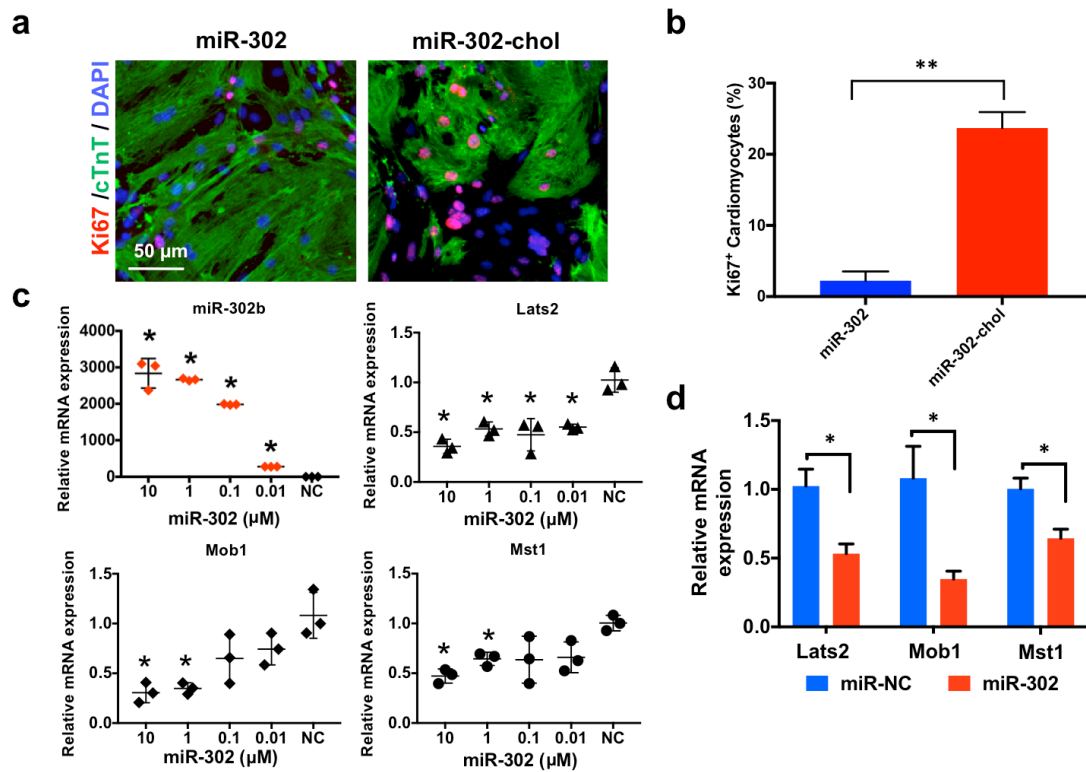


In the format provided by the authors and unedited.

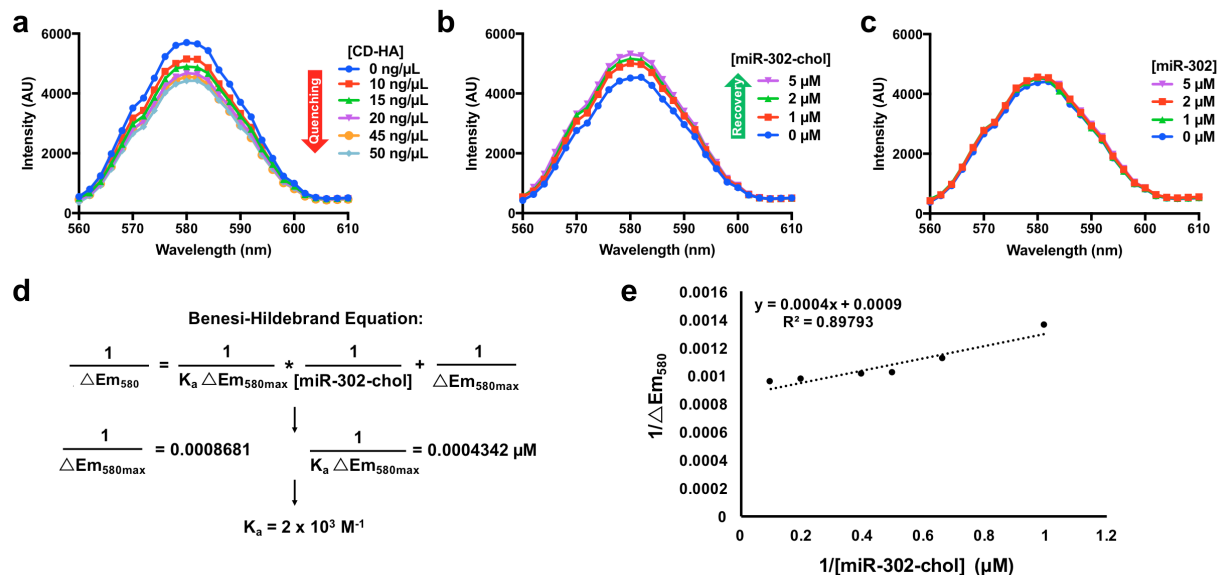
Sustained miRNA delivery from an injectable hydrogel promotes cardiomyocyte proliferation and functional regeneration after ischaemic injury

Leo L. Wang¹, Ying Liu², Jennifer J. Chung³, Tao Wang², Ann C. Gaffey³, Minmin Lu², Christina A. Cavanaugh², Su Zhou², Rahul Kanade³, Pavan Atluri³, Edward E. Morrisey^{2*} and Jason A. Burdick^{1*}

¹Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, USA. ²Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA. ³Division of Cardiovascular Surgery, Department of Surgery, University of Pennsylvania, Philadelphia, PA, USA. Leo L. Wang and Ying Liu contributed equally to this work. *e-mail: emorrise@mail.med.upenn.edu; burdick2@seas.upenn.edu

