Supplement file- Materials and Methods

Materials

Dharma FECT transfection reagent, miRNA inhibitor, mina-mimics and non-targeting controls, anatagomir-29a, antagomir-29c and mis-sense antagomir were purchased from Dharmacon (Dharmacon, Inc IL) [13, 14]. Northern kits were purchased from Signosis (Sunnyvale, Ca). QRT-PCR kit was purchased from Ambion (Austin, TX); monoclonal anti-Mcl-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Akt and anti-Ser-473 P-Akt antibodies from Cell Signaling (Beverly, MA); PIO from Takeda (Lincolnshire, IL); monoclonal anti-β-actin antibody, anti-BX antibodies, GW9662 and protease inhibitor cocktail from Sigma (St. Louis, MO); H9c2 cells and complete growth medium from American Type Culture Collection (ATCC, Manassas, VA); Trizol Reagent from Invitrogen (Carlsbad, CA); MTT cell respiration assay kit from R&D system (Minneapolis, MN), dUTP nick end-labeling (TUNEL) assay kit from Roche (Indianapolis, IN), and Caspase-3 Colorimetric Protease Assay Kit from BioSource (Hopkinton, MA).

RT-PCR

Rats received same treatment as above. We used applied biosystems inventoried 20X assay mixes of primers and TaqMan MGB probe (FAMTM dye-labled) for the target genes CD36 and AP2. Additional details are provided in the "Methods" section of the supplementary file.
The pre-developed 18S rRNA (VICTM-dye labeled probe) TaqMan® assay reagent (P/N 4319413E) served as endogenous control. Real time PCR was performed with 40ng cDNA for both target genes and control. The reagent we used was universal PCR master mix reagent kit (P/N 4304437). The cycling parameters for real time PCR was: UNG activation 50^0C for 2min,

AmpliTaq activation 95^{0}C for 10 min, denaturation 95^{0}C for 15 sec and annealing/extension 60 ^{0}C for 1 min (repeat 40 times) on ABI7000. Duplicate CT values were analyzed in Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems). The amount of target (2-ΔΔCT) was obtained by normalized to endogenous reference (18s) and relative to a calibrator (average of the control samples).

Apoptotic cell detection by in situ end-labeling and nuclear staining for H9c2 cells

To detect programmed cell death, the apoptotic nuclei were labeled using terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer's instructions. In this procedure, apoptotic nuclei were stained green. The nuclei of cells were counterstained with DAPI (0.1 μg/ml). The labeled cells were counted under a fluorescence microscope. The percentage of apoptotic cells was calculated as the ratio of TUNEL-positive cells to the DAPI-stained total cells, counted in six different random fields.

Measurement of caspase -3 activity

The enzymatic activity of caspase-3 induced by various conditions was measured with the Caspase-3 Colorimetric Protease Assay Kit according to the manufacturer's instructions. H9c2 cells prepared after respective treatments were lysed in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1μM aprotinin, 1μM leupeptin, 2mM dithiothreitol, 10mM Tris/HCl, pH 8.0) on ice for 30min and centrifuged at 15,000×*g* for 15min. Assays were performed by incubating 200μg protein of cell lysate in 100μl of reaction buffer containing 5μl of caspase-3 substrate (4mM DEVD-pNA) in 96-well plates. The reaction buffer contained 1%NP-40, 20mM Tris–HCl (pH 7.5), 137mM *N*-acetyl-cysteine and 10% glycerol. Lysates were incubated at 37°C for 2h. Samples were incubated in the dark and measured with a micro-plate reader at an absorbance of 405 nm.

Western blot analysis

Samples were homogenized in lysis buffer (in mMol): 25 Tris·HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na_3VO_4, 1% Triton X-100, 2% SDS and 1% protease inhibitor cocktail. The lysate was centrifuged at 10,000*g* for 15min at 4°C. The resulting supernatants were collected. Protein (75μg) was fractionated by SDS-PAGE (4%-20% polyacrylamide gels) and transferred to PVDF membranes (Millipore, Bedford, MA). Samples were incubated overnight at 4^0C with Anti-Mcl-1 antibodies. Bound antibodies were detected using the chemiluminescent substrate (NEN Life Science Products, Boston, MA). Protein signals were quantified with an image-scanning densitometer, and normalized to the corresponding β-actin signal. Data are expressed as percent of the expression in the control group.

