Supplemental material

Expanded Supplemental methods

Rodents

Wild-type (WT) (C57BL/6, Jackson Laboratory #000664), αMHC-MerCreMer B6.FVB(129)-*A1cf*$^{Tg(Myh6\text{-}cre/Esr1*)1Jmk}$/J (Jackson Laboratory, 005657) and Rosa^{mTmG} (*Gt(ROSA)26Sor*$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J (Jackson Laboratories, 007576) mice and Sprague Dawley (SD) (Charles River, 400) rats were bred and maintained under pathogen-free conditions. Cre recombinase expression was induced in the αMHC-MerCreMer-Rosa^{mTmG} double transgenic mice by intra-peritoneal (ip) administration of tamoxifen (Sigma T5648, 20 mg/kg/day) for 5 days; mice were then rested at least 5 days prior to any experimentation. All procedures conformed to the animal welfare regulations of the Massachusetts General Hospital Sub-Committee on Animal Research Care (SRAC) and under the guidelines on the use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996), and approved by the committee on the Ethics of Animal Experiments of Shanghai University. All mice used for experiments were within 8-14 weeks of age.

Number of mice used per experiment is indicated in the figure legends. Investigators performing the data analyses were blinded to rodent genotype and treatment status.

miR-30d overexpressing and knock out (KO) transgenic rat

U6-Rno-mir-30d: miR-30d overexpressing transgenic rat

Insertion sequence frame: 572bp

ACTAGTgagcccgcgtctgaaccctccgcgccgccccggccccagtggaaagacgcgcaggcaaaacgcacca

cgtgacggagcgtgaccgcgcgccgagcgcgccaaggtcgggcaggaagagggcctatttcccatgattccttcata

tttgcatatacgatacaaggctgttagagagataattagaattaatttgactgtaaacacaaagatattagtacaaaatacgt

gacgtagaaagtaataatttcttgggtagtttgcagttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaa

agtatttcgatttcttggctttatatatcttgtggaaaggacgaaacaccgGTAGCTTGCTGTCAGAAAGTCTG

TGTCTGTAAACATCCCCGACTGGAAGCTGTAAGCCACAGCCAAGCTTTCAGTCAGA

TGTTTGCTGCTACTGGCTCTTCGCATGCATCTTTTTTTTTTTCTACAGGTGATATCTT

TTAACACAAATAAAATGTAGTAGTCCTAGGAGACGGAATAGAAGCTAGC**gtttaaaGA**

ATTC

1. blue： hU6 promoter
2. yellow: pre-rno-miR-30d, red part-mature sequence

miR-30d KO transgenic rat

WT sequence:

TAGGTAAGTTTGTATTGAATCTCTTGTTAGATAATTCAGAAGAAGTTACTCTATCCTC

CTTATTGTTTACTTTATTGTTTCTTTTTTCCCCTAAGATATCACAGTTGTCATTCAGCT

CTGCAGTTGTCATTCAGCTCTGCAGTTGTCAAAGTTAGTAGCACAAACAGTGACTG

ACCATTTGCTCTGTTCCTTTGTAGCTTGCTGTCAGA**AAGTCTGTGTCTGTAAACAT**

CCCCGACTGGAAGCTGTAAGCCACAGCCAAGCTTTCAGTCAGATGTTTGCTGCTA

CTGGCTCTTCGCATGCATCTTTTGGACAGTCTGGCCAAGGATTGGAGCTGAGGAG

GGTGAGAAGTACATGGGATG

KO sequence:

TAGGTAAGTTTGTATTGAATCTCTTGTTAGATAATTCAGAAGAAGTTACTCTATCCTC
CTTATTGTTTACTTTATTGTTTCTTTTTTCCCCTAAG----------
----------191bp----------

GGCTCTTCGCATGCATCTTTTGGACAGTCTGGCCAAGGATTGGAGCTGAGGAGGG
TGAGAAGTACATGGGATG

Construction Lentivirus for miR-30d

Considering the mature sequence of miR-30d (UGUAAACAUCCCCGACUGGAAG), the following sequences were designed and used to overexpress miR-30d. F: 5'-CCGGTGTAAACATCCCCGACTGGAAGCTCGAGCTTCCAGTCGGGGATGTTTACAT TTTTG-3' R:5' AATTCAAAAATGTAAACATCCCCGACTGGAAGCTCGAGCTTCCAGTC GGGGATGTTTACA-3'. Overexpressed fragments were ligated into PLKO.1-TRC vectors. For lentivirus packaging, 293T cells were co-transfected with psPAX2, pMD2.G and one target plasmids using Lipofectamine™2000 Transfection Reagent (Invitrogen). The medium was harvested at 48 and 72 hours after transfection. After centrifugation and filtration, the lentivirus was obtained.

AgomiR mediated miR-30d gain of function

PBS dissolved miR-30d agomiR or NC agomiR (RiboBio) were administered through vein injection (iv) at a 20nmol/mice dose for 3 consecutive days. The MI or Sham surgeries were performed the day following the last agomiR administration.

Locked Nucleic Acid mediated miR-30d loss of function

For miR-30d loss of function *in vivo* experiments, a Locked Nucleic Acid (LNA) based approach was utilized. Loading intra-peritoneal (ip) doses of 25 mg/kg LNA anti-miR-30d

or scramble LNA (Qiagen) were administered for 3 consecutive days, followed by maintenance weekly doses of 10mg/kg LNA anti-miR-30d or scramble. I/R or Sham surgeries were performed 10 days after first LNA loading dose.

Myocardial Infarction (MI) model

MI was generated by ligating the left anterior descending coronary artery (LAD) using a 7/0 silk thread while Sham was created by the same process but without LAD ligation. MI model was applied to miR-30d overexpressing and knock out transgenic rats and to mice treated with miR-30d overexpressing lentivirus or miR-30d agomiR.

Ischemia/Reperfusion model

The Ischemia/Reperfusion (IR) murine ischemic heart failure model was performed as follows: mice were anesthetized with isoflurane (1-2% oxygen) and their left anterior descending (LAD) coronary artery was ligated 2-3mm from its origin using a 6.0 silk prolene suture (Ethicon) for 30 minutes, followed by reperfusion. Control Sham was created by the same process but without LAD ligation [42]. Post-operatively, animals received buprenorphine 0.05-0.1 mg/kg SQ every 10-14 hours for 72 hours for analgesia. IR model was applied to male mice treated with LNA anti-miR-30d. Mice underwent echocardiography imaging prior to tissue harvesting 4-7 weeks after surgery.

Echocardiography

Echocardiograms were performed on anaesthetized mice before euthanasia using a GE Vivid 7 ultrasound machine (GE Healthcare) with a frequency of 14.0 mHz, 131.5 frames/s, and a depth of 1 cm for two-dimensional images. M-mode images were taken at the midpapillary muscle level and were used for measurements. Echocardiograms were analyzed using Vivid 7 software.

Histology

Masson's Trichrome staining

To evaluate the morphological changes and the extent of cardiac fibrosis, heart samples were fixed in 4% Paraformaldehyde (PFA) and then embedded in paraffin. Cardiac fibrosis was determined using Masson's Trichrome staining (Keygen) in 4%PFA fixed paraffin embedded 5µm heart cross-sections. Images were taken under light microscopy at 200× magnification for analysis. The fibrotic fraction was obtained by calculating the ratio of blue (fibrotic) to total myocardial area using Image J Software (National Institutes of Health).

TUNEL staining

For apoptosis and α-actinin co-staining, FFPE sections were first deparaffinized as indicated above, and permeabilized with Proteinase K (Agilent Technologies, S302080-2) for 5min at RT. Sections were then incubated with the In-Situ Cell Death Detection Kit (Sigma-Aldrich, 12156792910) for 1h at 37C, following manufacturer's instructions. Once genomic cleaved DNA labeling was finalized, sections were blocked for 1h at RT, and incubated overnight at 4°C with 1:250 anti-α-actinin antibody (Sigma-Aldrich Cat# A5044, RRID:AB_476737). The following day, sections were incubated for 2h at RT with 1:250 secondary antibody (Thermo Fisher Scientific Cat# A-21235, RRID:AB_2535804), and mounted with DAPI containing mounting media (Invitrogen P36935). Images were acquired in a Leica SP8 confocal microscope. Analysis was performed using Image J.

When OCT embedded frozen heart sections were utilized for the TUNEL staining, 10 µm-thick sections were first incubated with α-actinin (Sigma-Aldrich Cat# A7811, RRID:AB_476766) at 4°C overnight. After an incubation with corresponding secondary

antibody at room temperature for 2 h, apoptosis was determined by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay using in situ cell death detection Kit (Vazyme, China) in accordance with the kit manufacturer's instructions. Images were taken using fluorescence microscope under a magnification of 200×. Image J Software was used for analysis.

Heart tissue Immunofluorescence

For immunofluorescence staining, Formalin-fixed-paraffin-embedded (FFPE) heart sections were first deparaffinized (Xylene 3 x 5', 100% Ethanol 2 x 3', 95% Ethanol 1', 75% Ethanol 1', diH_2O 5'). Antigen retrieval was performed using the IHC-Tek™ Epitope Retrieval Steamer Set (IHC World, Cat. # IW-1102), followed by permeabilization with 0.5% Triton-X100 in PBS. Sections were blocked for 1h at RT and incubated overnight in anti-GFP (Biomatik, CAU29134) or anti-Vimentin (BioLegend Cat# 699301, RRID:AB_2716136) antibody (1:250) at 4°C. The following day, sections were incubated for 2h at RT with the secondary antibody (1:250) and mounted with DAPI containing mounting media (Invitrogen P36935). Images were acquired in a Leica SP8 confocal microscope.

TTC staining

After 24 hours of LAD ligation, 1 mL Evans blue (Sigma) was injected into inferior vena of anesthetized mice. The heart was then removed and stored at -20°C for 15min. The heart was sectioned into 5 transverse slices of 1 mm, and stained with 1% triphenyltetrazolium chloride (TTC, Sigma T8877) for 10 min at 37°C. Next, sections were

fixed in 4% formaldehyde and macroscopic photographs of the TTC stained sections were collected.

Alternatively, five minutes after inducing ischemia by ligating the LAD with 7-0 silk, 50 µL of fluorescent microspheres (10 µm FluoSpheres, Molecular Probes) were injected into the ventricular cavity to identify the perfused area and nonperfused area-at-risk (AAR) [42]. 24h post-surgery the intact heart was excised and stored at -20°C for 15min. The semi-frozen heart was then cut in 6-8 cross sections and immersed in 1% TTC for 2-3min at RT, followed by a 15min incubation shaking at 37°C. Tissues were fixed in 10% formalin and imaged under UV light (for area-at-risk visualization) and macroscopically for TCC stained infarcted area. The infarct size was quantified as the total infarct area divided by the area-at-risk using Image J Software (National Institutes of Health).

RNA sequencing bioinformatics analysis

RNA from NRVMs transfected with Adeno-miR-30d, Adeno-GFP or LNA Anti-miR-30d was sequenced on the Illumina Nextseq using Trueseq libraries (RS-200-0048; Illumina) [43]. The raw sequence image files from the sequencer were converted to the *fastq* format and underwent QC[44]. The sequencing quality scores were checked for quality to ensure they did not deteriorate at the read ends. Useful metrics such as the per base sequence quality, the per base sequence content, per base N content, sequence length distribution, adapter and k-mer content and sequence duplication levels were collected for each sequencing run using *fastqc*[10]. At this stage, any samples that did not pass QC were flagged and investigated later. The *fastqs* were then aligned to the *rattus norvegicus* genome (RNor 6.0, ENSEMBL version 92) using *STAR* [45] aligner[46]. The alignment files in the form of *bam* files were indexed for the ability to view them on Integrative Genome

Viewer (IGV) [46]. Alignment statistics such as average read length, percentage of uniquely mapped reads, percentage of multi-mapped reads and number of splice junctions were collated. Only reads that uniquely mapped to the genome were considered for read annotation with the *rattus norvegicus* ENSEMBL 92 gene transfer format (gtf) file using *Salmon* [47]*. DESeq2* [48] was used for differential gene expression analysis. Genes are considered differentially expressed if they are UP-regulated by FC > +1.5 or DOWN-regulated by FC < -1.5 with an adjusted p-value value < 0.05 (FC: fold change, p-value adjustment performed by Benjamini Hochberg multiple hypothesis testing).

Pathway analysis

Pathway analysis was performed using Gene Set Enrichment Analysis [49,50] (GSEA preranked mode) using the KEGG pathway database. For all genes included in the differential expression analysis using DESeq2 (previously described) a metric was computed as the product of logFC and $-\log_{10}$(P-value). The genes were ranked by this metric resulting in the most significantly overexpressed genes at the top of the list and the most significantly under-expressed genes at the bottom. A "running sum" statistic was calculated for each gene set in the pathway database, based on the ranks of the members of the set, relative to those of the non-members. The enrichment score (ES) was defined as the maximum sum of the running sum and the genes that made up this maximum ES contribute to the core enrichment in that pathway. All pathways with an FDR < 0.25 were considered to be significant.

Luciferase Assays to validate miR-30d's targets

To evaluate miR-30d's direct targets, the 3'UTR of the bioinformatically predicted targets were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, E1330) vector by PCR using mouse cDNA as template. The primers for each predicted target are indicated in the Supplemental Methods. For the Luciferase reporter assay, reporter constructs (2μg) and 25nM miR-30d mimic (Thermo Fisher Scientific, 4464066) or Scrm (Qiagen, 1027280) were transfected into HEK293 cells using FuGENE® HD (Promega, E2690). Luciferase activity was determined through the Dual-Luciferase reporter assay system (Promega, E1960) 48h post-transfection.

The primers for each predicted target were:

itga5 Forward 5'-CAGTCTAGAGCCCTCCTGATCTCAGACTCATG-3', Reverse 5'-CAGGTCGACCAGCACCATCCACGGTTAGTG-3'

itga6 Forward 5'- CAGGCTAGCAGGTCACCTGTGCTAACAGAGTG-3', Reverse 5'-CAGCTCGAGCCTGCCAGAAAGGCAATTACGG -3'

itgbl1 Forward 5'-CAGGCTAGCAACAACTTTAGTAAACTGATATAAGTTACACCATAAAGAATTGT - 3', Reverse 5'- CAGGTCGACTTTAGAGCCCTTGTGATCTGGGAAGC -3'

map4k4 Forward 5'-CAGGCTAGCAAACTATCTGATTAAAGTTTGACTTTGTCACAAGTCTA - 3', Reverse 5'-CAGCTCGAGTGAAGCAAATTATTACAAGACATTTATTACAAATGTCACAGAC -3'

itgaV Forward 5'- CAGCTCGAGAACATGATGTAGTTTTCAACAATAGCATGGATTT - 3', Reverse 5'- CAGACGCGTGGCAACAAACTCTTGTCTGCTCTTAGT -3'.

Itgb3 Forward 5'- CAGACGCGTTGAGACCATCTTCAGATGACGCCAG - 3', Reverse 5'-CAGGTCGACAGACGATACAGCTGAGCTTCAACG -3'.

