Wtip is required for proepicardial organ specification and cardiac left/right asymmetry in zebrafish

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Received January 1, 2016; Accepted June 2, 2016

DOI: 10.3892/mmr.2016.5550

Abstract. Wilm's tumor 1 interacting protein (Wtip) was identified as an interacting partner of Wilm's tumor protein (WT1) in a yeast two-hybrid screen. WT1 is expressed in the proepicardial organ (PE) of the heart, and mouse and zebrafish wtip knockout models appear to lack the PE. Wtip's role in the heart remains unexplored. In the present study, we demonstrate that wtip expression is identical in wt1a-, tfc21-, and tbx18-positive PE cells, and that Wtip protein localizes to the basal body of PE cells. We present the first genetic evidence that Wtip signaling in conjunction with WT1 is essential for PE specification in the zebrafish heart. By overexpressing wtip mRNA, we observed ectopic expression of PE markers in the cardiac and pharyngeal arch regions. Furthermore, wtip knockdown embryos showed perturbed cardiac looping and lacked the atrioventricular (AV) boundary. However, the chamber-specific markers amhc and vmhc were unaffected. Interestingly, knockdown of wtip disrupts early left-right (LR) asymmetry. Our studies uncover new roles for Wtip regulating PE cell specification and early LR asymmetry, and suggest that the PE may exert non-autonomous effects on heart looping and AV morphogenesis. The presence of cilia in the PE, and localization of Wtip in the basal body of ciliated cells, raises the possibility of cilia-mediated PE signaling in the embryonic heart.

Introduction

The heart is one of the first organs to form during development from the lateral plate mesoderm (1-3). The primitive heart tube consists of two layers: The endocardium and the myocardium (4). Progenitor cells in the lateral plate mesoderm migrate toward the midline, where they form the two-layered heart tube. Subsequent morphological changes are necessary to shape this primitive heart tube into the chambered heart organ and for the atrioventricular (AV) boundary to develop between chambers that provide directional flow. In addition to the endocardium and myocardium, a third cellular layer, the epicardium, develops from an extracardiac population of cells called the proepicardial organ (PE) (5-12).

The mesodermally-derived epicardium covers the myocardial layer and is known to provide signaling required for proper development of the heart (13-15). However, the molecular regulation of epicardial function is just beginning to be investigated. Wilm's tumor protein (wt1), vascular cell adhesion protein 1 and a4 integrin genes are essential for epicardium formation. Targeted mutagenesis of these genes in mice provided genetic evidence supporting a role in cardiac development (16-19). Additional evidence suggested that the PE contributes to the development of the coronary vessels, and is required for continued cardiac development and function (5-11,20,21).

Previous studies indicated that in zebrafish, similar to other vertebrates, the PE can be distinguished morphologically at 48 h post fertilization (hpf) as a group of cells located in close proximity to the ventral wall of the heart (22,23). The PE is characterized by the expression of wt1a, transcription factor 21 (tfc21), and T box 18 (tbx18) at 48 hpf (22,23). Specification of the PE requires a bone morphogenetic protein (BMP) ligand, which is not, as previously assumed, derived from the liver bud (23). Instead, cardiac-specific bmp4 signaling from the myocardium induces PE specification. Independent from Bmp4 signaling, tbx5 expression in the lateral mesoderm during the early somite stages is also required for PE specification (23). Previous studies in adult zebrafish hearts indicate that the epicardium forms a smooth surface covering the entire heart wall. Additionally, it was discovered that partial amputation of the adult heart elevated the expression of tbx18 and retinaldehyde dehydrogenase 2, which suggests that epicardial genes serve a critical role in the response to injury (24-26). The activated epicardial cells proliferate and undergo epithelial-to-mesenchymal transition, which requires platelet-derived growth factor (27) and β-catenin and retinoic
acid signaling (21). The developmentally activated epicardial cells quickly invade the proliferating myocardium at the site of injury and create a dense vascular network that is likely to encourage regeneration (24-27).

In addition to its role in response to heart injury, the epicardium has important regulatory functions in the normal development of the myocardium (17,18,28-31). Despite the central role of epicardial signaling during heart development and repair, the molecular signals driving specification of the PE remain poorly understood.

In chicks, the PE first forms in a bilaterally symmetrical fashion, and then develops asymmetrically. Disruption of genes required for specifying right-sidedness in the body, such as fibroblast growth factor 8 (fgf8) or snail homolog 1 (snail1), prevented PE specification (32). In mice, the dependence of PE specification on the left-right (LR) body axis is unclear. In this organism, both PE precursors develop equally on both sides, and later fuse at the midline to generate the PE (33). Establishment of the LR axis in the zebrafish heart depends on global LR cues that are generated by cilia during early gastrulation stages (34). Abnormal LR development can affect cardiac looping during vertebrate morphogenesis. Therefore, some cardiac defects that are associated with the abnormal positioning of the cardiac chambers or with vessel malformation are secondary to altered LR development (35). However, some patients demonstrate heterotaxy of the heart without other obvious LR defects, suggesting that ciliary function may also have an important role in cardiovascular morphogenesis itself (36-38). Slough et al (39) reported the presence of monocilia in several areas of the mouse embryonic heart, suggesting a role for cilia in cardiac morphogenesis. Furthermore, hearts in kinesin-3a (Kif3a) mutants developed abnormal endocardial cushions (ECCs) and thinner compact myocardium, and completely lacked cardiac cilia (39). Additionally, the compact myocardium was thinner in embryos mutant for polycystic kidney disease 2, a protein that functions as a mechanosensor in the kidney and node (39).