Area at Risk (AR) and myocardial infarct size (IS) determination

Three-month old mice received intravenous injection of 80mg/kg of antagomir-29a (**anta-29a**), antagomir-29c (**anta-29a**), mis-sense antagomir (**misanta-29a**), or vehicle (saline) once daily through their tail vein at normal pressure for 3 days. Last injection was given 16h before surgery. Similar regimen has been previously shown to effectively silence microRNAs up to 24h after injection [13, 14]. The expression of miR-29a or miR-29c at day 4 was determined by qRT-PCR. On the fourth day mice were anesthetized with intraperitoneal injection of ketamine (60mg/kg) and xylazine (6mg/kg), intubated and ventilated (FIO_2=30%). The chest was opened; the left coronary artery was encircled with a suture and ligated for 30min, followed by 4h reperfusion. After 4h, mice were re-anesthetized, the coronary artery was reoccluded, Evan's blue dye 3% was injected into the right ventricle and mice were euthanized under deep anesthesia. Hearts

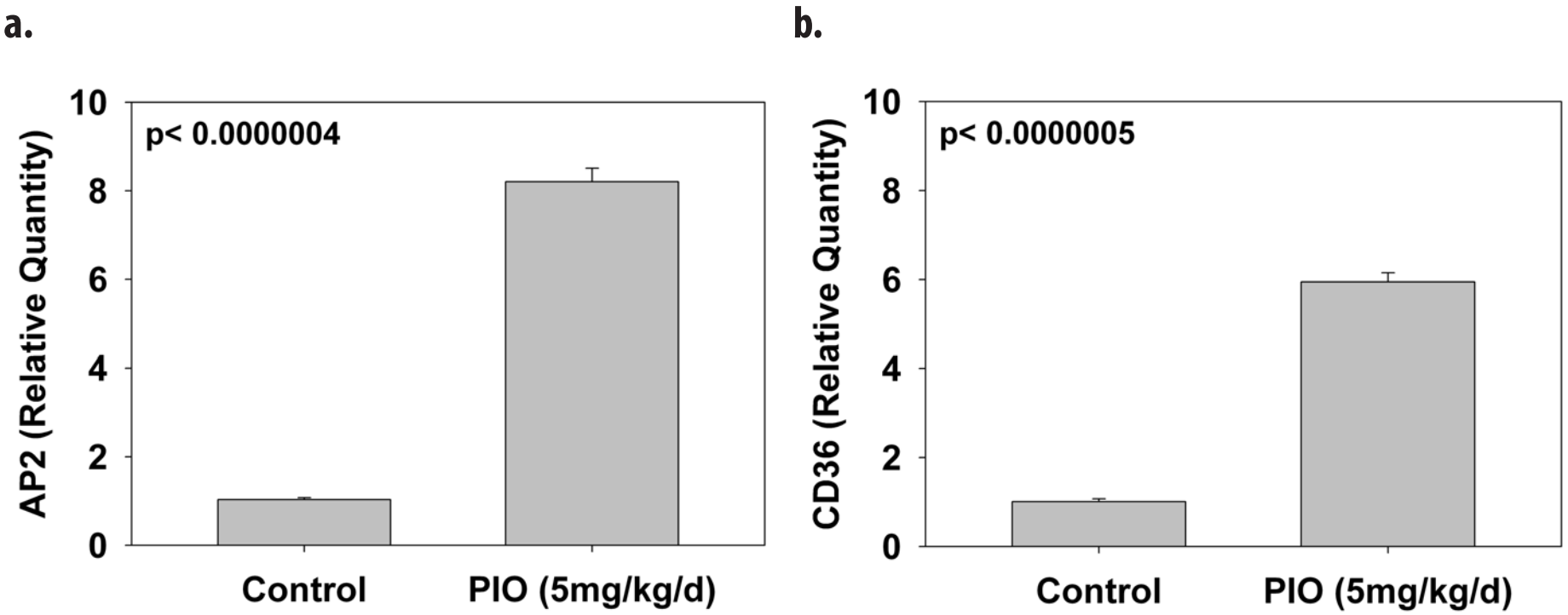
were excised and the left ventricle was sliced transversely into 6 sections. Slices were incubated for 10 minutes at 37°C in 1% buffered (pH=7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC). Slice were photographed in order to identify the area risk (**AR**, uncolored by the blue dye, red), the infarcted zones (unstained by TTC, white or yellow), and the non-ischemic zones (colored by the blue dye). The area of AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice. [12, 16, 17]

