Requirements of myocyte-specific enhancer factor 2A in zebrafish cardiac contractility

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Abstract Myocyte-specific enhancer factor 2A (MEF2A) regulates a broad range of fundamental cellular processes including cell division, differentiation and death. Here, we tested the hypothesis that MEF2A is required in cardiac contractility employing zebrafish as a model organism. *MEF2A* is highly expressed in heart as well as somites during zebrafish embryogenesis. Knock-down of *MEF2A* in zebrafish impaires the cardiac contractility and results in sarcomere assembly defects. Dysregulation of cardiac genes in *MEF2A* morphants suggests that sarcomere assembly disturbances account for the cardiac contractile deficiency. Our studies suggested that MEF2A is essential in cardiac contractility.

Keywords: MEF2A; Cardiac contractility; Zebrafish; Development; Morpholino; Morphant

1. Introduction

Heart failure is a complex disorder in which cardiac contractility is insufficient to adequately supply blood to other organs. This syndrome is a common complication that ensues from a wide variety of cardiovascular pathologies. Studies in failing human hearts show numerous morphological changes including degenerative alterations, mutation of contractile elements and marked disorganization of sarcomere. These changes suggest that defects of a contractile gene lead not only to decreased elastic properties of the sarcomere but also to disturbances in sarcomerogenesis [1–4].

The zebrafish, *Danio rerio*, offers several distinct advantages as a genetic and embryological model system, including the external fertilization, rapid development and optical clarity of its embryos. In addition, because of their small size, zebrafish embryos are not completely dependent on a functional cardiovascular system. Even in the total absence of blood circulation, they receive enough oxygen by passive diffusion to survive and continue to develop in a relatively normal fashion for several days, thereby allowing a detailed analysis of animals with severe cardiovascular defects [5–11]. By contrast, avian and mammalian embryos die rapidly in the absence of a functional cardiovascular system. Forward genetics in zebrafish has led to the identification of several mutations affecting cardiac contractility [10–13]. So far, in the large scale mutagenesis screens of zebrafish, no mutations were linked to *myocyte-specific enhancer factor 2A* (*MEF2A*) locus [8,10–14].

A loss-of-function mutation in the human *MEF2A* causes an autosomal dominant form of coronary artery disease [15,16]. These data have recently been put into question [17,18]. Deletion of *MEF2A* in mice results in sudden cardiac death as well as marked right ventricular dilation that is not explained by increased pressure of the pulmonary vasculature [19]. Despite of the sudden cardiac death phenotype, several differences exist between these mice and humans with mutations in *MEF2A*. More detailed phenotypic analysis of animals with *MEF2A* mutations will be required to uncover its biological function.

In the present study, we described that *MEF2A* is highly expressed in the heart and somites during zebrafish embryogenesis. And we provided the first evidence that MEF2A is required for zebrafish cardiac contraction. *MEF2A* knockdown results in sarcomere assembly defects, and the disturbances of sarcomere assembly could account for the cardiac contractile deficiency.

2. Materials and methods

2.1. Zebrafish embryos

Wild-type (AB* strain) zebrafish embryos were obtained from natural spawning of wild-type adults. Zebrafish were raised, maintained and staged as previously described [20,21].

2.2. RT-PCR analysis

Total RNA was extracted from different stages embryos and adult heart. RT-PCR was performed to amplify a 202 bp fragment, whose authenticity was confirmed by sequencing (*MEF2A* forward primer: ATG CCC ACT GCC TAC AAC TC, reverse primer: CAT TCT GGC TGG TGT TGA TG). Zebrafish β-actin was used as control for qualitative and quantitative assessment of the cDNA sample

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Abbreviations: MEF2A, myocyte-specific enhancer factor 2A; hpf, hours post fertilization; GFP, green fluorescent protein; MO, morpholino; vmhc, ventricular myosin heavy chain; amhc, atrial myosin heavy chain; cmlc2, cardiac myosin light chain 2; anf, atrial natriuretic factor; V, ventricle; A, atrium

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(*β-actin* forward primer: TAC AGC TTC ACC ACC ACA GC, reverse primer: AAG GAA GGC TGG AAG AGA GC).The size of the *β-actin* amplicon is 206 bp.

2.3. Morpholino modified antisense oligonucleotide and microinjections

We designed one morpholino modified antisense oligonucleotide (MO, Gene Tools, LLC) against the splice donor site of *MEF2A* exon 8 to interfere with splicing (GT-MO) and another MO directed against the 5′ sequence around the putative start codon to block *MEF2A* translation (ATG-MO). The sequences for the GT-MO and ATG-MO were the 5′-GTCGTTTGTGCTCACCAGAGTTGTA-3′ and 5′-ATCTGTATCTTCTTCCGTCCCATCT-3′, respectively. A standard control MO was designed for control microinjections. The sequence for the standard control MO was 5′-CCTCTTACCT-CAGTTACAATTTATA-3′. Wild-type embryos were injected at the one-to-two cell stage with 2.5–10 ng MO per embryo.

