In vivo, cardiac-specific knockdown of target protein, Malic Enzyme-1, in rat via adenoviral delivery of DNA for non-native miRNA

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ABSTRACT

This study examines the feasibility of using the adenoviral delivery of DNA for a non-native microRNA to suppress expression of a target protein (cytosolic $NADP^+$-dependent malic-enzyme 1, ME1) in whole heart *in vivo*, via an isolated-heart coronary perfusion approach. Complementary DNA constructs for ME1 microRNA were inserted into adenoviral vectors. Viral gene transfer to neonatal rat cardiomyocytes yielded 65% suppression of ME1 protein. This viral package was delivered to rat hearts *in vivo* (Adv.miR_ME1, 10^{13} vp/ml PBS) via coronary perfusion, using a cardiac-specific isolation technique. ME1 mRNA was reduced by 73% at 2-6 days post-surgery in heart receiving the Adv.miR_ME1. Importantly, ME1 protein was reduced by 66% ($p<0.0002$) at 5-6 days relative to sham-operated control hearts. Non-target protein expression for GAPDH, calsequestrin, and mitochondrial malic enzyme, ME3, were all unchanged. The non-target isoform, ME2, was unchanged at 2-5 days and reduced at day 6. This new approach demonstrates for the first time significant and acute silencing of target RNA translation and protein content in whole heart, *in vivo*, via non-native microRNA expression.

INTRODUCTION

The recent decade has witnessed prolific advances in the methodological approaches for the overexpression of target proteins via viral-based cDNA delivery schemes to heart, *in vivo* [1,2]. With the recent developments in RNA interference technologies (RNAi), it is highly anticipated that these viral-based delivery approaches can be extended for the suppression of target protein expression in the heart [3]. The approach has been overwhelmingly successful in isolated cell culture models [4-6], including several studies for the suppression of malic enzyme as targeted in this study [7-9]. However, there are only a few reports for successful suppression of a target protein in heart, *in vivo*, based on RNAi schemes [10-14]. In those few studies, suppression was achieved via viral-based delivery of DNA code for the expression of short hairpin RNA (shRNA) [10,14], or direct injection of "naked" shRNA or short interfering RNA (siRNA) [11-13]. In this study, we will examine the potential of inhibiting protein expression, in vivo, based on an artificial microRNA scheme (miRNA), which is suggested to provide a higher level of RNA interference and less toxic side effects compared to shRNA [15,16].

Two types of small ribonucleic acid molecules are central to production of siRNA and RNA interference; the short hairpin RNA (shRNA) and microRNA (miRNA) [17-19]. The shRNA serve ideally as single-target silencing tools, whereas naturally occurring miRNA typically regulate multiple targets, and multiple miRNAs are able to target the same mRNA [20]. The miRNA structure is similar to shRNA, they include a 3' and 5' flanking region, and the miRNA utilize the common pathways of shRNA synthesis used to generate siRNA [17-20].

In this study, the feasibility of using viral-based delivery of DNA code for non-native miRNA to limit RNA translation in the heart was assessed. The target sequence of the miRNA construct was not designed to mimic an indigenous form of miRNA. Instead, the artificial miRNA construct was designed to include an inhibitory sequence targeted to a single protein, cytosolic $NADP^+$ dependent malic-enzyme 1 (ME1). The DNA code for the siRNA sequence was annealed to a non-native micro-RNA template (miR_ME1), and packaged in an adenoviral vector (Adv_miR_ME1). Three separate miRNA sequences were examined, and the efficacy of inhibition was evaluated in isolated neonatal rat cardiomyocytes (see Supplemental Data). One package was selected and delivered to adult rat heart *in vivo*, via the open-chest isolated-heart retrograde perfusion technique as previously described by us [21-24]. Hearts were excised at 2-6 days post surgery, and the acute responses of protein and mRNA expression/suppression were assessed for target (ME1) and non-target proteins (ME2, ME3, calsequestrin, and GAPDH). Expression was compared among three separate control groups: untreated excised hearts, miR-scrambled treatment, and PBS

treatment. Malic enzyme-1 was the target protein of interest based off of on-going studies in our laboratory which examine the regulatory role of ME1 on glucose oxidation and redox state in healthy and diseased heart models [25,26].

MATERIALS AND METHODS

Construction, Production, and Purification of Adenoviral Vectors

Three separate miR-RNA sequences were designed using BLOCK-iT RNAi Designer (Invitrogen, Carlsbad, California) to target rat cytoplasmic NADP(+) dependent malic enzyme mRNA (NM_012600.2). Each sequence was synthesized and annealed into Invitrogen's miR-RNA template containing an upstream / downstream adapter and hairpin loop. Figure 1 depicts the three sequences investigated. The final miR-RNA chosen for *in vivo* studies (#1244) included the following 22 nt siRNA sequence: GTATAGCACTCCTCTGCAGAAC. Each miR-RNA sequence was cloned into pcDNA6.2-GW/miR expression vector (Invitrogen) with a CMV promoter and verified by Invitrogen. Then each of the miR-RNA sequences were recombined into a pAd/CMV/V5-DEST Vector (Invitrogen). The adenoviral vector was transfected into HEK 293A cells using Lipofectamine (Invitrogen). The adenovirus was amplified in HEK 293 cells, harvested, and purified by cesium chloride density gradient centrifugation as previously described [21,22].

Vector Evaluation in Primary Cardiomyocytes.

