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약학석사학위논문

Construction of *akap12a* or *β* specific knockout zebrafish using CRISPR/Cas9 system

CRISPR/Cas9 시스템을 이용한 *akap12a*와 *β*의 knockout 제브라피쉬의 구축

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분자의학 및 바이오제약학전공

한 송 이

ABSTRACT

Construction of *akap12a* or *β* specific knockout zebrafish using CRISPR/Cas9 system

Song-Yi Han
Department of Molecular Medicine
and Biopharmaceutical Sciences
The Graduate School of
Convergence Science and Technology
Seoul National University

AKAP12 (A-Kinase Anchoring Protein 12) is a scaffolding protein which regulates various biological processes. It has been reported that AKAP12 regulates the cytokinesis progression and involves in the formation of brain-barrier by regulating angiogenesis and tight junction formation. Also, AKAP12 has a tumor suppressor function

and relates to cell migration. AKAP12 has several binding partners such as PK (protein kinase) C and A, calmodulin, cyclins, phosphoinositides and β-1,4 galactosyltransferase. It has been identified that there are three types of AKAP12 isoforms (designated *α, β* and *γ*) with different promoters in human.

In zebrafish, two types of AKAP12 isoforms, AKAP12*α* and AKAP12*β*, have been reported. We confirmed that AKAP12*α* and AKAP12*β* were expressed similarly but distinctly.

However, each function of two isoforms is not known so far. To elucidate the role of AKAP12 isoforms, we constructed *akap12a* or *β* specific knockout zebrafish. To construct knockout zebrafish, we adopted CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nuclease 9) system which is powerful and efficient tool for genome modifications at the targeted genomic locus.

Here, we reported the construction of *akap12a* or *β* specific knockout zebrafish using the latest genome editing tool. To confirm the mutagenesis, we performed T7E1 (T7 endonuclease 1) assay. As a result, expected fragments were detected and indicated the CRISPR/Cas9-mediated modification. Also, we confirmed the presence of indel mutation by DNA sequencing of the *akap12a* or *β* specific target region. The results displayed that several bases were deleted or inserted.

Then, we identified developmental abnormalities of *akap12a* or *β* specific knockout zebrafish. Trunk defect, hemorrhage and disrupted

heart laterality were observed in *akap12a* or *β* specific knockout zebrafish. However, there was slight difference between *akap12a* knockout zebrafish and *akap12β* knockout zebrafish in quantitative analysis. In *akap12β* knockout zebrafish, abnormal embryos were more observed relatively.

Taken together, these data suggested that AKAP12*a* and AKAP12*β* performed similar functions, but there was slight difference in expression period and amounts. *akap12β* was expressed form the earlier stage and more abundantly. Moreover, *akap12β* knockout embryos showed more defects numerically.

keywords : AKAP12, AKAP12*a*, AKAP12*β*, CRISPR/Cas9, zebrafish
Student Number : 2013-24046

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INTRODUCTION

1. AKAP12 (A-Kinase Anchoring Protein 12)

AKAP12 was originally identified as an auto-antigen in cases of myasthenia gravis and thus called Gravin [1]. It was subsequently shown to be orthologous to a rodent protein, SSeCKS (*Src*-Suppressed C Kinase Substrate), a major PKC substrate and binding protein [2].

AKAP12 is a scaffolding protein which controls multiple biological processes through its ability to scaffold key signaling proteins such as PK (protein kinase) C and A, calmodulin, cyclins, phosphoinositides and β-1,4 galactosyltransferase [3, 4].

In previous studies, several roles of AKAP12 were demonstrated. AKAP12 regulates the cytokinesis progression and involves in the formation of brain-barrier by regulating angiogenesis and tight junction formation [5, 6]. Also, AKAP12 has a tumor suppressor function and relates to cell migration [7, 8] (Fig. 1).

In human and rodent, three isoforms of AKAP12 (designated α, β and γ) have been reported so far. They have different promoters, so they can be controlled independently [9].

In zebrafish, two types of AKAP12 isoforms, AKAP12α and AKAP12β, have been reported. During zebrafish embryogenesis, AKAP12 regulates the mesodermal cell behavior and maintain vascular integrity [10]. However, difference and similarity of two

isoforms remain unclear.

In this study, we confirmed that during the zebrafish embryogenesis, spatiotemporal expression of *akap12a* and *akap12β* is similar but distinct. These data suggested the possibility of different role of AKAP12 isoforms in zebrafish.

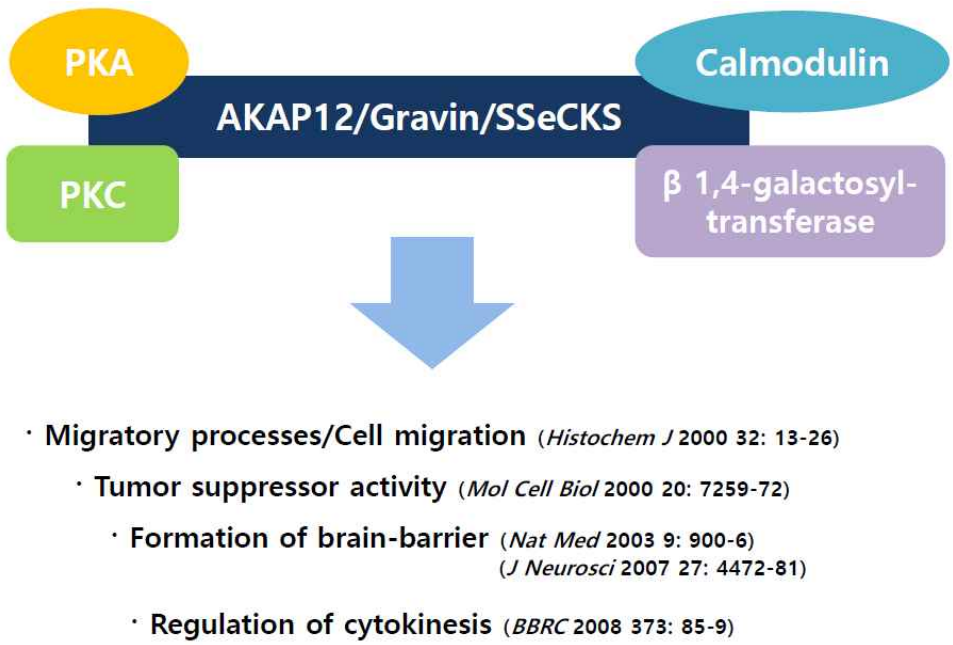

Figure 1. Schematic representation of AKAP12

2. CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nuclease 9) system

In many bacteria and most archaea, CRISPR (clustered regularly interspaced short palindromic repeats) form peculiar genetic loci, which provide adaptive immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner [11]. CRISPR-mediated immune systems rely on small guide RNAs for sequence-specific detection and silencing of foreign nucleic acids, including viruses and plasmids [12].

