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Fog1 is required for cardiac looping in zebrafish

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Abstract

To further our understanding of FOG gene function during cardiac development, we utilized zebrafish to examine FOG's role in the early steps of heart morphogenesis. We identified fragments of three *fog* genes in the zebrafish genomic database and isolated full-length coding sequences for each of these genes by using a combination of RT-PCR and 5′-RACE. One gene was similar to murine FOG-1 (*fog1*), while the remaining two were similar to murine FOG-2 (*fog2a* and *fog2b*). All Fog proteins were able to physically interact with GATA4 and function as transcriptional co-repressors. Whole-mount in situ hybridization revealed *fog1* expression in the heart, the hematopoietic system, and the brain, while *fog2a* and *fog2b* expression was restricted to the brain. Injection of zebrafish embryos with a morpholino directed against *fog1* resulted in embryos with a large pericardial effusion and an unlooped heart tube. This looping defect could be rescued by co-injection of mRNA encoding murine FOG-1, but not by mRNA encoding FOG-1 lacking the FOG repression motif. Taken together, these results demonstrate the importance of FOG proteins for zebrafish cardiac development and suggest a previously unappreciated role for FOG proteins in heart looping that is dependent on the FOG repression motif.

Keywords: Transcription; Repression; GATA; FOG; Zfpm2; Zfpm1; Heart; Cardiogenesis; Development; *Danio rerio*

Introduction

The GATA family of transcriptional activators contains 6 members, three of which are expressed predominantly in cells of the hematopoietic system (GATA1, 2, and 3) and three that are expressed predominantly in heart, gut, and lung (GATA4, 5, and 6) (Crispino, 2005; Heicklen-Klein et al., 2005; Molkentin, 2000; Pikkarainen et al., 2004; Sorrentino et al., 2005). There are several reports of mutations within these genes leading to human disease. Mutations in GATA1, for example, lead to familial dyserythropoietic anemia and thrombocytopenia (Mehaffey et al., 2001; Nichols et al., 2000), while mutations in GATA3 lead to HDR (hypoparathyroidism, sensorineural deafness, renal anomaly) syndrome (Nesbit et al., 2004; Van Esch et al., 2000). Some of the reported mutations result in a GATA protein that is unable to bind DNA, but other mutations result in GATA factors with intact DNA binding but impaired binding to their co-factors, the friend of GATA (FOG) proteins (Mehaffey et al., 2001; Nesbit et al., 2004; Nichols et al., 2000). These observations highlight the importance of FOG-GATA interactions for the development of specific organ systems and suggest that FOG proteins themselves may be important in human disease.

GATA factors are also known to be important for cardiac development. Cardiac development is a tightly regulated process requiring the combinatorial interaction of multiple transcription factors in a temporally and spatially restricted fashion (Brand, 2003; Bruneau, 2002; Cripps and Olson, 2002; Fishman and Chien, 1997). Work in flies, fish, and mice has demonstrated that GATA factors are required for proper heart formation and can physically interact with many other transcriptional regulators of cardiac development including Nkx2.5, SRF, MEF2, NFATc, Tbx5, and FOG proteins (Belaguli et al., 2000; Durocher et al., 1997; Garg et al., 2003; Molkentin et al., 1998; Morin et al., 2000; Svensson et al., 1999; Tevosian et al., 1999). This work has provided the

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foundation for understanding the molecular basis of human congenital heart disease. As with GATA1 and GATA3, mutations in GATA4 have been described in humans (Garg et al., 2003). These mutations disrupt GATA4's ability to bind to DNA and interact with the transcription factor Tbx5. Patients with such mutations have defects in cardiac morphogenesis characterized predominantly by atrial septal defects.

Like the GATA family, members of the FOG family of transcriptional co-factors are expressed in the heart during development and are required for proper heart formation across species from fruit flies to mice (Cantor and Orkin, 2005). All FOG proteins are multi-type zinc finger proteins that are characterized by their ability to bind to GATA factors and co-activate or co-repress transcription of target genes (Holmes et al., 1999; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Tsang et al., 1997). In mice and humans, there are two FOG genes, FOG-1 and FOG-2. Mice with a targeted disruption of FOG-1 die at embryonic day (ED) 11.5 of severe anemia secondary to a block in the differentiation of erythrocytes, making it difficult to ascertain the role of FOG-1 in cardiovascular development (Tsang et al., 1998). However, an endothelial lineage-specific disruption of the FOG-1 gene results in mice that die at ED 14.5 of congenital heart defects that include double outlet right ventricle, a common AV valve, and ventricular and atrial septal defects, demonstrating the importance of FOG-1 for cardiac development (Katz et al., 2003). Mice with a targeted disruption in the FOG-2 gene also die in mid-gestation of congenital heart malformations that include defects similar to those seen in the FOG-1 endothelial-specific disruption (double outlet right ventricle, a common AV valve, ventricular, and atrial septal defects) as well as left ventricular wall hypoplasia, and the failure to form coronary arteries (Svensson et al., 2000b; Tevosian et al., 2000). Together, these results suggest the importance of both FOG-1 and FOG-2 for murine cardiac development.

