The Effect of *Jazf*1 Overexpression in Zebrafish Cardiac Development

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ABSTRACT

***JAZF*1 (Juxtaposed with Another Zinc Finger gene 1) transcription factor are Zn-finger proteins that bind to the nuclear orphan receptor TAK/TR4 (Nakajima *et al.*, 2004). The nuclear orphan receptor TAK1/TR4 functions as a positive as well as a negative regulator of transcription. It was recently reported that congenital cardiovascular malformations are significantly more frequent in Neurofibromatosis 1 (NF1) patients with microdeletion syndrome than in those with classical NF1. JAZF1 was expressed in adult heart of patients with microdeletion syndrome. JAZF1 is highly conserved among various species include zebrafish.**

We hypothesized that the expression of zebrafish *Jazf*1 may lead to severe forms of congenital heart disease that allow the survival of newborns and adults. In this study, we created *Jazf*1 transgenic zebrafish which over-express zebrafish *Jazf*1 cDNA under control of the CMV promoter. Our results suggested that *Jazf*1 expression may play an important role in zebrafish cardiac development.

(Key words : *Jazf*1, zebrafish, cardiac development, heart looping)

INTRODUCTION

Heart development requires a precise and extremely complex series of molecular mechanisms to ensure the proper expression of cardiac transcription factors, and alterations in their expression can result in heart defects (Srivastava and Olson 2000; Clark *et al*. 2006; Nemer 2008). Congenital heart diseases are the most commonly observed human birth defects and are the leading cause of infant morbidity and mortality (Hoffman *et al*., 2002; Clark *er al*., 2006). However, the exact molecular mechanisms of congenital heart disease remain to be elucidated.

Heart development involves a complex genetic regulatory program. These programs designed to ensure the proper temporal and spatial expression of cardiac transcription factors (Srivastava and Olson, 2000). The activity of cardiac transcription factors can be negatively or positively regulated by zinc-finger protein (Svensson *et al*., 2000). Zinc-finger transcription factors are the most numerous family of transcription regulators.

Juxtaposed with another zinc finger gene 1 (JAZF1), a protein with an unknown function, is a basic protein with a molecular mass of 27.1 kDa containing three putative zinc finger motifs. A recent study indicated that JAZF1 acts as a strong repressor of DR1-dependent transcriptional activation by the testicular orphan nuclear receptor 4 (TR4) (Nakajima *et al*., 2004). The TR4 receptor functions as both a positive and negative regulator of transcription and controls many important physiological functions (Hirose *et al*., 1994; Lee and Chang 1995). The expression of TR4 transcripts is widely observed in various mouse tissues, including the central nervous, metabolic, and cardiovascular systems (Young *et al*. 1997; van Schaick *et al*., 2000; Bookout *et al*., 2006; Yang *et al*., 2006). JAZF1 interacts specifically with the ligand-binding domain of TR4 and functions as a TR4-selective cofactor that may play an important role in mediating transcriptional repression by TR4 (Nakajima *et al*., 2004).

Neurofibromatosis type 1 (NF1) microdeletion syndrome is a condition cause by haploinsufficiency of the NF1 gene and contiguous genes (Riva *et al*., 2000; Je-

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nne *et al.*, 2001). It was recently reported that congenital heart disease is significantly more frequent in patients with NF1 microdeletion syndrome than in those with classical NF1 (Venturin *et al.*, 2004b; Lin *et al.*, 2000). This syndrome is often characterized by the presence of a more severe phenotype displaying facial dysmorphisms, learning disabilities, mental retardation and cardiovascular abnormalities (Tonsgard *et al.*, 1997; Riva *et al.*, 2000; Venturin *et al.*, 2004b). The outcome of congenital heart disease in NF1 microdeleted patients is probably caused by the haploinsufficiency of one or more of the genes in the deletion interval. The 1.5 Mb NF1 microdeletion includes twelve genes for cardiovascular malformation. Only two genes, JAZF1 and Centaurin-alpha 2 (CENTA2) genes, are expressed in adult heart (Whitley *et al.*, 2002). JAZF1 was expressed in adut heart of patients with NF1 microdeletion syndrome. Human JAZF1 gene is highly conserved among various species include zebrafish. We hypothesized that the ectopic expression of zebrafish *Jazf*1 gene may lead to severe forms of congenital heart disease that allow the survival of newborns and adults in zebrafish.

