

# Regulatory Roles for miR-126 in Inflammatory Response and Programmed Death of Cardiac Myocytes Driven by Acidosis During Simulated Ischemia

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## ABSTRACT

During extended myocardial ischemia combinations of hypoxia and acidosis drive cardiac cell loss by apoptosis and necrosis that contribute importantly to myocardial infarction (MI). Here we investigated roles for miR-126 in stress kinase activation, induction of inflammatory cytokines and apoptosis caused by exposure of cultured cardiac myocytes to hypoxia/acidosis or acidosis alone. Key apoptotic pathway intermediates and inflammation markers IL-6, IL-8, and tumor necrosis factor (TNF  $\alpha$ ) were quantified by western blot analyses, while miR-126 expression was tracked by quantitative polymerase chain reaction (qPCR). Cause and effect of miR-126 was evidenced by knockdown (KD) with a miR-126-selective antimiR. Obligatory roles for acidosis in promoting apoptosis confirmed our previous reports that hypoxia alone does not confer a lethal signal. Acidosis with or without hypoxia increased apoptosis that was paralleled by elevated miR-126, increased phosphorylation of p38-MAPK and JNK, enhanced expression of IL-6, IL-8, and TNF  $\alpha$ , and downregulation of Bcl-2. All effects were prevented by prior transfection of myocytes with antimiR-126. The results support significant roles for miR-126 in regulating cardiac myocyte survival pathways and cell death during exposure to simulated ischemia and acidosis.

## Introduction

Cardiac myocyte cell death by apoptosis accompanies heart disease of both ischemic and nonischemic origin [1-3]. It has been demonstrated in the myocardium from failing human hearts [4,5], in patients with arrhythmogenic right ventricular

dysplasia [6], and in association with myocardial infarction, both within the infarcted area itself and in the surrounding viable tissue [7]. In animal models, increased apoptosis accompanies both phases of ischemia and reperfusion [8]. Our group reported

that severe chronic hypoxia alone does not cause apoptosis of cardiac myocytes in culture. Rather, coincident acidosis and/or reoxygenation is required to trigger apoptosis [9-11]. Important roles of inflammatory mediators in cardiac cell death by ischemia with or without reperfusion are well established [12-14]. Proton pumps and pH regulation are known to play important roles in apoptosis signaling [15,16]. Hypoxic cardiac myocytes generate excess  $H^+$  through increased anaerobic glycolysis, net hydrolysis of ATP, and  $CO_2$  retention. Protons are extruded from the myoplasm to the interstitial space by the combined action of 3 major ion-specific membrane transporters, including the  $Na^+/H^+$  exchanger,  $Na^+/HCO_3^-$  cotransporter, and vacuolar proton ATPase [17,18]. Increased activity of the  $Na^+/H^+$  exchanger can cause  $Ca^{2+}$  overload because the elevated intracellular  $Na^+$  is subsequently exchanged for  $Ca^{2+}$  via the  $Na^+/Ca^{2+}$  exchanger. Inhibition of  $Na^+/H^+$  exchange is protective against ischemic injury, while inhibition of the vacuolar ATPase promotes apoptosis, in part by shifting the proton load toward the  $Na^+/H^+$  transporter and thus increasing  $Ca^{2+}$  uptake, and in part by reducing the myocyte capacity to control  $[pH]_i$ . Acidosis has been shown to correlate with apoptosis in a number of other systems [19-21].

