KCTD10 is a member of the PDIP1 family, which is highly conserved during evolution, sharing a lot of similarities among human, mouse, and zebrafish. Recently, zebrafish KCTD13 has been identified to play an important role in the early development of brain and autism. However, the specific function of KCTD10 remains to be elucidated. In this study, experiments were carried out to determine the expression pattern of zebrafish KCTD10 mRNA during embryonic development. It was found that KCTD10 is a maternal gene and KCTD10 is of great importance in the shaping of heart and blood vessels. Our data provide direct clues that knockdown of KCTD10 resulted in severe pericardial edema and loss of heart formation indicated by morphological observation and crucial heart markers like amhc, vmhc, and cmlc2. The heart defect caused by KCTD10 is linked to RhoA and PCNA. Flk-1 staining revealed that intersomitic vessels were lost in the trunk, although angioblasts could migrate to the midline. These findings could be helpful to better understand the determinants responsible for the heart and blood vessel defects.

Keywords KCTD10; PDIP1; heart; blood vessel; zebrafish

Introduction

KCTD10 (potassium channel tetramerization domain-containing 10) belongs to the PDIP1 family, whose two other members are PDIP1 and TNFAIP1. All of them contain a BTB/POZ domain or a potassium channel tetramerization (K-tetra) domain in the N-terminus [1], and a proliferating cell nuclear antigen (PCNA)-binding motif in the C-terminus [2]. The most important functions of the BTB/POZ domain are acting as the substrate-specific adaptor for the Cul3 ubiquitin ligase and promoting the degradation of the target proteins [3]. KCTD10 interacts with PCNA [4,5] and a small subunit of DNA polymerase δ [2], and is induced by tumor necrosis factor-α (TNF-α). In human, KCTD10 down-regulation could inhibit cell proliferation [5]. Transcription factors Sp1 and AP-2α could bind to proximal promoter region of human KCTD10, and regulate its expression [6]. KCTD10 has a 3716-bp cDNA, encoding a 313 amino acid (aa) protein within the open reading frame. The KCTD10 gene is highly conserved during evolution among human, ox, mouse, chicken, Xenopus, and zebrafish.

Little is known about KCTD10’s function, except its DNA sequence, hereditary background, and putative structure. Both KCTD10 and KCTD13, the members of PDIP1 family, bind to POLD2, while KCTD13 and TNFAIP1 mediate target proteins ubiquitination through binding to Cul3 [7]. KCTD13 and TNFAIP1 are also related to the nervous system diseases. KCTD13 is a major driver for the neurodevelopmental phenotypes associated with the 16p11.2 copy number variation [8], and TNFAIP1 is found in the brain of Alzheimer disease patients [9].

In the present study, we focused on KCTD10’s role in early embryogenesis based on the zebrafish development model. The expression pattern and function of KCTD10 in zebrafish embryonic development were explored by whole-mount in situ hybridization. Knockdown of KCTD10 directly induced abnormalities in heart and blood vessels, and the rescue experiment could perfectly reverse the tendency, compensating the morphological defects. All of these results showed that KCTD10 is necessary for zebrafish heart and blood vessels development. It could be inferred that RhoA and PCNA, the two important regulators for heart development, are the targets of KCTD10, which may be the possible pathway for KCTD10 to fulfill its function.
Materials and Methods

Zebrafish and embryos maintenance and western blot analysis
Zebrafish were purchased from Institute of Hydrobiology, Chinese Academy of Science (Wuhan, China), and bred under 14/10 h day/night cycle at 28°C [10]. Embryos were collected and staged according to hours post-fertilization (hpf) and morphological features, then fixed with 4% paraformaldehyde in phosphate-buffered saline. To disturb the production of melanin pigments, embryos were raised in 0.003% 1-phenyl-2-thiourea (P3755; Sigma, St Louis, USA) before being fixed.

Western blot analysis was performed by using peroxidase-conjugated anti-PCNA (PC10, 1 : 500; Santa Cruz, Santa Cruz, USA) and anti-actin (A5441, 1 : 1000; Sigma). The whole process was done as previously described [11].

