

# Non-coding RNA and the Mitochondrion – Function and Pathology in the Heart

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### Abstract

Mitochondria regulate bioenergetics, apoptosis, cell cycle, calcium handling, and other pathways. In the heart, where energy demands are much greater, preservation of mitochondrial function is vital. Diabetes mellitus significantly impairs cardiac function, with changes in mitochondrial metabolism and integrity. Importantly, the mitochondria contains non-coding RNAs which could act in regulating both mitochondrial and cellular health, but it is currently unknown how regulation occurs.

We wanted to investigate the role of non-coding RNAs in the mitochondria, examining their mechanisms of import/export, binding capacity, and mitochondrial relevance. Understanding the non-coding RNA regulation of mitochondrial and cellular function during diabetes mellitus can profoundly impact our understanding of the pathology. Diabetic and nondiabetic human right atrial tissue and mouse whole heart were used for analysis. Subpopulations of mitochondria (interfibrillar, IFM and subsarcolemmal, SSM) were separated through differential centrifugation. RNA from mitochondrial subpopulations were sequenced through the Illumina HiSeq in two technical replicates. Crosslinking Immunoprecipitation mitochondrial subpopulations for relevant RNA-binding proteins, of Polynucleotide Phosphorylase (PNPase) and Argonaut 2 (AGO2), and the RNA-RNA Interactome were sequenced through the Illumina MiSeq.

### Introduction

Diabetes mellitus affects 29.1 million Americans and 25.9% of Americans age 65 and older. Adults with diabetes mellitus have two to four times the rate of mortality from heart disease as compared to those without diabetes mellitus, and 68 percent of diabetic patients age 65 and older die of heart disease (Aavik et al. 2015, Alipoor et al. 2017). While transcription factor regulation of gene expression, posttranslational modification, and substrate inhibition via negative feedback have shown to be foundational in regulating protein expression and activity, microRNA (miRNA) control of gene expression through inhibition of transcription has become recognized as a significant contributor to homeostasis (Asrih and Steffens 2013).

MiRNA are single-stranded noncoding RNA molecules, ~22 nucleotides long, that associate into a multi-protein RNA-induced silencing complex (RISC) which inhibits its target mRNA species from being translated into a functional protein (Bartel 2009). Differential expression of miRNAs have been characterized in a variety of cardiovascular conditions including atherosclerosis, myocardial infarction, and heart failure to modulate processes such as energy substrate metabolism, fibrosis, and cardiac remodeling (Pinti et al. 2017, Small and Olsen 2011, Zhang and Schulze 2016). Nuclear- and, more recently, mitochondrial-genome-encoded proteins which function in the mitochondria, have been shown to be regulated by miRNA (Baradan et al. 2017, Baseler et al. 2012, Das et al. 2012, Jagannathan et al. 2015). What still remains a highly relevant, and hotly debated, topic is the process involved in miRNA import into the mitochondrion. While it has been hypothesized that some miRNAs could be transcribed through the mitochondrial genome (Sripada et al. 2012), predominately, miRNA import is favored as the mechanism for miRNA accumulation in the mitochondrion. Research has demonstrated the presence of miRNA within the mitochondrion (Bandiera et al. 2011, Dasgupta et al. 2015, Kren et al. 2009, Shinde and Bhadra 2015, Zhang et al. 2014), and even how fluctuating concentrations within the organelle contributes toward the development of pathologies (Das et al. 2014, Das et al. 2012, Duarte et al. 2015, Shepherd et al. 2017, Sripada et al. 2017). Two currently proposed mechanisms of miRNA import into the mitochondria include the direct movement across the mitochondrial membrane through a chaperone, Argonaut 2 (AGO2) (Das et al. 2012, Zhang et al. 2014), and diffusion through small RNA import machinery, Polynucleotide Phosphorylase (PNPase) (Shepherd et al. 2017). Understanding the significance of miRNA in the mitochondria during diabetes, and how miRNA mitochondrial import occurs, provides the propensity for more completely understanding the pathology.

### Results



Figure 2: Changes in mitochondrial transcribed genes in the SSM and IFM diabetic and between non-diabetic human right atrial mitochondria. (A) Pathway Ingenuity Analysis (IPA) depiction of mitochondrial electron chain transport complexes and suggested to proteins changes significant decrease, red = significant increase in expression

In diabetes, the SSM have significantly decreased expression of mitochondrial transcribed mRNA. Additionally, the actions of PNPase (in regulating long-non coding RNAs) and AGO2 (in regulating small noncoding RNA) provides an interesting dynamic for non-coding RNA import. The RNA-RNA interactome of the mitochondria reveals IncRNA-miRNA sponging, suggesting a novel mechanism of non-coding regulation in the mitochondria. LncRNA and miRNA regulation in the mitochondria perpetuates diabetic cardiomyopathy, altering sponging capacity of miRNAs that target the mitochondrial genome.



