Published in final edited form as:
J Am Coll Cardiol. 2011 November 22; 58(22): 2270–2278. doi:10.1016/j.jacc.2011.08.041.

A MicroRNA Signature Associated with Recovery from Assist Device Support in Two Groups of Patients with Severe Heart Failure

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Abstract

Objective—Test the hypothesis that cardiac microRNAs (miRs) profiling in severe heart failure patients at the time of ventricular assist device (VAD) placement would differentiate those who remained VAD-dependent from those with subsequent left ventricular (LV) recovery.

Background—The relationship of myocardial miR expression to ventricular recovery is unknown.

Methods—We studied 28 patients with nonischemic cardiomyopathy requiring VAD support consisting of Test and Validation cohorts from two institutions; 14 with subsequent LV recovery and VAD removal and 14 clinically matched VAD-dependent patients. Apical core myocardium was studied for expression of 376 miRs by PCR-based array and RT-PCR methods. Samples from seven non-failing hearts were used in confirmatory studies.

Results—By PCR-array, ten miRs were differentially expressed between LV recovery and VAD-dependent patients in the Test cohort. RT-PCR confirmed lower expression in LV recovery patients for 4 miRs (-15b, -1.5 fold; -23a, -2.2 fold; -26a, -1.4 fold; and -195, -1.8 fold; all $p<0.04$ vs. VAD-dependent). The Validation cohort similarly showed lower miRs expression in LV recovery patients: -23a (-1.8 fold) and -195 (-1.5 fold) both $p<0.03$). Furthermore, miR-23a and

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No conflicts.

-195 expression in non-failing hearts was similar to LV recovery patients (both p<0.04 vs. VAD dependent). LV recovery patients also had significantly smaller cardiomyocytes by quantitative histology in both cohorts.

Conclusions—Lower cardiac expression of miRs-23a and -195 and smaller cardiomyocyte size at the time of VAD placement were associated with subsequent LV functional recovery. Differential expression of miRs at VAD placement may provide markers to assess recovery potential.

Keywords

cardiomyopathy; heart-assist device; microRNA; hypertrophy

INTRODUCTION

Despite the beneficial effects of mechanical unloading achieved after ventricular assist device (VAD) placement on molecular markers and cardiomyocyte structure and function in the failing heart (1–7), only a small number of VAD-supported patients recover sufficient function to allow permanent removal of the VAD (8–10). Current data suggest that an unappreciated percentage of VAD-supported patients have a potential for recovery but may not be adequately tested for the ability to permanently and successfully remove mechanical support (9, 10). Clinical variables may assist in the prediction of recovery (9–12) but there are few studies that identify biochemical markers associated with functional recovery sufficient to allow device removal (13, 14).

MicroRNAs (miRs) are short (21–23 nucleotides long) non-coding RNAs that may play potent and widespread roles in post-transcriptional regulation of gene expression. miRs affect diverse pathways including apoptosis, cell growth and proliferation, oncogene suppression and activation, and stem cell activation (15). Recent human and animal studies have shown significant alterations in cardiac miR expression patterns with heart failure, while others demonstrated miR involvement in key heart failure pathways (16–24).

In this study, we investigated patterns of cardiac miR expression at the time of VAD placement to identify patterns associated with functional recovery from severe heart failure in VAD-supported patients. We also investigated whether this ‘recovery potential’ expression profile was induced in failing hearts by introduction of VAD support. Finally, we assessed this miR profile in nonfailing control hearts.

METHODS

Patient Selection and Tissue Collection

All studies were performed under protocols approved by the University of Pittsburgh, Texas Heart Institute, and Cleveland Clinic Foundation Institutional Review Boards.

Test Cohort

The Test Cohort consisted of 14 heart failure patients with LV tissue samples (Cores) obtained and banked at the time of VAD placement. The **Recovered** group (**R**; n=7) consisted of patients who recovered sufficient cardiac function to allow LVAD removal. Recovered patients were compared with VAD implanted patients who remained VAD-dependent **(Dependent, D**, n=7**),** and who were retrospectively matched for clinical variables associated with recovery so as to closely approximate those variables in R group patients. Patients with ischemic heart disease were excluded from all groups reported in this study. Of the 7 explanted and discharged patients, 1 suffered unexplained death ~1.5 months

after discharge and 1 died ~ 3 months post-explantation due to *Staphylococcus aureus* mediastinitis with preserved cardiac function. The remaining 5 have survived free from heart failure more than 6 months.

