Chamber-specific Patterns of Epicardium Formation in Zebrafish

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CHAMBER-SPECIFIC PATTERNS OF EPICARDIUM FORMATION IN ZEBRAFISH

By

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The City University of New York
Abstract

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Adviser: Professor Nathalia G. Holtzman

The outer cardiac layer, the epicardium, coordinates the final steps of vertebrate heart development. This cardiac tissue arises from cells in the proepicardial organ (PEO) that forms around the base of the inflow tract. Its general location is conserved across species despite morphological differences. Cellular mechanisms of migration differ across species. Three strategies of PEO migration are described: 1) The floating cyst model - PEO cells released into the pericardial cavity are directed by fluid movements to migrate onto the myocardium; 2) Villi transfer - cardiac contractions may mediate multicellular PEO villi contact to the myocardium; and 3) Tissue bridge-mediated transfer - PEO cells migrate along a bridge to contact the myocardium. All currently described mechanisms suggest the same strategies for coverage of both cardiac chambers. Using zebrafish, we demonstrate distinct mechanisms of atrial and ventricular PEO migration. We introduce a novel concept of chamber-specific epicardium formation. This concept opens new avenues to investigate chamber-specific epicardium regulation and epicardial-derived cell fate.

During epicardium development, spatio-temporal differences in mRNA and transgenic reporter expression of conserved PEO genes \textit{wilms’ tumor 1} (\textit{wt1}) and \textit{transcription factor 21} (\textit{tcf21}) in zebrafish suggest two distinct PEO populations: atrial-specific (A PEO) or ventricle-specific (V
Transgenic PEO reporter \textit{wt1:GFP} is expressed widely in PEO cells onwards from 30 hours post fertilization (hpf) although some PEO cells decrease expression after adhesion to the myocardium. Transgenic PEO reporter \textit{tcf21:DsRed} is expressed in a subset of PEO cells around the inflow tract and in V PEO cells. \textit{tcf21:DsRed} begins expression in atrial epicardium at about 96 hpf. Complete embryonic epicardium is formed by 6 days post fertilization (dpf). In addition, we discovered PEO villi in close proximity to the ventricular myocardium surface. To demonstrate the cardiac contractility requirement for villi-mediated PEO migration, cardiac contractions were inhibited using two chemicals and one genetic mechanism and assayed for migrated cells. Cardiac contractions are required for ventricular epicardium formation. Surprisingly, we found atrial epicardium in embryos with inhibited cardiac contractions.

Our results indicate chamber-specific PEO cell migration mechanisms: Atrium-specific migration is independent of cardiac contractions as cells migrate directly on the atrial myocardium surface. Ventricle-specific migration is dependent on cardiac contractions. Our identification of chamber-specific PEO subpopulations and chamber-specific migration mechanisms establish a new chamber-specific perspective to investigate regulation of epicardium formation and regulation of epicardial-derived cells. Supplementary files include movies 1 and 2. Movie 1 exhibits a proepicardial villous moving along with the contracting ventricle. Movie 2 shows a floating group of proepicardial cells that cycles around the pericardial cavity during cardiac contractions.
Acknowledgments

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Chapter 1 – Introduction to proepicardium origin, migration and epicardium

Zebrafish is an ideal model organism to study heart development and its usefulness has been recently been reviewed1–3. Several features of zebrafish make them useful for study. Adults breed readily and their eggs are fertilized externally, therefore, ample embryos for observation and experimentation are easily collected. Optical clarity allows for live observation in the whole embryo during development2. In addition, more genetic and molecular tools have become available that allow examination of cells and tissues in vivo2. Zebrafish are vertebrate organisms that develop more rapidly than chick or mouse2. The small adult size allows for easy maintenance of many fish stocks. Several features of zebrafish particularly suit the study of the proepicardium: zebrafish can survive without cardiac contractions up to 7 days post fertilization (dpf). Oxygen-exchange by diffusion is sufficient for embryonic survival due to the small body size2. Hearts of zebrafish consist of two cardiac chambers, one ventricle and one atrium. This simple heart plan can help to elucidate cellular and molecular mechanisms that may be difficult to detect in other organisms like mouse or chick, in which multiple cardiac chambers may add a layer of complexity.

The outermost layer of the heart, the epicardium, contributes cells and signals to coordinate maturation of the underlying cardiac layers4–9. Epicardial cells produce retinoic acid and erythropoietin signals that promote cardiomyocyte proliferation. Additionally, epicardial cells can delaminate from the epicardium and invade into the myocardium to support maturation of coronary vasculature. Signals from the epicardium and from the myocardium coordinate coronary vascular development.
The subpopulation of epicardial cells that delaminate from the epicardium undergo epithelial-to-mesenchymal-transition (EMT) generating an epicardium-derived mesenchymal cell (EPDC) population\textsuperscript{10-13}. Epicardial EMT is regulated by several transcription factors. wilms’ tumor suppressor-1 (\textit{WT1}), transcription factor 21(epicardin/pod1/capsulin) (\textit{TCF21}), and T-box factor 18 (\textit{TBX18}) are expressed in the epicardium. \textit{WT1} regulates epicardial cell adhesions through transcriptional regulation of cell adhesion factors integrin alpha4 (\textit{ITGA4}) and E-cadherin\textsuperscript{14}. \textit{TCF21} transcriptionaly regulates \textit{ITGA4} and promotes EPDC differentiation\textsuperscript{15}. \textit{TBX18} is required for proper epicardium formation and regulation of signaling including Hedgehog, Wnt, VEGF, Angiopoetins, and TGF-\beta that in turn regulate coronary vascular development\textsuperscript{16}. Delamination from the epicardium and invasion of EPDCs into the subepicardial space to support coronary vessel maturation is regulated by myocardin-related transcription factors (MRTF) and serum response factor (SRF) in the epicardium\textsuperscript{17}.

EPDCs are reported to function as “cardiac stem cells”\textsuperscript{4} giving rise to coronary smooth muscle cells, pericytes, fibroblasts and cardiomyocytes\textsuperscript{10,11,18}. EPDCs are also involved in myocardial compaction and purkinje fiber development\textsuperscript{19}. Some EPDCs give rise to coronary endothelial cells (ECs). A subpopulation of epicardium in mouse expressing epicardial markers \textit{Semaphorin 3D (Sema3D)} and \textit{Scleraxis (Scx)} give rise to coronary endothelial cells\textsuperscript{20}. Endothelial precursors in the subepicardial space migrate in the same direction as epicardial growth until they coalesce into a primitive vascular plexus. This structure is remodeled and patterned to form a network. Vessel growth proceeds towards the base of the heart, and subsequently connects with the aorta. With perfusion, arteriogenesis begins through vascular wall matrix enrichment following with recruitment of smooth muscle cells and pericytes. As myocardial thickness increases, coronary vasculature expands and undergoes remodeling and
branching. Initial endothelial cell differentiation and delay of smooth muscle cell differentiation are likely regulated by epicardial retinoic acid signaling and myocardial VEGF signaling\textsuperscript{21}.

The epicardium and the myocardium contribute to the microenvironment of the subepicardial space. Epicardial-myocardial signaling during coronary vessel development has been extensively reviewed\textsuperscript{22,23}. A complex network of fibroblast growth factor (FGF) signaling between the epicardium and myocardium interconnects with Hedgehog signaling that promotes endothelial cell differentiation, retinoic acid signaling that promotes epicardial EMT and fibroblast proliferation, and notch signaling that regulates cell migration and differentiation that supports coronary vessel development\textsuperscript{22}. The contribution of proper epicardium development to the cell biology of cardiac maturation highlights its importance in fate specification and heart maturation. An understanding of embryonic epicardium development will be a helpful tool to begin to understand the regeneration potential of the adult epicardium.

Cardiac regeneration recapitulates embryonic developmental programs. Adult epicardium expresses embryonic epicardial genes and activates signals that coordinate embryonic cardiac maturation. Embryonic epicardial markers are upregulated in the adult epicardium after injury. \textit{Wt1} and \textit{Tbx18} are expressed in atrial and ventricular epicardium in the regenerating mouse heart\textsuperscript{24,25}. In zebrafish, after resection of the ventricular apex, new cardiomyocytes originate from progenitor cells that express epicardial markers \textit{raldh2} (also a component of retinoic acid signaling) and \textit{tbx18}\textsuperscript{40}. Epicardial cells close to the resection site invade the regenerating tissue and contribute smooth muscle cells for new blood vessels in a similar fashion as epithelial-to-mesenchymal transition and vasculogenesis during heart development\textsuperscript{40}. Recent studies of epicardium ablation in zebrafish indicate that Hedgehog signaling can regulate epicardial
regeneration\textsuperscript{26}. Based upon a small chemical screen FGF and BMP signaling may also play a role in epicardial regeneration\textsuperscript{26}.

Components of FGF, RA and notch signaling are also expressed in the epicardium of regenerating hearts. In regenerating zebrafish hearts, \textit{fgfr2}\textsuperscript{+} and \textit{fgfr4}\textsuperscript{+} epicardial cells migrate toward \textit{fgf17b}\textsuperscript{+} cardiomyocytes\textsuperscript{27}. Fgf inhibition via a heat shock inducible dominant-negative Fgfr1 receptor inhibits blood vessel regeneration\textsuperscript{28}. \textit{raldh2} is upregulated in ventricular epicardium after injury in both mouse and zebrafish\textsuperscript{25,29}. Components of notch signaling are also upregulated in epicardium after injury in zebrafish\textsuperscript{29}. Inhibition of notch signaling via a dominant negative inhibitor inhibits regenerative cardiomyocyte proliferation, despite upregulation of epicardial genes and appropriate epicardial EMT and EPDC differentiation\textsuperscript{29}.

A secreted peptide, Thymosin \(\beta\)4 (T\(\beta\)4) stimulates coronary vasculature and cardiac tissue growth both \textit{in vitro} and \textit{in vivo} . Treatment of murine adult epicardial explants with Thymosin \(\beta\)4 (T\(\beta\)4) can stimulate epicardial migration and differentiation into fibroblasts, smooth muscles and endothelial cells\textsuperscript{30}. T\(\beta\)4 is essential for embryonic coronary vasculogenesis, angiogenesis, and arteriogenesis. The adult murine epicardium has angiogenic and arteriogenic potential\textsuperscript{31}. In \textit{vivo} mice with myocardial infarctions injected intraperitoneally with T\(\beta\)4 activate adult epicardium and vessel growth. \textit{Tbx18} expression increases in the epicardium and the myocardium while \textit{Wt1} expression localizes to the subepicardial space. These patterns of expression recapitulate the embryonic epicardial program after cardiac injury\textsuperscript{32}. Collectively, these studies demonstrate that the epicardium is a major player in regenerating damaged cardiac tissue and blood vessels.

The epicardium develops from a population of cells known as the proepicardial organ (PEO)\textsuperscript{33}. Origins of the PEO may lie in the Lateral Plate Mesoderm (LPM), a source of cardiac
precursor cells in the developing zebrafish embryo. LPM patterning affects myocardial development and proepicardial cell specification. Disruption of migration in the LPM, genes expression in the LPM, or signaling to the LPM can disrupt cardiac structure and PEO specification. Bifid hearts arising from knockdown of casanova (casMO) or miles apart (milMO) that disrupt migration of cells in the LPM each develop their own PEO. PEO development associated with each heart indicates that the origins of the PEO lie in the LPM. Disruption of genes expressed in LPM, hand2 and tbx5a in zebrafish diminishes PEO specification. Thereby genetic patterning of the LPM affects PEO specification. Fgf8 signaling cascade in developing chick regulates Snai1 and Pitx2 in LPM. Downstream of Snai1 is Bmp4. Bmp4 in chick regulates PEO specification and proliferation. Similarly in zebrafish, Bmp signaling specifies PEO and promotes PEO proliferation. This body of evidence suggests that origin of the PEO may reside in the LPM.

The location of the PEO varies by cardiac morphology and anatomical differences across species: PEO grows out from septum transversum (ST) (mouse, rat, treeshrew, and Xenopus), pericardial epithelium (chick), pronephric glomerulus (lamprey), and between the atrium and the yolk (zebrafish). In general, the PEO is located near the sinus venosus (SV) region. Early observations of PEO describe mesothelial cell clusters or projections that grow towards the myocardium. Zebrafish has unique cardiac morphology that physically separates PEO near the SV region from PEO near the atrioventricular junction. In other vertebrate organisms such as chick and mouse, these two regions are very close together. This physical separation in zebrafish allows us to evaluate cell migration and genetic expression for differences between the two proepicardial cell populations. Proepicardium proliferates and
migrates to the myocardium at stages approximately or shortly after cardiac looping across species (Table 1).

