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## **Part I: Development of the heart and its vessels**

### **Chapter 2: Cardiac progenitor cells of the first and second heart fields**

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## **Abstract**

The heart forms from the first and second heart fields which contribute to distinct regions of the myocardium. This is supported by clonal analyses which identify corresponding first and second cardiac cell lineages in the heart. Progenitor cells of the second heart field and its sub-domains are controlled by a gene regulatory network and by signalling pathways which determine their behaviour. Multipotent cells in this field also can contribute cardiac endothelial and smooth muscle cells. Furthermore, skeletal muscles of the head and neck are related clonally to myocardial cells that form the arterial and venous poles of the heart. These lineage relationships, together with the genes that regulate the heart fields, have major implications for congenital heart disease.

## **2.1 Introduction and historical perspective**

The heart is the first organ to form and plays a vital role in pumping nutrients and removing waste as soon as the embryo reaches a size where passive diffusion no longer is sufficient to ensure cell survival. Early heart defects are a frequent source of mortality, estimated to account for as many as 30% of embryos/foetuses lost before birth in humans (1). As many as 0.8% of newborn children in developed countries have some form of congenital heart malformation. These figures reflect the complex process of cardiogenesis which involves the integration of different cell populations with precise spatial and temporal control as the heart undergoes morphogenesis (2) (Figure 1). In the mouse embryo, which provides a mammalian model suitable for genetic manipulation, the early heart tube forms and begins beating at about embryonic day (E) 8 which is equivalent to 3 weeks of human gestation. At this stage the tube is linear, with an anterior outflow tract and posterior inflow region, connecting to the circulatory system. As it grows, the cardiac tube rapidly bends leftwards and then loops to the right so that the mature outflow and inflow tracts, the arterial and venous poles, are aligned anteriorly. The cardiac chambers grow out from the looped tube in a process described as ballooning (3), with formation of septa and valves to control blood flow within the heart. By this stage neural crest cells of neurectodermal origin migrate into the cardiac tube which is composed of myocardium surrounded by an inner layer of endocardium and an outer epicardium, all of mesodermal origin.

Cardiomyocytes proliferate within the heart tube and it was thought previously that this accounted for heart growth, with different regions of the early tube prefiguring the future atria and ventricles, in a segmental model of cardiogenesis (4). However classic experiments in the chick embryo had already pointed to the addition of cells to the growing tube, notably to the inflow region (5). Investigators subsequently showed that cells are added to the outflow region of the avian heart from adjacent mesoderm (6, 7). At the same time, in 2001, addition of cells from pharyngeal mesoderm, marked by expression of a transgene integrated upstream of the *fibroblast growth factor (Fgf) 10* locus, was demonstrated to contribute to outflow tract and right ventricular myocardium in the mouse embryo (8). The transcription factor *Islet1* also marks these cells and its expression pattern, together with the *Islet1* mutant phenotype, indicated a contribution to the venous as well as the arterial pole of the heart (9). This was confirmed later by explant experiments and tracking of cells labelled by dye injection in cultured embryos (10), which also showed that the early heart tube has a mainly left ventricular identity (11). These findings regarding cardiac progenitor cells that form the developing heart tube were complemented by a different experimental approach examining myocardial cell lineages in the mouse embryo. Retrospective clonal analysis demonstrated that myocardium derives from two cell lineages. The first lineage is the source of left ventricular myocardium, whereas the second lineage is the source of the myocardium of the outflow tract and most of the right ventricle and also contributes to the atria which are derived from both lineages (12). Thus, cardiac progenitor cells of the first heart field (FHF) form the early cardiac tube, whereas cells of the second heart field (SHF) subsequently enter the developing tube and constitute the second lineage contribution to the heart (2) (Figure 2).

The distinction between these two cell populations is underlined by the early segregation of the first and second lineages, previously estimated to take place at the onset of gastrulation or earlier (12). This conclusion was drawn from a genetic approach to clonal analysis that depends on a random recombination event in a progenitor cell that converts an inactive *laacZ* reporter to an active *lacZ* sequence. In this case the reporter was targeted to an allele of the *cardiac actin* gene resulting in  $\beta$ -galactosidase expression in clonally related daughter cells in the myocardium. The size of the clone gives an estimation of the time of recombination. However this is only approximative. Other genetic approaches to clonal analysis have made it possible to more precisely estimate the timing of clonal segregation and also to follow all mesodermal cell types in the heart. One such approach was based on a conditional *confetti* multicolour reporter and brief induction of a Cre recombinase that activates a single coloured reporter in a progenitor cell and its descendants (13). In this case

the *Cre* is controlled by the *mesoderm posterior 1 homolog (Mesp1)* gene which is expressed in the primitive streak in gastrulating mesodermal cells that will contribute to all compartments of the heart (14). These experiments confirmed that two myocardial cell lineages form the heart, as previously described, and demonstrated that the first lineage can be distinguished by E6.25-E6.75, at the onset of gastrulation, whereas the second lineage that gives rise to SHF derivatives is marked when the *Cre* is induced at E6.75-E7.25. A similar genetic approach based on a mosaic analysis with double markers (MADM) also showed distinct segregation of two lineages (15). The common progenitor that gives rise to the two lineages is therefore present prior to activation of *Mesp1* early in gastrulation, before day 12 of human embryonic development. Left and right ventricular myocardium, derived from the FHF and SHF respectively, originates from *Foxa2* expressing progenitor cells (16). However recent genetic tracing experiments using inducible *Cre* recombinase have shown that *Foxa2* is activated independently in left and right ventricular progenitor cells at the time of gastrulation (17).