Neonatal rat ventricular myocytes (NRVM) isolation

NRVMs were isolated from 1-day old Sprague Dawley rat neonates following a collagenase II (Worthington Biochemical, LS004177) and pancreatin (Sigma-Aldrich, P3292) based digestion and purified via Percoll (GE Healthcare, 17-0891-01) gradient. Purified NRVMs were cultured in DMEM (Gibco/Thermo Fisher Scientific, 11995123), 5% penicillin/streptomycin, 5% L-glutamine, 5% Fetal Bovine Serum and 10% Horse Serum containing media. 24h post isolation NRVMs were transfected with either miR-30d mimic (Thermo Fisher Scientific, 4464066) or LNA anti-miR-30d (Qiagen, YI04100903) using Lipofectamine RNAiMax (Invitrogen/ Thermo Fisher Scientific, 13778150) in serum-free DMEM media and harvested 48h post-transfection for target gene expression quantification.

For Oxygen-Glucose Deprivation/Reperfusion (OGD/R) experiments, NRVMs were first transfected with miR-30d mimic and negative control mimic (or miR-30d inhibitor and negative control inhibitor) using Lipofectamine™2000 Transfection Reagent (Invitrogen) in low serum medium (1% FBS). After 24h, cells were placed in an anaerobic chamber with AnaeroPack (Mitsubishi Gas Chemical) and cultured in glucose/serum free DMEM media (Gibco) for 8h, followed by culturing in normal aerobic and normal culture medium environment for 12h.

In order to evaluate the NRVM apoptotic phenotype after silencing *map4k4,* miR-30d-5p, or both, freshly isolated NRVMs were transfected with LNA anti-miR-30d (100nM, Qiagen,

YI04100903), siRNA targeting *map4k4* (100nM, Sigma, Cat. #EMU076381), or both, using RNAiMax (Invitrogen/ Thermo Fisher Scientific, Cat. #13778150) as transfection reagent for 36h in serum-free DMEM media. Next, cells were exposed to hypoxia for 8h in glucose and serum free DMEM media (Thermo Fisher Scientific, Cat. #11966025), followed by 12h exposure to normoxia in complete DMEM media. For apoptotic cell quantification, cells were fixed in 4% PFA, permeabilized with 0.2% Triton X-100, blocked in 10% Donkey Serum and stained for TUNEL (Thermo Fisher Scientific, Cat. #C10618) and anti-cardiac Troponin (1:1000, Abcam, Cat. # ab47003). Pictures were acquired with a BioRad microscope (ZOE™ Fluorescent Cell Imager 1450031) (n=16 per condition), and the percent of apoptotic cells per field of image were quantified.

Adult mouse cardiac fibroblast isolation, transfection and staining

Adult mice were euthanized, their hearts were excised, minced, washed in PBS and then subjected to digestion at 37°C for 30 mins with agitation in a mixture of 0.25% trypsin and 5mg/ml Liberase TL (Roche, 5401020001). Digested tissue was then centrifuged at 1500rpm for 5 minutes and the pellet re-suspended into fibroblasts growth media (Lonza 4526 or Cell Biologics M2267) and plated on 0.1% gelatin coated plates ON at 37°C. After 24h, plates were washed, non-adherent cells discarded, and the adherent cells were grown in fibroblast growth media. Fibroblasts in passage 1 or 2 were used for experiments.

Adult cardiac fibroblasts were transfected with miR-30d mimic (Thermo Fisher Scientific, 4464066) or scramble (Qiagen, 1022076) using Lipofectamine 3000 (Invitrogen) and harvested 48h post-transfection for target gene expression quantification. For inducing cardiac fibroblasts to myofibroblast differentiation, 10ng/mL TGFβ1 (Thermo Fisher

Scientific, PHG9204) were added 24h post-transfection, for an additional 24h before harvest.

Adult mouse cardiac fibroblasts isolated from αMHC-MerCreMer-Rosa^{mTmG} mice subjected to IR surgery and cultured for 10 days, were formalin fixed for 10min, permeabilized with 0.5% Triton-X100 in PBS and blocked for 1h at RT. Staining with anti-GFP (Biomatik, CAU29134) or anti-Vimentin (BioLegend, 699301, RRID:AB_2716136) antibody (1:250) was performed overnight at 4°C, followed by 2h incubation at RT with the secondary antibody (1:250), and mounted with DAPI containing mounting media (Invitrogen, P36935). Images were taken with a Leica SP8 confocal microscope.

Neonatal rat cardiac fibroblast isolation, transfection and staining

Cardiac fibroblasts were obtained from 1 to 3-day-old Sprague Dawley (SD) rats' hearts and cultured in DMEM (Corning) with 10% fetal bovine serum (Biological Industries) and 1% penicillin/streptomycin (Keygen). Second passage cardiac fibroblasts were used for transfection with miR-30d mimic and negative control mimic (or miR-30d inhibitor and negative control inhibitor) using Lipofectamine™2000Transfection Reagent (Invitrogen) in low serum medium (1% FBS). After 24h, cells were cultured with 10 ng/mL recombinant human TGF-β (Sino Biological) for 24 h or 1μM AngII (Sigma) for 48h.

For EdU incorporation assay, fibroblasts were labeled with EdU (10μM) 24 h before harvesting and Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit (Ribobio, C10310-3, China) was used to reveal EdU incorporation.

Cardiac and cellular RNA isolation and qPCR

RNA was isolated from murine cardiac tissue through the Exiqon miRcury total RNA isolation kit following the manufacturer's instructions. For cellular RNA isolation from

NRVM and CFB, cells were initially lysed with Trizol (Thermo Fisher Scientific, 15596018), and consequently RNA was isolated via chloroform-isopropanol-ethanol protocol. Real-time reverse transcription (RT) quantitative polymerase chain reaction (qPCR) for myocardial tissue, NRVM and CFB mRNA was performed using 1µg RNA input through the Kapa Sybr Green system for qPCR (Kapa Biosystems). The sequences of the primers used for quantifying each mRNA are indicated in the Supplemental Materials. mRNA quantification was normalized to the housekeeping gene β-Actin and represented as fold change ($2^{-}\Delta\Delta^{Ct}$) versus Sham or Scrm conditions depending on the experimental set up.

For cardiac tissue, CFB and NRVM miRNAs, 200ng RNA input were used in the miRCURY LNA RT Kit (Qiagen, 339340) for RT. For miRNA qPCR the PCR Master Mix from Exiqon (203420-01) was utilized together with the miR-30d-5p miRCURY LNA miRNA PCR Assay from Qiagen (339306). miRNA quantification in heart tissue, cells and EVs was normalized to 5S miRCURY LNA miRNA PCR Assay (Qiagen, 339306), and represented as fold change ($2^{-}\Delta\Delta^{Ct}$) versus Sham, scramble or normoxia depending on the experimental set up.

Droplet digital PCR (ddPCR)

For each ddPCR reaction, 6 µL of cDNAs, 10 µL of QX200 EvaGreen ddPCR Supermix for mature miRNA (Bio-Rad, 186-4034) or ddPCR™ Supermix for Probles (No dUTP) (Bio-Rad, 1863024) for pre-miRNA, 1 µL of miR-30d-5p PCR primer set (Qiagen, 339306) or pre-miR-30d probe (Thermo Fisher Scientific, 4426961) and nuclease-free water were mixed, making a 20 µL reaction. A no template control (NTC), where nuclease-free water was added instead of cDNA samples, was also included in the assay. Each ddPCR assay

mixture was loaded into an 8-channel droplet generation cartridge (Bio-Rad, 1864008). Then, 70 µL of either QX200 Droplet generation oil for Evagreen (Bio-Rad,1864006) for mature miR-30d-5p reactions or Droplet generation oil for Probes (Bio-Rad, 1863005) for pre-miR-30d reactions were loaded into the appropriate wells and the cartridge was placed inside the QX200 droplet generator (Bio-Rad, 1864002). The resulting droplets were carefully transferred to a 96-well plate (Bio-Rad, 12001925), the plate was then heat-sealed with foil and placed in a conventional thermal cycler (Eppendorf). Thermal cycling conditions were as follows for mature miR-30d-5p: 95°C for 5 minutes, then 40 cycles of 95°C for 30 seconds and 56°C for 1 minute, and three final steps at 4°C for 5 minutes, 90°C for 5 minutes, and a 4°C indefinite hold. A ramp rate of 2°C/s was used in all the conditions. Thermal cycling conditions were as follows for pre-miR-30d: 95°C for 10 minutes, then 40 cycles of 95°C for 30 seconds and 56°C for 1 minute, and two final steps at 98°C for 10 minutes, and a 4°C indefinite hold. A ramp rate of 2°C/s was used in all the conditions. At the end of the PCR reaction, droplets were read in the QX200 droplet reader (Bio-Rad, 1864003) and analyzed using the Quantasoft™ version 1.7 software (Bio-Rad, 1864011).

Extracellular vesicle (EV) isolation and characterization

All protocols utilized and expanded below are in accordance with the MISEV (Minimal Information for Studies of Extracellular Vesicles) guidelines for EV research.

Exosome isolation from transgenic rat plasma with ExoQuick

For quantification of exosomal miR-30d levels in miR-30d overexpressing and KO rats, 300µL plasma were incubated with thrombin (SBI#EXOQ5TM-1) at the concentration of 5U/mL for 5 min at room temperature. Following centrifugation at 10,000rpm for 5 min at

4°C, the supernatant was transferred to a new EP tubes. Exosomes were isolated from 250μL plasma with ExoQuick Exosome Precipitation Solution (SB1#EXOQ20A-1) according to manufacturer's instructions. Exosome pellet was resuspended in 100μL PBS. Caenorhabditis elegans miR-39 (cel-miR-39) at a 50 pmol/L concentration was added as the spike-in control. The total exosomal RNA was isolated using miRNeasy Mini Kit (Qiagen, 217004). For quantitative miRNA analysis, the Bulge-Loop™ miRNA qRT-PCR Primer Sets (RiBoBio) were used for RT with iscript cDNA synthesis kit (BioRad#1708890) and miRNAs expression was detected by quantitative reverse transcription polymerase chain reactions (qRT-PCRs) with Takara TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara#RR420A) in the LightCycler® 480 System (Roche). Cel-miR-39 was used in as an endogenous control.

Cell culture media and mouse plasma EV isolation based on Size Exclusion Chromatography (SEC) through Izon

For EV isolation from plasma and cell culture media, SEC-based Izon Science Ltd°'s qEV Original 35nm column was utilized. For mouse plasma EV isolation, 500μL of plasma were loaded into the column, obtaining 12 fractions of 500μL/fraction. Adjacent fractions were pooled (1-2, 3-4, 5-6, 7-8, 9-10, 11-12) and concentrated using Amicon's Ultra-4 10KDa columns (EMD Millipore, UFC801024) spanned for 40 minutes at 5000rpm at RT. For isolating EVs from cell culture media of NRVMs, 20mL of conditioned media were pooled from 5 independent experiment and concentrated using Amicon's Ultra-15 10KDa columns (EMD Millipore, UFC901024) spanned for 40minutes at 5000rpm at RT. The concentration step resulted in 500μL solution of concentrated conditioned media, which was loaded in qEV Original 35nm columns, obtaining 12 fractions of 500μL/fraction.

Fractions 3-6, 7-10 and 11-13 were pooled and concentrated using Amicon's Ultra-4 10KDa columns (EMD Millipore, UFC801024) spanned for 40 minutes at 5000rpm at RT.

Western Blot for EVs isolated through SEC

The concentrated EV suspensions from plasma or cell culture media were lysed for protein extraction (1X Cell Lysis buffer – Cell Signaling Technology, 9803S-, 0.5% PMSF -Thermo Fisher Scientific, 36978-, 2% Protease and Phosphate Inhibitor (Thermo Fisher Scientific, 78441), in 1X PBS) for 1h rocking at 4°C and spanned down at maximum speed for 10 minutes. From the resulting suspension, the protein concentration was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227) and run for western blotting following the protocol detailed in the Supplemental Methods. The primary antibodies (1:1000) utilized for mouse plasma and NRVM culture media EVs are indicated in the Supplemental Methods.

RNA isolation and ddPCR of EVs isolated through SEC

For RNA isolation from concentrated exosomal fractions 7-10 derived from NRVMs the Norgen Biotek Corp. Exosomal RNA Isolation Kit (58000) was utilized following manufacturer's instructions. The resulting exosomal RNA (140ng RNA input) was reverse transcribed with Qiagen's miRCURY LNA RT Kit (339340) and copy numbers of mature mir-30d-5p were quantified through ddPCR (primer utilized hsa-miR-30d-5p LNA™ PCR primer set, UniRT, from Qiagen 339306/YP00206047): data is represented as fold versus normoxia.

Cell culture media EV-RNA isolation with exoRNeasy kit (Qiagen, 77044)

EVs from NRVM conditioned media were isolated, followed by vesicular RNA isolation, according to manufacturer's protocol (Qiagen, 77044). RT and qPCR reactions to determine miR-30d levels were performed as mentioned above for tissues and cells miRNAs.

EV isolation from cardiac tissue

EVs were isolated as previously described[20] with additional modifications. In brief, hearts were collected from mice 48h after surgery and perfused with ice-cold PBS (1x) prior to removing the atriums and right ventricle. The remaining heart tissue consisting of the left ventricle was submerged in 2mL ice-cold PBS (1x) and finely chopped on ice using fine sterile scissors. Samples were centrifuged at 400 x g for 15 minutes at 4°C and the supernatant was transferred to a new tube for another centrifugation at 2000 x g for 15 minutes at 4°C to remove any remaining cellular/tissue debris. The supernatant (~2mL) containing the EVs was carefully transferred to a new tube and filtered using 0.8µm membrane filter (Corning, CLS431221). For the extraction of RNA the exoRNeasy Serum/Plasma Midi (Qiagen, 77044) was used following the manufacturer's instructions.

DotBlot for detection of exosome markers in cardiac tissue EVs

Murine cardiac EV suspensions were subjected to a DotBlot to detect exosome markers. Briefly, 2µL of each sample set were loaded into a nitrocellulose membrane (BioRad, 1620168). Once samples were submerged and dried, the membrane was blocked for 1h with 5% BSA (Sigma Aldrich, A7906) in 1% TBST (Boston BioProducts, IBB-180X) for 1h at RT rocking. Primary antibody (1:1000, reference in Supplemental Methods) in 5% BSA in 1% TBST was incubated for 1h at RT rocking, followed by secondary antibody (1:5000)

in 1% TBST for 1h at RT rocking. Blots were developed with Femto SuperSignal (Thermo Fisher Scientific, 34095).

Nanoparticle Tracking Analysis

EV numbers and size distribution were measured by NanoSight LM10 system (Malvern Panalytical, Malvern, UK). In brief, EV samples were diluted 1:20 with filtered (0.22μm) ice-cold PBS (1x) to meet the instrument's optimal particles per frame. The pump was washed before and after each sample measurement with PBS (1x) to avoid any sample-to-sample contamination. All samples were measured twice, each time, three 20-second recordings were taken. The camera settings were adjusted according to the manufacturer's instructions and data were analyzed using the NanoSight NTA 2.3 software.

