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Supplemental Information

miR-133a Enhances the Protective Capacity of Cardiac Progenitors Cells after Myocardial Infarction

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SUPPLEMENTAL FIGURE AND LEGENDS

Supplemental Figure 1

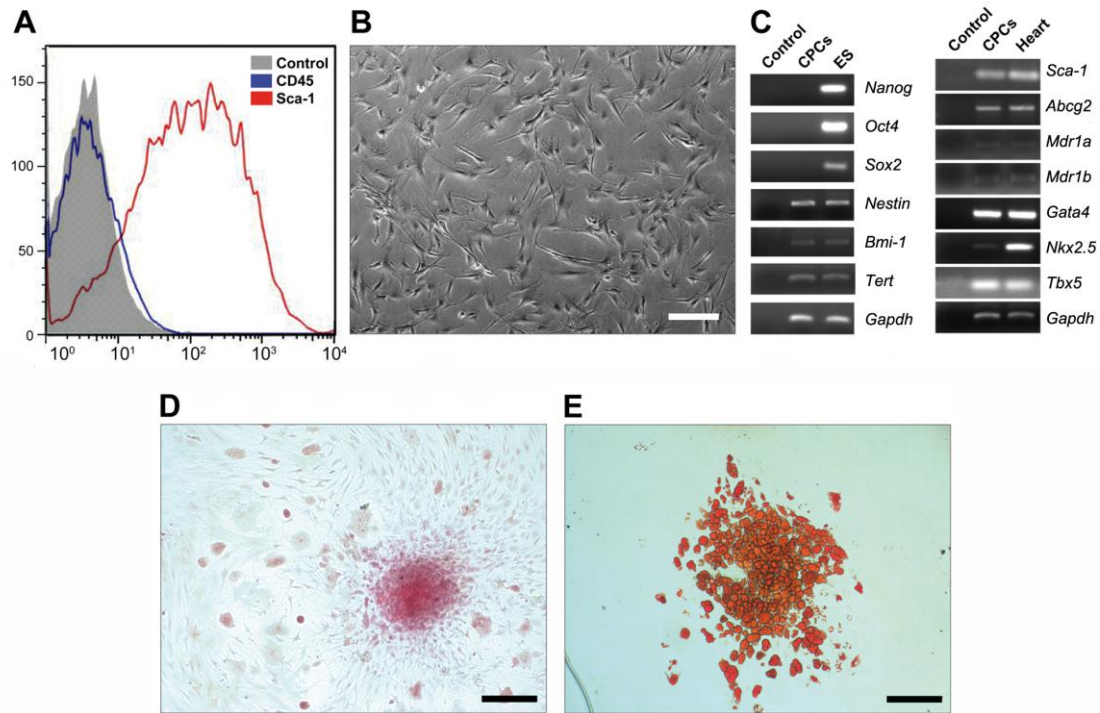


Figure S1. Characterization of CPCs. A, CPCs SCA-1⁺/CD45⁻ FACS characterization. B, Bright-field morphology of cultured CPCs. C, Expression profile (RT-PCR) of stem cell- and cardiac-related genes. D,E, Differentiation potential of SCA-1⁺/CD45⁻ CPCs. Cells were treated with differentiation medium for D, osteocytes or E, adipocytes, and stained with Alizarin Red and Oil Red O, respectively (bar, 200 μ m).

Supplemental Figure 2

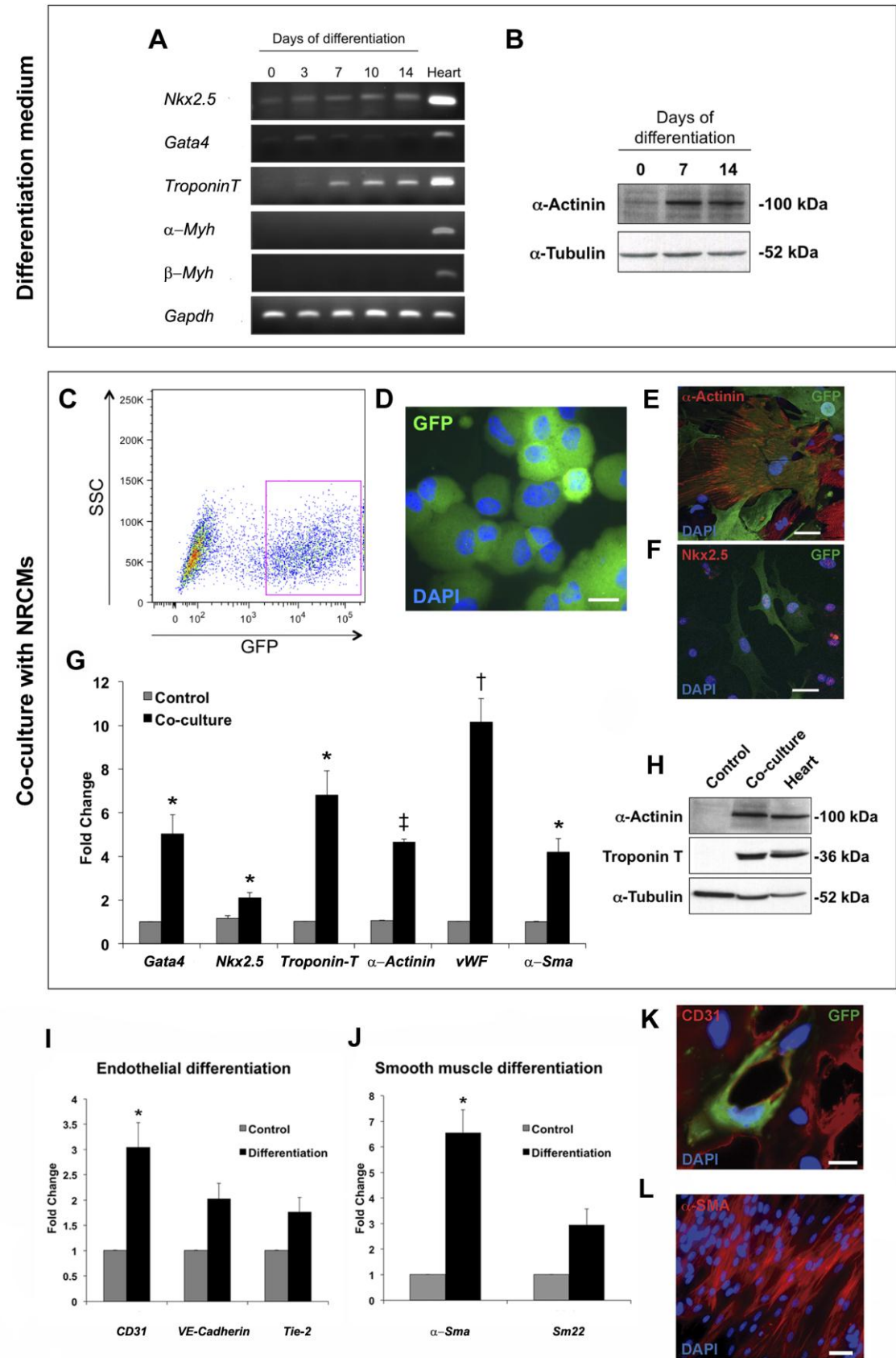


Figure S2. CPC differentiation. A, RT-PCR analysis of *Nkx2.5*, *Gata4*, *Troponin T*, α -*Myh* and β -*Myh* expression after culture of CPC-derived GFP⁺ cells in cardiac differentiation medium. Troponin T is detectable from day 7 of differentiation. Heart was used as a positive control. B, Western blot analysis of α -actinin during cardiac-induced differentiation. α -actinin is detectable from day 7 of differentiation. C, CPC-derived GFP⁺ cells were isolated from co-cultures with NRCMs by FACS. D, GFP expression was validated by cytopspin followed by microscopy (bar, 20 μ m). E, F, GFP⁺ CPCs were seeded with NRCMs, and after co-culture for 7 days, some GFP⁺ CPC-derived cells stained positive for α -actinin (E) and NKX2.5 (F) (bars, 40 μ m). G, Quantitative RT-PCR analysis of cardiac (*Gata4*, *Nkx2.5*, *Troponin T* and α -*Actinin*) and endothelial (*von Willebrand factor* (*vWf*) and α -*Sma*) gene expression in FACS-purified GFP⁺-CPCs from control cultures and from NRCM co-cultures (n=3 experiments). H, Representative Western blot analysis of troponin T and α -actinin in control CPCs and CPCs isolated from NRCM co-cultures. Heart was used as a positive control. I, RT-qPCR analysis of *CD31*, *VE-cadherin* and *Tie2* expression in control CPCs and endothelial-differentiated CPCs (n=3 experiments). J, RT-qPCR analysis of endothelial α -*Sma* and *Sm22* expression in control CPCs and smooth muscle-differentiated CPCs (n=3 experiments). **P*<0.05, †*P*<0.01, ‡*P*<0.001. Data are mean±SEM. K, GFP⁺ CPC-derived endothelial cells express CD31 after *in vivo* angiogenesis plug assay (bar, 20 μ m). L, CPC-derived smooth muscle cells express α -SMA after differentiation in specific medium (bar, 40 μ m).

