Akap12beta supports asymmetric heart development via modulating the Kupffer’s vesicle formation in zebrasfish

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INTRODUCTION

The vertebrate body plan is accomplished by left-right asymmetric organ development and the heart is a representative asymmetric internal organ which jogs to the left-side. Kupffer’s vesicle (KV) is a spherical left-right organizer during zebrafish embryogenesis and is derived from a cluster of dorsal forerunner cells (DFCs). Cadherin1 is required for collective migration of a DFC cluster and failure of DFC collective migration by Cadherin1 decrement causes KV malformation which results in defective heart laterality. Recently, loss of function mutation of A-kinase anchoring protein 12 (AKAP12) is reported as a high-risk gene in congenital heart disease patients. In this study, we demonstrated the role of akap12β in asymmetric heart development. The akap12β, one of the akap12 isoforms, was expressed in DFCs which give rise to KV and akap12β deficient zebrafish embryos showed defective heart laterality due to the fragmentation of DFC clusters which resulted in KV malformation. DFC-specific loss of akap12β also led to defective heart laterality as a consequence of the failure of collective migration by cadherin1 reduction. Exogenous akap12β mRNA not only restored the defective heart laterality but also increased cadherin1 expression in akap12β morphant zebrafish embryos. Taken together, these findings provide the first experimental evidence that akap12β regulates heart laterality via cadherin1. [BMB Reports 2019; 52(8): 526-531]

Keywords: AKAP12, Asymmetric development, Heart laterality, Kupffer’s vesicle (KV), Zebrafish
Previous AKAP12 studies did not focus on the differential role of the two AKAP12 isoforms, AKAP12α and AKAP12β. Although they only have a small difference in the N-terminal region (less than 5%), each AKAP12 isoform has an independent promoter (14) and shows distinct spatiotemporal mRNA expression during embryogenesis (12, 13). Interestingly, a recent study identified AKAP12 as a loss-of-function mutated gene in CHD patients, which has not been reported in previous reports (15). Here, we demonstrate that akap12β, not akap12α, is expressed in DFCs and that loss of akap12β leads to defective heart laterality due to decreased cdh1 expression. This report provides the first experimental evidence of a heart laterality regulatory mechanism by akap12β, which might also play a role in human heart heterotaxy.

RESULTS

Akap12β is the major isoform of akap12 during KV development in zebrafish

Akap12α and akap12β are two known isoforms of akap12 in zebrafish (Fig. 1A). During embryonic development, each isoform is differentially regulated due to a distinct promoter region (Fig. 1A, B). The expression of akap12β was first observed in the sphere stage, and that of akap12α was initiated in the bud stage, later than akap12β. Interestingly, akap12β morpholino (MO) injection significantly reduced the expression of pan-akap12 at 75% epiboly stage, while akap12α MO injection did not affect the pan-akap12 expression (Fig. 1C, D).

Next, we investigated the spatiotemporal expression of akap12 by in situ hybridization (ISH) (Fig. 1E-G). Mesodermal cells highly expressed akap12 mRNA as previously reported (11). Interestingly, akap12 was expressed in sox17-positive DFCs, known as the progenitor cells of KV. The observed akap12 was suggested to be akap12β, as sox17-positive DFCs, which originate from non-involuting endocytic marginal cells, are present at the 75% epiboly stage (Fig. 1E, F). Moreover, two color ISH for akap12 and dand5, the marker of KV, revealed that akap12 expression in KV was not detected at the 6 somite stage (ss) when KV formation by DFC collective migration is completed (Fig. 1G). Taken together, akap12β, but not akap12α, was transiently expressed in KV ascendant cells when the cluster of DFCs underwent collective migration.

Heart laterality is disrupted in akap12β morphants

Specific expression of akap12β in KV lineage cells motivated us to investigate whether akap12β regulates the left-sided heart orientation via fine-tuning the KV formation in zebrafish. First, the positioning of the heart was investigated by ISH for cmlc1 in akap12β morphants. Akap12β morphants exhibited mesocardia (~20%) and dextrocardia (~12%), while control morphants showed normal heart laterality (Fig. 2A, B). Then, we evaluated KV formation in akap12β morphants by ISH for spaw, which is expressed in the KV region. As shown in Fig. 2C, D, knockdown of akap12β disrupted KV formation in akap12β morphants, as shown by ISH for spaw and spaw-expressing KV lineage cells highlighted in red. Consequently, these results suggest that akap12β plays a role in human heart heterotaxy.
spaw, the nodal-related gene and a novel marker for KV, as the abnormal KV development is a frequent cause of disorienting heart laterality. Bilateral (19%) and right-sided (13%) spaw expression were observed in akap12β morphants while aberrant spaw expression was observed in only 6% of the control morphants (Fig. 2C, D).

