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Molecular cloning and developmental expression of *plakophilin 2* in zebrafish

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Abstract

Armadillo proteins are involved in providing strength and support to cells and tissues, nuclear transport, and transcriptional activation. In this report, we describe the identification and characterisation of the cDNA of the desmosomal armadillo protein plakophilin 2 in zebrafish. The 2448 bp coding sequence encodes a predicted 815 amino acid protein, with nine armadillo repeats characteristic of the p120-catenin subfamily. It shares conserved *N*-glycosylation, myristoylation, and glycogen synthase kinase 3, casein kinase 2, and protein kinase C phosphorylation sites with mammalian armadillo proteins including plakoglobin and β-catenin. Semi-quantitative reverse transcription polymerase chain reaction and whole mount *in situ* hybridisation show that it is expressed both maternally and zygotically. It is ubiquitously expressed during blastula stages but becomes restricted to epidermal and cardiac tissue during gastrulation. These results provide evidence that zebrafish plakophilin 2 is developmentally regulated with potential roles in cell adhesion, signalling, and cardiac and skin development.

Keywords: Plakophilin 2; Zebrafish; Desmosomes; Armadillo proteins; Development

Plakophilin 2 is an armadillo (*arm*) repeat protein belonging to the p120^{ctn} subfamily. In mammals it is the only plakophilin to be expressed in cardiomyocytes and it is also expressed in all simple, complex, and stratified epithelia. It is found in desmosomal cell–cell junctions where it anchors cytoskeletal filaments to the cell membrane. It interacts directly with desmoplakin, plakoglobin, desmoglein 1 and 2, and desmocollin 1a and 2a. In addition to its role in desmosomes, plakophilin 2 can also interact with β-catenin to influence β-catenin/TCF signalling activity, and it can migrate to the cell nucleus [1].

Desmosomes form early in vertebrate development. In mice, they begin to assemble at the 32-cell stage corresponding to the formation of the blastocoele and are found at the contact sites of the trophectoderm cells [2]. The exact composition of desmosomes is tissue specific and assembly and disassembly are regulated by calcium, kinase/phosphatase activity, and crosstalk with adherens junctions [3,4]. In humans, arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heart muscle disorder characterised by arrhythmias and dilatation of the right ventricle of the heart, and the disease has been linked to sudden death in young people and athletes. Mutations in several desmosomal proteins have been linked to ARVC, but the majority of cases have mutations in plakophilin 2 [5–8]. Understanding the mechanisms behind the condition will aid in its treatment and prevention. A model for pathogenesis of ARVC suggests that impaired functioning of cell adhesion junctions during stress may lead to cardiomyocyte detachment and death accompanied by inflammation and replacement of cardiac muscle with fibrous and adipose tissue [9]. Knockout mice have been generated for several desmosomal proteins and those for arm repeat proteins, in particular, show severe cardiac defects [10]. In wildtype mice plakophilin 2 has been shown to be

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sequestered in the heart in precursor particles with desmoplakin which is a functionally essential component of desmosomes [11]. At E10.75 desmoplakin is co-localised with plakoglobin, β-catenin, plakophilin 2, and desmoglein. In plakophilin 2 knockout mice, desmoplakin did not co-localise with any of the junction proteins but was found to aggregate in the cytoplasm away from junctions. The depletion of plakophilin 2 in the cardiac muscle of mice results in lethal alterations in heart morphogenesis followed by cardiac rupture and blood leakage, and defects in the cellular junctions with embryonic lethality at midgestation [11].

Genetic studies in humans and knockout studies in mice have shown that plakophilin 2 is essential for maintaining the structural integrity of the heart. However, as the cardiac muscle alterations in knockout mice are embryonic lethal, a plakophilin 2 null line could not be generated. Zebrafish is a vertebrate model species with embryos that develop externally and thus can be easily monitored. They can also survive for several days without a functioning cardiovascular system and so morpholino knockdown studies overcome the limitations associated with the knockout mice. Heuser and colleagues have previously used zebrafish to show the role that another desmosomal protein, desmocollin-2, plays in ARVC [12]. However, because the majority of cases of ARVC involve mutations in plakophilin 2 it is necessary to examine the expression of this gene in zebrafish and to compare it to the human gene and protein before proceeding with knockdown experiments. In this study we cloned the full-length cDNA of zebrafish plakophilin 2, compared the putative protein sequence to the human plakophilin 2 and analysed its expression pattern throughout zebrafish embryonic development.