Supplemental Figure 3

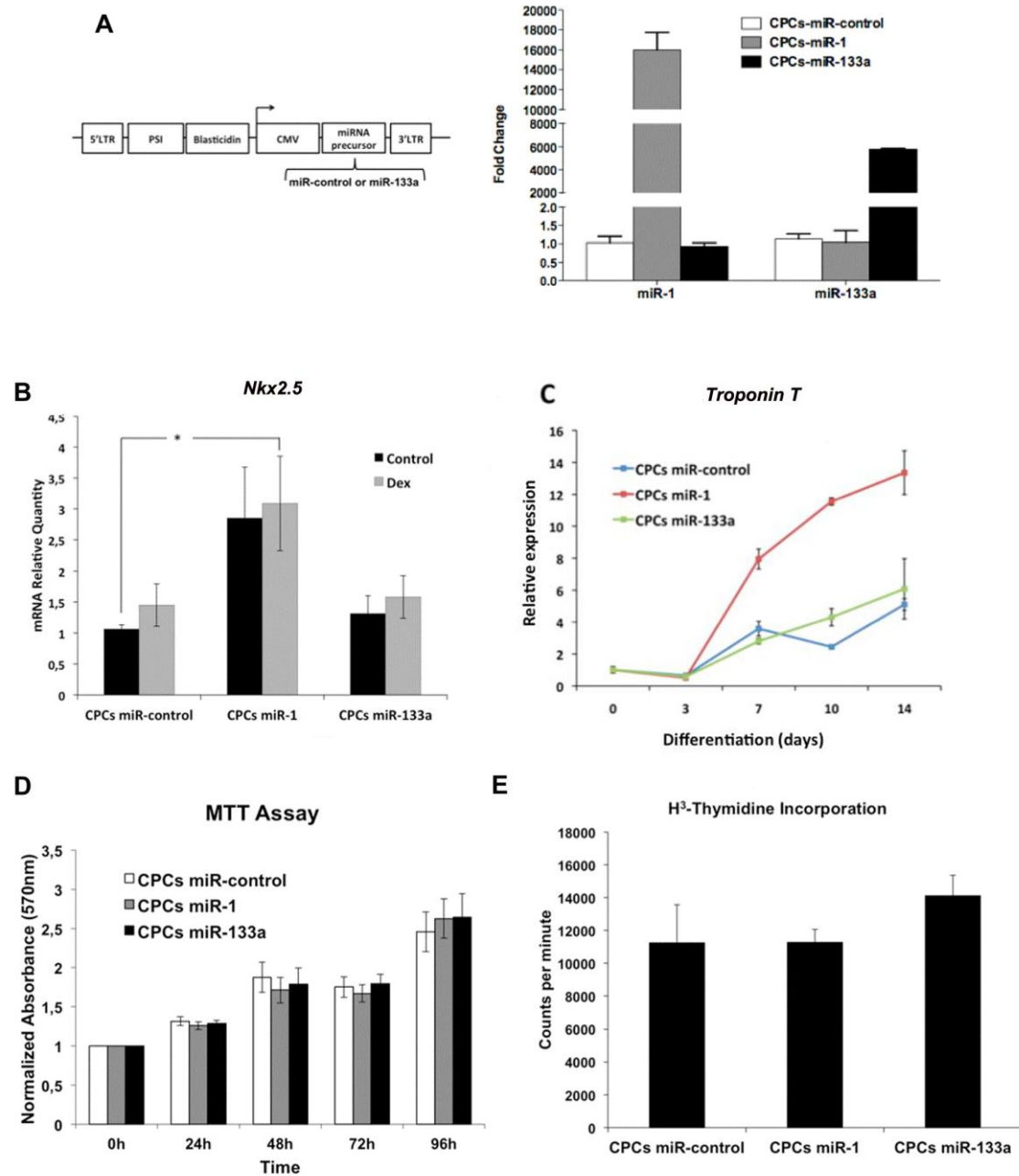


Figure S3. Effect of miR-1 and miR-133a overexpression on CPC differentiation and proliferation. Related to Fig 1. A, Retroviral vector used for overexpression of miR-1 miR-133a and miR-control (left); quantitative analysis of miRNA overexpression in CPCs (right, n=3 experiments). B, RT-qPCR expression analysis of *Nkx2.5* in undifferentiated versus CPCs cultured in differentiation medium (n=3 experiments). C,

Representative time-course RT-qPCR analysis of *troponin T* expression after induction of differentiation (n=3 experiments). D, Colorimetric MTT assay over 96 hours (n=2 experiments; n=3 replicates per condition). E, ^3H -thymidine incorporation assay at 48 hours (n=3 experiments). * $P<0.05$. Data are represented as mean $\pm$ SEM. Related to Fig 1.

Supplemental Figure 4

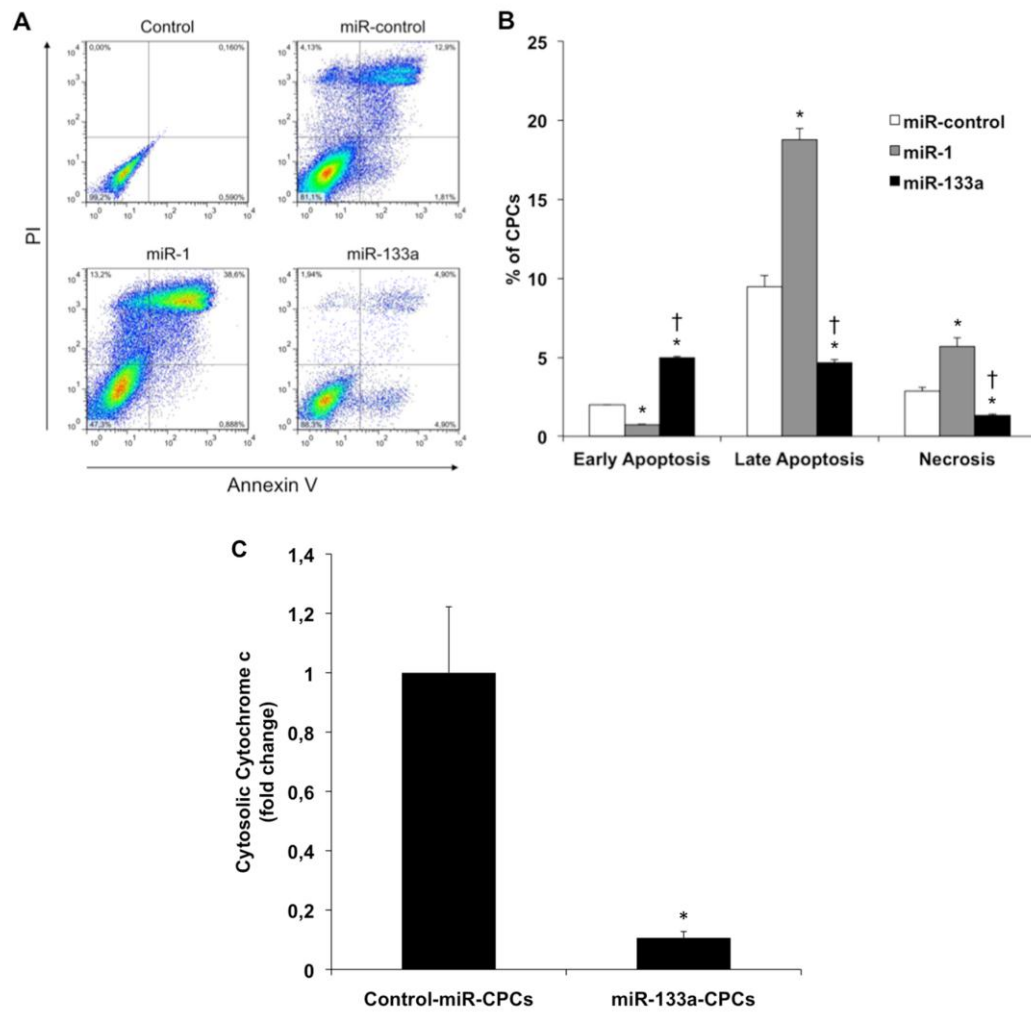


Figure S4. Effect of miR-1 and miR-133a overexpression on CPC apoptosis. Related to Fig 1. A, Representative FACS analysis of CPCs treated with H₂O₂ (200μM; 5 hours) and stained with annexin V and PI. B, Quantification of early apoptosis (annexin V⁺/PI⁻ cells), late apoptosis (Annexin V⁺/PI⁺ cells) and necrosis (annexin V⁻/PI⁺ cells) after H₂O₂ treatment (n=3 experiments; **P*<0.05 respect to miR-control, †*P*<0.005 respect to miR-1). C, Fold change quantification of cytosolic cytochrome c in CPCs after H₂O₂ treatment (n=3 experiments; **P*<0.05). Data are represented as mean±SEM. Related to Fig 1.

