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약학석사학위논문

Analysis of *akap12* in zebrafish: spatiotemporal expression of two isoforms and pancreas development

제브라피쉬의 *akap12* isoform 발현 및 췌장 발생에 대한 연구

2014년 2월

서울대학교 대학원

약학과 의약생명과학전공

정 진 경

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이 논문을 약학석사학위논문으로 제출함

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정 진 경

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2013년 12월

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Abstract

Analysis of *akap12* in zebrafish: spatiotemporal expression of two isoforms and pancreas development

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A Kinase Anchoring Protein 12 (AKAP12), also known as Gravin and src-suppressed C kinase substrate (SSeCKS), is a multivalent scaffolding protein that controls multiple biological processes. Its activity results from scaffolding signaling proteins such as protein kinase (PK) C and A, calmodulin, cyclins,

phosphoinositides, β-1,4 galactosyltransferase, Src, as well as the actin cytoskeleton. It has been demonstrated that there are three types of AKAP12 isoforms (designated α, β, and γ) in human with different promoters and known that the major types are AKAP12α and AKAP12β.

In zebrafish, the expression of AKAP12 is dynamically controlled. It is detected ubiquitously during gastrulation. During segmentation, the expression is restricted to the mesoderm, nervous system and blood vasculature. Later, it becomes localized to the head region. The composition and distribution of AKAP12 indicates its crucial role and function in development process.

However, similarity and difference of expression and distribution between AKAP12α and AKAP12β in zebrafish remain unclear. Here we investigated the biological significance of these two isoforms during zebrafish development.

During zebrafish embryogenesis, RT-PCR analysis detected the mRNA expression of AKAP12 isoforms at different developmental stages: AKAP12β is detected from the late blastula period; Expression of AKAP12α is initiated at bud stage, the late gastrula period. By knockdown of each isoform expression using morpholino-based antisense oligonucleotide, respective expression of AKAP12α and AKAP12β was identified. To clarify the pattern of expression clearly, we designed probes for each isoforms. As a result, zebrafish

AKAP12α, β were all found to be expressed similarly but distinctly. Taken together, these observations become essential for identifying the role and function of AKAP12α and AKAP12β that are involved in the zebrafish development.

In addition to identify spatiotemporal expression of the two isoforms, we uncovered the role of *akap12* in pancreas laterality. During pancreas development, the organ primordia undergo asymmetric movement to ensure proper function.

Yet the role of *akap12* in pancreas laterality is not well defined. In this study, we investigated the function of *akap12* in asymmetric movement of pancreas during development. In the absence of *akap12*, we identified disrupted pancreas morphology. In particular, *akap12* does not function directly in pancreas development. We confirmed the result by KV-specific knock-down technic. Collectively, our data provide the role of *akap12* in pancreas laterality.

keywords : AKAP12, AKAP12α, AKAP12β, pancreas, Left-right asymmetry, zebrafish

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Introduction

1. A-Kinase Anchoring Protein 12 (AKAP12)

AKAP12 was identified as an auto-antigen in myasthenia gravis thus known as Gravin [1, 2]. It is also called SSeCKS (*Src*-Suppressed C Kinase Substrate), because it is shown to be orthologous to a rodent protein.

AKAP12 is a multivalent scaffolding protein and controls several key proteins. Several binding partners that have been reported are PKA, PKC, calmodulin, and β-1,4-galactosyltransferase [3, 4].

It has been reported that AKAP12 conducts significant roles in biological processes. AKAP12 regulates the cytokinesis progression [5]. Also AKAP12 involves in the formation of brain-barrier by regulating angiogenesis and tight junction formation [6]. During embryogenesis, AKAP12 has a role for migratory processes by controlling cytoskeletal and tissue architecture [7] (Fig 1).

To date, three isoforms of AKAP12 has been demonstrated in human and rodent. It is designated α, β, and γ and known to have different promoters. Such different promoters allow AKAP12 to control each isoform independently [8].

In zebrafish, 2 types of AKAP12 isoform, AKAP12α and AKAP12β have been reported. Additionally, several roles of AKAP12 in zebrafish embryogenesis have been demonstrated:

AKAP12 functions for convergent extension of the mesoderm that is required for axis elongation [9]; AKAP12 regulates vascular integrity [10]. However, difference and similarity between two isoforms remain unclear.

In this study, we identified spatiotemporal expression of *akap12α* and *akap12β* during development in zebrafish using two strategies. It is confirmed that two isoforms are expressed similarly but distinctly. These observations become essential for identifying the role and function of AKAP12α and AKAP12β that are involved in the zebrafish development.