Three types (I - III) of CRISPR system have been identified across a wide range of bacterial and archaeal hosts, wherein each system comprises a cluster of *Cas* (CRISPR-associated) genes [13].

Recent work has shown that bacterial type II CRISPR system can be adapted to create gRNAs (guide RNAs) capable of directing site-specific DNA cleavage by the Cas9 nuclease *in vitro* [14].

In the CRISPR/Cas9 system derived from *Streptococcus pyogenes* (which is the system used in this study), the target DNA must immediately precede a 5′-NGG PAM (protospacer adjacent motif) [15] (Fig. 2).

Cas9 has been used to generate engineered eukaryotic cells carrying specific mutations via both NHEJ (non-homologous end joining) and HDR (homology-directed repair). Direct injection of gRNA and Cas9 RNA into eukaryotic cells has been enable to lead

the rapid indel mutation (deletion/insertion) at the double strand break site [16-19].

Here, we reported the construction of *akap12a* or *β* specific knockout zebrafish for demonstrating each function of two isoforms.

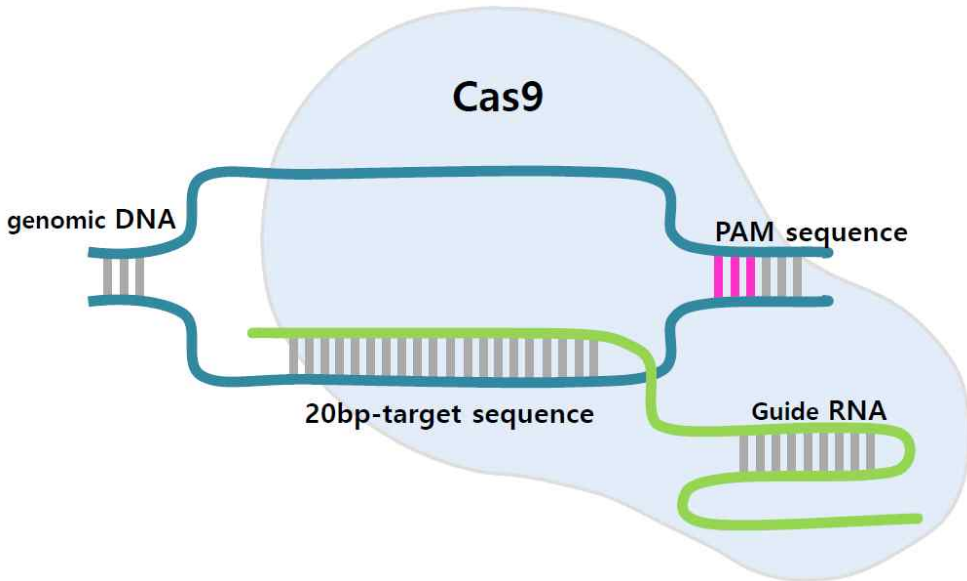

Figure 2. Schematic representation of CRISPR/Cas9 system

MATERIALS AND METHODS

1. Zebrafish maintenance

Wild-type zebrafish acquired from a commercial aquarium was maintained at 28.5℃ on a 14-hour light/10-hour dark cycle. Embryos were generated by natural mating and raised in Danieau' s solution. The embryos were treated with 1-Phenyl-2-thiourea (Sigma) to inhibit pigment formation at 12hpf. Other maintaining conditions of zebrafish were according to the Zebrafish book [20].

2. Preparation of anti-sense probes

To create anti-sense probes, the partial sequences of *akap12*, *cmlc1* were amplified using RT-PCR and cloned into pGEM-T Easy vector (Promega). Then, using digoxigenin (DIG)-labeling mix, SP6, T7 RNA polymerase (Roche), *in vitro* transcription was performed to obtain anti-sense probe.

3. Whole-mount *in situ* hybridization

The zebrafish embryos were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) for overnight at 4℃. 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, proteinase K (Roche) were treated for 1 min to 20 min at room temperature according to the developmental stages. Next, the embryos were transported to the pre-hybridization solution (50% formamide, 5X SSC, 5mg/ml yeast tRNA, 50μg/ml heparin, 0.1% Tween 20) and incubated for 3 hours at 70℃. After incubation step, the mixed solution, which contains anti-sense digoxygenin (DIG)-labled RNA probes, was added in substituted pre-hybridization solution and the embryos were incubated at 70℃ overnight. A series of washing steps (50% formamide/2X SSCT, 2X SSCT, 0.2X SSCT, PBST) were carried out and the embryos were blocked with anti-DIG-Alkaline phosphatase fragment diluted 1:2000 in blocking solution (0.5% Roche blocking reagent, 5% goat serum, PBT). After washing 10 times with PBST for 10 min, the embryos were submerged in staining solution (0.1M TrisCl, 50mM $MgCl_2$, 0.1M NaCl, 0.1% Tween 20, 1mM levamisole) three times for 5 min. NBT/BCIP stock solution (Roche) was used for color reaction and stained embryos were mounted in glycerol [21].

4. Isolation of zebrafish RNA and cDNA synthesis

Total RNA was isolated from the different stages of zebrafish embryos using the TRIzol (Ambion). To synthesize cDNA, two micrograms of RNA from each stages were used. The isolated RNA and 10 picomoles oligo-(dT) primer were incubated at 70℃ for 10 min and then 4℃ for 2 min. Additionally, reverse transcription was

carried out at 42℃ for 60 min with the mixture of 5X M-MLV reverse transcription buffer, 10mM dNTP mixture, and M-MLV reverse transcriptase (Promega).

5. Polymerase chain Reaction (PCR)

The synthesized cDNAs were utilized as a template for PCR reaction and specific primers were used:

for *akap12a*, F (5'-ATGGGAGCGACACCATCCGTGC-3') and
R (5'-TCATGCACTGTGACAACCTCTGTGGAG-3');
for *akap12β*, F (5'-ATGCTTGGGACAATAAC-3') and
R (5'-TCATGCACTGTGACAACCTCTGTGGAG-3');
for *β-actin2*, F (5'-GCAGAAGGAGATCACATCCCTGGC-3') and
R (5'-CATTGCCGTCACCTTCACCGTTC-3').

