Supplemental Material

SUPPLEMENTAL METHODS

Quantitative RT-PCR

Briefly, for each miRNA, a specific hairpin primer was used for reverse transcription to a DNA product amenable to amplification in quantitative PCR (qPCR) utilizing FAM-3 fluorescence detection on an Applied Biosystems 7900 HT Fast Real-Time PCR system (Foster City, CA). Primers were ordered from Ambion, Inc (Taqman miRNA assays). For each plasma sample, qPCR was performed for the candidate miRNA as well as for the spike-in control with three technical replicates. The number of cycles to reach threshold for a given sample relative to the spike-in control is related to the relative concentration of the candidate miRNA ($\Box C_t^{miRNA}$-$\Box C_t^{cel\text{-}miR\ 39}$). The difference of $\Box C_t$ values between any two samples is subsequently exponentiated to generate the relative fold-change between the samples (see **Statistical analysis**, below). The miR PCR assay was robust with excellent correlation between measurements obtained on different days (Fig. S1). In particular, we tested our PCR assay on the same isolated RNA six months apart, with excellent reproducibility between the two (R=0.88 for the correlation, Supplemental Fig. S6). This held true for samples at either extremes of miR-30d concentration, which vary in this graph by over 5 PCR cycles (a 64-fold variation in miR-30d concentrations), with excellent agreement. Finally, we calibrated our assay using a serial dilution of miR-30d over an 8-order of magnitude change and our assay maintained an excellent linear response (r=0.997 for the correlation, data not shown).

MiR-30d concentrations in canine cardiac samples and cultured CMs were determined relative to U6 snRNA as a housekeeping gene consistent with multiple other studies[1],[2]. To ensure that U6 functioned as an invariant housekeeping gene in this canine model, we also demonstrated that U6 levels were the most invariant compared to the levels of other housekeeping genes used in canine studies[3] (Fig. S5).

Reverse transcription of non-miRNA RNA species was performed using the multiscribe reverse transcription kit (Clontech Inc). Briefly, 1 µg of extracted RNA was used in an RT reaction using a 10X random priming mix to generate cDNA. qPCR was performed using the iQ SYBR Green Supermix (Bio-Rad Inc., Hercules CA) on a CFX384 real-time PCR system. Concentrations of all mRNAs were determined relative to GAPDH. Specific primer are below:
GAPDH forward 5'- AAC TCC CTC AAG ATT GTC AGC AA-3', reverse 5'- GGC TAA GCA GTT GGT GGT GC-3'; ANP forward 5'- AAG AAC CTG CTA GAC CAC CTG G-3', reverse 5'- GCT TCC TCA GTC TGC TCA CTC A-3'; α-MHC forward 5'- CCG AGT CCC AGG TCA ACA AG-3', reverse 5'- TCA TCG TGC ATT TTC TGC TTG G-3'; β-MHC forward 5'- GAG AGA TGG CTG CAT TTG GG-3', reverse 5'- GTC ACC GTC TTG CCA TTC TC-3'; MAP4K4 forward 5'- GCC TTA TGG GGA GTG AAT TT-3', reverse 5'- ACC CCT GCT TCT TCT CAA

CT-3'; lims1 forward 5'- CAA CTG CGG GAA GGA GCT AA-3', reverse 5'- GGC ATT CAC TAC TCG CCC TT-3'; JAK1 forward 5'- AGG CAA GAG TGC ATA GAG CG-3', reverse 5'- GGG TCT TGT CCT TGA GTG GG-3'; PGC1 forward 5'- GTA GGC CCA GGT ATG ACA GC-3', reverse 5'- CTC TCT GCG GTA TTC GTC CC-3'; CamKIV forward 5'- GAC TTC AAT CAA AGG CGG CG-3', reverse 5'- GAG GAT CCC GTT TAG AGC CG-3'; PPPIR14C forward 5'- GCT CCT CAA GGG AGG ATT CG-3', reverse 5'- TTT CTT CTT CCT CGC AGC CA-3'; MAP3K13 forward 5'- ACA GAC GTG GCA GAG TAA GC-3', reverse 5'- CTG CAG AGG CAA TGT CCA GA-3'; PODXL forward 5'- ATC CTG CCA TAA AGC CCC AC-3', reverse 5'- AGG GCT CCC CTT ACA AAA GC-3'.