In this study, we focused on the role of *Jazf*1 in cardiac development and heart ability in zebrafish. We *Jazf*1 overexpression zebrafish which express zebrafish *Jazf*1 cDNA under control CMV promoter.

MATERIALS AND METHODS

Cloning and Construction of Vector

PCR was carried out using mouse 13.5 day post-coitus (dpc) embryo cDNA and oligonucleotide primers designed to optain the open reading frame of the mouse *Jazf*1 gene. The primer sequences were forward primer *Jazf*1-F; 5′-AGC ACC ATG ACA GGC ATC G-3′ and reverse primer *Jazf*1-R; CAG CAC GCA ACT GCT GCA T-3′. The PCR for *Jazf*1 yielded a product of 745 bp. The *Jazf*1 ORF was sub-cloned into the pGEM-T vector (Promega, Madison, WI, USA) and pEGFP-N1 vector (Clontech, Palo Alto, CA, USA). All cloned cDNA vectors were confirmed by restriction enzyme disestion and DNA sequencing.

Microinjection of Mouse *Jazf*1 Gene into Zebrafish Embryos

The entire coding region of mouse *Jazf*1 cDNA was amplified by PCR and inserted into a pCS2+ vector. Capped mRNAs for *Jazf*1 were transcribed from linearized pCS2+ containg *Jazf*1 cDNA templates with SP6 RNA polymerase *in vitro* transcription kit (Roche, Mannheim, Germany) with mtG(5′)ppp(5′)G cap analog according to the manufacturer's instructions. Capped mRNAs for *Jazf*1 were injected at a concentration of 100 ng/ul into yolk cells direct beneath the blastomere at one-cell stage.

Imaging

Embryos were examined with Zeiss Axioskop and Zeiss imager Z1 and photographed with a Zeiss Axiocam HRc. Images were processed using Zeiss Axiovision and Adove Photoshop software (Adove System, San Jose, Caliponia, United States).

Data Analysis

Statistical analyzes were performed by one-way analysis of variance (ANOVA) or Student's *t*-test, using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA). Data are presented as means±SEM from at least three independent experiments. Statistical significance was set at $p<0.05$.

RESULTS

Protein Alignment of *Jazf*1 from Four Species

To predict the function of *Jazf*1, we performed amino acid sequence analysis using the EMBL-EBI ClusterW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) program. Protein alignment revealed that *Jazf*1 is highly conserved among Homo sapiens, *Mus musculus*, and *Rattus norvegicus*, with an average sequence identity among these three mammalian species of >99%. In contrast to general perceptions, *Jazf*1 is not exclusive to mammals, and a homolog of mammalian *Jazf*1 was also found in Danio rerio (Fig. 1).

*Jazf*1 Overexpression Vector Construct

*Jazf*1 gene amplified from E13 mouse embryo cDNA using PCR.

The primers used for tail biophy PCR are indicated as arrows. The 1052bo bands specific to the *Jazf*1 transgene are indicated (Fig. 2).

Randomization of Heart Looping in *Jazf*1 Overexpression Zebrafish Embryos

Because of *Jazf*1 is conserved between mouse and zebrafish, we microinjected *Jazf*1 overexpression vector construct into zebrafish embryos. Overexpression of *Jazf*1 results in heart looping abnormalities in zebrafish (Fig. 3).

During heart formation, zebrafish heart is come to be looped like S-shape, and ventricle is positioned to the left and atrium is positioned to the right on frontal views in wild-types at about 48hpf (Fig. 3A; yellow dash lines), but *Jazf*1 overexpression heart was not accomplished this developmental process. It showed linear or reverse shape, which indicated that the looping

