Macrophage migration inhibitory factor plays a permissive role in the maintenance of cardiac contractile function under starvation through regulation of autophagy

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Aims	The cytokine macrophage migration inhibitory factor (MIF) protects the heart through AMPK activation. Autophagy, a conserved pathway for bulk degradation of intracellular proteins and organelles, helps preserve and recycle energy and nutrients for cells to survive under starvation. This study was designed to examine the role of MIF in cardiac homeostasis and autophagy regulation following an acute starvation challenge.
Methods and results	Wild-type (WT) and MIF knockout mice were starved for 48 h. Echocardiographic data revealed little effect of starvation on cardiac geometry, contractile and intracellular Ca^{2+} properties. MIF deficiency unmasked an increase in left ventricular end-systolic diameter, a drop in fractional shortening associated with cardiomyocyte contractile and intracellular Ca^{2+} anomalies following starvation. Interestingly, the unfavourable effect of MIF deficiency was associated with interruption of starvation-induced autophagy. Furthermore, restoration of autophagy using rapamycin partially protected against starvation-induced cardiomyocyte contractile defects. In our *in vitro* model of starvation, neonatal mouse cardiomyocytes from WT and $MIF^{-/-}$ mice and H9C2 cells were treated with serum free-glucose free DMEM for 2 h. MIF depletion dramatically attenuated starvation-induced autophagic vacuole formation in neonatal mouse cardiomyocytes and exacerbated starvation-induced cell death in H9C2 cells.
Conclusion	In summary, these results indicate that MIF plays a permissive role in the maintenance of cardiac contractile function under starvation by regulation of autophagy.
Keywords	Starvation • MIF • Autophagy • AMPK • Rapamycin

1. Introduction

Both clinical and experimental evidence have shown that starvation, the so-called protein-calorie malnutrition, is capable of decreasing heart mass and promoting myocardial atrophy.[1] More importantly, acute and prolonged starvation has been demonstrated to overtly compromise myocardial contractile function.[2,3] Although the underlying mechanisms behind starvation-induced unfavourable changes in cardiac geometry and function still remain elusive, ample studies have indicated a pivotal role of autophagy in the maintenance of cardiac homeostasis during the period of starvation.[1,4]

Autophagy is an evolutionarily conserved cellular pathway governing long-lived protein and cytoplasmic organelle degradation and recycling.[5–7] Under physiological conditions, autophagy performs a house-keeping action for cardiomyocytes through degrading dysfunctional proteins and organelles, refreshing amino acid pool, and providing new sources of intracellular energy.[8–10] Although autophagy is maintained at a relatively low level under physiological conditions, it is susceptible to perturbation from a number of cardiovascular diseases including ischemia−reperfusion,[11] pathological hypertrophy,[8] and heart failure.[12] While the vital protective role of basal autophagy in the heart is highly appreciated, the role of autophagy in the heart under pathological conditions still remains controversial. For example, recent studies have indicated that suppression of autophagy is beneficial for cardiac hypertrophy.[13] Along the same line, activated autophagy has been proven to be detrimental for pressure overload-induced heart

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failure.[12] On the other hand, autophagy induction through treatment with rapamycin, a specific inhibitor of mTOR, has been shown to reverse the established cardiac hypertrophy triggered by pressure overload.[14,15]

Among various stimuli, starvation represents perhaps the most well-known physiological inducer of autophagy.[4,6] Under starvation, activated autophagy helps to maintain intracellular ATP levels and protein synthesis, and to promote cell survival by degrading membrane lipids and intracellular proteins and organelles.[16] Autophagy is essential during starvation period.[1,17] Suppression of autophagy significantly shortened survival in Atg5-deficient neonatal mice due to shortage of amino acids and energy.[17] In neonatal mouse hearts, food deprivation triggered autophagy, which was overtly diminished by Atg5 knockout.[17] Autophagy is pivotal for not only cardiomyocyte survival but also maintenance of cardiac function.[1,2,4] In adult mice, myocardial autophagy was drastically elevated following a 48 h starvation period.[1,4] Disruption of cardiac autophagy using the lysosomal inhibitor bafilomycin A1 may prompt overt cardiac dysfunction in starved adult mice.[1]

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine secreted by various tissues, including the myocardium.[18–20] Recent evidence has depicted cardioprotective roles for MIF in various pathological conditions, including burn injury,[21] type 1 diabetes,[22] and ischemia–reperfusion injury.[18–20] Several scenarios have been postulated for MIF-induced biological responses including activation of the AMPK and JNK signalling cascades.[18–20] Although AMPK is known to play a pivotal role in the initiation of autophagy,[6] little is known with regards to the role of MIF in autophagy regulation. To this end, this study was designed to examine the role of MIF in the regulation of myocardial contractile function under starvation. Given the crucial role of starvation in autophagy initiation, a special focus was given with regards to the potential role of autophagy in MIF knockout-induced changes in murine cardiac homeostasis under starvation.

2. Methods

2.1 Experimental animals and starvation

All animal procedures carried out in this study were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY, USA) and was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were housed in a climate-controlled environment ($22.8 \pm 2.0°C$, $45-50\%$ humidity) with a 12/ 12 light/dark cycle with *ad libitum* access to tap water and diet until experimentation. The MIF$^{-/-}$ mice were generated as described.[23] Genotyping was done using PCR. For starvation studies, 3-month-old adult male MIF$^{-/-}$ mice and C57BL/6 wild-type (WT) littermates were deprived of food for 48 h.[2,4] These mice were given free access to water.

2.2 MIF reconstitution

For MIF reconstitution, MIF$^{-/-}$ mice were given recombinant mouse MIF [rmMIF, 2 i.p. injections of 10 μg of lipopolysaccharide (LPS)-free rmMIF at 24 h intervals].[24] Prior to food deprivation, MIF$^{-/-}$ mice were given the first injection of rmMIF (i.p., 10 μg). Twenty-four hours later, fasted MIF$^{-/-}$ mice were given a second injection of rmMIF (i.p., 10 μg).