The WT1-interacting protein (Wtip) was originally identified as an interacting partner of WT-1 in a yeast two-hybrid screen (40). Recent studies and previous work by our group revealed the following roles for Wtip: i) Wtip interacts with the C-terminus of receptor tyrosine kinase-like orphan receptor 2 in yeast and mammalian cells (41); ii) Ajuba LIM proteins [Ajuba, LIM domain containing 1 (LIMD1), and Wtip] interact with Snail to remodel epithelial dynamics (42,43); iii) Wtip is a LIM domain protein of the Ajuba/Zyxin family, which is enriched in the basal bodies of cells in the pronephros and Kupffer's vesicle in zebrafish (44). In zebrafish, wtip deletion leads to pronephric cysts, hydrocephalus, body axis curvature and pericardial edema (44); iv) cryptic deletion in the human leads to pronephric cysts, hydrocephalus, body axis curvature Kupffer's vesicle in zebrafish (44). In zebrafish, a LIM domain protein of the Ajuba [Ajuba, LIM domain containing 1 (LIMD1), and Wtip] interact C-terminus of receptor tyrosine kinase-like orphan receptor 2 revealed the following roles for Wtip: i) Wtip interacts with the screen (40). Recent studies and previous work by our group encouraged regeneration (24-27).

### Materials and methods

**Zebrafish maintenance.** All animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center. *Danio rerio* (AB strain) were maintained and raised at 28.5˚C under a 14-h light/10-h dark cycle. Zebrafish embryos were kept in 0.5X E2 egg medium (7.5 mM NaCl, 0.25 mM KCl, 0.5 mM CaCl$_2$, 0.5 mM MgSO$_4$, 0.075 mM KH$_2$PO$_4$, 0.025 mM Na$_2$HPO$_4$, 0.35 mM NaHCO$_3$, 0.01% methylene blue). To suppress pigmentation of zebrafish embryos, 0.0045% 1-Phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO, USA) was added to egg medium as needed. Embryos and larvae were staged according to h post-fertilization (hpf) or days post-fertilization (dpf) (46).

**Morpholino and mRNA injections.** A translational blocking morpholino oligonucleotide (MO) targeted against the 5'UTR of wtip (wtipMO; 5'-GAT CCT CGT CGT ATT CAT CCA GTG ACC TGA AGG C-3'; 22), and randomized control MO (conMO; 5'-CCT TTT TTA TCC TCA ACC TAA GGT TCA AA-3') were obtained from Gene Tools, LLC (Philomath, OR, USA). A volume of 4.6 nl with a concentration of 0.225 mM wtipMO, 0.056 mM wtipMO, 0.25 mM wt1aMO, 0.0625 mM wt1aMO, and 92 pg wtip mRNA was injected at the one-cell stage using a nanoliter 2000 microinjector (World Precision Instruments, Inc., Sarasota, FL, USA). Zebrafish wtip mRNA was synthesized with T7 RNA polymerase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), after linearization of the pCR®-BluntII-TOPO®-wtip construct with HindIII (Thermo Fisher Scientific, Inc.). All mRNAs were purified using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Microinjections into 1-cell embryos were performed as described by Feng et al (47). To knockdown wtip specifically in dorsal fore-runner (DFCs), wtipMO was injected into the yolk cell of the ~1,000-cell stage embryos as previously described (48).