PCR was conducted using 10 picomoles of each primer in a T3000 thermocycler (Biometra). The reaction conditions were as follows:

for *akap12a*, initial denaturation at 94℃ for 5 min, followed by 28 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 5 min;

for *akap12β*, initial denaturation at 94℃ for 5 min, 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 5 min, followed by 18 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 45 sec.

6. Morpholino microinjection

Morpholino injection was performed at one-cell-stage to knockdown

AKAP12 effectively [22]. Injeted doses were 2ng for *akap12a*, 6ng for *akap12β*, and 6ng for standard control. Splice-blocking morpholinos were purchased from Gene Tools, LLC (Oregon, USA). *akap12a* morpholino targets against the splice-donor site of exon 1 of *akap12a* and *akap12β* morpholino targets the site of exon 3 of *akap12β*. For the control, standard control morpholino was used. The sequence of each morpholinos are as follows:

akap12a MO (5'-TACCTTGCCATCTGCGGTTTCTCCA-3');

akap12β MO (5'-TCTTACCTGTTAGAGTTATTGTCCC-3');

control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3').

7. Construction of gRNA

Small guide RNA was assembled by a method derived from Chen [23]. The target sequence of *akap12a* or *β* specific was designed by E-CRISP.

akap12a F (5'-TAGGGAGCGACACCATCCGTGCAG-3')

R (5'-AAACCTGCACGGATGGTGTCGCTC-3')

akap12β F (5'-TAGGGCAAAAGTTTTTGAAATGCT-3')

R (5'-AAACAGCATTTCAAAAACTTTTGC-3')

Annealed oligonucleotides corresponding to the target sequence were produced from Bioneer Corp. The customized pT7-gRNA vector (Addgene ref 46759) was digested with BglⅡ and Sal I then annealed nucleotides were inserted into the pT7-gRNA vector using T4 DNA ligase (Promega).

8. RNA synthesis

To produce gRNA, the template DNA was linearized by BamH I digestion, and treated with mixture of 100μl/ml Proteinase K (Roche) and 0.5% of SDS at 50℃ for 20 min. Then, the linearized plasmid was synthesized by *in vitro* transcription using MEGAshortscript T7 kit (Invitrogen). The pT3TS-nCas9n vector (Addgene ref 47929) optimized for zebrafish expression was created by Chen [23]. To generate Cas9 RNA, the template DNA was linearized by Xba I digestion, and treated with mixture of 100μl/ml Proteinase K (Roche) and 0.5% of SDS at 50℃ for 20 min. Next, the linearized plasmid was synthesized by *in vitro* transcription using mMESSAGEmMACHINE T3 kit (Invitrogen).

9. mRNA microinjection

The mixture was composed of 150ng/μl of Cas9 RNA and 100ng/μl of gRNA with appropriate phenol red and nuclease free water in accordance with a total volume. Injected doses were 150pg for Cas9 RNA and 100pg for gRNA. The mixture of gRNA and Cas9 RNA was injected at one-cell-stage and raised in Danieau' s solution.

10. T7 Endonuclease 1 Assay

After injection with mixture of gRNA and Cas9 RNA, a few embryos were selected randomly in 3dpf and genomic DNA was isolated from whole embryos. The specific site including target

sequence was amplified by PCR from the genomic DNA. Oligonucleotide primers for PCR were designed as follows:

for *akap12a*, F (5'-GGCGCATTTACTCGAACTGT-3') and
R (5'-TCGCACCTCTGCTGTATGTC-3');

for *akap12β*, F (5'-CTCACCCAGAAACCGAGAAG-3') and
R (5'-ACACCCAAGAACCACTGTCC-3').

The reaction conditions were as follows; for *akap12a* and *β*, initial denaturation at 94℃ for 5 min, followed by 34 cycles at 94℃ for 45 sec, 60℃ for 1 min, 72℃ for 1 min 30 sec using mixture of 150ng of genomic DNA and 10 picomoles of each primer. The reannealing process consisted of initial denaturation at 95℃ for 4 min, followed by cooling to 85℃ at -2℃ per second and further to 25℃ at -0.1℃ per second. The half of reannealed amplicon was digested with the mixture of 0.3μl of T7 endonuclease 1 (BioLabs) and 10X NEB buffer 2 (BioLabs) in a total volume of 20μl at 37℃ for 30 min. The reaction products were resolved using 1.2% agarose gel electrophoresis.

11. Microscopy

Expression patterns of *in situ* hybridization were photographed by Zeiss Stemi 2000C using a AxioCam ICC-1 camera and processed with Axiovision software.

RESULT

1. Expression of *akap12* during zebrafish development

To confirm the spatiotemporal expression of AKAP12 mRNA in zebrafish, we conducted whole-mount *in situ* hybridization. *akap12* is detected ubiquitously at the early stages (Fig. 3A, B). During segmentation period, *akap12* is localized at the specific region such as mesoderm, adaxial cells, notochord, vascular and nervous system (Fig. 3C-E). At 2 and 3 dpf, the expression is restricted to the head region (Fig. 3F, G).

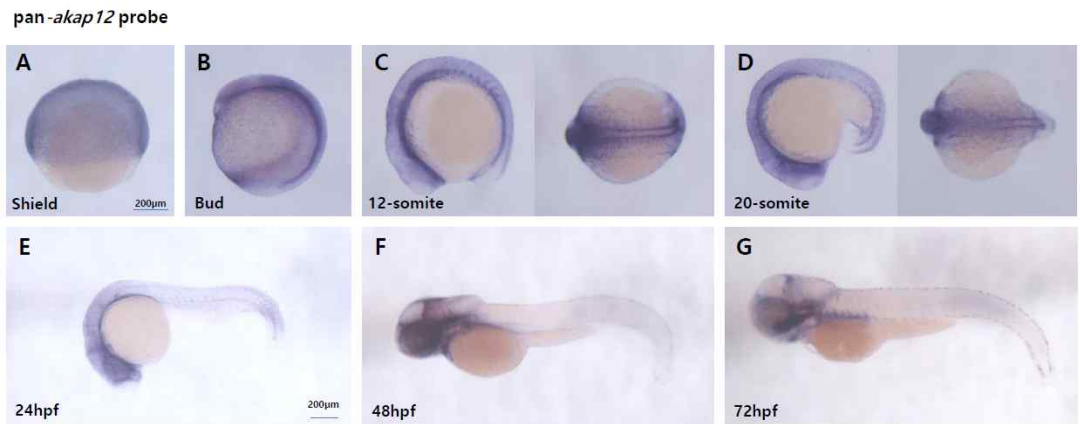

Figure 3. Spatiotemporal expression pattern of *akap12*

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

2. Two isoforms of *akap12* in zebrafish

The *akap12* gene of zebrafish is located in chromosome 20. In zebrafish, two types of *akap12* isoforms, *akap12a* and *akap12β*, have been reported. Significantly, there is a conserved region. The sequence of *akap12a* and *akap12β* shows 98% identity. *akap12a* is composed of three exons with two variants and the conserved region, while *akap12β* is composed of two exons with a variant and the conserved region. The full-length of zebrafish *akap12a* is 4791 bp and *akap12β* is 4602 bp (Fig. 4).

