SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 5931-5937, 2017

Dynamic expression analysis of *armc10*, the homologous gene of human *GPRASP2*, in zebrafish embryos

CHUNYU LIU[1*], CHANGSONG LIN[1*], JUN YAO[1], QINJUN WEI[1], GUANGQIAN XING[2] and XIN CAO[1]

[1]Department of Biotechnology, School of Basic Medicinal Sciences, Nanjing Medical University, Nanjing, Jiangsu 211166; [2]Department of Otolaryngology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029, P.R. China

Received August 30, 2016; Accepted May 23, 2017

DOI: 10.3892/mmr.2017.7357

Abstract. G protein-coupled receptor-associated sorting protein 2 (GPRASP2), a member of the GASP family, has been reported to be involved in the modulation of transcription. However, few studies have revealed the role of *GPRASP2* in the development and progression of diseases. As a model organism, zebrafish have been widely used to investigate human diseases. In the present study, zebrafish armadillo repeat-containing 10 (*armc10*), an orthologous gene of human *GPRASP2* was identified, and the spatial and temporal expression patterns of *armc10* in zebrafish during early embryonic development were revealed. Bioinformatics analyses showed that ARMC10 protein sequences were highly conserved. Reverse transcription polymerase chain reaction analysis and whole mount *in situ* hybridization revealed that zebrafish *armc10* was maternally expressed and was detected at a weak level up to 12 h post-fertilization (hpf), however, its expression increased to a high level at 24 hpf. At the 75% epiboly stage and 12 hpf, *armc10* was widely expressed in the embryo. At 24 hpf, *armc10* mRNA was expressed in the nervous system of the zebrafish head. When the embryo was 2 days old, the wide expression of *armc10* was maintained in the nervous system of the zebrafish head. At 72 hpf, the mRNA expression of *armc10* was located specifically in the otic vesicles in addition to the nervous system of the head. At 96 hpf, the expression of *armc10* was maintained in the otic vesicles and the nervous system of the head. The results of the present study provided novel insight into the spatial and temporal mRNA expression of *armc10* in zebrafish, for the further investigation of nervous system diseases.

Correspondence to: Professor Xin Cao, Department of Biotechnology, School of Basic Medicinal Sciences, Nanjing Medical University, 101 Longmian Road, Nanjing, Jiangsu 211166, P.R. China
E-mail: caoxin@njmu.edu.cn

Dr Guangqian Xing, Department of Otolaryngology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu 210029, P.R. China
E-mail: xing-gq@163.com

[*]Contributed equally

Key words: zebrafish, armadillo repeat-containing 10, dynamic expression, G protein-coupled receptor-associated sorting protein 2

Introduction

G protein-coupled receptor (GPCR)-associated sorting protein 2 (GPRASP2), located at the chromosome region Xq22.1, is a member of the GPCR-associated sorting protein (GASP) family, comprising 10 members, which were identified by sequence homology searches (1,2). It exhibits significant functions in modulating the activity of GPCRs (3), which triggers numerous cellular events, including the modification of secondary messenger levels (4), receptor desensitization and internalization (5), and modification of gene transcription (6,7). For example, *GASP-1* interacts with cytoplasmic tails of several GPCRs, including D2 dopamine receptor, δ opioid receptor 1, β-2 adrenergic receptor and D4 dopamine receptor (8), and has been reported as an important breast cancer tumor and serum biomarker (9). *GPRASP2* has been identified as a non-synonymous rare variant involved in the regulation of neurite outgrowth and other synaptic functions (10), and is an essential component of the Hedgehog-induced ciliary targeting complex, which regulates the translocation of Smoothened into the primary cilia (11). In addition, the knockdown of *GPRASP2* has been shown to enhance hematopoietic stem cell repopulation (12). However, previous studies have shown that current understanding of the association between *GPRASP2* and diseases remains limited.

Armadillo repeat-containing 10 (Armc10), a 343-amino acid protein, which contains six ARM repeats, is a member of the Armc10/Armadillo repeat-containing X-linked protein (Armcx) family of proteins, which exhibit a variety of functions in embryogenesis and tumorigenesis, including cell migration, cell proliferation, tissue maintenance, tumorigenesis, signal transduction and maintenance of overall cell structure (13,14). The *armc10* gene is widely expressed in several species, and zebrafish *armc10* has been found to be a homologous gene of *GPRASP2* in our previous synteny analysis study (15).

