper cellular differentiation, migration, and apoptosis results in congenital heart defects (CHD), which are a major cause of childhood morbidity and mortality and remains a substantial challenge even in countries with advanced health-care systems. The incidence of CHD is approximately 8 per 1000 live births,² making CHD the most common birth defect. Mendelian and chromosomal syndromes account for

about 20% of all cases of CHD. The genetic mechanisms underlying nonchromosomal or non-Mendelian “sporadic” CHD, which account for the remaining 80%, are poorly understood.

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Tetralogy of Fallot (TOF) is the most common form of cyanotic CHD, with an incidence estimated at 5 to 7 per 10 000 live births, thus representing 5% to 7% of all congenital heart lesions. The occurrence of CHD in the offspring of mothers with TOF is approximately 3.1%,³⁻⁵ supporting a genetic contribution. TOF is characterized by a malalignment

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of the conal septum, leading to a rightward deviation of the aorta. This results in a large ventricular septal defect and varying degrees of right ventricular outflow tract narrowing.

Sporadic CHD, which accounts for the majority of CHD, has proven intractable to genetic investigation. These sporadic events are most often inherited from unaffected parents, suggesting incomplete penetrance.⁶ Variable penetrance can be explained, at least in part, by differences in the genetic buffering capacity between individuals.^{7,8} In addition, *de novo* events, including sequence alteration or copy number changes, can impact gene function or alter dosage and contribute to mutational load. Furthermore, recessive mutations, if homozygous, may further destabilize regulatory networks.

Recently, it has become apparent that microRNAs (miRNAs) play an important role in cardiac signaling and transcriptional pathway regulation.⁹ MiRNAs are important posttranscriptional inhibitors of gene expression, resulting from the degradation of target mRNA or inhibition of translation. It is estimated that the human genome may contain up to 1000 miRNAs. A powerful aspect of miRNA function is the ability of individual miRNAs to coordinately regulate multiple target genes, encoding proteins with related functions (eg, stem cell differentiation, neurogenesis, and skeletal and cardiac muscle development and function).¹⁰ Furthermore, individual mRNAs can be targeted by multiple miRNAs, allowing for enormous combinatorial complexity and regulatory potential.

Cardiac development depends on the correct spatiotemporal expression of particular miRNAs. MiR-1 promotes embryonic stem cell differentiation toward a cardiomyocyte lineage, whereas miR-133 inhibits differentiation into cardiac muscle. MiR-1 targets the Notch ligand δ -like-1 (Dll-1)¹¹ and the transcription factor Hand2,¹² which is required for right ventricular growth and cardiomyocyte expansion. Furthermore, miR-1 knockout mice had thickened ventricles resulting from hyperplasia. Deletion of miR-1-2 causes lethal ventricular septal defects.¹³ MiR-133a-1/133a-2 double knockout in mice results in inappropriate expression of serum response factor and cyclin D2, leading to late embryonic and neonatal lethality due to ventricular septal defects and chamber dilatation.¹⁴ Deletion of either miR-106b-25 or miR-106a-363 combined with the miR-17 to miR-92 null allele can result in embryonic lethality, accompanied by severe ventricular septal defects, atrial septal defects, and thin-walled myocardium.¹⁵ Additionally, Kuhn et al reported that 5 human chromosome 21-derived miRNAs (miR-99a, let-7c, miR-125b-2, miR-155, and miR-802) are overexpressed in hearts from subjects with Down syndrome and CHD.¹⁶ Also, miR-196a, which is an upstream regulator of Hoxb8 and Sonic hedgehog homolog, has been associated with sporadic CHD.¹⁷ Taken together, these studies confirm a significant role for miRNAs in vertebrate heart development.

In addition, miRNAs have been shown to influence expression of key genes that regulate alternative splicing in the mouse.¹⁸ There are clear spatial and temporal transcript splicing transitions that are conserved in the vertebrate heart during fetal and postnatal development.^{19,20} Proper transition from a fetal transcript splicing pattern to a postnatal pattern is required for correct heart development.²⁰ Thus, in addition to

regulating mRNA levels, miRNAs may play an indirect role in regulating heart development by impacting transcript processing of developmentally important genes; however, the extent and mechanism by which miRNAs contribute to human CHD remains unknown.