Results:

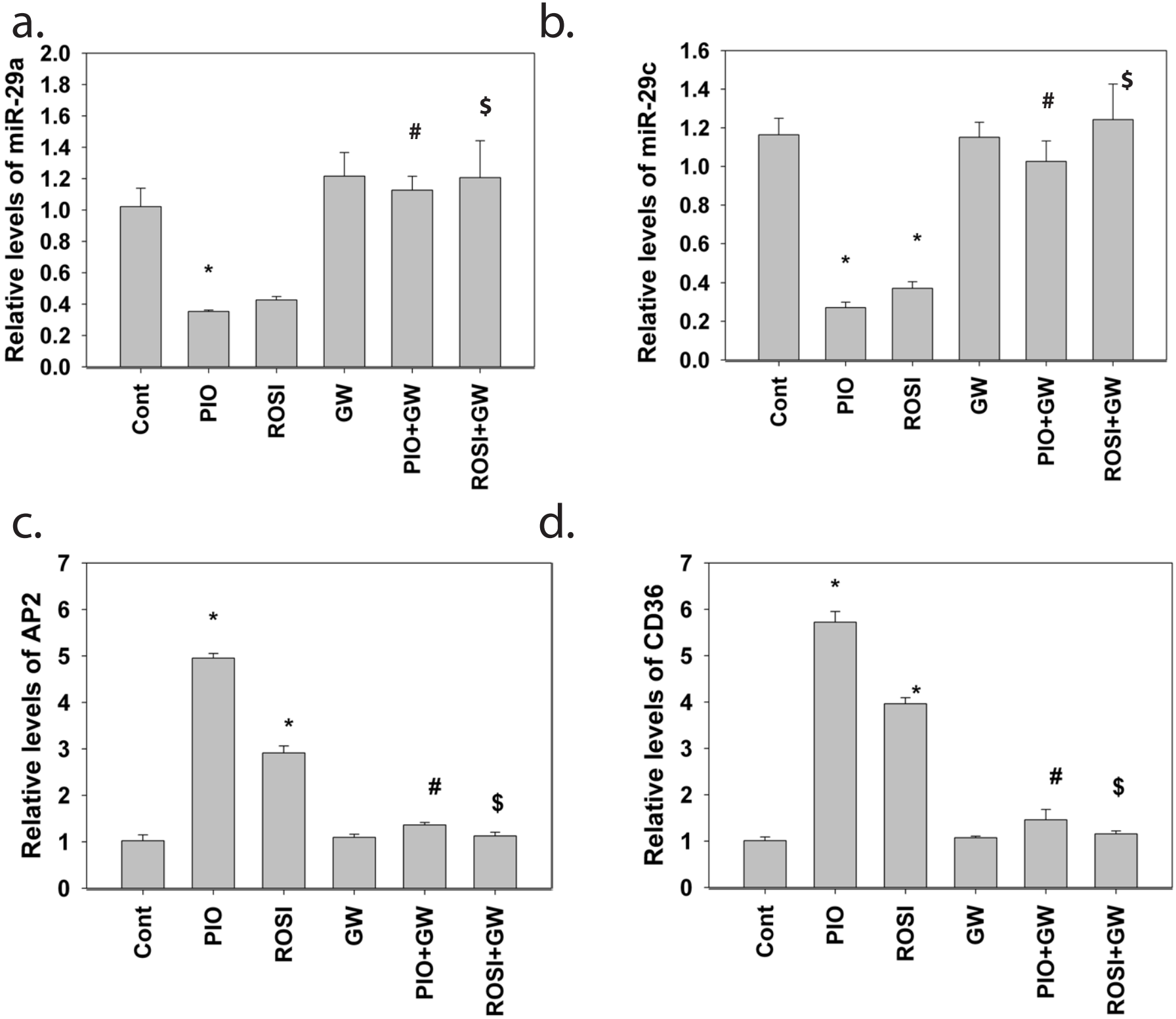
Supplement Table 1:

