Inactivation of Myosin Binding Protein C Homolog in Zebrafish as a Model for Human Cardiac Hypertrophy and Diastolic Dysfunction

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Background—Sudden cardiac death due to malignant ventricular arrhythmia is a devastating manifestation of cardiac hypertrophy. Sarcomere protein myosin binding protein C is functionally related to cardiac diastolic function and hypertrophy. Zebrafish is a better model to study human electrophysiology and arrhythmia than rodents because of the electrophysiological characteristics similar to those of humans.

Methods and Results—We established a zebrafish model of cardiac hypertrophy and diastolic dysfunction by genetic knockdown of myosin binding protein C gene (mybpc3) and investigated the electrophysiological phenotypes in this model. We found expression of zebrafish mybpc3 restrictively in the heart and slow muscle, and mybpc3 gene was evolutionally conservative with sequence homology between zebrafish and human mybpc3 genes. Zebrafish with genetic knockdown of mybpc3 by morpholino showed ventricular hypertrophy with increased myocardial wall thickness and diastolic heart failure, manifesting as decreased ventricular diastolic relaxation velocity, pericardial effusion, and dilatation of the atrium. In terms of electrophysiological phenotypes, mybpc3 knockdown fish had a longer ventricular action potential duration and slower ventricular diastolic calcium reuptake, both of which are typical electrophysiological features in human cardiac hypertrophy and heart failure. Impaired calcium reuptake resulted in increased susceptibility to calcium transient alternans and action potential duration alternans, which have been proved to be central to the genesis of malignant ventricular fibrillation and a sensitive marker of sudden cardiac death.

Conclusions—mybpc3 knockdown in zebrafish recapitulated the morphological, mechanical, and electrophysiological phenotypes of human cardiac hypertrophy and diastolic heart failure. Our study also first demonstrated arrhythmogenic cardiac alternans in cardiac hypertrophy. (J Am Heart Assoc. 2013;2:e000231 doi: 10.1161/JAHA.113.000231)

Key Words: animal model • cardiac alternans • diastolic dysfunction • heart failure with normal ejection fraction • hypertrophy • zebrafish

The myosin binding protein C (MYBPC) gene (mybpc3) encodes myosin binding protein C, a key constituent of the thick filaments localized to doublets in the C-zone of the A-band of the sarcomere. By binding to myosin, titin, and actin, MYBPC contributes to maintaining the structural integrity of the sarcomere and regulates cardiac contractility and relaxation.1–4 Mutations of mybpc3 gene have been demonstrated to be associated with a risk of cardiac hypertrophy and represent one of the common causes of hypertrophic cardiomyopathy.2–4 Recently, it has also been demonstrated that genetic variants in human mybpc3 gene are associated with susceptibility to diastolic heart failure without overt cardiac hypertrophy.5 Therefore, the function of MYBPC is closely related to cardiac structural and function and may be a new therapeutic target in the treatment of cardiac hypertrophy and diastolic dysfunction.

Diastolic heart failure or heart failure with a normal ejection fraction (HFNEF) is one of the most important and common cardiovascular diseases. Clinically, the most common cause of diastolic heart failure is left ventricular hypertrophy, resulting either primarily from hypertrophic cardiomyopathy or secondarily from hypertension and aortic
stenosis. One of the common causes of death in patients with left ventricular hypertrophy is malignant ventricular arrhythmia.6,7 Sudden cardiac death (SCD) due to malignant ventricular arrhythmia is the most devastating manifestation of cardiovascular diseases. The Framingham Heart Study reported that left ventricular hypertrophy was associated with an increased risk of SCD in a community-based cohort.7 Because the hemodynamic pathophysiology of cardiac hypertrophy is well known, the fundamental electrophysiological mechanism of SCD or malignant ventricular cardiac hypertrophy is not completely understood.8 Although there are several murine models of cardiac hypertrophy, such as genetic ablation of mybpc3 or aortic banding in mice,4,9 the electrophysiological phenotypes of murine hearts are completely different from those of human heart due to a very high heart rate and very short action potential duration (APD), which hinder the evaluation of cardiac repolarization.10 Because the electrophysiological phenotypes of large animal heart are closer to those of human heart,11 currently there has been no well-established model of cardiac hypertrophy or diastolic dysfunction in large animals.