MicroRNAs (miRs) are endogenous, single-stranded, non-coding RNAs that range in length from 18 to 24 nucleotides and regulate gene expression primarily by binding to the 3' untranslated region of target genes [22,23]. The miR-126 locus generates 2 mature miRNAs, miR-126-3p and miR-126-5p [24]. Previous work linked miR-126 with vascular integrity and angiogenesis and described its downregulation, and possible biomarker role in AMI [25-28]. MiR-126 is enriched in endothelial cells (EC) and EC progenitors, and has been assigned essential roles in cardiac developmental as well as in the recovery of the heart from injury by activating survival kinases ERK1/2 and Akt and increasing pro-angiogenic signaling [29-31]. Roles for miR-126 have also been described in the regulation of cardiac myocyte proliferation and inflammation, including Interleukins in non-endothelial  $H_9c_2$  cells [32]. Aberrant expression of miR-126 associated with vascular pathologies including but not limited to cancers, diabetes and ocular disease has revealed roles in a range of biological processes including apoptosis, autophagy, pathological angiogenesis, cell migration and proliferation [33,34]. Roles for miR-126 in the inflammatory responses of cardiac myocytes subjected to simulated ischemia have not been fully described. We previously reported that hypoxia/acidosis but not hypoxia alone presents a powerful apoptotic stimulus for cultured cardiac myocytes [10,11,35,36]. Therefore, to further define the molecular pathway of cell death in this model we quantified miR-126 levels, markers of inflammation, survival kinases and apoptosis pathway components in cardiac myocytes subjected to acidosis with or without hypoxia and in the

presence and absence of anti-miR-126. The results identify miR-126 as a regulator and potential biomarker of ischemic cardiac injury.

## Materials and Methods

### Reagents

Antibodies were obtained from the following vendors: IL-6 (sc-130326); IL-8 (sc-32817); TNF $\alpha$  (sc-130349); Bcl-2 (sc-7382); p38 (sc-33688); phosphorylated (p-) p38 (sc-166182); JNK (sc-7345); p-JNK (sc-293136) from Santa Cruz biotechnology, GAPDH from Cell Signaling Technology. Human pre-microRNA expression constructs Anti-miR-126 was from System Bioscience LLC.

### Cardiac Myocytes Culture and Exposure to Acidosis Condition

All procedures involving animals were performed in accordance with Southern Medical University guidelines for the care and use of animals. Cardiac myocytes were prepared and cultured from hearts of 1–3-day-old neonatal rat pups as previous described [10,11,35]. Briefly, after 3–5 days in culture contracting monolayers were placed in serum-free medium and exposed to treatments 72h later. Cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 0.1 mg/ml G-418 and 10% fetal bovine serum in a humidified atmosphere with 5%  $CO_2$  at 37°C. Our conditions for exposure to hypoxia (0.5%  $O_2$ /5%  $CO_2$ ) and measurement of pH are described in detail elsewhere [10,11,36].

### Cell Transfection

Myocytes were transfected with anti-miR-126 plasmid and control plasmid respectively by lipofectin reagent as described by the manufacturer (Thermo Fisher Scientific). MiR-126 expression was confirmed by qPCR assay at 24h after transfection.

### Apoptosis Assay

Apoptosis assays were performed using an ApopTag peroxidase in situ apoptosis detection kit (Cat# S7100, Millipore, Billerica, MA, USA), according to the manufacturer's recommendations. Briefly, fixed cells were quenched in PBS with 3% hydrogen peroxide and incubated in equilibration buffer, working strength TdT enzyme, anti-Digoxigenin conjugate, followed by color developer peroxidase substrate and counterstain with 0.5% (w/v) methyl green. Apoptotic cells were quantified under phase contrast microscopy.

### RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cell pellets using Trizol by standard procedures (Invitrogen); reverse transcription of miR-126 expression was performed using a MultiScribe RT kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR conditions were as follows: 3-min incubation at 95°C, followed by 40 cycles of 95°C x 15-sec, 60°C for 30-sec, and 72°C for 45-sec,

followed by 5-min incubation at 4°C. Primer sequences were as follows: miR-126 forward, 5'-TATAAGATCTGAGGATAGGTGGGTCC CGAGAACT-3'; reverse, 5'-ATATGAATTCTCTCAGGGCTATGCCGCT AAGTAC-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3', U6 reverse 5'-AACGCTTCACGAATTTGCGT-3'.

