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ORIGINAL ARTICLE

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

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Human myofibrillogenesis regulator 1, 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 [^{3}H]-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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Keywords: cardiomyocyte; hypertrophy; myofibrillogenesis regulator; sarcomere organization

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.[4,5] Sarcomere organization has a direct and profound influence on cardiac function.[6] 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-line[9] 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

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heart by PCR with the primers 5′-GTGGGATCTCACCATGGCGGC-3′ and 5′-CGCTCCTCAGGTCTGCAC-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 TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and DNAstar (DNASTAR Inc., Madison, WI, USA). The sequences we selected and synthesized were tkrevdkdrvkqmkarqnmrlsn and tgeyesqrfrassqsapspdvgsgvqt, 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.[12] 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 $2.5–3.0\times10^5$ 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 transfection with pcDNA3.1-hMR1 (MR-1) and (3) vector control transfection with pcDNA3.1 (vector). (4) The improved duplexed stealth RNAi technology[13,14] was used for silencing assay (RNAi). The sequence of the selected target against rat MR-1 was 5′-CGACAGCUAACAAGGCUUCCCAGAA-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 Lipofectamin2000 (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.

Table 1 Time course

	Time points after transfection (h)			
Treatment or measurement	*8*	*16*	*24*	*48*
Overexpression MR-1 (WB)	√	√	√	√
Hypertrophy effect				
ANF/BNP transcription (RT-PCR)	√	√	√	√
[^{3}H]-Leucine incorporation	√	√	√	√
Calculation of cell size	√	√	√	√
F-/G-actin assays				
F/G-actin fractionation	—	√	—	—
F-actin organization	√	√	√	—
Myomesin-1 and MRLC				
Subcellular localization (ICF)	√	√	√	√
Expression at protein level (WB)	—	—	√	—
Expression at mRNA level (RT-PCR)	—	—	—	√

Abbreviations: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; ICF, immunocytofluorescence; MRLC, myosin regulatory light chain; RT-PCR, reverse transcriptase polymerase; WB, western blot.
'√' means the time points at which measurements were taken.

[^{3}H]-Leucine incorporation

Total protein synthesis rate in cardiomyocytes was evaluated by incorporation of [^{3}H]-Leucine (Amersham, Cambridge, England). Cultured cardiomyocytes were plated in 24-well plates at 2×10^4 cm^{-2}. After transfection for 0, 4, 12 and 36 h, cardiomyocytes were incubated with [^{3}H]-Leucine (10 μCi per well) for 8, 12, 12 and 12 h. [^{3}H]-Leucine incorporation was determined as described.[2] Briefly, cells were washed by pre-cooling 0.01 mol l^{-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 (ccpm)) was determined by using a liquid scintillation counter (PerkinElmer-wallac1450, Phoenix, AZ, USA).

Calculation of cell size

Cardiomyocytes were plated into 24-well plates at 1×10^4 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.[15]

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-α-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 ×60 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 cm^{-2}. After transfection for 16 h, cells were lysed with actin stabilization buffer containing 10 mmol l^{-1} Tris (pH 7.4), 2 mmol l^{-1} $MgCl_2$, 1% Triton X-100, 0.2 mmol l^{-1} dithiothreitol and 15% glycerol. Soluble (G-actin) and insoluble (F-actin) fractions were separated by centrifugation (12 800 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 phosphate dehydrogenase (GAPDH), atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), myomesin-1, rMR-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.[2] The soluble supernatant of extracts was determined by the Bradford method.[16] 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 l^{-1} Tris-HCl (pH 7.6), 137 mmol l^{-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-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.