Supplemental Figure 5

A

Category	Functions	Functions Annotation	p-Value	# Molecules
Cell Death	cell death	cell death	9,19E-13	319
Cell Death	apoptosis	apoptosis	4,27E-11	248
Cardiovascular Disease	heart disease	heart disease	2,67E-09	164
Cell Death	cell death	cell death of organ	1,55E-08	106
Cardiovascular Disease	ischemia	ischemia	2,96E-07	130

B

Category	Diseases/Functions Annotation	p-Value	Molecules
Cell Death/ Survival	cell death	4.61E-19	353
Cell Death/ Survival	apoptosis	1.07E-17	289
Cell Death/ Survival	necrosis	5.63E-15	277
Cell Death/ Survival	neuronal cell death	4.68E-09	80
Cell Death/ Survival	apoptosis of neurons	2.26E-08	55
Cell Death/ Survival	cell death of brain cells	2.91E-07	36
Cell Death/ Survival	cell death of tumor cell lines	3.07E-07	157
Cell Death/ Survival	cell death of central nervous system cells	3.26E-07	38
Cell Death/ Survival	cell death of connective tissue cells	6.53E-07	71
Cell Death/ Survival ¹	cell death of blood cells	1.51E-06	75
Cell Death/ Survival	apoptosis of tumor cell lines	4.90E-06	122
Cell Death/ Survival	cell death of fibroblasts	1.27E-05	32
Cell Death/ Survival	cell death of immune cells	1.53E-05	69
Cell Death/ Survival	apoptosis of connective tissue cells	5.91E-05	33
Cell Death/ Survival ¹	cell death hematopoietic progenitor cells	6.05E-05	28

¹ *Abcc1, Adcyap1, Adora2a, Alk, Arrb1, Bcl2l1, Bcl2l11, Bmf, Bnip3l, Camk2g, Ccnd2, Cd300a, Cd4, Cdc42, Cflar, Cnr1, Cnr2, Col1a1, Csf2, Dapk2, Def6, Dicer1, Dnaja3, E2f1, Egfr, Ezr, F2r, Faim, Fcgr1a, Fcgr3a, Fgfr1, Fli1, Fnip1, Foxo1, Gcnt1, Gimap1, Gimap5, Hrk, Ikbke, Il6r, Inpp5d, Irak3, Irf2, Itga4, Itga5, Itpkb, Klrc4, Klrk1, Klrk1, Ltbr, Mapk3, Meis1, Mt1e, Nf1, Nfat5, Nfatc2, Nfya, Nlrp3, Notch1, Npc1, Nr5a1, Perp, Pml, Prdx6, Prkcd, Rras, Sh3bp2, Sirpa, Slc46a1, Slc6a6, Smad6, Syk, Tcf7, Thpo, Trem1, Vdr, Vipr2a, Wnt5a*

C

Category	Diseases or Functions Annotation	p-Value	Molecules
Cardiac Proliferation ²	proliferation of heart cells	1.43E-03	11
Cardiac Proliferation ²	proliferation of cardiomyocytes	1.85E-03	10
Cardiac Arrhythmia	bradycardia	5.02E-03	6
Cardiac Proliferation	proliferation of ventricular myocytes	8.18E-03	3
Cardiac Arrhythmia	arrhythmia	8.80E-03	20
Cardiac Fibrosis ³	fibrosis of heart	1.08E-02	12
Cardiac Arrhythmia	supraventricular arrhythmia	1.12E-02	14
Cardiac Arrhythmia	arrhythmia of heart ventricle	1.17E-02	9
Cardiac Arrhythmia	paroxysmal supraventr. tachycardia	1.40E-02	5
Cardiac Necrosis/Cell Death ⁴	apoptosis of cardiomyocytes	1.65E-02	16
Increased Red Blood Cells	increased quantity of red blood cells	1.97E-02	11
Cardiac Fibrosis ³	fibrosis of left ventricle	2.83E-02	4
Cardiac Arrhythmia	tachycardia	3.42E-02	9
Cardiac Necrosis/Cell Death ⁴	cell death of cardiomyocytes	3.53E-02	17
Cardiac Necrosis/Cell Death	cell death of heart	3.68E-02	18

² *Agt, Dicer1, Dusp1, Egfr, Foxo1, Igf2, Mapk11, Meis1, Ncoa6, Nfat5, Rbpj*

³ *Adora1, Adra2aA, AgtT, Cnr2, Dicer1, Dio3, Igf1r, Ikbke, Mapk11, Nf1, Stk4, Vdr*

⁴ *Adra1a, AgtT, Bcl2l1, Bcl2l11, Bnip3l, Cnr2, Dicer1, E2f1, Fstl1, Gnaq, Mtpn, Prkcd, Sirt1, Slc8a1, Sptlc2, Stk4, Ube4b*

Figure S5. Bioinformatics analysis of Top Biological and Toxic Functions involving predicted miR-133a targets. A and B, Bioinformatics analysis of putative Top Disease and Biological Functions affected by miR-133 (IPA analysis software). IPA analysis predicted *cell death* (highlighted in yellow) as the main biological function affected at

organ and cellular levels, followed by *cardiovascular disease* (A). From all genes (106) included in the *cell death* category, we selected those related to injured heart, blood cells and progenitor precursor populations (highlighted in light green) (B). B¹, The total list of genes included in the categories named as *cell death of blood cells* and *cell death of hematopoietic precursors* (genes present in both lists are in bold letter). C, Bioinformatics IPA analysis of putative Top Toxic Functions affected by miR-133. Targets involved in fibrosis and cell death of heart and in proliferation and apoptosis of heart cells and cardiomyocytes, were listed with high p-values. C², List of genes included in the categories named as *proliferation of heart cells* and *proliferation of cardiomyocytes* (genes present in both lists are in bold letter). C³, List of genes included in the categories named as *fibrosis of heart* and *fibrosis of left ventricle* (genes present in both lists are in bold letter). C⁴, List of genes included in the categories named as *apoptosis of cardiomyocytes* and *cell death of cardiomyocytes* (genes present in both lists are in bold letter).

