약학석사 학위논문

The role of AKAP12 in axon generation during motor neuron development of zebrafish

제브라피쉬의 운동뉴런 발생시기에 **axon**의 생성에 미치는 **AKAP12**의 역할

2017년 2월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약 전공

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2017년 2월

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부위원장 _____ (인)

위 원 _____ (인)

ABSRACT

The role of AKAP12 in axon generation during motor neuron development of zebrafish

제브라피쉬의 운동뉴런 발생시기에 **axon**의 생성에 미치는 **AKAP12**의 역할

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The Graduate School of

Convergence Science and Technology

Seoul National University

AKAP12 is a scaffolding protein which interacts with multiple molecules, such as PKA, PKC, and Calmodulin. Tumor suppressor activity, mediation of cell migration, and formation of blood-brain barrier are some of the various functions that are carried out by AKAP12. During the development of zebrafish, AKAP12 is known

to be expressed in the slow muscle cells and their precursors, the adaxial cells. It also plays a key role in the movement of adaxial cells from the notochord to the lateral surface. However, it is believed that the interaction between motor neurons and muscle cells is crucial for proper generation of motor neurons in zebrafish. Therefore, this study was conducted to identify the role of AKAP12 during motor neuron generation in zebrafish.

We observed an uncontrolled, branch-like patterned motor neuron generation in AKAP12 morphants generated by microinjection of AKAP12 splice-blocking morpholinos. However, AKAP12 is expressed in muscle cells, not in neuron cells. Therefore, it regulates motor neuron generation in a cell non-autonomous manner. Thus, we hypothesized that the AKAP12 affects the motor neuron by controlling the secretion of a downstream molecule. We screened for the molecule and observed that the amount of HSPG around the somite cell is upregulated in AKAP12 morphants. HSPG is a proteoglycan that exists in ECM and promotes generation of axons by controlling many receptor-ligand interactions. AKAP12 spatiotemporally regulates the expression of HSPG. Therefore, AKAP12 morphants which express less AKAP12 could not inhibit HSPG, so HSPG expression was disregulated.

However, it has been reported that several genes have different phenotypes between morphants and knockouts. Therefore, we constructed AKAP12 knockout zebrafish using TALEN and confirmed whether they show similar phenotypes compared to the morphants. In selecting the AKAP12 knockout, we used T7E1 and melting curve of qPCR, and these selected knockouts also showed uncontrolled motor neuron sprouting as the morphants.

In conclusion, we found that AKAP12, which is expressed in the muscle precursor cells, controls the HSPG, and when this muscle precursor cell migrates, the remaining HSPG regulates motor neuron generation near the notochord.

Key words : motor neuron, axon, generation, zebrafish, development, AKAP12, AKAP12α, AKAP12β, morphant, knockout, HSPG

Student number : 2015-26011

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INTRODUCTION

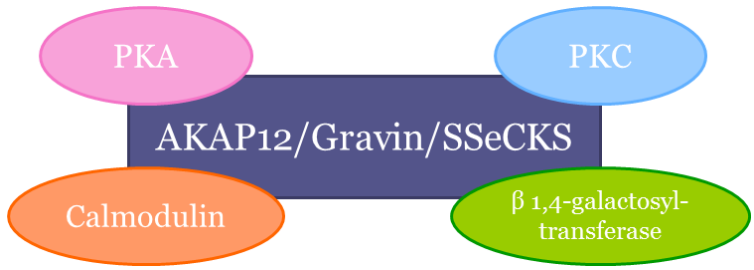
1. AKAP12 (A-Kinase Anchoring Protein 12)

The A-Kinase Anchoring Protein 12 (AKAP12), also known as Gravin, was originally recognized as the auto-antigen of myasthenia gravis. AKAP12 is also orthologous to SSeCKS, Src-Suppressed C Kinase Substrate, which is a protein expressed in rodents.

AKAP12 is a scaffolding protein that is related to various biological processes. It can also act as a tumor suppressor, control for cytoskeleton remodeling, and mediate cell migration. AKAP12 also gets involved in the development of brain-barriers by regulating angiogenesis and controlling tight junction formation. These activities are regulated by the interaction between AKAP12 and various signaling molecules such as Calmodulin, PKA, PKC, β-1,4-galactosyltransferase, F-actin, Src, and phospholipids. There are three isoforms of AKAP12 in humans and rodents, AKAP12α, AKAP12β, and AKAP12γ. Each of them have different promoters and can be controlled independently.

In zebrafish, AKAP12 is engaged in the regulation of complicated cellular behaviors, such as the locomotor activity and the morphogenesis of muscles. AKAP12α and AKAP12β are the two isoforms of AKAP12 which are expressed in zebrafish. And these two sequences are known to be 98% identical. The length of AKAP12α is 4791bp and AKAP12β is 4602bp. AKAP12α consists of three exons, two variants, and one conserved region, whereas AKAP12β contains two exons, one variant and one conserved region.

A



- Tumor suppressor activity
- Migratory processes / Cell migration
- Formation of brain-barrier
- Regulation of cytokinesis

B

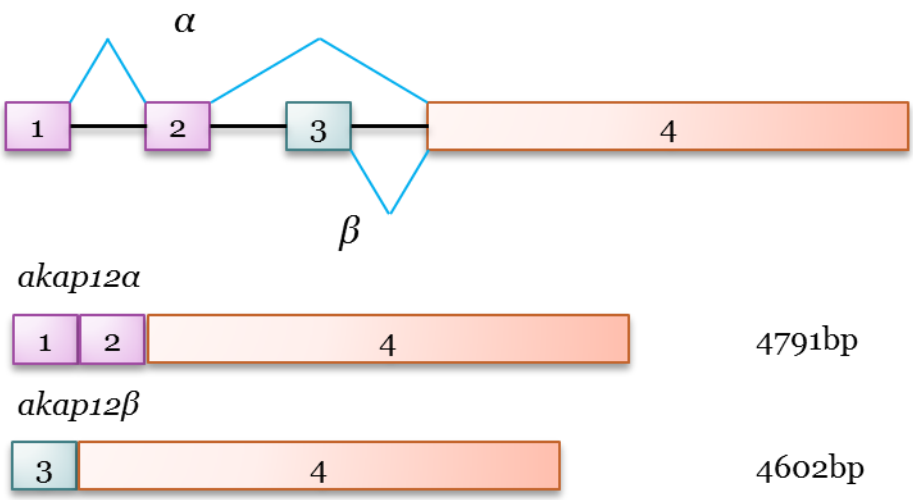


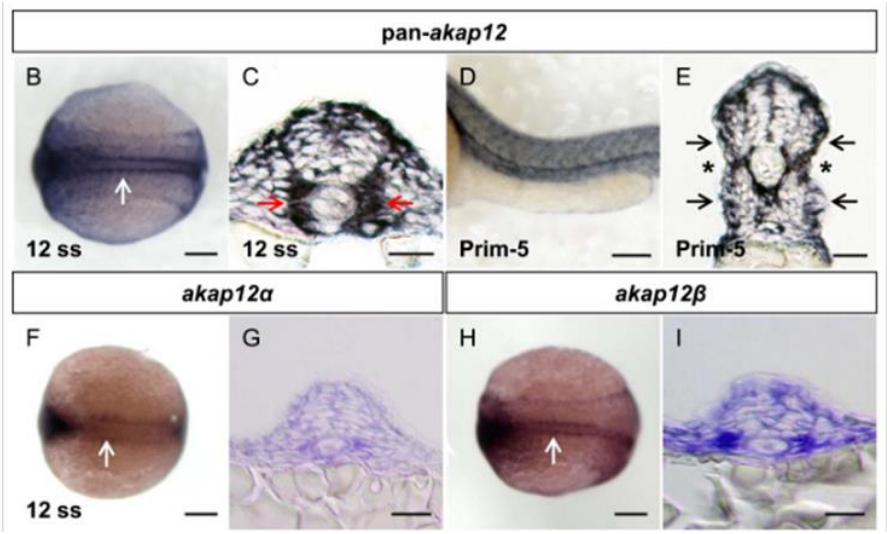
Figure 1. The representation scheme of AKAP12

(A) AKAP12 is a scaffolding protein that regulates various biological processes. (B) The two isoforms of AKAP12 in zebrafish.

2. AKAP12 and muscles in zebrafish

AKAP12 controls the movement of muscular tissues during the muscle development and it has been confirmed to be expressed in the muscle. Experimental data shows whole-mount *in situ* hybridization of AKAP12 in zebrafish (Fig. 2A). In 12ss stage (15hpf), AKAP12 was expressed in the adaxial cells near the notochord, which are precursors of the muscle fibers. However, in prim-5 stage (24hpf), AKAP12 was not expressed near the notochord. Instead, the expression of AKAP12 actually occurred in the lateral surface. This is because muscle precursors (Fig. 2B, red, near notochord) become slow muscles (Fig. 2B, red, lateral surface) during the muscle generation (somitogenesis). Another reason is that AKAP12 is expressed in the muscular tissues. Thus, the location of the expression of AKAP12 changes. The red segments in Figure 2B show where AKAP12 is being expressed. During the developmental stage, neurogenesis (Fig. 2B, green) occurred and these neurons sprout out from the original sites of the adaxial cells. Therefore, the experimental hypothesis was that there must be a relationship between motor neurons and the AKAP12.