Materials and methods

Sequence analysis. Using the mouse plakophilin 2 protein sequence (NP_080439), predicted zebrafish plakophilin 2 gene sequences were found in the National Centre for Biotechnology Information (www.ncbi.gov) and the Ensembl genome assembly (Zv6) (www.ensembl.org/Danio_rerio) databases using the BLAST N program. Primers (forward primer: 5′-CTTGTGGGCGTGGCCTAATG-3′; reverse primer: 5′-AAGGTGTGTCCATTCAACGC-3′) were designed to amplify the sequence by RT-PCR (95 °C, 5 min; 95 °C, 60 s; 55 °C, 60 s; 72 °C, 3 min; 35 cycles; 72 °C, 10 min) from 48 h post-fertilisation (hpf) embryos and this PCR product was subcloned into pGEM-T Easy vector (Promega). Five overlapping regions of the cDNA were sequenced in the forward and reverse direction by Agowa Sequencing Service, Berlin, Germany—region 1: 694 bp (5′-CTTGTGGGCGTGGCCTAATG-3′, 5′-AGAGCCCAAGCATAAACCAG-3′) 2: 670 bp (5′-GCCGCCTCTATTTCACAGAG-3′, 5′-GGCATCTGGACTGTTGAAGC-3′) 3: 637 bp (5′-GGCTGTAAATCTGCTGACAC-3′, 5′-GTGCAGGATGCAGACACAGT-3′) 4: 633 bp (5′-TCCGTGGCACTATTGCAGAC-3′, 5′-GTATGTGGCAGATGGTGACG-3′) 5: 472 bp (5′-AAATGTGGAGCAGCCCATAG-3′, 5′-AAGGTGTGTCCATTCAACGC-3′). The predicted amino acid sequence was analysed using Predictprotein software (www.predictprotein.org), Motif Scan (http://scansite.mit.edu) and the PSIPRED Protein Structure Prediction Server (bioinf.cs.ucl.ac.uk/psipred/psiform.html) which predict secondary and tertiary protein structures based on previously characterised sequences. The ClustalW program was used to align the sequences [13].

Semi-quantitative reverse transcription-polymerase chain reaction. Single-stranded cDNA was synthesised using SuperScript Reverse Transcriptase (Invitrogen) from total RNA extracted from 128-cell, sphere, shield, 75% epiboly, 3 somite, 18 somite, 24, 48, and 72 hpf stage zebrafish embryos using TRI reagent (Molecular Research Center). Five hundred nanograms of cDNA from each stage was used for semi-quantitative RT-PCR (95 °C, 30 s; 65 °C, 30 s; 72 °C, 40 s; 38 cycles) using primers yielding a 670 bp product (417–1086 bp of the coding sequence; forward primer: 5′-GCCGCCTCTATTTCACAGAG-3′; reverse primer: 5′-GGCATCTGGACTGTTGAAGC-3′). Aliquots (5 μl) were removed after 13, 18, 23, 28, 33, and 38 cycles. In positive controls the plasmid containing plakophilin 2 cDNA was used as template DNA while in negative controls template DNA was replaced with water. The aliquots and 1 μl of positive and negative control reactions were electrophoresed on a 1.5% agarose gel alongside a 100 bp ladder (New England Biolabs). The annealing temperature for the β-actin control was 54 °C using the following primers, 5′-ACGCTTCTGGTCGTACTA-3′ and 5′-GATCTTGATCTTCATGGT-3′.

Whole mount in situ hybridization. Embryos were fixed in 4% paraformaldehyde (Sigma, UK). A 670 bp RNA probe was generated from the centre of the zebrafish plakophilin 2 sequence (704–1372 bp of the coding sequence; primers—5′-TGGAATCGGAAAAGCACACC-3′ and 5′- GAGGTTTGACCGCTTCTCTG-3′). The antisense probe was synthesized by linearising the plakophilin 2 plasmid with NcoI and transcribing with Sp6 RNA polymerase. PstI and T7 RNA polymerase were used to synthesise the control sense probe. All probes were made using a Digoxigenin RNA labelling kit (Roche Diagnostics). The procedure was as described by Westerfield [14] and Hauptmann [15]. Each *in situ* hybridization was performed in duplicate, i.e., two batches of 30 embryos. Embryos were incubated with antisense or sense probes under similar conditions and were visualized and photographed using a Nikon Eclipse E600 microscope and DXM1200F digital camera.

