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Synergistic induction of miR-126 by hypoxia and HDAC inhibitors in cardiac myocytes

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ABSTRACT

HDAC inhibitors are under clinical development for the treatment of hypertrophic cardiomyopathy and heart failure although the mechanisms of protection are incompletely understood. Micro-RNA 126, an endothelium-specific miR has been assigned essential developmental roles in the heart by activating survival kinases ERK1/2 and Akt and increasing pro-angiogenic signaling. Here we provide the first evidence that hypoxia and HDAC inhibitors selectively and synergistically stimulate expression of miR-126 in cardiac myocytes. MiR-126 expression was increased 1.7-fold ($p < 0.05$) after 1 h of hypoxic exposure and this was further enhanced to 3.0-fold ($p < 0.01$) by simultaneously blocking HDAC with the pan-HDAC inhibitor Tricostatin A (TSA). TSA alone did not increase miR-126. In parallel, hypoxia and TSA synergistically increased p-ERK and p-Akt without effecting VEGF-A level. Knockdown of miR-126 with si-RNA eliminated inductions of p-ERK and p-Akt by hypoxia, whereas miR-126 overexpression mimicked hypoxia and amplified p-ERK and p-Akt in parallel with miR-126. The results suggest that miR-126 is a hypoxia-inducible target of HAT/HDAC and its activation in cardiac myocytes may contribute to cardioprotection by activating cell survival and pro-angiogenic pathways selectively during ischemia.

1. Introduction

Chronic cardiac hypertrophy subsequent to myocardial infarction is considered to be maladaptive and a pharmaceutical target to prevent heart failure (reviewed in [1,2]). In animal models, pan-HDAC inhibitors including Trichostatin A (TSA) suppress cardiac hypertrophy that is induced pharmacologically, physically or genetically and confer significantly improved ventricular performance [3–5]. Because of this, pan-HDAC inhibitors are in development to treat heart failure. The mechanism of action is not clear, and the field has been especially confusing because early studies on HDACs predicted the opposite effect [6–11]. Class IIa HDACs for example block hypertrophy by inhibiting MEF2-mediated gene transcription, and targeted gene knockout of HDAC-5 or -9 leads to profound cardiac hypertrophy [6–8]. Therefore the suppressive effects of pan-HDAC inhibitors must be attributed to other targets distinct from class IIa and MEF2. In fact it turns out that class IIa HDACs are not sensitive to pan-HDAC inhibitors [12,13] but there are multiple other possible targets that may convey the response (reviewed in [2]). Pan-HDAC inhibitors induce the expression of the anti-hypertrophic transcription factor kruppel-like factor 4 [14,15], and block the expression of hypertrophy-related genes B-type natriuretic peptide (BNP) and the sodium–calcium exchanger (NCX1) by promoting increased acetylation of transcription factors YY1 and Nkx2.5, respectively [16,17]. Pan-HDAC inhibitors may also inhibit excessive autophagy following AMI, activate p38, and block cardiac fibrosis and inflammation [18,19]. Therefore the anti-hypertrophic actions of pan-HDAC inhibitors are most likely conferred through the net effect of multiple pathways and no single over riding mechanism has been identified. Interestingly, HDAC inhibitors also reduce cell death and prevent maladaptive ventricular remodeling in models of cardiac ischemia [20–22].

Micro-RNAs (miRs) are small non-coding 18–25 nucleotide RNAs that regulate the expression of more than 70% of mammalian genes (reviewed in [23,24] and play key roles in numerous physiological/developmental and pathological processes. Altered expression of miRs has been linked with each of the major pathways associated with hypertrophy/heart failure including thyroid hormone (miRs 208a, miR-208b, miR-499), IGF1/Akt (mir-1,