## **2.2 The First Heart Field**

The first differentiated cells expressing cardiac muscle markers are detected prior to formation of the heart tube, in a region under the head folds that spans the mid-line of the embryo, known as the cardiac crescent. The early heart tube forms as a result of fusion of the crescent at the midline. This process reflects morphogenetic movements of the underlying endoderm as the embryo develops, but also depends on genes expressed in cardiac mesoderm which control cell migration and fusion of the crescent. Not much is known about the underlying cell biology in mammals, however a number of mutations have been identified that perturb the process. Failure to migrate leads to cardiac bifida where two hearts begin to develop on either side of the embryo. Mutation of the gene encoding the transcription factor GATA-binding protein 4 (*Gata4*) provides a classic example of this phenotype (2). The early heart tube expresses transgenic markers for left ventricular myocardium and dye labelling of these cells followed by embryo culture showed that they will mainly contribute to the left ventricle (11). Markers of cells in the crescent that distinguish the first from the second heart field have been elusive. For example, the transcription factor T-box (*Tbx*) 5, implicated in Holt-Oram syndrome (18) is present in FHF cells, but also marks sub-domains of the SHF (19) and is expressed dynamically in the forming heart tube before showing left ventricular localisation. The *hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (Hcn4)* gene which is expressed later in the conduction system, is transiently activated

specifically in the cardiac crescent and thus provides an interesting early marker (20). However it is probably expressed in myocardial cells at this stage rather than in the FHF progenitors. Induction of a *Mesp1-green fluorescent protein (GFP)* transgene at the early (E6.5) and later (E.7.5) time points, when progenitors for the first and then the second heart fields can be distinguished (13), makes it possible to separate these cell populations by flow cytometry and to perform transcriptome analysis. This approach has already identified a number of differentially expressed genes and should lead to novel markers. Such experiments also open the way to functional analysis, not only for FHF cells present in the crescent but also at earlier stages as they progress through the primitive streak, exit it and then migrate anteriorly to take up their position under the head folds.

### **2.3 The Second Heart Field**

The cardiac progenitor cells of the SHF (Figure 1) are located medially to the cardiac crescent, extending posteriorly. As the crescent fuses to form the early heart tube these cells come to lie behind the tube and also anteriorly, adjacent to the outflow tract, as well as posteriorly, adjacent to the sinus venosus and venous pole as it develops. Initially the tube is not completely closed and there is continuity with the underlying dorsal mesocardium which is attached to the pericardial wall, with some differentiating cells entering the heart dorsally from the SHF(12). Subsequently the dorsal mesocardium breaks down, as the tube closes. The cardiac tube becomes suspended in the pericardial cavity, with cells of the SHF, continuing to feed from the dorsal pericardial wall into the poles of the heart to form myocardium. Recent dynamic imaging of heart tube formation in mouse embryo culture has identified a pause in heart tube growth that precedes the onset of SHF cell addition to the heart tube (21). This delay may be necessary to accommodate the morphogenetic transition linked with dorsal mesocardial breakdown.

SHF deployment continues over several days in the mouse embryo, with myocardium at the base of the pulmonary and caval veins still being formed as late as E12.5 (40 days of human development). The SHF provides a pool of cardiac progenitor cells which are maintained as a proliferating, non-differentiated population. Transcription factors and signalling pathways that mark these cells play a critical role in regulating this behaviour (22). Temporally regulated genetic labelling, using inducible Cre recombinase expressed in progenitor cells, or differentiated myocardium, illustrates the progressive addition of SHF cells to the RV and OFT, as well as to the venous pole (23, 24). Mutations in regulators of SHF development lead to major arterial pole defects and also to venous pole defects which

may be more subtle, reflecting the fact that the outflow tract is derived exclusively from the second myocardial cell lineage, and thus is dependent entirely on the SHF contribution. In newborn children with a congenital heart defect, 30% have a malformation of the arterial pole of the heart. Fortunately this can usually be corrected surgically and the child survives. However a small proportion of babies die after surgery and re-examination of the records indicates that in a number of cases there was also a venous pole defect that had not been detected (25). It is therefore important to check for subtle venous pole defects which can be repaired at the same time as surgery is performed to correct a defect at the arterial pole.

### **2.3.1 Regulation of proliferation and differentiation**

Cells in the SHF are highly proliferative so that the pool of cardiac progenitor cells is maintained as the heart grows. FGF, canonical WNT, Notch and Hedgehog signalling promote proliferation (22, 26). Differentiation of SHF cells is triggered as they enter the poles of the heart. BMP signalling plays an important role in this process, antagonising FGF signalling (27, 28), and promoting the deployment and differentiation of cells that will contribute to the arterial pole. Notch and non-canonical WNT signalling are implicated also in cardiac differentiation (22, 26). At the venous pole of the heart, canonical WNT, Hedgehog and BMP signalling orchestrate the critical balance between proliferation and differentiation, where Hedgehog signalling promotes the migration of cells to the heart tube as well as their proliferation (29, 30). These pathways are activated by signals from surrounding tissues as well as by cell autonomous signalling within the SHF (31). Thus the midline structures (neural tube and notochord) are sources of canonical WNT and Hedgehog signalling, promoting SHF proliferation. Hedgehog signalling also comes from the endoderm, while FGF signals come from surrounding endoderm and ectoderm as well as from the SHF itself. Neural crest cells, which migrate through the SHF into the arterial pole, mediate BMP inhibition of FGF signalling, such that in the absence of neural crest, excessive SHF cell proliferation results in reduced cardiomyocyte differentiation and defective growth of the heart. BMP signals are also produced by cells in the SHF, promoting neural crest survival, and by cardiomyocytes, promoting recruitment to the poles of the heart.

