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RESEARCH LETTER

Nano-miR-133a Replacement Therapy Blunts Pressure Overload–Induced Heart Failure

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Noncoding RNAs for curative purposes have officially entered the clinical field, as shown by the recent US Food and Drug Administration and European Medicines Agency approval of the first 3 liposome-based siRNA drugs: Onpattro (patisiran) for the treatment of a rare life-threatening form of amyloidosis, Givlaari (givosiran) for acute hepatic porphyria, and Leqvio (inclisiran) for lowering cholesterol.[1] For all 3 drugs, the liver is the primary target. In cardiac medicine, although several noncoding RNA candidates, especially microRNAs (miRNAs), have been proven to have great therapeutic potentials, the emergence of noncoding RNA therapeutics has not yet translated into any approved drugs. In fact, miRNA-based clinical trials for heart disease are still limited to only diagnostic/prognostic uses of circulating miRNAs; no clinical trials for the potential use of miRNA mimic–based drugs for cardiac treatment are ongoing. A major issue challenging patient-friendly application of an miRNA-based therapy for myocardial treatment is the lack of an efficient delivery system, allowing cardiac-enriched uptake, controlled drug release, reduced unwanted off-target effects, low-toxic side effects, and low-dose administration. Gene therapy via cardiotrophic adeno-associated viral vectors is at present the only valid approach, as shown in recent preclinical trials. However, concerns about uncontrolled long-term expression, difficulties in fine-tuning dosage, immune reaction, risk of random gene integration, and potential cancerogenicity, as well as the need for invasive cardiac administration, still hamper broad, reliable, and safe use in the clinical settings. Here, we provide proof of concept for an unconventional and effective nanotechnology-based inhalation approach for delivery of a synthetic miRNA mimic via biocompatible and biodegradable calcium phosphate–based nanoparticles (CaPs)[2] preferentially to cardiomyocytes without significant accumulation in other myocardial cells or organs (Figure [A and B]).

The cardiac-enriched miR-133a, which is inversely related to failing heart conditions in human and animals, represents a potential therapeutic molecule to counteract the progression of pathological cardiac remodeling. In a mouse model of ventricular pressure overload induced by transverse aortic constriction (TAC), miR-133a rapidly decreases as early as day 1 after TAC and gradually recovers toward basal levels at 6 to 8 weeks after TAC.[3] In a transgenic mouse model for inducible expression of miR-133a, we previously demonstrated that restoration of miR-133a levels mitigates the pathological effects of TAC.[4] However, effective and clinically oriented interventions aimed at facilitating cardiac delivery of exogenous synthetic miR-133a or other therapeutic miRNA mimics are currently not available. Here, inhalable CaPs loaded with synthetic miR-133a mimic (CaP-miR) were tested as a replacement therapy approach for the specific restoration of miR-133a levels in cardiomyocytes just within the precise time frame when the miRNA is pathologically repressed. TAC mice were treated with CaP-miR delivered by intratracheal nebulization once a day on alternate days for 4 consec-

