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The Cardiac Transcriptome and Dilated Cardiomyopathy Genes in Zebrafish

Running title: *Shih et al.; Zebrafish Cardiac Transcriptome and DCM Genes*

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Abstract:

Background - Genetic studies of cardiomyopathy and heart failure have limited throughput in mammalian models. Adult zebrafish have been recently pursued as a vertebrate model with higher throughput, but genetic conservation must be tested.

Methods and Results - We conducted transcriptome analysis of zebrafish heart and searched for fish homologues of 51 known human dilated cardiomyopathy (DCM)-associated genes. We also identified genes with high cardiac expression and genes with differential expression between embryonic and adult stages. Among tested genes, 30 had a single zebrafish orthologue, 14 had 2 homologues, and 5 had 3 or more homologues. By analyzing the expression data on the basis of cardiac abundance and enrichment hypotheses, we identified a single zebrafish gene for 14 of 19 multiple-homologue genes and 2 zebrafish homologues of high priority for *ACTC1*. Of note, our data suggested *vmhc* and *vmhcl* as functional zebrafish orthologues for human *MYH6* and *MYH7*, respectively, which are established molecular markers for cardiac remodeling.

Conclusions - Most known genes for human DCM have a corresponding zebrafish orthologue, which supports the use of zebrafish as a conserved vertebrate model. Definition of the cardiac transcriptome and fetal gene program will facilitate systems biology studies of DCM in zebrafish.

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Key words: dilated cardiomyopathy, transcriptome, fetal gene program, zebrafish

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Introduction

The identification of *DMD* (*Duchenne muscular dystrophy*) and *ACTC1* (*Actin, alpha cardiac muscle 1*) as the first causative genes for X-linked and autosomal-dominant dilated cardiomyopathy (DCM), respectively, opened the door for genetic studies of idiopathic heart failure.[1,2] Although mutations in dozens of genes have been linked to DCM,[3] current genetic testing panels only have a 37% yield.[4] Therefore, the discovery of novel DCM genes and/or modifier genes for cardiomyopathy remains a major challenge. The rapid advance of genomic technologies, such as genomewide linkage analysis, genomewide association studies, whole-exome sequencing, and RNA sequencing (RNA-seq), have accelerated the discovery process. Several candidate genes typically result from such studies, which necessitates further experimental validation and mechanistic studies. Mouse is the most prevalent animal model. However, the high cost of mouse and other mammalian models imposes substantial restrictions. More affordable alternative vertebrate animal models with higher throughput are desirable.

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Zebrafish (*Danio rerio*) is a nonmammalian vertebrate model widely used to study developmental genetics and functional genomics. Genetic resources and tools with higher throughput create novel research opportunities for cardiomyopathy, including 1) the capacity to conduct large-scale mutagenesis screening using as mutagens N-ethyl-N-nitrosourea,[5] virus,[6] or transposons[7]; 2) the complete sequence of the zebrafish genome; 3) the development of TILLING technology (targeting induced local lesions in genomes), which has been used to generate mutations for about half the genome (http://www.sanger.ac.uk/Projects/D_rerio/zmp/)[8]; 4) morpholino technology, which can be used to quickly knock down genes during embryogenesis[9]; and 5) genome editing technology using TALENs (transcription activator-like effector nucleases) and/or CRISPRs (clustered regularly interspaced short palindromic repeats),

which can be used to generate targeted gene knockouts, conditional gene knockouts, large-fragment genome deletions, or gene knockins.[10]

The positional cloning of *titin* and *tnnt2* as causative genes for *pickwick* and *silent heart*, 2 embryonic lethal mutants identified from an N-ethyl-N-nitrosourea–based mutagenesis screen, initiated efforts to apply zebrafish as a model for studying human cardiomyopathy.[11,12] Additional studies in zebrafish embryos have been conducted to annotate functions of known cardiomyopathy genes, such as *actn2*, *myh*, *myl*, and *lamn*,[13-15] to discover new cardiomyopathy genes such as *nexilin*, *lama4*, and *ilk*,[16-18] to mimic the cardiac remodeling process,[19] and to screen compounds of potential therapeutic value.[20] However, fish embryos cannot be used to recapitulate the full spectrum of human cardiomyopathy, a disease progressing from an initial compensated phase to a later decompensated stage. Accordingly, adult zebrafish models for cardiomyopathy have been recently pursued. Cardiomyopathy-like phenotypes have been reported in adult fish stressed by either chronic anemia and/or doxorubicin.[21,22] However, there are still concerns about the conservation of zebrafish cardiomyopathy models, and gene-based adult models have yet to be generated.

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Toward the goal of establishing adult zebrafish as a vertebrate model for large-scale genetic studies of cardiomyopathy, we conducted transcriptome studies of zebrafish heart and assessed the conservation of the zebrafish model by seeking zebrafish orthologues of human DCM-associated genes.[3,23] In mammals, many fetal genes, which are quiescent in the adult stages but can be reactivated during heart failure, have been used as molecular markers for the pathogenesis of cardiomyopathy.[24] Therefore, we also defined the cardiac transcriptome in both larva and adult zebrafish and conducted differential expression analysis between larva and adult heart.

DOI: 10.1161/CIRCGENETICS.114.000702

Materials and Methods

Fish Husbandry

Wild-type WIK fish were used for this study. The study was approved by the Mayo Clinic Institutional Animal Care and Use Committee.