Supplemental Figure 6

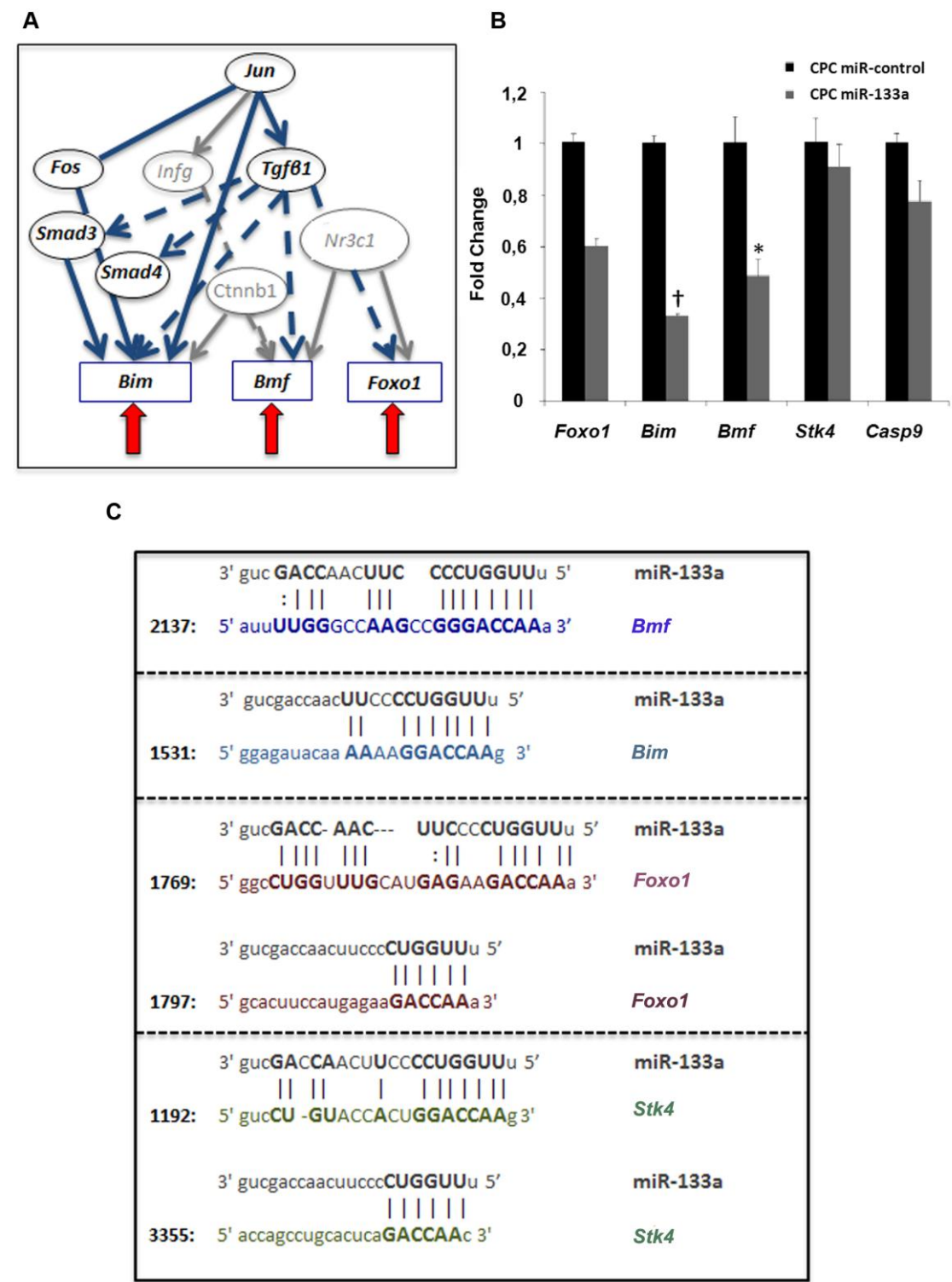


Figure S6. Bioinformatics analysis of predicted up-stream regulator pathways and validation of miR-133 selected targets. Related to Fig 1. A, IPA bioinformatics analysis looking for putative up-stream regulator pathways that could be affected based

on the list of 106 predicted targets list for miR-133a included in cell death category. Solid arrows and lines show direct relationship among genes, dashed-line arrows show indirect relationships among genes, grey shaded arrows and genes are not directly involved in the main pathway highlighted in bold letter, red solid arrows point to predicted activated targets upon activation of the upstream-regulator pathway in bold letter. B, RT-qPCR expression analysis of our target selection in miR-control-CPCs vs miR-133a-CPCs, after H₂O₂ treatment; caspase 9 was used as a positive control (5 hours; n=3 experiments; †*p*<0.00005, **p*<0.005). Data are represented as mean±SEM; values are relative to the expression level of the corresponding gene in miR-control-CPCs. C, 3'-UTR binding sites for miR-133a in *Bmf*, *Bim*, *Foxo1* (2 sites) and *Stk4* (2 sites) (obtained from microRNA.org). Related to Fig 1.

Supplemental Figure 7

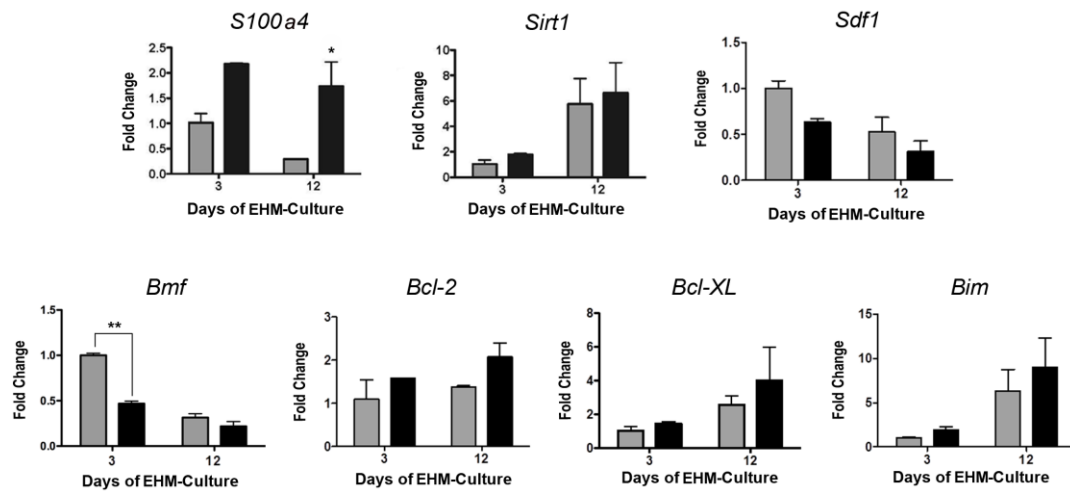


Figure S7. miR-133a-EHM differential expression profile. Related to Fig 7. RT-qPCR expression analysis of *S100a4*, *Sirt1*, *Sdf1*, *Bmf*, *Bcl-2*, *Bcl-XL* and *Bim* in control-miR-CPCs (grey bars) and miR-133a-CPCs (black bars) in EHM co-cultures at day 3 and day 12 (n=3 independent experiments, * $p < 0.05$, ** $p < 0.01$). Data are represented as mean $\pm$ SEM.

SUPPLEMENTAL TABLES

Table S1. Primary antibodies. Related to Figures 2, 4, 5, 6 and 7.

Primary Ab	Manufacturer	Species	Dilution (application)
Sarcomeric actin	Dako (M 0874)	Mouse	1:100 (IHC)
CD31	BD Pharmingen (553370)	Rat	1:100 (IF)
CD45-eFluor450	eBioscience (48-0451)	Rat	1:100 (FCyt)
CD45-PE	BD Pharmingen (553081)	Rat	1:100 (FCyt)
GFP	Abcam (ab290)	Rabbit	1:100 (IF)
Laminin	Abcam (ab11575)	Rabbit	1:50 (IF)
NKX2.5	Santa Cruz Biotech (sc-14033)	Rabbit	1:50 (IF/FCyt)
Sca-1-FITC	BD Pharmingen (553335)	Rat	1:20 (FCyt)
Sca-1-PE	BD Pharmingen (553336)	Rat	1:100 (FCyt)
Tropomyosin	Sigma (T2780)	Mouse	1:100 (IF/FCyt)
Troponin T	Abcam (ab8295)	Mouse	1:100 (IF); 1:1000 (WB)
α -SMA	Sigma (A2547)	Mouse	1:1000 (IF)
α -Tubulin	Calbiochem (CP60)	Mouse	1:2000 (WB)

IF: Immunofluorescence. WB: Western blot. FCyt: Flow cytometry.

Table S2. Primers for RT-PCR and real time RT-PCR. Related to Figures 1, 5, 6 and 7.

