Low Temperature Mitigates Cardia Bifida in Zebrafish Embryos

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Abstract

The coordinated migration of bilateral cardiomyocytes and the formation of the cardiac cone are essential for heart tube formation. We investigated gene regulatory mechanisms involved in myocardial migration, and regulation of the timing of cardiac cone formation in zebrafish embryos. Through screening of zebrafish treated with ethylnitrosourea, we isolated a mutant with a hypomorphic allele of mil (s1pr2)/edg5, called s1pr2mil (as10). Mutant embryos with this allele expressed less mil/edg5 mRNA and exhibited cardia bifida prior to 28 hours post-fertilization. Although the bilateral hearts of the mutants gradually fused together, the resulting formation of two atria and one tightly-packed ventricle failed to support normal blood circulation. Interestingly, cardia bifida of s1pr2mil embryos could be rescued and normal circulation could be restored by incubating the embryos at low temperature (22.5°C). Rescue was also observed in gata5 and bon cardia bifida morphants raised at 22.5°C. The use of DNA microarrays, digital gene expression analyses, loss-of-function, as well as mRNA and protein rescue experiments, revealed that low temperature mitigates cardia bifida by regulating the expression of genes encoding components of the extracellular matrix (fibronectin 1, tenascin-c, tenascin-w). Furthermore, the addition of N-acetyl cysteine (NAC), a reactive oxygen species (ROS) scavenger, significantly decreased the effect of low temperature on mitigating cardia bifida in s1pr2mil embryos. Our study reveals that temperature coordinates the development of the heart tube and somitogenesis, and that extracellular matrix genes (fibronectin 1, tenascin-c and tenascin-w) are involved.

Introduction

During zebrafish cardiac development, heart progenitors are located near the ventral lateral margins at 5 hours post-fertilization (hpf). By 12 hpf, both myocardial and endocardial progenitors appear bilaterally under the future hindbrain region. Bilateral cardiomyocytes then migrate toward the midline and fuse to form a cone-shaped heart at the 21-somite stage (ss). Next, the heart cone begins to elongate, and forms a single heart tube at 24 hpf [1–3].

Previous studies have demonstrated that mutations in several genes cause defective migration of myocardial precursors, including genes involved in myocardium formation [4,5], endoderm formation [5–7], extracellular matrix composition [8], and siphingosine-1-phosphate signaling [9–11]. Studies on fibronectin mutants of mice and chick embryos incubated with fibronectin antibodies demonstrated that fibronectin is the major extracellular matrix component that directs myocardial migration to the midline [12,13]. In zebrafish, fibronectin has been shown to be required for the proper formation of adherens junctions between myocardial precursor cells; this in turn maintains epithelial integrity, which is essential for myocardial migration [8]. Increased

levels of fibronectin 1 (fn1) mRNA were previously observed in hand2 mutants; a genetic reduction of fn1 gene dosage in hand2 mutants partially rescued the cardia bifida phenotype, thereby implicating fibronectin in the development of this disorder [14].

The mil/edg5 gene encodes a G protein-coupled receptor which binds to sphingosine-1-phosphate in the lateral plate mesoderm [10], while tshp1/tshp2 encodes the siphingosine-1-phosphate transporter in the yolk syncytial layer, required for transport of sphingosine-1-phosphate to the mesoderm [9,11]. Mutations in either gene have been reported to cause cardia bifida. Injection of fibronectin into the midline of mil 14-ss embryos could partially rescue the cardia bifida, and caused partial fusion of bilateral cardiomyocytes [15]. Decreased levels of fibronectin were also detected at the midline in embryos injected with edg5-morpholino antisense oligomers (MO) [11]. The findings of these studies indicate that the cardia bifida exhibited by mil mutant embryos can be partially attributed to decreased cell-fibronectin interactions. However, both the regulatory mechanisms that control genes involved in myocardial migration, and the developmental processes required for heart tube formation, remain unclear.
In this study, we screened zebrafish treated with ethynitroso-
source (ENU), and isolated a hypomorphic allele of mil (s1pr2)/ 
edg5, designated as s1pr2m10 (as10). Embryos with the s1pr2m10 
allele exhibited decreased mil-edg5 expression, and this was 
associated with cardiac bifida at 24 hpf. Interestingly, the cardiac 
bifida phenotype of s1pr2m10 embryos could be rescued (restoring 
normal circulation) by incubating the embryos at a low 
temperature (22.5 °C). A similar rescue was observed in gata5 
and bon cardia bifida morphants raised at 22.5 °C. DNA 
microarray, MO knockdown, and rescue experiments with mRNA 
protein revealed that low temperature mitigates cardia bifida by 
regulating the expression of extracellular matrix genes, particularly 
in the anterior lateral plate mesoderm, midline, and pharyngeal 
arch regions.

Materials and Methods

Ethics Statement
All animal procedures were approved by the Animal Use and 
Care Committee of Academia Sinica (Protocol # RFi-
ZOOH520100065).

Zebrafish Maintenance and Staging, and Low 
Temperature and N-acetyl Cysteine (NAC) Treatment

Adult zebrafish strains, including AB, SJJD, 
Tg(−7.5bmp4:GFP)2, Tg(mnf2:EGFP), cnl2:2H2AF2micCherry72, 
s1pr2m10 (as10), and mil (s1pr2m10+/+/edg5) were raised under 
standard conditions, as previously described [16]. To perform 
the rescue experiments, embryos were incubated at 22.5 °C from 
the one-cell zygote stage onwards. Two staging systems for 
zebrafish embryos were used in this study, the somite stage (ss), 
which is determined by counting the somite number, and the 
conventional temporal stage, which is determined by hours post-
fertilization (hpf). We also followed the previously described 
morphological criteria for staging [17]. One-cell zygotes of as10 
were incubated at 22.5 °C and treated with 50 or 150 μM N-acetyl 
cysteine (NAC) (Merck) from the tailbud stage onwards. NAC-
treated embryos were continuously incubated at 22.5 °C and 
allowed to develop to the 26 ss stage; the position of migrating 
cardiomyocytes was examined at this stage.

Mutagenesis and Isolation of s1pr2m10 Mutants

Random mutagenesis was conducted using 
Tg(−7.5bmp4:GFP)2 transgenic fish, as previously described 
[18]. ENU-treated Tg(−7.5bmp4:GFP)2 males were outcrossed 
to untreated homozygous Tg(−7.5bmp4:GFP)2 females to 
generate a larger stock of F1 founders, and mutant screening was 
performed using the early pressure protocol to generate parthe-
nogenic homozygous F2 embryos [18].

