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Combined Deep microRNA and mRNA Sequencing Identifies Protective Transcriptomal Signature of Enhanced PI3K α Signaling in Cardiac Hypertrophy

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Abstract

The perturbation of myocardial transcriptome homeostasis is the hallmark of pathological hypertrophy, underlying the maladaptive myocardial remodeling secondary to pathological stresses. Classic and novel therapeutics that provide beneficial effects against pathological remodeling likely impact myocardial transcriptome architecture, including miRNA and mRNA expression profiles. Microarray and PCR-based technologies, although employed extensively, cannot provide adequate sequence coverage or quantitative accuracy to test this hypothesis directly. The goal of this study was to develop and exploit next-generation sequencing approaches for comprehensive and quantitative analyses of myocardial miRNAs and mRNAs to test the hypothesis that augmented phosphoinositide-3-kinase-p110 α (PI3K α) signaling in the setting of pathological hypertrophy provides beneficial effects through remodeling of the myocardial transcriptome signature. In these studies, a molecular and bioinformatic pipeline permitting comprehensive analysis and quantification of myocardial miRNA and mRNA expression with next-generation sequencing was developed and the impact of enhanced PI3K α signaling on the myocardial transcriptome signature of pressure overload-induced pathological hypertrophy was explored. These analyses identified multiple miRNAs and mRNAs that were abnormally expressed in pathological hypertrophy and partially or completely normalized with increased PI3K α signaling. Additionally, several novel miRNAs potentially linked to remodeling in cardiac hypertrophy were identified. Additional experiments revealed that increased PI3K α signaling reduces cardiac fibrosis in pathological hypertrophy through modulating TGF- β signaling and miR-21 expression. In conclusion, using the approach of combined miRNA and mRNA sequencing, we identify the protective transcriptome signature of enhanced PI3K α signaling in the context of pathological hypertrophy, and demonstrate the regulation of TGF- β /miR-21 by which enhanced PI3K α signaling protects against cardiac fibrosis.

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Disclosures

None declared

Keywords

Next-generation sequencing; miRNA; transcriptome; cardiac hypertrophy; PI3K α

1. Introduction

Pathological cardiac hypertrophy, a maladaptive response of the myocardium to increased biomechanical stresses, is associated with increased propensity to develop interstitial fibrosis, heart failure, life-threatening arrhythmias and sudden cardiac death [1, 2]. Physiological hypertrophy in response to exercise training, in contrast, is characterized by normal cardiac structure and maintained cardiac function, and is not associated with increased risk of heart failure, arrhythmias or sudden cardiac death [2, 3]. Two distinct signaling mechanisms, G-protein coupled receptor (GPCR) signaling [2, 4, 5] and phosphoinositide-3-kinase-p110 α (PI3K α) signaling [3], have been linked to the development of pathological and physiological hypertrophy, respectively. In addition, it has been demonstrated that augmentation of PI3K α signaling is beneficial in the setting of pathological hypertrophy and heart failure [6, 7], resulting in improved cardiac function and survival. The mechanisms through which enhanced PI3K α signaling is protective, however, have not been delineated.

A hallmark of pathological hypertrophy is that the myocardial transcriptome landscape, including mRNAs, miRNAs, long noncoding RNAs and other RNA species, is dramatically altered [8, 9], suggesting that perturbation of transcriptome homeostasis is critically coupled to the disease process. We hypothesized that augmented PI3K α signaling provides protective effects by preventing or reversing the changes in myocardial miRNA and mRNA expression associated with pathological hypertrophy. Exploiting a molecular and bioinformatic pipeline optimized for transcriptome profiling with next-generation high-throughput sequencing, we examined the impact of increased PI3K α signaling on myocardial transcriptome structure in the setting of pathological hypertrophy. These analyses identified multiple miRNAs and mRNAs that were abnormally expressed in pathological hypertrophy and normalized with enhanced PI3K α signaling. TAC-induced pathological remodeling is blunted with enhanced PI3K α signaling and is associated with the reduced expression of multiple miRNAs linked to cardiac pathology and diseases, including miR-199a-5p, miR-214 and miR-212. In addition, the combined miRNA and mRNA analyses here revealed that increased PI3K α signaling modulates TGF- β signaling and miR-21 expression, effects that will result in reduced fibrosis. Taken together the data presented here provide new insights into the link between PI3K α signaling and cardiac miRNA regulation, as well as into the mechanisms through which enhanced PI3K α signaling mitigates maladaptive remodeling induced by pathological stresses.

2. Methods

An expanded Methods section is available in the Online Data Supplement.

All high-throughput sequencing data have been submitted to the NCBI gene expression and hybridization array data repository (GEO ID: GSE35350)

(<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pvwltsikocuyglw&acc=GSE35350>)

2.1 Experimental Animals

Eight to twelve-week-old male WT FVB mice and a transgenic mouse line with cardiac specific expression of constitutively active PI3K α (caPI3K α) [10] were subjected to

transverse aortic constriction (TAC) to produce pressure overload-induced left ventricular hypertrophy (LVH) [11]. Seven days after surgery, sham-operated and TAC- WT and *caPI3K α* animals were examined with echocardiography, followed by collection of cardiac tissues. LV weight to tibia length (LVW/TL) ratios were recorded at the time of tissue collection. The echocardiographic and LVW/TL measurements from all of experimental animals are provided in Supplemental Table S1.