Validation Cohort

The Validation cohort tissue provided by the Texas Heart Institute arose from 7 Recovered (R), and 7 Dependent (D) patients (as defined for the Test cohort, and matched for clinical variables). All 7 explanted patients survived and were free of heart failure symptoms for more than 6 months.

Patient Selection; Pre- and Post-VAD

Paired LV samples, collected at the time of VAD implantation **(Pre-VAD)** and cardiac transplantation **(Post-VAD),** were obtained from an independent third group of 6 patients (University of Pittsburgh) with severe heart failure from nonischemic cardiomyopathy.

Nonfailing Hearts

Non-failing human heart tissue (NF) was obtained from the Cleveland Clinic Foundation. Transmural samples of the lateral LV wall were obtained from unmatched donors whose hearts were not suitable for transplantation despite normal ventricular structure and function as measured by echocardiography. Non-failing samples were clinically matched with the Test and Validation Cohorts for age and gender (n=7, age 36±10 years, 50% female).

PCR and Histological Analysis

A screening miR PCR based array was performed on the Test cohort, followed by confirmatory individual quantitative PCR in the Test and Validation cohorts (25). miRs confirmed altered in both cohorts were tested in non-failing hearts. Measurement of cardiomyocyte size was performed in both cohorts (6, 26). Details of procedures and data analysis are provided in the Supplemental Methods section.

Statistical Analyses

miR expression data are presented as fold up or down regulation in the D group versus R (26). A negative value indicates lower expression in the R group. For confirmatory PCR, data is expressed as fold up or down expression in D versus R, or between Pre- and Post VAD samples, or normalized to the mean of NF samples for NF versus R or D. Results were compared between groups by a non-parametric one-way analysis of variance (Kruskal-Wallis test). Upon detection of overall significance, limited hypothesis driven post-hoc analyses were performed using Mann-Whitney U test. For Pre- versus Post-VAD, a paired t-test was employed. All data are reported as mean ± SD. Significance was accepted at $p<0.05$.

RESULTS

Patient Characteristics

Within each cohort (Table 1), parameters at the time of VAD implantation were not significantly different between R and D groups. However, there were significant differences between the cohorts: the Validation cohort had more male patients, greater LV diameters, a longer duration of heart failure prior to VAD implantation, longer time on VAD support, and more patients on rotary VAD support. Table 1 also lists clinical parameters while on VAD support, and follow up LV ejection fraction 6 months after VAD explantation in the Recovered groups of both cohorts. Test and Validation cohorts differed in the duration of support and EF at 6 months post-explantation.

Screening Real Time PCR Array

In the Test cohort, 6 of 7 patients in each group were first analyzed by Real Time PCR Array for miR expression. Of 376 miRs studied in the Test cohort, 141 were expressed at a level of ≤35 Ct in at least 1 VAD core sample; 108 were detected in every VAD core sample at a level of ≤35 Ct. Ten miRs were differentially expressed between the R and D groups ($p<0.05$), (Figure 1 and Table 2). miRs previously reported to play roles in heart failure relevant pathways were not differentially expressed (miRs-1, -21, -133a, -133b and -208) (15–24).

Real Time PCR Confirmation; Test Cohort

The expression of 9 differentially expressed miRs was examined using Taqman PCR in 6 patients in both groups of the Test cohort. Of the 9 miRs studied, miRs-15b, -23a, -26a and -195 were confirmed as significantly altered between R and D groups ($p \leq 0.05$) (Table 2 and Figure 2). The 10^{th} miR (miR-181b) could not be confirmed in the Test cohort due to limitation of RNA availability but was tested in the Validation cohort (see below). In the seventh patient from each group of the Test cohort, Taqman PCR was used to measure the expression of eight miRs (-1, -15b, -21, -23a, -26a, -133a, -133b, -195), and the reference small RNA RNU48.

Real Time PCR Confirmation; Validation Cohort

The 4 miRs confirmed to be differentially expressed in the Test cohort, along with miR-181b, were similarly measured in the Validation cohort. miRs-23a and -195 had significant differential expression (Table 2 and Figure 3). Expression of miRs -15b, -26a, and -181b was not significantly different, although the direction of change was the same as in the Test cohort. Combining the two datasets demonstrated that miR-15b was also differentially expressed ($p<0.03$).