RNA Preparation

Fifty hearts from zebrafish larvae 4 days post fertilization (dpf) were dissected and pooled. This stage was selected because of the maturity of heart structures and the accessibility of the hearts for surgical removal. Five hearts consisting of both atrium and ventricle were dissected and pooled from 6-month-old WIK fish. Muscle samples consisting of both fast-twitch and slow-twitch fibers were dissected from an adult fish. Tissues were homogenized using a mortar and pestle (Fisher Scientific), and total RNA was extracted using TRIzol (Sigma) according to the manufacturer's instructions. RNA quality was assessed using a 2100 Bioanalyzer Instrument (Agilent Technologies) in the Mayo DNA Sequencing Core Facility.

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Preparation of Libraries

RNA libraries were prepared according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina). The liquid-handling EpMotion 5075 robot (Eppendorf) was used for TruSeq library construction. All AMPure bead (Beckman Coulter) clean up, mRNA isolation, end repair, and A-tailing reactions were completed on the 5075 robot. Reverse transcription and adaptor ligation steps were performed manually. The adapter-modified DNA fragments were then enriched by 12 cycles of polymerase chain reaction (PCR) using primers included in the Illumina Sample Prep Kit. Concentration and size distribution of the libraries were determined on an Agilent Bioanalyzer DNA 1000 chip. Sample concentration was confirmed using Qubit fluorometry (Invitrogen). Libraries were loaded onto paired-end flow cells at concentrations of 8

DOI: 10.1161/CIRCGENETICS.114.000702

to 10 pM to generate cluster densities of 700,000/mm^2 following Illumina's standard protocol using the Illumina cBot and cBot Paired-End Cluster Kit version 3. The flow cells were sequenced as 51×2 paired-end reads on an Illumina HiSeq 2000 using TruSeq SBS Sequencing Kit version 3 and HCS v2.0.12 data collection software. Base-calling was performed using Illumina's RTA version 1.17.21.3.

Mapping of Sequence Reads

The generated FASTQ sequence reads from an Illumina HiSeq instrument were aligned to the latest available zebrafish genome assembly (Zv9) by TopHat.[25] At most, 2 mismatches were allowed for the first 32 bases in each alignment. Reads with more than 2 mismatches or that mapped to multiple genomic locations (an alignment score <4) were deleted. In all, 32,677 genes were annotated in Ensembl Zv9. The raw read counts for genes were generated by HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) for further downstream analyses.

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Differential Gene Expression Analysis

For analysis of differential gene expression, we eliminated genes without any reads across all samples. Statistical testing was done using the R package DESeq.[26] Because the primary goal of our analysis was to explore the underlying cause of differentially expressed genes between different tissues, an adjusted *P* value/false discovery rate (to adjust for multiple testing using the Benjamin-Hochberg method) cutoff of 0.01 was used to select significantly changed genes. In addition, we used the following 2 criteria to identify genes differentially expressed in embryo and adult: 1) the number of reads per kilobase per million reads (RPKM) for the gene was ≥0.3 in either the embryonic transcriptome or the adult transcriptome; and 2) the change in the expression level was at least 2-fold.

Functional Annotation Bioinformatics Microarray Analysis (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) was used to assign genes into pathway categories.[27]

Quantitative Reverse Transcriptase PCR

The Superscript III First-Strand Synthesis System (Invitrogen) was used to generate cDNA from 500 ng RNA. Quantitative reverse transcriptase PCR (qPCR) was carried out using a Roche LightCycler 480 QPCR apparatus in 96-well QPCR plates (Roche Diagnostics Corp). The expression of the genes was normalized using the expression level of *gapdh* or *actb2* by –ΔΔCt (cycle threshold) values. Nine RNA samples (3 each for embryo heart, adult heart, and adult muscle) were analyzed in triplicate by qPCR. The primers are listed in Supplemental Table 1.

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Data Analysis

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We used reads per kilobase of transcript per million mapped reads (RPKM) as the normalization method to calculate gene expression in each library of each tissue. In the differential gene expression analysis, the R package DESeq was used to normalize the gene raw read counts by considering both library size and expression distribution. To overcome the overdispersion problem in RNA-seq data, a modified negative binomial distribution model was used.

Results

Definition of Cardiac Transcriptome in a Zebrafish Heart

Using the standard paired-end RNA-seq protocol, we obtained more than 74, 114, and 130 million reads for embryonic hearts, adult hearts, and adult muscles, respectively (Supplemental Table 2). More than 75% of reads could be mapped to the Zv9 zebrafish genome. The reads were highly consistent within the 3 biological repeats for each experimental condition, as indicated by the correlation analysis (Supplemental Figure 1).

DOI: 10.1161/CIRCGENETICS.114.000702

Two previous transcriptome studies used RPKM cutoffs of 3 or greater[28] or 0.3[29] to delineate expression levels in the heart. Using the cutoff of 3 RPKM, 5,345 genes, or 16% of the 32,677 genes in the zebrafish genome, met the criteria in an adult zebrafish heart, and 6,169 genes, or 19% of the genome, met the criteria in the embryonic zebrafish heart. Using the cutoff of 0.3 RPKM, 14,797 genes or 45% of the genome and 15,217 genes or 47% of the genome are expressed in an adult heart and an embryonic heart, respectively. Previously, it has been shown that the 200 most abundant mRNAs in mouse heart, which account for less than 1% of all 25,000 mouse gene transcripts, make up approximately 65% of the total cardiac mRNA pool.[30] In zebrafish, the 200 most abundant genes made up about 66% and 61% of the total mRNA pool in the embryonic heart and the adult heart, respectively.

