MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs

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Prompt coronary catheterization and revascularization have markedly improved the outcomes of myocardial infarction, but have also resulted in a growing number of surviving patients with permanent structural damage of the heart, which frequently leads to heart failure. There is an unmet clinical need for treatments for this condition, particularly given the inability of cardiomyocytes to replicate and thereby regenerate the lost contractile tissue. Here we show that expression of human microRNA-199a in infarcted pig hearts can stimulate cardiac repair. One month after myocardial infarction and delivery of this microRNA through an adeno-associated viral vector, treated animals showed marked improvements in both global and regional contractility, increased muscle mass and reduced scar size. These functional and morphological findings correlated with cardiomyocyte de-differentiation and proliferation. However, subsequent persistent and uncontrolled expression of the microRNA resulted in sudden arrhythmic death of most of the treated pigs. Such events were concurrent with myocardial infiltration of proliferating cells displaying a poorly differentiated myoblastic phenotype. These results show that achieving cardiac repair through the stimulation of endogenous cardiomyocyte proliferation is attainable in large mammals, however dosage of this therapy needs to be tightly controlled.

The heart of mammals immediately after birth3 and those of urodeles and fish throughout life4–5 are capable of spontaneous regeneration. In these cases, new tissue formation occurs through the partial de-differentiation of already existing cardiomyocytes, followed by their proliferation6–7. In adult mammals, however, cardiomyocyte proliferation is only marginally increased after myocardial infarction (MI)8, but remains far below clinically relevant levels. Therefore, empowering the endogenous capacity of cardiomyocyte proliferation after damage is a potential strategy for achieving cardiac repair.

Previous studies have shown that cardiomyocyte proliferation is under the control of the microRNA (miRNA) network9–12. In particular, high-throughput screening work revealed that a few human miRNAs, including hsa-miR-199a-3p, can stimulate rodent cardiomyocyte entry into the cell cycle and cardiac regeneration after MI in mice11. We therefore investigated whether these findings could be translated to MI in pigs, a clinically relevant large-animal model.

We identified *AAV* serotype 6 (*AAV*6) as the most effective vector to transduce pig cardiomyocytes after intramyocardial injection (Extended Data Fig. 1a, b). We generated an *AAV*6 vector expressing the hsa-miR-199a-1 pri-miRNA gene under the control of the constitutive CMV promoter; the sequences of miR-199a-3p and miR-199a-5p, encoded by this pri-miRNA, are identical in rats, mice, pigs and humans (Extended Data Fig. 1c, d). MI was induced in 25 pigs by 90-min occlusion of the left anterior coronary artery followed by reperfusion. The pigs were randomly divided into two groups receiving either 2 × 10^{15} empty *AAV*6 (*AAV*6-control) particles or the same dose of *AAV*6-miR-199a (Fig. 1a, Extended Data Fig. 1e) injected into the left ventricle (LV) wall. An additional group of sham-operated animals served as a control. The levels of transduction and transgene expression were robust and persistent over time in the injected areas, as assessed by both quantitative PCR and in situ hybridization (Fig. 1b, c, Extended Data Fig. 2). Both miRNA strands were expressed at comparable levels (Extended Data Fig. 3a). We also verified that a few of the known miR-199a targets were effectively downregulated in the treated animals. These included two factors in the Hippo pathway—the upstream inhibitor TAO kinase 1 (TAOK1)13,14 and the phospho-YAP E3 ubiquitin-ligase β-transducin repeat containing protein (β-TrCP)15—and the actin cytoskeleton regulatory protein coflin-216 for miR-199a-3p, and HIF1α for miR-199a-5p17 (Extended Data Fig. 3b). Target sites for these miRNAs are conserved in pigs (Extended Data Fig. 3c–f). Viral DNA spread and levels of miR-199a transgene expression remained essentially restricted to the injected myocardium (Extended Data Fig. 3g, h).

Morphological and functional assessment was performed using cardiac magnetic resonance imaging (cMRI) based on late gadolinium enhancement (LGE) images (LGE-cMRI). Two days after MI, the gadolinium-retaining region, defined as either infarct mass or size, was not significantly different between the *AAV*-control and *AAV*-miR-199a groups (*n* = 12 and 13, respectively), in agreement with the measurements of oedema extension based on enhanced T2-weighted signals18 (Fig. 1d, e). Four weeks after MI, scar mass and scar size were approximately 50% smaller in pigs treated with *AAV*-6-miR-199a than in the control group (mean shown in Fig. 1f; paired analyses in the same pigs shown in Extended Data Fig. 4a). Representative LGE-cMRI images of five cross-sectional planes (1–5, Fig. 1g) of hearts from two representative pigs per group at days 2 and 28 after MI are shown in Fig. 1h. A marked reduction in scar size (identified by red counterstain) at day 28 is visible in the pigs that received *AAV*-6-miR-199a (the original images without counterstain are shown in Extended Data Fig. 4b). For two other representative animals, gross anatomy of cardiac slices with corresponding LGE-cMRI images at day 28 are shown in Extended Data Fig. 4c. Concordant with the cMRI data, the fibrotic area in the infarcted region was significantly reduced at 28 days (Fig. 1i, j). The infarct region included a core fibrotic area and a surrounding grey zone, comprising a mixture of viable myocardium and fibrotic regions (Fig. 1k). At 28 days after MI, the core was smaller in the *AAV*-6-miR-199a group (*P* < 0.05; Fig. 1l), also suggestive of a process of regeneration driven by miR-199a in the infarct border zone.

Functional data measured by cMRI showed that the LV ejection fraction was recovered after 28 days in the animals injected with *AAV*-6-miR-199a, whereas it remained more than 20 percentage points below...
The image contains a research article with figures and text describing the results of a study on the effect of miR-199a treatment on heart function in pigs. The text is presented in paragraphs and figures, detailing the methods, results, and conclusions of the study. The article discusses the impact of miR-199a on cardiomyocyte proliferation and the overall cardiac function of pigs treated with AAV6-miR-199a. The figures illustrate the changes in infarct size, cardiac MRI images, and other relevant data. The study suggests that miR-199a treatment leads to improved cardiac function, with a decrease in infarct size and an increase in cardiomymocyte proliferation, supported by statistical analyses and experimental data. The article concludes with implications for further research and potential therapeutic applications.
miR-199a delivery improves global and regional cardiac function. a–d, LV ejection fraction (a), LV stroke volume (b), LV end-systolic volume (c) and LV end-diastolic volume (d) measured by cMRI in non-infarcted controls and infarcted pigs at days 2 and 28 after MI with injection of AAV6-control or AAV6-miR-199a. Data are mean ± s.e.m.; the number of animals per group and time point is indicated. *P < 0.05 versus AAV6-control at the same time point; †P < 0.05 versus sham; ‡P < 0.05 versus day 2; two-way ANOVA with Bonferroni post hoc correction. The number of analysed animals is indicated in the figure. e, Example of cardiac short-axis image with the tagging grid in diastole and systole. f, Subdivision of the LV short axis in eight circumferential segments (left) and their correspondence with the infarct core, border zone and the remote zone (right). The syringe indicates the infarct border injected with AAVs. IS, infarct core; S, septal, AS, anteroseptal; A, anterior; AL, anterolateral; L, lateral; IL, inferolateral; I, inferior. g, h, Eight-segment curves corresponding to LV radial (LV Er) (h) and circumferential (LV Ecr) (i) strain at 28 days after MI. Data are mean ± s.e.m. *P < 0.05 versus AAV6-control; †P < 0.05 versus sham; two-way ANOVA with Bonferroni post hoc correction. The number of animals for the analysis is indicated in j and k. i, Graph showing example of calculation of the AUC in arbitrary units (AU). j, k, AUC for Erj (j) and Ecr (k). Data are mean ± s.e.m.; the number of animals per group is indicated. *P < 0.05 versus AAV6-control; †P < 0.05 versus sham; one-way ANOVA with Bonferroni post hoc correction. I, Eight-segment curves corresponding to LV end-systolic wall thickening (LVWT) 28 days after MI. Data are mean ± s.e.m. *P < 0.05 versus AAV6-control; †P < 0.05 versus sham; two-way ANOVA with Bonferroni post hoc correction. The number of analysed animals is shown in m, m, AUC for LVWT. Data are mean ± s.e.m.; the number of animals per group is indicated. *P < 0.05 versus AAV6-control; †P < 0.05 versus sham; one-way ANOVA with Bonferroni post hoc correction.

and re-expressed during zebrafish heart regeneration. 