To further examine the underlying molecular pathogenesis of *GPRASP2, zebrafish* at different embryonic stages were

used in the present study as a model organism to perform whole mount *in situ* hybridization (WISH) and reverse transcription polymerase chain reaction (RT-PCR) analysis of zebrafish *armc10,* the homologous gene of human *GPRASP2*. The results revealed the spatial and temporal expression patterns of *armc10* in zebrafish during early embryonic development and assist in further understanding the role of *GPRASP2* in embryogenesis and disease pathogenesis.

Materials and methods

Zebrafish care and maintenance. Zebrafish (Tübingen line) were provided by China Zebrafish Resource Center (Wuhan, China). The zebrafish care and experimental procedures were performed in accordance with the regulations set forth by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China). Zebrafish were maintained under 14 h light/10 h dark cycles and fed twice daily in a static water system at 28.5˚C. The vessels used for collecting embryos were placed at the four corners of the hydrostatic system fish tank 1 day prior to collecting embryos. The vessels were removed from the water following exposure to light for 30 min the subsequent day. The embryos were then raised at 28.5˚C in an incubator following collection and washing. The embryonic stages were defined as described previously (16).

RNA purification and cDNA synthesis. Total RNA was extracted from 80 embryos at 24 h post-fertilization (hpf) using TRIzol reagent (Sangon Biotech Co., Ltd., Shanghai, China). Following extraction, 1 μg of RNA was reverse transcribed into cDNA using RT Prime mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The primers were designed based on the sequences of *armc10* (ENSDARG00000062960) provided by the Ensembl database (http://asia.ensembl.org/index.html) to clone the coding sequence of *armc10*. The primers used were as follows: *armc10* F1, 5'-TGGGAGATGGCAGATGAT-3' and R1, 5'-AGGAGC CGTCCAGTAAAA-3'; *armc10* F2, 5'-CTCTGCTGGGGA TTGTGG-3' and R2, 5'-GAGAGTCCGGTCTCCTCCTC-3'. The RT product was used as a template for nested-PCR with 10 μl 2X PCR Mastermix (Beijing TransGen Biotech Co., Ltd., Beijing, China), 1 μl cDNA, 2 μl F/R primers and 7 μl H_2O. The conditions for the nested-PCR were as follows: 95˚C for 3 min, and 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 1 min, followed by incubation for 10 min at 72˚C.

Probe synthesis. The cDNAs of the 3'untranslated region (3'UTR) of zebrafish *armc10* was used to amplify templates for the synthesis of *armc10* antisense RNA probes using the following primer pair: F2-*armc10*-utr 5'-CTCTGCTGGGGA TTGT GG-3' and R2-*armc10*-utr 5'-GAGAGTCCGGTCTCC TCCTC-3'. The sequence was then cloned into the pGEM-T Easy vector with T7 and SP6 RNA polymerase promoter sequences for *in vitro* transcription.

The templates used for synthesizing *armc10* antisense RNA probes were generated by PCR amplification using pGEMT-*armc10* as templates. RNA probes were generated by *in vitro* transcription from the T7 RNA promoter, incorporating DIG-11-UTP (Roche Diagnostics, Indianapolis, IN, USA) nucleotides, using Sp6 RNA polymerase with the MAXIscript kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The DNA template was removed from the synthesized probe by DNaseI treatment and the probe was purified using LiCl-based precipitation. The probe was dissolved in DEPC-treated water and stored at -80˚C.

Sequence analysis. The full-length sequence of zebrafish was obtained from the GenBank database (https://www.ncbi.nlm .nih.gov/genbank/). The coding sequence data of zebrafish *armc10* were analyzed using Jellyfish 1.1 (http://www .jellyfishsoftware.com/) (17). Multiple sequence alignment of the amino acid sequences was performed using ClustalX2 (http://www.clustal.org/) to identify the evolutionarily conserved regions of ARMC10 among animals. Mega 6.0 (http://www.megasoftware.net/) was used to construct a phylogenetic tree of the evolution of ARMC10. Synteny analysis was performed using the Ensembl database.