A



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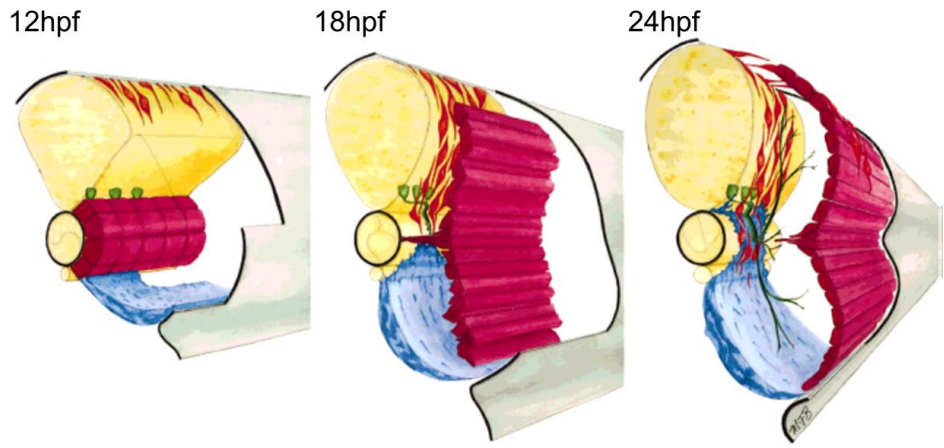


Figure 2. Somitogenesis in zebrafish and AKAP12

(A) In 12ss stage, AKAP12 is expressed in adaxial cells, which are muscle precursors. At prim-5, AKAP12 is expressed at lateral surface which becomes the slow muscle.(Differentiation. 88(4-5):106-16, 2014) (B) At the early stages, muscle exists near the notochord as adaxial cells (red, 12hpf). Adaxial cells eventually move to the lateral surface and become slow muscles. During the time interval, the generation of motor neuron starts near the notochord (green).(Developmental Dynamics. 219:287-303, 2000)

3. Zebrafish motor neuron

Depending on the developmental stage, the motor neurons of zebrafish can be classified into primary motor neurons and secondary motor neurons. In the primary motor neuron, there exists three somata: CaP (caudal primary), MiP (middle primary), and RoP (rostral primary). The elongation of the axon from each soma begins at 12hpf and continues until 30hpf. The secondary motor neuron development follows until 48hpf. This study aims to investigate the defection of primary motor neuron. This is because at early developmental stages, not only is it easier to observe motor neurons but also expressions of AKAP12 and muscle generation are believed to be heavily influenced during such stages.

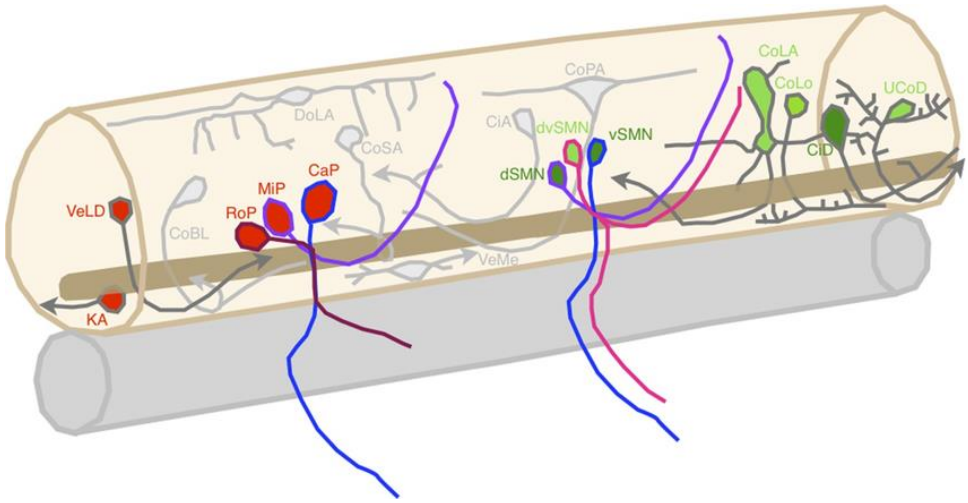


Figure 3. Zebrafish motor neurons

The primary and secondary motor neuron of zebrafish. The primary motor neuron consists of CaP (caudal primary motoneuron), MiP (middle primary motoneuron), and RoP (rostral primary motoneuron).(J Comp Neurol. 522(4):861-75, 2014)

4. HSPG (Heparan Sulfate Proteoglycan)

HSPG is a proteoglycan that contains one or more heparan sulfate chains covalently attached. It is located in the extracellular matrix and also at the surface of the cell membrane. HSPG regulates several receptor–ligand interactions by acting as a co–receptor, or by controlling the movement and the localization of ligand and receptors.

There are various extracellular factors that regulate the intrinsic factors of neuron generation. In particular, it is known that HSPG significantly affects the axonal growth during neurogenesis. HSPG is responsible for stabilizing receptor oligomers, which influence the growth of neurons. This results in an irregular distribution of the tyrosine phosphatase activity. This leads to production of microdomains that have high phosphotyrosine levels supporting the neuronal extension. In addition, these factors promote the regeneration of neurons when neurons get damaged.

Furthermore, the defect of increased HSPG is similar to that of AKAP12 morphants. Thus, this study elucidates the relationship between AKAP12 and HSPG during motor neuron development.

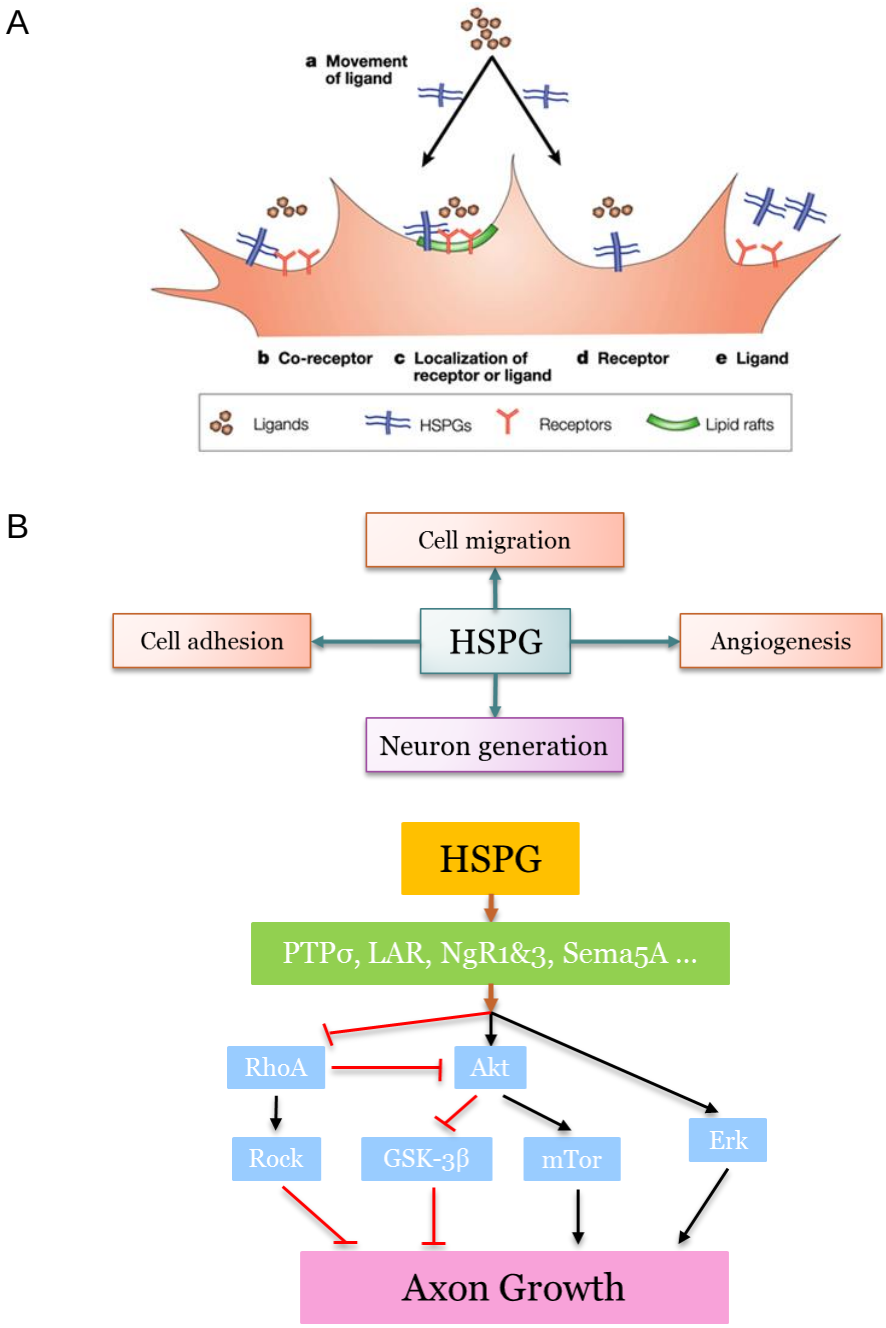


Figure 4. HSPG regulates motor neuron generation

(A) Models for guidance of axons by HSPG. HSPG regulates motor neuron generation by controlling many ligand–receptor interactions. (Nat Rev Genet. 5(12):923–35, 2004) (B) The role of HSPG and the schematic diagram explaining molecular mechanisms for HSPG promotion on neuronal growth and the downstream signaling.

MATERIALS AND METHODS

1. Zebrafish maintenance

The condition in which wild-type zebrafish were kept at was 28.5℃ on a 14-hour light/10-hour dark cycle. Embryos were gained through natural mating and raised in Danieau's solution. In order to inhibit pigment formation at 12hpf following the gastrulation step, they were treated with 1-Phenyl-2-thiourea (Sigma). Other maintenance conditions were followed by the zebrafish book.

2. Isolation of Zebrafish RNA and cDNA synthesis

The TRIzol (Ambion) was used to isolate total RNA from each zebrafish embryo developmental stages. Two micrograms of RNA were taken from each stage in order to synthesize cDNA. The isolated RNA was incubated together with ten picomoles of oligo-(dT) primer at a temperature of 70℃ for ten minutes and then at 4℃ for two minutes. Reverse transcription was followed under the temperature of 42℃ for 60 minutes. During this reverse transcription, the mixture of 5X M-MLV reverse transcription buffer, M-MLV reverse transcriptase (Promega), and 10mM dNTP mixture was used.

