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Tissue factor pathway inhibitor-2 is critical in zebrafish cardiogenesis

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ABSTRACT

Human tissue factor pathway inhibitor-2 (Tfpi-2) is an extracellular matrix-associated Kunitz-type serine proteinase inhibitor and plays an important role in various cellular processes. We have previously shown that zebrafish Tfpi-2 (zTfpi-2) mainly expressed in the brain and heart of zebrafish, and it is involved in the development of central nervous system. Here, we identified zTfpi-2 as an evolutionarily conserved protein essential for zebrafish heart development, as embryos depleted of zTfpi-2 failed to undergo cardiogenesis. Changes of cardiogenic markers, *vmhc*, *amhc* and *bmp4*, confirmed zTfpi-2 knockdown caused cardiac defects, including retrenched ventricle, enlarged atrium and malformation of atrioventricular boundary. The sarcomeric organization was also disrupted by embryonic depletion of zTfpi-2, thus establishing the functional role of zTfpi-2 in cardiac contractility. In addition, hematopoietic defects were detected in the zTfpi-2-deficiency embryos. Importantly, injection of *ztfpi-2* mRNA attenuated those cardiac and hematopoietic defects. Taken together, this study demonstrated a critical role of zTfpi-2 during embryonic cardiac development, as well as an important regulator of hematopoiesis.

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1. Introduction

Tfpi-2, also known as placenta protein-5 (PP5), is a 32-kDa serine proteinase inhibitor consisting of three Kunitz-type proteinase inhibitor domains (KD1, KD2, KD3) homologous with tissue factor pathway inhibitor (Tfpi). Tfpi and Tfpi-2 are secreted into the extracellular matrix (ECM) by a wide variety of human cells and belongs to the serine protease inhibitor superfamily [1]. Data have showed that Tfpi plays an important role in the regulation of coagulation via its inhibition of the TF-pathway [2]. Systemic homozygotic deletion of Tfpi-KD1 results in intrauterine lethality, presumably circulatory insufficiency, suggesting that Tfpi is indispensable to the development and viability of mice [3].

In 1994, Miyagi et al. [4] have reported that Tfpi-2 is expressed in several tissues, such as liver, heart, kidney, skeletal muscle, pancreas and placenta. In another study, expression of *Sciaenops ocellatus* Tfpi-2 was detected, in increasing order, in spleen, muscle, gill, brain, liver, kidney, blood, and heart [5]. Our previous study also showed that zTfpi-2 displayed an expression gradient, with high levels of expression in the brain and heart [6]. Because Tfpi-2 inhibits several serine proteases in the ECM, it is thought that it may play a regulated role in processes of histogenesis, embryonic development and wound healing [7]. Recently, it is also identified that Tfpi-2 can mediate the invasion and migration of human cancer cells as a new tumor suppressor gene [8], inhibit plaque disruption in atherosclerosis [9] and regulate inflammation and apoptosis [10]. However, its physiological significance in the development of heart remains to be explored.

In vertebrates, the heart is the first organ formed in the developing embryo. The zebrafish, *Danio rerio*, offers several distinct advantages as an embryological and genetic model system [11]. Their optical clarity, external embryogenesis, and fast development allow for analysis of gene activities during heart development [12]. In addition, zebrafish embryos are not completely dependent on a functional cardiovascular system. Even in the total absence of blood circulation, they receive enough oxygen by passive diffusion to survive, thereby allowing a detailed analysis of animals with severe cardiovascular defects [13].

The Notch pathway is a versatile regulator of cell fate specification, growth, differentiation, and patterning processes in metazoan organisms [14]. Notch activity affects how cells respond to intrinsic or extrinsic developmental cues that are necessary to unfold specific developmental programs. Our previous data have indicated that the Notch pathway is involved in zTfpi-2-mediated the central nervous system (CNS) development [6]. Notch signaling is also critical in mammalian cardiogenesis, as mutations in this signaling

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pathway are linked to human congenital heart disease [15]. Furthermore, Notch signaling can repair myocardial injury by promoting myocardial regeneration, protecting ischemic myocardium, inducing angiogenesis, and negatively regulating cardiac fibroblast-myofibroblast transformation [16,17].