Positional Cloning for s1pr2m10

s1pr2m10 heterozygous mutant fish in the AB background were 
outcrossed to the wild-type SJJD background to generate mapping 
stocks. Simple sequence length polymorphism (SSLP) markers 
were used to perform the linkage analyses. For the low-resolution 
mapping analysis, s1pr2m10 was mapped to chromosome 3 between 
markers Z3925 and Z39291 (2.77 Mb). To construct a high-resolution map around the 
s1pr2m10 locus, 900 s1pr2m10 homozygous mutant embryos and 
custom-designed SSLP markers were used. The minimum 
recombinants included markers CUS7-400021 (1/900) and 
CUS7-400022 (2/900). The sequences of the primer pair for 
CR38-83127 were F-GTCAGTGTC- 
CATCCTCCTGTT and R-TGGTGACCCATATCTTTT- 
CATTTG.

Ligation-mediated Polymerase Chain Reaction (PCR)

Ligation-mediated PCR was performed to identify an insertion 
in intron 1 of the mil-edg5 gene. Genomic DNA isolated from 
s1pr2m10 mutants was digested with NlaIII, and ligated to an Nla 
linker prepared by annealing Nla oligo 1 (GTAATACGACT-
CATATAGGGGTCGCTTAAAGGAGCATTG) and Nla oligo 2 (GTCCTTAAAGCCGGAG). The first PCR was 
used as a template for PCR with primers either flanking or within the insertion (Figure S1B). The 
sequences of the three primers were as follows: F1:AAAA- 
CATCTGACCGCTACTTCTC; R1:ATAAGAGTGCT- 
CATGTGTTTGAC; and R1:GGCTGAGTCATTTACTT- 
CAGGA.

Quantitative Real-time Reverse-transcription Polymerase 
Chain Reaction (qRT-PCR)

qRT-PCR was conducted using 2x SYBR green PCR master 
mix (Roche) with a Roche Light Cycler 480 II thermal cycler. The 
following genes were amplified using the indicated primers: edg5 
(F: GGAGGCTACCTCCTGGAAGG and R: AGCG 
CCAGCAGAAGTACAC; fn1 (F: GACAGCGCCAGAAGTACAC and R: TGGGATTTTTACAGGTG); 
tnc (F: CAGTCAGGAGAGAGGACTAT and R: GTG CCGTGATTTTACAGGTG); tnc (F: 
CAGTCAGGAGAGAGGACTAT and R: GTG CCGTGATTTTACAGGTG); 
DAGGAGGTAGGAGAGGACTAT and R: GTG CCGTGATTTTACAGGTG).

Morpholino Antisense Oligomer (MO)-mediated 
Knockdown

The sequences of MOs used in this study include: 
bon MO [19] (GACTGGCAATGTGTCATGCTTCT); edg5 MO [20] 
(AGACGCGAGTACGACATGGAGG); edg5 5 mm 
(AAGCCTACTCACTTACAGGAGG); fn1 MO [8] 
(TTTTCTCACAGTGGCTATTGAAAC; gata5 MO [20] 
(TGGGAATTTTACCTATATGAGG); tnc MO [22] (GAGAG 
GACCTCAGGAGACCTC; tnc 5 mm (GACGAG 
GACATGTCAGAAGACTCC); tnc MO [22] (TATATGGGCT 
CCAGTGTAACCTTGG); tnc MO [22] 
(AAGAGGCTACCTCCTGGAAGGACCTC); and tnc 5 mm 
(AAGAGGCTACCTCCTGGAAGGACCTC).
Whole-mount in Situ Hybridization, Histological Analysis, and Photography

Embryos treated with 0.003% phenylthiocarbamide were subjected to whole-mount in-situ hybridization, using digoxigenin- or fluorescein-labeled antisense RNA probes and either alkaline phosphatase-conjugated anti-digoxigenin or anti-fluorescein antibodies as previously described. Various templates derived from the pGEM-T vector were linearized, and the following antisense RNA probes were generated, using the restriction sites and promoters in parentheses: amhe (Neo I/sp6), amnl2 (Neo I/sp6), foxa1 (Sal I/T7), fhu1 (Neo I/sp6), tec (Apa I/sp6), and tec (Sac II/sp6). Paraffin sectioning (5 μm) and haematoxylin and cosin staining were performed using standard procedures. All images were taken using a Zeiss AxiCam HRC camera mounted on a Zeiss Imager M1 microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described [23]. In brief, embryos were fixed in 2% paraformaldehyde at 4°C overnight. Fixed embryos were embedded in 4% low-melting point agarose and sectioned with a vibratome (Leica VT1000M) to sections of 200 μm. The sections were washed with 1% Triton X-100 in PBS (PBSTx) at room temperature, before being incubated in blocking solution (10% lamb serum in PBSTx). Sections were then incubated with an anti-fibronectin antibody (Sigma) at a dilution of 1:100 for 1.5 days at 4°C. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) was used as a secondary antibody, at a dilution of 1:200. The sections were washed with blocking solution and PBSTx at room temperature. After staining, sections were examined with a Leica Confocal Microscope (TCS SP5 MP).

DNA Microarray, Gene Ontology (GO), and Pathway Analyses

Total RNA was extracted from 22-ss wild-type and s1pr2as10 mutant embryos raised at either 28.5 or 22.5°C using an RNAeasy Plant Mini Kit (Qiagen), and 10 μg of RNA was treated with RNase-free DNase I. The RNA quality was verified with a Bioanalyzer 2100 (Agilent). DNA microarray analysis was performed by NimbleGen using Zebrafish Gene Expression 12×135K arrays (NimbleGen) containing 38,489 genes. The 1,844 genes that exhibited a 1.5-fold or greater change in expression between embryos raised at 28.5 and 22.5°C for either s1pr2as10 homozygous mutants or wild-type were clustered into 5 groups using GeneSpring GX software (Agilent). The same strategy was applied to the 264 genes that exhibited a 1.5-fold or greater change in expression. The identified genes were subjected to gene ontology (GO) analysis using a Batch-Genes tool based on a zebrafish or human database (GOEAST, http://omicslab.genetics.ac.cn/GOEAST/tools.php), for which the p values were smaller than 0.1 [24]. Pathway analysis was performed on these genes using the website of the Database for Annotation, Visualization and Integrated Discovery (DAVID), http://david.abcc.ncifcrf.gov/, for which p values were smaller than 0.1 [25,26].

