Exosomally derived Y RNA fragment alleviates hypertrophic cardiomyopathy in transgenic mice

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Cardiosphere-derived cell exosomes (CDCexo) and YFI, a CDCexo-derived non-coding RNA, elicit therapeutic bioactivity in models of myocardial infarction and hypertensive hypertrophy. Here we tested the hypothesis that YFI, a 56-nucleotide Y RNA fragment, could alleviate cardiomyocyte hypertrophy, inflammation, and fibrosis associated with hypertrophic cardiomyopathy (HCM) in transgenic mice harboring a clinically relevant mutation in cardiac troponin I (cTnI^{Gly146}). By quantitative PCR, YFI was detectable in bone marrow, spleen, liver, and heart 30 min after intravenous (i.v.) infusion. For efficacy studies, mice were randomly allocated to receive i.v. YFI or vehicle, monitored for ambulatory and cardiac function, and sacrificed at 4 weeks. YFI (but not vehicle) improved ambulation and reduced cardiac hypertrophy and fibrosis. In parallel, peripheral mobilization of neutrophils and proinflammatory monocytes was decreased, and fewer macrophages infiltrated the heart. RNA-sequencing of macrophages revealed that YFI confers substantive and broad changes in gene expression, modulating pathways associated with immunological disease and inflammatory responses. Together, these data demonstrate that YFI can reverse hypertrophic and fibrotic signaling pathways associated with HCM, while improving function, raising the prospect that YFI may be a viable novel therapeutic candidate for HCM.

INTRODUCTION

Cardiosphere-derived cells (CDCs) have been tested clinically and preclinically in a variety of cardiovascular conditions.¹ The prevailing concept of CDC therapy posits that transplanted cells produce and secrete extracellular vesicles (e.g., exosomes; CDCexo) with therapeutic payloads to target cells in a paracrine manner.²–⁴ Although CDCs and CDCexo are themselves promising therapeutic candidates, they suffer from a number of practical and conceptual limitations. CDCs vary in potency, require regular recourse to source tissue, and are fragile living entities that need careful storage and handling.¹ While CDCexo are more durable,¹ parental CDCs are necessary for exosome production and isolation. To circumvent these limitations, we have leveraged RNA-sequencing (RNA-seq) technology to mine CDCexo cargo and identify distinct molecular entities with therapeutic activity.⁵,⁶ The most plentiful RNA species in CDCexo is a fragment of the human Y4 gene (designated EV-YF1, or more simply YF1).³ This exosomally abundant non-coding RNA (ncRNA) has bioactivity in vitro and in vivo:⁵ macrophages exposed to YFI increased the production of interleukin-10 (IL-10) and protected cardiomyocytes against oxidative stress. Additionally, YFI reduced cardiomyocyte apoptosis and decreased infarct size in a rat model of myocardial infarction⁷ and attenuated cardiac hypertrophy and fibrosis in mice with angiotensin II (Ang II)-induced hypertension.⁵ These findings motivated us to determine whether YFI might exert therapeutic benefits in a clinically relevant mouse model of hypertrophic cardiomyopathy (HCM).

HCM is the most common genetic illness affecting the heart and generally involves mutations of sarcomeric genes, including cardiac troponin I (cTnI).⁷,⁸ Cardiomyocyte hypertrophy and fibrosis are the cardinal histologic abnormalities underlying the cardiac dysfunction and arrhythmias that plague HCM.⁹ Here, we test the hypothesis that YFI can reverse the pathological manifestations of hypertrophy and fibrosis in a mouse model of HCM. These mice harbor a mutation within the inhibitory domain of cTnI (R146G in mice [cTnI^{Gly146}]; R145G in humans), which enhances contractile function and depresses relaxation coincident with cardiomyocyte hypertrophy and fibrosis; patients with this mutation exhibit severe global ventricular hypertrophy. Using an array of in vitro and in vivo assays, we show that YFI suppresses, indeed reverses, cardiomyocyte hypertrophy and fibrosis via immunomodulation and macrophage polarization.