An extensive gene regulatory network operates in the SHF to control cardiac progenitor cell behaviour (22). Key transcription factors modulate signalling pathways, illustrated by *Tbx1* interference with the BMP signalling cascade to delay differentiation (31, 32) or *Tbx1* activation of enhancers that control *Fgf8* and *Fgf10* expression in the SHF (31-33). *Islet1* also activates the *Fgf10* enhancer (33) and has a SHF enhancer which is itself

controlled by forkhead box (Fox) c2 (22). In addition, *Hopx*, encoding a homeodomain only transcription factor, has been shown to play a role in balancing pro-proliferative Wnt and pro-differentiation BMP signalling as progenitor cells enter the elongating OFT(34).

Some genes expressed in the SHF are specific to the progenitor cell population in this cardiac context. *Foxc2*, for example, marks SHF progenitors and *Islet1* has long been considered to be a key marker of this field. However more sensitive reporters now indicate that *Islet1* is also expressed at a low level in FHF progenitors, although the *Islet1* mutant phenotype suggests that it is mainly functional in the SHF (22).

Genes that are involved in myocardial differentiation also are expressed in the SHF. These include genes coding for the transcription factors, NK2 homeobox 5 (*Nkx2-5*), *Gata4*, *Mef2c*, serum response factor (SRF), *Tbx5* and the chromatin remodelling protein SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 3 (*Smarcd3*; also called 60kDa BRG-1/Brm associated factor subunit C1; *Baf60c*) which marks a subset of early *Mesp1*-positive cardiac progenitors (22). These genes are expressed in differentiating cells of the cardiac crescent and in regions of the heart, where combinations of them activate sarcomeric genes. Ectopic expression of *Gata4*, *Tbx5* and *Smarcd3* in non-cardiogenic regions of the mouse embryo has been shown to activate cardiac muscle formation (35), indicating the role of these factors in cardiac progenitor cell determination as well as in cardiac differentiation. Their targets in the SHF are not well understood, although transcriptome analysis of *Nkx2-5* expressing cells gives some insights (36, 37). Notably *Nkx2-5* functions in a negative feedback loop with *Bmp2/Smad1* signalling that controls cardiac progenitor cell behaviour, which is probably of importance in the etiology of *Nkx2-5* related human congenital heart defects (36). Different combinations of factors are one reason why targets may differ between cardiac progenitors and myocardium. Another consideration is the level of expression which may affect function. The potential importance of modulating the level of these factors is indicated by *Tbx1* down-regulation of SRF and *Tbx5* in the SHF (19, 32). In the *Tbx1* mutant, abnormal cardiac differentiation of SHF progenitors in the dorsal pericardial wall probably reflects this effect as well as de-regulation of the balance between BMP and FGF signalling (31). The SHF enhancer of the *Fgf10* gene provides an example of how levels and combinations of transcription factors can be functionally important (33). In this enhancer both *Islet1* and *Nkx2-5* bind to the same sites. In the SHF, *Islet1* levels are high and compete successfully for binding, leading to activation of *Fgf10*, which also depends on *Tbx1*. *Islet1* has been shown to interact with *Smarcd3* and acts as a pioneer factor driving lineage commitment (38). In the heart, on the other hand, *Nkx2-5* levels are very high and

displace *Islet1* (33). This leads to down-regulation of *Fgf10* in keeping with the repressive function of *Nkx2-5* observed for SHF genes, including *Islet1*, in the heart (36). Since *Nkx2-5* also functions as a transcriptional activator in the myocardium this dual role again depends on combinatorial effects of other factors.

Most transcription factors and components of signalling pathways that play a role in cardiogenesis are not cardiac specific. This is in contrast to skeletal muscle where the myogenic regulatory factors of the myogenic differentiation (MyoD) family are only expressed during myogenesis (39). Analysis of mutant phenotypes is informative for the heart only when the gene does not play a vital role during early embryogenesis. *Islet1* or *Mesp1*, for example, are mainly important for formation of the heart at early stages of development, whereas *Fgf8* is required for gastrulation and conditional mutation of *Fgf8* directed to cardiac progenitors is essential in order to understand its role in the SHF. *Mesp1*-Cre lines (14) have proved to be very valuable in targeting all cardiac progenitors, whereas an enhancer of the *Mef2c* gene targets cells in the SHF (40). It follows that in the human population, many mutations in cardiac regulatory genes may be early embryonic lethal or result in multiple effects as seen in a number of syndromes where the heart is affected. This is the case, for example, of mutations in *TBX1* which give rise to 22q11.2 deletion (or DiGeorge) syndrome (41). This includes major defects at the arterial pole of the heart, such as common arterial trunk or persistent truncus arteriosus as well as craniofacial abnormalities, due to the function of *Tbx1* in the SHF as well as in endoderm and ectoderm in the pharyngeal region which is important for cardiac and cranial neural crest. Unless there is some cardiac specific aspect of function, mutations in enhancers that direct gene transcription to the cardiac progenitors of the first or second heart fields are the best candidates for defects that are restricted to the formation of the heart. Many such enhancers, that specifically direct transcription in the SHF, have now been identified for genes such as *Fgf8*, *Fgf10*, *Islet1*, *Nkx2-5* or *Mef2c* (22). They are regulated by combinations of SHF transcription factors, as for *Fgf10* (33).