### Protein Assay

Proteins were quantified by BCA Protein Assay Kit (Pierce, Appleton, Wisconsin). and detected by standard Western blots stained with horseradish peroxidase labelled secondary antibodies.

### Statistical Analysis

All experiments were repeated at least three times. Data are presented as the mean and standard deviation. Statistical comparisons were performed using ANOVA followed by paired, one-tailed t test, using InStat software for Macintosh (GraphPad Software Inc, San Diego, CA USA). Statistical difference between groups was calculated by using Student's t-test. A p-value <0.05 was considered to be statistically significant.

## Results

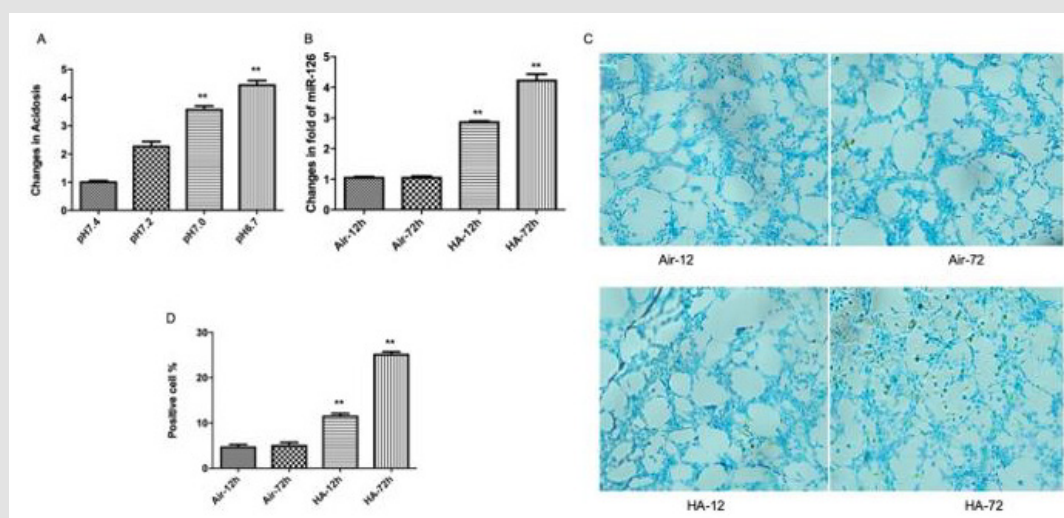
### Hypoxia/Acidosis Augments miR-126 Expression Coincident with Increased Apoptosis

Control and starting media pH were 7.4. Progression to pH 7.2 indicated mild acidosis after 12h hypoxia/acidosis that declined to pH 6.7 after 72h (Figure 1a). MiR-126 expression was significantly increased by hypoxia/acidosis at 12h, and maximally increased by >4-fold at 72h (Figure 1b). Apoptosis assays confirmed linearly increased apoptotic cells during 72h incubations. (Figure 1c,d). Inflammatory cytokines TNF $\alpha$ , IL-6, and IL-8 were quantified in lysates from cardiac myocytes subjected to identical conditions described above. As shown in (Figure 1e-1h), levels of TNF $\alpha$  peaked

after only 12h of hypoxic/acidosis incubation whereas the levels of IL-6, and IL-8 were maximally increased after 72h coincident with the maximal induction of miR-126 (all p<0.01).