Real Time PCR Confirmation; Comparison with NF Heart Tissue

The 2 miRs (-23a and -195) found differentially expressed in both Test and Validation Cohorts were measured in NF LV tissue. Expression of both miRs-23a and -195 was significantly increased in the D groups compared with NF or R samples, but was similar between NF and R group in both cohorts (Figure 4).

Cardiomyocyte Size Determination

Fluorescent microscopy of wheat germ agglutinin stained cardiomyocytes from the Test cohort demonstrated that the R group had a significantly smaller cardiomyocyte cross-sectional areas when compared with the D group (Figure 5a). Independent examination by light microscopy of H+E stained samples show that the R group had significantly smaller cardiomyocyte diameters when compared with the D group (Figure 5b).

Effects of VAD Support on Selected miRs

To determine whether miRs that were differentially expressed between R and D Core samples were also responsive to mechanical unloading, the expression of select miRs was measured in a separate set of six paired cardiac samples obtained at the time of VAD implantation and subsequent cardiac transplantation. Patient characteristics are listed in Table 3.

VAD support did not alter expression of any of the miRs found to be differentially expressed between the R and D groups (Table 4). To assess whether mechanical unloading alters expression of other miRs, additional miRs previously shown to play a role in heart failure (15–24) were analyzed by real-time PCR. While miRs-208 and -21 showed altered

expression in response to mechanical support (Table 4), miRs-1, -133a, -133b did not. None of these miRs, however, were differentially altered between R and D groups.

DISCUSSION

We report, in two separate patient cohorts, a pattern of myocardial miR expression at the time of VAD placement that is associated with subsequent LV functional recovery. The main findings in this study are: 1) In patients with advanced non-ischemic heart failure requiring VAD support, cardiac expression of miR-23a and -195 prior to LVAD implantation differs between hearts with eventual recovery of ventricular function and those with persistent VAD dependence. 2) This differential expression is associated with remission of heart failure independent of clinical parameters previously associated with recovery, such as LV size, duration of heart failure, and duration of VAD support. 3) Remission of heart failure is associated with a smaller cardiomyocyte size at the time of VAD placement. The experimental protocol and main findings are summarized in Figure 6.

As myocardial transcriptomic biomarkers can associate with outcomes in heart failure (27), we hypothesized that biomarkers (such as miR expression patterns) exist at the time of VAD implantation that are associated with the potential for recovery. We analyzed cardiac miR expression in patients selected to match clinical variables between the Recovered and Dependent groups within each cohort (Test and Validation). However, the two cohorts differed significantly in clinical features typically associated with the development of VAD-independence. These differences between the Test and Validation cohorts make the observation of a conserved relationship between miR expression and the development of VAD-independence particularly noteworthy.

Several reports have identified specific miRs that modulate pathologic processes relevant to heart failure; hypertrophy (16, 18, 19, 23), cell death (28), fibrosis (20, 29), vascular proliferation (30), and arrhythmias (17, 31). Relevant to our observations, it is notable that a) overexpression of miR-195 occurs in pressure overload rodent hearts and that transgenic cardiac overexpression of miR-195 induces heart failure (16), and b) elevated miR-195 expression occurs in rodent and human failing hearts (21, 22, 24). Similarly, miR-23a can mediate cardiomyocyte hypertrophy (32), and is differentially expressed between failing and non-failing hearts (16, 24). Congruent with the reported function of miRs -23a and -195, our data demonstrate that hearts that eventually recover function have smaller cardiomyocyte sizes (6, 33). Intriguingly, while VAD support has been shown to decrease cardiomyocyte size (1), our data demonstrates that VAD support alone does not induce a miR expression profile associated with functional recovery. Thus a differential miR expression in failing hearts that eventually recovered VAD-independence suggests that underlying pathophysiologic processes exist at the time of VAD placement that differ between those hearts that recover function and those that do not. We did not detect differences between the Recovered and Dependent hearts in the expression of other miRs that are differentially expressed between failing and non-failing hearts (i.e. miRs-1, 21, -29, -125b, -130, and 133 (21, 24)). The fact that they did not differ with the ability to recover function suggests that cellular processes associated with a more limited set of miRs (-23a, -195, and possibly -15b) are critical in defining the potential to recover cardiac function after mechanical unloading.