# Methods and Materials

#### Mitochondrial Subpopulations

Subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) subpopulations were isolated as previously described following the methods of Palmer et al. (Palmer et al. 1977) with minor modifications by our laboratory (Dabkowski et al. 2010; Baseler et al. 2011, 2013; Croston et al. 2013; Thapa et al. 2015). Mitochondrial pellets were resuspended in KME buffer (100mM KCI, 50mM MOPS and 0.5mM EGTA pH 7.4) and utilized for all analyses.

#### Cross-linking Immunoprecipitation (CLIP)

CLIP was performed as previously described (Jagannathan et al. 2015), with some modifications. Briefly, human right atrial appendage and animal whole heart mitochondrial subpopulations were irradiated 5 times with 400 mJ/cm2 (~15 cm distance from the UV source) on ice using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA). Anti-Ago2 and anti-PNPASE were used for protein pulldown. After RNA 3'-end biotinylation labeling (Thermo Scientific, Pittsburgh, PA), samples were loaded into SDS-PAGE. The membrane above the protein of interest was excised, RNA isolated, samples pooled (n = 5 per group) and submitted for Next-Generation Sequencing

#### RNA Preparation for Next-Generation Sequencing

RNA was purified either from human right atrial appendage total isolated mitochondria or human right

gene name	baseMean	log2FoldChange	padj	gene name	baseMean	log2FoldChange	padj	(P < 0.05) $(R)$ Tables
MT-ND1	8635	-2.02	0.0065	MT-ND1	9212	-1.07	0.389	$(P_{adj} \leq 0.03)$ . (B) Tables
MT-ND2	9731	-2.79	0.0003	MT-ND2	8472	-1.54	0.060	depicting changes in the
MT-ND3	4893	-2.10	0.0012	MT-ND3	5675	-0.81	0.611	expression of
MT-ND4	16168	-2.40	0.0022	MT-ND4	15036	-1.23	0.247	
MT-ND4L	2224	-2.98	0.0001	MT-ND4L	1703	-1.78	0.064	mitochondrial transcribed
MT-ND5	14731	-1.54	0.0203	MT-ND5	14329	-0.67	0.683	genes in the SSM and
MT-ND6	3855	-1.30	0.0320	MT-ND6	3681	-0.40	0.836	
MT-CYB	7529	-1.97	0.0031	MT-CYB	7220	-0.81	0.601	IFM. Counts are read as
MT-CO1	46321	-1.75	0.0091	MT-CO1	48111	-0.53	0.820	the baseMean value.
MT-CO2	7772	-1.65	0.0147	MT-CO2	9313	-0.66	0.677	
MT-CO3	13068	-1.77	0.0236	MT-CO3	14940	-0.56	0.820	
MT-ATP6	6837	-2.00	0.0060	MT-ATP6	8083	-0.89	0.499	
MT-ATP8	1279	-2.78	0.0001	MT-ATP8	1229	-1.18	0.410	