<table>
<thead>
<tr>
<th>Heart development event</th>
<th>Mouse/Rat</th>
<th>Chick/Quail</th>
<th>Xenopus</th>
<th>Zebrafish</th>
<th>Sturgeon</th>
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<tbody>
<tr>
<td>Cardiac looping</td>
<td>E 8.5&lt;sup&gt;47&lt;/sup&gt;</td>
<td>HH 16-18&lt;sup&gt;48,49&lt;/sup&gt;</td>
<td>Stage 35&lt;sup&gt;50,51&lt;/sup&gt;</td>
<td>36&lt;sup&gt;52&lt;/sup&gt; – 48 hpf&lt;sup&gt;53&lt;/sup&gt;</td>
<td>8 dph&lt;sup&gt;54&lt;/sup&gt;, (Singleman, unpublished)</td>
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<tr>
<td>PEO-epicardium development</td>
<td>E 9.5&lt;sup&gt;55&lt;/sup&gt; - 14.5&lt;sup&gt;59&lt;/sup&gt;</td>
<td>HH 14 - 17&lt;sup&gt;56&lt;/sup&gt;</td>
<td>Stage 39 - 46&lt;sup&gt;15&lt;/sup&gt;</td>
<td>40 – 120 hpf&lt;sup&gt;35,36,57,58&lt;/sup&gt;</td>
<td>4 – 25 dph&lt;sup&gt;59&lt;/sup&gt;</td>
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Table 1: Development of epicardium with context of cardiac looping across species.
E=embryonic day, HH=Hamburger-Hamilton stages, hpf=hours post fertilization, dph=days post hatch

Molecular regulation of proepicardium and epicardium has been under study for the last few decades. Various transcription factors function in the epicardium across species<sup>60</sup>. The most conserved genes expressed in PEO are transcription factors wilms’ tumor suppressor-1 (WT1) and transcription factor 21(epicardin/pod1/capsulin) (TCF21). WT1-null mice and wt1MO in zebrafish disrupt epicardium formation<sup>35,61</sup>. WT1 regulates PEO formation in embryoid bodies<sup>62</sup>. WT1 expression plasmid activates human integrin alpha4 gene (ITGA4) gene reporter construct in Human embryonic kidney (HEK) 293 cells; levels of ITGA4 protein in homozygous WT1-null mouse epicardium is significantly reduced<sup>14</sup>. WT1 has also been shown to control retinoic acid signaling from the epicardium to the myocardium<sup>63</sup>. In vitro, WT1<sup>+</sup> cells generate epithelial sheets<sup>64</sup>. Therefore wt1 promotes formation the PEO and the epicardium<sup>35,62</sup>, and regulates cell adhesion required for the epicardium<sup>14,35,64</sup>, and regulates retinoic acid signaling from the epicardium to the myocardium that supports myocardium maturation<sup>63</sup>.  


Another conserved PEO gene *TCF21*, a basic helix-loop-helix transcription factor, has roles in cell migration, differentiation, proliferation and invasion behavior throughout embryonic development. *Tcf21* genetically interacts with *Tbx1* in pharyngeal mesoderm regulatory network and cardiac morphogenesis.\textsuperscript{65} *tcf21*MO in *Xenopus* and *Tcf21*-null mouse epicardium disrupt epicardial adhesion to the myocardial surface and myocardium maturation.\textsuperscript{15,66} These results suggest that *tcf21* may control epicardial adhesion to the myocardium. Additionally *Tcf21* regulates epithelial-to-mesenchymal transition (EMT) to smooth muscle (SM) and fibroblast lineages.\textsuperscript{66} In zebrafish, *tcf21* function in pharyngeal mesoderm has been studied in relation to craniofacial myogenesis and pharyngeal arch artery development.\textsuperscript{67,68} *tcf21* depletion by morpholino resulted in poor development of craniofacial muscles. *tcf21*-null zebrafish demonstrated that *tcf21* in pharyngeal mesoderm was dispensable for blood vessel development.\textsuperscript{68} *tcf21* function in proepicardial migration and epicardial adhesion to the myocardium in zebrafish was previously unknown. Work presented in this thesis will demonstrate the role of *tcf21* during epicardial development in zebrafish.

**PEO asymmetry**

The proepicardium can be asymmetrically patterned prior to epicardium formation. In a review of PEO asymmetry across species, Schlüeter and Brand described that in most studied organisms, PEO begins as a bilateral and symmetrical structure, then proliferates in a right-sided manner to contact the myocardium.\textsuperscript{69} Schlüeter and Brand, also noted that Zebrafish PEO asymmetry was previously unknown. In the following work, we explore the left/right asymmetry in zebrafish. In a literature review of right-sided PEO asymmetry, right-sided PEO contacts the myocardium at the ventricle. These findings are summarized in the table below.
Organism | Bilateral | Asymmetry
--- | --- | ---
Lamprey | Pronephric glomerulus extension\(^{46}\) | Right-sided ventricular contact\(^{46}\)
Sturgeon | At sinus venosus\(^{59}\) | PEO on right-side of SV is more robust than on left side of SV PEO\(^{59}\)
Zebrafish | - | Right-sided PEO clusters\(^{57+}\)
Xenopus | - | Right-sided SV PEO and tissue bridge formation\(^{44}\)
Chick | Bilateral at SV, but initially right-sided, left-side develops, then diminishes\(^{70}\) | Right-sided PEO proliferation\(^{69–71}\)
Quail | - | Right sided-PEO proliferation\(^{48*}\)
Rat | Bilateral extension of PEO\(^{48}\) | Right-sided PEO contacts the ventricle\(^{48}\)
Mouse | Initially bilateral PEO\(^{72}\) | No asymmetry – fuses along midline\(^{72}\)
Dogfish | PEO extends bilaterally\(^{46}\) | Only contacts the ventricle on the right-side\(^{46}\)

Table 2: Bilateral PEO symmetry and right-sided asymmetry across species.

\(^{+}\)Images from Peralta et al. show PEO clusters on the right-side of the SV, however proepicardial asymmetry is not clearly addressed. \(^{*}\)Nesbitt et al. describe that quail PEO is left-sided however, the image is a dorsal view of the quail embryo and therefore the PEO is right-sided. Comparison to quail heart anatomy in a recent article\(^ {73}\) supports this orientation. Pombal et al. also note right-sided contact of proepicardium to the myocardium in quail.

Molecular mechanisms underlying PEO asymmetry in chick may elucidate key factors underlying PEO asymmetry across some species\(^ {37,70–72}\). PEO asymmetry in chick develops through proliferation of the right-sided proepicardium and apoptosis of the left-sided proepicardium\(^ {72}\). An FGF signaling cascade regulates proliferation of right-sided proepicardium...
in chick. Proepicardial cells express bmp receptors at HH16. Fgf8 in the endoderm\textsuperscript{37–39} initiates a signaling cascade\textsuperscript{74} that is detected by bmp receptors on the surface of proepicardial cells\textsuperscript{40}. In mouse, FGF8 is a left-side determinant\textsuperscript{75}. FGF-BMP signaling may be a conserved mechanism of right-sided proepicardium proliferation in organisms with right-sided PEO asymmetry. In zebrafish, heat shock induced dominant negative bmp receptors at 36 hpf and bmp4 mutants score for reduced PEO tbx18 and tcf21 expression at 57 hpf. BMP signaling also regulates proepicardial proliferation in zebrafish\textsuperscript{36}. Similar molecular regulation of proepicardial proliferation to chick indicates that zebrafish may exhibit a right-sided PEO asymmetry. Our work determines PEO left/right symmetry/asymmetry in zebrafish.

**Proepicardium migration mechanisms**

To form the epicardium, proepicardial cells must migrate from the PEO onto the myocardium. Early observations of epicardium identified patches of epicardial tissue that form on the myocardium opposite to the mesothelial projections or villi\textsuperscript{33,43,45,76}. Proepicardial cells form multi-cellular villous processes that contact the myocardium during cardiac contractions\textsuperscript{43,45,56,59,77–80}. Observations in many species indicate that proepicardial cysts released from the PEO float through pericardial fluid to reach the myocardial surface\textsuperscript{41,43,48,57,79}. Both proepicardial villi and vesicles contribute to epicardium formation\textsuperscript{41,43}. In some species, villi attach to the myocardium and mature into tissue bridges\textsuperscript{44,78}. Proepicardial cells migrate along a tissue bridge that forms between the PEO and the myocardial surface\textsuperscript{44,59,78}. Epicardium spreads on the myocardium in a caudal to cranial direction\textsuperscript{41,81}. Some species use more than one of these mechanisms. Chick exhibit proepicardial villi prior to tissue bridge formation\textsuperscript{40,56,71,78}. Mouse exhibit proepicardial villi and floating cysts\textsuperscript{77,82}. Mechanisms across many species are summarized in Table 3. In zebrafish, recently two mechanisms have been described: floating cyst
and cellular bridge\textsuperscript{57,58}. Both mechanisms require cardiac contractions for proepicardial migration\textsuperscript{57,58}. We propose that a villous mechanism reconciles findings of floating cyst, cellular bridge, and requirement for cardiac contraction. Recent studies of epicardial development in zebrafish are limited to ventricle. We propose a novel mechanism for atrial epicardium formation.

<table>
<thead>
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<th>Organism</th>
<th>Floating Cell</th>
<th>Villi</th>
<th>Tissue Bridge</th>
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<tbody>
<tr>
<td>Lamprey</td>
<td>-</td>
<td>-</td>
<td>+\textsuperscript{46}</td>
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<tr>
<td>Sturgeon</td>
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<td>+\textsuperscript{59}</td>
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<td>+\textsuperscript{58}</td>
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<tr>
<td>Xenopus</td>
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</tr>
<tr>
<td>Chick</td>
<td>-</td>
<td>+\textsuperscript{40,56,71}</td>
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<tr>
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<td>+\textsuperscript{48}</td>
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<td>Rat</td>
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<td>Mouse</td>
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Table 3: Mechanisms of proepicardial migration across species. (-) not observed, (+) observed.

**PEO adhesion to the myocardium**

PEO cells adhere to the myocardium through cell-cell or cell-matrix adhesions. To adhere PEO to the myocardium in mouse, cell surface proteins integrin alpha-4 ITGa4 (transmembrane protein on proepicardial cells) and vascular cell adhesion molecule-1 (VCAM-1) (transmembrane protein on cardiomyocytes that extends extracellularly) bind together. ITGa4 in
the proepicardium can bind with VCAM-1 or fibronectin (FN1) expressed in mouse myocardium\textsuperscript{85,86}. In proepicardial explants, \textit{Itga4} genetic null PE cells do not bind VCAM-1, but do bind FN1\textsuperscript{82}. ITGa4 and VCAM-1 protein binding are required for proper epicardium formation in mouse as loss of either results in lack of epicardium or epicardial blistering, which is indicative of an adhesion defect\textsuperscript{82,85–87}. VCAM-1 also modulates epicardium cell-cell adhesions and inhibits TGFβ stimulated EMT in cultured chick and rat epicardial mesothelial cells\textsuperscript{88}. VCAM-1 may be necessary to maintain epithelial epicardium on the myocardial surface. Proepicardial Itga4 binds to myocardial VCAM-1 to facilitate proepicardial adhesion to the myocardium. VCAM-1 stabilizes epicardium on the myocardial surface. In the absence of VCAM-1, ITGa4 can bind to FN to facilitate adhesion to the myocardium.

Connexin43 (\textit{Cx43}) may also have a role in ventricular proepicardium migration and adhesion. In zebrafish, \textit{cx43} is expressed in the outflow tract and in pericardium dorsolateral to the myocardium, but absent in the myocardium\textsuperscript{89}. In Cx43 knock out mice, epicardial blisters form on the ventricular myocardium similar to ITGa4 or VCAM-1 deficiency\textsuperscript{90}. Epicardial explant cultures from Cx43 knock out mice have defective actin cytoskeleton organization, altered ZO-1 distribution, altered cell invasion behavior, and changes in cell migration and cell shape\textsuperscript{90}. These results indicate cell polarity defects\textsuperscript{90}. Proepicardial cell polarity may be important to ventricular proepicardium migration and adhesion.

N-cadherin is necessary for adhesion both among cardiomyocytes and between the epicardium and myocardium. N-cadherin mutant \textit{glass onion (glo)} and \textit{N-cadherin (cadherin2)} morphants exhibit altered cardiomyocyte organization\textsuperscript{91}. Similarly, conditional N-cadherin knockout mice exhibited defects in intercalated disc structure; additionally, N-cadherin-depleted hearts experienced a significant decrease in Cx43\textsuperscript{92}. The epicardium blisters in N-cadherin-
deficient mice and the underlying ventricular myocardium is thinner\textsuperscript{93}. N-cadherin also plays a role in left-right patterning of chick heart\textsuperscript{94}. Together, N-cadherin likely plays an early role in cardiogenic patterning and adhesion among cardiac and proepicardial cell lines.