Digital Gene Expression (DGE) Analysis

Total RNA was extracted from 22-ss s1pr2as10 mutant embryos raised at 28.5 or 22.5°C using an RNAeasy Plant Mini Kit (Qiagen), and 4 μg was treated with RNase-free DNase I. The RNA quality was verified with a Bioanalyzer 2100 (Agilent). DGE analysis was performed by BGI through next-generation sequencing (NGS). In brief, Oligo(dT) beads were used to enrich mRNA from total RNA, and the mRNA was then reverse transcribed into double-stranded cDNA, using reverse transcriptase and DNA polymerase. The cDNA was digested with Ndel and ligated to Illumina adaptor 1. The cDNA was then cut at 17 bp downstream of the CAYG site using Mmul, and its 3’ end was ligated to Illumina adaptor 2. PCR was performed using primers GX1 and GX2, which target adaptors 1 and 2, respectively. The resulting 95-bp fragments were isolated by 6% TBE polyacrylamide gel electrophoresis (PAGE), and the DNA was purified and subjected to Illumina sequencing using a Genome Analyzer II (Illumina).

Results

Phenotypic Characterization of the s1pr2as10 Mutation

In order to screen for mutants with heart morphogenetic defects, we conducted a ethyl nitrosourea mutagenesis screen of the Tg(−7.5bmp4:GFP) mutations, which expresses GFP in the heart after 24 hpf. Through this screen, we isolated the s1pr2as10 mutant line (Figure 1B), which is a recessive embryonic lethal mutation with several phenotypes, including pericardial edema (Figure 1B'), small eyes, and tail blisters (Figure 1 B''). Blood circulation was not established during the embryonic and larval periods. We subsequently crossed s1pr2as10 into a Tg(ctl2:EGFP, cml2:HZ2AF znCherry) zebrafish generation to background to generate higher fluorescence intensity in the bilateral cardiomyocytes. In contrast to wild-type (WT) myocardial precursors, which migrated toward the midline and fused to form a single heart tube by 24 hpf (Figure 1C'), myocardial precursors in the s1pr2as10 mutants remained in the bilateral lateral plate mesoderm (LPM) at 24 hpf (Figure 1D). Although the s1pr2as10 bilateral myocardial precursors eventually reached the midline, they formed a threecamerated heart with two atria and one tightly-packed ventricle from 48 hpf (Figure 1F and 1H); this is in contrast to WT hearts, which contain a single atrium and ventricle (Figure 1E and 1G). These results indicate that the newly identified s1pr2as10 mutation delays the fusion of bilateral myocardial precursors, leading to the formation of non-functional three-chambered hearts.

Genetic Mapping of the s1pr2as10 Mutant Gene

By performing positional cloning with simple sequence length polymorphism markers, we established that s1pr2as10 is located on chromosome 3 near the mil/edg5 locus (Figure S1A). Sequencing of the mil/edg5 coding regions within cDNA or genomic DNA of s1pr2as10 mutant embryos did not predict any changes in the amino acid sequence (data not shown). Next, we examined the non-coding regions of the mil/edg5 locus via ligation-mediated polymerase chain reaction (PCR) and identified a large insertion (more than 1,000 base pairs from linkage group 11) in the first intron of mil/edg5, near exon 1 (data not shown). To confirm this insertion, we performed PCR with three primers, including F1 and R1 outside the insertion and I1 within the insertion (Figure S1B and S1C). A 426-bp DNA fragment amplified by primers I1 and R1 was detected only in the s1pr2as10 heterozygote and homozygote mutant embryos. To examine the impact of the insertion on mil/edg5 gene expression, we performed quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) to compare the expression levels of mil/edg5 between the s1pr2as10 mutants and their WT siblings during and after cardiogenesis. Significantly reduced mil/edg5 mRNA levels were detected in the s1pr2as10 mutants prior to 96 hpf (Figure S1D). These results strongly suggest that the s1pr2as10 cardiac defect is caused by hypomorphic mil/edg5 gene expression and that s1pr2as10 may represent a new allele of mil/s1pr2as10. Complementation experiments further demonstrated that s1pr2as10 failed to complement a mil mutant at 24 hpf (Figure 1L and 1M vs. 1K). In summary,
**s1pr2as10** is a hypomorphic allele of *mil* that is caused by an insertion into intron 1.

**s1pr2as10** Mutants Exhibit a Weaker Phenotype than *mil* Mutants

Next, we carefully compared the cardiac phenotypes of *s1pr2as10* and *mil* mutations. We observed that the average distance between bilateral hearts at 24 hpf in *s1pr2as10* mutant embryos was shorter than that of *mil* mutants (Figure 1J and 1K). In addition, while the bilateral hearts persisted until at least 48 hpf in *mil* mutants, the myocardia fused by 48 hpf in *s1pr2as10* mutants (Figure 1F). Both of these observations indicate that the cardia bifida of *s1pr2as10* mutants is weaker than that of *mil* mutants. We subsequently conducted time-lapse analyses of *cmlc2*-stained embryos to investigate the timing of bilateral heart fusion in the *s1pr2as10* mutant (Figure 1N). At 24 hpf, the majority of *s1pr2as10* mutant embryos contained two clusters of laterally positioned myocardial precursors, whereas by 28 hpf, over 80% of mutant embryos contained myocardial precursors in contact with one another (Figure 1O). To demonstrate that the *s1pr2as10* mutant phenotype could be attributed to the low levels of *mil*/*edg5* mRNA, we injected different doses of *edg5*-MO or *edg5*-5mm MO into *Tg(cmlc2:EGFP, cmlc2:H2AFZmCherry)* cy13 transgenic fish at the one-cell zygote stage. To quantitatively assess the severity of cardiac abnormalities, we grouped the cardiac phenotypes into three classes; Class I represented a normal heart tube, Class II represented cardiomyocytes either in close proximity or in contact, and Class III represented the most severe cardia bifida (Figure S2A). At 24 hpf, dose-dependent cardia bifida was detected in embryos injected with 1 to 16 ng of *edg5*-MO, but not in embryos injected with 30 pg *mil*/*edg5* mRNA resulted in a partial rescue of cardia bifida at 24 hpf, as compared to mutant embryos injected with LacZ (Figure S3). Overall, these data indicate that the hypomorphic migration defect of myocardial precursors in *s1pr2as10* mutant embryos was a result of decreased levels of *mil*/*edg5* mRNA.