Canine Dyssynchronous heart failure model and sample preparation

In the canine model of dyssynchronous heart failure (HF_{DYS}) and CRT, adult mongrel dogs (Bruce Rotz Kennels, Shippensberg, PA) underwent left bundle branch ablation followed by either six weeks of rapid atrial pacing (200 beats per min) to induce HF_{DYS}, or three weeks of rapid atrial pacing followed by three weeks of biventricular pacing at the same rate (CRT). At terminal study, dogs were anaesthetized and samples collected as previously described 50-100 mg aliquots from the septal and lateral walls of each dog's left ventricle were used for experiments. All procedures were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions and followed USDA guidelines[4].

At the time of experiment, tissue samples were thawed on ice, weighed, and then placed in either a guanidinium hydrochloride-containing solution (Cell disruption buffer, Ambion Inc.) for RNA extraction, or 1x cell lysis buffer (Cell Signaling) containing 1x phosphatase inhibitor (Thermo Scientific), 1x protease inhibitor (Calbiochem) and 1 mM phenylmethanesulfonyl fluoride (Cell Signaling), for Western blot analysis. Tissue was mechanically disrupted for 5 minutes by a 5mm steel bead (Qiagen, Inc., Valencia, CA) using the Qiagen TissueLyzer LT disruptor at 5Hz. Following disruption, RNA was extracted using the mirVANA PARIS kit according to manufacturer's protocol. Protein samples were lysed for 1 h, sonicated, and centrifuged at 10,000 g for 5 min. An aliquot of the resulting supernatant, containing 50 µg protein, was incubated with 1x SDS-Sample buffer (Boston BioProducts) at 65°C for 5 min.

Myocyte stretch, preparation and exosome isolation

In brief, CMs were cultured on a silicone membrane and subjected to either 20% stretch for 6 h or 10% stretch for 24 h at 3 Hz. The culture media was then collected and extracellular vesicles (EVs) were isolated using multiple centrifugation steps as previously described The isolated fraction was incubated with 20 µm/L acridine orange for 90 min at room temperature protected from light. Exosomes and microvesicles were pelleted by ultracentrifugation at 100,000 g for 2 h and washed with PBS to remove excess acridine orange. This was repeated twice. The final pellet was re-suspended in 1 mL PBS and sorted

based on size (<200 nm) and fluorescence using the Propel Lab's Nano-View forward scatter detector integrated onto a Beckman Coulter MoFlo XDP cell sorter. Sorted samples were re-pelleted as described above and RNA was extracted using the miRVANA PARIS RNA isolation kit (Ambion). Cell lysates were collected as described above.

Neonatal rat myocyte isolation, preparation, immunohistochemistry

CMs were cultured in DMEM (Gibco) containing 10% horse serum (Gibco), 5% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) for 24 hrs, and subsequently with serum-free, antibiotic-free DMEM. Forty-eight hours after plating, cells were transfected using Lipofectamine 2000 (Invitrogen Inc.) with miR-30d (90 pmol/mL; Ambion Inc.) or a negative control mimic (Ambion Inc.). Cells were harvested by mechanical scraping 48 h after transfection and RNA was isolated using the miRVANA PARIS RNA isolation kit (Ambion). Alternatively, cells were lysed in protein lysis buffer and prepared for Western blot analysis as described above.

