Plakoglobin has both structural and signalling roles in zebrafish development

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A B S T R A C T

Plakoglobin, or gamma-catenin, is found in both desmosomes and adherens junctions and participates in Wnt signalling. Mutations in the human gene are implicated in the congenital heart disorder, arrhythmogenic right ventricular cardiomyopathy (ARVC), but the signalling effects of plakoglobin loss in ARVC have not been established. Here we report that knockdown of plakoglobin in zebrafish results in decreased heart size, reduced heartbeat, cardiac oedema, reflux of blood between heart chambers and a twisted tail. Wholemount in situ hybridisation shows reduced expression of the heart markers nkx2.5 at 24 hours post fertilisation (hpf), and cmlc2 and vmhc at 48 hpf, while there is lack of restriction of the valve markers notch1b and bmp4 at 48 hpf. Wnt target gene expression was examined by semi-quantitative RT-PCR and found to be increased in morphant embryos indicating that plakoglobin is antagonistic to Wnt signalling. Co-expression of the Wnt inhibitor, Dkk1, rescues the cardiac phenotype of the plakoglobin morphant. β-catenin protein expression is increased in morphant embryos as it is its colocalisation with E-cadherin in adherens junctions. Endothelial cells at the atrioventricular boundary of morphant hearts have an aberrant morphology, indicating problems with valvulogenesis. Morphants also have decreased numbers of desmosomes and adherens junctions in the intercalated discs. These results establish the zebrafish as a model for ARVC caused by loss of plakoglobin function and indicate that there are signalling as well as structural consequences of this loss.

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Introduction

Plakoglobin, or gamma-catenin, is a member of the catenin family of cell adhesion proteins, containing the 42 amino acid armadillo repeat, the product of the segment polarity gene in Drosophila (Peifer and Wieschaus, 1990; Peifer et al., 1992, 1994). It is one of only a few proteins found in both desmosomes and adherens junctions. At the cell membrane, it associates with the cytoplasmic tail of cadherin molecules and links them to the actin filament network in adherens junctions or to the intermediate filament network in desmosomes. The latter are found in tissues that undergo mechanical stress such as epidermis and heart (for review Carrod and Chidgey, 2008).

In addition to their structural role in cell junctions, cytosolic plakoglobin and β-catenin participate in canonical Wnt signalling. In the absence of a Wnt signal, they are phosphorylated and targeted for degradation by the ubiquitin pathway. Binding of Wnt to a Frizzled receptor results in stabilisation of the catenin degradation complex and translocation of the catenin to the nucleus where it binds to members of the T-cell specific factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996). The signalling role of plakoglobin was first indicated when its overexpression in Xenopus resulted in anterior axis duplication (Karnovsky and Klymkowsky, 1995). However, the mechanism by which plakoglobin exerts a signalling role is still uncertain. Studies in keratinocytes have shown that plakoglobin from adherens junctions can directly move to the nucleus and bind to transcription factors (Hu et al., 2003). However, in a number of cell lines, nuclear plakoglobin prevented binding of TCF-4 or LEF-1 to DNA resulting in a lack of transcriptional activity, whereas studies in a β-catenin deficient cell line have shown plakoglobin to have direct TCF/LEF transcriptional activity (Zhurinsky et al., 2000; Miravet et al., 2002; Maeda et al., 2004). In addition, plakoglobin may also have an indirect transcriptional activity as a result of replacing β-catenin in adherens junctions and allowing translocation of β-catenin to the nucleus (Li et al., 2007).

A mutation leading to the insertion of serine at position 39 in the N-terminus of plakoglobin causes arrhythmogenic right ventricular cardiomyopathy (ARVC), which is characterised by irregular heartbeat and loss of myocardial tissue with replacement by adipose and fibrous tissue in the right ventricle (Asimaki et al., 2007), whereas a two base pair (bp) deletion causing truncation of plakoglobin C-terminal results in Naxos disease, which presents as palmoplantar keratoderma and woolly hair in addition to the ARVC (Mckoy et al., 2000). Mutations in
other desmosomal proteins are also associated with ARVC (Rampazzo et al., 2002; Gerull et al., 2004; Pilichou et al., 2006).

In an effort to model these lethal human diseases, plakoglobin knockout mice were generated (Bierkamp et al., 1996; Ruiz et al., 1996). However, they showed embryonic lethality at day E10.5 or E12-15 due to ruptured ventricles. It is thought that the lack of proper desmosomes in cardiac tissue prevents it from withstanding the onset of cardiac contraction. In some genetic backgrounds, plakoglobin knockout mice survived until late gestation but exhibited skin blistering (Bierkamp et al., 1996). These studies focused only on the structural role of plakoglobin. Due to this embryonic lethality in mice, we chose the zebrafish model system to examine plakoglobin function because zebrafish embryos can survive for a few days without a functioning cardiovascular system (reviewed by Stainier, 2001). Previous studies in zebrafish have identified zebrafish plakoglobin and localised the protein expression to cell–cell adhesion junctions in follicle cells of the ovary (Cerdà et al., 1999). We have previously localised plakoglobin protein expression in embryos from 1-cell to 72 hours post fertilisation (Martin and Grealy, 2004). We hypothesised that plakoglobin has a signalling role in addition to its structural role in embryo development.

Here we report that the loss of plakoglobin results in embryos with severe cardiac defects. Increased colocalisation of adherens junction proteins, E-cadherin and β-catenin, in the epithelial layer of morphant embryos confirms that plakoglobin co-operates with β-catenin in adhesion. Loss of plakoglobin results in an increase in Wnt target gene expression indicating that plakoglobin competes with β-catenin in Wnt/β-catenin signalling. The atrioventricular endothelial cells of morphant embryos have an abnormal morphology indicating aberrant valvulogenesis. Adhesion junctions of the intercalated discs in the absence of plakoglobin are lower in number and have a more diffuse ultrastructure. These results indicate that plakoglobin has both signalling and structural roles in zebrafish development.