2.3 Echocardiographic assessment

Cardiac geometry and function were evaluated in anaesthetized (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) mice using the two-dimensional guided M-mode echocardiography (Philips SONOS 5500) equipped with a 15-6 MHz linear transducer (Phillips Medical Systems, Andover, MD,

USA). The chests were shaved and mice were placed in a shallow left lateral position on a heating pad. Using the two-dimensional (2D) parasternal short-axis image obtained at a level close to papillary muscles as a guide, a 2D guided M-mode trace crossing the anterior and posterior wall of the left ventricular (LV) was obtained at a sweep speed of 50 mm/s. The echocardiographer was blind to the treatment of the mice. Caution was taken to avoid excessive pressure over the chest, which could induce bradycardia and deformation of the heart. LV anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using the method adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic (ESD) diameters using the equation $(EDD - ESD)/EDD*100$. Estimated echocardiographically derived LV mass was calculated as $[(LVEDD + \text{septal wall thickness} + \text{posterior wall thickness})^3 - LVEDD^3] \times 1.055$, where 1.055 (mg/mm^3) denotes the density of myocardium. Heart rates were averaged over 10 consecutive cycles.[25]

2.4 Isolation of murine cardiomyocytes

Hearts were rapidly removed from anaesthetized mice and mounted onto a temperature-controlled (37°C) Langendorff system. After perfusion with a modified Tyrode's solution (Ca^{2+} free) for 2 min, the heart was digested with a Ca^{2+}-free KHB buffer containing liberase blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN, USA) for 20 min. The digested heart was then removed from the cannula and left ventricle was cut into small pieces in the modified Tyrode's solution. A yield of at least $60-70\%$ viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Rapamycin was obtained from EMD Biosciences (EMD Biosciences, 553210). Rapamycin was dissolved in DMSO and used at 5 μM.[25] To evaluate the role of autophagy in cardiomyocyte mechanics, a cohort of cardiomyocytes isolated from the four different groups was incubated with rapamycin for 4 h prior to assessment of cardiomyocyte contractile function and intracellular Ca^{2+} handling properties.

2.5 TUNEL staining

Mice hearts were frozen immediately after euthanasia, and 7 μm thickness sections were obtained using a Leica, cryomicrotome (Model CM3050S, Leica Microsystems, Buffalo Grove, IL, USA). Sections were stained with *in situ* terminal dUTP nick end-labelling (TUNEL) staining kit (Roche Diagnostics Corporation, 11684795910) to detect apoptotic cells according to the manufacturer's instructions.[26] In addition, cardiomyocytes were stained with the Desmin antibody (Cell Signaling Technology, #4042). Nuclei were stained with DAPI. Cells were imaged with an inverted laser-scanning confocal microscope at $\times 100$ magnification (Zeiss 710, Thornwood, NY, USA). The percentage of TUNEL-positive nuclei was quantified using an ImageJ software.

2.6 MIF mRNA interference

For MIF silencing, H9C2 cells were cultured in the antibiotic-free DMEM medium supplemented with 10% FBS. After overnight incubation, cells were transfected with rat MIF-specific small interference RNA (siRNA) commercial agent (Thermo SCIENTIFIC Dharmacon RNAi Technologies, L-080124-00-0005). Control cells were treated with the non-targeting scrambled control (Sc) siRNA. To maximally deplete, H9C2 cells were treated with MIF siRNA agent for 72 h at 37°C prior to further experimentation.[2,27,28]

2.7 Neonatal mouse cardiomyocyte (NMCM) culture

Cardiomyocytes were isolated from 2-day-old mouse neonates.[29] Briefly, ventricles were dissected, and minced in PBS. Tissues were digested with Blendzyme 4 (45 μg/mL, Roche). After centrifugation, cells were resuspended in DMEM containing 20% foetal bovine serum (GIBCO) and were plated in a culture flask. After 2 h, non-attached cells were seeded on fibronectin-coated culture chamber slides for 24 h in DMEM, with 20%

FBS and 25 μM arabinosylcytosine. For *in vitro* starvation, neonatal mouse cardiomyocyte (NMCM) cells were rinsed with PBS three times and were treated with serum free-glucose free DMEM for 2 h as described.[2] To visualize the formation of autophagic vacuoles, adenovirus encoding GFP-LC3 was employed.[27] Rapamycin (100 nM, in DMSO)[28] was used to induce autophagy in NMCM cells. 3-Methyladenine (3-MA, Sigma, M9281-500MG) at a concentration of 2.5 mM[28] was employed to inhibit autophagy. The formation of LC3B puncta was examined with an inverted laser-scanning confocal microscope at ×100 magnification (Zeiss 710, Thornwood, NY, USA).

2.8 Cell viability assay

H9C2 cell viability was evaluated following different treatments by the thiazolyl blue tetrazolium bromide (MTT) assay as described.[26]

2.9 Data analysis

The D'Agostino-Pearson omnibus test was used to determine the normality of sample data. Data were expressed as Mean $\pm$ SEM. Statistical significance ($P < 0.05$) was estimated by one-way analysis of variation (ANOVA) followed by a Tukey's test for *post hoc* analysis or two-way repeated-measures of ANOVA when appropriate. All statistics was performed with GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA).

Additional methods are in the Supplemental Information.

3. Results

3.1 Starvation-induced cardiac dysfunction via a MIF-dependent pathway

Although MIF was initially reported to be a proinflammatory cytokine,[30] recent evidence has revealed an important role for MIF in cardiac homeostasis under both physiological and pathological conditions,[18–20] To evaluate if MIF participates in the regulation of cardiac function under

starvation, WT and MIF knockout mice were deprived of food for 48 h. Echocardiographic assessment revealed negligible changes in cardiac geometry and function following starvation. Interestingly, genetic MIF deficiency unmasked unfavourable changes in both cardiac geometry and function following starvation including decreased wall thickness and septal thickness, increased left ventricular end-systolic diameter, and LVEDD as well as reduced fractional shortening (*Figure 1A−E*). These findings suggest a crucial role of MIF in the maintenance of cardiac geometry and function under starvation.

3.2 MIF deficiency is associated with reduced contractile and intracellular Ca²⁺ responses in cardiomyocytes following starvation

Consistent with echocardiographic findings, starvation failed to affect cardiomyocyte contractile properties including resting cell length, maximal velocity of shortening/relengthening ($\pm$ dL/dt), peak shortening (PS), time-to-peak shortening (TPS), and time-to-90% relengthening (TR_{90}). However, MIF deficiency unmasked cardiomyocyte contractile dysfunction following starvation manifested as significantly decreased resting cell length, lessened $\pm$ dL/dt and PS as well as prolonged TPS and TR_{90}. Reconstitution of $MIF^{-/-}$ mice with rmMIF protected starvation-induced cardiomyocyte mechanical anomalies in $MIF^{-/-}$ mice, as evidenced by normalized resting cell length, $\pm$ dL/dt, PS, TPS, and TR_{90}. Cardiomyocyte contractile function in fed $MIF^{-/-}$ mice was unaffected by rmMIF (*Figure 2A−F*). These results indicated a detrimental effect of MIF deficiency on cardiomyocyte contractile homeostasis under starvation.