Nanoflow using Alexa 647 conjugated anti-miR-30d molecular beacon

Conditioned NRVM media and plasma from αMHC-MerCreMer - Rosa^{mTmG} mice (diluted 1/100) were incubated with 50nM of Alexa 647 conjugated anti-miR-30d molecular beacon (PNA Bio) at 37°C for 30min. Following the incubation, samples were run in a Beckman Coulter MoFlo XDP cytometer, and data was analyzed using FlowJo software. Data from a control experiment, showing the increase in signal of miR-30d-Alexa 647 in media from NRVMs transfected with miR-30d is shown in Supplemental Methods Figure 1A-B. Additionally, an SSC-FSC plot for beads of different sizes is shown in Supplemental Methods Figure I C, which was used as reference for gating on EVs ranging from 100-500nm.

Human plasma EV-RNA isolation and library preparation for EV-RNA sequencing

EV- RNA was isolated from 1 mL plasma from patients with heart failure and controls using the ExoRNeasy Serum/Plasma Midi kit (Qiagen, 77044). Libraries were constructed and amplified from approximately 3 ng RNA using the NEXTflex small RNA-Seq Kit V3 for Illumina Platforms (Bioo Scientific, a PerkinElmer company; 5132-06). Size selection of libraries was performed by gel electrophoresis on a 10% TBE gel (Invitrogen, EC6275BOX) with excision of the 140 to 160 nucleotide bands (corresponding to 21–40nt RNA fragments). Libraries were sequenced on an Illumina HiSeq platform for single read 50 cycles at the NextGen Sequencing Core at Massachusetts General Hospital.

Human plasma EV-RNA Sequencing analysis

We utilized the exceRpt analysis pipeline [51] that is composed of a cascade of serial computational filters and alignments as our analytical core. First, the 3' adapter sequences were removed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html), sequence reads with consistently low-quality reads were removed. Quality reads were mapped using STAR [45] against NCBI's UniVec/Vecscreen database to filter out common laboratory contaminants as well as all primary endogenous ribosomal RNAs (5S, 5.8S, 18S, 28S, and 45S). The remaining reads were then aligned to the host genome and transcriptome. Transcript abundance are calculated using both raw read counts and normalized reads per million (RPM). Mapped transcripts are then annotated with miRBase.

Western blotting

Murine heart lysates were prepared by digesting the tissues in 1X lysis buffer, protease and phosphatase inhibitor and PMSF (Keygen) in a Qiagen Tissue Lyser for 8min at 50rpm at 4°C, rocked for 1h at 4°C and sonicated. The Pierce™ BCA protein assay

(Thermo Fisher Scientific, 23227) or Takara's BCA protein assay was performed to quantify lysates' protein concentration, and 20µg of each were used for 4-20% or 10% SDS-PAGE electrophoresis. Gels were transferred to PVDF or nitrocellulose membranes (Millipore or BioRad) and blocked with 5%BSA (Sigma) for 1h at RT. Primary antibodies were incubated ON at 4°C rocking at a 1:1000 concentration. The primary antibodies used are indicated in western blot antibodies section below. Secondary HRP-antibodies (Agilent Cat# P0448, RRID:AB_2617138, Agilent Cat# P0447, RRID:AB_2617137) were incubated for 1h at RT rocking. Blots were developed using the Supersignal Femto developer (Thermo Scientific, 34095) or High-sig ECL Western Blotting Substrate (Tanon, 180-5001).

CM isolation and non-CM cell sorting after IR

10-week-old C57BL/6J (Jackson Lab, 000664) male mice were subjected to IR surgery, and hearts were harvested for cardiac resident cell isolation 24 and 72h post-surgery. Briefly, CM were first isolated using the Langendorff perfusion apparatus, by perfusing the cannulated heart (perfusion buffer: 13.7mM NaCl, 0.4mM KCl, 0.1mM MgCl, 1mM HEPES, 0,033mM NaH_2PO_4, 1mM Glucose, 0.5mM Taurine), followed by enzymatic digestion with perfusion buffer containing Collagenase D (Worthington Biochemical, Cat. #LS004189), Collagenase B (Worthington Biochemical, Cat. #LS004177) and Protease XIV (Sigma-Aldrich, Cat. #P5147). Once digested, the hearts were minced, and the CM and non-CM were isolated. The cardiac cell suspension was filtered through a 250µM tissue strainer (Thermo Fisher,87791), and the CM were collected by gravity, while non-CM cells were collected from the supernatant.

For non-CM cell sorting, the CM-free cell suspension was washed in 1xPBS+ 1% BSA + 1mM EDTA, followed by staining for live/dead cells using the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit, for UV excitation (Thermo Fisher Scientific, L34961), in a 1:1000 dilution in 1xPBS+ 1% BSA+ 1:200 Fc Block (BD Biosciences, 553142) for 30 minutes light protected. Cells were then washed twice with 1xPBS+ 1% BSA + 1mM EDTA and stained for non-CM cell specific markers for 40 minutes in ice light protected: Anti-CD31-PE (BioLegend, 102408, 1:200), anti-CD45-APC/Cy7 (BioLegend, 103116, 1:200) and Anti-MEF-SK4-APC (Miltenyi Biotec, 130-102-900, 1:50). Stained cells were washed twice and sorted with a FACSAriaII sorter. CD31+MEF-SK4-CD45- cells were considered endothelial cells, CD31-MEF-SK4+CD45- cells were considered cardiac fibroblasts, CD45+CD31-MEF-SK4- were considered leukocytes and CD31-MEF-SK4-CD45- were classified as other (all cardiac cells except CM, endothelial cells, fibroblasts and leukocytes). Flow cytometry data was analyzed using FlowJo software.

CM, as well as sorted non-CM cells, were lysed in Trizol (Thermo Fisher Scientific, 15596018) for RNA isolation and analysis as described above. The primer sequences utilized to determine the purity of each cardiac cell type are indicated in the Supplemental Methods. mRNA quantification was normalized to the housekeeping gene β-Actin and represented as fold change ($2^{-\Delta\Delta^{Ct}}$) versus the main cell type expressing the quantified gene in each case.

<u>AGO2-immunoprecipitation in human plasma or conditional cell culture medium from NRVMs</u>

Antibody biotinylation: AGO2 (Abcam, Cat#ab57113 for immunoprecipitating human AGO2; Sigma, Cat#SAB4200085 for immunoprecipitating rat AGO2) antibodies were desalted using the Zeba spin desalting column (Thermo Fisher Scientific, Cat#89882) to remove sodium azide and biotinylated using the EZ-Link™ Sulfo-NHS-LC-Biotin reagent (Thermo Fisher Scientific, Cat# A39257) by following the manufacturer's manual. The excess free biotin was removed by filtering the biotinylated antibody mixture through another Zeba spin desalting column and the concentration of biotinylated antibody was measured using Nanodrop UV spectrophotometer.

Magnetic beads preparation: Dynabeads MyOne Streptavidin C1 (Invitrogen, Cat#65001) suspension was washed three time with 1 volume 0.01% Tween-20/PBS. For blocking, the beads were washed 2 times with 1 volume 0.1% BSA/PBS and resuspended with 1 volume of 0.1% BSA/PBS.

Immunoprecipitation: Cell culture medium was concentrated using Amicon® Ultra-15 Centrifugal Filter Units – 10KDa (Sigma, Cat#UFC901008) prior to use.

1ml human plasma or concentrated cell culture medium was diluted 1:1 with double filtered 1X PBS and was incubated with 6 μg biotinylated antibody for 20 min at RT with rotation. Then, 39 μL of blocked Dynabeads was added to the mixture and rotated for 25 min at RT. The mixture was then washed three times with 0.1% BSA and subjected to small RNA extraction or protein isolation.

Lipoprotein fractionation from human plasma and conditional cell culture medium from NRVMs

The density of cell-free human plasma or concentrated cell culture medium was adjusted to 1.063 g/mL with KBr and centrifuged at 100,000 g for 20 h at 4 ℃ and the top 20%

fraction containing LDL/VLDL was collected. The remaining material was adjusted to 1.21 g/mL with KBr, and after centrifugation for another 20 h using the same parameters, the top 20% fraction containing HDL was collected. The collected LDL/VLDL fraction and HDL fraction were then subjected to either small RNA isolation or protein extraction.

RNA extraction from Dynabeads, EV and Lipoprotein fractions: RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Cat# 217184) following manufacturer's protocol. In brief, the Dynabeads resuspended in 200 ul PBS, lipoprotein fractions (400 ul) and combined EV fractions (~250 ul after Amicon 4 10kDa concentration) were subjected to phenol/chloroform extraction step for RNA extraction using Qiazol Lysis Reagent (Qiagen, Cat#79306) followed by chloroform. The aqueous phase was used as input into the miRNeasy procedure and the RNA was eluted in 14 mL of nuclease-free water. The eluted RNA was subjected to reverse transcription (8 ul RNA input in 20 ul reaction volume) using the miRCURY LNA RT Kit (Qiagen, Cat# 339340). miR-30d levels were assessed using ddPCR as described before.

Protein Extraction: Dot blot was performed on the Ago2, lipoprotein and EV fractions from the pooled plasma. 100 ul RIPA buffer (Thermo Fisher Scientific cat# 89900) with Halt™ Protease and Phosphatase Inhibitor Cocktail (1X, Thermo Fisher Scientific cat# 78440) was added to Ago2 Dynabeads, lipoprotein fractions (~200 ul after Amicon 4 10 KDa concentration) and combined EV fractions (~250 ul). Protein lysate samples (5 ul) were spotted onto the nitrocellulose membrane. The membrane was let dry for 1hr and then was blocked by soaking in 5% BSA in TBS-T for 1 hour at room temperature. The membrane was incubated with the following primary antibodies overnight at 4^{o} C: Ago2 (Sigma-Aldrich, cat# SAB4200085, at 0.5 ug/mL dilution), Apolipoprotein B/ApoB

Antibody (Novus Biological, cat# NB200-527, 1:1000), ApoE (Santa Cruz, cat# sc-13521, 1:200), ApoA1 (Thermo Fisher Scientific cat# MA515391, 1:500), Alix (Biolegend, cat# 634501, 1:250). Secondary HRP-antibodies (Dako) were incubated for 1h at RT rocking. Blots were developed using the Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific cat# 34095).

qRT-PCR Primers:

map4k4 (mouse, rat): Forward 5'-GAAGAGAGGCGAGAAAGATGAG-3', Reverse 5'-GCACATTGACGATGGAACTTG -3',

itga5 (mouse, rat): Forward 5'- GGCCAGCTCCGTCTATGATG-3', Reverse 5'-TGGATGTCTGAGCCATTAAGGA-3';

actb (mouse): Forward 5'- GTGACGTTGACATCCGTAAAGA - 3', Reverse 5' - GCCGGACTCATCGTACTCC - 3'.

Tnt (mouse): Forward 5'- GTAGAAGAGGTTGGTCCTGATG -3', Reverse 5'-CTTCCTCCTTCTTCCTGTTCTC -3'

Pecam1 (mouse): Forward 5'- GCTCTCGAAGCCCAGTATTT -3', Reverse 5'-TTCTCGCTGTTGGAGTTCAG -3'.

Cd45 (mouse): Forward 5'- GCAAGTGGAGGCACAGTATATC-3', Reverse 5'-GCTCACTCTCTTTGCTCATCTC -3'.

Postn (mouse): Forward 5'- GTCGTGGACAAACTCCTCTATC-3', Reverse 5'-CACCGTTTCGCCTTCTTTAATC -3'.

18S: Forward 5'- GACAGGATTGACAGATTGATAG-3', Reverse 5'- AATCGCTCCACCAACTAA -3'.

Postn (rat): Forward 5'- CGGCTATATGAGAATGGAAGGG-3', Reverse 5'- CACCGTTTCGCCTTCTTTAATC -3'.

FN1 (rat): Forward 5'- CGAGGTGACAGAGACCACAA-3', Reverse 5'- CTGGAGTCAAGCCAGACACA -3'.

Ctgf (rat): Forward 5'- GACCCAACTATGATGCGAGCC-3', Reverse 5'- CCCATCCCACAGGTCTTAGAAC -3'.

Acta2 (rat): Forward 5'- ACCATCGGGAATGAACGCTT-3', Reverse 5'- CTGTCAGCAATGCCTGGGTA -3'. Western Blot antibodies:

For *in vivo* and *in vitro* rodent experiments:

- BAX (Cell Signaling Technology Cat# 2772, RRID:AB_10695870 or ABclonal Cat# A11931, RRID:AB_2758874)
- BCL-2 (Santa Cruz Biotechnology Cat# sc-7382, RRID:AB_626736 or ABclonal Cat# A11025, RRID:AB_2758373)
- Cleaved Caspase-3 (Cell Signaling Technology Cat# 9664, RRID:AB_2070042)
- Caspase-3 (Cell Signaling Technology Cat# 9662, RRID:AB_331439), MAP4K4 (Proteintech Group Cat# 55247-1-AP, RRID:AB_10836939)
- Integrin α5 (Santa Cruz Biotechnology Cat# sc-166665, RRID:AB_2280538)
- TGFβ1 (Thermo Fisher Scientific Cat# MA5-15065, RRID:AB_10984297 or Cell Signaling Technology Cat# 3711, RRID:AB_2063354)

- p-Smad2/3 (Cell Signaling Technology Cat# 8828, RRID:AB_2631089)
- Smad2/3 (Cell Signaling Technology Cat# 8685, RRID:AB_10889933)
- α-SMA (Sigma-Aldrich Cat# A2547, RRID:AB_476701),
- GAPDH (Cell Signaling Technology Cat# 2118, RRID:AB_561053)

Plasma EVs:

- Alix (BioLegend, Cat. # 634501)
- CD9 (BioLegend, Cat. # 124802)
- Cardiac Troponin T (Thermo Fisher Scientific, Cat. # MA5-12960)
- Cre (Sigma Millipore, Cat. # 69050)
- Cre (Sigma Aldrich, Cat. # C7988)
- GFP (Biomatik, Cat. # CAU29134).

NRVM culture media EVs:

- Alix (Cell Signaling Technology, Cat. # 92880S)
- CD9 (Novus Biologicals, Cat. # NBP2-67310)
- CD63 (Novus Biologicals, NBP2-68077)
- Cardiac Troponin T (Thermo Fisher Scientific, Cat. # MA5-12960).

<u>Dot Blot:</u>

- Alix (BioLegend, Cat. # 634501)
- CD9 (BioLegend, Cat. # 124802)
- CD63 (Millipore-Sigma, Cat. #SAB4301607)
- Cardiac Troponin T (Thermo Fisher Scientific, Cat. # MA5-12960)

- Grp94 (Cell Signaling Technology, Cat. # 2104T)
- Calnexin (Novus Biologicals, Cat. #NB300-518)
- Ago2 (Sigma-Aldrich, Cat# SAB4200085)
- Apolipoprotein B/ApoB Antibody (Novus Biological, Cat# NB200-527)
- ApoE (Santa Cruz, Cat# sc-13521)
- ApoA1 (Thermo Fisher Scientific, Cat# MA515391)

Tissue miRNA Atlas: samples preparation and small RNA library

RNA Isolation: fresh frozen tissue was sliced into pieces that were no greater than 0.5mm in thickness on dry ice to maintain integrity and placed in 1mL of RNA Later ICE. The RNA later-Ice immersed tissue was incubated for at least 16 hours at -20C to allow for absorption. The tissue was immediately placed in a glass 16 mm x 100 mm Covaris tube and mixed with 400 uL of lysis/binding buffer from the mirVana miRNA Isolation Kit (ThermoFisher Scientific, Cat. # AM1560). The Covaris was set to treat each sample at a peak power of 452, a duty factor of 17.4, and cycles/burst of 280. Each sample was then treated for 15-30 seconds in a Covaris S220 sonicator depending if the tissue was visibly lysed. The lysate was transferred to a new pre-chilled 2mL Eppendorf tube with 40uL of miRNA homogenate solution, mixed gently with flicking and incubated on ice for 3-5 minutes. Total RNA was extracted according to manufacturer's protocol with the Total exosome RNA and protein isolation kit (ThermoFisher Scientific, Cat# 4478545). RNA was eluted using 100uL of 95C ultrapure water. Total RNA was treated with DNase, Turbo DNA-free kit (ThermoFisher Scientific Cat. #AM1907). DNA free Total RNA was cleaned

and concentrated using Zymo clean and concentrator kit (Zymo Research, Cat. #R1016) and eluted in 28ul of ultrapure water.