Recently, zebrafish has been proved to be a good model in which to study human cardiac electrophysiology, especially cardiac repolarization, because its heart rate and action potential morphology strikingly resemble those of human heart.10,12 Accordingly, in the present study, based on the role of MYBPC on cardiac hypertrophy and diastolic dysfunction,2–5 we sought to establish a zebrafish model of human cardiac hypertrophy and diastolic heart failure by genetic knockdown of mybpc3. We first systematically characterized the status and expression pattern of mybpc3 gene in zebrafish, which had never been reported before. Then we tried to recapitulate the structural, mechanical, and electrophysiological phenotypes of human cardiac hypertrophy and diastolic heart failure in this zebrafish model.

Methods

Cloning of Zebrafish mybpc3 cDNA

For amplifying mybpc3 cDNA, 3 primer sets [(Mybpc3-1 forward: 5′-ACACTCAACCAGATGCCAG-3′ and Mybpc3-1 reverse: 5′-TCAGTGGCGTCTCTCATCTC-3′), (Mybpc3-2 forward: 5′-TGACTGAAGATGGACAGAAGA-3′ and Mybpc3-2 reverse: 5′-TTCCTCTCTAGTCTACAC-3′), and (Mybpc3-3 forward: 5′-CTCCACCAGGACTCTT-3′ and Mybpc3-3 reverse: 5′-ACGTCTCTCTCACTTGT-3′)] were designed according to a partial DNA fragment of mybpc3 previously.13,14 The presumptive Mybpc3 amino acid sequences were determined using the Wisconsin Sequence Analysis Package v.10.0 (GCG). The Gap program of that package was used for multiple comparisons. The Clustalw molecular evolution genetic program was used for our phylogenetic tree analysis (http://www.ebi.ac.uk/clustalw/).

Zebrafish Embryos, Whole Mount In Situ Hybridization, Antibody Labeling, and Cryosection

The procedures for zebrafish culture, embryo collection, fluorescent observation, whole mount in situ hybridization, antibody labeling, and cryosection have been described previously.13–15 The designation of developmental stages of zebrafish followed those of Kimmel et al.16 According to the guidelines of zebrafish anesthesia and euthanasia,15 there is no evidence of higher order cognition in zebrafish during the first week of development. During the first week of development, embryonic movements are simple reflexes that do not provide evidence for a capacity for suffering. Thus, during the first week, zebrafish larvae have not reached the point in brain development where stimuli can be experienced as aversive.

Both mybpc3 and cmlc2 were used as probes.17 They were digoxigenin labeled after their partial DNA fragments were cloned.

Preparation and Microinjection of Morpholino

Mybpc3-morpholino (MO) (5′-CTCTGGCATCCTGTGAGTG-TCCC-3′; Gene Tools) was established according to zebrafish mybpc3 cDNA sequence for blocking translation. Negative control MO (5′-CTCTTACCTCAGTACATTTATA-3′) was designed according to the random nucleotide sequences. All of these were prepared at stocking concentrations of 1 mmol/L and diluted with double-distilled water to the proper concentrations (4.5 ng/2.3 nL).

Microscopic Observation of Zebrafish Embryos

All embryos were observed at specific stages under a microscope (DM 2500; Leica) equipped with Nomarski differential interference contrast optics and a fluorescent module with a GFP filter cube (Kramer Scientific). Photographs of embryos at specific stages were taken with a CCD (DFC490; Leica). Measurement of myocardial wall thickness in the embryo heart was performed as previously reported,18 using a camera with high temporal (14 frames/s) and spatial (5184 × 3456 pixels) resolutions (Canon EOS-1DX).
Evaluation of In Vivo Systolic and Diastolic Functions of the Zebrafish Embryo Heart

We evaluated the systolic and diastolic functions in the zebrafish embryo heart using the automatic video edge-detection system. In brief, SoftEdge (IonOptix Corporation) was used to measure real-time length via contrast analysis of digitized image data. Edge detection was based on image intensity, and image contrast was enhanced using the video gain and offset controls. The signal was calibrated using a standard millimeter graticule. The calculated values were verified by conventional velocity measurement obtained from 2-dimensional images using a camera with high temporal (14 frames/s) and spatial (5184 × 3456 pixels) resolutions (Canon EOS-1DX). Velocity values were expressed as micrometers per second for myocardial wall velocity in systole and diastole to represent the systolic and diastolic functions, respectively.