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Using differential gene expression analysis, we identified 2,795 upregulated genes that exhibit high expression in the embryonic heart but low expression in the adult heart and 3,175 downregulated genes with low expression in the embryonic heart but high expression in the adult heart. Among the upregulated genes, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis identified several related to cell cycle and DNA replication (Supplemental Table 3) ($P<.05$). The downregulated pathways ($P<.05$) included those involved in carbohydrate metabolism (Supplemental Table 3). We also discovered decreased expression of genes in the calcium signaling pathway at 4 dpf, which were activated in adult heart (Supplemental Table 4). Some of these genes were orthologues of known "fetal genes" involved in calcium handling,[31] including *atp2a2a* (orthologue of *SERCA2A*, –3.57-fold change), *slc8a1a* (orthologue of *NCX1*, –2.46-fold change), and *slc8a2a* (orthologue of *NCX2*, –5.62-fold change).

In mammals, the expression of some sarcomeric genes switches from fetal to adult isoforms after birth. Similarly, in zebrafish we detected 25 genes with high expression in the

larval stage that were downregulated at the adult stage (Supplemental Table 5A) and 17 genes with low expression during the embryonic stage that were upregulated at the adult stage (Supplemental Table 5B). qPCR analysis of 11 genes in each group (Supplemental Figure 2A) showed high correlation with the fold changes.

Identification of Zebrafish Homologues of 51 DCM-Associated Genes

Previous human genetic studies suggested 51 genes as causing or conferring susceptibility to inheritable DCM.[3,23,32] Our survey of the orthology data in the zfin Data report (http://zfin.org/downloads/human_orthos.txt) and Ensembl (http://www.ensembl.org/index.html) identified zebrafish homologues for 46 of these genes. For the 5 DCM genes that did not have a clear homologue, we searched either the literature or the NCBI database for further information. Results were found for 3 of these genes. For the human gene *SCN5A*, *scn5laa* and *scn5lab* have been suggested as orthologues in zebrafish[33]; these are currently named *scn12aa* and *scn12ab*, respectively, in Ensembl. Ensembl suggests that *dsc2l* may be the zebrafish orthologue of Desmocollin 2.[34] Although 3 *DSC* genes exist in humans that exhibit tissue-specific expression patterns (*DSC1*, *DSC2*, and *DSC3*), there is only 1 *dsc2l* gene in zebrafish. Although no zebrafish orthologue of phospholamban was suggested from either zfin or Ensembl, a phospholambanlike gene (NM_001201561.1) was annotated in the NIH database (http://www.ncbi.nlm.nih.gov/nuccore) which exhibits 50% protein identity to the human counterpart. Insufficient information was found for the other 2 genes. There are several candidate zebrafish homologues for *FOXD4*, but the closest homologue is *foxd5*. Because the gene is indicated as a potential orthologue with a low confidence score in Ensembl, we consider the zebrafish orthologue of *FOXD4* to remain unclear. Finally, we found no zebrafish orthologue for cardiotrophin 1, which might encode a cytokine that only exists in mammals.

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Of the 49 total zebrafish homologues for human DCM genes that we identified, 30 had a single orthologue in zebrafish. Identities of the proteins encoded by these orthologues ranged from 10.14% to 86.73% (mean, 59%) (Table 1). The other 19 DCM genes had multiple zebrafish homologues (Supplemental Table 6): 14 with 2 homologues and 5 with 3 or more homologues.

Assessment of Cardiac Abundance of the 30 DCM Genes With a Single Orthologue

It is posited that genes with higher abundance in a particular organ must have important roles in this organ. This abundance hypothesis motivated efforts to generate a cardiac expressed sequence tag collection,[35] to define genes with higher expression levels in the heart via RNA-seq analysis,[28] and to enrich cardiac mutant lines via an expression-based strategy.[36] Given that DCM-associated genes have been shown to have pivotal roles in cardiac diseases, we set out to test the abundance hypothesis by assessing the expression level of the 30 genes with a single zebrafish orthologue.

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When RPKM ≥0.3 was used as the cutoff, 28 orthologues (93%) met the criteria in both adult and embryonic heart; *dmd* and *nebl* did not meet the criteria (Table 1). We then compared the expression level of the 30 DCM orthologues with those of the whole genome. The average RPKM value of the orthologues for the 30 DCM genes was significantly higher than that of the whole genome (mean, $1.0\times10^{0.77}$ vs $1.0\times10^{-0.33}$; $P<.001$) in adult heart (Supplemental Figure 3A). Similarly, the average expression level of the orthologues for the 30 genes was significantly higher in embryonic heart than in the whole genome (mean, $1.0\times10^{0.56}$ vs $1.0\times10^{-0.18}$) (Supplemental Figure 3B).

When RPKM ≥3 was used as the cutoff, 19 of 30 genes (57%) were considered highly expressed in adult heart, and 17 of 30 genes (63%) were highly expressed in embryo heart. Together, our expressional analysis of 30 DCM genes supports the abundance hypothesis.

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Moreover, our data support that RPKM ≥0.3 is a reasonable cutoff to define the zebrafish cardiac transcriptome.

Prioritization of Zebrafish Homologues for 19 DCM-Associated Genes With Multiple Homologues

To facilitate future genetic studies of the 19 DCM-associated genes with multiple homologues, we attempted to prioritize the corresponding 59 zebrafish homologues according to their cardiac expression profile. We applied the abundance hypothesis for this prioritization process—among multiple homologues of the same DCM-causative gene, the homologue with higher abundance in the heart is given higher priority. Moreover, we applied a cardiac enrichment hypothesis in our prioritization process—among multiple homologues of the same DCM gene, the homologue exhibiting higher expression in the heart than in the muscle was given higher priority.

