Myofibrillogenesis regulator 1 induces hypertrophy by promoting sarcomere organization in neonatal rat cardiomyocytes

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Human myofibrillogenesis regulator 1 (hMR-1) is a novel 17-kDa protein, is closely involved in cardiac hypertrophy. We studied the molecular mechanism that links MR-1 to hypertrophic response. Hypertrophic hallmarks such as cell size and [³H]-leucine incorporation were significantly increased when MR-1 was transfected into cardiomyocytes for 48 h. However, sarcomere organization was promoted when MR-1 was transfected for 8 h. The finding that cardiac hypertrophy was induced long after increase of sarcomere organization indicates that the promoted sarcomere organization may be one of the crucial factors causing hypertrophy. Furthermore, when MR-1 was transfected into cardiomyocytes, the nuclear localization of myomesin-1 was shifted to the cytoplasm. Transfection with small ubiquitin-like modifier-1 (SUMO-1) mimicked the effect of MR-1 inducing translocation of myomesin-1. However, transfection with SUMO-1 in MR-1-silenced cardiomyocytes failed to induce translocation and sarcomere organization, even though SUMO-1 expression was at the same level. Overexpression of MR-1 may induce cardiomyocyte hypertrophy via myomesin-1-mediated sarcomere organization.

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INTRODUCTION

Human myofibrillogenesis regulator 1 (hMR-1) is a novel characterized human functional gene cloned from a human skeletal muscle complementary DNA (cDNA) library. This 755-bp length gene is located on the human chromosome 2q35 and encodes a 142-amino-acid protein. MR-1 is highly expressed in the myocardium, skeletal muscle, kidney and liver.¹ Our previous studies showed that MR-1 was significantly upregulated in the hypertrophic myocardium of rats subjected to abdominal aorta stenosis and in angiotensin II-stimulated neonatal rat cardiomyocytes;² transfection with siRNA abolished angiotensin II-induced hypertrophy. These studies suggest that MR-1 is involved in cardiac hypertrophy.²,³ However, the specific mechanism involved has not been clearly demonstrated.

A highly ordered and precise organization process of contractile proteins is critical for myofibrillogenesis and differentiation of striated muscle cells.⁴ Sarcomere organization has a direct and profound influence on cardiac function.⁵ Therefore, understanding the mechanism of sarcomere organization in cardiac hypertrophy is essential. As crucial structural and regulatory proteins of sarcomere, myomesin-1 and myosin regulatory light chains (MRLCs) were found to interact with MR-1 directly in a previous yeast two-hybrid screen assay and in vitro GST pull-down assay.¹

M-line structure has essential roles in sarcomeric assembly and stabilization.⁷ Myomesin-1 is thought to be the most prominent structural component of the sarcomeric M-line. The 185-kDa myomesin-1 is encoded by MYOM-1 gene and is a member of the Ig-fibronectin superfamily.⁸ Myomesin-1 promotes the proper orientation and incorporation of its C-terminus into the developing M-line⁹ and directly binds to the thick filament component myosin, titin, obscurin and MURFs. Myomesin isoforms exhibit an almost spatio-temporal expression pattern,¹⁰ which suggests a regulatory role in precise targeting of numerous proteins and coordinated sarcomeric assembly. Myomesin-1 locates in the cytoplasm in adult cardiomyocytes, where it functions in sarcomeric structures, but is distributed in the nucleus in neonatal cardiomyocytes.¹¹ Modification of myomesin-1 by small ubiquitin-like modifier (SUMO) is critical for the translocation of myomesin-1 from the nucleus to the cytoplasm.¹¹

We investigated whether MR-1 induces cardiac hypertrophy by regulating myomesin-1-mediated sarcomere organization through SUMOylation of myomesin-1.

METHODS

Plasmid constructs

The open reading frame of hMR-1 gene deposited in GenBank database (accession number AF417001) was cloned from a cDNA library of the human...
heart by PCR with the primers 5′-GTGGGATCTCAAGATGGCCGGC-3′ and 5′-CCGCTCCTACGCTGTCACC-3′. hMR-1 full-length gene was linked by using pGEM-T Easy (Invitrogen, Carlsbad, CA, USA) and subcloned into pcDNA3.1/Myc-His(−)B (Invitrogen).

**Antibody preparation**

Rabbit anti-MR-1 polyclonal antibody was obtained from polypeptide-immunized New Zealand rabbits. Peptides were analyzed and selected by using TMHHM (http://www.cbs.dtu.dk/services/TMHHMM-2.0/) and DNAstar (DNASTAR Inc., Madison, WI, USA). The sequences we selected and synthesized were threelhckvkgakqarymphm and tgyesprassapqygvqy, respectively. This self-prepared antibody detects human original or rat original antigens, available for western blot or immunocytofluorescent assays.