To further evaluate the possible mechanisms of action underlying starvation and MIF knockout-induced myocardial changes, intracellular
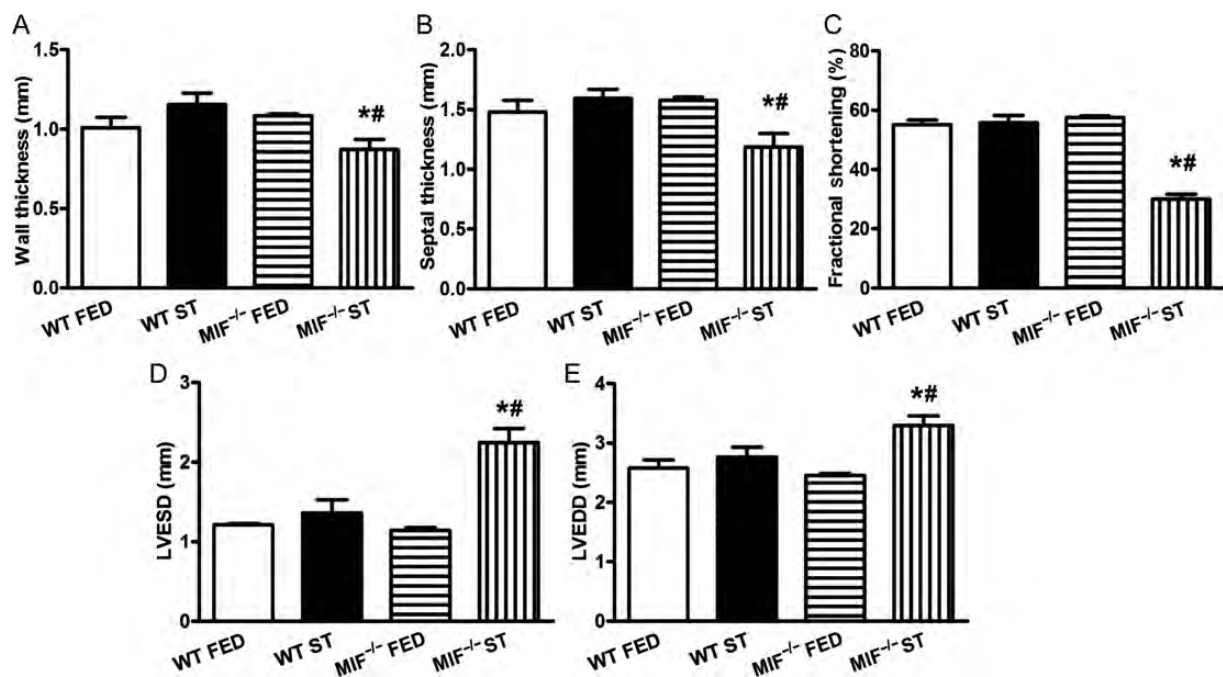

Figure 1 Echocardiographic parameters in WT and $MIF^{-/-}$ mice normally fed or starved (ST) for 48 h. (*A*) Wall thickness; (*B*) septal thickness; (*C*) peak shortening (PS, normalized to resting cell length); (*D*) LV end-systolic diameter (LVESD); and (*E*) LV end-diastolic diameter (LVEDD). Mean $\pm$ SEM, $n = 8-9$ mice per group, *$P < 0.05$ vs. FED group, #$P < 0.05$ vs. WT ST group.
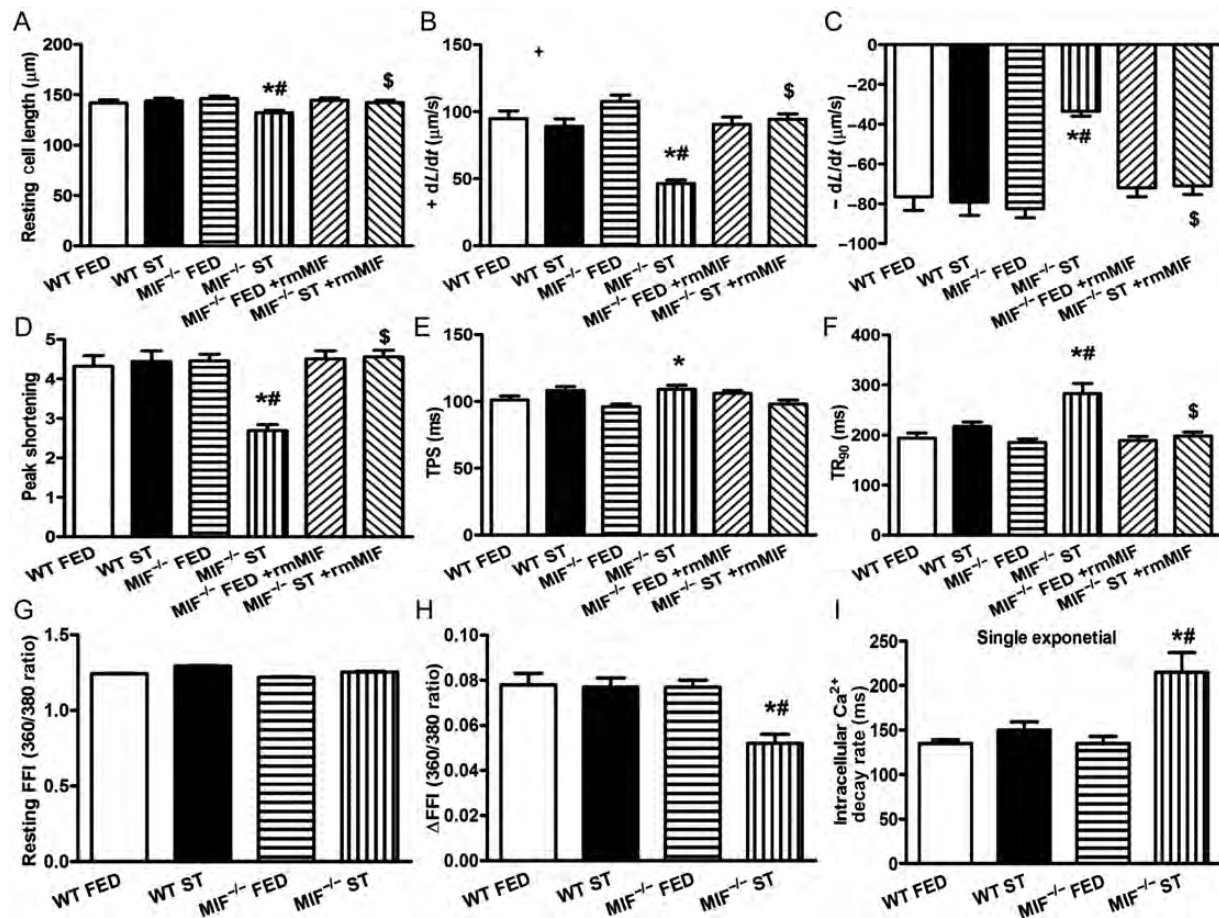
Figure 2 Contractile function and intracellular Ca^{2+} handling properties of cardiomyocytes isolated from WT and $MIF^{-/-}$ mice normally fed or starved (ST) for 48 h with or without rmMIF reconstitution. (*A*) Resting cell length; (*B*) maximal velocity of shortening ($+dL/dt$); (*C*) maximal velocity of relengthening ($-dL/dt$); (*D*) peak shortening (PS, normalized to resting cell length); (*E*) time-to-PS (TPS); and (*F*) time-to-90% relengthening (TR_{90}); (*G*) resting fura-2 fluorescence intensity (FFI); (*H*) electrically stimulated rise in FFI (ΔFFI); and (*I*) single exponential intracellular Ca^{2+} decay rate. Mean $\pm$ SEM, $n = 100–130$ cells, $*P < 0.05$ vs. FED group, $^{\#}P < 0.05$ vs. WT ST group, $^{\$}P < 0.05$ vs.MIF knockout ST group.