### **2.3.2 Patterning the SHF**

The SHF is patterned on the anterior/posterior axis where regional heterogeneity is apparent. The anterior part of the SHF is marked by genes that are not expressed throughout the field (22). This is the case for *Fgf8* or *Fgf10* and also for the SHF *Mef2c* enhancer which is not active in the posterior SHF. This anterior/posterior distinction correlates with the contribution of cells expressing these genes to the arterial or venous pole respectively. This is illustrated in the anterior SHF also, where *Tbx1* is mainly expressed in cells that will

contribute to myocardium at the base of the pulmonary trunk (31), in contrast to *Fgf8/10* or the *Mef2c* enhancer which mark progenitors of the right ventricle as well as outflow tract myocardium, including the pulmonary trunk and the aorta. Differences in the origin of myocardium at the base of these arteries are reflected by differential gene expression (19). Pulmonary trunk myocardium, for example, is marked by *semaphorin 3c* (*Sema3c*) gene expression. In *Tbx1* mutants pulmonary trunk myocardium is reduced (31), which is probably the early abnormality that underlies the development of tetralogy of Fallot and other cardiac defects in 22q11.2 deletion (DiGeorge) syndrome patients. Use of an inducible *Mef2c* enhancer-driven *Cre* has shown that these different regions of the OFT arise from temporally distinct progenitor populations (42). Future myocardium at the base of the aorta arises early, at E7.5, while future pulmonary trunk myocardium is progressively added to the arterial pole from E8.5. Continued addition of sub-pulmonary myocardium from the SHF up to E12.5 has been proposed to drive arterial pole rotation, essential for correct alignment of the ventricular outlets, through a pulmonary-push mechanism (43).

The posterior SHF is marked also by distinct gene expression profiles. Homeobox (*Hox*) genes, differentially expressed along the axis of the embryo, are classically involved in anterior/posterior patterning. Anterior members of the *Hox* gene clusters such as *Hoxb1* are expressed in the posterior SHF, under the control of retinoic acid signalling. When this signalling pathway is perturbed, *Hox* gene expression is affected and the posterior boundary of the SHF is modified (44). *Tbx5* is expressed in cardiac progenitor cells in the posterior SHF. In addition to atrial myocardium, the dorsal mesenchymal protrusion also derives from the posterior SHF, from cells expressing *Tbx5* and *Islet1*, and its development depends on Hedgehog and BMP signalling (29, 30). This structure is important in the context of congenital heart malformations since it provides the muscular base of the atrial septum and anomalies in its formation from the posterior SHF leads to atrial and atrioventricular septal defects. *Tbx18* is expressed in a particular lateral domain of the posterior SHF which is distinguished also by lack of *Nkx2-5* and *Islet1* expression (45). This domain, which has been proposed to constitute a third heart field, contributes caval vein myocardium, which is marked by continuing *Tbx18* expression. Retrospective clonal analysis distinguishes sub-lineages of the second myocardial cell lineage (equated with the SHF) which contribute to myocardium at the arterial or venous poles of the heart. However this clonal analysis does not distinguish a particular sub-lineage contributing to caval vein myocardium only, as might be expected if this arises from a third heart field (46).

The distinction between anterior and posterior domains of the SHF is made on the basis of gene expression, however cells may move between these domains during SHF deployment. This was first indicated by genetic tracing of cells that had expressed *Hoxb1* in the posterior SHF (44), leading to labelling of myocardial derivatives at the venous pole. Unexpectedly *Tbx1*-dependent myocardium at the base of the pulmonary trunk was labelled also (19). In keeping with this result, dye labelling of small groups of cells in the posterior domain of the SHF showed, after embryo culture, that some labelled cells were located in the outflow tract and had therefore moved anteriorly, expressing markers of the anterior SHF, as indicated by *Fgf10* reporter gene expression (47). Most cells do not appear to move extensively within the SHF and clonal analysis suggests coherent, rather than dispersed, cell growth as the heart forms (2). However lineage studies for the venous pole of the heart showed unexpected clonality between myocardium at the left side of the venous pole (pulmonary vein, left caval vein and left atrium) and pulmonary trunk myocardium at the arterial pole (46). This is consistent with anterior movement of a sub-set of progenitor cells located in the left side of the posterior SHF from which left venous pole myocardium derives. Cre genetic tracing experiments show that the classic anterior SHF markers, *Tbx1* (48) and the *Mef2c* SHF enhancer (49), are transiently expressed in progenitor cells giving rise to the atrial septum and dorsal mesenchymal protrusion. Indeed, loss of function of *Tbx1* impacts not only the anterior SHF but also the posterior SHF, resulting in venous as well as arterial pole defects (19). The development of the dorsal mesenchymal protrusion is affected, leading to ostium primum atrial septal defects, a class of atrioventricular septal defect. In addition to roles in promoting proliferation and limiting differentiation in the SHF, *Tbx1* is required for the segregation of cells to the arterial and venous poles of the heart from a common early progenitor population expressing *Tbx1*, the *Mef2c*-enhancer transgene and *Hoxb1* (19). From E8-8.5, *Tbx5*, a regulator of FHF and venous pole progenitor cells, is activated in a subpopulation of *Tbx1* expressing cells in the dorsal pericardial wall that will contribute to atrial septal structures (50). Transient coexpression of *Tbx1* and *Tbx5* is followed by downregulation of the anterior SHF program (Figure 3). Distinct *Tbx1* expressing arterial pole and *Tbx5* expressing venous pole progenitor populations thus emerge in the dorsal pericardial wall. Retinoic acid is required to activate *Tbx5* in *Tbx1* expressing cells and blocking this signalling pathway during the E8-8.5 developmental window also leads to ostium primum atrial septal defects (50). *Tbx5* in the posterior SHF is critically required to drive the expression of canonical Wnt ligands, *Wnt2* and *Wnt2b* in venous pole progenitor cells (51). In addition, *Hoxb1*, a direct target of retinoic acid signalling, has been shown to negatively