Materials and methods

Zebrafish maintenance

Zebrafish, AB strain, Tg(jhl1:EGFP) and Tg(TOP:GFP)w25 (Lawson and Weinstein, 2002; Dorsky et al., 2002) were obtained from the Zebrafish International Resource Centre and maintained under standard conditions at 28 °C (Westerfield, 1995).

Amplification of plakoglobin transcripts

Transcripts of plakoglobin were amplified from 24 hpf zebrafish cDNA using primers (Supplementary Data, Table 1) with the following conditions; 95 °C, 5 min; 95 °C, 60 s; 55 °C, 60 s; 72 °C, 3 min; 35 cycles; 72 °C, 10 min. Products were subcloned in pGEM-TEasy vector (Promega) and sequenced in the forward and reverse directions by Agowa Sequencing Service, Germany. The predicted amino acid sequence was analysed using Predictprotein software (www.predictprotein.org), and the PSIPRED Protein Structure Prediction Server (bioinf.cs.ucl.ac.uk/psipred/psiform.html).

Plakoglobin knockdown

A morphofish against the AUG region common to plakoglobin-1a and plakoglobin-1b was designed by and purchased from Gene Tools, Oregon (5′-GAGCCCTCCATCGAATTCCAT-3′). A five bp mismatch control morphofish was also used (5′-GAGCCCTCGGCA-TCTGGAATTCCAT-3′). Morphofishes were resuspended in nuclease-free water at a stock concentration of 1 mM. Plakoglobin morphofish, control morphofish or phenol red vehicle control (1 nl) was injected at the 1- to 2-cell stage.

Western blotting and immunoprecipitation

Deyolked and dechorionated embryos were collected as described by Link et al. (2006) with protease inhibitor cocktail (Sigma) in all solutions. Lysates were snap frozen in liquid nitrogen, thawed on ice and 50 μl of lysis buffer (400 mM NaCl, 20 mM Tris pH 8.0, 20% Glycerol, 2 mM DTT and 10% protease inhibitor cocktail) were added per 100 embryos. To aid yolk removal, Freon (100 μl) was added to lysates which were vortexed for 30 s and centrifuged at 4°C for 15 min at 11,337 × g. Protein (25 μg) was resolved by 7.5% reducing SDS-PAGE followed by western blotting. Antibodies used included anti-plakoglobin (1:1000, BD Transduction Laboratories), anti-α-tubulin (1:2000, Sigma), anti-β-catenin (1:1000, Sigma), and anti-FLAG antibody (1:2000, Sigma). A cross-linking anti-mouse IgG antibody (1:2000, Sigma) was used with the plakoglobin antibody. Secondary antibodies of goat anti-mouse IgG (1:8000, Sigma) and goat anti-rabbit IgG (1:140,000, Sigma) were used. The cross-linking anti-mouse IgG antibody and Protein A beads (Sigma) were used to immunoprecipitate proteins. Following separation by SDS-PAGE, bands were excised and sequenced by ESI-tandem mass spectrometry (Dr. S. Liddell, University of Nottingham).

Preparation of mRNA for rescue experiments

Full length open reading frames (ORF) of plakoglobin-1a and plakoglobin-1b were amplified using primers that included a FLAG-tag label at the 5′ end of each construct (Supplementary Data, Table 1). A second set of primers for each transcript was used to alter 5 bp in the morpholino target region without altering the resulting protein sequence using a Quick-change site-directed mutagenesis kit (Stratagene). Both constructs were subcloned into pCS2+ vector. Capped mRNA was synthesized using mMessage mRNA kit (Ambion) according to manufacturer’s instructions.

Wholemount in situ hybridisation (WISH)

Antisense probes were synthesized from linearised plasmid using SP6, T7 or T3 polymerase and a DIG-labelling RNA kit (Roche). Embryos were fixed in 4% paraformaldehyde (PFA), dehydrated and hydrated through a methanol series and hybridized with probe as per Westerfield (1995) and Hauptmann (1999). The bound probe was detected with BM-Purple AP-Substrate (Roche).

O-dianisidine staining

Dechorionated embryos were incubated in O-dianisidine staining solution (2.45 mM O-dianisidine, 0.01 M sodium acetate, pH 4.5, 0.65% H2O2, and 40% ethanol) for 15 minutes in the dark at room temperature. Embryos were fixed in 4% PFA, briefly washed through a methanol series and mounted for viewing (Ransom et al., 1996).

Immunofluorescence

For sectioning, embryos were embedded in 4% agarose, sectioned by vibratome and blocked in blocking solution (0.3% Triton X-100, 4% BSA, PBS). Antibodies used included anti-plakoglobin (1:100, BD Transduction Laboratories), anti-desmocollin 2 and 3 (1:100, Zymed), anti-E-cadherin (1:100, Sigma), monoclonal anti-β-catenin (1:500, Sigma) and polyclonal anti-β-catenin (1:200, Sigma). Isotype-specific Alexa Fluor-633 or -546 secondary antibodies were also used (Molecular Probes). Embryos were examined using Zeiss LSM 510 laser scanning microscope and colocalisation was quantified using Zeiss LSM 5 software.

Semi-quantitative RT-PCR

Total RNA from morphofish injected embryos was extracted by TriReagent (MRC) and was reverse transcribed. The cDNA was used...
as template in semi-quantitative PCR as described by O’Boyle et al. (2007). All primer sequences are provided in Supplementary Data, Table 1.

Transmission electron microscopy (TEM)

Dechorionated embryos at the desired stage were fixed in 2.5% gluteraldehyde / 2.5% PFA and post fixed in osmium tetroxide. The embryos were dehydrated in an ethanol series and embedded in a low viscosity resin (Agar Scientific Ltd.). Embryos were sectioned transversely using a Reichert Jung Ultracut instrument and ultrathin sections of interest were selected using light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and sections of interest were selected using light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H7000 electron microscope.

Statistics

Results were expressed as mean±SD. Normality was tested for using Shapiro–Wilkes. Differences between groups were evaluated by one way ANOVA and Student–Newman–Keuls. Chi-square tests were used to compare the distribution pattern of adhesion junctions. A P-value of less than 0.05 was considered statistically significant.