Other noncoding RNA (ncRNA) species have received relatively little attention in terms of their regulatory role in development; however, there is evidence that other classes of ncRNA may also play critical roles in cellular function and organismal development. A significant class of evolutionarily conserved ncRNA is the small nucleolar RNAs (snoRNAs). The snoRNAs primarily guide chemical modification (methylation and pseudouridylation) of specific nucleotides in other RNAs: mainly ribosomal RNAs, transfer RNAs, and small nuclear RNAs (snRNAs) (ie, spliceosomal RNAs). There are 2 main classes of snoRNAs: the C/D box snoRNAs, which are associated with nucleotide specific methylation, and the H/ACA box snoRNAs, which are associated with pseudouridylation.^{21,22} SnoRNAs are ancient in evolutionary origin, with homologs in yeast and plants. In most vertebrate species, snoRNAs reside in the introns of other genes and require splicing to initiate snoRNA maturation.²³

In addition, a subset of snoRNAs target spliceosomal RNAs, are associated with cajal bodies in the nucleus, and are known as small cajal-associated RNAs (scaRNAs). Most scaRNAs target spliceosomal RNAs that are part of the primary spliceosome, referred to as the U2 spliceosome. The U2 spliceosome is composed of ribonucleoprotein particles that contain the spliceosomal RNAs (U1, U2, U4, U5, and U6); however, there is a second spliceosome, the U12 spliceosome, which mediates the excision of a minor class of evolutionarily conserved introns that have noncanonical recognition sequences that differ from those of the U2 spliceosome. The U12 spliceosome shares the U5 snRNA with the U2 spliceosome and also contains the snRNAs U11, U12, U4atac, and U6atac that functionally correspond to U1, U2, U4, and U6 in the U2 spliceosome.²⁴ The U12 spliceosomal RNAs contain biochemically modified nucleotides, but they are much less frequent than in their U2 counterparts.

Studies of snoRNA-directed modification of ncRNA in bacteria and lower eukaryotes have shown that nucleotide modifications are important for stabilization, maturation, turnover, and localization of the target ncRNAs.^{25,26} Recently, the first examination of the developmental significance of snoRNAs in vertebrates was reported in zebrafish.²⁷ The suppression of specific snoRNAs impaired the biochemical modification of targeted nucleotides in the 28S rRNA, leading to severe morphological defects and embryonic lethality in zebrafish. These data provide the first evidence that nucleotide modification in rRNA plays an essential role in vertebrate development.

To our knowledge, there are no reports of genome-wide assessment of noncoding RNA in tissue derived from human heart affected with CHD. Therefore, we collected myocardium from a cohort of children sharing the same congenital heart defect, TOF. We present here the pattern of ncRNA expression in myocardium from children with TOF compared with tissue from normally developing age-matched comparison subjects, and fetal myocardium.

Table. Age and Sex of Subjects

	N	Sex	Mean Age (Range)	Analyzed by Array	Analyzed by qRT-PCR
TOF	16	11 M/5 F	276 d (98–510)	16	16
Control	8	3 M/5 F	142 d (28–382)	8	8
Additional TOF subjects used for validation	8	4 M/4 F	292 d (167–425)	0	8

QRT-PCR indicates quantitative reverse transcription polymerase chain reaction; TOF, tetralogy of Fallot; d, days.

Materials and Methods

Subjects

Our subjects were children <1 year of age with TOF, requiring surgical reconstruction. The diagnosis and anatomy were obtained by echocardiography and angiography, and confirmed at the time of surgery. Informed consent was obtained from a parent or legal guardian after reviewing the consent document and having their questions answered. All proper institutional review board approvals were obtained for this study. All microarray analyses were run on samples from 16 infants with nonsyndromic TOF (ie, no 22q11.2 deletion), 8 infants with normally developing hearts, and 3 fetal samples (Table). We also recruited an additional 8 subjects with nonsyndromic TOF to allow for independent validation via quantitative reverse transcription polymerase chain reaction (RT-PCR).

We obtained 3 fetal hearts (≈90 days gestation) through the National Institute of Child Health and Human Development-supported tissue retrieval program from the Central Laboratory for Human Embryology at the University of Washington (Seattle, Wash.). The fetal hearts were dissected by 1 of the surgeons who also performed many of the reconstructions of the conotruncal defects (J.E.O.), to ensure the tissue analyzed was from a similar location as the tissues removed during surgery.

Comparison tissues from 8 normally developing infants (3 males, 5 females) were obtained from LifeNet Health (<http://www.lifenethealth.org>). LifeNet Health is a nonprofit regenerative medicine company that provides bio-implants and organs for transplantation. The control subjects were matched for age to the study population, and all control subjects expired owing to noncardiac-related causes. LifeNet Health follows the following protocol for tissue recovery: (1) If the donor is placed in a refrigerated morgue within 12 hours of asystole, tissues can be recovered for up to 24 hours and placed in a 1°C to 10°C sterile isotonic solution; or (2) if the donor is not refrigerated, tissues can be recovered for up to 15 hours and placed in a 1°C to 10°C sterile isotonic solution. All donor tissue was de-identified, no donor confidential information was disclosed, and consent was obtained to use the tissue for research.