**Cardiomyocyte culture and transfection**

All procedures were performed in accordance 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) and approved by the local animal care and use committee. Primary cultures of cardiac cardiomyocytes from 1-day-old Sprague–Dawley rats were prepared as described previously. Briefly, ventricular tissue was enzymatically dissociated and the resulting cell suspension was enriched. The dispersed cells were pre-plated for 1.5 h to minimize fibroblast contamination. Cells were plated at 2.5–3.0×10^5 cells ml^-1 onto poly-d-lysine-coated coverslips (Sigma, St Louis, MO, USA), well plates or dishes and cultured in Dulbecco’s modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% neonatal bovine serum (PAA, Linz, Austria), 3.7 g sodium bicarbonate and 100 μg ml^-1 ampicillin.

Cardiomyocytes were randomly divided into the following groups for treatment: (1) untransfected normal control (control), (2) overexpression by transient transfection with pcDNA3.1-hMR1 (MR-1), and (3) vector control transfection. The sequence of the selected target against rat MR-1 was 5′-CGAGACGUACAAGGCUUCCCCAGAA-3′. Transient transfection with plasmid pcDNA3.1-hMR1, pcDNA3.1-SUMO-1, pcDNA3.1 and the interfering siRNA was performed 24 h after plating using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. For each transfection sample in 24-well/60-mm dish format, 1.5/15 μg plasmid or 20 pmol/200 pmol stealth siRNA was used. The time course of the experiments is shown in Table 1.

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**[3H]-Leucine incorporation**

Total protein synthesis rate in cardiomyocytes was evaluated by incorporation of [3H]-Leucine (Amersham, Cambridge, England). Cultured cardiomyocytes were plated in 24-well plates at 2×10^5 cells cm^-2. After transfection for 0, 4, 12, and 36 h, cardiomyocytes were incubated with [3H]-Leucine (10 μCi per well) for 8, 12, 12 and 12 h. [3H]-Leucine incorporation was determined as described. Briefly, cells were washed by pre-cooling 0.01 mol^-1 phosphate buffer saline three times, and formic acid was added for 30 min at room temperature. The cell lysis buffer was wholly transferred to a scintillation bottle and incubated with 2 ml scintillation fluid for 15 min. Radioactivity (calibrated counts-per min (cpm)) was determined by using a liquid scintillation counter (PerkinElmerWallac1450, Phoenix, AZ, USA).

**Calculation of cell size**

Cardiomyocytes were plated into 24-well plates at 1×10^4 cells cm^-2. After transfection for 16–48 h, cell morphology was observed under a microscope, and cell surface area was determined and analyzed by using Image Pro-Plus 4.1 (Media Cybernetics, Silver Spring, MD, USA) as described.

**Immunocytofluorescence**

Cardiomyocytes grown on coverslips and transfected for 8–48 h were fixed in precooling methanol at −20 °C for 5 min and 4% paraformaldehyde at room temperature for another 15 min, then blocked by the addition of 10% donkey serum in phosphate-buffered saline containing 0.1% Triton X-100 for 30 min. We identified cells by indirect immunofluorescent staining with anti-MR-1 rabbit polyclonal antibody (1:50), anti-myomesin-1 goat polyclonal antibody (1:100) and anti-a-actinin mouse monoclonal antibody (1:400; Sigma-Aldrich, St Louis, MO, USA) overnight at 4 °C, then Texas red-conjugated donkey anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA), fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat (Santa Cruz Biotechnology) or Alexa Fluor 488-donkey anti-mouse IgG (Molecular Probes, Eugene, OR, USA). The coverslips were mounted on glass slides with mounting medium and DAPI (Vector Laboratories, Burlingame, CA, USA). Images were obtained under a confocal scanning microscope (Zeiss LSM-510 Meta, Jena, Germany). An Alexa 630 oil immersion objective with a numerical aperture of 1.4 was used. Distances between neighboring Z disks were measured and analyzed using Image Pro-Plus.

**Quantification of F/G-actin**

Cardiomyocytes were plated in 60-mm dishes at 3×10^4 cells cm^-2. After transfection for 16 h, cells were lysed with actin stabilization buffer containing 10 mmol^-1 Tris (pH 7.4), 2 mmol^-1 MgCl2, 1% Triton X-100, 0.2 mmol^-1 dithiothreitol and 15% glycerol. Soluble (G-actin) and insoluble (F-actin) fractions were separated by centrifugation (12800 g, 1 min) at 4 °C. Each fraction was resolved by 10% SDS-PAGE and subjected to western blot analysis with pan-actin antibody (1:500, Cell Signaling Technology, Beverly, MA, USA).