Failure of PEO to adhere to the myocardium results in epicardial blistering and defective epicardium formation. This phenotype is common to loss of function of par-3 family cell polarity regulator (Par3, Pard3a) \textit{Par3\textsuperscript{84}}, \textit{TCF21}, \textit{Fibronectin}, \textit{VCAM-1}, \textit{α4-integrin}, \textit{Cx43}, \textit{N-cadherin} and retinoic acid signaling. Common to all of these molecules is the establishment and maintenance of cell adhesion and cell polarity. Cell polarity is sensitive to cell-cell and cell-ECM adhesion\textsuperscript{95}. Changes in cell polarity can also affect cell migration\textsuperscript{96}. Disruption of cell polarity through aPKC (now called protein kinase C, iota, previously known as aPKC and \textit{heart and soul}) and stardust cell polarity complexes disrupt PEO morphogenesis in zebrafish\textsuperscript{35}. \textit{par3-par6-aPKC} form a cell polarity protein complex\textsuperscript{95}. Disruption of \textit{Par3} in mouse disrupts epicardium formation\textsuperscript{84}. \textit{tcf21} knockdown in \textit{Xenopus} results in abnormal concentrated aPKC in proepicardial cell processes and an aggregation of laminin (a basal membrane ECM protein) on the myocardial surface between myocardium and epicardium. Similarly, in \textit{Fibronectin} and \textit{Cx43} knockout, abnormal ZO-1 (normally localizes to cell-cell junctions) distribution and actin cytoskeleton organization indicate cell polarity disruption\textsuperscript{90,97}. VCAM-1 binds to α4-integrin, that mediates Rho-GTPase activity, thereby VCAM-1 and α4-integrin affect cell polarity\textsuperscript{88,96}. Loss of N-cadherin detaches epicardium from the myocardium and thereby affects cell-cell interactions\textsuperscript{93} that may modulate cell polarity. \textit{RXRa}\textsuperscript{−/−} mice also have abnormal epicardium\textsuperscript{98}. Retinoic acid signaling regulates \textit{Tcf21} in epicardium\textsuperscript{9}, and can regulate N-cadherin\textsuperscript{99}, and \textit{Fibronectin}\textsuperscript{82}. Retinoic acid signaling may be a master regulator of proepicardial cell adhesion
and cell polarity. Cell polarity links proepicardial migration defects and epicardial adhesion to the myocardium.

**Epicardial cell fates**

Epicardial-derived cells (EPDCs) differentiate into several cell types that promote cardiac maturation and repair. PEO cells give rise to epicardium, and thereby, EPDCs. Epicardial cell lineage may shed light on chamber-specific proepicardial populations and may uncover clues to regulation of cardiac maturation and repair. Proepicardial markers and their spatio-temporal expression have long been used to infer cellular lineage. Recently, genetic approaches using Cre-recombinase trace cell lineage in a time and tissue-dependent manner. This system was used to trace epicardial cell lineage through proepicardial genes \( TBX18, TCF21, \) and \( WT1 \). \( Tbx18 \) lineage was traced to cardiomyocytes in both atrium and ventricle in mouse\(^{55} \). *In vitro*, \( Tbx18^+ \) mouse PEO can differentiate into cardiomyocytes and smooth muscle myosin heavy chain-positive smooth muscle\(^{55} \). Adult mouse \( Tbx18 \)-lineage traced hearts indicate a coronary artery smooth muscle lineage. \( Tbx18 \) cell lineage does not contribute to coronary endothelial cells\(^{55} \). While \( Tbx18 \)-lineage was found in atrial myocardium, \( Tbx18 \)-deficient mice experienced no atrial myocardium loss\(^{100} \). Therefore, \( Tbx18 \) is not required for atrial myocardium development in mouse. In chick, \( Tbx18 \) expression was found in the sinus horn, atrial epicardium, and atrial endocardium. In zebrafish, \( tbx18 \) regulatory sequences were induced in a subset of cardiomyocytes during development or regeneration\(^{101} \). \( tbx18 \)-lineage can contribute to cardiomyocytes, coronary artery smooth muscle, sinus horn, atrial epicardium, and atrial endocardium.

\( tcf21 \)-lineage cells in zebrafish contribute to smooth muscle in the outflow tract, but not coronary vascular smooth muscle. \( tcf21 \)-traced cells (perivascular cells) envelop smooth muscle
cells within large ventricular vessels. Labeled larvae were also grown to adulthood to assess tcf21-lineage through ventricular resection and consequential regeneration. Adult fish were labeled prior to ventricular resection and assessed for tcf21 contribution to the regenerating heart. In both cases, traced cells resembled vascular support cells like those observed in matured larval labeled fish. Lineage-labeled cells did not co-localize with myocardial markers MHC or Mef2. tcf21-lineage does not contribute to cardiomyocytes in zebrafish. In mouse, Tcf21-lineage traced coronary vascular smooth muscle (cVSMCs) and cardiac fibroblasts. tcf21-lineage can contribute to smooth muscle in the outflow tract, perivascular cells, cVSMCs, and cardiac fibroblasts.

Wt1-lineage to cardiomyocytes is controversial due to two research labs reporting different findings in tamoxifen-inducible Wt1CreERT2 mice. Zhou et al. reported that Wt1GFPCre/+ line traces lineage to cardiomyocytes in the linear heart tube and that tamoxifen-inducible Wt1CreERT2 line also traces cardiomyocytes in mouse. Rudat and Kispert argue that Cre-recombination is random and sporadic because it occurs prior to establishment of proepicardium. Rudat and Kispert find that tamoxifen-inducible Wt1creERT2 mouse line mediated poor and variable recombination in the epicardium in their hands - and concluded that Wt1-lineage cardiomyocyte fate is not substantiated. Zhou et al. acknowledge that epicardium contribution to cardiomyocytes requires further investigation. Additionally, they find that wt1 lineage cells have a coronary endothelial cell fate. A regeneration study in Wt1Cre crossed to R26R reporter in mouse with β-Gal staining identified Wt1 cell lineages in regenerating heart: subepicardial mesenchyme, mesenchymal cells, fibroblasts, myofibroblasts, coronary endothelium, and cardiomyocytes. In zebrafish, wt1 regulatory sequences were induced in some cardiomyocytes during development. Therefore, wt1-lineage cells can contribute to coronary endothelium, fibroblasts, and perhaps cardiomyocytes.
Each proepicardial gene delineates a unique set of EPDCs. Cardiomyocyte fate is common to \textit{tbx18} and \textit{wt1} lineages. Fibroblast fate is common to \textit{tcf21} and \textit{wt1} lineages. \textit{tbx18} plays a role in cells that line the atrium. Chamber-specific cell-fates of epicardial-derived cells remain largely unexplored.

**Summary**

Many questions remain to be answered about epicardium formation. Zebrafish offer new insights through temporally distinct PEO near the SV and near the atrioventricular junction. Early proepicardial migration in zebrafish prior to 48 hpf and PEO villi were previously undescribed\textsuperscript{35,36,57,58,101,105}. Study of early zebrafish PEO cell migration may hold keys to understanding PEO cell migration mechanisms and PEO cell populations. Contractions have previously been demonstrated as necessary for proepicardium migration\textsuperscript{57,58}. PEO cell migration in the absence of cardiac contractions was previously undescribed. Determination of asymmetry in zebrafish PEO may offer additional information to the evolution of the PEO across species.

Molecular regulation of PEO is under active study. Epicardial function of PEO gene \textit{tcf21} has been characterized in mouse and \textit{Xenopus}, but in zebrafish was previously unknown. \textit{tcf21} function in zebrafish may provide additional insights to molecular regulation of proepicardium. Molecules that mediate proepicardial adhesion to the myocardium have previously been described. However, chamber-specificity of proepicardial-myocardial adhesion was previously unknown. Different adhesion protein complexes on cardiac chambers would add to molecular mechanisms of proepicardial migration.

PEO-lineage of EPDCs is well described across species. EPDC differentiation is highly specialized and compartmentalized. However, no studies compare EPDC cell fate between atrial
and ventricular epicardium. To better understand embryonic developmental programs and regenerative programs of epicardial cells, identification of chamber-specificity of EPDCs is important. Using zebrafish, we begin to address and answer some of these questions. We propose that in zebrafish, PEO near the SV region migrates directly on the atrial myocardium and also generates a villous population near the AV region that is dependent upon contractility to migrate to the ventricle.
Chapter 2 - Chamber-Specific Patterns of Proepicardium Migration in Zebrafish

Abstract

Dynamic cell migrations direct tissue morphogenesis. Proepicardial cells migrate from an extra-cardiac location onto the myocardial heart surface to form epicardium and direct cardiac maturation. More than one mechanism may occur within a species. Here we report unique chamber-specific proepicardial cell migration mechanisms in zebrafish. In addition to floating cysts and a cellular bridge mechanism, we demonstrated that zebrafish have a villi-mediated mechanism and that cardiac contractions are required for ventricular proepicardium migration. *tcf21* mediates ventricular proepicardium adhesion in zebrafish. Transgenic reporters for proepicardium and myocardium, were used to construct a model of proepicardium migration from the linear heart tube stage to mature larval heart. A novel atrial proepicardium migration mechanism was determined by inhibiting cardiac contractions with pharmacological or morpholino treatment. *tcf21*’s role in ventricular proepicardium adhesion was determined through morpholino-mediated knock down and with a null mutant. Zebrafish exhibit a ventricle-specific right-sided asymmetry in proepicardium. *tcf21* promotes ventricular proepicardium migration. Chamber-specific mechanisms of proepicardial cell migration form the epicardium: a villous-mediated ventricular mechanism and a novel atrial mechanism independent of cardiac contractions.
Introduction

The epicardium is the outer tissue layer of the heart that overlies the myocardium. Proper myocardial maturation requires the proper development of the epicardium in vertebrates. The epicardium originates from an extra-cardiac population of cells, known as the proepicardial organ (PEO). The PEO is composed of a cluster of mesothelial or mesenchymal cells projecting from the septum transversum or similar tissue adjoining the sinus venosus (SV) in most species. Initial studies of PEO morphology in zebrafish report one cluster of PEO cells near the sinus venosus (SV) at 40 hpf and another cluster of cells on the dorsal pericardium opposite to the atrioventricular myocardium (AV) at 48 hpf. This additional cluster at the atrioventricular junction is undescribed in other organisms. The presence of two physically separated populations suggests the presence of two distinct PEO populations, one at the SV and another at the AV. Other species may also have two populations, but heart morphology in other organisms may prevent physical separation of the populations. Zebrafish allow analysis of differences in the migration and gene expression of each distinct population. Patterns of conserved PEO gene expression in zebrafish can be compared to previous studies in other organisms to provide additional information about the evolution of epicardium formation across species.

The most conserved PEO genes by expression across species are wilm’s tumor-1 (WT1), and transcription factor 21 (TCF21). Both encode transcription factors that are known to play roles in cell proliferation, epithelial-to-mesenchymal transition (EMT), cell migration, and cell differentiation. WT1 is required for epicardium formation. In zebrafish, morpholino knockdown of wtl disrupts tcf21 (mRNA) expression at 48 hpf. PEO cells have a rounded morphology and fail to properly adhere to the myocardium. wtl (gene) likely regulates...
expression of tcf21 (gene) and proepicardial cell adhesions. tcf21 (gene) interacts in transcription factor pathways and regulates transcription of many genes. Detached epicardium in Tcf21-deficient mice and tcf21-depleted Xenopus embryos suggest that tcf21 is required for proper adhesion to the myocardium. In zebrafish, tcf21MO knockdown affects craniofacial myogenesis. What is the role of tcf21 in proepicardial migration in zebrafish? We examined epicardial coverage in splice-site morpholino mediated tcf21-depleted embryos and in tcf21-null mutant embryos (tcf21+/– mutants were identified by defects in craniofacial myogenesis) to determine tcf21 function in proepicardial migration.

We sought to carefully examine the physically separated PEO populations in zebrafish that offer a unique perspective to study PEO asymmetry. In most species, the PEO expands on the right side and contacts the myocardium on the right side with the exception of mouse, in which fusion at the midline occurs. Zebrafish PEO asymmetry has previously been undescribed. During the course of examining proepicardial migration and genetic expression, we gathered data to determine left-right PEO asymmetry in zebrafish.

