**Materials and methods**

**General**

Reagents and chemicals were obtained from commercial sources with the highest purity available, used without further modification unless stated otherwise. Water used was purified on an EMD Millipore Milli-Q Integral Water Purification System.

**Mice**

All animal studies were performed in accordance with institutional guidelines and regulations of the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences. All animal experiments were performed on adult C57Bl6J male mice from Charles River Laboratories (8-9 weeks of age).

**Surgery**

Mice were anesthetized with an intra-peritoneal injection of ±100µl of mixed Ketamine-Xylazine (67 mg/kg Ketamine and 8.3 mg/kg mouse weight Xylazine), intubated and ventilated using a rodent ventilator (UNO® Microventilator UMV-03). The anesthesia was maintained with 1.5%-2% isoflurane. Mice were placed in a supine position on a heated plate to maintain a body temperature between 38°C and 39°C. Hair was removed from the thorax with Veet® hair removal cream and the skin was disinfected with iodine and 70% ethanol. The skin was incised left of the midline, pectoral muscles were retracted, exposing the ribs. An incision was made between the 2nd and 3rd rib after which the left ventricle (LV) was visible. For myocardial infarction (MI) procedure a 7-0 silk suture was tied around the left anterior descending coronary artery (LAD) to permanently occlude the artery. For ischemia reperfusion (IR) injury, a 3 mm polyethylene (PE) tube was placed over the LAD. A suture was then tied around the LAD and PE tube. The PE tube was removed after one hour of ischemia and the ligature was cut to allow for reperfusion via the LAD. For shamsurgeries, the surgeries were identical to MI and IR surgeries apart from that the LAD was not occluded. For the intramyocardial injections, mice were injected twice in the left ventricle (LV) at different positions on the (possible) infarct-site with 10µl PBS or UPy-gel using Hamilton syringes with 31 gauge 30° beveled needles. After injections, the ribcage was sutured (5-0 silk), the muscle layers were repositioned, and the skin was closed using a wound clip. The mice recovered on the heated plate whilst receiving oxygen supply. Once recovered, they were disconnected from the ventilator and given a single subcutaneous injection Buprenorphine (Temgesic®, 0.05-0.1mg/kg mouse weight) and were housed under standard conditions until tissue collection would take place.

**EdU labeling**

To assess cardiomyocyte proliferation, infarcted animals (n=4-6 per group) received ethynyl-29-deoxyuridine (EdU, Life Technologies; 350 μg per animal intraperitoneally) every other day from day 1 until day 11 after MI (6 injections total).

**Synthesis of cationic UPy-Amine**

1,2 CDI was used to activate the Boc-NH2-PEG11-OH, following Cbz-protected dodecyl diamine addition with DIPEA. Purification was done using C18 column chromatography. Triethylsilane was used to deprotect the Cbz-protecting group. Subsequently UPy-hexyl-isocyanate and DIPEA were added, of which the resulting product was purified using C18 column chromatography. 1H-NMR (400 MHz, CDCl3): δ – 13.10 (s, 1H), 11.84 (S, 1H), 10.08 (s, 1H), 5.84 (s, 1H), 5.04 (s, 1H), 4.87 (s, 1H), 4.68 (s, 1H), 4.45 (s, 1H), 4.20 (t, 2H), 3.6-3.7 (m, 42H), 3.53 (t, 2H), 3.1-3.35 (m, 10H), 2.23 (s, 3H), 1.15-1.7 ppm (m, 37H); LCMS: [M] calcd 1165.47; found 533.5 [M+2H-Boc]2+, 594.3 [M+H+Na]2+. The compound was stored in the freezer and small portions were deprotected with 4M HCl in dioxane before use.

**Hydrogel preparation**

10 wt% UPy-PEG hydrogel was prepared by dissolving the UPy-PEG hydrogelator powder in PBS (pH 11.6) and stirring at 70 °C for 1 hour using a magnetic stirrer. For a total volume of 100 µL hydrogel, 10 mg of UPy-PEG hydrogelator powder and 90 µL PBS (pH 11.6) was used. The viscous solution was cooled to room temperature with resulting pH of 9.0. UPy-Cat hydrogels were prepared by first dissolving the precursors UPy-Amine and UPy-PEG in water/ACN (50:50) mixture, which was freeze-dried for complete molecular mixing. Subsequently, the UPy-Amine precursor powder was dissolved in PBS at 70 °C for 1 hour.