We analyzed the expression of the donor tissue to determine if there were identifiable effects related to the postmortem interval, as previously described for muscle.²⁸ Sanoudou et al observed that postmortem specimens (up to 46 hours) had a significant and prominent upregulation of 89 genes that were members of pathways involved with protein biosynthesis, oxidative stress, apoptosis and cell cycle regulation. We examined the behavior of these genes in our surgically acquired TOF tissue and our normal tissues (data not shown). We did not observe any systematic activation of the genes reported by Sanoudou et al in the control tissue (with up to 15 hours postmortem interval) relative to the TOF tissue (essentially no postmortem interval). Only 1 of their 89 genes was significantly changed in expression between our tissue samples. The expression of the others was randomly distributed and did not meet statistical significance. In addition, RNA quality assessment, using a Bioanalyzer 2100, indicated no evidence of RNA degradation in any samples used for expression analysis. Taken together, these data suggest that the method of organ collection used by LifeNet Health maintained the viability of the tissue and induced no postmortem-associated alteration in gene activity.

RNA Preparation

RNA was extracted from ≈10 mg of frozen tissue from the right ventricle, using a mirVana miRNA isolation kit (Applied Biosys-

tems/Ambion) according to manufactures instruction. The control tissues (cryo-preserved pulmonary homografts) were thawed per protocol in sterile conditions. One author (J.E.O.) aseptically dissected samples from the normally developing control and fetal hearts to ensure the tissues from the right ventricle were from equivalent locations. Tissue samples obtained during patient surgery (attending surgeons J.E.O. and G.K.L.) were immediately de-identified and frozen. All tissue samples removed during surgery were excised by the performing surgeon for clinical indications, using standard-of-care procedures. Although a subset of patients were previously palliated with a modified Blalock-Taussig shunt, the right ventricular outflow tract region from which the tissue was harvested had not undergone any previous surgical manipulation.

Microarray Analysis

The miRNA microarrays were Affymetrix GeneChip microRNA-1.0 (Affymetrix Inc). The GeneChip microRNA-1.0 array contains probes for 847 human miRNAs and 922 human snoRNAs (the snoRNAs are inclusive of scaRNAs). The exon arrays were Affymetrix HuEx-1_0-st-v2. The raw data for the miRNA arrays have been deposited in the Gene Expression Omnibus (miRNA arrays accession No. GSE35490). All arrays were run at the University of Kansas Medical Center-Microarray Facility (KUMC-MF) according to the manufacturer's protocols. KUMC-MF is supported by the University of Kansas School of Medicine, KUMC Biotechnology Support Facility, the Smith Intellectual and Developmental Disabilities Research Center (HD02528), and the Kansas IDeA Network of Biomedical Research Excellence (RR016475).

All statistical analyses were performed using statistical software: Partek Genomics Suite software version 6.6 (Partek Inc), SPSS, and Ingenuity Pathways (Ingenuity Systems, Inc). Raw data (CEL files) were uploaded into Partek Genomics Suite for normalization and statistical analysis. Robust Multichip Analysis (RMA) was used for background correction, followed by quintile normalization with baseline transformation to the median of the control samples. Only probes with intensity values >20% of background value, in at least 1 of the conditions, were included for additional analysis. A Student *t* test with a Benjamini and Hochberg multiple test correction for false discovery rate (FDR) was used to determine significance. Probes were filtered using an FDR-adjusted *P* value ≤0.05.

The Ingenuity Pathways Analysis (IPA) version 9.0 (Ingenuity Systems, Inc) was used to explore networks, canonical pathways, predefined functional categories, and potential miRNA interactions with their predicted targets (using integrated access to TargetScan Human, version 5). IPA contains hundreds of pathways and identifies significant associations between the experimental data set and canonical pathways, functional categories, or disease associations within the database, by comparing the ratio of the number of molecules from the data set that map to a given pathway divided by the total number of molecules that map to the pathway. A Fisher exact test was used to calculate a *P* value, determining the probability that the association between the genes in the dataset and the pathway was explained by chance alone. All biological functions and/or diseases in IPA's database were considered for the analysis without bias. Significance was defined as a *P* value ≤0.05.

We used hierarchical clustering within Partek software to group subjects with similar expression patterns of snoRNAs. No *a priori* grouping was applied. The resulting dendrogram was used to assess individual and group relationships among the subjects.