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By applying these 2 criteria to 28 homologues for the 14 DCM genes with 2 homologues, we could prioritize 1 homologue over the other for 10 DCM genes (Table 2) if the combined score for these candidate genes was determined to be more than 5, which was our arbitrary cutoff value. However, we could not prioritize homologues for the other 4 DCM genes, *ANKRD1*, *LDB3*, *TMPO*, and *TTN*. In contrast to cardiac expression of *ANKRD1* in human, the expression of both *ankrd1a* and *ankrd1d* was low in zebrafish heart (RPKM<0.3). For *LDB3* and *TTN*, both *ldb3a/b* and *ttna/b* were enriched in the muscle but not in the heart. For *TMPO*, both *tpmoa* and *tpmob* had comparable cardiac and skeletal expression levels. Thus, more experiments are needed to determine if their functions are redundant in DCM pathogenesis.

We then applied the 2 criteria to analyze 31 homologues for the 5 DCM genes with 3 or more homologues (Table 2). Among the 3 homologues of *TNNT2*, only *tnnt2a* scored much higher than the others and thus is recommended. Compared with 3 *TNNI* homologues in

mammals, including *TnI-fast*, *TnI-slow*, and *TnI-cardiac*,[37] there are 14 troponin I homologues in zebrafish, including 2 tandem duplications of *tnni2a.1-4* and *tnni2b1-2*. None of them is assigned as the zebrafish *TNNI3* orthologue in the zfin database, whereas some of them are recommended by Ensembl with low confidence. According to our RNA-seq data, *tnni1b* was the major cardiac isoform with both high abundance and cardiac-enriched expression and thus is recommended. Our data also suggested that *tnni2a.3* and *tnni2b.2* were the 2 major skeletal muscle isoforms. No *ACTC1* orthologue is reported in the zfin database, and only *acta1b* is listed as an orthologue in Ensembl. Our RNA-seq data indicated that *actc1a* was the major actin isoform and *acta1b* was the minor isoform in both embryonic and adult hearts. In contrast, *actc1b* is a skeletal muscle-enriched isoform that accounts for 99.79% of total actin at the mRNA level in adult muscle. Therefore, both *actc1a* and *acta1b* should be considered when studying *ACTC1*-based DCM in zebrafish. qPCR of 10 genes in each condition showed high correlation of the fold changes between the 2 methods (Supplemental Figure 2B) and confirmed the relative expression of *TTNI3* and *ACTC1* homologues by semiquantitative reverse transcriptase PCR (Supplemental Figure 4).

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Prioritization of Zebrafish Myosin Heavy Chain Homologues

Zebrafish have 9 *MYH* homologues. Seven homologues share more than 80% protein identity with *MYH6* and *MYH7*, but *myh7ba* and *myh7bb* share 67% and 66% identities, respectively, with *MYH7* (Supplemental Table 6). In a 4 dpf embryonic heart, *myh6* (also termed *amhc*, *atrial myosin heavy chain*), *vmhc* (*ventricle myosin heavy chain*), and *vmhcl* (*ventricle myosin heavy chain like*) represent the 3 most abundant homologues (12.13%, 31.36%, and 53.36%, respectively), whereas in an adult heart, *vmhcl* contributes to 88% of the total *myh* transcripts. According to the cardiac enrichment hypothesis, *myh6* and *vmhcl* are highly enriched in heart

compared with muscle (Table 2, Figure 1A and B).

Because previous studies indicated that *myh6/amhc* expresses specifically in the atrium, while *vmhc* only expresses in the ventricle,[15] we checked chamber specificity for these promising *myh* homologues (Figure 1C). Consistent with its embryonic expression, *myh6/amhc* retained its atrium-restricted expression pattern and contributed to more than 90% of the myosin transcripts in atrium. *vmhcl* expressed specifically in the ventricle and contributed to more than 90% of the myosin transcripts in the ventricle. In contrast to its abundant expression in the embryonic ventricle, *vmhc* was almost undetectable (less than 0.5%) in either atrium or ventricle of the adult. The expression of *myh7ba* and *myh7bb* were also quite low (less than 5%) in both chambers.

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In humans, both *MYH7* (α-MHC) and *MYH6* (β-MHC) are expressed in myocardium and cause cardiomyopathy when mutated.[38,39] These genes are in tandem on chromosome 14, with *MYH6* located 5.3 kb downstream of *MYH7* (Figure 1D), and their expression is developmentally regulated. *MYH6* is mainly expressed in embryonic heart, whereas *MYH7* becomes the predominant adult isoform.[40] Similarly, mouse *Myh7* and *Myh6* are located in tandem on chromosome 14, with *myh6* located 5.3 kb downstream of *myh7*. Different from human, *Myh7* is mainly expressed in the embryonic rodent heart, whereas *Myh6* becomes the predominant adult isoform.[41]

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In zebrafish, *vmhcl* and *vmhc* are located in tandem on chromosome 2, with *vmhc* approximately 4.4 kb downstream of *vmhcl*. Similar to that in human, *vmhc* mainly expresses in embryo, whereas *vmhcl* becomes the predominant adult isoform. To determine whether *vmhcl* and *vmhc* respond to cardiac stresses, we assessed their expression in doxorubicin-induced cardiotoxicity.[21] *vhmc* expression was significantly activated and peaked at 3 dpi, and *vmhcl* expression was decreased. The ratio of *vmhc/vmhcl* was increased by 25-fold compared with that

in an uninjected fish heart (Figure 2A). Consistent with fetal gene responses in mammals, we also detected gene activation for *nppa* and *nppb*, 2 established molecular markers for cardiac remodeling (Figure 2B), and downregulation of genes in calcium-handling pathways, including *pln, ryr2b, atp2a2*, and *slc8a1a*, after doxorubicin treatment (Figure 2C). Interestingly, *nppa* expression recovered to basal levels at 2 weeks post injection, whereas *nppb* remained activated at least until 3 weeks post injection.