Results

We used the previously published plakoglobin sequence to amplify the ORF of zebrafish plakoglobin (Cerdà et al., 1999). However, in amplifying the ORF and a region of the 3′ untranslated region by PCR, we obtained two products; one of 2.9 kb, the expected size for plakoglobin, and a second product of 1.9 kb. Both products were subcloned and sequenced and these sequences were compared to Zebrafish ensembl database release 7. Following these sequence searches, we changed the annotation of the gene previously known as plakoglobin to plakoglobin-1a. The shorter transcript was annotated as plakoglobin-1b (GenBank accession no. AM998809). Plakoglobin-1b shares 99% identity with plakoglobin-1a over the 1248 bp of its ORF. The plakoglobin-1a transcript contains an additional 1089 bp in the 3′ end which are absent from the plakoglobin-1b transcript. The predicted protein product of plakoglobin-1b has an expected molecular weight of 42 kDa compared to 83 kDa for plakoglobin-1a. The predicted protein contains only four armadillo repeat domains compared to 13 repeats for plakoglobin-1a. A comparison of this transcript variant with the transcripts of armadillo-related proteins in other species by ClustalW showed plakoglobin-1b to be equally similar to the human plakoglobin transcript variant 1 and transcript variant 2 sequences (score 49) (Franke et al., 1989). In Drosophila, two variants of the armadillo protein have been reported (Peifer and Weischaus, 1990; Loureiro and Peifer, 1998). Again, the plakoglobin-1b transcript is equally similar to the armadillo and the neural armadillo sequences by ClustalW (score 39).

Phenotype generated by knocking down plakoglobin in zebrafish

To examine the role of plakoglobin in zebrafish, we injected 2.5, 5 or 7.5 ng of plakoglobin morpholino and analysed the phenotypes at 24-, 48-, and 72 hpf (Figs. 1A–F). Embryos were classified depending on their phenotype into normal, mild, intermediate or severely affected. At 24 hpf, mildly affected plakoglobin morpholino injected embryos had delayed midbrain-hindbrain border formation. Intermediately affected embryos also had reduced heart size with either oedema or a kinked tail. Severely affected embryos had all these defects. At 48 hpf and 72 hpf, mildly affected embryos had kinked tail, oedema and a...
reduced heartbeat. Intermediately affected embryos also had severe oedema, reduced heart size, blood pooling, reduction in blood circulation and reflux of blood between the chambers of the heart. Severely affected embryos typically had a complete absence of blood in a heart that failed to loop. At 24 hpf, at least 90% of embryos were affected at all doses. However, the majority of embryos receiving the lowest dose appeared normal by 48 hpf, whereas over 65% of those receiving the two higher doses remained affected at 48 and 72 hpf (Fig. 1G). A 5 bp mismatch morpholino was injected as a control and at the 2.5 and 5 ng doses these embryos were phenotypically indistinct from sibling wildtype embryos, whereas 3% of embryos injected with 7.5 ng of control morpholino showed non-specific defects at 72 hpf. Therefore, we chose 5 ng as the dose for further experiments. At this dose, morphant embryos exhibited a range of cardiac phenotypes, from mild oedema, to reduced heart size and a failure to loop correctly (Figs. 1H–K; Supplementary data, videos 1–4). In addition, the mean heart rate of morphants was reduced compared to control morpholino injected embryos at 48 hpf (106.2±0.58 beats per minute (bpm) vs 117.5±0.91 bpm respectively, P<0.05). The knockdown of plakoglobin in morpholino injected embryos was confirmed by western blotting (Fig. 1L). A band of approximately 83 kDa, the expected size for zebrafish plakoglobin-1a, was detected in control morpholino injected, phenol red injected and wildtype embryos. No band was detected in the plakoglobin morphant embryos, indicating that the morpholino inhibits plakoglobin-1a protein translation. A second band of approximately 70 kDa was detected with the plakoglobin antibody. This band was isolated by immunoprecipitation and sequenced by mass spectrometry and identified as vitellogenin-1, an abundant yolk precursor protein. These results indicate that the morpholino prevents translation of plakoglobin-1a mRNA resulting in morphant embryos.

The morphant phenotype can be rescued by co-injection of plakoglobin-1a RNA

To confirm that the observed phenotype was specific to the loss of plakoglobin-1a, FLAG-tagged zebrafish plakoglobin-1a mRNA with a

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**Fig. 2.** Plakoglobin-1a RNA rescues the morphant phenotype in a dose-dependent manner. (A) In each experiment, 5 ng of control morpholino (yellow) or plakoglobin morpholino was injected on its own (purple) or with 62.5 pg (white), 125 pg (green) or 250 pg (black) of plakoglobin-1a RNA. At 72 hpf, injection of plakoglobin morpholino alone resulted in 71% affected embryos while co-injection of 62.5 pg of morpholino and plakoglobin-1a resulted in 67% affected embryos. Co-injection of 125 pg of plakoglobin-1a resulted in only 11% of embryos exhibiting a morphant phenotype. Co-injection of 250 pg of plakoglobin-1a RNA with control morpholino was injected into embryos at 1– to 2-cell stage. At 72 hpf, the (C) morphant phenotype, including cardiac oedema (arrows), was rescued in (D) plakoglobin morpholino and plakoglobin-1a co-injected embryos. (E) Translation of injected plakoglobin-1a mRNA was confirmed by western blotting of 24 hpf embryo lysate with an anti-FLAG antibody. A band of approximately 83 kDa was detected corresponding to the expected size of FLAG tagged plakoglobin-1a (arrow). Blotting with an anti-plakoglobin antibody confirmed knockdown of plakoglobin in morphant embryos and partial rescue of expression in co-injected embryos (arrowhead). An anti-α-tubulin antibody was used as a loading control.
mutated morpholino-binding region was co-injected with the morpholino. A dose response study determined the optimal dose to be 125 pg of plakoglobin-1a (Fig. 2A). This dose normalised the plakoglobin morphant phenotype (Figs. 2B–E) whereas embryos co-injected with 62.5 pg showed the morphant phenotype, while those receiving 250 pg were severely disrupted or dead. Western blot analysis using an anti-FLAG antibody detected a band of approximately 83 kDa in co-injected embryos that was not detected in control morpholino injected or morphant embryos indicating the translation of the injected plakoglobin-1a (Fig. 2E). These results confirmed that the morphant phenotype is specific to the loss of plakoglobin-1a as co-injection of plakoglobin-1a mRNA with the morpholino resulted in normal embryos.