To elucidate the biological functions of Tfpi-2 in early heart development, anti-sense morpholino oligonucleotides (MO) were designed and performed to specifically block the expression of zTfpi-2 in zebrafish. Effects of both the translation-blocker, ATG-MO, and the splice-inhibitor, SP-MO, were verified in our previous study [6]. In this report, we found that significant heart defects arose in zTfpi-2-deficient embryos during zebrafish embryogenesis. Importantly, the defects of zTfpi-2 morphants could be rescued by coinjection of *ztfpi-2* mRNA, further supporting the specificity of the two MOs. Our results revealed that zTfpi-2 may play an important role in the physiology and pathology of heart. Moreover, our data also implicated that zTfpi-2 can act as a regulator of hematopoiesis in zebrafish, as knockdown its expression was linked to notable hematopoietic defects.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Zebrafish were maintained and bred under standard conditions [11]. Embryos were staged according to the method reported by Kimmel et al. [11]. To block pigment formation in the embryos, 0.003% phenylthiourea was added to the media at 18 hpf and replenished every 24 h thereafter.

2.2. Morpholino antisense oligonucleotides and mRNA microinjections

MOs were obtained from Gene Tools, LLC (U.S.) and resuspended in nuclease-free water [18]. Embryos were injected at the one-cell stage with 1 nl of MOs. For *ztfpi-2*, the sequence of the splice-inhibiting MO (SP-MO) and the translation-blocking MO (ATG-MO) were as follows: 5′-GAAAATGAACGTACTTGGTATCCTG-3′ and 5′-GCTCCGAGTAAATCACACGCCATTG-3′, respectively. The sequence of standard control (Scon-MO) and the five mismatch control (Mcon-MO) for the SP-MO are listed below: 5′-CCTCTTACCTCAGTTACAATTTATA-3′; 5′-GAtAATcAACcTACTTGcTAT CgTG-3′.

Rescue experiment was performed as previously described [6]. All injections were performed at least three separate times.

2.3. Whole mount in situ hybridization (WISH)

Plasmids encoding zebrafish *vmhc*, *amhc*, *bmp4* and *gata1* were provided by Ting Jin, and *α-E1-globin* was provided by Duan Juan. Digoxigenin-labeled RNA probes were synthesized using the DIG RNA Labeling kit (Roche). WISH was carried out as described previously [19], and staining was performed with an alkaline phosphatase substrate kit (Vector Laboratories).

2.4. Quantitative real time PCR (qPCR)

Total RNAs was extracted from 20 zebrafish embryos using the Trizol reagent (Invitrogen). RNA was reverse-transcribed using PrimeScript RT reagent kit (TaKaRa). The real-time quantitative RT-PCR analyses were performed according the manufacturer's instructions. The data were collected using the ABI 7500 system (Applied Biosystems) and analyzed by the sequence detection software 3.1 (Applied Biosystems). The relative expression values were normalized to the internal control, β-actin. The qPCR primer sequences are listed in Table 1.

2.5. Histology and electron microscopy

To section the embryos, whole mount *in situ* hybridized embryos were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4 °C. The embryos were then washed with PBST three times for 5 min each to remove the fixative. This procedure was followed by a two-step embedding process: the first step involved a 1.5% agarose in 5% sucrose solution to adjust for embryo orientation, and the other step used O.C.T. compound (Tissue-Tek, Sakura) to allow for sectioning. For transmission electron microscopy, embryos were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer.

2.6. O-dianisidine staining

Embryos at 48 hpf were dechorionated with pronase, and stained with 1 ml freshly prepared staining buffer (0.6 mg/ml O-dianisidine; 0.5 ml of 0.1 M NaAc, pH 4.5; 0.65% hydrogen peroxide; 40% ethanol), followed by incubation in the dark for 15 min. The reaction was stopped by adding PBST. Images were acquired using Olympus system, and analyzed with Image-pro plus software.

2.7. Photography

Stained embryos were examined with Olympus BX61 and SZX12 microscopes and photographed with a DP 70 digital camera. Images were processed using Adobe Photoshop software.

2.8. Statistical analysis

Quantitative data were expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance followed by the LSD post hoc test for multiple comparisons. *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Knockdown of zTfpi-2 caused abnormal development in heart

Morphologically, embryos injected with SP-MO or/and ATG-MO looked normal until 12 hpf. At 24 hpf, zTfpi-2-deficiency embryos

Table 1
Primer sequences for qPCR.