Detection of armc10 mRNA using RT-PCR analysis and WISH. The distribution of the mRNA expression of *armc10* was examined using RT-PCR analysis, as previously reported (18). The embryos were staged as previously described (16). The sequences of primers used to detect the presence of *armc10* cDNA during embryogenesis were *armc10*, F2 5'-CTCTGC TGGGGATTGTGG-3' and R2, 5'-GAGAGTCCGGTCTCC TCCTC-3'. PCR analysis was performed using, 10 μl 2X PCR Mastermix (Beijing TransGen Biotech Co., Ltd.), 1 μl cDNA, 2 μl F/R primers and 7 μl H_2O and the conditions were as follows: 95˚C for 3 min, and 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 1 min, followed by 10 min at 72˚C. The sensitivity of the RT-PCR analysis was controlled by performing amplification of zebrafish *β-actin* using the same cDNA as a template (19). The primers of *β-actin* were as follows: *β-actin*, forward 5'-CCAGACATCAGGGAGTGA-3' and reverse 5'-GATACCGCAAGATTCCATAC-3'.

WISH was performed as previously described (16,17). To prevent the development of melanin pigmentation at later stages, 0.003% 1-phenyl-2-thiouera was added at 24 hpf. The concentration of the probe used in hybridization was 1.0 ng/μl for *armc10*. Images were captured using a stereoscopic microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Results

Analysis of the zebrafish armc10 gene. On examining the zebrafish genome (*armc10*; ensembl.org), it was found that the zebrafish *armc10* gene (XM_009297973) is located on chromosome 25, has six exons and encodes a 348 amino acid protein. The Armc10 protein contains a transmembrane domain at the N-terminus (aa7-29), a putative cleavage site (aa30-36) and a flanking basic region close to the transmembrane region, similar to that found in translocase of outer mitochondria membrane 20 and B-cell lymphoma 2, which predicts putative targeting to the outer mitochondrial membrane (20). Full-length Armc10 contains six Arm domains arranged in a DUF634 domain (aa 85-337), which are partially deleted in certain isoforms (21). Multiple sequence alignment of the amino acid sequences of ARMC10 derived from six different species shows a high level of conservation in the ARMC10