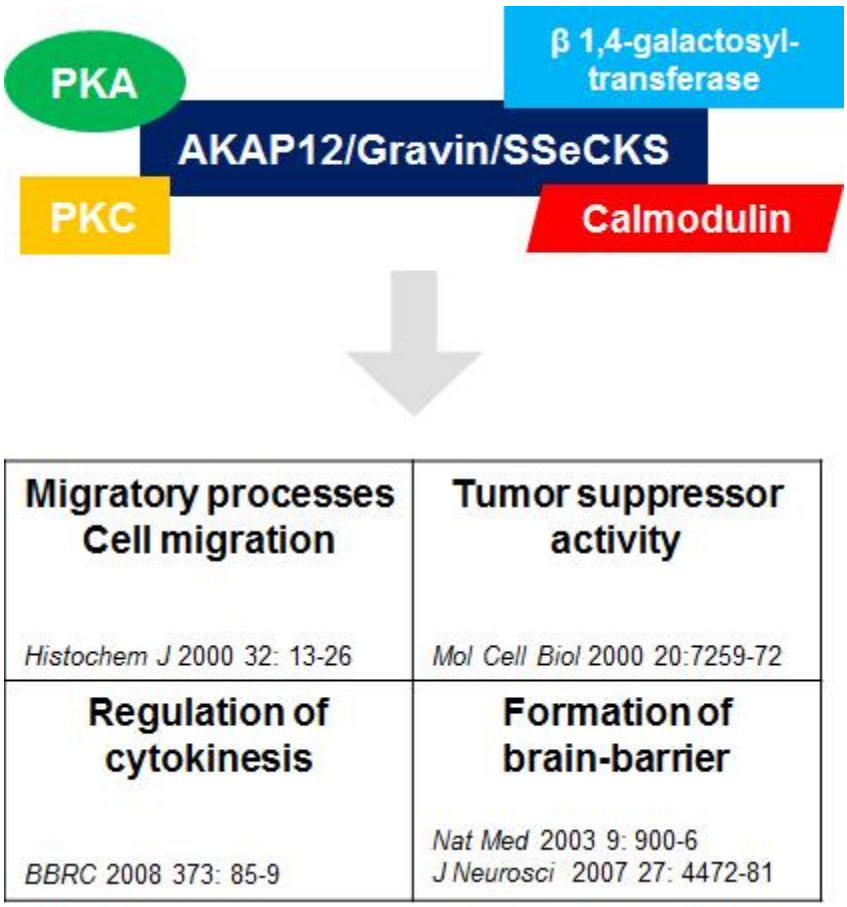

Figure1. Schematic representation of AKAP12

AKAP12, which functions as a scaffolding protein, controls diverse biological processes. Several binding partners that have been reported are PKA, PKC, calmodulin, and β 1,4−galactosyltransferse.

2. Endocrine pancreas development in zebrafish

The vertebrate pancreas is a bifunctional organ. It consists of exocrine and endocrine tissue: the exocrine tissue constitutes major part of the organ and is composed of duct cells and acinar glands which secrete digestive enzymes; the endocrine tissue is composed of five types of cells (designated α, β, γ, δ, and ε cell) which produce hormones [11, 12].

In zebrafish, pancreas is originated from the endoderm layer. Expression of pancreatic hormone in zebrafish is initiated at the 12-somite stage with insulin that is produced by β cell. Fusion of insulin positive cells is gradually progressed, and by 20-somite stage, the insulin positive cells form a one-cell-thick layer. Afterwards they are organized into a cluster. At 24 hpf, the cluster becomes localized at the midline [13].

Other cell types appear later than β cell: *somatostatin*-expressing δ cells at 19 hpf; *glucagon*-expressing α cells at 21 hpf [14].

By 24 hpf, these endocrine cells cluster into the primary islet, followed by fusion of ventral bud designated to be a exocrine part and dorsal bud designated to be a endocrine part at 48 hpf. Then it is situated on the right side of the embryo.

3. Left-right asymmetry in zebrafish

In vertebrates, establishment of Left-right (L-R) asymmetry is a significant in organogenesis. Brain, heart, and visceral organs such as pancreas and liver are located in asymmetric positions to function properly.

The zerafish model has been widely adopted for the developmental study because of accessibility [15, 16]. In zebrafish embryos, laterality organ for establishing asymmetry is Kupffer' s vesicle (KV). KV is a ciliated organ and regulates chirality by generating fluid flow [17, 18].

The L-R patterning process includes several steps. Initially, bilateral symmetry breaks at cleavage stages. Then the transient organ, KV is formed in early somitogenesis, followed by transmission of the asymmetric signal to the lateral plate mesoderm. Finally, the asymmetric information is sent to the organ primordia to direct their position asymmetrically [19].

To date, it has been demonstrated that AKAP12 regulates L-R asymmetric development of zebrafish by interfering KV [20]. However, it remains still unclear that which organ is affected. In this study, we investigated pancreas, one of the organs that show chirality, and found that the developmental defect of pancreas results from defected KV. Our findings in this study indicate that AKAP12 have a role in pancreas laterality.

Material and Method

1. Zebrafish maintenance

Wild-type AB zebrafish and Tg(*ins*:GFP) acquired from the Zebrafish Organogenesis Mutation Bank (ZOMB) were used in this study. Zebrafish were maintained at 28.5℃ on a 14-hour light/10-hour dark cycle as previously described [21]. Embryos were generated by natural pairwise mating and raised in Danieau's solution. The embryos were staged according to the hours post fertilization (hpf) based on the standard developmental stage [22]. The embryos were treated with 1-Phenyl-2-thiourea (Sigma) to inhibit pigment formation at 12 hpf.

2. Isolation of zebrafish RNA and cDNA synthesis

Total RNA from the different stages of zebrafish embryos were extracted using the TRIzol reagent (Ambion) standard protocol and resuspended in nuclease-free water.

Three micrograms of RNA were used for cDNA synthesis. The isolated RNA and 10 pmol oilgo dT primer were incubated at 70℃ for 10 min and then 4℃ for 2 min. After the reaction, the mixture of 5X M-MLV reverse transcription buffer, 10 mM dNTP mixture, and M-MLV reverse transcriptase (Promega) was added. Then reverse transcription was performed at 42℃ for 60min; inactivation of the enzyme was conducted at 70℃ for

15min.

3. Polymerase Chain Reaction

The cDNAs synthesized were used as the template for PCR reaction. The following couple of primers were used: for *akap12α*, F1 (5'-ATGGGAGCGACACCATCCGTGC-3') and R1 (5'-TCATGCACTGTGACAACCTCTGTGGAG-3'); for *akap12β*, F1 (5'-ATGCTTGGGACAATAAC-3') and R1 (5'-TCATGCACTGTGACAACCTCTGTGGAG-3'); for *β-actin2*, F1 (5'-GCAGAAGGAGATCACATCCCTGGC-3') and R1 (5'-CATTGCCGTCACCTTCACCGTTC-3'). PCR was carried out using 10 pmoles of each primer in a T3000 thermocycler (Biometra).

Reaction conditions are as follows: for *akap12α*, initial denaturation at 94℃ for 5min, followed by 28 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 5 min; for *akap12β*, initial denaturation at 94℃ for 5min, followed by 24 cycles at 94℃ for 45 sec, 55℃ for 45 sec, 72℃ for 5 min; for *β-actin2*, initial denaturation at 94℃ for 5min, followed by 18 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 45 sec.