Results and discussion

Isolation and nucleotide sequence analysis of zebrafish plakophilin 2

Using the mouse plakophilin 2 sequence we ran a BLAST search in the Ensembl zebrafish database for a homologous zebrafish sequence. A 1488 bp cDNA fragment was found in Contig BX001012 on chromosome 4 encoding a 495 amino acid protein. This sequence was then used to search the NCBI database. A 2841 bp mRNA sequence predicted to be a zebrafish sequence similar to plakophilin 2 (XM_691368) and encoding an 815 amino acid hypothetical protein (XP_696460) was found. Primers designed to the 5′- and 3′-untranslated regions (UTRs) of this sequence were designed to amplify a 2824 bp product. The cDNA was sequenced fully in both directions and deposited in the EMBL nucleotide sequence database—Accession Number AM906170.

The cDNA consists of a 2448 bp coding sequence flanked by a 205 bp 5′-UTR and a 171 bp 3′-UTR. The genomic organisation of zebrafish plakophilin 2 was predicted from the Ensembl database showing 13 exons with 12 introns, similar to mammalian plakophilins 1–3 which have 13–15 exons.

Amino acid sequence analysis of zebrafish plakophilin 2

The complete open reading frame of the plakophilin 2 cDNA encodes an 815 amino acid protein with a calcu-

lated molecular weight of 90 kDa and an overall p*I* of 7.3. The zebrafish plakophilin 2 protein sequence shares 35% identity with the human sequence [16], 37% identity with mouse plakophilin 2 [17], and 33% identity with a predicted sequence for chick (XP_416362). As expected, the greatest variation is in the long N-terminal domain, which shares 20% identity with the human plakophilin 2, whereas the short C-terminal domain has 53% identity (Fig. 1A and C). We deduced the helical arrangement of the arm repeats in human and zebrafish plakophilin 2 based on the work of Choi and Weiss on plakophilin 1 [18]. The zebrafish arm repeat domains are very similar to those of the human protein. They both have nine arm repeat domains with an insert between repeats five and six, similar to arm proteins of the p120^{ctn} subfamily in other species. Each repeat follows the arm repeat consensus sequence seen in plakophilin 1 and, excepting the first repeat, they contain three α-helices. In general, the third helix (H3) is more highly conserved. Five of the H3 helices in each protein contain the LXNL motif near their C-terminus. Four of the human and five of the zebrafish H3 helices also contain the terminal serine found in β-catenin (Fig. 1B) [18].

Further analysis of the zebrafish plakophilin 2 sequence predicts the presence of several motifs important in cell–cell adhesion, strength, and cell signalling. Glycosylation modifies and regulates a variety of transcription factors, nuclear pores, and cytoskeletal proteins. The stability of adherens junctions is differentially affected by the degree and complexity of *N*-glycosylation of E-cadherin [19]. There are six conserved predicted *N*-glycosylation sites (consensus sequence NX(S/T)) located in H3 of arm repeats 3, 4, 5, 6, and 7 of the human and zebrafish proteins. Five of these overlap with the LXNL helical motif and are also seen in H3 of the corresponding arm repeats of plakophilin 1 [18]. We found that other arm proteins, including β-catenin and plakoglobin, also have the *N*-glycosylation consensus sequence in H3 of the arm domains. Plakoglobin has been shown experimentally to be *O*-glycosylated on serine and threonine residues [20].