regulate differentiation in the posterior SHF during heart tube elongation and is required, together with its paralogue *Hoxa1*, for development of the dorsal mesenchymal protrusion and atrial septation (52).

In addition to patterning on the anterior/posterior axis, the SHF also shows left/right patterning. This is driven by the Nodal signalling pathway which leads to expression of the transcription factor paired-like homeodomain 2 (*Pitx2*) in cells on the left side of the SHF. *Pitx2* is highly expressed in the posterior SHF where it promotes a left myocardial cell fate by repressing the acquisition of right atrial identity (10). It also tends to repress proliferation so that the left SHF and sinus venosus are less proliferative than the right. At later stages *Pitx2* has been implicated, in conjunction with *Nkx2-5*, in initiating the formation of pulmonary vein myocardium at the left venous pole (53). *Pitx2* in the left anterior SHF also plays a role in the formation of the arterial pole of the heart (54, 55). The rotation of the outflow tract, which is essential for the correct juxtaposition of the pulmonary trunk and aorta, depends on *Pitx2* and, prior to this, genetic interactions with *Tbx1* have been demonstrated in the SHF (56). *Foxh1* is another transcription factor that plays a role in the development of the anterior SHF (57) and mutations in *FOXH1* have been linked to arterial pole defects in humans (58). This reflects a modification of left/right signalling since *Foxh1* functions in the Nodal pathway. Upstream of Nodal, Hedgehog signalling is important for the establishment of left/right asymmetry in the embryo and *Shh* mutants have arterial pole defects resembling tetralogy of Fallot (59). These mutants have bilateral expression of *Pitx2* and left atrial isomerism (60). Nodal expressing progenitor cells in the left SHF have been shown to direct cardiac looping to the right by coordinating asymmetry at the cardiac poles, acting upstream of proliferative and differentiation pathways (61).

Recent studies have investigated the cell biology and biomechanics of SHF cardiac progenitor cells in the dorsal pericardial wall. SHF cells constitute an atypical epithelium with apical junctions and mesenchymal-like basolateral cell domains characterised by dynamic filopodia (Figure 4) (62). N-cadherin mediated epithelial adhesion in the SHF is required to prevent premature progenitor cell differentiation (63). In the absence of *Tbx1*, SHF cells are more rounded and have elevated apical markers and reduced filopodial structures (62). Consistent with these observations, *Tbx1* has been shown to promote focal adhesion and regulate expression of the focal adhesion protein Paxillin (64). *Tbx1* also regulates expression of the gene encoding the non-canonical Wnt ligand *Wnt5a* in the SHF (65). Like *Tbx1*, *Wnt5a* is required for bidirectional SHF cell addition to the cardiac poles and has been proposed to promote progenitor cell intercalation in the dorsal pericardial wall, contributing, potentially

together with elevated proliferation, to a pushing force that may drive SHF deployment (66-68)}. Increased adhesion in the anterior SHF close to the arterial pole has been proposed to create an additional pulling force (69). Indeed, patterned epithelial tension has been documented across the dorsal pericardial wall during SHF deployment, with elevated tension in *Tbx1*-expressing cells in the lateral posterior DPW where cells are oriented towards the elongating outflow tract (Figure 4) (70). Increased tension is associated with nuclear YAP/TAZ localization that may in turn promote proliferation in a biomechanical regulatory loop driving rapid heart tube elongation. In addition, planar cell polarity pathway genes have been shown to regulate the epithelial status of SHF cells as they differentiate in a transition zone at the cardiac poles and are required for maximal heart tube elongation (71).

#### **2.4 Non-myocardial derivatives of the first and second lineages: Endocardium, epicardium and vascular endothelial and smooth muscle cells**

As the heart tube forms, other cell types of mesodermal origin, in addition to myocardial cells, participate in its development. The endocardium, marked by *Nfatc1* expression, constitutes the inner layer of the cardiac tube and chambers as they develop (22). It provides an important source of signals that promote ventricular trabeculation and also is important for valve formation, initiated by delamination of endocardial cells from the cardiac cushions. The source of the endocardium has been controversial. It derives from cells that had expressed *Islet1* and *Nkx2-5*, suggesting a SHF origin.