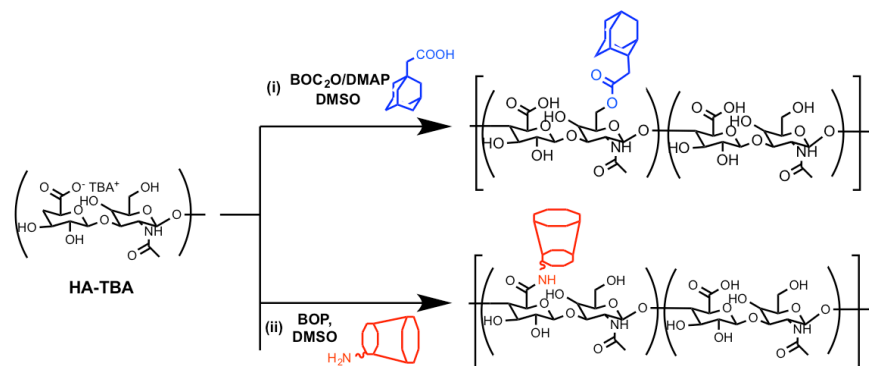


Supplementary Figure 1. Cholesterol-modification of miR-302 mimics. Proliferation of neonatal mouse cardiomyocytes by un-modified (miR-302) and cholesterol-modified (miR-302-chol) miR-302b and miR-302c mimics (200 μ M), using a) co-staining and b) quantification of cTnT and Ki67 to denote cardiomyocyte proliferation. Cardiomyocytes were treated with miR-302 for 24 hours; staining was performed after 48 hours. Quantification was based on counting of Ki67⁺cTnT⁺ co-stained cells relative to total cTnT⁺ cells per high power field (mean $\pm$ SD, n=3, **p<0.01). c) qPCR quantification of miR-302b, Lats2, Mob1 and Mst1 in neonatal cardiomyocytes following dose response to cholesterol-modified miR-302 demonstrated a dose dependent knockdown of Hippo components (mean $\pm$ SD, n=3, *p<0.05 compared to miR-NC). d) Quantification of miR-302 knockdown by qPCR at 1 μ M (mean $\pm$ SD, n=3, *p<0.05 compared to miR-NC).

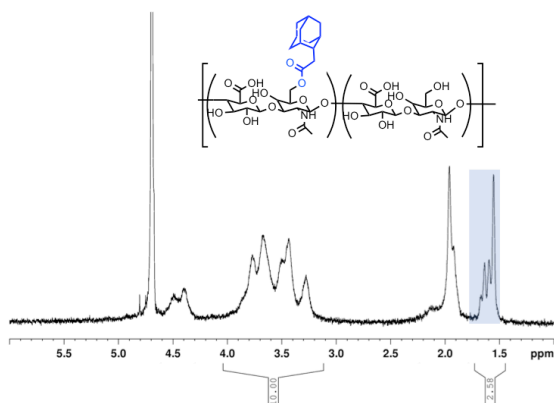


Supplementary Figure 2. Cholesterol-modified miR-302 interaction with CD-HA. a) Rhodamine B fluorescence was quenched by CD-HA due to hydrophobic interaction. Increasing amounts of CD-HA saturated quenching at 50 ng/μL. b) At a saturating concentration of CD-HA (50 ng/μL), cholesterol-modified miR-302 interacted with CD-HA/Rho B complexes to displace and unquench rhodamine, recovering fluorescence in a dose-dependent fashion. c) Unmodified miR-302 did not lead to fluorescent recovery of CD-HA/Rho B complexes. d) The Benesi-Hildebrand equation was used to determine the binding constant between the cholesterol-modified mimic and CD-HA, approximating the guest-host binding affinity. e) At each dose of cholesterol-modified miR-302, the change in fluorescence (ΔE_{m580}) was calculated and plotted as $1/(\Delta E_{m580})$ on the y-axis with $1/[miR-302-cho]$ on the x-axis. The y-intercept of the best-fit line allowed for the calculation of $\Delta E_{m580max}$, which was used to calculate the K_a value from the slope $[1/(K_a * \Delta E_{m580max})]$.

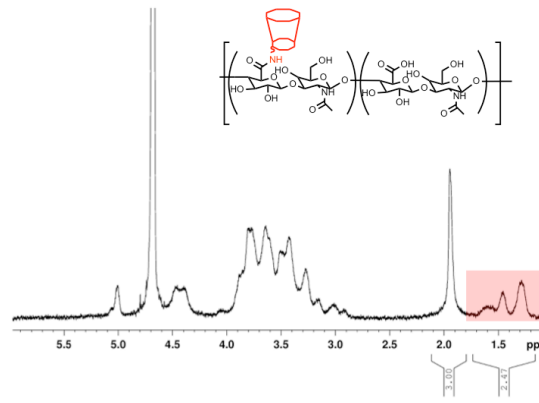
a



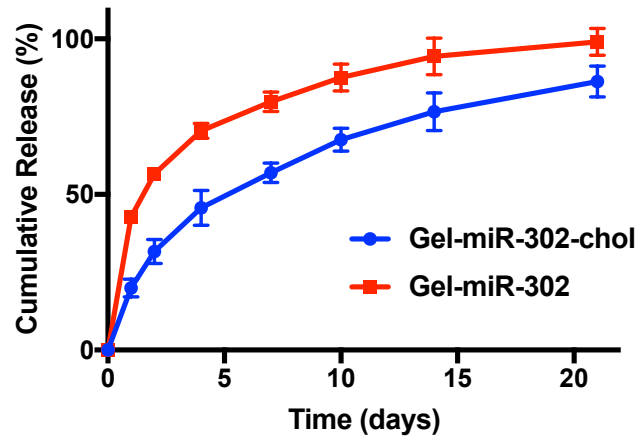
b ^1H NMR Adamantane-Modified -Hyaluronic Acid (AD-HA)



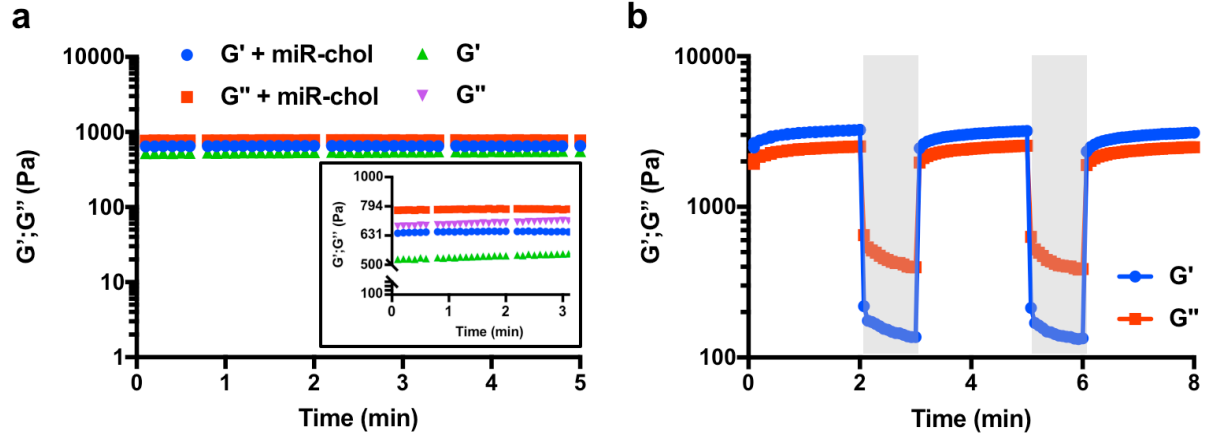
c ^1H NMR Cyclodextrin-Modified Hyaluronic Acid (CD-HA)