Malic enzyme-1 knockdown via the three vectors was first verified in isolated cardiomyocytes. Neonatal cardiomyocytes were isolated from 1-day-old Sprague-Dawley rats, as previously described [27]. In brief, cells were plated at a density of 240 cells/mm^2 in MEM (Sigma-Aldrich, St. Louis, Missouri,) containing Hanks' salts, 5% calf serum, vitamin B12, 1% Penicillin/Streptomycin/Amphotericin B, and 0.1mol/L bromodeoxyuridine at 37°C, in the presence of 5% CO_2. After 24h, the medium was replaced with similar serum-free medium with 1% transferrin and 1% insulin. Cells were infected, with adenoviral vector (35 pfu/cell) containing one of the three miR-RNA sequences. The control group was infected with an adenovirus containing DNA code for a scrambled miR-RNA sequence. Cells were harvested in media 24h after viral exposure for western blot analysis of ME1 protein expression. A high MOI (35 pfu/cell) was selected in order to maximize the RNA interference process to within the timeframe of a viable cell culture preparation (ie., < 1 wk).

Vector Delivery to Rat Heart, In Vivo

Adenovirus or virus-free PBS solution was delivered to the heart *in vivo* by coronary perfusion as described in our previous reports [21,22]. In brief, 3 month old male Sprague-Dawley rats (350g) were anesthetized, intubated, and placed on an ice pad to cool the core body temperature to 30^{o}C. The chest was opened at the second intercostal space. All vessels to/from the heart were cross-clamped simultaneously, and the heart was retrograde perfused in vivo for 7 min with calcium-free Tyrode solution through catheters position in the aortic root (delivery) and right ventricle (efflux). At the time of adenoviral injection, 0.2 ml of Adv.miR-ME1 or Adv.miR-scrambled (10^{13} vpu/ml in PBS) was first delivered through the catheter position in the aortic root. This allowed the adenovirus to circulate down the coronaries. Next, the efflux catheter positioned in the right ventricle was removed, and an additional 0.5 ml/kg of adenovirus (~0.2 ml) was delivered to the aortic root at 300 ± 100 mmHg of peak pressure. After 90 s, catheters were repositioned in the right and left ventricles, and unsequestered virus was flushed from the heart with Krebs buffer containing calcium (1.5 mM). The heart rate recovered, the cross-clamp was removed, the chest was closed, and the rats recovered. We previously reported that the percentage of cardiomyocytes transduced *in vivo* by this technique is 58% [21,22]. After 2-6 days, hearts were excised for the protein and mRNA analysis by western blots and PCR.

Western blots

Neonatal Cardiomyocytes. Cells were harvested in lysis buffer containing 20mM tris (hydroxymethyl) aminomethane, 100mM sodium chloride, 1mM EDTA, 0.5% Triton-X, and protease inhibitor (Sigma P8340). Following a 15 min digestion period on ice, samples were centrifuged (10 min, 3,000 rpm) and the supernatant was collected for BCA protein assays to determine protein concentration. Samples of equal protein mass were loaded onto a 4-12% Bis-Tris NuPAGE gel and transferred overnight onto a polyvinylidene difluoride membrane. Membranes were probed with a monoclonal anti-ME1 (4μg/mL, Abcam, Cambridge, UK) primary antibody, and calsequestrin was used to normalize protein loading (1:2000, Thermo Fisher Scientific, Waltham, Massachusetts). Membranes were visualized after enhanced chemiluminescence treatment (Thermo Fisher Scientific) and densitometric analysis of band intensity was used to determine changes in protein expression.

Adult Cardiac Tissue. Two to six days after Adv.miR-ME1 (n=6), PBS (n=5), or Adv.scrambled (n=5) delivery to coronaries in vivo, rat hearts were excised and retrograde perfused with a bolus of PBS to flush blood from the coronaries and ventricles [21,22]. Hearts were then freeze-clamped for Western blot analysis. Because the exogenous gene is heterogeneously expressed throughout the heart [11], whole ventricles were pulverized via a pestle and mortar, and homogenized in RIPA buffer [0.5 mM Tris-HCl, 1.5 mM NaCl, 2.5% deoxycholic acid, 10 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 10% glycerol, 1% Triton X-100, and protease inhibitor]. Samples remained on ice for 15 min before being centrifuged (10 min, 3,000 rpm) and the supernatant was collected for BCA assay to determine protein concentration. Samples of equal protein mass were loaded in gels following the same general procedures described above. Primary antibodies for ME1 (3.33μg/mL, Abcam), ME2 (5μg/mL, Abcam), ME3 (6.7μg/mL, Abcam), GAPDH (1:10,000 Thermo Fisher Scientific), and calsequestrin (1:2000, Thermo Fisher Scientific) were used to assess the level of protein expression.

Measurement of RNA reduction by PCR.

Total mRNAs were extracted from adult cardiac tissue using Trizol reagent (Life Technologies). The RNAs were DNase-treated and reverse transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems, Life Technologies) on a Veriti Gradient PCR Thermocycler. A quantitative RT-PCR was carried out on an ABI ViiA7 using TaqMan primers and probes targeting ME1, ME2, and ME3. GAPDH served as the internal control. Primers for ME1, ME2, ME3, and GAPDH were designed by Roche (Hoffmann, Louisiana). A second primer and probe for ME1 (IDT, Coralville, Iowa) was designed to sit at the proposed cut site of the miR-RNA and had the following sequence: the forward primer 5'-TGCTTTGAGTAATCCGACCAG-3', reverse primer: 5'-ACTGGATCAAAAGGACTGCC-3', and probe: 5'-AGTGCTATAAAGTGACCAAGGGCCG-3'.