For hydrogels loaded with antimiR, the antimiR was added from a stock solution and mixed into the still-warm hydrogel before allowing it to cool. The added volume was taken into account when the precursor hydrogelator was prepared.

**Rheology**

Rheological measurements of the hydrogels were performed on an Anton Paar Physica MCR501 Rheometer. Release studies were performed using Millicell Hanging Plate inserts PIEP12R48. All rheological measurements were performed at 37 °C unless stated otherwise, in combination with a P-PTD 200 evaporation blocker preventing the sample from drying. A cone-plate geometry of 25 mm was installed with fixed distance of 0.049 mm and shear viscosity was recorded as function of shear rate (100 to 0.1 s-1, 10 points per decade). A plate-plate geometry of 25 mm was used with plate distances ranging from 0.45 to 0.6 mm. viscous liquid precursor was pipetted on the bottom plate and HCl (1 M) was pipetted on multiple places on top to initiate gelation. After curing times of 2 – 2.5 hours storage and loss moduli were recorded as function of angular frequency (100 to 0.1 rad s-1, 22 measurement points) at 1% strain and as function of strain (0.1 to 1000%, 22 measurement points) at 1 rad s-1.

**AntimiR design**

AntimiR-195 was designed and synthesized by miRagen®. It is an LNA/DNA mixmer: a 16-mer complementary to miR-195 and partially complementary to the remaining miR-15 family members.

**Release of antimiR**

For the *in vitro* antimiR release study, 100 µL of the hydrogels with antimiR were added in Millicell plate inserts. These were put in a 24-well plate and at set time points the PBS was collected, antimiR concentration was determined and fresh PBS was added to the wells. AntimiR concentration was measured with UV absorption at 261 nm. Experiments were performed in duplicate. For antimiR release from UPy-Cat, hydrogels were dissolved before the final measurement to confirm that all antimiR was measured.

**Echocardiography**

Cardiac function and dimensions were evaluated by 2-dimensional echocardiography using a Vevo® 2100 Ultrasound system (Visual Sonics). Mice were sedated with 5% isoflurane and anesthesia was maintained with 1-2% isoflurane.M-mode tracings from short axis view were used to measure internal diameter, anterior and posterior wall thicknesses at end diastole and end systole. The mean value of at least 9 cardiac cycles were used to determine the measurements for each animal. LV trace measurements from short axis view in M-mode were used to calculate fractional shortening (FS) and ejection fraction (EF) and calculated by the Vevo® LAB 1.7.1 software as: FS(%) = 100\* [(LVID;d – LVID;s)/ LVID;d], EF (%) = 100\* [(LV Volume;d – LV Volume;s)/ LV Volume;d]. Blood flow was assessed using pulsed wave (PW)-mode, assisted by Color Doppler mode in aortic arch view.

**Tissue collection**

For cardiac tissue collection, mice were euthanized by cervical dislocation. The chest was opened to expose the heart. The heart was removed, washed in ice cold PBS and weighed. For RNA analysis, the atria and right ventricle were removed and the left ventricle (including the septum) was snap frozen in liquid nitrogen.

**RNA extraction and real-time PCR analysis**

To isolate RNA, we used TRIzol® reagent (Life Technologies) following the manufacturer’s instructions. The RNA concentration was assessed by a NanoDrop Spectrophotometer. Complementary DNA (cDNA) was synthesized from a total of 500ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad, #1708891). Real-time PCR (RT-PCR) for coding genes was performed using gene specific primers according to the instructions described by the IQ™ SYBR Green Supermix (Bio-Rad, #170-8885). The real time PCR protocol was as follows: 95ºC for 15min, followed by 40 cycles at 95ºC for 15s, 60ºC for 30s and 72ºC for 30s. Gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) or hypoxanthine-guanine phosphoribosyltransferase (*Hprt*).

