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Short communication

# Miro1 as a novel regulator of hypertrophy in neonatal rat cardiomyocytes

Carolina Conejeros[a], Valentina Parra[b,c,d], Gina Sanchez[a], Zully Pedrozo[c,d,e,*], Ivonne Olmedo[a,c,*]

[a] Programa de Fisiopatología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Chile
[b] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile
[c] Red para el Estudio de Enfermedades Cardiopulmonares de alta letalidad (REECPAL), Chile
[d] Advanced Center for Chronic Diseases (ACCDiS), Universidad de Chile, Chile
[e] Programa de Fisiología y Biofísica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Chile

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ABSTRACT

Cardiac hypertrophy is an adaptive response to manage an excessive cardiac workload and maintain normal cardiac function. However, sustained hypertrophy leads to cardiomyopathy, cardiac failure, and death. Adrenergic receptors play a key role in regulating cardiac function under normal and pathological conditions. Mitochondria are responsible for 90% of ATP production in cardiomyocytes. Mitochondrial function is dynamically regulated by fusion and fission processes. Changes in mitochondrial dynamics and metabolism are central issues in cardiac hypertrophy. Stimulating cardiomyocytes with adrenergic agonists generates hypertrophy and increases mitochondrial fission, which in turn is associated with decreased ATP synthesis. Miro1 is a mitochondrial outer membrane protein involved in mitochondrial dynamics and transport in neurons. The objective of this work was to evaluate whether Miro1 regulates cardiomyocyte hypertrophy through changes in mitochondrial dynamics. In neonatal rat ventricular myocytes, we showed that phenylephrine induced cardiomyocyte hypertrophy and increased Miro1 mRNA and protein levels. Moreover, alpha-adrenergic stimulation provoked a mitochondrial fission pattern in the cardiomyocytes. Miro1 knockdown prevented both the cardiomyocyte hypertrophy and mitochondrial fission pattern. Our results suggest that Miro1 participates in phenylephrine-induced cardiomyocyte hypertrophy through mitochondrial fission.

## 1. Introduction

Heart hypertrophy is a beneficial adaptation in response to an excessive cardiac workload that serves to maintain normal cardiac function. However, despite being initially adaptive against hemodynamic overload, long-term cardiac hypertrophy often progresses to heart failure [1]. At cellular level, cardiomyocyte hypertrophy is associated with increased cell size, enhanced protein synthesis, changes in intracellular $Ca^{2+}$ levels, oxidative stress, and a switch towards a fetal gene expression profile. Together, these changes lead to drastic alterations in cardiac function and cardiac metabolism [2].

Mitochondria occupy approximately 30% of the cardiomyocyte mass and are responsible for 95% of ATP production in these cells. These organelles also regulate $Ca^{2+}$ homeostasis and, in some circumstances, can trigger cell death. Mitochondria are therefore crucial in coordinating energy transduction in cardiomyocytes [3,4]. Mitochondrial function is dynamically regulated by fusion and fission processes, which take on a key role under metabolic stress [5,6]. The literature suggests that mitochondria are indeed central to the pathophysiology of the hypertrophied heart [4]. Left ventricular hypertrophy is associated with mitochondrial structural deformities, including increased mitochondrial volume and disrupted mitochondrial network architecture [7]. Furthermore, in vitro studies have demonstrated that treating cultured neonatal rat ventricular myocytes with norepinephrine, a classic inducer of cardiac hypertrophy, promotes mitochondrial fragmentation and decreased mitochondrial function by promoting the migration of the fission protein Drp1 to the mitochondria [8].