Primer	Sense	Antisense
<i>Abcg2</i>	5'-AGCAGCAAGGAAAGATCCAA-3'	5'-CCCATCACAACGTCATCTTG-3'
<i>Bcl-2</i>	5'-GGTCTTCAGAGACAGCCAGG-3'	5'-GATCCAGGATAACGGAGGCT-3'
<i>Bcl-XL</i>	5'-GCTGCATTGTTCCCGTAGAG-3'	5'-GTTGGATGGCCACCTATCTG-3'
<i>bFgf</i>	5'-TTCATAGCAAGGTACCGGTTG-3'	5'-AGAAGAGCGACCCACACG-3'
<i>Bim</i>	5'-GCTCCTGTGCAATCCGTATC-3'	5'-GCCCCTACCTCCCTACAGAC-3'
<i>Bmf</i>	5'-CTGTTCAAGGCGAGGTTTT-3'	5'-AGTTCCATCGGCTTCATACG-3'
<i>Bmi-1</i>	5'-TTTTATGCTGAACGACTTTTAACTT-3'	5'-GCTCAGTGATCTTGATTCTGGT-3'
<i>CD31</i>	5'-AGTTGCTGCCCATTCATCAC-3'	5'-CTGGTGCTCTATGCAAGCCT-3'
<i>Flk-1</i>	5'-TCCAGAATCCTCTTCCATGC-3'	5'-AAACCTCCTGCAAGCAAATG-3'
<i>Gata4</i>	5'-CCATCTCGCCTCCAGAGT-3'	5'-CTGGAAGACACCCCAATCTC-3'
<i>Gfp</i>	5'-CCACATGAAGCAGCACGAC-3'	5'-GTGCTCAGGTAGTGGTTG-3'
<i>Gusb</i>	5'-ACTCCTCACTGAACATGCGA-3'	5'-ATAAGACGCATCAGAAGCCG-3'
<i>Hgf</i>	5'-CTTCTCCTTGGCCTTGAATG-3'	5'-CCTGACACCACTTGGGAGTA-3'
<i>Igf-1</i>	5'-CACTCATCCACAATGCCTGT-3'	5'-TGGATGCTCTTCAGTTCGTG-3'
<i>Mdr1b</i>	5'-ATCTTCTGAGGTTCCGCTCA-3'	5'-ATACGCCAACAGCAGGTTTC-3'
<i>Nanog</i>	5'-CCAACCCAACTTGAACAAC-3'	5'-GAAGTTATGGAGCGGAGCAG-3'
<i>Nestin</i>	5'-CTCGGGAGAGTCGCTTAGAG-3'	5'-ATTAGGCAAGGGGGAAGAGA-3'
<i>Nkx2.5</i>	5'-GGCTTTGTCCAGCTCCACT-3'	5'-CATTTTACCCGGGAGCCTAC-3'
<i>Oct4</i>	5'-TCTTCTGCTTCAGCAGCTTG-3'	5'-GTTGGAGAAGGTGGAACCAA-3'
<i>Rat Anp</i>	5'-GCCTTTTGGCTCCCAGGCCA-3'	5'-TCCAGGTGGTCTAGCAGGTTCTTG-3'
<i>Rat Bnp</i>	5'-GGCTGTGACGGGCTGAGGTTG-3'	5'-GGTGGTCCCAGAGCTGGGGAAAG-3'
<i>Rat Gusb</i>	5'-ACTCCTCACTGAACATGCGA-3'	5'-ATAAGATGTACCAGAAGCCA-3'
<i>Rat α-Mhc</i>	5'-GCGGACATTGCCGAGTCCCAG-3'	5'-TGGGATAGCAACAGCGAGGCTC-3'
<i>Rat β-Mhc</i>	5'-AGGGCGGACATTGCCGAGTC-3'	5'-CAGGCATCCTTAGGGTTGGGTAGC-3'
<i>S100a4</i>	5'-TTTGTGGAAGGTGGACACAA-3'	5'-CAGCACTTCCTCTCTCTTGG-3'
<i>Sca-1</i>	5'-GGCAGATGGGTAAGCAAAGA-3'	5'-CAATTACCTGCCCCTACCCT-3'

Primer	Sense	Antisense
<i>Sdf1</i>	5'-CAGCCGTGCAACAATCTGAAG-3'	5'-CTGCATCAGTGACGGTAAACC-3'
<i>Sirt1</i>	5'-GACACAGAGACGGCTGGAAC-3'	5'- CAGACCCTCAAGCCATGTTT-3'
<i>Sm22</i>	5'-GACTGCACTTCTCGGCTCAT-3'	5'-CCGAAGCTACTCTCCTTCCA-3'
<i>Sox2</i>	5'-AAAGCGTTAATTTGGATGGG-3'	5'-ACAAGAGAATTGGGAGGGGT-3'
<i>Tbx5</i>	5'-TGGTTGGAGGTGACTTTGTG-3'	5'-GGCAGTGATGACCTGGAGTT-3'
<i>Tgfb1</i>	5'-CAACCCAGGTCCTTCCTAAA-3'	5'-GGAGAGCCCTGGATACCAAC-3'
<i>Tie2</i>	5'- TTTCGGCATCAGACACAAGA-3'	5'- CCGGCTTAGTTCTCTGTGGA-3'
<i>Troponin T</i>	5'-ACCCTCAGGCTCAGGTTCA-3'	5'-GTGTGCAGTCCCTGTTTCA-3'
<i>VE-Cadherin</i>	5'-CGTTGGACTTGATCTTTCCC-3'	5'-CGCCAAAAGAGAGACTGGAT-3'
<i>Vegfa</i>	5'-AATGCTTTCTCCGCTCTGAA-3'	5'-GCTTCCTACAGCACAGCAGA-3'
<i>vWF</i>	5'-CTCACACAGAGCCACAAAGG-3'	5'-AACTGCGAGAGCTCTTCTGG-3'
<i>α-Actinin</i>	5'-TGTTCTCGATCTGTGTCCCC-3'	5'-CATGCAGCCTGAAGAGGACT-3'
<i>α-Mhc</i>	5'-CTTCATCCATGGCCAATTCT-3'	5'-GCGCATTGAGTTCAAGAAGA-3'
<i>α-Sma</i>	5'-GTTCAGTGGTGCCTCTGTCA-3'	5'-ACTGGGACGACATGGAAAAG-3'
<i>β-Mhc</i>	5'-GAGCCTTGGATTCTCAAACG-3'	5'-GTGGCTCCGAGAAAGGAAG-3'

Table S3. Echocardiographic data in the rat MI model. Related to Fig 2.

	Vehicle (n=5)	Control-miR-CPCs (n=6)	miR-133a-CPCs (n=11)
Baseline (before MI)			
LVEDD (mm)	5.12 ± 0.13	5.63 ± 0.19	5.13 ± 0.25
LVESD (mm)	3.16 ± 0.16	3.60 ± 0.21	2.81 ± 0.27
LVAd (mm ²)	29.87 ± 0.98	24.16 ± 1.47	22.21 ± 1.35
LVAs (mm ²)	8.60 ± 0.33	7.11 ± 0.56	6.04 ± 0.43
AWdT (mm)	1.79 ± 0.04	1.13 ± 0.04	1.64 ± 0.09
AWsT (mm)	2.78 ± 0.04	1.92 ± 0.19	2.51 ± 0.13
FS (%)	38.24 ± 0.84	36.25 ± 1.81	46.17 ± 3.44
FAC (%)	71.16 ± 0.39	70.48 ± 1.63	72.22 ± 1.94
AWTF (%)	35.58 ± 1.18	39.19 ± 4.75	34.76 ± 1.26
2 weeks (after treatment)			
LVEDD (mm)	7.17 ± 0.34	6.33 ± 0.52	6.57 ± 0.21
LVESD (mm)	5.64 ± 0.36	4.20 ± 0.36	4.15 ± 0.26
LVAd (mm ²)	43.80 ± 1.86	37.06 ± 2.75	33.39 ± 1.42
LVAs (mm ²)	28.36 ± 1.39	16.73 ± 2.88	13.24 ± 0.92
AWdT (mm)	1.29 ± 0.20	1.66 ± 0.15	1.66 ± 0.06
AWsT (mm)	1.64 ± 0.26	2.40 ± 0.12	2.43 ± 0.15
FS (%)	21.62 ± 1.83	33.65 ± 1.23 ^a	37.24 ± 2.48 ^a
FAC (%)	35.15 ± 1.05	56.23 ± 5.11 ^a	60.63 ± 1.80 ^a
AWTF (%)	21.19 ± 1.07	31.68 ± 3.30 ^a	31.52 ± 1.52 ^a
4 weeks (after treatment)			
LVEDD (mm)	7.65 ± 0.23	6.67 ± 0.39	7.06 ± 0.24
LVESD (mm)	6.11 ± 0.25	4.72 ± 0.54	4.66 ± 0.33
LVAd (mm ²)	50.89 ± 4.96	46.15 ± 4.35	41.34 ± 2.13
LVAs (mm ²)	39.35 ± 4.44	27.33 ± 4.40	17.23 ± 1.75
AWdT (mm)	1.42 ± 0.40	1.54 ± 0.19	1.71 ± 0.08
AWsT (mm)	1.67 ± 0.41	2.08 ± 0.20	2.47 ± 0.15
FS (%)	20.28 ± 1.32	30.19 ± 4.52	34.72 ± 2.68 ^a
FAC (%)	23.15 ± 0.61	42.37 ± 6.42 ^a	59.08 ± 2.51 ^{ab}
AWTF (%)	16.71 ± 2.06	25.12 ± 5.92	29.97 ± 2.41 ^a

LVEDD: left ventricular end-diastolic diameter. LVESD: left ventricular end-systolic diameter. LVAd: left ventricular area at diastole. LVAs: left ventricular area at systole. AWdT: anterior wall diastolic thickness. AWsT: anterior wall systolic thickness. FS: fractional shortening, calculated as $FS (\%) = [(LVEDD - LVESD)/LVEDD] \times 100$. FAC: fractional area change, calculated as $FAC (\%) = [(LVAd - LVAs)/LVAd] \times 100$. AWTF: anterior wall thickening fraction, calculated as $AWTF (\%) = [(AWdT - AWsT)/AWdT] \times 100$. ^a, P<0.05 versus vehicle. ^b, P <0.05 versus control-miR-CPCs.