Perkin Elmer Small RNA Library Preparation: small RNA libraries were generated using NEXTflex Small RNA Library Prep Kit v3 following the manufacturer's instructions with 16 cycles of PCR amplification and 100% adapter. Following PCR amplification, libraries between 140 and 160bp in size were gel purified using 6% TBE gels followed by ethanol precipitation and resuspension in 11ul of ultra-pure water.

Small RNA library QC and Pooling: gel purified libraries were quantified using Agilent 2100 Bioanalyzer with High-Sense DNA chips. Equimolar amounts of libraries were pooled and quantified by Bioanalyzer with High-Sense DNA chips. Pooled libraries were normalized and denatured at a working concentration range of 6-8pM with 5% PhiX spike-in for flow cell cluster generation.

Analysis: fastq files were run through the excerpt small RNA pipeline[51], miRNA counts were normalized in R using DESeq2 and mean ± SD were plotted per tissue.

PEARL-HF Study Population

The PEARL trial was a single-center, prospective prognostic and echocardiographic study of chronic HF under medical and device therapy, led by Drs. Das and Shah, NCT02632656). The primary aim of the PEARL-HF study was to test the hypothesis that circulating extracellular RNAs were associated with echocardiographic and clinical outcomes related to heart failure. For this research, we selected 18 participants based on availability of adequate plasma for EV isolation. Standard clinical and demographic characteristics were measured via questionnaire and physical examination at study enrollment. Venous blood was drawn via peripheral venipuncture, centrifuged (2000g x

10 min x 2), and stored at −80°C for future analysis. Our local MGH institutional review board approved all study procedures, and all subjects gave written informed consent before enrollment.

Human studies in Framingham Heart Study

Whole blood mRNA expression profiling was performed as previously noted in 2446 Framingham Heart Study (FHS) Offspring cohort participants (using the Affymetrix exon array ST 1.0) [52]. A total of 17,318 transcripts (mRNAs) were included, and a "robust multichip analysis" (RMA) algorithm via Affymetrix Power Tools (APT) was used to arrive at signal values (e.g., log-2 transformed expression intensity) for the purposes of normalization. Plasma circulating miRNA expression analysis has previously been described by our group [53]. In cases where whole blood mRNA and plasma circulating miRNA were both available, co-expression association analyses were conducted (N=2295) using linear mixed effects models (package *lmekin in R)* [54]. mRNA was the dependent variable, and miR-30d-5p was the independent variable, with further adjustments for age, sex, technical aspects of mRNA profiling [55], imputed cell counts [55], and family structure. A false discovery rate (FDR) of 10% (Benjamini-Hochberg) was used for statistical significance to identify mRNAs associated with miR-30d-5p level to carry forward to enrichment analysis.

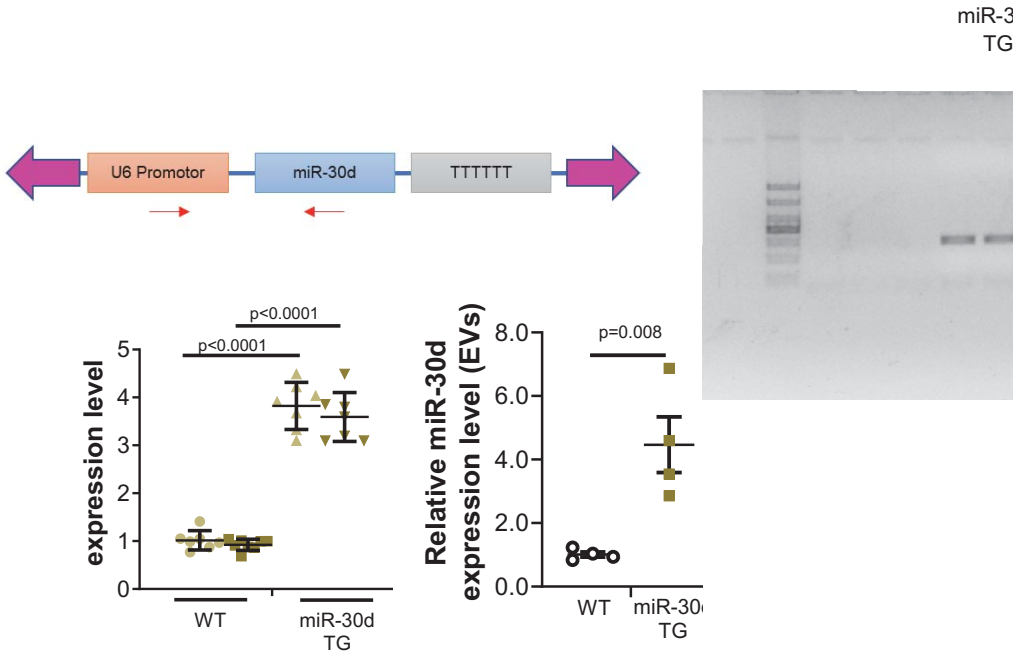
Enrichment Analysis

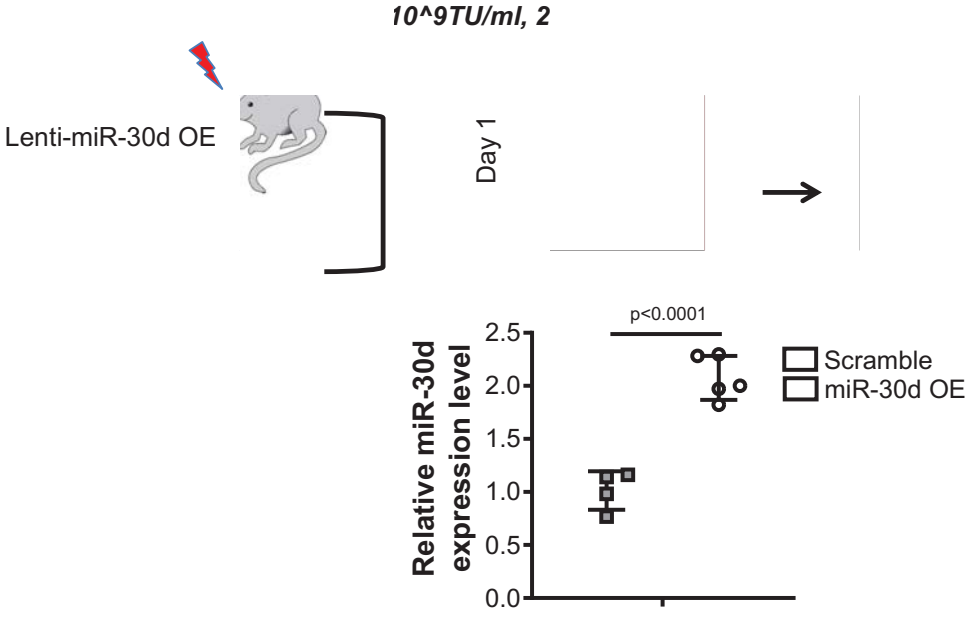
A relaxed FDR threshold of 10% was used to define a set of 72 genes with HGNC symbols. Functional enrichment analysis was performed on these 72 genes using Enrichr [56]. Results from Gene Ontology: Biological Process, Reactome [57] and WikiPathways [58]

were exported and selected high-scoring terms (i.e., -log(Pvalue)*Zscore) were combined into a single barplot visualization in R.

Statistical Analysis: Data are expressed as the mean ± SD. Normality of the data was assessed with Shapiro-Wilks test. In data sets with normal distribution, statistical analyses were done by parametric unpaired *t* test or by parametric ANOVA corrected using Tukey's multiple comparison test. Correction for multiple hypothesis testing was done for each individual hypothesis, but not across the entire data set which may be a limitation. In data sets that did not pass the normality test, statistical analyses were performed by unpaired non-parametric Mann Whitney *t* test or by non-parametric ANOVA test corrected using Dunn's multiple comparison test. Statistical analyses were done using GraphPad Prism software (GraphPad). P values of each statistical comparison are indicated in each graph, and the statistical analysis used for P value calculation are indicated in the respective figure legend.

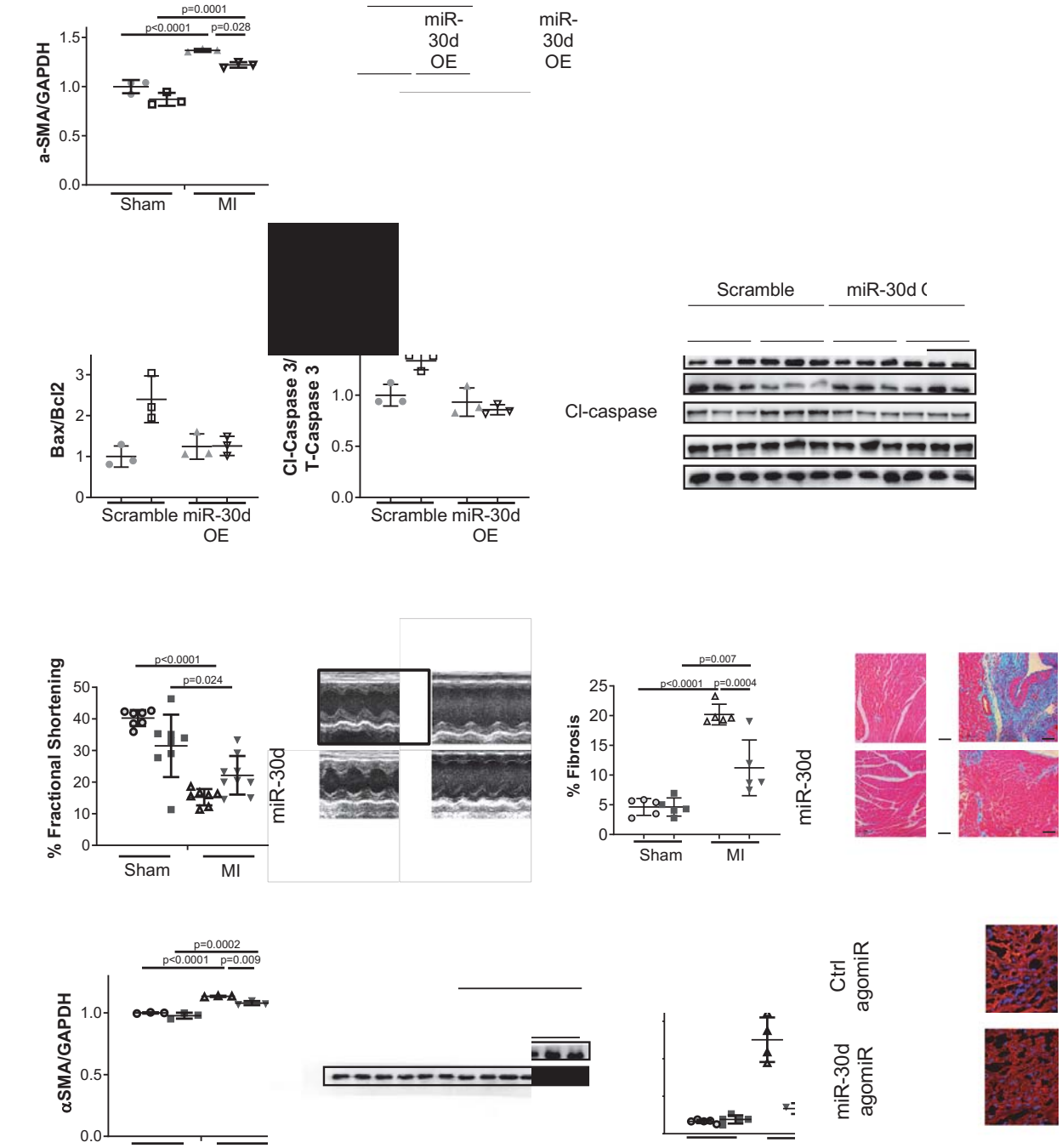
Supplemental Figure I





Supplemental Figure I. Generation of transgenic rat overexpressing miR-30d and experimental design of mice treated with miR-30d overexpressing lentivirus. A. Genetic constructs of miR-30d overexpressing (OE) male rats. **B.** Agarose gel electrophoresis, where the band corresponding to the miR-30d overexpressing insert sequence is shown (flanked by red arrows in A). **C.** On the left, relative mature miR-30d expression level quantification in the heart of WT and miR-30d overexpressing transgenic rats 3 weeks after MI surgery; a specific Bulge-Loop primer for mature miR-30d was first used for cDNA transcription, followed by PCR using specific primers to quantify mature miR-30d. Data was normalized to 5S rRNA. WT Sham n= 7, WT MI n=8, miR-30d TG Sham n=7, miR-30d TG MI n=7. Data normality determined with Shapiro-Wilk test. P values calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. On the right, miR-30d expression in plasma exosomes was quantified in WT and miR-30d overexpressing transgenic rats. Cel-miR-39 spike-in was used as an endogenous control. WT=4, miR-30d TG= 4. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. **D.** Experimental design of mice treated with miR-30d overexpressing Lentivirus or Scramble Lentivirus, undergoing MI or Sham surgeries. Bar graph shows relative miR-30d expression level quantification in the heart of Scramble or miR-30d overexpressing lentivirus treated mice. Scramble lentivirus n= 4, miR-30d OE lentivirus n=5. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. Data are represented as mean ± SD.

