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► **To cite this version:**

Claudio Cortes, Alexandre Francou, Christopher de Bono, Robert G. Kelly. Epithelial properties of the second heart field. *Circulation Research*, 2018, 122 (1), pp.142-154. 10.1161/CIRCRESAHA.117.310838 . hal-02117787v2

**HAL Id: hal-02117787**

**<https://hal.science/hal-02117787v2>**

Submitted on 29 Apr 2020

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# Epithelial Properties of the Second Heart Field

Claudio Cortes, Alexandre Francou, Christopher De Bono, Robert G. Kelly

**Abstract:** The vertebrate heart tube forms from epithelial progenitor cells in the early embryo and subsequently elongates by progressive addition of second heart field (SHF) progenitor cells from adjacent splanchnic mesoderm. Failure to maximally elongate the heart results in a spectrum of morphological defects affecting the cardiac poles, including outflow tract alignment and atrioventricular septal defects, among the most common congenital birth anomalies. SHF cells constitute an atypical apicobasally polarized epithelium with dynamic basal filopodia, located in the dorsal wall of the pericardial cavity. Recent studies have highlighted the importance of epithelial architecture and cell adhesion in the SHF, particularly for signaling events that control the progenitor cell niche during heart tube elongation. The 22q11.2 deletion syndrome gene *Tbx1* regulates progenitor cell status through modulating cell shape and filopodial activity and is required for SHF contributions to both cardiac poles. Noncanonical Wnt signaling and planar cell polarity pathway genes control epithelial polarity in the dorsal pericardial wall, as progenitor cells differentiate in a transition zone at the arterial pole. Defects in these pathways lead to outflow tract shortening. Moreover, new biomechanical models of heart tube elongation have been proposed based on analysis of tissue-wide forces driving epithelial morphogenesis in the SHF, including regional cell intercalation, cell cohesion, and epithelial tension. Regulation of the epithelial properties of SHF cells is thus emerging as a key step during heart tube elongation, adding a new facet to our understanding of the mechanisms underlying both heart morphogenesis and congenital heart defects. (*Circ Res.* 2018;122:142-154. DOI: 10.1161/CIRCRESAHA.117.310838.)

**Key Words:** cell adhesion ■ cell shape ■ epithelium ■ heart development ■ mesoderm ■ progenitor cells

Folding and remodeling of coherent sheets of polarized cells, or epithelia, are central morphogenetic events during organogenesis.<sup>1,2</sup> The vertebrate heart forms from cardiac progenitor cells located in bilateral epithelial sheets that fuse in the ventral midline of the early embryo.<sup>3</sup> Subsequent growth of the heart tube occurs by addition of extracardiac progenitor cells from adjacent cardiopharyngeal mesoderm.<sup>4,5</sup> Perturbation of the addition of these cells, known as the second heart field (SHF), results in a failure to fully elongate the heart tube and underlies a spectrum of common forms of congenital heart defects (CHD). Failure to maximally extend the embryonic outflow tract (OFT) at the arterial pole of the heart results in OFT alignment defects including double outlet right ventricle, overriding aorta, and tetralogy of Fallot.<sup>6-8</sup> At the venous pole, the SHF gives rise to the dorsal mesenchymal protrusion; abnormal development of this structure contributes to atrioventricular septal anomalies, including ostium primum defects.<sup>6,8-10</sup> Studies of SHF development have identified multiple signaling pathways and transcription factors that control progenitor cell proliferation and differentiation.<sup>6,7</sup> Mutation of these regulatory components, as well as exposure to environmental stress such as hypoxia, perturbs SHF development leading to CHD.<sup>6,11</sup> In contrast, the cell biology of the SHF has received less attention. Here, we review recent studies that have

investigated the epithelial properties of SHF cells and how their perturbation affects cardiac development. After a brief introduction to epithelial polarity and the epithelial features of early cardiac progenitor cells, we will focus on evidence that polarity of SHF cells in the dorsal wall of the pericardial cavity is essential to maintain proper intercellular signaling in the progenitor cell niche and to maximally extend the heart tube. In particular, we will examine how the 22q11.2 deletion (DiGeorge) syndrome gene *Tbx1* regulates progenitor cell status by controlling cell shape and dynamic basal filopodia in the SHF. The following section will consider PCP (planar cell polarity) factors, including noncanonical Wnt ligands. Several of these genes have been implicated in epithelial architecture in the dorsal pericardial wall and in the transition zone during progenitor cell differentiation at the cardiac poles. Finally, we will discuss mechanisms of heart tube elongation based on PCP and epithelial tension in the SHF. Together these findings identify control of the epithelial properties of cardiac progenitor cells as an additional regulatory step during SHF development.

## Epithelial Polarity

At its simplest, formation of the heart from epithelial progenitor cells can be considered to be a process of cardiac origami by which a 2-dimensional sheet is folded into a

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*Circulation Research* is available at <http://circres.ahajournals.org>

DOI: 10.1161/CIRCRESAHA.117.310838

### Nonstandard Abbreviations and Acronyms

<b>BMP</b>	bone morphogenetic protein
<b>CHD</b>	congenital heart defect
<b>EMT</b>	epithelial to mesenchymal transition
<b>FGF</b>	fibroblast growth factor
<b>OFT</b>	outflow tract
<b>PCP</b>	planar cell polarity
<b>SHF</b>	second heart field
<b>VEGF</b>	vascular endothelial growth factor

complex 3-dimensional structure.<sup>12,13</sup> Epithelial remodeling during morphogenesis is driven by a combination of cell and tissue-wide forces.<sup>1</sup> Individual cells within an epithelium are polarized such that cytoplasmic and membrane components are distributed in stereotypical and spatially discrete domains (Figure 1A and 1B). Polarization defines an apicobasal axis characterized by tight junctions, which create a diffusion barrier separating apical from basolateral membrane domains, and adherens (or adhesion) junctions regulating contact with adjacent cells.<sup>12</sup> The apical domain of polarized cells is established and maintained by protein complexes including aPKC, Par3, and Crumbs isoforms, whereas a Scribble-positive basolateral domain is in contact with a basal lamina and the extracellular matrix (Figure 1B). Calcium-dependent cadherin adhesion proteins are enriched in the plasma membrane of adherens junctions and coupled intracellularly to catenin proteins including  $\beta$ -catenin, which is also a major intracellular component of the canonical Wnt signaling pathway. In turn, catenin proteins form a complex that can physically bind F-actin, transducing cadherin extracellular interactions to the intracellular actin cytoskeleton.<sup>14</sup> At the cellular level, epithelial morphogenesis is regulated by forces emerging from the interplay between the actomyosin cytoskeleton and cell–cell adhesion complexes together with cell–matrix interactions.<sup>15</sup> Epithelial morphogenesis is also driven by tissue-wide forces such as tension and spatially controlled patterns of proliferation that propagate across the epithelium, including externally generated forces driving global epithelial shape.<sup>1</sup> Polarization takes place not just apicobasally (perpendicular to the epithelium) but is also established in the plane of the epithelium, termed PCP, and is regulated by a set of PCP proteins and the noncanonical Wnt signaling pathway (Figure 1C).<sup>16,17</sup> Initially characterized based on its role in sensory bristle and eye development in the fruit fly *Drosophila*, PCP signaling defines cell position within the epithelial sheet with respect to neighboring cells. PCP has been shown to regulate diverse processes during vertebrate and invertebrate development, playing a central role in epithelial patterning and morphogenesis, including polarized cell intercalation and the coordination of epithelial polarity with embryonic axes.<sup>16</sup>