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miR-133), calcineurin/NFAT (miR-23a, miR-133, miR-199a), and TGF-β/Smad (208a, miR-208b, miR-499, miR-1, miR-29) signaling pathways. MiRs specifically associated with ischemia and reperfusion include miRNA-1, miRNA-21, miRNA-24, miRNA-29, miRNA-92a, miRNA-126, miRNA-133, miRNA-320, miRNA-199a, miRNA-208, and miRNA-195 (reviewed [25]). These miRs have been linked with diverse functions including programmed cell death, angiogenesis, ion channel expression, cell cycle, eNOS and survival kinases [25]. MiRNA-126 is a pro-angiogenic miR, strongly expressed in the heart endothelium that targets Sprouty-related protein-1 (Spred-1), PIK3R2, a regulatory subunit of PI3K, and RGS16, a member of the regulator of G protein signaling family [26–28]. Downregulation of these targets activates survival kinases ERK and Akt and enhances the actions of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [27,29,30]. MiR-126 is encoded within intron 7 of the Egfl7 gene and presumably co-regulated with the transcript of Egfl7. Egfl7 is an extracellular matrix protein that is also preferentially expressed in endothelial cells and their progenitors where both the host gene and miR-126 have been positively linked with angiogenesis and cell survival. Egfl7 gene transcription is induced by hypoxia in endothelial cells and by hypoxic preconditioning in the brain [31,32]. A parallel regulation of miR-126 would be predicted although has not been shown. Here we present novel observations that hypoxia and TSA induce miR-126 expression in cardiac myocytes and this is associated with enhanced activation of ERK and Akt, the known effectors of miR-126 signaling.

2. Materials and methods

2.1. Reagents

Antibodies were obtained from the following vendors: VEGF-A from Santa Cruz biotechnology (Santa Cruz, CA), histone-3, acetylated histone-3, ERK1/2 from Cell Signaling Technology (Boston, MA), Actin from Chemicon (Danvers, MA). Trichostatin A (TSA) and DMSO from Sigma (St. Louis, MO, USA). HAT/HDAC kits from BioVision (Mountain View, CA). Human pre-micro RNA expression construct Lenti-PremiR-126 and Anti-miR-126 were from System Bioscience LLC.

2.2. Cardiac myocytes culture and exposure to hypoxia

Cardiac myocytes were prepared from hearts of 1–3 days-old neonatal rat pups as previous described [33,34]. After 3–5 days in culture contracting monolayers were placed in serum-free medium and exposed to treatments 72 h later. Our conditions for exposure to hypoxia (0.5% O_2/5% CO_2) are described in detail elsewhere [33,34]. Cells were pretreated for 1 h with TSA (100 nmol/L) or DMSO with or without lentivirus infection as described previously [35].

2.3. RNA analysis

Total RNA was isolated from myocardial tissue using Trizol reagent, and analyzed by real-time PCR using TaqMan probes (Applied Biosystems, Foster City, CA, USA) as described previously [36]. All values were expressed relative to a mean expression value for the 22,000+ transcripts on each microarray.

2.4. Quantitative RT-PCR

A quantitative real-time reverse transcription-PCR assay from Ambion was used to quantify miR-126 expression [36]. 5S-rRNA was used as control. Each PCR reaction was carried out in triplicate in a 25 μl volume using SYBR Green Assay Master Mix (Applied Biosystems) for 3 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s in a Bio-Rad I Cycler (Bio-Rad Laboratories). miRNA levels were quantified based on the ratio of miRNA/5S-rRNA.

2.5. Western Blot and HDAC/HAT activity assays

Our procedures for Western blotting are described in detail elsewhere [33,34]. Total HAT activity and total HDAC activity were quantified using commercially available kits (BioVision #K332-100 for HAT activity and #K331-100 for HDAC activity activity (Mountain View, CA USA), as per the directions of the manufacturer.

2.6. Statistical analysis

Data are expressed as mean ± SEM. 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).

3. Results

3.1. HDAC and HAT activity under acute hypoxia

Neonatal cardiac myocytes were cultured as described in methods and exposed to hypoxia or continued normoxia for 1 h or 4 h. HDAC and HAT activities were measured as described in Section 2. Both activities were significantly increased after 1 h of hypoxia (Fig 1a and b; $p = 0.0047$ and 0.0067, respectively) and elevated HAT, but not HDAC was sustained at 4 h. Western analyses of cell lysates confirmed a significant enhancement of histone H3 acetylation in lysates from hypoxic cultures at both times (Fig 1c and d). As would be predicted, the pan-HDAC inhibitor, Tricostatin A (TSA) reversed the effects of hypoxia on HDAC activity and conferred a small but significant increase of HAT activity, presumably by blocking the reverse reaction (Fig 1e and f). These results indicate that acute hypoxia increases both HAT and HDAC activities of cardiac myocytes, effects that should neutralize each other and net acetylation of affected targets, however it is predicted that acetylation of these targets will be selectively increased in the presence of HDAC inhibitors.