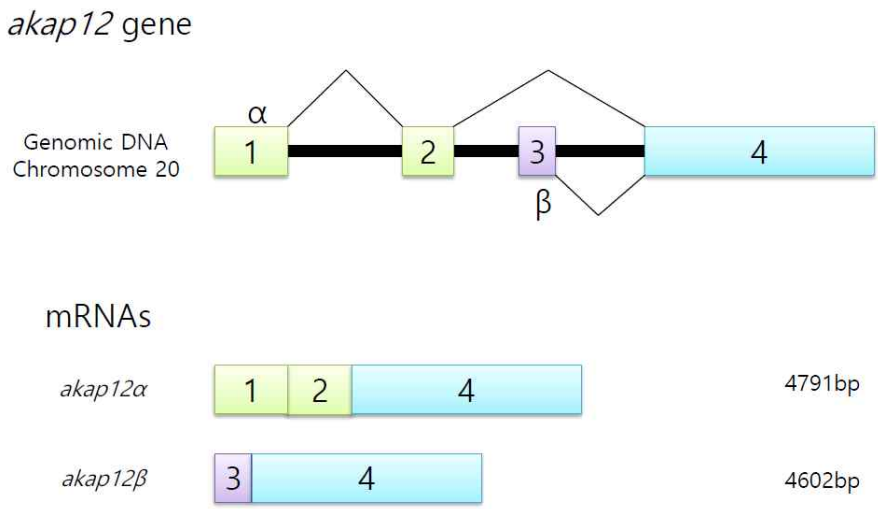

Figure 4. Organization and isoforms of *akap12* gene in zebrafish

Organization of two isoforms of *akap12*.

3. Identification of spatiotemporal expression of *akap12 a, β*

To identify the mRNA expression of *akap12* during development, we carried out RT-PCR analysis using synthesized cDNA at different stages. The results revealed that *akap12a* and *akap12β* were expressed at different developmental stages. The expression of *akap12 a* was initiated from the bud stage, but *akap12β* was detected from the sphere stage (Fig. 5).

Next, we injected morpholino oligonucleotides (MO) targeting the splice donor site to identify the spatiotemporal expression of *akap12* two isoforms specifically. *akap12* gene consists of exon 1, 2, 3 and 4. Exon 4 is a conserved region. *akap12a* MO was designed to target the exon 1 and *akap12β* MO was designed to target the exon 3 (Fig. 6A).

The MO injected embryos were collected after 2 dpf. To confirm the efficiency of *akap12* knock-down, we performed RT-PCR analysis. Consequentially, the expression of *akap12a* and *akap12β* was successfully suppressed (Fig. 6B).

Using the morphants, we carried out whole-mount *in situ* hybridization with pan-*akap12* probe. Through this method, we were able to identify *akap12a* expression using *akap12β* morphants and *akap12β* expression using *akap12a* morphants (Fig. 6C). Comparing the expression, the expression was detected from different stages corresponding to the RT-PCR analysis (Fig. 5). During embryogenesis, the expression pattern between two isoforms was

similar. However, it is confirmed that *akap12β* forms were more abundant according to the intensity of the signal (Fig. 6C).

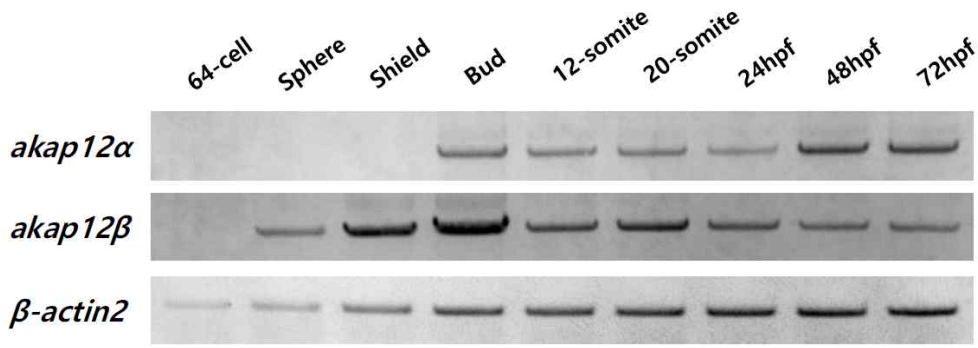

Figure 5. *akap12* mRNA expression during zebrafish development

RT-PCR analysis of *akap12* expression at different developmental stages. The expression of *akap12α* was detected from the bud stage, but *akap12β* was initiated from the sphere stage.

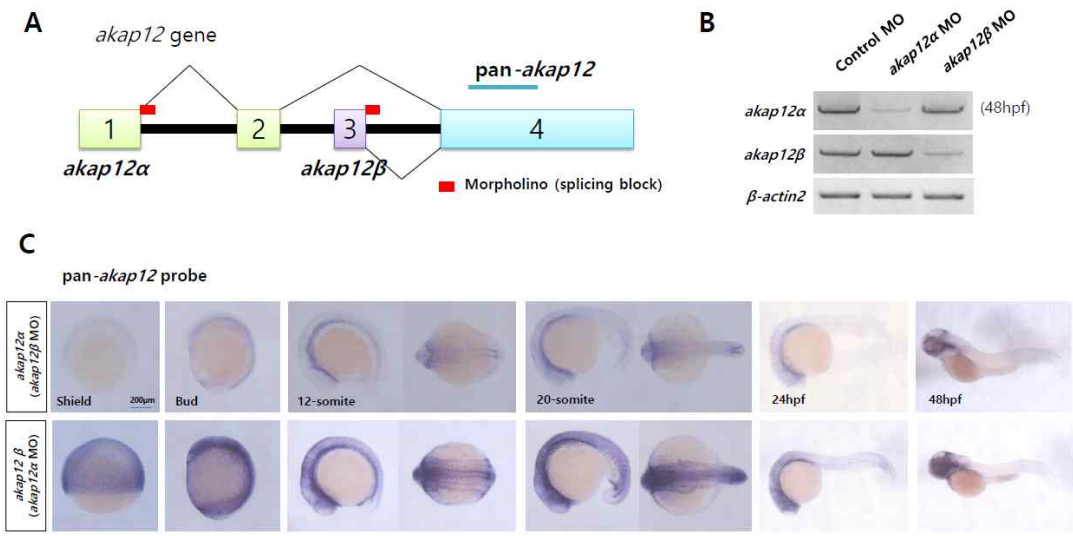

Figure 6. Identifying spatiotemporal expression pattern of *akap12 α* or *β* specifically using knock-down embryos