2.2 Small RNA Library Construction and Sequencing

Total RNA from the LV of individual animals was isolated with Trizol (Life Technologies) using described methods [12, 13]. The quality and integrity of the RNA samples prepared were confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies). Small RNA libraries were prepared using TrueSeq Small RNA Sample Prep Kits (Illumina) in accordance with the manufacturer's instructions. Briefly, 3' and 5' adapters were sequentially ligated to small RNAs (from 1 μ g total RNA), followed by a reverse transcription reaction to create single stranded cDNAs, which were then amplified (by PCR) and barcoded using a common primer and a primer containing unique six-base index sequence. The amplified libraries were size-selected/gel-purified and quantified using Qubit dsDNA HS Assay kit (Life Technologies). Six to eight barcoded libraries were pooled in equimolar (10 nmol/L) amounts and diluted to 8 pmol/L for cluster formation on a single flow cell lane, followed by single-end sequencing on an Illumina HiSeq 2000 sequencer. A total of 15 small RNA libraries were sequenced (see results) using two HiSeq2000 flow lanes, which provides >5X sequence coverage of the mouse cardiac miRNome for each sample, based on the estimation of 100–200 million sequence reads generated per lane.

2.3 Small RNA Sequencing Data Analyses

Sequencing data from samples pooled in the same flow cell lane were separated (demultiplexed) using CASAVA 1.6 software (Illumina). A total of 165.5 million reads were obtained from sequencing 15 small RNA libraries (Table 1). The sequence reads were analyzed using the miRanalyzer program [14, 15], where the raw sequencing data were transformed and filtered to keep only sequences containing 17–26 bases. Filtered reads were then successively mapped (using Bowtie) [16] to: (1) the miRBase [17] v.18 mouse database (allowing up to two mismatches), to detect known miRNAs; (2) the RefSeq and Rfam database, to detect contamination from other RNA species; and (3) the mouse genome to detect potential novel miRNAs. The sequences matching known miRNAs were clustered and counted; the read counts of each of the known miRNAs were then normalized to the total counts of sequences mapped to the miRBase v.18 database and presented as PMMR (sequences per million mapped reads).

2.4 Discovery of Novel miRNAs

Sequence reads that are counterparts of known mature miRNAs, but that have not been previously reported in the miRBase database, were identified and defined as novel miRNA-star (miRNA*). Sequence reads that did not match known miRNAs or known transcripts from the RefSeq and Rfam databases were mapped against the mouse genome (M37). Once aligned, sequence reads that shared characteristics of miRNAs were identified and clustered using the computational algorithms provided by miRanalyzer [14]. These clusters were further filtered to exclude contamination from repetitive sequences and from mRNA or noncoding RNA degradation products. Only clusters with a read depth ≥ 100 reads were chosen for further analyses. The filtered candidate miRNAs and their precursors were further examined using the following criteria [18, 19]: (1) non-homologous to any known non-coding RNAs; (2) predicted with stem-loop hairpin structure with a $-\Delta G \leq -15$; (3) mature miRNA length of 17–26 bases mapped to the stem of the predicted hairpin structure without extension into the loop or outside of the stem-loop fold; and (4) lack of a bulged secondary

structure larger than 50% of the mature sequence within the stem of the hairpin. The structures of putative miRNA precursors were examined using the RNA-folding prediction software, RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). Candidate miRNAs (and their precursors), fulfilling the described criteria, were grouped as putative novel miRNAs. The putative novel miRNAs were then checked against the latest miRBase v.18 database (released in Nov 2011). The miRNA dataset obtained was also analyzed against an earlier version of the miRBase, miRBase v.16. Identifying putative novel miRNAs absent from miRBase v.16 but reported in miRBase v.18 provides an internal control to validate this analytic approach (see Results).

2.5 Quantitative Reverse-Transcription PCR

Total RNA isolated from the LV of individual animals was reverse transcribed into cDNA using NCode VILO miRNA cDNA synthesis Kit (Life Technologies). qRT-PCR was carried out using EXPRESS SYBR GreenER miRNA qRT-PCR Kit (Life Technologies) with NCode universal reverse primer and forward primers designed specifically for each of the miRNAs (Supplementary Table S2). All data, analyzed using the threshold cycle relative quantification ($\Delta\Delta C_T$) method, were normalized to the value measured for 5S rRNA. Transcript analyses of atrial natriuretic factor (*ANF*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were carried out by regular SYBR Green qRT-PCR, as previously described [12, 13].

2.6 Biochemical Analyses

Western blots analyses of *Sprouty-1* and GAPDH expression were carried out using protein lysates prepared from WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC LV using previously described methods [13].

2.7 RNA Library Preparation and Sequencing

RNA libraries were prepared using TrueSeq RNA Sample Prep Kits (Illumina) in accordance with the manufacturer's recommendations. In brief, 3 μ g of total LV RNA was twice oligo(dT) selected using poly-T oligo-attached magnetic beads. The poly-A(+) RNA was then eluted, fragmented and reverse transcribed into first strand cDNA using random hexamers, followed by second-strand cDNA synthesis. Double-stranded cDNAs were end-repaired and adenylated (singly) at the 3' ends. Barcoded adapters containing unique six-base index sequences and T-overhangs were ligated to the cDNA samples prepared from each of the individual mouse LV samples. Individual cDNA libraries were PCR amplified and purified; five to six barcoded libraries were pooled in equimolar (10 nmol/L) amounts and diluted to 4 pmol/L for cluster formation on a single flow cell lane, followed by single-end sequencing on an Illumina HiSeq 2000 sequencer. To provide a minimum of 1X sequence coverage of the mRNome, we were only able to sequence 11 (see results) mRNA libraries in two HiSeq2000 flow cell lanes. This number is smaller than the 15 samples sequenced for the miRNA analyses because the size of the mouse cardiac mRNome is much larger than that of miRNome.

2.8 RNA Sequencing Data Processing

After demultiplexing the sequencing data, adapter sequences were removed and the individual libraries were converted to the FASTQ format. Sequence reads were mapped to the mouse genome (mm9) with Bowtie [16], allowing up to two mismatches. Sequence reads aligned to the mouse genome were imported into Partek Genomics Suite version 6.5 (Partek, St Louis, MO) for sequence read clustering, counting and annotation. The RefSeq transcript database was chosen as the annotation reference and subsequent data analyses were focused on sequence reads mapping to coding exons. The read counts of each known

transcript were normalized to the length of the individual transcript and to the total mapped read counts in each sample and expressed as RPKM (reads per kilobase of exon per million mapped reads) [8, 20]. Sequence reads mapped to different isoforms of individual genes were pooled together for subsequent comparative analyses.