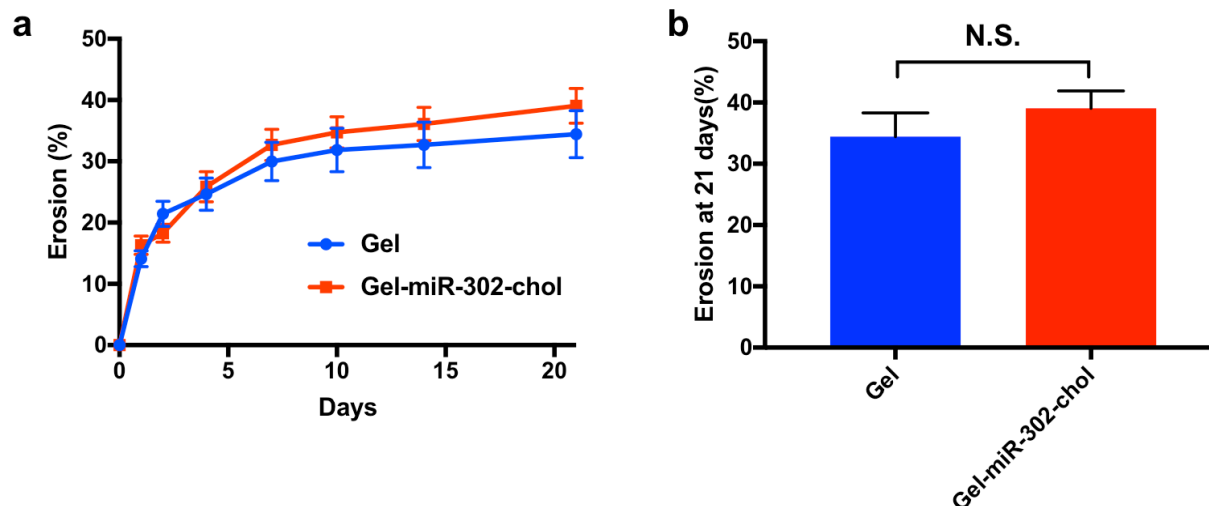
Supplementary Figure 3. Synthesis and ^1H NMR spectra of AD-HA and CD-HA. a) AD-HA was synthesized from the esterification between 1-adamantaneacetic acid and the primary alcohol of HA by di-tert-butyl dicarbonate (Boc_2O). CD-HA was synthesized from the amidation reaction between aminated cyclodextrin (mono-(6-hexanediamine-6-deoxy)- β -cyclodextrin) and the carboxylic acid of HA by (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP).¹ b) Adamantane functionalization was determined to be ~20% of HA disaccharides from integration of the ethyl multiplet of adamantane ($\delta=1.42\text{--}1.70$, 12 H) relative to the HA backbone ($\delta=3.10\text{--}4.10$, 10 H). c) Cyclodextrin functionalization was determined to be ~20% of HA disaccharides by integration of the hexane linker ($\delta=1.22\text{--}1.77$, 12 H) relative to the methyl singlet of HA ($\delta=2.1$, 3 H).



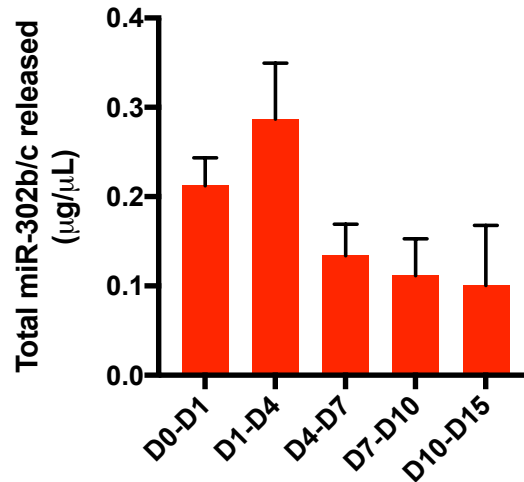
Supplementary Figure 4. Release of cholesterol-modified and un-modified miR-302. Hydrogels (100 μ L) were formed with or without cholesterol-modified miR-302 (210 μ M of miR-302b and 210 μ M of miR-302c) at 5 wt% in microcentrifuge tubes. PBS was added above hydrogels in microcentrifuge tubes and collected over three weeks to quantify total miR-302 release. After 21 days, hydrogels were dissolved in adamantane solution to determine the remaining miR-302 content and all values were normalized to cumulative miR-302. miR-302 release was slower when modified with cholesterol due to complexes between cholesterol and CD (mean $\pm$ SD, n=3 hydrogels per group).