Proepicardial cells migrate from the PEO to the myocardium. Historically, three main migration mechanisms have been described: proepicardial villi, floating proepicardial cysts, and tissue bridge. All three mechanisms facilitate transfer of proepicardial cells from the PEO to the myocardium. PEO produces multi-cellular villi that project towards the myocardium. Tips of proepicardial villi are thought to contact the myocardium during cardiac contractions. Previous findings suggest that villous mediated migration is conserved across species. Proepicardial cells detached from PEO and myocardium are called floating cysts. Floating cysts are also described across species. Tissue bridges form stable connections between the PEO and the myocardium through an extracellular matrix bridge.
Recent reports in zebrafish describe a floating cyst mechanism\textsuperscript{57} and a cellular bridge mechanism\textsuperscript{112}. Both mechanisms require cardiac contractions\textsuperscript{57,58}. A bridge mechanism suggests stable contact between the PEO and the myocardium through extra-cellular matrix adhesions. Plavicki et al. demonstrate \textit{activated leukocyte cell adhesion molecule} (ALCAM) expression between PEO cells and the myocardium. However, this contact may be transient. The stability of ALCAM binding varies\textsuperscript{113}. ALCAM is expressed during dynamic cell growth or cell migrations; reviewed by Swart\textsuperscript{114}. It is likely that ALCAM binding is transient between PEO cells. A villous mechanism for PEO migration could reconcile the limitations of the floating cyst and cellular bridge mechanisms.

Both villous and proepicardial cyst mechanisms are previously described in mouse\textsuperscript{77,84}. In mouse, proepicardial cells adhere to the myocardium via adhesions of proepicardial alpha4 integrin (ITGa4) to myocardial vascular cell adhesion molecule-1 (VCAM-1)\textsuperscript{82,85,86}. Using tissue-specific markers to identify PEO and myocardium we examined PEO morphology near the SV and the atrioventricular junction for the presence of proepicardial villi in zebrafish.

We find chamber-specific PEO populations, atrial PEO (A PEO) and ventricular PEO (V PEO). A PEO is \textit{wt1b:GFP}\textsuperscript{+} and \textit{tcf21:DsRed}\textsuperscript{−} whereas V PEO is both \textit{wt1b:GFP}\textsuperscript{+} and \textit{tcf21:DsRed}\textsuperscript{+}. SV PEO contains both A PEO and V PEO cells. PEO cells at the AV junction are V PEO cells. Loss of \textit{tcf21} in zebrafish embryos reduces ventricular epicardium formation while atrial epicardium formation remains unaffected. \textit{tcf21} expression also aids in identifying right-sided asymmetry of V PEO. Zebrafish initially exhibit bilateral PEO asymmetry in the SV PEO and subsequently, right-sided asymmetry in the V PEO. V PEO cells form villous protrusions in zebrafish. Cardiac contractions are required for V PEO migration. We demonstrate a novel mechanism of PEO cell migration that proceeds in the absence of cardiac contractions – an
atrial-specific mechanism of A PEO cell migration in which PEO cells directly migrate from the SV PEO to adjacent atrial myocardium.

RESULTS

Chamber-specific proepicardium migration

To examine spatial and temporal differences between the two PEO populations, we employed transgenic embryos that visualize the myocardium and proepicardium, Tg(my7:DsRed; wt1b:GFP)\textsuperscript{115,116} and Tg(my7:GFP; tcf21:DsRed)\textsuperscript{101} embryos. myl7:DsRed or myl7:GFP provides cardiac context by labeling myocardium and wt1b:GFP or tcf21:DsRed was used to visualize proepicardium. Serluca reports identical patterns of wt1 and tcf21 expression in zebrafish PEO, PEO near the SV at 40 hpf and an additional PEO cluster near the AV myocardium at 48 hpf\textsuperscript{35}. We closely examined proepicardial cells in both regions at both time points, then extended our study to cover embryonic epicardium formation. We found differences in timing of epicardium formation and in PEO gene expression between the atrium and the ventricle.

Identification of atrial-specific proepicardium migration

Initially, we observe a population of wt1b:GFP\textsuperscript{+} cells located near the venous pole of the heart as early as 35 hpf. This population consists of rounded, mesenchymal cells and epithelial-like bands of cells surrounding the sinus venosus (SV PEO) (Fig.1A)(n=8; 3 independent clutches). This SV PEO population is similar to the wt1a\textsuperscript{+} PEO population reported by Serluca at 40 hpf with some variation likely due to differences between wt1a and wt1b expression; wt1b is more robustly expressed in proepicardium than wt1a\textsuperscript{105}. SV PEO begin to cover the adjacent surface of the atrial myocardium as early as 35 hpf (Fig. 1A). The atrium is almost fully covered
with wt1b:GFP+ cells at 40 hpf (A PEO)(Fig. 1B)(n=7; 2 independent clutches). After migrating to the surface of the atrial myocardium, atrial proepicardial cells decrease wt1b:GFP+ expression; Markedly, only very rarely are wt1b:GFP+ cells on the ventricle in early stages of heart development 35-45 hpf (Fig. 1A,B). One cell was present on the ventricle of one 40 hpf embryo out of 15 embryos 35-45 hpf (Fig. 1B). Thus atrial proepicardium migration precedes ventricular proepicardium migration. Our findings suggest that A PEO progressively migrates from the SV PEO to the atrial myocardium from 35 hpf – 40 hpf, prior to ventricular proepicardium migration.

**Ventricle-specific proepicardium migration**

Close examination of the proepicardial cells near the AV myocardium at 47-49 hpf reveals multicellular villi (indicated by yellow arrows) that express wt1b:GFP (Fig. 1I, J, and L). 15/36 embryos exhibit villi. The cells on the pericardial wall preceding V PEO coverage of the ventricular myocardium likely form villi (Fig.1C). Villi are also found to the right of the sinus venosus (Fig.II-L) and extend towards the ventricular myocardium (Fig. 1L). Spherical cells adhere to the ventricular myocardium across from a villous, arrowhead (Fig.1J). A high-speed movie captures a villous moving with the beat of the ventricular myocardium surface (white arrow) (Fig.1K, Movie 1). These findings suggest that during cardiac contractions, villi transfer cells to the ventricle.

Additional ventricular proepicardial cells adhere to the ventricle at approximately 55 hpf (Fig.1D)(n=9; 4 independent clutches). The ventricle is partially covered with epicardium at 65 hpf (Fig1. E)(n=5; 4 independent clutches). Complete coverage of the ventricular myocardium occurs as early as 72 hpf (Fig.1F)(n=19; 6 independent clutches). At 96 hpf, the cardiac chambers are more in apposition and more epicardial cells cover the ventricle than at earlier
stages of heart looping (Fig.1G)(n=14; 4 independent clutches). As the rounded proepicardial cells flatten on the myocardium, \textit{wt1b}:GFP expression decreases. \textit{tcf21}:DsRed is more easily detected in epicardium at later stages. Complete embryonic epicardium formation occurs 96 hpf – 6 days post fertilization (dpf)\textsuperscript{35,36,105}. Similarly, we find that epicardium almost completely covers the myocardium in \textit{Tg(myl7:GFP; tcf21:DsRed)} embryos at 6 dpf (Fig. 1H)(n=9; 3 independent clutches). We report that ventricular proepicardium migration occurs after atrial proepicardium migration. We find that the ventricular epicardium forms 47 hpf – 6 dpf.

Our findings suggest that two PEO populations, A PEO and V PEO, diverge from an SV PEO population. A PEO migration precedes V PEO migration. The presence of villi and transition in cardiac contractions suggest a ventricle-specific villous mechanism of proepicardial migration mediated by cardiac contractions.

**Cardiac contractions are required for ventricular proepicardium coverage**

Cells of the ventricular proepicardial villi likely transfer cells to the ventricular myocardium in a cardiac contraction-dependent manner. To demonstrate the requirement for cardiac contractions for ventricular proepicardium migration, we inhibited heart contractions and assayed for epicardial coverage. Heart contractions were inhibited by pharmacological agents 2, 3-Butane Dione Monoxime (BDM) and blebbistatin at 55 hpf at which time atrial proepicardium migration, proepicardial villi formation, and the transfer to the ventricular myocardium had begun (Fig.1D). (At 55 hpf, 2/9 embryos show 2-5 ventricular epicardial cells, 7/9 show 0-1 ventricular epicardial cell). Knockdown of \textit{tnnt2a} mediated by silent heart-morpholino (\textit{sihMO}) abolished cardiac contractions throughout cardiac development. Note very few or absent \textit{wt1b}:GFP\textsuperscript{+} cells on the ventricle (Fig.2A'-D'). Three models of cardiac contraction inhibition yielded significantly less ventricular epicardium than their controls (Fig.2B-G) and significantly
less than hearts with normal cardiac contractions (Fig. 2E). Previous reports indicate that wt1b:GFP can be expressed in some ventricular myocardial cells\(^1\). We did observe some ventricular cells co-expressing wt1b:GFP and myl7:DsRed (data not shown). However, ventricular epicardium quantification only included wt1b:GFP\(^+\), myl7:DsRed\(^-\) cells. Proepicardial villi formed in embryos with tntt2a knockdown (Fig.3). Villi formation does not require cardiac contraction.

**Defective ventricular proepicardium migration in wea mutants**

To assess changes in the pattern of ventricular epicardium that correspond to erroneous cardiac contraction, we observed proepicardial transgenic reporter expression in wea mutant background. In the tcf21:DsRed embryos at 72 hpf, wildtype cardiac contraction siblings exhibit tcf21:DsRed\(^+\) cells near the SV and exhibit normal progression of ventricular epicardium formation (Fig.4A-A’’) whereas, in mutants with partial atrial contractions, V PEO cells fail to form ventricular epicardium (Fig.4B-B’’). We have demonstrated that cardiac contractions are required for ventricular epicardium formation. Ventricular proepicardial coverage is disrupted in embryos with partially contracting hearts. Disrupted V PEO migration may be due to the distended heart tube and pericardium in wea mutants.

**Novel atrial proepicardium migration mechanism**

To confirm a distinct contraction-independent mechanism of atrial proepicardium migration, we observed earlier stages of atrial migration in control and tntt2a knockdown embryos. The atrial migration has begun by 35 hpf in control and tntt2a knockdown embryos (Fig.5A,B). The atrium is nearly completely covered with atrial epicardium by 42 hpf in control
and tnni2a knockdown embryos (Fig.5C,D). These findings suggest that atrial proepicardium migration progresses independently of cardiac contractions.

Previous findings in zebrafish indicate that wt1b:GFP is expressed in some atrial cardiomyocytes\textsuperscript{101,105}. We also observe that some atrial myocardial cells express both wt1b:GFP and myl7:DsRed. However, complete overlap of expression occurs in only a few cells, and not throughout the linear heart tube. We find that often overlap of these markers is not in the cell’s entirety and co-localization tends to occur in some portions of the cytoplasm. Atrial myocardium is much thinner than ventricular myocardium. It is likely that the observed partial overlap correspond to parts of distinct atrial myocardial and atrial epicardial cells that are so close together that some portions cannot be resolved by confocal microscopy.

Atrial epicardium was gauged by the distance of the furthest wt1:GFP\textsuperscript{+} atrial epicardial cell from the SV that was continuous in a sheet conformation with other wt1:GFP\textsuperscript{+} atrial epicardial cells. No significant difference was found between atrial epicardium of control and sihMO embryos. We demonstrate a novel mechanism of proepicardial migration that occurs prior to ventricular migration and does not depend on villi formation, floating cysts, or cellular bridge formation. The atrial proepicardium likely migrates directly through a crawling mechanism.

**PEO asymmetry**

To detect asymmetry, we examined PEO in transgenic embryos and by whole mount \textit{in situ} hybridization for nascent mRNA transcripts of wt1a and tcf21. Early observations of wt1b:GFP expression indicate bilateral symmetry of the PEO near the SV (Fig.1 A,B)(n=8). By \textit{in situ} hybridization, we find that wt1a is expressed in bilateral SV PEO at 45 hpf (Fig.6A).
Additionally, *wt1a* is expressed in a right-sided expanse of PEO cells (Fig. 6A,B). A subset of cells at the right side of the SV PEO and a set of cells near the AV junction express *tcf21* (Fig. 2C). At 63 hpf, *tcf21* expression is limited to the ventricular epicardium (Fig. 2D).