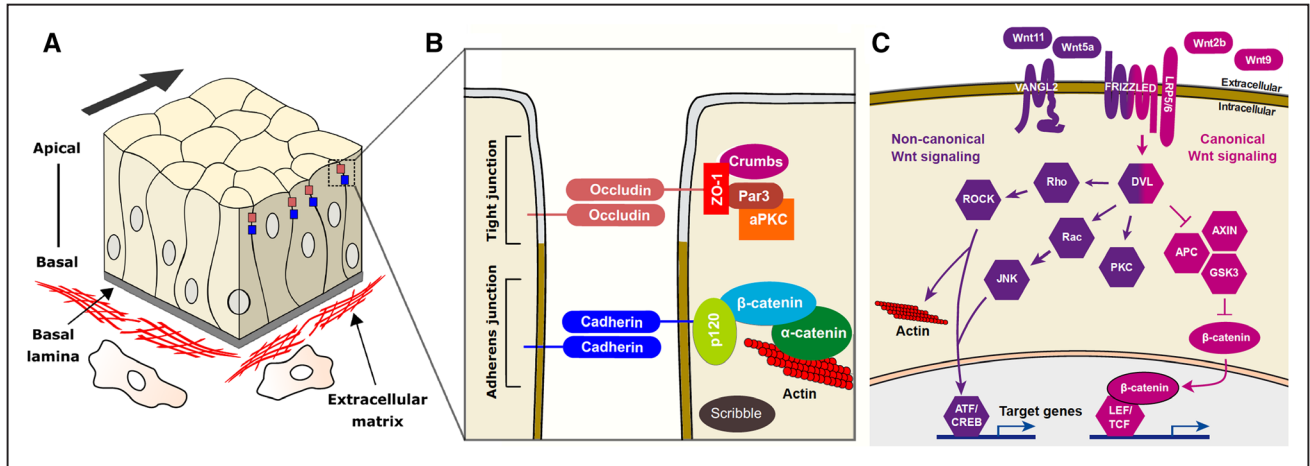
### Early Heart Development

Transitions either from epithelial to mesenchymal (EMT) or mesenchymal to epithelial states are essential developmental steps associated with progressive fate restriction and

organogenesis, as well as during cancer and fibrosis.<sup>18</sup> EMT and mesenchymal to epithelial transition can be partial, allowing epithelial sheet migration.<sup>19</sup> During heart development, cardiac progenitor cells undergo iterative cycles of EMT and mesenchymal to epithelial transition; for example, EMT in OFT valve primordia or during epicardial development corresponds to tertiary EMT events in these lineages.<sup>18</sup> Cardiac identity is specified at or before gastrulation, as epithelial epiblast cells in the primitive streak undergo EMT to give rise to nascent mesoderm. EMT is followed by migration of cardiac progenitor cells to anterior lateral regions of the embryo to form the bilateral heart-forming regions.<sup>20</sup> EMT of cardiogenic mesoderm is regulated at the transcriptional level by the basic helix-loop-helix factors *Mesp1* and *Mesp2*, and *Mesp1* is further required for polarized progenitor cell migration, through the activation of genes encoding the guanine nucleotide exchange factor RASGrp3 and PCP protein *Prickle1*.<sup>21</sup> Recent evidence has shown that *Mesp1* is activated independently in first heart field progenitor cells, which give rise to the early heart tube, and the SHF, highlighting the early segregation of these lineages.<sup>22,23</sup>

As the embryonic coelom forms in lateral mesoderm, cardiac progenitor cells undergo a mesenchymal to epithelial transition and become restricted to splanchnic epithelium adjacent to future foregut endoderm. Progressive restriction of the adhesion molecule N-cadherin has been implicated in sorting splanchnic from somatic mesoderm, adjacent to surface ectoderm, and N-cadherin remains expressed apically in epithelial cardiac progenitor cells.<sup>24</sup> The acquisition of apicobasal polarity occurs in an anterior to posterior direction and is accompanied by membrane  $\beta$ -catenin expression and lateral accumulation of  $\text{Na}^+, \text{K}^+$ -ATPase. In addition, basal enrichment of  $\beta 1$ -integrin is required for migration across a fibronectin-rich extracellular matrix.<sup>24,25</sup> Epithelialization may demarcate the transition between dispersive and coherent growth phases of myocardial progenitor cells,<sup>26</sup> and experiments in avian embryos have shown that this step is essential for early heart formation and myocardial differentiation in the first heart field.<sup>27</sup> Differentiation initiates as right and left cardiac primordia converge and fold ventrally in the anterior midline of the embryo, concomitant with foregut closure, to give rise to beating cardiomyocytes in the cardiac crescent and early heart tube<sup>28,29</sup> (Figure 2). Interactions with the underlying endoderm play critical roles in myocardial induction, midline convergence, and early heart tube formation.<sup>3,31,32</sup>

Insight into the genetic regulation of the epithelial properties of early myocardial cells has come from analysis of the zebrafish embryo. Polarized epithelial organization and myocardial cell coherence mediated by the apical determinants PKC $\iota$  and MPP5 are required for the correct formation of the zebrafish heart tube.<sup>33</sup> Furthermore, proper deposition of the extracellular matrix component fibronectin is required for both establishment of junctions and the epithelial organization of zebrafish myocardial precursors.<sup>34</sup> The transcription factor *hand2* is necessary for setting up apicobasal polarity in these cells; in *hand2* mutant embryos, the junctional protein ZO1 is mislocalized, apical proteins are absent, and fibronectin deposition is enhanced.<sup>35,36</sup>



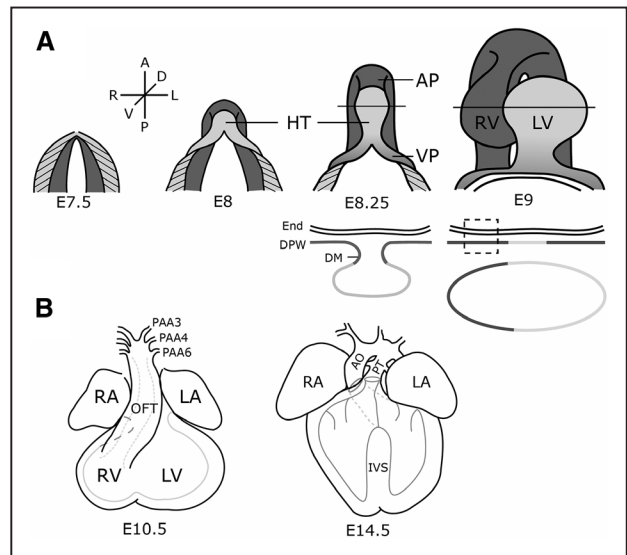
**Figure 1. Features of polarized epithelial cells and Wnt signaling pathways.** **A**, Basic features of epithelial cells showing stereotypical apicobasal cell domains and underlying mesenchymal cells. Planar cell polarity, orthogonal to the apicobasal axis, is indicated by the large arrow. **B**, Detailed schema of the boxed region in **A** showing tight junctions separating apical and basolateral membrane domains and adherens junctions, focusing on elements discussed in the text. **C**, Cartoon showing intersections between  $\beta$ -catenin Wnt signaling, noncanonical Wnt signaling, and planar cell polarity pathway components referred to in this review. DVL indicates dishevelled; GSK3, glycogen synthase kinase 3; JNK, Jun N-terminal kinase; LEF, lymphoid enhancer binding factor; LRP, lipoprotein receptor-related protein; and TCF, T-cell factor.