Raising *s1pr2as10* Embryos at low Temperature Rescued the Cardiac Phenotype

The hearts of *s1pr2as10* mutant embryos raised at a standard temperature (28.5°C) contained two atria and one tightly-packed ventricle, as visualized by *cmlc2* and *amhc* double-staining; most of these embryos lacked blood circulation at 72 hpf (Figure 2A). However, we found that the *s1pr2as10* mutants raised at a lower temperature (22.5°C) presented with a less severe phenotype (Figure 2B). More than 85% of the *s1pr2as10* mutant embryos...
raised at 22.5°C had hearts with one atrium and one ventricle at 72 hpf, as compared to only 12% of the embryos raised at 28.5°C (Figure 2C). In addition, normal blood circulation was detected at 72 hpf in 69% of the s1pr2as10 mutant embryos raised at 22.5°C, but in only 9% of those raised at 28.5°C (Figure 2D). Rescued s1pr2as10 homozygous mutants grew to adulthood. A high proportion of the offspring of rescued mutants were also rescued when raised at 22.5°C (Table S1). However, the tail blister phenotype of the s1pr2as10 mutant embryos could not be rescued by development at 22.5°C (Figure 2B). This result indicates that incubation at low temperature specifically affects myocardial migration, and that s1pr2as10 is not simply a temperature-sensitive allele of mil/edg5. We subsequently performed temperature shift experiments, by incubating s1pr2as10 mutants from different stages at 28.5°C or 22.5°C. We found that the critical time window in which low temperature could rescue cardiobifida in s1pr2as10 mutants was from ~16 to the 22 somite stage (ss) (Figure S4).

Low Temperature Shifts the Timing of Cardiac Cone Formation

To examine why the cardiac defects of the s1pr2as10 mutants were rescued by low temperature, we conducted time-lapse experiments to observe bilateral myocardial precursor migration at different somite stages. In Tg(cmlc2:EGFP, cmlc2:H2A.FZmCherry)cy13 transgenic embryos raised at 28.5°C, the bilateral myocardial precursors fused at the midline and became cone-shaped at the 21-ss (Figure 3B, red boxes in WT at 28.5°C). Surprisingly, the timing of cardiac cone formation was shifted to the 19 ss when the embryos were raised at the lower temperature (Figure 3B, red boxes in WT at 22.5°C). Although the overall growth rate at 22.5°C was about 2-fold slower than that at 28.5°C [27], low temperature does not seem to cause any morphological defects in WT and s1pr2as10 embryos (Figure 3A). Similarly, the bilateral cardiomyocytes in the s1pr2as10 mutant embryos fused at the 26 ss at low temperature (Figure 3B, red boxes in s1pr2as10 22.5°C); this result was in contrast to the delayed fusion (28 hpf) observed at 28.5°C (Figure 1O). In situ hybridization using a cmlc2 probe revealed that 77% WT and 84% s1pr2as10 mutant embryos raised at low temperature formed a fused cardiac cone at the 19 ss and 26 ss, respectively (Figure 3C). In contrast, the majority of WT and s1pr2as10 mutant embryos raised at standard temperature presented with horseshoe-shaped hearts or bilateral hearts at 19 ss and 26 ss, respectively (Figure 3C). However, cardiobifida of mil mutant embryos cannot be rescued by low temperature treatment (Figure S5A). Since defects in endoderm formation also result in cardiobifida [7,28], we induced cardiobifida by injecting different doses of gata5 MO or bon MO. We found that the cardiobifida phenotypes of embryos injected with 10 ng of gata5 MO or bon

Figure 2. Development at low temperature rescues the s1pr2as10 cardiac phenotype. (A) s1pr2as10 mutants raised at 28.5°C contained hearts with two atria (dark purple, amhc staining) and one ventricle (red, cmlc2 staining), and presented with tail blisters at 72 hpf. (B) s1pr2as10 mutant embryos raised at 22.5°C contained hearts with a single atrium and ventricle, and exhibited blood circulation at 72 hpf (arrow). (C) Percentages of s1pr2as10 mutant embryos raised at 22.5°C contained hearts with a single atrium and ventricle, and exhibited blood circulation at 72 hpf (arrow). (D) Percentages of s1pr2as10 mutant embryos raised at either 28.5 or 22.5°C displaying blood circulation at 72 hpf. The error bars indicate the standard error. Statistical significance was determined using Student’s t-test. *** indicates p<0.001.

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MO can be significantly rescued by being raised at low temperature (Figure 3D and 3E) while cardiia bifida defects in embryos injected with 20 ng of gata5 MO were not rescued by low temperature treatment (Figure S2B). Although the rescue effect was not statistically significant, a reduced percentage of cardiia bifida phenotype was notable in embryos injected with 20 ng of bon MO. (Figure S2B). In addition to cardiia bifida, cells of the anterior gut tissues also failed to migrate to the midline in gata5/fau mutants [29]. We found that the distance between the two lateral anterior gut tubes was also significantly reduced in embryos injected with 10 ng gata5 MO and incubated at low temperature, as compared to those incubated at standard temperature (145 µm vs. 160 µm) at the prim-25 stage (Figure S6). Thus, low temperature alters the timing of cardiac cone formation in WT, s1pr2as10 mutant (which exhibit hypomorphic migration defects) and other cardiia bifida morphants, resulting in a rescue of the cardiia bifida phenotype that is independent of the genetic background.

Gene Expression Profiles of Zebrafish Embryos Raised at 28.5 and 22.5 °C

Previous reports have demonstrated that changes in temperature affect developmental processes and gene expression in zebrafish embryos [29,30]. We performed DNA microarray (Figure 4) and digital gene expression (DGE) (Table S2) analyses to further investigate how low temperature mitigates cardiia bifida. By combining these two analyses, we identified several candidate gene groups and demonstrated that genes encoding extracellular matrix (ECM) proteins are important for preventing cardiia bifida. DNA microarray and qRT-PCR revealed that colla5 edg5 levels were not affected by raising WT or s1pr2as10 embryos at different temperatures (28.5 °C or 22.5 °C) (Figure S7).