Real Time Quantitative Polymerase Chain Reaction

To validate gene expression, quantitative RT-PCR (qRT-PCR) was performed on a subset of genes/transcripts using Taqman assays (Applied Biosystems, Inc) according to the manufacturer's instructions. Briefly, 2 ng of total RNA from each sample was reverse-transcribed using a miRNA-specific Taqman miRNA reverse transcription. The cDNA was then PCR-amplified using the human Taqman miRNA assay system for the following human miRNAs: miR-1201, miR-122, miR-421, miR-1275, and miR-27b and the snoRNAs U26 and ACA26. Additional snoRNAs (HBII-382, mgU2-25/61, U92, and ACA35) and the spliceosomal RNAs U2, U6, U4, and U12 were analyzed by qRT-PCR using specific primers (primers are listed in online-only Data Supplement Table 1) and SYBR Green to detect the amplicon, as previously described.^{29,30} For each sample, real time qRT-PCR was performed in triplicate on an ABI 7000 sequence detection instrument. The point at which the intensity level crossed the PCR cycle threshold (C_T) was used to compare individual reactions. *RNU24* and *GAPDH* showed no significant variation in intensity level among our samples on the microarrays. The expression of the 2 was also significantly correlated ($P < 0.01$). Therefore, we used *RNU24* and *GAPDH* to normalize our qRT-PCR results. Results were calculated as fold change relative to control subjects.

Results

The ncRNA microarrays contained probes for 847 human miRNAs and 922 snoRNAs. We set our detection threshold at 20% above the background signal intensity, which resulted in the detection of 598 miRNAs and 550 snoRNAs in either the control or TOF tissue samples. Of the detectable miRNAs, 61 had a significant change in intensity using a Benjamini correction for multiple testing (online-only Data Supplement Table 2). There were 135 unique snoRNAs with a significant change in intensity using a Benjamini correction (online-only Data Supplement Table 3).

MiRNAs

There are several miRNAs that have previously been associated with heart development (reviewed by Liu and Olson, 2010), none of which had a change in expression in our TOF samples, including miR-1 and miR-133 (online-only Data Supplement Figures 1 and 2, respectively). In contrast, other less studied miRNAs had highly significant changes in expression in the TOF tissue samples. There was a high degree of consistency in expression level in the TOF tissues relative to control myocardium among the 61 significantly altered miRNAs, of which miR-421 and miR-1201 are representative examples (online-only Data Supplement Figures 3 and 4). We chose 5 miRNAs with the largest change in expression between TOF and normal tissues, and also having the greatest consistency among samples, to validate by qRT-PCR (miR-1275, -27b, -421, -1201, and -122). These 5 miRNAs were validated on all 16 TOF samples used for array analysis and in an additional 8 independent TOF samples (online-only Data Supplement Table 4A). The qRT-PCR analysis was in agreement with the array data for all TOF samples analyzed. The 8 samples not analyzed by arrays were in agreement with the 16 samples analyzed by array (Data Supplement Table 4A).

Several recent publications have indicated a similarity between miRNA expression changes associated with heart disease and a fetal pattern of expression.^{18,31–33} Therefore, we compared the expression pattern of the 61 miRNAs with

significantly changed expression in TOF myocardium to the expression pattern in fetal myocardium (online-only Data Supplement Table 2). Of the 61 miRNAs significantly changed in TOF tissue relative to the controls, there were 30 that had changed in the same direction in the fetal heart tissue compared with the control tissue, and 15 of those were significant in the fetal tissue samples (with a Benjamini correction for multiple testing). Three were confirmed by qRT-PCR (miR-1275, -421, and -1201) (online-only Data Supplement Table 4A). The similarity of expression pattern between tissues from infants with nonsyndromic TOF and fetal tissues is illustrated by the expression patterns for miR-421 and miR-1201 (online-only Data Supplement Figures 3 and 4).