Plakoglobin-1b cannot compensate for the loss of plakoglobin-1a in morphant embryos

To investigate the relative contribution of plakoglobin-1b in zebrafish development a FLAG-tagged plakoglobin-1b construct was co-injected with the plakoglobin morpholino. The plakoglobin antibody could not be used as plakoglobin-1b lacked the antibody epitope. We altered the morpholino binding region to make the plakoglobin-1b mRNA resistant to the plakoglobin morpholino. This mRNA (62.5 pg, 125 pg or 250 pg) was co-injected with 5 ng of plakoglobin morpholino. Co-injection of plakoglobin-1b at any of the doses failed to rescue the morphant phenotype (P<0.05 vs. control; Figs. 3A–D). Plakoglobin-1b injected embryos were subject to western blotting with an anti-FLAG antibody to detect the protein and confirm the translation of the injected mRNA. A band of approximately 42 kDa, the predicted size of plakoglobin-1b, was detected (Fig. 3E). Embryos co-injected with both plakoglobin morpholino and plakoglobin-1b RNA were phenotypically morphant indicating that plakoglobin-1b failed to rescue the effects of plakoglobin knockdown.

Role of plakoglobin in cardiac development

As the cardiac phenotype in morphant embryos was the most prevalent, we used several cardiac marker genes to identify the stage at which cardiac development was perturbed in plakoglobin morphants. First, we examined cardiac precursor migration and heart tube formation using nkx2.5, one of the earliest cardiac precursor markers.
Its expression was reduced in plakoglobin morphant embryos at 24 hpf but in the correct location, to the left of the midline, indicating that the loss of plakoglobin results in fewer cardiac precursors but does not affect precursor migration or heart tube formation (Fig. 4B). To investigate heart chamber formation we examined expression of chamber specific genes, cardiac myosin light chain2 (cmlc2) and ventricular myosin heavy chain (vmhc; Yelon et al., 1999). These were both reduced in 48 hpf morphant embryos compared to control morpholino injected embryos (Figs. 4C–F). Expression of these chamber markers also indicated that only the severely affected morphant hearts failed to loop correctly. The abnormal reflux of blood between the chambers of the heart of morphant embryos could be as a result of disrupted valve formation.

We examined this using bone morphogenetic protein-4 (bmp-4) and notch1b. Both genes are expressed throughout the anterior-posterior axis of the heart prior to 36 hpf and, in normal embryos, expression becomes restricted to future cardiac valve regions by 37 hpf for bmp4 and 48 hpf for notch1b (Walsh and Stainier, 2001; Westin and Lardelli, 1997). At 30 hpf, expression of notch1b was similar to controls in 73% of plakoglobin morpholino injected embryos (38/52, data not shown). At 48 hpf, notch1b expression failed to restrict in 79% of morphant embryos and continued to be expressed throughout the entire heart (Fig. 4H). Bmp-4 expression at 30 hpf was increased throughout the embryo in 86% of morphant embryos (49/57, data not shown) and at 48 hpf had failed to restrict to the valve forming regions in 92% of morphant embryos (Fig. 4J). Therefore, the absence of plakoglobin results in an increase in bmp-4 and notch1b expression and a loss of the restriction of these genes that is required for cardiac valve formation.

**Cardiac valve morphology is altered in morphant embryos**

Following the expanded expression of genes important in cardiac valve development, we examined cardiac valve morphology by TEM in morphant embryos. Using electron microscopy, the structure of 72 hpf control and morphant hearts was examined. In control hearts, the cuboidal endothelial cells in the future valve region between the atrium and ventricle were detected (Figs. 5A and C). However, in morphant hearts, no cuboidal cells were observed. Instead the endothelial cells in the area between the atrium and ventricle had an irregular morphology (Figs. 5B and D). The number of cell junctions between the endothelial cells in morphants (24 junctions in 33 borders) was reduced compared to control embryos (65 junctions in 34 borders; P<0.001; see supplementary Table 2). These results indicate that endothelial cells have an aberrant morphology with reduced adhesion, indicating abnormal valvulogenesis in the absence of plakoglobin.

**Haematopoiesis and angiogenesis in plakoglobin morphants**

We wished to examine if the reduced circulation and blood pooling observed in plakoglobin morphant embryos were as a result of abnormal cardiac function or abnormal haematopoiesis. Firstly, we confirmed the morphological defects by O-dianisidine staining of haemoglobin in 48 hpf embryos (Figs. 6A, B). The levels of immature haematopoiesis were compared between control and morphant embryos (Figs. 6C–I). The numbers of embryos with the displayed phenotype were (A) 62/65 (B) 55/63 (C) 72/75 (D) 53/72 (E) 68/70 (F) 40/72,(G) 77/80, (H) 67/85, (I) 74/78 and (J) 68/74.
blood cells in the heart were compared in morphant and control embryos by labelling E-cadherin, which is expressed by developing erythrocytes (Armeanu et al., 1995). Confocal microscopy of 72 hpf embryo heart sections showed a reduction in the amount of E-cadherin-labelled immature blood cells in the morphant heart compared to control embryos (Figs. 6C, D). To investigate whether this reduced blood cell expression was due to altered blood development, we examined expression of the haematopoiesis markers stem cell leukaemia (scl) and gata1. Stem cell leukaemia encodes a basic helix–loop–helix transcription factor and knockdown of scl in zebrafish results in a lack of haemangioblasts (Patterson et al., 2005). The gata1 transcription factor is downstream of scl and is a marker for primitive erythroid lineage (Detrich et al., 1995). These markers for haematopoiesis had normal expression patterns at 20 somites in morphant embryos (data not shown). This suggests that the reduction in blood cells was not due to disrupted blood formation. We next examined if the reduced blood circulation was due to defects in the vasculature of the morphant embryo. Transgenic fltl:EGFP fish

Fig. 5. The structures of control and morphant cardiac valves were examined. (A) In control hearts, endothelial cells in the atrioventricular boundary have cuboidal morphology. (B) In morphant hearts, endothelial cells have a squamous morphology. (C) Higher magnification of the boxed area in (A). (D) Higher magnification of the boxed area in (B). Atria are indicated by (A) and ventricles by (V). The number of embryos with the displayed phenotype were (A) 2/2 and (B) 2/2. Scale bar is 10 μm for A and B and 2 μm for C and D.