To investigate whether the specific downregulation of akap12β in the KV lineage cells such as DFCs also disrupts heart laterality, the akap12β MO was injected into the yolk at the 128 to 512-cell stage (DFC MO) for exclusive reduction of akap12β in the KV lineage cells including DFCs (16). DFC-specific injected MO is restricted in the boundary between the blastomeres and yolk where the KV lineage cells exist since the marginal blastomeres are connected to the yolk cell by a cytoplasmic bridge (Supplementary Fig. S1). Disrupted heart laterality (∼41%) and aberrant spaw expression (∼25%) were also observed in DFC-specific akap12β morphants, whereas low rate defects were identified in DFC control morphants (Fig. 2E-H).

(Akap12β regulates collective migration of DFCs)

Next, we validated the notochordal expression of lefty1, which functions as the midline molecular barrier to restrict nodal activity to the left lateral plate mesoderm (17). However, normal expression of lefty1 was observed in the notochord of both control and akap12β morphants, regardless of dorsal curvature (Fig. 3A, B). Then, we examined dand5 expression, which is a molecular barrier of spaw by surrounding KV. The control morphants showed mostly normal dand5 expression with a horseshoe shape, however, discontinuous dand5 expression was observed in ∼58% of akap12β morphants (Fig. 3C, D). Moreover, a sox17:EGFP-positive DFC cluster, which gives rise to the KV, was identified as fragmentation of DFC clusters in 30% of DFC akap12β morphants, while minimal DFC fragmentation was observed in only ∼1% of DFC control morphants (Fig. 3E, F). Then, we confirmed the DFC fragmentation in DFC akap12β morphants by ISH for foxi1a at 75% epiboly stage. Fragmentation of foxi1a-positive DFC clusters was observed in ∼39% of DFC akap12β morphants, while a single non-fragmented DFC cluster was identified in DFC control morphants (Fig. 3G, H).

Taken together, these data suggest that reduced akap12β expression in DFCs results in the disruption of asymmetric signals and heart laterality due to failure of DFC collective

Fig. 3. Failure of collective migration of DFCs in akap12β morphants. (A) Visualization of lefty1 by ISH in 18 ss embryos of control and akap12β morphants. (B) Stacked bar graph (control morphants; n = 74, akap12β morphants; n = 92). (C) Representative images of dand5 by ISH in 6 ss embryos of control and akap12β morphants. (D) Stacked bar graph (control morphants; n = 86, akap12β morphants; n = 110). (E) Visualization of DFC clusters by immunostaining of sox17:EGFP in 75% epiboly embryos of DFC-specific morphants. (F) Stacked bar graph (DFC control morphants; n = 82, DFC akap12β morphants; n = 77). (G) Representative images of DFC clusters by ISH for foxi1a in 75% epiboly embryos of DFC-specific morphants. (H) Stacked bar graph (DFC control morphants; n = 22, DFC akap12β morphants; n = 36).

Fig. 4. Malformation of KV integrity in akap12β morphants and restoration of defective phenotypes in akap12β morphants by exogenous akap12β mRNA. (A) Immunostaining of Cdh1 and sox17:EGFP in 75% epiboly embryos of DFC control and akap12β morphants. Scale bar, 10 μm. (B) qRT-PCR analysis of cdb1 mRNA in 75% epiboly embryos of DFC control and akap12β morphants. (C) Representative images of Cldn5a-immunostained KV lumen (upper) and whole embryo (lower) in 6 ss embryos of DFC control and akap12β morphants. Scale bar, 20 μm. (D) The lumen area surrounded by Cldn5a is shown as the means ± SD. *P < 0.05, (DFC control morphants; n = 30, akap12β morphants; n = 35). (E) NKI Visualization of DFC clusters by immunostaining of sox17:EGFP in 75% epiboly embryos of control, akap12β, and akap12β mRNA-injected akap12β morphants. (F) Stacked bar graph (control morphants; n = 32, akap12β morphants; n = 30, akap12β mRNA-injected akap12β morphants; n = 35). (G) Visualization of KV lumen by immunostaining for Cldn5a in 6 ss embryos of control, akap12β, and akap12β mRNA-injected akap12β morphants. Scale bar, 20 μm. (H) The lumen area surrounded by Cldn5a is shown as the means ± SD. ***P < 0.001, **P < 0.01, (control morphants; n = 10, akap12β morphants; n = 10, akap12β mRNA-injected akap12β morphants; n = 11).
migration.