### Inhibition of Hypoxia/Acidosis-Induced Apoptosis and Inflammation by KD of miR-126

To investigate a possible causal-effect of enhanced miR-126 expression on the regulation of inflammation markers and cardiac myocyte apoptosis by hypoxia/acidosis and aerobic acidosis, we repeated the experiments of (Figures 1 & 2) in the presence of anti-miR-126 to knockdown (KD) miR-126 expression in hypoxic/acidotic and aerobic-acidosis treated myocyte cultures (Figure 2a). Under conditions of miR-126 KD, apoptotic indices were decreased, consistent with a role for miR-126 in promoting apoptosis in these models (Figure 2b, 2c). In parallel, expression levels of IL-6, IL-8, and TNF $\alpha$  were each significantly decreased by miR-126 KD. Therefore, elevated miR-126 expression appears to be a precondition both for induction of inflammatory factors and increased apoptosis in response to hypoxia/acidosis and aerobic acidosis (Figure 2d-2g). To investigate the timeline and possibly differential regulation of inflammation and apoptosis by miR-126 in this model, we blocked miR-126 expression at different stages after induction of hypoxia/acidosis. As shown in (Figure 3a-3d), despite quantitative differences in the responses of individual pro-inflammatory cytokines to incubation time and media pH, in all cases miR-126 KD conferred a greater relative suppression at the earlier stages of acidosis compared with the more severe late-stage acidosis of pH 6.7, (Figure 3e-3h). These results provide evidence for a graded effect of acidosis on inflammatory cytokine induction as well as a requirement for elevated miR-126 in the cytokine activation pathways.



**Figure 1:** Hypoxia/acidosis augments miR-126 expression coincident with increased apoptosis and effect on inflammatory responses. Cultured cells were treated from acidosis-12 hours to 72 hours (A). RT-PCR showing the expression level of miR-126 (B); Cell apoptosis assay using ApopTag peroxidase in situ detection kit (C,D); Western blot revealing the expression levels of TNF- $\alpha$ , IL-6 and IL-8 proteins (E-H). D Data are expressed as mean+SEM. \*\*P<0.01 compared between HA-12 h and HA-72 h.

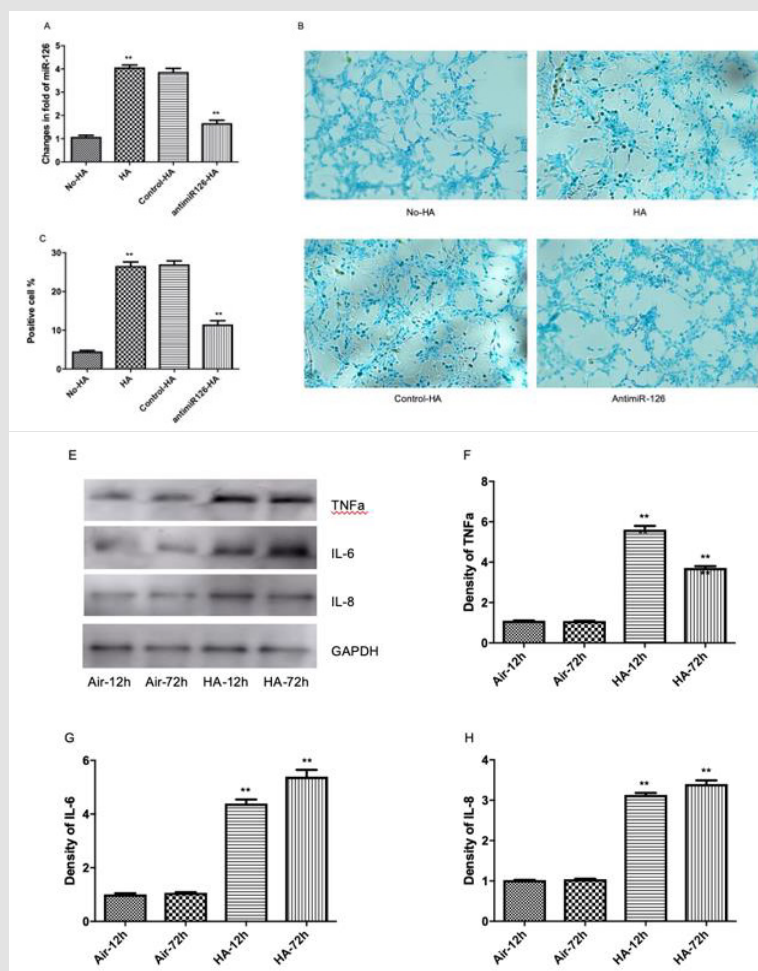
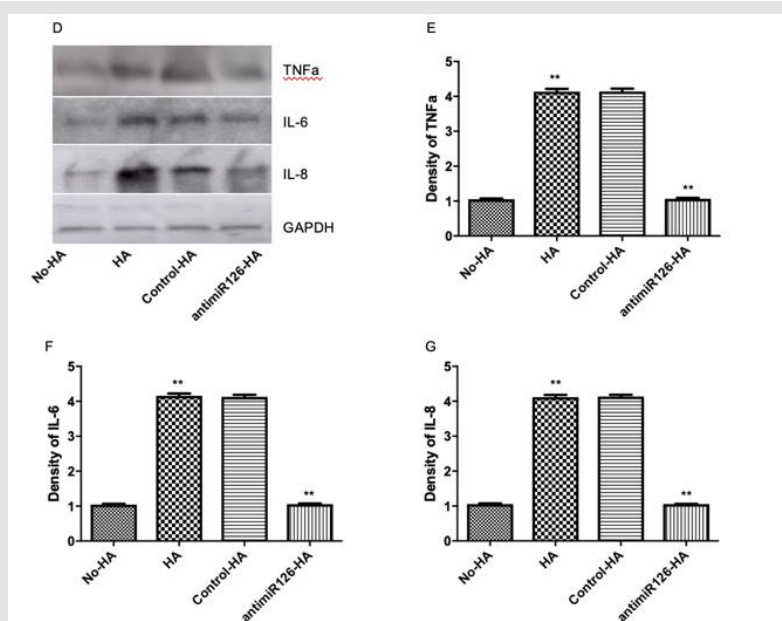