A novel subgroup of Rho-GTPases, named Miro (for mitochondrial Rho), were described in 2003 [9]. Miro1 is the primary regulator of anterograde mitochondrial movement along microtubules in the axons and dendrites, through an indirect interaction with a motor protein called kinesin KIF5B [10]. Miro1 is highly expressed in the heart [9]; however, little is known regarding its role in mitochondrial dynamic and/or metabolism. Since mitochondrial dysfunction and dynamics seem to play a key role in cardiac hypertrophy, the main aim of our

* Corresponding authors at: Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago de Chile, 8380453, Chile.
*E-mail addresses:* zpedrozo@med.uchile.cl (Z. Pedrozo), ivonneolmedo@med.uchile.cl (I. Olmedo).

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work was to elucidate the participation of Miro1 in cardiomyocyte hypertrophy.

In this study, we evaluated mitochondrial morphology in the pathological model of hypertrophied cultured neonatal rat ventricular myocytes, triggered by phenylephrine (PE). After 48 h of treatment, PE induced cardiomyocyte hypertrophy, upregulation of Miro1 mRNA and protein content, associated with mitochondrial fission. Miro1 knockdown prevented the PE-induced hypertrophy and mitochondrial fission. These results suggest that Miro1 is involved in the PE-induced mitochondrial morphological changes in vitro.

## 2. Materials and methods

### 2.1. Bioethics

All studies conformed to the Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by our Institutional Ethics Review Committee.

### 2.2. Cell culture, transfection, and stimuli

Cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 1 to 3-day-old neonates as described previously [11]. To generate Miro1 knockdown, NRVMs were transfected with a siRNA specific to Miro1 (SMARTpool Mix, Dharmacon, 120 nmol/L) according to the manufacturer's protocol. To over-express Miro1, NRVMs were transduced with the Ad-r-RHOT1 adenovirus (Vector Biolab) at a multiplicity of infection of 10 plaque-forming units per cell. Empty vector (cytomegalovirus, CMV) was used as a control for transduction. Miro1 knockdown and over-expression were evaluated by Western blot and qRT-PCR. Experiments began 16 h after protein knockdown or adenoviral transduction. To induce hypertrophy, NRVMs were exposed to 50 μM of phenylephrine (Sigma-Aldrich Corp., St. Louis, MO, USA) for 48 h.

### 2.3. Quantitative real-time qRT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. mRNA levels were normalized to 18S rRNA. The primers used were as follows: Miro1 (for: CAAATGAAAGCGGCTGGATAAC, rev: AGCCTAG ATAGCCCAGATACTC), Miro2 (for: GTGGTGTGTGTGGTGTACGA, rev: TGCCATTCACGAGGGGAATC), β-MHC (for: AAGCAGCAGTTGGATGA GCG; rev: CCTCGATGCGTGCCTGAAGC), ANP (for: CTTCTTCCTCTTC CTGGCCT rev: TTCATCGGTCTGCTCGCTCA), BNP (for: TCCTTAATCT GTCGCCGCTG; rev: AGGCGCTGTCTTGAGACCTA), and 18S (for: AAC TCCCTCAAGATTGTCAGCAA, rev: CAGTCTTCTGAGTGGCAGTGATG) [16]. A ΔΔCt method was used to calculate relative transcript abundance.

### 2.4. Protein extraction and Western blot analysis

Proteins were extracted from NRVMs using cold T-PER buffer (Thermo Scientific, Rockford, IL, USA) in the presence of a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Western blot was performed as described previously [11] using a monoclonal primary antibody against Miro1 (1:4000, Abcam Cambridge, MA, USA, catalog number #ab83779) and GAPDH (1:5000, Sigma-Aldrich Corp., St. Louis, MO, USA, catalog number #G8795) as a loading control. Blots were quantified with Image Lab software.

### 2.5. Cardiomyocyte hypertrophy evaluation

Cell area was determined by epifluorescence microscopy analysis (Nikon, C2+ Confocal Microscope) of TritonX-100 permeabilized cells stained with rhodamine phalloidin (1:400; F-actin staining) as previously described [8]. At least 40 cells from randomly-selected fields were analyzed using ImageJ software (NIH, USA).