Gene	Forward 5′ to 3′	Reverse 5′ to 3′
vmhc	AGCTTGAGGCAGAAAGAGCTGCTA	AAGGTCTCTGCGGAGCTTCTGAAA
amhc	AAACCACTCACCCACACTTTGTGC	AGCTGGTGCATGACCAAACAGTTG
bmp4	GCTTCGCGTCTACAGGCAACAAAT	TTTCCCATTTGGAGGTGTTGTGCC
scl	ACGCCGCTCGCCACTATTAACAG	TGAGTCCTGGTCCTCAGTAAAG
gata1	GTCCAGTTCGCCAAGTTTAC	GGGTTGTAGGGAGAGTTTAG
α-E1-globin	TGCTCTCTCCAGGATGTTG	TCCGGCATTAAGGTCATC
β-Actin	ATGCCCCTCGTGCTGTTTTC	GCCTCATCTCCCACATAGGA

showed smaller head, slower vermicular motion of heart and reduced spontaneous movement. The obvious cardiac abnormalities were noted when embryos developed to 48 hpf, including un-consumed yolk sac, heart edema, reduced heart rate (HR) and less globin pigment. Compared to wild type (Wt) and the two MOs control (Mcon-MO and Scon-MO), embryos injected with SP-MO showed severe cardiac abnormalities (Fig. 1A and B). The HR dramatically reduced in SP-MO-injected embryos (76 ± 14/min, $n = 20$) than Mcon-MO-injected embryos (138 ± 9/min, $n = 20$). Embryos injected with ATG-MO showed the similar phenotype, and the HR was decreased (81 ± 11/min, $n = 20$) than Scon-MO control (142 ± 8/min, $n = 20$). Additionally, the two MOs co-