RNA probe preparation and whole-mount in situ hybridization
KCTD10 cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: forward, 5'-TCCCAGAGCTCGACATTAT-3' and reverse, 5'-CCGGTACTCTACTGTAGGTTGGTGATGCTAGTGTTGGTGGTGAC-3'. The PCR product was cloned into pGEM-T easy plasmid (Promega, Madison, USA) and sequenced. Using digoxigenin (DIG)-labeling reagent (Roche, Basel, Switzerland), the construct was linearized by XhoI and transcribed with SP6 for the antisense probe, or I followed by T7 transcription for the sense strand, directly against the initiation codon to block translation of KCTD10 at the mRNA level. MOs were diluted to a concentration of 1 mM with RNase-free water for storage, and adjusted to appropriate concentration before use. Glass microinjection needles (0.07 mm of inner diameter at the tip) were used to inject MOs into the zebrafish yolks at one- and two-cell stage [13].

Capped mRNAs were also synthesized in vitro with KpnI linearized T easy-KCTD10 plasmid and the T7 RNA polymerase except for the DIG-labeling. For co-injection of mRNA and MOs, the prepared mRNA was injected prior to MOs injection.

Results

Bioinformatic analysis of zebrafish KCTD10 gene
We retrieved the zebrafish KCTD10 cDNA sequence from the NCBI databases and did the preliminary analysis. The full-length of KCTD10 cDNA is 3716 bp, in which the open reading frame encodes a 313 aa protein. In addition, a deduced BTB domain is located in the N terminus (data not shown).

Comparison of the protein sequences of KCTD10 among human, chimpanzee, ox, mouse, chicken and frog reveals that the zebrafish KCTD10 is evolutionally conserved, which shares 94.57% homology with human, while 93.93% with chimpanzee, 91.37% with ox, 93.29% with mouse, 94.89% with chicken, 92.33% with Xenopus, respectively (Fig. 1). Only few residues were changed among different species, suggesting its conserved functions in different organisms.

Expression profile of KCTD10 in zebrafish development
To clarify the expression pattern of KCTD10 mRNA during the early embryogenesis, total RNA was extracted from zebrafish embryos at different developmental stages. RT-PCR was carried out to identify the exact expression pattern from 2 to 5 dpf. The results showed that KCTD10 was primarily.


expressed from 2 to 10 hpf (Fig. 2A). The highest expression level occurred from 2 to 6 hpf. In subsequent stages, the transcription level was deceased to basal level at 16 hpf. After 16 hpf, \textit{KCTD10} transcription was increased again, reaching a peak at 24 hpf, and then decreased at 36 hpf. Finally, the \textit{KCTD10} mRNA kept a steady growth until 5 dpf. In adult tissues, \textit{KCTD10} was ubiquitously detected in various tissues, but relatively abundant in brain, heart, liver, gill, fin, and testis (Fig. 2B). These data indicated that \textit{KCTD10} may take part in the development of organs including heart, brain, and liver.

To some extent, the function of a gene can be predicted by studying its expression distribution. We then analyzed the spatiotemporal expression pattern of \textit{KCTD10} at different stages using \textit{in situ} hybridization. The results showed that \textit{KCTD10} was sustained throughout the whole process from fertilization to 5 dpf. The early appearance of \textit{KCTD10} may give a hint of its maternal origin, or at least its regulative potential.

Initially, high level of \textit{KCTD10} began to express in a diffuse manner all around the embryo (Fig. 3A–J). High expression level of \textit{KCTD10} was detected mainly in the brain and trunk. After a 24 hpf, \textit{KCTD10} was intensified in the brain region while it was kept at a moderate level in the trunk (Fig. 3K,K2). At 36 and 48 hpf, \textit{KCTD10} expression was strengthened in the eyes, midbrain–hindbrain boundary, pharyngeal arch, and hatching gland, etc. (Fig. 3L,L2,M,M2). At the same time, the expression level in somites was diminished. From 72 to 5 dpf, the high expression level of \textit{KCTD10} was mainly located on the head, ear vesicle, and viscus...
Hence, it can be inferred that KCTD10 is likely to be implicated in the formation of eyes, brain, ear vesicle, and viscus during development.

Knockdown of KCTD10 in the developing embryos

In order to determine the role of KCTD10, a targeting MO oligonucleotide was designed against the translation initiation site of KCTD10 mRNA, so as to block the translation of KCTD10 protein. We tested the efficiency of synthesized KCTD10-MO according to the fluorescent signal by co-injection of KCTD10-MO with the KCTD10-EGFP plasmid, which encodes a KCTD10-EGFP fusion protein that can be detected under a fluorescence microscope (Fig. 4A,D), into fertilized zebrafish eggs. The fluorescence intensity of KCTD10-EGFP will be lowered because of MO binding to KCTD10 mRNA. Consequently, significant decrease of EGFP signal was observed in the co-injected embryos (Fig. 4C,F) compared with embryos injected solely with KCTD10-EGFP plasmid (Fig. 4B,E). These results suggested that KCTD10-MO did knockdown KCTD10 mRNA, and did not impair embryo integrity.