Extraction of Zebrafish Embryo Heart and In Vitro Electrophysiological Recordings

The heart of the embryo was dissected from the thorax en bloc by using fine forceps and then transferred to the recording chamber. Only spontaneously beating whole hearts were studied. All experiments were performed at room temperature. The recording chamber was superfused with solution containing (in mmol/L) NaCl 140, KCl 4, CaCl2 1.8, MgCl2 1, glucose 10, and HEPES 10, pH 7.4. Action potentials were recorded by the microelectrode and disrupted patch method, as previously reported. Action potentials were measured by using an amplifier (Axopatch 200B; Axon Instrument) and digitized with a 12-bit analog-to-digital converter (Digidata 1440A Interface; Molecular Devices). Resting action potentials were first recorded and then triggered by incrementally injecting pulses of depolarizing current. For calcium transient recording, the embryo hearts were stained with Fluo 3–acetoxymethyl ester (5 μmol/L; Molecular Probes) and then recorded with an inverted confocal microscope (LSM 510; Carl Zeiss) as previously recorded. For fluorescence excitation, the 488-nm band of an argon laser was used. Emission was recorded using a long-pass LP 515 filter set.

Measurement of APD and Calcium Transient Alternans and Calcium Transient Decay Rate

Alternans of APD (APD-ALT) and calcium transient (Ca-ALT) were determined by measuring differences in local APD and calcium transient amplitude on consecutive beats as previously reported. The APD-ALT (in ms) and Ca-ALT (in %) were plotted against the pacing rates (PRs).

The calcium transient decay rate was measured as previously reported. The calcium level was reported as F/F0, where F0 was the resting or diastolic fluorescence level. To quantify the calcium transient decay rate, the decay portion of the calcium transient (from 30% to 100% of decline phase) was fit to a single exponential function whose time constant, τ, was used to measure calcium transient decay rate.

Statistical Methods

All data were expressed as mean ± SD. Continued data from independent group were compared by using the Mann-Whitney U test. The Cochran-Armitage test was used to test a trend of the dose–response relationship between frequencies of phenotypic abnormalities and dose of Mybpc3-MO injection. A P value <0.05 was considered statistically significant.

Results

Cloning of Zebrafish mybpc3 cDNA and Comparison of Deduced Amino Acid Sequences

In searching the GenBank database, we found 1 putative zebrafish mybpc3 sequence (NM_001044349). After RT-PCR and DNA sequencing, we found there exist 3 mybpc3 transcription variants (mybpc3-tv1, -tv2, and -tv3) during zebrafish early embryogenesis (Figure 1A). After sequence cross-comparison, we found there are 5 differences between human and zebrafish mybpc3 transcripts (indicated by arrows 1 to 5). For example, all zebrafish mybpc3 transcription variants lack the second exon (arrow 1) but possess 2 extra exons (arrows 3 and 5); zebrafish mybpc3-tv2 and -tv3 have an extra exon (arrows 4 and 2) in comparison with mybpc3-tv1. The deduced zebrafish Mybpc3-tv1 amino acid sequence consists of a 1276–amino acid polypeptide. The zebrafish Mybpc3-tv1 polypeptide shares sequence identities of 63%, 61%, 62%, 63%, 61%, 68%, and 70% with the reported Mybpc3 of human, chimpanzee, bovine, mouse, rat, chicken, and Xenopus, respectively. We used the Clustalw program to determine the phylogenic similarities between zebrafish Mybpc3-tv1 and other known species. The phylogenetic tree generated by the program showed that zebrafish Mybpc3-tv1 possesses a unique pattern, different from those of higher vertebrates (data not shown).

