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**Title:**

**Shaping the mouse heart tube from the second heart field epithelium**

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**Abstract**

As other tubular organs, the embryonic heart develops from an epithelial sheet of cells, referred to as the heart field. The second heart field, which lies in the dorsal pericardial wall, constitutes a transient cell reservoir, integrating patterning and polarity cues. Conditional mutants have shown that impairment of the epithelial architecture of the second heart field is associated with congenital heart defects. Here, taking the mouse as a model, we review the epithelial properties of the second heart field and how they are modulated upon cardiomyocyte differentiation. Compared to other cases of tubulogenesis, the cellular dynamics in the second heart field are only beginning to be revealed. A challenge for the future will be to unravel key physical forces, driving heart tube morphogenesis.

## Introduction

The four-chambered mammalian heart develops from a single heart tube, emerging in the early embryo, at E8.5 in the mouse or the third week of gestation in humans. Tubulogenesis progresses while the tube elongates anteriorly at the arterial pole and posteriorly at the venous pole, by gradual incorporation of cardiac progenitors from the adjacent mesoderm. Two clonally distinct mesodermal populations of progenitors [1], known as the first and second lineages, arise sequentially in the primitive streak [2] and are part of the splanchnic layer of the lateral plate mesoderm. Recent single cell transcriptomics have clarified markers of these cell populations and mapped their location in the first (FHF) and second (SHF) heart fields [3,4] (Fig1). Whereas the tubular primordium derives from the FHF, it elongates by progressive incorporation of progenitor cells from the FHF and SHF. Abutting the septum transversum at the boundary with the extra-embryonic mesoderm, the FHF is initially the most anterior and lateral cardiac domain and is displaced ventrally and posteriorly as the gut closes. *Hand1* and *Mab21l2* label the domain referred to as the juxta-cardiac field, which contains first lineage progenitors [3,4]. In contrast the SHF, classically labelled by *Isl1* and *Tbx1*, is initially more medial and becomes displaced dorsal to the heart tube, forming the dorsal pericardial wall. The SHF becomes the only anterior mesodermal source of cells, with a specific contribution to the outflow tract, the arterial pole of the tubular heart. Genetic alterations in SHF cells, for example by *Tbx1* [5] or *Vangl2* [6] inactivation, have been shown to cause severe congenital heart defects, including conotruncal malformations such as common arterial trunk, double outlet right ventricle and tetralogy of Fallot. The outflow tract undergoes morphogenetic processes, such as rotation and septation, which are essential for the separation and alignment of the great arteries (aorta and pulmonary trunk) relative to the ventricles (Fig.1). Anomalies in these processes dramatically impact heart function, by a failure to separate the double blood circulation and correspond to around 30% of congenital heart defects [7]. Better

understanding of how the outflow tract develops from the SHF is thus an important medical issue.

The SHF provides an example of a transient epithelium of progenitor cells, which is important to shape an organ [8]. Here, we review the epithelial properties of the SHF, how it transitions to the myocardium and discuss the dynamics of cell behaviour in the SHF shaping the heart tube.

## **1- Characteristics of the second heart field epithelium**

The dorsal pericardial wall has been described by pioneer ultrastructural analyses in the mouse [9] as an epithelial layer, composed of “more or less” cuboidal cells, because of their curved shape. Typical features of epithelial cells, including stable cell-cell junctions, apico-basal polarity and interaction with basement membrane [10] have been validated by more recent immunolabelling [11,12] (Fig.2). Lateral cell-cell junctions are detected by CDH1/2, CTNNB1 as well as TJP1. Apical domains are labelled by PRKCZ, CRB3, PAR3, with a primary cilium protruding towards the pericardial cavity. Apical cell surfaces are enriched in cortical actin and MYH10, and have a polygonal morphology [13,14]. Actomyosin accumulation is also associated with lateral cell-cell junctions, forming a ring-like structure on the cell apical side [14,15]. Baso-lateral domains are characterized by SCRIB and ITGB1, with basal filopodia protruding in the underlying basement membrane, itself composed of LAMININ, FN1 and COL1 and facing the endoderm. The presence of filopodia and the pointed basal shape has been seen as a more mesenchymal signature and so the SHF has been proposed to be an atypical epithelium [11]. However, basal filopodia were described in other epithelia in association with dynamic Notch and Fgf signaling [16–18].