Most of the KCTD10-MO-treated embryos were proved to have different abnormalities, while the NC-MO (negative control MO)-treated embryos hardly showed any abnormality. We categorized these phenotypes into three grades based on severity [14]. Grade I: abnormal morphology did not begin to emerge until 36 hpf including expanded heart, pericardial edema, lower heart rate, and slight peristaltic pumping. The retarded development returned to normal after 24 hpf, and the embryos basically obtained a 5-day life span during which time their trunks were well-developed. The more KCTD10-MO was injected, the severer morphological defects occurred in the embryos (Fig. 5). Grade II: starting from 24 hpf, embryo’s trunks were curved in various degrees and could not stretch with very weak blood circulation. The heart remained a straight tube, and the pericardial

Figure 3. Expression pattern of KCTD10 during the developing period in zebrafish embryos (A–J) Initially, high level of KCTD10 began to express in a diffuse manner all around the embryo (K,K2) After 24 hpf, the KCTD10 signal was intensified in the brain region while was kept a moderate level in the trunk. (L,L2,M,M2) At 36 and 48 hpf, KCTD10 expression was strengthened in the eyes, midbrain–hindbrain boundary, pharyngeal arch, and hatching gland. (N–P,N2–P2) From 72 to 5 dpf, the high expression level of KCTD10 was mainly located on the head, ear vesicle, and viscus.
cavity was kept expanding. The atrium and ventricle could not be easily distinguished from each other (Fig. 5). Grade III: KCTD10-MO-treated embryo had obviously smaller head and trunk than those in the control, and its tail was twisted. The head was no longer transparent from 16 hpf. Although somites still developed, the boundaries of somites became blurry and some embryos were unable to form regular somites (Fig. 5). There was not a single embryo that could survive after 5 days due to the deformed heart, which had always been tubular with barely perceived heartbeat [15]. In addition, the digest of the yolk sac was incomplete.

We injected three different doses of MO in trial. With no surprise, higher doses of MO always lead to higher death rate (Table 1). When injected with 2 ng MO, 84.4% of the embryos were in Grade I and 5 ng MO injection resulted in 31.7% of the embryos in Grade II, while there were 78.5% embryos in Grade III with the injection of 8 ng MO. There was clearly a correlation between the dose of KCTD10-MO and the severity of treated zebrafish embryonic phenotypes. With the same amount of NC-MO injection, there were similar phenotypes in embryos as those in wild-type embryos, totally different from the KCTD10-MO-treated embryos. We suggested that KCTD10-MO contributed to these phenotypes, at least partly, if not all.

With the co-injection of MOs and its targeting mRNA synthesized in vitro, the MO-induced morphological defects were alleviated. Through rescue experiments, it was found that when co-injected 8 ng MO with 0.2 ng mRNA, the percentage of Grade III was remarkably reduced, and the mortality was reduced at 5 dpf (Fig. 6). On the other hand, the deformity was profoundly relieved. These results suggested that MO effectively blocked the translation of KCTD10 and hence induced various morphological defects during development.

Expression of cardiac markers in KCTD10 morphants
To further explore the mechanism of heart defect in KCTD10 morphant, we determined the expression of three heart chamber markers including amhc, vmhc, and cmlc2 [16]. The three markers are exclusively expressed in the atrium, the ventricle, and in both the chambers, respectively [14]. In situ hybridization demonstrated that the expression of amhc was enhanced with its expression region narrowed in contrast to the controls from 24 to 48 hpf (Fig. 7A,B), suggesting the delayed development and impaired maturation of cardiomyocytes with the knockdown of KCTD10. In the mean time, the vmhc signal was also more confined to the abnormal ventricle in the KCTD10-MO-injected embryos (Fig. 7A,B), revealing the defects in cardiac ventricle and outflow tract [17,18]. Furthermore, the expression of cmlc2 showed clearly that in the KCTD10 morphants, both the atrium and the ventricle were hypogenetic as a linear tube (Fig. 7A,B) indicating that the dysmorphic hearts failed to loop and form a functional atrioventricular canal [12].