3. Polymerase Chain Reaction(PCR/qPCR)

The synthesized cDNA that were used as a template for PCR reaction and the specific primers are stated as follows:

for AKAP12α, 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').

10 picomoles of each primer were used for PCR in a T3000 thermocycler (Biometra). The reactions were performed under the following conditions:

for AKAP12α, initial denaturation at 94℃ for 5 min, another 28 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 5min;

for AKAP12β, initial denaturation at 94℃ for 5 min, another 24 cycles at 94℃ for 45 sec, 55℃ for 45 sec, 72℃ for 5min;

for β-actin2, initial denaturation at 94℃ for 5 min, another 18 cycles at 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 45 sec.

4. Preparation of anti-sense probes

In order to produce anti-sense probes, specific parts of the AKAP12 sequence were amplified through RT-PCR and were cloned into pGEM-T easy vector (Promega). *In vitro* transcription was then carried out using digoxigenin (DIG)-labeling mix or POD-labeling mix, T7 RNA polymerase (Roche), and SP6.

5. Whole-mount *in situ* hybridization

Zebrafish embryo fixation was performed using 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) with pH of 7.4, at the temperature of 4℃. Embryos that have been fixed were washed with PBS-T (1X PBS with 0.1% Tween 20) for 5 minutes. After embryos have been washed two times, they were dehydrated with methanol and were stored at -20℃ for 2 hours. They were rehydrated in series of 75%, 50%, and 25% methanol in PBS-T. Following that, the embryos were treated with proteinase

K (Roche) at room temperature for 1 to 20 minutes, in accordance with the developmental stages. After such treatment, they were transported to a pre-hybridixation solution (50% formaide, 5X SSC, 50ug/ml heparin, 0.1% Tween 20, 5mg/ml yeast tRNA). Following the incubation at 70℃ for 3 hours, the mixed solution was added and were incubated overnight again at 70℃. The solution contained anti-sense digoxygenin (DIG)-labeled RNA probes in pre-hybridization solution. The embryos went through a series of washing steps (50% formamide/2X SSCT, 2X SSCT, 0.2% SSCT, PBST) and then they were blocked with anti-DIG-Alkaline phosphatase fragment diluted 1:2000 in blocking solution (PBT, 0.5% Roche blocking reagent, 5% goat serum). The embryos were washed 10 times with PBST for 10 minutes and then submerged for 5 minutes in the staining solution (0.1M TrisCl, 50mM MgCl2, 0.1M NaCl, 0.1% Tween 20, 1mM levamisole) three times. For color reaction, NBT/BCIP stock solution (Roche) was used. The stained embryos were then seated in glycerol.

6. Morpholino injection

Using 2ng AKAP12α morpholino oligomer (MO), 6ng AKAP12β MO, and 6ng control oligos at the one-to-four cell stage, zebrafish embryos were injected. The morpholino sequences (5'-3') are AKAP12α splice-blocking MO, 5'-TACCTTGCCATCTGCGGTTTCTCCA-3', AKAP12β splice-blocking MO, 5'-TCTTACCTGTTAGAGTTATTGTCCC-3', and control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

7. Immunohistochemistry

Embryo fixation was carried out in 4% paraformaldehyde at 4℃

overnight, and then the embryos were washed multiple times in 1X PBS with 0.1% Tween 20 for whole-mount immunohistochemistry. They were blocked using 5% bovine serum albumin and 10% normal goat serum in PBDTT that consists 0.5% Triton X-100, 1X PBS, 1% dimethyl sulfoxide, and 0.1% Tween 20. The embryos were then incubated with primary antibodies (anti-HSPG(10E4), anti-primary motor neuron axon(znp-1,1:100) overnight at 4℃. Using PBDTT, the embryos were washed and incubated with sepcies-matched Alexa 488-conjugated or 546-conjugated secondary antibodies. Zeiss LSM 700 confocal microscope with ZEN 2011 software was used to image specimens.

8. T7 Endonuclease 1 assay

After gDNA is obtained from zebrafish, PCR is the first thing that needs to be carried out. The target sequence was amplified using PCR from the genomic DNA. The following are the designed oligonucleotide primers for PCR:

for AKAP12, F (5'-CCCTGAAACAATAGAAGTGCACCA-3') and

R (5'-TTGTGGGCGATTCAGCTGGCA-3');

The conditions in which the reaction was carried out were as follows: for AKAP12α and AKAP12β, initial denaturation at 94℃ for 5 minutes, followed by 34 cycles at 94℃ for 45 seconds, 60℃ for 1 minute, 72℃ for 1 minute 30 seconds using mixture of 150ng of genomic DNA and 10 picomoles of each primer. The conditions in which the reannealing process was performed were as follows: initial denaturation at 95℃ for 4 minutes, followed by cooling to 85℃ at -2℃ per second and again to 25℃ at -0.1℃ per second. Half of the reannealed amplicon was digested for 30 minutes with

the mixture of 10X NEB buffer 2 (BioLabs) and 0.3ul of T7 endonuclease 1 (BioLabs) in a total volume of 20ul at 37℃. Using 1.2% agarose gel electrophoresis, the reaction products were resolved.

9. PCR melting point

Using undiluted gDNA, a real-time PCR (qPCR) amplification was performed in a reaction that contains Fast SYBR Green Master mix with ROX (Applied Biosystems) in 10 μl total volume and final concentration of 0.7 μM for each primer. The reactions were carried out in the StepOnePlus Real-Time PCR System (Applied Biosystems). The conditions for PCR cycles are as follows: 95 ° C for 20 seconds, 40 cycles at 95 ° C for 1 second, 60 ° C for 20 seconds, 95 ° C for 15 seconds, 60 ° C for 1 minute, and a gradient from 60 ° C to 95 ° C with continuous detection at 0.015 ° C/sec increment for 15 minutes. StepOnePlus Software was used to analyze and export the results for further analysis. Each sample was processed with technical triplicates.

10. Microscopy

Zeiss Stemi 2000C with AxioCam ICC-1 camera was used to photograph expression patterns of *in situ* hybridization. The results were then processed with Axiovision software. Zeiss LSM700 confocal microscope was used to attain fluorescent images.

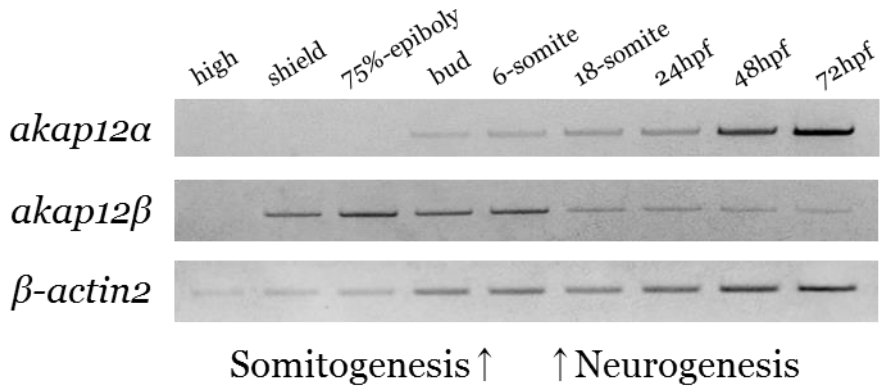
RESULTS

1. AKAP12 expression during developmental stage

In zebrafish, it has been reported that there are two AKAP12 isoforms: AKAP12α and AKAP12β. Because AKAP12α and AKAP12β have different promotors, their expression levels are also different. In order to verify the expression patterns of AKAP12 in embryogenesis, a RT-PCR analysis was conducted using cDNA that have been synthesized at different embryogenesis stages. Consequently, the expression level of AKAP12α was first observed at the bud stage(10hpf) and the amount of expression level increased throughout development. The expression pattern of AKAP12β began at the shield stage(6hpf) and this pattern decreased with time. The graph of AKAP12α plus AKAP12β showed a similar expression pattern to that of AKAP12β. This implies that AKAP12β has a dominant expression level over AKAP12α (Fig. 5). Moreover, since AKAP12 is expressed before the initiation of somitogenesis(10hpf) and neurogenesis(16hpf), AKAP12 can affect those two developmental processes.

In order to verify the spatiotemporal expression levels of AKAP12 mRNA in zebrafish, a whole-mount *in situ* hybridization was carried out (Fig. 6). AKAP12 was ubiquitously observed at early stages. After the somitogenesis starts, AKAP12 is expressed in the somite, especially at adaxial cells and slow muscles.

A



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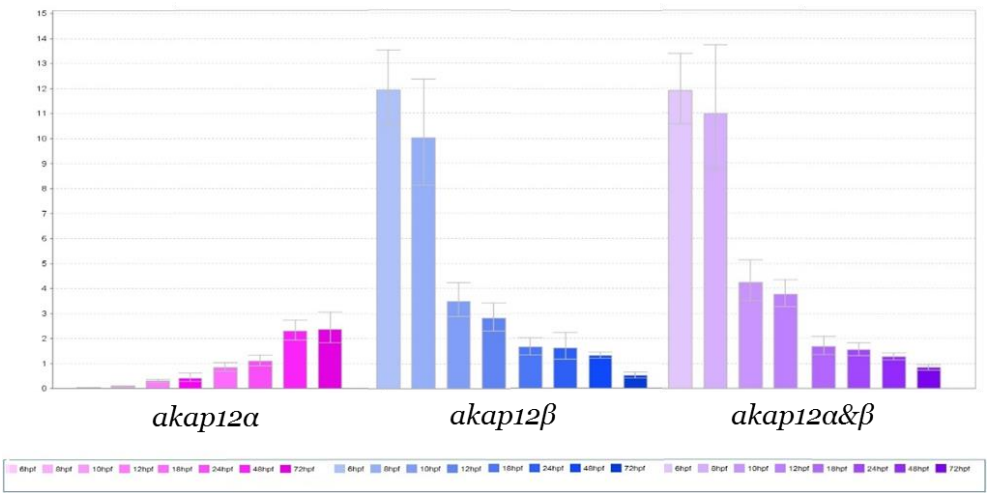


Figure 5. AKAP12 expression during the developmental stage

(A) AKAP12α is expressed at the bud(10hpf) stage and its expression level increases with time. AKAP12β is expressed at the shield(6hpf) stage and decreases with time. This expression time is sufficient to have influence on the somitogenesis and the neurogenesis.