### Second Heart Field

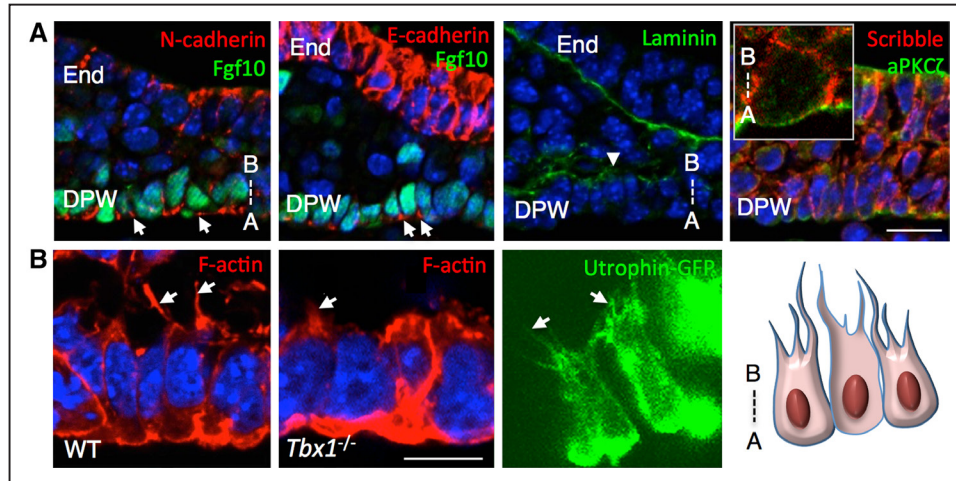
In birds and mammals, the early heart tube is a myocardial trough separated from an endocardial lining by cardiac jelly. The early heart tube is attached along its entire length to splanchnic mesoderm in the dorsal pericardial wall by the dorsal mesocardium, where the folds of the right and left splanchnic epithelia converge (Figure 2A).<sup>37</sup> Rupture of the dorsal mesocardium isolates the tubular heart in the pericardial cavity where it remains attached to splanchnic mesoderm at the arterial and venous poles. The cardiac poles converge as rightward looping and heart tube elongation take place.<sup>38</sup> During this process, the heart tube elongates by progressive addition of SHF cells from the dorsal pericardial wall (Figure 2A).

SHF cells are characterized by the expression of genes encoding transcription factors such as *Isl1*, *Six2* and *Tbx1* and the growth factor *Fgf10* (fibroblast growth factor 10).<sup>30,39,40</sup> The SHF lies within a larger field of cardiopharyngeal mesoderm that also encompasses craniofacial skeletal muscle progenitor cells in the mesodermal core of the pharyngeal arches.<sup>5,41–43</sup> Vital dye labeling and genetic lineage analyses have identified an early proliferative center in posterior cardiopharyngeal mesoderm from which cells segregate into anterior and posterior SHF domains to contribute to the arterial and venous poles of the heart.<sup>44–48</sup> Progressive addition of SHF cells at the arterial pole gives rise to right ventricular and OFT myocardium and smooth muscle cells associated with the great arteries.<sup>39,40,49–51</sup> For example, SHF cells derived from *Six2* expressing progenitor cells can be labeled, using inducible Cre-lox technology, as they progress across the dorsal pericardial wall and into the arterial pole of the heart at different times after induction.<sup>40</sup> Myocardial differentiation of SHF cells is regulated by a balance between prodifferentiation signals, such as BMP (bone morphogenetic protein) signals, and proliferation pathways, such as canonical Wnt and FGF signaling as well as Hedgehog signaling from pharyngeal endoderm.<sup>6,7,52</sup> The SHF contains multipotent cardiovascular progenitor cells, which give rise to smooth muscle and endothelial cells as well as myocardium, although how and when cell fate choices

are regulated *in vivo* are not fully understood.<sup>22,53,54</sup> A transition zone or zone of commitment has been identified at the junction between the dorsal pericardial wall and distal OFT where



**Figure 2. Early heart development and the second heart field (SHF).** **A**, Cartoon showing the formation of the linear heart tube by folding of epithelial sheets in splanchnic mesoderm and subsequent heart tube elongation by addition of SHF progenitor cells (dark shading) to the arterial pole (AP) and venous pole (VP) of the heart. Transverse sections illustrate the connection between the heart tube and dorsal pericardial wall that is lost on rupture of the dorsal mesocardium (DM). The box indicates the region of the dorsal pericardial wall (DPW) and underlying endoderm (End) shown in Figure 3A. Anterior (A)–posterior (P), right (R)–left (L), and dorsal (D)–ventral (V) embryonic axes are indicated. **B**, The fully elongated and looped embryonic heart (E10.5) and the 4-chambered heart after septation (E14.5). Maximal elongation of the outflow tract is essential for correct alignment of the right and left ventricles with the pulmonary trunk and ascending aorta. AO indicates aorta; HT, heart tube; IVS, interventricular septum; LA, left atrium; LV, left ventricle; OFT, outflow tract; PAA, pharyngeal arch artery; PT, pulmonary trunk; RA, right atrium; and RV, right ventricle. Figure derived from Kelly et al.<sup>30</sup>



**Figure 3. Epithelial properties of second heart field (SHF) cells.** **A**, Immunofluorescence of transverse sections at the level indicated by the box in Figure 2A, showing that the SHF is an atypical epithelium composed of cells with apically restricted N-cadherin and E-cadherin containing adherens junctions (arrows), a discontinuous basal lamina (arrowhead) and distinct apical and basolateral membrane domains labeled by aPKC $\zeta$  and Scribble, respectively. Note the lateral accumulation of E-cadherin and continuous basal lamina in epithelial cells in overlying pharyngeal endoderm (End). **B**, The basolateral membrane of SHF cells in the dorsal pericardial wall (DPW) is characterized by *Tbx1*-dependent filopodial protrusions (arrows) enriched in filamentous actin labeled with Phalloidin (red) or Utrophin-GFP (green fluorescent protein; green). The typical features and morphology of SHF epithelial cells as shown in the bottom right panel. Scale bars: A, 20  $\mu$ m; B, 10  $\mu$ m. A indicates apical; and B, basal. Adapted from Francou et al<sup>64</sup> with permission. Copyright ©2014, The Company of Biologists Ltd.

progenitor cell markers such as *Isl1* and *Tbx1* are downregulated and the myocardial transcriptional program activated in cardiomyoblasts expressing the transcription factor *Hoxp*.<sup>52,55,56</sup> VEGF (vascular endothelial growth factor) has been proposed to regulate the allocation of SHF cells to endocardial versus myocardial lineages within the transition zone.<sup>57</sup> Failure of proper deployment of SHF cells to the arterial pole results in OFT shortening. This leads to insufficient OFT length for correct rotation and alignment of the ascending aorta and pulmonary trunk with the left and right ventricles during cardiac septation (Figure 2B).<sup>38,58</sup> SHF defects thus underlie common forms of outlet CHD, such as double outlet right ventricle, overriding aorta, and tetralogy of Fallot. Furthermore, signal exchange between the SHF and cardiac neural crest cells, essential for division of the OFT into the ascending aorta and pulmonary trunk, is required to coordinate arterial pole development.<sup>6</sup> At the venous pole, SHF cells contribute to atrial and venous pole myocardium. This includes the dorsal mesenchymal protrusion, or vestibular spine, which forms the muscular base of the primary atrial septum; abnormal development of this structure results in atrioventricular septal defects.<sup>9,10,59</sup> Thus, perturbation of these late adding progenitor cells leads to a spectrum of CHD affecting both cardiac poles. For example, loss of function of *Tbx1*, the major gene implicated in 22q11.2 deletion (or DiGeorge) syndrome, leads to decreased proliferation and elevated differentiation in the SHF, resulting in reduced SHF contributions to both poles of the heart and consequent OFT and atrioventricular septal CHD.<sup>54,60,61</sup>