RESULTS

Biodistribution

To assess the biodistribution of YFI in a mouse model of HCM, we delivered vehicle (saline) or YFI (0.15 μg/g of body weight, as used previously in other mouse models)⁵ acutely (via retro-orbital [r.o.] injection) to 4-month-old cTnI^{Gly146} mice. Thirty min or 4 h later, we measured levels of YFI in various tissues by quantitative PCR. Figure 1A shows that, at 30 min, YFI was detectable above background in...
Mobility and cardiac function in HCM mice
To assess the therapeutic efficacy of YF1 in a mouse model of HCM, we delivered vehicle (saline) or YF1 (0.15 μg/g of body weight) bi-weekly (via r.o. injection) to 4-month-old cTnIGly146 mice over the course of 4 weeks (Figure 2A). Untreated, nontransgenic littermates (wild-type, WT) served as a control. While body weight (BW) measurements did not differ between groups (Figure 2B), YF1 significantly attenuated cardiac left ventricular (LV) wall thickness over the course of treatment (therapeutic efficacy was evident as early as 1 week following treatment, Figures 2C and 2D). At endpoint, we observed improvements in grooming behavior (qualitative observations, Figure 2E) and maximal walking capacity by treadmill (Figure 2F). Additionally, overall heart weight, but not lung weight, was normalized following YF1 treatment. Together, these data demonstrate that infusion of a single defined ncRNA, YF1, can apparently improve the well-being of cTnIGly146 mice.

Cardiomyocyte hypertrophy and fibrosis are reduced with YF1 treatment
Structural remodeling of the LV in HCM is characterized by an increase in cardiomyocyte size and interstitial fibrosis. To examine the impact of YF1 on these processes, we performed histological and molecular analyses on cardiac tissue. We observed striking decreases in interstitial LV fibrosis (Figure 3A) and cardiomyocyte hypertrophy (Figure 3B). Consistent with the histology, western blot analyses revealed significant downregulation of JNK phosphorylation, cJun expression, and Smad2 phosphorylation (Figures 3C and 3D); no significant changes were observed in P38 or P65 signaling (Figure S1). These data help rationalize the benefits of YF1, given that the JNK and Smad pathways figure prominently in hypertrophy- and fibrosis-related signaling.

Peripheral inflammation parallels structural changes in the heart
HCM is characterized by low-grade systemic inflammation. To assess whether YF1 modulates this response, we performed flow cytometric analyses of blood at endpoint. While no change was observed in the percentage of CD4+ and CD8+ lymphocytes, vehicle-treated HCM mice had reduced levels of total CD3+ lymphocytes compared to WT or YF1-treated HCM mice (Figures 4A and 4B). We also noted a significant increase in Ly6C+ myeloid cells in vehicle-treated HCM, which trended to decrease with YF1 treatment (Figure 4C). Decreased neutrophil (Ly6C Ly6G+) and increased monocyte (Ly6C-Ly6G+) populations in HCM mice receiving vehicle were normalized by YF1 treatment (Figure 4C).

YF1 suppresses inflammatory cytokines and myeloid cell mobilization to the heart
Myeloid cells mobilize to the circulation in response to chemokine gradients following injury. Thus, we analyzed serum levels of known cytokines and chemokines by protein array. Of the 40 cytokines tested (Figure S2), YF1 significantly reduced CXCL1 and TNFR1 (Figure 4D) in HCM serum, but none of the other cytokines was changed much. While TNFR1 is associated with TNF-induced inflammation and cardiovascular disease, the neutrophil chemokine CXCL1 correlated with inflammatory cell mobilization (Figure 4C) and was investigated further. Cxcl1 gene expression was significantly reduced in heart tissue from YF1-treated HCM mice (Figure 4E). To determine the source of CXCL1 expression, we cultured cardiomyocytes (neonatal rat ventricular myocytes, NRVMs) and bone marrow-derived macrophages (BMDMs) in vitro. In response to Ang II stimulation, NRVMs, but not BMDMs, robustly upregulated Cxcl1 expression (Figure 4F). Next, we examined whether YF1 suppresses inflammation within the heart. Decreases in proinflammatory cytokines Il1b and Il6 (Figure 5A) and monocyte marker Cd14 (Figure 5B) were accompanied by fewer macrophages in the myocardium (Figures 5C and 5D). Together, these data demonstrate that YF1 attenuates CXCL1 expression in cardiomyocytes, reduces myeloid cell peripheral blood mobilization, suppresses proinflammatory cytokine expression, and inhibits macrophage infiltration in the heart.