Table 1: Body weight, AR and IS

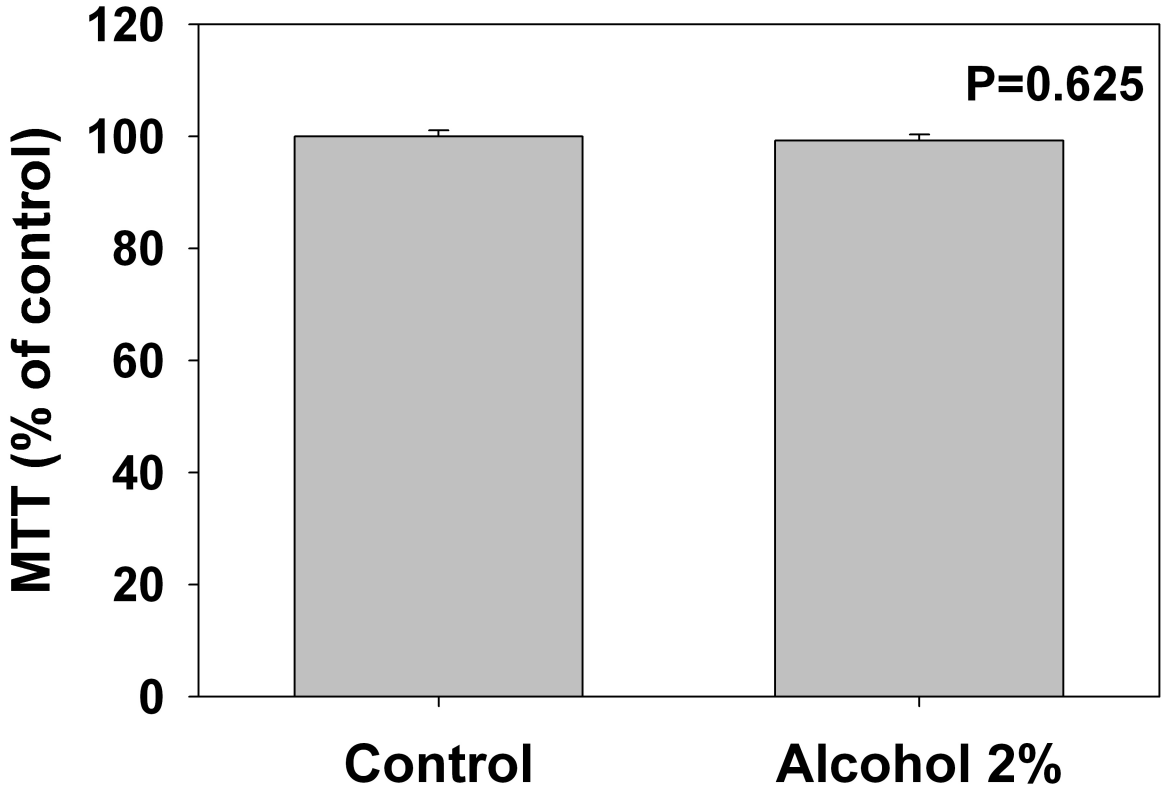
	Control N=6	**anta-29a N=6**	**anta-29c N=6**	**misanta-29 N=6**	**P value**
Body weight (g)	25.7±0.4	26.2±0.4	25.8±0.3	25.7±0.3	0.749
AR (% of LV weight)	39.6±0.9	39.7±0.6	42.5±1.2	40.0±0.6	0.087
IS (% of LV weight)	14.7±1.6	7.0±0.6	6.0±0.5	16.8±0.5	P<0.001



Supplement Figure 1: rt-PCR shows that AP2 (a) and CD36 (b) levels were increased in rat hearts after 7-day pretreatment with PIO (5 mg/kg/d)(n=4 in each group).