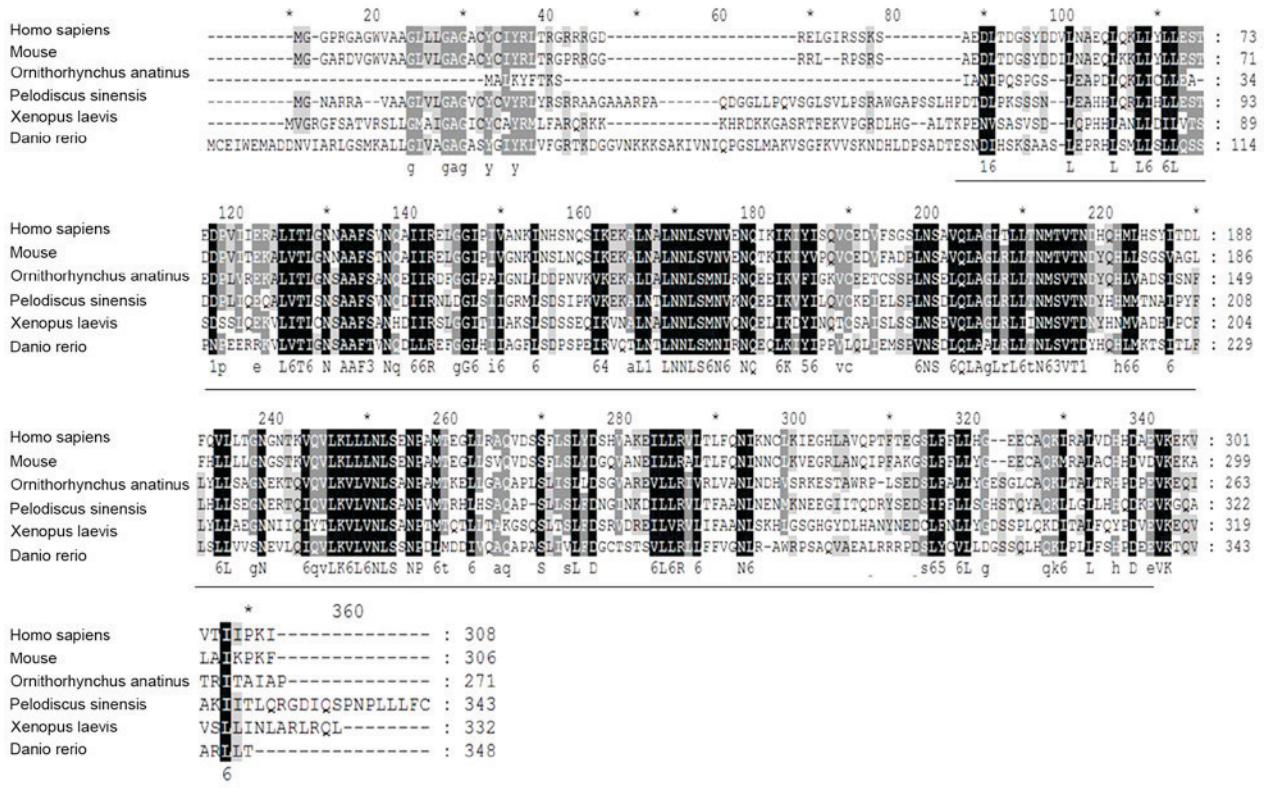

Figure 1. Amino acid sequence alignment of *armc10* from several species. Sequences were aligned using ClustalX2. The armadillo domains are indicated by underlining. Conserved residues are shown in black (100% conservation), dark grey (80% conservation) and light grey (60% conservation). An absence of shading denotes residues with 60% conservation. *armc10,* armadillo repeat containing 10.

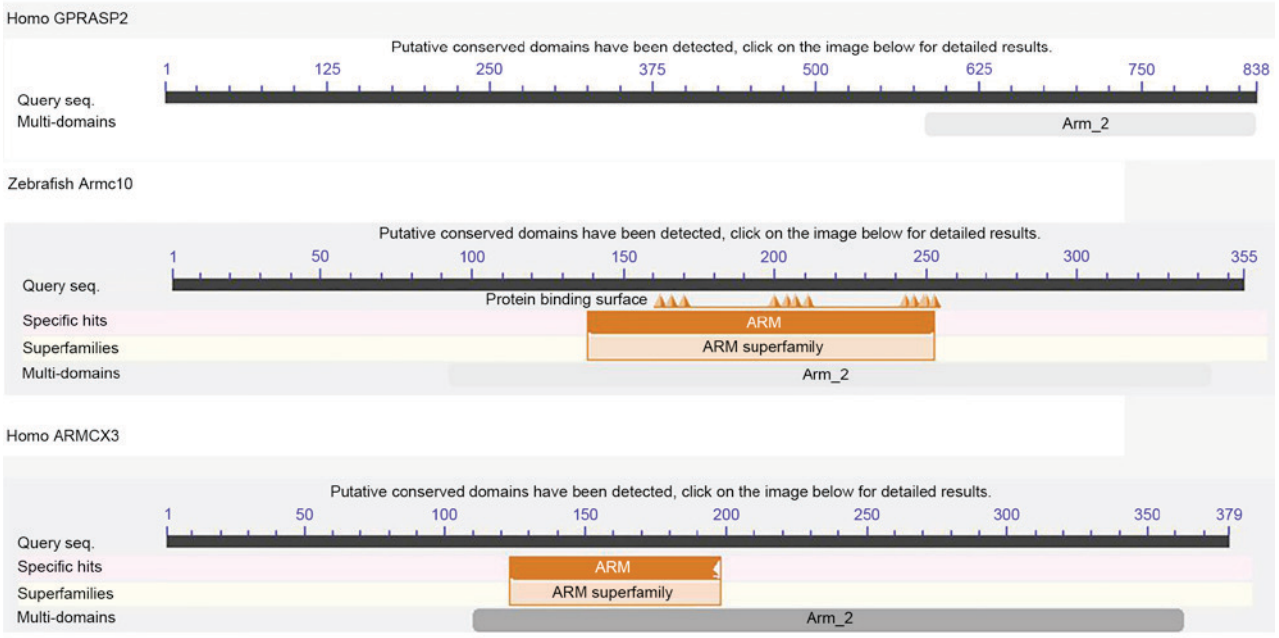

Figure 2. Analysis of conservative structure of protein functional domains. The ARM superfamily was present in the zebrafish Armc10 and Homo ARMCX3 sequences. Armc10, armadillo repeat-containing 10; ARMCX3, armadillo repeat-containing X-linked protein 3.

protein sequences among different species (Fig. 1). Typically, conserved Arm domains of ~253 amino acids (22) were found to be distributed in ARMC10 (Fig. 1). The existence of ARM_2 multi-domains also confirmed the presence of the amino acid residues (Fig. 2).