The SHF is a transient reservoir of heart progenitors, containing a few thousand cells (unpublished observations at E8.5, T. Bønnelykke, A. Desgrange, S. Meilhac). It is formed after gastrulation: clonal analyses of *Mesp1* expressing cells showed that cells of the second cardiac lineage ingress through the primitive streak between E6.75-E7.25 [2]. Live-imaging showed that SHF cells begin to differentiate into cardiomyocytes as a second wave, after

FHF cells have formed a tube primordium, from a stage equivalent to E8.5f [19] (Fig. 1). At this stage, the pericardial cavity has formed, the SHF is located in the dorsal pericardial wall, and also extends posteriorly up to the second somite as indicated by fate mapping [20] (Fig.1). Inducible genetic tracing with *Isl1* suggests that the SHF is exhausted after E9.5 [21]. ISL1 and NKX2-5 double positive cells reminiscent of cardiac precursors are found at E10.5, but in the anterior pharyngeal mesenchyme rather than the epithelial dorsal pericardial wall [22]. The epithelium of the dorsal pericardial wall is in contact with mesenchymal cells underneath [9], which also express SHF markers such as *Fgf10*, *Isl1* and *Tbx1* [11,12] and cardiac markers including *Gata4* [23]. It has been proposed that these mesenchymal cells incorporate posteriorly into the epithelium of the dorsal pericardial wall [24], a process which depends on retinoic acid signalling [25]. Some mesenchymal cells underneath the dorsal pericardial wall have a neural crest origin and are important for the proliferation and differentiation of SHF cells [26–28]. In addition to the myocardium, SHF cells contribute to other cardiac cell types, including endocardial, epicardial, lymphatic and smooth muscle cells [2,29].

Along with apico-basal polarity, the SHF expresses several components of the core Planar Cell Polarity (PCP) pathway, including VANGL2, PRICKLE1 and DVL [6,24,30]. However, a polarised localisation of these proteins in the plane of the dorsal pericardial wall epithelium has not been reported, potentially because of the technical challenge. Thus, planar polarity of the SHF epithelium remains uncharacterised. PCP ligands are expressed in the posterior SHF (WNT5A) and myocardium (WNT5A, WNT11) [24,31]. Conditional mutants show that *Vangl2* and *Wnt5a* are required within the SHF for the elongation of the outflow tract [6,24].

The SHF is regionalised, along the antero-posterior and left-right axes. The anterior domain expresses *Tbx1*, *Fgf8/10*, whereas the posterior domain expresses *Tbx5*, *Hoxb1*. This subdivision, regulated by retinoic acid signalling [32], separates progenitors integrating the heart tube by the arterial and venous poles respectively. The left side of the SHF transiently expresses the left determinant *Nodal*, which is important for the asymmetric shaping of the embryonic heart tube, in a process referred to as looping [33]. Clonal analyses

showed that left progenitors also have specific fates, such as the left atrium and pulmonary trunk [20,34].

Thus, the SHF in the dorsal pericardial wall is an epithelium, which provides progenitor cells to the heart tube in an orchestrated manner, following the integration of patterning and polarity cues.

## **2- Transition from second heart field progenitors to myocardial cells**

The boundary between the SHF and the myocardium is double. At a molecular level, it corresponds to the switch from progenitor markers (*Isl1*, *Tbx1*) to the expression of cardiomyocyte-specific genes, including *Nkx2-5*, *Asb2*, *Hand2* and that encoding the contractile apparatus (*Tnnt2*, *Tnni3*, *Acta2*, *Actc1*, *Myh1*, *Myl7*, ...) [6,35–37] (Fig.2). At a morphological level, the boundary separates the tubular myocardium from the planar dorsal pericardial wall. Thus, the transition from the SHF to the myocardium associates cell differentiation with tubulogenesis, in the sense of morphogenetic events elongating and shaping the heart tube.