Reduction of cdh1 by akap12β knockdown disrupts DFC cluster integrity

Fragmented DFC clusters are the symbolic phenotype of disrupted cell collectivity between DFCs which is maintained by Cdh1-based adherence junction (18). Thus, we evaluated Cdh1 expression in akap12β morphants by immunostaining at 75% epiboly stage. DFC control morphants showed high Cdh1 expression at intercellular surfaces between the DFCs. However, DFC akap12β morphants displayed significantly reduced Cdh1 expression within DFCs (Fig. 4A). Furthermore, not only protein expression, but also mRNA expression was decreased in akap12β morphants (Fig. 4B). These data suggest that the reduced Cdh1 expression within DFCs by akap12β downregulation could lead to KV malformation due to DFC fragmentation. Then, we examined how DFC fragmentation affects KV formation in akap12β morphants. Interestingly, we identified that the size of KV in DFC akap12β morphants was relatively smaller than that of DFC control morphants using differential interference contrast images of living embryos. Consistent with these observations, KV apical lumen area encompassed by Cldn5α was significantly reduced in DFC akap12β morphants (0.63-fold vs. DFC control morphants, Fig. 4C, D).

Finally, akap12β mRNA was injected with MOs to rescue the phenotypes of akap12β morphants. Fragmentation of DFC clusters was restored by exogenous akap12β. Akap12β mRNA injected akap12β morphants showed a higher rate of normally formed single non-fragmented DFC cluster (~76%) than akap12β morphants (~57%) (Fig. 4E, F). Consistently, Exogenous akap12β mRNA also increased the KV lumen area in akap12β morphants (1.75-fold vs. akap12β morphants, Fig. 4G, H). Taken together, these data suggest that the loss of cell collectivity within DFCs reduces KV size and this reduction finally results in heart laterality defects in akap12β morphants.

DISCUSSION

The current study investigated the role of akap12β and akap12α in heart laterality establishment, providing the first experimental evidence that akap12β, not akap12α, might play a role in human heart heterotaxy. We showed that akap12β is the major isoform of akap12 during embryogenesis and is expressed in DFCs, ascendant cells of KV. Knockdown of akap12β led to the reduced cdh1 expression in DFCs which resulted in loss of cell collectivity within DFCs. Finally, fragmented DFC clusters gave rise to smaller KV and malformed KV failed to establish proper heart laterality.

AKAP12 was first identified as an autoantigen in myasthenia gravis, so it was named Gravin (7). In the present study, we investigated the specific role of akap12β in heart laterality regulation. In akap12α morphants, a single sox17:EGFP-positive DFC cluster was preserved and reduction of cdh1 was not observed during KV development (Supplementary Fig. S2 and S3A, B). In this regard, Gelman et al. examined the expression of AKAP12 isoforms in the internal organs including the heart of human and mouse and showed by immunostaining that Akap12 is expressed in the heart during fetal stage (19). Besides, Streb et al. reported that Akap12α and Akap12β are differentially regulated by independent promoters in different tissues and cells (14). In the recent study, two kinds of de novo loss of function mutations of AKAP12 within the exon shared by both isoforms were identified as high-risk mutations in CHD patients of left ventricular obstruction and heterotaxy such as dextrocardia, respectively (15). Interestingly, sudden death was observed in 3% to 4% of 4-month-old Akap12-null mice which carry the deletion of the common exon of Akap12 isoforms and cardiomegaly was commonly identified in all those cases by autopsy (20). Accordingly, we hypothesized that AKAP12α might control the pure heart development considering it as the major isoform of AKAP12 in the heart (14) and AKAP12β might regulate proper heart positioning depending upon our current investigation, respectively. Further investigation is necessary to define the exact role of AKAP12 isoforms in heart development.

Our group reported that AKAP12 regulates junctional protein expression such as E-Cadherin, VE-Cadherin, Claudin-1, Occludin, and ZO-1 in diverse systems (8-10, 12). In zebrafish, Cdh1-mediated cell adhesion between adjacent DFCs is essential for their collective migration followed by KV morphogenesis (2, 18). In this regard, we also observed reduction in cdh1 mRNA and protein expression in akap12β-deficient zebrafish. Moreover, exogenous akap12β mRNA not only maintained a single non-fragmented DFC cluster and the size of KV but also restored cdh1 mRNA expression in akap12β morphants (Fig. S3C). However, we focused on the role of akap12β in the establishment of heart laterality in the current study so further investigations including mechanism(s) of independent expression of akap12 isoforms and regulation of cdh1 expression by akap12β should be necessary.