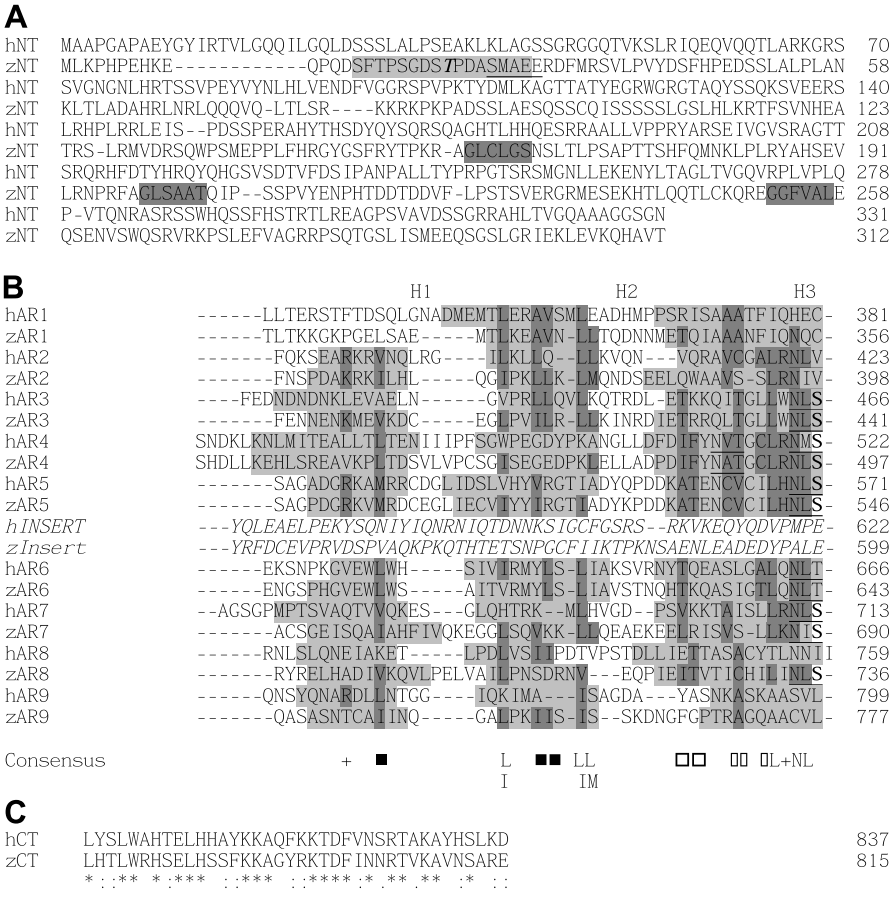

Fig. 1. Comparison of the human and zebrafish plakophilin 2 protein sequences. The human sequence is denoted by 'h' and the predicted zebrafish sequence by 'z'. 'NT' refers to the amino-terminal domain, 'AR' to the arm repeats, and 'CT' to the carboxy-terminal domain. (A) Amino terminal domain. The putative GSK3 site is indicated by light shading with the GSK3 target residue in bold italics. The priming CK2 site is underlined. Predicted myristoylation motifs are indicated by dark shading. (B) Sequence alignment of the nine arm repeats and insert. Each helix is indicated by light shading with the consensus residues in darker shading, and the consensus is shown underneath. Consensus residues are indicated by closed squares for large hydrophobic residues, open squares for general hydrophobic residues, open rectangles for small hydrophobic residues, and + for basic residues. Glycosylation motifs are underlined, and the terminal serine is in bold type. (C) Carboxy-terminal domain. Identical residues are indicated by asterisks, conserved substitutions by colons, and semi-conserved substitutions by stops.

As there is no known consensus sequence for *O*-glycosylation, we could not investigate if plakophilin 2 had potential *O*-glycosylation sites. However, there are several serines and threonines that could be *O*-glycosylated. The balance between *N*- and *O*-glycosylation, as well as the degree and complexity of the glycans, may play a role in targeting arm proteins to the membrane for their junctional role or alternatively to the cytosol or nucleus for their signalling role.

Early in the N-terminal domain of zebrafish plakophilin 2 there is a glycogen synthase kinase 3 (GSK3) site. The minimal consensus sequence for a GSK3 site motif is **S/T**-X-X-X-S/T(P), where the GSK3 target is indicated in bold, X represents any amino acid and the target is primed by prior phosphorylation of a S/T four residues C-terminal to it by another kinase, e.g., casein kinase 2 [21,22]. Fiol and colleagues suggest that there is a series of this motif (**S/T**-X-X-X-**S/T**-X-X-X-S/T). Once the last S/T is primed by phosphorylation by another kinase, GSK3 can sequentially phosphorylate the other S/Ts. The end of the GSK3 site in the zebrafish plakophilin 2 sequence contains a casein kinase 2 (CK2) phosphorylation site (Fig. 1A). GSK3 plays an important part in the canonical Wnt pathway which includes arm proteins β-catenin and plakoglobin, by targeting them for degradation [23]. There are several other predicted CK2 phosphorylation sites in the zebrafish plakophilin 2 protein sequence, including five in the arm domains. CK2 phosphorylation in the arm domains of β-catenin has been shown to increase its stability and resistance to degradation by the proteasome, thus favouring its signalling role [24]. Protein kinase C (PKC) phosphorylation is important for protein–protein interactions involving the cytoskeleton and signalling complexes. It has been shown to be involved in altering desmosomal adhesion in keratinocytes in response to calcium and to influence the assembly of desmosomes at the cell surface [25–27]. Several of the PKC sites in the zebrafish sequence have corresponding motifs in the human plakophilin 2 sequence (SLR position 126–128 bp; TKK 315–317 bp; TRR 429–431 bp; TPK 581–583 bp; TVK 806–808 bp; Consensus sequence S/T-X-R/K).