(A) Organization of *akap12* gene. Specific morpholinos interfere with splicing. The pan-*akap12* probe is designed to detect conserved region of *akap12* gene. (B) Confirmation of the efficiency of *akap12* knock-down by RT-PCR analysis. (C) With the knock-down embryos, whole-mount *in situ* hybridization was performed to identify the expression of two isoforms.

4. CRISPR/Cas9-mediated mutagenesis of *akap12a* or *β* in zebrafish

To ascertain roles of *akap12a* or *β* specifically in zebrafish development, we constructed *akap12a* or *β* specific knockout zebrafish using CRISPR/Cas9 system. The optimized gRNA vector (pT7-gRNA) and Cas9 vector (pT3TS-nCas9n) for genome modification of zebrafish were produced by Chen [23]. 20 bp *akap12a* -targeted sequence adjacent to a PAM (Protospacer-adjacent motif) site was cloned into gRNA vector. Equally, 20 bp *akap12β*-targeted sequence adjacent to a PAM site was cloned into another gRNA vector (Fig. 7). The gRNA and Cas9 RNA were generated by *in vitro* transcription and co-injected into embryos at one-cell-stage. The gRNA was injected with 100pg and Cas9 RNA was injected with 150pg. The RNA injected embryos were selected randomly after 3 dpf to isolate the genomic DNA form whole embryos. Then, we confirmed the mutagenesis using T7E1 (T7 endonuclease 1) assay. As a result, cleaved band suggested that CRISPR/Cas9-mediated mutation of *akap12a* or *β* occurred specifically in zebrafish (Fig. 8A, B). Next, we further confirmed the presence of indels in the embryos by sequencing the *akap12a* or *β* specific target region (Fig. 8C, D).

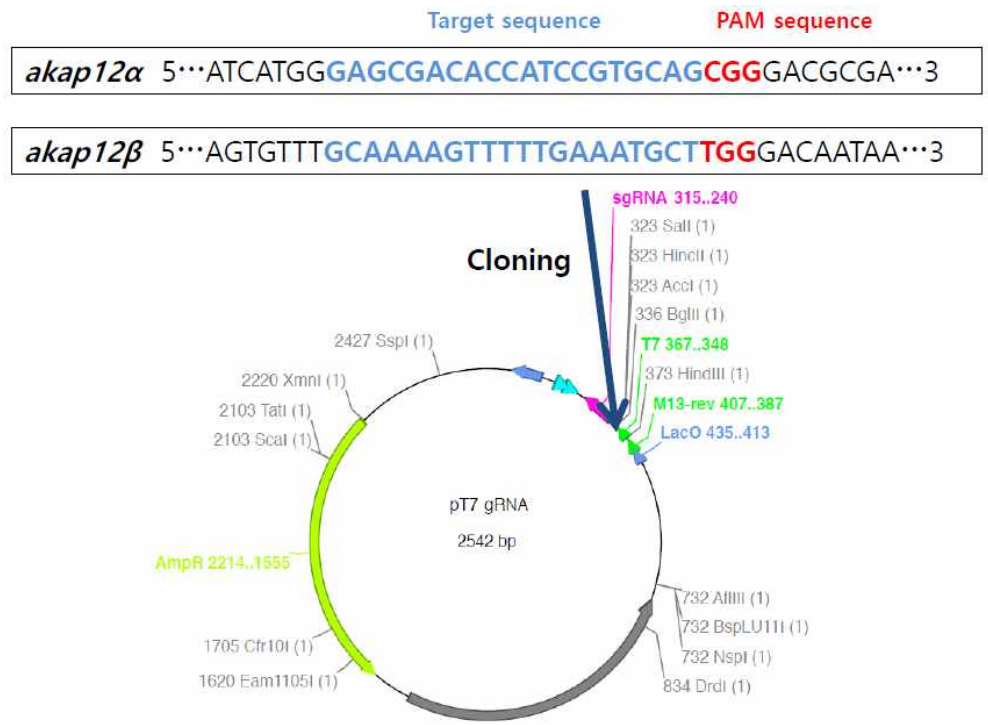

Figure 7. Construction of gRNA complementary to *akap12α* or *β*

Each of 20 bp *akap12α*-targeted sequence and *akap12β*-targeted sequence adjacent to the PAM site was cloned into the pT7-gRNA vector separately.