Discussion

Transcriptome Analysis in a Zebrafish Heart

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In this study, we defined the transcriptome of zebrafish heart. Analysis of the expression of zebrafish orthologues for 30 DCM-causative genes showed that more than 60% of the genes are in the top 16% for expression level, and 93% are in the top 45% for expression level. These data provide quantitative support to the abundance hypothesis; that is, genes with higher abundance in a particular organ have more important roles in that organ. Accordingly, the following strategy could be used to improve the efficiency of large-scale genetic studies of DCM. One could initially focus on ≈5,000 genes (≈16% of the genome, identified using RPKM ≥3) as a pilot study, which is predicted to uncover about half of DCM-related genes. If successful, one could extend the study to the additional ≈10,000 genes (≈45% of the genome, identified using RPKM ≥0.3) in an effort to uncover most of the remaining DCM-related genes.

By comparing the cardiac transcriptome of a 4 dpf larval fish and an adult fish, we defined the fetal gene program in a zebrafish heart. Among the approximately 5,000 genes we identified with differential expression, many upregulated genes and their related pathways reflect high cell proliferation in the larval stage. Our pathway analysis also uncovered activated genes in the Hedgehog and the TGF-beta signaling pathway (Supplemental Tables 7 and 8), which have

been implicated in cardiogenesis.[42] Several genes related to calcium handling and muscle contraction were identified, and their mammalian orthologues have been used as molecular markers for cardiac remodeling. Interestingly, we noted downregulated genes in carbohydrate metabolism in the larval stage, which differs from mammals.[43] This discrepancy probably reflects the difference between a zebrafish heart that develops *ex utero* and consumes the yolk composed of lipid and triacylglycerol and a mammalian heart that develops *in utero* and mainly consumes glucose delivered via the circulating blood.

Transcriptome Analysis Facilitates the Prioritization of DCM-Associated Genes in Zebrafish

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Of the 51 human DCM-associated genes, 19 had more than 1 homologue in zebrafish. Because of a genome duplication event in teleost fish,[44] approximately 15% (3,105/20,479) of human genes have more than 1 orthologue in zebrafish. We applied the cardiac abundance hypothesis to assess the expression of different homologues for each DCM-associated gene, which appears to be highly effective. The expression of *actn2, cryabb, desma, scn12ab, synbe2b*, and *tnnt2* contributes to more than 99% of the transcripts within the gene family, which strongly suggests their predominant roles in the heart. We also used the cardiac enrichment hypothesis to prioritize homologues, which partially reflects the subfunctionalization of the duplicated genes. Among the 59 fish homologues, 22 are cardiac-enriched genes and 20 are muscle-enriched genes; many of them exhibit more than 100-fold greater expression levels (Supplemental Table 9). By combining the 2 self-evident hypotheses, our scoring system allows us to effectively prioritize homologues for DCM genes, based on a combined score higher than 5. Although ultimate proof requires evidence from genetic manipulation of each homologue to model DCM pathogenesis, we advise starting with the recommended homologues.

Our systematic analysis of DCM-associated genes underscores the current status of the zfin database and Ensembl. Additional new homologues for the 51 DCM genes may be identified in the future. Some genes may be improperly annotated in the current genome. For example, *actc1b* should be renamed to reflect its identity as a skeletal muscle actin, not a cardiac actin, whereas *acta1a* and *acta1b* should be classified as cardiac muscle actins, not skeletal muscle actins. Combining the evidence from chromosome localization and cardiac expression profiles during development and cardiac stress, our data suggest *vmhcl* and *vmhc* as zebrafish functional orthologues for *MYH7* and *MYH6*, respectively. More experimental evidence is required, however, before they are annotated *myh7* and *myh6*, respectively, since no synteny was found between zebrafish and human (data not shown). Also, *amhc*, not the currently used *myh6* in zfin, is a more proper name for this atrium-specific homologue of *MYH*.

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Limitations

We did not observe developmental expression changes between *nppa* and *nppb* (1.84- and 1.44-fold changes between larval and adult stage, respectively), despite both genes being molecular markers for cardiomyopathy[20-22] and in DOX-induced cardiotoxicity (Figure 2B). This observation could be explained by dynamic expression of *nppa* and *nppb* during cardiogenesis, which peaks at day 2 and then decreases.[20] In the future, transcriptomes should be analyzed at more time points, especially during cardiogenesis. In addition, total RNA was extracted from whole hearts, which contain heterogeneous cell populations. It is plausible that genes with either cell type–specific or tissue-restrictive expression patterns are underrepresented. To address this concern, specific cell types could be isolated by fluorescence-activated cell sorting, which can then be subjected to RNA-seq analysis. Alternatively, single-cell–based RNA-seq technology can be explored.[45]

Zebrafish is a Conserved Model for Studying DCM

Recently, it has been shown that 71.4% (14,623/20,479) of human protein-coding genes have at least 1 related zebrafish homologue and that 82% of human disease-associated genes have a zebrafish counterpart.[46] Here, we demonstrated that 96% of DCM-associated genes have corresponding homologues in zebrafish, supporting our central hypothesis that zebrafish is a conserved nonmammalian model suitable for studying cardiomyopathies. In fact, as a teleost fish, zebrafish is among the lowest-level vertebrate models with a chambered heart structure and conserved cellular layers including epicardium, myocardium, and endocardium.[47]

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Our systematic identification and prioritization of fish homologues of DCM-associated genes will facilitate genetic studies in zebrafish, including the generation of gene-based DCM models. Besides mutants that have already been generated by TILLING, mutants for the remaining genes can be easily generated by using TALEN and/or CRISPR genome editing technology.[10] It is anticipated that the efficient zebrafish model will contribute to the validation of genetic variants discovered in human patients, the elucidation of pathological pathways, and the development of novel therapeutics for DCM.