Ca^{2+} handling was evaluated using the fura-2 fluorescence technique. Our data showed that intracellular Ca^{2+} flux properties were unaltered by starvation in WT mice. In contrast, hearts from MIF knockout mice displayed a reduced rise in intracellular Ca^{2+} in response to electrical stimulus (ΔFFI) and prolonged intracellular Ca^{2+} clearance without affecting resting intracellular Ca^{2+} levels following starvation (*Figure 2G–I*). These data suggested that MIF deficiency is capable of unmasking intracellular Ca^{2+} mishandling in cardiomyocytes following starvation.
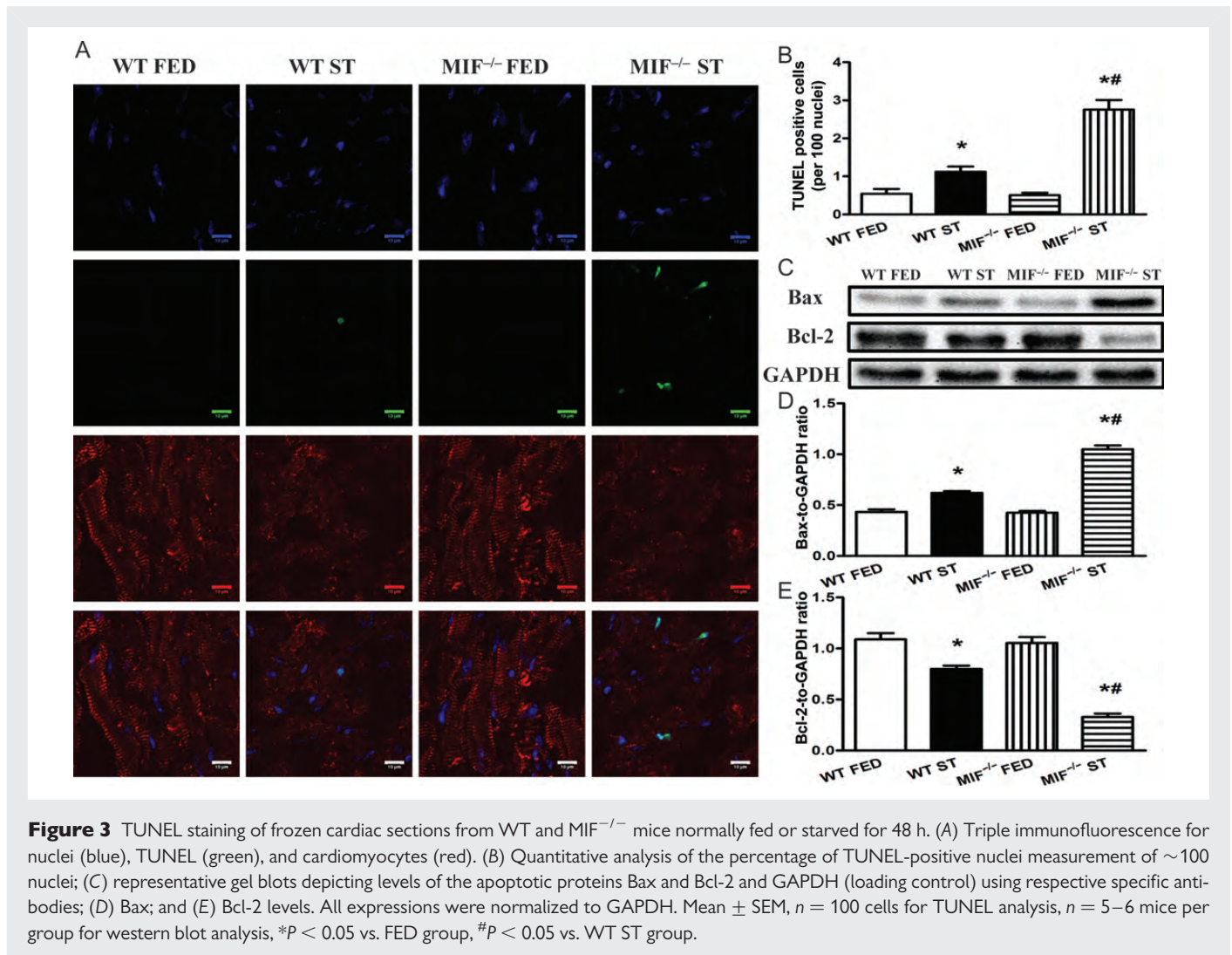
3.3 Expression of SERCA2a, Na^{+}-Ca^{2+} exchanger, and phospholamban

To explore the possible mechanisms of action underlying MIF- and starvation-induced changes in cardiomyocyte contractile function and intracellular Ca^{2+} homeostasis, western blot analysis was performed to evaluate the protein levels of the essential intracellular Ca^{2+} regulatory proteins SERCA2a, Na^{+}-Ca^{2+} exchanger (NCX), phospholamban (PLB), and phosphorylated PLB (p-PLB). Our data revealed that myocardial levels of SERCA2a, NCX, PLB, and p-PLB were not significantly

affected by starvation in WT mice. However, starvation dramatically down-regulated levels of SERCA2a, NCX, and p-PLB, but up-regulated PLB level in the heart from $MIF^{-/-}$ mice (Supplementary material online, *Figure S1A–E*).