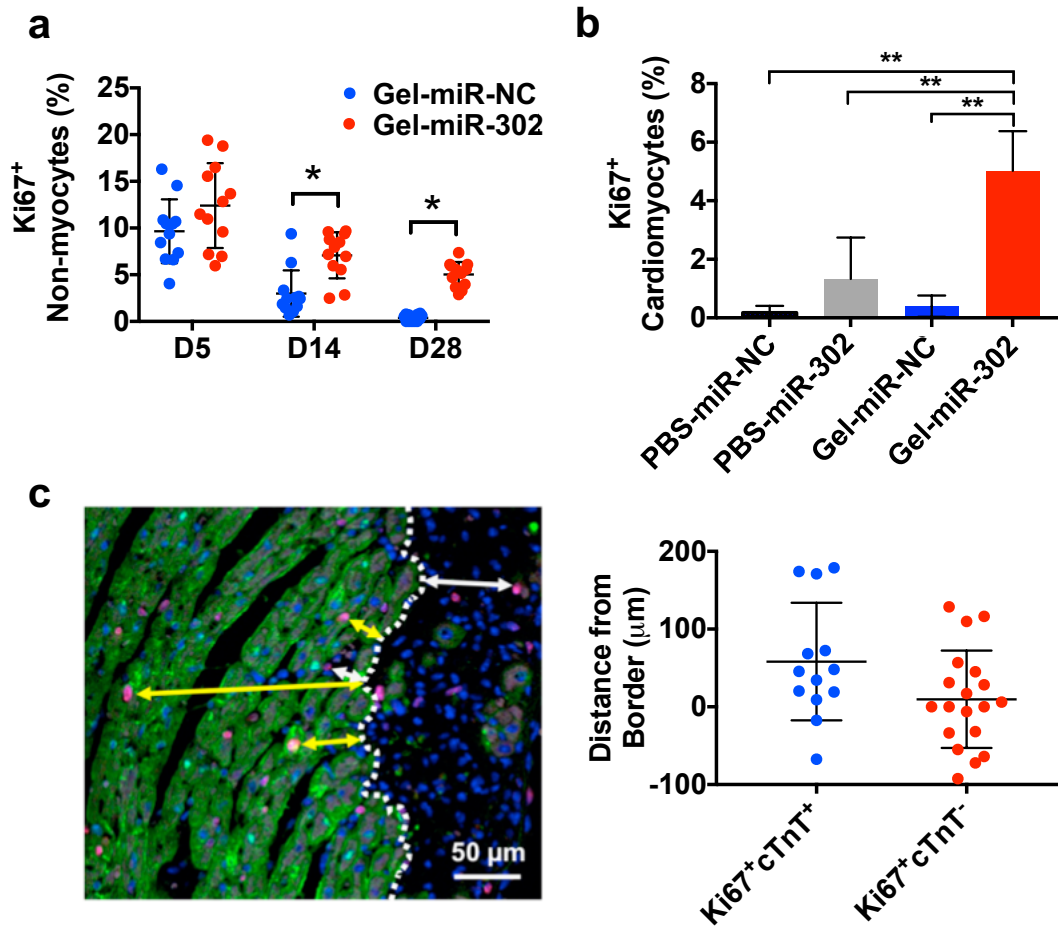
Supplementary Figure 5. Shear oscillatory rheology of gel-miR-302 complexes. a) Time sweep of storage (G') and loss (G'') moduli (1 Hz, 0.5% strain) of hydrogels (5 wt%) alone or with encapsulated cholesterol-modified miR-302 (210 μM of miR-302b and 210 μM of miR-302c). miR-302 inclusion minimally affected moduli. b) Alternating low (0.5%) and high (250%, grey shading) strain of gel-miR-302 complexes at 20 Hz. G' declined below G'' in response to high strain, indicating flow and predominantly liquid properties ($G'' > G'$) at high strains. Upon cessation of strain, G' and G'' recovered to initial mechanics ($G' > G''$).



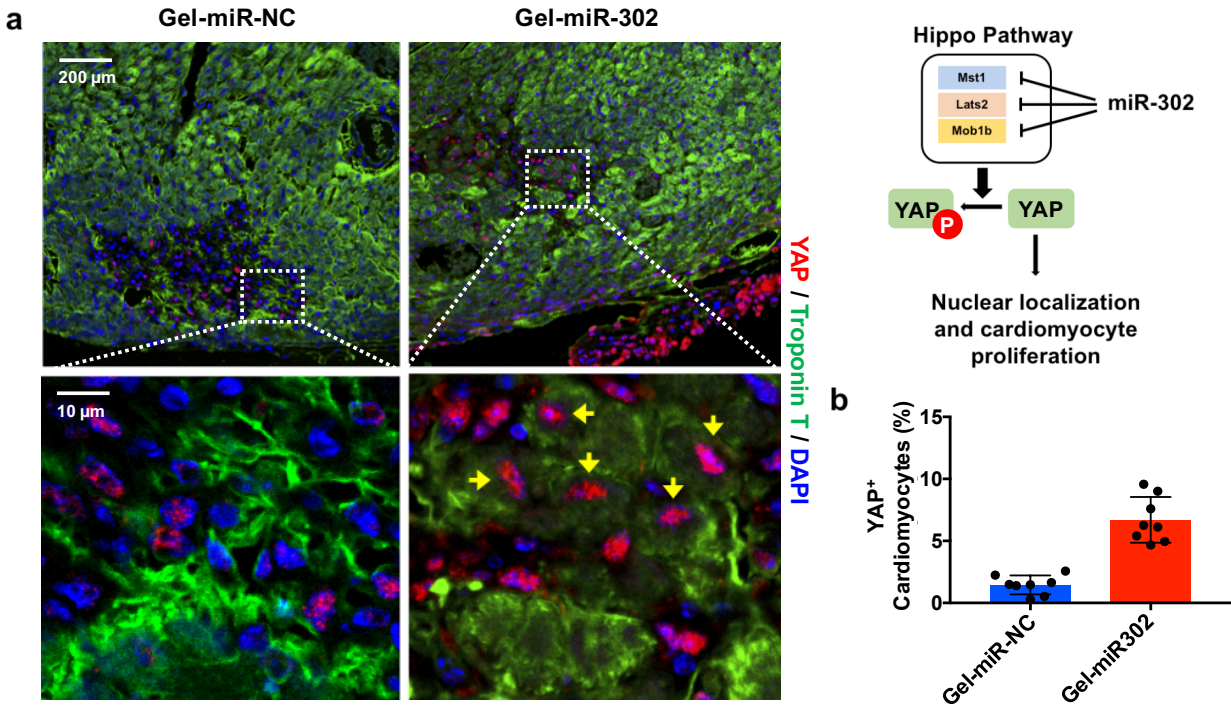
Supplementary Figure 6. Gel-miR-302 complex erosion. a) Hydrogels (100 μ L) were formed with or without cholesterol-modified miR-302 (210 μ M of miR-302b and 210 μ M of miR-302c) at 5 wt% in microcentrifuge tubes. PBS was added to hydrogels, collected over three weeks, and quantified for hyaluronic acid content through a uronic acid colorimetric assay. b) Erosion by three weeks indicated minimal difference when the miR-302 was included (mean $\pm$ SD, n=3, N.S. = not significant).