The SHF contribution to the heart tube has been reported to occur via a transition zone (Fig.2) [9], which co-express progenitor (*Isl1*) and cardiomyocyte (*Nkx2-5*, high *Hand2*, low *Tnni3/Acta2/Myh1*) markers [6,36,37]. Single cell transcriptomics also identified populations of intermediate differentiating cardiomyocytes [3,4]. In the transition zone, epithelial cells of the SHF become more cohesive, forming a transient columnar epithelium (Fig.2), before integration into the multi-layered myocardium, which retains apico-basal polarity in the outer most layer. Increased cohesion is provided by the expression of CDH1 and CTNNA, relocalisation of CDH2 around the cell contour, increased LAMININ and focal adhesion markers ITGB1, VCL and ILK [11,12,36,38,39]. This is accompanied by the detachment of the tissue from the endoderm and novel interaction with the endocardium [19] (Fig.2).

The incorporation of SHF cells to the heart tube can be impaired by different means. Decreased proliferation in the dorsal pericardial wall, in *Fgf8/10* and *Hes1* mutants [40–42] or decreased replenishment of the epithelium from

underlying mesenchymal cells in *RARα1<sup>-/-</sup>*, *RXRβ<sup>-/-</sup>* mutants [25] reduces the outflow tract by shortage of cells. Impairment of cardiomyocyte differentiation, in *Nkx2-5*, *Tbx20*, *Mef2c* mutants, also prevents the elongation of the heart tube [43–45]. In addition, the epithelial architecture of SHF is required for the growth of the outflow tract. This is evidenced upon alterations in apico-basal polarity (PRKCZ activator) [11], alterations in the PCP pathway (*Wnt5a*, *Vangl2*, *Rac1*, *Prickle1* mutants) [6,24,30,38,46] and decreased cell adhesion (*Cdh2*, *Hand2* mutants) [36,39]. An upstream regulator of the epithelial architecture of SHF is the transcription factor TBX1, which directly activates *Wnt5a* [47] and is also required for apico-basal polarity [11,12]. In *Vangl2*, *Cdh2* and *Tbx1* mutants, in which the SHF architecture is impaired, myocardial differentiation is abnormally detected in the transition zone, in some instances extending in the anterior dorsal pericardial wall [6,11,39] (Fig.3). As this is associated with a shorter outflow tract, it suggests uncoupling of cardiomyocyte differentiation from tubulogenesis. It remains to be determined whether mutant cardiomyocytes were not able to detach from the endoderm or to reach the tube pole. Another regulator of epithelium remodelling is the actin cytoskeleton. Its involvement in the SHF is best exemplified by the expression of FLNA, an actin binding protein which stiffens the cytoskeleton. Its degradation by the ubiquitin ligase ASB2 in cardiomyocytes and in the transition zone is required for the elongation of the heart tube [35].

In summary, the progression of cells along the cardiomyocyte differentiation trajectory is accompanied by changes in cell shape and tissue cohesion. This tight coordination is required for the elongation of the heart tube. Mapping of intermediate differentiating cardiomyocytes suggests different sites of cell incorporation into the heart tube [3,4]. Whether a columnar epithelium is observed in all sites, and also applies to the first myocardial lineage, remains to be systematically investigated. The PCP pathway appears as an important player of the SHF tissue architecture, as indicated by mutant phenotypes. In addition, WNT5A was shown to be inversely correlated with tissue cohesion [38] and VANGL2 to change subcellular localisation, from membranous to cytoplasmic upon cardiomyocyte differentiation [6]. Classical effectors of the PCP pathway include small GTPases (RHOA, RAC1 and CDC42), formins

(DAAM1/2), atypical protein kinase C (PRKCZ/I), JNK kinases (MAPK8/9), non-muscular myosins (MYH9/10/14), which modulate the actomyosin cytoskeleton, cell adhesion, apico-basal polarity and extracellular matrix assembly [48]. Many of them have been shown to be required for heart morphogenesis, but their interplay with the PCP pathway in the SHF is still poorly understood. The PCP pathway can also coordinate cell behaviour during morphogenesis, as we will discuss in the next section.