Mega 6.0 was used to construct a phylogenetic tree of the evolution of ARMC10 using amino acid sequences from 32 species (Fig. 3). The results showed that sequences belonging to the same family or order were formed in a cluster. The zebrafish *armc10* sequence formed one clad with that of

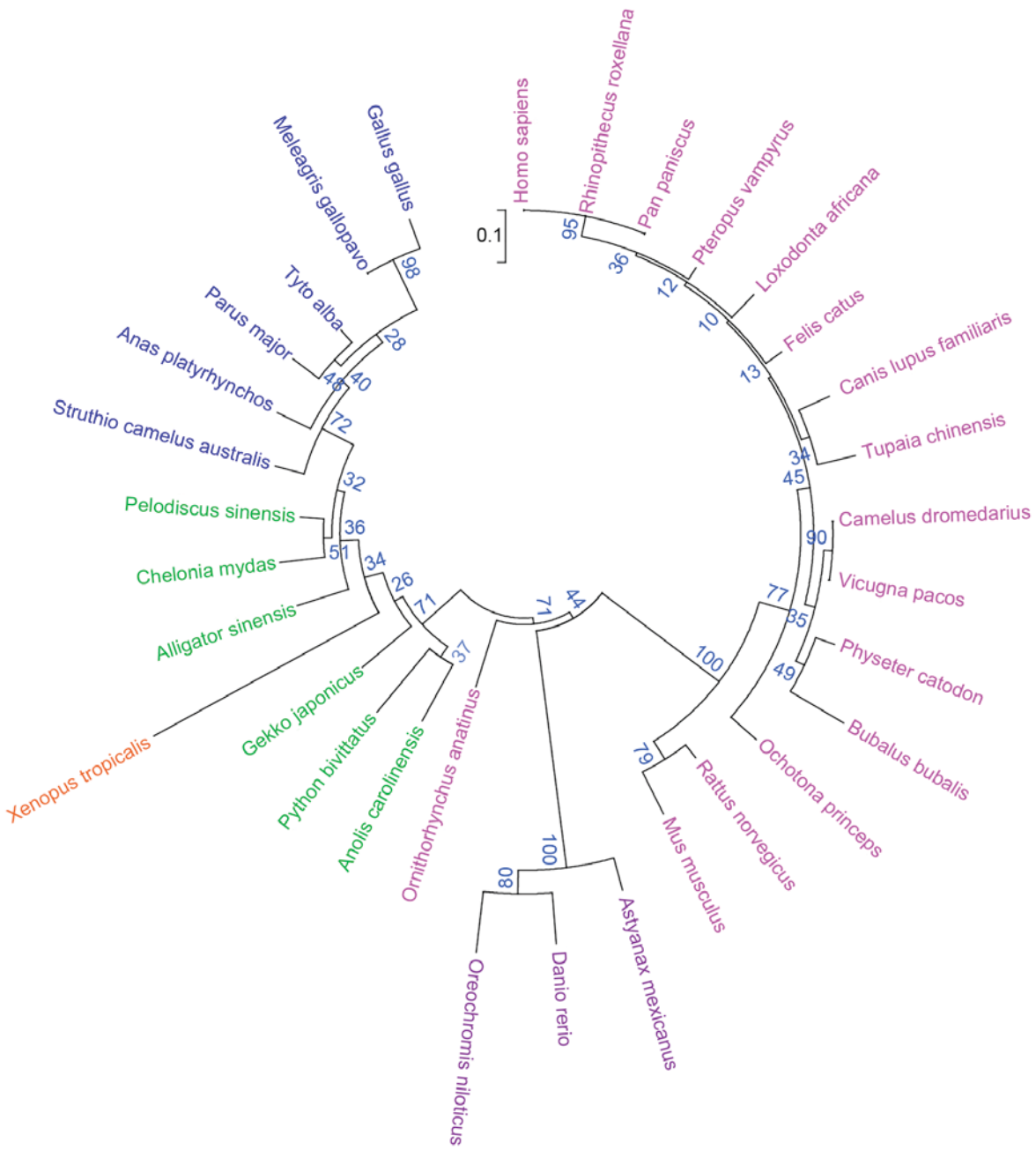

Figure 3. Phylogenetic tree of the zebrafish ARMC10 amino acid sequence with other species. A phylogenetic tree was constructed using the neighbor-joining method with Mega 6.0. Species belonging to same class were labeled in the same color: Pink, mammalia; violet, actinopterygii; green, reptilian; blue, aves; brown, amphibia. The ARMC10 sequences were retrieved from the GenBank database. ARMC10, armadillo repeat containing 10.