### Epithelial Properties of SHF Cells

Although molecular characterization of the SHF initiated in 2001, prior work from Virágh and Challice<sup>62</sup> had suggested that splanchnic mesodermal cells in the dorsal pericardial wall contributed to growth of the arterial pole of the mouse heart. On the basis of observations using light and transmission electron microscopy, these authors proposed that cuboidal

epithelial cells in splanchnic mesoderm transform into cardiac muscle at the arterial pole of the heart.<sup>62</sup> Subsequently, Waldo et al<sup>51,63</sup> described avian SHF cells in the dorsal pericardial wall, termed the secondary heart field, as constituting a pseudostratified columnar layer of epithelial cells. More recently, epithelial features of SHF cells in the dorsal pericardial wall of mouse embryos have been characterized by immunofluorescence.<sup>56,64,65</sup> SHF cells have a smooth apical surface facing the pericardial cavity, with accumulation of *Crumbs3*, *Par3*, and aPKC $\zeta$  and apical monocilia.<sup>64</sup> SHF cells are also characterized by apically positioned ZO1-positive tight junctions adjacent to N-cadherin-positive adherens junctions and an atypical mesenchymal-like basolateral membrane labeled by Scribble (Figure 3).<sup>64</sup> The basolateral domain of epithelial SHF cells is characterized by filopodial-like protrusions containing filamentous actin and microtubules.<sup>64,66</sup> It remains unclear whether the onset of epithelialization of SHF cells proceeds through a similar sequence of events to first heart field cells and how this upstream process is regulated. However, spatiotemporal analysis revealed that the epithelial properties of SHF cells progressively mature during progenitor cell deployment.<sup>56,64,67</sup> For example, apically restricted E-cadherin-containing adherens junctions and a basal lamina are apparent in the anterior SHF as cells approach the transition zone, consistent with increasing epithelial cohesion before differentiation.<sup>56,64,67</sup> In the following section, we will focus on the epithelial cell biology of SHF cells in the dorsal pericardial wall and its significance for maintaining the progenitor cell niche.

### Dynamic Filopodia and Insights Into the Regulation of Cell Biology by *Tbx1*

The dynamic properties of basal filopodia in murine SHF cells in the dorsal pericardial wall were explored using time-lapse confocal imaging of thick slice cultures of mouse embryos, revealing that the filopodia are highly dynamic and contact

underlying mesenchymal cells, neighboring cells in the epithelium as well as ventral foregut endoderm (Figure 3).<sup>64</sup> Filopodia have been proposed to mediate long-distance signaling during vertebrate morphogenesis, such as, for example, Hedgehog signaling in avian limb mesenchyme.<sup>68,69</sup> Signaling filopodia have also been observed on the basal side of epithelial cells in avian somites where they have been associated with retrograde transport of the receptor Fzd7<sup>70</sup> and deposition of fibronectin and Hedgehog signaling.<sup>71</sup> Whether basal filopodia play a role in transducing intercellular signaling events in the SHF remains to be determined; however, they are enriched in phosphotyrosine, consistent with activation of membrane receptor tyrosine kinases.<sup>64</sup> Alternatively, basal filopodia in the SHF may be involved in cell movement into or out of the epithelium. Indeed, intercalation of *Wnt5* expressing mesenchymal cells may contribute to growth of the epithelium in the posterior region of the dorsal pericardial wall, as will be discussed in detail below.<sup>66</sup> On the other hand, basal filopodia may indicate cell movement out of the epithelium into underlying mesenchyme where cells may adopt noncardiac fates, for example, contributing to endothelium of the pharyngeal vasculature.<sup>72</sup> Localized EMT of coelomic somatic mesoderm has been shown to generate mesenchyme at the onset of limb outgrowth.<sup>73</sup> Filopodia may also be involved in cell movement during epithelial sheet migration through dynamic focal adhesions with the extracellular matrix and underlying endoderm, a process that has been implicated in epithelial tube formation in *Drosophila*.<sup>74</sup> Finally, basal protrusions in differentiated cells in the OFT are required for the process of myocardialization that generates the muscular outlet septum,<sup>75</sup> and basal filopodia in the dorsal pericardial wall could potentially prefigure this activity. Further dynamic imaging and functional studies are necessary to resolve these questions.

Observation of *Tbx1* null embryos revealed that loss of Tbx1 severely impairs filopodia formation and also reduces the basolateral membrane domain of SHF cells, increasing the circularity of cells in the dorsal pericardial wall (Figure 3B).<sup>64</sup> Among the epithelial proteins analyzed, only aPKC $\zeta$  was quantitatively modified in *Tbx1* mutant embryos, being upregulated and losing its apical localization. As an apical determinant, upregulation of aPKC $\zeta$  could contribute to the reduction of the basal domain of *Tbx1* mutant SHF cells, with a concomitant deregulation of basal domain-dependent signaling. In support of this model, embryo culture in the presence of a physiological activator of aPKC $\zeta$  phenocopied the epithelial defects seen in *Tbx1* null embryos, leading to increased circularity and reduced filopodia in SHF cells; furthermore, aPKC $\zeta$  activator-treated embryos showed decreased proliferation and ectopic differentiation in the dorsal pericardial wall and a shortened OFT.<sup>64</sup> This is consistent with a signaling role for basal filopodia in regulating proliferation and differentiation in the SHF. Moreover, these results suggest that the regulation of the epithelial properties of SHF cells by Tbx1 may be a primary role of this transcription factor in heart development. In support of this conclusion, recent genome-wide identification of loci regulated by Tbx1 and histone 3 lysine 4 monomethylation identified pathways involved in cell morphology, adhesion, and regulation of the actin cytoskeleton, rather than those directly involved in cell proliferation and differentiation.<sup>76</sup> In

additional support of a role for Tbx1 in regulating cell shape, zebrafish *tbx1* has been shown to modulate cardiomyocyte cell shape during looping morphogenesis, through regulating the expression of genes encoding the noncanonical Wnt ligand Wnt11 and adhesion molecule *alcama*.<sup>77</sup> Together these data point to the regulation of basic cell biological mechanisms underlying Tbx1 function in the SHF with important implications for the origin of 22q11.2 deletion syndrome patient phenotypes. Detailed characterization of Tbx1 target genes in the SHF, including identification of the mechanisms by which Tbx1 regulates aPKC $\zeta$  activity, will be required to further explore this. The extent to which other transcriptional regulators of SHF development similarly control the epithelial features of these progenitor cells remains to be determined. Candidates include *Hand2*, which controls apicobasal polarity of cardiac progenitor cells in the zebrafish embryo and is required in the murine SHF for progenitor cell survival.<sup>35,78</sup> SHF cell survival is also compromised, and *Hand2* is downregulated in embryos lacking the transcription factors *Msx1* and *Msx2*, leading to a spectrum of OFT CHD.<sup>79</sup>