3.4 MIF deficiency exacerbates starvation-induced myocardial cell death

Starvation is an important trigger for myocardial apoptosis.[1,26] To evaluate the impact of MIF deficiency on starvation-induced cell death, if any, TUNEL assay was performed. While the TUNEL-positive cells were extremely low in the fed WT and MIF knockout mice, starvation significantly promoted apoptosis in WT mouse hearts, and this effect was accentuated in the MIF knockout group (*Figure 3A* and *B*). Consistently, western blot analysis revealed that starvation markedly increased the proapoptotic protein Bax while down-regulated antiapoptotic protein Bcl-2. Although MIF knockout alone failed to affect the levels of Bax and Bcl-2, it exacerbated starvation-induced changes in Bax and Bcl-2 (*Figure 3C* and *D*). These observations suggest that MIF deficiency exacerbates starvation-induced cell death in murine hearts.

Figure 3 TUNEL staining of frozen cardiac sections from WT and MIF$^{-/-}$ mice normally fed or starved for 48 h. (A) Triple immunofluorescence for nuclei (blue), TUNEL (green), and cardiomyocytes (red). (B) Quantitative analysis of the percentage of TUNEL-positive nuclei measurement of ∼100 nuclei; (C) representative gel blots depicting levels of the apoptotic proteins Bax and Bcl-2 and GAPDH (loading control) using respective specific antibodies; (D) Bax; and (E) Bcl-2 levels. All expressions were normalized to GAPDH. Mean $\pm$ SEM, $n = 100$ cells for TUNEL analysis, $n = 5$–6 mice per group for western blot analysis, $*P < 0.05$ vs. FED group, $^{\#}P < 0.05$ vs. WT ST group.

3.5 MIF deficiency attenuates starvation-induced cardiac autophagy activation

Given the pivotal role of autophagy in the maintenance of cardiac geometry and function during starvation,[1,2] we examined the role of autophagy in starvation-induced cardiac anomalies in MIF knockout mice. As expected, starvation promoted autophagy manifested by the conversion of LC3BI to LC3BII (Figure 4A–D). LC3BII is an accepted marker for autophagosome formation,[31] although higher autophagosome accumulation may result from either increased autophagosome formation (autophagy initiation) or interrupted autophagosome degradation (autophagosome clearance). To discern the involvement of autophagy initiation and degradation, levels of p62, a well-defined autophagy adaptor preferentially degraded by autophagosomes,[32,33] were monitored. Our data revealed that p62 levels were significantly suppressed in starved mouse hearts, with a less pronounced decrease in MIF knockout mice (Figure 4A and E). Starvation-induced activation of cardiac autophagy was further confirmed by increased expression of Beclin1 and Atg5, known molecular markers for autophagy.[17] Although MIF deficiency alone did not affect cardiac autophagy, it significantly attenuated starvation-induced autophagy in the hearts as evidenced by lessened LC3BII, Beclin1, and Atg5 levels (Figure 4A–G).

AMPK is a key mediator of autophagy downstream of MIF,[6,18] and AMPK activation was examined in the WT and MIF knockout mice with or without the starvation challenge. Our data revealed that starvation activated AMPK in hearts from WT mice although such effect was significantly attenuated in the MIF knockout hearts (Figure 4A, H, I). As a downstream target for AMPK, mTOR activity (evaluated using phosphorylation of mTOR and its downstream target S6K) was drastically dampened by starvation in hearts from WT mice, the effect of which was partially restored in hearts from MIF knockout mice (Figure 4A, J–M). These results suggest that the unfavourable effects of MIF deficiency on cardiac homeostasis in response to starvation are associated with dysregulated AMPK-mTOR autophagy signalling in the heart.

3.6 Rapamycin treatment rescues cardiomyocytes contractile anomalies in starved MIF$^{-/-}$ mice

Although our data revealed that MIF deficiency attenuated starvation-induced autophagy, a permissive role of autophagy in starvation-induced cardiac dysfunction may not be established. To better corroborate the role of autophagy in starvation-induced responses, cardiomyocytes from fed and starved MIF$^{-/-}$ mice were incubated with rapamycin, an inducer of autophagy, for 4 h prior to assessing cardiac mechanical
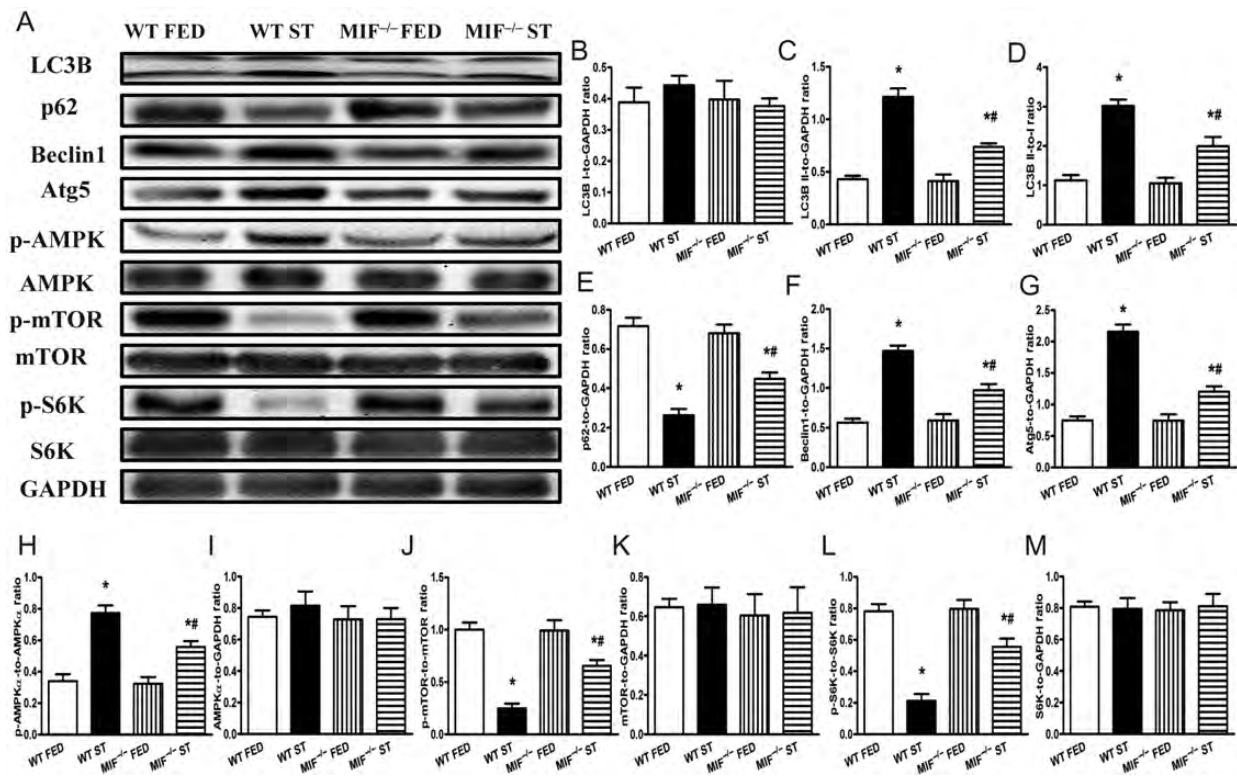
Figure 4 Expression of autophagy signalling molecules in hearts from WT and MIF$^{-/-}$ mice normally fed or starved for 48 h. (*A*) Representative gel blots depicting levels of autophagic markers including LC3BI/II, p62, Beclin1, Atg5, AMPKα phosphorylation at Thr^{172}, AMPKα, mTOR phosphorylation at Ser2448, mTOR, p70 S6kinase phosphorylation at Thr389, and p70 S6K. GAPDH was used as the loading control; (*B*) LC3B I expression; (*C*) LC3B II expression; (*D*) LC3B II-to-LC3B I ratio; (*E*) p62 expression; (*F*) Beclin1 expression; (*G*) Atg5 expression; (*H*) AMPKα phosphorylation at Thr^{172} (pAMPKα-to-AMPKα ratio); (*I*) pan AMPKα expression; (*J*) mTOR phosphorylation at Ser2448 (pmTOR-to-mTOR ratio); (*K*) total mTOR expression; (*L*) p70 S6kinase phosphorylation at Thr389 (pS6K-to-S6K ratio); and (*M*) total S6K. Mean ± SEM, $n = 5-6$ mice per group, $*P < 0.05$ vs. FED group, $^{\#}P < 0.05$ vs. WT ST group.