Figure 2A.



**Figure 2:** Inhibition of hypoxia/acidosis-induced apoptosis and inflammation by KD of miR-126. Cultured cells were treated from acidosis 72 hours with or without transfected with anti-miR-126 plasmids. RT-PCR showing the expression level of miR-126 (A); Cell apoptosis assay using ApopTag peroxidase in situ detection kit (B-C); Western blot revealing the protein expression levels (D-G). Data are expressed as mean+SEM. \*\*P<0.01 compared between No-HA and HA.



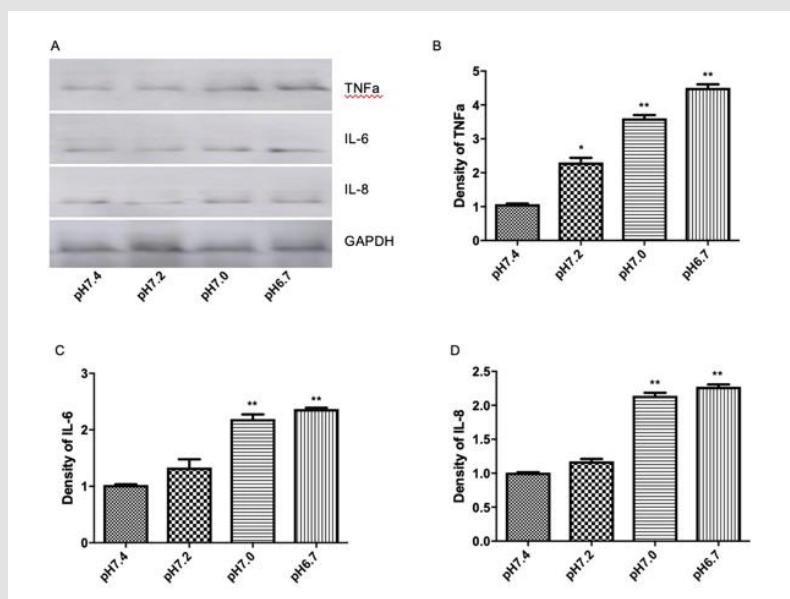
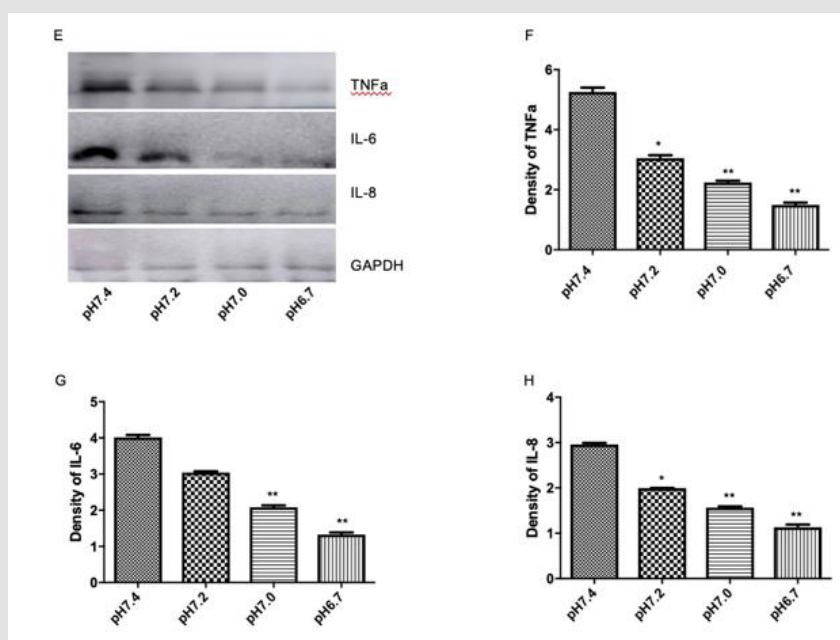


Figure 3A.