We then examined the putative targets of these miRNAs, either experimentally validated or predicted with a high degree of statistical confidence, using consensus predictions from TargetScan (<http://www.targetscan.org/>), microRNA.org (<http://www.microrna.org/microrna/home.do>), and MicroCosm Targets (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>). There was a distinct pattern of enrichment of the targets as we focused on genes with increasingly direct links to cardiac development. We determined consensus potential targets for the 61 differentially expressed miRNAs with all genes currently in the databases (>17 000 genes). Matches were predicted with high probability for 6216 genes or 34.9%. We used the IPA database to derive a list of genes which contained the word *cardiac* in their description (2237 IPA genes). In addition, we derived a refined list of genes with validated roles in heart development or function (597 validated genes) using the 3 online databases: Cardiovascular Gene Ontology Annotation Initiative (http://www.ebi.ac.uk/GOA/CVI/prioritized_gene_list.html); CHD Wiki (http://homes.esat.kuleuven.be/~bioiuser/chdwiki/index.php/Main_Page); and HuGE Navigator (version 2.0) (<http://www.hugenavigator.net/HuGENavigator/startPagePhenoPedia.do>). Finally, in order to focus on genes that are more likely to impact cardiac development, we used IPA to derive a list of 229 genes detectable on our arrays from 5 networks with experimentally confirmed roles in regulating vertebrate heart development: Wnt signaling; Notch; Sonic hedgehog homolog; cardiomyocyte differentiation via bone morphogenetic protein receptors; and factors promoting cardiogenesis in vertebrates. (The online-only Data Supplement Table 5 provides details of all derived gene lists.) TargetScan predicted that 998 of the IPA genes (998/2237; 44.6%) and 273 of the validated list of genes (273/597; 45.7%) were potentially targeted by the 61 miRNAs with changed expression in TOF. Our consensus target list predicted that 188 of the genes in the 5 networks (188/229; 77%) are putatively targeted by at least 1 of the 61 miRNAs with altered expression in TOF myocardium. It is extremely unlikely ($P < 0.0001$) that the proportion of targeted genes in the 5 networks associated with heart development (77%) is greater than the proportion of targeted genes in the general database (34.9%) simply by chance. The lower 95% confidence limit for the rate of target genes in the 5 networks associated with heart development is 72.2%. Both calculations indicate statistical significance for enrichment of genes targeted by the miRNAs in the 5 networks associated with heart development.

Genes important for regulating cardiac development

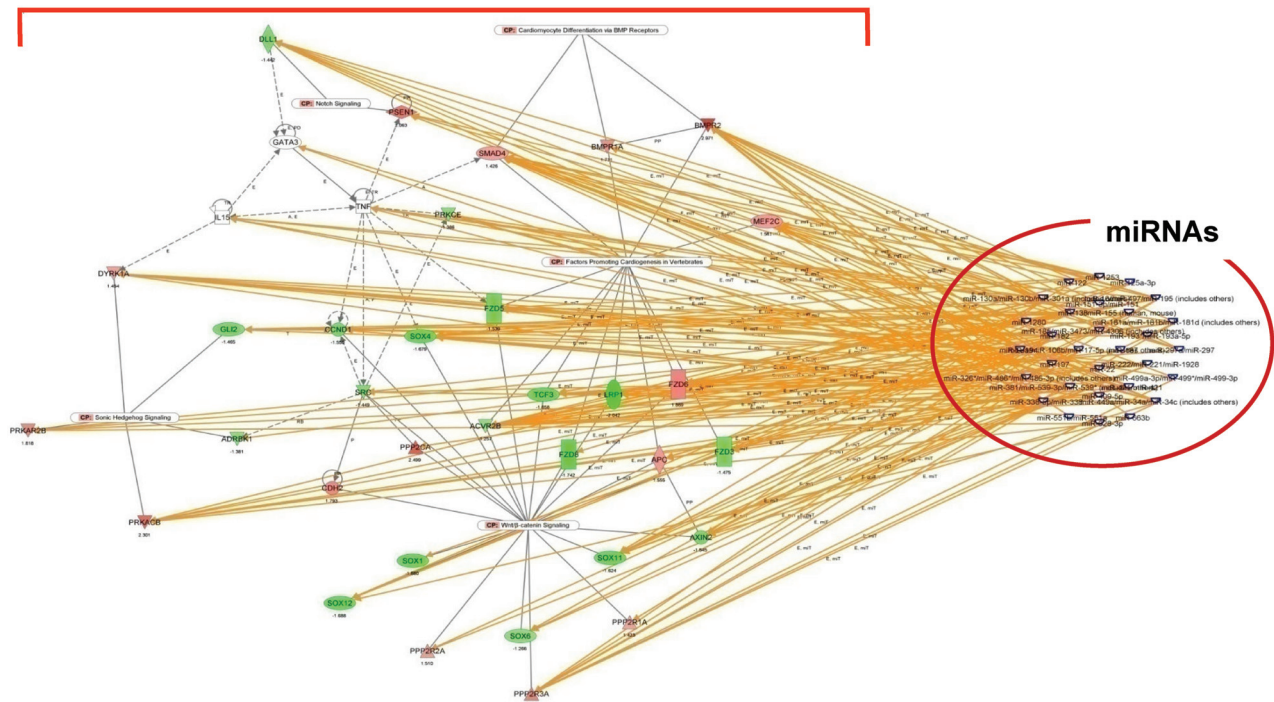


Figure 1. Significant negative correlations between microRNAs and putative target genes. Orange lines connect microRNAs to target genes with significant negative correlations. All microRNAs and target genes had significant changes in expression in tetralogy of Fallot (TOF) relative to controls.