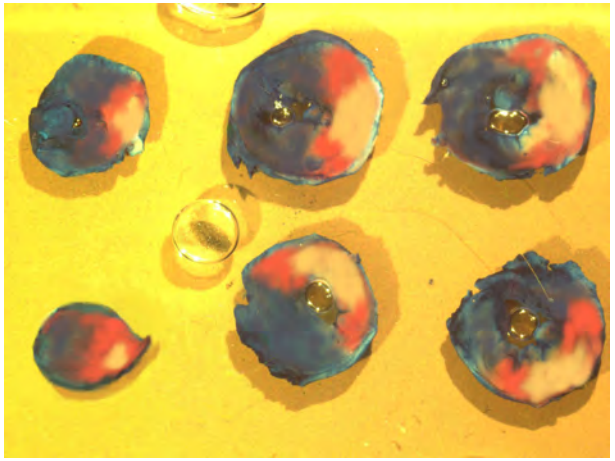

a.
Relative levels of miR-29a
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
$
Cont
PIO
ROSI
GW
PIO+GW
ROSI+GW
b.
Relative levels of miR-29c
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
#
$
Cont
PIO
ROSI
GW
PIO+GW
ROSI+GW
c.
Relative levels of AP2
7
6
5
4
3
2
1
0
*
*
#
$
Cont
PIO
ROSI
GW
PIO+GW
ROSI+GW
d.
Relative levels of CD36
7
6
5
4
3
2
1
0
*
*
#
$
Cont
PIO
ROSI
GW
PIO+GW
ROSI+GW

Supplement Figure 2. qRT-PCR analyses of Mir-29a (***a***), MiR-29c (***b***), and real time-PCR analyses of AP2 (***c***), and CD36 (***d***) expression in H9c2 cardiomyocytes after incubation with PIO, ROSI, GW9662, and their combination. Note: Mean levels in control group were defined as 100%. The small housekeeping RNA U6 was used as a loading control. Four independent experiments were performed. *P<0.05 compared with the control group. # P<0.05 versus PIO. $ p<0.05 versus ROSI. The difference in miR-29a levels between the control and rosiglitazone group was not statistically significant (p=0.063).

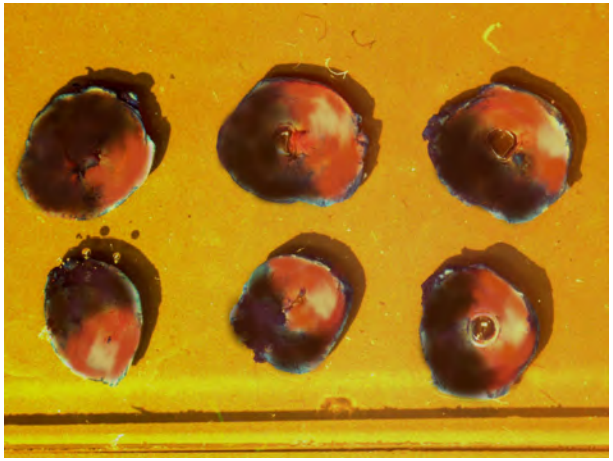


Supplement Figure 3: H9c2 cells were incubated with or without alcohol 2% in normoxyemic conditions (NSIR). Cell viability was assessed by MTT assay. Eight independent experiments were conducted for each group. Alcohol 2% did not affect cell viability.