**In situ hybridization.** Whole-mount in situ hybridization was performed as previously described (49,50), using the following probes: wtip, wt1a, tcf21, tbx18, bmp4, LR determination factor 1 (lefty1), lefty2, southpaw (spaw), cardiac myosin light chain 2 (cm2c2), α-cardiac myosin heavy chain (amhc), ventricular MHC (vmhc) and natriuretic peptide A (nppa) (34,44,51-54). Polymerase chain reaction (PCR) DNA templates were used for wt1a, tcf21, bmp4, lefty1, lefty2, spaw, cm2c2, amhc, vmhc and nppa. DNA templates were used for wtip and tbx18. We used the following primers to prepare the PCR DNA template for reverse transcription (RT)-PCR and 2nd PCR for wt1a (765 bp): wtip-5IF1 5'-CGG GTG GAA ACG GTA ACT GTA-3'; wt1a-1161RI 5'-TCT GCA GTT GAA GGG CCT TTC-3'; wt1a-240F2 5'-GCA CTT CTC CGG ACA GTC AC-3'; and wt1a-1004R2T7 5'-GTT AAT...
ACG ACT CAT TAT AGG GAG AAC CTG CGA CCA CAG TCT-3'. For tfc21 (682 bp), we used the following primers:
tfc21-68F1 5'-TCA TCT CGA GCT GTA AGA-3';
tfc21-6851R1 5'-CAT GTT GCC TGG AAC CAG-3';
tfc21-6875F1 5'-TTC ACC CTC CAC CCT CTT TCT-3';
tfc21-6875R1 5'-GTT GAA GAA GAA AGC-3'; and
tfc21-8502R2T7 5'-GGT AAT ACG ACT CAC TAT AGG ATG CGA GTG AGG ATG TTG TCC TCT-3'. The primers used for leftf1 (875 bp) were: leftf1-8751F1 5'-AGG CGT TGC TGA AGA AAC TGG-3'; leftf1-1065R2 5'-AAT ATT GTC CAT TGC GCA GCC TTC-3'; leftf1-1553F2 5'-GAT CCC AAC GCA CGT AAA GAA-3'; and leftf1-1027R2T7 5'-GGT GAA GGG AAG-3'. Primers were generated for monoclonal antibody (MAB) 4848-1 (IgG1 [H+L]), 1:1,000 dilution; EMD Millipore, Billerica, MA, USA. The following secondary antibodies were obtained from Covance, Inc., Denver, PA, USA, anti-γ-tubulin (GTU-88; 1:800 dilution; Sigma-Aldrich), anti-acetylated α-tubulin (6-11B-1; 1:800 dilution; Sigma-Aldrich), and anti-centrin (20H5; 1:1,000 dilution; EMD Millipore, Billerica, MA, USA). The following secondary antibodies were obtained from Thermo Fisher Scientific, Inc.: Goat anti-rabbit Alexa Fluor®488 (IgG [H+L]), goat anti-mouse Alexa Fluor®488 (IgG2b), goat anti-mouse Alexa Fluor®488 (IgG1 [γ1]), goat anti-mouse Alexa Fluor®488 (IgG2a [γ2a]) and goat anti-mouse Alexa Fluor®546 (IgG1 [γ1]). Whole-mount immunohistochemistry samples were dehydrated with a graded series of methanol, embedded in JB4 resin (Polysciences, Inc., Warrington, PA, USA), and cut into 5-7 µm sections using an RN2255 microtome (Leica Technology, Exton, PA, USA). The sections were stained with DAPI (546 [IgG1 (α)], 1:100 dilution; Sigma-Aldrich), anti-acetylated α-tubulin (6-11B-1; 1:800 dilution; Sigma-Aldrich), and anti-centrin (20H5; 1:1,000 dilution; EMD Millipore, Billerica, MA, USA). The following secondary antibodies were obtained from Thermo Fisher Scientific, Inc.: Goat anti-rabbit Alexa Fluor®488 (IgG [H+L]), goat anti-mouse Alexa Fluor®488 (IgG2b), goat anti-mouse Alexa Fluor®488 (IgG1 [γ1]), goat anti-mouse Alexa Fluor®488 (IgG2a [γ2a]) and goat anti-mouse Alexa Fluor®546 (IgG1 [γ1]). Whole-mount immunohistochemistry samples were dehydrated with a graded series of methanol, embedded in JB4 resin (Polysciences, Inc., Warrington, PA, USA), and cut into 5-7 µm sections using an RN2255 microtome (Leica Technology, Exton, PA, USA). The sections were stained with DAPI (Kirkgeard & Perry Laboratories, Inc., Gaithersburg, MD, USA), mounted in Fluorescent Mounting Media (Kirkgeard & Perry Laboratories, Inc.), and were imaged with a FV-1000 confocal laser-scanning microscope (Olympus America, Inc., Center Valley, PA, USA).

Histological analysis. Embryos were fixed with histology fixative (1.5% glutaraldehyde, 4% formaldehyde, 3% sucrose in 0.1 M phosphate buffer (PB, pH 7.3)) overnight at 4°C. Fixed embryos were then dehydrated with a graded series of methanol and embedded in JB4 resin (Polysciences, Inc., Warrington, PA, USA). Sections (4 µm) were cut with an RN2255 microtome (Leica Technology) and stained with Harris hematoxylin and special eosin II (BBC Biochemical, Mount Vernon, WA, USA). Once the sections were mounted in Polymount (Polysciences, Inc.), the stained sections were imaged with a Provis AX-70 microscope (Olympus America, Inc.) equipped with a RETIGA EXi digital camera (QImaging, Surrey, Canada).

Results

Wtip expression in the cardiac region is identical to PE marker genes. WTI is expressed in the PE and glomerulus podocytes in
mammals, birds, zebrafish and medaka (22,57-61). In addition to wtla gene expression in zebrafish PE (22), the expression patterns of other PE markers, including tcf21 and tbx18, are well documented at 48 hpf, 57 hpf, and 4 days post-fertilization (dpf) (22,23). It was previously demonstrated that zebrafish embryos depleted for wtip via anti-sense morpholino (wtipMO) developed pericardial edema (44).