### Signaling Pathways and the Epithelial SHF Niche

Progressive SHF deployment is orchestrated by intercellular signaling events, including autocrine signaling and signal exchange with surrounding pharyngeal ectodermal and endodermal epithelia and neural crest-derived mesenchyme. Signaling pathways implicated in SHF development include Wnt, Notch, FGF, BMP, Hedgehog, retinoic acid, and noncanonical ( $\beta$ -catenin independent) Wnt pathways (reviewed in <sup>6,7</sup>). Epithelial properties of SHF cells, such as filopodia with a potential signaling role, are likely to be important in determining the SHF progenitor cell niche during heart tube extension. Primary cilia also play major roles in signal transduction and have been implicated as a cause of CHD.<sup>80,81</sup> In particular, cilia are required to establish embryonic laterality pathways that direct cardiac looping morphogenesis.<sup>82</sup> Primary cilia are observed on the apical surface of SHF cells and in other cell types during heart development, including valve mesenchyme and endocardium<sup>64,83,84</sup>; their potential role in the SHF remains to be investigated, for example, by conditional loss of function of regulators of ciliogenesis.

Cell–cell adhesion has been implicated in defining the SHF progenitor cell niche. N-cadherin-deficient embryos have defects in myocardial growth and adhesion and fail to fully elongate the heart tube.<sup>85</sup> Soh et al<sup>65</sup> inactivated N-cadherin in the SHF using conditional mutagenesis and observed that the resulting embryos had a hypoplastic OFT and right ventricle. This was associated with decreased proliferation and elevated differentiation in the SHF. Loss of N-cadherin led to a reduction in cellular  $\beta$ -catenin levels and a decrease in components of the canonical Wnt signaling pathway, a major driver of proliferation in the SHF; by conditionally restoring  $\beta$ -catenin signaling, the authors could partially rescue the mutant phenotype.<sup>65</sup> N-cadherin is also required for heart development in the zebrafish; mutation of N-cadherin in the *glass onion* mutant results in morphological and differentiation defects and altered myocardial cell shape, although a specific requirement in SHF cells has not been described.<sup>86</sup> Adherens junction

proteins have been shown to regulate progenitor cells in other tissues. For example, E-cadherin, also expressed in the anterior dorsal pericardial wall, is regulated by FGF signaling in the mouse incisor where it both maintains the dental stem cell niche and promotes collective epithelial cell migration<sup>87</sup>; interestingly, this process is *Tbx1* dependent.<sup>88</sup> In additional support of a role for adhesion-mediated interactions in SHF development, Zeng and Yelon<sup>89</sup> have demonstrated that the cell adhesion molecule *Cadm4* restricts the size of the zebrafish OFT downstream of FGF signaling, by regulating the size of the progenitor cell pool. Together these results highlight the importance of cell adhesion in the SHF for both signal transduction and maintenance of the progenitor cell niche.

### PCP in the Transition Zone

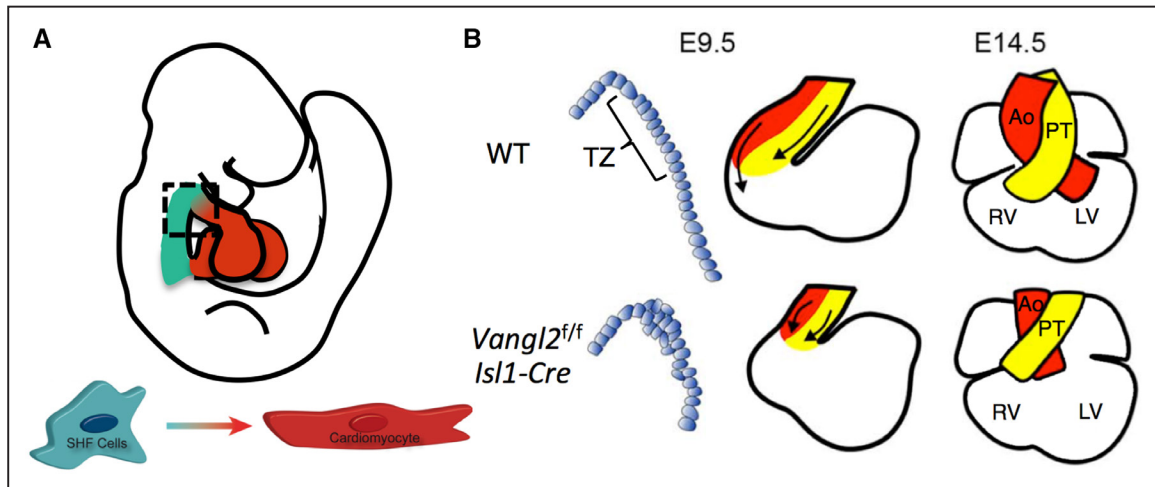
PCP proteins have been implicated in OFT CHD, including alignment defects such as double outlet right ventricle, although the underlying mechanisms have remained unclear. Two PCP proteins, *Vangl2* and *Prickle1*, have recently been shown to be required for normal establishment of cell polarity in the transition zone as SHF cells differentiate at the arterial pole of the heart (Figure 4A).<sup>56,90</sup> In the case of *Vangl2*, conditional gene inactivation in the SHF using *Isl1-Cre* resulted in a shortened OFT at midgestation and double outlet right ventricle at fetal stages.<sup>56</sup> Detailed analysis of epithelial properties in the transition zone at earlier stages revealed disruption of cell polarity, including mislocalization of N-cadherin, E-cadherin, and Scribble, together with loss of apical enrichment of aPKC $\zeta$  and an expanded basolateral domain. These changes suggest perturbation of both apicobasal and planar polarity in the transition zone. Abnormal positioning of cells within the plane of the epithelium and epithelial thickening is likely to cause the OFT shortening and alignment defects in SHF conditional *Vangl2* mutant embryos (Figure 4B).<sup>56</sup> These changes were accompanied by precocious activation of differentiation markers in the transition zone suggesting early loss of the progenitor cell phenotype, further illustrating the importance of epithelial architecture in controlling SHF cell differentiation. Similarly, a missense mutation in the gene encoding the PCP protein *Prickle1* in the *Beetlejuice* mutant mouse leads to a shortened OFT and consequent alignment defects.<sup>90</sup> Again, aPKC $\zeta$  localization was disrupted, associated with a thickened epithelium in the transition zone. The small GTPase *Rac1* has been implicated in apicobasal and PCP pathways and the regulation of cytoskeletal dynamics; conditional loss of function in the SHF has shown that *Rac1* is required for maximal proliferation and epithelial organization in the SHF, including apical localization of aPKC $\zeta$ , as well as OFT lengthening and alignment.<sup>91</sup> Interestingly, in polarized neural progenitor cells, apical aPKC $\zeta$  has been shown to negatively regulate the activity of the cyclin-dependent kinase inhibitor p27, thus promoting proliferation and blocking differentiation.<sup>92</sup> Whether this mechanism underpins PCP OFT phenotypes remains to be seen. Together, these phenotypes highlight the importance of PCP in maintaining epithelial architecture in the transition zone.