There are several scenarios by which lower miR-23a and-195 expression may be associated with functional recovery. Patients that recovered may have had a) less severe heart failure, b) a different disease etiology that allows greater likelihood of recovery, or c) genetic variants that underlie decreased expression of particular miRs that mediate pathophysiologic processes of heart failure, or the response to mechanical unloading. The observation that Recovered patients had miR-23a and -195 levels that resembled that of non-failing hearts

and a smaller cardiomyocyte size than Dependent hearts raises the important possibility that the Recovered patients had less severe heart failure. Although we cannot exclude this possibility, we think that this is unlikely given that the standard clinical measures of heart failure (Table 1) were identical in Recovered and Dependent groups from both cohorts. Regarding the role of disease etiology, cardiac miR expression profiles differ according to heart failure etiologies (24). However we carefully matched the Recovered and Dependent groups for clinical parameters and etiology so as to prevent bias with diseases (such as myocarditis, peripartum cardiomyopathy) having a higher capacity for recovery (Supplemental Material Table s1). Finally, as the 'Recovery' population is only a small percentage of all VAD-supported patients, it is plausible that these patients possess infrequent miR gene variants whereby decreased miR expression is associated with recovery from heart failure, or beneficial response to mechanical unloading. Indeed, expression quantitative trait loci have been found for ~20% of miRs expressed in fibroblasts (34), and miR SNPs have been associated with congenital heart diseases susceptibility (35) and dilated cardiomyopathy incidence (36).

A major limitation of this observational study is that the list of differentially expressed miRs does not provide insight into the mechanistic processes associated with recovery. One approach to find insight into mechanistic processes would be to use a target prediction programs (i.e. Targetscan or Pictar) to develop a list of possible mRNA targets. However when analyzing miRs-23a and -195, we found little congruency in either the list of potential mRNA targets identified by the two target prediction programs, or the biologic pathways/processes identified through multiple pathway analysis programs (data not shown). The lack of convergent results may reflect either the current limitations of these bioinformatic methods, or the need to identify additional miRs that are differentially expressed between the Recovered and Dependent groups. Indeed, due to the rarity of VAD-supported patients who recover cardiac function, (8–10), the number of patients studied is small, and likely underpowered for detection of all differentially expressed miRs.

In summary, we report cardiac miR expression differences that may identify patients which have the greatest potential for recovery after VAD support. Such patients may have less severe disease, unrecognized disease etiologies that allow for recovery, or genetic variants that relate miR expression to functional recovery. This 'recovery' expression profile is not induced by mechanical unloading, suggesting that the patients' potential for recovery is already established at the time of VAD placement. If confirmed in a larger cohort, such information could help guide clinical decisions regarding utilization of mechanical support devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING SOURCES: Research supported by funds from the Cardiovascular Institute, University of Pittsburgh (Drs Ramani and McTiernan), the Kaufman Center for Heart Failure, Cleveland Clinic Foundation (Dr. Moravec), and the National Institutes of Health (5R01HL086918-0, Dr. Gorcsan; RO1-HL/AG 61483, Dr. Taegtmeyer).

We thank Marc Simon MD, University of Pittsburgh, for providing clinical data; the clinical co-coordinators (Karen Janosko, RN, MSN; Lori DeGore, RN; Kathleen Lockard, RN MBA; Kristin Shoemaker, RN, BSN; Tracie Sabatine, RN, BSN, Pittsburgh, and Sylvia Carranza, BS, Houston), surgical staff, and the mechanical device bioengineers for their assistance.

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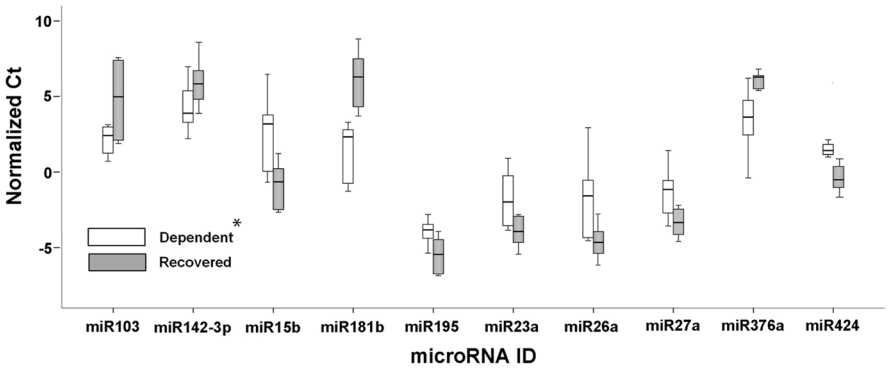

Figure 1. miRs differentially expressed between Recovered and Dependent patients as detected by RT-PCR Array in Test cohort

Threshold detection values after normalization to small RNAs U6 and RNU44 expression. Box and whisker plot, where the range of values are represented by the whiskers, the upper and lower quartile by the box, and the median value by the bar. N=6 per group; *, $p<0.05$.