2.9 Sequencing Data Analyses and Statistical Methods

Gene symbols, PMMR and RPKM values were imported into MultiExperiment Viewer (MeV v4.7.4) for comparison of miRNA and mRNA expression values, computation of significant levels/false discovery rates, preparation of heat-map, hierarchical clustering and self-organizing trees analyses. Gene ontology analyses were performed using g:Profiler (<http://biit.cs.ut.ee/gprofiler/>) [21]. Correlation coefficients and linear regression for comparisons of miRNA/mRNA expression between biological replicates were calculated using Excel (Microsoft). The statistical significance of differences among experimental groups was evaluated by one-way analysis of variance (ANOVA), followed by post-hoc Tukey's multiple comparison correction. In some cases, Student's *t* test or Mann-Whitney *U* test were used to evaluate the differences between groups. A two-tailed *P* value <0.05 was considered statistically significant.

3. Results

3.1 Enhanced PI3K α Signaling Mitigates Pressure Overload-Induced Pathological Remodeling

Transverse aortic constriction (TAC) in WT animals, as expected, resulted in marked pressure overload-induced LV hypertrophy, evident in the ~30% increase in LVW/TL ratio (Figure 1A and Supplemental Table S1) and a significant (~12 fold, *P*<0.001) increase in expression (Figure 1B) of ANF, a molecular marker of pathological hypertrophy [2]. Cardiac specific expression of constitutively active PI3K α (caPI3K α) also produced marked LV hypertrophy (Figure 1A and Supplemental Table S1), although in this case ANF expression was not increased (Figure 1B), observations consistent with the development of physiological hypertrophy [10]. Enhanced PI3K α signaling in TAC, however, significantly (*P*<0.001; ~5 fold) attenuated the TAC-induced increase in ANF expression (Figure 1B), consistent with previous reports suggesting that enhanced PI3K α signaling is protective and mitigates the maladaptive, pathological myocardial remodeling secondary to pressure overload [6, 7].

3.2 Deep Sequencing of miRNAs in WT Sham and WT+TAC, caPI3K α sham and caPI3K α +TAC LV

Using the 15 barcoded small RNA libraries prepared from the mouse LV samples (4 WT sham, 5 WT+TAC, 3 caPI3K α sham and 3 caPI3K α +TAC), a total of 165,496,374 reads were generated from 2 flow cell lanes on the HiSeq2000 sequencer (Table 1), on average producing >10 million reads per library. The read lengths obtained from the libraries showed a Gaussian distribution with a peak at 22 nucleotides (Supplemental Figure 1), consistent with the enrichment of miRNAs. More than 152 million of the sequence reads (92.2%) were aligned to the mouse genome (mm9), ~88 million (53.3%) mapped to known miRNAs, and ~11 million (6.7%) to RNA species other than miRNA. Approximately 53 million (32.3%) of the remaining reads mapped to unannotated genomic regions, and ~10 million of these were subsequently reclassified to be putative miRNAs (see below). In each sample, the read counts of each of the known miRNAs were then normalized to the total counts of sequences mapped to the miRbase v.18 database and presented as PMMR (sequences per million mapped reads). Scatter plots of the normalized read counts of all known miRNAs showed high degrees of correlation ($\beta=0.95$, $R^2=0.99$, Supplemental Figure 2A) between biological replicates, indicating high levels of reproducibility for these miRNA-Seq experiments. The

expression levels of several of the miRNAs identified in WT sham LV were also examined with qRT-PCR and the relative abundances (in C_T) of the individual miRNAs were found to be well correlated with the results from miRNA-Seq (Supplemental Figure 2B).

Using the criterion that a miRNA sequence must be detected in at least 2 small RNA libraries, the sequence reads were mapped to 393 mature miRNAs reported in miRbase v.18 (Table S3). A wide range of miRNA expression levels, ranging from 0.1 to 222655.7 PMMR, was observed. MiR-22 was the most abundant mature miRNA identified, corresponding to 18–20% of all of the miRNA sequence reads. The relative expression levels of the 20 most abundant mature miRNAs are shown in Figure 1C. Interestingly, 13 of these 20 miRNAs, including muscle-specific miR-1 and miR-133a [22] and endothelial cell-specific miR-126-5p [23], have been reported previously to be highly abundant in the mouse heart [24]. The 10 most abundant miRNAs accounted for >70% of all miRNA sequence reads in all LV samples (Figure 1D). In addition to known mature miRNAs, 13 novel miRNA* (mature-star miRNA, mature miRNAs derived from the less-expressed, opposite arm) sequences were identified for known miRNA genes without prior annotation in miRBase v.18 (Table 2 and Supplemental Table S4). Comparative analyses revealed that 78 miRNAs were differentially expressed in the 4 sets of LV samples analyzed ($P < 0.01$ at a false-discovery rate of 0.05). A complete list of the differentially expressed miRNAs, with mean PMMR values, fold differences (compared to WT sham LV) and P values, is presented in Supplemental Table S5. Consistent with previous studies demonstrating normal cardiac structure and function in the caPI3K α mouse model of physiological hypertrophy [6, 10], unsupervised hierarchical clustering analyses revealed that the miRNA expression profiles in WT sham and caPI3K α sham LV samples were similar (Supplemental Figure 3). In contrast, the miRNA expression signature of WT+TAC LV was dramatically different from both WT sham and caPI3K α sham LV, segregating the WT+TAC from both the WT sham and the caPI3K α sham LV samples (Supplemental Figure 3). Consistent with previous miRNA studies in murine models of pressure overload-induced pathological hypertrophy, the expression levels of the miR-146 family [25], as well as miR-199a-5p [26, 27], miR-21 [25–27], miR-212 [28] and miR-214 [25–27] were upregulated, whereas miR-133a [25, 27] was downregulated in WT+TAC, compared with WT sham, LV. Interestingly, when comparing the miRNA expression profiles of WT+TAC and caPI3K α +TAC LV, it is clear that the aberrant expression pattern of miRNAs observed with TAC-induced LVH was blunted substantially with increased PI3K α signaling (Supplemental Figure 3 and Supplemental Table S5).