Low Temperature Mitigates Cardiia Bifida by Altering ECM Gene Expression

Based on the DNA microarray and DGE analyses, we established that several genes involved in the ECM were affected by low temperature; these genes included tenascin-C (tnc), tenascin-W (tnw), and collagen type II, alpha-1a identified through the DNA microarray analysis (Figure 4), and fibronectin 1 (fn1), collagen, type XI alpha 2 and s1pr2as10 through the DGE analysis (Table S2). We first focused on fn1, which was previously found to be important for myocardial migration [8]. An increased level of fn1 was initially identified via DGE in s1pr2as10 mutant embryos raised at 22.5 °C (Table S2). Whole mount in situ hybridization and immunocytochemistry revealed increased expression of fn1 mRNA and protein in the midline regions and bilateral anterior LPM of both 22-ss WT and s1pr2as10 mutant embryos raised at 22.5 °C (Figure 3B and 3D and Figure S8). However, levels of fn1 at the tail bud or yolk regions were not affected by incubation at low temperature (Figure 3E–3H). qRT-PCR revealed an approximately 1.3-fold increase of fn1 mRNA in s1pr2as10 mutant embryos raised at 22.5 °C, as compared to those raised at 28.5 °C. However, this temperature-mediated up-regulation was not statistically significant (Figure 5). The failure to detect a significant change in WT and s1pr2as10 mutant embryos raised at low temperature may be due to the inability of temperature to affect fn1 expression at the tail bud and yolk regions (Figure 5E–5H).

As incubation at low temperature raised fn1 expression and rescued the cardiia bifida phenotype in s1pr2as10, we hypothesized that fn1 up-regulation may be required for the rescue of heart development. To test this hypothesis, we injected fn1-MO into s1pr2as10 mutants raised at 22.5 °C. As shown in Figure 3J, 26ss mutant embryos injected with 1.25 or 2.5 ng fn1-MO displayed severe cardiia bifida, even after being raised at the low temperature. In addition, we injected human fibronectin protein into the midline region (Figure 5K) of 14–16 hpf s1pr2as10 mutant embryos raised at 28.5 °C, to determine whether elevated levels of fibronectin can rescue the cardiia bifida phenotype. We found that the percentage of mutant embryos with class III severe cardiia bifida was reduced from 60% to 42% (Figure 5K). Together, these results indicate that incubation at low temperature mitigates cardiia bifida in s1pr2as10 mutants by promoting fn1 expression in the midline and bilateral anterior LPM regions.

Two Tenascin Genes, Tenascin-C and Tenascin-W, Play Important Roles in myocardial migration in embryos raised at low temperature

In addition to fn1, expression levels of two other extracellular matrix genes, tenascin-C (tnc) and tenascin-W (tnw), were affected by incubation at low temperature. The tnc gene can produce four different isoforms through differential splicing. By using RNA probes against exons common to all tnc isoforms for in situ hybridization, we detected increased expression of tnc at the pharyngeal arches and in the cells between the brain and eyes of 22-ss WT and s1pr2as10 mutant embryos raised at 22.5 °C as compared to those raised at 28.5 °C (Figure 6B and 6D vs. 6A and 6C). However, expression of tnc in the somite region was not affected by the temperature of incubation (Figure 6E–6H). Accordingly, the use of qRT-PCR to measure tnc mRNA did not uncover significant differences between WT and s1pr2as10 mutant embryos raised at 22.5 °C as compared to those raised at 28.5 °C (Figure 6I). We subsequently used a previously published tnc-MO1 that can prevent the translation of all tnc isoforms [22] and designed a tnc–5 mm MO based on the tnc-MO1 sequence for use as controls. Additionally, we used the previously published tnc-MO2, which blocks splicing of tnc exon 1 [22]. Mutant s1pr2as10 embryos incubated at 22.5 °C and injected with either tnc-MO1 or tnc-MO2 presented with more severe cardiia bifida phenotypes, as compared to tnc–5 mm injected embryos (Figure 6J and Figure 6K). These results indicate that low temperature rescues s1pr2as10 cardiac phenotype partially due to increased expression of tnc.

DNA microarray analysis also revealed decreased expression of another tenascin gene, tnc, in 22-ss WT and s1pr2as10 mutant embryos raised at 22.5 °C. Whole-mount in situ hybridization and qRT-PCR confirmed that tnc mRNA expression was reduced in all tissues, including the LPM, of both 22-ss WT and s1pr2as10 mutant embryos raised at 22.5 °C (Figure 6M, 6O and 6P). We subsequently designed morpholinos (tnc-MO1 and tnc-MO2) to the 5’ untranslated region (UTR) of tnc to prevent its translation; we also designed a tnc–5 mm based on the tnc-MO1 sequence as a control. In order to test the efficiency and specificity of tnc-MOs, we fused EGFP in frame with the tnc 5’UTR under the control of the CMV enhancer/promoter, and co-injected this construct with tnc-MO1, tnc-MO2, or tnc–5 mm into one-cell zygotes. As shown in Figure S9D and E, we detected EGFP expression in the majority of embryos co-injected with tnc–5 mm and CMV-tncUTR-EGFP plasmid. In contrast, the majority of embryos (85%) co-injected with tnc-MO1 and CMV-tncUTR-EGFP did not express GFP. However, 30% of embryos co-injected with tnc-MO2 and CMV-tncUTR-EGFP expressed GFP, indicating that tnc-MO1 is more efficient at blocking EGFP translation.
This may be a consequence of \textit{tnw-MO2} targeting a region further upstream from that of \textit{tnw-MO1}.

We then injected either \textit{tnw-MO1} or \textit{tnw-MO2} into \textit{s1pr2as10} mutants raised at 28.5°C, and observed partial rescue of the cardia bifida phenotype at 24 hpf (Figure 6Q and Figure S9B). Injection of \textit{tnw-MO1} resulted in greater rescue of the cardia bifida phenotype than with \textit{tnw-MO2}; this may be attributed to the reduced efficiency of \textit{tnw-MO2} in blocking translation. Furthermore, some of the rescued embryos contained a single heart tube and displayed normal circulation (data not shown). Interestingly, injection of \textit{tnw} mRNA at the one-cell stage caused a highly severe cardia bifida phenotype in \textit{s1pr2as10} mutants, which was observed even at 22.5°C (Figure 6R).