Oreochromis niloticus (bootstrap value 77). Higher bootstrap values were observed among the mammalians, including *Rattus norvegicus, Ochotona princeps, Camelus dromedarius and Homo sapiens, Pan paniscus* and *Rhinopithecus roxellana*). The *Tyto alba*, *Parus major* and *Anas platyrhynchos* species formed a clad, separating it from that of reptilia (*Pelodiscus sinensis, Chelonia mydas* and *Alligator sinensis*). The tree indicated that the ARMC10 protein underwent natural selection during evolution in accordance with the requirements of the environment.

Through blasting of the current zebrafish database in Ensemble with zebrafish *armc10*, the present study found that human *GPRASP2* was a homologous gene of zebrafish *armc10*. Synteny analysis indicated that human *GPRASP2* (NP_001171805) and human *ARMCX3* (NP_775104) were paralogous genes with 12.2% identity (Fig. 4A), whereas human *ARMCX3* (NP_775104) exhibited 25% amino acid identity with zebrafish *armc10* (Fig. 4B). Human *GPRASP2* also shared 8.68% identity with zebrafish *armc10* (Fig. 4C). Therefore, human *GPRASP2* and zebrafish *armc10* were considered homologous genes.

Expression of armc10 during zebrafish embryonic development. To analyze the spatio-temporal expression patterns of *armc10*, the present study performed RT-PCR analysis and WISH at stages of zebrafish development from the cleavage stage until 96 hpf. The results of the RT-PCR analysis demonstrated that *armc10* was expressed throughout