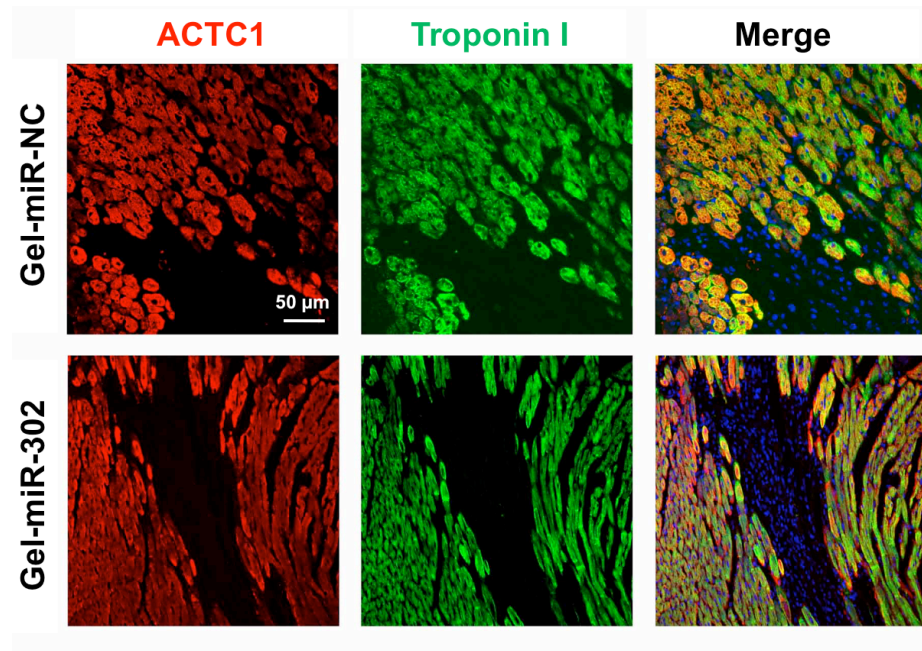
Supplementary Figure 7. Absolute miR-302 quantification from gel-miR-302 releasates. Hydrogels (100 µL) were formed with or without miR-302 (210 µM of miR-302b and 210 µM of miR-302c) at 5 wt% in microcentrifuge tubes. Release of miR-302 from gel-miR-302 at various collection intervals is expressed as total miR-302 concentration. Release from D0-D1, D1-D4, D4-D7 corresponded to approximate concentrations of ~5-10 µM miR-302 in releasate solution, which was subsequently added to neonatal cardiomyocytes for proliferation assays (mean $\pm$ SD, n=3). Exact concentrations of miR-302 (µM) expressed in releasates were as follows: D0-D1 = 7.5 µM, D1-D4=10.2 µM, D4-D7=4.9 µM, D7-D10 = 4.0 µM, D10-D15 = 3.6 µM.



Supplementary Figure 8. *In vivo* cell proliferation after gel-miR-302 injection. a) Mice were selected to receive miR-NC or miR-302 injections with hydrogel (gel-miR-NC or and gel-miR-302) or PBS (PBS-miR-NC and PBS-miR-302) in non-infarcted heart tissue inferior and lateral to the LAD at the left atrial appendage. At D5, D14 or D28, hearts were sectioned and stained for Ki67. Quantification of Ki67 positive non-myocytes (Ki67⁺cTnT⁻) was performed around both injection sites for three mice per group (mean $\pm$ SD, n=3 mice per group, *p<0.05). b) Quantification of Ki67 positive cardiomyocytes (Ki67⁺cTnT⁺) surrounding injection sites at five days, demonstrating increased proliferation from gel-miR-302 compared to all controls (mean $\pm$ SD, n=3 mice per group, **p<0.01). c) In gel-miR-302 treated sections, proliferating cells were quantified from the injection border at five days, where distance away from injection site (toward cTnT⁺ area) was positive and distance into injection site (toward cTnT⁻ area) was negative. Yellow and white arrows indicate representative proliferating cardiomyocytes (Ki67⁺cTnT⁺) and non-myocytes (Ki67⁺cTnT⁻) surrounding injection site (mean $\pm$ SD, n=3 gel-miR-302 mice).

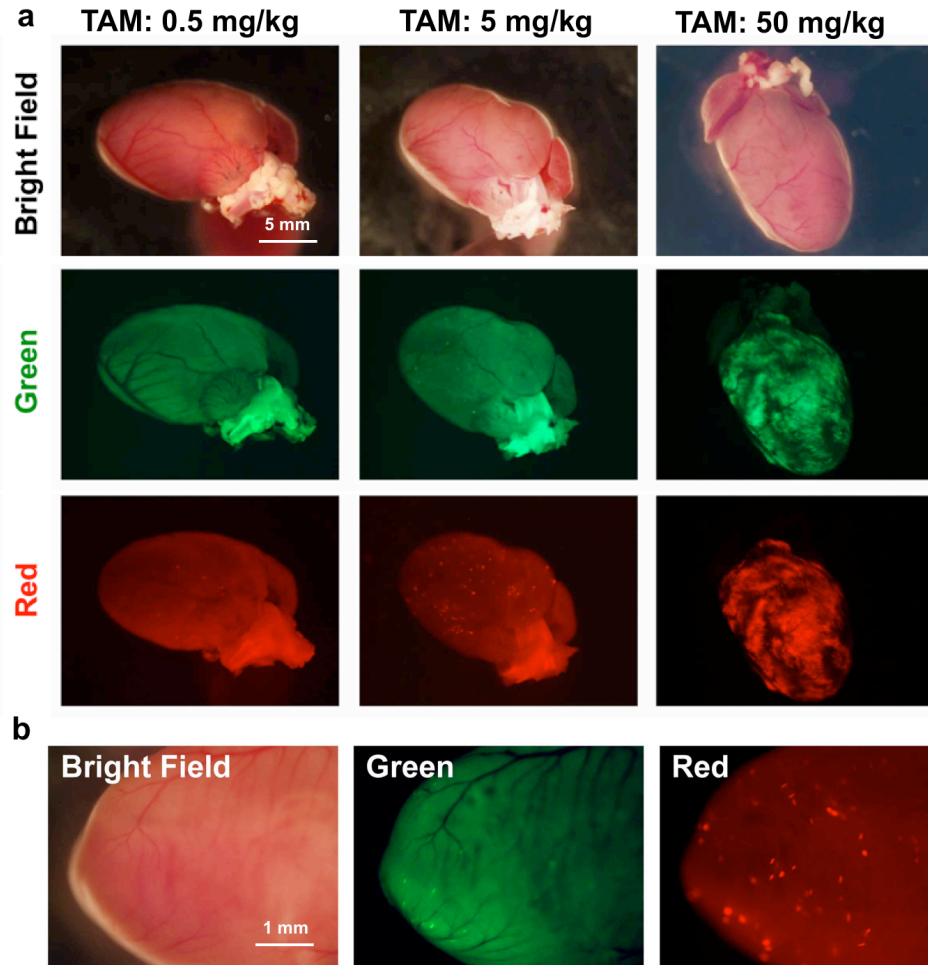


Supplementary Figure 9. YAP expression after gel-miR-302 injection. Gel-miR-302 injections were made inferolateral to the proximal LAD without ligation of the LAD in mice. a) Sections were co-stained with YAP at five days post-treatment with gel-miR-NC or gel-miR-302. YAP localizes to the nucleus in response to miR-302 stimulation to interact with TEAD transcription factors to cause cardiomyocyte proliferation.² b) Quantification of total YAP surrounding both injection sites, which demonstrated increased total and nuclear YAP (yellow arrows) in gel-miR-302 treated groups (mean $\pm$ SD, n=3 mice per group, **p<0.01.)