A similar pattern of expression is found in transgenic embryos. *tcf21*:DsRed⁺ PEO cells also appear more abundant on the right side of the SV PEO than the left (Fig. 6F). Right-sided *tcf21*:DsRed⁺ PEO cells connect to a cluster of PEO cells on the ventricle and *tcf21*:DsRed⁺ PEO cells partially cover the ventricle (Fig. 6G). Ventricular epicardium coverage is almost complete as early as 72 hpf (Fig. 6H).

Double proepicardial transgenic embryos highlight the ventricular proepicardial population. At the outset of ventricular proepicardium migration, *wt1b*:GFP⁺ and *tcf21*:DsRed⁺ cells are located at the right side of the SV and on the ventricular surface closest to the right side of the SV (Fig. 6IJ). These findings show a right-sided expansion of ventricular proepicardium.

**Reconciliation of ventricular migration mechanisms**

Villi are often located on the right side of the sinus venosus. Peralta et. al report that floating cells originate from a cluster of proepicardial cells near the venous pole. Upon re-examination of images presented in their report, this venous pole cluster is located on the right side of the sinus venosus. Proepicardial cysts circulate with pericardial fluid during normal cardiac contractions from approximately 48-72 hpf⁵⁷. Findings of cyst-mediated proepicardial colonization of the myocardium are limited to the ventricle⁵⁷. We also observed floating proepicardial cells in the pericardial cavity. *tcf21*:DsRed⁺ proepicardial cysts in the pericardial cavity are indicated by pseudocolor in sequential stills from a high-speed movie (Fig. 6K and L and Movie 2). Distinct *tcf21*:DsRed expression in ventricular proepicardium combined with the
right-sided position of villi and \( tcf21:DsRed^+ \) floating cells that only adhere to the ventricle suggest that proepicardial cysts likely arise from villi and that cyst-mediated PEO cell migration to the myocardium is likely ventricle-specific. Our results also suggest a ventricle-specific role of \( tcf21 \) in proepicardial migration.

**\( tcf21 \) promotes ventricular epicardium formation**

\( tcf21 \)MO has previously been characterized to affect craniofacial myogenesis in zebrafish\(^67\). In the \( tcf21 \) mutant – pharyngeal arch arteries develop from endothelial cells from the dorsal head vasculature. Thus it has been shown that \( tcf21 \) is required for craniofacial myogenesis\(^67,68\), and not required for pharyngeal arch artery development\(^68\).

To test a potential ventricle-specific role of \( tcf21 \), \( tcf21 \) knockdown was mediated by a splice-site morpholino, \( tcf21 \)MO-2\(^67\) and \( tcf21 \) null mutant (\( tcf21^{+/–} \))\(^68\). Knockdown with \( tcf21 \)MO reduces ventricular epicardium to about half the ventricular epicardium of control \( Tg(myl7:GFP; tcf21:DsRed) \) embryos (Fig.7B) while normal cardiac morphology and contraction function are maintained (Fig.7D and E). In \( tcf21 \) knockdown embryos, the ventricular epicardium has a more rounded cell morphology than the flat epithelial-like morphology of the controls (Fig.7B’ and D’).

A similar ventricular epicardium defect phenotype was found in \( tcf21 \) mutants. Wildtype siblings \( tcf21^{+/+} \) or \( tcf21^{+/+} \) exhibit normal ventricular epicardium formation at 72 hpf (n=5). \( tcf21^{+/–} \) mutant siblings exhibit defects in ventricular epicardium formation at 72 hpf (n=2). Additional embryos were fixed and immunostained for myocardium with MF20. Wildtype siblings exhibit normal ventricular epicardium (n=3)(Fig.8A). Mutant siblings exhibit moderate (n=3) (Fig.4B) to severe defects in ventricular epicardium formation (n=3) (Fig.8C).
*tcf21* knockdown in *Xenopus* and mouse reduces cell adhesions between the proepicardium and myocardium\textsuperscript{15,66}. The rounded ventricular epicardium morphology that occurs with loss of *tcf21* in zebrafish suggests a similar adhesion deficiency. Alternatively, *tcf21* may mediate mesenchymal-to-epithelial (MET) transition in ventricular proepicardium. *tcf21* is not required for specifying ventricular PEO but may play a role in ventricular proepicardium adhesion or epithelial transition. *tcf21* knockdown does not appear to affect atrial proepicardium migration in comparison to control (Fig. 7A and C). *tcf21* is dispensable for atrial proepicardium migration. Our findings suggest that *tcf21* regulates ventricular epicardial adhesion to the myocardium.
Ventricle-specific proepicardial adhesion to the myocardium

Proepicardial cell migration requires cell-cell adhesions. Cell-cell adhesions between proepicardial cells affect villous integrity while proepicardial adhesions to the myocardium facilitate transfer from the PEO to the myocardium surface. Once on the myocardium, epicardium-myocardium and epicardium-epicardium adhesions maintain the epicardial layer on the myocardial surface.

Adhesion molecules facilitate cell-cell and cell-matrix adhesions. Vascular cell adhesion protein-1 (VCAM-1) and integrin alpha-4 (ITGa4) are known to mediate proepicardial adhesion in mouse epicardium formation. VCAM-1 is detected on mouse cardiomyocytes\textsuperscript{85}. ITGa4 is expressed on mouse proepicardial cells\textsuperscript{86}. Functional loss of either VCAM-1 or ITGa4 disrupts epicardium formation\textsuperscript{85–87}. In the absence of ITGa4, VCAM-1 can bind to fibronectin expressed on cardiomyocytes\textsuperscript{85,117}. Although VCAM-1 and ITGa4 have been shown to facilitate epicardium formation in mouse, limitations of present studies omit description of chamber-specific epicardial adhesion mechanisms.

Since we have identified chamber-specific proepicardial populations and migration mechanisms in zebrafish, we asked whether Vcam1 and Itga4 might have a chamber-specific role in ventricular epicardium adhesion. To address cell adhesion mechanisms underlying epicardium formation in zebrafish, we conducted \textit{in situ} hybridization with \textit{vcam-1} and immunostaining for Vcam1 and Itga4 in zebrafish. Knowledge of chamber-specific proepicardial populations and migration mechanisms allowed us to look for potential chamber-specificity of adhesion mechanisms that may have been over-looked in other species. It is likely that cell-
surface receptors on proepicardial cells may be chamber-specific. Therefore, it is possible that A
PEO and V PEO partner with different adhesion molecules on the myocardial surface.

To determine chamber-specific patterns of adhesion molecule expression, we evaluated
expression of vcam-1 in zebrafish by in situ hybridization. At 49 hpf, when proepicardial
markers wt1a and tcf21 are expressed at the right side of the sinus venous and at the
atrioventricular junction, vcam-1 appears to be expressed in the atrium and ventricle in (Fig.9A-
C). However, vcam-1 was not detected in the heart region at earlier stages. At the same stage as
proepicardial expression near the atrioventricular junction, vcam-1 localizes to the periphery of
the atrioventricular junction (Fig.9D-F). These patterns of expression suggest that vcam-1 has a
role in proepicardial migration at the atrioventricular junction or in valve formation. Shortly
thereafter, vcam-1 is detected at the ventricle (Fig.9H). vcam-1 localizes to the ventricle (Fig.9K)
in a similar pattern as tcf21 and the shape of the outer curve of the ventricle (Fig. 9J). This
pattern suggests that vcam-1 mRNA localizes to the ventricular myocardium and supports a role
for vcam-1 in ventricular proepicardium adhesion.

In mouse, VCAM-1 protein is localized to the myocardium. Considering vcam-1
expression in ventricular myocardium in zebrafish, we would also expect Vcam1 to localize to
the ventricular myocardium in zebrafish. Our findings indicate that Vcam1 is localized caudal
and dorsal to the atrial myocardium at 50 hpf (Fig.9M) but not within the atrial myocardium
itself. This localization suggests expression of Vcam1 on proepicardial cells at the SV PEO and
on V PEO cells. At 55 hpf, Vcam1 localizes to the ventricular myocardium and partially to the
atrial myocardium near the AV junction (Fig.9N). Immunostaining correlates with in situ
localization of Vcam1 to the ventricular myocardium. Additionally, Vcam1 might be expressed
on V PEO cells.
In mouse, ITGa4 localizes to the surface of proepicardial cells and mediates adhesion to the myocardium. In zebrafish, we find that Itga4 is localized between *wt1b:GFP* cells on the pericardial wall opposite to the atrioventricular junction and within the pericardial cavity with predominance on the ventricular side at 55 hpf (Fig.9O). Itga4 localization between ventricular proepicardial cells suggests a ventricle-specific role of ITGa4 in proepicardial adhesion between proepicardial cells and in proepicardial adhesion to the myocardium.

In zebrafish, we find that *vcam-1* mRNA and protein localize specifically to the ventricular myocardium. Ventricle-specific expression of *vcam-1* suggests a role of *vcam-1* in ventricle-specific proepicardial adhesion to the myocardium. Itga4 is detected on ventricular proepicardium cells and also detected in a region near the ventricular myocardium. Itga4 likely mediates cell-cell adhesions within ventricular proepicardial villi and also mediates ventricular proepicardium adhesion to the ventricular myocardium. Together, these results suggest that ventricle-specific proepicardium adhesion to the myocardium is mediated by Vcam1 and Itga4 binding.
Figure 1: Spatio-temporal distinctions of PEO cell migration and proepicardial villi. (A,B, D-H,L) Ventral view. (C) Skewed ventral view. (I-K) Lateral view. (A,B,D) Homozygous *wt1b*:GFP embryos, GFP (green) and MF20 (magenta) (C,E-G) *myl7*:DsRed (magenta); *wt1b*:GFP (green) in live embryos. (H) Dissected *myl7*:GFP; *tcf21*:DsRed heart. (I-K) Multicellular proepicardial villi (yellow arrows), white arrowhead and arrows indicate transferred proepicardial cell and villi respectively. (L) Optical slice from confocal micrograph, of live *myl7*:DsRed; *wt1b*:GFP embryo, proepicardial villous (yellow arrow). hpf=hours post fertilization, v=ventricle, a=atrium.
Figure 2: Cardiac contractions are required for ventricular PEO migration. (A-D) Dissected myl7:DsRed (magenta); wt1b:GFP (green) hearts at 100 hpf. (A’-D’) Optical sections through ventricle. (A”-D”) Optical sections through atrium. (E-G) Mean % wt1b:GFP ventricular epicardium cells. Error bars represent mean +/- standard error. (E) N=9,10; p<0.0001; α=0.05  (F) N=9, 10; p=0.0001; α=0.05  (G) N=10, 12; p<0.0001; α=0.05.
Figure 3: Ventricular PEO villi form in *sih*MO embryos. (A-C) Live embryos imaged at 48 hpf. (A) Some *wtlb*:GFP+ cells on the ventricle. (B,C) Blue arrowhead indicates *wtlb*:GFP+ or *tcf21*:DsRed+ ventricular proepicardial villi.

Figure 4: Defective ventricular proepicardium migration in *wea* mutants at 72 hpf. (A-A”) Wildtype sibling. (B-B”) Partial atrial contractor (*wea* mutant). (A,B) *myl7*:GFP. (A’,B’) *tcf21*:DsRed. (A”,B”) Merge express *tcf21*:DsRed at the SV PEO and an ectopic aggregate of PEO cells on the left pericardial wall. (A-A”) Normal ventricular epicardium (n=2). (B-B”) Ventricular epicardium is absent in *wea* partial contraction mutants (n=3).
Figure 5: Novel atrial PEO migration mechanism independent of cardiac contractions. Homozygous wt1b:GFP embryos, GFP (green) and MF20 (magenta) (A-D) Ventral views. (A’-D’) optical sections through atrium. (E) mean % atrial epicardium at 42 hpf, (measurement details provided in methods), error bars represent +/- s.e.m, (no significant difference by two-tail t-test, equal variance), control (n=10), sihMO (n=12).

Figure 6: Right-sided Asymmetry of the ventricular PEO population. (A, C-L) Ventral views. (B) Lateral view. (A-D) Whole mount double in situ hybridization. (F-H) myl7:GFP in
blue, and \textit{tcf21}:DsRed in magenta, live embryos. (I,J) \textit{tcf21}:DsRed in magenta, and \textit{wt1b}:GFP in green, live embryos. (K,L) Stills from high speed movie, floating \textit{tcf21}:DsRed\textsuperscript{+} cells in magenta, live embryo. (A,B) \textit{myl7} probe in red and \textit{wt1a+kts} probe in blue (n=6). (C,D) \textit{tcf21} probe in blue (C) \textit{tcf21} expression in PEO cells at right side of SV and near the AV (n=2). (D) \textit{tcf21} expression in PEO cells on the ventricle (n=1). (E) Schematic of right-sided expansion of V PEO, blue represents myocardium, \textit{tcf21}:DsRed\textsuperscript{+} cells in magenta, and \textit{wt1b}:GFP\textsuperscript{+} cells in green, R=right, L=left. (F) right-sided PEO expansion (n=2). (G,H) Partial to full ventricular epicardium coverage (n=7). (I) Right-sided ventricular proepicardium expresses \textit{tcf21}:DsRed and \textit{wt1b}:GFP (n=4). (J) Ventricular epicardium expresses \textit{tcf21}:DsRed and \textit{wt1b}:GFP (n=2). (K,L) floating \textit{tcf21}:DsRed\textsuperscript{+} cells in the pericardial cavity during cardiac contractions in a live embryo.