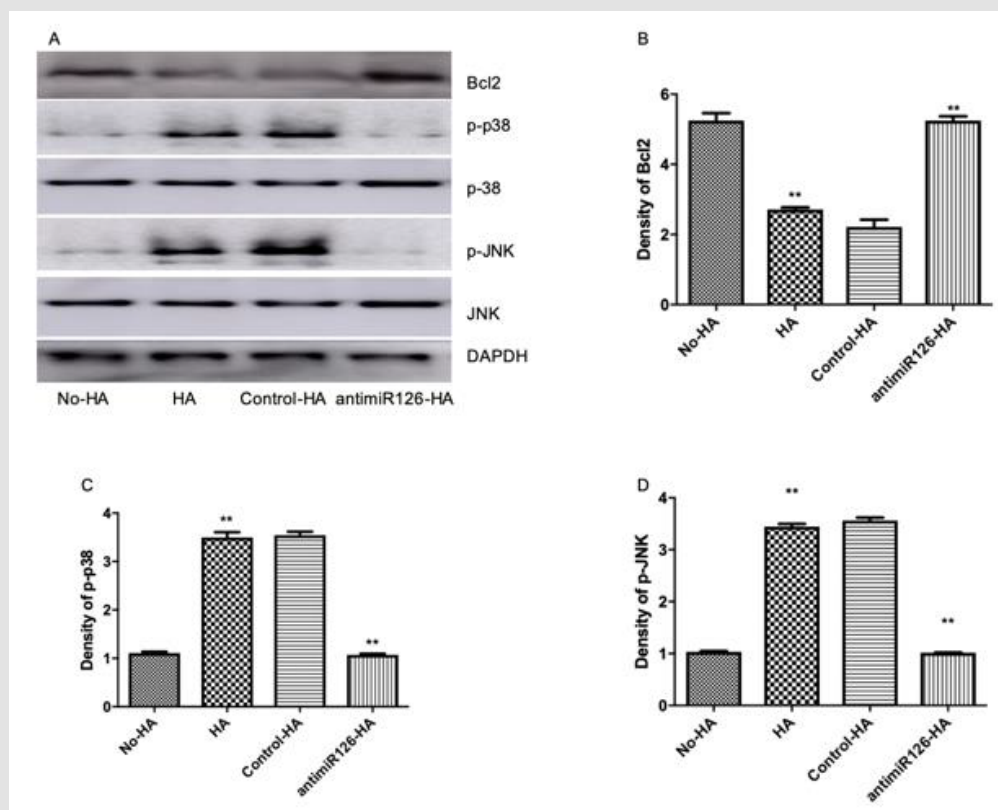


**Figure 3:** Roles for Bcl2 and MAPK/JNK in miR-126-mediated inflammation and apoptosis. Data are expressed as mean + SEM; Cell apoptosis assay using ApopTag peroxidase in situ detection kit (I-L);\*\*P<0.01, \*P<0.05 compared when pH=7.4, pH=7.2, pH=7.0 and pH=6.7.

### Roles for Bcl2 and MAPK/JNK in miR-126-Mediated Inflammation and Apoptosis

Previous work from others and ourselves established key roles for Bcl-2, mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) in apoptosis pathways of cardiac myocytes exposed to redox stress, acidosis and simulated ischemia with and without reperfusion [35-39]. Regulatory roles for miR-126 have also been described in cancer cells undergoing Bcl-2-mediated

apoptosis [40-42]. Therefore, we investigated a possible role for Bcl-2 in the miR-126/pH sensitive apoptotic pathway of cardiac myocytes described herein. As shown in (Figure 4a,4b), Bcl-2 levels decreased markedly in cardiac myocytes subjected to hypoxia/acidosis or aerobic acidosis, and the effects were blocked by KD of miR-126 in parallel with reduced apoptosis. The results suggest negative regulation of anti-apoptotic Bcl-2 by miR-126 in this model that is consistent with some previous results [41,43,44].



**Figure 4:** Regulation of Bcl2 expression by miR-126 mediated inactivation of MAPK/JNK signaling. Western blot revealing the protein expression levels (A-D); Data are expressed as mean + SEM. \*\*P<0.01 compared between No-HA and HA.

We quantified the expression levels of p38-MAPK and JNK in hypoxic/acidotic and aerobic-acidotic cardiac myocytes in the presence and absence of miR-126 KD. As shown in Figure 4a-4d, phosphorylation of p38 and JNK increased in myocytes that were exposed to both conditions of acidosis, effects that were