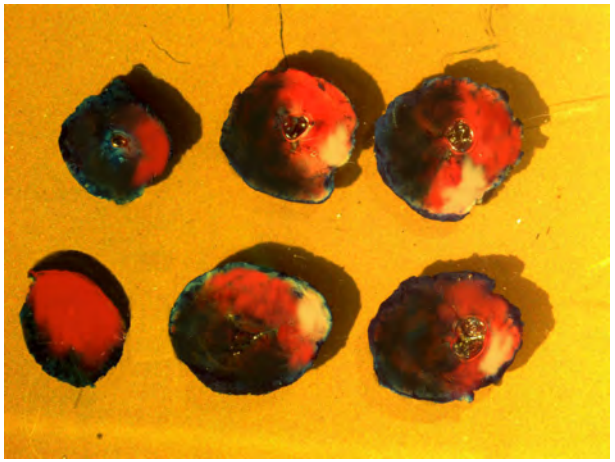
Control



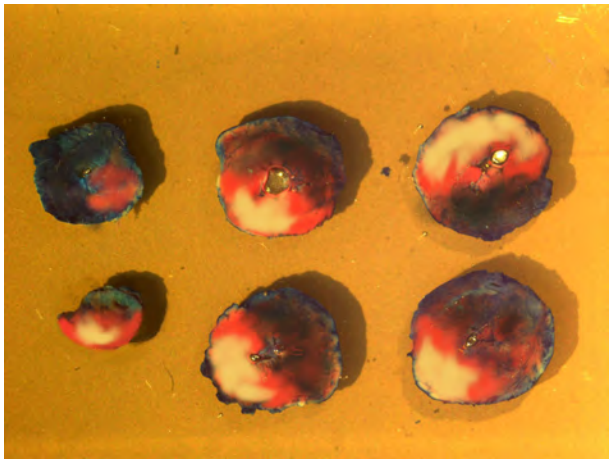
anta-29a



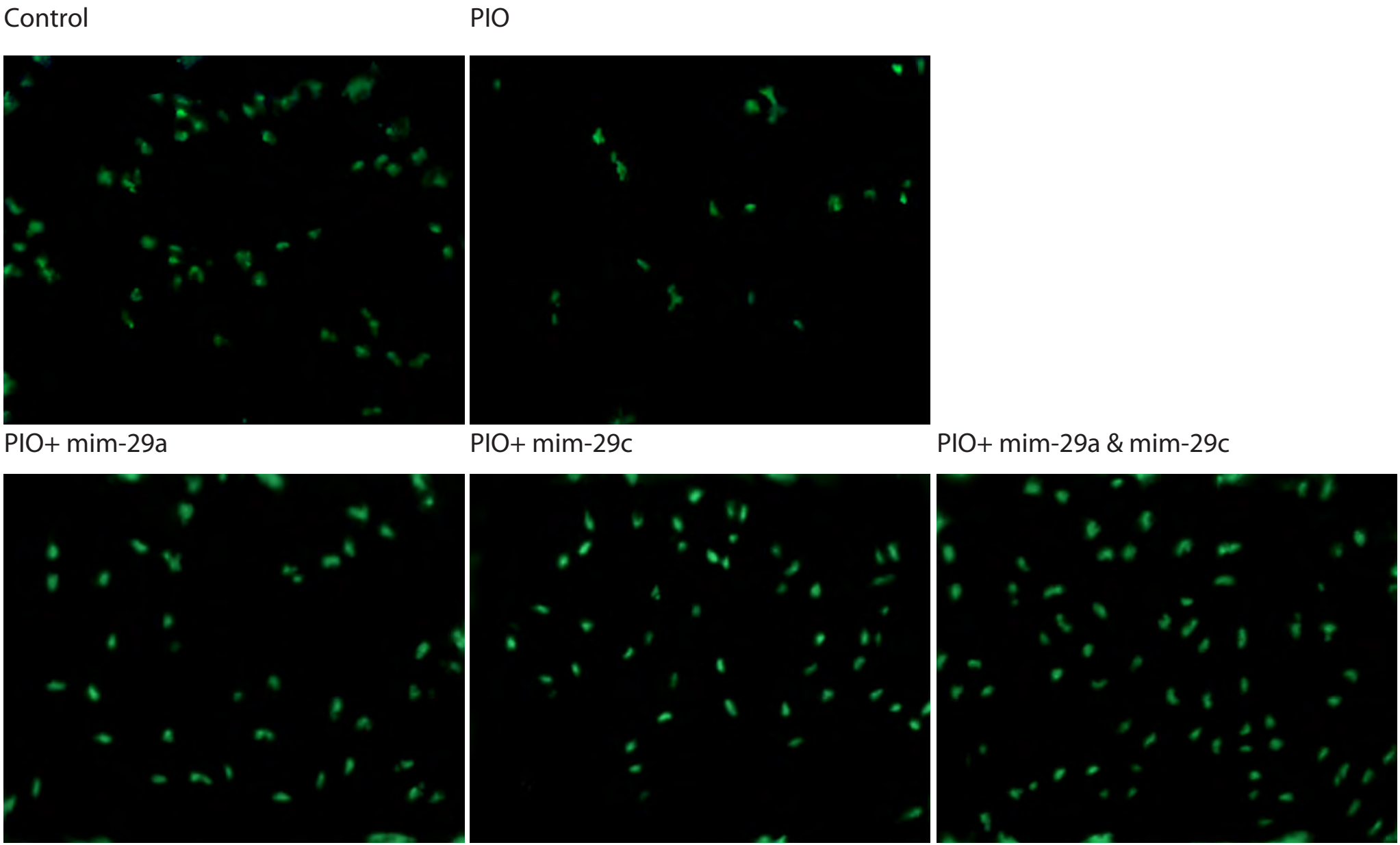
anta-29c



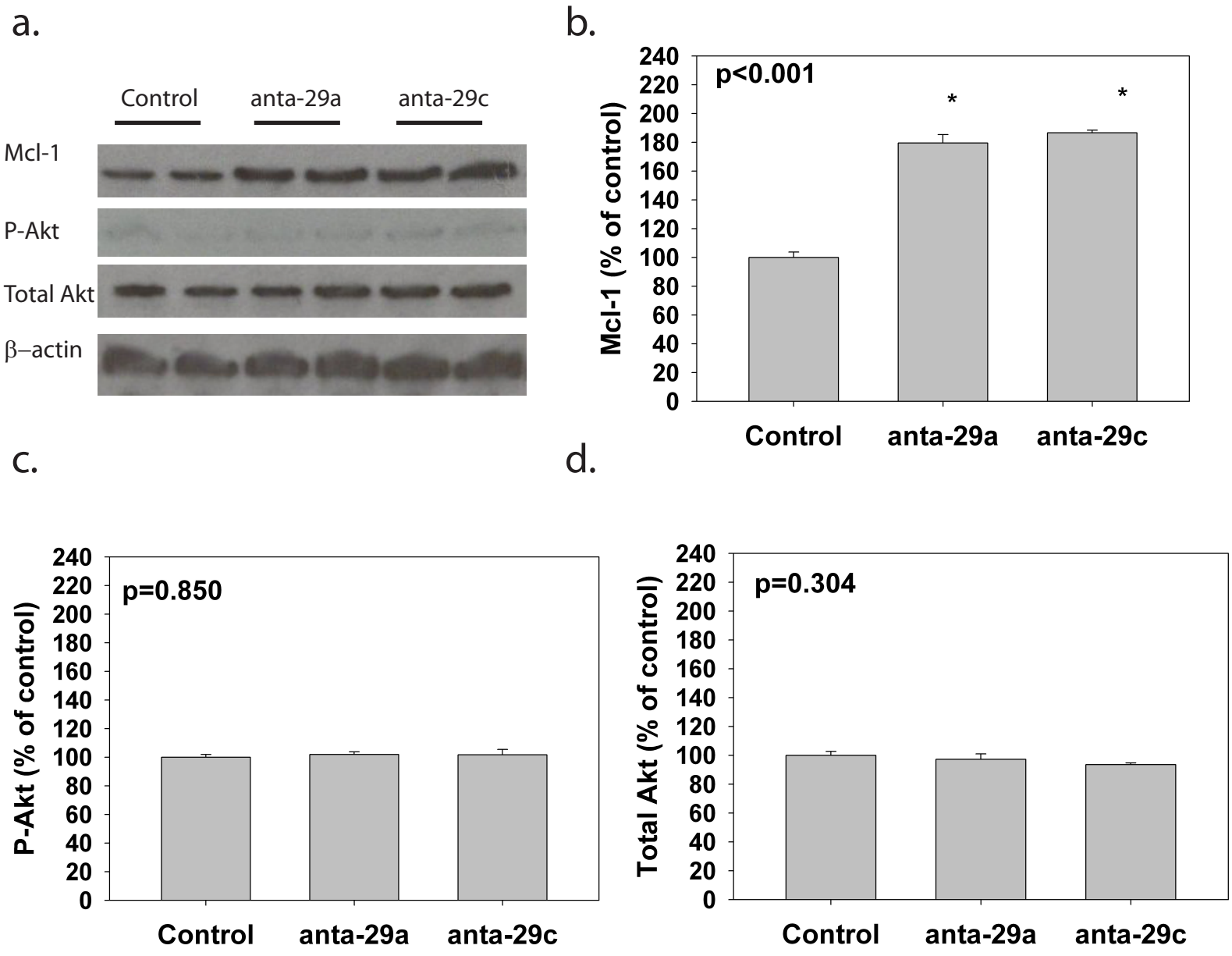
misanta-29



Supplement Figure 4: Hearts of mice treated with vehicle (control), anta-29a, anta-29c, or misanta and exposed to 30 min coronary artery ligation followed by 4h reperfusion. The ischemic AR is composed of the areas unstained by the blue dye and appears red (viable tissue stained by TTC) and white/yellow (infarcted zone).