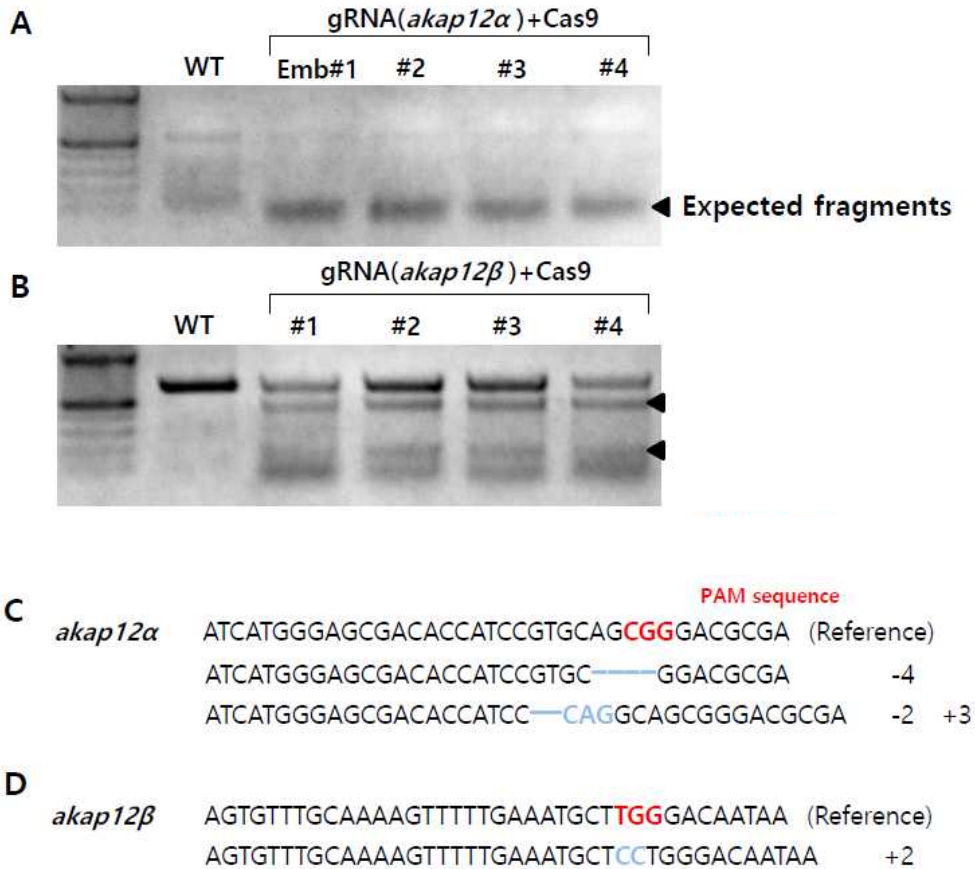

Figure 8. Confirmation of mutagenesis using T7E1 assay and DNA sequencing

(A, B) The result of T7E1 assay showed CRISPR/Cas9-mediated mutation of *akap12α* or *β* specific. The arrowheads indicated expected cleaved band. (C, D) Confirmation of the presence of indel mutation by sequencing the *akap12α* or *β* specific target region. The several bases were deleted or inserted.

5. Identification of developmental abnormalities of *akap12a* or *β* knockout zebrafish

The noticeable trunk defect was observed in *akap12a* or *β* knockout zebrafish. There were three types of trunk defect which were normal-like phenotype, curved trunk and curved trunk with short body length (Fig. 9A-D). The graph indicated that more embryos showed trunk defect relatively in *akap12β* knockout zebrafish (Fig. 9E).

Secondly, the striking hemorrhage was detected in *akap12a* or *β* knockout zebrafish. There were two types of hemorrhage which were hemorrhage adjacent to the heart and in the brain (Fig. 10A-C). The graph suggested that more embryos displayed hemorrhage relatively in *akap12β* knockout zebrafish (Fig. 10D).

Lastly, to further investigate the effect of knocking-out *akap12a* or *β* in zebrafish, we performed whole-mount *in situ* hybridization with a *cmlc1* probe (heart marker) (Fig. 11A-H). As a result, in *akap12a* or *β* knockout zebrafish, considerable number of embryos showed disrupted heart laterality in similar ratio (Fig. 11I).

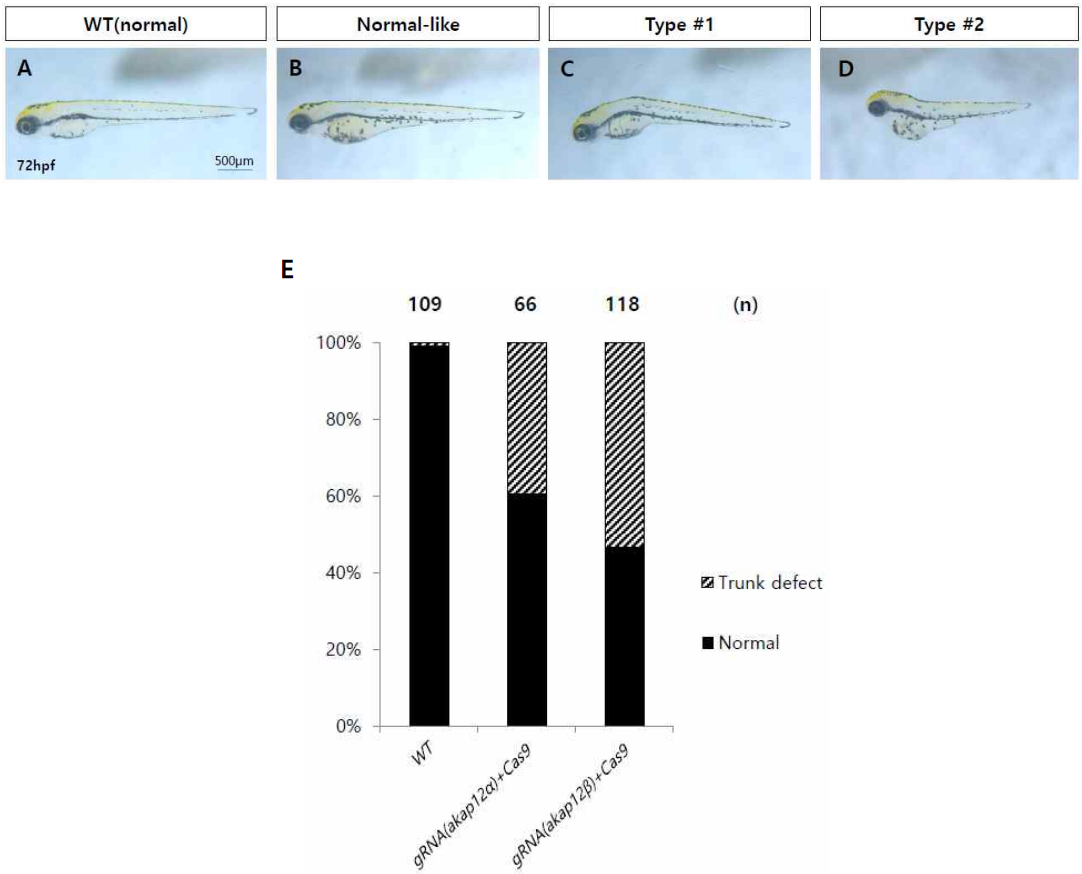

Figure 9. Trunk defect in *akap12α* or *β* knockout zebrafish

(A-D) Comparing the wild-type, three types of trunk defect (normal-like phenotype, curved trunk and curved trunk with short body length) were induced. (E) The graph indicated the percentage of embryos with trunk defect (39.4%, n=26/66 in *akap12α* KO and 53.4%, n=63/118 in *akap12β* KO).