Statistical Analysis

Data is presented as mean ± standard error. Data set comparisons were performed with Student's unpaired, two-tailed parametric t-test, and confirmed by a non-parametric test (Mann-Whitney Rank Sum Test, or Kruskal Wallis Test). Differences in mean values were considered statistically significant at a probability level of less than 5% ($p < 0.05$). Reported p values are for the Student's t-test, unless otherwise stated.

Animal Use Approval

All protocols and procedures involving vertebrate animals were approved by the Animal Care Policies and Procedures Committee at the University of Illinois in Chicago (Institutional Animal Care and Use Committee accredited), and animals were maintained in accordance with the *Guide for the Care and Use of laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

RESULTS

Efficacy of miR-ME1 inhibition in primary cardiomyocytes.

The protein content for the target protein, cytosolic malic enzyme-1, was assessed by western blot analysis 24 hrs after infection of isolated neonatal rat cardiomyocytes with adenoviral vectors containing the DNA templates encoded for the endogenous expression of a non-native miR-ME1 or miR-Scrambled. Three separate coding sequences for miR-ME1 and one scrambled control sequence were evaluated. All three target sequences resulted in a marked reduction of ME1 expression in the isolated cardiomyocytes relative to the scrambled miR-RNA control group. The results are presented in the Supplemental Data section. Sequence #1244 showed the greatest knockdown, at 35% of the control value. This viral package was selected for large-scale amplification and subsequent *in vivo* experiments.

Efficacy of miR-ME1 inhibition, *in vivo*.

The open-chest isolated-heart perfusion technique, previously described by us [21-24], was used here to deliver adenoviral vectors ($1x10^{13}$ vp/ml PBS) containing the DNA code for the endogenous expression of a non-native miR_ME1 (n=9). Sham operated controls received similar treatment with virus-free PBS (n=6). The efficacy of this strategy to inhibited ME1 expression was assessed by western blot and PCR analysis at 2-3 days and 5-6 days post-treatment. The results are illustrated in Figure 2. Consistent with our earlier reports for this open-chest adenoviral delivery procedure [21-24], survival was >80% post-surgery. Body weights were similar between groups at the time of heart excision (PBS: 348 ± 12 g; Adv.miR_ME1 348 ±9, $p>0.05$), as were heart weights (PBS: 2.36 ± 0.20 g; Adv.miR_ME1 2.21 ± 0.08, $p>0.05$). At 2-3 days post-treatment, the mRNA level for ME1 was reduced by 76% in the Adv.miR_ME1 group relative to PBS treated controls. ME1 protein content was not significantly

different between groups at 2-3 days post-surgery, though the trend was lower in the Adv.miR_ME1 group (reduced 15%, $p>0.05$). Importantly, at 5-6 days post-treatment, ME1 mRNA was reduced by 70% ($p=0.07$) and protein content was significantly reduced by 66% ($p<0.0002$ for a Student's parametric t-test; $p=0.02$ for the Mann-Whitney Rank Sum non-parametric test) in the Adv.miR-ME1 group relative to the PBS treated controls. This is the first demonstration that adenoviral delivery of DNA, encoding a sequence for the endogenous expression of a non-native miRNA, results in suppression of the targeted protein in the heart, *in vivo*.

ME1 expression for three control group strategies.

There is no ideal control group for gene transfer studies. Whether a scrambled, PBS, GFP, luciferase, or empty virus strategy is included as a control group, expression of some endogenous proteins are altered relative to untreated hearts [28,29]. Here, we assessed the content of ME1 in hearts from rats undergoing the open-chest isolated-heart perfusion procedure and receiving either a bolus of virus-free PBS (n=3) or the Adv.miR-scrambled vector (n=3). The results were compared to the level of ME1 protein measured from untreated hearts excised directly from a third group of rats (n=3). Western blots and densitometry graphs normalized to the endogenous calsequestrin content are illustrated in Figure 3. The ratio for ME1 content, relative to calsequestrin, was not significantly different between groups. However, the data suggests a non-statistical trend between groups. Compared to the untreated group, the PBS group shows an increased trend in ME1 expression, and the scrambled group shows a downward trend in ME1 expression. As described in the Discussion, this variability between the three control groups was expected based on earlier gene transfer reports from our group and others [14,23,29]. Importantly, whether we compared the Adv.miR-ME1 treated animals to the untreated excised group, the PBS group, or the Adv.miR-scrambled group, ME1 protein expression was reduced significantly by 5-6 days via this Adv.miRNA interference strategy.

Expression of non-target proteins.

We assessed whether the strategy to inhibit ME1 affected the expression of the non-target proteins, calsequestrin and GAPDH, and the non-target mitochondrial isoforms of malic enzyme, ME2 and ME3. When selecting the code for ME1 inhibition, we performed a BLAST search to confirm that the selected 19-23 nt sequence was specific to ME1 and not these off-target proteins. The western blots and densitometry graphs are illustrated in Figure 4 and 5 for the

content of the off-targets proteins from hearts excised 5-6 days following Adv.miR_ME1 (n=6) or PBS treatment (n=3). Calsequestrin and GAPDH protein expression were not different between groups (Figure 4). The non-target isoform of malic enzyme, ME3, was also not different between groups (Figure 5). ME2 expression was not statistically different between the Adv.miR-ME1 and PBS control at 5-6 days post-treatment, though the trend for ME2 expression was lower in the Adv.miR-ME1 group. Close examination of the westerns reveal that the expression at 5 days was unchanged, whereas at 6 days the protein content was reduced.