(B) The expression level of AKAP12α plus AKAP12β showed similar pattern to that of AKAP12β.

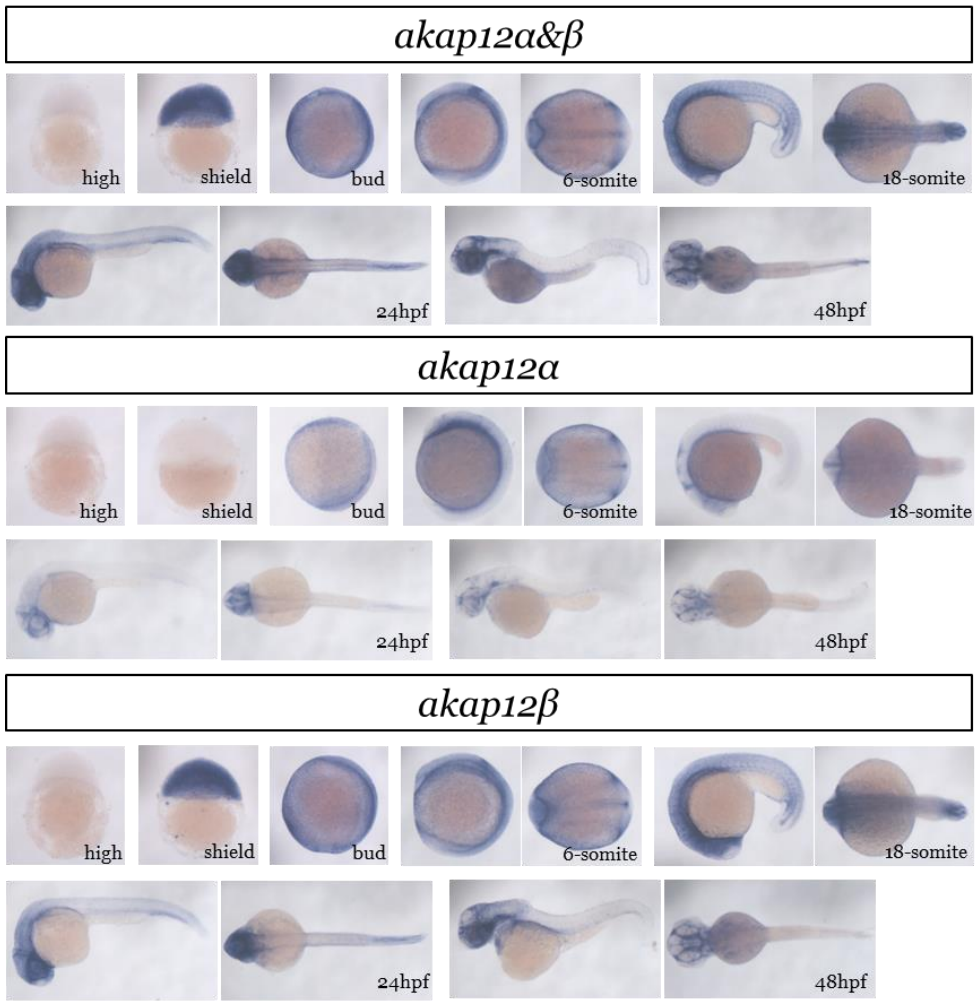


Figure 6. Spatiotemporal expression patterns of AKAP12

Spatiotemporal expression patterns of AKAP12α plus AKAP12β. AKAP12 is ubiquitously expressed at early stages and also expressed at somite when somitogenesis starts. Moreover, we were able to identify the expression pattern of AKAP12α by using AKAP12β morphants and the expression pattern of AKAP12β by using AKAP12α morphants

2. Motor neuron generation error occurs in AKAP12 morphants

In order to find out the role of AKAP12 in zebrafish motor neurons, morpholino oligonucleotides (MO) were injected to target the splice donor site. AKAP12 gene contains exon 1, 2, 3, and 4. Among those exons, exon 4 is the conserved region. Exon 1 was targeted by AKAP12α MO, and exon 3 was targeted by AKAP12β MO. The MO-injected embryos were collected at 27hpf (Fig. 7A). To identify the efficiency of AKAP12 knockdown, we performed the RT-PCR analysis, which confirmed that the expression of AKAP12α and AKAP12β have been significantly suppressed. Moreover, in order to confirm the spatiotemporal expression level of AKAP12 mRNA in morphants, we carried out a whole-mount *in situ* hybridization with the pan-AKAP12 probe. This probe was targeted at exon 4, which is the common site of AKAP12α and AKAP12β. Through this experiment, we were able to identify that the expression of AKAP12 is reduced in morphants (Fig. 7B).

With these morphants, we performed an immunohistochemistry staining to investigate the motor neuron of zebrafish. We observed that pathfinding errors had occurred in the motor neuron generation of the morphants. Also, the number of branching showed a statistically significant difference (Fig. 8).

To confirm this defect, we used a transgenic zebrafish line that induces fluorescence at the primary motor neuron (*Tg(mnx1:GFP)*). We injected each morpholino in *Tg (mnx1:GFP)*. As a result, more sprouting was observed compared to the control group (Fig. 9).

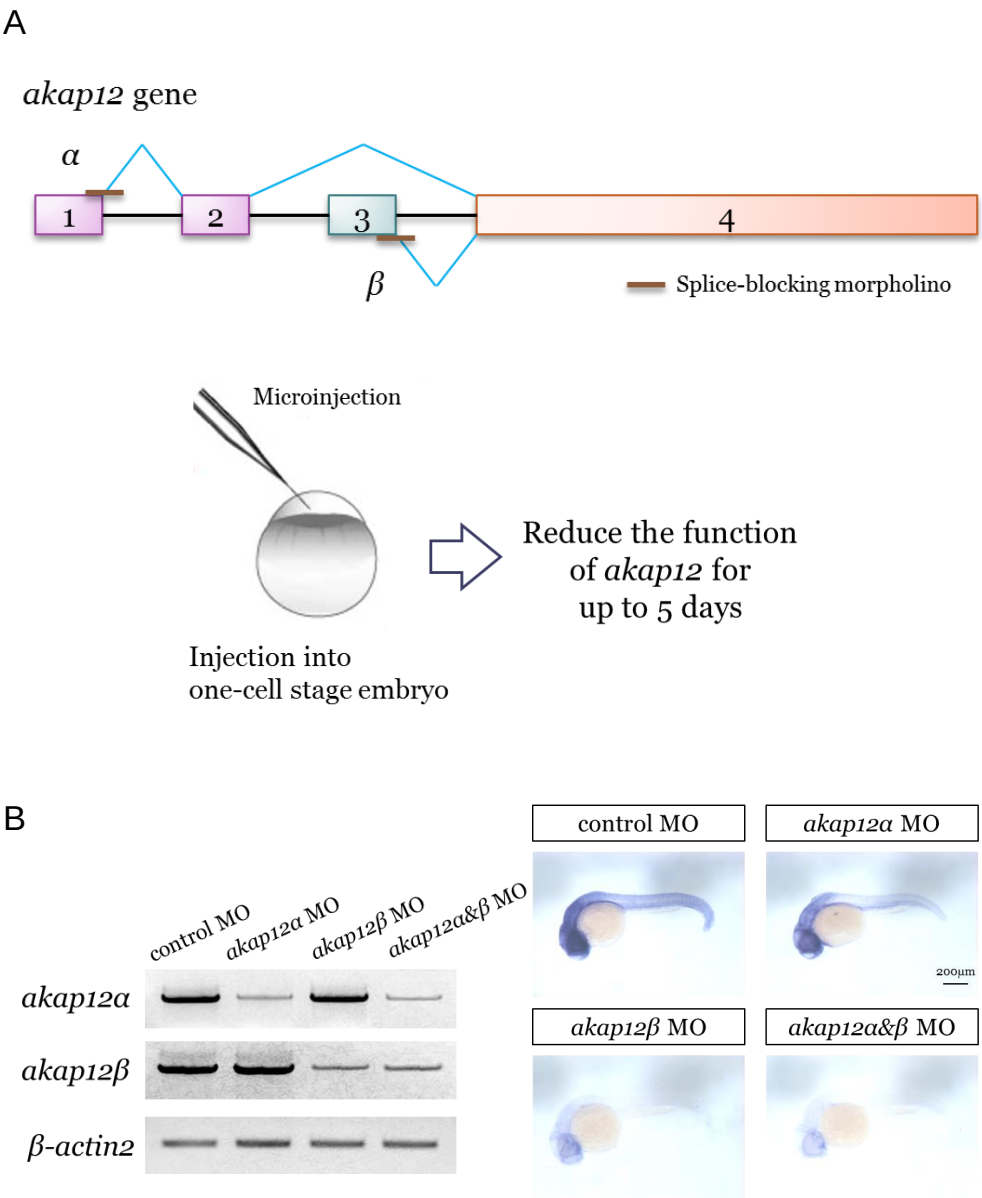


Figure 7. Morphants generated by microinjection of splice-blocking morpholino

(A) The morpholino inhibits splicing process. AKAP12α morpholino targets exon1-intron1 site. AKAP12β morpholino targets exon3-intron3 site.