Table 2 Primer sequences for RT-PCR

	Sequences (forward and reverse)	*Product length (bp)*
hMR-1	Pr1: 5′-GTGGGATCTCACCATGGCGGC-3′	755
	Pr2: 5′- CCTCAGGTCTGCAC-3′	
GAPDH	Pr3: 5′-TGCTGAGTATGTCGTGGAG-3′	288
	Pr4: 5′-AAAGAGGAAAGGCTGTGAAAC-3′	
ANF	Pr7: 5′- AGGCTCCTTCTCCATCACCAA-3′	346
	Pr8: 5′-CGCCCTCAGTATGCTTTTCA-3′	
BNP	Pr9: 5′-TTTGGGCAGAAGATAGACCG-3′	239
	Pr10: 5′-TGGCAAGTTTGTGCTGGAAG-3′	
Myomesin-1	Pr11: 5′-AGTTAACTGGTCCCACAATGGG-3′	366
	Pr12: 5′-GAGTGGGCTCGTTGATCTGC-3′	
MLC-2	Pr13: 5′-GCGAAAGACAAAGATGACTGA-3′	364

Abbreviations: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde phosphate dehydrogenase; hMR-1, human myofibrillogenesis regulator 1; MLC-2, myosin regulatory light chains.

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, was increased 0.9-fold (3333.5 ± 106.1 *vs.* 1789.3 ± 83.0 ccpm; $P<0.05$) (Figure 1c) and cell size was significantly increased by 1.0-fold that of the vector control ($18\,487.9 \pm 3804.9$ *vs.* $8998.2 \pm 1427.7\ \mu m^2$, $P<0.05$) (Figure 1d).

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 α-actinin were double stained, respectively, in the 48-h-normal-

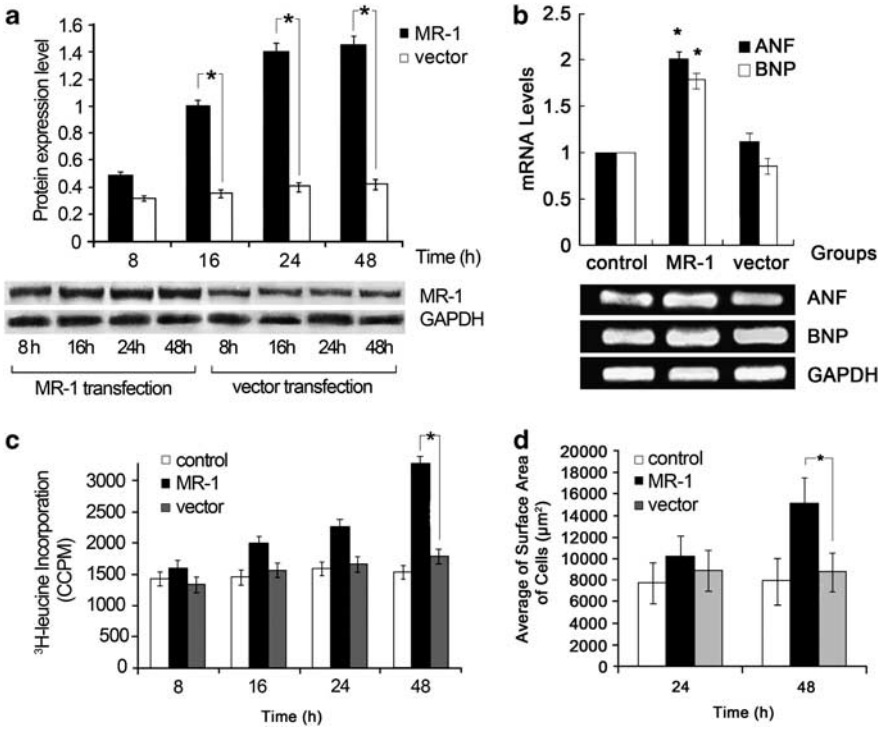

Figure 1 The relative amounts of MR-1 expression levels and assessment of hypertrophic hallmarks in cardiomyocytes transfected with MR-1. (**a**) MR-1 was overexpressed in neonatal rat ventricular cardiomyocytes at 16–48 h when transfected with pcDNA3.1-hMR1 plasmid (MR-1) compared with the culture transfected with pcDNA3.1 (vector). $^*P<0.05$ *vs.* vector. (**b**) mRNA levels of atrial natriuretic factor (ANF) and brain natriuretic protein (BNP) normalized to that of glyceraldehyde phosphate dehydrogenase (GAPDH) after 24-h transfection with MR-1. (**c**) Incorporation of [^{3}H]-Leucine reflecting the velocity of protein synthesis after up to 48-h transfection with MR-1. $^*P<0.05$ *vs.* vector. (**d**) Mean area of cell surface with MR-1 transfected for 24 and 48 h.