To investigate possible roles for Wtip in PE specification and development, whole-mount in situ hybridization was used (Fig. 1). Wtip mRNA was detected at 48 hpf (Fig. 1A) and 4 dpf (Fig. 1E) in zebrafish embryonic hearts. At 48 hpf, wtip-positive cells at the pericardial surface of the yolk were observed to be broadly dispersed at the AV-to-sinus venous region (Fig. 1A, yellow arrowhead marks the AV junction). By 4 dpf, wtip-positive cells appeared to have spread over the heart to cover the myocardium (Fig. 1E, yellow arrowhead). To determine the identity of the cells expressing wtip, the common PE marker genes wtla (Fig. 1B and F, yellow arrowheads), tcf21 (Fig. 1C and G, yellow arrowheads) and tbx18 (Fig. 1D and H, yellow arrowheads) were evaluated at 48 hpf (Fig. 1A-D) and 4 dpf (Fig. 1E-H). At 48 hpf, wtla, tcf21 and tbx18 expression was detected at the level of the sinus venous and adjacent to the AV junction (Fig. 1B-D, yellow arrowheads). At this stage, cardiac expression typically appears punctate, as would be expected for the isolated clumps of PE, which have not yet formed a uniform layer. In addition, tcf21 expression occurred in the pharyngeal arch (Fig. 1C, black arrows), and tbx18 expression, ectopic expression in the pectoral fin (Fig. 1D, asterisk). By 4 dpf, the wtla-, tcf21- and tbx18-positive cells spread over the heart to cover the myocardium (Fig. 1F-H, yellow arrowheads). Therefore, the wtip-positive cells exhibited an expression pattern similar to the known PE marker genes wtla, tcf21 and tbx18, and corresponded in physical appearance to extracardiac cell populations, indicating their PE identity (22,23).

To further assess a possible cell autonomous role for Wtip in the PE, the subcellular localization of this protein in the PE was examined using a zebrafish-specific anti-Wtip antibody (44). In a previous study, Wtip protein was observed to be enriched in the basal body, a structure that resides at the base of cilia in the pronephros, Kupffer’s vesicle (KV) and other ciliated tissues (44). While cilia have not been reported in the zebrafish embryonic heart, they have been discovered in mouse (39) and chick hearts (62). Accordingly, the possibility that Wtip may localize to the basal body at the base of cilia in the zebrafish embryonic heart was considered. Therefore, double immunostaining was performed with antibodies against zebrafish Wtip and the cilia marker acetylated α-tubulin (Fig. 1I and J), or the basal body marker γ-tubulin (Fig. 1K-M) and basal body marker centrin (Fig. 1N-P), in 48 hpf PE. Sagittal section staining suggested that Wtip was localized to the basal body of cilia located on PE in the 48 hpf embryos (Fig. 1I-P). The specificity of the localization pattern was verified by knockdown of endogenous wtip expression with wtipMO (44), which abolished Wtip immunostaining (Fig. 1J). Together, these data provide the first evidence, to the best of our knowledge, that the zebrafish embryonic heart develops cilia and basal bodies (Fig. 1I-P). In addition, these data indicate that Wtip is expressed in the basal bodies of cells in the PE of the zebrafish embryonic heart.

Wtip signaling is required for PE specification. wt1 null mutant mice develop cardiac abnormalities (16), and the epicardium fails to form properly (19). The wt1 gene is expressed in both the epicardial lineage and in kidney podocytes, and this pattern is well conserved in mammals, zebrafish and medaka (22,59,61,63). To test whether Wtip is required for PE formation and specification during zebrafish embryonic heart development, a wtip morpholino antisense oligonucleotide (MO) was used to inhibit translation of wtip transcripts (44).

First, the PE formation in 48 hpf wtip knockdown embryos was examined (Fig. 2) using the PE-specific markers tcf21 (Fig. 2B, C and G) and tbx18 (Fig. 2E, F and H). Injection of wtipMO produced embryos with body axis curvature, pronephric cysts, hydrocephalus and pericardial edema. Approximately 99% of injected embryos (n=64) developed pericardial edema. In wtip knockdown embryos, the expression of tcf21 and tbx18 in the heart was found to be significantly reduced (97/108 embryos had wtip expression in the PE, whereas all control embryos had wtip expression in the PE) (25/36, 69.4% for tcf21 and 14/27, 51.9% for tbx18; Fig. 2B, E, G and H, yellow arrowheads) or absent (11/36, 30.6% for tcf21 and 13/27, 48.1% for tbx18; Fig. 2C, F, G and H, yellow arrowheads). By contrast, despite modest developmental delay (2 h) in these embryos, tcf21 expression in the pharyngeal arch (Fig. 2B and C, black arrows) and tbx18 expression in the pectoral fin were unaffected (Fig. 2E and F, asterisk). These data indicate that Wtip signaling is essential for early epicardial development and is required to specify the PE.