We also injected \textit{fn1-MO}, \textit{tnw-MO1}, or \textit{tnw} mRNA into wild type embryos to investigate their effects on myocardial migration. We found that the percentage of \textit{tnw-MO1}-injected embryos (26%) which developed a horseshoe-shaped heart at 22 ss was similar to that of control embryos (27%) (Figure S10A and S10B). The percentage of \textit{tnw} mRNA-injected embryos with a horseshoe-shaped heart at 22 ss was greater (38%) than that of control, and 12% of these embryos also exhibited cardia bifida (Figure S10D). The percentage of embryos with horseshoe-shaped hearts at 22 ss was increased further by \textit{fn1-MO1}-injection (69%), and 9% of these embryos exhibited cardia bifida (9%) (Figure S10C); these results indicate that fibronectin plays a more important role in regulating myocardial migration than tenasin-C or tenasin-W. However, the majority (93%) of

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**Figure 3. Low temperature shifts the timing of formation of the cardiac cone.** (A) Lateral views of 22-ss wild-type (WT) and \textit{s1pr2as10} mutant embryos raised at 28.5 or 22.5°C. (B) Time-lapse analyses of the medial migration of bilateral cardiomyocytes during the −17–30 ss in \textit{Tg(cmlc2:EGFP, cmlc2:H2AFZmCherry)103} transgenic fish (WT) or \textit{s1pr2as10} mutant embryos raised at 28.5 or 22.5°C. Five WT or \textit{s1pr2as10} mutant embryos were analyzed for each stage. The images outlined in red indicate the stage at which cardiac cone formation took place under each condition. (C) \textit{In situ} hybridization of 19-ss wild-type (WT) or 26-ss \textit{s1pr2as10} mutant embryos raised at 28.5 or 22.5°C with a \textit{cmlc2} RNA probe. The number of embryos displaying \textit{cmlc2} staining/the total number of embryos analyzed is shown for each panel. (D) Different degrees of myocardial migration defects were observed in \textit{gata5} and \textit{bon} morphants at 24 hpf. Class I (single heart tube), Class II (cardiomyocytes in close proximity but not in contact), and Class III (two separate hearts) defects are shown. (E) Percentages of each class of myocardial migration defects in 10 ng \textit{gata5-MO} or \textit{bon-MO}-injected embryos raised at 28.5 or 22.5°C. Scale bar = 100 μm. Statistical significance was determined using Student’s t-test. * indicates p<0.05. doi:10.1371/journal.pone.0069788.g003
embryos injected with a mixture of *tnc*-MO1, *fn1*-MO, and *tnw* mRNA exhibited cardia bifida at 22 ss (Figure S1Oc); this result further demonstrates that tenasin-C and fibronectin positively regulate, while tenasin-W negatively regulates, myocardial migration in zebrafish embryos.

Together, these data indicate that *tnc* and *tnw* play opposing roles in zebrafish myocardial migration, and that low temperature mitigates cardia bifida by altering the expression of ECM genes: *fn1* and *tnc* are up-regulated, and *tnw* is down-regulated.

Figure 4. Gene ontology (GO) and pathway analyses for differentially expressed genes identified by DNA microarray analysis. GO and pathway analyses were conducted on different groups of genes using two strategies. (A) Genes with a greater than 1.3-fold change in expression between embryos raised at 28.5 and 22.5°C were used in strategy 1. GO analysis was performed using the zebrafish database. (B) Genes with a greater than 1.5-fold change in expression between embryos raised at 28.5 and 22.5°C were used in strategy 2. GO analysis was performed using the human database. The GO and pathway analyses were performed on genes from both Groups I and II or on genes from Groups III, IV and V. Box and Whisker plots for each group indicate the median (line within the box), 25th and 75th percentiles (top and bottom of the box), and 10th and 90th percentiles (Whiskers/error bars). The P-value from GO and pathway analyses are smaller than 0.1.

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Reactive Oxygen Species (ROS) Mediate Mitigation of Cardia Bifida by Low Temperature

Cold exposure was previously shown to increase expression of peroxisome proliferator-activated receptors (PPARs) and uncoupling proteins (UCPs) in the zebrafish brain, to prevent oxidative damage and to maintain metabolic balance and homeostasis [31].

We investigated whether ROS mediates mitigation of the cardia bifida phenotype in s1pr2<sup>as10</sup> mutants by low temperature treatment. To test this hypothesis, we treated s1pr2<sup>as10</sup> mutant embryos with either 50 or 150 μM of N-acetyl cysteine (NAC), a ROS scavenger, at the tailbud stage, and incubated the embryos at 22.5 °C. As shown in Figure 7, we detected a dose-dependent...
NAC on the percentage of slp2a\textsuperscript{as10} mutant embryos with a class I single heart tube at 26 ss when incubated at low temperature; class I heart tubes were reduced by NAC to 46–32% as compared to 67–73% in untreated slp2a\textsuperscript{as10} mutant embryos. This result suggests that ROS may mediate mitigation of cardiac bifida by low temperature.

**Discussion**

Previous reports have demonstrated that changes in temperature affect various aspects of development and gene expression [29,30]. In this study, we have demonstrated that temperature also affects the timing of cardiac cone formation. We have specifically demonstrated that low temperature (22.5°C) mitigates cardiac bifida in both slp2a\textsuperscript{as10} and tnc\textsuperscript{as10} and bon mutant embryos. DNA microarray and digital gene expression analyses revealed that low temperature regulates expression of several extracellular matrix (ECM) genes to control myocardial migration.

An increase in fnl expression at the midline region and bilateral anterior lateral plate mesoderm (LPM) was identified in both 22-ss WT and slp2a\textsuperscript{as10} mutant embryos raised at 22.5°C (Figure 5B, and 5D and Figure S8); Injection of slp2a\textsuperscript{as10} mutant embryos with fnl-MO abolished the rescue of cardiac bifida by low temperature (Figure 5J), while injection with human fibronectin protein decreased the percentage of slp2a\textsuperscript{as10} mutant embryos raised at 28.5°C with class III severe cardiac bifida at 24 hpf (Figure 5K).

