In Vivo Assessment of Cardiac Morphology and Function in Heart-specific Green Fluorescent Zebrafish

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Background/Purpose: The zebrafish (*Danio rerio*) is a new animal model for cardiac research. Zebrafish possessing a green fluorescent heart facilitates the dynamic observation of cardiac development, morphology, and function *in vivo*. However, the effect of an excessive expression of green fluorescent protein (GFP) in cardiac muscle on the heart function of zebrafish has not been reported.

Methods: We cloned a 1.6 kb polymerase chain reaction (PCR) product containing the upstream sequence (870 bp), exon 1 (39 bp), intron 1 (682 bp), and exon 2 (69 bp) of the zebrafish cardiac myosin light chain 2 gene. A germ line-transmitted zebrafish possessing a green fluorescent heart was generated by injecting this PCR product fused with the GFP gene with ends consisting of inverted terminal repeats of an adeno-associated virus.

Results: Green fluorescence was intensively and specifically expressed in the myocardial cells located around both the heart chambers. Two lines with different GFP expression were bred (A26 and A277). The luminance of A277 was brighter than that of A26 (1.7-fold). The 4 days postfertilization (dpf) cardiac function and morphology were similar between these two groups. However, the 8 dpf cardiac growth seemed to be retarded in the A277 group. The 8 dpf heart rate, stroke volume, and cardiac output were also significantly lower in the A277 group.

Conclusion: Excess expression of GFP seems to exert some detrimental effects on zebrafish hearts. [*J Formos Med Assoc* 2007;106(3):181–186]

Key Words: cardiac function, green fluorescent protein, heart-specific promoter, zebrafish

The zebrafish (*Danio rerio*) is a new animal model for cardiac research.[1–3] Its heart contains four components (sinus venosus, atrium, ventricle, and bulbus arteriosus). Although it is equipped with a prototypic vertebrate heart, the studies of genetic control for zebrafish development can reveal some hints to solve human problems.[4] In response to the lack of a transgenic line of zebrafish labeled with heart-specific fluorescence *in vivo* to serve as a research model, we have successfully developed a cardiac-specific promoter containing the upstream sequence of the zebrafish cardiac myosin light chain 2 gene (*cmlc*2) and generated several germ lines of transgenic zebrafish that have green fluorescent protein (GFP)-tagged hearts.[5] However, excess cardiac expression of GFP has been reported to cause dilated cardiomyopathy in mice.[6] Therefore, we performed this study to evaluate the morphology and functioning of zebrafish hearts with different expressional levels of green fluorescence.

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Materials and Methods

Zebrafish breeding

Zebrafish of AB strain (wildtype) and transgenic strain were cultured and maintained according to procedures described by Westerfield.[7] Embryonic stages were recorded as hours postfertilization (hpf) and days postfertilization (dpf) following Kimmel et al.[8]

Gene construct, gene transfer, and germ lines of transgenic fish

In the previous report,[5] we described the primers which were designed to clone an 1.6 kb polymerase chain reaction product, in which the upstream sequence (870 bp), exon 1 (39 bp), intron 1 (682 bp), and part of exon 2 (69 bp) of the zebrafish *cmlc*2 were included, and the plasmid construct pICMLE which the 1.6 kb fragment was fused with GFP cDNA to form a cassette. This cassette was flanked on both sides by the 145 bp inverted terminal repeats derived from an adeno-associated virus (AAV-ITR). This plasmid was linearized with *Not*I and microinjected into the one-cell fertilized eggs of zebrafish at a concentration of 25 ng/nL. The procedures for microinjection and germ lines screening were described in the previous report.[5] Embryos derived from F2 heterozygotic transgenic lines, A26 and A277, which represented the low and high levels of GFP expression, respectively, were selected for this study.