3.2. Hypoxia and HDAC inhibition synergistically induce miR-126 expression

The host gene of miR-126, epidermal growth factor-like domain 7 (Egfl7) is a hypoxia-regulated gene with anti-inflammatory properties [31,32]. On the assumption that the intron-encoded miR-126 is under the same regulation as the host Egfl7 gene, we investigated the effects of hypoxia and HDAC inhibition on the expression levels of miR-126 in cardiac myocytes. As shown in Fig. 2a, miR-126 levels were significantly increased after 1 h exposure of cardiac myocytes to hypoxia and the effect was amplified by coincident treatment with TSA. To confirm the effects of hypoxia on miR-126 expression, cells were infected with lentiviral vectors expressing antagomiRs or premiRs respectively of miR-126. As shown in Fig. 2b, antagomiR treatment eliminated miR-126 induction by hypoxia in the presence or absence of TSA, whereas infection with the premiR augmented the effect and this was further enhanced by TSA as expected.

3.3. Synergistic actions of hypoxia and TSA on ERK and Akt

The downstream effectors of miR-126 include survival kinases ERK and Akt that in turn regulate the pro-angiogenic activities of

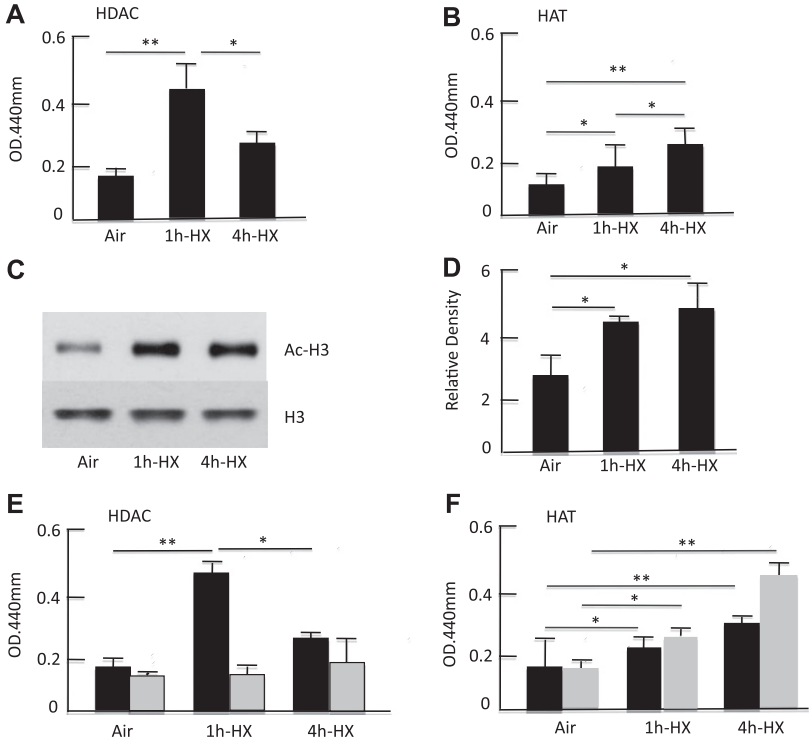

Fig. 1. Activation of HDAC and HAT by acute hypoxia. Cardiac myocytes were subjected to air or hypoxia for 1–4 h as described in Refs. [33] and [34]. HDAC (A) and HAT (B) activities were measured as described in Section 2. (C) Cell lysates were subjected to Western Blot analysis for acetylated histone H3 and the relative acetylation quantified by densitometry (D). (E, F) Isolated cardiac myocytes were treated with vehicle (DMSO; solid bars) or the pan-HDAC inhibitor (TSA, 100 nM; light shade)) 1 h before hypoxia, and DHAC (E) or HAT activity (F) determined as above. Data are means ± SEM. Significance by student's *t*-test ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$).