(B) AKAP12 expression is reduced in morphants

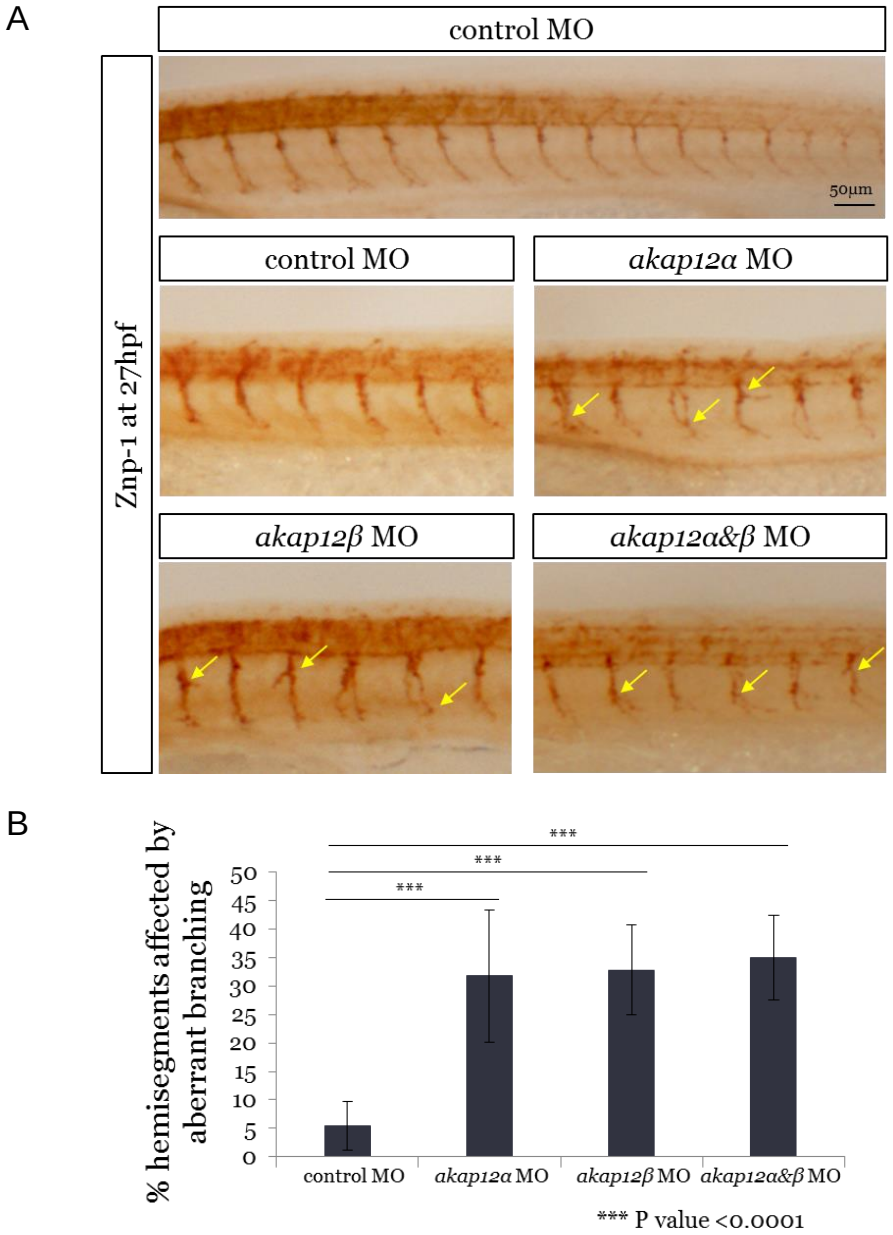


Figure 8. Uncontrolled motor neuron generation of AKAP12 morphants

(A) Immunohistochemistry of znp-1 at 27hpf in control morphants and AKAP12 morphants. Pathfinding error occurred in AKAP12 morphants as opposed to the control group(yellow arrows).

(B) Percentage of 27hpf embryos showed uncontrolled motor neuron brancing. *** P value <0.0001.

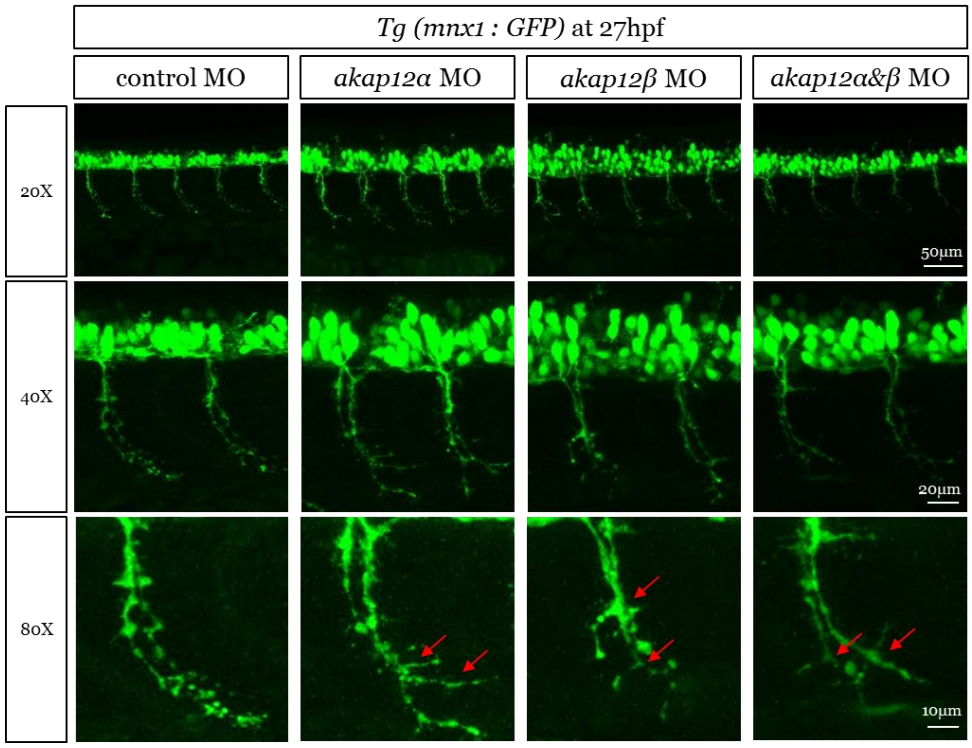


Figure 9. Uncontrolled motor neuron generation of AKAP12 morphants in transgenic zebrafish

To confirm motor neuron sprouting, we chose *Tg(mnx1:GFP)* which induces fluorescence at primary motor neurons. AKAP12 morphants showed more motor neuron axon branching compared to the control group(red arrows).

3. Construction of AKAP12 knockout zebrafish

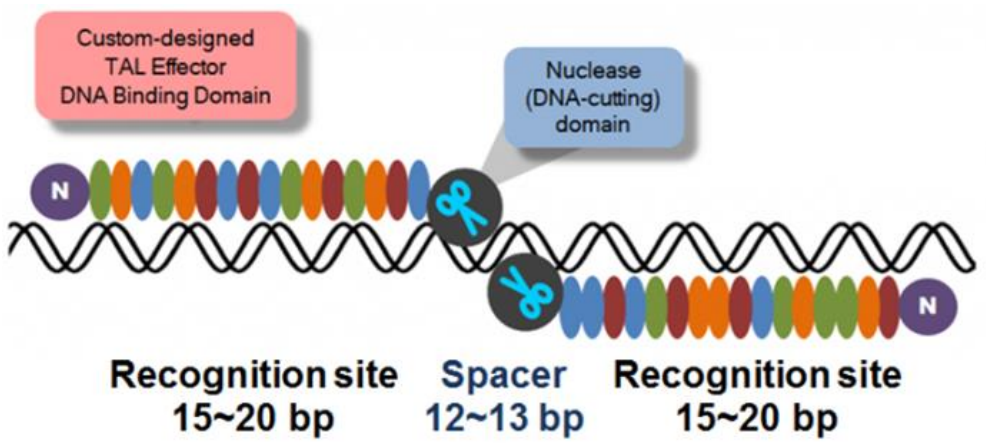
We constructed the TALEN knockout line to elucidate what would happen if we used AKAP12 knockout mutants instead of morphants. TALEN(Transcription Activator-Like Effector Nuclease) is a restriction enzyme that is designed to cut at specific sequences of DNA (Fig. 10). They are made by fusing a DNA-binding domain to a DNA cleavage domain. With this restriction enzyme, we could cut the common sequence of AKAP12α and AKAP12β, the exon 4. We tried to obtain the homozygous knockout(*akap12(-/-),* homo, knockout, mutant) by mating TALEN heterozygous knockout(*akap12(+/-)*, hetero) that were already stocked in the laboratory. During this process, we demonstrated two different methods to identify the knockout.

First method is to use the T7E1. T7E1(T7 Endonuclease 1) is an enzyme that cuts through the bubble of DNAs that are not perfectly matched. Thus, in the case of heterozygous knockout, since there are two different kinds of sequences (knockout and wild types), bubbles are formed after melting and reannealing step of PCR. T7E1 cuts through the bubble, and this creates 2 or 3 bands. Because the cutting point was set to be exactly in the middle, two bands represent the heterozygous knockout and one band represents the wild type or the homozygous knockout (Fig. 11).

This was once again confirmed by using the melting curve of qPCR. We designed PCR length so that the melting point of knockout is about 1℃ lower than that of the wild type. This is because knockouts have some missing sequences compared to the wild type. As heterozygous knockouts have both the knockout gene

and the wild type gene, their melting point was between that of the knockout and the wild type (Fig. 12).