The infarcted zone is unstained by the TTC and appears yellow/ white.

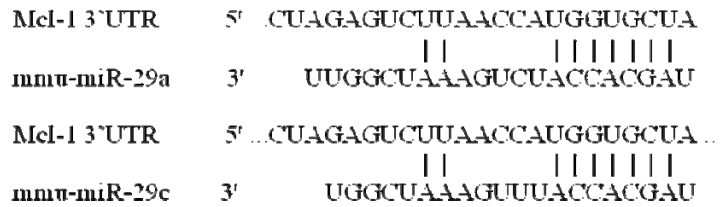


Supplement Figure 5: Representative samples of TUNEL staining of H9c2 cells exposed to SIR. Transfection with mim-29a or mim-29c, respectively, partially blocked the effect of PIO. Co-transfection with mim-29a and mim-29c completely blocked the anti-apoptotic effect of PIO.

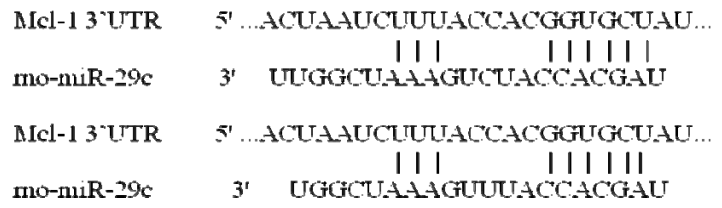


Supplement Figure 6: Effects of anta-29a and anta-29c on myocardial levels of total Akt, P-Akt and Mcl-1 in hearts of sham-operated mice not exposed to ischemia/ reperfusion. a. samples of immunoblots of Mcl-1, P-Akt, total Akt and β-actin; b. densitometric analysis of Mcl-1 levels; c. densitometric analysis of Ser-473 P-Akt levels; d. densitometric analysis of total Akt levels. * $p<0.05$ versus control.

Mice



Rat



Supplement Figure 7. The predictive binding sites on 3'-UTRs of mice or rats Mcl-1 interact specifically with miR-29a and -29c. (TargetScan 4.2)