GATA4 normally localizes in the nucleus, where it promotes transcription of cardiac genes, but is also found in the cytoplasm during embryonic development. Presence of these cells persisted at 28 days and was restricted to the injected infarct border (Extended Data Fig. 7).

We investigated some of the molecular correlates of cardiac repair and improved cardiac function. The ratio between transcripts for adult α-myosin and fetal β-myosin heavy chains was maintained by AAV6-miR-199a (Extended Data Fig. 8a). Cardiomyocyte cross-sectional area was not different between treated and control pigs 30 days after myocardial infarction (Extended Data Fig. 8b, c). At the same time point, there were no differences in the levels of pathological muscle and vascular markers, including desmin, myogenin, endothelin receptor type B receptor and Wilms’ tumour protein 1 (WT1) (Extended Data Fig. 8d). The increase in gene expression for both atrial and brain natriuretic peptide (ANP and BNP) was blunted in the pigs treated with AAV6-miR-199a (Extended Data Fig. 8e), and no difference was detected in vessel density (Extended Data Fig. 8f).

A subset of infarcted animals treated with AAV6-control (n = 9) and AAV6-miR-199a (n = 10) was followed beyond the first month from treatment. Three of the AAV6-miR-199a pigs continued to show persistent beneficial effects on cardiac morphology and function at eight weeks, with progressive reduction of the cardiac scar (Fig. 1a, Extended Data Fig. 9a). cMRI images over time of a second pig, and gross cardiac morphology after euthanasia at two months, are shown in Extended Data Fig. 9b, c. Despite this progressive morphological and functional improvement until seemingly complete restoration, seven of the ten pigs in the AAV6-miR-199a group died from sudden death at weeks 7–8, in the absence of preceding clinical signs (Fig. 4b).

In two of these pigs, a subcutaneously implanted miniaturized recorder documented the final phases of ECG preceding sudden death, showing tachyarrhythmia events that had evolved into ventricular fibrillation (Extended Data Fig. 10a, b). mRNA levels of 14 different ion channels or associated proteins involved in various types of arrhythmogenic conditions did not reveal significant differences between miR-199a-treated and control animals, arguing against the possibility that miR-199a might directly affect channels that control cardiac electric activity (Extended Data Fig. 10c). Thus, these tachyarrhythmias might be consequent to the generation of areas of poorly differentiated cardiomyocytes that, by progressively growing in size, eventually determine fatal re-entry electrical circuits. Alternatively, they might arise because of the simultaneous expression, from the same vector and in addition to pro-regenerative miR-199a, of the miR-199a-3p strand, which is known to exert deleterious effects in the heart.

Examination of tissue sections from AAV6-miR-199a-injected pigs, stained with haematoxylin and eosin, revealed the occasional presence of small clusters of cells infiltrating the myocardium (Fig. 4c, Extended Data Fig. 11). These cells were negative for markers of inflammatory (CD45) or haematopoietic and endothelial (CD34) cells, or for markers that identify differentiated muscle (desmin, sarcomeric α-actinin and HHF35) or epicardial (WT1) cells. They were proliferating (positive for Ki67) and expressed a few antigens that are present during early myogenic development, including GATA4, myogenin (the reactivation of which characterizes rhabdomyosarcoma cells), caldesmon (expressed at high levels in leiomyoma and leiomyosarcoma) and the endothelin receptor type B (expressed in smooth muscle cells). Of note, in situ hybridization revealed that these clusters were negative for miR-199a while being surrounded by cardiomyocytes that expressed this miRNA (Fig. 4d). Thus, these cells had either lost the AAV6 vectors during replication in AAVs do not integrate into the host cell genome or they arose as a consequence of an altered microenvironment induced by AAV6-miR-199a-expressing cardiomyocytes.

miRNAs are appealing as genetic tools to stimulate cardiac proliferation, as this leverages their capacity to regulate the levels of multiple genes simultaneously. Our study shows that cardiac AAV6-miR-199a delivery reduces infarct size, diminishes cardiac fibrosis and improves contractile function in infarcted pigs by stimulating cardiomyocyte de-differentiation and proliferation. However, uncontrolled, long-term
expression of this miRNA eventually resulted in sudden cardiac death of most test subjects. Therefore, cardiac administration of pro- proliferative miRNAs can stimulate cardiac repair after MI but the treatment needs careful dosing. This is beyond the current capabilities of virus-mediated gene transfer but could be achieved through cardiac delivery of naked, synthetic miRNA mimics.  

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-1191-6.

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Fig. 3 | AAV6-miR-199a administration induces cardiomyocyte proliferation. a, Schematic representation of the protocol for BrdU administration. b, Representative KI67 immunohistochemistry of the infarct border zone 12 days after surgery, and relative quantification. The bottom panels show higher magnification images of the outlined regions in the top panels. Data are mean ± s.e.m.; the number of animals per group is indicated. *P < 0.05; two-sided Student’s t-test. Scale bars, 100 μm. c, d, Representative images of BrdU (c) and phosphorylated histone H3 (d) immunostaining in the infarct border zone 12 days after MI, with relative quantifications. The bottom panels show higher magnification images of the outlined regions in the top panels. Data are mean ± s.e.m.; the number of animals per group is indicated. *P < 0.05; Student’s two-sided t-test. Scale bars, 100 μm. e, Aurora B immunofluorescence, showing localization in midbodies (arrow) in hearts from pigs treated with AAV6-miR-199a, 12 days after MI. Scale bars, 20 μm. f, Distribution of the number of total and BrdU+ nuclei per cardiomyocyte in pigs treated with AAV6-control or AAV6-miR-199a 12 days after surgery. Data are mean ± s.e.m. from four pigs with at least eight sections analysed per pig. g, Representative images of multinucleated cardiomyocytes with BrdU+ nuclei. WGA, wheat germ agglutinin staining of cardiomyocyte sarcolemma. Scale bars, 100 μm. h, Representative images of connexin-43 (CX43, red) and phospho-Aurora B (pH3, blue-green) immunofluorescence in heart sections from pigs treated with AAV6-miR-199a, 12 days after infarction. Scale bar, 100 μm. i, Representative immunohistochemistry of GATA4+ cells in pigs inject with AAV6-control or AAV6-miR-199a 12 days after treatment. The bottom panels show higher magnification images of the outlined regions in the top panels. j, Quantification of cells showing cytoplasmic localization of GATA4. Data are mean ± s.e.m.; the number of animals per group is indicated. Quantification from at least seven high-resolution images acquired from at least eight different regions of each heart. *P < 0.05; Student’s two-sided t-test. Scale bars, 100 μm.
Fig. 4 | Long-term expression of miR-199a induces progressive cardiac regeneration but causes sudden death. a, LGE-CMRI representative images, from apex to base, of one AAV6-control and one AAV6-miR-199a-treated pig heart at 1, 4 and 8 weeks after MI. The infarct area is counterstained in red; the corresponding original images without counterstaining are shown in Extended Data Fig. 9a. Similar cardiac repair results were observed in three pigs treated with miR-199a that survived two months after treatment. b, Kaplan–Meier curve (log-rank test) showing mortality after MI and vector administration. The number of animals per group is indicated. c, Haematoxylin and eosin (H&E) staining or immunostaining for the indicated antigens of the same cell cluster in consecutive tissue sections from an infarcted heart injected with AAV6-miR-199a at eight weeks after treatment. Scale bars, 100 μm. d, In situ hybridization of miR-199a-3p, scrambled control and U6 LNA probes in pig heart sections with infiltrating cell clusters. Scale bar, 100 μm.
METHODS

Production and purification of recombinant AAV vectors. hsa-miR-199a was amplified from human genomic DNA isolated from HeLa cells, using the QiAamp DNA mini kit (Qiagen), according to the manufacturer’s instructions, as previously described. The amplified sequence was cloned into the pZac2.1 vector (Gene Therapy Program, Penn Vector core, University of Pennsylvania, USA), which was used to produce recombinant AAV vectors in the AAV Vector Unit at IGCEB Trieste, as described previously. In particular, AAV2 serotype 6 vectors were generated in HEK293T cells, by co-transfecting the plasmid vector together with the packaging plasmid pDP6 (PlasmidFactory). Cells for AAV production were free from mycoplasma contamination.