To determine whether Wtip signaling is sufficient to induce PE marker gene expression, wtip mRNA was over-expressed (Fig. 3). In embryos with ubiquitous wtip mRNA expression, ectopic tcf21 (34/34, 100%; Fig. 3B, black arrows) and tbx18 (31/31, 100%) expression was observed in the heart region (Fig. 3D, black arrows) compared with control tcf21 (n=26; Fig. 3A) and tbx18 (n=26; Fig. 3C) expression in the PE region of the heart (yellow arrowheads indicate sinus venous). In all of the wtip mRNA-overexpressing embryos, ectopic clusters of the tcf21- and tbx18-positive cells were observed, which appeared more widespread and numerous than the clusters seen in wild-type embryos. Occasional ectopic clusters were located in or near the craniofacial area (Fig. 3B and D, black arrows), and were consistently observed in the cardiac region. Overexpression of wtip mRNA in embryos did not affect tbx18 expression in the pectoral fin (Fig. 3C and D, black asterisk).

Wtip and wtla functionally interact during PE development. Mouse wtip was originally identified as an interacting partner of wt1 in a yeast two-hybrid screen (40). However, the implications of the potential interaction between Wtip and WT1 in PE formation have not been reported. The co-expression of wtla (Fig. 1B and F, yellow arrowhead; 22) and wtip (Fig. 1A and E, yellow arrowhead) in PE control embryos, along with the similar cardiac phenotypes generated by knockdown of either gene, support the notion that these two genes may function together in zebrafish PE formation. In wt1a morphants (Fig. 4), the expression of PE markers was reduced (7/47, 14.9% for tcf21, Fig. 4M; and 3/33, 9.1% for tbx18, Fig. 4N) or absent (40/47, 85.1% for tcf21, Fig. 4G and M, black arrows; and 30/33, 90.9% for tbx18; Fig. 4M and N, black arrows). As described (Fig. 2B, C, E-H), in wtip morphants, PE markers were similarly reduced (21/30, 70% for tcf21, Fig. 4M; and 13/25, 52% for tbx18, Fig. 4N) or absent (9/30, 30% for tcf21, Fig. 4C and M, black...
To further investigate the potential for Wtip and WT1 interaction, a combined knockdown of wtip and wt1a was performed using sub-threshold doses of both morpholinos to assess whether a genetic interaction between these proteins would alter the expression of the PE markers tcf21 and tbx18. Prior to this, the appropriate sub-threshold doses for each morpholino was determined: i) wt1aMO, n=28 for tcf21 (Fig. 4I and M, yellow arrowhead) and n=27 for tbx18 (Fig. 4I and M, yellow arrowhead) and ii) wtipMO, n=36 for tcf21 (Fig. 4E and M, yellow arrowhead) and n=35 for tbx18 (Fig. 4F and M, yellow arrowhead). Sub-threshold doses for either morpholino alone had no effect on tcf21 (Fig. 4E, I and M, yellow arrowhead) or tbx18 (Fig. 4F, J and N, yellow arrowhead) expression in the PE: wt1aMO, 26/28, 92.9% for tcf21 (Fig. 4I and M, yellow arrowhead) and 25/27, 92.6% for tbx18 (Fig. 4I and N, yellow arrowhead); wtipMO, 34/36, 92.9% for tcf21 and 33/35, 94.3% for tbx18 (Fig. 4E, F, M and N, yellow arrowhead). However, in wtip and wt1a double morphants, tcf21 and tbx18 expression was severely reduced (3/27, 11.1% for tcf21, Fig. 4M; and 3/32, 9.4% for tbx18, Fig. 4N) or absent (24/32, 88.9% for tcf21, Fig. 4K and M, black arrow; and 29/32, 90.5% for tbx18, Fig. 4L and N, black arrow) in the cardiac region. These data indicate that Wtip is genetically associated with Wt1a, and that together they influence PE specification.

Wtip is required for cardiac looping and valve formation, but not for chamber patterning. Cardiac looping is a morphogenetic process in vertebrates. This process re-shapes the linear heart tube by bending the cardiac chambers into close juxtaposition. In zebrafish, cardiac looping begins around 30 hpf as the heart tube folds, gradually bringing the atrium...
and ventricle into a side-by-side position by 48 hpf (15,37). The continued bending of the heart tube, accompanied by chamber ballooning (~48-58 hpf) (64), and concentric growth within the chambers (65), substantially remodels the shape of the heart by 72 hpf. Functionally, the organ changes rapidly over this period, as heart rate and cardiac output increase steadily (3,66,67). Blood flow, which initially incorporates some back-flow through the A V junction, becomes consistently unidirectional as endocardial cushions and A V valves develop (68). Transgenic Tg(myl7:EGFP) embryos, which express GFP in the embryonic heart, provided easy visualization of the heart (Fig. 5A and B). As a simple rubric to describe the progression of cardiac looping in wtip morphants, the cardiac ‘looping angle’ was measured, defined as the degree of difference in the anterior/posterior (A/P) body axis and the plane of the AV junction (Fig. 5C) (55). At 48 hpf, control hearts exhibited an average looping angle of 16°, whereas looping angles in embryos injected with wtipMO were significantly larger, indicating that they were less looped (Fig. 5A-D). These data indicate that cardiac looping was impaired by Wtip depletion.