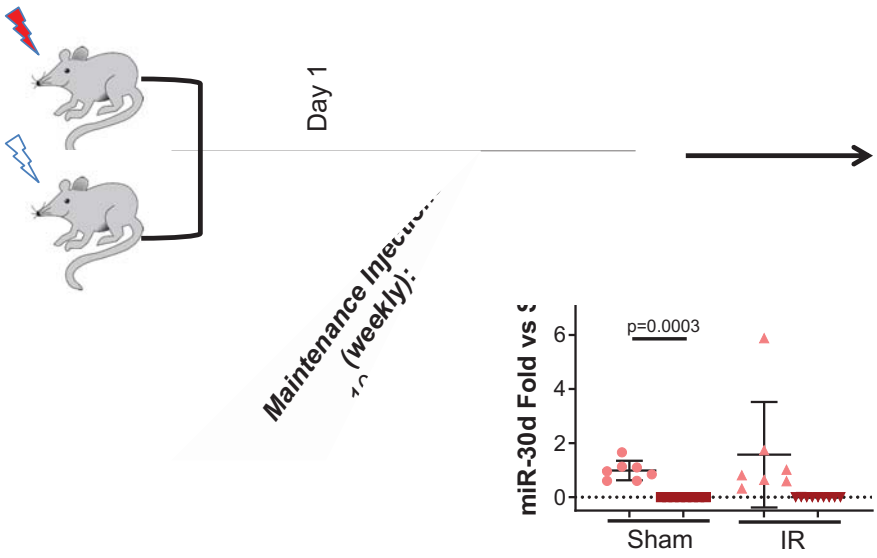
Supplemental Figure II

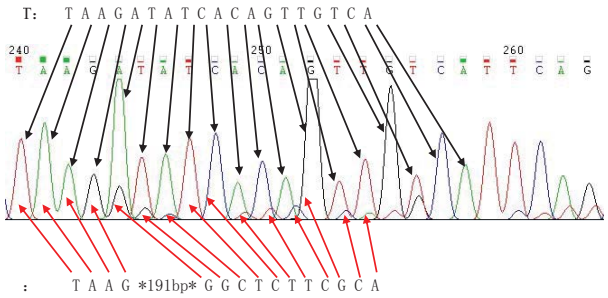


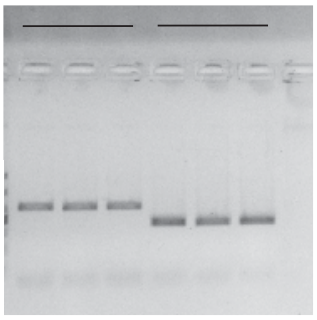
Supplemental Figure II. Lentivirus and AgomiR mediated miR-30d overexpression results on improved cardiac remodeling phenotype after MI. (A-B) C57BL/6 mice treated with 10^9TU/mL miR-30d overexpressing lentivirus or scramble lentivirus, were subjected to Sham or MI surgery and their cardiac remodeling phenotype was analyzed. **A.** Expression of α-SMA, marker or fibroblast to myofibroblast transition, was determined through western blot; quantification of fold change vs scramble lentivirus is graphed on the left and representative blot is shown on the right. Sham scramble lentivirus n=3, Sham miR-30d OE lentivirus n=3, MI scramble lentivirus n=3, MI miR-30d OE lentivirus n=3. **B.** Protein levels of key molecules regulating apoptotic pathways were quantified through western blotting for Bax, Bcl2, cleaved-Caspase 3 and total Caspase 3; data is graphed on the left as fold change vs scramble lentivirus and representative blots are shown on the right. Sham scramble lentivirus n=3, Sham miR-30d OE lentivirus n=3, MI scramble lentivirus n=3, MI miR-30d OE lentivirus n=3. **(C-F)** 10-week-old C57BL/6 mice were treated iv with either 20nmol miR-30d agomiR or control agomiR for three consecutive days through tail vein injection, followed by MI surgery on the fourth day. 4 weeks after the surgery, cardiac function was determined through non-invasive echocardiography prior to euthanasia and organ harvest. **C.** Cardiac function determined by percent Fractional Shortening; quantification is graphed on the left and representative M-mode images are shown on the right. Sham Control agomiR n=7, Sham miR-30d agomiR n=8, MI Control agomiR n=7, MI miR-30d agomiR n=9. **D.** Collagen deposition determined by staining heart sections for Masson's Trichrome; results are graphed on the left and representative bright field images are shown on the right. Sham Control agomiR n=5, Sham miR-30d agomiR n=5, MI Control agomiR n=5, MI miR-30d agomiR n=5. **E.** α-SMA

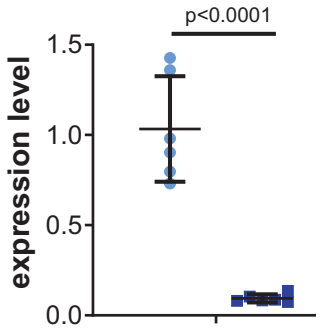
protein expression was determined by western blot, as a marker of fibroblast to myofibroblast transition. Quantification in graphed on the left, and representative blot is shown in the right. Note that the blot in Fig 6B for TGF-β and GAPDH was reprobed for α-SMA and GAPDH in this blot. Sham Control agomiR n=3, Sham miR-30d agomiR n=3, MI Control agomiR n=3, MI miR-30d agomiR n=3. **F.** Cardiac myocyte apoptosis determined by TUNEL and α-actinin co-staining of cardiac cross-sections, is quantified as % of TUNEL+ cardiac myocytes per field; data is graphed on the left and representative pictures are shown on the right. White arrows point to representative apoptotic cardiac myocytes. Scale bar 25μM. Sham Control agomiR n=5, Sham miR-30d agomiR n=5, MI Control agomiR n=4, MI miR-30d agomiR n=5. Data normality determined with Shapiro-Wilk test. P values calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. Data are represented as mean ± SD.

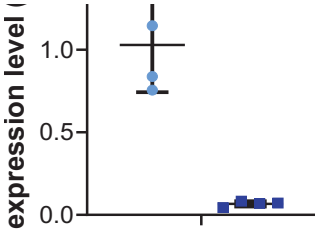
Supplemental Figure III





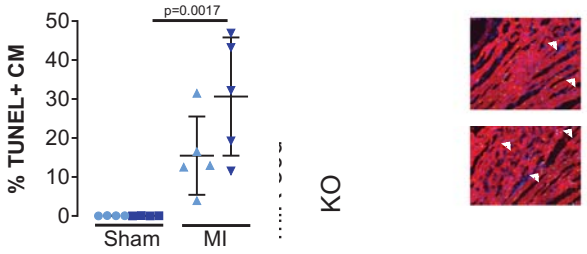






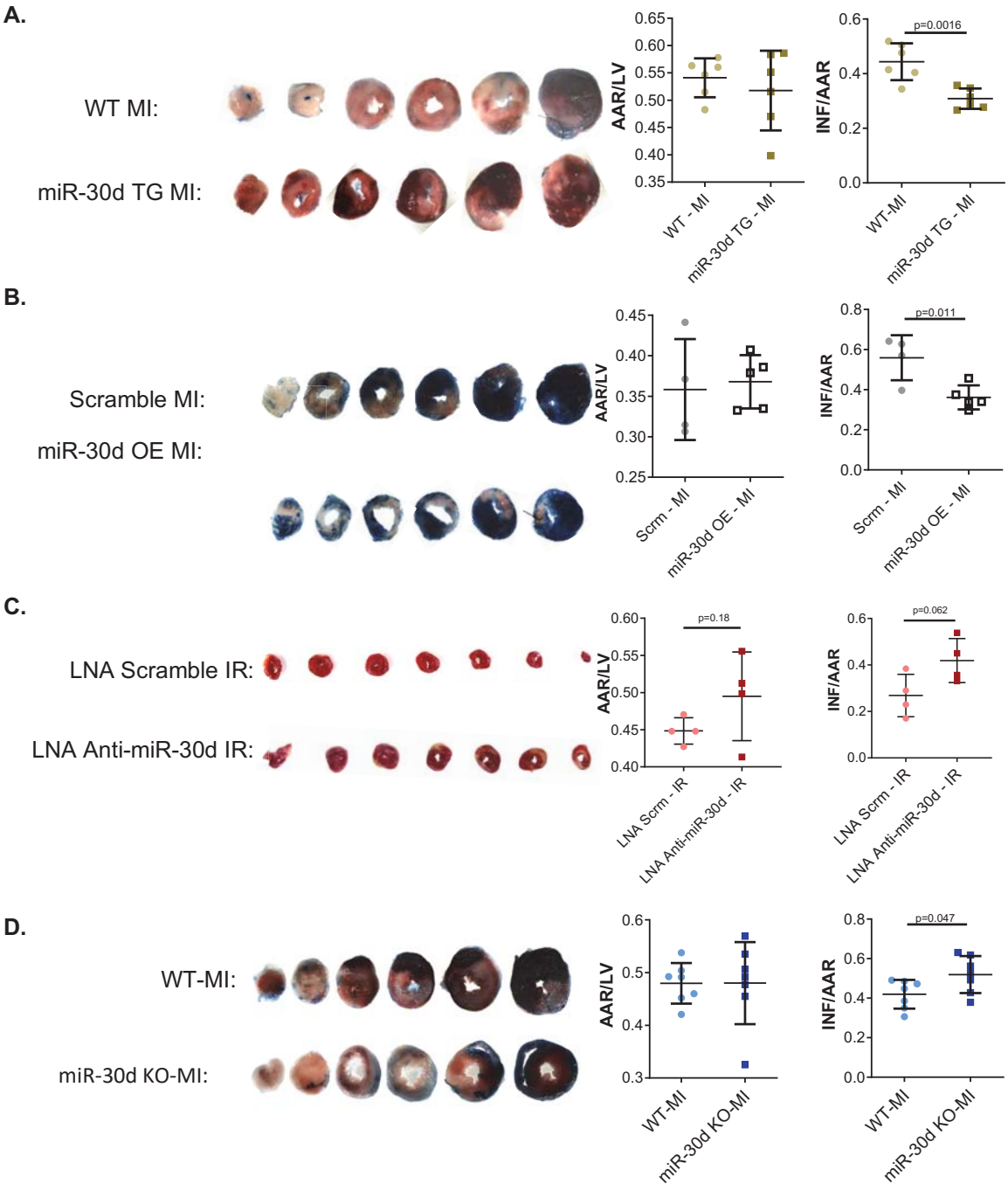
Supplemental Figure III. Experimental design of mice treated with LNA Anti-miR-30d and generation of transgenic miR-30d knock out (KO) rat. A. LNA mediated miR-30d loss of function experiments schematic. Loading 25mg/kg ip doses of LNA were given for three consecutive days, followed by maintenance 10 mg/kg ip doses. Sham or IR surgeries were performed on day 10 from the first LNA dose. The bar graph represents the cardiac miR-30d levels determined by qPCR at the time of harvest. Sham LNA Scrm n=7, Sham LNA Anti-miR-30d n= 8, IR LNA Scrm n= 7, IR LNA Anti-miR-30d n=9. P value calculated with unpaired non-parametric Mann Whitey t-test. **B-D.** Genetic approach for generating miR-30d KO rats in which **(B-C)** Genomic WT (top) sequence and KO (bottom) sequence with a 191 base deletion that corresponds to the entire miR-30d sequence are shown. **(D)** On the left, quantification of relative miR-30d levels in the heart of KO rats as compared to WT rats. WT n= 6, miR-30d KO n=6. On the right, miR-30d expression in plasma exosomes was quantified in WT and miR-30d KO rats. Cel-miR-39 spike-in was used as an endogenous control. WT n= 4, miR-30d KO n=4. Data normality determined with Shapiro-Wilk test. P values calculated with unpaired parametric t-test. Data are represented as mean ± SD.

Supplemental Figure IV



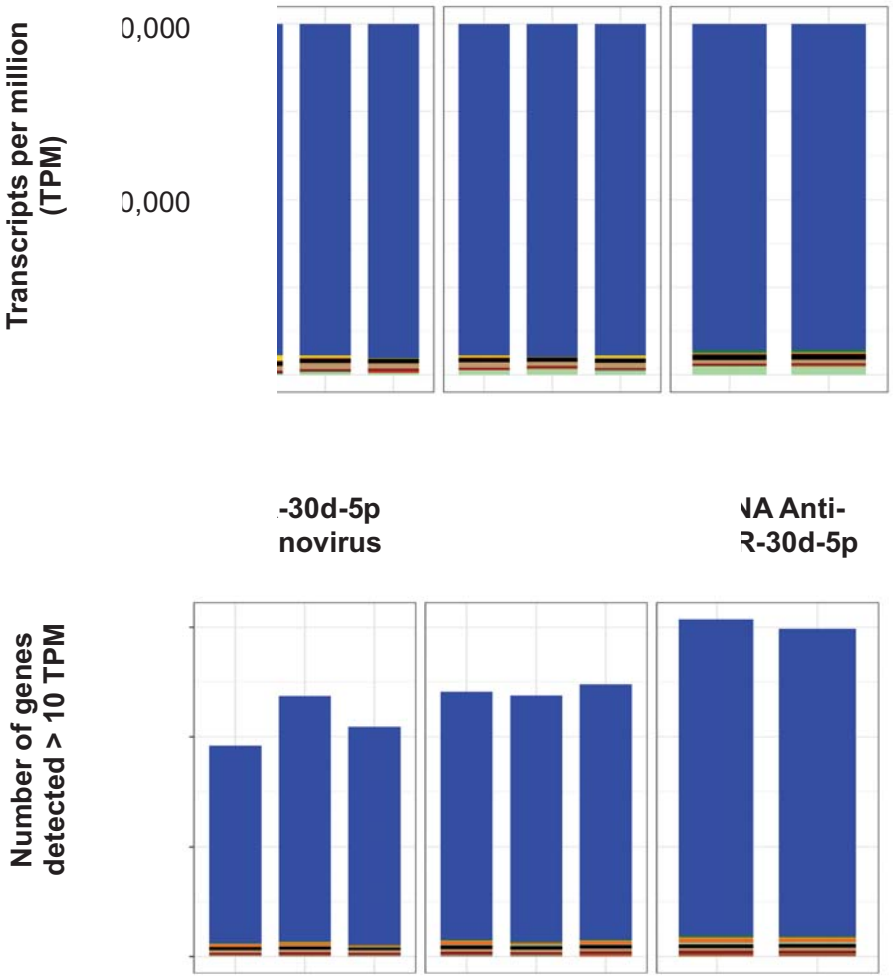
Supplemental Figure IV. CM apoptosis in miR-30d KO rats after MI. CM apoptosis determined 24 hours after surgery by TUNEL and α-actinin co-staining of heart cross-sections, is quantified as % of TUNEL+ CM per field; data is graphed on the left and representative pictures are shown on the right. Arrows point to representative apoptotic CM. WT Sham n=4, miR-30d KO Sham n=4, WT MI n=5, miR-30d KO MI n=5. Data normality determined with Shapiro-Wilk test. P value calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. Data are represented as mean ± SD.