For hypertrophy imaging, 48 h after transfection, cells were fixed in 4% Paraformaldehyde (PFA) for 5 min, followed by permeabilization by 0.5% Triton-X 100 in PBS for 5 min. Cells were then washed twice in PBS and blocked for 1 h at RT in 5% BSA in PBS. Cells were incubated with sarcomeric α-actinin antibody (1:500, 5% BSA in PBS, Sigma) for 1 h, washed three times for 5 min each in PBS and incubated in the dark with anti-mouse 488 secondary antibody (1:500, 5% BSA in PBS, Invitrogen). Finally, cells were washed three times for 5 min each in PBS and mounted with hard-set DAPI mounting media. Cells were imaged the following day using a Zeiss upright confocal microscope and cell size was analyzed using Image-J software.

Western Blotting

Samples were separated using SDS-PAGE on a Criterion TGX 4-20% gel (Bio Rad), and transferred to nitrocellulose membrane (Bio Rad). Membranes were probed overnight with specific primary antibodies. All antibodies were purchased from Cell Signaling with the exception of the MAP4K4 antibody, which was purchased from LifeSpan BioSciences Inc. Secondary antibody against rabbit IgG conjugated with horseradish peroxidase was used for detection (Dako, Denmark). Membranes were developed using super signal west pico chemiluminescent substrate and super signal west femto maximum sensitivity substrate (Thermo scientific), and imaged on a Chemidock MP imaging system (Bio-Rad). Where multiple proteins of similar size were analysed, membranes were stripped by incubation with stripping buffer (0.76 g Tris, 2 g SDS, 700 µl β-mercaptoethanol in 100 ml) for 40 min, and re-probed with the primary antibody of interest.

Supplemental References

1. Huang ZP, Chen J, Seok HY, Zhang Z, Kataoka M, Hu X, Wang DZ. Microrna-22 regulates cardiac hypertrophy and remodeling in response to stress. *Circ Res*. 2013;112:1234-1243
2. Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T, Urbich C, Boon RA, Dimmeler S. Atheroprotective communication between endothelial cells and smooth muscle cells through mirnas. *Nat Cell Biol*. 2012;14:249-256
3. Matkovich SJ, Wang W, Tu Y, Eschenbacher WH, Dorn LE, Condorelli G, Diwan A, Nerbonne JM, Dorn GW, 2nd. Microrna-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. *Circ Res*. 2010;106:166-175
4. Chakir K, Daya SK, Tunin RS, Helm RH, Byrne MJ, Dimaano VL, Lardo AC, Abraham TP, Tomaselli GF, Kass DA. Reversal of global apoptosis and regional stress kinase activation by cardiac resynchronization. *Circulation*. 2008;117:1369-1377

SUPPLEMENTAL TABLES

Supplementary Table S1: Demographics and clinical characteristics of derivation cohort.

	Non-Responders (n=6)	Responders (n=6)
Age	52 +/-9	55+/-9
Male Gender (%)	100	100
Percent DCM	100	100
LBBB (%)	100	100
QRS duration	130	160
NYHA Baseline	3	3
LV lead position (lateral or posterolateral)	100	100
Baseline EF	24+/-7	29+/-2
6 month LVEF	24+/-6	48+/-4
LVEF Change at 6 months	1+/-7	19+/-4

Abbreviations: DCM: dilated cardiomyopathy; LBBB: left bundle branch block; RV: right ventricle; NYHA: New York Heart Association; LV: left ventricle; LVEF: left ventricular ejection fraction.

Supplementary Table S2: Comparison of multiple regression models combining clinical covariates and microRNAs

A

Covariate	Model 1			Model 2			Model 3		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
miR-30d	2.34	1.10-5.00	0.03	2.52	1.07-5.94	0.03	2.52	1.07-5.94	0.03
Pre-implant QRS	1.00	0.98-1.03	0.78	1.02	0.99-1.05	0.32	1.02	0.99-1.05	0.32
LVEF	1.02	0.90-1.16	0.74	0.99	0.87-1.13	0.92	0.99	0.87-1.13	0.91
Prior revascularization	-	-	-	0.15	0.03-0.79	0.03	0.15	0.03-0.81	0.03
Serum creatinine	-	-	-	-	-	-	0.94	0.18-5.02	0.94