DISCUSSION

As with shRNA, delivery of non-native miRNA constructs have been used to suppress target protein expression in cell cultures [4-6]. In whole animal models [30,31], pioneering studies in the cancer field have described impressive therapeutic effects with these non-native miRNAs, yet the approach had not been tested in heart, *in vivo*. In this study, we examined whether adenoviral delivery for gene transfer to whole heart *in vivo*, could inhibit the expression of the target protein, malic enzyme-1, using a strategy for induced expression of a non-native miRNA. We selected a 19-23 nt sequence for ME1, which initially resulted in a 66% reduction in ME1 expression in isolated neonatal cardiac myocytes. Subsequently, adenoviral delivery of the DNA sequence to the heart, *in vivo*, resulted in a 70% reduction in mRNA levels for ME1 2-6 days post-surgery. In support of the proposed strategy, the target protein, ME1, was acutely reduced by 65% at 5-6 day post-surgery relative to all control groups examined (ie., PBS, miR-scrambled, and untreated hearts).

Translational interference by non-native miRNA.

Figure 6 illustrates the molecular pathway involved in the down regulation of protein expression by mRNA interference. Whereas shRNA and siRNA target RISC cleavage of the mRNA, indigenously produced miRNA can either target mRNA cleavage or mRNA translational inhibition [20,32]. Cleavage requires the miRNA sequence to fully complement the 19-23nt target of the mRNA [17,20]. An incomplete match between the two complementary sequences yield inhibition of translation rather than cleavage [17,20]. Our target sequence was fully complementary, and we anticipated mRNA degradation. However, preliminary PCR data for ME1 mRNA levels revealed no change in content at 2-6 days following Adv.miR-ME1 delivery, *in vivo* (data not shown). This initial PCR analysis was performed with a probe for malic enzyme-1 mRNA that was not designed to be specific to the

target site of miRNA recognition. We designed a second probe, which was specific to the targeted sequence. For this second probe, PCR analysis revealed a ~73% reduction in mRNA content for ME1 at both 2-3 days and 5-6 days post-surgery. These results suggest that the mRNA was either cleaved or blocked at the target site, whereas the full strand of mRNA (as detected by the first probe) was not degraded within this acute timeframe.

In this study, we found the mRNA level was knocked down ~70% at both 2-3 days and 5-6 days post gene transfer *in vivo*, whereas the protein was not knocked down until the later endpoint. This delayed response is indicative of the natural turnover rate of the endogenous ME1 pool. The delay also indicates that mRNA measures cannot be used as indirect markers of protein knockdown in siRNA based studies. Protein expression does not necessarily follow changes in mRNA content because of differences between the rates of protein turnover and mRNA translation. Similarly, changes in protein expression cannot be defined based on *in vitro* measurements of activity, because enzymatic flux is not solely linked to protein content [33,34].

Viral-based delivery strategies to the heart, *in vivo.*

Our results for miRNA support proof-of-concept results (ie., RNAi in vivo) for both direct injection and intracoronary delivery of the shRNA and siRNA constructs to the heart, *in vivo* [10-14]. Morgan and colleagues directly injected naked siRNA to silence the expression of the Na+/H+ exchanger in the left ventricle of mouse myocardium [13]. Naked siRNA is stable *in vivo* for 72 hrs [12], and after 48-72 hrs the mRNA levels were reduced by 80% in the left ventricle, protein expression was down 30%, and the effects were confined to the heart. Similarly, Huang and Wu directly injected non-viral short hairpin RNA plasmid intramyocardially to inhibit prolyl hydroxylase-2 (PDH2) expression in vivo [11], and reported peak inhibition by day 14, which returned back to baseline levels by week 4. Gupta et al directly injected lentiviral vectors expressing myotropinin shRNA in the myocardium of mice, which resulted in 50% down regulation of the target gene expression in mice at 6 weeks [10]. While these direct injection approaches to RNA interference provided inhibition of target gene in heart without collateral infection of the peripheral tissue (ie., liver), silencing was restricted to the site of injection in the heart, or just proximal. Global gene transfer to the heart requires methods of intracoronary or intravenous viral vector delivery.

Poller and colleagues demonstrated the feasibility of inhibiting RNA translation in heart via viral-based delivery of code for short hairpin RNA by intracoronary and intravenous vector delivery [28]. In 2009 they

examined silencing mRNA for phospholamban in whole heart of mice and rat by both an adenoviral vector (AdV_shRNA) delivered to the coronaries (acute response), and a tail vein injection of adeno-associated viral vector (AAV_shRNA) (chronic response). Phospholamban protein expression was significantly reduced by both viral-based approaches and cardiac function was enhanced. Thus, our results for the inhibition of RNA translation via the microRNA approach support these earlier results for protein knockdown via the short hairpin RNA in heart, *in vivo*.

One important distinction between our work, and this earlier global work by Poller, relates to the infection of peripheral tissue. In the Pollar study, tail vein injection of rAAV9_GFP in mice revealed equal gene transfer to both heart and liver [28]. Indeed, others have shown that this systemic injection of AAV vectors generally transduce to the liver more effectively than to cardiac and skeletal muscles [35]. Non-target gene transfer to the liver can be reduced by using a cardiac specific AAV serotype (such as AAV9) or a cardiac specific promoter [36]. However, higher doses of these vectors are required to achieve the same level of RNA inhibition in heart, and problems with shRNA-related toxicity, endogenous microRNA oversaturation, and liver failure are still observed [35,37]. Similarly, the conventional aortic cross-clamp method used to delivery adenoviral vectors in this Poller study was also not cardiac specific. Nevertheless, the problem with specificity is avoided by delivering the viral vectors via the fully isolated heart *in vivo* approach as described in this study. We previously demonstrated that the isolated-heart perfusion technique, as utilized here, is cardiac specific [21,22]. That is, we are able to flush any unsequestered virus from the heart prior to releasing the isolating cross-clamp, thereby eliminating viral accumulation or gene transfer to peripheral tissue.