again blocked by KD of miR-126. The results suggest that hypoxia/acidosis activates MAPK/JNK signaling in a manner that depends on miR-126 expression and correlates with downregulated Bcl-2 expression. Therefore, apoptosis driven by acidosis with or without concurrent hypoxia of cardiac myocytes requires increased expression of miR-126 and MAPK/JNK, and suppression of Bcl-2.

## Discussion

Apoptotic, necrotic and autophagic cell death pathways play central roles in tissue injury caused by ischemia-reperfusion during myocardial infarction (MI) and are important therapeutic targets [14,45,46]. During ischemia caused by coronary artery occlusion, affected myocardial tissues downstream of the occlusion become progressively hypoxic and acidotic [11,35,47]. In extreme cases of prolonged ischemia, lactic acidosis and associated proton generating pathways can cause intracellular pH to fall below 6.4 [47-49]. Here, to simulate the effects of in vivo graded ischemia we subjected cardiac myocytes to conditions previously established by our laboratory to mimic no-flow ischemia, progressive acidosis

and caspase-dependent apoptotic cell death [10,11]. Under these conditions we found that miR-126 was induced by >4-fold and the apoptotic index increased to >25% after 72h of hypoxia/acidosis and a medium pH of 6.7. The increased level of apoptosis was accompanied by sustained inductions of inflammatory cytokines, TNF $\alpha$ , IL-6 and IL-8, elevated phosphorylation of p38-MAPK and JNK and decreased expression of anti-apoptosis Bcl-2. KD of miR-126 by delivery of a selective antimir prior to hypoxia/acidosis, normalized expression of cytokines, eliminated the enhanced phosphorylation of p38-MAPK and JNK, significantly abrogated apoptotic cell death, and re-established control levels of Bcl-2.