B

Covariate	Model 1			Model 2			Model 3		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
miR-142-5p	1.98	1.15-3.40	0.01	2.28	1.22-4.28	0.01	2.47	1.26-4.85	0.009
Pre-implant QRS	1.01	0.98-1.04	0.46	1.03	0.99-1.06	0.14	1.03	0.99-1.07	0.12
LVEF	1.00	0.89-1.12	0.94	0.95	0.84-1.09	0.48	0.97	0.85-1.10	0.59
Prior revascularization	-	-	-	0.09	0.01-0.66	0.02	0.08	0.35-0.60	0.02
Serum creatinine	-	-	-	-	-	-	2.11	0.35-12.6	0.41

C

Covariate	Model 1			Model 2			Model 3		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
miR-766	1.81	1.01-3.25	0.05	1.97	1.00-3.90	0.05	1.99	1.00-3.96	0.05
Pre-implant QRS	1.00	0.98-1.03	0.84	1.01	0.99-1.04	0.34	1.02	0.99-1.05	0.33
LVEF	1.01	0.89-1.13	0.93	0.98	0.86-1.11	0.73	0.98	0.86-1.11	0.75
Prior revascularization	-	-	-	0.14	0.03-0.75	0.02	0.13	0.02-0.74	0.02
Serum creatinine	-	-	-	-	-	-	1.16	0.24-5.58	0.86

Supplementary Table S3: Age and gender adjustments for miR-30d as a predictor of CRT response.

Effect	Point Estimate	95% Wald Confidence Limits		P value
Age	1.006	0.949	1.066	0.85
female	9.063	0.885	92.802	0.06
log miR-30d	3.101	1.325	7.257	0.009

Supplemental Figure Legends

Supplemental Fig. S1. Reproducibility of qRT-PCR assay for plasma microRNAs. Levels of miR-30d were measured in human plasma samples six months apart, with excellent correlation as indicated in the figure.

Supplemental Fig. S2. Correlation of plasma miR-30d with miR-142-5p levels. R=0.73 for the correlation and is highly significant with $p<0.001$.

Supplemental Fig. S3. Probability of 10% improvement in LVEF against log (miR-30d concentration) for the combined cohorts (n-61, p=0.02, logistic regression)**.** Inset shows baseline differences in miR-30d in the second cohort of 21 patients alone (p=0.08).

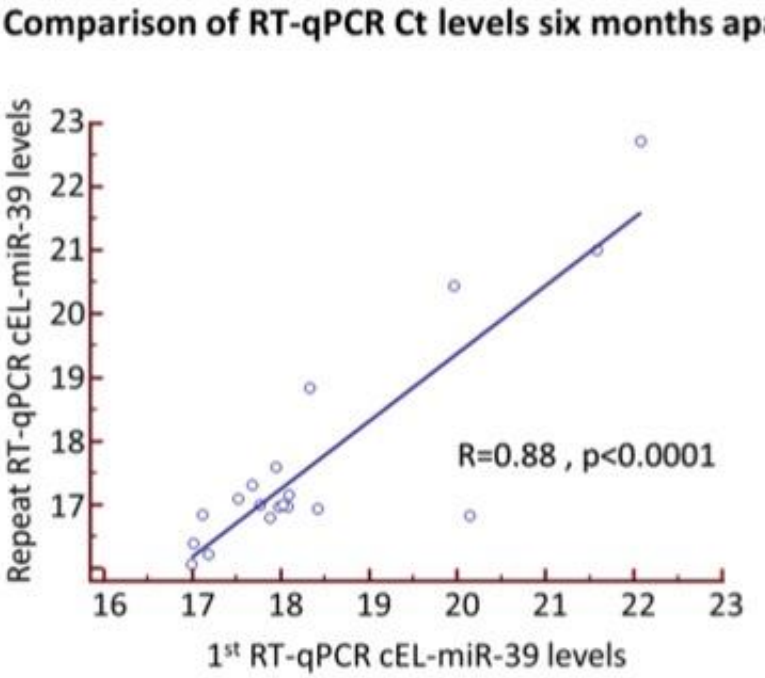
Supplemental Fig. S4. mRNA levels of cardiac markers α- and β- MHC are absent whereas connective tissue growth factor levels are enriched in fibroblast cultures compared to cardiomyocyte cultures.