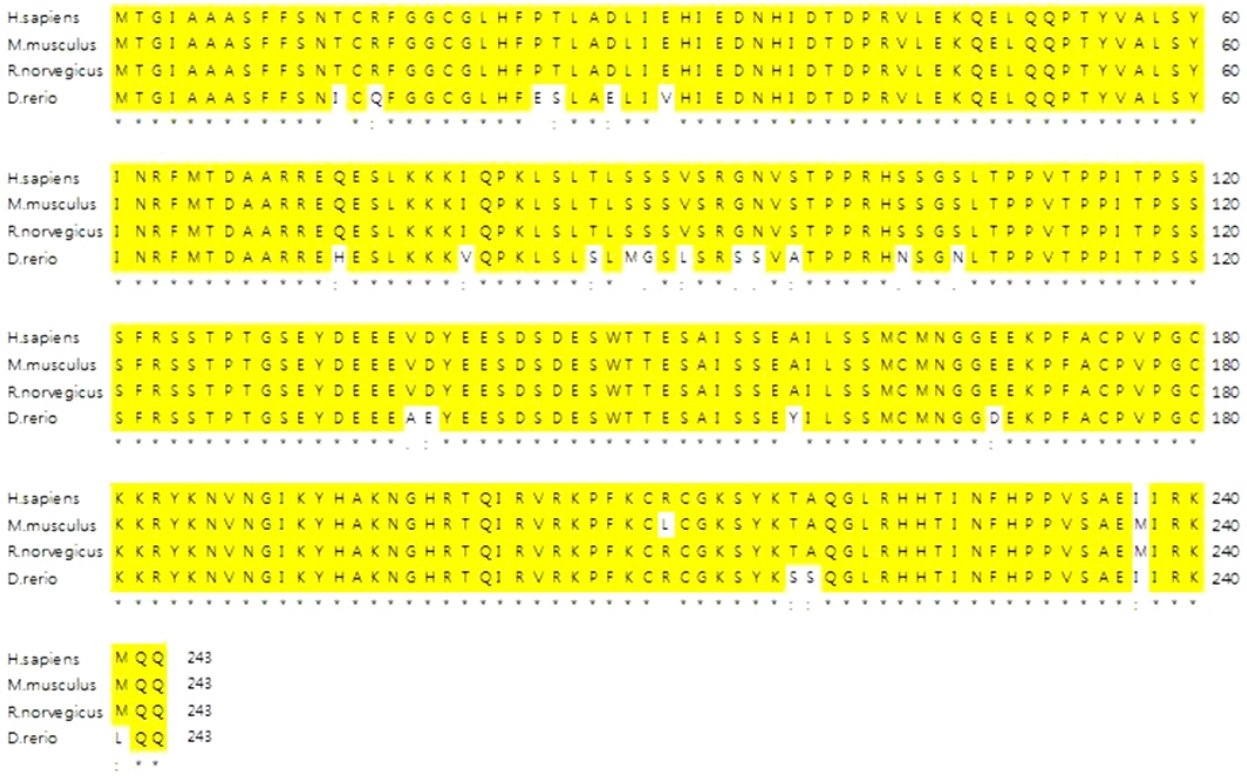


Fig. 1. Protein alignment of *Jazf*1 from four species. Sequences were obtained from GenBank entries and were aligned using the EMBL-EBI ClusterW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) program. *Jazf*1 is highly conserved among homo sapiens, *mus musculus*, *rattus norvegicus* and *danio rerio*.

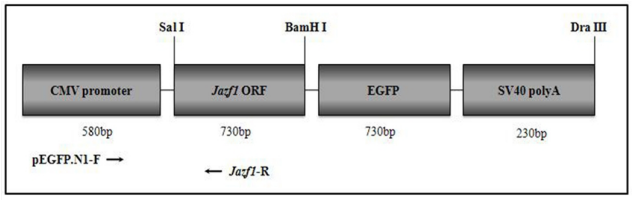


Fig. 2. Cloning and construction of vector. The primers used for tail biopsy PCR are indicated by arrows.