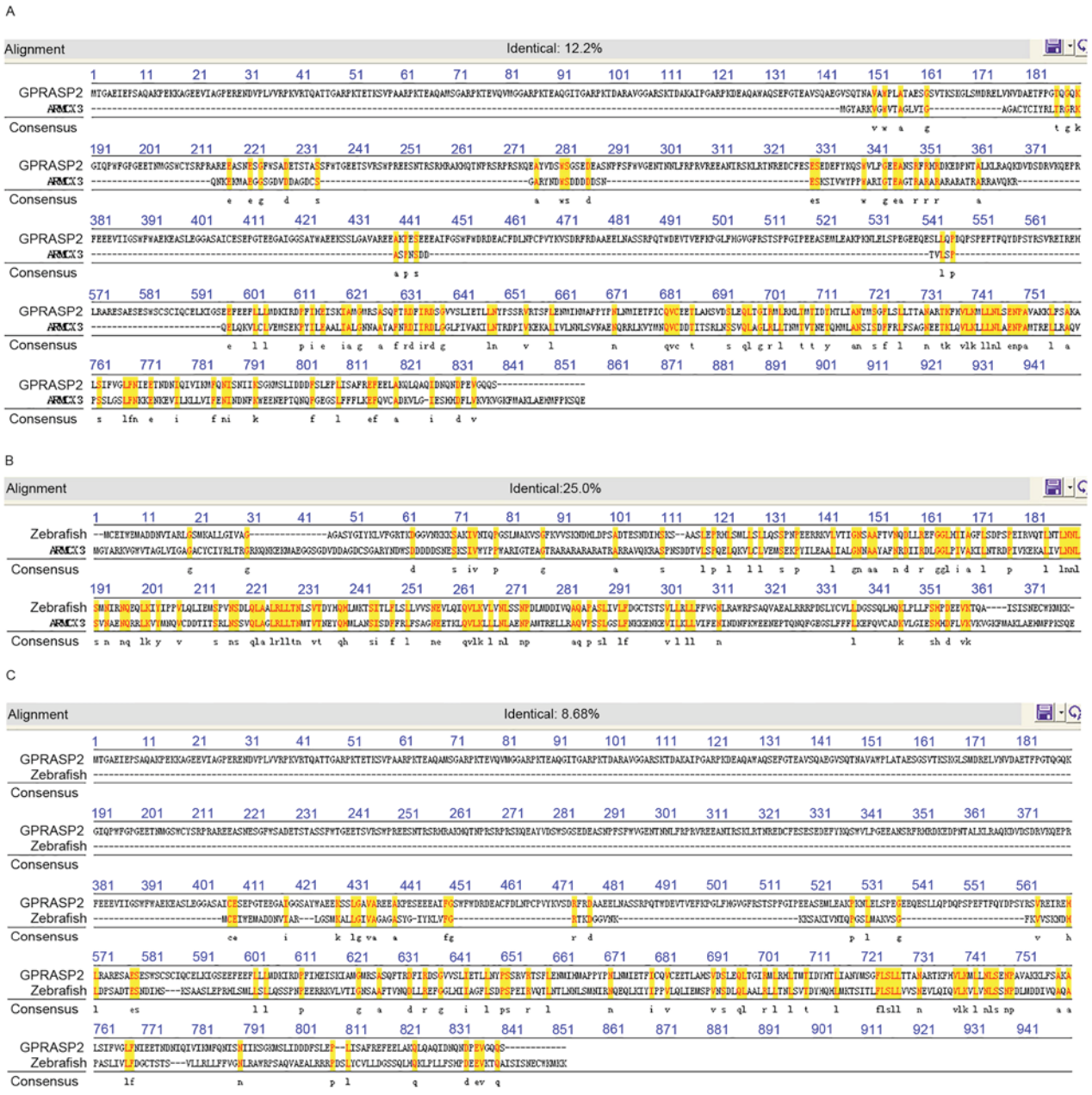

Figure 4. Amino acid sequence alignment of human GPRASP2 and other proteins. (A) Human GPRASP2 with human ARMCX3; (B) human ARMCX3 with zebrafish armc10; (C) human GPRASP2 with zebrafish Armc10. The same amino acids are marked in yellow. GPRASP2; G protein-coupled receptor-associated sorting protein 2; ARMCX3, armadillo repeat-containing X-linked protein 3; Armc10, armadillo repeat-containing 10.

early development. However, at the cleavage (two-cell) stage, 75% epiboly stage and at 12 hpf, the expression of *armc10* was weak. The embryos showed higher mRNA expression levels of *armc10* from 24 hpf (Fig. 5). Consistent with the results of the RT-PCR analysis, WISH revealed that the hybridization signal of *armc10* was detected at the two-cell stage, indicating that *armc10* was maternally expressed (Fig. 6A). At the 75% epiboly stage and at 12 hpf, *armc10* was widely expressed in the embryos (Fig. 6B and C). At 24 hpf, *armc10* mRNA was expressed in the nervous system of the zebrafish head (Fig. 6D). When the embryos were 2 days old, *armc10* maintained its wide expression in the nervous system of the zebrafish head (Fig. 6E). At 72 hpf, the *armc10* mRNA was specifically expressed in otic vesicles in addition to the nervous system of the head (Fig. 6F). At 96 hpf, the expression of *armc10* remained in the otic vesicles and the nervous system of the head (Fig. 6G).

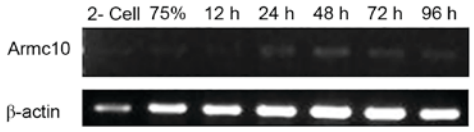

Figure 5. Temporal expression of zebrafish *armc10*. Temporal expression of zebrafish *armc10* was analyzed using reverse transcription-polymerase chain reaction analysis. Zebrafish *armc10* was first detected at the two-cell stage and persisted throughout development. β-actin was used as a control. *armc10*, armadillo repeat-containing 10.