**Figure 7:** Knockdown of \textit{tcf21} decreases ventricular epicardium adhesions. (A, C) Homozygous \textit{wt1b}:GFP embryos, GFP (green) and MF20 (magenta) at 42 hpf. (B, D) \textit{myl7}:GFP in blue, and \textit{tcf21}:DsRed in magenta, live embryos at 72 hpf. (B’, D”) Enlarged images from B and D. (E) Mean % of ventricular epicardium cells, error bars represent +/- standard error, N=10, 6; p<0.05 (\(\alpha=0.05\))

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**Figure 8:** \textit{tcf21}\textsuperscript{+/+} decreases ventricular epicardium adhesions. \textit{tcf21}:DsRed expression at 3.5 dpf. (A) Wildtype siblings \textit{tcf21}\textsuperscript{-/-} or \textit{tcf21}\textsuperscript{+/+}. (B) Moderate ventricular epicardium phenotype of \textit{tcf21}\textsuperscript{-/-}. (C) Severe phenotype of \textit{tcf21}\textsuperscript{-/-}.
Figure 9: Vcam1 may mediate ventricular proepicardium adhesion (A-L) in situ hybridizations in whole embryos. (H) Composite image of several focal planes taken of the same embryo. (M-O) Immunostaining. (A,B,D,E) Proepicardial gene expression on the right side of the SV and near the AV junction. (C) Diffuse vcam-1 expression within the ventricular and atrial myocardium. (F) vcam-1 near the AV junction corresponds to V PEO. (G) myl7 gives myocardial context. (H) vcam-1 on the ventricular myocardium. (I,J) Proepicardial gene expression in the ventricular epicardium. (K) vcam-1 on the ventricle corresponding to ventricular epicardium. (L) myl7 gives myocardial context, cardiac chambers are more side-by-side at this stage. (M) Vcam1 at SV caudal to the atrial myocardium and near the pericardium close to the ventricular myocardium. (N) Vcam1 on the ventricular myocardium and part of the atrial myocardium closest to the AV junction. (O) Itga4 on V PEO cells near the AV junction and diffuse expression near the ventricle.
Materials and Methods

Zebrafish

Transgenic zebrafish were used to visualize proepicardium and myocardium. \textit{wt1b:GFP} [\textit{Tg(wt1b:EGFP)}]^{115}, \textit{myl7:DsRed} [\textit{Tg(myl7:Dsred)}]^{116}, \textit{tcf21:DsRed} [\textit{Tg(tcf21:DsRed2)}]^{101}, and \textit{myl7:GFP} [\textit{Tg(myl7:GFP)}]^{118} have been described previously. \textit{weak atrium (wea)} (\textit{myh6}\textsuperscript{sk7/sk7})^{119} possess a autosomal dominant mutation in the \textit{my6} gene that confers a partial contraction or non-contraction phenotype in the atrial myocardium. \textit{wea} embryos were maintained in 200mM Mannitol/egg water solution to reduce pericardial edema. \textit{tcf21}\textsuperscript{−/−} are previously described\textsuperscript{68}. Zebrafish were raised and maintained in the Queens College; CUNY animal facility with IACUC approved animal procedures and protocols. Embryos were kept in egg water at 28.5°C and staged according to hours post fertilization (hpf) and days post fertilization (dpf)\textsuperscript{120}.

\textit{in situ} hybridization

To visualize patterns of proepicardial gene expression in the whole embryo (Chapter 2, Fig.6; Chapter 2, Fig.9; Appendix I, Fig.1.) \textit{in situ} hybridization protocol was followed as previously described\textsuperscript{121} and slightly modified for double \textit{in situ} hybridization: include both probes in hybridization step. BMP staining was conducted first, then quenched with 0.1M glycine 0.1% tween. 5 washes in 1X PBT, then blocking step was repeated as conducted for anti-digoxygenin – this time for anti-fluorescein. 1:2000 dilution pre-absorbed anti-flourescein in 2mg/ml BSA/PBT. Followed regular staining protocol until last NTMT wash. Then Stain with fast red: 1 tablet in 2ml 0.1M Tris-HCl pH 8.2. Shook 1-3 min. Stained up to 48 hpf in fast red stain (I placed in 4C overnight) (protect from light). Times for bleach and Proteinase K that I suggest are
summarized in table 1 below. Probes used: *wt1a*\textsuperscript{122}, *wt1b*\textsuperscript{122}, *wt1a+kts*\textsuperscript{122}, *wt1b+kts*\textsuperscript{122}, *tcf21*\textsuperscript{35}, *tbx18*\textsuperscript{123}, *vcam-1* (ZGC *vcam1* cDNA, clone id: 8149942, catalog number: MDR1734, accessions: BC133177, DV597009), *itga4* (cDNA, clone id: EXELIXIS4282900, catalog number: EDR4422-98356070, accession: EE991779) and *myl7*\textsuperscript{124}.

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Table 1: Bleach and Proteinase K for *in situ* hybridization.

**Preparing Preabsorbed Antibody** (optional, but useful for probes with lots of background expression)

To reduce background staining in *in situ* hybridization, antibodies were preabsorbed in zebrafish embryos. Works best for embryos at the same stage or older than the stage of interest. (7 dpf fish worked well for 54-55 hpf and 62-63 hpf in situ). Similar to *in situ* protocol\textsuperscript{121} collect, fix, and wash, dehydrate, permeabilze, rehydrate and wash embryos. Use hydrated embryos to preabsorb the antibody in a 1:100 PBT dilution (5 µl anti-Dig in 495 µl PBT) overnight at 4°C. Pipette off the antibody solution and store in a fresh tube labeled 1:100 Anti-Dig/PBT at 4°C. Discard embryos. Dilute the antibody to 1:2000 (of original anti-Dig) for use in *in situ* hybridization

**PCR Protocol**
The plasmid containing *vcam-1* did not contain a restriction site for linearization to serve as a DNA template for RNA probe synthesis. *vcam-1* was amplified from its plasmid using primers:

VCAM T7 2 5’-TAATACGACTCACTATAGGGACCTGCAGTTCTCAGTTTTAGGG-3’ and

VCAM T3 2 5’-AATTAACCCCTCACTAAAGGGAGGATCAACAGATCTGACTTC-3’.

Primers were prepared at 1:10 dilution and plasmid template DNA was diluted to 1:100 prior to addition to PCR mix. Cycle in Thermocycler: 95°C for 4 min; 35X (95°C for 30s, 55°C for 30s, 72°C for 2 min); final extension 72°C for 7 min.

**Immunofluorescence**

Antibodies were used to detect and visualize protein localization in embryos. (Chapter 2, Fig.1,2,7, and 9) Immunostaining was conducted as previously described\(^{125,126}\) with the following modifications. For antibody detection of MF20 and GFP, embryos were fixed in 2% pfa overnight, rinsed with phosphate buffered saline solution (PBS), and blocked with 10% sheep serum, 0.2% saponin in BSA/PBS for 1 hour at room temperature. Primary antibody (1:10 MF20, 1:500 Anti-GFP (IgG rabbit or mouse) in 0.2% saponin/PBS was applied overnight at 4°C. Embryos were washed with 0.2% saponin/PBS, then incubated in 1:500 secondary antibody in 0.2% saponin/PBS Alexa Fluor anti-IgG2b-546 and either Alexa Fluor anti-IgG (rabbit)-488 or Alexa Fluor (mouse) anti-IgG-488 overnight at 4°C. Embryos were washed with 0.2% saponin/PBS and stored in PBS at 4°C. For Vcam1 and Itga4, embryos were fixed in 1% formalin and antibodies were prepared at 1:500 dilution. Alexa Fluor anti-IgG (rabbit)-488 or Alexa Fluor anti-IgG (rabbit)-546 were used as secondary antibodies. Phalloidin and DAPI staining were conducted as previously described\(^{127,128}\).

**Microscopy**
Embryos were mounted for live high-speed imaging on glass slides with electrical tape bridges, egg water with tricaine, and covered with a coverslip, similar to viewing chambers\textsuperscript{129}. For confocal imaging, embryos were embedded in 0.7-1% agarose on glass-bottom dishes, similar to agar mounting\textsuperscript{129}. Transgenic fluorescent and immunofluorescent embryos were observed under a fluorescent dissecting microscope (M\textsuperscript{2} BIO, Zeiss) or (M125, Leica) and confocal microscope (LC5, Leica). Whole-mount in situ hybridization embryos were observed under a dissecting microscope (M\textsuperscript{2} BIO, Zeiss) and photographed with a CCD (Axiocam MRe, Zeiss). 3D projections of confocal images were generated using Imaris software. Image processing included background subtraction and smoothing with a median filter. Red color was adjusted to magenta and intensities of fluorescence were adjusted using the display adjustment feature.

**Quantification and statistical analysis**

\textit{wt1}:GFP\textsuperscript{+} cells adhering to the myocardium were manually counted from individual optical slices, optical sections and the 3D projections through Imaris for (Chapter 2, Fig. 2).

\textit{tcf21}:DsRed\textsuperscript{+} cells adhering to the myocardium were counted by generating spots in Imaris (Chapter 2, Fig.7). 3D projections were flattened to two dimensions through snapshot in Imaris; ImageJ was used to take measurements of atrial length (AL) and atrial GFP (AGFP). (AL) was defined as the length of the curve of the atrial myocardium from the center of the sinus venosus to the center of the atrioventricular junction. (AGFP) was defined as the furthest point along the AL that a \textit{wt1}:GFP\textsuperscript{+} cell is detected. Atrial coverage (AC) was defined as (AGFP)/(AL) for % atrial epicardium in (Chapter 2, Fig.5). Two-tail t-test with a 95% confidence interval was used to compare control to experimental groups in all quantifications.

**Agarose pseudo sections**

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Gelatin embedding plastic moulds were used to embed previously fixed fish, and allowed to set in 1% agarose. Embryos were sectioned by hand with a blade into two pieces along the midline – cutting dorsal to ventral in one quick smooth motion while looking through dissecting microscope. They were imaged with cut surface against a glass bottom slide.

**Pharmacological cardiac contraction inhibition**

Pharmacological agents were used to inhibit cardiac contractions. 20mM 2,3-butanedione monoxime (BDM) (Sigma B-0753, Sigma–Aldrich, St. Louis, Missouri, United States) and 50µM Blebbistatin (+/- Blebbistatin (EMD) (203390) in 0.25% DMSO were used to stop heart contractions as described previously\(^{130-133}\). Control embryos were pulsed in solutions until heart stopped then transferred to egg water/175mM mannitol solution for recovery. Additional 0.25% DMSO control was conducted for Blebbistatin.

**Heart dissections and preparation for confocal microscopy**

Hearts were dissected to assess epicardial coverage. Embryos fixed overnight in 4% PFA were washed with PBT. Embryos were held in place with 2% or 3% methylcellulose and hydrated with PBT. Hearts were dissected similar to larval heart dissections\(^{134}\). Forceps were used to simultaneously hold the fish and pull the jaws dorsally. The bottom of the pericardial cavity was cut in order to excise the pericardial cavity from the body. The heart was further dissected from the pericardial cavity. Dissected hearts were placed in a small amount of methylcellulose on a rectangular coverslip and hydrated with some PBT. A ring of petroleum jelly was used to contain the PBT solution and cushion the heart, then another coverslip (square) was applied to sandwich the heart. The sides of the coverslips were sealed with clear nail polish. Hearts were imaged with
confocal microscope (LC5, Leica) with 20X objective at magnifications 2.5X-4X, the system was optimized for capture of optical sections.