Viral stocks were obtained by PEG precipitation and two subsequent CsCl gradient centrifugations. Titration of AAV viral particles was performed by real-time PCR quantification of the number of packaged viral genomes, as described previously; the viral preparations had titres between 1.3 × 103 and 10 × 1013 viral genomes per ml.

Open chest surgery and MI. Three-to-four-month-old male farm pigs, weighing 28–32 kg, were sedated with a cocktail of 4 mg/kg tiletamine hydrochloride and 4 mg/kg zolazepam hydrochloride injected intramuscularly, intubated and mechanically ventilated with positive pressure. Inhalatory anaesthesia was maintained by a mixture of 1–2% isoflurane dissolved in 40% air and 60% oxygen. Electrocardiogram (ECG), heart rate and arterial pressure were constantly monitored. A thoracotomy was performed in the left fourth intercostal space and then the pericardial sac was opened to expose the heart. A small group of animals (n = 3) received direct intramyocardial injections of 1 × 1012 viral genomes of AAV6, AAV8 and AAV9 suspended in PBS and carrying the reporter gene eGFP in three separate sites of the LV anterior wall, one vector serotype for each site, to compare their transduction efficiency.

MI was induced by coronary occlusion in 25 pigs anaesthetized and operated as described above. In brief, 30 min before coronary occlusion, pigs were medicated with 4.3 mg/kg of amiodarone in 500 ml of 0.9% sodium chloride to prevent arrhythmias. The left anterior descending coronary artery (LAD) was isolated from surrounding tissue distal to the first diagonal branch, encircled by a suture thread (Extended Data Fig. 1e); the two ends of the suture were threaded through a plastic tube and tightened to achieve occlusion of the vessel, confirmed by the presence of regional myocardial cyanosis, ST segment elevation in the ECG and ventricular arrhythmias, which were more pronounced within the first 30–45 min. The LAD occlusion was removed after 90 min to start the reperfusion phase.

After 10 min of reperfusion, the surviving animals were randomized in two groups receiving: 2 × 1013 empty AAV6 (AAV6-control; n = 12) or 2 × 1013 AAV6-hsa-miR-199a-3p (AAV6-miR-199a; n = 13). The viral particles were suspended in 2 ml of PBS and delivered by 20 direct intramyocardial injections equally spaced along the border zone (100 μl per injection). The latter was visualized as the majority of the myocardium (pale compared to the normally perfused myocardium; Extended Data Fig. 1e). Some of the injection sites were tagged with coloured epicardial stitches to detect and sample the corresponding myocardial tissue post-mortem for histological analysis. An additional group of sham-operated animals was operated in the same manner, but the LAD was not ligated (sham; n = 6). In this experimental setting, the delivery of the vector at the time of MI allows avoiding a second surgery a few days/hours after MI, which would importantly increase animal mortality.

At the end of the study, animals were anaesthetized and euthanized by injection of 10% KCl to stop the heart at diastole. The excised hearts were sectioned through four horizontal planes and each section was then subdivided into sub-sections for further histological and molecular analyses as shown in Extended Data Fig. 2a, b. In brief, each heart was sectioned in four 1-cm thick slices, starting from the apex towards the base. Then, each slice was divided into 2–8 regions (indicated by letters). In all quantifications, we considered at least 8 sectors of the four heart sections. Sectors H, T and C corresponded to the infarct border zone, where the vectors were administered, while sector L was considered representative of the remote zone, since it was on the same plane but on the opposite position (posterior) relative to sector T. Each region was then divided into 2 pieces (for RNA analysis and histology, respectively) by a transversal cut in order to keep both the endocardial and pericardial borders visible in each piece. For all quantifications, the same regions were chosen in animals injected with either control or miR-199a vectors.

The protocol for the animal studies (no. 76/2014 PR) was approved by the Italian Ministry of Health and was in accordance with the Italian law (D.lgs. 26/2014).

LV assessment with cMRI. Cardiac magnetic resonance imaging was performed at 2 days and 4 weeks after MI. Animals were sedated with a cocktail of 4 mg/kg tiletamine hydrochloride and 4 mg/kg zolazepam hydrochloride injected intramuscularly. One animal was monitored with a continuous intravenous infusion of propofol (30–40 mcg/kg/min) at spontaneous respiration. Pigs were placed in a right lateral position with the heart at the isocentre on MRI unit. ECG was monitored continuously.

cMRI images were acquired with a clinical 1.5 T scanner (Signa Excite HD; GE Medical Systems), using a non-breath-hold ECG gated, multi-NEX steady-state free precession pulse sequence (fast imaging employing steady-state acquisition). The heart was scanned along two long axis views (vertical and horizontal) and with a set of short axis views covering the entire LV from atrioventricular valve plane to the apex. The following parameters were used: field of view 30 cm, slice thickness 8 mm, no gap between each slice, repetition time 3.7 ms, echo time 1.6 ms, views for segment 2, flip angle 45°, bandwidth 125 Hz, 30 phases, matrix 224 × 224, reconstruction matrix 256 × 256, NEX 3, free breathing. Myocardial oedema at 2 days after MI was identified using T2-weighted short-tau inversion-recovery fast spin echo pulse sequence. The sequence parameters were field of view 30 cm; slice thickness 8 mm, TR 2 R-R intervals, TE 100 ms, TI 150 ms, matrix 256 × 256 (ref. 35). The main functional characteristics of pigs treated with either AAV6-control or AAV6-miR-199a as detected by cMRI at different time points are summarized in Extended Data Table 1.

Tagging-cMRI images were acquired with an electrocardiography-gated, segmented K-space, fast gradient recalled echo pulse sequence with spatial modulation of magnetization to generate a grid pattern. Nonselective radiofrequency pulses separated by spatial modulation of magnetization-encoding gradients allowed tag separation of 10 mm. Three sets of short-axis at basal, middle and apical level views were acquired with a grid of tags line with 45° and 135° angulation. The number of views per phase was optimized based on heart rate. The following parameters were used: field of view 30 cm, slice thickness 8 mm, no gap between each slice, repetition time 8 ms, echo time 4.3 ms, flip angle 15°, bandwidth 31 Hz, 30 phases, matrix 192 × 192, reconstruction matrix 256 × 256, NEX 3.

To identify the scar and quantify the extension of post-infarction fibrosis, delayed enhanced images were acquired in two-dimensional T1-weighted segmented inversion recovery gradient-echo–sequence 5–10 min after administration of gadoteric acid (Gd-DOTA 0.2 mmol/kg intravenous) in short- and long-axis views correspondent to those of cine-cMRI. The following parameters were used: field of view 30 cm, slice thickness 8 mm, no gap between each slice, repetition time 4.6 ms, echo time 1.3, flip angle 20°, matrix 224 × 192, reconstruction matrix 256 × 256, NEX 3.