Off target protein expression.

While the expression of ME1 was not statistically different in heart samples from three control groups examined (ie., excised heart, PBS, and scrambled miRNA), there was some variance between groups. Western blots indicated the trend for higher ME1 expression in the PBS group relative to the content in hearts excised from non-treated animals, and lower in the group receiving scrambled miRNA. The expression of the other off target proteins, calsequestrin and GAPDH, were unchanged. This observation was not entirely unexpected. That is, in earlier adenoviral gene transfer studies performed with isolated cardiomyocytes and whole intact heart, the expression of the off-target protein, SERCA2a, was down regulated following gene transfer for both Adv.GFP (ie., GFP overexpression) and Adv.shGFP (GFP inhibition) compared to non-treated controls [28]. Whereas the expression of GAPDH, the Na^+-

Ca^{+2} exchanger, and calsequestrin were unaffected in these studies. The mechanism for this effect is unclear. Nevertheless, ME1 expression was inhibited via the miRNA treatment strategy performed under the current study relative to all three control groups.

We also examined whether the expression of two off-target isoforms of malic enzyme were affected by the ME1 miRNA treatment. Malic enzyme-1 is localized to the cytosol, and is a $NADP^+$ dependent enzyme involved in anaplerosis in the diseased heart [25,26]. Two other isoforms of malic enzyme are localized to the mitochondria; the NAD^+ dependent malic-enzyme-2 (ME2), and the $NADP^+$ malic-enzyme-3 (ME3). While ME3 was unaffected by the treatment, western blot analysis for ME2 reveal a trend toward reduction in expression at day 6. The mechanism for this observation, in light of this new and rapidly advancing technology, is unclear, especially since ME2 is a NAD^+ not $NADP^+$ dependent isoform. Others have also observed changes in non-target isoform protein expression [18,19], and it is speculated as to whether these observations are linked to a compensatory mechanism to account for a change in the expression of the target protein, or a response via some unknown endogenous miRNA pathway.

Experimental implications.

An important consideration implicates a known immune/inflammatory response to the adenovirus at 7 days following the treatment [38, 39]. The inflammatory response is not an issue if the target protein is knocked down and experimental measures are completed prior to day 7. In the case of the target enzyme examined in this study, a robust knockdown was observed at day 5-6. We could predict a moderate level of knockdown at day 4. If experimental studies are performed during the inflammatory event, a control group receiving a similar adenoviral dose of empty adenovirus or Adv.miR-scrambled should be included to account for inflammatory effects. Alternatively, immunosuppressors or immunodeficient rats could be considered in order to avoid the anti-adenoviral vector immune responses [39]. Furthermore, the AAV does not induce an inflammatory response. While AAV is ideal for long-term therapeutic treatment strategies, it is not ideal for mechanistic based studies where an acute knockdown of a single protein is desired. The adenoviral gene delivery approach provides the advantage of an acute knockdown with minimal confounding compensatory events characteristic of AAV and transgenic models.

Conclusion

While the siRNA approach has evolved as a powerful tool to down-regulate the expression of a target protein for elucidating the molecular basis of cardiovascular disease in studies of isolated cardiomyocytes, there have been far fewer attempts in whole animal models. This is a very important consideration for studies of metabolic enzymes (such as ME1), because isolated myocytes in culture, whether adult or neonatal, fail to represent the metabolic demand and mitochondrial activity of cells functioning in an intact myocardium. Therefore, extending the RNA interference approach to the intact beating heart is an important advancement and technique for molecular based studies performed under physiologically relevant conditions.

LIST OF ABBREVIATIONS

AAV	=	Adenoassociated virus
Adv	=	Adenovirus
BCA	=	Bicinchoninic acid - protein assay
cDNA	=	Complementary DNA
CMV	=	Cytomegalovirus
GAPDH	=	Glyceraldehyde-phosphate dehydrogenase
GFP	=	Green fluorescence protein
HEK	=	Human embryonic kidney cells
ME1	=	Malic Enzyme 1 (cytosolic isoform, NADP+ dependent)
ME2	=	Malic Enzyme 2 (mitochondrial isoform, NAD+ dependent)
ME3	=	Malic Enzyme 3 (mitochondrial isoform, NADP+ dependent)
MEM	=	Minimum essential medium
miR	=	Micro RNA
miRNA	=	Micro RNA
PBS	=	Phosphate buffered saline
PDH2	=	Prolyl hydroxylase-2

RIPA = Radio-immunoprecipitation assay buffer

RISC = RNA-induced silencing complex

RNAi = RNA interference

RPM = revolutions per minute

RT-PCR = Real time - polymerase chain reaction

SERCA = Sarcoendoplasmic reticulum calcium ATPase transporter

shRNA = Short hairpin RNA

vpu/ml = Viral particles units per milliliter

CONFLICTS OF INTEREST

There are no competing financial interests in relation to this work.

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Contributions. E. Douglas Lewandowski: designed research/study, interpreted results, edited manuscript. J. Michael O'Donnell: designed research/study, analyzed / interpreted results, wrote manuscript. Asha Kalichira: performed research/study, collected data, analyzed data, prepared figures. Jian Bi: performed research/study.