properties.[25] Although rapamycin failed to affect contractile function in cardiomyocytes from fed MIF$^{-/-}$ mice, it protected MIF$^{-/-}$ mice from starvation-induced cardiomyocyte mechanical anomalies. Rapamycin treatment mitigated starvation-induced decrease in ± d*L*/d*t* and PS, as well as prolonged TPS and TR$_{90}$. Nonetheless, rapamycin failed to restore decreased resting cell length (*Figure 5*). These data demonstrate that rapamycin rescues starvation-induced cardiac dysfunction in MIF$^{-/-}$ mice.

3.7 Rapamycin rescues MIF knockout-induced loss of autophagy under starvation

To further confirm the role of MIF in starvation-induced autophagy activation, neonatal cardiomyocytes (NCM) from WT and MIF knockout mice were transfected with an adenovirus expressing GFP-LC3 for 24 h prior to starvation induced by serum and glucose deprivation for 2 h treatment in the absence or presence of rapamycin or the autophagy inhibitor 3-MA. Evaluation of autophagosome formation using GFP-LC3 puncta revealed that starvation induced autophagy in NCM from WT mice (*Figure 6A*, *B*, *K*). Although rapamycin stimulated autophagy in NCM from WT mice under normal condition, it failed to further induce autophagy in these cells under starvation (*Figure 6A*, *C*, *D*, *K*). Blockade of autophagy using 3-MA dramatically suppressed autophagy

activation in NCM isolated from WT mice treated with starvation or rapamycin (*Figure 6D*, *E*, *K*). Consistently, MIF depletion significantly suppressed starvation-induced accumulation of GFP-LC3 puncta in NCM from MIF knockout mice (*Figure 6F*, *G*, *K*). Interestingly, rapamycin treatment induced a similar level of autophagosome formation in NCM isolated from WT or MIF knockout mice (*Figure 6C*, *H*, *K*). Moreover, rapamycin rescued autophagosome formation in starved NCM isolated from MIF knockout mice (*Figure 6G*, *I*, *K*). Not surprisingly, the beneficial role of rapamycin in autophagy induction in starved NCM from both WT and MIF knockout mice was abolished by the autophagy inhibitor 3-MA (*Figure 6E*, *J*, *K*). These data showed that MIF depletion suppressed starvation-induced autophagy in cardiomyocytes, which was rescued by rapamycin.

3.8 Rapamycin promotes cell survival in starved, MIF-deficient H9C2 cells

Starvation has been demonstrated to promote apoptosis in cardiomyocytes *in vitro*.[26] Our data indicate that MIF deficiency reduced cardiomyocyte survival during starvation by attenuating starvation-induced autophagy activation. To evaluate whether the detrimental effect of MIF depletion on cell survival is mediated by autophagy, H9C2 cells with or without MIF knockdown were treated with rapamycin. In accordance with our previous findings, starvation significantly decreased
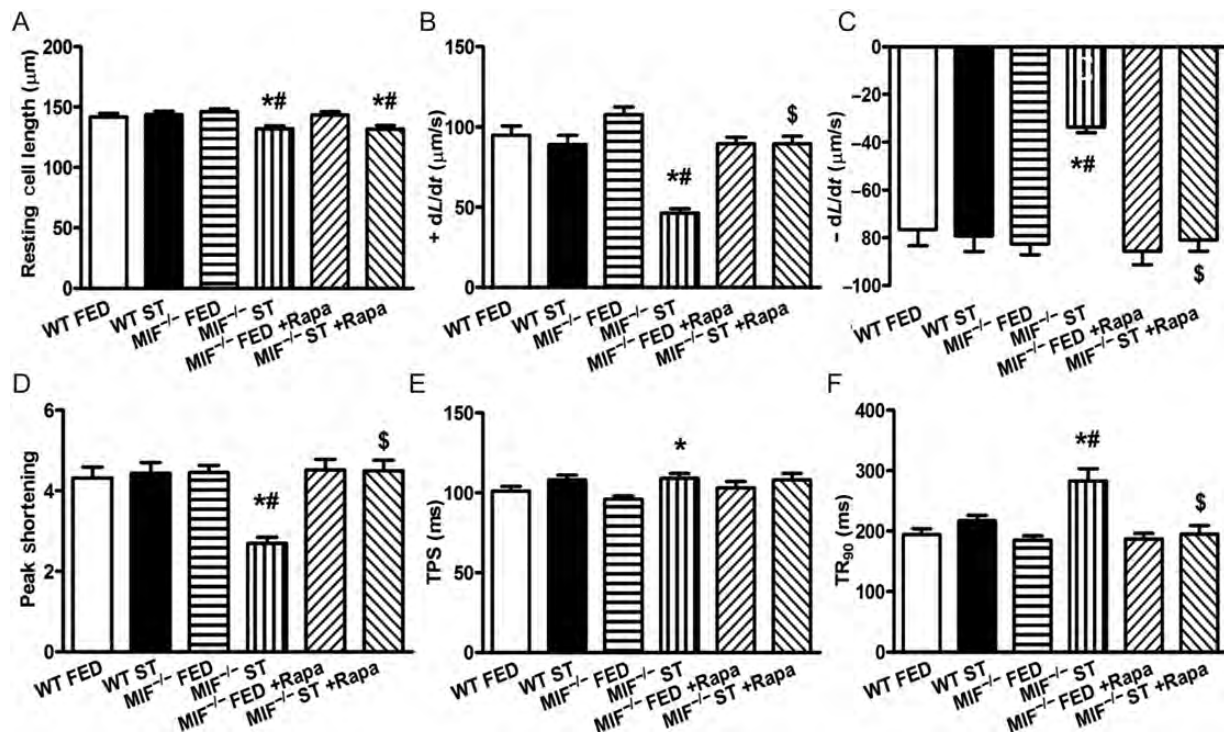
Figure 5 The effect of rapamycin (Rapa) on contractile function of cardiomyocytes isolated from WT and MIF$^{-/-}$ mice normally fed or starved for 48 h. (A) Resting cell length; (B) maximal velocity of shortening ($+dL/dt$); (C) maximal velocity of relengthening ($-dL/dt$); (D) peak shortening (PS, normalized to resting cell length); (E) time-to-PS (TPS); and (F) time-to-90% relengthening (TR$_{90}$). Mean $\pm$ SEM, $n = 100-130$ cells, $*P < 0.05$ vs. FED group, $^{\#}P < 0.05$ vs. WT ST group, $^{\$}P < 0.05$ vs. MIF knockout ST group.