To identify miRNAs that were abnormally expressed in pathological hypertrophy but partially or completely normalized with increased PI3K α signaling, an unsupervised, self-organized tree analysis for the differentially expressed miRNAs (in Supplemental Table S5) was conducted (Supplemental Figure 4). A total of 23 miRNAs fitting this “protective PI3K α miRNome” expression pattern were identified and clustered (Figure 2). Most of these miRNAs were upregulated 2 fold in WT+TAC, compared with WT sham, LV, and were normalized (to WT sham levels) or markedly reduced (compared to WT+TAC levels) with increased PI3K α signaling. A number of the miRNAs identified in the “protective PI3K α miRNome” have been shown to cause or to contribute to various myocardial pathologies, including cardiac hypertrophy (miR-146b, miR199a-5p, miR-21, miR-212, miR-214), heart failure (miR-146b, miR199a-5p, miR-21, miR-212, miR-214), myocardial ischemia (miR-134, miR-199a-5p, miR-21, miR-214, miR-379), cardiac fibrosis (miR-21) and fetal gene program activation (miR-21, miR-212). A complete list of the 23 miRNAs identified and references to previously identified cardiac and extra-cardiac functions of these miRNAs is provided in Supplemental Table S6. Taken together, these data indicate that enhanced PI3K α signaling ameliorates the adverse effects of pathological stress on multiple pathology-linked miRNAs, resulting in normalized myocardial miRNA expression.

3.3 Identification of Novel miRNAs Expressed in Mouse Ventricular Tissue

One advantage of miRNA-Seq is the opportunity to discover novel miRNAs. Using a computational algorithm on the basis of miRNA biogenesis [14], we identified ~10 million unannotated mapped sequence reads that share characteristics of miRNAs. These sequence reads generated 896 alignment clusters. Of these, 543 had read counts ≥ 10 and these clusters were further filtered to exclude contamination from repetitive sequences and mRNA or noncoding RNA degradation products. Clusters with a read depths ≥ 100 were chosen for further analyses. A total of 246 of these identified clusters were subjected to subsequent analyses for miRNA precursor characteristics, including predicted folding structures and negative ΔG criteria (see Methods). Using the combined read depth and precursor characteristics criteria, we identified 11 novel miRNAs that were not reported in the latest miRBase v.18. To test the robustness of the analysis, the dataset was re-analyzed against miRBase v.16 and additional 15 miRNAs that are absent in the miRBase v.16 but reported in miRBase v.18 were identified (Supplemental Table S7), validating the analytical approaches used. Among the 11 novel miRNAs (Supplemental Table S8) identified in mouse LV, 3 were differentially regulated in WT+TAC, compared with WT sham, LV (Figure 3A). The predicted secondary structure and precursor sequence of one of these novel miRNAs (miR-cluster 16) are illustrated in Figure 3B. The expression of miR-cluster 16 and the two other novel miRNAs in WT sham and WT+TAC LV were also confirmed using qRT-PCR (Figures 3C and 3D).

3.4 Parallel Messenger RNA Expression Profiling in Mouse LV with mRNA Sequencing

To complete the functional transcriptome profiling, mRNA sequencing (mRNA-Seq) experiments were conducted in parallel to the miRNA-Seq. Eleven barcoded mRNA libraries were prepared from mouse LV samples (4 WT sham, 3 WT+TAC, 2 caPI3K α sham and 2 caPI3K α +TAC). A total of 242,851,107 sequence reads were generated from 2 HiSeq2000 flow cell lanes (Table 1), with an average sequencing depth of > 22 million reads per library. Among the 242 million sequence reads obtained, 84.5% (~205 million reads) were aligned to mm9 mouse genome, among which ~140 million reads (57.6% of total reads) were mapped within exon regions (Table 1). Read counts mapped to different isoforms of individual genes were pooled together to calculate the RPKM (reads per kilobase of exon per million mapped reads) value of each gene [20]. These mRNA-Seq experiments were highly reproducible among biological replicates (Supplemental Figure 5). Using the cutoff value of 3 RPKM, which corresponds to expression level ≈ 1 copy per cell [20], we identified a total of 8989 genes that were expressed at ≥ 3 RPKM in at least one LV sample (Supplemental Table S9); 2638 genes were found to be differentially expressed ($P < 0.05$, FDR of 0.05) among the four groups of LV samples (Supplemental Table S10). As an internal control, the expression level of the *pik3ca* gene was increased by 15–20 fold in every caPI3K α , compared to WT, LV sample, consistent with the overexpression of the caPI3K α transgene in these animals. The caPI3K α sham LV share similar transcriptome signatures with WT sham LV, whereas the transcriptional signature of WT+TAC LV was distinct (Figure 4) from WT and caPI3K α sham LV. In WT+TAC LV, for example, the activation of the fetal gene program (including *nppa*, *nppb* and *acta1*) [29, 30] and increased expression of cardiac fibrosis genes (including *col3a1*, *col5a2*, *col8a1*, *col15a1*, *col6a1*) were evident. Interestingly, consistent with the notion that enhancing PI3K α signaling in pathological hypertrophy is protective, the expression levels of many of the genes identified as abnormally expressed in WT+TAC LV (compared with WT sham) were attenuated or normalized (in caPI3K α +TAC LV) with increased myocardial PI3K α signaling (Figure 4).