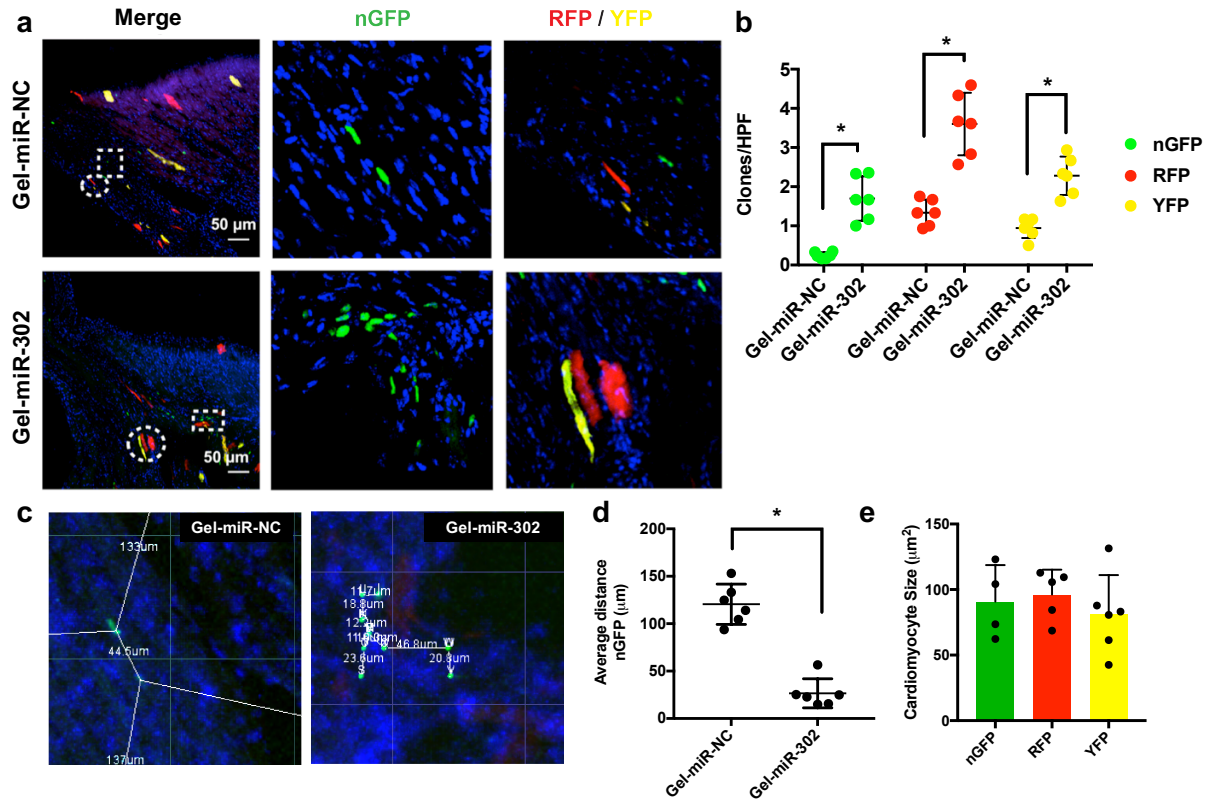


Supplementary Figure 10. Cardiomyocyte maturity after gel-miR-302 injection.

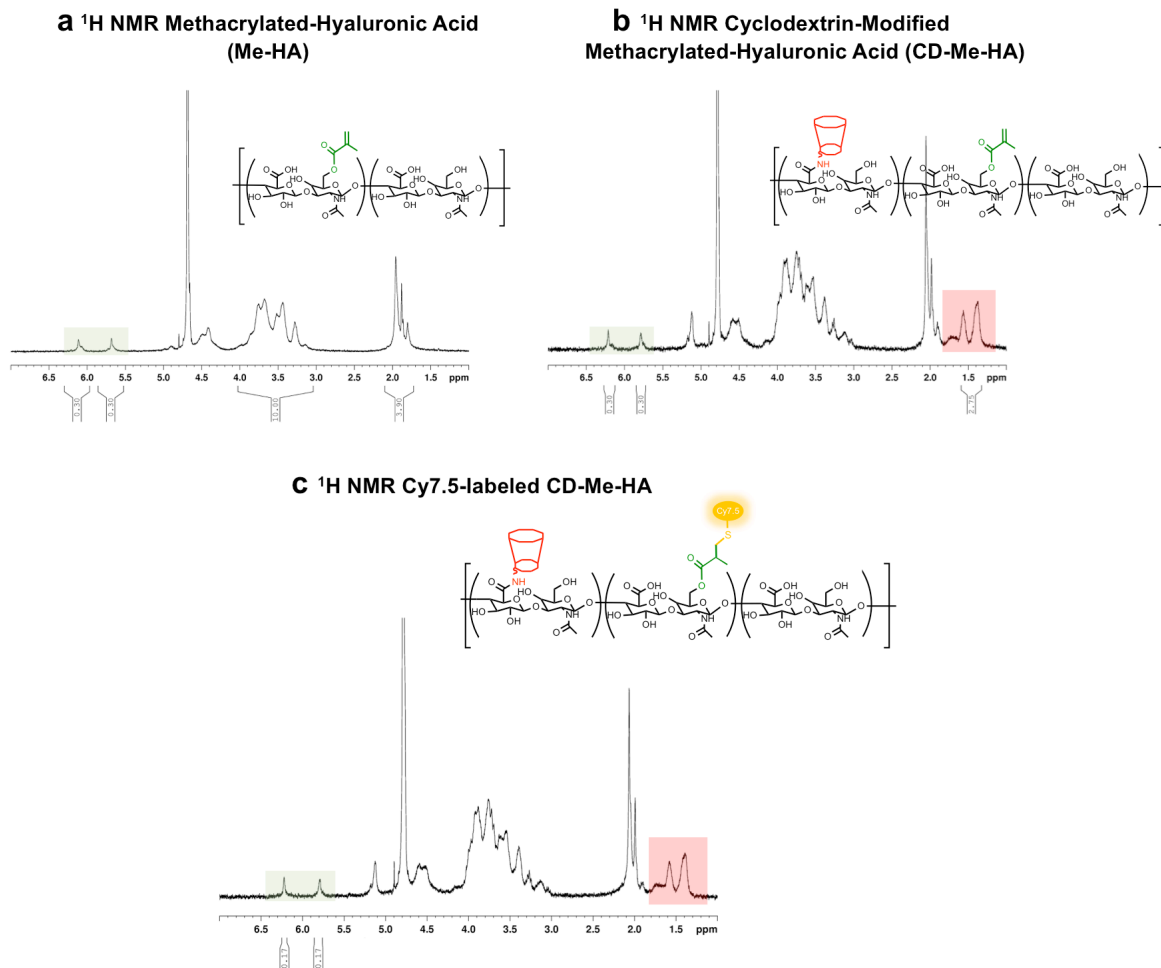
Gel-miR-302 injections were made in mice without infarction. Sections were co-stained with cardiac muscle α -actin (ACTC1) and cardiac troponin I at five days post-treatment with gel-miR-NC or gel-miR-302. ACTC1 marked cardiomyocytes during all stages of maturation (including immaturity) whereas troponin I stained only mature cardiomyocytes. Complete overlap of the cardiac markers suggested retention of a mature phenotype following gel-miR-302 injection.