**Reverse transcriptase-PCR**

After transfection for 8–24 h, total RNA isolation and RT-PCR involved use of the EasyScriptFirst-Strand cDNA Synthesis SuperMix Kit (TransGen, Beijing, China) according to the manufacturer’s instructions. Primers for glyceraldehyde dehydrodehydrogenase (GAPDH), atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), myomesin-1, tMR-1 and homologous original hMR-1 are in Table 2. The PCR products were separated on 1.5% agarose gel and photographed for analysis.

**Western blot analysis**

After transfection for 8–48 h, cardiomyocytes were lysed, and protein extraction was as described. The soluble supernatant of extracts was determined by the Bradford method. Samples of 50 μg protein were prepared and separated on 12% and 8% acrylamide gels for characterization of MR-1 (17 kDa), MRLC (16 kDa) and myomesin-1 (185 kDa). The separated proteins were electrophoretically transferred to nitrocellulose membranes, blocked with 5% bovine serum albumin in Tris-buffered saline Tween 20, containing 20 mmol^-1 Tris-HCl (pH 7.6), 137 mmol^-1 NaCl and 0.1% Tween 20. The membranes were incubated with the antibodies anti-MR-1 (1:100), anti-myomesin-1 (1:200), anti-a-actinin (1:400) and anti-GAPDH (1:400) at room temperature for 16 h. Membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibodies (1:10000) for 1 h at room temperature and then washed three times. Bands were visualized using the enhanced chemiluminescence kit (ECL, Amersham) and exposed to X-ray film.
anti-SUMO-1 (1:500; Cell Signaling Technology) and anti-GAPDH (1:500; Santa Cruz Biotechnology) overnight at 4 °C. After incubation for 2 h with horseradish peroxidase-conjugated secondary antibodies, the reaction was visualized by using an enhanced chemiluminescence kit (Santa Cruz Biotechnology). The integrated optical density (IOD=mean intensity × area) of proteins was quantified by using Image-Pro Plus. The relative level of analyzed protein expression was normalized to that of GAPDH.

**Statistical analysis**

Each experiment was performed at least in triplicate. Cardiomyocytes were pooled from three to four different rat litters, and data from three to four experiments were pooled and analyzed by using SPSS v13.0 (SPSS Inc., Chicago, IL, USA); data are presented as mean±s.d. Differences between two groups were analyzed by two-sample t-test for independent samples and among groups by one-way analysis of variance with Newman–Keuls post-test analysis. \( P<0.05 \) was considered statistically significant.

**RESULTS**

**Overexpression of MR-1 is sufficient for hypertrophy in cardiomyocytes**

We first examined the effect of the MR-1-overexpression model and found successful overexpression of MR-1 in MR-1-transfected cardiomyocytes, with an increase in expression by 1.6-fold at 8 h, 2.8-fold at 16 h, 3.4-fold at 24 h and 3.4-fold at 48 h as compared with pcDNA3.1 (vector)-transfected cultures (Figure 1a, \*\( P<0.05 \)). In determining hypertrophy, three hypertrophic hallmarks, that is, \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b). At 48 h of transfection, protein-synthesis velocity, as determined by \[^{3}H\]-leucine incorporation, mean area of cell surface and expression levels of ANF and BNP, were used. Compared with the vector control with MR-1 transfection for 24 h, ANF and BNP mRNA expression was increased by 1.1- and 0.9-fold, respectively (\( P<0.05 \)) (Figure 1b).

MR-1 is incorporated into sarcomeres and is involved in sarcomere organization

To directly assess whether MR-1 is involved in sarcomere organization, MR-1 and sarcomere A-band marker MRLC and Z-line marker \( \alpha \)-actinin were double stained, respectively, in the 48-h-normal-
Overexpression of MR-1 promotes rapid organization of sarcomeres

We previously found that MR-1 is involved in sarcomere organization; therefore, to determine whether MR-1 affects sarcomere organization, 1-day-cultured cardiomyocytes that were cultured or transfected for another 8–24 h were stained for polymerized actin by phalloidin-FITC and the ratios of myocytes containing well-organized sarcomeres were semi-quantified. The normal control displayed a stress fiber-like structure, which is similar to the vector control (Figure 3A). Transfection with MR-1 caused rapid sarcomere organization from 8 h. More than two-thirds of the cell area showed well-organized sarcomeres after MR-1 transfection as compared with the vector control. The ratio was increased by 0.6-fold at 8 h (34.5 ± 5.5% vs. 21.6 ± 7.6% in vector; \( P < 0.05 \)), 1.0-fold at 16 h (58.1 ± 4.3% vs. 29.1 ± 5.3%; \( P < 0.01 \)) and 1.3-fold at 24 h (62.1 ± 5.4% vs. 26.4 ± 4.8%; \( P < 0.01 \)).