Epithelial properties in the distal OFT<sup>91,92</sup> are also regulated by the Wnt ligand *Wnt11*. *Wnt11* promotes cardiogenesis via noncanonical ( $\beta$ -catenin independent) Wnt signaling.<sup>93</sup>

*Wnt11* null embryos have shortened OFTs and double outlet right ventricle; cytoarchitecture is altered in the OFT of mutant hearts, including a diminished basal lamina and reduced Scribble expression.<sup>94</sup> Interestingly, *Wnt11* regulates *TGF $\beta$ 2* expression in the SHF and distal OFT, and similar polarity defects were observed in *TGF $\beta$ 2* mutant embryos, which also have outflow alignment defects.<sup>94,95</sup> Conditional mutagenesis has recently shown that *Wnt11* is required in differentiated cardiomyocytes for OFT development, whereas deletion of *Wnt11* in the SHF using *Isl1-Cre* leads to both OFT and atrioventricular septal defects. The latter result from reduced proliferation in the posterior region of the SHF, leading to hypoplasia of the dorsal mesenchymal protrusion and subsequent failure to properly septate the heart.<sup>96</sup> Defective filopodial activity during OFT myocardialization has been observed in both PCP and *Rac1* mutant embryos, potentially as a consequence of abnormal epithelial polarity in the transition zone.<sup>91,97</sup> Furthermore, the small GTPase RhoA and Rock1 kinases have been shown to be downstream mediators of the *Vangl2* myocardialization phenotype.<sup>97</sup> Loss of protrusive activity of OFT cardiomyocytes was also observed in hearts lacking *Daam1*, a formin-related protein implicated in PCP-driven actin cytoskeletal modification.<sup>98</sup> Defective myocardialization has been observed in other mouse mutants including *Gjal* null embryos; loss of *Gjal*, encoding Connexin 43, also affects directional cell migration in epicardial cells.<sup>99</sup> During subsequent development, polarity and cell shape changes play critical roles in multiple aspects of cardiomyocyte growth within the embryonic and fetal heart, including looping morphogenesis, epicardial development, chamber-specific growth patterns, and in the formation and compaction of trabeculae.<sup>50,100–102</sup> PCP signaling has been implicated in many of these processes. For example, Scribble, *Vangl2*, and *Wnt11* are all required for correct localization of N-cadherin in ventricular cardiomyocytes, leading to defects in cardiomyocyte adhesion and organization in mutant hearts.<sup>103,104</sup> The extent to which the establishment of polarity in the progenitor cell population prefigures cell shape-driven processes in the later heart remains to be explored.

### Wnt5a Function in the SHF and an Intercalation/Cohesion-Based Model for SHF Deployment

*Wnt5a*, like *Wnt11*, encodes a noncanonical ( $\beta$ -catenin independent) ligand of the Wnt family of proteins. Noncanonical Wnt ligands make use of a different set of transducers and targets for signaling and have also been shown to inhibit canonical Wnt signaling<sup>105</sup> (Figure 1). *Wnt5a* is expressed in splanchnic mesoderm, and loss of *Wnt5a* leads to OFT alignment and septation defects associated with reduced neural crest cell contributions.<sup>106</sup> Loss of 1 *Wnt5a* allele increased the severity and incidence of OFT shortening and alignment defects of *Vangl2* mutant embryos, demonstrating genetic interaction and further implicating PCP in OFT morphogenesis.<sup>66</sup> *Wnt5a*;*Wnt11* double mutant embryos have an early cardiac phenotype with failure of SHF deployment and elevated canonical Wnt signaling.<sup>107</sup> Canonical Wnt signaling plays a biphasic role during SHF development, promoting proliferation and inhibiting differentiation.<sup>108</sup> Noncanonical Wnt signaling



**Figure 4. Planar cell polarity in the transition zone.** **A**, Cartoon depicting the transition zone at the arterial pole of the heart (boxed) where second heart field (SHF) progenitor cells differentiate as they enter the outflow tract (OFT). **B**, *Vangl2* is required for epithelial organization in the transition zone (TZ) and maximal OFT elongation. Failure of this process in SHF conditional mutant *Vangl2* but not wild-type (WT) embryos results in a failure to correctly align the left ventricle and ascending aorta leading to double outlet right ventricle (RV). Ao, aorta; LV, left ventricle; and PT, pulmonary trunk. Panel **B** adapted from Ramsbottom et al<sup>66</sup> with permission. Copyright ©2014, Ramsbottom et al.

thus inhibits canonical Wnt signaling as SHF cells differentiate in the transition zone. Further analysis has shown that canonical Wnt signaling in the SHF is inhibited by *Wnt5a* and *Wnt11* through induction of nonapoptosis-related caspase-3 activity and Akt degradation.<sup>109</sup> Dissecting the relative importance of PCP signaling versus inhibition of canonical Wnt signaling in heart development downstream of *Wnt5a* and *Wnt11* activity is challenging, especially given the roles of  $\beta$ -catenin in both canonical Wnt signaling and cell adhesion.

Further evidence of the importance of PCP in SHF development has come from analysis of Disheveled proteins, cytoplasmic scaffolding proteins operating at the junction between PCP and canonical Wnt signaling pathways.<sup>16</sup> *Dvl1* and *Dvl2* are required for OFT elongation and subsequent ventriculoarterial alignment.<sup>110</sup> Using a BAC (bacterial artificial chromosome) transgenic rescue approach, Sinha et al<sup>66</sup> showed that OFT defects in *Dvl1/2* mutant embryos result from disruption of the PCP functions of Disheveled. As in the case of *Vangl2*, conditional loss-of-function experiments showed that *Dvl1/2* function is required in the SHF lineage for proper cardiac morphogenesis. However, whereas *Vangl2* and *Prickle1* are required for epithelial architecture in the transition zone, loss of *Dvl1/2* modulates the epithelial integrity of SHF cells in the posterior region of the dorsal pericardial wall.<sup>66</sup> A similar epithelial phenotype was observed in the absence of *Wnt5a*. In both *Dvl1/2* and *Wnt5a* null embryos, compact clusters of cells, rather than a discrete epithelium, were observed in posterior splanchnic mesoderm, leading to the proposal that *Wnt5a*-regulated PCP signaling is required for intercalation of mesenchymal cells into the dorsal pericardial wall.<sup>66</sup> Consistent with this hypothesis, a reduction in filamentous actin containing filopodial-like cell protrusions was observed in splanchnic mesoderm of *Wnt5a* and *Dvl1/2* mutant embryos.<sup>66</sup> This is coherent with the absence of a basal lamina in the posterior dorsal pericardial wall of wild-type embryos.<sup>64</sup> These observations provide a PCP-based mechanistic model for SHF deployment: *Wnt5a*-driven PCP may recruit mesenchymal cells into the posterior dorsal pericardial wall,

renewing the epithelium as cells contribute to the arterial pole and at the same time creating a pushing force displacing the epithelial sheet toward the arterial pole.<sup>66</sup> The direct role of filopodia in the *Wnt5a* null phenotype versus loss of polarity remains to be elucidated. Labeling experiments in avian embryos have revealed that cells in the posterior region of the dorsal pericardial wall are deployed as a cohort in a *Wnt5a*-dependent process toward the OFT, further reinforcing this model.<sup>111</sup> Interestingly, retinoic acid signaling has also been implicated in renewal of SHF progenitor cells during heart tube elongation, suggesting the coordinated involvement of multiple signaling pathways in this region of the SHF.<sup>112</sup>