Fig. 6. Reduced circulation in morphant embryos. (A, B) Lateral view of O-dianisidine stained 48 hpf (A) control and (B) morpholino injected embryos. Blood was present in the anterior blood vessels, the heart, the common cardinal vein, the dorsal aorta, and posterior cardinal vein. In the morphant embryo, blood was pooled as it exited the common cardinal vein and there was a reduction in blood cells in the anterior blood vessels. (C, D) Laser-scanning immunofluorescence microscopy images of transverse sections of 72 hpf embryo hearts. Fluorescence images with anti-E-cadherin antibody (red), a marker for epithelium and haematocytes, in (C) control morpholino injected embryo. Haematocyte numbers were reduced in (D) morphant heart. Hearts are outlined by white dotted lines. (E–H) The endothelial specific transgenic fltl:EGFP embryos injected with (G) control or (H) plakoglobin morpholino were analysed under light (E and F) and fluorescence microscopy (G and H) for vascular defects at 72 hpf. There was no alteration in vasculature in morphant embryos despite evident blood pooling (arrow).
were injected with the plakoglobin morpholino or control morpholino to examine angiogenesis. The vasculature was examined at 24 hpf, 48 hpf and 72 hpf. Vascular development was normal in plakoglobin morphants (Figs. 6E–H). This indicates that any circulation defects or blood pooling were not as a result of defects in vasculature. From our study of blood and vasculature formation, we can deduce that haematopoiesis and angiogenesis are not affected in morphant embryos but the later abnormal cardiac valve function results in defects to blood circulation. These results indicate that loss of plakoglobin results in reduced cardiac chambers with impaired cardiac valve formation. Although morphants have normal vasculature, these defects result in reduced blood circulation and oedema.

**Plakoglobin is antagonistic to Wnt/β-catenin signalling**

The increased expression of *bmp-4* at 30 hpf indicated that signalling was altered in morphant embryos. To investigate signalling at earlier stages in the morphant embryos we examined the expression of known Wnt target genes, bozozok and *bmp-4*, and other signalling pathway components. At the shield stage, *bmp-4* expression was increased in 86% of morphant embryos (Fig. 7B). However, *bmp-7* expression was similar in morphant and control injected embryos (n = 57) (data not shown). *Bmp2b* was upregulated in 62% of shield stage morphant embryos (Fig. 7D). Wnt/β-catenin signalling is required for organiser formation in vertebrate embryos. We examined this using early dorsal markers *bozozok* (*boz*) and *squin* (*sqt*) in control-injected and morphant embryos. Bozozok, a homeodomain protein and direct target of β-catenin, is expressed in the dorsal yolk syncitial layer and dorsal blastomeres, and co-operates with squint, a nodal related protein, in organiser formation (Shimizu et al., 2000; Solnica-Krezel and Driever, 2001). Bozozok expression was increased in 62% and *sqt* in 78% of sphere stage plakoglobin morphant embryos (Figs. 7F and H). The relative transcript levels of *bmp-4*, *bmp-2b*, *boz* and *sqt* were examined by semi-quantitative RT-PCR amplification from shield and sphere stage plakoglobin and control morpholino injected embryos. Lane order: 100 bp ladder, PCR cycle 13, 18, 23, 28, 33, positive plasmid control, negative template control and 1 Kb ladder. β-actin was used as a template control. Expression of *bmp4*, *bmp2b* and *sqt* were detected five cycles and *boz* was detected ten cycles earlier in morphants than control embryos. Analysis of GFP expression in morpholino injected TOPdGFP fish. (J) In control morpholino injected fish, GFP expression is detected at the atrioventricular boundary (black arrowhead). (K) In morphant embryos, GFP is expressed throughout the heart (white arrowhead). The number of embryos with the displayed phenotype were (J) 31/39 and (K) 34/43. Co-expression study of Dkk1 and control or plakoglobin morpholino. (L) Control morpholino injected embryos at 72 hpf with normal hearts (165/165). (M) Plakoglobin morphants at 72 hpf with abnormal hearts including oedema, reflux of blood between chambers (88/149). (N) Co-injection of 20 pg of DKK1 and control morpholino resulted in oedema and reflux of blood between chambers (30/128). (O) Co-injection of 20 pg of DKK1 and plakoglobin morpholino with normal cardiac phenotypes (10/162).
PCR and all showed increased levels of expression in morphant embryos. Bmp-4 and bmp-2b appeared at cycle 28 in morphants vs cycle 33 in controls; boz appeared at cycle 23 in morphants vs cycle 33 in controls and sqt appeared at cycle 23 in morphants vs cycle 28 in controls (Fig. 7I). The increased expression of Wnt target genes, such as bozozok and BMP4, in morphant embryos confirms that plakoglobin is antagonistic to Wnt/β-catenin signalling. To ascertain if this increase in Wnt/β-catenin signalling in morphant embryos results in the cardiac phenotype, we examined GFP expression in the TCF reporter TOPGFP transgenic line (Dorsky et al., 2002). GFP expression in control embryos was detected only at the atrioventricular boundary, indicating Wnt/β-catenin activity in these cells (Fig. 7J). However in morphant embryos, Wnt/β-catenin activity was detected throughout the heart indicating an increased level of Wnt signalling in the heart (Fig. 7K). To further examine the relationship of plakoglobin and Wnt/β-catenin signalling, we co-injected dickkopf-1 (Dkk1), an inhibitor of Wnt signalling, and examined the resulting phenotype (Hashimoto et al., 2000). At 24 hpf, 85% (109/128) of embryos co-injected with control morpholino and Dkk1 displayed the Dkk1 overexpression phenotype of an enlarged head, increased eye size and shortened body axis, with 17% of embryos also showing somite defects. While 68% (112/163) of plakoglobin morpholino and Dkk1 co-
injected embryos displayed increased head and eyes, none displayed somite defects. At 72 hpf, control morpholino injected embryos had normal cardiac phenotype while plakoglobin morpholino injected embryos displayed the morphant phenotype (Figs. 7L and M). By 72 hpf, 24% of control morpholino and Dkk1 co-injected embryos exhibited cardiac defects including failure to loop and reflux of blood between chambers (Fig. 7N). However, in plakoglobin morpholino and Dkk1 co-injected embryos, only 6% of embryos exhibited cardiac defects (10/162; P<0.001 vs plakoglobin morpholino; Fig. 7O). These results indicate that in the absence of plakoglobin, Wnt/β-catenin signalling is increased. Co-injection of DKK1 rescues this defect. These results highlight the importance of the antagonistic signalling role of plakoglobin in embryo development.