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FIGURES

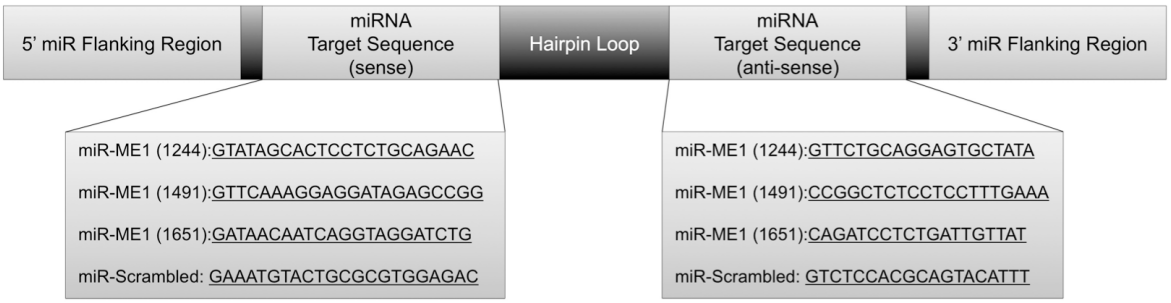

Figure 1: The general structure of the miRNA sequences placed into the adenoviral vector include a 5' and 3' flanking region, hairpin loop, and the target sequence. Three separate target sequences were designed and examined in this study to target ME1 knockdown. Preliminary studies in isolated neonatal cardiomyocyte indicated sequence #1244 resulted in the greatest knockdown of ME1, and was selected for *in vivo* studies. A fourth sequence was designed as a scrambled control.

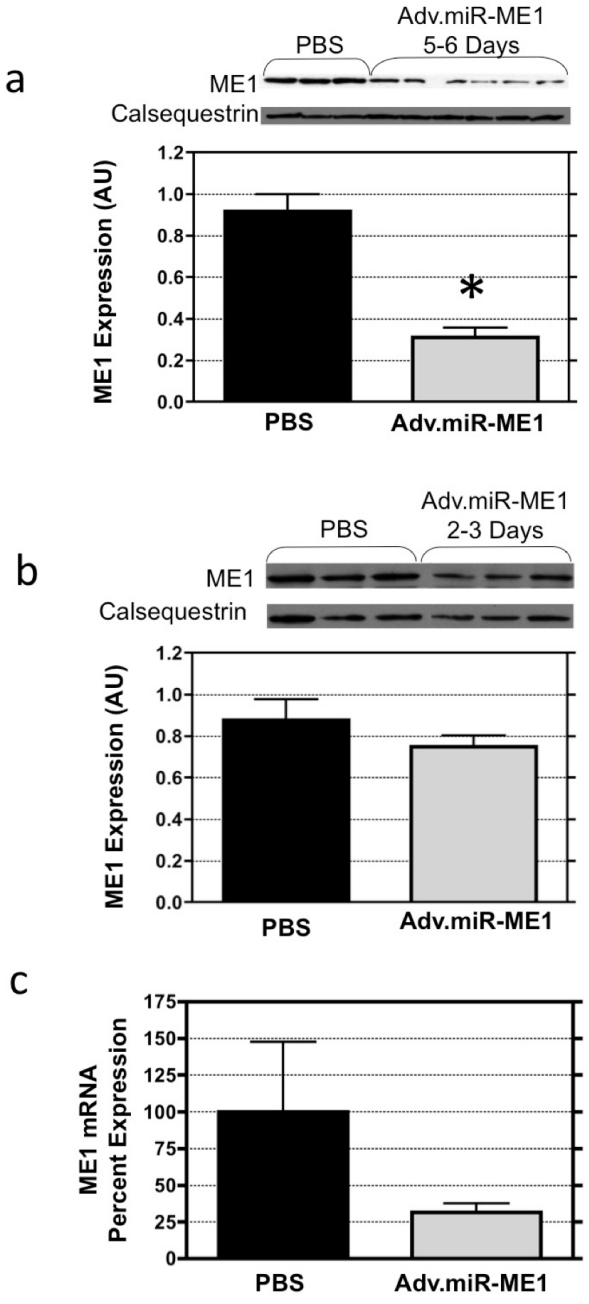

Figure 2: Protein and mRNA expression of ME1 in heart (a) 5-6 days and (b) 2-3 days following delivery of Adv.miR-ME1 to rat heart via isolated-heart retrograde perfusion of the coronaries, in vivo. ME1 content was not significantly reduced at 2-3 days post-treatment (n=3) compared to PBS controls (n=3). However, at 5-6 days post-treatment, ME1 expression was reduced by 65% (n=6, $p<0.0002$). (c) mRNA content was reduced at both 2-3 days and 5-6 days post Adv.miR-ME1 treatment.