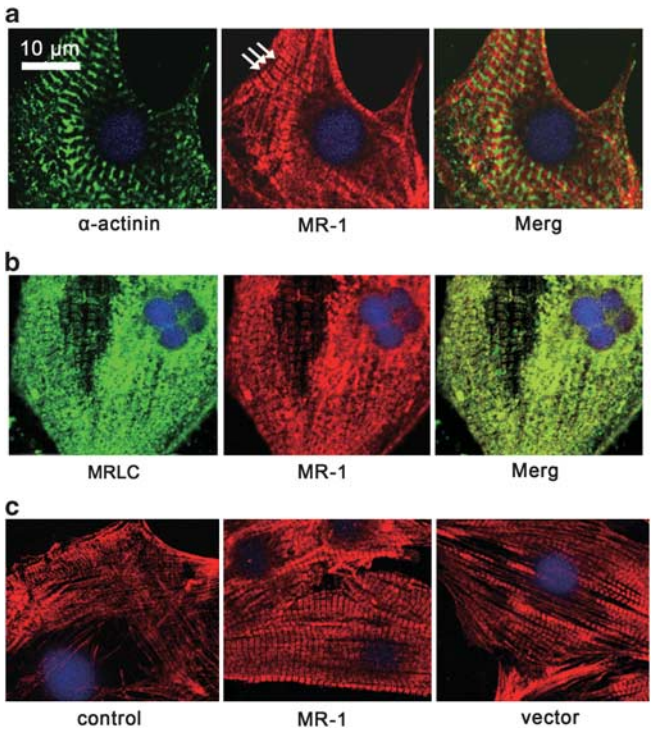

Figure 2 Subcellular localization and organization of MR-1 in cardiac myofibrils indicated by antibody of MR-1, α-actinin and MLC-2. (**a**) Double immunofluorescent staining of MR-1 (red) with α-actinin (green) showing regular striated pattern of MR-1 (red) in the areas where α-actinin (green) is negatively stained. (**b**) MR-1 is co-localized with MLC-2 and merged in A-band. (**c**) Staining of MR-1 in neonatal rat cardiomyocytes. Control: the punctated, thin, rudimentary filaments of MR-1 are shown in the normal cultured cardiomyocytes. MR-1: striated pattern of MR-1 staining shown in a cardiomyocyte transfected with MR-1. Vector: both nonstriated and striated MR-1 filaments shown in one cardiomyocyte transfected with vector plasmid.

cultured cardiomyocytes. The staining of MR-1 mainly existed in the area with negative staining for α-actinin (Figure 2a) but positive staining for MRLC (Figure 2b). MR-1 was co-located with MRLC. A dark zone was observed in the middle of the brightly stained MR-1 band (Figure 2b, arrow), clearly indicating the H-zone of a sarcomere. Thus, MR-1 could be incorporated into sarcomere A-bands between Z-lines.

Next, we observed the spatial–temporal features of subcellular distribution of MR-1 by staining with MR-1 antibody. In cardiomyocytes cultured for 1 to 3 days, most of the cells maintained an intact myofibrillar structure or punctated, thin, rudimentary filaments (Figure 2c—control). With prolonged time, the distribution pattern of MR-1 changed regularly. Most of the cardiomyocytes contained well-arranged striated bundles composed of regular-repeat positive-stained units. Transfection of MR-1 caused a rapid organization of MR-1. A cardiomyocyte cultured for 1 day and transfected for another 16 h exhibited a highly ordered, striated MR-1 pattern (Figure 2c—MR-1). These two main patterns of MR-1 may also be found in one cardiomyocyte; for example, a cardiomyocyte transfected with vector plasmid for 16 h showed the combination of MR-1 structures (Figure 2c—vector), which suggests an unfinished process of myofibrillogenesis in that cell.

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.* $21.6 \pm 7.6\%$ in vector; $P > 0.05$), 1.0-fold at 16 h (58.1 ± 4.3 *vs.* $29.1 \pm 5.3\%$; $P < 0.01$) and 1.3-fold at 24 h (62.1 ± 5.4 *vs.* $26.4 \pm 4.8\%$; $P < 0.01$).