Macrophages are required for YF1 activity
To test whether YF1 elicits cardioprotection via direct or indirect (immunomodulation of macrophages) effects, we stimulated NRVM
with Ang II and, 2 days later, exposed the NRVM to vehicle, YF1, or media conditioned by YF1-treated macrophages. The following day, NRVM were washed and collected for RNA extraction and gene-expression analyses (Figure 6A). YF1 had no effect on cardiomyocyte hypertrophy, while media conditioned by YF1-treated macrophages reduced the expression of \textit{Nppa} and \textit{Nppb} (Figure 6B). To better understand the effects of YF1 on macrophages, we exposed BMDM to vehicle or YF1 in the presence or absence of Ang II stimulation. RNA-seq revealed a dramatic shift in gene-expression profile following YF1 treatment in the presence or absence of Ang II stimulation (Figure 6C); these gene-expression changes were lost if transfection reagent was omitted when YF1 was administered (Figure S3A). The gene-expression changes were associated with several biological pathways, as determined through ingenuity pathway analysis, including pathways associated with immunological diseases and inflammatory disorders (Figures S3B and S3C). Additionally, YF1 led to increased expression of the sodium-dependent dicarboxylate transporter \textit{Slc13a3}, independent of Ang II stimulation (Figures 6D and 6E). \textit{Slc13a3} is a high-affinity transporter, which binds a broad range of metabolic substrates (e.g., succinate and glutarate) and may skew the metabolic status of macrophages toward M1- or M2-like phenotypes.\textsuperscript{17-19} These data reveal that YF1 alters the transcriptional landscape in unstressed and stressed macrophages and, specifically, upregulates a potent modulator of macrophage phenotype.

**YF1 reverses fibrosis in aged animals with HCM**

Until now, the data presented were from young adult mice (4-month-old). It seems probable that therapeutic efficacy of YF1 may wane in long-established disease. To test this idea, we implemented the same treatment protocol (2 injections per week for a period of 4 weeks) as...
described earlier, but in 18-month-old cTnI<sup>Gly146</sup> mice (Figure S4A). Despite a small sample size (n = 3/group), YF1 treatment trended toward an improvement in walking distance, but did not alter BW (Figure S4B). Over the course of treatment, YF1 led to a significant decrease in interventricular septum thickness at end-diastole (IVS<sub>d</sub>) and trended toward a decrease in LV posterior wall thickness at end-diastole (LVPW<sub>d</sub>) (Figure S4C). No changes in heart weight or lung weight were observed (Figure S4D). Interestingly, YF1 treatment dramatically reduced fibrosis in 2 of the 3 animals treated with YF1 (Figure S4E). While these data do not have the statistical power to conclusively demonstrate a therapeutic effect, the improvements in IVS<sub>d</sub> and fibrosis in 18-month-old cTnI<sup>Gly146</sup> mice are encouraging and complement the dataset collected with 4-month-old cTnI<sup>Gly146</sup> mice (Figures 2, 3, 4, 5, and 6).

**DISCUSSION**

HCM remains a major unmet medical need. It is the most common genetic disorder affecting the heart and accounts for a sizable fraction of morbidity and mortality in young people, particularly athletes. The only widely accepted therapeutic options for HCM involve localized destruction of heart muscle (by alcohol ablation or surgical myectomy) to relieve outflow obstruction, and/or the use of implantable cardioverter-defibrillators to prevent arrhythmic death. Both modalities are invasive, associated with considerable risks and
side effects, and variably effective. Thus, new therapeutic approaches are desperately needed. A circuitous route, punctuated by serendipity, led us to test an exosomally abundant ncRNA as a therapeutic candidate in a model of HCM.