4. Preparation of anti-sense probes

Using some samples of zebrafish embryos obtained from different developmental stage, partial sequences of *akap12*, *akap12α*, *akap12β*, and *preproinsulin (ins)* were amplified. The

primer pairs for cloning is as follows: for *akap12α*, F1 (5' -AAGGATCCATGGGAGCGACACCATCCGTG-3') and R1 (5' -AAGTCGACTCATTCAGCCGGTGGGTTCTCCT-3'); for *akap12β*, F1 (5' -ACATCATAGAGAAAGAGAGG-3') and R1 (5' -CTGTTAGAGTTATTGTCCCA-3'). These PCR products were cloned in pGEM-T Easy Vector (Promega). They were sequenced on both strands to confirm specificity. Then the antisense *akap12*, *akap12α*, *akap12β*, and *ins* digoxigenin (DIG)-labeled probes were obtained by *in vitro* transcription. With digoxygenin RNA labeling kit (Roche), the antisense probes were synthesized using SP6 polymerase.

5. Whole-mount *in situ* Hybridization

5.1 *in situ* Hybridization

Whole-mount *in situ* hybridizations were carried out as described previously [23]. The embryos were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) at 4℃ for overnight. Fixed embryos were washed twice with PBS-T (1X PBS with 0.1% Tween 20) for 5min and dehydrated with methanol and stored at -20℃ for at least 2 hours. Dehydrated embryos were rehydrated via 75%, 50%, and 25% methanol series in PBS-T. After rehydration, the samples were treated with proteinase K (Roche) at room temperature according to the developmental stages for permeabilizing the embryos, followed by fixation with 4% PFA in PBS at room

temperature for 20 min. After three times of washing with PBS-T, the embryos were blocked in hybridization buffer (50% formamide, 5X SSC, 5 mg/ml yeast tRNA, 50 μg/ml heparin, and 0.1% Tween 20) at least for 2 hours at 65℃, and replaced with hybridization containing 5 ng/μl antisense probe, and then incubated at 65℃ for overnight. Then, embryos were rinsed at 65℃ with 50% formamide/2x saline sodium citrate with 0.1% Tween-20 (SSCT), 2x SSCT, 0.2x SSCT, and PBST. To remove endogenous alkaline phosphatase, embryos were treated with 0.1M glycine with 0.1% Tween-20 (pH 2.2) for 45 min. Blocking step was performed with blocking solution (0.5% Roche blocking reagent, 5% goat serum in PBT) at least for 3 hours at room temperature. Afterwards anti-DIG-AP Fab solution (Roche, diluted 1:2000 in blocking solution) was replaced and incubated at 4℃ for overnight. Washing 10 times with PBST for 20min, the embryos were washed in staining solution (100 mM Tris-Cl, 50 mM $MgCl_2$,100mM NaCl,1mM levamisole, and 0.1% Tween-20). Signals were visualized by NBT/BCIP stock solution (Roche). The samples were mounted in 75% glycerol and photographed.

5.2 Fluorescent *in situ* hybridization

Whole-mount fluorescent *in situ* hybridization was performed as described previously [24, 25] using *akap12* probe. After hybridization with the probe at 65℃ for overnight, the probe was

washed extensively at 65℃. Then, samples were blocked with blocking solution, followed by replacing anti-DIG-POD solution (Roche, diluted 1:500 in blocking solution). Washing 8 times with PBST, embryos were incubated 30 min in TSA Plus TMR Solution (Perkin Elmer) and then washed with PBT 6 times. The samples were mounted in 75% glycerol and photographed.

6. Whole-mount immunohistochemistry

At 50hpf, Tg(*ins*:GFP) embryos were fixed with 4% PFA in PBS at 4℃ for overnight and dehydrated with methanol at -20℃ at least for overnight. After rehydration by adding serial methanol with PBST, collagenase (1 mg/ml) in PBST was treated for 60min, followed by quickly washing with PBST twice, and PBDTT (1XPBS, 1% DMSO, 0.5% Triton X-100, and 0.1% Tween-20) twice. Samples were treated with blocking solution (5% BSA, 10% normal goat serum in PBDTT) for 3 hours. Then, primary antibodies were treated at 4℃ for overnight. After washing with PBDTT for 5 times, embryos were blocked with 1/5 diluted blocking buffer for 3 hours and incubated with secondary antibodies in 1% BSA/PBDTT at 4℃ for overnight. Primary antibodies used were: mouse anti-GFP (Santa Cruz Biotechnology) at 1:1000; mouse anti-glucagon (Sigma) at 1:500. Antibodies were visualized with Alexa-Flour-488 conjugated secondary antibodies (1:500; Molecular Probes).

7. Morpholino injection

Splice-blocking morpholinos were obtained from Gene Tools, LLC (Oregon, USA). Two morpholinos targeting *akap12α,β* were used. *akap12α* MO (5' -TACCTTGCCATCTGCGGTTTCT CCA-3') targets against the splice-donor site of exon 1 of *akap12α* and *akap12β* MO (5' -TCTTACCTGTTAGAGTTATT GTCCC-3') targets the site of exon 3 of *akap12β*. For the control, standard control morpholino was used (5' -CCTCTTAC CTCAGTTACAATTTATA-3'). Injection was performed at the one-cell stage as reported previously [26]. Injected doses were 2 ng for *akap12α*, 6 ng for *akap12β*, and 6 ng for control.

8. Microscopy

Expression patterns of in situ hybridization were photographed by Zeiss Stemi 2000C using a AxioCam ICC-1 camera and processed with Axiovision software. Fluorescent images were acquired on a Zeiss LSM700 confocal microscope.

