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Haploinsufficiency of *Hand1* improves mice survival after acute myocardial infarction through preventing cardiac rupture

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ABSTRACT

Previous studies have demonstrated a significantly lower level of *Hand1* in ischemic cardiomyopathy than in normal heart tissue. The role of decreased Hand1 in myocardial infarction remains unclear. This study was designed to investigate the effects of haploinsufficiency of *Hand1* on mouse heart after myocardial infarction.

8-10 weeks old male heterozygous *Hand1*-deficient ($Hand1^{+/-}$) mice and wild-type littermates (control) were subjected to sham operation or ligation of the left anterior descending coronary artery to induce acute myocardial infarction (AMI). $Hand1^{+/-}$ mice have low incidence of left ventricular free wall rupture in the first week after operation than control mice. Then we found lower MMP9 activity and less cardiomyocytes apoptosis in $Hand1^{+/-}$ than in control mice. All of these contribute to the protection role of haploinsufficiency of *Hand1* after AMI.

1. Introduction

Myocardial infarction occurs when the blood flow cannot satisfy the metabolic demand of the myocardium and cause damage to heart muscle. Sudden death occurs within hours of onset of symptoms due to arrhythmias or pump failure. After this critical period, cardiac rupture becomes a major fatal reason in the first week after acute myocardial infarction (AMI). It accounts for 5–31% of in-hospital mortality [1,2].

Hand1 is a basic helix-loop-helix (bHLH) transcription factor which belongs to the twist family. Germ-line *Hand1* null alleles died between embryonic day 8.5 and 9.5 because of deficiencies in extra embryonic mesoderm and placenta [3]. Aside from the development of extra embryonic vasculature, *Hand1* also plays an essential role in heart development and it is one of the five core transcription factors in heart development [4–7]. Hand1 expressed majorly in cardiomyocytes of left ventricle (LV). Cardiac specific *Hand1* deletion caused defects in the LV and endocardial cushions, and exhibited dysregulated ventricular gene expression [8].

Previous studies in our group and others demonstrated that *Hand1* expression was decreased in adult rodent hearts compared with those in embryos and cardiac-specific over-expression of *Hand1* caused arrhythmias and even heart failure in adult mice [9,10]. Recent research found that cardiomyocytes metabolism shifts from glycolysis to lipid oxidation soon after birth and Hand1 negatively regulates genes involved in lipid metabolism [11]. So, less Hand1 is beneficial for adult heart. Researchers have also shown about half loss of *Hand1* in hypertrophic and ischemic cardiomyopathy, but whether the half loss of *Hand1* can influence ischemic cardiomyopathy was previously unknown [12]. In this study, we explored the relationship between Hand1's expression level and ischemic cardiomyopathy.

2. Materials & methods

2.1. Animals

Mice were housed in groups in accordance with the regulations on mouse welfare and ethics of Nanjing University with 12-h dark-light cycles and free access to food and water. *Hand1* heterozygous

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mice were provided by Eric N. Olson (UT Southwestern Medical Center), and these mice were maintained on a C57/BL6 genetic background. These mice were generated by replacing the first coding exon of *Hand1* with a β-galactosidase reporter gene [3]. Primers for genotyping were as below: *Hand1*a, 5′ GGC GGT GCT AAT TGG GCT G 3'; *Hand1*b, 5′ GCA AAA TTC TAT GTT CAC TCA GCA ATG 3′ and *Hand1*c, 5′ CGC ATC GCC TTC TAT CGC C 3'. The wild-type band was 750 bp, and the mutant band was 400 bp.

The animal experiment was approved by Nanjing university ethics review board and the animal procedures were performed according to the NIH Guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Myocardial infarction (MI) surgery

MI models were generated following the method reported previously in mice [13,14]. Briefly, 8- to 10-week-old male mice (weighing between 25 and 30 g) underwent left coronary artery ligation. First, mice were anesthetized intraperitoneally with avertin (2, 2, 2-Tribromoethanol) at a dose of 0.4–0.75 mg/g. Second, the thoracic cavity was opened under a volume-cycled rodent respirator (model 683, Harvard Co.), which provides positive pressure ventilation at 2–3 ml/cycle and a respiratory rate of 140 cycles/min. The left anterior descending coronary was then ligated with a 7-0 silk suture approximately one mm from the tip of the left auricle. Finally, the chest was closed with continuous 6-0 prolene suture, followed by a 4-0 polyester suture to close the skin.

2.3. Cell apoptosis assay

DNA fragmentation was detected in situ using the TUNEL assay as described previously [15]. Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemical). Nuclear density was determined by manual counting of TO-PRO-3-stained nuclei in four fields for each animal using the 40× objective, and the number of TUNEL-positive nuclei was counted as the mean of four 40× objective fields.