Quantification of GFP intensity by software

Zebrafish 4 dpf embryos derived from transgenic lines were fixed with 4% paraformaldehyde in 1X phosphate buffered saline for 16 hours. Hearts were incubated directly into optimal cutting temperature compound without washing, and were serially cryosectioned with a thickness of 10 μm (MICROM HM5000). The sections were observed and photographed under a fluorescent microscope (MZ FLIII; Leica Microsystems, Germany) and a Nomarski microscope (DMR; Leica Microsystems).

The software "photoshop 5.0" (Adobe) was used to quantify the GFP intensity shown on the digital photograph. When the region of interest for the green heart was set, the option "histogram" in the command list was selected. The channel was changed to "green" and there were 255 green color scales in this system. Each pixel was selected to fit the suitable level of green color. The numbers of pixels in the region of interest were automatically calculated. The sum of green level for each pixel represented the GFP intensity of the picture.

Evaluation of morphology and function of zebrafish hearts

Zebrafish embryos of 4 dpf and 8 dpf derived from transgenic lines were anesthetized with 2-phenoxyethanol 70 μL/200 mL. The fish were observed with an Axioplan microscope (Zeiss, Germany) with Nomarski optics and fluorescence. Images were captured with a Sony digital camera using the Ulead VedioStudio 4.0 SE software. The cardiac images were recorded as audio video interleave (AVI) files for 30 seconds and stored in computer. Then the AVI file was converted to bmp files sequentially according to the method described previously.[9] One bmp file contained one frame of the cardiac motion. The cardiac motion was viewed frame by frame to define the systolic and diastolic phase. The ventricular volume was calculated using the formula for a prolate spheroid:[10]

$$V = \frac{4}{3} \times \pi \times a \times b^2$$

Where a and b are the long and short radii, respectively (one-half of the measured long and short axes) of the ventricle (Figure 1). The stroke volume (SV) and cardiac output (CO) were calculated as:

$$SV = (\text{end-diastolic volume} - \text{end-systolic volume})$$

$$CO = SV \times \text{heart rate}$$

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical differences among the groups were obtained by using the Student's *t* method. It was considered statistically significant if $p < 0.05$.

Results

Transgenic germ lines of zebrafish with GFP-tagged hearts

When the *Not*I-cut pICMLE fragments were micro-injected into one-cell fertilized eggs of zebrafish, we found 324 founders that displayed heart-specific
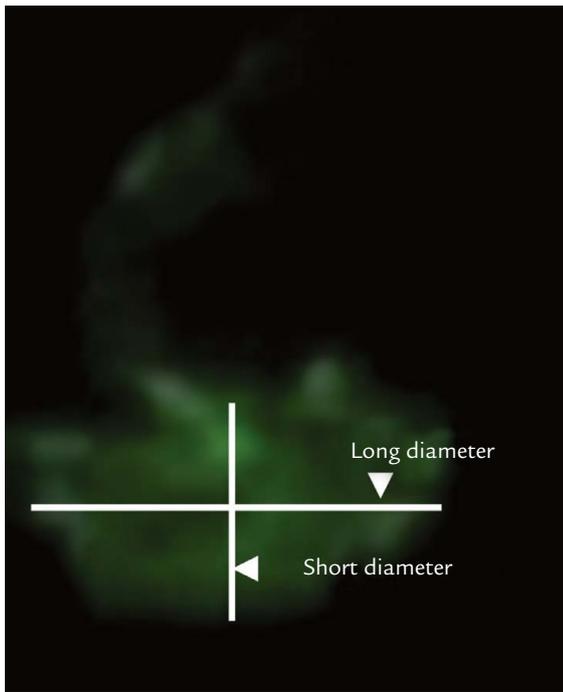

Figure 1. The ventricle of zebrafish is prolate spheroid in shape. The long axis was measured as the longest transverse distance of the ventricle. The short axis was measured as the longest vertical distance of the ventricle. The cardiac motion was viewed frame by frame to define the systolic and diastolic phase.

GFP expression. Among them, 37 fish were produced siblings that expressed GFP specifically in hearts. Two lines were selected for this study: A26 and A277, in which the expression of GFP-labeled hearts of F2 heterozygotic transgenic fish were at the low and high level, respectively. The GFP intensity of A277 was 1.7-fold more than that of A26 (Figure 2).