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; $^*P < 0.05$, $n=3$) 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.[17] 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 *vs.* $1.47 \pm 0.04\,\mu m$ at 8 h to 1.79 ± 0.07 *vs.* $1.53 \pm 0.06\,\mu m$ at 16 h to 1.93 ± 0.08 *vs.* $1.72 \pm 0.04\,\mu m$ 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

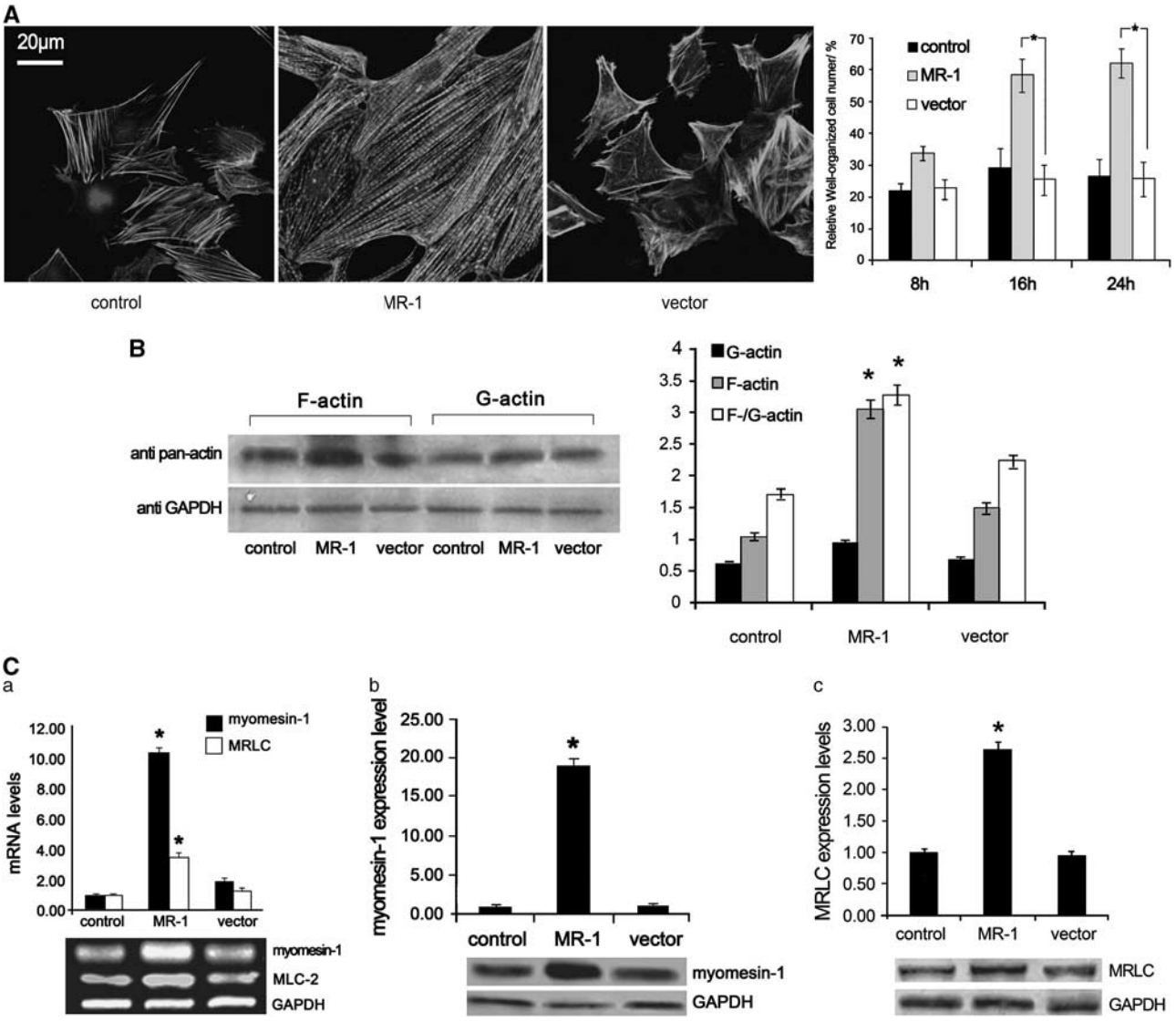

Figure 3 Transfection with MR-1 causes rapid sarcomere organization in neonatal rat cardiomyocytes. (**A**) 1-d cultured cardiomyocytes were transfected or normally cultured for another 24 h, fixed and specifically labeled with phalloidin-FITC. The figure shows the ratio of well-organized cardiomyocytes when MR-1 was transfected in cardiomyocytes for 16–24 h. $^*P<0.01$ *vs.* vector. (**B**) The ratio of polymerized actin to G-actin in MR-1-transfected cardiomyocytes at 16 h as measured by F/G-actin fractionation and then western blot analysis. $^*P<0.05$ *vs.* vector. (**C**) RT-PCR and western blot analysis of mRNA and protein levels of myomesin-1 and MLC-2 normalized to that of glyceraldehyde phosphate dehydrogenase (GAPDH) at 16 h (a) and western blot results (b, c). $^*P<0.01$ *vs.* vector. A full color version of this figure is available at the *Hypertension Research* journal online.

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 $59.8 \pm 6.9\%$ in the SUMO-1 group to $22.1 \pm 6.4\%$ 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

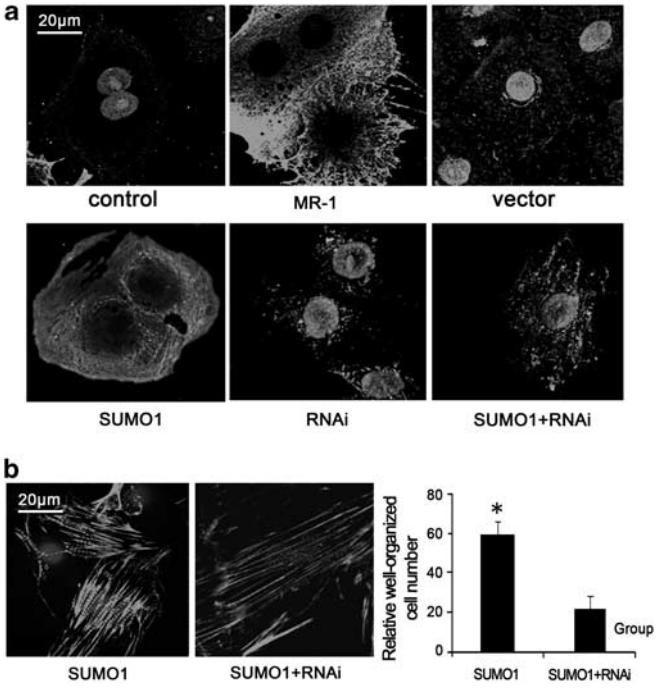