Myristoylation has a role in targeting proteins to the cell membrane and has been shown to be important in stabilising adherens junction proteins [28]. Plakophilin 2 is important in anchoring the cytoskeleton to the cell membrane, thus the predicted myristoylation sites in the N-terminal domain of the zebrafish protein are consonant with this function (Fig. 1A).

Spatial and temporal expression of plakophilin 2 throughout zebrafish embryonic development

The temporal expression of plakophilin 2 was examined by semi-quantitative RT-PCR (Fig. 2). The plakophilin 2 transcripts are detected in all stages examined from pre-midblastula transition (MBT), when the zygotic genome is activated, until 72 hpf. Therefore, plakophilin 2 is both maternally and zygotically expressed [29]. This is as expected as desmosomes are formed early in development when they are required to provide strength and stability to the growing embryo [2].

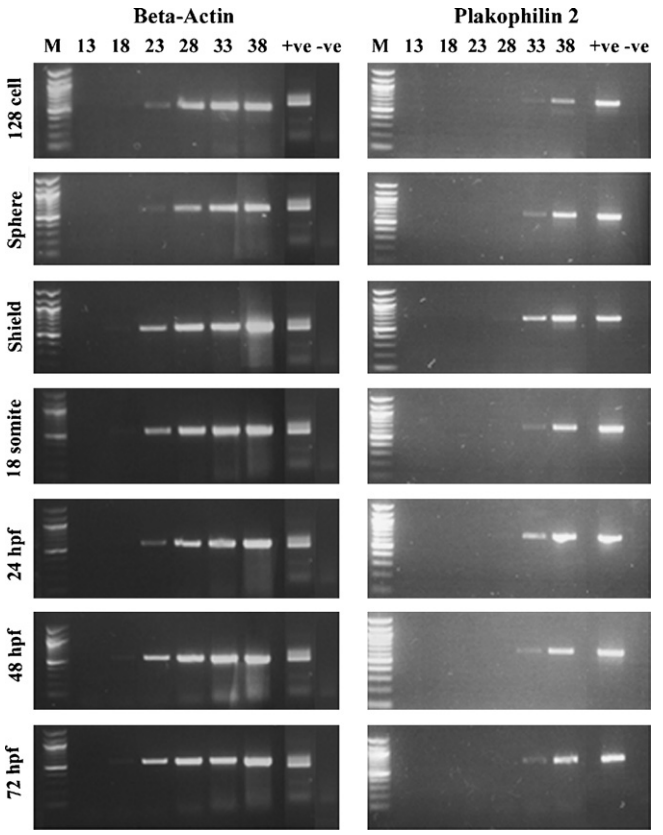

Fig. 2. Relative expression levels of plakophilin 2 throughout development. Semi-quantitative RT-PCR amplification of plakophilin 2; lane loading from left to right in the following order: 100 bp DNA marker, cycle 13, 18, 23, 28, 33, 38, positive control, negative control lacking template DNA. β-Actin control samples were expressed at a similar level throughout development.

The expression pattern of plakophilin 2 in the developing zebrafish embryo was examined using whole mount *in situ* hybridisation. Beginning early in development, at the end of cleavage, plakophilin 2 transcripts are expressed throughout the embryo. It is expressed throughout the developing epidermis until the 18 somite stage (Fig. 3). As embryonic development progresses, the expression pattern becomes restricted to the anterior of the embryo and in particular the epidermal tissue of the neural structures (Fig. 3L–K). At 48 hpf concentrated expression in the heart, ventral to the head and anterior to the yolk (Fig. 3M), was observed. These expression patterns are similar to those seen for plakoglobin and desmocollin 2 in zebrafish [12,30]. Expression of desmocollin 2 also becomes restricted to the heart at 48 hpf [12].