During myocardial precursor migration, fibronectin secreted by the endoderm and/or endocardial precursors is deposited at the midline and laterally around the myocardial precursors, to maintain the integrity of myocardial epithelia, and mediate interactions between the endoderm and migrating myocardial precursors [8,15]. It has been demonstrated that knockdown of mil/edg5 results in cardiac bifida and decreased levels of fibronectin [11]. Furthermore, injection of the midline with fibronectin partially rescues the cardiac bifida phenotype in mil mutant embryos [15]. All of these findings suggest that both mil/edg5 and low temperature play a positive role in regulating myocardial migration. Proper deposition of extracellular matrix proteins is also important for normal cardiac function after formation of the four-chambered heart, and excess collagen and fibronectin deposition results in cardiac fibrosis in cardiac hypertrophy.

Reactive oxygen species (ROS) have been shown to up-regulate expression of profibrotic genes, such as col2a1, col1a1, and fn1, thereby resulting in direct extracellular matrix deposition in a transgenic mouse model, and resulting in cardiac hypertrophy [32].

Tenascins are a family of large multimeric extracellular matrix glycoproteins involved in cell adhesion, migration and proliferation [33,34]. Tenasin-C has been shown to either promote or inhibit cell adhesion and migration, depending on the cell type. Addition of tenasin-C stimulated the migration of human adult dermal fibroblasts cultured within matrices comprised of fibro-nectin [35]. Smooth muscle cells cultured on tenasin-C substrate exhibited increased expression of \( \alpha \)-integrin and elevated phosphorylation of focal adhesion kinase (FAK), followed by enhanced migration [36]. Tenasin-C also promoted bovine retinal endothelial cell migration by enhancement of FAK phosphorylation [37]. On the other hand, high levels of tenasin-C produced by gloma tumour tissues actively hindered T-cell migration, resulting in an accumulation of T cells in the peritoneal region [38]. Although the function of tenasin-W and its gene regulation has not been well-characterized, it has been shown to confer anti-adhesive properties [39]. Addition of soluble tenasin-W was reported to inhibit the formation of lamellipodia with stress fibers and focal adhesion complexes in a murine myoblast cell line (C2C12) cultured on fibronectin. Various factors, including inflammatory cytokines, growth factors, oxidative stress, and hypoxia, have been shown to induce fnl expression [40]. In neonatal rat cardiomyocytes, mechanical strain increased fnl expression by activating nuclear factor-kB through ROS [41]. Here, we observed up-regulation of fnl and down-regulation of tnc in embryos raised at low temperature (Figures 5 and 6). MO knockdown of tnc mRNA or protein rescue confirmed the opposing roles of tenasin-C and tenasin-W in mitigating cardiac bifida in zebrafish embryos. Moreover, we found that the addition of N-acetyl cysteine (NAC), a ROS scavenger, decreased mitigation of cardiac bifida in slp2a\textsuperscript{as10} mutant embryos raised at 22.5°C (Figure 7A).

Based on our results and previously published studies, we propose a model describing how low temperature mitigates cardiac bifida in zebrafish embryos (Figure 7B). Low temperature may enhance ROS production in cranial neural crest cells, endocardial and myocardial cells, and endoderm (in a similar manner to ROS induction in zebrafish brain upon cold exposure [31]), as these tissues are more vulnerable to oxidative stress [42–47]. The presence of ROS in the endoderm and anterior LPM may then up-regulate fnl expression, resulting in the secretion of fibronectin in the midline and bilateral anterior LPM (Figure 5 and Figure S8). Meanwhile, ROS production in the cranial neural crest derived-bilateral pharyngeal arches may activate tnc expression, thereby promoting secretion of tenasin-C near migrating bilateral cardiomyocytes (Figure 6). Increased levels of secreted tenasin-C may enhance integrin expression and promote FAK phosphorylation in migrating bilateral cardiomyocytes, thereby facilitating their interaction with fibronectin, formation of focal adhesion complexes, actin polymerization, and protrusion [37,48–50]. Increased fibronectin around migrating cardiomyocytes can further promote the integrity of myocardial epithelia, which is a prerequisite of myocardial migration [8]. Since tenasin-W is known to inhibit cell adhesion to fibronectin, ROS may also down-regulate tnc expression, consequently preventing secretion of tenasin-W from scattered epithelial cells in the head region, and thereby allowing migrating cardiomyocytes to interact with...
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Figure 7. Reactive oxygen species (ROS) mediate mitigation of cardia bifida in zebrafish embryos incubated at low temperature. (A) s1pr2as10 mutant embryos raised at 22.5 °C were treated with 50 or 150 µM N-acetyl cysteine (NAC) at the tailbud stage, and then were incubated at 22.5 °C. Different degrees of myocardial migration defects were observed at the 26 ss. Class I (single heart tube), Class II (cardiomyocytes in close proximity but not in contact), and Class III (two separate hearts). (B) A proposed model showing how low temperature mitigates cardia bifida in zebrafish embryos. Statistical significance was determined using Student’s t-test. * indicates p<0.05.

Supporting Information

Figure S1 Genetic mapping of the s1pr2as10 mutant gene. (A) Meiotic map of the s1pr2as10 locus. The numbers above the line indicate the number of meiotic recombination events out of the total number of meioses. (B) An enlarged view of the mil/edg5 locus shows the insertion in intron 1. Three primers (F1, R1, and II) were used to confirm the insertion. (C) A 182-bp DNA fragment amplified using primers F1 and R1 was detected in wild-type (WT) and s1pr2as10 heterozygous mutants, whereas a 426-bp DNA fragment amplified using primers II and R1 was detected in s1pr2as10 heterozygous and homozygous mutant embryos. (D) Expression of mil/edg5 mRNA was reduced in s1pr2as10 mutants prior to 96 hpf, as revealed by qRT-PCR. WT (+, W1; s1pr2as10 mutant (−, M). The error bars indicate the standard error. (TIF)

Figure S2 Dosage curve of an edg5-morpholino antisense oligomer (MO) and its association with cardia bifida. (A) Tg(cmlc2:EGFP, cmlc2:H2AFZ:mCherry)113 embryos injected with different doses of edg5-MO and edg5-5 mM exhibited varying degrees of myocardial migration defects, ranging from Class I (a single heart tube) to Class II (cardiomyocytes either in close proximity or in contact) and Class III (separated cardiomyocytes) at 24 hpf. Scale bar = 100 µm. (B) Percentages of Class I, II and III myocardial migration defects in Tg(cmlc2:EGFP, cmlc2:H2AFZ:mCherry)113 embryos injected with 1–16 ng of edg5-MO at 24 hpf. (TIF)