Loss of plakoglobin does not increase β-catenin mRNA expression but increases protein expression

As Wnt signalling is increased in morphant embryos, we wished to examine if the loss of plakoglobin resulted in changes in β-catenin mRNA or protein expression. β-catenin protein expression was examined by western blotting using an antibody that recognises both β-catenin-1 and β-catenin-2. Total β-catenin protein was increased in plakoglobin morphant embryos (Supplementary Fig. 1A). Protein levels were normalised to α-tubulin. The relative levels of total β-catenin to α-tubulin in morphant embryos was 147.66±15.67 relative densitometric units compared to 78.10±3.84 relative densitometric units for control injected embryos (P<0.05, Supplementary Fig. 1B, n=3). β-catenin mRNA was then examined to determine if this increase was due to increased transcription or translation of the β-catenins. β-catenin-1 and β-catenin-2 mRNA localisation was normal in morphant embryos as examined by WISH (Supplementary Figs. 1C–F). The relative transcript levels of β-catenin-1 and β-catenin-2 were examined by semi-quantitative RT-PCR. β-catenin-1 was first detected at cycle 28 in both control and plakoglobin morphant embryos. β-catenin-2 was first detected at cycle 33 in both control and morphant embryos. β-actin was detected at cycle 23 in both the plakoglobin morphant and control injected samples. These results show that β-catenin-1 and β-catenin-2 levels are similar in morphant and control injected embryos (Supplementary Fig. 1G) indicating that increased expression of β-catenin in morphant embryos was not due to increased transcription but may be due to increased translation or increased stability of the β-catenin protein.

β-catenin compensates for loss of plakoglobin in adherens junctions but not in desmosomes

We examined the effect of the loss of plakoglobin and the increased β-catenin protein expression on the localisation of other adherens junction proteins by confocal immunofluorescence microscopy, β-catenin and E-cadherin had distinct localisation patterns in control-injected 72 hpf embryos (Fig. 8G). However, in plakoglobin morphant embryos these two adhesion proteins were extensively co-localised (Fig. 8H). Quantification of co-localisation, using Pearson’s correlation coefficient, confirmed the increase in co-localisation of E-cadherin and β-catenin in morphant embryos compared to control embryos (P<0.05, Fig. 8S). Embryos co-injected with morpholino and plakoglobin-1a had a similar level of co-localisation to control embryos, indicating that co-injecting plakoglobin-1a with morpholino could rescue the morphant phenotype (Fig. 8I). However, co-injection of plakoglobin-1b failed to rescue the phenotype and co-localisation was similar in co-injected plakoglobin-1b and morpholino to morphant embryos (Fig. 8R). Therefore, in the absence of plakoglobin-1a, β-catenin increased its binding with E-cadherin in adherens junctions. We next examined the effect of loss of plakoglobin on desmosomal proteins. β-catenin is not normally expressed in desmosomes and there was little co-localisation of β-catenin and the desmosomal cadherins, desmocollin 2 and 3 in control injected embryos. In plakoglobin morphant embryos, there was no change in the expression pattern of β-catenin or desmocollins 2 and 3 (Supplementary Figs. 1H–J).

**Fig. 9.** Loss of plakoglobin results in fewer adhesion junctions and with an altered structure. (A and B) Electron microscopy of the intercalated discs of zebrafish embryo hearts at 72 hpf. (A) In control morpholino injected embryos, desmosomes (black arrowheads) were in series. (B) In morphant embryos, diffuse adhesion junctions (white arrowhead) were detected. (C) A table of indicating the cell–cell borders numbers, the number of adhesion junctions, and the number of each type of junction in control and morphant embryos. Unclassified indicates adhesion junctions that could not be definitively classed as adherens junction, desmosome or tight junction. N=2 embryos per sample. Scale bar is 100 nm.
Figs. 2A–F). There was no statistical difference in colocalisation of the proteins in control morpholin and plakoglobin morpholin injected embryos (Supplementary Fig. 2G). Controls for the desmocollin antibody are included in Supplementary Fig. 2. These results indicate that β-catenin compensates for the loss of plakoglobin in adherens junctions but not in desmosomes in plakoglobin morphant embryos.

**Loss of plakoglobin results in reduced and altered adhesion junctions**

We next examined the ultrastructure of adhesion junctions in the intercalated discs of control and morphant 72 hpf embryos by electron microscopy. In control embryos, adherens junctions and desmosomes, with their distinctive dense tripartite composition, could be easily distinguished and were often observed in a series at the cell borders (Fig. 9A). However in morphant embryos (Fig. 9B), there was a striking loss in the number and proportion of adherens junctions and desmosomes, despite similar numbers of cell–cell borders (p<0.05) (Fig. 9C). Adhesion junctions, when present, were more diffuse in structure than either the adherens junctions or desmosomes observed in control embryos and therefore were difficult to distinguish. These results indicate that the absence of plakoglobin results in a reduced number of adhesion junctions.