To compare their transduction efficiency.

The parameters for episode detection were set as follows: fascicular ventricular tachycardia (interval 300 ms, duration 12/16 beats), ventricular tachycardia (interval 360 ms, duration 16 beats), bradycardia (interval 2000 ms, duration 4 beats), asystole (duration 3 s) and atrial fibrillation (all episodes).

cMRI image analysis. Randomized images were analysed in a blinded manner by a group of experts (Medical Imaging Department, Assisi University Hospital, Italy), using commercially available research software package (Mass 6). In the acute phase of MI, the region corresponding to infarct-related oedema was defined based on a signal intensity times higher than the mean SI of normal myocardium on T2-weighted short-axis images and the oedema size expressed as a percentage of total LV mass.

Global LV functional parameters (end-diastolic volume and end-systolic volume, ejection fraction) and left ventricular regional wall thickening (LVWT) were measured as previously described. LV endocardial and epicardial borders were manually traced on all short-axis cine images at the end-diastolic and end-systolic frames to determine the end-diastolic and end-systolic volumes, respectively, as well as ejection fraction and cardiac mass. The same software was used to calculate LVWT. In brief, the middle slice (area of interest), orthogonal to LV long axis, at 30% of its length starting from the apex, was divided into 8 equal circumferential segments (Fig. 2f). The inferoseptal segment at the connection of the right ventricle with the LV was defined as a reference point for the ventricular segmentation. Eight segments were plotted to generate the curve and subsequently calculate the AUC (Fig. 2i).

The analysis of tagged cardiac images was performed using a custom software based on the previously published method (see ‘Code availability’). The two-dimensional maximal circumferential (ECc) and radial strain (ERg) were evaluated along short-axis LV slices (basal, middle and apical), divided into 8 equal circumferential segments, starting from the reference point of the ventricular segmentation (Fig. 2f). The values for ECc and ERg, obtained for each segment, were plotted to generate curves, as in the case of LVWT (Fig. 2g, h, respectively) and, subsequently, the AUC was calculated to integrate all the values along the LV circumference (scheme in Fig. 2i).

LV volumetric assessment was performed with the first-pass technique. The first pass regional signal intensity/time curves, expressed as arbitrary unit/time, relative to different LV regions were generated using the Mass 6 software. Perfusion was evaluated semiquantitatively with signal intensity/time curves by
calculating the maximal upslope corresponding to the maximal signal intensity change during the wash-in phase for 18 segments obtained by sectioning the LV along three parallel short-axis planes, each divided in 6 segments.

To detect post-infarction fibrosis and to determine its size, the LV short-axis stack of LGE images was first assessed visually for the presence of LGE. The quantification of LGE was then performed on all LGE-positive studies by manually adjusting the full-width half-maximum method to differentiate the dense infarct core from the heterogeneous grey zone as previously described. The infarct core was defined as an area with SI >50% of maximal SI of enhanced myocardium. The grey zone of the infarct periphery was defined as the myocardium with SI < peak of remote myocardium but <50% of maximal SI of the high SI myocardium. Finally, infarction core and the grey zone were quantified as a percentage of the total myocardium and as a percentage of the total infarct size.

DNA and RNA isolation and quantification. Total DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer’s instructions and used as a template to detect and quantify vector DNA by real-time PCR. Primers and TaqMan probe (Applied Biosystems), recognizing the CMV promoter driving miR-199a expression, were as described. The pig housekeeping 18S rRNA gene was used for normalization (Thermofisher Scientific).

Total RNA, including the small RNA fraction, was isolated from pig tissue fragments using the mirNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA treatment was performed during RNA isolation according to the manufacturer’s protocol. For gene expression analysis, total RNA was quantified by Nanodrop and reverse transcribed using hexameric random primers followed by qRT–PCR. The housekeeping gene GAPDH was used for normalization.

For miR-199a-3p quantification, total RNA was reverse transcribed using miRCURY LNA PCR synthesis kit (Exiqon) and qRT–PCR was performed with pre-designed miRCURY LNA PCR primer sets (Exiqon) and miRCURY LNA SYBR Green master mix according to the manufacturer’s instructions. miRNA expression was normalized on the expression levels of 5S rRNA.

Histological and immunofluorescence analyses. The hearts were briefly washed in PBS, weighted, sectioned as shown in Extended Data Fig. 2a, b, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology or immunofluorescence. Haematoxylin and eosin and Masson’s trichrome staining (Bioptica) were performed according to standard procedure and analysed for morphology; the extent of fibrosis was measured on ×4 magnification images using ImageJ.

For immunostaining, pig heart sections were deparaffinized in xylene and hydrated. Antigen retrieval was performed by boiling samples in sodium citrate solution (0.1 M, pH 6.0) for 20 min. Sections were allowed to cool down and permeabilized for 20 min in 1% Triton X-100 in PBS, followed by blocking in 1% BSA (Roche). Sections were then stained overnight at 4°C with the following primary antibodies diluted in blocking solution, recognizing the following antigens: sarcomeric α-actinin (Abcam), Ki67 (Cell Signaling), histone H3 phosphorylated at serine 10 (Millipore), aurora B kinase (Abcam), GATA4 (Abcam), desmin (Roche), myogenin (Cell Marque), endothelin receptor B (Abcam), WT1 (Cell Marque), CD34 (Roche), CD45 (Roche). Sections were washed with PBS and incubated for 2 h with the respective secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 647 (Life Technologies). Nuclei were stained with Hoechst 33342 (Life Technologies). Alternatively, after endogenous peroxidase inhibition with 3% H2O2, sections were incubated with appropriate biotin-conjugate secondary antibody (Abcam) in TBS–BSA 1% for 1 h at room temperature. Following signal amplification with avidin–biotin complex–HRP (VECTASTAIN), DAB solution (VECTOR) was applied for 3 to 10 min. Haematoxylin (Biotica) was further used to stain nuclei.

For BrDU incorporation analysis, after section permeabilization, DNA denaturation was obtained by incubating 10 min in 1 M HCl on ice and 20 min in 2 M HCl at 37°C. Sections were further incubated with 0.1 M sodium-borate buffer pH 8.4 for 12 min at room temperature, washed three times with PBS and then blocked for 1 h in 10% horse serum in PBS. Tissue sections were stained overnight at 4°C with α-actinin antibody (Abcam) in 5% horse serum in PBS and in anti-BrDU (Abcam). Washes and secondary antibody incubation were performed as described above.

To measure cardiomyocyte cross-sectional area, lectin WGA (Vector Laboratories) was diluted 1:100 in PBS and added with the secondary antibody to sample sections and incubated as described above. Capillary density was determined after staining histological sections with lectin WGA together with α-SMA antibody (Sigma) diluted in PBS.

In all quantifications of immunofluorescence and immunohistochemistry images, we considered, for each animal, at least 8 sectors belonging to all four heart sections shown in Extended Data Fig. 2a, b. For each region considered, histological analysis was performed by acquiring 7 high-resolution images at 20× magnification, which were quantified by blinded researchers.

In situ hybridization. miRNA in situ hybridization (ISH) was performed using LNA probes for miR-199a-3p and U6 snRNA, as well as an oligonucleotide with the same nucleotide content as the anti-miR-199a probe but in a scrambled sequence. Experiments were performed using a miRNA ISH kit for formalin-fixed paraffin-embedded (FFPE) tissues (Qiagen) according to the manufacturer’s protocol. In brief, FFPE heart tissue slides were deparaffinized in xylene, treated with protease-K (15 µg/ml) for 10 min at 37°C and incubated with hsa-miR-199-3p (20 nM), scramble (20 nM) and U6 probes (2 nM) for 1 h at 57°C in a hybridizer. After washing with SSC buffer, miRNA expression was detected using an anti-DIG alkaline phosphatase (AP) antibody (1:800) (Roche Diagnostics) supplemented with goat serum (Jackson Immunoresearch) and NBT-BCIP sub-

Statistical analysis. No statistical methods were used to predetermine sample size. Data are presented as mean ± s.e.m. Statistical analysis was performed using commercially available software (GraphPad Prism). Data were first checked for normal distribution, then differences among groups were compared by one-way and two-way ANOVA followed by the Bonferroni post hoc test. Comparisons between two groups were made using the unpaired t-test. For survival analysis, a Kaplan–Meier survival curve was generated and log-rank statistics test was rendered. The AUC was obtained using the trapezoidal rule and statistical comparisons performed by one-way and two-way ANOVA. For all statistical analyses, significance was accepted at P < 0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All relevant data are included in the paper, the Extended Data and Supplementary Information. Source data for Figs. 1–3 are available in the online version of the paper.