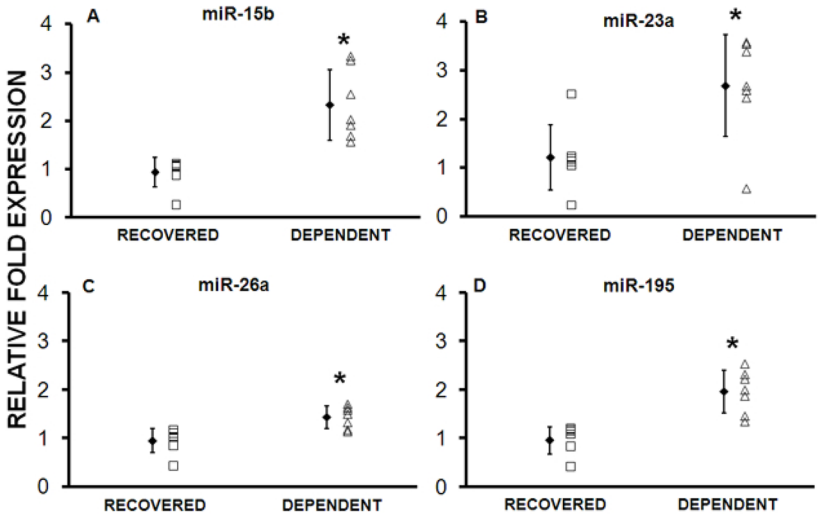

Figure 2. Recovered patients in the Test Cohort have decreased expression of selected miRs

Individual miRs found differentially expressed in the screening array were tested using individual PCR. miRs-15b, -23a, -26a and -195 were confirmed to be differentially expressed. *p<0.04 (n=7 per group)

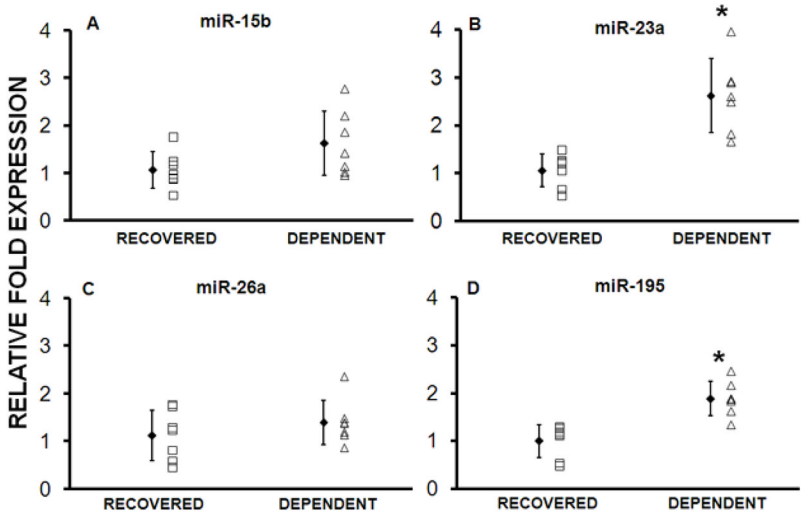

Figure 3. Recovered patients in the Validation Cohort have decreased expression of miRs-23a and -195

miRs confirmed to be differentially expressed in the Test cohort were tested in the Validation cohort (n=7 per group). miRs-23a and -195, but not -15b and 26a were differentially expressed. *p<0.03