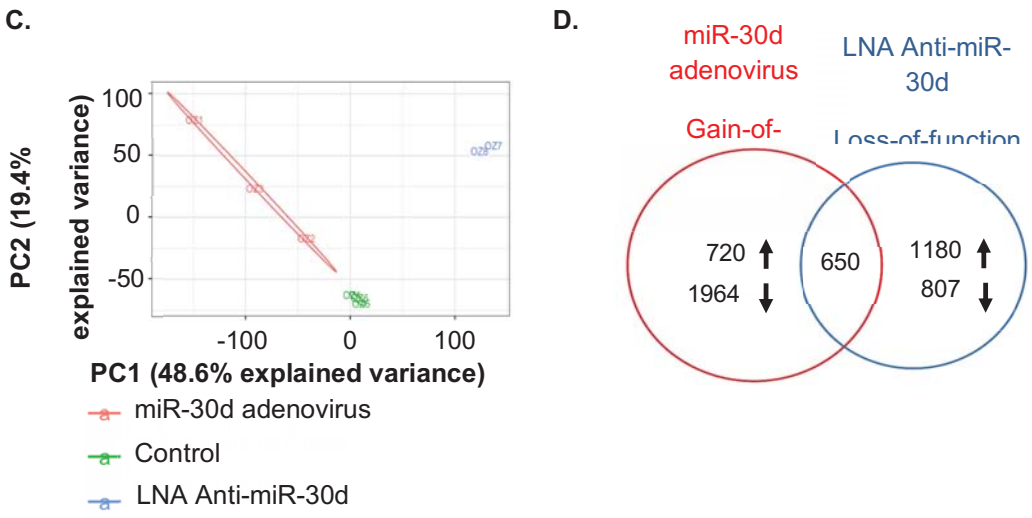
Supplemental Figure V



Supplemental Figure V. Infarct size (INF) relative to Area at Risk (AAR) determined by staining cardiac cross-sections with TTC (triphenyltetrazolium chloride) 24h post-MI or IR surgery. A-B. Representative infarct size pictures of **(A)** miR-30d overexpressing transgenic rats (WT MI n=6, miR-30d TG MI n=6) and **(B)** Lentivirus mediated miR-30d overexpressing mice (Scrm MI n=4, miR-30d OE MI n=5) are shown on the left and INF/AAR quantification on the right. **C-D.** Representative infarct size pictures of **(C)** LNA mediated anti-miR-30d loss of function mice (LNA Scrm IR n=4, LNA Anti-miR-30d IR n=4) and **(D)** miR-30d KO rats (WT MI n=7, miR-30d KO MI n=7) are shown on the left and INF/AAR quantification on the right. Data normality determined with Shapiro-Wilk test. P values calculated with unpaired parametric t-test. Data are represented as mean ± SD.

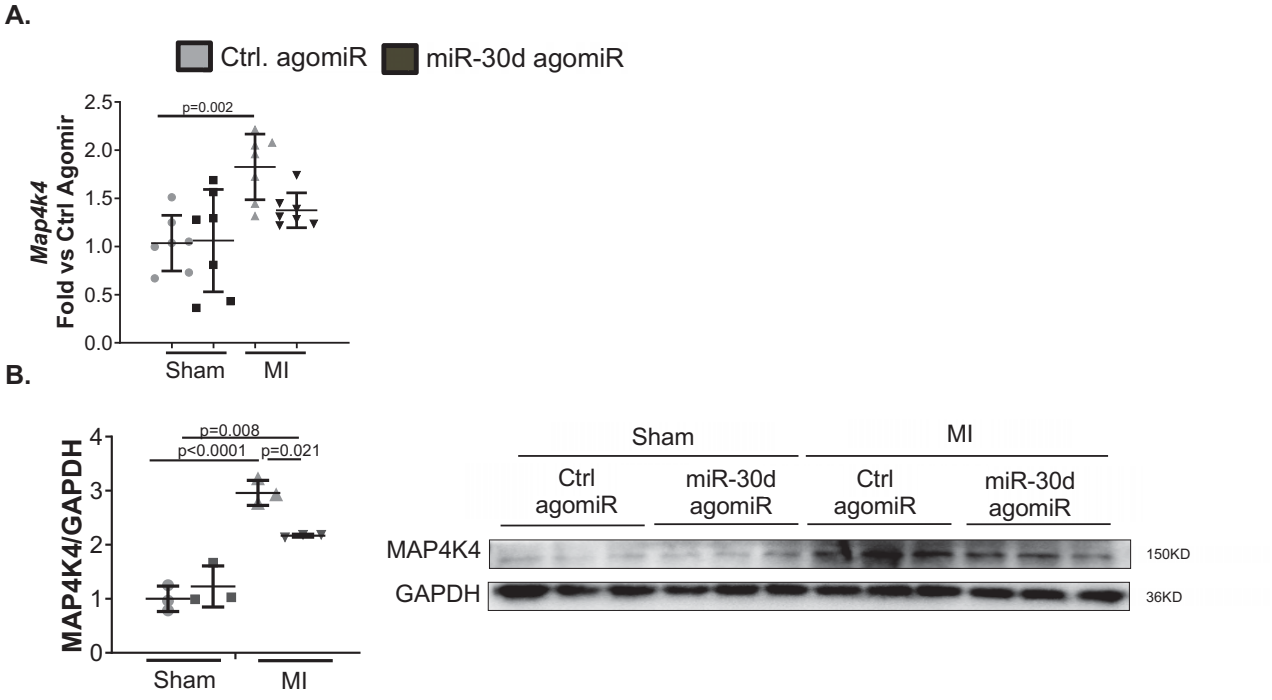
Supplemental Figure VI





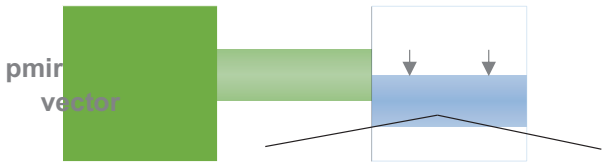
Supplemental Figure VI. Distribution of reads and genes detected by RNA sequencing. A. Stacked bar plot representing the distribution of total reads aligned to the rat genome belonging to various RNA biotypes. **B.** Stacked bar plot displaying number of genes detected having at least 10 TPM belonging to various RNA biotypes. **C-D.** Neonatal Rat Ventricular Myocytes (NRVMs) were transfected with miR-30d overexpressing adenovirus or LNA anti-miR-30d, and their RNA was sequenced in order to determine pathways regulated by it. (**C)** Principal Components Analysis showing the difference in overall gene expression profiles of the transfected NRVMs. (**D)** Venn Diagram showing the number of genes significantly dysregulated (abs(log_2FC)>=1 and p_{adj}<0.05) between the miR-30d overexpression and loss-of-function experiments.

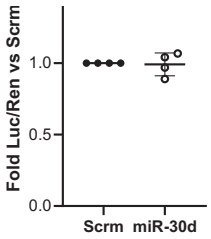
Supplemental Figure VII

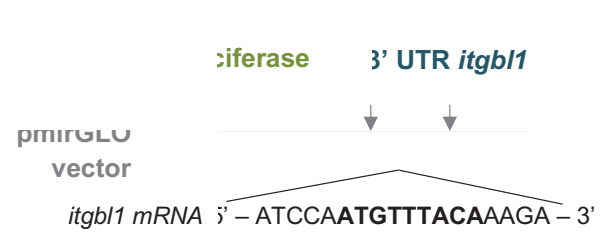


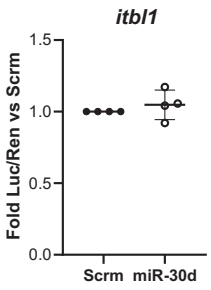
Supplemental Figure VII. AgomiR mediated miR-30d overexpression results on decreased MAP4K4 expression after MI. A-B. (A) mRNA (Sham Ctrl agomiR n=7, Sham miR-30d agomiR n=7, MI Ctrl agomiR n=7, miR miR-30d agomiR n=7) and **(B)** protein (Sham Ctrl agomiR n=3, Sham miR-30d agomiR n=3, MI Ctrl agomiR n=3, miR miR-30d agomiR n=3) expression of *Map4k4* in the heart of mice treated with miR-30d or control agomiR undergoing MI surgery. Data normality determined with Shapiro-Wilk test. P value calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. Data are represented as mean ± SD.

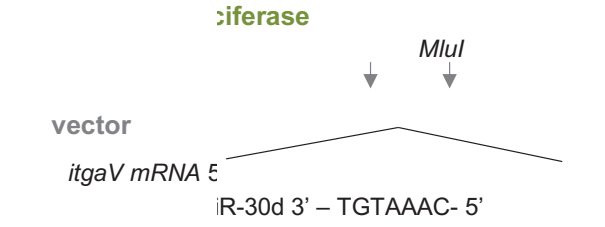
Supplemental Figure VIII

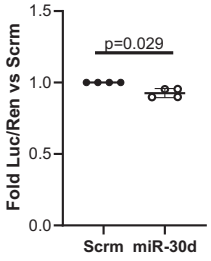




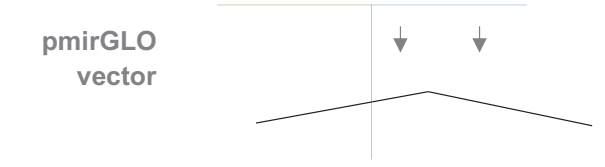


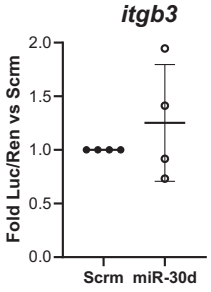






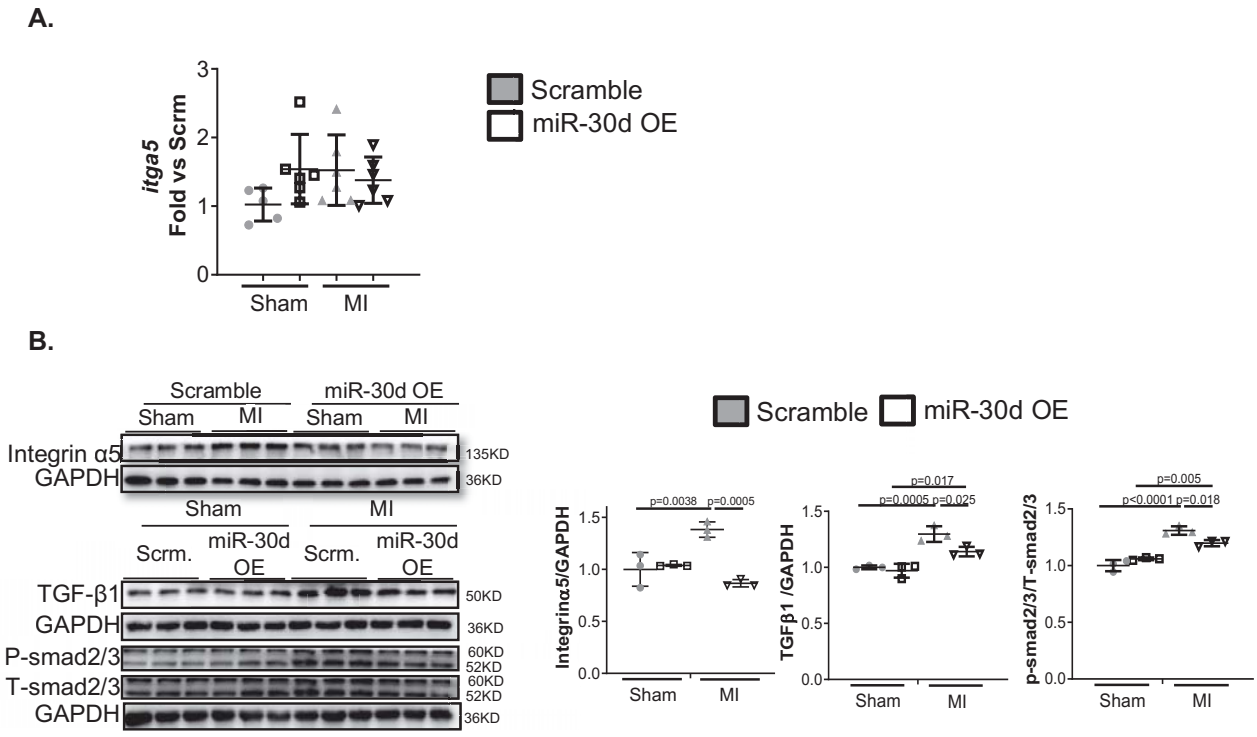
D.





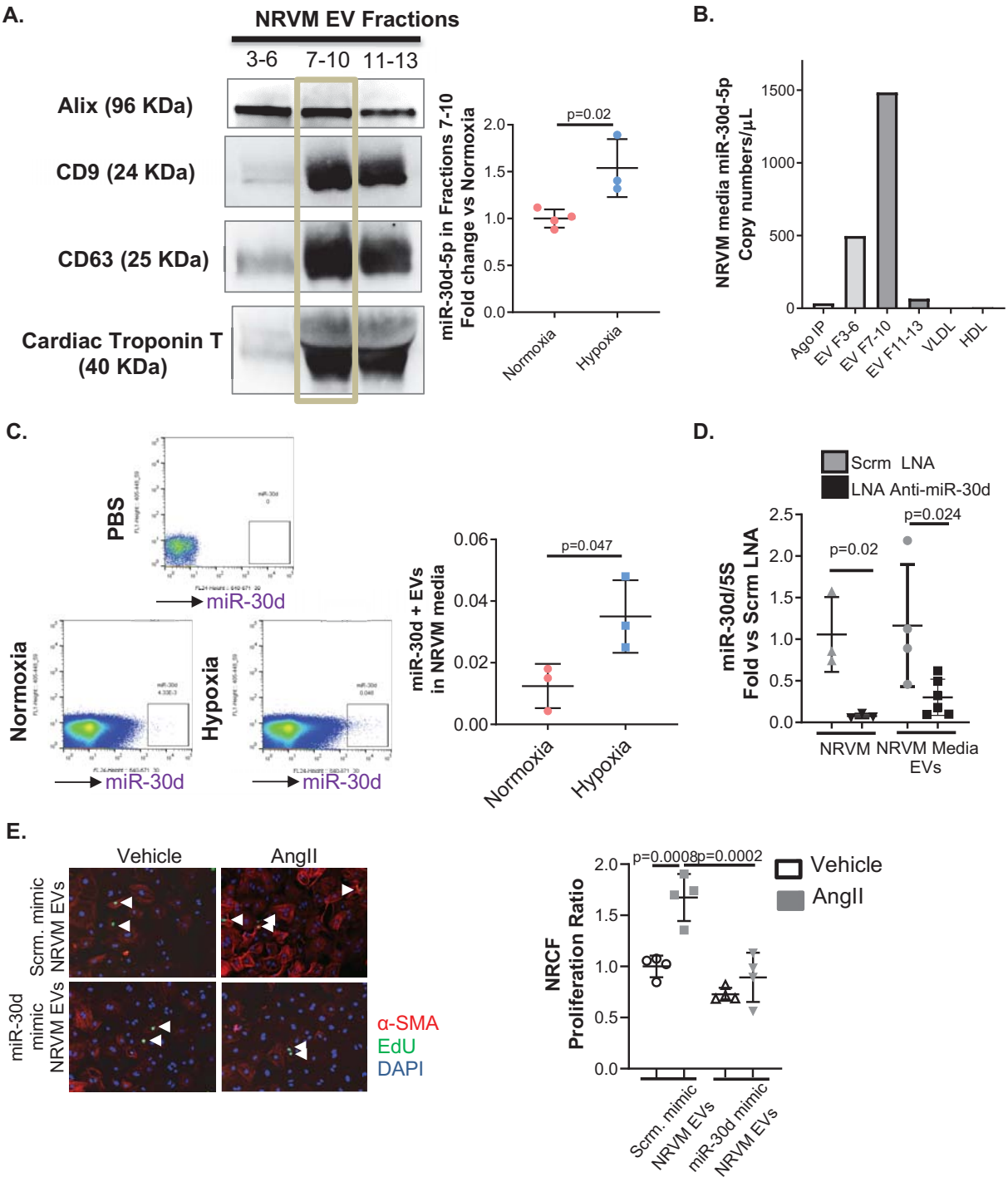
Supplemental Figure VIII. pmirGLO Vector design for each potential miR-30d target. Specific 500bp 3'UTRs of genes regulated by miR-30d were cloned into pmirGLO vectors, and their luciferase expression in the presence of miR-30d was quantified. Scrm mimic n=4, miR-30d mimic n=4. P value calculated with unpaired non-parametric Mann Whitney t-test. Data are represented as mean ± SD.