### 2.6. Mitochondrial dynamics analysis

After treatment, NRVMs were incubated for 30 min with MitoTracker Green FM (400 nM, Thermo Scientific, Rockford, IL, USA) in Krebs solution. Confocal image stacks were captured with a Carl Zeiss LSM 700 Laser Scanning Microscope, using ZEN 2011 image capture and analysis software and a Plan-Apochromat 63×/1.4 Oil DIC objective, as previously described [5]. Images were deconvolved with ImageJ, and then *Z*-stacks of thresholded images were volume-reconstituted. The number and individual volumes of each object (mitochondria) were quantified using the ImageJ-3D Object Counter plugin. Each experiment was performed at least four times, and 16–25 cells per condition were quantified. The criterion for mitochondria fission was decreased mitochondrial volume accompanied by increased number of mitochondria, whereas increased mitochondrial volume and decreased number of mitochondria were considered to represent mitochondrial fusion [5,8].

### 2.7. Statistical analysis

Data are shown as mean ± SEM of the indicated number (n) of independent experiments. Data were analyzed using a Student's unpaired *t*-test or two-way ANOVA followed by a Tukey test to compare means among ≥3 groups. Differences were considered significant at $p < .05$.

## 3. Results and discussion

### 3.1. Phenylephrine increases Miro1 content in neonatal rat ventricular myocytes

NRVMs were treated with phenylephrine (PE) 50 μM for 48 h, and then cell area was evaluated using rhodamine phalloidin staining. After treatment, the cell area increased significantly, by 50% as compared to control (Fig. 1A-C). Moreover, PE significantly increased mRNA levels for β-MHC, ANP, and BNP compared to control cells (Fig. 1D-F). These results are consistent with those described for PE-induced cardiomyocyte hypertrophy in vitro [12]. More importantly, NRVMs treated with PE for 48 h showed increased Miro1 mRNA levels and protein content (Fig. 1G and H, respectively). This is the first report of the effect of PE on Miro1 expression in an in vitro model of cardiomyocyte hypertrophy. Cardiomyocytes remained alive for the complete treatment period (data not shown). Studies performed in primary neuronal cultures and H9c2 cells have linked Miro1 with mitochondrial fusion at physiological $Ca^{2+}$ concentrations and, on the contrary, promoted mitochondrial fission at high concentrations of the ion, suggesting that the Miro1 protein may function as a calcium-sensitive “switch” for mitochondrial dynamics [13].

A recent study showed that the motor protein KIF5B induced in vitro changes in mitochondrial function and intracellular distribution of mitochondria in neonatal rat cardiomyocytes treated with PE. The authors showed that KIF5B was upregulated in cardiac hypertrophy generated by transverse aortic constriction and in hypertensive transgenic Ren2 rats. In vitro depletion of KIF5B prevented mitochondrial redistribution from perinuclear to cell periphery and the hypertrophic response observed after stimulation with PE [12]. Since KIF5B interacts with Miro1, it is possible that Miro1 also is involved in cardiomyocyte hypertrophy.