At the end of these processes, we performed sequencing. Using this system, with hetero x hetero mating, we were able to obtain 4 zebrafish knockout individuals. By mating these zebrafish, we obtained knockout embryos (Fig. 13).



akap12a&β TALEN

```
           TALEN-left                          TALEN-right
CTGACAGTGAAGGAAGCAGAGAGTGGTGCTGATGCAGCCACTGAGGAAAAGAAAGAGGAACCTGCA  (WT)
CTGACAGTGAAGGAAGCAGAGAGTGGTGCTGATG----------GAAAAGAAAGAGGAACCTGCA  (-11)


RFVGFKFTLKKDKNEKTEPVQLLTVKEAESGADAATEEKKEEPAAEEDRSVEEKSPETTENEAKAEEVTEKA  (WT)
RFVGFKFTLKKDKNEKTEPVQLLTVKEAESGADGKERGTCSRGGQVCGRKITRNHRK* (-11)
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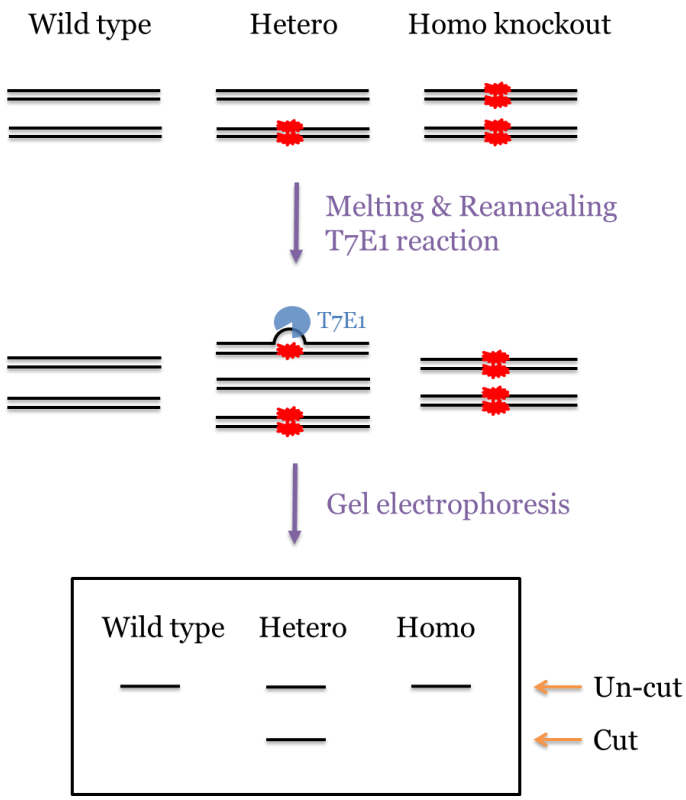
Figure 10. Transcription Activator-Like Effector Nuclease

TALEN system was used to obtain AKAP12 knockouts. Heterozygotes are obtained after cutting at the exon4 site and several mating. (https://www.extremetech.com/extreme/215283-gene)

A



B

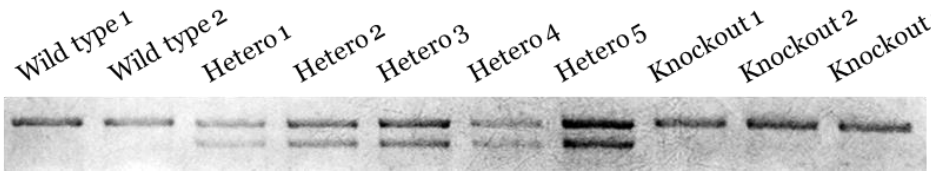
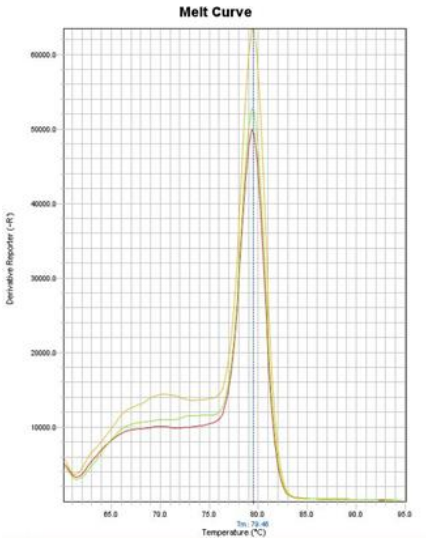


Figure 11. T7 Endonucleae 1 (T7E1)

(A) After the melting and the reannealing step, T7E1 cleaved mismatched DNA bubbles.

(B) The wild type and the homozygous knockout showed one band and the heterozygous knockout showed two bands at the DNA gel.



Wildtype 1	Wildtype 2	Knockout 1	Knockout 2	Knockout 3
80.66	80.5	79.46	79.61	79.31
80.51	80.35	79.46	79.46	79.31
80.51	80.35	79.46	79.46	79.31
Hetero 1	Hetero 2	Hetero 3	Hetero 4	Hetero 5
79.91	79.91	80.21	79.76	79.91
79.91	80.06	79.91	79.76	79.91
80.06	80.06	80.06	79.91	80.06

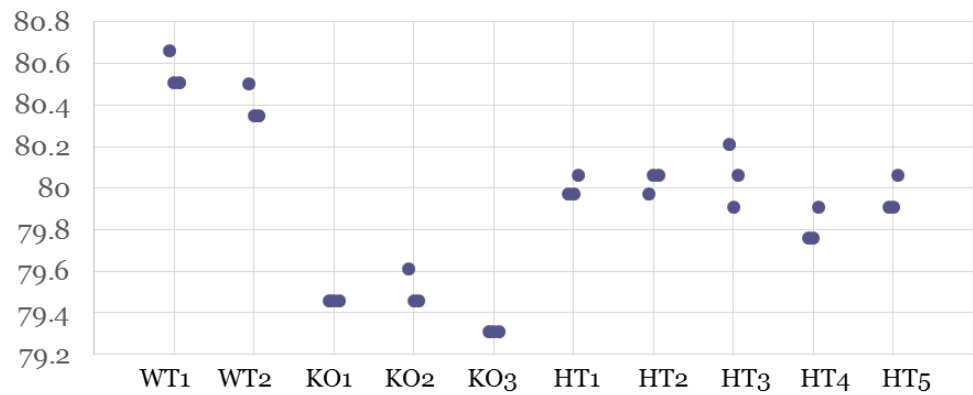
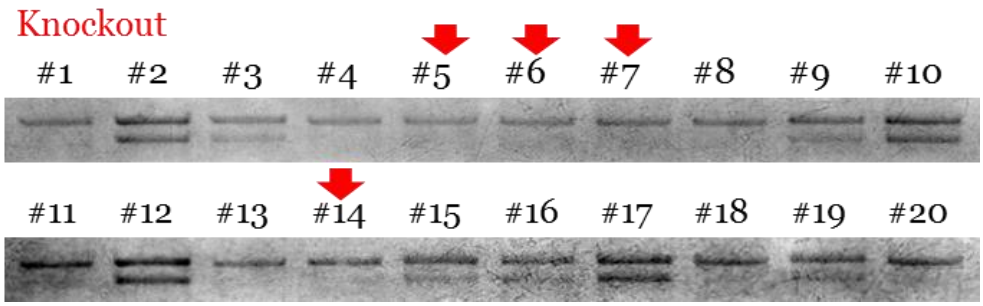


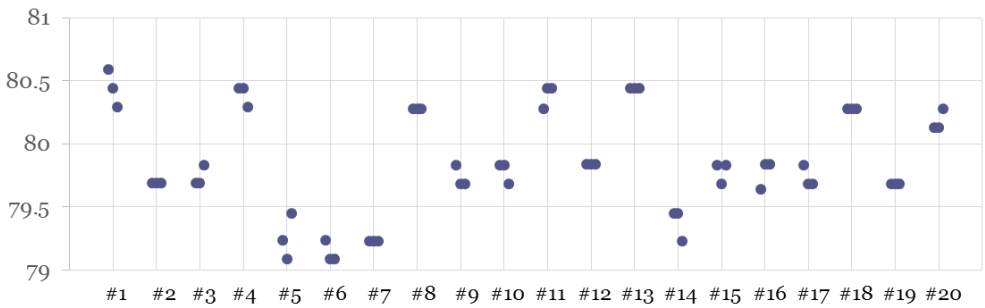
Figure 12. Melting curve of qPCR

After performing qPCR, the melting curve was measured. As a result, the melting point of the homozygote was found to be 1℃ lower than that of the wild type. The heterozygous knockout has a middle value between the melting point of the homozygous knockout and the wild type.

A



B



C

akap12a&β TALEN