Table S4. Morphometric data in the rat MI model. Related to Fig 3.

	Vehicle (n=5)	Control-miR-CPCs (n=7)	miR-133a-CPCs (n=7)
LV area (mm ²)	23.39 ± 4.59	21.33 ± 2.81	25.92 ± 2.44
LV cavity (mm ²)	20.02 ± 4.77	7.73 ± 2.14	5.30 ± 1.66
Risk region area (mm ²)	12.61 ± 2.80	8.36 ± 0.74	8.34 ± 0.85
Scar region area (mm ²)	10.45 ± 1.83	6.74 ± 0.86	4.65 ± 0.96
Infarcted wall thickness (mm)	0.60 ± 0.15	0.46 ± 0.02	0.80 ± 0.05
Risk area (% of LV)	53.42 ± 3.25	41.82 ± 4.30	31.77 ± 4.10 ^a
Scar area (% of risk region)	45.55 ± 3.48	33.32 ± 3.71 ^a	17.28 ± 4.03 ^{a,b}
Viable myocardium (% of risk region)	14.85 ± 4.04	20.90 ± 4.40	48.26 ± 8.81 ^{a,b}

LV, left ventricle. ^a *P* <0.05 versus vehicle; ^b *P* <0.05 versus control-miR-CPCs.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

Cardiac progenitor cells (CPCs) were isolated from adult female C57BL/6 mice. Neonatal rat cardiomyocytes (NRCMs) were isolated from newborn Wistar rats. Myocardial infarction was conducted in adult male Wistar rats or adult female C57BL/6 mice. The corresponding local ethics committee approved animal studies. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Isolation, culture and surface marker characterization of Sca-1⁺ CPCs

C57BL/6 mice between 8 and 12 weeks of age were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg). After cannulation of the aorta, the heart was removed and perfused *ex vivo* through the coronary system during 10-20 minutes with a solution of 4mg/mL collagenase type II (Worthington). The digested hearts were minced and mechanically disaggregated, and the resulting cell suspension was passed through a 70µm filter. The fraction of small live cells was obtained by density gradient centrifugation with OptiPrepTM (Axis-Shield), and the SCA-1⁺ hematopoietic lineage negative fraction was purified by magnetic cell sorting using a lineage cell depletion kit and Sca-1 microbeads (Miltenyi Biotec). The isolated cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10% ES-qualified FBS (Invitrogen), 20 ng/mL bFGF (Peprotech), 40 ng/mL EGF (Sigma), 10³ U/mL LIF (Millipore), 1% insulin-transferrin-selenium (Invitrogen), 1% penicillin/streptomycin (Lonza) and 1% L-glutamine (Lonza). Cell surface markers were analyzed by flow cytometry in a BD

FACSCanto II flow cytometer using BD FACSDiva software (Becton Dickinson). SCA-1⁺ CPCs were stained with antibodies in ice-cold PBS, 0.5% BSA, 2 mM EDTA.

Myocardial infarction and cell injection

Myocardial infarction models were as described previously, with small variations (Arminan et al., 2010, Fischer et al., 2009). Briefly, male Wistar rats (6 weeks old) or female C57BL/6 mice (12 weeks old) underwent left thoracotomy under general anesthesia with 2.5% sevoflurane, and were ventilated with a rodent respirator. In the rat model animals were treated with 20 mg/kg/day of cyclosporine A (Novartis) for 3 days before the procedure until sacrifice. Myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery. At five minutes post ligation, 10⁵ CPCs (control-miR–CPCs or miR-133a–CPCs suspended in 21µl PBS as vehicle) or vehicle were injected by a blinded surgeon into three sites around infarct border zone. In the rat model, the cell suspension contained red fluorescent microbeads (6.25x10⁶ beads/animal; Molecular Probes). Animals that underwent thoracotomy without artery ligation or cell injection were used as sham controls. After injections, the chest was closed and animals allowed recovering.

Cardiac function was monitored by echocardiography 1 day before the ligation (baseline) and 2 and 4 weeks after surgery, using the Vivid 7 echocardiography system (GE Healthcare, Little Chalfont, UK). Vivid S5 10.1.x software (GE Healthcare) was used to measure fractional area change (FAC) from the 2D-Mode of the short-axis view and fractional shortening (FS) from the M-mode of the short axis view.

Neonatal rat cardiomyocyte *in vitro* assays

Neonatal rat cardiomyocytes (NRCMs) were obtained from 0-2 day old Wistar rats. Hearts were minced and cells separated by digestion with trypsin (Invitrogen, NY, USA) and collagenase type II (Worthington) followed by differential plating. After 2 days, seeding onto a NRCM monolayer, followed by co-culture for 7 days, induced CPC differentiation. Apoptosis in serum starved NRCMs was monitored by TUNEL staining (Cell Death Detection Kit; Roche, Basel, Switzerland) of troponin-positive cells. NRCM apoptosis (caspase-3/9) and hypertrophy (cardiomyocyte area; ImageJ, NIH) were also measured in response to H₂O₂ (50 µmol/L, 2 hours) or angiotensin II (ANGII, 100 nmol/L, 72 hours; Sigma) in the presence of CPC conditioned medium (CM).

Differentiation of CPCs toward the cardiac, smooth muscle and endothelial lineages

Cardiac differentiation

Differentiation was promoted by seeding CPCs at 2×10^4 cells/cm² in culture medium, and replacing medium after 24 hours with α -MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 10nM dexamethasone. At 0, 3, 7, 10 and 14 days, samples were collected for gene expression and western-blot analysis.

For co-culture differentiation experiments, NRCMs were seeded at 7.5×10^4 cells/cm² on gelatin-coated plates or slides. Medium was changed the next day, and after 72 hours, GFP⁺ CPCs were seeded onto the NRMC monolayer at 3×10^3 /cm² in cardiomyocyte medium without cytosine β -D-arabinofuranoside. Co-culture was continued for 7 to 10

days. For immunofluorescence analysis cells were fixed in 4% PFA. For gene expression and western blot analysis cells were collected by trypsinization, and CPC-derived (GFP⁺) cells were isolated by cell sorting with a FACS Aria SORP (Becton Dickinson).

Smooth muscle differentiation

For smooth muscle differentiation CPCs were seeded at 2×10^4 cells/cm² in culture medium, and after 24 hours medium was replaced with medium supplemented with 100nM oxytocin. After 3 days, medium was changed to DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 10mM β -glycerophosphate, 1 μ M dexamethasone and 0.2 mM ascorbic acid. After 7 days cells were fixed in 4% PFA and analyzed by immunofluorescence.

Endothelial differentiation

For endothelial differentiation *in vitro*, CPCs were seeded at 2×10^4 cells/cm² in culture medium, and after 24 hours medium was replaced with DMEM/F12 supplemented with 2% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/mL VEGF, 20 ng/mL IGF-1, 10 ng/mL EGF, 50 ng/mL ECGF and 1 μ g/mL hydrocortisone. Cells were collected 4 days later for gene expression analysis.

Endothelial differentiation *in vivo* was evaluated by Matrigel angiogenesis plug assay. Matrigel mixed with 10^5 GFP⁺ CPCs was injected subcutaneously into the backs of 12 week-old C57BL/6 female mice. After 7 days the animals were sacrificed and the implants formed were fixed and paraffin embedded, and 5 μ m sections were analyzed by immunofluorescence.

CPC proliferation

MTT metabolic assay. Control-miR–CPCs, miR-1–CPCs and miR-133a–CPCs were seeded at 5×10^3 cell/cm² in 24 well plates, and the MTT assay was performed at 24, 48, 72 and 96 hours after plating in 3 independent wells per condition, using the Cell Proliferation Kit I (MTT) from Roche.

³H-thymidine incorporation. Control-miR–CPCs, miR-1–CPCs and miR-133a–CPCs were seeded at 5×10^3 cell/cm² in 24 well plates, and the next day medium was switched to DMEM/F12 supplemented with 1% penicillin/streptomycin and 1% L-glutamine. Twenty-four hours later, medium was replaced with fresh medium supplemented with 1 µCi/mL [6-³H]-thymidine (Perkin-Elmer). After 48 hours, cells were fixed and collected, and ³H-thymidine incorporation was measured by standard scintillation counting.