The self-organizing tree algorithm was applied to identify genes that were downregulated or upregulated in pathological hypertrophy, and normalized or reversed with increased PI3K α signaling, i.e., the “protective PI3K α mRNome”. These genes are presented in Supplemental

Table S11 and S12. A total of 249 genes were found to be down-regulated in pathological LVH and normalized with increased PI3K α signaling (Figure 5A). Gene ontology analysis of these 249 genes revealed an enrichment in mitochondrial, cellular catabolism, oxidation-reduction and lipid metabolism genes (Figure 5B), consistent with previous reports suggesting the critical role of PI3K α signaling in maintaining homeostatic mitochondrial biogenesis and lipid metabolism [31]. The expression levels of 156 other genes, however, were found to be upregulated in WT+TAC LV, and reversed with enhanced PI3K α signaling (Figure 5C). In this case, many genes linked to development, stress responses and the extracellular matrix were identified (Figure 5D), consistent with previous suggestions that PI3K α signaling attenuates the activation of the fetal gene program and the fibrotic changes associated with pressure overload-induced LVH [6, 7]. Interestingly, a number of genes involved in immune and inflammatory responses including *Il6*, *Il6st*, *Nfkb1*, *Tlr4*, *Vcam1*, *Icam2*, *Tnfrsf1a* and *Tnfrsf1b*, were also upregulated with TAC-induced pathological hypertrophy (Supplemental Figure 6), consistent with previous reports linking innate immunity/inflammatory responses and cardiac hypertrophy/LV dysfunction [32–34]. Interestingly, the upregulation of these inflammatory markers by TAC was also markedly blunted with increased PI3K α signaling (Supplemental Figure 6).

3.5 miR-21 in the Inhibition of Cardiac Fibrosis by Enhanced PI3K α Signaling

Enhanced PI3K α signaling has been shown to reduce cardiac fibrosis in pathological LVH induced by pressure overload [6]. Indeed, the upregulation of fibrosis genes, including collagens, fibronectin (*fbn1*), fibrillin (*fn1*) and connective tissue growth factor (*ctgf*), in TAC-induced LVH was largely abrogated with enhanced myocardial PI3K α signaling (Figure 6A). Interestingly, miR-21, which has been shown to promote tissue fibrosis in pulmonary [35] and cardiac [36] disease, was upregulated in response to TAC, and this upregulation was attenuated with increased PI3K α signaling (Figure 2A,B). It has been shown that miR-21 promotes cardiac fibrosis through translational suppression of *Sprouty-1*, which inhibits the pro-fibrotic ERK-MAPK signaling pathway [36]. Consistent with this, *Sprouty-1* protein expression was significantly ($P<0.05$) decreased in WT+TAC, compared with WT sham, LV. The downregulation of *Sprouty-1* protein in response to TAC, however, was reversed with increased PI3K α signaling (Figure 6B). In addition, components of the TGF- β pathway, a potent miR-21 activator [37, 38], including *tgfb2*, *tgfb1* and *tgfb2*, were upregulated with TAC, and suppressed (in caPI3K α +TAC LV) with increased PI3K α activity (Figure 6A). These results suggest that enhanced PI3K α signaling in the context of TAC inhibits cardiac fibrosis through reducing TGF- β signaling and the repression of the pro-fibrotic miR-21.

4. Discussion

4.1 miRNA-Seq Revealed Protective miRNA Expression Signature of Enhanced PI3K α Signaling in Pathological Hypertrophy

The comparative analyses of miRNA expression in WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC LV samples allowed the identification of the “protective PI3K α miRNome” in the setting of pathological hypertrophy. As described above and detailed in Supplemental Table S6, increased expression of many of these miRNAs has been previously linked to various myocardial pathologies. For example, miR-199a-5p, miR-214 and miR-212 have all been shown to be upregulated in murine models of pathological hypertrophy [25–27] and in human failing hearts [27, 39, 40]. In addition, overexpression of miR-199a-5p, miR-214 or miR-212 in murine myocytes reportedly led to hypertrophic growth and activation of the fetal gene program [27, 28]. The observation that enhanced PI3K α signaling suppresses the expression of these disease-causing miRNAs suggests that the anti-pathological-hypertrophy effects of increased PI3K α signaling are mediated, at least

in part, by normalizing the expression of these “pathological miRNAs”. Previous attempts to modulate the pathological expression of these miRNAs have consisted largely of eliminating or reducing them, one or two miRNAs at a time. Interestingly, however, this approach seems to have only very small to moderate beneficial effects, observations that likely reflect the inherent complexity of the signaling networks involved [41, 42]. The observation that enhanced PI3K α signaling results in the marked and simultaneous suppression of multiple “pathological miRNAs” suggests that modulating the expression of multiple disease-causing miRNAs concurrently might be a novel therapeutic strategy to treat the hypertrophied or failing heart. This strategy has the potential of impacting multiple signaling pathways simultaneously which could have a greater effect on disease progression and/or severity than reducing the expression of individual miRNAs.

Interestingly, it was recently reported that miR-222, 34a and 210 are upregulated in a mouse model of chronic myocardial infarction (MI) [43]. In addition, the upregulation of these miRNAs in response to MI was shown to be augmented with reduced cardiac PI3K α activity and attenuated with increased cardiac PI3K α activity [43]. Specifically, miR-222, 34a and 210 were upregulated in WT LV with MI (compared to WT sham) by ~1.3–1.4 fold, and the upregulation of these miRNAs was attenuated (~1.1–1.2 fold) in caPI3K α LV with MI [43]. The results of the studies detailed here revealed increased expression (~1.8 fold) of both miR-222 and 34a in WT LV with TAC, and these increases were attenuated partially (to a level of ~1.4 fold) with increased PI3K α signaling. In contrast, miR-210 expression was not significantly different in WT or caPI3K α LV in the absence and in the presence of TAC (Supplemental Table S3). Taken together, these observations are consistent with the suggestion that, similar to MI, miR-222/miR-34a are upregulated in response to TAC, and that enhanced PI3K α signaling ameliorates the upregulation of miR-222/miR-34a in both settings (i.e., in TAC-induced LVH and MI) [43]. Neither of miR-222 or miR-34a, however, was included in the “protective PI3K α miRNome” reported here due the stringent statistical criteria used. The differences in the miR-210 expression pattern (upregulated with MI but unchanged with TAC-induced LVH), however, could be attributed to the differences of experimental models (chronic MI vs acute TAC-induced LVH) used in the present study and in the study by Lin et al [43].