Code availability

Tagged cardiac images were analysed using custom software called ‘tagging tool’, based on the previously described method. This software was implemented by the UOC Magnetic Resonance of Fondazione Toscana Gabriele Monasterio, Pisa, Italy. To request access to this software please contact G.D.A. (aquaro@ftgm.it).

This software is only for use in animal studies and clinical use in humans is not permitted.

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Extended Data Fig. 1 | Transduction of swine hearts after MI with AAV vectors. **a, b,** AAV6 is the most effective serotype for porcine heart transduction. The graphs show viral genomes (a) and eGFP mRNA (b) levels one month after direct intramyocardial injection of $1 \times 10^{12}$ viral genome particles of AAV6, AAV8 and AAV9 vectors carrying the eGFP transgene (these three AAV serotypes have been reported to transduce post-mitotic tissues at high efficiency). Data are mean ± s.e.m.; the number of animals per group is indicated. **c,** Nucleotide sequence of the miR-199a-1 precursor. Mature miR-199a-5p and miR-199a-3p sequences are shown in green and their seed sequences are shown in blue and red, respectively. **d,** Mature miR-199a-5p and miR-199a-3p sequences are conserved in human, mouse, rat and pig. The miRNA seed sequences are shown in blue for miR-199a-5p and in red for miR-199a-3p. **e,** Representative photograph taken during porcine surgery and vector injection. After thoracotomy, the pericardial sac was opened, the LAD was exposed and occluded below its first branch for 90 min. Ten minutes after reperfusion, AAV6-control or AAV6-miR-199a were injected into the infarct border zone.
Extended Data Fig. 2 | Systematic assessment of miR-199a-3p expression after AAV6-mediated transduction. 

**a**, Schematic representation of pig heart sectioning for histological and molecular studies. After arrest in diastole, the heart was excised and the pericardial sac removed. AAV injection sites, which were marked with coloured epicardial sutures during surgery, were further traced with a green waterproof paint. Four 1-cm thick transversal slices were cut starting from the base to the apex (1–4). Each slice was subsequently divided into 2–8 regions, each one labelled with a capital letter, and then into additional sub-regions (letters plus numbers) for targeted molecular and histological analyses. Sectors H, T and C corresponded to the infarct border zone, where the vectors were administered, whereas sector L was considered representative of the remote zone. 

**b**, Injection and infarct border segments for each slice were divided into smaller fragments (dashed lines) to accurately assess the levels of expression of the transgene at 12 days after transduction. The syringe indicates the injection sites.

**c**, For each slice and segment, the graphs show real-time PCR quantifications of the mature miR-199a-3p expressed as fold change over endogenous levels (AAV6-control). One representative animal is shown out of four analysed in the same systematic manner with comparable results.

**d**, In situ hybridization of pig heart sections for the detection of miR-199a expression at the single-cell level. Each of the sectors indicated in b was tested by in situ hybridization using LNA probes detecting miR-199a-3p or U6 snRNA, or a probe with the same nucleotide composition as the one against miR-199a-3p but with a scrambled sequence (scramble). Expression of miR-199a-3p was robust in cardiomyocytes and specific for the injected areas throughout the LV. One representative animal is shown out of four analysed in the same systematic manner with comparable results. Scale bar, 100 µm.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Downregulation of miR-199a target genes in transduced heart tissue and organ distribution of the AAV6-miR-199a vector. 

a, Real-time PCR quantification of both strands of miR-199a in AAV6-control- and AAV6-miR-199a-injected pig hearts (n = 4 and n = 10, respectively) normalized to endogenous 5S rRNA. Data are mean ± s.e.m. 

b, mRNA levels of predicted and annotated target genes of miR-199a in AAV6-control- and AAV6-miR-199a-treated pig hearts (n = 4 per group) one month after MI and viral transduction. Data are mean ± s.e.m.; *P < 0.05 versus AAV6-control; two-sided t-test. 

c–e, Predicted target sites of miR-199a-3p in the 3′-untranslated region (UTR) sequences of swine cofilin-2, TAOK1 and βTRC according to TargetScan release 7.2. All three of these genes are verified to be direct targets of this miRNA in rodents; the corresponding 3′ UTR target sites for cofilin-2 and TAOK1 are conserved in swine; for βTRC, two alternative target sites in swine are shown. Other miR-199a-3p target genes originally identified in mice (in particular, homer1 and Clic5) are not conserved in the swine genome. In the pig genome, βTRC also has an additional predicted target sequence for miR-199a-5p, which is indicated. 

f, Predicted target site of miR-199a-5p in the 3′ UTR of pig HIF1A mRNA. 

g, Quantification of viral genomes in the indicated organs one month after intracardiac injection of AAV6-miR199a. Data are expressed as fold change over liver levels after normalization for cellular DNA content using 18S DNA as a reference (mean ± s.e.m., n = 4 per group). The levels of viral DNA in the myocardium of the injected animals were more than 18 times higher than in the liver and more than 40 times higher than in other organs (spleen, kidney and lung). 

h, Levels of miR-199a-3p RNA in the indicated organs one month after intracardiac injection of AAV6-miR-199a. Data are shown as fold change over endogenous miRNA levels in the liver of control animals after normalization for cellular 5S rRNA (n = 4 per group). Data are mean ± s.e.m. The amount of hsa-miR-199a-3p RNA was not elevated in any analysed organ, except for the heart. No overt signs of pathology, including hyper-proliferation (assessed by Ki67 staining) were observed.
Extended Data Fig. 4  | miR-199a improves global heart function and decreases infarct mass one month after treatment. a, Graphs showing percentage changes in infarct mass, infarct mass over LV mass and ejection fraction, as indicated, between 2 and 28 days after MI and AAV6-control or AAV6-miR-199a delivery, measured by cMRI. The number of analysed animals were 7 and 8, 7 and 8, and 7 and 9 for infarct mass, infarct mass over LV mass and ejection fraction for the two groups, respectively. Top graphs, cumulative values for all pigs. Data are mean ± s.e.m.; *P < 0.05; two-tailed t-test; bottom graphs, data from individual pigs. b, Infarct healing one month after AAV6-miR-199a injection. The LGE-cMRI images (from apex to base, a to e) are the same as in Fig. 1h, but without red counterstain. The red arrows show the infarcted area in the central plane. c, Gross anatomy of cardiac slices with corresponding LGE-cMRI images in representative AAV6-control- and AAV6-miR-199a-treated pig hearts, at 28 days after MI. d, Heart rate in sham and infarcted animals injected with AAV6-control and AAV6-miR-199a at one month after treatment. Data are mean ± s.e.m.; the number of animals per group and time point are indicated.
Extended Data Fig. 5 | AAV6-miR-199a induces cardiomyocyte proliferation in vivo. 

**a**, Representative images of Ki67 and α-actinin immunofluorescence staining of the infarct border (sector H) or remote (sector L) zones of AAV6-control- and AAV6-miR-199a-treated animals ($n = 4$ and $n = 6$, respectively; analysis is from at least seven high-resolution images acquired from at least eight different regions of each heart), 12 days after MI. Scale bars, 100 µm. At least six animals were treated.