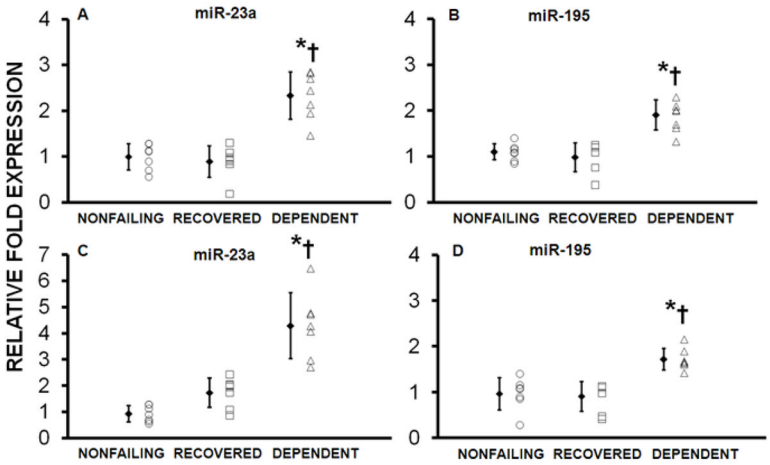

Figure 4. Expression of miRs 23a and 195 was similar in Nonfailing hearts compared with Recovered, but was significantly increased in Dependent vs. Nonfailing

Fig 4A and B represent data from the Test Cohort, while Figs 4C and D are from the Validation cohort. *p<0.04, Dependent vs. Recovered; †p<0.03, Dependent vs. Nonfailing control Hearts.

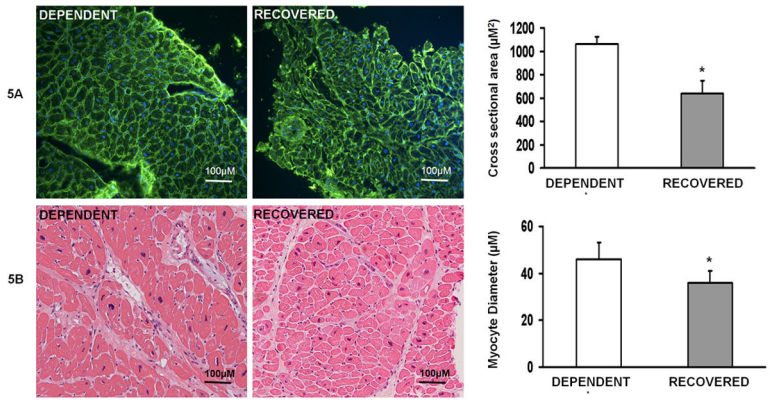

Figure 5.

Figure 5a. Recovered patients have smaller cardiomyocytes at the time of VAD implantation.

Representative images (20X) of wheat germ agglutinin (green; nuclei blue) stained VAD core samples from Test Cohort Recovered and Dependent samples, with calculated cross sectional area in μM^2. (n=3 per group; *p=0.01). **5b**. Representative (H+E) images and measurements of cardiomyocyte diameter (μM) in the Validation Cohort Recovered and Dependent samples (n=7 per group, *p = 0.01).