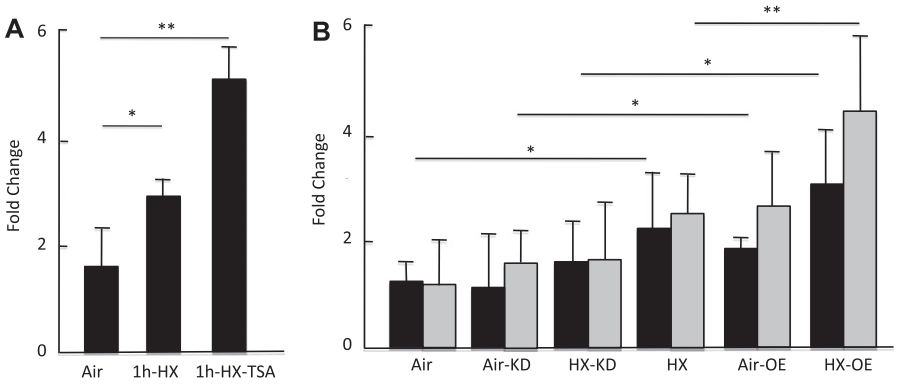

Fig. 2. Synergistic induction of miR-126 by hypoxia and HDAC inhibitor. (A) Cardiac myocytes were treated with vehicle (DMSO) or 100 nM TSA for 1 h before exposure to hypoxia. Endogenous miR-126 levels were measured by quantitative real time (rt)-PCR. (B) Cardiac myocytes were transfected with miR-126 antagomiR (KD) or premiR (OE) as described in Section 2, treated with vehicle or TSA and exposed to aerobic or hypoxic incubation as in (A). Solid bars: DMSO; light shade: TSA. Data are means ± SEM. Significance by student's *t*-test ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$).

vascular endothelial growth factor (VEGF), possibly by targeting downstream VEGF receptors (reviewed in [37]). Therefore we quantified the activities of these intermediates to determine whether they were activated by hypoxia and TSA in parallel with miR-126. As shown in Fig. 3a, after 1 h of exposure to hypoxia the levels of p-ERK-1/2 and p-Akt were each significantly increased and the increases were further augmented by coincident TSA treatment. We did not observe any significant change of VEGF expression following treatment with hypoxia or TSA over this time period (data not shown). A recent report suggested that TSA interferes negatively with HIF-1α-regulated genes including VEGF. During the time frame of our analyses 1–4 h, we did not see significant changes in VEGF expression. In light of the previous study it is possible that longer times of exposure to TSA may reduce VEGF expression as well as other hypoxia/HIF-1-regulated genes that exert positive and negative survival signals; genes such as the programmed death regulator Bnip3, glucose transporters and glycolytic enzyme genes may be important in this context. Our results conform that hypoxia and TSA mediate parallel inductions/activations of miR-126, ERK-1/2 and Akt during acute exposure.

3.4. Hypoxia/TSA-induction of ERK and Akt is regulated by miR-126

To confirm cause and effect between miR-126 and the hypoxia/TSA-mediated inductions of survival kinases we used lentiviral infection to deliver negative and positive miR-126 oligonucleotides prior to exposure of cardiac myocytes to hypoxia and TSA. As shown in Fig. 4a and b, knockdown (KD) of miR-126 eliminated the augmented activity of ERK and Akt seen by exposure of cells to hypoxia with or without TSA. Conversely, overexpression (OE)