Supplemental Fig. S5 Levels of common housekeeping genes in the canine model of HF_{DYS} and CRT. Levels of the previously published RPL 32 and RSP 19 both compare favorably with U6 snRNA as a housekeeping gene with levels that do not vary in HF_{DYS} and CRT dogs.

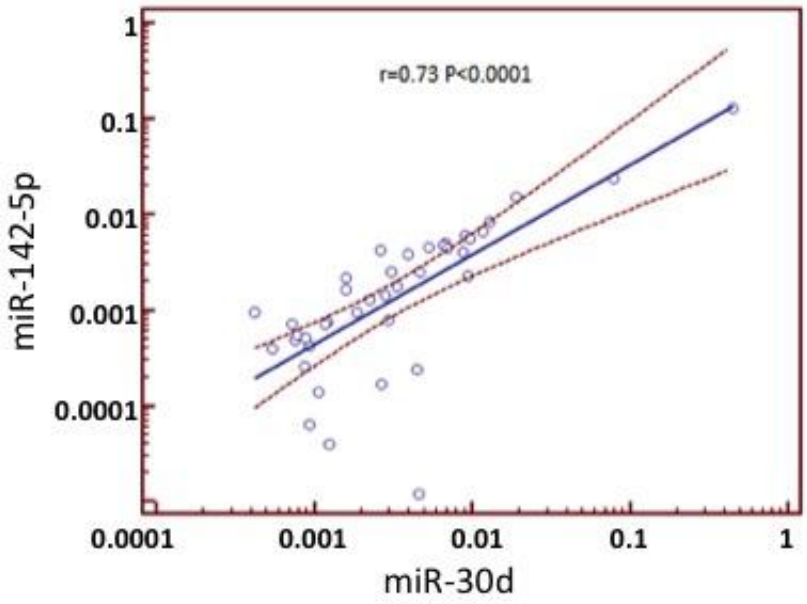
Supplemental Fig. S6 miR 142-5p regulation in canine DHF and CRT model. Levels of 142-5p are highest in the lateral wall of DHF dogs, as assayed by qRT-PCR, and are normalized by CRT.

Supplemental Fig. S7. Transfection of miR-30d mimic into cultured ventricular cadiomyocytes. miR-30d transfection results in a dose dependent increase in microRNA delivery to the cells.

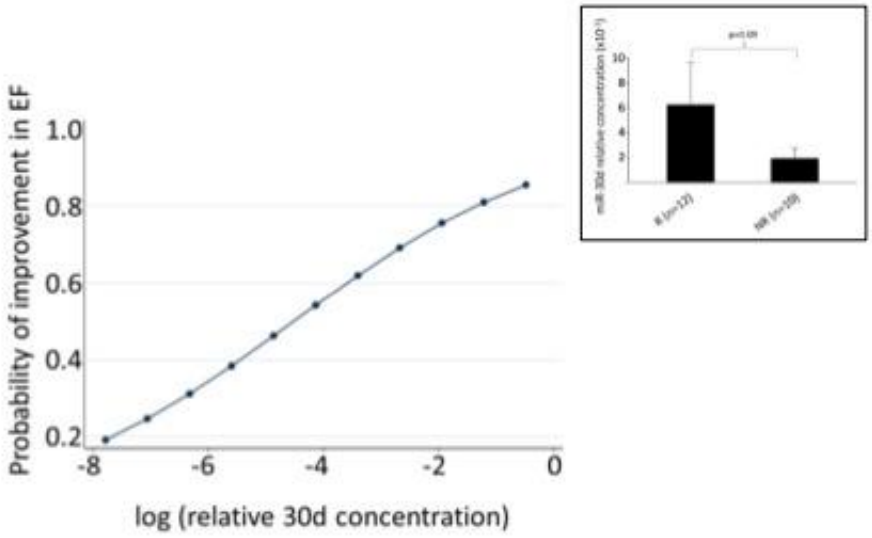
Supplemental Figure S1: Reproducibility of qRT-PCR over time