### **3- Cell dynamics of the second heart field: shaping the heart tube**

Several tubular organs develop from an epithelial sheet of cells, providing examples of the cellular mechanisms driving this major tissue deformation [49], relevant to the embryonic heart tube. When the heart tube emerges at E8.5e, it is a bulging open structure. This corresponds to the detachment of the cardiac cell layer from the endoderm, during which columnar epithelial cells of the heart field become more rounded as they express myocardial differentiation genes [19]. Whether this initial budding depends on a change in cell adhesion between the cardiac mesoderm and the endoderm, or rather on a mechanism of buckling remains undetermined. The tube then closes dorsally, implying convergence of the left and right sides of the heart field epithelium [19], a process affected in *Aldh1a2* mutants, in which the heart tube remains open [50]. Another aspect of tube closing is the breakdown of the dorsal mesocardium at the midline of the dorsal pericardial wall at stage E8.5i-j, under the control of Shh and matrix metalloproteinases [51].

Tubulogenesis continues after closure, at both the arterial and venous poles. The mechanism of tube elongation from the epithelial SHF remains unclear. Does it involve the same process as initial heart tube budding by detachment from the endoderm? The observation of abnormal myocardial differentiation in the dorsal pericardial wall of *Cdh2* or *Tbx1* mutants, associated with a shorter outflow tract [11,39], is compatible with this hypothesis (Fig.3). Alternatively, tubulogenesis may rely on tissue folding at the edge between the SHF and the tube, which is reminiscent of folding in other organs. During the formation of the mouse neural tube, folding of the epithelial neural plate



depends on apical cell constriction. This involves apical actomyosin contraction, regulated by SHROOM3, that interacts with actin and ROCK, downstream of the PCP pathway [52,53]. Invagination of the fly mesoderm is similarly driven by apical cell constriction, but as a consequence of the delamination process of cells undergoing epithelial-mesenchymal transitions [54]. In the mouse SHF, the PCP pathway is required for the elongation of the heart tube [6,24,30,38,46]. Inactivation of *Shroom3*, which is notably expressed in the splanchnic mesoderm around the outflow tract, leads to malformation of the arterial pole [55]. However, apical constriction has not yet been described in cardiac progenitor cells. Another mechanism classically associated with tube elongation, in the mouse neural tube or frog kidney tubule [56,57], is convergent extension. This is mediated by mediolateral cell intercalation, based on cell crawling with polarised cellular protrusions, as well as cell junction shrinkage upon polarised actomyosin contractions [58]. The PCP pathway, which controls actomyosin contractility, cell migration and junction shrinking, is an important regulator of convergent extension [56–58]. For the elongation of the fly salivary gland tube, cell intercalation in the epithelium has been shown with a more complex pattern, being circumferential around the tube opening [59]. Evidence of convergence in the SHF, and consequent extension of the tube, has been provided by time lapse analysis of Dil injections in the chick [60] (Fig.3). In the mouse, clones of myocardial cells are distributed in clusters along the axis of the tube, which is a sign of intercalation [61]. Measurement of the SHF domain expressing *Mef2cAHF-Cre*, and inducible genetic tracing of posterior SHF cells expressing *Wnt5a*, also support convergent extension mechanisms, which are impaired in mutants for the PCP pathway component WNT5A [15]. At a cellular level, cell shape elongation, oriented cell divisions and polarised apical actomyosin [14], the latter potentially oriented by the PCP pathway [15], have been observed. This correlates with measures of tension; yet whether tension is a cause or consequence of morphogenesis remains undetermined. Alternatively, PCP mutant phenotypes have been interpreted in terms of pushing forces from inferred posterior incorporation of mesenchymal cells into the SHF epithelium and pulling forces from increased adhesion at the transition zone [62]. To discriminate between hypotheses of tubulogenesis, experimental

challenge and computer simulations of forces will be required, as well as dynamic analyses of cell intercalation [58].

A physical constraint in the SHF can be deduced from the measurement of the distance between the poles. This distance is fixed over a day, at E8.5 [51]. How the position of the poles is controlled, while SHF cells are dynamically incorporated into the heart tube, is unknown. It remains to be tested whether the epithelial architecture of the dorsal pericardial wall provides rigidity to set the distance between the heart tube poles. This is an important determinant of heart morphogenesis, as the fixed pole distance promotes buckling of the growing tube, resulting in the formation of a helical structure referred to as the heart loop [51] (Fig. 1).