Result

Part Ⅰ. Spatiotemporal expression of *akap12* isoforms during development in zebrafish

1. Comparison of AKAP12α and AKAP12β

Location of zebrafish *akap12* gene is in chromosome 20. In zebrafish, it is reported that there are two isoforms of *akap12*, *akap12α* and *akap12β*. Importantly, there is a conserved region: 98% identity was showed between the region of *akap12α* and *akap12β*. The full-length of zebrafish *akap12α* is 4791 bp and composed of three exons with two variants and the conserved region; *akap12β* is 4602 bp and composed of two exons with a variant and the conserved region (Fig. 2).

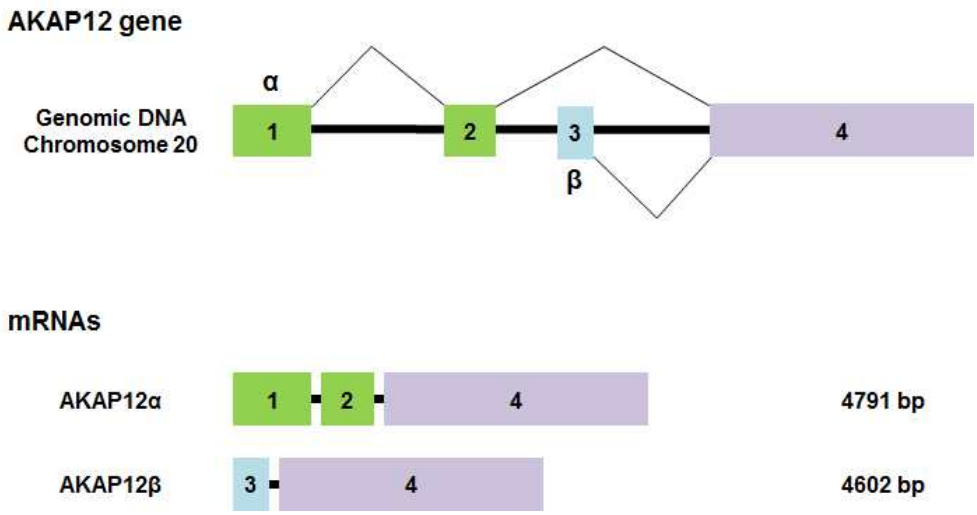

Figure2. Organization and isoforms of zebrafish *akap12* gene

Organization of two isoforms of akap12.

2. Expression of *akap12* during zebrafish development

To confirm the expression pattern of *akap12* in embryogenesis, we conducted RT-PCR analysis using synthesized cDNA at different stages. The result showed that *akap12α,β* mRNA expression is detected from different stages. The expression of *akap12α* is initiated from the bud stage. On the other hand, *akap12β* is detected from sphere stage and increased gradually, and then decreased at 24 hpf (Fig. 3).

Next we carried out whole-mount in situ hybridization to determine the spatiotemporal expression of AKAP12 mRNA in zebrafish. Expression of *akap12* changed dynamically. At the early stage by the late gastrula period, *akap12* is seen ubiquitously (Fig. 4A,B). During segmentation period, *akap12* is localized to the specific region: mesoderm; adaxial cells; notochord; vascular system; and nervous system (Fig. 4C-E). At 2 dpf and 3 dpf, the expression is restricted to the head region. (Fig. 4F,G)

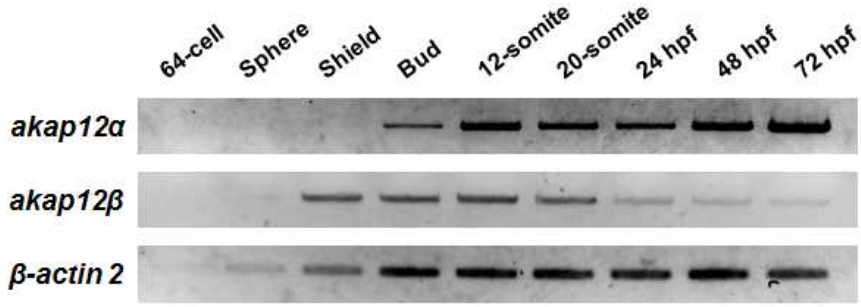

Figure3. *akap12* mRNA expression during zebafish development

RT-PCR analysis of *akap12* gene expression at different developmental stages. Two isoforms, *akap12α* and *akap12β* is detected from distinct stages.

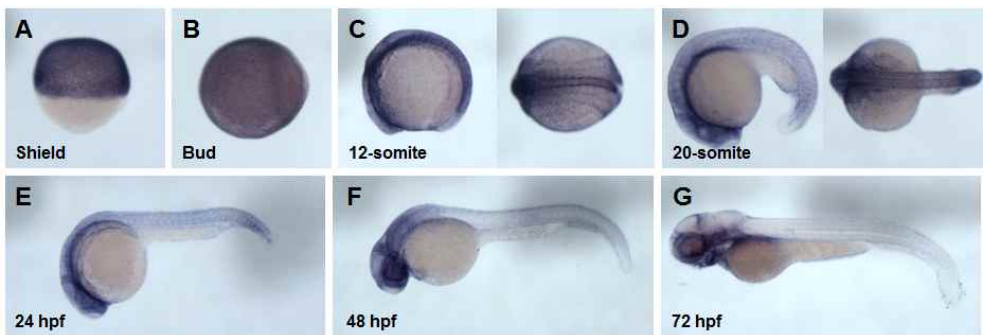

Figure4. Spatiotemporal expression pattern of akap12

Whole-mount *in situ* hybridization using pan-*akap12* probe. (A,B) *akap12* expression is observed ubiquitously during gastrula stage. (C-E) During segmentation period, the signal is in the mesoderm, adaxial cells, notochord, vasculature, and nervous system. (F,G) *akap12* is abundant in the head region.

3. Identification of spatiotemporal expression of *akap12α,β*

3.1 Identifying *akap12 α, β* specifically by using *akap12*-knockdown embryos

To identify the spatiotemporal expression of *akap12* two isoforms specifically, we injected morpholino oligonucleotides (MO) targeted to the splice donor site. AKAP12 gene consists of exon 1, 2, 3, and 4. Exon 4 is conserved region. *akap12α* MO was designed to target the exon 1; *akap12β* MO was designed to target the exon 3 (Fig. 5A). The MO injected embryos were collected after 3 dpf. To confirm the efficiency of *akap12* knock-down, we carried out RT-PCR analysis. As a result, the expression of *akap12 α* and *akap12β* were suppressed successfully (Fig. 5B).