The path of discovery revealed that CDCs work by secreting exosomes that suppress or reverse fibrosis, attenuate myocyte apoptosis, stimulate angiogenesis, effect immunomodulation, and promote cardiomyocyte cell-cycle re-entry. Despite a recent claim that the effects of cell therapy are nonspecific and mimicked by infusion of dead cells or zymosan, we have shown: (1) transplanted dermal fibroblasts do not mimic the effects of transplanted CDCs; (2) transplanted CDCs

Figure 4. YF1 suppresses granulocyte and monocyte peripheral blood mobilization
(A) Representative flow plots depicting gating strategy. (B) Quantification of changes in cell populations in (A). (C) Representative flow plots and quantification of myeloid cell populations. (D) Representative image of protein array and quantification. Boxes depict proteins with significant changes in expression; CXCL1 denoted by solid border, TNF R1 denoted with dotted border. (E) Gene-expression analysis of Cxcl1 expression in heart tissue. (F) Gene-expression analysis of Cxcl1 in angiotensin II (AngII) treated and untreated cardiomyocytes (NRVM, neonatal rat ventricular cardiomyocytes) and macrophages (BMDM, bone marrow-derived macrophages). Graphs depict mean ± SEM with n = 4–5/group. Statistical significance was determined using two-tailed, unpaired, Student’s t test or one-way ANOVA followed by Tukey’s multiple corrections test. *p < 0.05.
and becomes available for clinical use, will there be a clinical need for YF1? In the absence of data, the answer remains conjectural, but it is noteworthy that the mechanisms of action differ fundamentally; mavacamten is an inhibitor of crossbridge cycling, which attenuates sarcomeric hyper-contractility, while YF1 acts via immunomodulation. Thus, it is at least theoretically possible that the two therapeutic modalities may eventually prove additive, or synergistic, in terms of therapeutic benefit.

MATERIALS AND METHODS

Animal model
All studies were performed at Cedars-Sinai Medical Center in accordance with the Institutional Animal Care and Use Committee guidelines. Female and male mice (4-month-old and 18-month-old), as detailed within the manuscript, were used for in vivo experimental procedures.

Transgenic cTnIGly146 mouse embryos (line 121) were generously donated by Jeffrey Robbins from Cincinnati Children’s Hospital. The Cedars-Sinai Rodent Genetics Core implanted cTnIGly146 embryos in female FVB surrogate mice and successfully recovered the cTnIGly146 transgenic mouse line. Genotype was confirmed by the Core and within our lab with specific forward (5′-GGTGGACAAAGTGGATGAAGA-3′) and reverse (5′-TGCCACGGAGGTCA-3′) primers (Figure S1).

All mice (cTnIGly146 and FVB [WT, control]) were housed in a pathogen-free facility with a 14 h/10 h light/dark cycle with food and water provided ad libitum. In vivo experimental protocols were performed on 1- to 12-month-old female and male mice. Cardiac hypertrophy was monitored by non-invasive transthoracic echocardiography. At the designated time point, animals received vehicle (saline) or YF1 (0.15 mg/g body weight) via r.o. injection; 2 times per week over the course of 4 weeks (Figure 2A).

Echocardiography
Cardiac function and morphology were assessed by transthoracic two-dimensional and targeted M-mode echocardiography (Vevo3100, VisualSonics). Three representative cycles were captured.
for each animal per time point, and measurements for LV ejection fraction (LVEF), end-diastolic interventricular septal thickness (IVSd), and end-diastolic LV posterior wall thickness (LVPWd) were obtained and averaged.

**Treadmill**
Mice were placed inside an Exer-3/6 rodent treadmill (Columbus Instruments) at a 5-degree elevation. Animals were acclimated to the device by leaving them undisturbed for 30 min and then engaging the belt at a slow pace (10 m/min) for 20 min. After the acclimation period was complete, the exercise protocol began; shock grid activated (0.15 mA, 1Hz) and belt speed increased (1 m/min after each minute of exercise). Mice resting on the shock grid for >10 s reached maximal exercise capacity and were removed from the device.