The SHF is patterned on the left side by the secreted factor NODAL, which biases asymmetries in cell differentiation, cell proliferation and extra-cellular matrix composition [33]. This manifests as an asymmetric folding and rotation of the arterial pole, and asymmetric cell ingression at the venous pole [51]. Without *Nodal*, asymmetries at the arterial and venous poles are reduced and their orientation randomised, resulting in abnormal looping of the heart tube and ultimately a spectrum of heart malformations of the heterotaxy syndrome. Asymmetric cell differentiation continues at later stages, in the pharyngeal mesenchyme, proposed to act as a pulmonary push underlying the spiralling of the great arteries [22]. Additional patterning of the SHF in anterior and posterior domains distinguishes cells that incorporate the arterial and venous poles respectively. However, how cells move in opposite directions remains poorly understood.

Thus, the SHF not only provides cells but is the site where several processes important to shape the heart are controlled. The cell dynamics and coordinated orientation involved in tissue convergence, extension, folding, concomitant ingression at different sites will require further investigation. In addition to controlling the correct elongation of the heart tube, cell dynamics in the SHF show asymmetries, which are important to modulate tube shape. The rightward looping of the tube positions cardiac chambers relative to each other [63]. The rightward rotation of the arterial pole is initiated at the beginning of heart looping and continues after, resulting in the spiralling of the great arteries

(Fig.1). Finally, the poles of the heart undergo asymmetric movements towards the midline, to connect to each ventricle. These processes altogether contribute to the left-right partition of the double blood flow in the heart [64].

## **Conclusions**

The SHF in the dorsal pericardial wall is as a transient epithelium, in which heart progenitors integrate patterning and polarity cues. The progression of progenitors towards differentiation is tightly coupled with morphogenetic processes, transforming the epithelial sheet into a tube. Genetic evidence accumulated in the last few years demonstrate the importance of the SHF epithelial architecture for shaping the heart tube. Anomalies in the SHF epithelium have important functional impact, by impairing the heart structure that is essential to channel the double blood circulation.

Whereas gene patterning and the regulation of differentiation are well advanced following progresses in sequencing and mouse genetic engineering, a challenge for the future is to unravel the cellular dynamics and physical forces underlying heart tube morphogenesis, exploiting developments in imaging, quantitative analyses, computer modelling and mouse embryo culture [65,66]. Stem cell cultures have shown the ease of differentiating cardiomyocytes. Yet, in vitro engineering of a functional cardiac tissue, with an oriented architecture, or production of a heart tube in organoids has proven difficult. Recent improvements in organoids are able to better reproduce morphogenesis by controlling physical conditions and extra-cellular matrix [67]. This controllable system would provide a new tool to understand how the epithelial state is important for morphogenesis and patterning of the heart tube.

## **Conflict of interest statement**

Nothing declared.

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## Figure legends

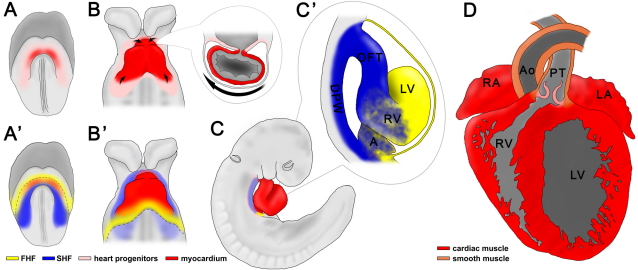
**Figure 1 Developmental stages of the heart.** A) Heart progenitors (pale red) and differentiated cardiomyocytes (dark red) are observed at the cardiac crescent stage (late headfold, E8.5c) in the mouse embryo. A') Progenitors are from the first heart field (FHF, yellow) or second heart field (SHF, blue) populations. B) The tubular heart at E8.5f contains differentiated cardiomyocytes and grows by incorporation of progenitor cells at the tube poles (arrows). At this stage, the rightward rotation of the arterial pole (arrow in the inset) is shown on a transversal section. B') at E8.5f, SHF and FHF progenitors are located behind and posteriorly to the tube, respectively. Drawings showing the regionalization of cardiac progenitor pools in A'-C' are based on published transcriptomic profiles [3,4] and clonal analyses [1]. C) The dorsal pericardial wall (DPW), containing the SHF, is located behind the looped heart at E9.5. C') Functional segments can be distinguished along the heart tube: outflow tract or arterial pole (OFT), right (RV) and left (LV) ventricles and primitive atria (A). D) The postnatal mouse heart is four-chambered. The arterial pole of the heart is split in two great arteries, the aorta (Ao) and pulmonary trunk (PT). RA, right atrium ; LA, left atrium.