Using the morphants, we carried out *in situ* hybridization using a pan-*akap12* probe. By this method, we were able to localize *akap12α* expression by *akap12β* morphants and *akap12β* expression by *akap12α* morphants (Fig. 5C). Comparing the expression, the expression is detected from different stages corresponding to the RT-PCR analysis (Fig. 3). As the embryos develop, expression pattern indicates that two isoforms are expressed similarly. According to the intensity of the signal, it is identified that *akap12β* forms are more abundant.

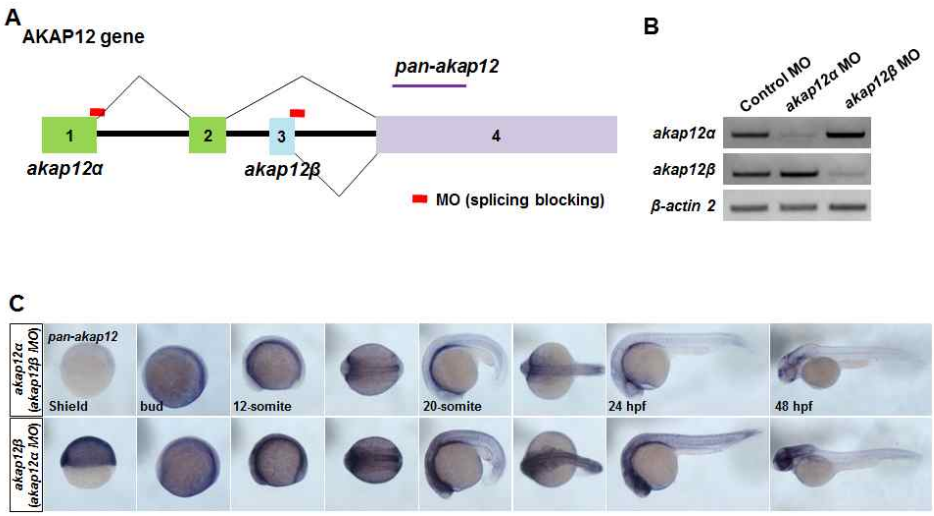

Figure5. Strategy for identifying *akap12* α, β specifically by knock-down embryos

(A) Organization of AKAP12 gene. Specific morpholinos are targeted to interfering splicing. The pan-*akap12* probe used for *in situ* hybridization is designed to detect conserved region of AKAP12 gene. (B) By one-cell stage injection, *akap12* α, β knock-down embryos are obtained. It is confirmed by RT-PCR analysis. (C) Using knock-down embryos, *in situ* hybridization was performed to detect expression of each isoform.

3.2 Identifying *akap12 α, β* specifically by specific antisense probes

We could identified specific expression of *akap12 α, β* during zebrafish embryogenesis by knock-down of *akap12* previously, however, this method possessed several limitations. First, we could not rule out the developmental defect. Second, since the morpholino effect is decreased gradually, specific expression cannot be detected especially at the late embryogenesis. Thus, we developed another strategy. We attempted to construct specific antisense probes (Fig. 6A). *akap12α* probe was designed to detect *akap12α*-specific exon 1 giving a product of 213 bp. However, since *akap12β*-specific exon encodes only 8-amino acid, we designed *akap12β* antisense probe include untranslated region of akap12*β* giving a product of 191 bp.

As a result of *in situ* hybridization using *akap12α, β*-specific antisense probes, similarity and distinctiveness revealed more clearly (Fig. 6B). Expression pattern correlates exactly with the previous result using the *akap12* knock-down embryos (Fig. 5C). During the first day of development, beginning of each isoform was detected at different stage. However, differences between two isoforms are revealed apparently. At 20-somite stage, in dorsal view, ventral mesoderm region is observed only in *akap12β*. At 24 hpf, we saw several differences in trunk region. In developmental stage, ventral mesoderm becomes lateral mesoderm, which is related to formation of cardiovascular

and lymphatic systems. In agreement with this, *akap12β* is exhibited in intersegmental vessel of trunk region predominantly. On the other hand, *akap12α* expression is strong in notochord. Signal is restricted to the head region at 48 hpf. In this stage, we found that *akap12β* expression is more substantial in both retina and midbrain-hindbrain boundary (MHB). MHB is designated cell populations that affect cell fate in surrounding neural plate cells [27]. This region is a significant organizing center that has a role in midbrain and cerebellar induction, as well as patterning.

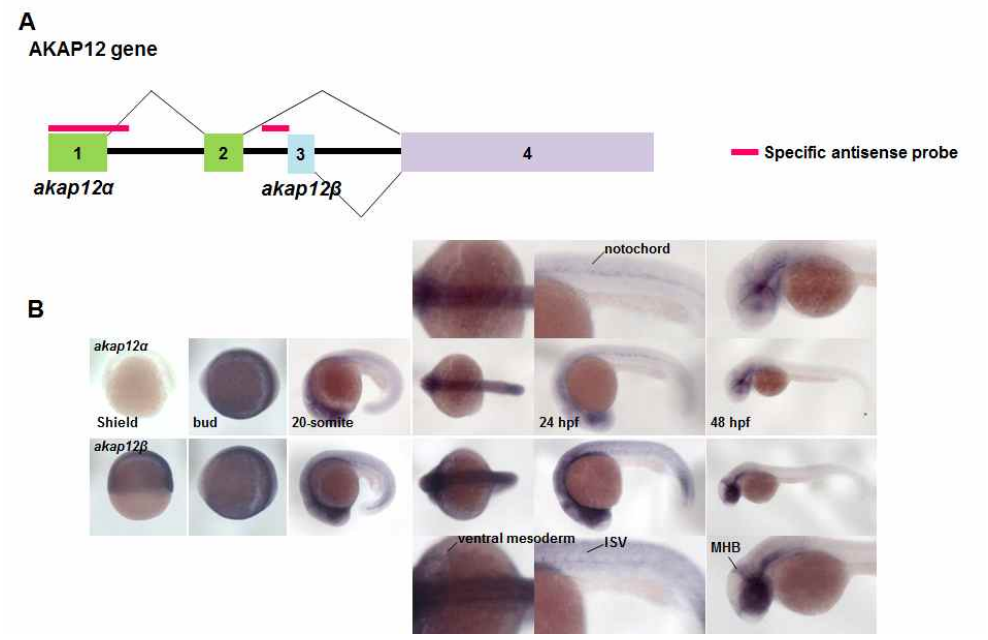

Figure6. Strategy for identifying akap12α, β specifically using specific antisense probes