In addition, at 24 hpf, normal heart tube elongated to the left ventricle side as shown in amhc and cmlc2 staining [19]. In KCTD10 morphants, their expression was still located at the midline. Appearance of heart tube was obviously delayed. At 20 hpf, the signal of vmhc can be discerned in the ventricular precursors at the apex of shallow cardiac...
However, after moving to the midline, the vmhc expression was maintained in certain position without further expansion. After 2 days of development, wild-type embryos normally accomplish heart tube looping, and a distinct cardiac chamber begins to form in order [20], with the ventricle occurring to the right of the atrium [21]. As shown in cmlc2 staining (Fig. 7C), a relatively high portion (11 out of 32) of KCTD10 knockdown embryos get the reversed pattern of heart looping, whose ventricles were on the left of atria. These data showed that the cardiac left–right twist was disturbed in the KCTD10-MO-treated group. All these phenomena showed above were achieved when embryos were treated or untreated at the same stages. Therefore, MO-induced delay can be excluded. These evidence suggested that KCTD10 plays an important role in the development of heart muscle.

Inhibition of zebrafish KCTD10 influences the early angiogenesis
KCTD10-MO injection causes the majority of embryos showing developmental defects in the blood stasis and weak circulation at 24 hpf. It is reasonable to suppose that KCTD10 may be responsible for the blood development in zebrafish. Analysis of flk-1, a blood marker gene [22], showed a characteristic loss of intersegmental expression in KCTD10-MO-injected embryos (Fig. 8A), indicating the defective angiogenic sprouting. In contrast, there was no significant difference between embryos injected with NC-MO and the wild-type embryos at 24 hpf (Fig. 8B,C). These results strongly supported that in the KCTD10-MO-injected embryos, the differentiation of blood vessels was halted.

KCTD10-controlled heart formation is linked to RhoA and PCNA
It has been previously reported that RhoB interacts with TNFAIP1 and induces apoptosis [23], and RhoA in zebrafish...
is a key regulator of cardiomyocyte differentiation [24]. Besides, PCNA is also a popular marker for cell proliferation [25] and heart regeneration [26]. We categorized embryos into four groups: uninjected, NC-injected, MO-injected, and MO/mRNA-co-injected embryos. After injection, embryos with heart defects were picked out and counted. The abnormality rate was calculated. It was showed that the heart abnormalities induced by KCTD10 knockdown could be rescued by co-injection of RhoA mRNA (Fig. 9A), which indicated that KCTD10, as a BTB protein, may be partly responsible for RhoA degradation, thereafter affecting the normal cardiogenesis. We then performed immunofluorescence to detect PCNA at the protein level. Unfortunately, unlike heart regeneration after injury, almost all cells are PCNA-positive during the heart development. Then, the protein was extracted and western blot analysis was performed. The results showed that PCNA was slightly increased in the KCTD10-MO group (Fig. 9B), suggesting that PCNA, as an indicator of cell proliferation, was also the target of KCTD10. These results may suggest that the function of KCTD10 is linked to the RhoA and PCNA signaling.

Discussion

In this study, we characterized the role of the zebrafish KCTD10 in the heart and blood vessels development. Specific targeting of KCTD10 by MOs results in embryos with defective hearts and detained sprouting of new blood vessels. Because KCTD10 gene is highly conserved, the function of KCTD10 is likely to be consistent among different vertebrates.

Zebrafish is a perfect model organism which can help us quickly distinguish the morphological changes. In the initiation, higher mRNA level can be observed by in situ hybridization in the early developmental stages; therefore, KCTD10 might be regarded as a maternal gene. Later, its abundance in several tissues starts to differentiate. It is noteworthy that KCTD10 mRNA is going to concentrate on narrower regions like the head, heart, and viscus in the later developmental phases, consisting with the results by RT-PCR that KCTD10 mRNA is in relatively higher level in adult tissues such as the brain, heart, liver, testis, and so on. Morphological abnormalities happen to occur mainly in the heart, brain, trunk, and tail. As noted above, the functions of KCTD10 could be principally executed in the heart, brain, and tail.