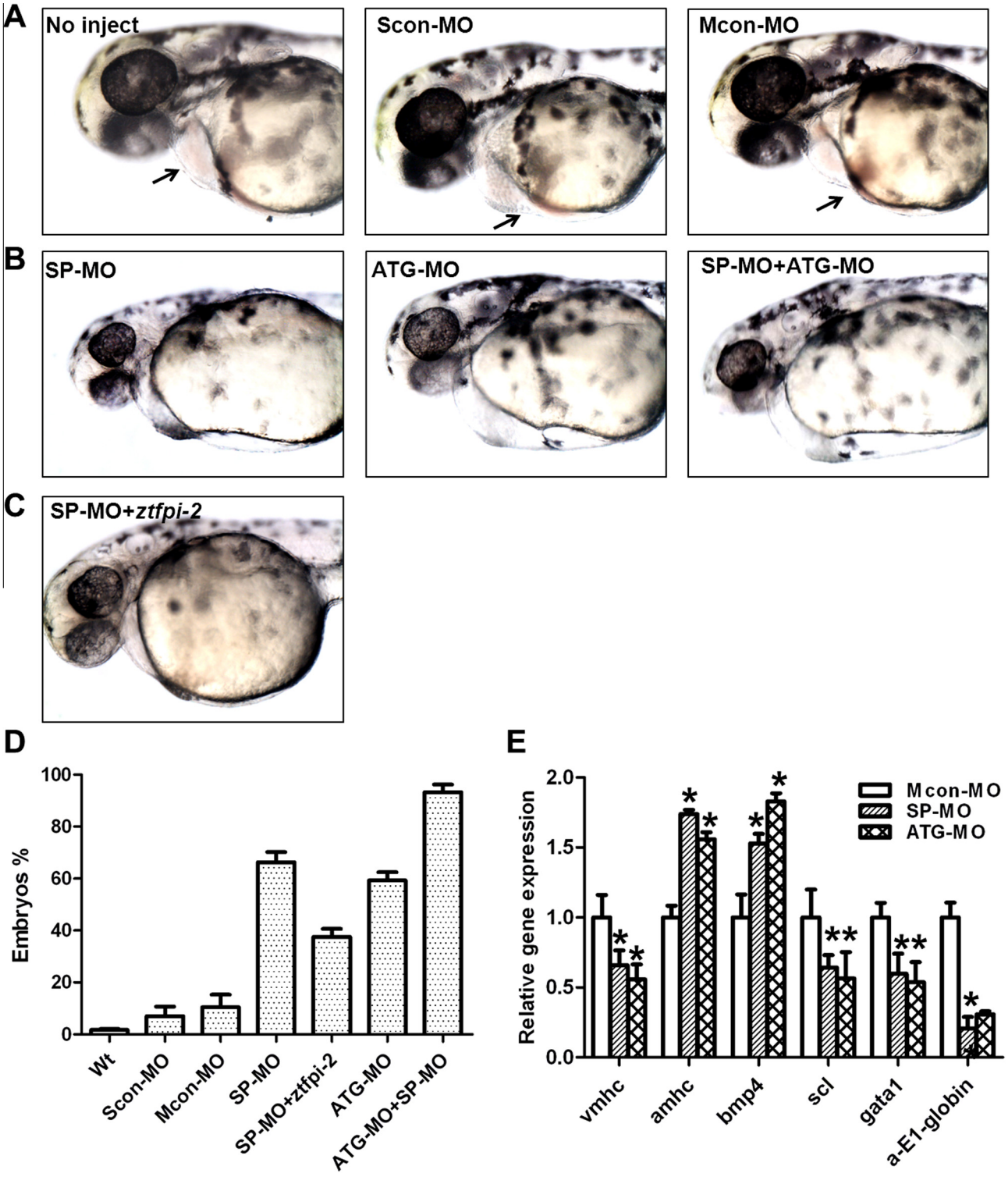

Fig. 1. Cardiac defects exhibited in zTfpi-2 morphants. Lateral views. (A) Embryos injected with 1 mM Mcon-MO and Scon-MO did not show any cardiac abnormality compared with no inject embryos at 48 hpf. (B) 0.5 mM SP-MO and 0.6 mM ATG-MO injected embryos displayed malformation of the heart with globin deficiencies (arrows); Co-injected SP-MO and ATG-MO resulted in more severe cardiac defects. (C) 75 pg *ztfpi-2* mRNA successfully rescued the cardiac defects. (D) Statistics of zebrafish phenotypic analyses. (E) Real-time quantitative PCR verification of marker changes when zTfpi-2 was knocked down at 48 hpf. Asterisks indicate a significant difference from the Mcon-MO group.