Figure S3 Injection of s1pr2as10 mutants with mil/edg5 mRNA partially rescued the cardia bifida phenotype. Injection of s1pr2as10 mutant embryos with mil/edg5 mRNA, but not LacZ mRNA, decreased the percentage of embryos with the Class III cardia bifida phenotype. Statistical significance was determined using Student’s t-test. * indicates p<0.05. (TIF)

Figure S4 The 16 ss to the 22 ss is the critical time interval for rescue of the s1pr2as10 mutant heart at 22.5 °C. s1pr2as10 mutants raised at 28.5°C (red line) or 22.5°C (blue line) at different stages were rescued to differing levels, as determined by the percentage of embryos containing one atrium and one ventricle at the protruding-mouth stage. (TIF)

Figure S5 Low temperature treatment cannot rescue the cardia bifida phenotype of mil mutants and 20 ng gata5 MO-injected embryos. (A) mil mutants embryos were incubated at 28.5°C or 22.5°C. Different degrees of myocardial migration defects were observed from prim 5-prim 15. (B) Percentages of each class of myocardial migration defect in 20 ng gata5-MO or bon-MO-injected embryos raised at 28.5 or 22.5°C at the 26 ss. Myocardial migration defects were observed at prim-5, Class I (single heart tube), Class II (cardiomyocytes in close proximity but not in contact), and Class III (two separate hearts). (TIF)

Figure S6 Anterior gut migration defects in gata5 morphants can be rescued by low temperature treatment. Embryos injected with 10 ng gata5 MO were incubated at 28.5°C (A) or 22.5°C (B). Embryos were harvested at prim-25 and stained with foxa1 RNA probe. (C) The distance between two lateral anterior gut tubes was significantly different between morphants incubated at 28.5°C or 22.5°C. Scale bars = 100 µm. The error bars indicate the standard error. Statistical significance was determined using Student’s t-test. *** indicates p<0.001. (TIF)
Figure S7 mil1/edg5 levels were similar in embryos raised at 28.5 C or 22.5 C. qRT-PCR measurement of mil1/edg5 levels in 22-ss WT or s1pr2+/− mutant embryos raised at 28.5 or 22.5 C. The error bars indicate the standard error. (TIF)

Figure S8 Low temperature increases fibronectin 1 expression in the midline region. Immunohistochemistry was used to demonstrate increased fibronectin 1 expression at the midline region (white arrows) of 22-ss wild type (WT) and s1pr2+/− mutant embryos raised at 22.5 C. Scale bars = 100 μm. (TIF)

Figure S9 Evaluation of the roles of tnc and tnw in the mitigation of cardia bifida, and of the efficiency and specificity of tnw MOs. (A) Knockdown of tnc with tnw-MO2 in s1pr2+/− mutants increased the percentages of 26-ss embryos raised at 22.5 C with the Class II and Class III cardia bifida phenotype. (B) Knockdown of tnc with tnw-MO2 in s1pr2+/− mutant embryos raised at 28.5 C partially rescued cardia bifida phenotypes at 24 hpf. Class I (a single heart tube) to Class II (cardiomyocytes either in close proximity or in contact) and Class III (separated cardiomyocytes). Statistical significance was determined using Student’s t-test. * indicates p < 0.05, ** indicates p < 0.01. (C) Diagram indicating the relative binding positions of two tnw-MOs in the 5′ untranslated region of tnw mRNA. (D) Green fluorescence can be detected in embryos co-injected with CMV-tnwUTR-EGFP and tnw-5mm at 24 hpf. Green fluorescence was not observed in the majority of embryos co-injected with CMV-tnwUTR-EGFP and tnw-MO1, while green fluorescence was detected in 30% of embryos co-injected with CMV-tnwUTR-EGFP and tnw-MO2. (E) Percentage of embryos expressing EGFP following co-injection of CMV-tnwUTR-EGFP with tnw-3mmMO, tnw-MO1 or tnw-MO2. The error bars indicate the standard error. (TIF)

Figure S10 Knockdown of fn1 or tnc and overexpression of tnw results in cardia bifida in wild type embryos. Wild type embryos at the one-cell zygote stage were injected with 5 ng tnw-MO1 (B), 2.5 ng fn1-MO (C), 100 pg tnw mRNA (D), or a mixture of tnw-MO1, fn1-MO, and tnc mRNA (E), and incubated at 28.5 C. Un-injected embryos were used as a control (A). Embryos were harvested at the 22 ss and stained with omlc2. (TIF)

Table S1 Circulation defect of s1pr2+/− mutant embryos from different genotypes could be partially rescued when raised at 22.5 C. The statistics of the offspring derived from different genotypes of s1pr2+/− mutant raised at 22.5 C or 22.5 C. Genotype labeled with +/+ or −/− indicated the heterozygous or homozygous s1pr2+/− mutants respectively. For example, among 213 embryos from heterozygous mutants intercross (+/+ x +/−) raised at 28.5 C, 162 embryos showed normal wild-type phenotype (a), 46 embryos contained tail blisters phenotype and established no blood circulation (b), and 5 embryos contained tail blisters with normal circulation (c). Meld ratio was calculated by number of embryos with tail blister phenotype divided by number of total embryos. Rescue of tail blister with circulation phenotype was observed in offspring derived from different genotypes of s1pr2+/− mutant raised at 22.5 C. Rescue percentage of tail blister with circulation phenotype was calculated by number of embryos showing tail blister with circulation phenotype divided by total number of embryos showing tail blister phenotype. (DOC)

Table S2 Up- and down-regulated genes in s1pr2+/− mutant at 22.5 C as determined by digital gene expression (DGE) analysis. Double-stranded cDNA from 22 ss s1pr2+/− mutant embryos raised at 28.5 C and 22.5 C were synthesized for next generation sequencing. The Up- and down-regulated genes at 22.5 C were subsequently analyzed by pathway analysis according to Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Several groups of genes were selected for further analysis. (DOC)

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Author Contributions

Conceived and designed the experiments: CL. SH. Performed the experiments: CL, WW, YW, YL. Analyzed the data: CL, CH, C. Huang, SH. Contributed reagents/materials/analysis tools: C. Huang, C. Hao. Wrote the paper: CL, SH.

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