Quantification of F/G-actin was employed further. The ratio of polymerized actin to total actin was significantly increased at 16 h by 3.3-fold in MR-1-overexpressed cardiomyocytes (Figure 3B; \( 4 P < 0.05 \), \( n=3 \)) as measured by F/G-actin fractionation and western blot analysis, which indicates that MR-1 induces polymerization of actin monomers and assembly of actin filament.

As key structural and regulatory proteins of sarcomere, myomesin-1 and MRLC mRNA expression was significantly increased by 6.7- and 3.2-fold, respectively, with MR-1 transfection at 24 h as compared with vector transfection (Figure 3Ca, \( P < 0.01 \)). Similarly, transfection with MR-1 at 24 h significantly increased the protein expression of myomesin-1 and MRLC by 24.4- and 3.0-fold, respectively (Figure 3Cb and c, both \( P < 0.01 \)).

The distance between Z-lines increases gradually during maturation of myofibrils. Therefore, we measured the distance between two neighboring Z-lines to determine whether MR-1 promotes sarcomerogenesis. We found that transfection with MR-1 enlarged the average distance between neighboring Z-lines as compared with the vector control, from 1.62 ± 0.06 μm to 1.47 ± 0.04 μm at 8 h to 1.79 ± 0.07 μm vs. 1.53 ± 0.06 μm at 16 h to 1.93 ± 0.08 μm vs. 1.72 ± 0.04 μm at 24 h (\( P < 0.05 \)), which indicates that MR-1 induces a rapid organization of sarcomeres.

**Molecular mechanism of MR-1-promoted sarcomere organization**

The exact mechanism of MR-1-promoted sarcomere organization was asked. Myomesin-1, which was thought to be a cytoskeletal protein, is also present in the nucleus of myocytes of newborn pups, resulting in differential regulation of several gene products. The shuttling of myomesin-1 suggests that myomesin-1 may have special roles in the differentiation of striated muscle in addition to regulating its contractile functions.11 Overexpression of MR-1 induces translocation of myomesin-1. Myomesin-1 is exclusively cytoplasmic in adult cardiomyocytes but was predominantly localized in the nucleus when expressed in primary cultured neonatal rat cardiomyocytes (Figure 4a—control). Transfection with vector did not affect the nuclear localization (Figure 4a—vector). Immunostaining revealed that in cardiomyocytes transfected with MR-1 for 24 h, myomesin-1 located in the nucleus shifted to the cytoplasm (Figure 4a—MR1), where it functions in myofibrillogenesis.

SUMOylation is involved in MR-1-regulated myomesin-1 translocation. The SUMOylation status of myomesin-1 is important.11 To determine whether SUMO causes translocation of myomesin-1 and sarcomere organization, pcDNA3.1-SUMO-1 was transfected into cardiomyocytes for 24 h. Similar to the effect of MR-1, translocation of myomesin-1 was detected (Figure 4a-SUMO-1). We next transfected the interfering stealth siRNA into cardiomyocytes to silence the original rMR-1 and found that most of the myomesin-1 signals were still localized in the nucleus (Figure 4a—RNAi). Furthermore, on co-transfecting rMR-1-stealth siRNA and SUMO-1 into cardiomyocytes, myomesin-1 signals were distributed in the
nuclear and peri-nuclear areas (Figure 4a—SUMO-1+RNAi), which reveals an attenuated translocation of myomesin-1. We also determined whether the SUMO-1-promoted sarcomere organization, as well as this ordered assembly, could be affected by silencing MR-1. The ratio of cardiomyocytes with well-organized sarcomeres was reduced from 59.8 ± 6.9% in the SUMO-1 group to 22.1 ± 6.4% with RNAi silencing (Figure 4b, *P < 0.05), which suggests that MR-1 is necessary for myomesin-1-mediated sarcomere organization.

MR-1 promotes SUMOylation without any increase in SUMO-1 level. To clarify whether MR-1 promoted SUMOylation of myomesin-1 by increasing the SUMO peptide expression, we measured SUMO-1 expression after MR-1 transfection for 24 h. Both mRNA and protein levels of SUMO-1 in MR-1-transfected cells were not increased as compared with the vector control (*P > 0.05), which indicates that MR-1 may regulate myomesin-1 by promoting its conjugation with SUMO peptides rather than by new synthesis of SUMO.

**DISCUSSION**

This study of the novel cloned MR-1, involved in cardiac hypertrophy, showed for the first time that overexpression of MR-1 directly induced hypertrophy in neonatal rat cardiomyocytes, with an upregulation of ANF and BNP, an increase in protein synthesis concomitant with an increase in cell size and increased organization of sarcomeres. Thus, MR-1 is necessary for cardiomyocyte hypertrophy. FITC-annexin V, which binds phosphatidyl serine on apoptotic cells, was measured by flow cytometry. It did not show any significant difference of live-cell ratio between MR-1-transfected myocytes and untransfected myocytes.
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