A Figure 3 B													
Figure 3: Heatma	n MiRN	Mirna		NA	MT tRNA		MT tRNA						
deniction of transcrir	MIR6087	1.39	MIR30D	-2.94	MT-TS2	-1.31	MT-TE	-0.53					
	MIR10B	-2.04	MIR100	-2.32	MT-TS1	1.21	MT-TS2	-1.00					
changes within th	e MIR150	-2.26	MIR30A	-3.03	MT-TE	-1.38	MT-TS1	1.76					
mitochondrion of huma	n <sub>MIR663AHG</sub>	2.41	MIR126	-2.88	MT-TH	-2.80	MT-TC	-0.24					
right atrial tissue. (A) Th	e MIR502	-2.12	MIR23A	-3.11	MT-TL1	-1.85	MT-TL1	-1.44					
top miRNAs differential	V MIR208B	-2.26	MIRLET7G	-2.35	MI-IQ	-1./3	MT-TV	-2.01					
expressed followin	MIR3687-2	1.88	MIR26B	-2.41	IVI I - I V NAT TV	-3.12		-1.07					
expressed following	в MIR3687-1	1.88	MIR23B	-3.29		-2.44	MT-TO	-2.50					
diabetic insult. (E	3) MIR301A	-2.59	MIR24-2	-2.68	MT-TM	-2.00	MT_TT	-1.10					
Mitochondrial transcribe	d MIR3648-2	2.00	MIR30B	-2.83	MT-TP	-1.18	MT-TK	-2.08					
tRNAs differential	y MIR3648-1	1.98	MIR148A	-3.01	MT-TC	-1.79	MT-TP	-0.47					
expressed followin	g MIR517A	-3.05	MIR378A	-1.76	MT-TN	-1.22	MT-TM	-1.69					
diabetic insult	MIR517B	-3.04	MIR181A1	-2.22	MT-TT	-0.76	MT-TN	-0.63					
diabetic insuit.	MIR539	-2.72	MIR191	-2.51	MT-TY	-1.50	MT-TY	-0.81					
	MIR488	-3.33	MIR181A2	-2.23	MT-TA	-0.52	MT-TW	-1.63					
					MT-TL2	-0.82	MT-TA	-0.54					
Ν	5 log2f	2 fold 5	MT-TW	-1.56	MT-TR	-2.87							
	MT-TI	-3.22	MT-TL2	-0.24									
	MT-TR	-2.85	MT-TI	-3.37									
		MT-TG	-3.20	MT-TG	-3.04								
		MT-TD	-2.78	MT-TD	-2.84								
					MIR30A MIR10R	Figure	4: Over	view of					



PNPase and Ago2 CLIP. Ago2-CLIP showed associations with miRNAs while the PNPase-CLIP showed associations with LncRNAs. This suggests that both PNPase and Ago2 could work independently, or in concert, to import nonocoding RNAs. CLIP = crosslinkingimmunoprecipitation.

Figure 1: Sample metrics for SSM and IFM of human right atrial mitochondria, differences expressed between diabetic and non-diabetic. (A) Principle Component Analysis (PCA) for sample distribution, revealing both intraand inter sample variance. (B) Raw count matricies for sample-to-sample distribution implementing the PoiClaClu package in R. (C) The MA-plot reveals the differentially expressed genes (red,  $P_{adi} \leq 0.05$ ) in comparison to genes with non-significant change between groups (grey). The most differentially expressed gene is circled in blue.

#### atrial appendage and animal whole heart protein-RNA pulldowns, using the miRNeasy Mini Kit (product no.: 217004, Qiagen, Hilden, Germany). Library preparation was performed using NEXTflex® Small RNA Sequencing Kit v3 (Illumina, Inc., San Diego, CA) for total mitochondria and TruSeq smallRNA Library Prep (Illumina, Inc., San Diego, CA) for protein-RNA pulldowns. Quality of RNA was determined using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA); degradation of cytosolic ribosomal RNAs (28S and 18S) are used as a measure of the total RNA Integrity Number (RIN). Each sample was run across two lanes of the Illumina Hi-Seq in Rapid Run mode for total mitochondrial miRNA and on one lane of the MiSeq for pooled CLIPed samples.

#### **Bioinformatics**

Generated Fastq files were further processed through HilSAT2 (total mitochondrial miRNA) and Bowtie (anti-AGO2 and anti-PNPASE RNA). Resulting BAM files were aligned to the reference genomes for human (Homo\_sapiens.GRCh38.91.gtf.gz) or mouse (Mus\_musculus.GRCm38.91.gtf.gz) were applicable. The R environment was used to asses sample distribution and differential gene expression through DESeq2.

#### **Statistics**

All measures of significance between the control and diabetic groups for the sequencing data are presented as adjusted *P*-values. Adjusted *P*-values are a composition of standard, unadjusted P-values and the stringency of the False Discovery Rate (FDR). Differential expression analysis through DESeq2 implements the Wald Test, using multiple testing against the null hypothesis that P-values are uniformly distributed across a data set, known as the Benjamini-Hochberg procedure. The FDR for this study was set at 0.05.

# Conclusions

The diabetic heart has been shown to suffer metabolic losses with the progress of the pathology. The mitochondrion, central to this etiology, is still poorly understand as to the regulator mechanisms and processes involved in its non-coding RNA transcriptome. We have identified major differences in mitochondrial transcribed genes and imported non-coding RNAs in the SSM and IFM. This process may be controlled centrally through mitochondrial (PNPase) and nuclear/cytosolic (Ago2) proteins. Further, understanding the transport mechanism of non-coding RNAs into the mitochondrion and how these non-coding RNAs interact with the mitochondrial transcriptome, protein regulation, and other essential processes within the mitochondrion, cell, and cardiac muscle.

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# Disclosures

The authors declare that they have no interests, financial or competing otherwise.