Biochemical and Biophysical Research Communications 456 (2015) 827–833

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tissue factor pathway inhibitor-2 is critical in zebrafish cardiogenesis

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ARTICLE INFO

Article history:
Received 29 November 2014
Available online 11 December 2014

Keywords:
Tfpi-2
Cardiac development
Notch pathway
Zebrafish

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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http://dx.doi.org/10.1016/j.bbrc.2014.12.017
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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′-GAtAATcAACcTACTTGcTATCgTG-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
a-E1-globin	TGCTCTCTCCAGGATGTTG	TCCGGCATTAAGGTCATC
β-Actin	ATGCCCCTCGTGCTGTTTTC	GCCTCATCTCCCACATAGGA

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