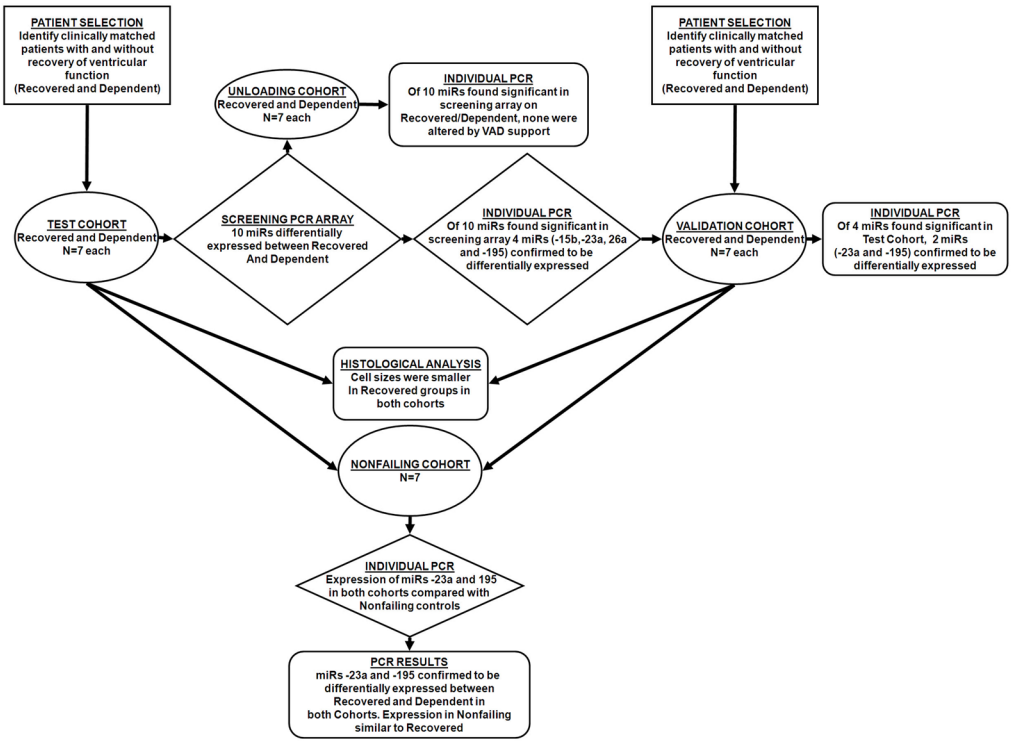

Figure 6.
Summary of experimental protocol and major findings.

J Am Coll Cardiol. Author manuscript; available in PMC 2012 November 22.

Table 1

Characteristics of patients demonstrating clinical similarity between Recovered and Dependent groups prior to VAD implantation, in both Test and Validation cohorts

Duration of congestive heart failure (CHF) was defined as the time in days from original presentation with heart failure until VAD placement. Days on VAD was defined as time to VAD explant (Recovered group) or Transplantation (Dependent group). All patients on pulsatile VAD support were on Thoratec (Pleasanton CA) extracorporeal devices. PCWP and cardiac index measured immediately prior to VAD implantation, while on inotropes.

	Clinical Characteristics At Baseline								
	Test Cohort			Validation Cohort			NonFailing Controls	Test Vs Validation Cohorts	All VAD Vs NonFailing Controls
	Recovered	Dependent	p Value	Recovered	Dependent	p Value		p Value	p Value
Number of Patients	7	7		7	7		7		
Age (years)	40±12	42±15	NS	27±8	33±9	NS	36±10	NS	NS
Percent Ischemic	0	0	NS	0	0	NS	0	NS	NS
Inotropic Support	100%	100%	NS	85%	100%	NS	0	NS	**<0.001**
Intraaortic Balloon Pump	43%	57%	NS	43%	14%	NS	0	NS	**<0.001**
Other Mechanical Support (TandemHeart)	0%	0%	NS	14%	14%	NS	0	NS	**<0.001**
Percent Male	50%	50%	NS	71%	71%	NS	50%	**<0.04**	NS
EF Prior to VAD (%)	18±5	15±5	NS	18±5	17±5	NS	65±10	NS	**<0.001**
LVEDd Prior to VAD (cm)	6.0±0.8	6.2±0.8	NS	7.0±1.0	7.0±1.0	NS	Data not available	**<0.02**	Data not available
PCWP Prior to VAD (mm Hg)*	26±9	27±10	NS	27±5	27±11	NS	Data not available	NS	Data not available
Cardiac Index Prior to VAD (L/min/m2)*	1.9±0.89	2.0±0.73	NS	1.6±0.24	1.8±0.41	NS	Data not available	NS	Data not available
Duration of CHF (days)	62±49	75±58	NS	680±1117	771±802	NS	NA	**<0.001**	NA
Type of VAD (% Rotary)	14%	14%	NS	71%	42%	NS	NA	**<0.01**	NA
Days on VAD	53±31	61±29	NS	433±250	369±167	NS	NA	**<0.001**	NA

	Clinical Characteristics At 6 Month Follow Up Post VAD Explantation							
	Test Cohort			Validation Cohort			NonFailing Controls	Test Vs Validation Cohorts
	Recovered	Dependent	p Value	Recovered	Dependent	p Value		p Value
Number of Patients	5	5		7	7		NA	
Betablocker use while on VAD	67%	83%	NS	100%	85%	NS	NA	NS
ACE Inhibitor use while on VAD	83%	83%	NS	85%	85%	NS	NA	NS

	Clinical Characteristics At 6 Month Follow Up Post VAD Explantation							
	Test Cohort			Validation Cohort			NonFailing Controls	Test Vs Validation Cohorts
	Recovered	Dependent	p Value	Recovered	Dependent	p Value		p Value
EF at 6 months post explantation (%)	55±5	N/A	-	43±11	N/A		NA	**<0.04**

NS: not significant;

NA: Not applicable.