Comparison of *Wnt5a* and *Tbx1* mutant embryos, where a reduction of filopodial activity was also observed,<sup>64</sup> revealed that a specific subpopulation of progenitor cells in the dorsal pericardial wall that normally contributes to future subpulmonary myocardium, similarly fails to deploy in both mutants.<sup>111,113</sup> Asymmetrical contribution of cells to the pulmonary trunk region continues through to embryonic day 12.5 in the mouse and has been proposed to drive ventral displacement of the pulmonary trunk during aorticopulmonary septation.<sup>114</sup> It remains to be seen whether this process, dubbed the pulmonary push, is mechanistically linked to PCP-driven pushing forces at earlier stages of development. Subpulmonary myocardium is derived from cells expressing *Hoxb1* in the posterior region of the SHF<sup>46</sup>; vital dye labeling and genetic lineage tracing experiments have shown that *Tbx1* is required for displacement of these cells toward the arterial pole of the heart from the 7-somite stage.<sup>61</sup> This corresponds to the time that *Tbx1* function, as defined by temporal deletion experiments, is required for OFT morphogenesis.<sup>115</sup> *Wnt5a* expression is reduced in the SHF of *Tbx1* null embryos and characterization of T-box binding sites at the *Wnt5a* locus identified *Wnt5a* as a direct target of *Tbx1*.<sup>116</sup> *Wnt5a* may thus mediate polarized cell behavior in the SHF downstream of *Tbx1*. Indeed, *Wnt5a* and *Tbx1* interact genetically, although a severe cardiac phenotype in double *Wnt5a* and *Tbx1* null



embryos is indicative of additive gene-specific functions rather than a purely epistatic relationship.<sup>116</sup> Consistent with this, the epithelial phenotype of *Tbx1* null embryos is less severe than that of *Wnt5a* null embryos.<sup>66,116</sup> Loss of both *Wnt5a* and *Tbx1* was associated with the upregulation of  $\beta$ -catenin in the dorsal pericardial wall, suggesting intersection with canonical Wnt signaling.<sup>116</sup> An antagonistic relationship between *Tbx1* and  $\beta$ -catenin signaling has recently been reported in the SHF that may contribute to the variable phenotypic expressivity of 22q11.2 deletion syndrome phenotypes.<sup>117</sup>

Li et al<sup>67</sup> further investigated the function of PCP in the SHF by transgenic misexpression of a conditional gain-of-function form of *Wnt5a*. Although this approach rescued cell polarity and the intercalation phenotype in the posterior region of the dorsal pericardial wall of *Wnt5a* null embryos, misexpression in the anterior dorsal pericardial wall of wild-type embryos blocked the deployment of SHF cells leading to a shortened OFT.<sup>67</sup> This phenotype seems to result from altered epithelial architecture in the anterior SHF, in particular a reduction in N-cadherin and  $\alpha$ -catenin levels, associated with a failure to increase cell cohesion close to the arterial pole. In wild-type embryos, increased cohesion in the anterior region of the dorsal pericardial wall is correlated with E-cadherin expression and the presence of a basal lamina.<sup>64</sup> Interestingly, the loss of cohesion in the anterior region of the dorsal pericardial wall following *Wnt5a* misexpression led to abnormal folds of epithelial cells that failed to integrate into the OFT.<sup>67</sup> Similar structures were previously observed in avian embryos after neural crest ablation, suggesting that PCP signaling in the SHF may be perturbed in the absence of neural crest-derived cells.<sup>63</sup> Thus, in addition to a *Wnt5a*-driven pushing force in the posterior region of the dorsal pericardial wall, downregulation of *Wnt5a* in the anterior dorsal pericardial wall may increase cell cohesion, creating a pulling force promoting SHF deployment (Figure 5A).<sup>67</sup> Combined pushing and pulling forces potentially act as motors to drive progenitor cell deployment across the dorsal pericardial wall into the OFT.

### Epithelial Tension in the SHF

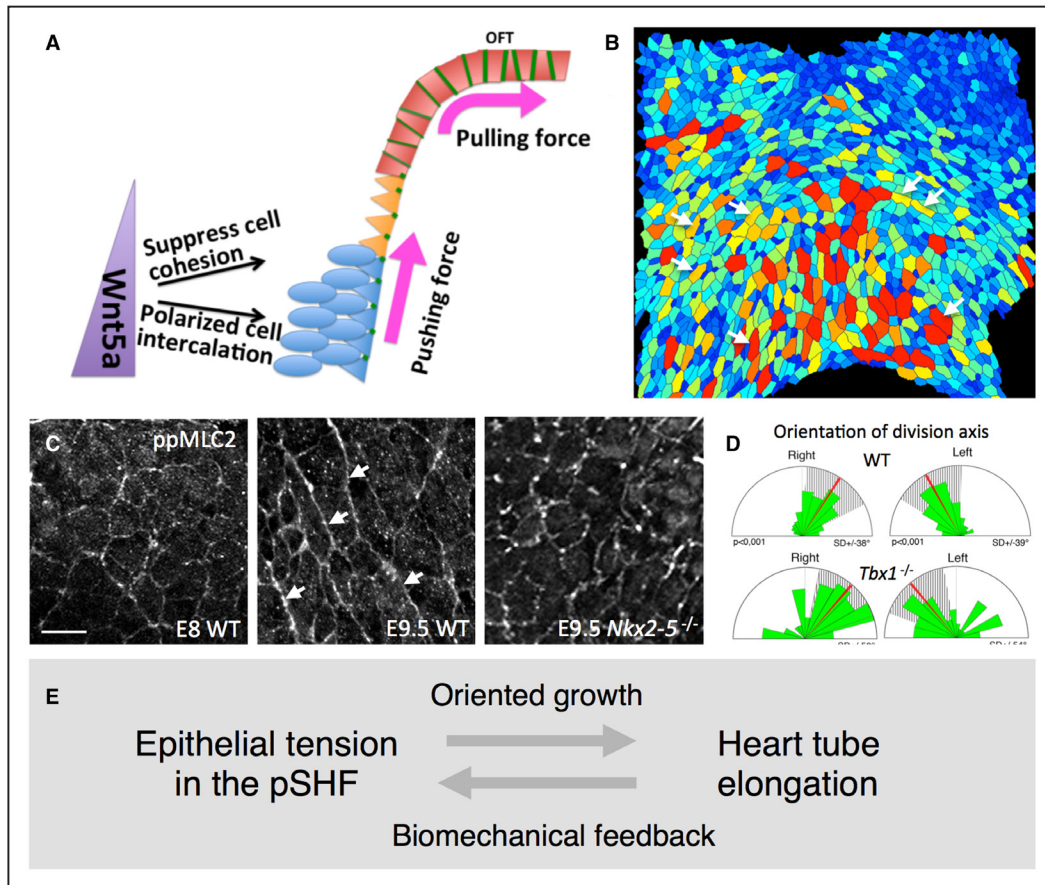
Differences between anterior and posterior domains of the dorsal pericardial wall have also been implicated in an epithelial tension-based model of SHF deployment.<sup>118</sup> Quantitative analyses of the apical surface area and orientation of SHF cells was performed after phalloidin staining of apical actin to individualize cells followed by data segmentation. This revealed that cells in the posterior region of the dorsal pericardial wall tend to have a larger apical surface than those in the anterior region. In addition, cells in the posterior dorsal pericardial wall are more elongated and oriented toward the arterial pole, consistent with epithelial deformation because of tissue-wide forces (Figure 5B). Greater deformation in the posterior region of the dorsal pericardial wall is consistent with increased epithelial cohesion close to the arterial pole. Analysis of active actomyosin complexes using an antibody detecting phosphorylated nonmuscle myosin light chain revealed accumulation along the long membranes of elongated cells, often forming cables covering several cell lengths, indicative of cell stretching and epithelial tension (Figure 5C). Interestingly,

oriented stretched cells and polarized accumulation of actomyosin were observed in the posterior region of the dorsal pericardial wall only after breakdown of the dorsal mesocardium, suggesting that tension across the epithelium coincides with restriction of SHF cell addition to the cardiac poles.<sup>118</sup> In support of such a hypothesis, cells were not stretched and actomyosin accumulated isotropically in the SHF of embryos lacking the cardiac transcription factor *Nkx2-5*, in which heart tube extension does not occur,<sup>119,120</sup> and altered patterns of cell elongation were observed in *Tbx1* null embryos where SHF deployment defects lead to hypoplasia of the distal OFT.<sup>118</sup>