**RNA synthesis**
YF1 (native and modified forms) were custom-synthesized (Integrated DNA Technologies). As reported previously, the 56-nucleotide sequence for YF1 is as follows:

\[ 5'-GGCUGGGCUCCGAUGGUAGGGUUAUCAGAACUUAUUAACAUUAGUGUCACUAAAGU-3'. \]

**Cell culture**

**BMDMs**
Bone marrow was isolated as previously described. Femurs were isolated from mouse, sterilized with 70% ethanol, flushed with PBS (containing 1% FBS and 2 mM EDTA), and filtered through a 70 \( \mu \)m filter. Cells were pelleted and red blood cells lysed with ACK Lysing Buffer (GIBCO). The resulting cells were resuspended in IMDM (containing 10% FBS and 10 ng/mL M-CSF [R&D Systems]) then plated and grown for 7 days to obtain macrophages.

**Transfection**
BMDMs were transfected with YF1 (50 nM) using Dharmafect 4 reagent (Dharmacon). After overnight incubation (~18 h), cells were washed and collected for RNA extraction (miRNeasy Mini Kit, QIAGEN). RNA concentration and purity were determined using a NanoDrop Spectrophotometer (Thermo Scientific).

**Cardiomyocytes**
NRVMs were isolated and cultured as reported. Isolated cardiomyocytes were incubated with Ang II (400 \( \mu \)M) in the presence of protease inhibitor (Sigma-Aldrich) to induce hypertrophy. 2 days later, vehicle, YF1, or conditioned media from YF1-treated...
macrophages was added. 18 h later, cardiomyocytes were collected, and RNA isolated for gene-expression analyses.

RNA analysis

**Biodistribution of EV-Y1 by qPCR**

Single doses of YF1 were administered as above, and mice (n = 8) were sacrificed either 30 min (n = 4) or 4 h (n = 4) afterward. For RNA isolation, spleen, liver, and heart were excised, blotted dry, and ~20 mg sampled; bone marrow was sampled by removing both femurs, cracking them open and flushing each with ~40 mL of PBS. After RNA isolation using miRNeasy Mini Kit (QIAGEN), reverse transcription was performed using mirScript II RT Kit (QIAGEN). qPCR was done using QuantiTect SYBR Green (QIAGEN) on QuantStudio 12K Flex instrument (Applied Biosystems) with the QuantiMir universal reverse primer, EV- Y1 forward primer (5'-GGCTGGTCC GATGGTAGTG-3'), and mouse U6 forward primer (5'-TGCC CCTGCGCAAGGATG-3') the housekeeping gene. Fold change was calculated using $2^{\Delta\Delta Cq}$, where $\Delta Cq$ compares the EV-Y1 Cq value to that of the same organ vehicle injected. To achieve quantitation, serial dilutions of EV-Y1 were added to homogenized tissues from each organ sampled in a healthy mouse. Cq value for EV-Y1 was plotted versus the log of the number of EV-Y1 in the sample. A second order polynomial analysis was performed using GraphPad Prism 5 and the equation was used to calculate the copy number of EV-Y1 in each organ.

**qPCR for transcripts**

cDNA was synthesized from mRNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. The resulting cDNA was standardized across samples and mixed with TaqMan Fast Advanced Master Mix (Applied Biosystems) and pre-designed TaqMan mouse primer sets (Life Technologies, Invitrogen): Tgfb1, Coll1a1, Nppa, Nppb, Cxcl1, Cdl4, Il1b, Il6, and Hprt. Samples were amplified (QuantiStudio 12K Flex Real-Time PCR system; Thermo Fisher Scientific) and analyzed by the ddCt method.

RNA-seq

The Cedars-Sinai Genomics Core performed total RNA-seq of macrophages with the Ion Total RNA-Seq Kit v2 (Life Technologies). Briefly, one microgram of total RNA was assessed for quality using a Bioanalyzer 2100 (Agilent Technologies), enriched with magnetic beads, fragmented, ligated with adapters, then reverse transcribed to make cDNA. The resulting cDNA was barcoded using Ion Xpress RNA-Seq Barcode 1-16 Kit and then amplified. RNA-seq libraries were assessed for concentration and size using the Qubit dsDNA HS Assay Kit (Life Technologies) and DNA 1000 Kit (Agilent Technologies), respectively. Samples were multiplexed and the pooled libraries were amplified onto Ion Sphere particles using an Ion PI Template OT2 200 Kit. Ion Sphere particles were then purified and prepared for sequencing (Ion Proton sequencer) using the Ion PI Sequencing 200 Kit. The raw sequencing signal was processed into FASTQ format and the adaptor was trimmed by built-in Torrent Suite software on the Proton sequencer. Alignment and reference annotation were performed as reported previously.