Next, it was examined whether the cardiac looping defect of wtip morphants was due to improper establishment of cardiac patterning. At 48 hpf, wtip morphants showed normal expression of the cardiac chamber-specific markers myl7 (cmlc2) (Fig. 5E-H), amhc (Fig. 5I-L) and vmhc (Fig. 5M-P). By 48 hpf, hearts in wild-type embryos have initiated cardiac looping, which shifts the ventricle to the right of the atrium (Fig. 5E, I and M). In the process of checking the heart patterning, a role for Wtip in differentiating the AV boundary
was identified. At 52 hpf, nppa was detected in both the
ventricle and atrium but was absent from the AV boundary
in wild-type embryos (Fig. 5Q, black arrows). Conversely,
nppa was detected throughout the heart, with no distinguish-
able exclusion from the AV boundary in wtip knockdown
embryos (Fig. 5R). Analysis of sections (Fig. 6) revealed a
thinned myocardial layer in the atrium, and increased sepa-
ratio of the myocardium and endocardium in the ventricle
(Fig. 6B and E). Rather than transitioning to the expected
cuboidal shape, AV endocardial cells in wtip knockdown
embryos retained a squamous appearance at 72 hpf, and
clear leaflets did not form (Fig. 6E). Importantly, the lack of
AV boundary, thinned myocardial layer in the atrium, and
increased separation of the myocardium and endocardium
in the ventricle phenotype of wtip morphants could all be
rescued by the co-injection of wtip mRNA, demonstrating
that the phenotypes specifically result from wtip knockdown
(Fig. 6C and F).

**Knockdown of wtip results LR asymmetry defects.** Ajuba
LIM proteins (LIMD1, WTIP, AJUBA) are categorized as
a family of LIM domain proteins, however, their functional
similarities remain unclear. Embryos depleted for the Ajuba
homolog in medaka (69) and zebrafish (70) exhibited develop-
mental abnormalities, including LR patterning defects. It
has been previously shown by *in situ* hybridization that wtip
is expressed in a broad range of tissues during the gastrula
period and early somitogenesis (44), including KV, which
is equivalent to the mammalian node in teleosts (71-73).
Furthermore, Wtip protein expression was investigated by
immunostaining using a zebrafish-specific Wtip antibody,
which localized to the basal bodies of pronephros, KV and
ciliated tissues (44). The wild-type heart typically 'jogs' to
the left and subsequently loops to the right (34). To inves-
tigate the establishment of the LR axis within the forming
zebrafish heart tube, bmp4 expression was examined (Fig. 7),
which is normally expressed predominantly on the left side
of the cardiac cone (118, 118, 100%; Fig. 7I and M) (34). It
was observed that wtip depletion caused embryos to exhibit
bilateral bmp4 expression (85/118, 72%; Fig. 7I and M). The
heart tube of wtip morphants never underwent looping as
occurred in control embryos at 48 hpf, which may reflect
altered left-right patterning of the body (Fig. 5A-D, G, K,
O and R). Next, it was checked whether Wtip regulates not
only bmp4, but also other early LR asymmetry markers in
zebrafish. The left1/2 and southpaw (spaw) genes encode
TGF-β superfamily proteins that are expressed in the left
lateral plate mesoderm in wild-type embryos. These early
laterality markers are essential for the establishment of LR
asymmetry in zebrafish (53). To investigate whether Wtip
regulates zebrafish LR patterning by modulating the forma-
tion and/or function of KV, wtipMO was injected into the
yolk at the early blastula stage (128-cell stage) to target the
precursors of KV (DFCs) and left1/2 (Fig. 7A-D, K) and spaw
(Fig. 7E-H, L) expression patterns were examined. Injection
of wtipMO led to randomization of the LR axis, as shown by
left1/2 (Fig. 7A-D, K) and spaw (Fig. 7E-H, L) gene expres-
sion in the lateral plate mesoderm at 20 hpf. All control
embryos displayed a left-sided left1/2 (118/118, 100%) and
spaw (180/180, 100%) expression (Fig. 7A, E, K and L). By
contrast, wtip morphants exhibited the full range of possible
expression patterns: Left-sided expression (left1/2, 111/159,
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69.8%; spaw, 120/185, 64.9%), bilateral expression (left1/2, 6/159, 3.8%; spaw, 37/185, 20%), right-sided expression (left1/2, 4/159, 2.5%; spaw, 14/185, 7.5%), and no expression (left1/2, 38/159, 23.9%; spaw, 14/185, 7.5%; Fig. 7A-H, K and L). These data suggest that Wtip signaling is required for early LR asymmetry.