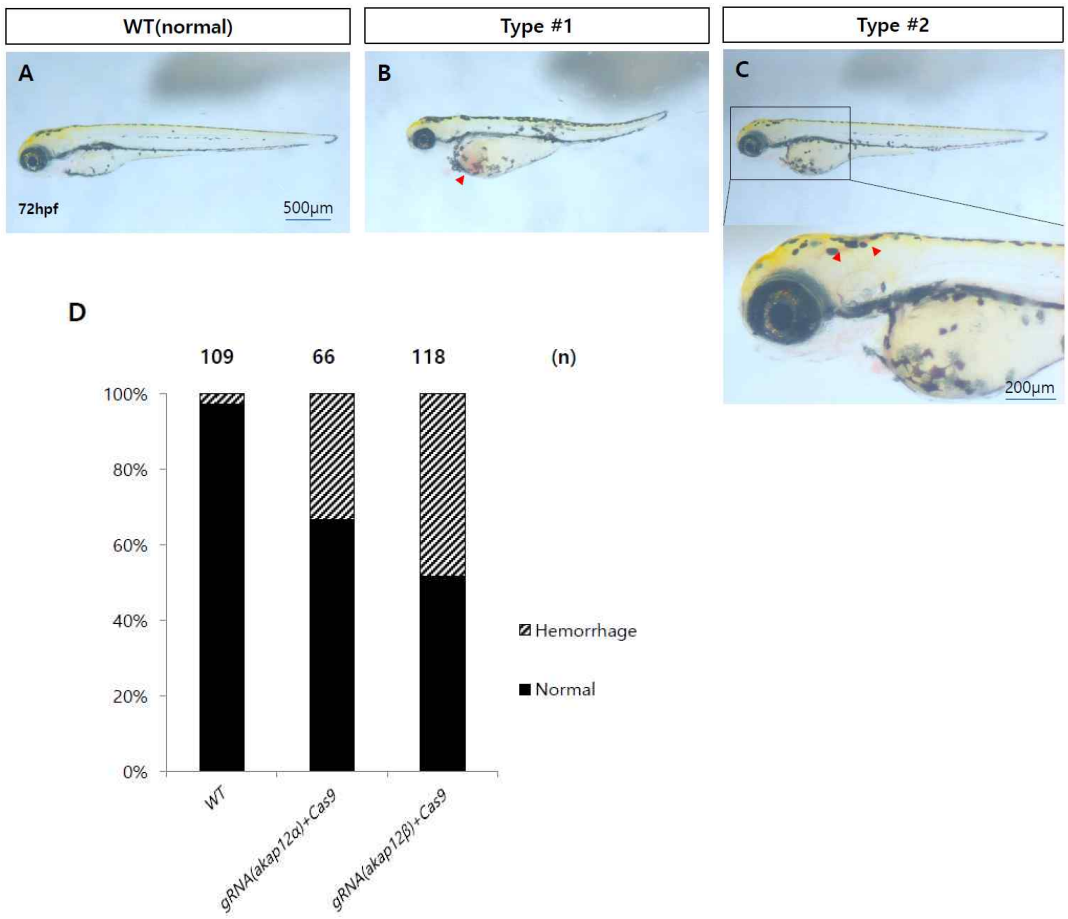

Figure 10. Hemorrhage in *akap12a* or *β* knockout zebrafish

(A-C) Comparing the wild-type, two types of blood leakage (adjacent to the heart and in the brain) were induced. (D) The graph displayed the percentage of embryos with hemorrhage (33.3%, n=22/66 in *akap12a* KO and 48.3%, n=57/118 in *akap12β* KO).

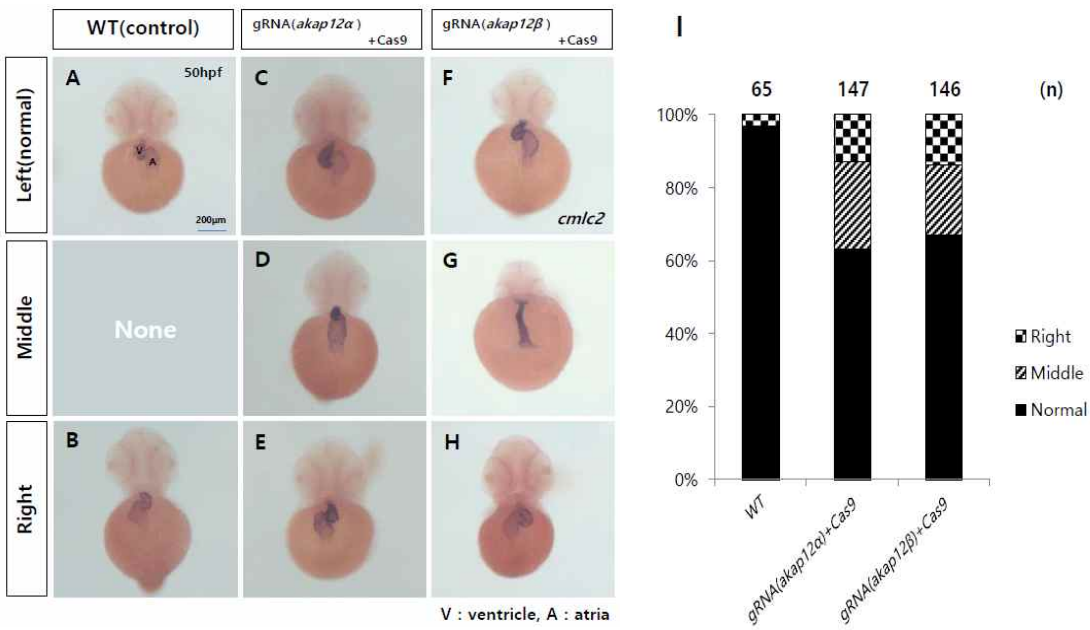

Figure 11. Disrupted heart laterality in *akap12α* or *β* knockout zebrafish

(A-H) Normal heart laterality (left) and disrupted left-right asymmetry (middle and right). (I) The graph showed the proportion of embryos with abnormal heart laterality (36.7%, n=54/147 in *akap12α* KO and 32.9%, n=48/146 in *akap12β* KO).

DISCUSSION

Here, we reported the construction of *akap12a* or *β* specific knockout zebrafish using CRISPR/Cas9 technology for identifying the each function of two isoforms.

In zebrafish, two types of *akap12* isoform, *akap12a* and *akap12β* have been reported. At first, we confirmed the spatiotemporal expression pattern of *akap12a* and *akap12β*.

Through the RT-PCR analysis, we verified that the initiation of mRNA expression was separated. The expression of *akap12a* was initiated from the bud stage, but *akap12β* was detected from the sphere stage (Fig. 5).