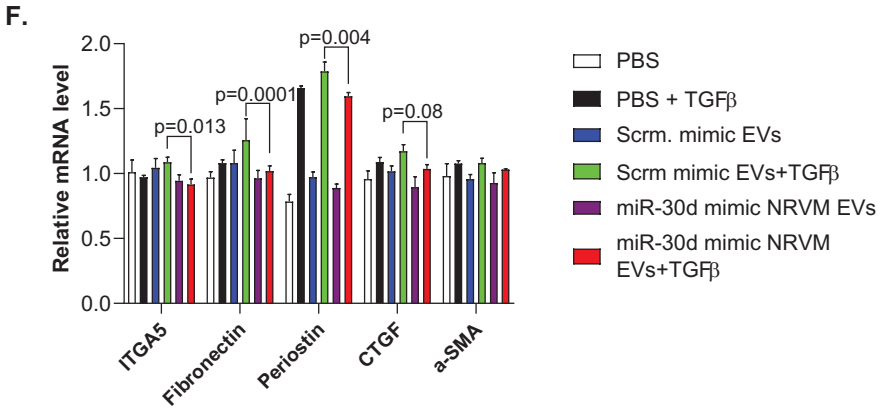
Supplemental Figure IX



Supplemental Figure IX. miR-30d regulates *itga5* and TGFβ1 mediated pro-fibrotic pathways after MI surgery. A. mRNA expression of *itga5* in the heart of lentivirus mediated miR-30d overexpressing or control mice after MI or Sham surgery. **B.** Protein expression of integrin α5, TGFβ1 and its downstream mediators Smad2/3 were determined through western blot in miR-30d overexpressing or control mice after MI or Sham surgeries. Representative blots are shown on the left and quantification on the right. Sham Scrm Lentivirus n=5, Sham miR-30d OE Lentivirus n=6, MI Scrm Lentivirus n=6, MI miR-30d OE Lentivirus n=6. Data normality determined with Shapiro-Wilk test. P values calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. Data are represented as mean ± SD.

Supplemental Figure X.



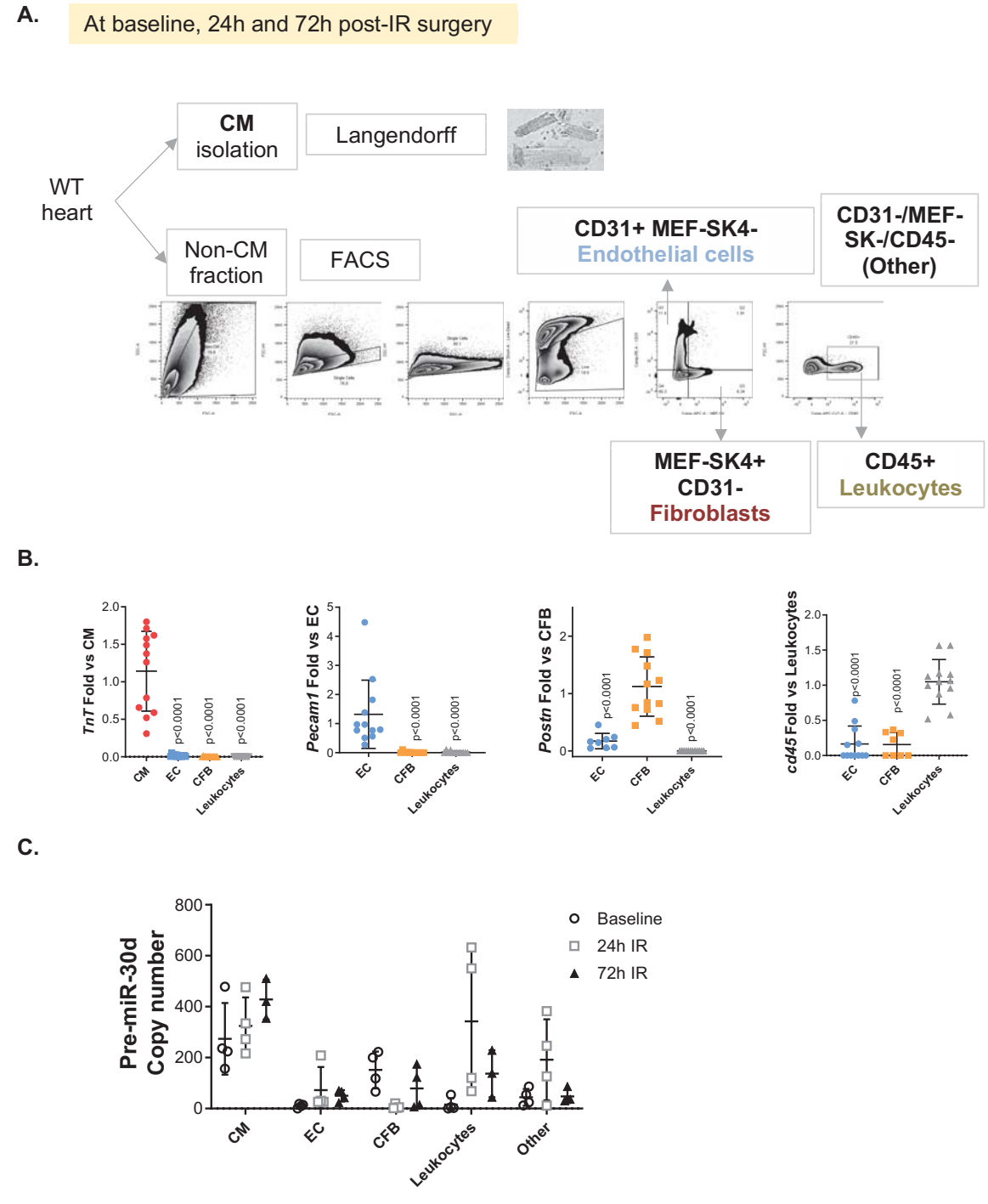


Supplemental Figure X. Validation of presence of miR-30d-5p in EVs derived from NRVMs. A. EVs from NRVM conditioned media (starting from 20mL media) were isolated with SEC-based Izon technology, after which fractions were pooled (fractions 3 to 6, fractions 7 to 10 and fractions 11 to 13). EV markers (Alix, CD9 and CD63), as well as cardiomyocyte marker Troponin T were detected through western blotting, confirming the presence of EVs in the fraction 7 to 10 pool. To validate the presence of miR-30d-5p in NRVM derived EVs, fractions 7 to 10 from normoxia or hypoxia (0.2% O_2) exposed NRVMs were pooled, treated with RNase (2mg/mL) for 30 minutes at 37°C and miR-30d-5p copy numbers were quantified using ddPCR. Data is represented as fold vs normoxia. Normoxia n=4, Hypoxia n= 3. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. **B.** EV fractions (3-6, 7-10 and 11-13) were isolated through SEC and RNA carriers were pulled down (Ago) or purified (VLDL and HDL) from 10mL pooled NRVM media. miR-30d expression levels were quantified through ddPCR in each of the compartments. **C.** Presence of miR-30d in NRVM released EVs in response to 24h normoxia or hypoxia (0.2% O_2), determined through nano flow cytometry, using a specific miR-30d detecting molecular beacon conjugated to Alexa 647

and NRVM conditioned media. Quantification of miR-30d+ EVs is represented on the right, Normoxia n=3, Hypoxia n= 3. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. **D.** NRVMs were transfected with 150pmol of scramble LNA of LNA anti-miR-30d-5p for 48h, and miR-30d-5p expression was quantified through qRT-PCR in the NRVM cellular compartment, as well as in the EVs released by NRVMs. Data is represented as fold vs scramble LNA treatment in the cellular or vesicular compartment, respectively. NRVM Scrm LNA n=3, NRVM LNA Anti-miR-30d n=3, EV Scrm LNA n=4, EV LNA Anti-miR-30d n=6. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. **E.** NRCFs were treated with EV fractions 7-10 derived from NRVMs transfected with 150pmol miR-30d mimic or scramble mimic for 48h. After additional 48h treatment of NRCF with 1μM AngII, cell proliferation was quantified through EdU staining of NRCF treated with NRVM-derived EVs and AngII (or vehicle): representative immunofluorescence pictures are shown on the left (white arrows pointing to proliferating fibroblasts) and proliferation quantification on the right. n=4. Data normality determined with Shapiro-Wilk test. P value calculated with ordinary ANOVA test, corrected using Tukey's multiple comparison test. **F.** NRCFs were treated with EV fractions 7-10 derived from NRVMs transfected with 150pmol miR-30d mimic or scramble mimic for 48h. After additional 24h treatment of NRCF with 10ng/mL TGFβ, transcriptional effects of miR-30d enriched EVs in NRCFs treated with TGFβ were quantified through qPCR. Profibrotic genes *itga5, FN1, POSTN, CTGF/CCN2* and *Acta2* expression was normalized to 18S and represented as fold vs PBS. n=3. Normality of data determined with Shapiro-Wilks test. P values calculated with ordinary

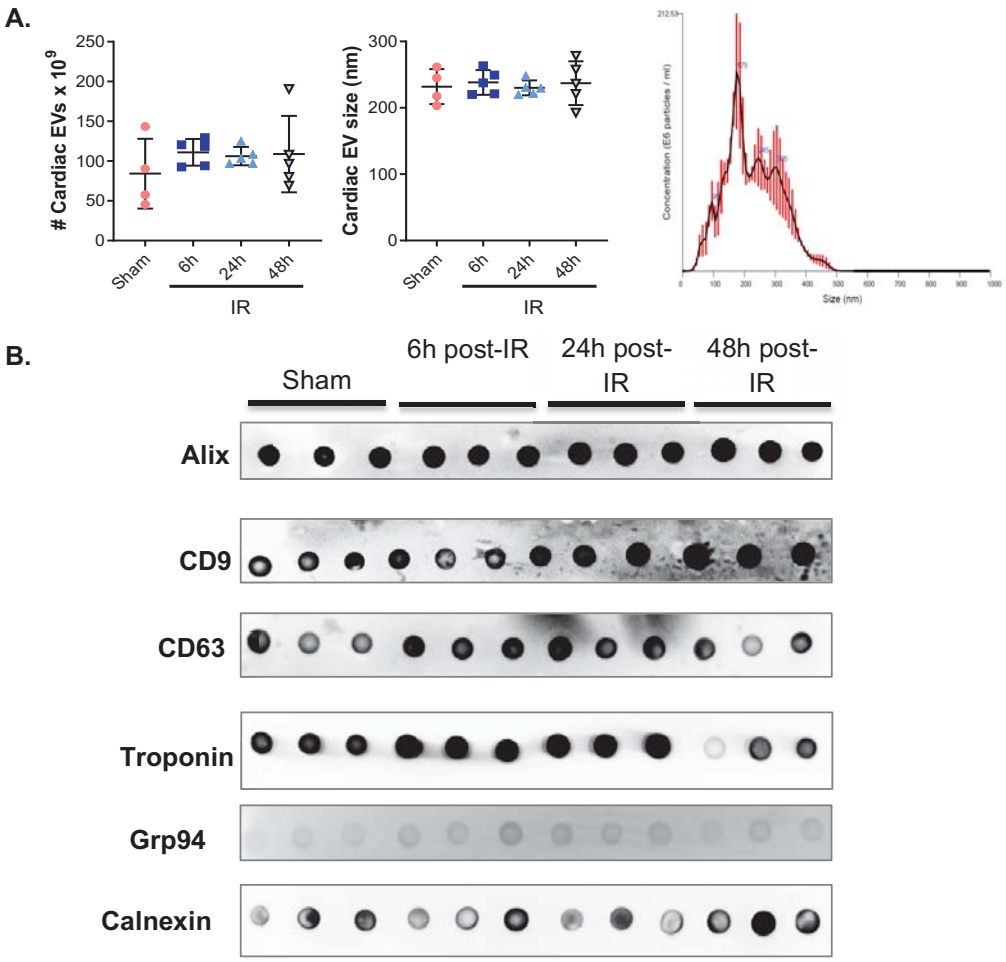
ANOVA test, corrected using Tukey's multiple comparison test. Data is represented as mean ± SD.

Supplemental Figure XI.



Supplemental Figure XI. Experimental strategy utilized for quantification of miR-30d expression levels by the different cardiac resident cells at baseline and acutely after cardiac ischemia. A. At baseline, 24h and 72h after IR-surgery, CM were isolated from murine hearts based on the Langendorff perfusion apparatus. The non-CM fraction was stained for EC, CFB and leukocyte markers and single-stained cells were sorted. Gating strategy utilized for non-CM cell sorting is detailed, where alive cells were selected from the singlets and cells positive for each marker were sorted. **B.** qRT-PCR validation of the purity of each cellular subset based on the mRNA expression of a variety of cell type-specific markers: troponin T for CM(CM n= 12, EC n=12, CFB n=12, Leukocytes n=12), *pecam1* (CD31) for EC (EC n=12, CFB n=12, Leukocytes n=12), periostin for CFB (EC n=8, CFB n=12, Leukocytes n=12), and *cd45* for leukocytes (EC n=8, CFB n=12, Leukocytes n=12). For each marker, data is represented as fold vs the main cell population expressing such marker. P value calculated with unpaired non-parametric Mann-Whitney t-test. **C.** Expression levels of pre-miR-30d were quantified through ddPCR at each time point foe the different cell subsets. Data is represented as copy numbers based on the same RNA input for condition. CM Baseline n=4, CM 24h IR n=4, CM 72h IR n=3, EC Baseline n=4, EC 24h IR n=4, EC 72h IR n=4, CFB Baseline n=4, CFB 24h IR n=4, CFB 72h IR n=4, Leukocytes Baseline n=4, Leukocytes 24h IR n=4, Leukocytes 72h IR n=3, “Other” Baseline n=4, “Other” 24h IR n=4, “Other” 72h IR n=4. Data is represented as mean ± SD.