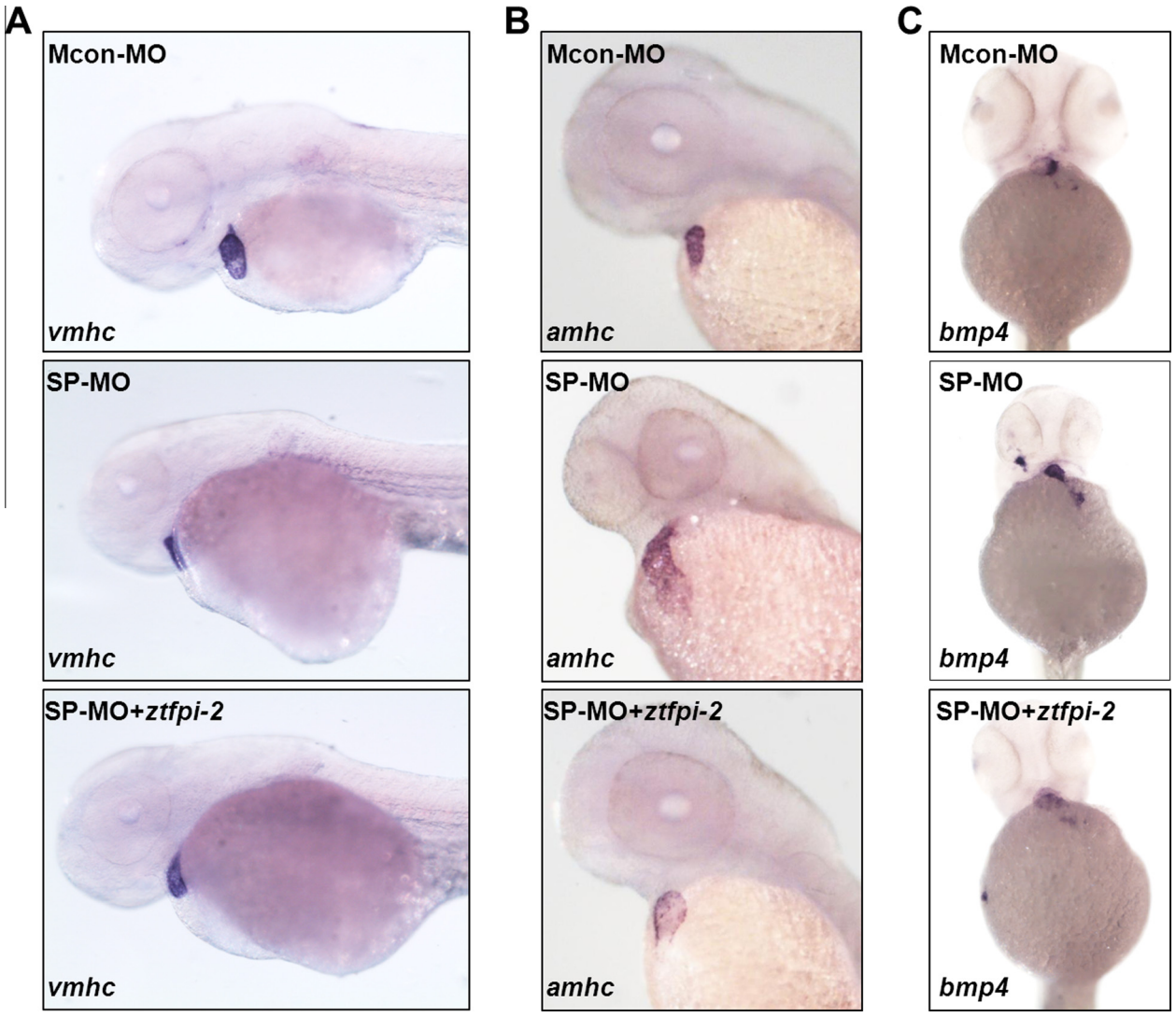

Fig. 2. WISH analysis of ventricle, atrium and atrioventricular boundary markers. Lateral views of zTfpi-2 morphant embryos indicated lower levels of *vmhc* (A) and higher levels of *amhc* (B). (C) Lateroventral views showed disorganization of *bmp4* in the cardiac region of zTfpi-2-deficient embryos at 48 hpf. While *ztfpi-2* mRNA could partially recover those defects.

injected resulted in synergism and more severe phenotypes with sharply decreased HR (63 ± 14/min, $n = 20$; Fig. 1B). Furthermore, morphological phenotypes were found to be restored in a large fraction by concomitant injection of *ztfpi-2* mRNA with substantially increased HR (103 ± 13/min, $n = 20$; Fig. 1C). While *ztfpi-2* mRNA is free of SP-MO target sequence, and the rescued morphology is more obvious, verifying the specificity of SP-MO in causing developmental deformity. Gross morphological phenotypes especially cardiac abnormalities were analyzed and statistic results were shown in Fig. 1D.

To further investigate the molecular basis for the cardiac abnormalities in zTfpi-2 inhibition embryos, we performed real-time PCR analysis of the gene expression profiles of heart from zTfpi-2 knockdown embryos at 48 hpf. From this analysis, expression alterations of cardiogenic markers were detected, *vmhc* for ventricle [20], *amhc* for atrium [21] and *bmp4* for atrioventricular junction [22]. In particular, the mRNA level of *vmhc* was obviously decreased while *amhc* and *bmp4* were significantly increased compared with the Mcon-MO group (Fig. 1E). Then, we examined the expression of the region-specific marker genes by *in situ* hybridization. WISH showed that zTfpi-2-defective embryos displayed atrophic ventricle with lower levels of *vmhc* (75%, $n = 35$; Fig. 2A), dilated atrial with overexpression of *amhc* (79%, $n = 32$; Fig. 2B), alongside malformation of atrioventricular junction with aberrance distribution of *bmp4* in the heart (81%, $n = 30$; Fig. 2C); whereas the rescued embryos recovered to some extents.

3.2. Effects of zTfpi-2 knockdown on sarcomeric organization

The functional defects especially the decreased HR in zTfpi-2 morphants suggested that the cardiac contractile apparatus is abnormal. To further confirm that zTfpi-2 plays an essential role in cardiac contractility, we set out to use transmission electron microscopy to explore the role of zTfpi-2 in sarcomere assembly. Transverse sections of zebrafish embryonic ventricle cells at 72 hpf are shown in Fig. 3A. At this stage, the control heart exhibited nascent sarcomeres containing both thick and thin filaments, and the nascent myofibrils assemble into higher order sarcomere structures (Fig. 3B). By contrast, SP-MO-injected embryos appeared the myofibrillar disorganization in the cardiac muscle (Fig. 3C). Detailed ultrastructural analysis of zebrafish cardiomyocytes revealed that the z-disc and other structures were irregular in zTfpi-2 inhibition embryos compared to controls. SP-MO and *ztfpi-2* mRNA co-injected embryos showed recovered myofibrils