Since miRNAs generally inhibit expression of target genes, we used expression data from exon arrays performed on the same 16 TOF and 8 control RNA samples to identify negative correlations with the expression of the 61 miRNAs by using Partek software. Expression of the network genes was derived from the mean value of all exon probes used in the exon array. One hundred and seventy-three of the genes from the 5 cardiac networks had a significant change in expression between the TOF samples relative to the controls. Interestingly, 44 cardiac network genes had significant negative correlations with 1 or more of the 61 miRNAs (only 33 of the set of 61 had negative correlations with the network genes; Pearson's correlation, $P < 0.05$; Figure 1). We tested the strength of this observation by choosing a random set of 61 miRNAs and looked for a correlation between the random group and the same 173 genes with changed expression. This resulted in a significant negative correlation of 6 of the network genes with 1 or more of the 61 randomly selected miRNAs. The proportion of genes having significant negative correlation with 1 or more of the 61 miRNAs in our collection (44/173) is significantly greater than the proportion of genes having significant negative correlation with 1 or more of the 61 randomly selected miRNAs (6/173; $P < 0.0001$).

We did the same assessment for negative correlation between the set of 61 miRNAs with our validated list of 575 genes known to be involved in heart development or function; 396 of which had a significant change in expression between TOF and control. We found a negative correlation between 73 of the validated list of cardiac genes (73/396; 18%) and 1 or more of the 61 miRNAs (only 40 of the set of 61 had negative correlations with the

network genes; Pearson's correlation, $P < 0.05$). The analysis of 61 random miRNAs with the 396 cardiac genes resulted in negative correlations with 3 cardiac genes on the list. The validated list of cardiac genes with a negative correlation with our collection of miRNAs is also significantly enriched compared with a random set of miRNAs ($P < 0.001$).

SnoRNAs

We detected 135 snoRNAs (including scaRNAs) that were statistically differentially expressed in the TOF samples using the Benjamini correction for multiple testing (online-only Data Supplement Table 3). Most of these snoRNAs (126) had decreased expression. Surprisingly, 115 were also decreased in the fetal myocardium relative to the controls. Only 9 of the snoRNAs had increased expression in the nonsyndromic samples, and 6 were increased in the fetal tissues relative to the control samples (online-only Data Supplement Table 3). A heat map and hierarchical clustering are shown for the snoRNAs in Figure 2. Note that TOF and fetal samples were clustered together based on snoRNA expression pattern reflecting the similarity in expression levels compared with the control samples.

We validated the expression of 6 snoRNAs by qRT-PCR (online-only Data Supplement Table 4B). We chose to validate U26 because of the recent report indicating that it was essential for vertebrate development.²⁷ The other 5 snoRNAs were chosen because they target small nuclear RNAs involved in the spliceosome. We used the snoRNAbase (<http://www-snoRNA.biotoul.fr/index.php>) to identify the targets of the snoRNAs with reduced expression in TOF. The

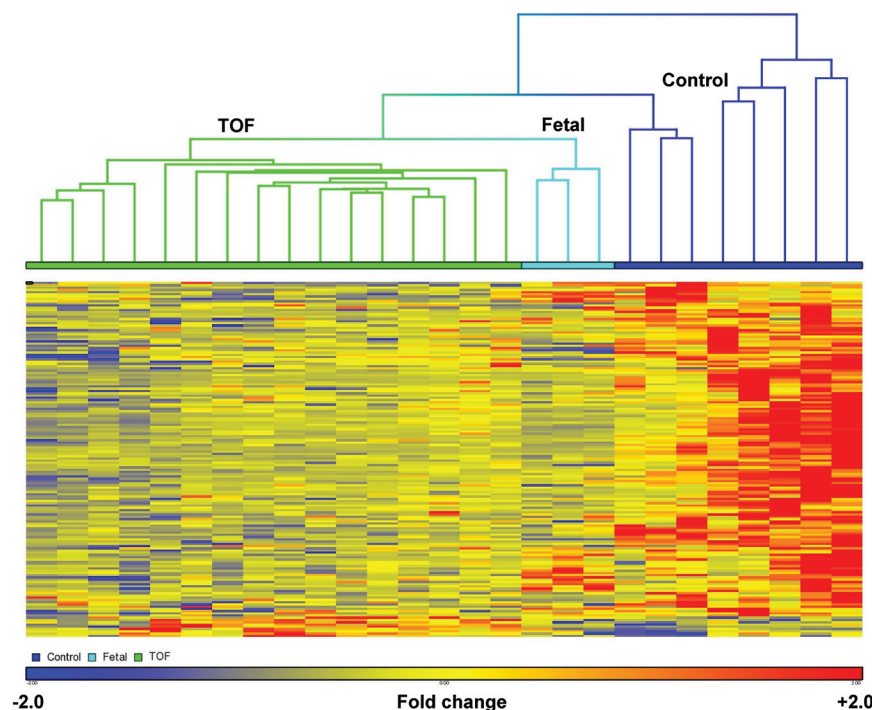


Figure 2. Clustering dendrograms derived from snoRNA that differ in expression between tetralogy of Fallot (TOF) and control myocardium.