**Discussion**

In this study, we have shown that plakoglobin-1a has both structural and signalling roles in zebrafish embryo development. Loss of plakoglobin-1a resulted in cardiac oedema, abnormal cardiac development and abnormal cardiac valve formation. β-catenin compensated for the loss of plakoglobin in adherens junctions but not in desmosomes. Loss of plakoglobin resulted in an increase in Wnt signalling in the heart and throughout the embryo.

The absence of plakoglobin in zebrafish resulted in cardiac defects which are broadly similar to those found when desmocollin-2 was knocked down in zebrafish (Heuser et al., 2006), or when plakoglobin was knocked down in mice (Bierkamp et al., 1996; Ruiz et al., 1996). All of these manipulations recapitulate ARVC in humans. Due to the embryonic lethality of plakoglobin null mice, Kirchhof et al. (2006) studied the effects of reduced plakoglobin in heterozygous plakoglobin deficient mice; these mice also had enlarged right ventricles with arrhythmias. Mutation of plakoglobin in humans also results in arrhythmias and replacement of cardiac muscle tissue by adipose tissue (McKoy et al., 2000). In addition, a few plakoglobin knockout mice in some genetic backgrounds exhibited skin blisters which were not detected in the zebrafish morphant (Bierkamp et al., 1996, 1999). However, the primary cardiac phenotype was similar in mice and zebrafish.

We have identified a transcript variant of plakoglobin in zebrafish, plakoglobin-1b. Splice variants of the human plakoglobin have been identified in carcinoma cell lines (Franke et al., 1989; Ozawa et al., 1995). However, this second zebrafish transcript was identified in wild type embryos. The armadillo protein in Drosophila has an isoform which functions in differentiating neurons, which may indicate a specific tissue expression of plakoglobin-1b at a later stage in embryo development (Loureiro and Peifer, 1998). As the transcript lacks the plakoglobin antibody epitope, its protein expression could not be localised in the embryo. It does not function redundantly with plakoglobin-1a as co-injection of plakoglobin-1b mRNA with morpholino failed to rescue the morphant phenotype or change the localisation of β-catenin and E-cadherin at adherens junctions. This may be due to the structure of the plakoglobin-1b protein. The amino terminal and first armadillo (arm) repeat of the human plakoglobin protein comprises the α-catenin binding domain. The first three armadillo repeats contain a desmoglein binding domain, while arm repeats 4 and 5 mediate E-cadherin binding (Chitaev et al., 1996; Troyanovsky et al., 1996). Plakoglobin-1b has these domains but the lack of arm repeats 6–13 may affect the targeting of the complex to adherens junctions and desmosomes. The region of arm repeats 6–10 has been identified as the minimum required for axis duplication in Xenopus mutation experiments and so indicates that the domain is important in plakoglobin signalling (Rubenstein et al., 1997). The absence of this domain suggests that plakoglobin-1b does not play a signalling role in zebrafish. This protein also lacks desmosomal cadherin binding domains that modulate the interaction of desmosomal cadherins (Wahl et al., 1996).

The presence of a second immunoreactive band in western immunoblotting with the plakoglobin antibody raised the possibility of the presence of additional transcript variants of plakoglobin-1a as it was not of the predicted size for plakoglobin-1b. However, the band was identified as vitellogenin-1, and vitellogenin was found to contaminate zebrafish lysates in proteomic analysis of zebrafish proteins (Link et al., 2006; Tay et al., 2006). The presence of this abundant yolk protein may hinder the identification of plakoglobin isoforms.

Plakoglobin morphant embryos have defective cardiac valves with increased expression of bmp4 and notch1b. Hurlstone et al. (2003) proposed a role for Wnt/β-catenin signalling in cardiac valve development. Loss of adenomatous polyposis coli (APC), a component of the catenin destruction complex, in zebrafish caused an upregulation of Wnt/β-catenin signalling, resulting in an excess of endocardium. In our study, the loss of plakoglobin also resulted in a loss of restriction of bmp4 and notch1b, increased Wnt signalling and altered cardiac valve formation. However we did not observe the excessive endocardial cushion outgrowth reported by Hurlstone and colleagues. Instead we found that whereas the endothelial cells of the atrioventricular canal of control embryos were cuboidal in shape, plakoglobin morphants lacked these cuboidal cells. This is similar to the findings of Beis et al. (2005) following ectopic expression of the Notch intracellular domain in all endothelial cells. These authors also found that loss of APC increased expression of cuboidal endothelial cells in the ventricle of APC mutant. They argue that increased notch signalling maintains squamous endothelial cells and prevents transition to the cuboidal shape. An alternative possibility is that increased notch signalling in the plakoglobin morphants caused increased endothelial to mesenchymal transition, which is characterised by loss of junctions and altered shape of endothelial cells, both of which were seen in the plakoglobin morphants. This effect was observed by Timmerman et al. (2004) in mice with increased Notch signalling. However, recent studies suggest that in zebrafish endocardial cushions do not form, but leaflets are formed directly by invagination (Scherz et al., 2008). In the light of this report, it appears that in plakoglobin morphants endothelial cells fail to transition from a squamous to a cuboidal shape.

Despite having transcriptional activity through the T-cell specific family of transcription factors, plakoglobin does not appear to play a role in haematopoiesis. Expression of haematopoietic markers scl and gata 1 appeared normal in morphant embryos. Later defects in circulation may be as a result of secondary effects due to a non-functioning cardiovascular system. This agrees with the findings of Koch et al. (2008) who showed that the conditional knockout of β-catenin in plakoglobin knockout mice did not affect haematopoiesis.