The analytical approach developed and used here allowed the identification of 13 novel miRNA* of known mature miRNA genes and 11 novel miRNAs. Importantly, of the >10 million sequence reads analyzed, up to 896 clusters were predicted to be putative mouse miRNAs. Of these, 246 clusters were detected at >100 reads, and only 11 putative miRNAs met all the criteria of “novel miRNAs”. There are two interpretations of the apparently low yield of novel miRNAs identified in these experiments. First, a large fraction of the “putative miRNA” reads may represent low-abundance hairpin species that were only occasionally processed by the Drosha-Dicer miRNA biogenesis machinery. It is certainly possible that most mouse miRNAs have already been identified and annotated. Consistent with the latter suggestion, recent studies using large-scale cloning approaches to identify novel mammalian miRNAs yielded only a few uncharacterized, low-abundance miRNAs [44, 45]. Importantly, however, we cannot exclude the possibility that the stringent criteria used here might have filtered out some miRNAs expressed at very low levels but that, nevertheless, are of biological significance. Regardless, additional experiments are needed to determine the functional roles of the novel miRNAs identified in the studies here.

In the miRNA sequencing experiments detailed here, a small number (~10–20) of highly abundant miRNAs were identified that account for the majority of the mature miRNA species expressed in the adult mouse LV (Figure 1C,D). Although this observation is similar to previously published miRNA-Seq findings in the mouse heart [24], the relative abundances of the highly expressed miRs in the previous report by Rao et al [24] were

different than those determined here. The most abundant miRNA in Rao et al [24], which accounted for ~40% of the total miRNA reads, was miR-1. Here, however, miR-1 ranked #20 in abundance and accounted for only ~1% of the total miRNA reads (Figure 1C and Supplemental Table S3). Although the use of different sequencing platforms or library construction approaches might be expected to cause minor read count differences [18], it is unlikely that either could contribute substantially to the differences observed in these two studies. It seems more likely the apparent discrepancy in the findings may reflect the fact that whole heart samples were used by Rao et al [24], whereas only LV samples were used in the studies here. Because of the highly sensitive and quantitative nature of deep sequencing experiments [46], regional differences in relative miRNA expression levels will impact quantitative analyses.

4.2 Combined miRNA-Seq and mRNA-Seq Analyses Provide a Transcriptome Framework to Explore Novel Mechanisms Underlying the Pathobiology of Cardiac Hypertrophy

The mechanisms through which miRNAs regulate biological process are translational inhibition or mRNA degradation of the miRNA targets [41, 47]. It has been argued recently that mRNA destabilization accounts for most of miRNA-mediated gene modulation [48, 49], and that most (up to 84%) of the protein level changes caused by miRNA regulation can be attributed to altered mRNA expression [50]. To understand the functional impact of miRNA regulation, therefore, it seems clear that it is necessary to examine miRNA and target mRNA expression levels concomitantly in the same biological samples. Combining miRNA-Seq and mRNA-Seq datasets allows direct assessment of the reciprocal expression of a given miRNA and its known/predicted target transcripts. Using this approach, for example, miR-21 was identified as part of the protective PI3K α miRNome. MiR-21 is an intronic miRNA residing in the TMEM49 gene [51] that has previously been shown to be upregulated in various cardiac diseases including pathological hypertrophy [25–27, 52], myocardial infarction [39], and heart failure [36]. It has also been demonstrated in failing rodent and human hearts that miR-21 levels are increased specifically in cardiac fibroblasts, enhancing pro-fibrotic ERK-MAPK activity through translational inhibition of the protein *Sprouty-1* [36], leading to increased extent of interstitial myocardial fibrosis. Previous studies have also shown that miR-21 expression is strongly upregulated by TGF- β signaling [37], which is implicated in myocardial injury, repair and fibrosis [53, 54]. Combining the known function and regulation of miR-21 described in the literature and the experimental results from the present studies, a mechanistic model was proposed (Figure 6C): upon pressure-overload, TGF- β signaling pathway is activated and promotes the expression of miR-21 in cardiac fibroblasts [36, 37], resulting in the translational suppression of the protein *Sprouty-1* [36], which tonically suppresses the activation of pro-fibrotic ERK-MAPK signaling [36]; ERK-MAPK signaling, therefore, is released from tonic inhibition by *Sprouty-1*, resulting in increased cardiac fibrosis. The augmentation of cardiac PI3K α signaling, however, abrogates the activation of TGF- β signaling, leading to blunted miR-21 expression and the maintenance of *Sprouty-1* expression, keeping the ERK-MAPK signaling inhibited and preventing the development of cardiac fibrosis. It is important to note here, however, that TGF- β -mediated miR-21 regulation is, almost certainly, not the only mechanism contributing to PI3K α -mediated attenuation of cardiac fibrosis in the context of pressure overload-induced pathological cardiac hypertrophy. It is possible, for example, that the impact of PI3K α signaling on cardiac fibrosis is also mediated through miRNA species in addition to miR-21, as well as through miRNA-independent mechanisms. Additional *in vitro* and *in vivo* experiments are needed to test these hypotheses directly and to delineate the detailed molecular mechanisms.