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DOI: 10.1161/CIRCGENETICS.114.000702

Table 1: Human DCM-Associated Genes (n=30) With 1 Zebrafish Orthologue

Human DCM-Associated Gene		Zebrafish Orthologue	Ensembl Gene ID	Identity	mRNA Expression, RPKM		
					Embryo Heart	Adult Heart	Adult Muscle
ABCC9	The subunit SUR2A of cardiac ATP-sensitive potassium channel	*abcc9*	ENSDARG00000015985	81.67%	3.37	24.61	4.27
BAG3	BCL2-associated athanogene 3	*bag3*	ENSDARG00000039486	32.52%	3.06	37.17	9.09
CAV3	Caveolin 3	*cav3*	ENSDARG00000024141	73.51%	8.50	11.90	20.82
CSRP3	Muscle LIM protein	*csrp3*	ENSDARG00000069975	71.65%	82.04	125.24	0.30
DMD	Dystrophin	*dmd*	ENSDARG00000008487	81.67%	0.37	0.24	7.80
DNAJC19	DnaJ(Hsp40) homolog, subfamily C, member 19	*dnajc19*	ENSDARG00000044420	77.59%	8.05	5.92	12.07
DSC2	Desmocollin 2	*dscl**	ENSDARG00000039677	30.00%	3.61	5.87	4.10
EMD	Emerin	*emd*	ENSDARG00000095774	20.87%	4.37	2.18	1.11
EYA4	Eyes absent homolog 4	*eya4*	ENSDARG00000012397	76.06%	1.49	2.16	12.05
FKTN	Fukutin	*fktn*	ENSDARG00000059437	64.44%	1.84	0.98	0.55
GATAD1	GATA zinc finger domain containing 1	*gatad1*	ENSDARG00000027612	73.23%	2.50	1.18	0.57
ILK	Integrin-linked kinase	*ilk*	ENSDARG00000056964	86.73%	12.24	10.03	4.48
LAMA4	Laminin a-4	*lama4*	ENSDARG00000020785	43.23%	13.40	7.77	6.71
LMNA	Lamin A/C	*lmna*	ENSDARG00000013415	64.91%	1.91	2.31	4.27
LAMP2	Lysosome-associated membrane protein 2	*lamp2*	ENSDARG00000014914	25.25%	10.10	26.71	6.49
MYBPC3	Myosin-binding protein C	*mybpc3*	ENSDARG00000011615	62.72%	198.23	464.24	4.47
MYPN	Myopalladin	*mypn*	ENSDARG00000076485	43.31%	0.22	3.24	8.77
NEBL	Nebulette	*nebl*	ENSDARG00000021200	10.14%	0.00	0.03	0.02
NEXN	Nexilin	*nexn*	ENSDARG00000057317	57.04%	47.98	81.55	23.33
PKP2	Plakophilin-2	*pkp2*	ENSDARG00000023026	54.26%	1.11	3.06	1.07

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DOI: 10.1161/CIRCGENETICS.114.000702

Table 1 (continued)

Human DCM-Associated Gene		Zebrafish Orthologue	Ensembl Gene ID	Identity	mRNA Expression, RPKM		
					Embryo Heart	**Adult Heart**	**Adult Muscle**
PLN	Phospholamban	*cardiac phospholamban-like*	ENSDARG00000069404	50.00%	96.91	290.34	1.91
PSEN1	Presenilin 1	*psen1*	ENSDARG00000004870	53.70%	6.33	2.63	1.13
PSEN2	Presenilin 2	*psen2*	ENSDARG00000015540	71.00%	2.91	1.74	2.50
RBM20	RNA binding protein 20	*rbm20*	ENSDARG00000087769	40.00%	2.62	12.08	6.99
SDHA	Succinate dehydrogenase complex, subunit A, Flavoprotein	*sdha*	ENSDARG00000016721	80.00%	44.39	91.56	44.49
SGCD	delta-Sarcoglycan	*sgcd*	ENSDARG00000009789	72.00%	4.46	2.95	6.86
TAZ	Tafazzin	*taz*	ENSDARG00000041421	60.27%	2.02	3.27	0.92
TCAP	Titin-cap or telethonin	*tcap*	ENSDARG00000007344	40.12%	0.35	141.31	161.93
TPM1	α-Tropomyosin	*tpm1*	ENSDARG00000087402	79.00%	16.84	1.64	78.93
VCL	Metavinculin	*vcl*	ENSDARG00000044968	84.00%	2.98	6.75	7.90

Abbreviations: DCM, dilated cardiomyopathy; RPKM, reads per kilobase per million reads.
[*] All human and zebrafish genes were 1-to-1 orthologues except *DSC2*, in which many human genes had 1 zebrafish orthologue.