Figure 4 MR-1 is necessary for myomesin-1 translocation and SUMO-1-induced promotion of sarcomere organization. (**a**) Immunofluorescent staining of myomesin-1 showing translocation of myomesin-1 in neonatal rat cardiomyocytes. Myomesin-1 predominantly localized in the nucleus in primary cultured neonatal rat cardiomyocytes (control). With MR-1 transfected into cardiomyocytes for 24 h, myomesin-1 translocated to cytoplasm (MR-1). Transfection with pcDNA3.1 plasmid showed localization of myomesin-1 similar to the control (vector). On transfection with small ubiquitin-like modifier-1 (SUMO-1), myomesin-1 shifted to cytoplasm similar to with MR-1 transfection. Myomesin-1 was localized in the nuclear area with RNAi silencing (RNAi). Transfection with SUMO-1 in MR-1-silenced cardiomyocytes showed that myomesin-1 localized in nucleus and peri-nuclear area (SUMO-1+RNAi). (**b**) Silencing of MR-1 attenuated SUMO-1-induced promotion of sarcomere organization as detected by FITC-labeled phalloidin. Sarcomere organization with SUMO-1 transfected into cardiomyocytes for 16 h (SUMO-1) and with RNA interference silencing (SUMO-1+RNAi), $*P<0.05$. A full color version of this figure is available at the *Hypertension Research* journal online.

until 48 h. During the same time course, the striated-like myofibril organization in MR-1-transfected myocytes did not differ from that in the untransfected cells (data not shown). These results suggested that MR-1 induces adaptive hypertrophy within 48 h. MR-1 is highly expressed in angiotensin II-induced hypertrophic rat cardiomyocytes.[6] Whether other hypertrophic stimuli such as endothelin or epinephrine induce MR-1 expression is still unclear and will be studied further.