Staining

**Tissue harvest and embedding**

Hearts were arrested in diastole following intra-ventricular injection of 10% potassium chloride. Hearts were excised, washed in PBS, embedded (Tissue-Tek O.C.T. Compound, Sakura), and stored at −80°C. All samples were cut (5 μm-thickness) by cryostat (Leica) and fixed (4% PFA in PBS) prior to staining.

**Picrosirius red**

Tissue sections were stained with Sirius Red (0.1% in Saturated Picric Acid; Electron Microscopy Sciences) according to the manufacturer’s protocol. Slides were mounted (DPX; Sigma-Aldrich), and then images acquired (ScanScope AT Turbo; Aperio) and analyzed (ImageJ software). Fibrosis was determined as the percent composition per field of view.

**Wheat germ agglutinin (WGA)**

Tissue sections were blocked (Protein Block, Agilent Dako) and stained with α-sarcemeric actinin (αSA). Alexa Fluor 546-conjugated secondary antibody, WGA (Alexa Fluor 488-conjugated, Invitrogen), and DAPI were applied prior to mounting (Fluoroshield with DAPI, MilliporeSigma). Tissue sections were visualized by confocal microscopy (Leica) and analyzed by ImageJ software. Cardiomyocyte size was determined by measuring the area of cardiomyocytes with centrally located nuclei.

**Immunohistochemistry**

Tissue sections were blocked (Protein Block, Agilent Dako) and stained with primary antibody. The appropriate fluorescently conjugated secondary antibodies (Invitrogen) were applied prior to mounting (Fluoroshield with DAPI, MilliporeSigma). Tissue sections were visualized by either confocal microscopy (Leica) or cell imaging multi-mode reader (Cytation 5, Biotek) and analyzed by ImageJ software.

**Immunoblotting**

**Protein extraction and isolation**

Tissue was collected, rinsed in PBS, placed in Allprotect tissue reagent (QIAGEN), and then stored at −80°C until use. Samples were minced, suspended in T-PER Buffer (containing Halt protease and phosphatase inhibitor, Thermo Fisher), homogenized (Bead Ruptor, OMNI), and then centrifuged. Protein supernatants were collected and concentrations measured (BCA Protein Assay Kit, Pierce).

**Western blot**

Protein samples were prepared for gel electrophoresis (NuPAGE 4%–12% Bis-Tris, Invitrogen) according to the manufacturer’s protocol. A normalized value between 10 and 30 μg was used for loading in each well. Proteins were then transferred to a Nitrocellulose Membrane, 0.45 μm (Thermo Scientific) for immunoblotting with antibodies. Bands were detected (Chemidoc Imaging System, Bio-Rad) with ECL Western Blotting Substrate (Pierce).
**Cytokine array**

Serum cytokine levels were detected using a Mouse Inflammation Antibody Array (AAM-INF-1, RayBiotech) according to the manufacturer’s protocol. Blood was collected and separated by centrifugation to collect serum. Membranes were blocked and incubated with serum overnight. The next day, the membranes were washed, incubated with Biotinylated Antibody Cocktail and then HRP-Streptavidin. Arrays were detected (ChemiDoc Imaging System, Bio-Rad) following the addition of detection substrate.

**Flow cytometry**

Peripheral blood immune cells were analyzed as reported previously. Briefly, blood was collected in heparinized tubes. Red blood cells were lysed with ACK lysis buffer (GIBCO), centrifuged, and then washed with fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% FBS and 2 mM EDTA). The resulting white blood cell suspension was stained with the fluorescently conjugated anti-inflammatory antibodies, washed, and analyzed (SA3800 Spectral Analyzer, Sony) based on their marker profile.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.04.014.

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**DECLARATION OF INTERESTS**

E.M. owns founder’s equity in Capricor, Inc. G.d.C. was a paid part-time consultant for Capricor, Inc. The other authors declare no competing interests.

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