(A) Organization of AKAP12 gene. Antisense probes which is designed to detect *akap12α*, *β* specific region was constructed. (B) *In situ* hybridization showing specific spatiotemporal expression of *akap12α*, *β*. Abbreviation: ISV, intersegmental vessel; MHB, midbrain-hindbrain boundary.

Part Ⅱ. The role of AKAP12 in pancreas laterality of zebrafish

1. Altered expression pattern of *ins* in *akap12* morphant

The migration of endocrine part of pancreas from midline to the right side has been accomplished at 50 hpf. Thus *preproinsulin (ins)* expression is observed on the right side of the wild-type larva (Fig. 7A). In *akap12* morphants, however, the expression pattern is altered: it exists in a split form; it remains in midline; it exists in a reversed position (Fig. 7B-D).

We observed defect in *akap12α* (38.9%, n=56/144) and *akap12β* morphants (31.3%, n=50/144), respectively, compared with control morphant (4.9%, n=8/144). Also, such a defect persisted in the late stage: at 3 dpf in *akap12α* (33.3%, n=9/27) and *akap12β* morphants (31.8%, n=7/22) compared with control morphant (3.7%, n=1/27); at 4 dpf in *akap12α* (46.4%, n=13/28) and *akap12β* morphants (25.9%, n=7/27) compared with control morphant (3.7%, n=1/27) (Fig. 7E). However, we could not identify difference between *akap12α* and *akap12β* morphants.

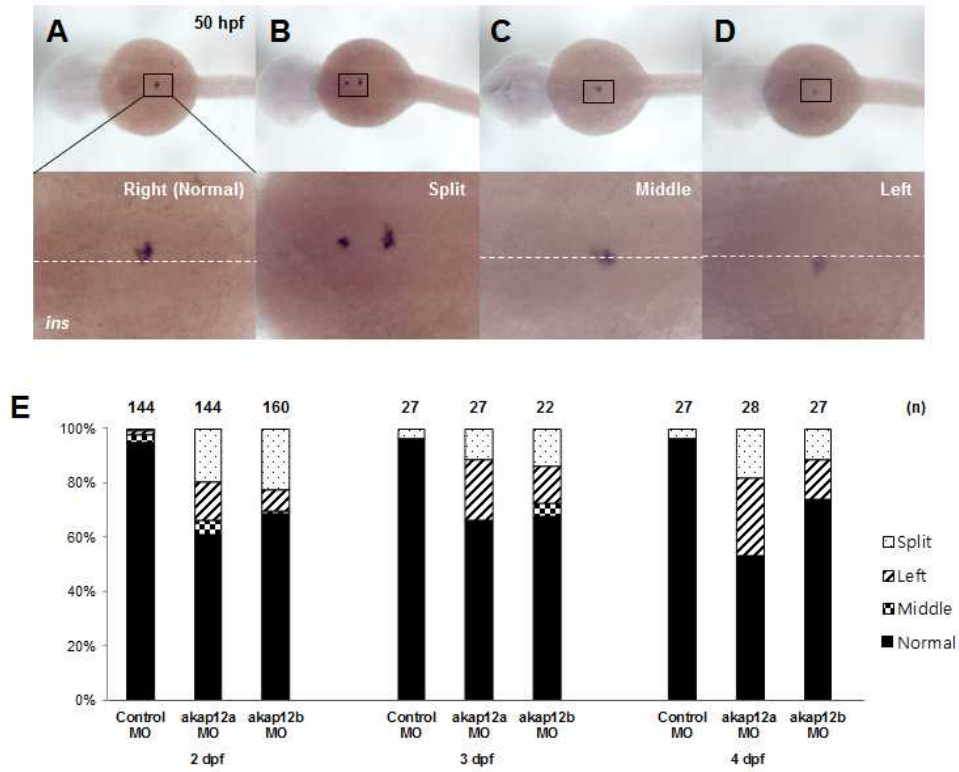

Figure7. Disrupted pancreas laterality in *akap12* morphants

(A–D) Dorsal view of 50 hpf (2 dpf) wild-type and *akap12* morphants displaying *ins* expression patterns. Normally *ins* is showed on the right side (A), however, in akap12 morphants, pancreas laterality was altered to be in a split form (B), on the middle (C), and on the left side (D). (E) Quantification of pancreas expression pattern in control, *akap12α*, and *akap12β* morphants at 2 dpf, 3 dpf, and 4 dpf.

2. AKAP12 has no direct effect on pancreas development.

The striking pancreas defects observed in *akap12* morphants prompted us to investigate whether AKAP12 regulate pancreas development directly. First, we investigated the expression of *akap12* in developing pancreas. Intense signal of *akap12* in *insulin* positive cells is not observed in co-localization images of *akap12* and *insulin* (Fig. 8A). At 50 hpf, *akap12* was seen to vasculature broadly.

Next, we examined the relationship between cell differentiation of pancreas and AKAP12. Previously, we confirmed impaired expression of ins, which indicates β cells, however, could not identified altered population. Thus, we inspected α cell population by Glucagon expression in the absence of *akap12* (Fig. 8B). As a result, there were no significant differences between control morphant and *akap12* morphants (Fig. 8C). AKAP12 is not required for α cell differentiation. These observations indicate that AKAP12 does not affect to the pancreas development directly.