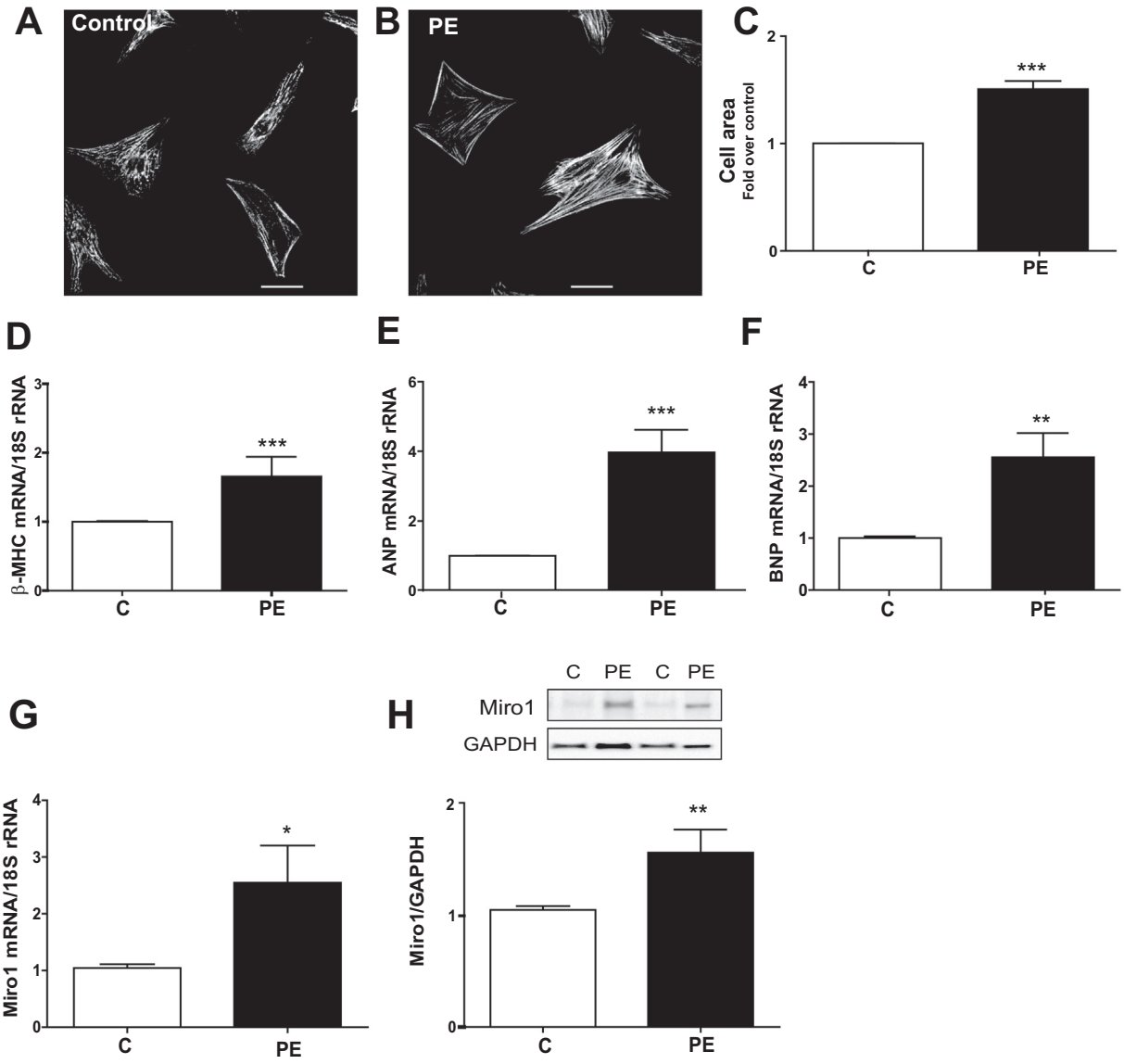


**Fig. 1.** Phenylephrine increases Miro1 content in neonatal rat ventricular cardiomyocytes. (A) Representative image of a control NRVM. (B) Representative image of a NRVM treated with phenylephrine 50 μM for 48 h. (C) Cell area of control and PE treated cardiomyocyte (mean ± SEM, n = 4, ***p < .0005 vs. control). (D) mRNA levels of β-MHC (beta-myosin heavy chain) (E) ANP (atrial natriuretic peptide) and (F) BNP (brain natriuretic peptide) in control and PE-treated groups (mean ± SEM, n = 4–7, ***p < .0001 vs. control). (G) Miro1 mRNA levels and (H) Miro1 protein content in cardiomyocytes treated with PE 50 μM for 48 h (mean ± SEM, n = 5–6, ***p < .0005 vs. control).