As one of transgenic lines described by Huang et al,[5] green fluorescence shown in A26 and A277 was intensively and specifically expressed in the myocardial cells located both around the heart chambers and the atrioventricular canal. Neither the epicardium nor the endocardium showed fluorescent signals. The GFP expression in the transgenic line faithfully recapitulated with the spatial and temporal expression of the endogenous *cmlc*2.

Cardiac function and morphology of zebrafish hearts with two different expressional levels of GFP

The cardiac function and morphology of the 4-dpf embryos derived from lines A26 and A277 were similar (Table 1). However, the cardiac growth of 8-dpf embryos seemed to be impaired in the A277 group, which had a high expression level of GFP in heart. In addition, the heart rate, SV, and CO were also lower in the 8-dpf embryos of the A277 group, compared with those in A26 embryos (Table 2).
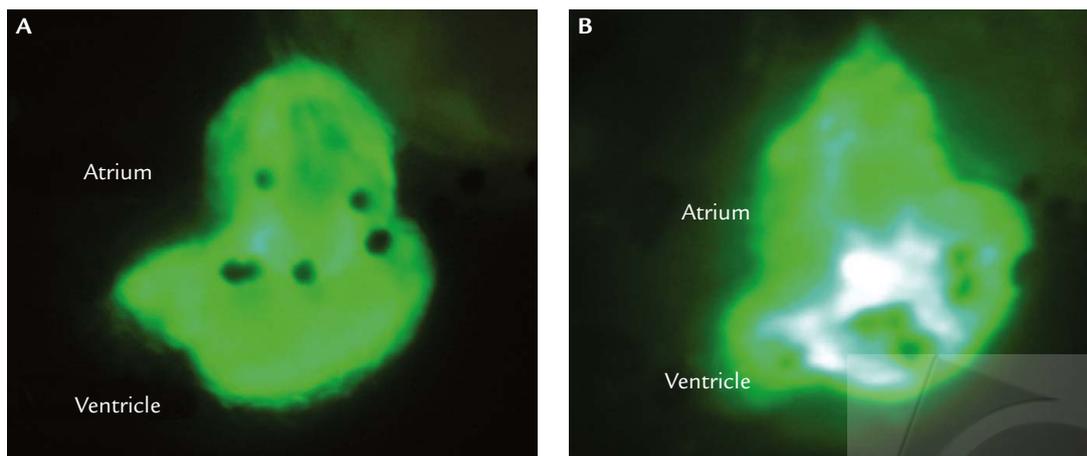
Figure 2. Two different lines of green fluorescent protein transgenic zebrafish were bred: (A) A26; (B) A277. The luminance of A277 was 1.7-fold brighter than that of A26.

Table 1. Cardiac morphology and function of the 4-days-postfertilization zebrafish embryos derived from two different expression levels of GFP

	A277 ($n = 10$)	A26 ($n = 10$)	p
Diastolic			
Long diameter (μm)	187 ± 17	174 ± 20	NS
Short diameter (μm)	103 ± 9	96 ± 10	NS
Systolic			
Long diameter (μm)	158 ± 18	146 ± 20	NS
Short diameter (μm)	80 ± 11	75 ± 7	NS
End-diastolic volume (μm³)	131,492 ± 29,537	106,970 ± 29,666	NS
End-systolic volume (μm³)	66,603 ± 19,580	53,986 ± 14,857	NS
Stroke volume (μm³)	64,890 ± 14,911	52,984 ± 18,327	NS
Heart rate (beats/min)	148 ± 18	140 ± 18	NS
Cardiac output (μL)	0.01 ± 0.002	0.007 ± 0.003	NS

Table 2. Cardiac morphology and function of the 8-days-postfertilization zebrafish embryos derived from two different expression levels of GFP