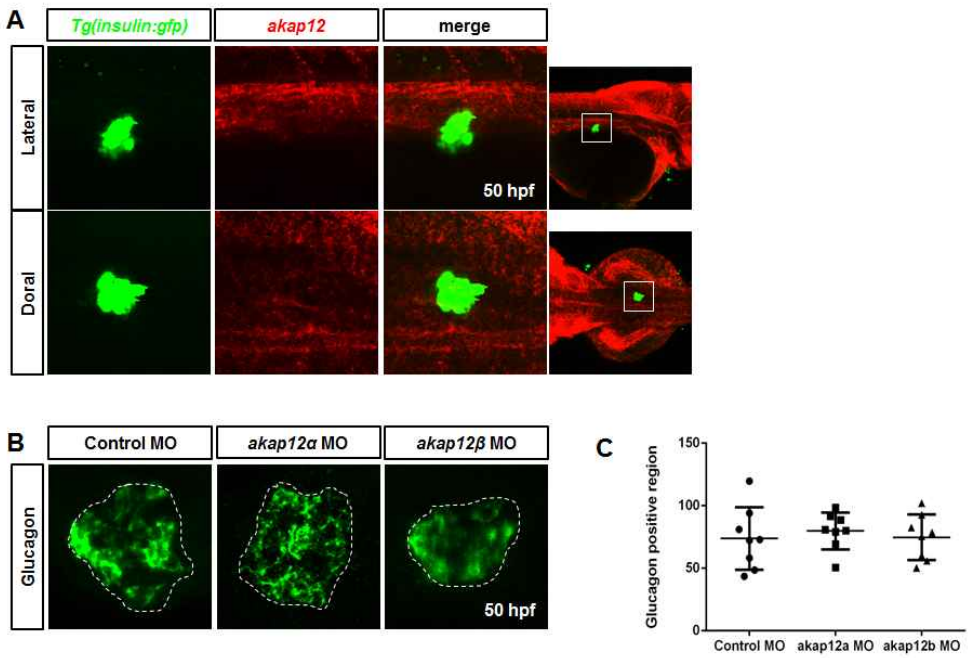

Figure8. AKAP12 does not affect to the pancreas development directly.

(A) Confocal images of 50 hpf wild-type embryos stained for *insulin* and *akap12*. (B) Confocal images of 50 hpf embryos displaying Glucagon expression in a dorsal view. The dotted lines are indicating Glucagon positive cell region. (C) Quantification of Glucagon positive cell region.

3. Pancreas defect results from the malformed KV.

We have previously shown that AKAP12 is independent on direct pancreas development. To analyze what caused pancreas defect, we used dorsal forerunner cell (DFC) –specific morpholino injection technic. In case of the progenitors of the DFC and KV, cytoplasmic bridge retains by 4 hpf, but that of other embryonic cells is closed by 2 hpf [28]. Thus, focusing on this gap, it is possible to deliver morpholinos to the progenitors of the DFC and KV specifically by injecting at the 256–512–cell stages (2.5–2.75 hpf) [29] (Fig. 9A). As a result, similar defect rate with the result of one–cell stage injection was showed in *akap12α* (50.0%, n=70/140) and *akap12β* morphants (42.2%, n=65/154) compared with control morphant (6.4%, n=9/140) (Fig. 9B). In summary, disrupted KV yields pancreas laterality defects.

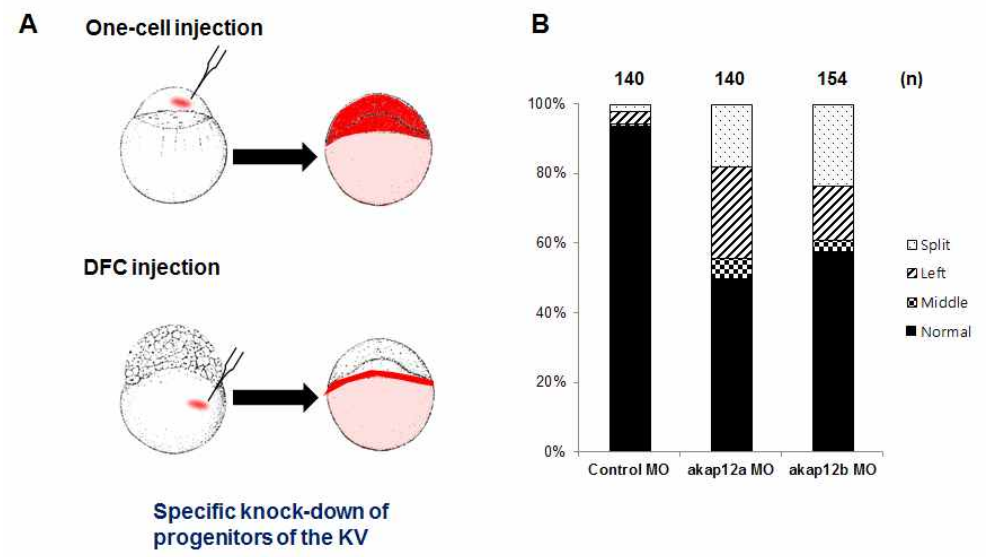

Figure9. Disrupted KV causes pancreas laterality defect.

(A) Comparison of one-cell injection and DFC injection technic. By performing DFC injection, specific knock-down of progenitors of the KV can be accomplished. (B) Quantification of pancreas expression pattern in control, *akap12α*, and *akap12β* morphants at 2 dpf by DFC injection.

Discussion

Here, we reported the analysis of AKAP12 during zebrafish embryonic development. First, we identified spatiotemporal expression pattern of two isoforms of *akap12α* and *akap12β*. Second, we showed the role of *akap12* in pancreas laterality.

Within the AKAP12 locus, the two isoforms are not modulated by alternative splicing, instead, each isoform has a distinct promoter [8]. In addition, RT-PCR analysis displayed that initiation of mRNA expression is separated. These suggest the possibility of different role of AKAP12α,β.