Table 2

List of miRs differentially expressed ($p<0.05$) on screening microarray, and confirmation of expression by individual Taqman Real-Time PCR with pre-amplification.Fold change refers to Recovered (R)/Dependent (D) ratio. A negative number indicates lower expression in the Recovered group compared with Dependent. Of the 10 miRs found differentially expressed by screening array, miRs-15b, -23a, -26a, and -195 showed decreased expression by confirmatory PCR. Of these 4, miRs-23a and -195 were also differentially expressed in the Validation Cohort. In the Test Cohort N= 6 each R and D for the Screening Array and N=7 each R and D for the Confirmatory PCR.

miR Expression								
Screening Microarray (Test Cohort)			**Confirmatory PCR (Test Cohort)**			**Confirmatory PCR (Validation Cohort)**		
miR ID	**Fold Change *R/D***	***p* -Value**	**miR ID**	**Fold Change *R/D***	***p* -Value**	**miR ID**	**Fold Change *R/D***	***p* -Value**
miR-181b	11.38	0.002	miR-181b*	N/A	N/A	miR-181b*	No difference	NS
miR-424	−4.38	0.027	miR-424	No difference	NS	miR-424	N/A	N/A
miR-376a	5.41	0.028	miR-376a	No difference	NS	miR-376a	N/A	N/A
miR-195	−2.91	0.029	**miR-195**	**−1.8**	**0.01**	**miR-195**	**−1.5**	**0.03**
miR-27a	−4.18	0.032	miR-27a	No difference	NS	miR-27a	N/A	NS
miR-23a	−4.52	0.032	**miR-23a**	**−2.2**	**0.03**	**miR-23a**	**−1.8**	**0.01**
miR-103	3.69	0.044	miR-103	No difference	NS	miR-103	N/A	N/A
miR-26a	−14.92	0.044	**miR-26a**	**−1.4**	**0.04**	miR-26a	No difference	NS
miR-142-3p	6.35	0.048	miR-142-3p	No difference	NS	miR-142-3p	N/A	N/A
miR-15b	−12.73	0.048	**miR-15b**	**−1.5**	**0.04**	miR-15b	−1.8	0.09

*miR-181b could not be validated by this method in the Test Cohort, but was not significantly altered in the Validation cohort.

NS, not significant;

N/A: not applicable. These measurements were not performed.

Table 3

Clinical characteristics of patients with paired samples obtained at the time of VAD placement (pre-VAD) and at time of transplant (Post-VAD).

Clinical Characteristics At Baseline	
Number of Patients	6
Age (years)	54±18
Percent Ischemic	0
Inotropic Support	100%
Intraaortic Balloon Pump	50%
Other Mechanical Support (TandemHeart)	0%
Percent Male	50%
EF Prior to VAD (%)	18±5
LVEDd Prior to VAD (cm)	6.6±1.2
PCWP Prior to VAD (mm Hg)	28±9
Cardiac Index Prior to VAD (L/min/m2)	1.8±0.69
Clinical Characteristics While On VAD	
Betablocker use while on VAD	83%
ACE Inhibitor use while on VAD	83%
Duration of CHF (days)	210±108
Duration of VAD support	144±67
Betablocker use while on VAD	67%
ACE Inhibitor use while on VAD	83%

Table 4

miRs differentially expressed in Recovered hearts by screening array (*) were not altered after VAD placement in a separate group with paired samples obtained before VAD implantation and at the time of transplantation.

miR ID	Selection Criteria	Fold change Pre/postVAD	*p* Value
miR-181b	*	-	-
miR-424	*	No difference	NS
miR-376a	*	No difference	NS
miR-195	*	No difference	NS
miR-27a	*	No difference	NS
miR-23a	*	No difference	NS
miR-103	*	No difference	NS
miR-26a	*	No difference	NS
miR-142-3p	*	No difference	NS
miR-15b	*	No difference	NS
miR-1†	Literature	No difference	NS
miR-21†	Literature	**1.6**	**0.04**
miR-133a†	Literature	No difference	NS
miR-133b†	Literature	No difference	NS
miR-208†	Literature	**1.9**	**0.02**

† miRs reported as playing key roles in heart failure (15–24) were also tested.

NS, not significant.