Recent studies have revealed that asymmetric distribution of hypoxia contributes to dorsoventral axis establishment during embryogenesis of sea urchin and that retinoic acid (RA) is involved (21-23). Moreover, our group previously reported that partial oxygen pressure regulates AKAP12 expression and that RA induces AKAP12 expression in CNS injury repair (9, 24) and we hypothesized that gradation of such factors could regulate the spatiotemporal expression of akap12 isoforms. Relating to cdh1 expression, our group reported that AKAP12 induced by reoxygenation and/or RA suppresses SNAI1, a master transcription factor for epithelial-mesenchymal transition, via the non-Smad pathway during the recovery of CNS injury and that AKAP12 knockdown increases SNAI1 expression in ARPE-19 epithelial cell line (24). Considering Sna1 as a well-known transcriptional repressor for E-cadherin expression (25) and the possible role(s) of Sna1 in asymmetric...
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development (26, 27), we suggest that reduced Cdhl expression in akap12β morphants might be mediated by enhanced Snai1 expression.

In addition to DFC collective migration, DFC numbers and ciliogenesis in KV are crucial for heart laterality (17). Our data indicated that proliferation of DFCs in akap12β morphants was comparable with control morphants and the number of cilia in akap12β morphants was similar to that in control morphants considering the size of the KV lumen (Supplementary Fig. S4, 5). Malformed KV affected not only heart laterality but also other asymmetric internal organs since we also observed aberrant pancreas positioning in akap12β morphants (Supplementary Fig. S6). These data, together with other current data, suggest that loss of function mutation of AKAP12 might be linked to diverse heterotaxy.

Given genetic evidences of AKAP12 loss of function mutation in certain CHD patients, our data indicating that akap12β, not akap12α, specifically regulates heart laterality via regulation of cdh1 expression in DFCs in zebrafish could be extended to the regulation of heart laterality and asymmetric development of internal organs in humans.

MATERIALS AND METHODS

Zebrafish
Tuebingen wild-type zebrafish and transgenic sox17:egfpψ1570 zebrafish (Tg(sox17:egfp)) were previously described (16). All zebrafish work was carried out in accordance with protocols approved by the Institutional Animal Care and Use Committees of Seoul National University.

Morpholino injection
The protocol of MO injection into zebrafish embryos was previously described (13). Briefly, splice-blocking MOs were injected into the yolk at one-cell stage for whole embryo knockdown or at 128-512-cell stage for DFC-specific knockdown as indicated. Translation-blocking MOs were used to rule out the off-target effects (Supplementary Fig. S7). MOs for akap12α and akap12β were previously described (13).

In vitro transcription
PCR-amplified akap12β was cloned into pCS2+ vector (28). 5’-capped and poly(A)-tailed mRNAs were generated using mMessage mMachine ultra kit (Ambion). 80-120 pg of akap12β mRNA was co-injected with akap12β MO. Sequences of primers for akap12β cloning are summarized in Supplementary Table S1.

RNA isolation and quantitative RT-PCR
The protocols of qRT-PCR and RT-PCR were previously described (29). Total RNA was isolated from zebrafish embryos at indicated stages with TRIzol reagent (Invitrogen) and cDNA was obtained from 2 μg of total RNA using MMLV reverse transcriptase (Promega). qRT-PCR was then performed using StepOnePlus RT-PCR system (Applied Biosystems) with RealHelix qPCR kit (NanoHelix). Relative mRNA expression levels were calculated by the comparative 2−ΔΔCt method. Actb2 and eef1a1l1 served as internal controls. Primer sequences for qRT-PCR are summarized in Supplementary Table S1.

Whole-mount ISH and immunostaining
The protocol of ISH was previously described (16). Specific regions of lefty1 and fox1a were cloned into pGEM T easy vector (Promega). ISH probe vectors for cmlc1, dan5, spaw, and pan-akap12 were previously described (12, 16) and primer sequences of lefty1 and fox1a for ISH probe vectors are summarized in Supplementary Table S1. The protocol of whole-mount immunostaining for Tg(sox17:egfp) embryos was described previously (16). Mouse anti-Cdh1 (1:200, BD Biosciences) and goat anti-mouse AF546 (1:1000, Invitrogen) were used for immunofluorescence. Stained embryos were mounted in glycerol and images were obtained by an AxioCam ICC-1 camera (Zeiss) on a Stemi 2000C (Zeiss) for immunohistochemistry and an LSM700 confocal microscope (Zeiss) for immunofluorescence, respectively, and processed using ZEN 2012 software (Zeiss).

Statistical analysis
Measurement of KV lumen area was described previously (16). The data are presented as means ± SD and analyzed with Prism 5 (GraphPad Software, Inc.). The data in Fig. 4B and 4D were analyzed by two-tailed Student’s t-test and the data in Fig. 4H were analyzed by one-way ANOVA test.

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CONFLICTS OF INTEREST
The authors have no conflicting interests.

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