We therefore examined the expression profile of 3 individual mybpc3 transcripts by the use of RT-PCR and found that mybpc3-tv1 signals are detectable from 18 to 96 hours postfertilization (hpf) (except for 48 hpf); mybpc3-tv2 signals are detectable at 18 to 48 hpf; and mybpc3-tv3 signals are detectable at 48 to 96 hpf (Figure 1B). These observations suggest that these 3 zebrafish mybpc3 transcripts have a unique profile during zebrafish early embryogenesis.
Zebrafish mybpc3 Expressed in Heart and Slow Muscle

We next determined the overall expressions of 3 mybpc3 mRNAs (combinations of mybpc3-tv1, -tv2, and -tv3) in zebrafish embryos by using whole mount in situ hybridization. During early embryonic development, mybpc3 expressions first became evident in the developing somites and heart tube (Figure 2A and 2B). At later stages, mybpc3 mRNA remained restricted to the heart and skeletal muscle throughout embryogenesis (Figure 2C and 2D). Cryosection results showed that mybpc3 mRNAs expressions are restricted in the ventricle and slow muscle but faint in the atrium (Figure 2E through 2J).

Loss of mybpc3 Leads to Cardiac Hypertrophy and Diastolic Heart Failure in Zebrafish

To investigate the role of MYBPC in vivo, we knocked zebrafish mybpc3 down by injecting morpholino-modified antisense oligonucleotides directed against translational start site (Mybpc3-MO) into one-cell–stage zebrafish embryos. We found that negative control MO or Mybpc3-MO–injected embryos showed normal heart morphogenesis during the first 24 hours of development (24 hpf). By 72 hpf, Mybpc3-MO–injected zebrafish showed pericardial edema and enlarged cardiac chambers as determined in bright field imaging (Figure 3A and 3B), which recapitulated typical features of human heart failure. In human heart failure, edema often occurs in dependent parts, such as bilateral lower legs. In embryonic fish, the pericardiac sac is the dependent part, and heart failure usually manifests as pericardial edema. The ventricular wall thickness was also increased in the Mybpc3-MO–injected zebrafish (diastolic wall thickness 15.1±0.8 μm in morphants and 13.4±0.7 μm in control embryos; P=0.009), resembling human ventricular hypertrophy. Immunostaining using both atrial and ventricular specific markers showed mild enlargement of the ventricle but significant enlargement of the atrium (Figure 3C and 3D), a typical feature of human diastolic dysfunction. Histologically, the Mybpc3-MO–injected heart showed ventricular hypertrophy (Figure 3E and 3F). Finally, increased expression of sarcomere proteins has been used as a marker of cardiac hypertrophy. Accordingly, we also found that Mybpc3-MO–injected hearts showed an increased expression of sarcomere protein, cardiac myosin light chain 2 (cmlc2), as determined with nonquantitative in situ hybridization (Figure 3G and 3H).
Interestingly, the frequencies of phenotypic abnormalities in consequence of Mybpc3-MO injection followed a dose-dependent manner (Table). At 2.0 ng, 36.1% (total cases in triplicate experiments, n = 88) of the surviving embryos (n = 244) displayed heart failure phenotypes, and at 3.0 ng, the percentage increased to 46.4% (n = 116; Table) (P = 0.011 for trend test).

We further characterized the alternation of mechanical function after loss of mybpc3 function. The ventricular systolic functions were comparable between the negative control MO or Mybpc3-MO–injected embryos, as determined by measurement of maximal ventricular systolic velocity (226 ± 64 μm/s in morphants and 214 ± 52 μm/s in control embryos; P > 0.05). However, Mybpc3-MO–injected embryos showed significantly impaired ventricular diastolic function, as determined by measurement of maximal ventricular diastolic relaxation velocity (213 ± 58 μm/s in morphants and 297 ± 65 μm/s in control embryos; P = 0.043). These features resembled those of human heart failure with preserved left ventricular ejection fraction (HFNEF) or diastolic dysfunction.24,25

Taken together, the Mybpc3-MO–injected fish showed typical features of human diastolic heart failure with dilated atrium, ventricular hypertrophy, and preserved ventricular systolic function.24,25

Mybpc3-MO–Injected Zebrafish Show Slower Diastolic Calcium Decay and Increased Susceptibility to Ca-ALT

Accumulating evidence has shown that APD-ALT arise primarily from Ca-ALT in both intact whole hearts31 and in vitro cardiomyocytes.21 In the next step, we tried to investigate the calcium dynamics in the Mybpc3-MO–injected hearts.