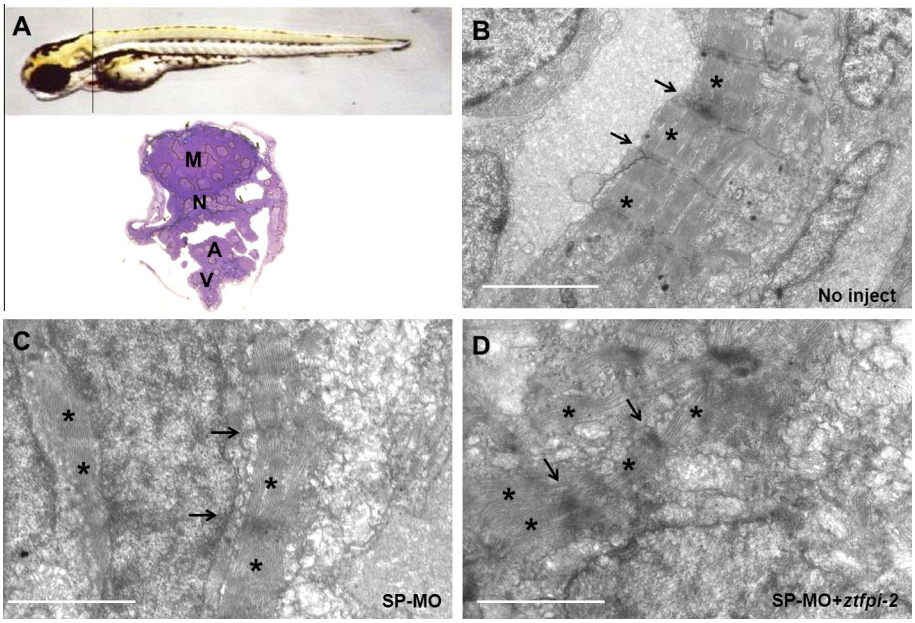

Fig. 3. zTfpi-2 was essential for sarcomeric organization. (A) Diagram showing the plane of section (top) and transverse sections of zebrafish embryo (bottom) at 72 hpf. M, myelencephalon; N, notochord; A, atrium; V, ventricle. (B) Transmission electron microscopy of control embryos, nascent myofibrils assembled into higher-order sarcomere structures (asterisks). Arrows pointing to the Z-disc of sarcomere. (C) zTfpi-2-defective embryos displayed less myofibrils and myofibrillar disorganization in the cardiac muscle. (D) SP-MO and *ztfpi-2* mRNA co-injected embryos showed recovered myofibrils and less disrupted sarcomeric structures. Scale bar, 1 μm.

(Fig. 3D), which indicated zTfpi-2 is involved in assembly of the cardiac contractile apparatus.

3.3. Hematopoietic development was impaired in zTfpi-2-deficient embryos

Recent findings highlight that hematopoietic cells express Notch receptors and their ligands, and Notch signaling affects the survival, proliferation, and fate choices of precursors at various stages of hematopoietic development [23]. Previously, Notch pathway was shown to be essential for Tfpi-2-mediated zebrafish CNS development, implying that knockdown of Tfpi-2 may impair hematopoietic process in zebrafish. At gross level, a notable decrease of red blood cells was appeared in the zTfpi-2-deficiency embryos (Fig. 1B). To address the effects of zTfpi-2 on hematopoiesis, we examined the expression of blood cell markers. QPCR showed that the expression of hemangioblast marker *scl*, primitive erythroid marker *gata1* [24], and mature erythrocyte marker *a-E1-globin* [25] were obviously decreased (Fig. 1E). To characterize the hematopoietic defects, the hemoglobin O-dianisidine staining of blood cells at 48 hpf embryos was used to assess their status. Marked suppression of hemoglobin production was observed in the precordium of zTfpi-2 morphants in comparison with strong hemoglobin staining in control embryos at 48 hpf (Fig. 4A), and the circulating blood flow seemed to be changed. WISH also showed that levels of *a-E1-globin* (88%, $n = 35$; Fig. 4B) and *gata1* (75%, $n = 36$; Fig. 4C) were significantly decreased in their specific region of zTfpi-2-defective embryos injected with the SP-MO at 24 hpf. However, *ztfpi-2* mRNA rescue with less deformity confirmed that the hematopoietic abnormalities were the result of specific inhibition of zTfpi-2 function, indicating that zTfpi-2 is required for the hematopoietic development.