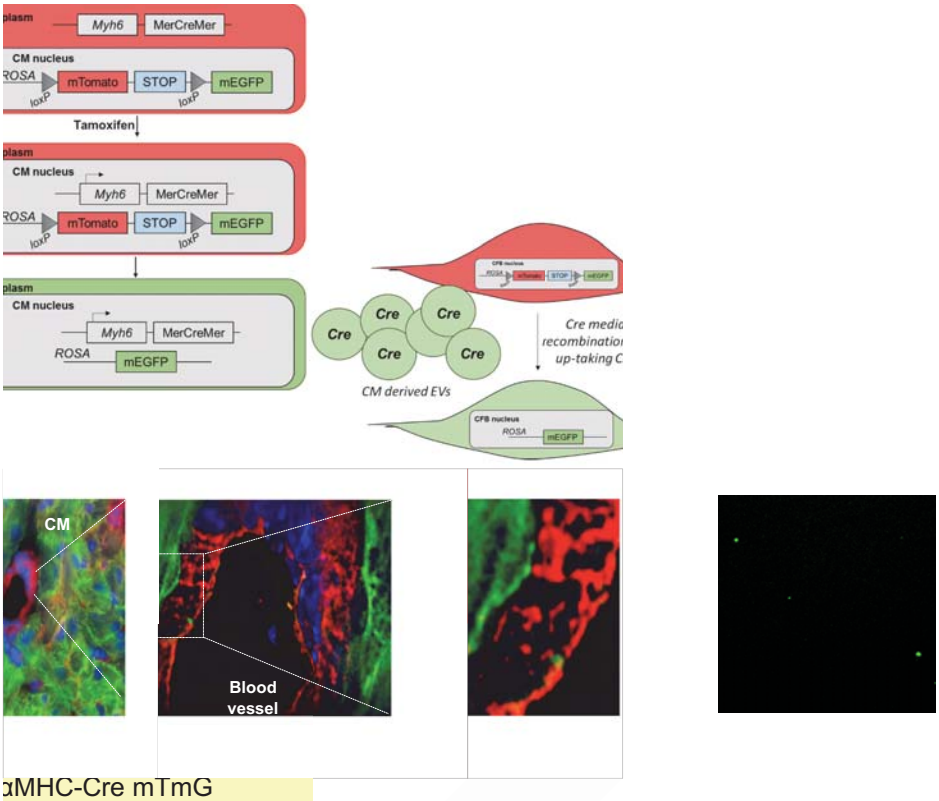
Supplemental Figure XII.

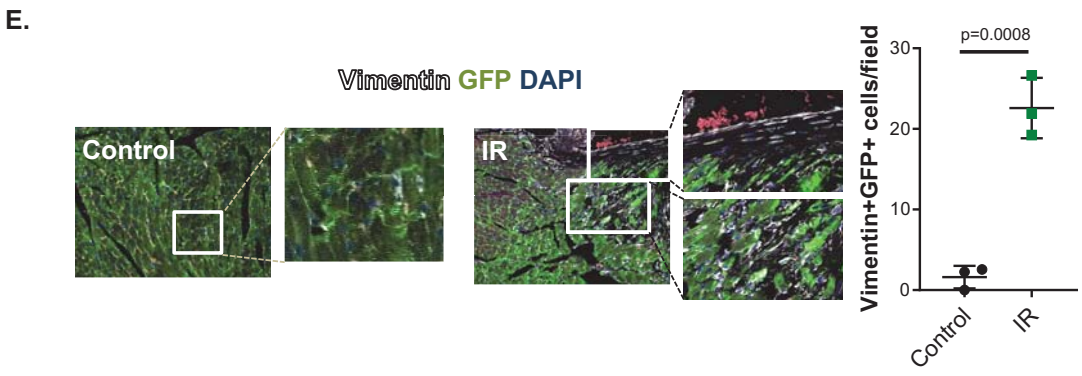


Supplemental Figure XII. Characterization of EVs isolated from the myocardium of WT mice undergoing IR surgery. A. Number of cardiac EVs per experimental time point and EV size distribution determined with NanoSight are graphed on the left. Representative size distribution plot is shown on the right. Sham n=4, 6h IR n=5, 24h IR n=5, 48h IR n=5. Data is represented as mean ± SD. **B.** Dot blot for EV markers (Alix, CD9, CD63) and myocardial source indicator (Troponin T) are shown for each

experimental time point. As negative controls, dot blot for Grp94 and calnexin are shown below. n=3.

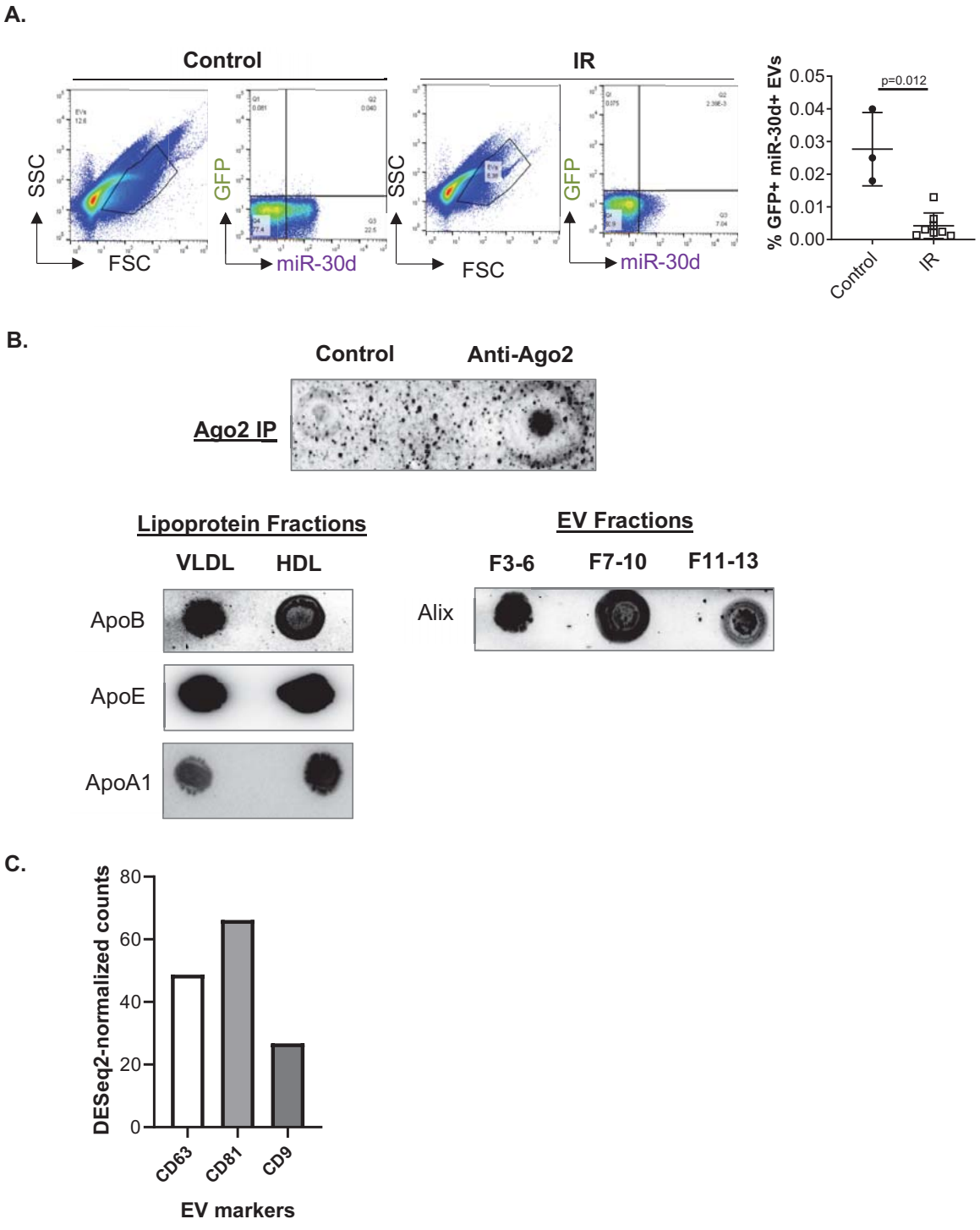
Supplemental Figure XIII.





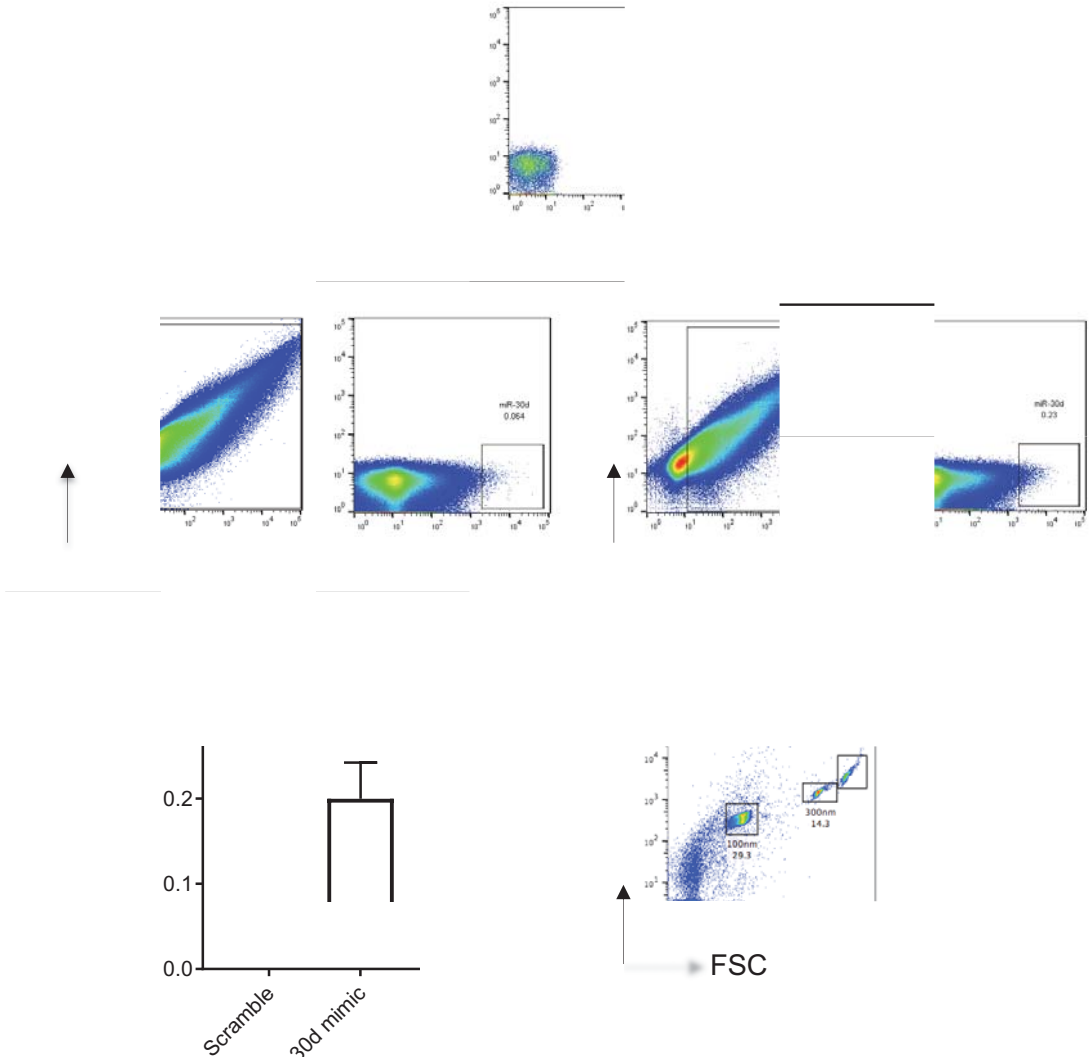
Supplemental Figure XIII. Characterization of the αMHC- MerCreMer - Rosa^{mTmG} mouse model for tracking communication between CM and other cell types through EVs. A. Explanatory model of the αMHC-MerCreMer - Rosa^{mTmG} mouse model, and Cre mediated recombination in cells that incorporate cardiomyocyte (CM) derived EVs that contain cre. **B.** Immunofluorescence pictures of cardiac cross-sections of αMHC-MerCreMer - Rosa^{mTmG} mice, where CM and CM-derived EVs are GFP+ and non-CM cells and EVs are tdTomato+. **C.** CM derived EVs in mouse plasma that show EVs of varying sizes expressing GFP. **D.** Detection of EV markers (Alix, CD9) in the EV fractions 7-8 and 9-10 mainly from plasma of αMHC-MerCreMer - Rosa^{mTmG} mice through western blot. In addition, CM source is confirmed by the expression of cardiac Troponin T and GFP, as well as the presence of Cre recombinase. **E.** Cardiac cross-sections of αMHC-MerCreMer - Rosa^{mTmG} mice undergoing IR were stained for vimentin, as fibroblast marker, and GFP+ Vimentin+ cells were imaged as indicator of fibroblast that upt ake cardiac myocyte derived EVs. Representative pictures are shown on the left and quantification of GFP+ Vimentin+ cells is quantified on the right. n=3. Data normality determined with Shapiro-Wilk test. P value calculated with unpaired parametric t-test. Data is represented as Mean ± SD.

Supplemental Figure XIV.



Supplemental Figure XIV. Expression of miR-30d-5p in small size EVs from plasma of αMHC- MerCreMer - Rosa^{mTmG} after IR. A. Presence of miR-30d in CM derived circulating EVs in plasma (diluted 1/100) of αMHC-MerCreMer - Rosa^{mTmG} mice subjected to IR is determined through nano flow cytometry, using a specific molecular beacon conjugated to Alexa 647. Representative FACS plots are shown on the left indicating miR-30d expression in small size GFP+ EVs. Quantification of GFP+ miR-30d+ EVs is represented on the right, Control n=3, IR n= 8. P value calculated with unpaired non-parametric Mann Whitney t-test. Data is represented as Mean ± SD. **B.** Detection of specific RNA-carrier markers in plasma through Dot blot. **C.** Normalized counts of miR-30d-5p in RNASeq performed in pull-downs of CD63, CD81 and CD9 from human plasma.

Supplemental Methods Figure I



Supplemental Methods Figure I. Confirmation of signal obtained with miR-30d-Alexa 647 molecular beacon in NRVM (Neonatal Rat Ventricular Myocyte) media. A-B. Media from NRVMs transfected with either scramble, or miR-30d mimic was incubated

with miR-30d-Alexa 647 molecular beacon for 30 minutes at 37°C and run in a Beckman Coulter MoFlo XDP cytometer. **(A)** Representative plots and **(B)** quantification are shown to validate the detection of strong signal in NRVMs transfected with miR-30d mimic, but not with either scramble siRNA. **C.** Beads of different sizes (100-500nm) were run as size reference for gating on small and large EVs.

Supplemental Table I. Table showing the percentage of reads unmapped, multimapped and uniquely mapped to the rattus norvegicus (Rnor 6.0) genome for the three sets of samples.

Group	*Replicate*	*Percentage of reads Unmapped*	*Percentage of reads mapped_to_multiple_loci*	*Percentage of reads uniquely mapped*	*Total input reads*
NRVM_miR-30d_adenovirus	1	10.92	11.57	77.52	34,579,367
NRVM_miR-30d_adenovirus	2	5.93	11.65	82.43	34,526,393
NRVM_miR-30d_adenovirus	3	16.76	10.35	72.9	23,217,346
Control	1	4.04	12.39	83.58	37,596,901
Control	2	7.61	11.59	80.79	21,995,298
Control	3	3.85	12.12	84.02	37,635,205
NRVM_LNA_Anti-miR-30d	1	6.45	10.49	83.06	26,452,382
NRVM_LNA_Anti-miR-30d	2	5.18	10.87	83.95	26,706,987

Supplemental Tables II-V: Submitted as online datasets.

Supplemental Table VI. Baseline patient characteristics of HF and control cohorts for EV-RNA sequencing study.

	Control (n=5)	**Heart Failure (n=18)**
Race	Caucasian 5 (100%)	Caucasian 15 (83%)
Male Gender	3 (60%)	14 (77%)
Age (years)	60.4 ± 3.5	61.58 ± 2
Baseline LVEF (%)	64.4 ± 2.7	33.69 ± 3.02

Supplemental Table VII: Submitted as online dataset.