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Table 2: Cardiac Abundance and Cardiac Enrichment for Zebrafish Homologues of 19 Human DCM-Associated Genes

Zebrafish Orthologue	Abundance, %	Score*	Cardiac Enrichment	Score†
ACTC1				
acta1a	1.32		128.55	√√√√
acta1b	20.22	√	248.67	√√√√
hm:zewp0073	3.77		157.02	√√√√
actc1a	74.58	√√√	178.41	√√√√
actc1b	0.12		0.00	
ACTN2				
actn2	99.66	√√√√	258.31	√√√√
CABZ01111872.1	0.34		14.23	√√√
ANKRD1				
ankrd1a	57.29	√√	0.08	
ankrd1b	42.71	√√	3.82	√√
CRYAB				
cryaba	0.00		NA	
cryabb	100.00	√√√√√	1.02	
DES				
desma	99.96	√√√√	2.68	√√
desmb	0.04		3.47	√√
DSP				
dspa	35.55	√	0.63	
dspb	64.45	√√√	2.88	√√
FHL2				
fhl2a	72.18	√√√	7.99	√√
fhl2b	27.82	√	9.28	√√
LDB3				
ldb3a	22.08	√	0.38	
ldb3b	77.92	√√√	0.37	
MYH6 and MYH7				
myh6	7.73		1082.61	√√√√√
vmhc	1.10		22.40	√√√
vmhcl	87.87	√√√√	1784.02	√√√√√
CR450736.2	0.00		0.00	
CU633479.5	0.00		0.02	
CU633479.6	0.00		0.00	
si:ch211-24n20.3	0.00		0.00	
myh7ba	3.00		10.62	√√√
myh7bb	0.30		8.73	√√
PDLIM3				
pdlim3a	17.32		13.16	√√√
pdlim3b	82.68	√√√√	6.19	√√
SCN5A				
scn12aa	0.08		1.10	
scn12ab	99.92	√√√√	111.39	√√√√

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Table 2 (continued)

Zebrafish Orthologue	Abundance, %	Score*	Cardiac Enrichment	Score†
SYNE1				
syne1a	69.95	√√√	3.38	√√
syne1b	30.05	√	1.19	
SYNE2				
syne2a	0.78		0.02	
syne2b	99.22	√√√√	2.28	√√
TMPO				
tmpoa	55.88	√√	1.13	
tmpob	44.12	√√	0.57	
TNNC1				
tnnc1a	89.20	√√√√	1639.25	√√√√√
tnnc1b	10.80		1.53	
TNNI3				
tnni1a	0.05		0.55	
tnni1al	0.01		0.01	
tnni1b	68.57	√√√	152.79	√√√√
tnni1c	0.30		0.03	
tnni1d	0.23		0.20	
tnni2a.1	0.03		0.10	
tnni2a.2	1.20		0.23	
tnni2a.3	0.18		0.00	
tnni2a.4	0.01		0.00	
tnni2b.1	0.00		0.01	
tnni2b.2	0.15		0.00	
zgc:112242	2.99		1.59	
zgc:101560	26.26	√	60.47	√√√
si:dkey-206m15.8	0.01		0.01	
TNNT2				
tnnt2a	99.92	√√√√	1427.04	√√√√√
tnnt2b	0.06		0.30	
tnnt2c	0.02		1.08	
TTN				
ttna	57.86	√√	0.26	
ttnb	42.14	√√	0.17	

Abbreviations: DCM, dilated cardiomyopathy; NA, not applicable.

* The abundance is defined as the percentage of expression level for each homologue among all homologues for the same DCM-causative gene (ie, RPKM of 1 homologue/[sum of RPKM of all homologues for the same DCM-causative gene]). The percentage of a homologue more than 20%, 40%, 60%, 80%, or 100% is assigned an abundance priority score of 1 (√) through 5 (√√√√√), respectively.

† The score for cardiac enrichment is calculated by the ratio of RPKM in heart to that in muscle. A ratio more than 2-, 10-, 100-, or 1,000-fold is assigned an enrichment priority score of 2 (√√) through 5 (√√√√√), respectively.

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Figure Legends:

Figure 1: Expressional Analysis of *MYH* Homologues in Zebrafish. A, Heat map of the average expression in $\log_{10}$(reads per kilobase per million reads [RPKM]) for *myh6/amhc, vmhc, vmhcl, myh7ba, myh7bb,* and *myhc4* (muscle-specific *myh*) in embryo heart, adult heart, and adult muscle. B, Validation of the RNA sequencing data in (A) showing expression levels using semiquantitative reverse transcriptase polymerase chain reaction (semi-qPCR). *gapdh* was used as control. C, Expression of *myh6/amhc, vmhc, vmhcl, myh7ba,* and *myh7bb* in adult atrium and ventricle as a percentage of total expression level from qPCR, normalized to the expression of *gapdh*. D, Schematics of genomic region for *MYH6/MYH7* homologues in human, mice, and zebrafish. Chr indicates chromosome.