**b**, High-magnification, representative images of phospho-histone H3 immunostaining in the infarct border zones of four different pigs treated with AAV6-miR-199a, 12 days after MI. Scale bar, 100 µm.
Extended Data Fig. 6 | Multinucleation and cardiomyocyte hypertrophy in miR-199a-treated pig hearts. a, Representative images of longitudinal sections stained with WGA to assess the number of nuclei per cardiomyocyte in the infarct border zone of AAV6-control- and AAV6-miR-199a-treated animals (n = 4 and n = 6, respectively; analysis is from at least seven high-resolution images acquired from at least eight different regions of each heart), 12 days after MI. The right panels show the estimated number of nuclei for each cardiomyocyte. Scale bar, 50 µm. b, Additional representative images of mono- or bi-nucleated BrdU+ cardiomyocytes in the infarct border zone of AAV6-control- and AAV6-miR-199a-treated animals, 12 days after MI. Scale bar, 50 µm. c, Cross-sectional area measurements of BrdU+ and BrdU− cardiomyocytes in AAV6-control- and AAV6-miR-199a-treated pigs 12 days after surgery. Data are mean ± s.e.m. from the analysis of four pigs. d, Representative images of BrdU+ and BrdU− cardiomyocytes. Scale bar, 50 µm. The right panels are high-magnification images of the indicated portions of the left images.
Extended Data Fig. 7 | Expression of GATA4 in cardiomyocytes in the infarct border zone of AAV6-miR-199a-treated pigs. a, Left, representative immunohistochemistry images of GATA4+ cells in AAV6-control and AAV6-miR-199a-injected pigs, 30 days after treatment. The bottom panels are high-magnification images of the indicated portions of the top images. The graph on the right shows the quantification of cells showing GATA4 cytoplasmic localization. Data are mean ± s.e.m.; the number of animals per group is indicated. Quantification is from at least seven high-resolution images acquired from at least eight different regions of each heart. *P < 0.05; two-sided Student’s t-test. Scale bars, 100 µm. b, c, Additional low- and high-magnification representative immunohistochemistry images of GATA4+ cells in the infarct border (sector H) or remote zone (sector L) of AAV6-control- and AAV6-miR-199a-injected pigs, 12 days (b) and 30 days (c) after treatment. Scale bars, 100 µm. d, AAV6-miR-199a treatment does not alter the levels of DAB2, SMARCA5 and DESTRIN mRNAs. The graphs show real-time PCR quantifications of the levels of the indicated genes in sham, AAV6-control- and AAV6-miR-199a-injected pig hearts, at 12 and 30 days after surgery; n = 3 per group. Data are mean ± s.e.m.; the number of animals per group and time point is indicated. *P < 0.05 versus AAV6-control at the same time point; two-sided t-test.
Extended Data Fig. 8 | Molecular correlates of miR-199a transduction. a, Real-time PCR quantification of the ratio between α- and β-myosin heavy chain mRNA in sham, AAV6-control- and AAV6-miR-199a-injected pig hearts, at 12 and 30 days after surgery in the H (border zone) and L (remote zone) cardiac sectors. Data are mean ± s.e.m.; the number of animals per group and time point is indicated. *P < 0.05 versus AAV6-control at the same time point; two-way ANOVA with Bonferroni post hoc correction. b, Lectin immunofluorescence (b) of sham, AAV6-control- and AAV6-miR-199a-treated pig sections, 30 days after MI and vector administration along with quantification (c) of cardiomyocyte cross-sectional area (μm²). Data are mean ± s.e.m.; the number of analysed animals is indicated. One-way ANOVA with Bonferroni post hoc correction. Scale bars, 50 μm. c, Low- and high-magnification (insets) representative images of infarcted hearts injected with AAV6-control or AAV6-miR-199a after immunohistochemistry to detect desmin (which is essential for maintaining structural and functional integrity of cardiomyocytes and was expressed at normally high levels), myogenin (which coordinates skeletal myogenesis and repair and was not expressed), endothelin-B receptor (which selectively stained arteriole smooth muscle cells) and WT1 (which was expressed at low levels in the vascular endothelium, but not in cardiomyocytes). Analysis was performed in at least seven high-resolution images acquired from at least eight different regions of the hearts of three pigs per group. Scale bars, 100 μm. e, Real-time PCR quantification of the levels of ANP and BNP in sham, AAV6-control- and AAV6-miR-199a-injected pig hearts, at 12 and 30 days after surgery. Data are mean ± s.e.m.; the number of animals per group and time point are indicated. NS, not significant; *P < 0.05 versus AAV6-control at the same time point. One-way ANOVA with Bonferroni post hoc correction. f, Representative sections of pig hearts treated with AAV6-control and AAV6-miR-199a at day 30 after infarction and vector injection stained with FITC–lectin to visualize vessels and with an α-SMA antibody to detect smooth muscle cells, along with quantification of lectin-positive vessels. No significant difference between the two MI groups was detected in capillary density at either 12 or 30 days. Data are mean ± s.e.m.; the number of animals per group is indicated. Analysis was performed in at least seven high-resolution images acquired from at least eight different regions of the heart. *P < 0.05; Student’s two-sided t-test. Scale bar, 100 μm.
Extended Data Fig. 9 | Long-term expression of miR-199a induces progressive cardiac regeneration. a, The LGE-cMRI images (from apex to base, a–c) are the same as those in Fig. 4a, but without red counterstain. The red arrows show the infarcted area in the central plane. b, cMRI images from a pig euthanized at week 8 after MI and AAV6-miR-199a treatment. Top, serial images from apex to base at day 2, week 4 and week 8; the infarct area is counterstained in red. Bottom, the same images without counterstaining. The green arrows show the pacemaker-lead attachment sites. c, Gross anatomy of cardiac slices of the pig shown in b after euthanization. The syringe indicates the injected area. The green arrows show the pacemaker-lead attachment sites. Similar cardiac repair results were observed in three pigs treated with miR-199a that survived two months after treatment.
Extended Data Fig. 10 | Recording of fatal arrhythmias in two infarcted pigs treated with AAV6-miR-199a-3p. Initiation of ventricular fibrillation recorded at the moment of death in two AAV6-miR-199a pigs by implanted miniaturized ECG recorders (Reveal, Medtronic, 9529).

a, A premature ventricular ectopic beat (red arrow) with a coupling interval of 380 ms during a slowing heart rhythm induced a fast ventricular tachycardia that degenerated in ventricular fibrillation.

b, A premature ventricular ectopic beat (red arrow) with coupling interval of 350 ms induced a fast ventricular tachycardia that quickly degenerated in ventricular fibrillation of different amplitudes resembling polymorphic ventricular tachycardia.

c, AAV6-mediated, long-term expression of miR-199a did not affect the expression levels of ion channels or associated proteins involved in known arrhythmogenic conditions. In the infarct border zone of pigs treated with AAV6-control or AAV6-miR-199a (n = 6 and n = 4, respectively) at 30 days after transduction, the expression levels of genes known to be involved in the pathogenesis of long QT syndrome (SCN5A, KCNE1, SNTA1, AKAP9 and ANK2), Brugada syndrome (CACNA1, CACNB2 and SCN1B), Carvajal syndrome (DSP), arrhythmogenic right ventricular cardiomyopathy (DSG2 and DSP) and catecholaminergic polymorphic ventricular tachycardia (CASQ2 and RYR2) were assessed. Additional investigated mRNAs were those coding for SERCA2A (which is encoded by ATP2A2 and also served as a positive control since it is depressed during heart failure and was found increased in miR-199a-treated animals), phospholamban (PLN) and connexins 40 and 43 (CX40 (which is encoded by GJA1) and CX43 (which is encoded by GJA5), respectively). The miR-199a-treated pigs in which analysis was performed included one pig that survived eight weeks (pig 50) and three pigs that died from sudden death at seven weeks (pigs 55, 66 and 67).