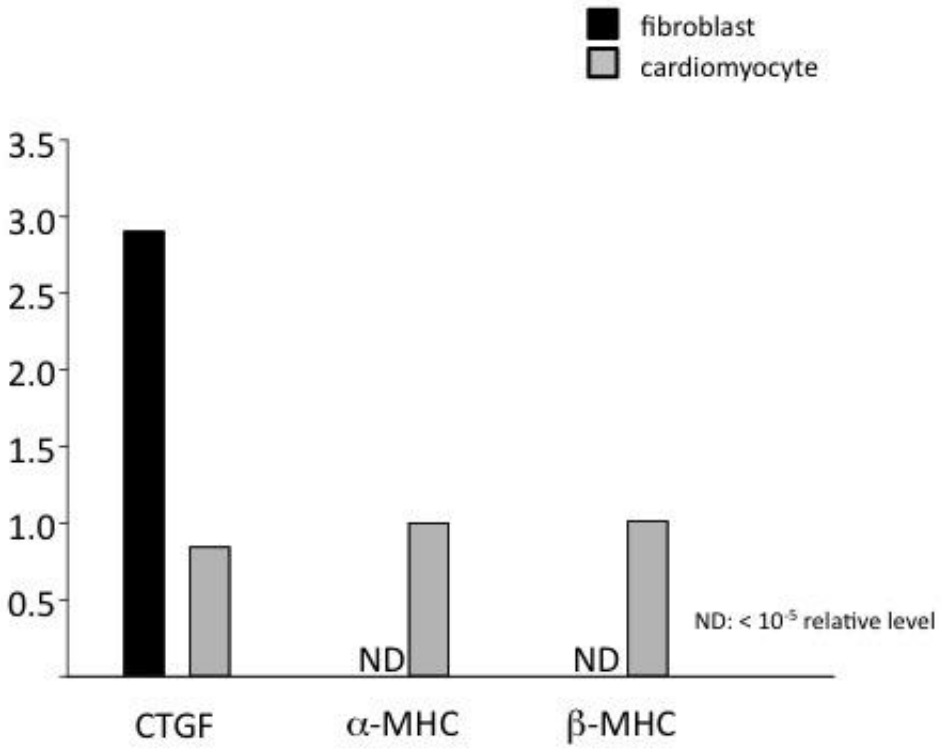
Supplemental Figure S2: co-regulation of miR-142-5p and miR-30d



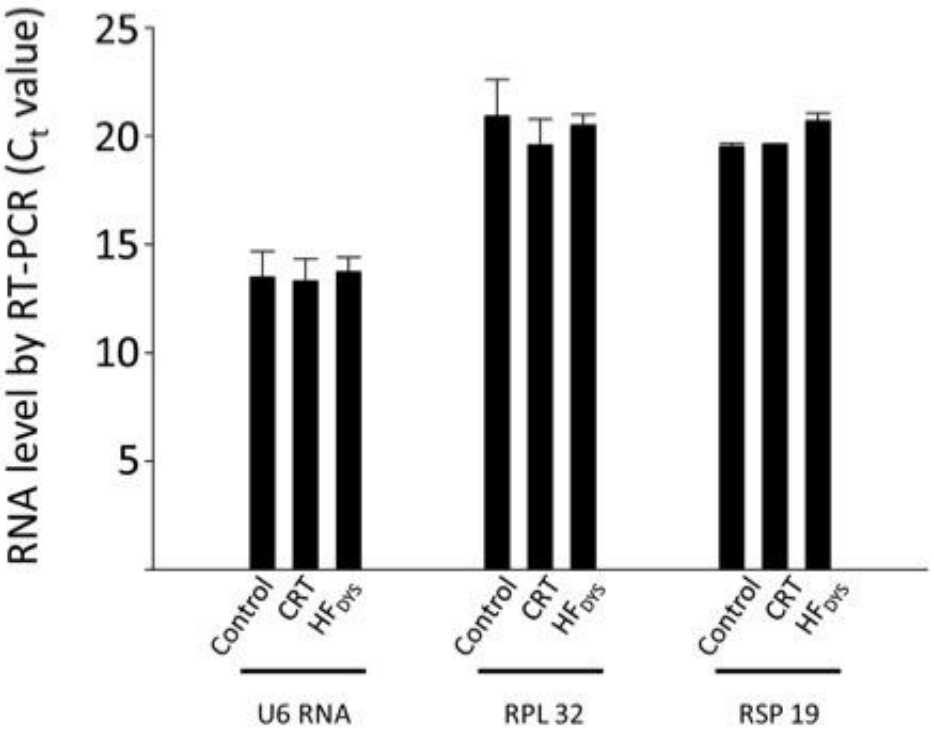
Supplemental Figure S3: miR-30d is correlated with improvement in LVEF: combined cohort