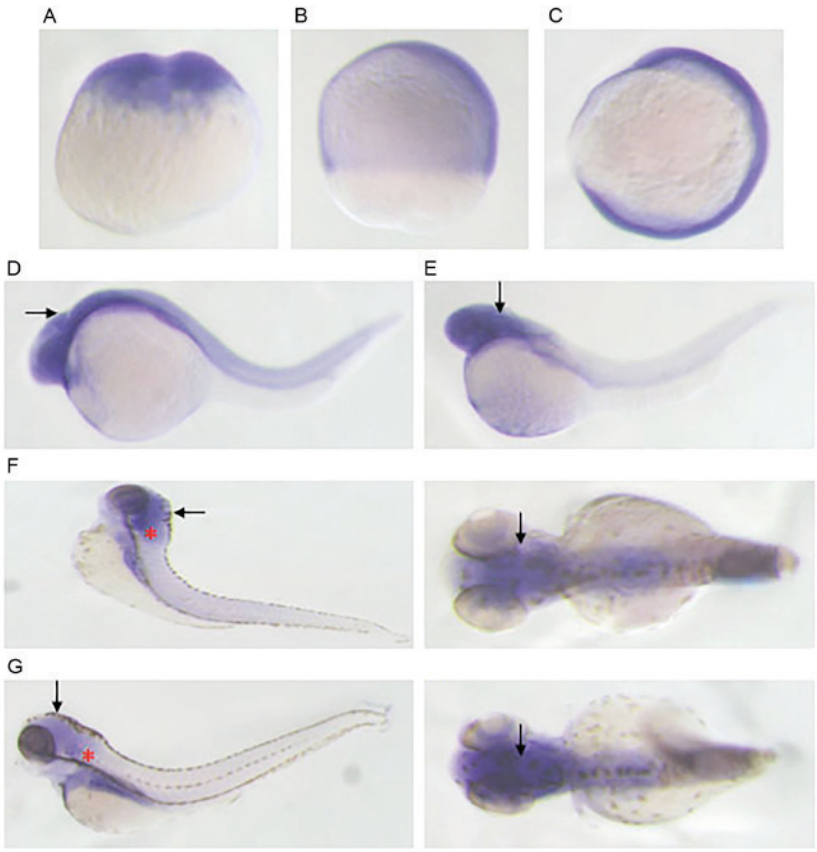

Figure 6. Expression of *armc10* during early embryonic development of zebrafish. Dorsal views at (A) cleavage stage (two-cell), (B) 75% epiboly, (C) 12 hpf, (D) 24 hpf, (E) 48 hpf. Views at (F) 72 hpf (left, lateral view; right, dorsal view) and (G) 96 hpf (left, lateral view; right, dorsal view). *armc10*, armadillo repeat-containing 10.

Discussion

In the present study, to further examine the potential molecular pathogenesis of *GPRASP2*, the characterization and expression pattern of the homologous *armc10* gene in zebrafish were examined. The results of the bioinformatics analyses showed a high degree of conservation of the ARMC10 protein sequences among different species. The high degree of evolutionary conservation was particularly reflected by the presence of amino acid residues, which are important for protein-protein interactions, including the N-terminus transmembrane domains and armadillo domains. The high conservation of the these domains is understandable, as it has been reported that the armadillo repeat domain is essential for protein-protein interactions (22-24) and is involved in diverse functions, including embryogenesis and tumorigenesis, by interacting with multiple binding partners (25). The phylogenetic analysis of ARMC10 using a phylogenetic tree demonstrated that the mammalian species formed a cluster with a higher bootstrap value and were closely associated with zebrafish, whereas variation was higher in lower organisms. It was concluded that ARMC10 gradually evolved from lower organisms with more variation, resulting in a more stable form in mammalian species. ARMC10 is also upregulated in hepatocellular carcinoma (26). Therefore, these results suggest a role for ARMC10 during embryogenesis and tumorigenesis.

In the present study, WISH and RT-PCR analysis were used to detect the expression of the zebrafish *armc10* gene during early embryogenesis. The results showed that *armc10* was detected at low levels prior to 12 hpf, and the expression levels became higher at 24 hpf, distributed primarily in the regions of the nervous system and otic vesicles. These results were consistent to a previous finding that *armc10* was widely expressed in adult nervous tissues, particularly in the forebrain regions of the cerebral cortex, hippocampus and thalamus (27). These sites of expression demonstrated that the expression of zebrafish *armc10* was dynamic during embryogenesis. The spatial and temporal expression map of *armc10*, together with reports that the levels of *armc10* regulate mitochondrial trafficking in neurons by controlling the number of moving mitochondria (21), suggest a role for *armc10* in the pathophysiology of neurological diseases. Coincidentally, the syntenic analysis performed in the present study revealed that human *GPRASP2* and zebrafish *armc10* were homologous genes. *GPRASP2* has also been reported to be involved in receptor endocytosis and postsynaptic signaling via its interaction with the disease protein huntingtin, and that polyQ-dependent alterations of the interaction can contribute to the pathogenesis of Huntington's disease (28). Therefore, the conservation of protein sequences between zebrafish and higher vertebrates demonstrated in the present study using syntenic and homologous analysis suggested that investigations of zebrafish *armc10* may provide important insights into these processes in humans.

Taken together, the present study established the gene expression map of *armc10* among different stages of zebafish

embryogenesis. The expression data compiled provided information relevant for future investigations of the role of *armc10* in the nervous system during zebrafish embryogenesis, and provided information to assist in examining the mechanism of *GPRASP2* associated with human nervous system diseases.

Acknowledgements

This study was supported by the grants from the National Natural Science Foundation of China (grant no. 31571302) and the Key Research and Development Program of Jiangsu Province (Social Development; grant no. BE2016762) to Professor Xin Cao; and grants from the Jiangsu Health Administration of China (grant no. LJ201120) and the Research Special Fund for Public Welfare Industry of Health, Ministry of Health of China (grant no. 201202005) to Dr Guangqian Xin.

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