The mRNA-seq experiments detailed here also uncovered functional transcriptome changes that suggest possible mechanisms underlying the protective effects of PI3K α signaling

against pathological hypertrophy (Figure 5). On the transcriptional level, pressure-overload impairs cardiac electrical and mechanical function through: (1) the suppression of the expression of genes required for normal cardiac functioning, including genes involved in regulating mitochondrial function, cellular oxidation-redox pathways, and lipid metabolism; and, (2) the upregulation of fetal genes, and genes involved in inflammation, stress-responses and regulation of the extracellular matrix. The results here demonstrate that increased PI3K α signaling ameliorates the aberrant expression of genes that are upregulated and genes that are downregulated in pathological cardiac hypertrophy, thereby providing multiple cardioprotective effects.

5. Conclusions

The results presented here utilized next-generation sequencing technologies for comprehensive cardiac miRNA and mRNA expression profiling in mouse LV and identified multiple miRNAs and mRNAs that were abnormally expressed in pathological hypertrophy and normalized with enhanced PI3K α signaling. Several novel miRNAs of potential significance in cardiac hypertrophy were also identified. Combined miRNA and mRNA analyses revealed that increased PI3K α signaling reduces cardiac fibrosis in pathological hypertrophy through modulating TGF- β signaling and attenuating miR-21 expression. In addition, the TAC-induced pathological remodeling was ameliorated with increased PI3K α signaling and was associated with the normalization of the pathological, disease-causing miRNAs including miR-199a-5p, miR-214 and miR-212. These results demonstrate not only the strength and robustness of next-generation sequencing in exploring the architecture and dynamics of the cardiac transcriptome in physiological and pathological hypertrophy, but also the potential of the combined approach to provide insights into molecular mechanisms underlying the cardioprotective effects of increased PI3K α signaling in the setting of pathological hypertrophy.

Highlights

- We exploited next-gen sequencing for a comprehensive cardiac mRNA and miRNA profiling
- We revealed the transcriptome signature of the cardiac protective effects of PI3K α
- Increased PI3K α signaling normalizes miRNA/mRNA expression with pathological LVH
- PI3K α reduces cardiac fibrosis through modulating TGF- β pathway and miR-21 expression
- We identified multiple novel miRNAs linked to remodeling in cardiac hypertrophy

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANF	atrial natriuretic factor
caPI3Kα	constitutively active phosphoinositide-3-kinase p110 α
FDR	false discovery rate
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GO	gene ontology
GPCR	G-protein coupled receptor
LV	Left ventricle
LVH	Left ventricular hypertrophy
LVW/TL	LV weight to tibia length ratio
MI	myocardial infarction
miRNA-Seq	microRNA sequencing
mRNA-Seq	mRNA sequencing
qRT-PCR	quantitative real time polymerase chain reaction
PMMR	sequences per million mapped reads
RPKM	reads per kilobase of exon per million mapped reads
TAC	transverse aortic constriction
WT	wild-type

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Figure 1. Functional impact of enhanced PI3K α signaling on pressure overload-induced LVH and LV miRNA abundance

(A) LV weight/tibia length (LVW/TL) ratios were determined in WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC (n=6 in each group) animals; individual and mean $\pm$ SEM values (* P <0.001, † P <0.05) are plotted. (B) Transcript expression of ANF, the molecular marker of pathological hypertrophy [2] is increased ~12 fold with TAC in WT LV. The increase in ANF expression in response to TAC is significantly (P <0.01) blunted in the LV of caPI3K α animals. (C) Expression levels of the 20 most abundant miRNAs in WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC LV are compared. The abundances of the individual miRNAs were expressed as percentages of individual miRNA read counts

contributing to the total read counts of known miRNAs in the library. (D)The 10 most abundant miRNAs account for >70% of the total miRNA reads in mouse LV.

Figure 2. miRNAs aberrantly expressed in pathological hypertrophy are normalized with enhanced PI3Kα signaling

(A) Heat map and hierarchical clustering of miRNAs found to be aberrantly expressed in TAC-induced pathological hypertrophy and normalized with augmented myocardial PI3Kα signaling. Green denotes low expression; red, high expression. (B) The average PMMR, fold change (compared to WT sham) and *P* values (ANOVA) of each miRNA identified are listed.

A

Novel miRNAs that are differentially downregulated in pathological hypertrophy

Cluster ID	Strand	Mature sequence	WT Sham Mean PMMR	WT TAC/ WT Sham	P value
16	+	CACGGCGGCTGGAATTCCC	247.4	0.34	0.026
4	+	ACCATCTGTGGGATTATGACTGAACG	825.2	0.29	0.005
54	+	GGGGGCGTGAATTATCGGGT	66.4	0.12	0.015

B

C

D

miR cluster 16 mature

5'-CAGGAACCUCAGUCAUUAUACACU**CACGGCGGCGUGGAUUUCC**AUCAAGUGAUCUGUGA**GGGAUUUCC**AGUGGGCCAUACGUGACAGAGCAGAAUGACGG-3'

miR cluster 16 star

Figure 3. Novel miRNAs identified in mouse ventricles are differentially expressed

(A) Three novel miRNAs (miR-cluster 16, 4 and 54), identified by miRNA-seq, were differentially expressed in WT+TAC, compared with WT sham, LV. (B) Sequence and predicted secondary structure of the miR-cluster 16 precursor. The linear sequence with the mature miRNA sequence in red and miRNA star sequence in blue is also illustrated. (C) Representative qRT-PCR amplification plots for miR-cluster 16, 4 and 54 in WT sham and WT+TAC LV. Dotted line indicates fluorescence threshold for determining C_T values. (D) qRT-PCR confirmed the differential expression of miR-cluster 16, 4 and 54 in WT sham (n=6) and WT+TAC (n=6) LV. (* $P<0.001$, † $P<0.05$)

Figure 4. Distinct mRNA expression profiles in pathological and physiological hypertrophy
Heat map and hierarchical clustering of the top 50 differentially regulated mRNAs (ranked by *P* value) in WT sham, WT+TAC, caPI3Kα sham and caPI3Kα+TAC LV.