Data are mean ± s.e.m. *P < 0.05 versus AAV6-control; Student’s two-sided t-test.
Extended Data Fig. 11 | See next page for caption.
Extended Data Fig. 11 | miR-199a induces formation of proliferating cell clusters with an early myoblast phenotype infiltrating the pig myocardium. Additional images of cell clusters infiltrating the infarcted hearts injected with AAV6-miR-199a after haematoxylin and eosin staining or immunostaining to detect the indicated antigens. These cells scored negative for the leukocyte common antigen CD45 and for CD34 (excluding their immune, haematopoietic or endothelial origin) and were highly proliferating, as inferred from almost complete positivity for Ki67. These cells also scored negative for markers of muscle differentiation, including desmin (identifying myogenic cells of cardiac, smooth and striated muscle), sarcomeric α-actinin (which labels Z lines in the cardiac and skeletal muscle sarcomere) and HHF35 (a monoclonal antibody recognizing muscle-specific α- and γ-actin); cells were also negative for WT1 (marking several malignancies and the epicardium). The infiltrating cells were positive for GATA4 (which is critical for proper mammalian cardiac development) and myogenin (the reactivation of which characterizes rhabdomyosarcoma cells) as well as the calmodulin-binding protein caldesmon (which regulates smooth muscle contraction and is expressed at high levels in leiomyoma and leiomyosarcoma) and the endothelin-B receptor, normally expressed in smooth muscle cells. The pig identity, treatment, time of analysis and cardiac sector from which the sample was taken are shown for each picture. Scale bars, 100 µm. Clusters of cells were never detected in control-injected animals, although in one animal injected with AAV6-miR-199a clusters of cells were detected in the absence of MI.
## Extended Data Table 1 | Functional and morphological parameters from cMRI analyses of the pig hearts

Animals are divided according to treatment (AAV-control and AAV-miR-199a) and day of analysis (day 2 and day 28).

| Day 2 | AAV6-Control | EDV (ml) | ESV (ml) | SV (ml) | EF (%) | scar (g) | LV mass (g) | scar (%) |
|-------|--------------|----------|----------|---------|--------|----------|-------------|---------|
| 1 Pig 43 | 39.65 | 14.87 | 25.00 | 63.05 | 9.00 | 47.90 | 18.79 |
| 2 Pig 46 | 62.20 | 24.00 | 38.20 | 61.41 | 13.06 | 57.72 | 22.63 |
| 3 Pig 51 | 65.36 | 23.94 | 41.42 | 63.37 | 12.57 | 48.27 | 26.04 |
| 4 Pig 52 | 57.32 | 20.78 | 36.54 | 63.74 | 14.92 | 63.39 | 23.53 |
| 5 Pig 68 | 58.00 | 21.80 | 36.20 | 62.41 | 7.94 | 50.59 | 15.69 |
| 6 ADD 03 | 61.11 | 19.45 | 41.66 | 68.17 | 5.10 | 52.03 | 9.80 |
| 7 ADD 07 | 71.18 | 27.92 | 43.26 | 60.77 | 23.40 | 69.56 | 33.64 |
| 8 ADD 13 | 70.82 | 27.15 | 43.67 | 61.66 | 13.82 | 70.67 | 19.56 |
| Mean | 60.70 | 22.49 | 38.24 | 63.07635 | 12.48 | 57.52 | 21.21 |

| Day 28 | AAV6-Control | EDV (ml) | ESV (ml) | SV (ml) | EF (%) | scar (g) | LV mass (g) | scar (%) |
|--------|--------------|----------|----------|---------|--------|----------|-------------|---------|
| 1 Pig 43 | 62.77 | 21.15 | 41.62 | 66.30 | 13.51 | 60.86 | 22.20 |
| 2 Pig 46 | 72.17 | 29.50 | 42.67 | 59.12 | 13.24 | 68.00 | 19.47 |
| 3 Pig 51 | 77.02 | 25.55 | 51.47 | 66.83 | 11.67 | 54.61 | 21.37 |
| 4 Pig 52 | 94.63 | 50.26 | 44.37 | 46.89 | 15.23 | 59.11 | 25.77 |
| 5 Pig 68 | 61.10 | 22.10 | 39.00 | 63.83 | 10.05 | 58.48 | 17.19 |
| 6 ADD 03 | 67.89 | 21.45 | 46.44 | 68.40 | 2.2 | 53.04 | 4.15 |
| 7 ADD 07 | 97.79 | 48.35 | 49.44 | 50.58 | 14.89 | 68.57 | 21.72 |
| 8 ADD 13 | 119.94 | 61.37 | 58.57 | 48.83 | 13.43 | 62.68 | 21.43 |
| 9 Pig 25 | 128.42 | 80.03 | 48.39 | 37.68 | 19 | 63.45 | 29.94 |
| 10 Pig 29 | 82.07 | 41.35 | 40.72 | 49.62 | 23.94 | 79.30 | 30.19 |
| 11 Pig 33 | 96.77 | 55.17 | 41.60 | 42.99 | 18.6 | 99.78 | 18.64 |
| 12 Pig 34 | 96.08 | 44.04 | 52.04 | 54.16 | 5.72 | 70.96 | 8.06 |
| Mean | 88.05 | 41.70 | 46.36 | 54.60 | 13.46 | 66.57 | 20.01 |

| Day 2 | AAV6-miR-199a | EDV (ml) | ESV (ml) | SV (ml) | EF (%) | scar (g) | LV mass (g) | scar (%) |
|-------|---------------|----------|----------|---------|--------|----------|-------------|---------|
| 1 Pig 54 | 69.15 | 30.59 | 38.56 | 55.76 | 18.52 | 54.19 | 34.18 |
| 2 Pig 55 | 60.10 | 28.37 | 31.73 | 52.79 | 10.38 | 59.96 | 17.32 |
| 3 Pig 66 | 64.18 | 20.71 | 43.46 | 67.72 | 14.17 | 58.66 | 24.16 |
| 4 Pig 67 | 73.17 | 23.98 | 49.19 | 67.23 | 10.62 | 61.15 | 17.37 |
| 5 Pig 69 | 49.40 | 17.02 | 32.38 | 65.55 | 14.76 | 51.21 | 28.82 |
| 6 ADD 05 | 70.20 | 19.74 | 50.68 | 72.19 | 7.21 | 53.59 | 13.45 |
| 7 ADD 06 | 57.64 | 20.56 | 37.07 | 64.31298 | 10.8 | 64.36 | 16.78 |
| 8 ADD 16 | 57.75 | 27.56 | 30.19 | 52.27706 | 16.08 | 63.7 | 25.24 |
| Mean | 62.70 | 23.57 | 39.16 | 62.23 | 12.82 | 58.35 | 22.16 |