For RT-PCR of microRNAs, cDNA was synthesized from 1µg total RNA using TaqMan microRNA Reverse Transcriptase kit (Applied Biosystems, #4366597). Real time PCR reactions were performed using TaqMan Universal Master Mix II (Applied Biosystems, #4440040), according to the manufacturer’s instructions and using specific probes for the *miR-15* family (ThermoFisher Scientific, #4427975, product IDs: 000389, 000390, 000391, 000494, 0001346) and *U6* (ThermoFisher Scientific, #4440887, product ID 001973). Fold changes in gene expression were calculated according to the 2-ΔΔCT-method and expressed as mean fold change ± SEM.

**RNA sequencing**

RNA integrity was analyzed using the Agilent 2100 Bioanalyzer system. RNA of each LV sample was used to prepare Illumina sequencing libraries with the TRUseq stranded mRNA kit (Illumina) and sequenced with 75bp read length with the Illumina NextSeq500 by the Utrecht Sequencing Facility. Subsequently, reads were mapped to the mouse genome (GRCm38). Differential expression was analyzed using R and the DESeq2 package. Genes with less than 1 reads per sample on average were discarded. Significantly regulated genes were defined as those with at least a 1.2-fold change over the control condition and a p-value < 0.01. For gene ontology analysis on significantly upregulated genes, all detected genes were used as a background.

**Data accessibility**

RNA sequencing data has been submitted to be publicly available through the NCBI Gene Expression Omnibus (GEO).

**Histology**

Hearts were fixed in 4% formalin at room temperature for 48h, embedded in paraffin and sectioned at 4μm. Sections were used for hematoxylin and eosin (H&E) stainingusing standard procedures. The short axis cross-section view and high magnification images were made using a Leica DM 4000 microscope and Leica LAS software. For immunohistochemistry, tissue sections went through a process of deparaffinization, rehydration, heat induced antigen retrieval and blocking with 1% BSA, the sections were incubated with specific primary antibodies overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibodies for 1 hour at room temperature, washed and sealed with a mounting medium containing DAPI (Vector Laboratories). To reveal EdU incorporation, tissue sections were further processed using the Click-IT EdU 555 Imaging kit according to the manufacturer’s instructions. Images were taken using the Leica TCS SPE confocal microscope. Antibodies used were mouse anti-α actinin (ACTN2, Sigma-Aldrich, #A7732) and the corresponding secondary fluorescent antibody anti-mouse Alexa-488 (Life Technologies).

**Primary cardiomyocyte culture**

Neonatal rat ventricular cardiomyocyte (NRVM) cultures were isolated by enzymatic dissociation of neonatal rat hearts, as described previously1. In short, hearts from 1-2-day old rat pups were collected, the atria were removed and the ventricular cells were enzymatically dissociated with trypsin (Life Technologies) in a water (37˚C) jacketed spinner flask. The single cell suspension was filtered and pre-plated to remove debris and non-myocytes respectively. Primary cardiomyocytes were initially maintained in Ham’s F10 medium (Gibco) supplemented with 5% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Life Technologies). The day after isolation, cardiomyocytes were switched to serum-free Ham’s F10 medium, supplemented with 1% penicillin/streptomycin and 1µl/ml insulin-transferrin-sodium-selenite supplement (Sigma-Aldrich, catalog number 11074547001). Pure cardiomyocytes were plated on 24-well plates with cover-slips (125,000 cells per well).

**Analysis of in vitro activity of antimiR-195 released from UPy-PEG**

Three-hundred µL UPy-PEG hydrogel, either pristine or with antimiR-195, was incubated with 1.5 mL cell culture medium in 2 mL Eppendorf tubes. Supernatants were collected, frozen and replaced at day 1, 3, 5, 7 and 14. NRVM were incubated for 48h with the medium incubated on UPy-PEG hydrogel. For immunofluorescence (IF) on cultured cardiomyocytes, cover-slips were incubated with blocking buffer with 1% Fish gelatin, and incubated with specific primary antibody (mouse anti-α actinin (Sigma-Aldrich, #A7732) and anti-KI67 (Abcam, ab15580)) for 25 minutes at room temperature. After washing with blocking buffer, the cover-slips were incubated with secondary antibodies (anti-mouse Alexa-488 and anti-rabbit Alexa-568 (Life Technologies)) for 25 minutes at room temperature, washed with MQ water and sealed with a mounting medium containing DAPI. The images were taken using the Leica TCS SPE confocal microscope.