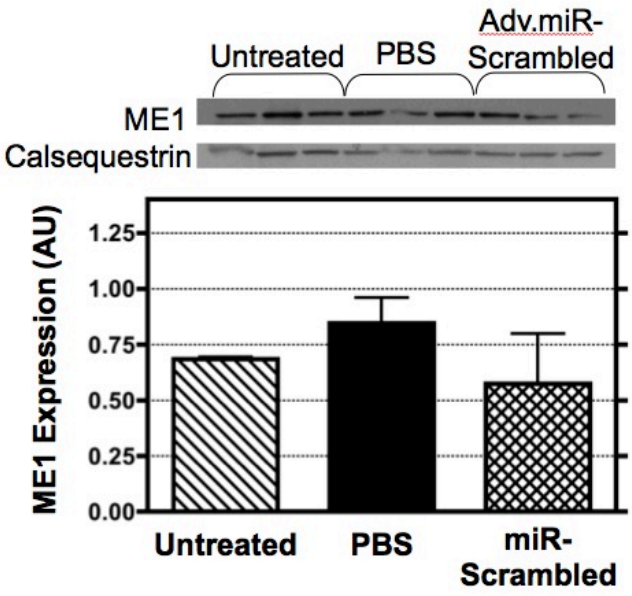

Figure 3: ME1 expression was measured by Western blot analysis for three different control conditions: untreated rat hearts, sham operated control rat hearts retrograde perfused with a bolus of virus-free PBS, and sham operated control rat hearts retrograde perfused with a bolus of PBS containing Adv.miR-scrambled. At 5-6 days post-treatment, the expression of ME1 was not significantly different between control groups.

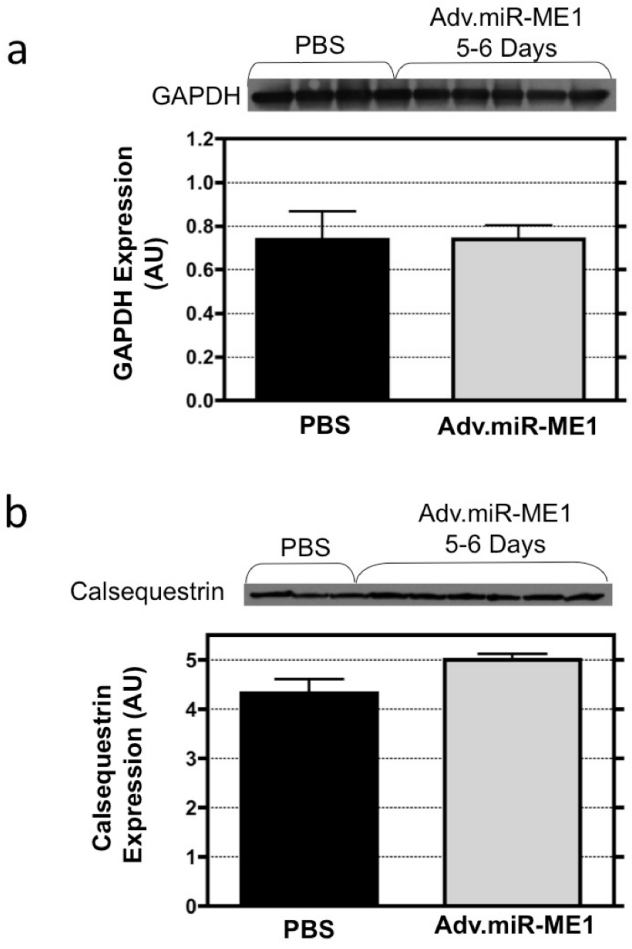

Figure 4: The expression of the non-target proteins, calsequestrin and GAPDH, were assessed by western blot analysis from heart receiving Adv.miR-ME1 or PBS treatment. At 5-6 day post treatment, calsequestrin and GAPDH protein expression were not different between groups. (The calsequestrin blot shown in panel b is the loading control shown in Figure 2a).

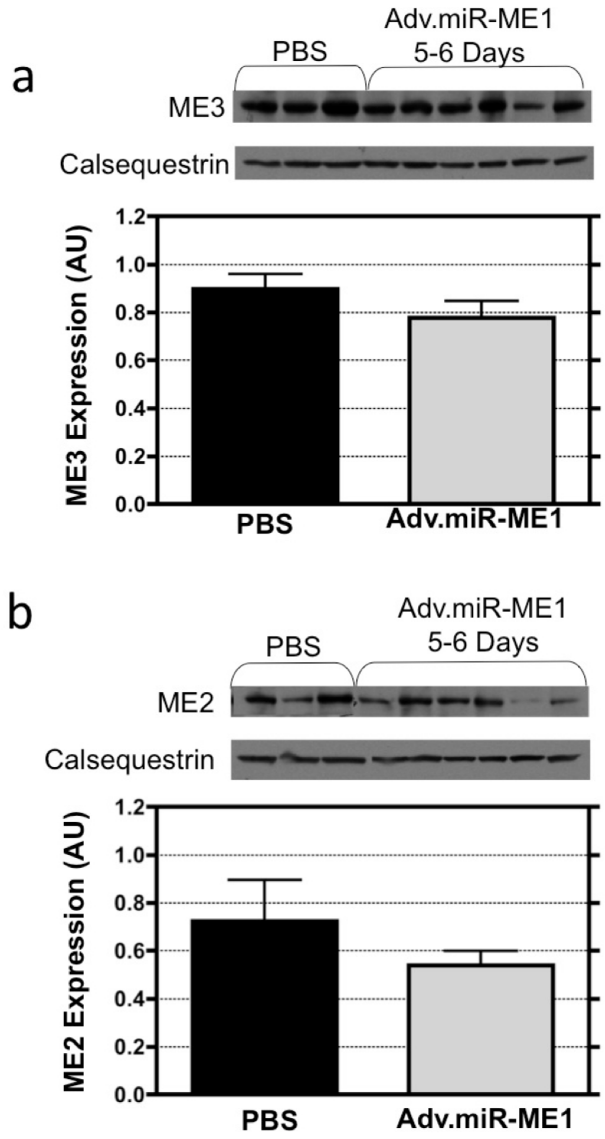

Figure 5: The expression of the non-target mitochondrial isoforms of malic enzyme (a) ME3, a mitochondrial $NADP^+$ protein, and (b) ME2, also a mitochondrial NAD^+ protein, were assessed in heart tissue by western blot analysis 5-6 days following Adv.miR-ME1 or PBS treatment. The expression of ME3 was not different between the two treatment groups. ME2 expression was also not statistically different between groups, though the expression was reduced at day 6.