We set two strategies to identify two isoforms. We injected *akap12α,β* specific morpholinos to obtain knock-down embryos. Using these embryos, we performed in situ hybridization with pan-akap12 probes (Fig. 5). We found the results were consistent to those of RT-PCR analysis (Fig. 3). However, because of imperfection caused by developmental defect and decreased morpholino effect, we adopted another strategy. By constructing *akap12α,β* specific morpholino, we could investigate two isoforms more clearly (Fig. 6). We confirmed the probe by comparing the expression in the corresponding position with the previous result. Interestingly, we observed the difference distribution of the two isoforms more apparently. By the gastrula period, since the expression is dispersed, the difference was not observed. In the segmentation period, at 20-somite stage, the most distinct expression was detected in

the ventral mesoderm. Only *akap12β* expression was displayed. In agreement with this result, the later stage, at 24 hpf, signal of *akap12β* was abundant in ISV. Ventral mesoderm is to be a lateral mesoderm which regulates vascular system. On the other hand, *akap12α* was predominant in notochord. At 2 dpf, the signal becomes restricted to the head region, and we discovered the difference in the retina and MHB.

Overall, *akap12α* and *akap12β* are expressed similarly but distinctly. Several roles of *akap12* in zebrafish development have been verified [9, 10]. In addition, knowledge of the expression of specific *akap12* isoforms in zebrafish may aid studies to reveal the role and function of AKAP12α and AKAP12β that are involved in the zebrafish development.

Additionally, we described a new role for *akap12* in the regulation of pancreas laterality. Pancreas functions to maintain blood sugar homeostasis, as well as secrete digestive enzymes, thus it is an essential organ. In the absence of *akap12*, we found aberrent pattern of pancreas laterality (Fig. 7). Yet the difference between two morphants, *akap12α* and *akap12β* morphant, is not clear. Since we could not discount the possibility of involvement of *akap12* in direct pancreas development, we inspected the interaction between *akap12* and pancreas during organogenesis and the requirement of *akap12* in a cell differentiation (Fig. 8). As a result, akap12 has no effect

on pancreas development directly. Finally, KV-specific knock-down of akap12 results in randomization of embryonic pancreas laterality (Fig. 9). Consequently, pancreas morphogenesis fails to occur correctly because of improperly formed KV. Our result suggests that akap12 functions in L-R asymmetry to modulate pancreas architecture.

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국문 초록

A kinase anchoring protein 12 (AKAP12) 는 생체 내에서 PKA, PKC, calmodulin 등의 작용을 조절하는 구조 단백질 (scaffolding protein) 이다. 선행 연구 결과, AKAP12는 세포질 분열을 조절하고 세포의 이동성을 조절하는 역할을 수행한다는 사실이 밝혀졌다. 본 논문에서는 AKAP12의 발생과정 상에서의 발현 및 기능을 제브라피쉬 실험동물모델을 이용하여 연구하였다.

인간과 설치류에서는 AKAP12의 동형 단백질에 대한 연구가 진행되어 각각의 발현 양상과 기능에서의 유사점과 차이점이 보고되었으나, 제브라피쉬에서는 이에 대한 연구가 진행되어 있지 않다.

발생 과정에서 *akap12* mRNA의 발현은 시기상에 있어 차이를 보이는데, *akap12α*의 경우 수정 후 10시간 후에 처음으로 나타나며 *akap12β*는 수정 후 4시간 후에 발현하였다. 시기적인 차이 이외에도 위치적인 차이가 존재할 가능성에 대해 확인하기 위해 두 가지 방법을 통해 알아보았다. Morpholino를 통해 각각의 akap12를 knock-down한 제브라피쉬 배아를 이용하여 발현을 확인하였으며, *akap12α*, *β*를 특이적으로 확인할 수 있는 probe를 구축하여 각각의 발현을 확인하였다. 이 때, 발현 시기의 차이는 존재하였으나 대체적으로 유사한 양상으로 발현하는 것을 확인하였고, 체절 간 혈관, 중뇌-후뇌 경계 등 특정 조직에서의 차이를 확인하였다. 이를 통해 제브라피쉬의 발달 과정에서 *akap12α*, *β* 각각의 역할의 유사성과 차이점을 규명함에 있어 연구 가능성을 제시할 것으로 생각된다.

한편, 발생 과정에서 뇌, 심장 및 간, 췌장 등의 내장 기관은 비대칭적으로 위치하여 올바른 기능을 수행하게 된다. 제브라피쉬에서 이러한 비대칭적 발생은 Kupffer' s vesicle 이라는 기관이 조절한다고 알려져

있다. 본 논문은 AKAP12가 췌장의 편측성에 미치는 영향에 대해 알아보고자 하였다.

먼저, *akap12*의 발현이 저해된 제브라피쉬 배아에서 췌장이 비정상적인 편측성을 나타내며, 이는 발생 후기에도 지속됨을 확인하였다. 이러한 이상이 췌장 발생 과정에서 *akap12*가 직접적인 영향을 주는 것인지 알아보기 위하여 *akap12*의 발현을 확인하였으나, 췌장에서 강한 발현을 확인할 수 없었다. 또한 췌장 세포의 분화에 대한 연관성을 조사하였으나 대조군 배아와 *akap12*-knockdown 배아에서 유의미한 차이를 확인할 수 없었다. 추가적으로 Kupffer's vesicle 에서만 특이적으로 *akap12*를 knockdown 시키는 기술을 이용하여 췌장의 편측성을 확인해 본 결과, 앞서 살펴보았던 결과와 유사한 비정상적 발현을 확인하였다. 즉, *akap12*가 비대칭적 발생 과정에 관여하며, 그 결과 췌장의 비대칭적 발생이 진행된다는 사실을 규명하였다.

주요어 : AKAP12, AKAP12α, AKAP12β, 췌장, 좌-우 비대칭, 제브라피쉬

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