	A277 ($n = 10$)	A26 ($n = 10$)	p
Diastolic			
Long diameter (μm)	220 ± 16	241 ± 15	< 0.05
Short diameter (μm)	104 ± 13	131 ± 16	0.001
Systolic			
Long diameter (μm)	188 ± 15	204 ± 14	< 0.05
Short diameter (μm)	81 ± 8	111 ± 17	< 0.001
End-diastolic volume (μm³)	159,597 ± 39,905	277,769 ± 78,301	0.001
End-systolic volume (μm³)	82,177 ± 20,407	169,450 ± 58,631	0.002
Stroke volume (μm³)	77,420 ± 26,126	108,317 ± 34,173	< 0.05
Heart rate (beats/min)	138 ± 22	182 ± 18	< 0.001
Cardiac output (μL)	0.01 ± 0.004	0.02 ± 0.005	0.002

Discussion

Zebrafish have a greater potential than other species as an experimental model for heart-related research for the following reasons: (1) their transparent embryos make it easy to observe organogenesis; (2) zebrafish have a 2-chambered heart that is similar in many ways to the 3- or 4-chambered heart in higher vertebrates;[11] (3) zebrafish hearts develop very rapidly and begin beating at 22 hpf, and formation with heart-tube looping is complete at 36 hpf;[12] (4) we can continuously observe the embryos' development and the reporter gene's expression at any stage without sacrificing fish samples; (5) zebrafish embryos can survive even without a functional cardiovascular system, making it possible to analyze cardiac defects without having to deal with the confounding context of dying embryos;[13] and (6) researchers have access to numerous cardiac morphogenetic and functional mutants as well as large-scale mutant screens.[14,15] Studies using zebrafish for cardiac

research have recently increased in number. These studies include those on the development and regeneration of hearts.[1,11,16,17] Zebrafish could also be used as a screening test for drug-induced repolarization abnormalities.[18]

Immunohistochemical detection, such as that accomplished by using MF-20 monoclonal antibody against myosin heavy chain, is commonly used to identify cardiovascular cells and to screen mutated hearts.[19–21] However, this antiserum is not originally for fish antigen and is also not highly cardiac specific. In addition, immunochemical work is laborious, costly, and cannot be dynamically done *in vivo*. Therefore, heart-specific green fluorescent zebrafish would be valuable as research models for tracing the development of heart, finding new heart-specific genes and functions, establishing biological indices of environmental pollutants, and studying the efficacy of therapeutic drugs.[5] GFP has been previously reported to be biologically inert.[22] However, excess cardiac expression of GFP in mice caused heart failure.[6] In the present study, 8-dpf cardiac function deteriorated and cardiac growth was retarded in the A277 line. Excess expression of GFP seemed to exert some detrimental effects on zebrafish hearts. Atmospheric oxygen is required for fluorescence to develop by GFP.[22,23] This process consumes oxygen and the consequence of oxidation by O_2 is hydrogen peroxide (H_2O_2).[24] The H_2O_2 is presumably released in 1:1 stoichiometry with mature GFP.[24] This byproduct might explain occasions when high-level expression of GFP can be deleterious. H_2O_2 stimulation induces c-Jun N-terminal kinase1 activation and apoptosis in rat cardiomyocytes.[25] Myocardial contractility is impaired by exposure to H_2O_2.[26,27] In the present study, SV and CO were lower in zebrafish with excess green fluorescence. The ventricular size (long and short diameter) was also smaller in this group. H_2O_2-induced apoptosis has been reported to affect the development of cardiomyocytes and this effect could be suppressed by growth hormone.[28] Our data were compatible with these cell culture results.

There were several limitations in our study. First, there were no zebrafish without GFP to serve as the control group. However, *in vivo* observation of cardiac function is impossible in zebrafish without GFP. Second, only two densities of GFP expression were described in this study. Therefore, no definite safety limit of GFP expression for cardiac development in zebrafish was determined in this study.

In conclusion, excess expression of GFP seems to exert some detrimental effects on developing zebrafish hearts.

Acknowledgments

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