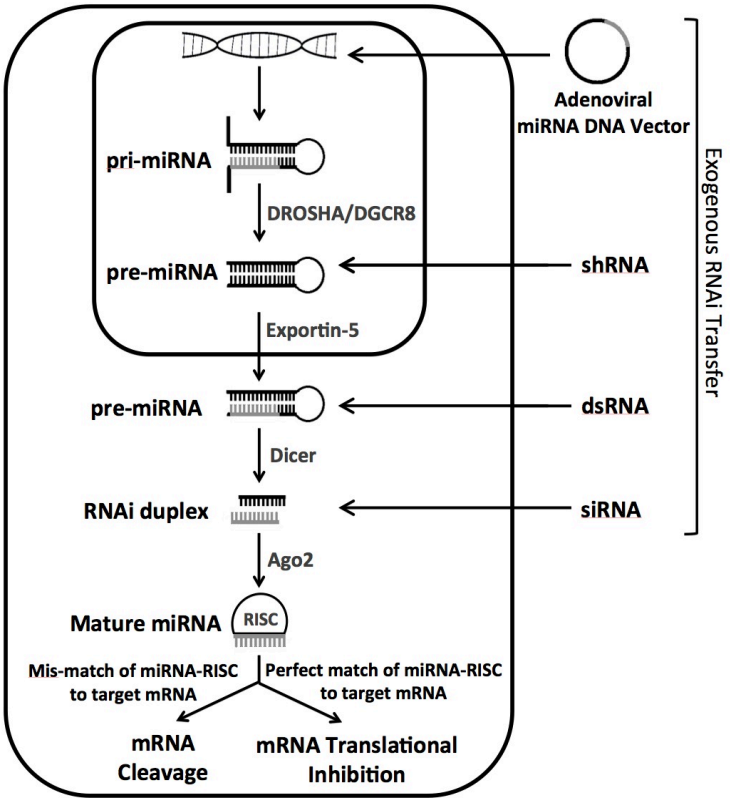

Figure 6: While a variety of approaches can be used to introduce RNAi into the cell, all are processed similarly and incorporate into the RNAi protein machinery. microRNAs start the furthest upstream in the pathway; DNA is taken up into the nucleus and transcribed into the primary miRNA (pri-miRNA) strand which contains the target strand, hairpin loop, and a 5' and 3' flanking region. The flanking regions are cleaved off by DROSHA/DGCR8, into the precursor miRNA (pre-miRNA). This structure resembles shRNAs introduced into the nucleus. The pre-miRNA is then exported out of the nucleus by Exportin-5 into the cytoplasm where it is cleaved again into its characteristic 19-23nt structure. Argonaute-2 facilitates unwinding of the duplex, and the functional guide strand is incorporated into the RISC complex. Perfect complementary of the guide strand to the mRNA target leads to mRNA cleavage by RISC. Partial complementary leads to suppression of mRNA translation.

SUPPLEMENTAL DATA

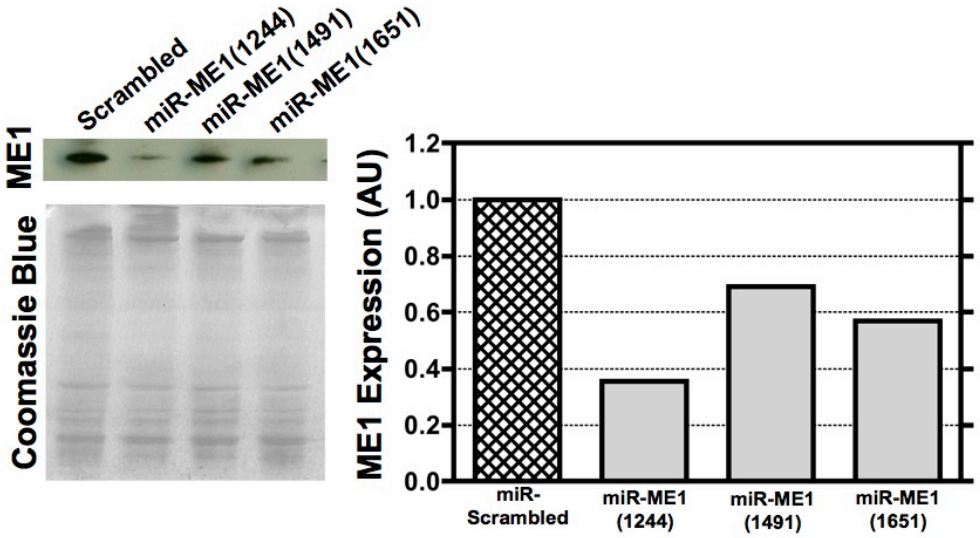

Figure 1: Preliminary assessment of ME1 protein expression was made in rat neonatal cardiomyocytes following delivery of three different Adv.miR-ME1 sequences or the Adv.miR-scrambled control. The Adv.miR-ME1 resulting in the greatest knockdown of protein expression (#1244) in the isolated cardiomyocytes was chosen for subsequent *in vivo* Adv.miR-ME1 experiments. (Gels were stained with Coomassie blue dye to verify equal protein loading).