Figure 5. Transcripts increased/decreased with pathological hypertrophy and normalized with enhanced PI3K α signaling are segregated to distinct functional groups

(A) Unsupervised hierarchical clustering of the transcripts that were downregulated in pathological hypertrophy and normalized with enhanced PI3K α signaling. (B) Gene ontology analysis revealed enrichment of genes that are required for normal myocardial functioning, including mitochondrial regulation, cellular catabolism, oxidation/reduction, electron transport, and lipid metabolism. (C) Unsupervised hierarchical clustering of the genes that were upregulated in pathological hypertrophy and normalized with enhanced PI3K α signaling; gene ontology analysis revealed enrichment of genes involved in

maladaptive myocardial responses to pathological stimuli, including genes involved in development, inflammation, stress responses and extracellular matrix formation (D).

Figure 6. Enhanced PI3K α signaling in pathological hypertrophy attenuates TGF- β and miR-21 expression, maintaining *Sprouty-1* protein expression and reducing fibrosis

(A) Averaged normalized read count of genes linked to fibrosis (*Ctgf*, *Col15a1*, *Col1a1*, *Col1a2*, *Col3a1*, *Col8a1*, *Fn1* and *Fbn1*) or the TGF- β pathway (*Tgfb1*, *Tgfb2*, *Tgfr1* and *Tgfr2*) in WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC LV. Normalized read count data were expressed relative to the mean WT sham value. (B) Representative Western blot of fractionated LV proteins from WT sham, WT+TAC, caPI3K α sham and caPI3K α +TAC LV (n=4-5 in each group) probed with anti-*Sprouty-1* and anti-GAPDH antibodies. The expression levels of each protein on each blot were measured and normalized to the expression of GAPDH in the same sample on the same blot. Protein expression data were

expressed relative to the mean value of WT sham LV samples. The mean $\pm$ SEM relative expression level of *Sprouty*-1 was significantly ($^{\dagger}P<0.05$) lower in WT+TAC than in WT sham, LV. The downregulation of the *Sprouty*-1 protein with TAC, however, was abrogated with enhanced PI3K α signaling. (C) Schematic of proposed mechanism underlying PI3K α -mediated suppression of cardiac fibrosis in pathological hypertrophy induced by TAC [36–38].

Table 1

Summary of miRNA- and mRNA-Seq read counts and mapping results

miRNA-Seq Read Counts	WT Sham (n=4)	WT TAC (n=5)	caPI3K α Sham (n=3)	caPI3K α TAC (n=3)	Total
Total	45901044	49701319	37441052	32452959	165496374
Mapped	41986529 (91.5)	45593199 (91.7%)	34304088 (91.6%)	30735651 (94.7%)	152619467 (92.2%)
Unmapped	3914515 (8.5%)	4108120 (8.3%)	3136964 (8.4%)	1717308 (5.3%)	12876907 (7.8%)
Known miRNAs	22981918 (50.1%)	27443912 (55.2%)	18360999 (49.0%)	19354436 (59.6%)	88141265 (53.3%)
Putative miRNAs	2514756 (5.5%)	2564454 (5.2%)	2696651 (7.2%)	2444635 (7.5%)	10220496 (6.2%)
Mapped to other RNA species	2968104 (6.5%)	2942701 (5.9%)	2950463 (7.9%)	2282158 (7.0%)	11143426 (6.7%)
Mapped to genome, unannotated	13521751 (29.5%)	12642132 (25.4%)	10295975 (27.5%)	6654422 (20.5%)	43114280 (26.1%)
mRNA-Seq Read Counts	WT Sham (n=4)	WT+TAC (n=3)	caPI3K α Sham (n=2)	caPI3K α +TAC (n=2)	Total
Total	47913934	45178741	84373729	65384703	242851107
Mapped	40884751 (85.3%)	38297928 (84.8%)	70954141 (84.1%)	55108361 (84.3%)	205245181 (84.5%)
Unmapped	7029183 (14.7%)	6880813 (15.2%)	13419588 (15.9%)	10276342 (15.7%)	37605926 (15.5%)
Mapped to exon regions	26279745 (54.8%)	26838716 (59.4%)	48917894 (58.0%)	37761525 (57.8%)	139797880 (57.6%)

TAC: trans-thoracic aortic constriction

Table 2

Novel miRNA* identified in mouse LV

Novel miRNA *	WT Sham Mean PMIR	WT TAC/ WT Sham	caPI3K α Sham/ WT Sham	caPDK α TAC/ WT Sham	caPI3K α TAC/ caPI3K α Sham
mmu-mir-145*	287.96	0.75	0.87	0.67	0.77
mmu-mir-322*	54.41	1.16	0.90	0.91	1.01
mmu-mir-425*	5.97	1.72	1.36	1.7	1.25
mmu-mir-1960*	1.55	0.94	1.03	0.81	0.79
mmu-mir-503*	1.31	1.36	0.79	1.94	2.46
mmu-mir-28*	1.26	1.46	1.12	0.53	0.47
mmu-mir-30d*	1.18	0.13	0.50	0.14	0.28
mmu-mir-382*	0.81	1.73	1.00	1.45	1.45
mmu-mir-153*	0.39	0.14	0.61	1.74	2.85
mmu-mir-3068*	0.22	0.97	0.48	0.00	0.00
mmu-mir-486*	0.20	0.00	3.96	0.69	0.17
mmu-mir-455*	0.19	1.23	0.00	0.00	N.A.
mmu-mir-140*	0.08	3.26	2.87	0.00	0.00
mmu-mir-143*	0.00	N.A.	N.A.	N.A.	1.05