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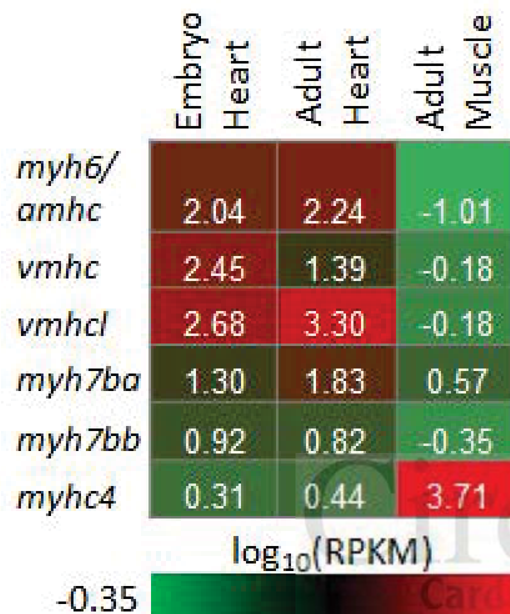
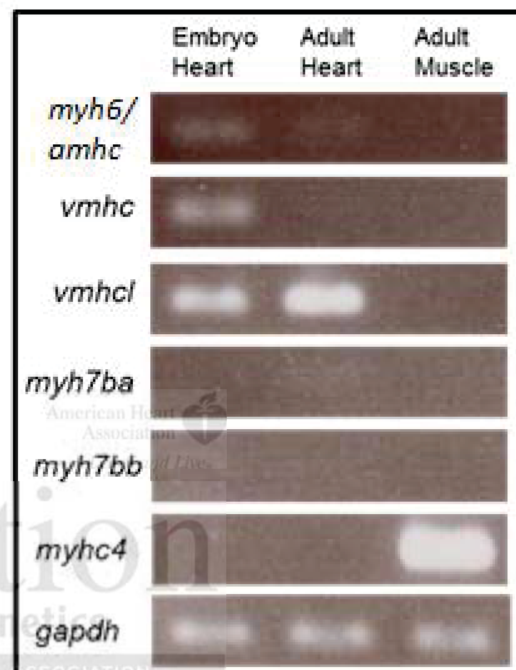
Figure 2: Transcripts of *vmhc* and *vmhcl* Respond to Doxorubicin. Adult zebrafish were injected with doxorubicin (20 mg/g), and gene expression in ventricle was determined using qPCR at 1 day, 3 days, 1 week, 2 weeks, and 3 weeks after injection. The expression level was normalized to that of *actb2*. Shown are expression levels of *vmhc* and *vmhcl* (A), *nppa* and *nppb* (B), and *pln, ryr2b, atp2a2*, and *slc8a1a* (C). In A, the ratio of *vmhc* to *vmhcl* was increased compared with that in an uninjected fish heart (green line), right scale. dpi indicates days post injection; wpi, weeks post injection. N=3 in each experimental group.

A



B



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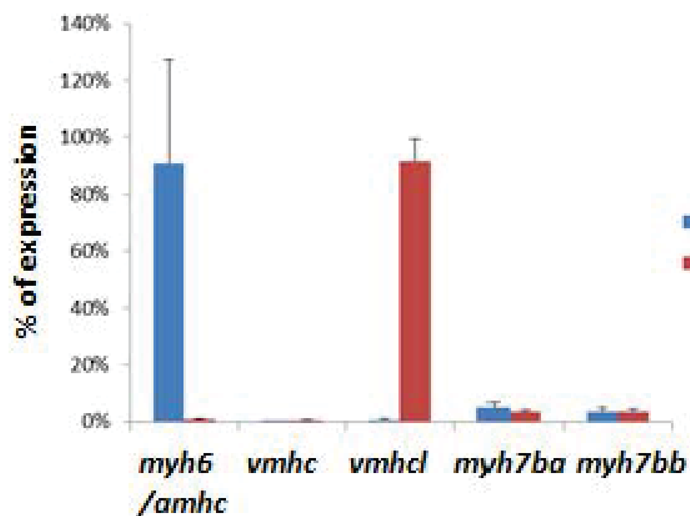
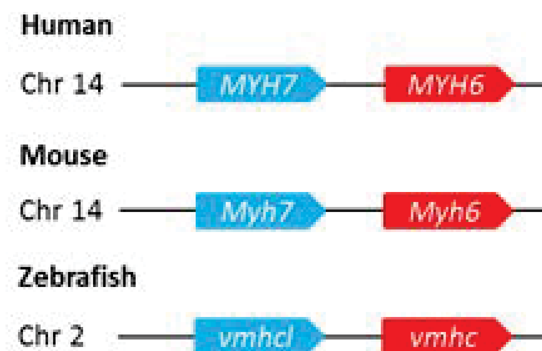
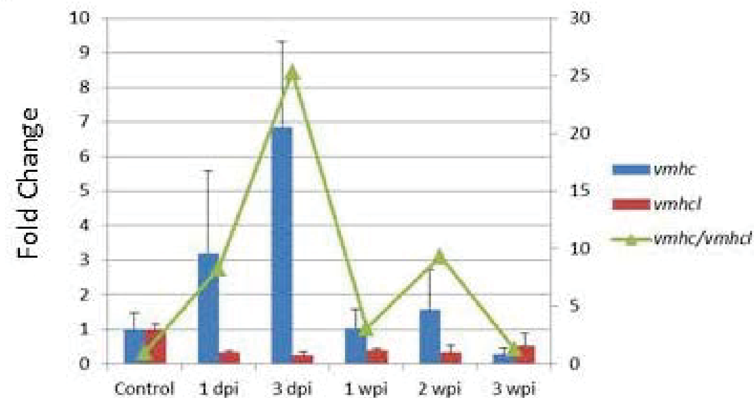
Journal of the American Heart Association

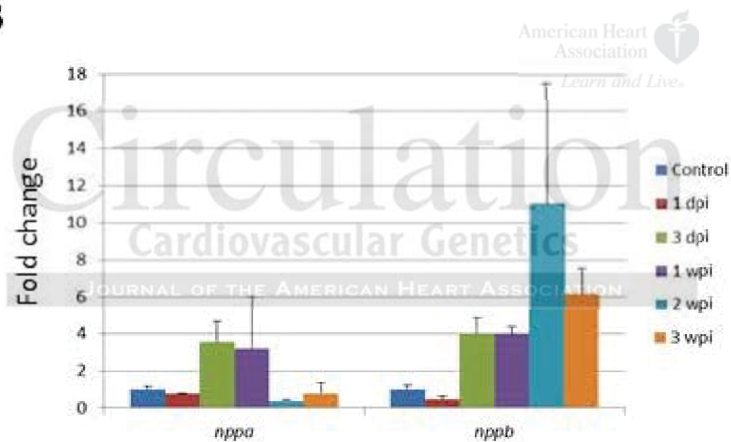
C



D







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