With pan-*akap12* probe, we performed whole-mount *in situ* hybridization using *akap12a* or *β* specific knock-down morphants. We were able to identify *akap12a* expression using *akap12β* morphants and *akap12β* expression using *akap12a* morphants by this method. Comparing the expression, the expression was detected from different stages corresponding to the RT-PCR analysis. During embryogenesis, the expression pattern between two isoforms was similar. On the other hand, it is confirmed that *akap12β* was expressed more abundantly according to the intensity of the signal (Fig. 6).

Overall, the expression of *akap12a* and *β* was similar but distinct. These results suggested the possibility of different roles of AKAP12 isoforms.

Subsequently, to further identify roles of *akap12a* or *β* specifically during zebrafish development, we constructed *akap12a* or *β* specific knockout zebrafish using CRISPR/Cas9 system.

The CRISPR/Cas9 system is used to facilitate efficient genome engineering in eukaryotic cells by simply specifying 20-nucleotide targeting sequence within its guide RNA [14]. CRISPR/Cas9-mediated genome editing is induced via non-homologous end joining (NHEJ) or homology-directed repair (HDR) [16].

With the gRNA and Cas9 RNA co-injected embryos selected randomly after 3 dpf, the genomic DNA was isolated form whole embryos. Then, we carried out T7E1 assay to confirm the mutagenesis. As a result, expected fragments indicated that CRISPR/Cas9-induced modification occurred in zebrafish. Also, we confirmed the presence of indel mutation in embryos by sequencing of *akap12a* or *β* specific target region. The results showed that several bases were deleted or inserted (Fig. 8).

These results indicated that *akap12a* or *β* specific knockout zebrafish was constructed successfully for further studies.

Co-injected embryos with gRNA and Cas9 RNA showed several defects. Firstly, the striking trunk defect was observed. There were three types of trunk defect which were normal-like trunk, curved trunk and curved trunk with short body length. The more embryos showed trunk defect relatively in *akap12β* knockout zebrafish (Fig. 9). Secondly, the conspicuous hemorrhage was detected in *akap12a* or *β* knockout zebrafish. There were two types of hemorrhage which were

hemorrhage adjacent to the heart and in the brain. The more embryos displayed hemorrhage relatively in *akap12β* knockout zebrafish (Fig. 10). Lastly, in *akap12a* or *β* knockout zebrafish, considerable number of embryos showed abnormal heart laterality in similar ratio (Fig. 11).

In summary, *akap12a* and *akap12β* looked like that they performed similar functions, but there was slight difference in expression period and amounts. Also, *akap12β* knockout zebrafish showed more defects quantitatively.

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국문 초록

AKAP12 (A-Kinase Anchoring Protein 12) 는 생체 내에서 PKC, PKA, calmodulin 등과 결합하여 다양한 생물학적 작용을 조절하는 구조 단백질 (scaffolding protein) 이다. 선행 연구들을 통해서, AKAP12가 세포질 분열 과정을 조절하고 뇌-혈관 장벽 형성에 중요한 역할을 한다는 사실이 밝혀졌다. 또한 AKAP12는 종양을 억제하고 세포의 이동성을 조절하는 역할을 수행한다는 사실이 밝혀졌다.

AKAP12의 동형 단백질에 대한 연구는 주로 인간과 설치류에서 진행되어 왔으며, 제브라피쉬에서는 이에 대한 연구가 부족하다.

본 연구에서는 제브라피쉬 발생 과정상에서의 AKAP12 동형 단백질의 발현 및 기능을 알아보고자 하였고 이를 위해 AKAP12 동형 단백질 각각에 대한 녹아웃 제브라피쉬를 구축하였다.

제브라피쉬의 배아 발생과정에서 RT-PCR을 이용하여 *akap12* mRNA의 발현 시기를 확인하였다. *akap12a*의 경우, 수정 후 10시간 뒤에 처음으로 발현되는 반면 *akap12β*는 수정 후 4시간 뒤부터 발현되기 시작하였다. 시기적인 차이 이외에 발현되는 위치에도 차이를 보이는지 확인하기 위하여 *in situ* hybridization을 수행하였다. 이 때, RT-PCR 결과와 부합하는 발현 시기에서의 차이를 확인하였고 발현되는 위치는 대체적으로 유사한 것을 확인하였으나 발현하는 양에서 차이를 보였다.

더 나아가 AKAP12의 동형 단백질에 대한 각각의 기능을 규명하기 위하여 최근에 널리 이용되고 있는 유전자 조작 기술인 CRISPR/Cas9 시스템을 이용하여 *akap12a*와 *β* 각각의 녹아웃 제브라피쉬를 구축하였다. 먼저, *akap12a*와 *β*를 각각 표적으로 하는 gRNA를 제작하였고 만들어

진 각각의 gRNA와 Cas9 핵산내부가수분해효소를 제브라피쉬 배아에 주입하였다. gRNA와 Cas9 핵산내부가수분해효소가 *akap12a*와 *β*에 선택적으로 작용하여 돌연변이를 유발했는지 확인하기 위하여 T7E1 assay를 수행하였다. 마찬가지로, DNA 염기서열 결정법을 이용하여 표적한 염기서열 상에서 결실이나 삽입돌연변이가 발생하는 것을 확인하였다.

*akap12a*와 *β* 각각의 녹아웃 제브라피쉬에서 발생 과정 중에 세 가지 종류의 결함이 나타나는 것을 확인하였다. 첫 번째로, 몸통이 휘어져 있거나 굽은 몸통과 동시에 짧아진 몸길이를 가지는 개체가 관찰되었다. 두 번째로, 심장 주변부나 머리 부분에서 출혈이 발생하는 개체가 확인되었다. 마지막으로, 심장의 좌-우 비대칭이 정상적으로 일어나지 않은 개체들을 확인하였다. 다만 차이가 있다면, *akap12a* 녹아웃 제브라피쉬에 비해 상대적으로 *akap12β* 녹아웃 제브라피쉬에서 더 많은 수의 개체들이 결함을 가지고 있는 것으로 확인되었다.

위 결과를 통해, AKAP12*a*와 AKAP12*β*는 서로 유사한 기능을 수행하는 것으로 생각된다. 그러나, 발현 시기와 양적인 측면에서 AKAP12*β*가 상대적으로 먼저, 더 많이 발현된다는 점과 결함을 가진 개체가 *akap12β* 녹아웃 제브라피쉬에서 더 많이 관찰된다는 점을 토대로 제브라피쉬 발생 과정 중에 AKAP12*β*가 좀 더 중요할 것이라고 판단하였다.

주요어 : AKAP12, AKAP12*a*, AKAP12*β*, CRISPR/Cas9, 제브라피쉬

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