Discussion

In humans, a wide range of cardiovascular defects is associated with kidney cystic disease, although the etiology of these abnormalities remains unclear. Similar to humans, pericardial edema in zebrafish is often observed in kidney cystic mutants.
or MO-mediated knockdown embryos (74,75). However, the specific roles of cilia and basal bodies in PE, heart morphogenesis and function during normal development, and congenital heart disease (CHD) remain unknown (37,76). Compared to myocardium and endocardium, relatively little is known about how PE develops and differentiates. The PE is an extra-cardiac source for several cardiac cell types, and increasing evidence suggests that the PE plays a role in heart development, repair of cardiac injury and ingrowth of coronary vessels (5-12). Despite the potentially central role of the epicardium in all of these

Figure 5. Wtip is required for AV boundary formation, but does not affect chamber patterning. Hearts of transgenic Tg(myl7:EGFP) embryos were visualized in 48 hpf (A) control or (B) wtip morphant embryos (1.035 pmol/embryo) to assess the extent of cardiac looping. (C) Diagram depicting the cardiac LA, defined as the degree of difference in the anterior/posterior body axis and the plane of the AV junction. (D) Wtip depletion decreases the mean LA by 48 hpf. The mean ± standard error is shown. *P<0.000359. n=10 embryos/treatment. 48 hpf whole-mount in situ hybridization in (E,F,I,J,M,N,Q) control and (G,H,K,L,O,P) wtip morphant embryos (1.035 pmol/embryo) of (E-H) myl7, (I-L) amhc, (M-P) vmhc and (Q,R) nppa. (E,G,I,K,M,O,Q,R) Ventral view and (F,H,J,L,N,P) lateral view. Wtip does not regulate chamber patterning, but regulates nppa expression to maintain chamber myocardium expression and exclusion from the myocardium of the AV boundary. Wtip, Wilm's tumor 1 interacting protein; AV, atrioventricular; myl7, myosin light chain 7; EGFP, enhanced green fluorescent protein; hpf, hours post fertilization; LA, looping angle; amhc, α myosin heavy chain; vmhc, ventricular myosin heavy chain; nppa, natriuretic peptide A; conMO, control morpholino oligonucleotide; A, anterior; P, posterios; avj, AV junction.
processes, the molecular signals driving the specification and morphogenesis of PE remain poorly understood. In the present study, the role of Wtip in PE development was investigated and genetic evidence presented that WT1, in association with Wtip, is required for PE specification. We further revealed a critical role for Wtip in heart looping and the establishment of the AV boundary. Based on the expression pattern of \textit{wtip} mRNA and protein, blocked Wtip function, and \textit{wtip} over-expression data, it is proposed that Wtip is required at early somite stages to provide LR asymmetry, and independently regulates later cardiac development, including heart-looping, PE specification and differentiation of the AV boundary.

Wtip was originally identified as an interacting partner of WT1 in a yeast two-hybrid screen (40). WT1 is expressed in the PE and glomerulus podocytes in mammals, birds, zebrafish and medaka (22,57-61). It was previously reported that depletion of the Wtip basal body was associated with the development of pronephric cysts, with it noted that these embryos also developed pericardial edema (44). Previously, cryptic deletion in the human \textit{wtip} gene was reported to cause hypospadias (urethra malformation in males) that can be associated with congenital heart disease (45). Therefore, we predicted that pericardial edema in \textit{wtip} knockdown embryos may be due to PE defects.

In the present study, a critical role of Wtip in PE specification during heart development was identified. It was demonstrated that \textit{wtip} shows cardiac expression in PE at 48 hpf and 4 dpf, in a pattern that specifically matches that of known PE-specific genes \textit{wt1a}, \textit{tcf21}, and \textit{tbx18} (22,23). Additionally, it was confirmed that the PE contains ciliated cells, and that Wtip localizes to the basal bodies within these cells in 48 hpf embryos, as was the case for ciliated cells in the pronephros and Kupffer's vesicle (44). This is the first evidence that the zebrafish embryonic heart develops cilia and basal bodies during embryonic development. Furthermore, it was observed that depletion of \textit{wtip} leads to the severe reduction or absence of PE formation and that Wtip and \textit{wt1a} cooperate for PE formation. By overexpressing \textit{wtip} mRNA, ectopic expression of PE-specific markers was observed in the cardiac region and in the pharyngeal arch area. Based on \textit{wtip} gene and protein expression patterns and on phenotypes generated by blocking Wtip function, it is proposed that Wtip is required initially to establish early LR asymmetry, and later to form the PE, modulate heart looping and promote AV differentiation.

Recently, the PE has been investigated for its role in normal heart morphogenesis (13,15,77) and additional capacity to form coronary vascular endothelial cells in mice (20). Zebrafish are now well established as a key model system for embryonic heart development and function, and possess a natural capacity for adult myocardial regeneration (78). To directly examine the possible critical roles for Wtip or the PE in response to injury and myocardial regeneration in adults, we would need to further consider using \textit{wt1a}, \textit{tcf21} or \textit{tbx18} transgenic lines (24-26).