Supplemental Figure S4: Purity of cell cultures

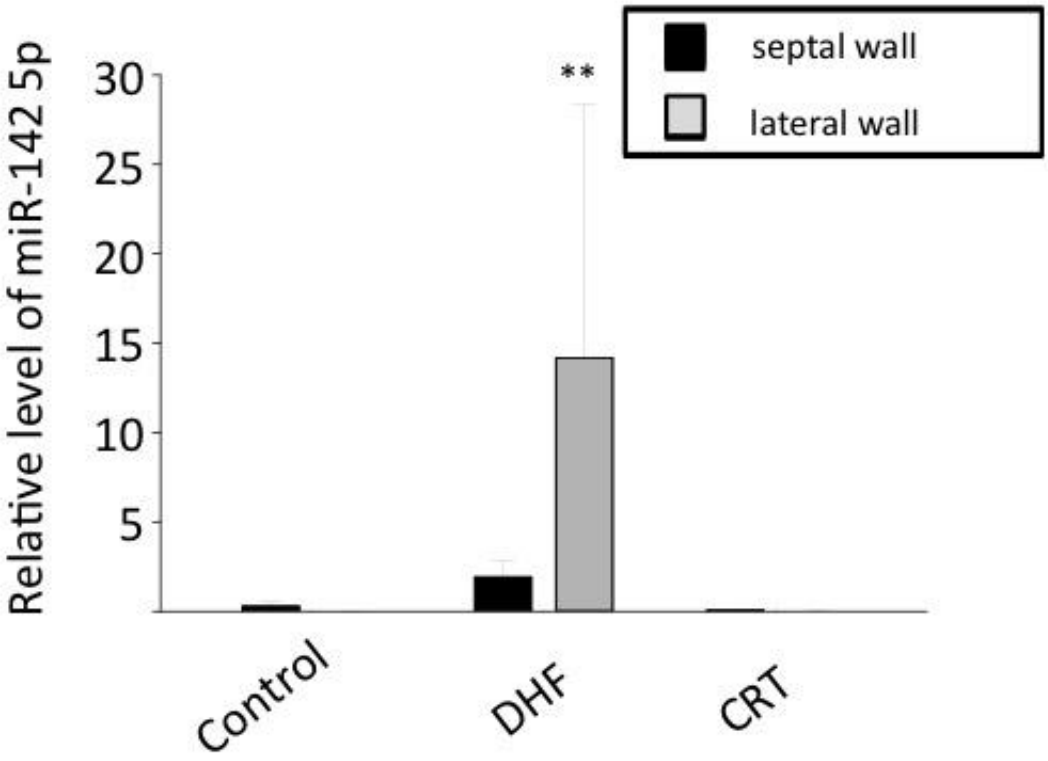


Supplemental Figure S5: U6 as a control for microRNA levels



p= NS for all pairwise comparisons

Supplemental Figure S6: Regulation of miR-142 in the canine DHF model



$p<0.05$ for comparison of lateral and septal walls

Supplemental Figure S7: miR-30d transfection