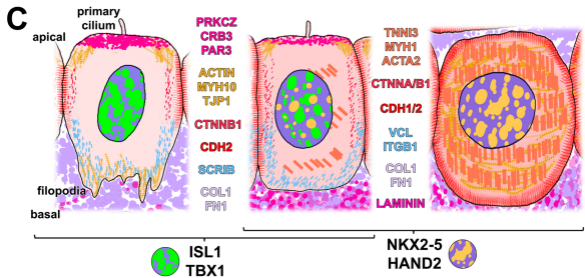
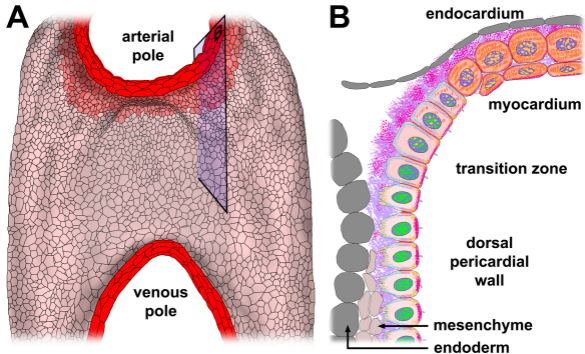
**Figure 2. Cellular architecture of the second heart field epithelium and transition to the myocardium.** A) Ventral view of the dorsal pericardial wall, showing the apical polygonal morphology of cells. The myocardium of heart poles is in dark red, and the transition zone in pale red. Drawing is based on [14]. B) Scheme of the regionalised properties of cardiac cells, highlighting four cell populations: mesenchymal and epithelial heart progenitors in the dorsal pericardial wall, cardiomyocytes in the distal outflow tract, and, in-between, cells of the transition zone. C) Schematic representation of the spatial distribution of polarity markers in SHF cells of the dorsal pericardial wall (left), transition zone (middle) and in differentiated cardiomyocytes

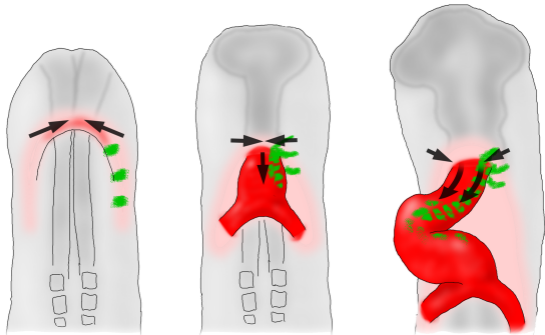


(right). Nuclear markers are indicated below. Drawing is based on several references, as cited in the text.

**Figure 3. Cell dynamics in the second heart field during arterial pole elongation.** A) The ingression of SHF progenitors into the developing arterial pole is consistent with convergent extension in the chick embryo. Drawings are made based on *in vivo* cell tracing experiments [60]. B) Lateral views of E9.5 wild-type (left) and mutant (right) embryos associated with disorganised SHF epithelium. Decreased length of the dorsal pericardial wall (double arrow) is observed in PCP [15] and *Tbx1* mutants [14]. *Tbx1* and *Cdh2* mutants as well as embryos treated with a PRKCZ activator show abnormal cardiomyocyte differentiation in the dorsal pericardial wall (asterisk) [11,14,39]. All these mutants further show outflow tract shortening (dotted line).





**A****B**