Although mutations in the FOG genes might be predicted to cause congenital heart disease, there is only one report to date of congenital heart disease associated with sequence variations in human FOG genes (Pizzuti et al., 2003). Interpretation of this work is hampered in part by incomplete knowledge of the important functional domains of FOG proteins. Such functionally important domains should be evolutionally conserved in species as diverse as mice and zebrafish. To identify such domains, and to provide a better foundation for understanding the molecular basis of congenital heart disease, we sought to identify and characterize *fog* genes in the *Danio rerio* genome. One of these genes, *fog1*, is similar to murine FOG-1 and expressed in the developing heart, intermediate cell mass (ICM), and nervous system of zebrafish embryos. The other two genes, *fog2a* and *fog2b*, are similar to murine FOG-2, and both are expressed in the developing brain. All zebrafish Fog proteins can bind to GATA4 via highly conserved zinc finger motifs and repress GATA-mediated transactivation of a cardiac-restricted promoter. Zebrafish depleted of Fog1 using an antisense morpholino develop congenital heart disease, with the developing heart tube failing to undergo cardiac looping. This phenotype could be rescued by overexpression of murine FOG-1 but not by a mutant version of FOG-1 lacking another conserved domain, the FOG repression motif, demonstrating the importance of this domain for Fog function in vivo.

Materials and methods

Zebrafish strains and care

The zebrafish strain used in this work was *AB. Zebrafish were cared for in accordance with the policies of the animal resources center at the University of Chicago. Zebrafish embryos were staged as described (Kimmel et al., 1995).

Isolation of fog cDNAs

Partial sequences encoding the C-terminus of three *fog* genes in zebrafish were obtained by a BLAST search of the zebrafish genomic database using the murine FOG-1 and FOG-2 protein sequences. To isolate the 5′ end of each *fog* cDNA, we first isolated total RNA from 96 h post-fertilization (hpf) zebrafish embryos using Trizol (Invitrogen, Carlsbad, CA). Next, rapid amplification of cDNA ends (RACE) was performed using a commercially available kit (CLONTECH, Palo Alto, CA) to isolate cDNA from the 5′ end of each *fog* gene. RACE products were subcloned into pGEM-T Easy (Promega, Madison, WI) and ten RACE clones per *fog* gene were sequenced to identify the longest transcripts. The full-length coding sequence for each of these transcripts was deposited into Genbank with accession numbers DQ015975 for *fog1*, DQ015976 for *fog2a*, and DQ015977 for *fog2b*. Expression vectors for each of the *fog* cDNAs were constructed by using the RT-PCR and primers (*fog1*, 5′-CGGGATCCTCAGGGTTCTCGTG-TTTACTGTGG and 5′-CGGAATTCCTAAACTGTGGGAATAGGTCAGCG; *fog2a*, 5′-CGGGATCCAGATACAGATACACACACGCGTGC and 5′-CCGCTCGAGAAGTGCTCCAGTAGCTAATGTCGG; *fog2b*, 5′- CGGAATT-CAGTTGTGCTGCTGCAGCTCTACGG and 5′-ATTTGCGGCCGCTG-CAGTTGTCAAGGGTGGACAACG) to amplify the entire coding region of each *fog* gene. These fragments were then inserted into the BamHI/EcoRI, BamHI/XhoI, or EcoRI/NotI sites of pcDNA3, respectively. All constructs were verified by sequencing.

In vitro binding reactions

The BL21 strain of *E. coli* was transformed with an expression construct encoding glutathione-S-transferase (GST) fused to the zinc fingers of murine GATA4 as described previously (Svensson et al., 1999). Fusion protein was purified using glutathione sepharose beads and incubated with in vitro translated, ^{35}S-labeled Fog proteins in binding buffer (150 mM NaCl, 50 mM Tris, pH7.5, 0.1% NP-40, 1 mM βME, 10 mM $ZnSO_4$, 0.25% BSA) as described previously (Svensson et al., 1999). Resultant complexes were washed extensively, resolved by SDS-PAGE, and visualized by autoradiography.

Transfections

NIH 3T3 fibroblasts were transfected using Superfect reagent (Qiagen, Valencia, CA) following the manufacturer's protocol. Briefly, 1.5×10^5 cells were plated onto 12-well plates 18 h prior to transfection. On the day of transfection, 1.5 μg of plasmid DNA was incubated with 3 μg of Superfect reagent in 75 μl of OptiMEM for 10 min, and then 400 μl growth media was added, and the entire mixture was applied to the washed cells for 3 h at 37°C, 5% CO_2. Following this incubation, cells were washed with PBS, and 1 ml of growth media was applied. Forty-eight hours after transfection, cells and media were harvested. Cell lysate was assayed for protein concentration (BioRad, Hercules, CA) and for β-galactosidase activity (Promega) using commercially available reagents. Corrected β-galactosidase activity was calculated by dividing the measured β-galactosidase activity by the protein concentration of the lysate. Human growth hormone concentration in the cell media was determined using an ELISA (Roche, Indianapolis, IN). Relative promoter activity was calculated by dividing the growth hormone concen-