The epicardium forms the outer layer of the cardiac tube (22). Epicardial cells delaminate and enter the underlying myocardium where they form the smooth muscle of the coronary blood vessels, as well as constituting the interstitial cells of the heart. The epicardium derives from the proepicardial organ, a transitory mesenchymal structure located near the venous pole of the heart. When cultured, epicardial cells can form myocardium, however whether this is the case in vivo is controversial. Cells in the proepicardial organ express *Islet1* and *Nkx2-5* and the latter is required for its formation. However the relation between the SHF and the epicardium remains unclear.

At the arterial pole of the heart the arterial tree derives from pharyngeal arch arteries. Pharyngeal arches (1, 2, 3, 4, and 6) are transitory extensions of the pharyngeal region that bulge out as pouches on either side of the embryo from E9. Their mesodermal core can be regarded as an extension of the SHF where cells express anterior SHF genes (22). Dye labelling of cells in arch 2, in cultured mouse embryos, has shown that they contribute outflow tract myocardium. The posterior arches give rise to endothelial cells of the arch

arteries, and as expected for SHF derivatives, targeted mutations in genes like *Fgf8/10* (72) or *positive regulatory domain I-binding factor 1 (Prdm1)* (73) lead to malformations of the arterial tree, as well as affecting aorta and pulmonary trunk development. Clonal analysis and genetic tracing has shown that SHF endothelial fates include coronary lymphatic endothelial cells that differentiate within a vasculogenic niche at the base of the pulmonary trunk (74). These cells contribute in particular to lymphatic endothelial cells on the ventral side of the heart, development of which is *Tbx1*-dependent (75).

Isolation of cells, marked by *Islet1* expression for example, from the embryo shows that they give rise to myocardial, smooth muscle and endothelial derivatives and this has also been reported at the single cell level (31, 76). Embryonic stem (ES) cell lines, when directed towards a cardiac fate, give rise to progenitors expressing *Islet1* and *Nkx2-5* (76). When cloned these cells prove to be multipotent again forming vascular and myocardial derivatives. However, this does not establish that cardiac progenitors *in vivo* give rise to more than one cell type. Clonal analysis using an inducible *Mesp1-Cre* and the conditional multicolour *confetti* reporter (13) has clarified this issue during cardiogenesis. Cells of the first myocardial cell lineage, that constitute the first heart field, were found to give rise only to myocardial cells. In contrast, cells of the second lineage that populate the second heart field were found to be multipotent with clones contributing to the endocardium and to endothelial or smooth muscle of blood vessels, as well as to myocardium. Most clones in the epicardium did not show labelling with myocardium, pointing to the existence of a unipotent population of epicardial progenitors.

Refinement of pluripotent stem cell approaches has enabled the differentiation of FHF and SHF lineages to be followed outside the embryo, providing an *in vitro* system to explore interactions during cardiogenesis. For example, the independent emergence of complementary FHF and SHF progenitor populations was followed using fluorescent reporter genes under the control of *Tbx1* and *Hcn4* regulatory elements in a precardiac organoid model, identifying *Cxcr4* as a marker of SHF cells (77). Subsequent genetic studies in mice have confirmed the importance of *Cxcr4* and *Cxcr2* chemotactic signalling in SHF deployment (78). Early steps in cardiogenesis have been recapitulated under conditions that structure organoid differentiation to mimic gastrulation and cardiac induction, including formation of a crescent-like structure expressing FHF markers and an adjacent SHF cell population (79). Comparison of the transcriptome of single cells isolated from these gastruloids with the situation in the embryo suggests that such pluripotent stem cell derived cardiac models will provide tractable systems to study the emergence of cardiac progenitor cells for high throughput analyses.

## 2.5 Insights into heart fields from single cell transcriptomics

Single cell RNA sequencing (scRNA-seq) provides a high resolution view of cell heterogeneity and transcriptome based lineage diversification of particular importance in dissecting developmental processes. Over the past five years the power of this technology has been brought to bear on the emergence of cardiac progenitor cells in the early embryo. In a study of *Mesp1*-expressing progenitor cells emerging from gastrulation, the divergence of molecularly distinct SHF cells and early differentiating FHF cells could be inferred within a multilineage primed progenitor population (Figure 4) (80). Early divergence of cells with characteristics of anterior and posterior SHF subpopulations was also observed in this study.

Subsequent scRNA-seq studies have confirmed and extended prior understanding of the two lineage model of heart morphogenesis. These studies have contributed to defining progenitor cell state transitions during cardiogenesis (81) and highlighted the importance of regional regulators of fate acquisition, identifying a role for *Hand2*, for example, in the right ventricular lineage, where it mediates retinoic acid antagonism in anterior progenitor cells (82). In addition, *Hand1*-expressing progenitor cells, originating at the embryonic-extraembryonic boundary, have been shown to contribute to the FHF and give rise to part of the left ventricle (83). In this study, distinct trajectories to a left ventricular fate were inferred with a second contribution from a predicted lineage related to SHF cells (83). This is consistent with prior evidence based on genetic tracing for *Mef2c-Cre* lineage contributions to the left side of the interventricular septum (15, 49). In another study a *Mab21l2* expressing progenitor cell population has been defined that contributes to the left ventricle and may correspond to late differentiating FHF progenitor cells from the *Hand1*-lineage that contribute to the growing heart tube across the lateral borders of the cardiac crescent, as opposed to the medial contribution of SHF cells (Figure 4) (84). The *Mab21l2* expressing progenitor cells have been termed the Juxtacardiac field and also contribute to epicardium. Trajectory analysis based on these datasets reveals FHF and SHF contributions to the arterial and venous poles of the developing heart, with the FHF contributing to the ventral venous pole and the SHF to the arterial and dorsal venous poles (Figure 4) (83-85). Recent scRNA-seq analysis of gastrulating embryos, coupled with high resolution immunofluorescence and live imaging, has shown that FHF and SHF lineages emerge from spatially and molecularly distinct pools of cells as mesoderm forms in the primitive streak at gastrulation (17). An ongoing challenge concerns relating transcriptionally defined trajectories from such studies to our understanding of clonal and genetic lineages during cardiac morphogenesis. An additional challenge is to

precisely map the embryonic location of different subpopulations of cells defined by single cell sequencing. The development of high resolution spatial transcriptomics approaches will contribute to resolving this latter point.