```
          TALEN-left                          TALEN-right
CTGACAGTGAAGGAAGCAGAGAGTGGTGCTGATGCAGCCACTGAGGAAAAGAAAGAGGAACCTGCA (WT)
CTGACAGTGAAGGAAGCAGAGAGTGGTGCTGATG----------GAAAAGAAAGAGGAACCTGCA (-11)
```

Figure 13. Construction of AKAP12 knockout zebrafish

(A) T7E1. After hetero x hetero mating, T7E1 was performed on 20 zebrafish.

(B) The melting curve. qPCR was performed and adequate samples were selected.

(C) Sequencing is performed and we have 4 knockout individuals.

4. AKAP12 mutant leads to uncontrolled motor neuron generation

We obtained homozygous knockouts which have been confirmed by T7E1, qPCR and sequencing, and could finally show the defect of AKAP12 knockouts. By mating knockout zebrafish, we acquired homozygous knockout embryos and these embryos were stained by using the immunohistochemistry technology. As a result, similar to the morphants, the mutants had more motor neuron axon sprouting than the wild type (Fig. 14).

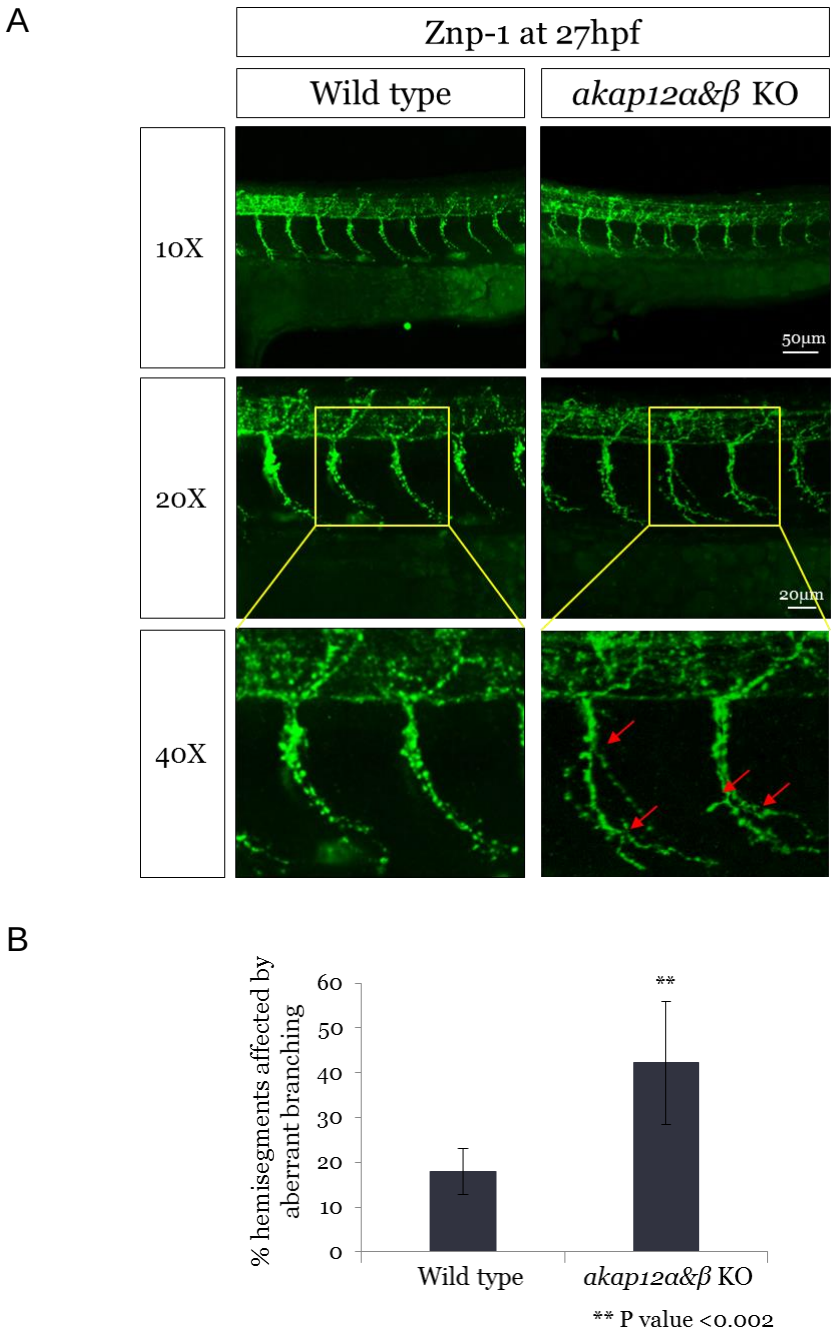


Figure 14. Uncontrolled motor neuron generation in knockout mutants

(A) AKAP12 knockout zebrafish showed uncontrolled motor neuron sprouting (red arrows). (B) Statistical results of motor neuron branching. **P value <0.002.

5. AKAP12 regulates HSPG which promotes zebrafish motor neuron generation

AKAP12 is expressed in the muscle, not in neurons (Fig. 2A). However, muscles move during somitogenesis. At 12hpf, the muscle exists as an adaxial cell near the notochord, and then slowly moves to the outer layer over time. It settles down in the lateral surface at 24hpf, which is totally apart from the notochord. Between the two periods, at around 18hpf, motor neurons are generated at the location where adaxial cell were (Fig 2B). Then, since AKAP12 is expressed in the muscle precursor and the muscle, AKAP12 does not exist near the motor neuron during the neuron generation (Fig. 15). So how is motor neuron generation affected by AKAP12?

We believe that there must be another molecule which is regulated by AKAP12. We hypothesized that when AKAP12 controls the molecule and moves away, the molecule, located near the notochord, assists proper pathfinding of motor neuron generation. In order to prove this hypothesis, we focused on HSPG, which is widely known to promote neuron generation.

When we stained AKAP12 morphants with HSPG, we found out that there was an increased and an ectopic expression of HSPG near the motor neurons in morphants (when) compared to the control group (Fig. 17). Therefore, we can conclude that AKAP12 inhibits the expression of HSPG. Since HSPG was not inhibited in AKAP12 morphants, neuron generation was promoted, thereby causing uncontrolled motor neuron generation.

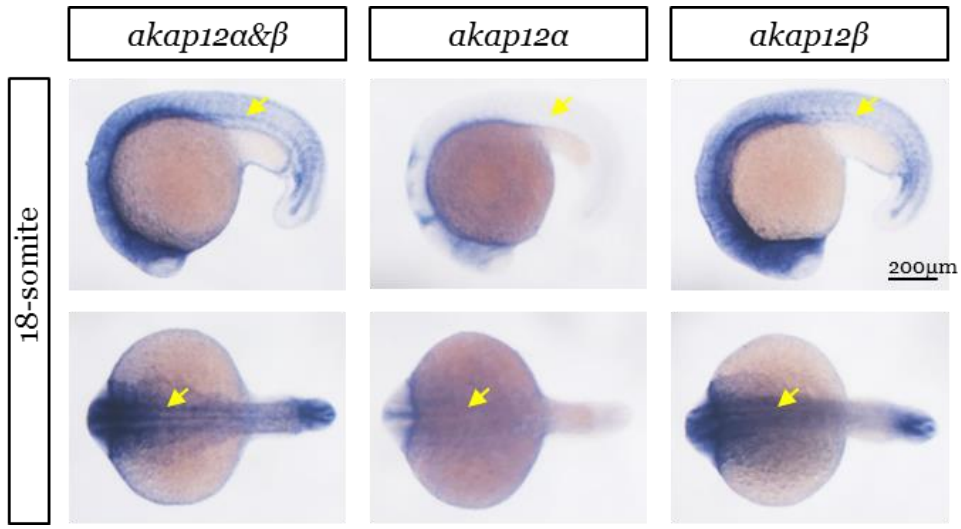


Figure 15. Expression of AKAP12 particularly in zebrafish somites

After somitogenesis, AKAP12 is expressed at the zebrafish somite (yellow arrows). In contrast, AKAP12 is not expressed at the motor neuron.

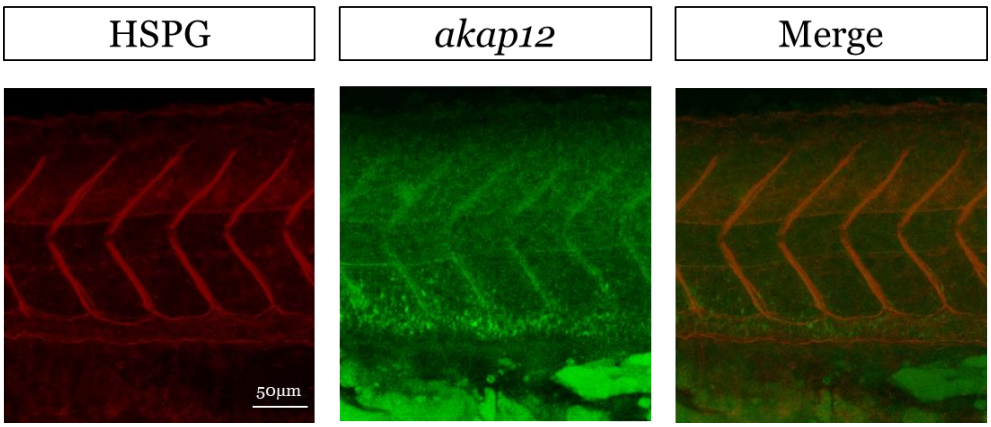


Figure 16. Expression of AKAP12 and HSPG

Double staining was performed to figure out whether AKAP12 can influence HSPG. Since AKAP12 and HSPG merged, AKAP12 is able to affect HSPG.

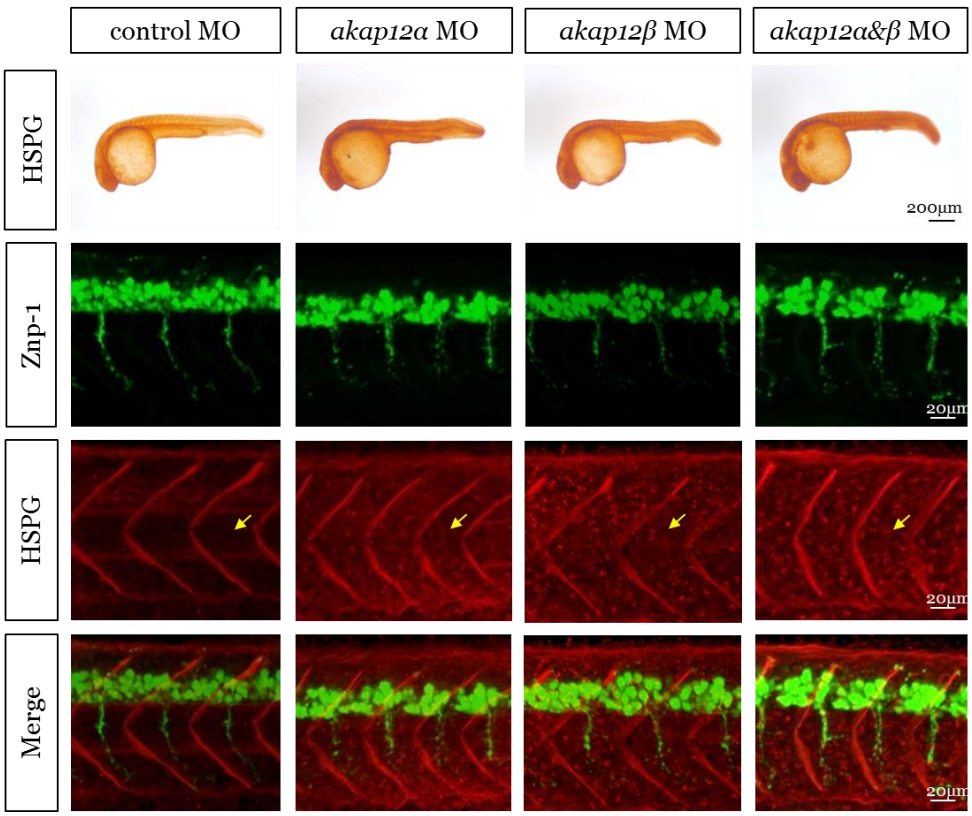


Figure 17. Downregulation of HSPG by AKAP12

We performed HSPG staining on AKAP12 morphants. Compared to the control group, HSPG is more expressed throughout the whole zebrafish. Especially near the motor neuron generation site, ectopic expression was shown (yellow arrows).

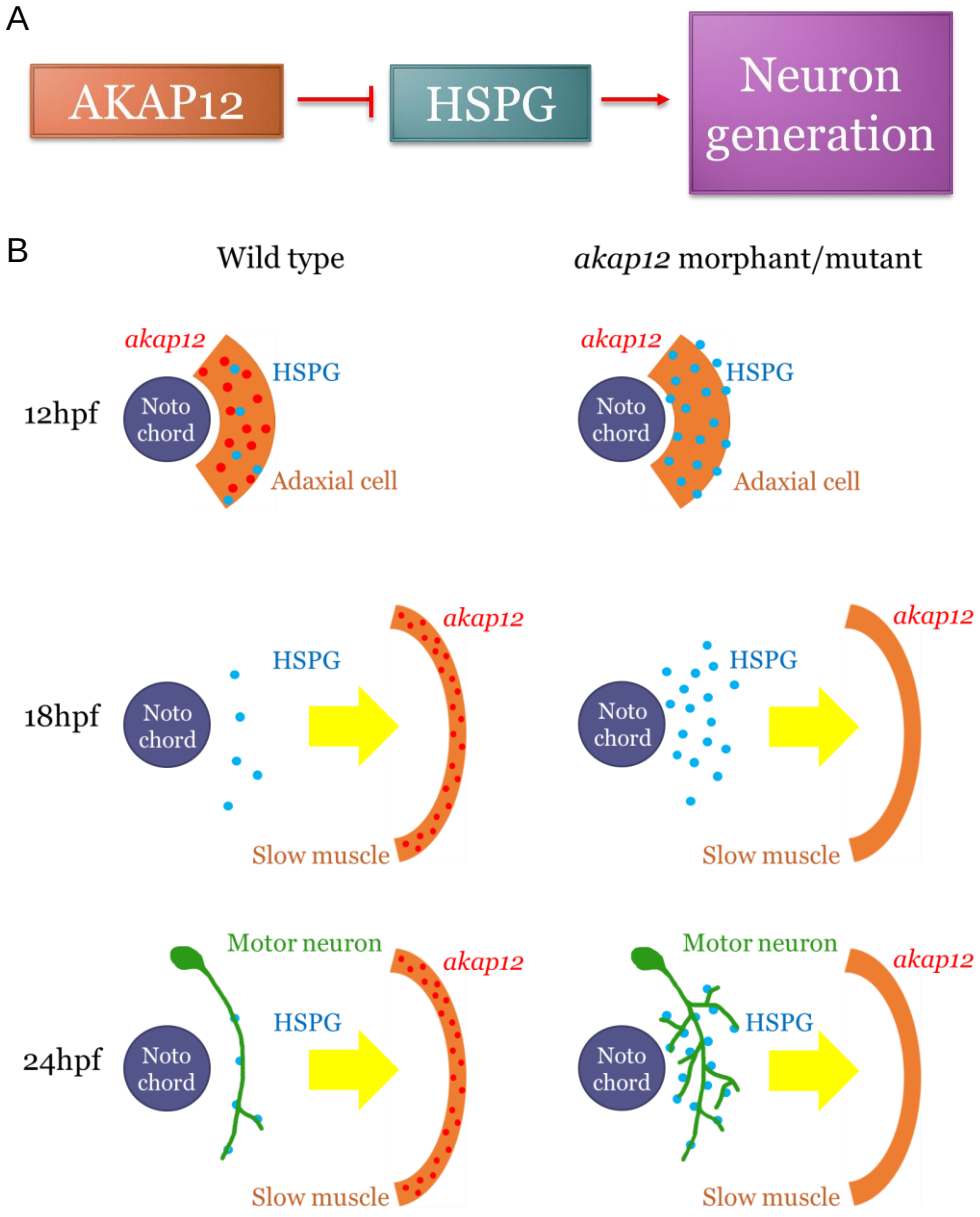


Figure 18. Conclusions