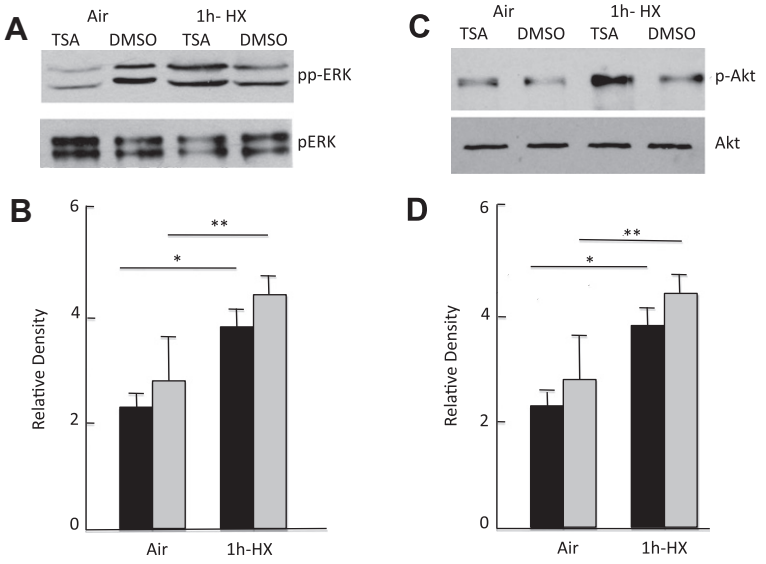

Fig. 3. Synergistic activation of ERK1/2 and Akt by hypoxia and TSA. (A, C) Cardiac myocytes were treated with vehicle (DMSO) or 100 nM TSA for 1 h before exposure to hypoxia as indicated. Lysate samples with equal protein were analyzed by western blot and quantified for ERK and phosphor-ERK (B) or Akt and phosphor-Akt (D). Solid bars: DMSO; light shade: TSA. Data are means ± SEM. Significance by student's *t*-test ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$).

of miR-126 augmented the hypoxia-mediated activities of both p-ERK-1/2 and Akt, again in the presence or absence of TSA. Fig. 4c shows there relative levels of endogenous miR-126 after the different treatments and illustrates the close correlation of miR-126 with p-ERK and p-Akt levels. We found that VEGF expression was not significantly affected by KD or OE of miR-126 under these conditions of hypoxia with or without TSA (data not shown). The results are consistent with a role for miR-126 in the up-regulation of ERK and Akt with possible downstream effects on survival signaling including that of VEGF.

4. Discussion

We have shown that miR-126 expression is activated in cardiac myocytes by hypoxia, an effect that is augmented by coincident inhibition of HDAC activity by the pan-HDAC inhibitor TSA. This is the first demonstration that miR-126, an endothelium-specific miR can be induced to express in non-endothelial cells, and the first demonstration that miR-126 is regulable by hypoxia and HDAC. Our cardiac myocyte cultures are 99% pure, the main contaminants are cardiac fibroblasts, and lysates from these cultures do not express detectable CD31 that eliminates the possibility that the effects are due to contaminating endothelial cells (data not shown). The elevated miR-126 levels seen in response to hypoxia in the presence or absence of TSA were reduced to basal levels by pre-treating cardiac myocytes with a lentiviral vector expressing miR-126 antagomiR but not by the premiR or scrambled sequence oligonucleotides, thus confirming the identity of the endogenous induced miR as miR-126. Our results show that HDAC and HAT activities were both increased in cardiac myocytes during 1–4 h of exposure to hypoxia. Inhibition of HDAC with TSA reduced

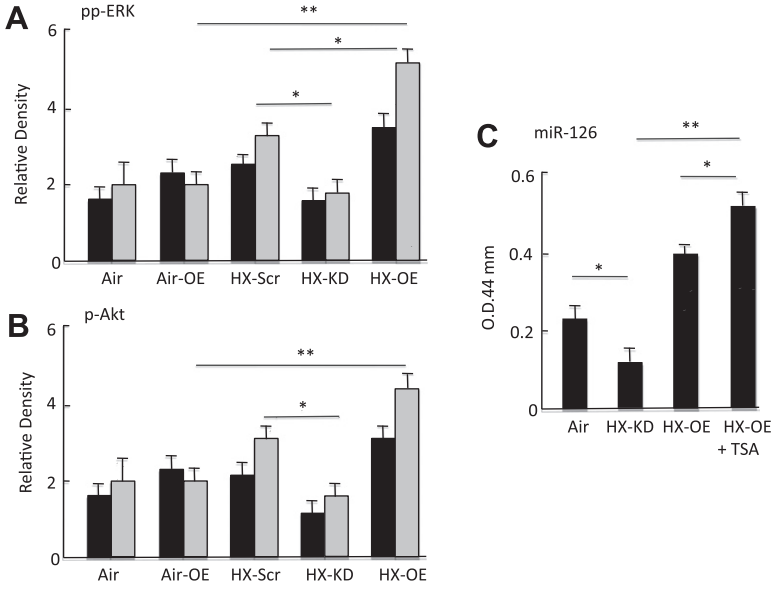

Fig. 4. miR-126 mediates p-ERK1/2 and p-Akt regulation by hypoxia and TSA. (A, B) Cardiac myocytes were transfected with miR-126 antagomiR (KD), premiR (OE) or scrambled (Scr) oligonucleotides as described in Section 2. Cultures were treated with vehicle or TSA and exposed to aerobic or hypoxic incubation as for Figs. 1–3. ERK, phosphor-ERK, Akt and phosphor-Akt were detected by Western Blots and quantified as described in Section 2. Solid bars: DMSO; light shade: TSA. (C) Endogenous miR-126 levels were determined quantitative rt-PCR. Data are means ± SEM. Significance by student's *t*-test ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$).