H9C2 cell survival, which was further exacerbated by MIF depletion. Interestingly, rapamycin significantly improved survival of starved H9C2 cells under the setting of MIF depletion without eliciting any effect itself (Supplementary material online, *Figure S2*). These results support a possible role for autophagy in the MIF depletion-triggered cell death during starvation.

4. Discussion

The salient findings from this work indicate that MIF deficiency unmasks starvation-induced myocardial and cardiomyocyte contractile defects, intracellular Ca^{2+} homeostasis and myocardial apoptosis. In addition, reconstitution with recombinant MIF effectively rescued starvation-induced cardiomyocyte contractile dysfunction in MIF knockout mice. Furthermore, our study reveals that the detrimental effect of MIF knockout during starvation was associated with suppression of starvation-induced autophagy in the heart. More importantly, the role of autophagy regulation in MIF knockout-mediated cardiac dysfunction during starvation was further substantiated by *in vitro* autophagy induction where rapamycin alleviated mechanical derangement in cardiomyocytes from starved MIF$^{-/-}$ mice. Furthermore, rapamycin protected against MIF deficiency-induced cell death under starvation, further substantiating a role for autophagy in MIF depletion-induced cardiac anomalies. Taken together, these observations favour the notion that MIF is permissive for the maintenance of myocardial function during starvation by promoting autophagy activation in the heart.

In our study, cardiomyocytes from starved MIF knockout mice displayed unchanged resting intracellular Ca^{2+} levels, decreased intracellular Ca^{2+} release in response to electrical stimulus, and delayed intracellular Ca^{2+} clearance. Although MIF deficiency did not affect intracellular Ca^{2+} homeostasis, it uncovered a reduced release of intracellular Ca^{2+} during starvation as well as worsened intracellular Ca^{2+} clearance deficit. Furthermore, we found that MIF knockout unmasked down-regulated intracellular Ca^{2+} regulatory proteins SERCA2a, NCX, and phosphorylation of PLB (which removes the 'locking' effect of PLB on SERCA), as well as up-regulated PLB level following starvation. These findings suggest that MIF deficiency disrupts intracellular Ca^{2+} handling during starvation. Accumulating studies from our lab have shown that alterations in intracellular Ca^{2+} homeostasis contribute to the cardiac mechanical responses,[25] further suggesting a pivotal role of MIF in maintaining intracellular Ca^{2+} handling in cardiomyocytes during starvation.

As a cytokine involved in innate cytokine, MIF is an important mediator participating in antimicrobial and stress responses. Recent studies have also depicted a role of MIF in the development of various cardiovascular diseases including type 2 diabetes mellitus,[34,35] atherosclerosis,[24,36,37] and coronary heart disease.[38] Accumulating studies have demonstrated that MIF is up-regulated in atherosclerotic lesions,[36,37] vascular endothelium, and smooth muscles may serve as triggers for atherosclerosis.[39] Subsequent reports have shown that MIF deficiency significantly retards atherosclerosis associated with insulin resistance.[24] More interestingly, recent studies revealed that MIF is expressed in cardiomyocytes to help the maintenance of cardiac homeostasis in various pathological conditions, including ischaemia/reperfusion[18–20] and type 1 diabetes.[22] In the course of ischaemia/reperfusion or type 1 diabetes, MIF deficiency is known to exacerbate
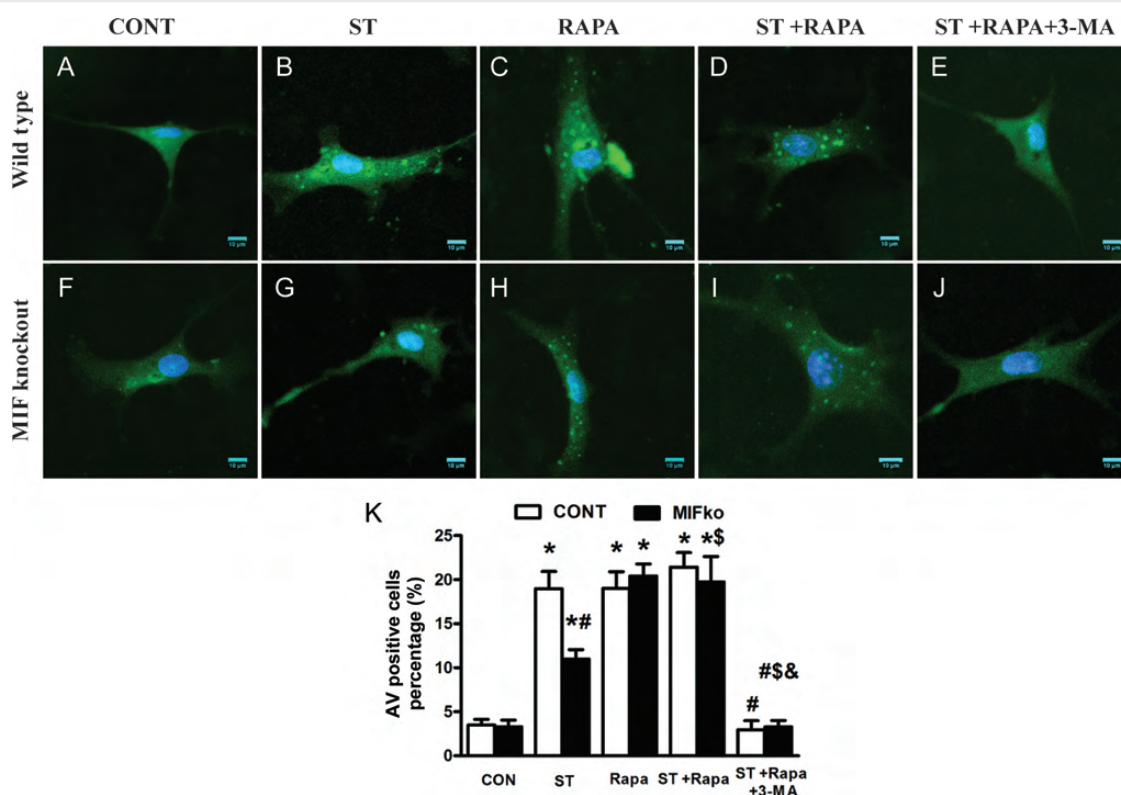
Figure 6 The effect of rapamycin (Rapa), 3-MA on starvation-induced autophagosome formation in neonatal cardiomyocyte (NCM) isolated from WT and MIF$^{-/-}$ mice. (A) NCM isolated from WT mice in normal culture medium with glucose and serum; (B) NCM isolated from WT mice in glucose and serum-free culture medium; (C) NCM isolated from WT mice in normal culture medium with rapamycin; (D) starved NCM isolated from WT mice treated with rapamycin; (E) starved NCM isolated from WT mice rapamycin and 3-MA; (F) NCM isolated from MIF$^{-/-}$ mice in normal culture medium; (G) NCM isolated from MIF$^{-/-}$ mice cultured in glucose and serum-free culture medium; (H) NCM isolated from MIF$^{-/-}$ mice treated with rapamycin in normal culture medium; (I) starved NCMs isolated from MIF$^{-/-}$ mice were treated with rapamycin; (J) NCMs isolated from MIF$^{-/-}$ mice were treated with rapamycin and 3-MA; and (K) percentage of cells with autophagosomes. Cells with 10 or more punctuate spots were scored as positive for autophagosomes. Mean ± SEM, $n = 300–400$ cells, $*P < 0.05$ vs. control (CONT) group; $^{\#}P < 0.05$ vs. starvation (ST) group; $^{\$}P < 0.05$ vs. MIFko +starvation (ST) group; $^{\&}P < 0.05$ vs. MIFko + starvation (ST) + rapamycin (Rapa) group.