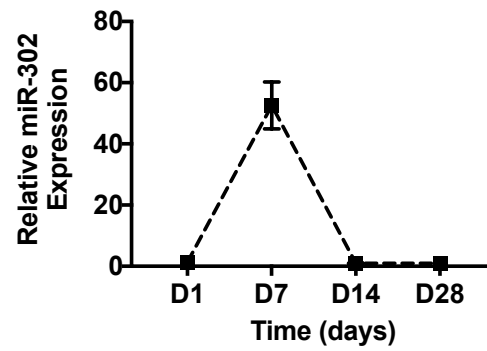
Supplementary Figure 11. Confetti hearts prior to ligation and gel-miR-302 injection. a) Tamoxifen titration indicated increasing nGFP and RFP reporter expression with increasing tamoxifen induction. b) Representative gross image of MHC-Confetti heart in green and red channels following tamoxifen induction (6.7 mg/kg) of reporter expression for 14 days but prior to gel-miR-302 injection.



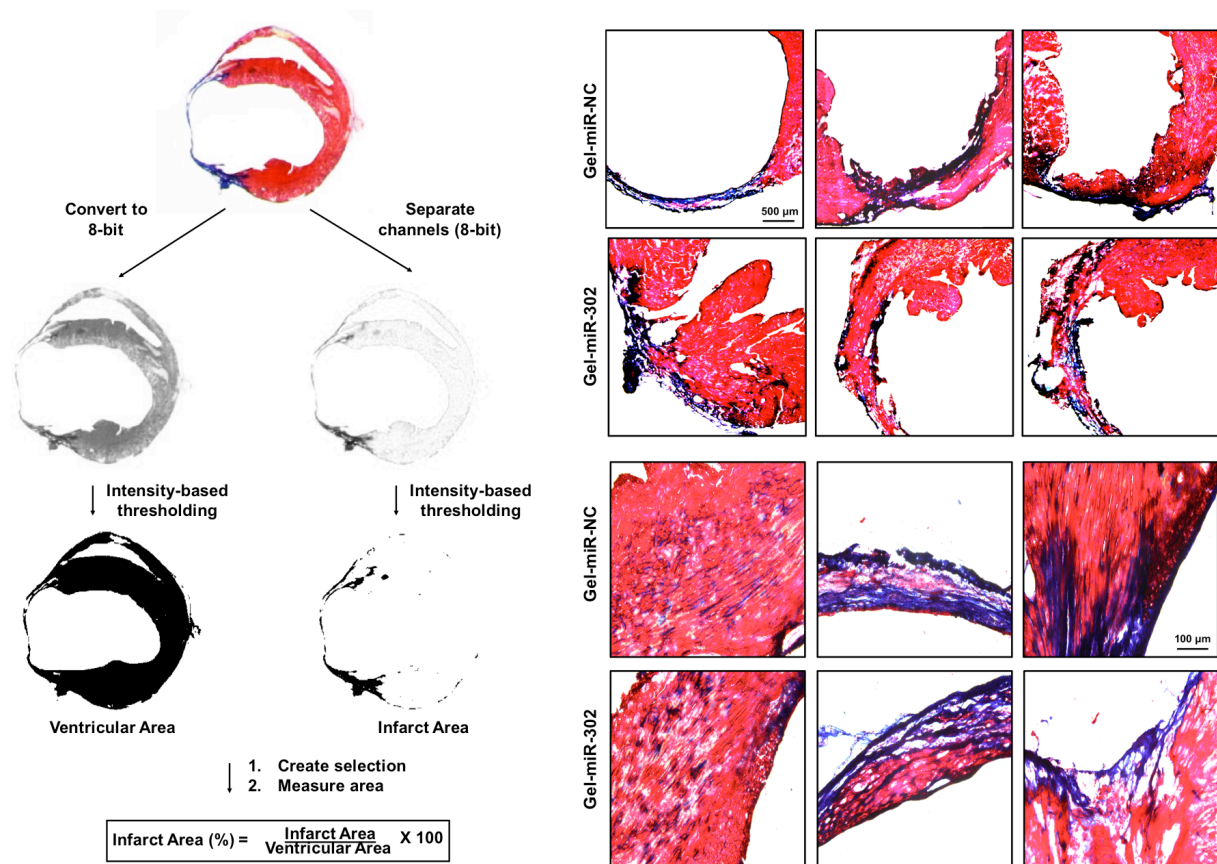
Supplementary Figure 12. Clonal proximity in Confetti hearts injected with gel-miR-302. a) Fluorescently-labeled cells were identified in border zones of infarcts with clones neighboring each other in gel-miR-302 treated sections. Neighboring nGFP, RFP and YFP cells are magnified to demonstrate clones. b) Quantification of clones suggested increased clones in gel-miR-302 treated groups for all three fluorescent reporter proteins. A clone was defined as a minimum of two neighboring cells within 50 μ m of each other (mean $\pm$ SD, gel-miR-NC, n=3; gel-miR-302, n=4, * $p < 0.05$). c) nGFP cells were found within 50 μ m of each other in gel-miR-302 sections, whereas they were greater than 100 μ m apart in gel-miR-NC sections. Measurements were performed by IMARIS from 3D confocal images. d) Quantification of distance between neighboring nGFP cells from IMARIS measurements (mean $\pm$ SD, gel-miR-NC, n=3; gel-miR-302, n=4, * $p < 0.05$). e) Quantification of cardiomyocyte size in each of nGFP, RFP and YFP channels using WGA to observe cardiomyocyte membranes. No evidence for hypertrophy was observed (mean $\pm$ SD, gel-miR-302, n=4).