| Day 28 | AAV6-miR-199a | EDV (ml) | ESV (ml) | SV (ml) | EF (%) | scar (g) | LV mass (g) | scar (%) |
|--------|---------------|----------|----------|---------|--------|----------|-------------|---------|
| 1 Pig 54 | 76.00 | 33.85 | 42.15 | 55.46 | 12.35 | 61.35 | 20.13 |
| 2 Pig 55 | 97.16 | 34.15 | 63.01 | 64.85 | 8.20 | 63.73 | 12.87 |
| 3 Pig 66 | 65.81 | 18.7 | 47.11 | 71.58 | 3.98 | 51.4 | 7.74 |
| 4 Pig 67 | 85.44 | 23.48 | 61.96 | 72.52 | 6.00 | 64.00 | 9.37 |
| 5 Pig 69 | 68.98 | 26.73 | 42.25 | 61.25 | 7.21 | 57.80 | 12.47 |
| 6 ADD 05 | 72.00 | 18.34 | 53.66 | 74.53 | 3.49 | 64.64 | 5.40 |
| 7 ADD 06 | 66.42 | 19.96 | 46.46 | 69.95 | 3.20 | 66.20 | 4.83 |
| 8 ADD 16 | 92.67 | 48.51 | 44.16 | 47.65 | 11.72 | 60.02 | 19.53 |
| 9 Pig 21 | 62.92 | 16.76 | 46.16 | 73.36 | 4.88 | 69.40 | 7.03 |
| 10 Pig 26 | 80.43 | 29.84 | 50.59 | 62.90 | 6.60 | 65.39 | 10.09 |
| 11 Pig 27 | 92.40 | 32.99 | 59.41 | 64.30 | 5.99 | 59.70 | 10.03 |
| 12 Pig 32 | 78.44 | 33.29 | 45.15 | 57.56 | 11.40 | 69.50 | 16.40 |
| 13 Pig 50 | 76.83 | 25.15 | 51.68 | 67.26 | 5.24 | 55.78 | 9.39 |
| Mean | 78.12 | 27.83 | 50.29 | 64.86 | 6.95 | 62.226 | 11.18 |
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|---|-----------|
| n/a | The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement |
|   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
|   | The statistical test(s) used AND whether they are one- or two-sided |
|   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
|   | A description of all covariates tested |
|   | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
|   | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
|   | For null hypothesis testing, the test statistic (e.g. \( F, t, r \)) with confidence intervals, effect sizes, degrees of freedom and \( P \) value noted |
|   | Give \( P \) values as exact values whenever suitable. |
|   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
|   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
|   | Estimates of effect sizes (e.g. Cohen’s \( d \), Pearson’s \( r \)), indicating how they were calculated |
|   | Clearly defined error bars |
|   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | Code Availability. Tagged cardiac images were analysed using the custom software named "Tagging Tool", based on a previously described method (ref 35.) This software was implemented by the UOC Magnetic Resonance of Fondazione Toscana “G. Monasterio”, Pisa, Italy. For any request to access to this software please contact GDA (email: aquaro@ftgm.it). This software is only for animal study and clinical use in humans is not permitted. |
| Data analysis | Data were analysed by GraphPad Prism 7.0 |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data
Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All relevant data are included in the paper and its Extended Data Information

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [x] Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/reporting#summary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
The sample size was predicted based on power analysis, assuming a power of 0.8, a 30% or higher difference in means with a standard deviation of 25% of the means and an alpha=0.05. Based on historical data, these standard deviation values are typical for most of the morpho-functional parameters that we considered in our study.

Data exclusions
No data were excluded

Replication
All molecular data were replicated at least three times by different investigators. Large animal cMCI data were obtained on the indicated number of pigs over a period of almost 2 years. Animals submitted to the same type of treatment during this period showed a similar response to treatment.

Randomization
Animal allocation to treatment groups (AAV6-Control, AAV6-miR199a, sham) was randomized

Blinding
The examiners performing MRI analyses and histological analyses were blind as to treatment group allocation of animals

Materials & experimental systems

Policy information about availability of materials

n/a Involved in the study

- [x] Unique materials
- [ ] Antibodies
- [ ] Eukaryotic cell lines
- [ ] Research animals
- [ ] Human research participants

Unique materials

Obtaining unique materials
Unique materials are AAV vectors generated for this study by the ICGEB AAV production facility – the corresponding plasmids used to generate these vectors will be rendered freely available

Antibodies

Antibodies used

Antibodies were against: sarcomeric β-actin (Abcam #ab9465, clone EA-53, 1:250 dilution, granted to work in paraffin-embedded pig tissue https://www.abcam.com/sarcomeric-alpha-actin-antibody-ea-53-ab9465.html); Ki67 (Cell Signaling #515, clone D3B8, 1:100 dilution, validated in paraffin-embedded pig tissue); histone H3 phosphorylated at serine 10 (Millipore #05-570, polyclonal, 1:250 dilution, validated in paraffin-embedded pig intestine); Aurora B kinase (Abcam #ab2254, polyclonal, 1:200 dilution, granted to work in paraffin-embedded pig tissue https://www.abcam.com/aurora-b-antibody-ab2254.html); GATA4 (Abcam #ab124265, polyclonal, 1:100 dilution, suitable for paraffin-embedded immunohistochemistry https://www.abcam.com/gata4-antibody-ab124265.html, validated in pig tissue); BrdU (Abcam, #ab6126, clone BU1/75 (ICR1), 1:250 dilution, suitable for paraffin-embedded immunohistochemistry https://www.abcam.com/brdu-antibody-bu175-icr1-ab6126.html, validated in pig tissue); Alpha-SMA (Sigma #C0621, clone 1A4); Wheat Germ Agglutinin (WGA; Vector Labs #FL1013, 1:100 dilution); Alexa Fluor 488, 555 or 647 (secondary antibodies; 1:50, Life Technologies). The following antibodies are in routine use for diagnostic purposes at the Pathology Unit at the University Hospital in Trieste (author: RB): desmin (Roche catalogue n. 0526700S001, clone DER11, prediluted), myogenin (Roche catalogue n. 05208290001, clone F50,
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) | HeLa and HEK293T cells were originally obtained from ATCC. Their use is irrelevant to the study, since the former was used a source of DNA to clone the miR-199a gene while the second is commonly used for the production of AAV vectors. No experimental data using these cell lines are described in the manuscript.

Authentication | Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.

Mycoplasma contamination | Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register) | None any commonly misidentified cell lines used in the study and provide a rationale for their use.

Research animals

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials | Three- to four-month old male farm pigs, weighting 28-32 kg, were used in this study.

Method-specific reporting

n/a Involved in the study

- [x] ChIP-seq
- [x] Flow cytometry
- [ ] Magnetic resonance imaging

Magnetic resonance imaging

Experimental design

Design type | Indicate task or resting state; event-related or block design.

Design specifications | Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures | State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s) | Cardiac functional, cardiac structural, cardiac perfusion, cardiac tagging

Field strength | 1.5 T

Sequence & imaging parameters | Spin echo pulse sequence. The sequence parameters: field of view 30 cm, matrix 224 x 224, reconstruction matrix 256 x 256, NEX 3, free breathing, slice thickness 8 mm, no gap between each slice, repetition time 3.7 msec, echo time 1.6 msec, views for segment 2, flip angle 45°, bandwidth 125 Hz, 30 phases.

Area of acquisition | Whole cardiac scan

Diffusion MRI | Not used

Preprocessing

Preprocessing software | Commercially available research software package (Mass 6, Leyden, The Netherlands) was used for cardiac functional and structural parameters, edema, myocardial perfusion, quantification of myocardial fibrosis. The analysis of tagged cardiac images was performed using a custom software tagging tool based on the method by Bogaert et al.
| **Normalization** | Parameters were normalized on body surface area, LGE and edema segmental analyses were indexed by left ventricle mass |
|-------------------|------------------------------------------------------------------------------------------------------------------|
| **Normalization template** | Not suitable |
| **Noise and artifact removal** | ECG gating for pulse sequence and the multiple averages (number of excitation; NEX) was used to reduce respiratory motion. |
| **Volume censoring** | Not suitable |

### Statistical modeling & inference

**Model type and settings**
Specify (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

**Effect(s) tested**
Differences among groups were compared by one- and two-way ANOVA followed by the Bonferroni post-hoc test.

Specify type of analysis:
- Whole brain
- ROI-based
- Both

Anatomical location(s)
The heart was scanned along two long axis views (vertical and horizontal) and with a set of short axis views covering the entire LV from atrioventricular valve plane to the apex.

**Statistic type for inference (See Eklund et al. 2016)**
Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

**Correction**
Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

### Models & analysis

n/a Involved in the study
- Functional and/or effective connectivity
- Graph analysis
- Multivariate modeling or predictive analysis

**Functional and/or effective connectivity**
Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

**Graph analysis**
Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

**Multivariate modeling and predictive analysis**
Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.