(A) From molecular point of view, AKAP12 downregulates HSPG and HSPG promotes motor neuron generation.

(B) Embryologically, adaxial cells which are near the notochord, express AKAP12 and this regulates HSPG. After adaxial cells move to the lateral surface with AKAP12, remaining HSPG regulates motor neuron generation.

DISCUSSION

Throughout the study, we could observe the expression of AKAP12 in zebrafish and confirm that when the expression of AKAP12 is reduced, uncontrolled motor neuron sprouting occurs. Moreover, we set proper selection methods to obtain the homozygous knockout and could confirm that AKAP12 knockouts also result in the same phenotype. Finally, through a simple mechanism study, we investigated that HSPG promotes the sprouting of motor neuron axon and AKAP12 inhibits the expression of those HSPG.

Considering both the experimental result and our knowledge on zebrafish development processes such as the somitogenesis and the neurogenesis, we could reach to the following conclusion (Fig. 18).

The expression of AKAP12 initiates around 6hpf and 10hpf in the adaxial cell, which is a muscle precursor. This tissue is located right next to the notochord, and expression of HSPG is regulated by the AKAP12 that is expressed at that location. However, after 12hpf, adaxial cell moves to the lateral surface and becomes a slow muscle until it reaches 24hpf. Since AKAP12 is expressed in the muscle, it no longer exists in the position where the muscle precursor used to be at. The motor neuron sprouting starts when the adaxial cell passes by the notochord at 16hpf. The sprouting of motor neurons takes place right next to the notochord where the adaxial cell was formerly located. Because the adaxial cell have already regulated HSPG by expressing AKAP12, motor neurons are generated through the pathway.

Therefore, when compared to the wild type, AKAP12

morphants and mutants were not able to regulate HSPG properly due to the lack of expression of AKAP12. As a result, HSPG regulated in an ectopic manner caused pathfinding errors during motor neuron generation. Thus, this led to branch-like pattern of the motor neuron generation.

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국문 초록

AKAP12는 PKA, PKC, Calmodulin과 같이 다양한 단백질들과 결합하는 scaffolding protein으로서 종양억제, 세포이동과 BBB의 형성 등 많은 역할이 밝혀져 있다. 또한, AKAP12는 제브라피쉬의 발생과정 중 적색근(slow muscle)과 그 전구세포(adaxial cell)에서 발현되며, adaxical cell이 척삭(notochord) 측면부에서 표면부(lateral surface)로 이동하는 과정에 중요한 역할을 한다. 일반적으로 근육은 운동 신경과 구조적, 기능적으로 밀접한 연관성을 가지며, 제브라피쉬 또한 운동 뉴런 발생과정에서 뉴런과 근육 조직의 상호작용이 중요한 역할을 한다고 생각된다. 따라서 제브라피쉬의 운동 뉴런의 발생과정에 미치는 AKAP12의 역할에 대해 확인해보았다.

AKAP12 splice-blocking morpholino의 microinjection을 통해 얻은 AKAP12 morphant에서 운동 뉴런의 무분별한 가지치기식 발생이 관찰되었다. 하지만 AKAP12는 근육에서 발현이 되며 뉴런에서는 발현이 되지 않기 때문에 adaxial cell에서 발현한 AKAP12가 다른 물질을 조절함으로써 운동 뉴런에 영향을 줄 것이라 추측하였다. 이에 somite cell 주변의 HSPG의 양을 측정한 결과, AKAP12의 morphant에서 HSPG의 양이 늘어난 것을 확인할 수 있었다. HSPG는 ECM에 존재하는 당단백질이며 많은 receptor-ligand 상호작용의 조절을 통해 뉴런의 발생과정에서 축삭 돌기(axon)의 발생을 촉진시킨다고 알려져 있다. 그러므로 이는, AKAP12 단백질의 발현을 억제시킨 morphant에서는 HSPG의 억제가 제대로 되지 않아 HSPG가 정상 위치가 아닌(ectopic) 곳에 발현이 되었고, 이것이 운동 뉴런의 발생을 촉진시켜 무분별한 뉴런의 가지치기식 발생이 일어난 것임을 시사한다.

그런데 몇몇 유전자의 경우 morphant와 녹아웃(knockout)의 표현형이 다르게 나타난다. 때문에 TALEN을 이용한 녹아웃을 제작해

동일한 현상이 일어나는지를 확인하고자 하였다. 녹아웃 개체는 T7E1과 qPCR의 melting curve를 이용하여 확인하였고 이 과정을 통하여 얻은 녹아웃을 관찰하였더니 morphant에서와 마찬가지로 운동뉴런에서 무분별한 발생이 일어났다.

결론적으로 근육 세포에서 발현이 되는 AKAP12가 HSPG를 조절하고, 근육 전구세포가 AKAP12와 이동한 후에는 그 자리에 발생되는 운동뉴런이 남겨진 HSPG의 영향을 받아 방향성이 조절되는 것임을 확인하였다.

주요어 : 운동 뉴런, 축삭돌기, 발생, 제브라피쉬, AKAP12, AKAP12α, AKAP12β, morphant, 녹아웃, HSPG
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