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scaRNAs regulate splicing and vertebrate heart development

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ABSTRACT

Alternative splicing (AS) plays an important role in regulating mammalian heart development, but a link between misregulated splicing and congenital heart defects (CHDs) has not been shown. We reported that more than 50% of genes associated with heart development were alternatively spliced in the right ventricle (RV) of infants with tetralogy of Fallot (TOF). Moreover, there was a significant decrease in the level of 12 small cajal body-specific RNAs (scaRNAs) that direct the biochemical modification of specific nucleotides in spliceosomal RNAs. We sought to determine if scaRNA levels influence patterns of AS and heart development. We used primary cells derived from the RV of infants with TOF to show a direct link between scaRNA levels and splice isoforms of several genes that regulate heart development (e.g., *GATA4*, *NOTCH2*, *DAAM1*, *DICER1*, *MBNL1* and *MBNL2*). In addition, we used antisense morpholinos to knock down the expression of two scaRNAs (scarna1 and snord94) in zebrafish and saw a corresponding disruption of heart development with an accompanying alteration in splice isoforms of cardiac regulatory genes. Based on these combined results, we hypothesize that scaRNA modification of spliceosomal RNAs assists in fine tuning the spliceosome for dynamic selection of mRNA splice isoforms. Our results are consistent with disruption of splicing patterns during early embryonic development leading to insufficient communication between the first and second heart fields, resulting in conotruncal misalignment and TOF. Our findings represent a new paradigm for determining the mechanisms underlying congenital cardiac malformations.

1. Introduction

Congenital heart disease (CHD) accounts for 25% of all birth defects and is a leading cause of death in children <1 year of age [1]. Nearly 80% of all CHD cases are idiopathic and multiple lines of evidence indicate a genetic contribution to CHD, but only relatively limited progress has been made in identifying the genetic basis of CHD [2,3]. Conotruncal defects, such as tetralogy of Fallot (TOF), result from disruption in the flow of tissue-specific information between the first and second heart fields at approximately 20 days of gestation. In the developing embryo, precise spatial and temporal signaling is required between the first heart field from which the left ventricle is derived and the second heart field (SHF) from which the right ventricle (RV) and conotruncal outflow tract are derived [4–10]. Abnormal rotation of the SHF causes conotruncal malformations such as TOF. Development of the conotruncal outflow tract is mediated by multiple transcription factors (e.g., *NKX2.5*, *GATA4*) and gene networks, including the Wnt and NOTCH pathways. Although studies of the developing vertebrate heart have provided a framework of regulatory control, they have failed to define the underlying causes of the majority of CHDs.

The importance of noncoding RNA (ncRNA) for heart development has recently been shown to depend on the correct spatiotemporal expression of particular microRNAs [11]. In addition, there are clear spatial and temporal transcript splicing transitions that are conserved in the vertebrate heart during fetal and postnatal development [12,13]. Some exons are constitutive and are present in every mature message; however, there are many alternatively spliced genes/exons which are variably retained or excluded from a mature transcript that dramatically increase the complexity of the transcriptome and thus the proteome. Alternative splicing (AS) is temporally and spatially controlled resulting in unique splice variants in different tissues and at different time points

Abbreviations: AS, alternative splicing; CHDs, congenital heart defects; RV, right ventricle; TOF, tetralogy of Fallot; scaRNAs, small cajal body-specific RNAs; CHD, congenital heart disease; SHF, second heart field; ncRNA, noncoding RNA; snRNAs, small nuclear RNAs; snoRNAs, small nucleolar RNAs; rRNAs, ribosomal RNAs; TA, truncus arteriosus; PA/IVS, pulmonary atresia with intact ventricular septum; LNA, locked nucleic acid; qRT-PCR, quantitative RT-PCR; KUMC-MF, University of Kansas Medical Center-Microarray Facility; IPA, Ingenuity Pathways Analysis; RMA, Robust Multichip Analysis; FDR, false discovery rate; MOs, morpholino antisense oligos; hpf, hours post fertilization; RPKM, reads per kilobase of exon per million mapped reads; SMN, survival of motor neuron; SMA, spinal muscular atrophy; RP, retinitis pigmentosa

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in the same tissue. The transition from a fetal to postnatal pattern of a conserved set of alternatively spliced isoforms was shown to be critical for mouse heart development [14]. Clearly, mRNA splicing plays a significant role in mammalian cardiac development, but the potential contribution to human heart pathology remains unknown.

The spliceosome facilitates pre-mRNA processing of most primary transcripts in eukaryotic genomes. The primary spliceosome, called the U2 spliceosome, is a multimegadalton ribonucleoprotein complex composed of numerous proteins and five small nuclear RNAs (snRNAs or spliceosomal RNAs: U1; U2; U4; U5; and U6). The conformation and composition of the spliceosome are highly dynamic and highly conserved across eukaryotes [15]. Elaborate RNA–RNA–protein interactions align the reactive subgroups and repeatedly rearrange as each intron is identified, intron–exon boundaries are located, and catalysis proceeds to remove each intron in every pre-mRNA.

As snRNAs mature they are themselves biochemically modified in a process directed by other small noncoding RNAs, the scaRNAs (small cajal body-specific RNAs). The scaRNAs are a subset of the small nucleolar RNA (snoRNA) family, which is a large family of conserved ncRNAs that primarily guide biochemical modifications of specific nucleotides (e.g., methylation and pseudouridylation) of ribosomal RNAs (rRNAs) and snRNAs. Without the specific modifications controlled by the scaRNAs, the spliceosome fails to function properly [16]. While the biochemical targets of snoRNAs have been clearly elucidated over the last 20 years, there is a paucity of information regarding the developmental significance of this abundant class of ncRNA.