**Microinjections**

Microinjections were carried out to inhibit cardiac contractions and to knockdown *tcf21*. Needles were pulled with Sutter P-97 at setting: Heat 475, Pull 60, Velocity 80, Time 150. Needles were clipped with blunt forceps to a diameter of about 0.01 mm. Droplet size was calibrated in mineral oil (Acros Organics). Injections were carried out as previously described\textsuperscript{135}. Morpholino oligos (MO) were obtained from Gene-Tools (Philomath, OR) *sih-MO (MO1-tnnt2a) (5’-CATGTTTGCTGATCTGACGCA-3’; Gene Tools)* was synthesized according to previous studies\textsuperscript{35,136}. Previous studies show that *sih*MO phenocopies *sih* mutation\textsuperscript{136}. *sih*MO was prepared at stocking concentrations of 1 mM and diluted with 0.04% phenol red to a working concentration of 100\(\mu\)M approximately 4ng was injected into *Tg(myl7:DsRed;wt1b:GFP)* embryos at one to four cell stage and examined at 24 to 96 hpf for myocardial function and survival. Standard control MO (5’-CCTCTTACCTCAGTTACAATTTATA) was designed according to the random nucleotide sequences (Gene Tools). ControlMO was prepared at stocking concentrations of 1mM and working concentration with 0.04% phenol red at 100\(\mu\)M. Approximately 4ng was injected into 1-4 cell cell embryos. ControlMO injected embryos were used as controls to compare to *sih*MO embryos. All *sih*MO embryos were screened under brightfield microscope for cardiac contractions. All of the *sih*MO embryos in this data set had no cardiac contractions.

*Tcf21MO2 (tcf21 mo2) (5’GTGTCTCACAGGGTGGACGATGT-3’; Gene Tools)* was synthesized according to previous studies\textsuperscript{67}. Tcf21MO was prepared at stocking concentrations of 1 mM and diluted with 0.04% phenol red to proper concentrations 100\(\mu\)M(tcf21MO).
Approximately 4ng was injected into Tg(myl7:DsRed; wt1b:GFP) and Tg(myl7:GFP; tcf21:DsRed) embryos at one to four cell stage and examined at 24 to 72 hpf for survival. Control embryos for tcf21MO experiment were the not injected siblings of tcf21MO embryos. All embryos in morpholino experiments were dechorionated at 24 hpf and maintained in 175mM mannitol/egg water to reduce pericardial edema. The two-sample t test was employed to identify significant changes between the treatment and control samples in the sihMO and tcf21MO experiments.
Discussion

In zebrafish, we find chamber-specific proepicardial populations that are not only spatially and temporally distinct, but also express a different set of proepicardial genes, and proepicardial asymmetry. *tcf21* is dispensable for atrial proepicardium migration, but promotes ventricular proepicardium migration. We find that cardiac contraction is required for villous-mediated, and other ventricle-specific proepicardial migration. We also discover a mechanism of migration that does not require cardiac contraction: a novel atrium-specific migration mechanism.

Figure 10: Cardiac chamber-specific PEO migration mechanisms. (A-F) Schematic of chamber-specific PEO migration mechanisms. (A,B,D) Atrial proepicardium migrates from the SV PEO onto the atrium to form atrial epicardium. (B,E) Atrial epicardium spreads to cover atrial myocardium. (B,C,F) Ventricular proepicardium forms villi - cardiac contractions are required for villous transfer and ventricular epicardium formation.

Chamber-specific proepicardial migration mechanisms are required to transfer cells from the PEO to the myocardium to form epicardium. We found a villous-mediated mechanism for proepicardial migration in zebrafish consistent with other studied species. This finding suggests that a villous mechanism is conserved across species. In zebrafish, this mechanism is ventricle-specific and requires contractions to populate the myocardium.
Ventricle-specific villous migration that requires cardiac contractions may be conserved across species. Our findings of ventricular proepicardial cells on opposite pericardial and myocardial surfaces combined with a requirement for contraction support a villous mechanism. We have shown that ventricular proepicardium is $tcf21$:DsRed$^+$ and $wt1b$:GFP$^+$. Floating PEO cells are also $wt1b$:GFP$^+$ or $tcf21$:DsRed$^+$. These cysts are likely detached cells that arise from V PEO villi. The transient nature of a villous mechanism fills the gap between proepicardial cysts and a cellular bridge structure for epicardium formation and partially accounts for the contraction requirement for all ventricular epicardium formation.

Atrial proepicardium migration initiates earlier than ventricular proepicardium migration. Although previous reports indicate that a small portion of cardiomyocytes are $wt1b$:GFP$^+^{101}$, we found that $wt1b$:GFP is predominantly expressed in epicardial cells overlying the atrial myocardium during atrial proepicardial migration 32-42 hpf. Expression of $wt1b$:GFP appears to downregulate as atrial proepicardial cells flatten on the surface of the myocardium. $wt1b$ may be required to initiate proepicardial migration and is downregulated as proepicardial cells spread and flatten on the myocardium in an epithelial-like state. This migration mechanism independent of cardiac contractions is novel in zebrafish and suggests that atrial proepicardium likely migrates via cell spreading. This finding raises questions as to atrium-specific proepicardium migration in other species. Cardiac contractions are required for V PEO migration. Although our studies mostly addressed villous-mediated migration, we found that inhibition of cardiac contractions inhibits most V PEO migration. This finding also suggests that villi, cysts and cellular bridge are all V PEO. This conclusion is consistent with the results from cyst-mediated and cellular bridge mechanism studies in which migration is only attributed to the ventricle – contribution to atrial coverage is not directly addressed in these studies. Cardiac contractions are
not required for villi formation but are required for villous transfer of V PEO cells. Intriguingly, atrial epicardium persists in *tnnt2a* knockdown embryos (Fig2D”). Atrial epicardium is not sufficient to populate the ventricular epicardium. The different requirements for cardiac contractions distinguish migration mechanisms of A PEO and V PEO to form the epicardium.

Plavicki et al. report that 60 hpf *sih*MO recipient ventricular explants co-cultured with 108 hpf control donor ventricular explants failed to form ventricular epicardium58. However, 60 hpf control recipient ventricles appear more mature than *sih*MO ventricles at 60 hpf. Immature ventricular cardiomyocytes may not accept more mature epicardial donor cells because epicardial-myocardial signaling is highly regulated. Alternatively, correct sarcomeric organization may be important for ventricular proepicardium migration.

We demonstrate a novel mechanism of proepicardial coverage in which cardiac contractions are dispensable. We find that A PEO cells migrate directly onto the adjacent atrial myocardium. Perhaps *wt1a* was not previously detected on the atrium at 40 hpf35 due to downregulation in atrial epicardium combined with overall less robust expression than *wt1b* in the proepicardium. This mechanism may be conserved across species. In most species, PEO near the sinus venosus is contiguous with the atrium. Incomplete loss of the epicardium82 may be explained by the persistence of this mechanism. Therefore, previous findings support that this novel mechanism of atrial proepicardium migration may be conserved across species. The atrial mechanism does not compensate for loss of a ventricular mechanism – this conclusion further delineates chamber-specific mechanisms of epicardium formation. The atrium is in close proximity to the SV PEO whereas the ventricle is more distant. The two populations have different strategies for migration to each chamber. Together the two mechanisms are likely more efficient than any one acting individually: spreading from one point onto the entire myocardium.
or achieving confluency from dispersed epicardial patches. Epicardial coverage at 6 dpf suggests that atrial epicardium and ventricular epicardium converge to form the enveloping epicardium.

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<th>Ventricular PEO</th>
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<td>Zebrafish&lt;sup&gt;(Khan, unpublished)&lt;/sup&gt;</td>
<td>Zebrafish&lt;sup&gt;57,101,105&lt;/sup&gt;</td>
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<td>Xenopus&lt;sup&gt;15,44&lt;/sup&gt;</td>
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Table 2: PEO gene expression across species by cardiac chamber specificity.

Support for conserved chamber-specific PEO migration mechanisms

We conducted a review of cardiac chamber-specific epicardium loss resulting from knock-down or knock-out of PEO genes across species. Tbx18 appears to be dispensable for A PEO migration and plays an early role in V PEO migration<sup>16,139</sup>. WTI likely plays an important
role in migration of A PEO and V PEO\textsuperscript{35,110}. \textit{TCF21} is dispensable for A PEO migration and may have a conserved role in V PEO migration\textsuperscript{9,15}.

We found a right-sided asymmetry in zebrafish PEO. Initially a bilateral proepicardial population at the sinus venosus, expands on the right side - giving rise to ventricular proepicardium. The transition of zebrafish proepicardium from bilateral to right-sided asymmetry is similar to chick, except that zebrafish maintain bilateral PEO symmetry at the SV. We identified early bilateral PEO symmetry at the SV PEO and late right-sided V PEO asymmetry in zebrafish. Initially bilateral SV PEO in zebrafish expands on the right side towards the ventricle, yielding a right-sided V PEO. Zebrafish exhibit an intermediate PEO morphology to chick, \textit{Xenopus}, and mouse. Chick and \textit{Xenopus} have a right-sided asymmetry associated with villi/tissue bridge formation to the AV or ventricular myocardium\textsuperscript{40,44}. Mouse exhibit villi (that do not form a bridge) that extend from the midline of the heart to the AV myocardium\textsuperscript{77}. Zebrafish have a right-sided asymmetry like chick and \textit{Xenopus}, and multicellular villi that can contact the AV myocardium and the ventricular myocardium like mouse. The rounded V PEO cells in \textit{tcf21} knockdown embryos support adhesion defects of V PEO to the ventricular myocardium similar to those found in \textit{Xenopus} and mouse\textsuperscript{9,15}. During migration, proepicardial cells adhere to the myocardium to form epicardium. \textit{tcf21} knockdown substantially reduces ventricular epicardium, but does not affect atrial proepicardium migration. A significant decrease in ventricular epicardium and rounded ventricular epicardial cell morphology suggest a reduction in proepicardial adhesions to the myocardium. Adhesion defects are consistent with findings in \textit{Xenopus} and mouse\textsuperscript{44,66}. \textit{tcf21} may transcriptionally regulate adhesion of ventricular epicardium. Since atrial proepicardium migration is not affected, proepicardial adhesions may also be chamber-specific. Types of adhesions may differ or regulation of adhesion (non-\textit{tcf21} regulated)
may differ between chambers. \textit{tcf21}:DsRed$^+$ atrial epicardium at later stages (120 hpf – 6 dpf) suggests a conserved role for \textit{tcf21} in epicardium maturation.

Proepicardial adhesion to the myocardium is required for epicardium formation. We found that \textit{vcam-1} and Vcam1 localize to the ventricular myocardium during V PEO migration and that Itga4 localizes in between V PEO cells. In zebrafish, our preliminary findings support a role for Vcam1 in V PEO adhesion and a role for Itga4 in cell-cell adhesion of villous proepicardial cells. V PEO adhesion to the ventricle is likely mediated by Itga4-Vcam1 interaction. Due to differing gene expression in proepicardial populations, it is likely that cell-surface receptors on the proepicardial cells and on the myocardium are chamber-specific. Chamber-specificity of epicardium adhesion is a novel concept.

Our findings are in line with mouse data of \textit{Itga4} null and VCAM-1 deficient mice$^{82,85,86}$. VCAM-1 and ITGa4 are required for proepicardial adhesion to the ventricular myocardium. However, partial atrial epicardium forms in \textit{Itga4} null and VCAM-1 deficient mice$^{82,85,86}$. ITGa4 and VCAM-1 are dispensable for atrial epicardium formation. Perhaps ECM interactions between atrial myocardium and atrial proepicardium are sufficient for atrial proepicardium migration. Based upon epicardial control of Fibronectin$^{82}$, Fibronectin along with other cell surface receptors may mediate atrial proepicardium-myocardium adhesion.

The current paradigm of epicardium formation refers to “epicardium” or its target “myocardium” as one collective tissue– our study supports a shift in thinking towards chamber-specificity of epicardium and myocardium. Chamber-specific proepicardial gene expression suggests pre-patterning of proepicardial cells.
Chapter 3 – Discussion and Future Directions

Potential role of directed cell migration in proepicardial coverage.

Two theories model directed cell migration of mesenchymal cells during embryonic development across organisms. Predominantly these cell migrations have been demonstrated in neural crest cells, but they may be conserved mechanisms of cell migration that occur in other tissues as well. I evaluate the possibility of these cell migration theories as they may apply to proepicardial cell migration: chase-and-run migration and contact inhibition locomotion (CIL).