## 2.6 Cardiopharyngeal mesoderm

Skeletal muscles of the trunk and limbs derive from somites, segments of paraxial mesoderm located on either side of the neural tube along the anterior/posterior axis of the embryo. Their formation depends on a gene regulatory network in which the transcription factor paired box 3 (Pax3) acts upstream of the myogenic regulatory factors of the MyoD family that are required for cell entry into the skeletal muscle programme (39). Head muscles have a non-somitic origin and although the myogenic regulatory factors also control myogenic determination and differentiation, Pax3 is not implicated in the upstream gene regulatory network (39). Extraocular muscles form from anterior pre-chordal mesoderm, but most head muscles derive from mesoderm of the first two pharyngeal (also referred to as branchial) arches, such that masticatory or facial expression muscles derive from the first or second arches respectively. The progenitors of these muscles have expressed genes, such as *Islet1*, that characterise the anterior SHF. Mutation of *Tbx1*, for example, leads to abnormalities in jaw muscles as well as to malformation of the arterial pole of the heart (86). Within the mesodermal core of the arches, cells that have activated myogenic regulatory factors are located in the more distal region compared to cardiac progenitors which continue to express SHF markers in the proximal pharyngeal mesoderm (87). These observations point to cardiopharyngeal mesoderm as a source of cardiac and skeletal muscle, with overlapping expression of SHF genes in the progenitor cells that give rise to the heart and head muscles (88). Retrospective clonal analysis shows that both types of muscle derive from a common progenitor. The *cardiac actin* gene, which was used to drive transcription of the *laacZ* reporter, is expressed in developing skeletal muscles as well as throughout the myocardium. First arch derived head muscles are clonally related to right ventricular myocardium, whereas second arch derived head muscles share a common clonal origin with the arterial pole of the heart, masticatory muscles on the left side of the head with myocardium at the base of the pulmonary trunk and those on the right side with myocardium at the base of the aorta (89). The clonal relationship between head muscles and the heart was also borne out by the observation of co-labelling of cells in the *Mesp1-Cre/confetti* system for genetically tracking clones (13). The results of a clonal analysis of myocardium at the venous pole of the heart indicated unexpected clonality between components of the left venous pole and pulmonary

trunk myocardium. This sub-lineage does not include head muscles, leading to the conclusion that two progenitor cell populations contribute to the myocardium at the base of the pulmonary trunk. Since defects in the formation of the pulmonary trunk are frequent in congenital heart disease, this dual origin is important for diagnosis and prognosis.

The neck constitutes a transition zone where some muscles are somite-derived and others, like the trapezius, sternocleidomastoid, or larynx muscles are marked by genetic tracing with regulatory elements of genes also expressed in the SHF (90). Clonal analysis of these muscles shows that they share common progenitors with venous pole myocardium, derived from the SHF (91). Again there is right/left lineage segregation such that muscles on the right side of the neck are clonally related to myocardium at the right venous pole and left neck muscles to the left venous pole. This sub-lineage includes pulmonary trunk myocardium at the arterial pole of the heart. SHF derived neck muscles show no clonal relationship with head muscles. These lineage relationships further underline the dual origin of pulmonary trunk myocardium (Figure 2). Esophageal skeletal muscle has also been shown to have a cardiopharyngeal genetic origin (92), although the lineage relationship with the heart and other craniofacial muscles remains to be determined. Extending the known derivatives of cells expressing SHF regulators, recent genetic lineage studies using the *Mef2-Cre* enhancer transgene and *Tbx1-Cre* have shown that the connective tissue of somitic-derived neck muscles is in part derived from cardiopharyngeal mesoderm (93). This finding highlights the integration of head and trunk developmental programs in the neck region. Moreover, defective attachment of somite-derived neck muscles is observed in *Tbx1* haploinsufficient mice, suggesting a role for cardiopharyngeal mesoderm in muscle patterning.

A discussion of the evolution of the heart and heart fields is outside the scope of this chapter. It is notable that other vertebrates have a SHF. In birds and fish this is an important source of cells for the arterial pole of the heart (31). A basic gene regulatory network, with similarities to that in the mammalian SHF, that involves genes including *Tbx1*, functions in cardiopharyngeal mesoderm in invertebrate chordates such as the ascidian *Ciona*, the sea squirt (94). Progenitor cells in this mesoderm also give rise to muscle and heart-like structures, pointing to the ancient origins of the relation between cranial muscles and the myocardium (88).