4. Discussion

The expression pattern of *ztfpi-2* suggested that it probably played an important role in the development of the zebrafish heart. Consistent with this, we have presented evidence from microarray analysis that marker genes required for the development and function of the heart, such as *gata5*, *gata6* and *nkx2.5*, altered greater than 2-fold after zTfpi-2 knockdown [6,26,27]. Our previous studies have revealed that early in embryogenesis Tfpi-2 is essential for zebrafish brain development, as its knockdown is linked to severe CNS defects. We have also found that Notch signaling pathway is involved in the function of zTfpi-2. Studies have shown that Notch signaling is an evolutionarily ancient, highly conserved pathway important for deciding cell fate, cellular development, differentiation, proliferation, apoptosis, adhesion, and epithelial-to-mesenchymal transition [14]. Signals exchanged between neighboring cells through the Notch receptor can amplify and consolidate molecular differences, which eventually dictate cell fates. In the vertebrate cardiovascular system, multiple Notch family receptors and several of their Jagged and Delta-like ligands are expressed during critical stages of embryonic and postnatal development [17]. Recent findings have demonstrated a critical role of Notch in zebrafish cardiac regeneration [28]. In addition, Notch signaling also regulates cardiomyocyte proliferation and early heart valve development [29].

Accumulating evidence indicates that Notch may protect the heart from an excessive and detrimental hypertrophic response and increase cardiomyocyte survival [30]. Notch inhibition leads to increased cardiomyocyte apoptosis, and exacerbates the development of cardiac hypertrophy and fibrosis [31]. Here, our results demonstrated that zTfpi-2 knockdown lead to retrenched ventricle, enlarged atrium and abnormal cardiac valve, therefore, will eventually induce heart failure. Compared to well-arranged myofibrils in the cardiomyocytes of Wt embryos, zTfpi-2 inhibition produced sarcomere assembly defects. The failure to generate normal sarcomeres possibly underlied the inability of the zTfpi-2 inhibited heart to generate significant systolic force. The disorganization of sarcomeres could be due to a specific function of zTfpi-2 in regulating the expression of a subset of sarcomeric genes, or other specific alterations in gene expression that secondarily leading to sarcomeric disorganization. Our existing data supported the latter hypothesis that members of Notch pathway probably participated