HDAC to baseline and caused almost 2-fold augmented HAT activity. Our results show for the first time that miR-126 when activated by hypoxia and TSA, exerts powerful regulation over ERK and Akt activities in cardiac myocytes. These effects were eliminated by KD of miR-126 and augmented by miR-126 overexpression by lentiviral delivery of the premiR (Fig 4). These results position miR-126 as a possible mediator of some of the cardioprotective effects of HDAC inhibitors associated with ischemic damage.

In addition to providing evidence that expression of miR-126 and perhaps its host Egfl7 can be induced in cardiac myocytes, our results provide another possible mechanism for the cardioprotective properties of pan-HDAC inhibitors. It is noteworthy that miR-126 and it downstream effectors ERK and Akt were induced by TSA only in hypoxic cardiac myocytes; TSA treatment had no apparent effect on normoxic cardiac myocytes. These results suggest that HDACs normally repress miR-126 expression under hypoxia. In agreement with this, previous work has shown that HDAC activity is activated by ischemia in the mouse heart and treatment of mice with TSA during or after ischemia-reperfusion conferred significant protected against infarction [21]. The same group reported that TSA also protected isolated cardiac myocytes form hypoxic injury in vitro [21]. In these studies, TSA was shown to block HIF-1α-related gene expression, and reduce VEGF expression, vascular permeability and apoptosis. The negative effects on HIF-1α-regulated genes are unexpected because HDAC inhibition is predicted to increase the expression of these genes by augmenting histone acetylation by p300-HAT. Indeed it has been shown that SIRT6, a class III HDAC negatively regulates HIF-1α-dependent gene transcription by antagonizing p300 [38,39]. Other studies have also shown that HDACs can activate HIF-1α-mediated transcription [40]. Therefore the actions of HDAC on HIF-1α are not clear and may be context-dependent. In our model we found that the level of VEGF did not change, possibly because of the short exposure time to TSA. However the activities of ERK and Akt increased rapidly, effects that reflect the rapid response time of micro-RNAs that do not rely on new protein synthesis. Such a rapid response time is consistent with roles for micro-RNAs in ischemic preconditioning and acute cardioprotection during AMI (reviewed in [25]). Our results suggest that miR-126 may play key role in TSA-mediated cardioprotection of the heart during ischemia-reperfusion as well as in cardiac myocytes subjected to hypoxia in vitro by activating survival kinases thereby blocking programmed death and tissue injury. In results not shown here we also found that TSA treatment decreased infarct size by 30% in a similar mouse model of acute myocardial infarction involving ischemia of 30 min and reperfusion for 24 h (Wei et al., unpublished). Other studies reported that TSA is also protective in a rat infarct model of permanent coronary artery occlusion [20], and TSA can mediated a preconditioning-like activity that was linked with hyperacetylation of MAPK-p38 [22].

It is possible that miR-126 has a mechanistic role in the protective actions of pan-HDAC inhibitors during late stage hypertrophy. Cardiac hypertrophy is initially adaptive with new muscle fibers that provide enhanced contractility. During late hypertrophy, the muscle mass outgrows the vasculature and nutrient delivery including oxygen is compromised so that the tissues become ischemic (reviewed in [41]). This state comprises decompensated hypertrophy and is often accompanied by angina especially during exercise because coronary reserve is compromised. TSA treatment of decompensated hearts that have regions of ischemia may activate miR-126 in endothelial cells as well as cardiac myocytes and provide enhanced angiogenesis in the former and enhanced survival of the latter. A similar response may be expected in the infarct border and remote zones that experience hypoxia during infarct expansion and negative remodeling following AMI [42].

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