## 2.7 Conclusions

The clonal relationships between progenitor cells that form the heart are important for understanding the potential links between different cardiac malformations. This is illustrated

by the SHF contribution to both the arterial and the venous poles of the heart (25). In the context of the SHF, perturbation of the two different sub-lineages that contribute to pulmonary trunk myocardium, one which also gives rise to head muscles and the other to the venous pole of the heart and neck muscles, will have different clinical manifestations. Effects on the myocardium derived from the second (SHF), as compared to the first (FHF) lineage, may also be associated with endocardial or cardio-vascular defects. These considerations are important for diagnosis and prognosis. Defects that reflect cell lineage relationships also relate to gene expression. *Tbx1* mutations, for example, impact pulmonary trunk myocardium and also affect head muscles. Genes that regulate the posterior SHF and affect the venous pole may also affect the formation of pulmonary trunk myocardium and in this case be associated with neck muscle defects, processes also perturbed in *Tbx1* mutant embryos. Perturbation of mesodermal FGF signalling (72) provides an example of how interference with cells of the anterior SHF, where this signalling pathway plays a key role in proliferation, affects both myocardium at the base of the great vessels and the formation of the arterial tree by affecting endothelial cells derived from the posterior arches.

Congenital heart defects due to mutations in genes coding for regulatory factors or signalling pathways that control cardiogenesis are likely to reflect perturbations in the later SHF contribution to the poles of the heart, since earlier effects are more likely to result in embryonic lethality. However perturbations in the FHF contribution to the atria or the right ventricle, for example, may be compensated by SHF derived myocardium leading to less drastic malformations. Furthermore, depending on the mutation and on genetic compensatory mechanisms, the cellular source, whether FHF or SHF, may be compromised only partially. Continuing work on the mouse genetic model, as well as on organisms as apparently far-removed as *Ciona* (95), should continue to provide insight into the origins of congenital heart defects, their diagnosis and prognosis, with the possibility of correction by post- or pre-natal surgery and in the future by gene or cell therapy, even perhaps *in utero*.

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## Figure Legends

**Figure 1.** Formation of the heart from the first and second heart fields.

Schematic representation of cardiogenesis showing the contribution of the first heart field (FHF – red), located in the cardiac crescent, to the myocardium of the left ventricle and other parts of the heart and the anterior second heart field (aSHF - light green) and posterior second heart field (pSHF - dark green) to the myocardium of the arterial and venous poles of the heart, as well as the right ventricle and atria. LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; OFT, outflow tract; ao, aorta; pt, pulmonary trunk; PV, pulmonary vein; RSV, right superior caval vein; LSV, left superior caval vein. E, embryonic day of mouse development.

**Figure 2.** A lineage tree for the heart.

Schematic representation of the contribution of first and second cell lineages to the mouse heart. FHF, first heart field; SHF, second heart field. Myocardial derivatives are in red and skeletal muscle in blue, with bold type indicating that this is the major source, for example the 1st lineage/FHF for the left ventricle or the 2nd lineage /SHF for head muscles, whereas not all neck muscles derive from this source. Other 2<sup>nd</sup> lineage (SHF) derivatives are indicated with dotted lines since their precise relation to each other or to the tree for myocardium and skeletal muscle is not yet clearly defined - endocardium in brown, endothelial cells in green and smooth muscle in purple. The approximate timing of lineage segregation from a common progenitor, present in the epiblast is indicated - E, embryonic day of mouse development.

**Figure 3.** Patterning the second heart field.

Left: Immunofluorescence showing Tbx1 (green) and Tbx5 (red) expression in the anterior (aSHF) and posterior (pSHF) SHF in sections corresponding to the boxed regions in the schema. Note the overlap present at E8.5 (arrowhead) and formation of a sharp boundary by E9.5 (arrow). Right, top: Expression of a conditional *lacZ* reporter gene at E14.5 showing *Mef2c-AHF-Cre* lineage contributions to atrial septal structures including the dorsal mesenchymal protrusion (arrowhead) and the right ventricle. Blocking retinoic acid signalling during the time window of Tbx5 activation results in loss of the dorsal mesenchymal protrusion and a primum type atrial septal defect (arrow). Right, bottom: diagram illustrating SHF cell segregation to alternate poles of the heart tube. Adapted from De Bono et al. (50). A,

atrium; AP, arterial pole; V, ventricle; RV, right ventricle; LV, left ventricle; RA, retinoic acid.

**Figure 4.** Morphology and single cell transcriptomic analysis of cardiac progenitor cells. Top, left: SHF cells, here expressing a utrophin GFP fusion protein at E9.5, constitute an atypical epithelium with dynamic basal filopodia (arrowheads). Adapted from Francou et al. (62). Top, middle: Segmented ventral view of the dorsal pericardial wall showing that cells, colour coded for surface area, are elongated in the posterior SHF and oriented toward the outflow tract (OFT). Adapted from Francou et al. (70). Top right: Scheme showing two-dimensional representation of single cell transcriptomes at E6.5; note the emergence of distinct cardiac progenitor cells, including anterior (aSHF) and posterior (pSHF) SHF populations, from a multilineage primed progenitor pool. Adapted from Lescroart et al. (80). FHF, first heart field; End, endocardium. Bottom, left: Cartoon showing the lateral and medial locations of the FHF/Juxtacardiac and SHF progenitor populations adjacent to the cardiac crescent at E7.5. Bottom, middle: trajectories from the FHF and SHF to myocardium based on single cell analysis of Tyser et al. and Zhang et al. (83, 84); bottom, right: a cartoon showing the deployment of cells to the elongating heart tube. Bottom panels adapted from Kelly (85).