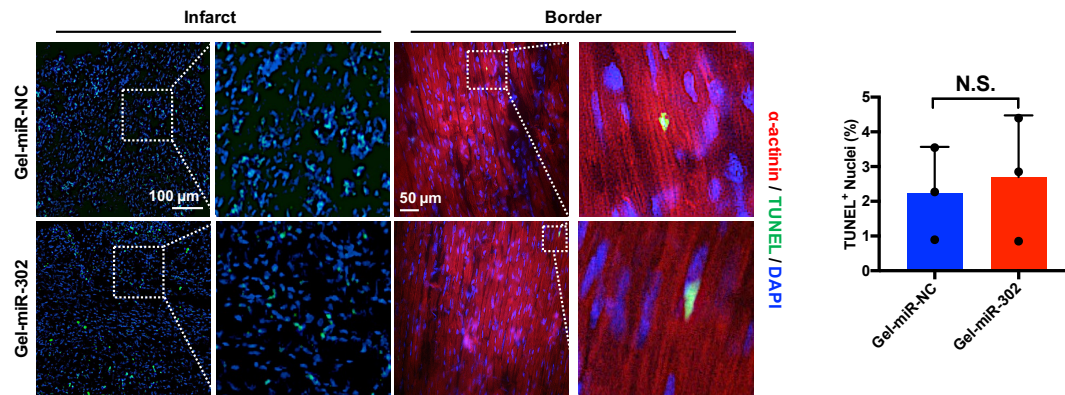
Supplementary Figure 13. ^1H NMR spectra of Me-HA, CD-Me-HA, and Cy7.5-labeled CD-Me-HA. Me-HA was synthesized as previously described.³ a) Methacrylate functionalization was determined by $\sim 30\%$ by integration of methacrylate alkene protons ($\delta=5.5\text{--}6.5$, 2 H) relative to the methyl singlet of HA ($\delta=2.1$, 3 H). b) CD-Me-HA was synthesized as previously described by formation of the TBA salt and subsequent amidation reaction between aminated cyclodextrin (mono-(6-hexanediamine-6-deoxy)- β -cyclodextrin) and the carboxylic acid of HA by (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP).³ Cyclodextrin functionalization on Me-HA was determined to be $\sim 20\%$ by integration of the hexane linker ($\delta=1.22\text{--}1.77$, 12 H) relative to the alkene protons of methacrylate ($\delta=5.5\text{--}6.5$, 2 H). c) Cy7.5-labeling of CD-Me-HA was performed through a Michael addition reaction between a thiolated Cy7.5 peptide (GCKKG-Cy7.5) and CD-Me-HA at pH 8 as previously described.³ Functionalization of CD-Me-HA with Cy7.5 was determined by integrating the alkene protons of methacrylate ($\delta=5.5\text{--}6.5$, 2 H) relative to the hexane linker of CD-HA ($\delta=1.22\text{--}1.77$, 12 H). The reduction in alkene protons suggests an approximate Cy7.5 modification of 13% of disaccharides in CD-Me-HA.



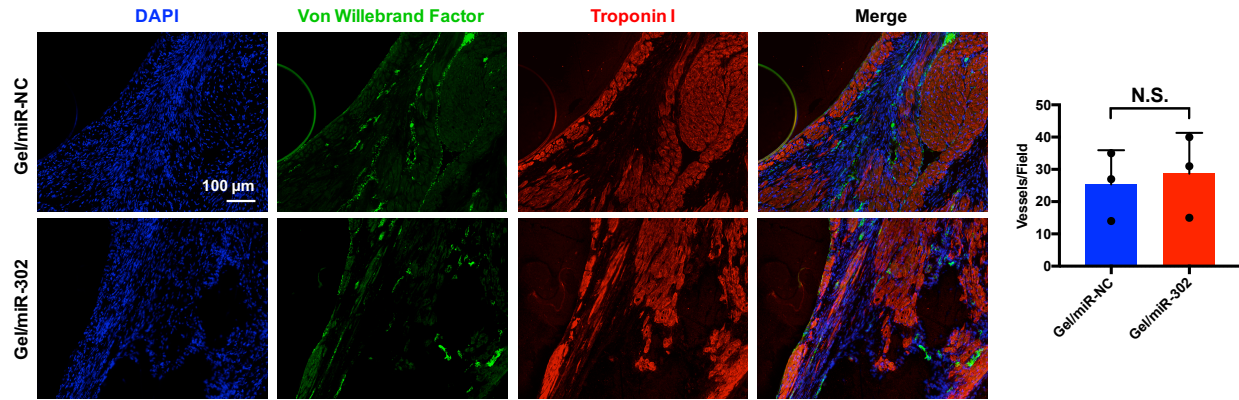
Supplementary Figure 14. miR-302 expression in the lungs after gel-miR-302 injection. qPCR quantification of miR-302 in the lungs at various timepoints following MI and gel-miR-302 injection into the heart, normalized to gel-miR-NC, indicating minimal miR-302 expression in lungs at D7 (mean $\pm$ SD, n=3 mice per timepoint).



Supplementary Figure 15. Method for ImageJ quantification of infarct size and representative hi-magnification Masson's images. Infarct size was quantified on ImageJ from separate channels with manual thresholding to separate infarct from non-infarcted tissue. Representative sections at low and high magnification of gel-miR-NC and gel-miR-302 treated hearts at day 28 are shown.



Supplementary Figure 16. Apoptosis at day 28 after MI and gel-miR-302 injection. Following infarction and gel-miR-302 or gel-miR-NC injection, mice were sacrificed after 28 days and hearts were explanted and processed for IHC. TUNEL was used to stain for apoptosis in the infarct site and α -actinin was used to stain cardiomyocytes and to delineate infarct tissue. There was no significant difference in TUNEL staining in gel-miR-NC or gel-miR-302 treated mice in infarct tissue (mean $\pm$ SD, n=3 mice per group, N.S. = not significant).



Supplementary Figure 17. Vascular density at day 28 after MI and gel-miR-302 injection. Following infarction and gel-miR-302 or gel-miR-NC injection, mice were sacrificed after 28 days and hearts were explanted and processed for IHC. Sections were stained with cardiac troponin I to mark cardiomyocytes, or von Willebrand Factor (vWF) to mark endothelium. vWF quantification was performed in the infarct on all vessels larger than 5 μm. There was no significant difference in vascular density in gel-miR-NC or gel-miR-302 treated mice in infarct tissue. (mean ± SD, n=3 mice per group, N.S. = not significant).

Supplementary References:

1. Rodell, C. B., Kaminski, A. L. & Burdick, J. A. Rational Design of Network Properties in Guest–Host Assembled and Shear-Thinning Hyaluronic Acid Hydrogels. *Biomacromolecules* **14**, 4125–4134 (2013).
2. Tian, Y. *et al.* A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci. Transl. Med.* **7**, 279ra38 (2015).
3. Rodell, C. B. *et al.* Shear-Thinning Supramolecular Hydrogels with Secondary Autonomous Covalent Crosslinking to Modulate Viscoelastic Properties In Vivo. *Adv. Funct. Mater.* **25**, 636–644 (2014).