The calcium transient of the Mybpc3-MO–injected hearts showed characteristic features as seen in mammalian

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**Figure 2.** mybpc3 expression patterns during zebrafish embryogenesis. A through D, Whole mount in situ hybridization of 24-hpf-, and 48-hpf-embryos shows that mybpc3 expresses in heart and somites. E and F, Cryosectioning showed that the expressions mybpc3 were restricted in ventricle, atrium, and slow muscles. G through J, By 72 hpf, mybpc3 signals were still detectable in ventricle and atrium but were down-regulated in muscle. A indicates atrium; hpf, hours postfertilization; mybpc, myosin binding protein C; V, ventricle.
hypertrophic and failure hearts.\textsuperscript{29,32} The rising time was longer, and the peak calcium was smaller (Figure 6). The decay rate of calcium transient was also slower, with a larger time constant, indicating defective calcium reuptake and cycling.\textsuperscript{21,23} Defective intracellular calcium cycling may directly contribute to Ca-ALT.\textsuperscript{21,23,31} We then sought to investigate whether Mybpc3-MO–injected hearts also had increased susceptibility to Ca-ALT.

Incremental pacing was performed to evaluate the relationship between Ca-ALT and PR in control and Mybpc3-MO–injected hearts (Figure 7). Like in the study of APD-ALT, in Mybpc3-MO–injected hearts, there was a leftward shift in the Ca-ALT–to–PR relationship, indicating greater susceptibility to Ca-ALT. Interestingly, the developments of APD-ALT and Ca-ALT were closely related and coupled, and both are highly rate dependent.

In summary, the Mybpc3-MO–injected hearts showed characteristic electrophysiological features with greater susceptibility to arrhythmogenic cardiac ALT as those seen in mammalian hypertrophic and failure heart.

Discussion

In the present study, we have systematically characterized the expression of \textit{mybpc3} in zebrafish and demonstrated that loss of \textit{mybpc3} in zebrafish recapitulates the morphological, mechanical, and electrophysiological phenotypes of human cardiac hypertrophy and diastolic heart failure. We have also first shown that \textit{mybpc3} gene is evolutionally conservative and is an essential gene for the cardiac development of vertebrates, because loss of \textit{mybpc3} leads to severe cardiac phenotypes. This is also the first study to demonstrate an increased susceptibility to arrhythmogenic cardiac ALT in a zebrafish model of cardiac hypertrophy and diastolic dysfunction.

In the characterization of \textit{mybpc3} gene expression in zebrafish, we found sequence homology between human and zebrafish \textit{mybpc3} genes. Furthermore, ablation of \textit{mybpc3} gene expression results in similar phenotypes in zebrafish and human hearts. These results implicate that zebrafish heart is a feasible model system in which to dissect the detailed pathophysiological mechanism of human cardiovascular diseases. Previously, Norton et al\textsuperscript{33} reported a similar approach to induce heart failure in zebrafish to study the function of \textit{BCL2-associated athanogene 3} gene in humans. However, in this study, only mechanical phenotypes were characterized. The most devastating phenotype of human heart failure is ventricular arrhythmia or SCD. Therefore, correlating the electrophysiological phenotype to mechanical phenotype is important and clinically relevant. Notably in the present study, we characterized both the mechanical and electrophysiological phenotypes in the zebrafish embryo heart and successfully established an animal model and platform in which to study the electrophysiological mechanism of SCD for human cardiac hypertrophy and diastolic heart failure.