downregulated snoRNAs primarily targeted 4 ncRNAs: the ribosomal RNAs 28S and 18S, and 2 spliceosomal RNAs, the snRNAs U2 and U6 (online-only Data Supplement Table 6). The downregulated snoRNAs targeted 61 (of 119 total modified nucleotides) in 28S and 38 (of 80 total) nucleotides in 18S rRNAs. Six snoRNAs targeted 10 nucleotides (of 13 total) in U2, and 6 snoRNAs targeted 5 nucleotides (of 8 total) in U6. Since snoRNAs are necessary for the processing and stability of their target snRNAs, we assessed the expression by qRT-PCR of U2, U6, and 2 spliceosomal RNAs not targeted by the downregulated snoRNAs, U4 and U12. U2 and U6 had significantly reduced expression in the 16 TOF samples compared with the 8 control samples; U2 was reduced 1.8-fold in TOF ($P=0.04$), and U6 was reduced 3.2-fold in TOF ($P<0.0001$). The spliceosomal RNAs U4 and U12 did not differ in expression level between TOF and control myocardium.

We used our exon array data to compare splicing variants in genes targeted by the 2 different spliceosomes between myocardium from normally developing infants and infants with TOF. We created a list of 464 genes that contain exons with U12 splicing recognition sequences using the U12 database (U12DB) (<http://genome.imim.es/cgi-bin/u12db/u12db.cgi>). Our analyses of splicing variation in TOF myocardium revealed a substantial disturbance of splicing in genes from networks known to be critical for heart formation (manuscript in preparation). Of the 229 genes in the 5 networks associated with heart development found on our exon arrays, 117 (117/229; 51%) had significantly altered splicing patterns. Interestingly, >25% of these changes were in common with fetal tissues (manuscript in preparation). We then used our exon array data to determine the level of alternative splicing in the U12 exons. We found 464 genes with U12 type exons on our array, and 35 (35/464, 7.5%) of them had significant alternative splicing events in TOF myocardium.

These 2 proportions, 51% versus 7.5%, are significantly different ($P<0.0001$), indicating that splicing variation was significantly higher in U2 type exons in cardiac network genes compared with U12 type exons in myocardium from infants with TOF. This suggests that the reduction of scaRNAs targeting U2 and U6 had a significant impact on U2-type splicing that was much more pronounced than in U12-type splicing.

Discussion

The origins of most CHD are thought to be multifactorial, implying contributions from anomalous gene expression and processing, epigenetic factors, as well as environmental contributions. In spite of the expanding knowledge of the genetic mechanisms involved in vertebrate cardiac formation, there is a lack of understanding regarding the origin of most sporadic CHD.

MiRNAs

Previous reports have clearly shown that several miRNAs play a central role in regulating vertebrate heart development (eg, miR-1, miR-133); however, these miRNAs do not appear to make a major contribution to sporadic TOF based on our analysis. This is perhaps because altering these miRNAs might result in changes in heart development that are incompatible with life; however, in our cohort there were samples which had expression of miR-133 differing from normal by >1 standard deviation, so we cannot rule out the possibility that overexpression or underexpression of miR-133 in these individuals may have contributed to their CHD.

The miRNAs with altered expression in the nonsyndromic TOF myocardium appear to preferentially target genes that are known to contribute to cardiac development. Most of the targeted genes may interact with multiple miRNAs, resulting in a complex regulatory mechanism. Of the 61 miRNAs with

altered expression, 11 putatively target 126 of the genes in the 229 genes from the 5 pathways derived from the IPA database that are explicitly involved in heart development. Thus, these miRNAs are candidates for further investigation. Little is known about the role these miRNAs play in heart development, but miR-30 has been shown to regulate connective tissue growth factor, thus potentially playing a role in structural changes in the heart.³⁴

In examining potential relationships between miRNAs with altered expression and genes with altered expression in TOF myocardium, we saw a clear trend toward increasing proportions of genes putatively targeted by the altered miRNAs. Target scan predicted that 77% of the genes from the 5 cardiac developmental pathways were targeted by the miRNAs altered in TOF myocardium compared with 34.9% of the list of all genes in the database. Although not mechanistically validated yet, these data suggest that dysregulation of these miRNAs may have contributed to the cardiac defect. Most of these miRNAs have not been previously studied for their potential role in cardiac development and function. It seems likely that individually these miRNAs may have a small influence on the regulation of cardiac development, however, the cumulative role they play warrants further investigation.