Chase and run migration requires a signal that mediates chemotaxis. Chemotaxis is the “chase” part. Stromal cell-derived factor 1 (Sdf-1) and its G-protein-coupled receptor CXCR4 are known for chemotactic migration in mouse and zebrafish. Neural crest cells presenting Cxcr4 migrate towards sdf1 expressing placodes. When neural crest cells (mesothelial) contact placodal cells (epithelial cells), placodal cells are repulsed. Repulsion is based upon Planar Cell Polarity (PCP) and N-cadherin signaling. PCP signaling inhibits Rac activity at the cell contact. Inhibition of Rac in turn leads to a collapse of cell protrusions and focal adhesions, and generates an asymmetry within the placode cluster. The placode cluster migrates away from neural crest cells, whereas, the NC cells migrate due to attraction to Sdf1. Thereby, both populations coordinately migrate. Epicardial cells express cytokine cxcl12a (Sdf1) after injury and inhibition of its receptor CXCR4 impedes regeneration of the myocardium. Epicardial cells appear aggregated at the myocardial surface in CXCR antagonist or cxcr4b−/− hearts. Growing myocardium may express CXCR4 during embryonic development. Epicardial spreading on the
myocardium may be mediated by Sdf1-CXCR chemotactic migration and possibly chase-and-run between pro-epicardium and epicardium.

Contact inhibition locomotion (CIL) promotes dispersion of cells. Upon contact, cells repolarize and migrate away from each other. CIL: cell contact-->collapse of cell protrusions-->loss of polarity-->repolarization. Three requirements underlie CIL: 1) cells do not overlap; 2) two adjacent cells do not make protrusions on top of one another; 3) collision between two cells will cause them to change velocity if CIL occurs. Par3 is required for CIL between neural crest cells in *Xenopus* and zebrafish. Although CIL mediates dispersal of cells, mesenchymal neural crest cells seem to collectively migrate. This effect may be due to coAttraction. NC cells secrete complement factor C3a and express its receptor C3aR at their surface. A group of NC cells secreting C3a may establish a local gradient. NC cells that migrate away from the group are attracted back through C3a-dependent chemotaxis. C3aR signaling activates Rac1 that mediates the polarization of the single NC cell towards the group.

If CIL occurs in epicardial cells, then inhibition of par3 would cause overlapping/aggregation of proepicardial cells and prevent them from migrating/spreading. This may be occurring in Par3 mice. Therefore, CIL may be a mechanism by which ventricular proepicardium migration occurs. Contact inhibition locomotion and coAttraction may be a part of ventricular proepicardium coverage because disruption of Par3 and cell polarity affects ventricular proepicardium coverage, but not atrial proepicardium coverage. Loss of tcf21 may affect proepicardial cell polarity thereby decreasing ventricular proepicardium migration. It is possible that disrupting tcf21 may disrupt CIL of proepicardial cells.
Epicardial derived cell lineages

We have demonstrated molecular distinction of A PEO and V PEO cells: A PEO is \(wt1b^{+}\)GFP\(^+\) and \(tcf21^{+}\)DsRed\(^-\); V PEO is both \(wt1b^{+}\)GFP\(^+\) and \(tcf21^{+}\)DsRed\(^+\). When in development are these populations specified? How are they regulated? Since disruption of \(tbx5a\) and \(bmp\) signaling reduce \(tbx18\) and \(tcf21\) PEO in zebrafish, they may be important regulators of V PEO specification and proliferation. Disruption of \(tbx5a\) prior to LPM patterning affects PEO. Knockdown of \(bmp\) signaling at 36 hpf reduces PEO. These conclusions together with our findings suggest that \(tbx5a\) plays an early role in specifying PEO close to LPM patterning and that \(bmp\) signaling promotes proliferation of V PEO after 36 hpf.

We detect \(tcf21^{+}\)DsRed expression in atrial epicardium as early as 96 hpf. \(tcf21^{+}\)-lineage tracing initiated at 72-96 hpf indicates \(tcf21^{+}\) atrial epicardium lineage. Therefore, the portion of atrial epicardium that delineates from \(wt1b^{+}\)-lineage or \(tcf21^{+}\)-lineage is unclear. To further confirm our atrial epicardium mechanism and clarify atrial epicardium lineage, the \(tcf21^{+}\)-lineage line would have to be induced at 2-3 dpf rather than at 3-4 dpf. It is also possible that \(tcf21^{+}\)-lineage cells add and further mature the immature atrial epicardium initially laid out by \(wt1b^{-}\)-lineage cells. Another way to test \(tcf21\) lineage in atrial epicardium would be to test \(tcf21\) null mutant embryos for \(tcf21^{+}\)DsRed\(^+\) atrial epicardium at 6 dpf.

Our preliminary studies of \(tcf21\) knockdown in \(ET27:EGFP\) zebrafish indicate that epicardial cells may differentiate into vascular support cells within the ventricle. However, \(ET27:EGFP\) is reported to localize to subendothelium in the BA/outflow tract\(^{144}\). Therefore it is unknown whether vascular smooth muscle is a normal cell fate for \(ET27:EGFP^{+}\) epicardial cells or whether this cell fate is a result of \(tcf21\) loss like smooth muscle differentiation of \(tcf21^{-}\)-
lineage cells in other species. Double transgenic ET27:EGFP; tcf21:DsRed and additional markers such as MLCK would be needed to confirm. ET27:EGFP expression inside the ventricle appears similar to aPKC expression in tcf21-depleted heart in Xenopus – rounded nuclei within the ventricular myocardium. Additional markers would be needed to verify the identity of these cells – pericytes, smooth muscle cells, or another cell type altogether.

Myocardial lineage of proepicardial cells is controversial. Through our observations, the pattern of atrial wt1b:GFP expression resembles FHF. However, I only observe early wt1b:GFP expression in few atrial cardiomyocytes at linear heart tube stage similar to previous reports of mouse wt1-lineage. Others have also reported expression of wt1 and tbx18 in some cardiomyocytes in zebrafish. Therefore, cardiomyocytes may arise from WT1 or TBX18 PEO lineages in zebrafish and mouse. Recent reports indicate that the regulatory region of wt1b:GFP includes an element that regulates myocardium. Therefore, it is possible that regulation of wt1 or tbx18 affects PEO or myocardium cell fate. Perhaps wt1 and tbx18 lineage cells have some plasticity to become epicardium or myocardium depending on regulation. Recently, atrial cardiomyocytes have been shown to contribute to ventricular myocardium repair. I speculate that these atrial cardiomyocytes may arise from tbx18 or wt1 lineages. Thereby tbx18 and wt1 may have a role in ventricular cardiomyocyte regeneration. tcf21 epicardial lineage in zebrafish may represent a portion of epicardial-derived cells. Because the epicardium is heterogeneous in its genetic expression and lineage – there is still potential for epicardial cells to contribute to cardiomyocytes other than tcf21-lineage cells.

Cardiac precursors and proepicardial precursors have common origins in the lateral plate mesoderm. How proepicardial cells differentiate from LPM is unknown; how A PEO and V PEO differentiate from PEO is also unknown (Fig.1). I speculate that tbx5a may have a role in
specifying PEO through field antagonisms and cardiac chamber-identity similar to its role in determining FHF/SHF ratio and functional cardiac boundaries\textsuperscript{145}. Loss of \( tbx5a \) results in expansion of FHF myocardium\textsuperscript{145}. Loss of \( tbx5a \) also results in loss of \( tbx18 \) and \( tcf21 \) PEO\textsuperscript{36}. Expansion of FHF decreases SHF myocardium and PEO. Along with cardiomyocyte identity of \( wt1 \) and \( tbx18 \) labeled cells, a common precursor population of SHF and PEO is possible. BMP and WNT signaling regulate cardiomyocyte and pre-epicardium specification in human pluripotent stem cells\textsuperscript{64}. 
Figure 1. Persisting questions of proepicardial lineage. How proepicardial cells delineate from lateral plate mesoderm is unknown.

Different EPDC outcomes are possible in the atrium vs. in the ventricle. Present studies only address EPDCs in the ventricle although differentiation of EPDCs appears very compartmentalized. Regeneration studies have been conducted in the ventricle, but only recently atrial cardiomyocyte contribution to myocardium regeneration has been discovered\textsuperscript{146}. More studies of the atrial epicardium and its derivatives may uncover new pathways for regeneration.
Appendix I – *tbx18* contribution to zebrafish epicardium

**Rationale**

While developing support for the model of proepicardial migration, I looked to the expression of conserved proepicardial genes in zebrafish. One of these is genes is *tbx18*. I conducted *in situ* hybridization to observe the pattern of *tbx18* expression in the proepicardium.

![Figure 1: tbx18 contributes to both atrial and ventricular proepicardium](image)

(A-D) Whole-mount *in situ* hybridization with *tbx18* probe. (A) Arrowhead indicates PEO at the atrioventricular junction. (E) *wt1a+kts* expression in dissected heart and surrounding jaw at 96 hpf for comparison with D. Note the dorsal view of the heart in E, whereas D is a ventral view.

**Results**

Varied *tbx18* expression was found in the heart at 74 hpf. *tbx18* presented in a ring surrounding the atrioventricular junction, arrowhead (Fig.1A). *tbx18* localized to the ventricle (Fig.1B). *tbx18* expression on the ventricle and the atrium (Fig.1C). Sparse *tbx18* expression at 5 dpf on the ventricle and bulbus arteriosus (Fig.1D). Dissection from *wt1a+kts in situ* at 96 hpf to help appreciate the shape of ventricle and bulbus arteriosus in 3D (Fig.1E).

**Conclusions**

*tbx18* is likely expressed in both atrial and ventricular proepicardium populations. I took some liberty to interpret the *in situ* hybridizations as there is no contextual marker present. Without myocardial context I cannot rule out *tbx18* contribution to other layers of the heart such as the endocardium and the myocardium.
Appendix II – ET27 and ET30A chamber-specific patterns of expression

Rationale

The Korzh lab recently generated two cardiac enhancer trap lines in zebrafish that mark proepicardium: [ET27, ET(krt4:EGFP)\textsuperscript{sqet27} and ET30A, ET(krt4:EGFP)\textsuperscript{sqet30A}]\textsuperscript{144}. The ET27 line is characterized by a transgenic insertion in \textit{par-3 family cell polarity regulator} (\textit{pard3a}) gene. Par3 is part of a cell polarity protein complex. ET30A is characterized by a transgenic insertion in \textit{potassium voltage-gated channel, subfamily H (eag-related), member 5a} (\textit{kcnh5}). I evaluated whether these transgenic proepicardial lines indicated chamber-specific patterns of epicardium formation.

Results

To evaluate chamber-specific patterns of epicardium formation, ET27 and ET30A embryos were observed under a fluorescent microscope and under a confocal microscope. By confocal microscopy, I found ET27:EGFP expression in atrial and ventricular endocardium and also in ventricular proepicardium in ET27 (Fig.2A,B). Outer epithelial EGFP expression is easily visible under fluorescence (Fig.2C,C’). EGFP is expressed in ventricular epicardium and proepicardium and atrial myocardium in ET30A (Fig.2D). Interestingly, it is abundantly expressed in ventricular proepicardium close to the atrioventricular junction (Fig.2E). Fluorescence in the outer epithelium impedes early observation of atrial proepicardium without confocal microscopy (Fig.2F,F’).
Figure 2: Chamber-specific patterns of ET27 and ET30A expression. (A, B, D, E) Optical sections from live confocal images of embryos. (C, C’, F, F’) Brightfield and fluorescent micrographs of live embryos. (A) EGFP+ cells in the ventricular epicardium, endocardium and myocardium. (B) EGFP+ cells in ventricular epicardium and atrial endocardium. (D) EGFP+ cells in the ventricular epicardium and atrial myocardium. (E) EGFP+ cells in V PEO. Note fluorescence in outer epithelium in (C’) - also in pericardial wall (A and B). (D, E) EGFP expression in ET30A embryos. (H, H’) Outer epithelium expresses EGFP in ET30A similar to ET27 (C,C’).

Conclusions

We asked whether ET27 and ET30A proepicardial lines support chamber-specific proepicardium migration. We find that the ET27 labels atrial and ventricular endocardium and ventricular proepicardium while ET30A labels ventricular proepicardium and epicardium and atrial myocardium. EGFP expression is ventricle-specific to proepicardium and epicardium in both lines at about 2-3 dpf. EGFP expression in the atrial endocardium if ET27 and in the atrial myocardium of ET30A line does not add to atrial proepicardium expression. Therefore, both lines have limitations that do not make them preferable over wt1b:GFP for atrial proepicardium studies at 2-3 dpf. ET27 and ET30A are not useful for fluorescent observation of atrial proepicardium due to their outer epithelial expression and expression in other cardiac lineages such as endocardium and myocardium. Perhaps, confocal imaging of earlier stages of both lines
would reveal a more robust expression in atrial proepicardium. Despite limitations of atrial proepicardium, both lines maybe useful for observation of ventricular proepicardium. Expression patterns of both epicardial marker lines ET27 and ET30A support chamber-specific patterns of epicardium formation.


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