cardiac dysfunction including cardiomyocyte mechanical and intracellular Ca^{2+} derangements.[18–20,22] The mechanism through which MIF exerts its cardioprotective effect is believed to be dependent upon binding and activation of its cardiac receptor CD74.[19,20] More importantly, the cardioprotective property of MIF seems to rely on promoting AMPK activity[18,20] and inhibiting JNK/MAPK.[19] Along the same line, results from our current study indicated that MIF deficiency unmasks cardiac contractile and intracellular Ca^{2+} handling defects following starvation. Our data further revealed an essential role of AMPK inhibition in MIF knockout-induced cardiac anomalies in response to starvation.

Our data suggest that the detrimental effect of MIF deficiency on cardiac homeostasis following starvation may involve autophagy derangement. Our *in vivo* observations indicate that starvation dramatically promotes cardiac autophagy, similar to previous findings.[1,4] However, MIF deficiency markedly inhibited starvation-induced autophagy induction in murine hearts. The important role of autophagy in the maintenance of cardiac homeostasis under starvation has been well established.[1,2] Bafilomycin A1, an inhibitor of autophagy at the step of lysosomal degradation of autophagosomes, dramatically suppressed cardiac function in mice starved for 3 days.[1] In mice with FoxO1 or Beclin1 knockout, cardiac function was significantly suppressed during

starvation due to inhibition of cardiac autophagy.[2] Consistent with these findings, our data suggested that the detrimental effect of MIF deficiency in cardiac function under starvation may result from suppressed autophagy induction in response to starvation.

Although it is not exactly clear at this point how MIF activated cardiac autophagy during starvation, AMPK is speculated to play an important role in MIF-induced autophagy. The energy sensor AMPK is a well-known activator of autophagy by inactivating mTORC1, an inhibitor of autophagy pathway.[10] In addition, AMPK promotes autophagy in cardiomyocytes by directly activating autophagy genes,[40,41] in line with the notion that starvation may activate AMPK in the heart.[17] Consistently, our data showed a remarkable rise in AMPKα phosphorylation in conjunction with suppressed mTORC1 activity (as evidenced by phosphorylation of mTOR and its downstream target S6K) following starvation, the effect of which was blunted by MIF knockout.

Perhaps the most significant findings from our study were that rescuing cardiomyocyte autophagy using rapamycin significantly improved cardiomyocyte contractile and intracellular Ca^{2+} properties from starved MIF knockout mice. As a specific inhibitor of mTOR, rapamycin is a potent inducer of autophagy.[25,28] Our *in vivo* results suggest that the detrimental effect of MIF deficiency in cardiac function following starvation was associated with suppression of starvation-induced cardiac

autophagy. The beneficial effect of rapamycin in our experimental setting is consistent with our recent observation that rapamycin ablates ageing-induced cardiac dysfunction through autophagy induction.[25] Induction of autophagy in response to starvation may be responsible for improved intracellular Ca^{2+} handling (SERCA2a, NCX, PLB, intracellular Ca^{2+} release, and clearance) and ultimately preserved myocardial function.[42] Furthermore, our *in vitro* data showed that starvation markedly increased the formation of autophagic vacuoles, a process that was partially inhibited by MIF depletion. This MIF depletion-induced inhibition of autophagy following starvation was rescued by rapamycin treatment. More importantly, MIF depletion exacerbated starvation-induced cell death in H9C2 cells, the effect of which was significantly improved by treatment with rapamycin. A schematic diagram is provided to layout starvation-induced myocardial contractile responses through MIF, AMPK-mediated signalling, and subsequently activation of autophagy (Supplementary material online, *Figure S3*).

In conclusion, the findings from our study provide the first evidence that MIF exerts a permissive role in the maintenance of cardiac contractile function following starvation. Our data revealed a pivotal role of autophagy inhibition in MIF knockout-induced cardiac anomalies following starvation. This is supported by the beneficial effects of rapamycin and recombinant MIF on cardiomyocyte contractile function and/or intracellular Ca^{2+} properties in starved MIF knockout mice. Although it is still premature to discern the precise mechanism through which MIF regulates myocardial autophagy following short-term starvation, our current study provides a better understanding for the role of MIF and autophagy in the maintenance of myocardial function following starvation. Further investigation is warranted to elucidate the therapeutic value of MIF and autophagy in the management of starvation-induced cardiac dysfunction.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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