CPC apoptosis

Before H₂O₂ treatment, CPCs were incubated overnight with serum-free DMEM/F12. The next day, cells were treated with 200 µmol/L H₂O₂ for 0, 2 or 5 hours, and samples were collected for gene expression analysis, protein lysate isolation or apoptosis quantification. The percentage of living cells was measured by FACS after staining with annexin V-FITC and propidium iodide (PI) (0.01%), with an apoptosis detection kit (BD Pharmingen). Caspase-3 activity was measured by fluorescence intensity with the CaspGLOW Green caspase-3 staining (MBL International). Cytochrome c release was measured with the Rat/Mouse Cytochrome c Quantikine ELISA Kit (R&D Systems).

Osteogenic and adipogenic differentiation of CPCs

CPCs were seeded at 2×10^4 cells/cm² in culture medium, and after 24 hours, medium was replaced by the corresponding induction medium. Osteogenic medium was α-MEM

supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 10 mM β -glycerophosphate, 0.1 μ M dexamethasone and 0.2 mM ascorbic acid. Adipogenic medium was α -MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 0.5 μ M hydrocortisone, 0.5 mM IBMX (3-isobutyl-1-methyl xanthine) and 60 μ M indomethacin; in all cases, induction medium was replaced every 3–4 days, and on day 21 cells were processed for histochemical analysis. Cells were fixed in 70% ethanol (1 hour, 4°C) and stained (5 minutes). For osteogenic assays, cells were stained with 40 mM Alizarin Red, pH 4.1. Cells cultured in adipogenic medium were stained with 2% Oil Red O.

Conditioned media

Control-miR–CPCs and miR-133a–CPCs were seeded at 80% confluence, and after 24 hours cells were washed with PBS and culture medium was replaced with M199 Glutamax supplemented with 10mM HEPES, 0.1mM MEM non essential amino acids and 1% penicillin/streptomycin (apoptosis and hypertrophy experiments). After 48 hours, conditioned medium was collected, centrifuged to eliminate cell debris, and stored at -80°C until use. The exosomal fraction was isolated by differential ultracentrifugation as described previously (Thery et al., 2006). Briefly, cleared conditioned medium was centrifuged for 10 minutes at 2,000g, the supernatant was collected and centrifuged for 30 minutes at 10,000g, and the supernatant was again collected and centrifuged at 100,000g for 70 minutes to pellet the exosomes, which were recovered and washed to eliminate contaminating proteins.

Synthetic miRNA transfection and Bim 3'UTR luciferase reporter assay

To confirm *Bim* as a target of miR-133a, a 1 kb fragment of the mouse *Bim* 3'UTR containing the putative binding site for miR-133a at nucleotides 1545-1551 was amplified by PCR from C57BL/6 mouse genomic DNA using the primers FW: 5'-

CTCGAGTGGGCTCACACACCGCTTGC-3' and RV: 5'-GCGGCCGCACAGGGCC TCATGGGAACCA-3'. To confirm *Bmf* as a target of miR-133a a 1 kb fragment of the mouse *Bmf* 3'UTR containing the putative binding site for miR-133a at nucleotides 2153-2160 was amplified as described above using the primers FW: 5'-CTCGAGTGCAAGGAGAGGGGACCCAAG-3' and RV: 5'-GCGGCCGCAGTAGA GCAGTTTGGCCCCTGA-3'. Fragments were subsequently cloned into a psiCHECK-2 vector (Promega) downstream of the *Renilla* luciferase reporter gene.

For luminescence assays, 293T cells were seeded at 10^5 cell/cm² in 24 well plates, and transfected 24 hours later using Lipofectamine 2000 (Invitrogen) with 250 ng of the reporter vector and 30 nM of the different LNA's: a scramble miRNA, a synthetic miR-133a or a miR-133a inhibitor (antimiR, all from Ambion). The ratio of *Renilla* to firefly luciferase luminescence was measured 24 hours later with the Dual luciferase assay system (Promega). Normalized *Renilla* to firefly ratios were determined in absence, presence or inhibition of miR-133a.

Bioinformatic analysis of predicted targets for miR-133a

Analysis and selection of targets predicted to be affected by miR-133 was developed by means of Ingenuity Analysis Pathway Software (IPA), which identifies mRNA targets for microRNAs using predicted microRNA-mRNA binding relationships from TargetScan, plus experimentally validated relationships from TarBase and curated by direct research from the biomedical literature. We first generated an initial dataset of predicted targets for miR-133a based on the results of 10 different algorithms (DIANAmT, miRanda, miRDB, miRWalk, PICTAR4, PICTAR5, PITA, RNA22, RNAhybrid and TargetScan). We accuated this list by selecting common targets predicted by at least 5 algorithms and this second dataset was analysed by Ingenuity

Pathway Analysis (IPA) software. The final selection of targets to test was complemented with a detailed review of the literature.

Protein lysates and Western blotting

Cells were lysed in RIPA buffer containing protease inhibitors (Roche) and, after centrifugation, supernatants were collected and protein quantified by the DC protein assay (Biorad). Protein (30µg per lane) was loaded on 4-12% gels (Biorad) and Western blotting was performed in the mini protean III system (Biorad). Membranes were blocked and incubated overnight with primary antibodies against α -actinin (1:500, Sigma) and troponin T (1:1000, Abcam), diluted in 5% non-fat milk in PBS, 0.1% Tween 20 (Sigma). Next day, membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako), and blots were developed with the ECL kit (GE Healthcare).

FACS analysis

Cells were stained with antibodies against NKX2.5 (1:50, Santa Cruz Biotech), α -actinin (1:100, Sigma), troponin I (1:100, Santa Cruz Biotech) or tropomyosin (1:100, Sigma), in ice-cold PBS, 0.5% BSA, 2 mM EDTA for 30 minutes (Table S1). Cells were then incubated with Cy3/FITC/PE-conjugated secondary antibodies (Jackson ImmunoResearch). Secondary antibodies alone (goat anti-rabbit IgG or anti-mouse IgG, Cy3 conjugated) or combined with irrelevant primary antibodies were used as negative controls for gating purposes. Staining was analyzed by flow cytometry in a BD FACSCanto II flow cytometer using BD FACSDiva software (Becton Dickinson). In some cases staining was visualized by microscopy after cell cytopsin.

Immunocytochemistry

Coverslips or chamber slides (Nunc) from differentiation or apoptosis experiments were washed with PBS, and the cells were fixed in 4% paraformaldehyde (PFA) at room temperature, permeabilized with 0.1% Triton-X, blocked with 10% goat serum and incubated overnight at 4°C with primary antibodies in 1% goat serum. Primary antibodies used are listed in supplementary table 1 (Table S1). The next day, cells were washed and incubated for 45 minutes at room temperature with secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 649 (Invitrogen) or with Cy3 (Jackson ImmunoResearch). Incubation with secondary antibody alone did not produce any detectable background signal. Slides were mounted in Vectashield with Dapi (Vector labs) and analyzed by confocal microscopy (Leica SP5, Leica).

RNA Isolation, RT-PCR and real time RT-PCR

Total RNA was isolated with the miRNeasy Mini Kit (Qiagen). For protein-coding genes, cDNA was obtained with Superscript III Reverse Transcriptase (Invitrogen), and sequences were amplified by PCR using the primers listed in Table S2; *Gapdh* or *Gusb* were used as endogenous normalization controls. PCR amplification and Quantitative PCR were performed with Taq DNA Polymerase (Roche) and Power SYBR-Green Master Mix (Applied Biosystems), respectively. miR-1 and miR-133a transcripts were quantified by real-time RT-PCR using the corresponding TaqMan Gene Expression Assays (Applied Biosystems), and U6 was used as an endogenous normalization control. PCR products were quantified with an ABI PRISM 7900 quantitative PCR system (Applied Biosystems) and analyzed with SDS v2.3 software (Applied Biosystems).

Echocardiography

Transthoracic echocardiograms were obtained by a blinded echocardiographer to assess cardiac function. In the rat model echocardiography was performed 1 day before the ligation (baseline) and at 2 and 4 weeks after surgery, under general anesthesia (2.5% sevoflurane), using an echocardiographic system (Vivid 7; GE Healthcare) equipped with a 10-MHz linear-array transducer. In the mouse model, echocardiography was performed 1 week and 12 weeks after surgery, under general anesthesia (2.5% sevoflurane), using the Vevo 770 Imaging System (VisualSonics). The hearts were imaged two-dimensionally in long-axis view (at the level of the greatest LV diameter) and in the short-axis view (at the level of the papillary muscle). In the rat model, fractional area change (FAC) was calculated from the 2D-mode of the short axis view while fractional shortening (FS) was calculated from the M-mode of the short axis view with Vivid S5 10.1.x (GE Healthcare). In the mouse model fractional area change (FAC) and left ventricular ejection fraction (LVEF) were calculated from the long-axis view with VisualSonics V1.3.8 (VisualSonics). Measurements of cardiac parameters and formulas for calculations are described in supplementary table 3. Animals with extremely small infarcts or showing poor echocardiography images were excluded.