Previous immunohistochemistry verified that MR-1 was localized in cardiac myofibrils and interacted with several sarcomeric contractile proteins[1] and provided evidence that MR-1 might induce cardiac hypertrophy by promoting myofibrillogenesis. We first detected MR-1 incorporated into the sarcomere A-band, colocalized with MRLC. Thus, MR-1 was involved in sarcomere assembly and cardiomyocyte differentiation by serving a structural role. For more understanding, we measured the hallmarks of myofibrillogenesis, such as F/G-actin ratio, ratio of well-organized cardiomyocytes, and levels of myomesin-1 and MRLC. In addition, the periodicities of M-lines remained constant at approximately 2.0 μm throughout sarcomerogenesis, whereas distances between Z-lines increased from 1.1 μm in early sarcomere organization to approximately 2.0 μm in more mature structures.[17] We thus measured the mean distance between Z-lines by immunostaining with antibody for the Z-line protein α-actinin. Early after transfection, from 8 to 16 h overexpressed MR-1 caused a rapid increase in the ratio of polymerized actin to total actin, ratio of well-organized cardiomyocytes and average distance between two neighboring Z-lines, as well as expression of sarcomeric myomesin-1 and MRLC. Interestingly, these significant alterations occurred long before the hypertrophic phenotypes such as cell size and [^{3}H]-Leucine incorporation, both of which were significantly increased with transfection of cardiomyocytes for 48 h. Therefore, sarcomere organization may be a substantial basis of cardiac hypertrophy.

We next wondered how MR-1 regulates sarcomere organization. Overexpression of MR-1 promoted myomesin-1 translocation and increased the expression of myomesin-1,[1] which has special roles in assembly and stabilization of sarcomere and differentiation of striated muscle.[7,11] The characteristic upregulation and translocation of myomesin-1 suggests that MR-1 may promote sarcomere organization by regulating myomesin-1. The 185-kDa M-line molecule myomesin-1 is a high-molecular-weight protein originally identified because of its ability to interact with titin and myosin.[18–21] Myomesin-1 is thought to be the most prominent structural component of the sarcomeric M-line because of its essential role in sarcomere assembly and stabilization.[22,23]

To explain why myomesin-1 is targeted to the nucleus only in neonatal cardiomyocytes but not in adult cardiomyocytes, SUMO-involved translocation of myomesin-1 was considered. SUMO is a novel post-translational protein that contains 101 amino-acid residues and modifies other proteins through the SUMOylation pathway. SUMOylation modification has been implicated in the regulation of subcellular localization of proteins. This process involves the covalent attachment of a SUMO-peptide to the lysine residues within the consensus sequence WKXE of the target proteins.[24,25] Reddy KB hypothesized that myomesin-1 is required for differential expression of yet unknown sarcomeric proteins.[11] Once this expression is achieved, SUMOylated myomesin-1 is exported out of the nucleus and incorporated into the sarcomeric structures. However, SUMO-1 and/or SUMO-conjugating machinery are not fully expressed in all neonatal cardiomyocyte nuclei, where most of the myomesin-1 is still localized.[17] We found that overexpression of SUMO-1 promotes translocation and expression of myomesin-1 and assembly of sarcomeres, which mimicked the effect of overexpression of MR-1. However, SUMO-1-induced translocation and upregulation of myomesin-1 were attenuated when SUMO-1 was co-transfected with rMR-1-stealth siRNA, which indicates that MR-1 is required in SUMO-1-involved myomesin-1 shifting. Myomesin-1 might be regulated by an MR-1-involved SUMOylation mechanism. Furthermore, we also measured the mRNA and protein levels of SUMO-1 after transfection and found that overexpression of MR-1 did not affect the SUMO expression. Thus, SUMOylation of myomesin-1 was not promoted by the SUMO-conjugating machinery, rather it was promoted by the increase of SUMO peptides. Overexpression of MR-1 may induce cardiomyocyte hypertrophy by promoting SUMOylated myomesin-1-mediated sarcomere organization.

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