SnoRNAs

The snoRNAs we examined had a very dramatic shift in expression in the TOF myocardium relative to control myocardium. The canonical function of snoRNAs is the biochemical modification of rRNA or spliceosomal RNAs; however, the effect of snoRNA dysregulation on development is poorly understood. In mammals (and humans, in particular), >90% of snoRNAs are encoded within introns, usually of genes associated with the ribosome. This colocalization suggests coordinated expression designed to foster coordinated function. The genes containing the dysregulated snoRNAs did not have changed expression in TOF relative to control tissue (data not shown), suggesting that snoRNA processing or stability was altered.

The similarity in snoRNA expression pattern among tissues from nonsyndromic subjects and fetal tissue samples was surprising. The direction of change was nearly identical and most reached statistical significance in the fetal tissues (online-only Data Supplement Table 3). It has been shown that miRNAs participate in coordinating gene expression and splicing during development of the fetal mouse heart.¹⁸ Other ncRNAs may play similar roles. Clustering of the subjects based on the ncRNAs that had differential expression in the myocardium from infants with sporadic TOF reveals a similarity among the tissue from sporadic TOF and the fetal tissues. The dramatic shift observed in ncRNA expression pattern may have resulted from failure of expression to progress properly during fetal heart development, perhaps, contributing to deterioration of spatially and temporally correct gene expression and splicing of mRNA leading to TOF.

The snoRNAs that were reduced in our TOF samples target spliceosomal RNAs of the U2 spliceosome but not the U12 spliceosome. In order to assess the specificity of the snoRNA association with splicing variation, we compared splicing between genes containing U2-type exon/intron recognition

sequences and U12 exon/intron recognition sequences. Significant differences in splice forms between the TOF and control samples occurred in 8% of the 464 U12 type genes on our array; whereas, 51% of the 229 genes in the 5 networks of cardiac genes were alternatively spliced in the TOF samples compared with control samples. These 2 proportions are highly different ($P<0.0001$). Furthermore, U2 and U6 snRNAs were reduced 1.8- and 3.2-fold in TOF myocardium, respectively, relative to control myocardium. Taken together, these observations suggest a link among reduced levels of scaRNAs that target the snRNAs U2 and U6, alternative splicing, and the developmental defect in infants with TOF.

Our data provide a first glimpse of the alteration in expression of ncRNAs in myocardium from children with TOF. The scope of the change in ncRNA suggests fundamental regulatory changes in the damaged myocardium as a response to TOF, and may contain changes that also contributed to the defect. Noncoding RNA is capable of broad regulatory influence, as well as targeted sequence/gene specific control. A better understanding of the regulatory role played by noncoding RNAs may lead to biomarkers with therapeutic relevance. The complexity of the genomic organization of ncRNAs and their regulatory nature does not lend itself to easy interpretation, but our report suggests areas in need of further research, including the origin of the altered expression of the ncRNAs, as well as the downstream impact on target genes in myocardium from infants with CHD. It appears clear that a comprehensive understanding of the origin of sporadic CHD will require a careful investigation of all aspects of noncoding RNA.

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Disclosures

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CLINICAL PERSPECTIVE

Congenital heart defects represent 25% of all birth defects. Idiopathic congenital heart defects represent 80% of cases, but in spite of increased understanding of the human genome, there has been limited progress made in identifying the genetic basis of idiopathic congenital heart defects. The importance of noncoding RNAs, especially microRNAs (miRNAs), for developmental regulation of the vertebrate heart has recently become apparent; however, there is little known about the expression of noncoding RNA in the human heart with developmental anomalies. We characterized expression of miRNAs and small nucleolar RNAs (snoRNAs) in right ventricular myocardium from infants with nonsyndromic tetralogy of Fallot, normally developing infants, and fetal myocardium. We found 61 miRNAs and 135 snoRNAs with significantly changed expression in myocardium from children with tetralogy of Fallot. The pattern of ncRNA expression in tetralogy of Fallot myocardium had a surprising resemblance to the fetal pattern. Putative targets of miRNAs with altered expression were enriched for genes of importance to cardiac development. SnoRNAs guide the biochemical modification of ribosomal and spliceosomal RNAs. Targets of the differentially expressed snoRNAs included 2 essential spliceosomal RNAs: U2 and U6. Importantly, in tetralogy of Fallot myocardium, we observed splicing variants in 51% of genes that are critical for cardiac development. Taken together, these observations suggest a link between levels of snoRNA, spliceosomal function, and heart development. This is the first report characterizing noncoding RNA expression in congenital heart defects. The striking shift in expression of ncRNAs reflects a fundamental change in cell biology, likely impacting expression, transcript splicing, and, possibly, contributing to the cardiac defect.