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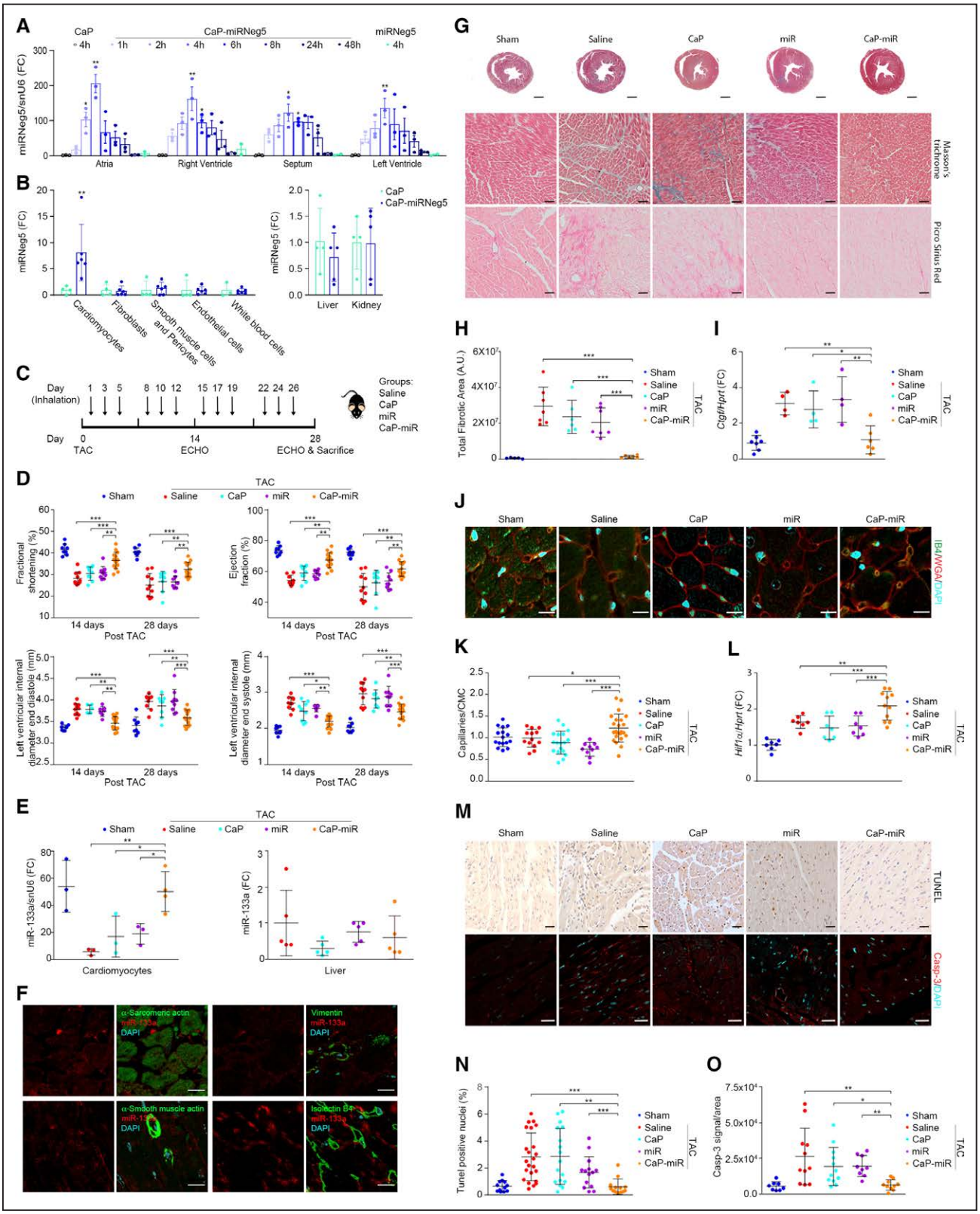

Figure. Inhalation of miR-133–loaded nanoparticles contrasts cardiac dysfunction in pressure overload–induced heart failure. **A** and **B**, Analysis of synthetic miRNeg5 (miRCURY Negative Control 5, GeneGlobe ID YM00479904, Qiagen) levels in 8-week-old C57BL/6J male mice treated by a single intratracheal administration as indicated. **A**, Quantitative reverse transcription (qRT)–polymerase chain reaction (PCR) for miRNeg5 (normalized to U6) on heart RNA at different time points after administration (n=3). **B**, Droplet digital PCR (ddPCR) for miRNeg5 on total RNA from **(left)** fluorescence-activated cell (FACS)–sorted myocardial cells and **(right)** peripheral tissues 8 hours after treatment (n=4–6). miRNeg5 molecules were normalized to cell number and expressed as fold change (FC) (*Continued*)