We previously detected 135 snoRNAs (including 12 scaRNAs) of more than 900 snoRNA probes on an ncRNA array which were statistically differentially expressed in the right ventricles of infants with TOF relative to controls [17]. Most of these snoRNAs (126, 93%) had decreased expression. Remarkably, 115 (91% of 126 with reduced expression) had similarly reduced expression in the fetal myocardium relative to the control tissue [17]. Two snRNAs, U2 and U6, also had reduced expression levels in TOF and fetal tissue relative to control tissue. The 12 scaRNAs that were moderately reduced in TOF myocardium targeted only the snRNAs, U2 and U6. Furthermore, we observed alternative splice isoforms of genes that were enriched in genetic pathways that are known to be critical for heart development. This suggests the possibility of the failure of adequate regulation of the scaRNA level early in gestation and a potential impact on spliceosomal function through alteration of U2 and U6.

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 ncRNAs [18,19]. However, similar studies in vertebrates have not been described until our recent report of the developmental significance of snoRNAs in zebrafish [20]. Impaired rRNA modification, even at a single site, led to severe morphological defects and embryonic lethality in zebrafish which suggests that rRNA modifications play an essential role in vertebrate development. Our studies highlight the importance of posttranscriptional modifications and their role in ncRNA function in higher eukaryotes. However, there are currently no reported studies of the role that scaRNAs play in regulating vertebrate development. Here we report the first investigation of scaRNA regulation of spliceosomal function and vertebrate development.

2. Material and methods

2.1. Derivation of primary cells

Tissue samples were collected at the time of surgical correction of TOF, truncus arteriosus (TA), and pulmonary atresia with intact ventricular septum (PA/IVS). All infants were less than one year of age and cytogenetic testing verified that none of the subjects had 22q11.2 deletions. Informed consent was obtained from a parent or legal guardian after reviewing the consent document and having their questions answered (IRB # 11120627). Detailed subject descriptions were previously published [17,21]. Primary cell cultures were derived from RV tissue of infants with TOF. The RV tissue was immediately immersed in DMEM (Invitrogen/Gibco, Grand Island, NY) plus 10% fetal calf serum (Sigma/Safc, St. Louis, MO) and 1% penn/strep (Gibco). The tissue was minced and most of the media was removed, leaving only enough to keep the tissue from drying out. After 24 h, additional media was added and cells were growing robustly after 3 to 4 days. Media was exchanged every 48 h. In addition, we obtained a primary neonatal cardiomyocyte cell culture derived from normally developing human neonatal cardiac tissue from Celprogen (San Pedro, CA Cat#36044-21). These cells were also grown in DMEM plus 10% fetal calf serum and 1% penn/strep.

2.2. Transfection of scaRNA plasmids into primary cells

The expression vectors pCGL-SCARNA4 (ACA26)and pCGL-SCARNA1 (ACA35)were a generous gift from Dr. Tamas Kiss, Universite Paul Sabatier [22]. The scaRNAs were cloned into an intron sequence between hemoglobin exons 3 and 4 so that they would be correctly processed in vivo and expression was driven with the CMV promoter. We replaced SCARNA1 with the corresponding sequences from the scaRNAs:SNORD94, SCARNA8, SNORD67, and SCARNA23. The scaRNAs were transfected into the primary cell lines derived from infants with TOF according to the manufacturer's protocol. Briefly, 2 μg of plasmid DNA was diluted in 200 μl of serum free media and added to 2 μl of the Poly Magnetofectant (a magnetic nanoparticle transfection reagent; Oz Bioscience, France), vortexed and incubated for 20 min at room temperature. The transfection mixture was added dropwise to 2×10^5 cells in 1.8 ml of media containing 10% serum in a single well of a 6 well plate. The culture plate was set on top of a plate magnet (Oz Biosciences) for 20 min, and returned to the incubator. After 72 h, the cells were trypsinized, pelleted and stored at −80 °C until processed for RNA extraction.

2.3. scaRNA knockdown

We used antisense LNA oligos (locked nucleic acid oligos, Exiqon Life Sciences, Woburn MA) to suppress the scaRNAs in primary cardiomyocytes as has been done previously in immortalized cell cultures [23,24]. Briefly, the LNA oligo protocol is as follows, 50 μM LNA oligo in 100 μl serum free media is mixed with 12 μl HiPerfect transfection reagent (Qiagen, Valencia, CA) and incubated for 20 min at room temperature. The transfection mixture was added to 2×10^5 cells in 2.3 ml of media with 10% serum in a single well of a 6 well cell culture plate. After 48 h, the cells were pelleted and stored at −80 °C until processing. Identifying LNA oligos for effective knockdown of the scaRNA was an empirical process. Two to four oligos were tested for each scaRNA to determine which were most effective at knocking down the target scaRNA.

2.4. RNA isolation and qRT-PCR (human tissue)

RNA was extracted from ~2×10^6 cells using a mirVana miRNA isolation kit (Invitrogen/Ambion) according to the manufacturer's instruction. Briefly, an equal quantity of total RNA (1 μg) together with random and oligo dT primers was reverse transcribed using Superscript III (Invitrogen by Life Technologies, Carlsbad, CA) according to the manufacturer's directions. Quantitative RT-PCR (qRT-PCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's directions as previously described [21]. The reaction was carried out in an ABI7000system (Applied Biosystems, Foster City, CA) beginning with 10 min at 95 °C. The intensity of the SYBR Green fluorescence was measured at the extension step of each cycle. At least three replicates were performed on each sample for each gene. The primers for each scaRNA, snRNA and gene are given in Table S1. A dissociation curve was generated for all