Our findings indicate that the loss of plakoglobin results in an increase in β-catenin protein expression. Although this is not due to increased transcription, it may be due to increased translation or an increase in protein stability due to less degradation via the ubiquitin pathway. This is in contrast to the findings in plakoglobin knockout mice where there was no increase in β-catenin levels in keratinocytes (Bierkamp et al., 1999). These authors also found that β-catenin localised to the desmosomes in mice lacking plakoglobin, which we did not find in plakoglobin morphant zebrafish embryos. However, we found increased colocalisation of β-catenin with E-cadherin, suggesting β-catenin increases its role in adherens junctions to compensate for the loss of plakoglobin. Similarly, in the absence of β-catenin in mouse embryos, plakoglobin becomes more co-localised with E-cadherin (Huelsken et al., 2000). In β-catenin deleted cell lines,
plakoglobin can compensate for lack of β-catenin at adherens junctions (Fukunaga et al., 2005). Cardiac specific deletion of β-catenin in adult mouse cardiomyocytes resulted in increased plakoglobin expression at intercalated discs and did not affect any other adherens junctions proteins (Zhou et al., 2007). These results, together with the increased co-localisation of β-catenin and E-cadherin in plakoglobin morphants confirm that β-catenin and plakoglobin share a function in adhesion junctions. Therefore, β-catenin can fulfill some of roles of plakoglobin in the cell by compensating for the loss of plakoglobin in adherens junctions.

Morphant embryos showed a striking loss of desmosomes and adherens junctions compared to control embryos, with a concomitant increased expression of diffuse junctions, which were not seen in the controls. These likely represent altered adherens junctions and desmosomes. These results are in general agreement with findings when desmosomal proteins were ablated. In desmocollin-2 zebrafish morphants, desmosomes were also reduced in number and lacked the midline structure between the cell–cell membranes (Heuser et al., 2006). Ultrastructural studies in the plakoglobin knockout mouse also showed a reduced number of desmosomes with an altered structure and the absence of the cytoplasmic plaque (Bierkamp et al., 1999). Another study of the intercalated discs of plakoglobin knockout mouse showed the presence of extended adhesive structures that contained both desmosomal and adhesion junction components rather than distinctive desmosomes (Ruiz et al., 1996). These results indicate the loss of plakoglobin disrupts adhesion junction structure in morphant embryos.

The absence of plakoglobin results in an increase in Wnt target gene expression. Bozozok acts downstream of β-catenin and is required for organiser formation and development of axial mesoderm (Fekany et al., 1999; Leung et al., 2003). Here we show that, in the absence of plakoglobin, expression of these genes is increased. The absence of the characteristic phenotype associated with overexpression of the genes may be explained by the negative feedback loop such as for bozozok (Solnica-Krezel and Driever, 2001). Crosstalk between signalling pathways is a complex control mechanism for organ formation. The current study shows that an increase in Wnt signalling resulted in an increase in BMP and Notch signalling pathways. In mice, a mutation in APC resulted in increased Wnt signalling and also increased Notch signalling (van Es et al., 2005). Wnt signalling has been shown to increase BMP-2 signalling resulting in mesenchymal differentiation (Nakashima et al., 2005). Our study shows that inhibition of the increase in Wnt signalling in morphant embryos by co-injecting Dkk1 rescued the cardiac phenotype. Studies by Hurlstone et al. (2003) have shown that inhibition of endogenous Wnt/β-catenin signalling, by Dkk1 overexpression, results in an absence of endocardial cushion formation. Recent studies have uncovered the importance of Wnt signalling at particular time points in cardiac development. While previously considered an inhibitor of cardiac formation, Wnt signalling has been found to have an important pro-cardiac role (Lickert et al., 2002). Expression of Wnt signalling prior to gastrulation promoted cardiac differentiation, but was inhibitory to cardiac formation if expressed after gastrulation, indicating the critical temporal-specific expression of Wnt in cardiac formation (Naito et al., 2006; Ueno et al., 2007). It is likely that the continual upregulation of Wnt signalling observed in plakoglobin morphant embryos disrupted this important temporal-specific expression of Wnt and thereby altered cardiac formation.

Heuser et al. (2006) have previously used zebrafish with disrupted desmosomes as a model for ARVC. The failure of plakoglobin to localise to desmosomes when mutated and when other desmosomal components are mutated has been proposed to identify a common mechanism for ARVC (Tsatsopoulou et al., 2006). Garcia-Gras et al. (2006) have shown that a reduction in Wnt signalling can result in fibrous and lipid replacement of cardiac tissue similar to ARVC in cardiac restricted desmoplakin knockout mice. They have proposed a mechanism for ARVC in which mutated desmosomal genes result in the release of plakoglobin from desmosomes and translocation to the nucleus, suppressing Wnt signalling. Loss of Wnt signalling resulted in myoblasts changing to adipocytes in vitro (Ross et al., 2000). It is unclear how mutations in plakoglobin itself, which results in ARVC, could be incorporated into this model. We propose that the loss of both the structural and the signalling roles of plakoglobin results in ARVC, and that the signalling defects are due to increased Wnt signalling.

However, the use of zebrafish as a model system for the study of ARVC has a number of limitations. The use of morpholinos restricts examination of phenotype to early developmental stages and subsequent steps in disease progression, such as replacement of cardiac tissue with fatty tissue cannot be examined. Another limitation is the possible presence of a second copy of a gene due to genome duplication in zebrafish after separation of fish from the mammalian lineage. Recent studies have identified differences in the structure of adhesion junctions in cardiomyocytes of lower vertebrates compared to mammals. Fish cardiomyocytes contain discrete structures of adherens junction component and desmosomal components compared to the composite junctions detected in higher vertebrates (Pieperhoff and Franke, 2008). However, formation of these composite junctions occurs late in mammalian development, mainly post-natally, and at the stages examined in the current study separate adherens junctions and desmosomes are present in mammalian embryonic hearts similar to those in zebrafish (Pieperhoff and Franke, 2007). Thus, plakoglobin knockdown in zebrafish provides an excellent model to investigate the underlying molecular mechanisms of ARVC.

From this study, we can conclude that plakoglobin co-operates with β-catenin in adherens junctions but acts antagonistically with it in Wnt signalling. We have provided evidence for the possible mechanism of ARVC involving plakoglobin. We identified both signalling and structural roles for plakoglobin in zebrafish embryo development.

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Appendix A. Supplementary data


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