Knockdown of KCTD10 resulted in failed heart looping and serious pericardial edema. Heart development is regulated by a delicate and tricky system in which many transcription factors are involved. The expressions of amhc, vmhc, and cmlc2 were disturbed by KCTD10, suggesting KCTD10’s importance in early specification of cardiomyocytes. In addition, the cardiac left–right twist was in disorder at 48 hpf in KCTD10-MO-injected embryos, which confirmed that KCTD10 affected the transcript level of these genes in the early developmental stage. Flk-1 is the receptor of vascular endothelial growth factor, and a notable marker for the migration of angioblasts from ventral mesoderm to
Flk-1 is in charge of formation of vascular system, like dorsal artery, axial vein, cranial vessels, and intersomitic vessels [27]. At 24 hpf, the expression of flk-1 was found at the midline, suggesting that KCTD10 did not interrupt the angioblast migration to the midline. However, KCTD10 was not displayed in the intersomitic vessels. Curved trunk and tail showed developmental retardation in the blood vessels sprouting, not to mention the blurred outlines of the somites.

Our findings showed that KCTD10 is essential in cardiogenesis and angiogenesis. However, the precise mechanism involved in defective heart looping and blood vessel sprouting is still not clear. Transcription factors such as Nkx2.5, GATA4, GATA2, bmp4 and signal molecules like Wnt11 and Notch do participate in the process of heart development [28]. It is well known that many genes in this process have a complex interaction network, and a single gene is regulated by several factors. Hence, more work is required to determine which signaling pathway KCTD10 mediates during heart development and angiogenesis.

Figure 7. In situ hybridization analysis of cardiac chamber markers in KCTD10-MO-injected embryos  Expression of amhc, vmhc, and cmlc2 in the morphants, control embryos, and un-injected embryos at 24 hpf (A) and 48 hpf (B). Embryos are shown in the ventral view, anterior to the left (A,B). Cmlc2 staining of the KCTD10-MO-treated embryos at 48 hpf. (C) The cardiac left–right asymmetry is disturbed. Ventral views, heads to the top (C). V, ventricle; A, atrium. White line indicates division between the atrium and the ventricle.

Figure 8. Down-regulation of KCTD10 affects the expression of flk-1 (A) flk-1 expression in a KCTD10-MO-injected embryo at 24 hpf, showing a characteristic lack of intersegmental expression. (B) flk-1 expression in a NC-MO-injected embryo at 24 hpf, intersegmental vessels were barely affected. (C) In situ hybridization analysis of flk-1 expression in a wild-type embryo at 24 hpf. Arrowheads show the intersegmental vessels. wt, wild type. KCTD10 affects zebrafish heart and blood vessel development
As stated above, one gene’s unfound features might be traced to other similar genes, so it is necessary to review the functions of PDIP1 and TNFAIP1.

The N-terminal region is an extremely conserved K-tetra (also named the T1 domain) [1], which is distantly associated with the BTB domain [29]. Many proteins are degraded by a BTB/POZ protein which forms a large complex including a single Cul molecule [30]. Those complexes carry out the function of labeling the ubiquitin tails to the target protein and conveying it to the proteasome. Moreover, the K-tetra domain is structurally similar to the BTB/POZ domain, so it is natural to presume that the K-tetra domain might also mediate the degradation of some proteins [31]. Hence, KCTD10 might regulate some proteins through its BTB/POZ domain by binding to an E3 ligase, like cullin 3. There are already some research reporting that KCTD10 interacts with PCNA and P50. However, the effect of KCTD10 on PCNA and P50 has not been reported, although KCTD10 cannot interact with these two DNA replication proteins simultaneously. It gives us an insight into the possible mechanisms that KCTD10 would take part in. We are interested in the fact that both AP-2α and TNF-α regulate the expression of KCTD10. One principal function of TNF-α is to induce cell apoptosis, and AP-2α is an important factor in development. We hypothesize that KCTD10 may perform some functions related to apoptosis and development, and its downstream factors would be PCNA and polymerase δ. If so, it makes sense how KCTD10 can influence the process of embryonic development. Initial results showed that KCTD10 does cause PCNA and RhoA degradation on a small scale, and the heart defects caused by KCTD10 can be rescued by supplementation with RhoA mRNA. In the future, we will investigate these two independent signaling pathways.

Given the fact that knockdown of KCTD13 is lethal to brain formation in zebrafish and TNFAIP1 expression is closely associated with Aβ in brains from Alzheimer disease patients, it is still unclear what role KCTD10 plays during the brain development. We did detect the delayed brain development in the KCTD10-MO-treated group. It seems that smaller brain is easily observed as the amount of KCTD10-MO injection increases. However, we believe that KCTD10 most probably participates in neurogenesis in a certain way, otherwise it cannot provide a convincing explanation why visible brain defects occur at such a high frequency.

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References

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