Figure Continued. over empty calcium phosphate–based nanoparticle (CaP) control. **C**, Schematic overview of mouse treatments. MiR-133a is an miRCURY LNA miRNA Mimic (GeneGlobe ID YM00470572, Qiagen). **D**, Echocardiographic analyses of treated and untreated mice subjected to transverse aortic constriction (TAC; pressure gradients >70 mm Hg) or sham (n=8–15). **E**, ddPCR for miR-133a on (**left**) FACS-isolated cardiomyocytes (normalized to U6) and (**right**) liver (normalized to the saline control condition) from mice treated as indicated (n=3–5). **F**, Fluorescence in situ hybridization for miR-133a on heart sections from CaP-miR–treated mice 4 weeks after TAC and sham. Green indicates α-sarcomeric actin (cardiomyocyte marker), vimentin (fibroblast marker), smooth muscle actin (smooth muscle marker), or isolectin B4 (IB4; endothelial marker); red, miR-133a; and cyan, DAPI (nuclei). Scale bar: 10 μm. **G**, Hematoxylin and eosin– (scale bar: 1 mm), Masson trichrome– (scale bar: 100 μm), and Picro Sirius Red– (scale bar: 100 μm) stained heart sections from mice 4 weeks after TAC or sham, treated as indicated. **H**, Quantification of fibrotic area (n=5 – 7). **I**, qRT-PCR for the fibrotic gene *Ctgf* on left ventricular (LV) RNA normalized to *Hprt* (n=4 – 7). **J**, IB4- (isolectin for endothelial cells), wheat germ agglutinin– (WGA; for plasma membrane staining), and DAPI- (nuclei) stained heart sections. Scale bar: 10 μm. **K**, Quantification of capillary density per cardiomyocyte. **L**, qRT-PCR for *Hif1a* on LV RNA normalized to *Hprt* (n=7–11). **M**, Terminal deoxynucleotidyl transferase dUTP nick-end labeling– (TUNEL; scale bar: 200 μm) and cleaved caspase-3– (casp-3; scale bar: 20 μm) stained heart sections. **N** and **O**, Quantification of percentage TUNEL-positive nuclei (**N**) and Casp-3–positive signal per signal area (**O**). All data are represented as mean±SD. Statistical analyses were performed with Prism 8 using the Kruskal-Wallis test with the Dunn multiple-comparisons test (**A** and **N**), Mann Whitney test (**B**), 2-way mixed-effects ANOVA with Tukey multiple-comparisons test after a Shapiro-Wilk test (**D**), and 1-way ANOVA with Dunnett multiple-comparisons test after a Shapiro-Wilk test (**E**, **H**, **I**, **K**, **L**, and **O**). A.U. indicates arbitrary units. **P*<0.05. ***P*<0.01. ****P*<0.005. miR indicates microRNA.

Nonstandard Abbreviations and Acronyms

CaP	calcium phosphate–based nanoparticle
miRNA	microRNA
TAC	transverse aortic constriction

utive weeks starting from the day of TAC (Figure [C]). Saline solution, miR-133 alone (miR), and unloaded CaPs alone served as controls. CaP-miR showed a protective effect against heart failure progression with preservation of left ventricular internal diameter, ejection fraction, and fraction shortening at 14 and 28 days after TAC (Figure [D]). This improvement was associated with effective restoration of physiological levels of miR-133a in fluorescence-activated cell sorting–isolated cardiomyocytes 28 days after TAC as measured by digital polymerase chain reaction (Figure [E], left). No significant accumulation of miR-133a in the liver of CaP-miR133–treated mice versus nontreated mice was found (Figure [E], right). Furthermore, in situ hybridization showed an enrichment of miR-133a in cardiomyocytes from CaP-miR133–treated TAC mice (Figure [F]). Histological analyses revealed reduced fibrosis (Figure [G–I]) in CaP-miR–treated TAC mice, whereas no beneficial effects were observed in mice administered unloaded CaPs or pristine miR-133a. In addition, CaP-miR–treated TAC mice showed increased capillary density per cardiomyocyte, suggesting that intensification of oxygen supply may play a role in preventing heart failure progression (Figure [J–L]). CaP-miR–treated TAC mice consistently showed a marked decrease in the activated programmed cell death (Figure [M and O]), supporting the notion that inhibition of hypoxia-induced apoptosis[5] contributes to counteract the pathological effects of TAC. In conclusion, we report that (1) inhalable CaP-miR represents an effective delivery approach for targeting the heart and facilitating the release of miRNA mimics within cardiomyocytes and (2) the delivered miR-133a is functional for the beneficial restoration of cardiac performance and prevention of remodeling in a mouse model of ventricular pressure overload.

MiR-133a is a well-established diagnostic circulating biomarker in patients with heart failure, and increased circulating miR-133a levels are inversely correlated with downregulation of the miRNA at the cardiomyocyte level as a result of a stress-induced secretion. Therefore, circulating miR-133a may represent an important biomarker for stratification of patients for a future replacement therapy that is based on inhalable miR-133a–loaded CaPs. Thus, by providing the proof of concept for an innovative application of inhalable nanoparticles for miRNA mimic delivery to the heart, our approach represents an important step forward for noncoding RNA–based precision medicine interventions of the diseased heart.

All procedures involving animals were approved by institutional and national authorities. The data that support the findings of this study are available from the corresponding author on reasonable request.

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Disclosures

None.

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