### *3.2. Miro1 is necessary to cause mitochondrial fission in phenylephrine-induced cardiomyocyte hypertrophy*

It is known that fission and fusion processes are able to regulate mitochondrial function under metabolic stress. To evaluate whether Miro1 was able to regulate mitochondrial fission or fusion, cultured NRVMs were treated with PE and stained with the mitochondrial probe MitoTracker Green. Miro1 protein content and mRNA levels were decreased after Miro1 siRNA transfection (Fig. 2A) with no change in Miro2 mRNA levels (Fig. 2B). The results shown in Fig. 2C-D demonstrate that PE induced a fragmented (fission) pattern in cardiomyocytes. Miro1 knockdown (siMiro1) prevented the appearance of the fission pattern observed with PE as compared to the control, suggesting that Miro1 is associated with changes in mitochondrial dynamics during cardiomyocyte hypertrophy. The data also show that siMiro1 prevented the increase in β-MHC mRNA levels and cellular area (Fig. 2E-F, respectively) observed after treatment with PE for 48 h. We also overexpressed Miro1 (Fig. 2G) to evaluate whether the increase in Miro1 protein levels induce cardiomyocyte hypertrophy. Our results show that Miro1 over-expression was not able to induce cardiomyocyte hypertrophy and did not interfere with PE-induced hypertrophy (Fig. 2H). Taken together, these data suggest that Miro1 may participate in cardiomyocyte hypertrophy induced by activation of the α1-adrenergic receptor but is not able to induce the pathological response intrinsically. Classically, Drp1 has been described as a protein that is important for regulating mitochondrial fission in the heart [14]. In NRVMs, α1 adrenergic receptor and further calcineurin activation leads to Drp1 activation, which in turn causes mitochondrial fission and further cardiomyocyte hypertrophy [8]. Moreover, it was recently reported that leptin, an adipokine related to obesity, also induces cardiomyocyte hypertrophy through enhanced mitochondrial fission via a calcineurin-mediated pathway [15]. Miro1 may be associated with Drp1 activation or exert its function through a different pathway; additional experiments will be necessary to study these hypotheses.

Finally, given that changes in mitochondrial dynamics are related to changes in mitochondria metabolism, future experiments would be