2.4. Microinjection of the MEF2A promoter construct in zebrafish embryos

The *MEF2A*-green fluorescent protein (GFP) construct was made by linking the 1.7-kb *MEF2A* promoter with the GFP reporter gene. The construct was linearized and dissolved in 5 mM Tris–HCl, pH 8.0, 200 mM KCl, 0.05% phenol red to a final concentration of 50 ng/ml. About 3 nl of the DNA sample was microinjected into the cytoplasm of the one-to-two cell stage embryos. By using a fluorescence microscope, we observed the green fluorescence in embryos.

2.5. In situ hybridization, immunofluorescence and photography

In situ hybridization experiments with *Titin* antisense were performed as previously described [22]. Whole mount immunofluorescence experiments were performed as previously described [22], using the monoclonal antibodies MF20 and CH1, which recognizes a sarcomeric myosin heavy chain epitope and a tropomyosin epitope. MF20 and CH1 developed by Dr. Donald A. Fischman and Dr. Jim Jung-Ching Lin were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of the Biological Sciences, Iowa City IA 52242. Stained embryos were examined with Olympus BX61 and SZX12 microscopes, and photographed with a DP70 digital camera. Images were processed using Adobe Photoshop software.

2.6. Western blot

Western blot were performed as previously described [21]. The blots were incubated with the primary antibody diluted in TBST (1:200 dilution for rabbit polyclonal anti-human MEF2A antibody (C-21;Santa Cruz) and 1:500 dilution for mouse monoclonal β-actin antibody (AC-40; Sigma)). After incubating with an anti-rabbit or anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (diluted 1:1000) at room temperature for 1 h, the blots were visualized by ECL kit according to the manufacturer's instructions.

2.7. Heart rates measurement and ventricular contractility analysis

Embryos were anesthetized and transferred to a recording chamber perfused with modified Tyrode's solution (136 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.3) at 48 h post fertilization (hpf). Heart rates were accounted under a dissect microscope. Cardiac contractions were recorded with a video camera (JVC, TK-C1381) as described [23]. The lengths of ventricles in diastolic and systolic conditions were measured to calculate the ventricular shortening fraction (VSF). Values are presented as mean ± S.D.

$$\mathrm{VSF} = \frac{\text{ventricular length at diastole} - \text{ventricular length at systole}}{\text{ventricular length at diastole}}.$$

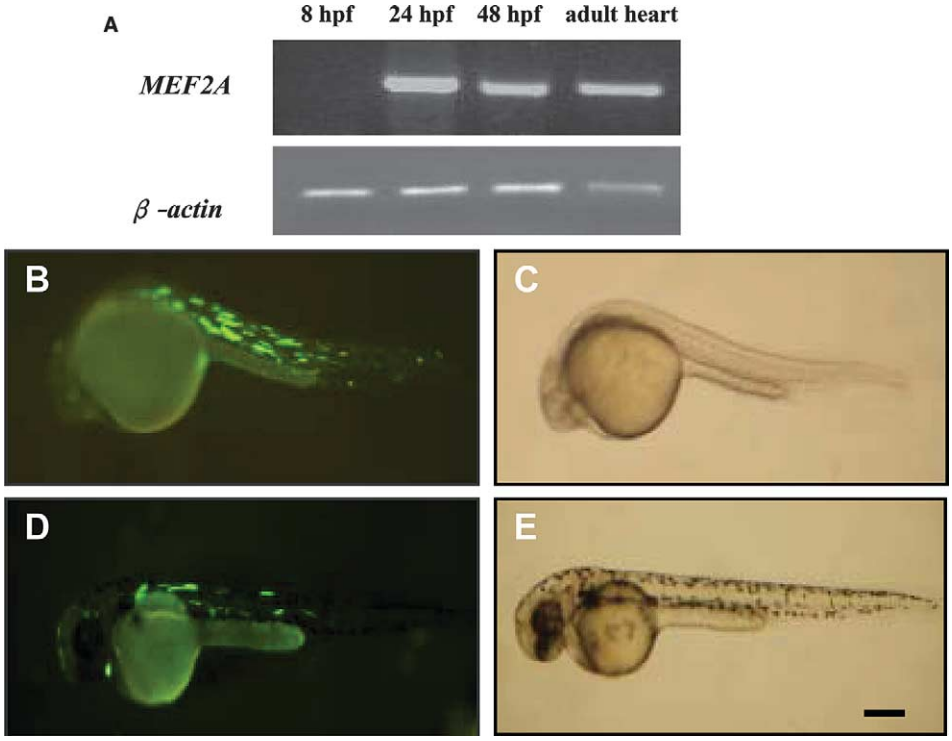

Fig. 1. *MEF2A* is highly expressed in the heart and somites during zebrafish embryogenesis. (A) RT-PCR analysis was performed with RNA samples isolated from different stages embryos and adult heart. *MEF2A* specific products were amplified from RNA isolated from 24 hpf, 48 hpf whole embryos and adult heart. The *β-actin* primers amplified a single fragment in all the samples. (B–E) Transient expression patterns of GFP fluorescence observed in *MEF2A*-promoter–GFP transgenic embryos. (B,C) Lateral views at the 24 hpf, anterior to the left. GFP expression is observed in skeletal muscle cells. (D,E) Lateral views at 48 the hpf, anterior to the left. GFP expression is observed in both heart and somites. (B,D: fluorescent field. C,E: bright field.) Scale bar, 100 μm.