Elevated proliferation was observed in the posterior region of the dorsal pericardial wall, consistent with identification of a caudal proliferative center in the avian SHF.<sup>45,118</sup> Epithelial topology directly influences growth direction, and stretched cells have been shown to divide on their longest axis.<sup>121,122</sup> Indeed, after cell division in the dorsal pericardial wall, daughter cells were observed to be distributed on the same axis as that of cell elongation (Figure 5D), leading to oriented patterns of clonal growth in the dorsal pericardial wall that potentially enhances cell deployment toward the OFT.<sup>118</sup> Epithelial tension and mechanical cues can promote proliferation through the Hippo signaling pathway.<sup>123</sup> Nuclear accumulation of the transcription factors YAP and TAZ, which act as mechanotransducers in multiple cell types, was observed in the posterior region of the dorsal pericardial wall. Blocking the interaction of YAP with the transcriptional cofactor Tead1 in embryo culture decreased proliferation in the dorsal pericardial wall and led to shortening of the OFT.<sup>118</sup> These results suggest a working model by which rapid SHF deployment both creates and is promoted by tissue-level tensile forces through biomechanical feedback and passive stretching of SHF cells (Figure 5E).<sup>118</sup> Genetic analysis of the role of YAP/TAZ in the SHF and direct measurement of tensile forces in the SHF will be required to validate this model. Additional potential sources of cell deformation in the progenitor epithelium, such as myocardial contraction within the heart tube or tensile forces in the underlying endoderm, remain to be evaluated, as does potential intersection with PCP-driven pushing and pulling forces.<sup>31,124</sup> Finally, an important yet little-explored aspect of the epithelial properties of SHF cells concerns left–right differences, evident from patterns of proliferation and active actomyosin distribution in the dorsal pericardial wall.<sup>118,125</sup> Asymmetrical cell behavior in the gut mesentery has been shown to drive asymmetrical morphogenesis through *Daam2* regulation of adhesion–cytoskeletal interactions.<sup>126</sup> Future analysis of the asymmetrical epithelial properties of cells in the dorsal mesocardial wall during heart tube elongation may provide information into the regulation of looping morphogenesis.

### Conclusions and Perspectives

Regulation of the epithelial properties of cardiac progenitor cells is emerging as a critical step in controlling the epithelial sheet folding or cardiac origami that drives early heart morphogenesis. We have seen how epithelial architecture in the SHF, including cell–cell adhesion, dynamic basal filopodia, and cell shape, is essential for signaling during progressive heart tube elongation, both in the dorsal pericardial wall and in the transition zone at the cardiac poles. Analyses of these epithelial properties have provided mechanistic insights into the processes



**Figure 5. Epithelial-based models for second heart field (SHF) deployment.** **A**, Regional *Wnt5a* expression promotes cell intercalation in the posterior SHF conferring a pushing force, whereas increased epithelial cohesiveness in the anterior SHF confers a pulling force. Together, these 2 forces promote SHF cell deployment toward the arterial pole of the heart. **B**, Segmented apical view of the dorsal pericardial wall epithelium at mouse embryonic day 9.5 (E9.5), with color-coded apical surface area, showing cell elongation in the posterior region (arrows) consistent with regional epithelial stress. **C**, Active actomyosin complexes visualized using a diphosphorylated nonmuscle myosin (ppMLC2) antibody are isotropically distributed before rupture of the dorsal mesocardium (left). ppMLC2 subsequently accumulates on the long membrane of elongated cells in the SHF (arrows, center); in contrast, active ppMLC2 distribution remains isotropic in *Nkx2-5* mutant embryos (right). **D**, The axis of cell division is oriented toward the arterial pole on both the left and the right sides of the dorsal pericardial wall in wild-type but not in *Tbx1* mutant embryos. **E**, Model linking epithelial tension in the SHF to heart tube elongation through biomechanical feedback. Scale bar: 10  $\mu\text{m}$ . OFT indicates outflow tract. Panel **A** reproduced from Li et al<sup>67</sup> with permission (Copyright ©2016, Elsevier Inc); panels **B–D** adapted from Francou et al<sup>118</sup> with permission (Copyright ©The Author(s), 2017).

driving SHF cell deployment, including cell intercalation and cohesive and tensile forces. Further analysis of these emerging biomechanical models for SHF deployment will require the development of dynamic imaging protocols. The contribution of multiple model systems will be essential. For example, the discovery of the zebrafish SHF opens up the possibility of high-throughput forward genetic approaches to identify novel regulators of the epithelial features of SHF progenitor cells. In addition, characterization of cardiopharyngeal mesoderm and second heart progenitor cells in the protochordate *Ciona intestinalis* allows high-resolution analysis of cardiac progenitor cell biology during migration and differentiation.<sup>127–129</sup> An outstanding question concerns the link between epithelial status and progenitor cell fate. How are different SHF cell fates encoded in the epithelium, including smooth muscle and endothelial fates, as well as patterning of arterial versus venous pole myocardial progenitor cells? Are cell fate decisions within the larger field of cardiopharyngeal mesoderm linked with exchange between the SHF epithelium and overlying cardiopharyngeal mesenchyme?

Are epithelial changes in the transition zone, such as apical constriction, directly linked with activation of the myocardial transcriptional program? Ongoing investigation of these questions, in part through further dissection of PCP and *Tbx1* function in the SHF, will provide new insights into the processes underlying CHD. Finally, it will be important to evaluate the significance of the epithelial status of cardiac progenitor cells for optimally directing pluripotent stem cells to cardiogenic fates.

### Note Added in Proof

Since completion of this review, 2 articles have highlighted the importance of actomyosin-dependent mesenchymal to epithelial transition and EMT-driven processes in early heart development and right-sided posterior SHF contributions to heart looping, respectively.<sup>130,131</sup>

### Acknowledgments

We are grateful to our colleagues in the Kelly group and Stéphane Zaffran for comments on the article and to Deborah Henderson

(University of Newcastle) and Jianbo Wang (University of Alabama) for Figures 4B and 5A, respectively.

### Sources of Funding

Work in RK's group is supported by the Fondation pour la Recherche Médicale (DEQ20150331717), the Agence National pour la Recherche (Transcardiac project ANR-13-BSV2-0003-02), the Association Française contre les Myopathies, and a grant from the Fondation Leducq (Transatlantic Network of Excellence 15CVD01).

### Disclosures

None.

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