A previous study reported that cells with primary 9+0 cilia were found in both the embryonic and the adult human heart (79). Previous studies revealed that monocilia are found in the mouse embryonic heart at embryonic day (e) e9.5-e12.5, by which time blood flow is present (39,76). The data suggest that in zebrafish embryos, cilia occur in the PE by 48 hpf, a similar stage to what has been observed in the chick embryonic heart (62). Previous reports suggest
that mice lacking cardiac cilia developed abnormal ECCs and compact myocardium (CM) at e9.5 (39,76). Notably, our observations in zebrafish suggest that wtip knockdown embryos lack a differentiated AV boundary, which is similar to the findings in mice, which lack ECCs (39,76). In addition, Wtip and cilia markers are co-expressed in the endocardium and myocardium prior to when the PE is formed in the zebrafish heart (data not shown), which may provide a connection to the AV valves that are simultaneously developing from the endocardium and myocardium. Previous studies have reported the timing and distribution of cilia in the embryonic mouse heart (39,76). At e9.5, cilia on the endocardial cells are predominantly located on the luminal surface, while cilia in the mesenchymal cells of the developing cushions are randomly oriented. By e12.5, atrial and ventricular septation is underway; only few cilia are found on the atrial endocardial layer. In the ECCs, however, cilia are found on the endothelial surface and are visible in the mesenchymal cells. The present study is the first, to the best of our knowledge, to report cilia on PE cells. Cilia in the endothelium may sense the flow during embryonic heart development. PE cells may be sensing information about their environment that relates to pericardial fluid or heartbeats, or potentially PE cilia may be involved in sensing biochemical information, such as the movement of fluid.

In mice, cilia are required for cardiac development, independently from their function in the development of LR asymmetry (39,76). Thus, it is possible that cardiac cilia function as mechanosensors, integrating information regarding flow, cardiac function and morphogenesis. If so, the prediction is that mutations in gene products that localize to cilia and/or basal bodies may affect heart morphogenesis and
function. In zebrafish, many cystic mutant phenotypes accompany LR asymmetry defects originating in early somitogenesis stages, visible as altered spaw and left1/2 gene expression in the lateral plate mesoderm and by altered bmp4 expression in the zebrafish heart.

In control embryos, the progression of cardiac looping is a morphogenetic process. The cardiac looping angle is defined as the degree of difference in the anterior/posterior (A/P) body axis and the plane of the AV boundary (55). The present study was unable to detect alterations in the expression of early patterning and chamber identity genes, suggesting that additional causes must be responsible for the malformation in the heart. Of note, chamber-specific markers amhc and vmhc were not altered, however, nppa was detected throughout the heart, with no distinguishable exclusion from the AV boundary in wtip knockdown embryos. Cardiac morphogenesis was significantly morphologically and molecularly affected at 48 and 72 hpf in wtip knockdown embryos, and gene restriction at the AV boundary was lost.

A number of the cardiac morphology mutants described in the original zebrafish large-scale mutagenesis screens have been well characterized, and the mutated genes have been identified. However, whether any of these mutants exhibit defects in cardiac cilia formation or function remains unknown, since the presence of cilia in the heart was not formerly described. Cardiac cilia are beginning to be characterized in other animal model systems, including chickens (62) and mice (39).

As it was unknown whether basal bodies could affect heart morphogenesis and function, it was unclear whether heart defects could also be associated with early embryonic defects, such as LR patterning, which are thought to involve ciliary function.

The zebrafish system offers unique advantages for studying cell biology during vertebrate organogenesis, as zebrafish embryos develop externally and are practically transparent throughout early development, thereby allowing non-invasive observation. Previous studies in zebrafish indicate that endocardial cells in the AV differentiate before the onset of epithelial-to-mesenchymal transformation, thereby defining a previously unappreciated step during AV valve formation (80,81). New zebrafish mutants exhibiting AV valve development will provide a unique set of tools with which to further understand the genetic basis of PE cell behavior and its effect on heart development (80,81).

Importantly, AV-associated phenotypes have been observed in mutant mouse models (81). The combination of genetics and pharmacological studies in animal models and the cell biology should provide novel insights into the developmental biology of cardiac organogenesis and provide relevant information to predict and understand human valvular and septal malformations.

The current study provides the first genetic evidence, to the best of our knowledge, for a novel role for the Wtip basal body protein in regulating the PE cell fate during development. In addition, our tools for wtip knockdown will be valuable for future studies to determine how Wtip signaling, possibly mediated via cilia, could indirectly modulate cardiac looping or AV development. The present study may additionally provide an avenue for understanding how the PE stimulates a response to cardiac injury, leading to its eventual repair.

Acknowledgements

The authors would like to thank all members of the Obara laboratory for the helpful discussion, and Kathy Kyler and Hiroyuki Matsumoto for critical reading of the manuscript. The authors would like to acknowledge the Zebrafish Information Resource Center for providing fish. Professor Tomoko Obara acknowledges financial support from the University of Oklahoma Health Sciences Center (OUHSC). Professor Tomoko Obara was supported by NIH grants R21-DK069604 and R01-DK078209, and the Oklahoma Center for the Advancement of Science and Technology (OCAST) grant HR14-082. The present study is supported in part by the Diabetes Histology and Image Acquisition and Analysis Core Facility at OUHSC (NIH: COBRE-IP20RR024215).

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