**Anti-PEG ELISA quantification of UPy-PEG in cardiac tissue**

Total hearts were used for cardiac ELISA assays. Tissue samples were homogenized in 1 mL PBS in lysing matrix tubes (FastPrep®) using the Fastprep®-24 sample preparation system. The standard curve was prepared by post-mortem injection of 20 µL UPy-PEG in a control heart, adding 1 mL of PBS and homogenizing it in the same manner as the other samples. This sample was diluted with homogenized cardiac tissue without added hydrogel in 10-fold steps to create a standard curve. Coating buffer (5.3 g Na2CO3 + 4.2 g NaHCO3/Liter, pH = 8, adjusted pH with 1N HCl) and phosphate-citrate buffer (17.4 g K2HPO4 + 21 g citric acid/Liter) were prepared. The ELISA plates (Nunc MaxiSorp C96, ThermoFisher Scientific, #430341) were coated with 50 µL (5 µg/mL diluted in coating buffer) AGP4 capture antibody per well and incubated overnight at room temperature. Plates were washed with PBS after which 200 µL blocking solution (5% BSA in PBS) was added for two hours at room temperature. Plates were washed 5 times. Tissue samples and standard curve samples were diluted (1:1) in dilution buffer (2% BSA in PBS) and 50 µL of diluted sample was added in three replicates to the plates. Plates were incubated for two hours at room temperature and were then washed one time with PBS-C (0.05% CHAPS in PBS) and two times with PBS for five minutes each, shaking gently on an analog shaker (VWR). The detection antibody, 6.3-biotin, was diluted in dilution buffer (0.05 µl/mL) and 50 µl was added to the plate. After one hour of incubation at room temperature, plates were washed one time with PBS-C and two times with PBS for five minutes each with gentle shaking. Streptavidin-HRP was diluted in dilution buffer (0.1 µg/mL) and 50 µl per well was added for one hour at room temperature. After one hour of incubation at room temperature, plates were washed three times with PBS-C (0.05% CHAPS in PBS) and two times with PBS for five minutes each with gentle shaking. 5 mg ABTS was added to 10mL phosphate citrate buffer and kept in the dark. Following washing, 2 µl of H2O2 was added to the ABTS substrate solution after which 100 µL per well was added to the plate. Peroxidase activity was measured in a microplate reader at 405 nm every 5-10 minutes up until 30 minutes after the ABTS solution was added. Concentration of PEG in tissue was measured by comparison against a standard curve of tissue samples with known concentrations of PEG (tissue samples subjected to *ex vivo* injection of UPy-PEG gel) using 4-parameter logistic regression (Myassays.com). Antibodies were obtained from Steve Roffler at Academia Sinica.

**Statistical analysis**

Values are presented as mean ± SEM. Outliers were identified, and subsequently excluded, using Grubbs’ test2 (GraphPad). Statistical significance was evaluated using one-way ANOVA (GraphPad) or two-way ANOVA (R), followed by post-hoc tests with correction for multiple testing according to the Benjamini-Hochberg procedure.

\*, \*\*, \*\*\*, \*\*\*\* indicate p < 0.05, 0.01, 0.001, 0.0001 compared to empty vehicle, respectively. †, ††, †††, †††† indicate p < 0.05, 0.01, 0.001, 0.0001 for UPy-PEG-antimiR versus PBS-antimiR, respectively.

**References**

1. van Rooij E, Fielitz J, Sutherland LB, Thijssen VL, Crijns HJ, Dimaio MJ, Shelton J, De Windt LJ, Hill JA and Olson EN. Myocyte enhancer factor 2 and class II histone deacetylases control a gender-specific pathway of cardioprotection mediated by the estrogen receptor. *Circ Res*. 2010;106:155-65.

2. E. Grubbs F. Procedure for Detecting Outlying Observations in Samples. *Technometrics*. 1974;11:53.