2.4. Gelatin zymography

After AMI, control and *Hand1*$^{+/-}$ mice hearts were harvested, and the infarct area, border area and non-infarct area were separated and frozen at −80 °C to detect the activity of matrix and metalloproteinase (MMP)-2 and −9, as described previously [16]. Briefly, tissue samples were homogenized in 500 μL buffer (50 mmol/L Tris, 0.2% Triton X-100, 10 mmol/L $CaCl_2$ and 2 mol/L guanidine hydrochloride, pH 7.5). Samples of 30 μg were applied to non-denaturing 10% polyacrylamide gels containing 1 mg/mL gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100, incubated overnight at 37 °C in zymography buffer (50 mmol/L Tris and 10 mmol/L $CaCl_2$) and stained with Coomassie Brilliant Blue for 2 h and decolorized. Gelatinolytic activity was visualized as white areas in the gel.

2.5. Histology and immunofluorescence staining

The hematoxylin & eosin (H&E) and immunofluorescence (IF) protocols were performed as described previously [17]. Briefly, the heart samples were washed with cold 1% PBS and then fixed in 4% PFA at 4 °C for 2 h. The samples were processed by (1) 3 × 5 min of washing in PBS; (2) 1 h each of incubation in 75%, 85%, 95% and then 3 × 1 h of incubation in 100% ethanol at room temperature (RT); (3) 3 × 5 min of incubation in xylene at RT; (4) 1 h of incubation in paraffin/xylene (1:1) at 65 °C; and (5) 3 × 30 min of incubation in fresh paraffin at 65 °C. The processed samples were then embedded in paraffin, sectioned (6 μm thick), and the sections were stained with hematoxylin and eosin following the standard protocol. Immunofluorescence (IF) staining was performed using wheat germ agglutinin (WGA) (dilution 1:500, Promega) at room temperature for 15 min to stain the cell membranes. Fluorescence microscopy images were visualized using a Research Fluorescence Microscope (Olympus) equipped with a digital camera.

2.6. Quantitative PCR

Total RNA was extracted from the left ventricle of mice using TRIzol reagent solution (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using Invitrogen's reverse transcription kit. Q-PCR was run with SYBR according to the SYBR® Premix Ex TaqTM II (TaKaRa) protocol using an ABI PRISM Fast Real-time PCR System 7300. Q-PCR primers were designed according to Harvard's primer bank or previous publications.

Hand1: F: 5′-TTC CCC TCT TCC GTC CTC TTA C-3'; R: 5′-AAT TCA GCA ACG AAT GGG AAC-3'; *MMP-2*: F: 5′-CAA GTT CCC CGG CGA TGT C-3'; R: 5′-TTC TGG TCA AGG TCA CCT GTC-3'; *MMP-9*: F: GCT GAC TAC GAT AAG GAC GGC A-3'; R: 5′-TAG TGG TGC AGG CAG AGT AGG A-3'; *GAPDH*: F: 5′-AGG TCG GTG TGA ACG GAT TTG-3'; R: 5′-TGT AGACCATGTAGTTGAGGTCA-3'. mRNA expression levels were normalized to *GAPDH* expression and calculated using the comparative CT method [18].

2.7. Echocardiography (Echo)

Echo was performed with Vevo 770 UBM (VisualSonics, Toronto, ON, Canada). Briefly, the mice were anesthetized with avertin I.P. (0.4–0.75 mg/g). The body temperature of the mice was maintained between 36 and 38 °C on a heat plate. The heart rate was maintained between 380 and 480 beats/min, and the heart function was examined by a RMV 707B transducer.

2.8. Determination of the infarct size

The hearts were cross-sectioned into 6-μm-thick slices, and stained by Masson's trichrome staining according to the protocol. (http://www.ihcworld.com/_protocols/special_stains/masson_trichrome.htm). The infarct size was determined by the area of infarction correlated to the area of the left ventricle (including LV-septum) in four different slices from the base to the apex of the heart, with 500 μm between each slice. Total infarct size was calculated by multiplying the mean percent value of the circular infarct area with the quotient: vertical extension of the infarct area/total ventricular extension.

2.9. Western blot

Proteins were extracted from LVs of mice hearts through homogenization in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS and 1 mM PMSF). Protein concentration in samples was determined using a Bradford assay (Bio-Rad, CA, USA) and measuring optical density at 595 nm using a 96-well plate reader. Proteins were resuspended in 5X Laemmli sample buffer (50 mM Tris, 4% SDS, 10% glycerol, 5% Mercaptoethanol, 0.01% Bromophenol Blue). Samples were heated to 95 °C for 5 min. Then 50 μg of proteins were separated according to molecular weight on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a semi-dry transfer system (Biorad). Membranes were blocked in 0.1% Tween 20 in PBS supplemented with 5% fat-free powdered milk for 40 min and then incubated with