Importantly we found that experimental manipulation of medium acidosis in parallel cardiac myocytes cultured aerobically in the absence of hypoxia reproduced almost identical results with respect to all measured parameters. This is consistent with our previous reports that hypoxia alone does not confer apoptosis without coincident acidosis or subsequent reoxygenation [9-11]. Indeed, we detected only minimal apoptosis even when cardiac myocytes were subjected to 6 days of pH neutral hypoxia [9]. Together the results support a pathway wherein miR-126 plays a central role in cardiac myocyte death caused by exposure to acidosis with or without concurrent hypoxia by regulating signaling pathways of inflammatory cytokine production, p38-MAKP/JNK

and Bcl-2. The findings are consistent with our previous reports that hypoxia with concurrent acidosis, but not hypoxia alone confers lethal cardiac myocyte injury through BH3-only pathways [9-11,35,36,50]. The results are also consistent with our previous report, and that of others that miR-126, while generally considered to be endothelial-specific is induced by hypoxia in non-endothelial cells including cardiac myocytes and regulates pro-survival kinases and inflammatory cytokines [32,51]. Acidosis appears to be the critical regulator of miR-126 expression in this model and the implications of the studies extend beyond those of ischemic cardiac injury to other conditions of acidosis including rapidly growing solid tumors. However, the main clinical relevance is cardiac ischemia, a pathophysiological state that can include extreme acidosis.

Previous work has described both positive and negative roles for miR-126 in the regulation of angiogenesis, apoptosis and cell proliferation in different cells and tissues including the heart, tumors and retinal cells primarily by targeting protein kinase signaling pathways including PTEN/PI3K/Akt, mTOR, ERK/MAPK, Notch [52-60]. These studies document the diverse targets of miR-126 and illustrate context-dependent actions with differential effects on cell survival and proliferation that may be determined in part by the metabolic state of the responding tissue [44,61,62]. As discussed in a recent review by Nammian et al. [44], miR-126 may confer opposite consequences depending on the biological context. Elevated miR-126 was shown to inhibit proliferation and activate apoptosis of human lung endothelial and HeLa cells respectively [41,43]. Context dependency was also reported in rats where upregulated miR-126 conferred increased levels of inflammatory factors and enhanced cardiomyocyte apoptosis, whereas simultaneous overexpression of E2F3 (a miR-126 target) with miR-126 had the opposite effect [55]. Our studies indicate increased expression of miR-126 in cardiac myocytes exposed to acidosis with or without hypoxia that promotes activation of p38-MAPK and JNK and increased production of pro-inflammatory cytokines TNF $\alpha$ , IL-6 and IL-8, suppression of Bcl-2 and consequent elevation of apoptosis. Whereas neonatal cardiac myocytes may not always fully reproduce the responses of adult myocytes to experimental manipulations, our previous work as well as that of others established that similar molecular pathways of injury by simulated ischemia with acidosis and/or reoxygenation is also seen in adult cardiac myocytes and intact heart as well as in astrocytes [63,64], but not cardiac fibroblasts [10], suggesting that it is a biologically relevant model.

## Conclusions

We describe central roles for miR-126 in the responses of cardiac myocytes to acidosis whether the intracellular pH autoregulates in response to metabolic changes caused by chronic hypoxia or by experimental manipulation of extracellular pH. miR-126 levels were increased similarly by both conditions

and paralleled by activations of inflammatory cytokines, stress kinases p38-MAPK and JNK, suppression of Bcl-2 and extensive apoptotic death of cardiac myocytes. Cause and effect of miR-126 was confirmed by demonstration that increased apoptosis and all signaling intermediates were abolished by KD of miR-126 with a selective antagomir.

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