We have successfully cloned the zebrafish plakophilin 2 cDNA and have shown that the putative protein is similar to the human plakophilin 2, with potential roles

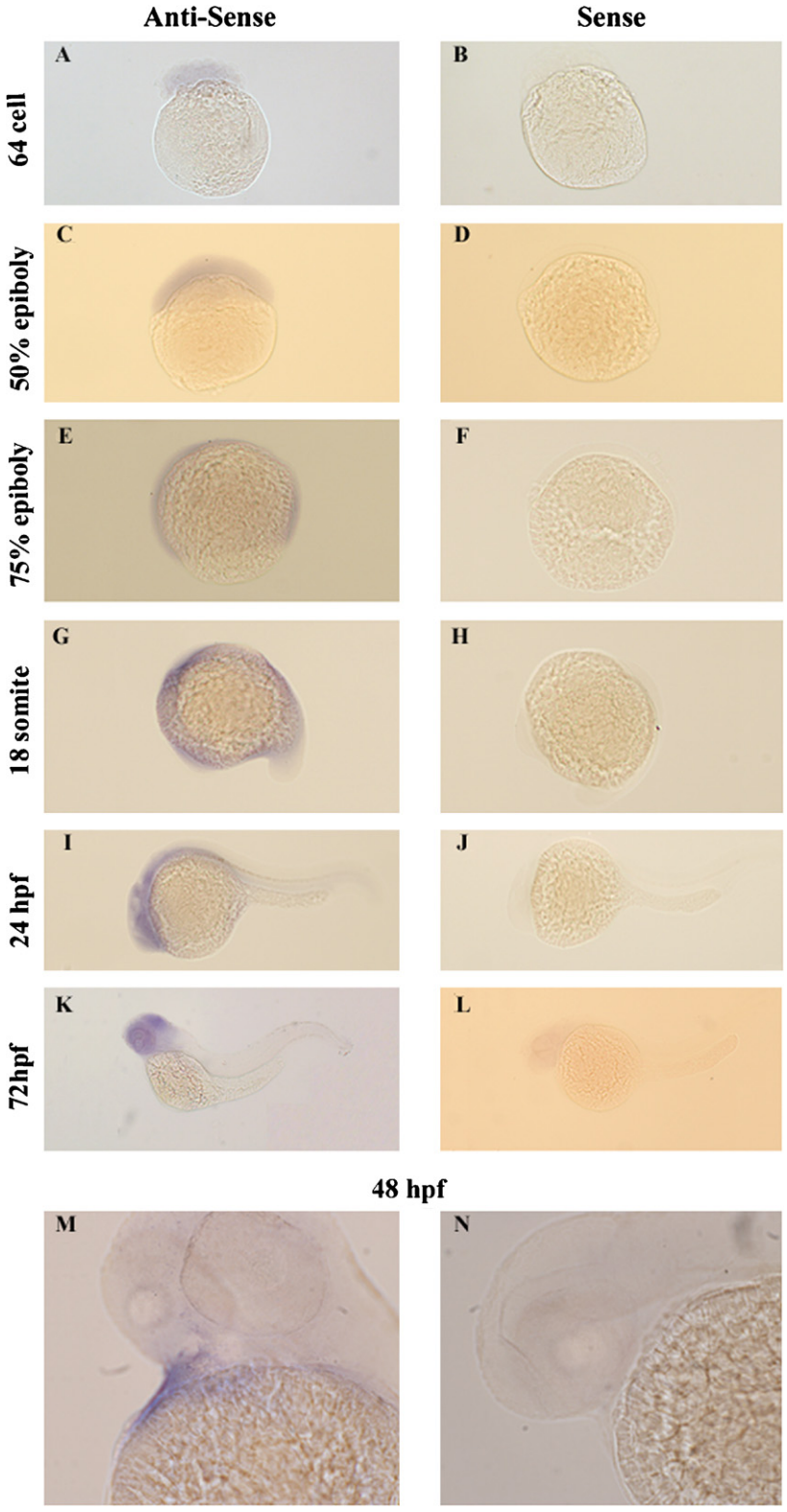

Fig. 3. Expression pattern of plakophilin 2 in zebrafish embryos. (A–L) Whole-mount in situ hybridization of zebrafish embryos at 64-cell, 50% epiboly, 75% epiboly, 18 somite, 24 hpf, 72 hpf with antisense probes in the left-hand panel and control sense probes in the right-hand panel. Lateral view, anterior to left. (M,N) Enlarged view of anterior of embryo at 48 hpf.

in cell adhesion and signalling. The mRNA is expressed throughout zebrafish embryonic development and it is present in epidermal and cardiac tissue at the later stages examined. Therefore zebrafish plakophilin 2 is an excellent candidate for knockdown studies to model ARVC.

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