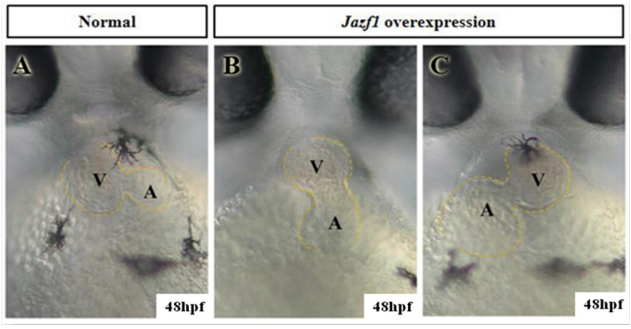


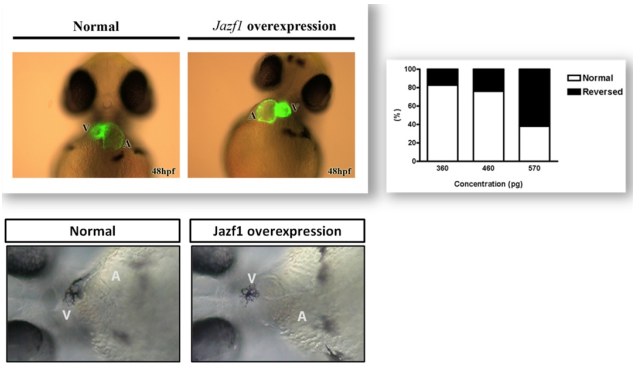
Fig. 3. Effects of *Jazf*1 overexpression on zebrafish embryos. Overexpression of *Jazf*1 results in heart looping abnormalities. At 48 hpf (A~C), compared to the clearly looped heart (S shapes) of wild-type embryos (A), the heart of *Jazf*1 overexpression embryos (B, C) remained central and reversal in a tubular structure. A, atrium; V, ventricle.

process is defected by *Jazf*1 overexpression gene during cardiac development (Fig. 3B, C; yellow dash lines). For further clear observation, we generated a *Tg(cmlc2:GFP)* /*Jazf*1 overexpression transgenic zebrafish model. The GFP expression in developing heart distinctly showed the S-shape heart morphology in wild-type, but mutants displayed reverse heart morphology (Fig. 4) similar to the optical observation.

*Cmcl*2 is functional gene for normal differentiation and roles of the myocardial cells. (Chen *et al*., 2008).

DISCUSSION

Patients with microdeletion syndromes often have congenital heart defects (Gelb, 2001). It was recently reported that congenital heart disease is also significantly found in Neurofibromatosis type 1 (NF1) microdeleted patients. The structural heart malformations in the NF1 deleted patients included pulmonic stenosis, atrial/ventricular septal defects, valve defects, hypertrophic cardiopathy and patent ductus arteriosus (Venturin *et al*., 2004b; Dorschner *et al*., 2000; Tonsgard *et al*., 1997). JAZF1 was expressed in adult heart of patients with NF1 microdeletion syndrome. Heart development in-



Injection	N	Amount (pg)	Heart looping		Die
			Normal	Reversed	
1	27	460	19(76.0%)	6(24.0%)	2
2	44	570	11(37.9%)	18(62.1%)	15
3	51	360	43(87.8%)	6(12.2%)	2
4	34	360	24(75.0%)	8(25.0%)	2
Total	156		97(71.9%)	26(28.1%)	21
Control (normal)	141		132(93.6%)	9(6.4%)	

Fig. 4. Randomization of heart looping in *Jazf*1 overexpression zebrafish embryos. Overexpression of *Jazf*1 results in heart looping abnormalities. At 48 hpf, compared to the clearly looped heart (S shapes) of normal embryos, the heart of *Jazf*1 overexpression embryos remained central and reversal in a tubular structure. Fluorescence images of normal and *Jazf*1 overexpression heart using *Tg(cmlc2:GFP)* transgenic fish. At 48 hpf, the fluorescence expression presented that the normal heart is the S-shape, but, eventually, *Jazf*1 overexpression heart displays the reverse S-formed morphology. A, atrium; V, ventricle; N, number.

volves a complex genetic regulatory program. These programs designed to ensure the proper temporal and spatial expression of cardiac transcription factors (Srivastava and Olson, 2000). The activity of cardiac transcription factors can be negatively or positively regulated by the zinc-finger protein (Svensson *et al.*, 2000). Zinc-finger transcription factors are the most numerous family of transcription regulators. JAZF1 transcription factors are Zn-finger proteins that bind to the nuclear orphan receptor TAK1/TR4. The nuclear orphan receptor TAK1/TR4 functions as a positive as well as a negative regulator of transcription. We hypothesizes that the expression of JAZF1 may lead to severe forms of congenital heart disease. JAZF1 is highly conserved among various species include mouse. So, we focused on the role of *Jazf*1 in cardiac development and heart ability in zebrafish.

In this study, we created *Jazf*1 overexpression zebrafish which express mouse *Jazf*1 cDNA under control of the CMV promoter. Our results suggested that *Jazf*1 expression may play an important role in mouse cardiac development and heart function.

In summary, in this study we describe the identification of *Jazf*1 as a modulator of cardiac development. Future studies have to determine the role of *Jazf*1 in the heart formation signaling pathway.

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