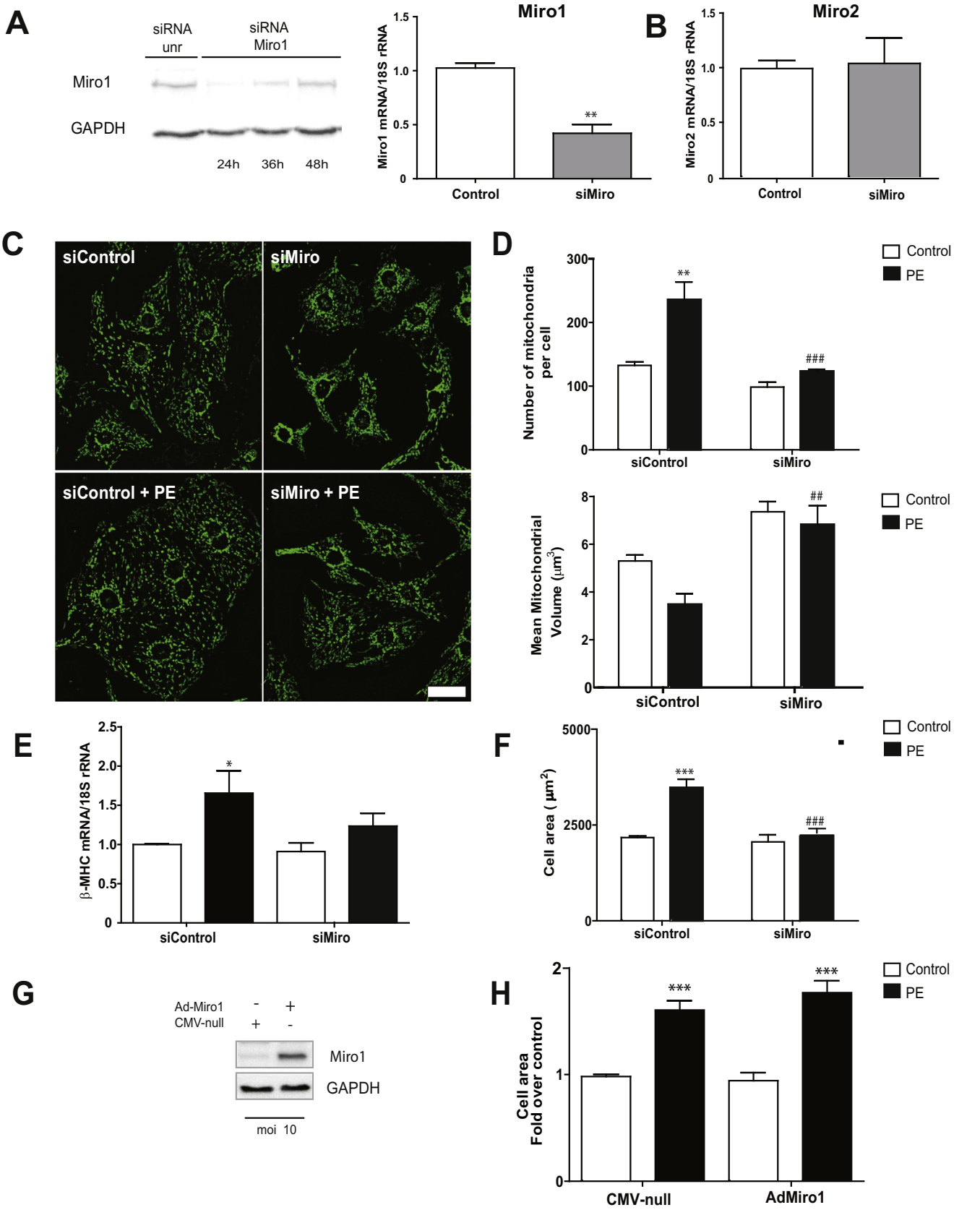


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**Fig. 2.** Miro1 is necessary to cause mitochondrial fission in phenylephrine-induced cardiomyocyte hypertrophy (A) Miro1 protein (left) and mRNA (right) content in presence of specific siRNA (siMiro) (mean ± SEM, n = 4, **p < .001 vs. control). (B) Miro2 mRNA levels in presence of siMiro1 (mean ± SEM, n = 4). (C) Representative confocal images of the mitochondrial network in cardiomyocyte treated with PE (50 μM, 48 h) scale bar: 20 μm. (D) Mitochondrial morphology of cardiomyocytes treated with PE (50 μM, 48 h). Images were subjected to 3D reconstruction to determine the number and volume of mitochondrial particles. (E) mRNA levels of β-MHC (mean ± SEM, n = 6–7, *p < .05 vs. control) and (F) Quantitative analysis of cell area. Number of mitochondria per cell, mitochondrial volume and cell area, were evaluated with ImageJ software (mean ± SEM, n = 4, **p < .01, ***p < .001 vs. control; ##p < .01, ### p < .001 vs. PE). (G) NRVMs over-expressing Miro1 (Ad-Miro1). (H) Cell area in NRVMs over-expressing Miro1 with or without PE (mean ± SEM, n = 3–4, ***p < .001 vs. control).

helpful to evaluate the contribution of Miro1 to metabolic phenotype changes in mitochondria in response to PE in cardiomyocytes.

## 4. Conclusion

These data suggest that Miro1 is necessary for cardiomyocyte hypertrophy and mitochondrial fission in PE-induced cardiomyocyte hypertrophy.

## Disclosures

None.

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