Morphology and histology

Rats were sacrificed 4 weeks after MI and the hearts fixed for histology. Eight sections per heart, covering the organ from apex to base, were stained with Masson's trichrome. Computer planimetry analysis of the Masson's trichromic images (Image Pro-Plus 5.1, Media Cybernetics, MD, USA) was used to calculate left ventricle (LV) morphometric parameters (LV cavity area, LV total area, risk region area, scar area and LV anterior wall thickness) for each section.

GFP⁺ cells in the myocardium were detected by immunohistochemistry with an anti-GFP antibody (Abcam, Cambridge, United Kingdom). α -SMA⁺ blood vessels were quantified by α -SMA staining (anti- α -SMA; Sigma, MO, USA) and cardiomyocyte cross-sectional area was measured by analysis of Laminin staining (anti-Laminin; Abcam) with ImageJ (NIH). To study cardiomyocyte proliferation, rats were injected with EdU (5-ethynyl-2'-deoxyuridine; 10 mg/kg/day) for 5 days prior to sacrifice. The EdU signal was detected in 3 sections per heart using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Molecular Probes, NY, USA), and sections were stained with anti-troponin T antibody. The number of total EdU⁺ cells and EdU⁺ cardiomyocytes in the border zone was measured with ImageJ (NIH).

Immunohistochemistry

Rat heart cryosections were washed with PBS at room temperature and permeabilized with 0.1% Triton-X. Mouse heart paraffin-embedded sections were deparaffinized, rehydrated, washed with PBS, and antigens were retrieved with sodium citrate. For tyramide amplification, slides were incubated with 0.3% H₂O₂. Tissue sections were then blocked with 10% goat serum or Perkin-Elmer blocking buffer (in the case of tyramide amplification) and incubated overnight at 4°C in blocking solution with primary antibodies. All primary antibodies used are specified in Table S1. Slides were washed and incubated for 1 hour at room temperature with secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 649 (Invitrogen), to Cy3 (Jackson ImmunoResearch), or to HRP (Dako). Incubation with secondary antibody alone did not give any detectable background signal. Slides were mounted in Vectashield with DAPI (Vector labs) and analyzed by confocal microscopy (Leica SP5, Leica).

Detection of GFP⁺ cells. CPCs (or their progeny) were detected in heart sections either by direct visualization of GFP protein fluorescence or by immunostaining with an ant-

GFP antibody; the anti-GFP antibody signal was amplified with tyramide (TSA, Perkin-Elmer). To evaluate the cardiac fate of transplanted CPCs and derived GFP⁺ cells, sections were also incubated with an anti-Troponin T or anti-Tropomyosin antibody.

α -Sma⁺ blood vessel quantification. To study arteriolar density in the rat model, 3 sections from each heart were stained with antibodies against troponin T and α -SMA, and the number of α -SMA⁺ blood vessels/mm² was quantified in 4 image fields per section of the remote (healthy) myocardium and 6 image fields per section in the border zone.

Cardiomyocyte proliferation. Rats were injected daily with EdU (10 mg/kg/day) for 5 days prior to sacrifice. EdU signal was detected in 3 sections per heart using the Click-iT EdU Alexa Fluor 647 imaging kit (Molecular Probes), and sections were stained with troponin T antibody. The number of EdU⁺ total cells and EdU⁺ cardiomyocytes in the border zone was quantified in 10 image fields per section using ImageJ (NIH).

In vivo cardiomyocyte hypertrophy. To analyze cardiomyocyte hypertrophy in the border zone of the infarct, two sections from each rat heart were stained with antibodies against Troponin T and Laminin, and cross-sectional area of cardiomyocytes was calculated in 4 image fields per slide using ImageJ (NIH).

NRCM *in vitro* hypertrophy assay

NRCMs were seeded at 1.2×10^5 cells/cm² on gelatin-coated slides, and the next day medium was switched to medium without FBS. After overnight incubation, cells were cultured in CPC-conditioned medium supplemented with 100 nM ANG II for 72 hours. Cells were fixed in 4% PFA and stained with troponin T primary antibody, and cell area was measured in 3 image fields per slide in 3 independent slides with NIH ImageJ software.

NRCM apoptosis assays

Co-culture with CPCs and serum starvation

NRCMs were seeded at 10^4 cells/cm² on gelatin-coated slides. The medium was changed the next day, and after 24 hours 6×10^4 CPCs/cm² were seeded onto the monolayer in cardiomyocyte medium free of FBS or horse serum. Co-culture was continued for 3 or 7 days, when cells were fixed with 4% PFA and stained with anti-troponin T (Abcam). Apoptotic cardiomyocytes were quantified by TUNEL staining of troponin T⁺ cells in 10 image fields per slide on 3 independent slides using the fluorescein cell death detection kit, (Roche).

Apoptosis experiments with H₂O₂

NRCMs were seeded at 3×10^5 cells/cm² on gelatin-coated 96 well optical plates. The medium was changed the next day with medium containing 2% FBS, and after 48 hours cells were incubated overnight with conditioned medium from CPCs. The next day, 50 mM H₂O₂ was added and, after 2 hours Caspase 3 and Caspase 9 activities were measured by fluorescence intensity with the CaspGLOW Green caspase-3 staining and CaspGLOW Green caspase-9 staining kits (MBL International).

Engineered Heart Muscle (EHM) assays

EHM generation

Engineered Heart Muscle (EHM) was generated as described (Zimmermann et al., 2000; Zimmermann et al., 2002). Neonatal rat heart cells were prepared by Trypsin/DNase digestion. For the construction of CPC-EHMs, rat heart cells were preplated for 60 min in DMEM (Biochrom), 10% FBS, 2 mmol/L Glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen), to enrich for

cardiomyocytes (Fig 7). Enriched cardiomyocytes were then mixed with CPCs (3:1) in a hydrogel containing 0.8 mg pH-neutralized rat-tail collagen, 10% v/v basement membrane protein from Engelbreth-Holm-Swarm tumor exudates (BD Biosciences), and concentrated culture medium (2xDMEM, 20% horse serum, 4% chick embryo extract, 200 U/ml penicillin, and 200 µg/ml streptomycin, all from Invitrogen). Subsequently, the EHM-mixture was cast into custom made-molds. EHM culture medium consisted of DMEM (Biochrom), 10% horse serum, 2% chick embryo extract, 100 U/ml penicillin, and 100µg/ml streptomycin. EHMs were transferred to stretch devices on culture day 7 for continuous culture under phasic load (from 100 to 110% of slack length at 2 Hz) for additional 5 days.

Isometric Force Measurements

On day 12, EHMs were transferred to organ baths containing oxygenated Tyrode's solution at 37°C. EHMs were preloaded under electrical stimulation (200 mA, 5 ms, 2 Hz). Twitch tension was measured under increasing calcium concentrations (0.2–2.8 mmol/L). Contractile activity and twitch parameters were evaluated using AMON software.

Flow cytometry

Neonatal rat heart cells, before and after preplating, were fixed in ice-cold 70% ethanol. Staining for α -Actinin (Sigma) was performed in the presence of 0.5% Triton-X at 4°C for 45 min. Alexa 546-coupled anti-mouse secondary antibody was applied for 30 min at 4°C. Samples were run on a LSRII SORP cytometer (BD Biosciences) and analyzed with Cyflogic software.

EHM histology

EHMs were fixed in neutral buffered 4% formaldehyde/1% methanol, pH 7.4 overnight, followed by extensive washing with PBS. For whole mount staining, EHMs were incubated in blocking buffer (PBS, 0.5% Triton-X, 5% goat serum, 1% BSA) overnight, followed by 2 nights incubation with primary antibodies. Primary antibodies applied were against α -actinin (1:1000; Sigma), active caspase-3 (1:250; Promega) and GFP (1:500; abcam). After extensive washing, appropriate secondary antibodies and DAPI (1 μ g/ml) were incubated overnight followed by extensive washing.

EHM sections were imaged with a Zeiss LSM 710 confocal microscope. For cell quantification, at least 8 random sections per EHM were imaged and analyzed by an independent observer using ImageJ software.

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