**Figure 3.** Knockdown of \textit{mybpc3} in zebrafish embryos leads to cardiomyopathy. A and B, Morphology of the embryonic zebrafish heart (72 hpf) with Mybpc3-MO injection or control. Arrow indicates the position of atrium. C and D, Embryos derived from control or Mybpc3-MO–injected group were stained with different antibodies MF20 (red) and S46 (green), and results reveals that \textit{Mybpc3} knockdown causes enlarged cardiac chambers, especially the atrium. Dashed and solid lines marked the morphology of atrium and ventricle, respectively. E and F, Hematoxylin and eosin (H&E) staining shows that \textit{mybpc3}-morphants have ventricular hypertrophy and thinner atrial wall. G and H, Whole mount in situ hybridization using a cardiac-specific marker \textit{cmlc2} as probes and showing that inactivation of \textit{mybpc3} causes increased heart size. H', Vental view of H. A indicates atrium; hpf, hours postfertilization; MO, morpholino; mybpc, myosin binding protein C; V, ventricle.
Previous studies have shown that loss of mybpc3 in mammalian hearts leads to cardiac hypertrophy,3,4 which is often demonstrated by cardiac imaging, such as echocardiography or magnetic resonance imaging. This kind of imaging modality is not feasible in the embryonic zebrafish heart. However, in the present study, we provided several lines of evidence to demonstrate cardiac hypertrophy in the small embryonic zebrafish heart, such as increased myocardial wall thickness and increased expression of sarcomere protein cmlc2 by nonquantitative measurement.26,27 Other convincing data to prove the presence of ventricular hypertrophy in our zebrafish model are marked diastolic dysfunction and dilated atrium. It has been shown in humans that left ventricular hypertrophy is often accompanied by ventricular diastolic dysfunction and dilated atrium.24,25 Therefore, the present zebrafish model of mybpc3 knockdown may be a good model in which to study the pathophysiology of human cardiac hypertrophy or diastolic dysfunction, such as the electrophysiological abnormalities as demonstrated in the present study. Although there have been several murine models of cardiac hypertrophy,4,9,10 using zebrafish as a model system provides major advantages of easy genetic manipulation, high throughput, and efficient data profiling. Moreover, accumulating evidence has shown that the electrophysiological phenotypes of zebrafish heart are much closer to those of human heart than are the murine hearts.10,12

It has been demonstrated that cardiac ALT, that is, beat-to-beat alternation of cardiac repolarization or APD, is a highly sensitive marker of susceptibility to SCD.30,34,35 Beat-to-beat alternation of APD, or APD-ALT, produces a repolarization gradient or spatial heterogeneity of refractoriness and facilitates the occurrence of conduction block and the initiation of reentrant arrhythmia. Accumulating evidence has showed that this arrhythmogenic APD-ALT may arise from defective calcium reuptake kinetics and Ca-ALT.21,30,31 In the present study, we showed that zebrafish hearts with mybpc3 knockdown displayed defective calcium cycling. Accordingly, in addition to cardiac hypertrophy and diastolic dysfunction, these hearts showed increased susceptibility to Ca-ALT and APD-ALT. Again, these results prove the concept that zebrafish heart could be used as a model system in which to study the molecular mechanism of ventricular arrhythmia or SCD related to cardiac hypertrophy. For example, selective gene manipulation may be performed in the future to test whether hypertrophy-related arrhythmogenic cardiac ALT could be abolished or prevented. Then, this gene may become the target for subsequent drug development to treated hypertrophy-related SCD.
We also first demonstrated that arrhythmogenic cardiac ALT could be induced in the hypertrophic heart, providing the possible electrophysiological mechanism of increased risk of SCD in patients with cardiac hypertrophy. Our results also implicate the possibility of increased susceptibility to SCD or severe ventricular arrhythmia in patients with diastolic heart failure or HFNEF, which has never been addressed before. Our results also imply that screening of mybpc3 genetic variation may be one of the strategies for SCD risk stratification in patients with diastolic heart failure or HFNEF. Chen et al also reported a mouse model with conditional knockout of mybpc3 in the heart. The cardiac phenotypes of this mouse model were very close to those of our zebrafish model, such as cardiac hypertrophy, cardiac diastolic dysfunction, and preserved systolic function. This may be another model in which to study the electrophysiological mechanism of SCD in cardiac hypertrophy or HFNEF. However, no electrophysiological phenotypes had been characterized in this murine cardiac hypertrophy model. As mentioned previously, it is very difficult to study cardiac repolarization in the mouse heart because of the extremely short APD.

**Conclusions**

We characterized the functional role of mybpc3 gene in terms of cardiac morphological, mechanical, and electrophysiological phenotypes and established the fundamental role of MYBPC in the mechanism of cardiac hypertrophy, diastolic dysfunction, and arrhythmogenic cardiac ALT. The results of the present study provide the rationale to develop novel...
therapy targeting MYBPC to prevent SCD in cardiac hypertrophy or diastolic heart failure.

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Disclosures
None.

References


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