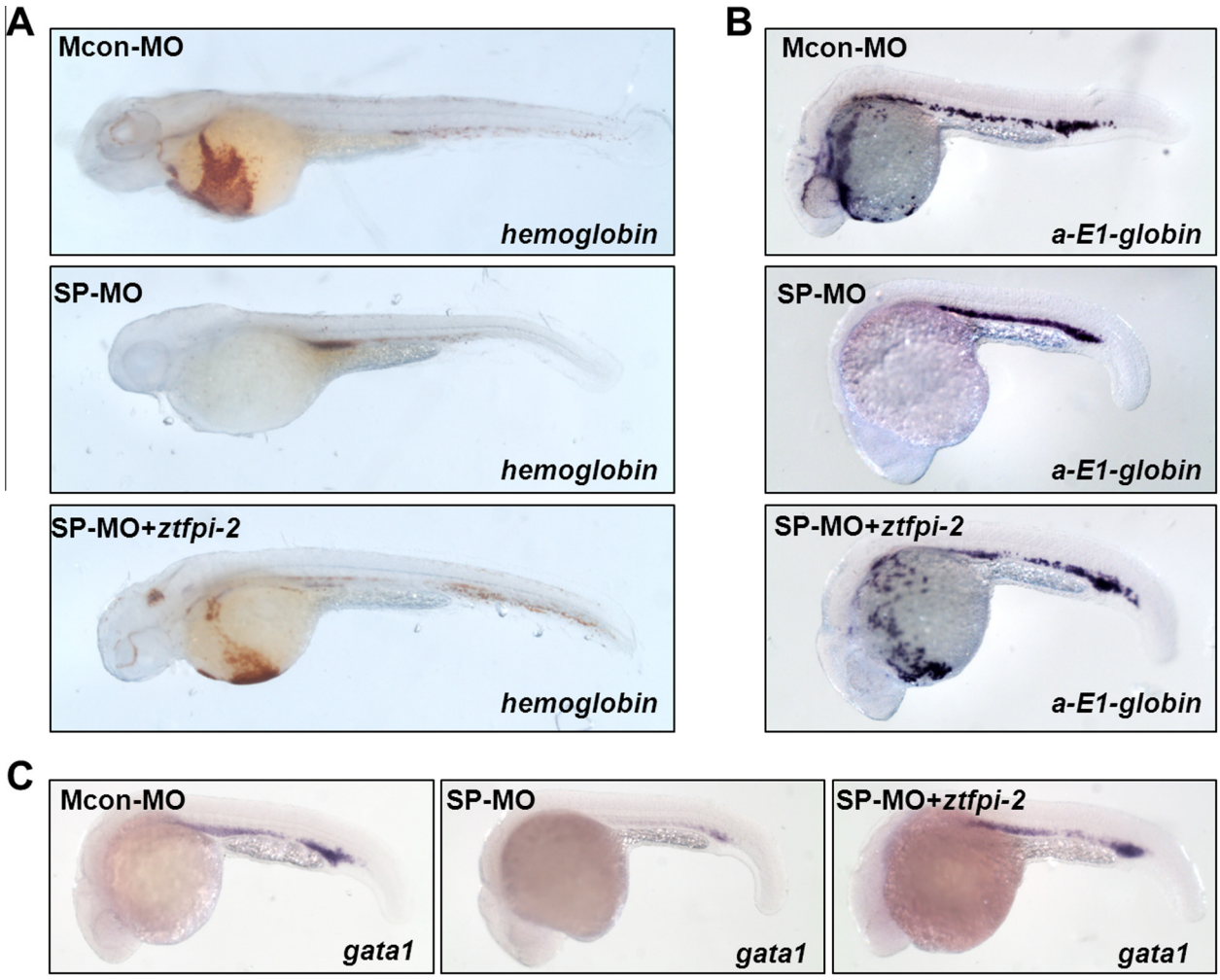

Fig. 4. zTfpi-2 deficiency impaired hematopoiesis in zebrafish. (A) Knockdown of zTfpi-2 led to decreased hemoglobin staining by O-dianisidine at 48 hpf. WISH showed, at 25 hpf, zTfpi-2 inhibition embryos had a lower expression of *a-E1-globin* (B) and *gata1* (C) than controls. These changes could be restored by co-injection of SP-MO with *ztfpi-2* mRNA.

in zTfpi-2-mediated cardiomyogenesis. The challenge for future studies will be to better understand the mechanisms of Notch pathway regulation by Tfpi-2, as well as its relevance to cardiovascular disorders.

Functional studies in mice, fish, tumor models, and cell culture systems have shown that the angiogenic growth of the blood vessel network, the proliferation of endothelial cells, and the differentiation of arteries and veins are controlled by Notch signaling [17,32]. We also examined the expression of marker genes involved in the development of blood vessels to determine whether the zTfpi-2 knockdown lead to the formation of abnormal blood vessels. Consistent with the recent findings, the angiogenesis related markers (*flk-1*, *tie1* and *tie2*) were found to be dramatically altered in the blood vessels of zTfpi-2-deficient embryos (data not shown). These data implied that Tfpi-2 also affected blood vessel organization in zebrafish embryos, probably through Notch pathway.

The expression of hemangioblast marker *scl*, primitive erythroid marker *gata1* and mature erythrocyte marker *a-E1-globin* were significantly reduced in zTfpi-2 morphants, indicating that the deficiency of zTfpi-2 caused abnormal proliferation of hemangioblast and differentiation of erythroid progenitors. There is accumulating evidence that Notch pathway plays an instructive role in the development and maintenance of the hematopoietic system. In hematopoietic stem cells, Notch signaling has the propensity to expand the stem cells, promote their self-renewal, and influence their survival [33]. These data are consistent with our findings that down-regulation of zTfpi-2 might prevent hematopoiesis.

Taken together, our results underscored the use of zebrafish as a model for investigating essential roles of Tfpi-2 in the process of cardiogenesis in particular and the biology of hematopoiesis in general. Advances in these fields will undoubtedly aid in the implementation of strategies for cardiovascular and hematopoietic disease.

Acknowledgments

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