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The heart field transcriptional landscape at single cell resolution

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Summary

Organogenesis requires the orchestrated development of multiple cell lineages that converge, interact and specialize to generate coherent functional structures, exemplified by transformation of the cardiac crescent into a four-chambered heart. Cardiomyocytes originate from the first and second heart fields, that make different regional contributions to the definitive heart. In this review a series of recent single cell transcriptomic analyses, together with genetic tracing experiments, are discussed, that provide a detailed panorama of the cardiac progenitor cell landscape. These studies reveal that first heart field cells originate in a juxtacardiac field adjacent to extraembryonic mesoderm and contribute to the ventrolateral side of the cardiac primordium. In contrast, second heart field cells are deployed dorsomedially from a multilineage-primed progenitor population via arterial and venous pole trajectories. Refining our knowledge of the origin and developmental trajectories of cells that build the heart is essential to address outstanding challenges in cardiac biology and disease.
Introduction

Identifying the origins of the constituent cells of an organ and the regulatory events by which cell fates are acquired are central goals of developmental biology. The heart primordium, or cardiac crescent, forms ventrally to foregut endoderm in the early embryo and is transformed into a four-chambered definitive heart through a complex series of morphogenetic events. These include formation and extension of a heart tube with distinct venous and arterial poles, rightward looping, chamber growth and cardiac septation. Defects in these processes result in a spectrum of congenital heart defects (CHD) that affect 1 in 100 live births 1. Early differentiating mesodermal progenitor cells, known as the first heart field (FHF), give rise to left ventricular and atrial myocardium. Cells from adjacent pharyngeal mesoderm, known as the second heart field (SHF), contribute progressively during looping morphogenesis to the right ventricle and outflow tract at the arterial pole of the heart as well as to atrial myocardium, including atrial septal structures, at the venous pole 2. There is a major interest in dissecting the control of cardiac progenitor cell deployment in order to understand the mechanisms leading to genetic diseases that affect different regions of the heart. These include outflow tract CHD such as tetralogy of Fallot, atrial and ventricular septal defects as well as regional ventricular defects, for example hypoplastic left ventricle or arrhythmogenic right ventricular cardiomyopathy 1. Moreover, understanding the origins of myocardial diversity is essential to optimize pluripotent stem cell differentiation into specific subtypes of cardiomyocytes for modeling heart disease, drug testing and the development of regenerative therapies.

Biology, in particular developmental biology, is currently being transformed by single cell transcriptomics, a technique that provides unprecedented insights into biological complexity, including cell heterogeneity and differentiation trajectories 3. This revolutionary approach provides snapshots of the transcriptional state of thousands of cells distributed across an epigenetic landscape, a metaphor proposed by Waddington 4, revealing the contours that channel cell fate to differentiated attractor states. Diffusion mapping of the transcriptome of neighboring cells within the landscape allows inference of developmental trajectories along pseudotime from precursor to differentiated cell fates as well as cell location in pseudospace with respect to embryonic axes, subject to validation, for example by spatiotemporal mapping of gene expression and genetic tracing experiments 3,5. First applied to cells within the developing heart in 2016 6,7, a number of recent studies have reported single cell RNA sequencing (scRNA-seq) of cardiac progenitor cells in the early mouse embryo (Figure 1A) 8-16. These experiments provide a valuable resource in the form of rich datasets and online browsers for mining the mechanisms controlling heart development. Insights into the intrinsic and extrinsic regulators of cardiac cell fate decisions emerging from these as well as single cell epigenomic studies have been recently discussed in a number of excellent reviews 17-19. Here we evaluate the impact of these scRNA-seq studies, together with genetic tracing experiments using Cre-Lox technology, on current understanding
of progenitor cell contributions to the heart, with focus on myocardial cell lineages and murine heart development.

Prospective genetic and retrospective lineage analyses have shown that FHF and SHF cells arise sequentially at gastrulation and independently activate expression of the gene encoding the upstream cardiovascular transcription factor, Mesp1. On leaving the primitive streak, FHF cardiac progenitor cells migrate bilaterally to the most anterior lateral splanchnic mesoderm, overlying future foregut endoderm (Figure 1B, C). As the endoderm folds to form the foregut, cardiac progenitor cells from the left and right sides of the embryo converge ventrally to form a coherent epithelial sheet and differentiate into functional cardiomyocytes in the cardiac crescent in response to positive and negative intercellular signaling cues. SHF cells migrate to more medial splanchnic mesoderm where they continue to proliferate and contribute to the inner curvature of the growing cardiac primordium across the dorsal mesocardium (Figure 1B, C). As the heart tube forms, the dorsal mesocardium breaks down isolating SHF cells in the dorsal wall of the pericardial cavity and restricting their addition to the arterial (anterior) and venous (posterior) poles of the heart tube. Heart tube elongation is coincident with cardiac looping and the SHF, patterned by embryonic left right signaling as the cardiac crescent forms, has been identified as a major driver of rightward looping. Clonal analysis has shown that the FHF and SHF largely correspond to two distinct cell lineages defined by their contributions to left ventricular and outflow tract myocardium respectively. Heart tube elongation is complete at midgestation in the mouse, equivalent to the fifth week of human development, and is followed by chamber growth by a process of ballooning morphogenesis. Four cardiac chambers emerge through formation of ventricular and atrial septa and division of the embryonic outflow tract into the pulmonary trunk and ascending aorta.

Insights from scRNA-seq into the early origins of the heart fields

How have the new scRNA-seq datasets impacted on the above understanding of heart fields? Single cell studies of early mouse embryos have shown that lineage specific epigenetic and gene expression patterns emerge at gastrulation and that, compared with other mesodermal fates, cardiac lineages bifurcate rapidly, consistent with the requirement for early functional specialization of the heart. Two studies of precardiac cells in nascent mesoderm have confirmed early lineage diversification and revealed unexpected heterogeneity in the substructure of cardiac progenitor populations. In a scRNA-seq study of Mesp1 expressing cells, Lescroart et al found that FHF and SHF progenitor populations emerge from a molecularly heterogeneous population of nascent mesoderm (Figure 2A). Moreover, this study identified distinct populations of SHF cells with anterior and posterior transcriptional signatures shown by trajectory analysis to have diverged from a common multilineage-primed progenitor population of Mesp1-expressing cells. A second study, combining scRNA-seq with
temporally regulated genetic tracing and live imaging, revealed that progenitor cell populations are prepatterned in spatially and molecularly distinct regions of the primitive streak. Sequential populations of cells leaving the primitive streak were identified, containing left ventricular, atrioventricular canal and epicardial progenitor cells, followed by right ventricular progenitors. Subsequently outflow tract and atrial progenitors migrate along distinct medial and lateral routes from more anterior and posterior regions of the streak respectively (Figure 2B). Together these experiments reveal that molecularly distinct subdivisions of the FHF and SHF, giving rise to different regions of the later heart, originate early in spatially and temporally distinct regions of the primitive streak. Where and when cardiac progenitor cells gastrulate thus defines the position of progenitor cells within nascent mesoderm, and determines future regional contributions to the heart. These findings bring new molecular insight into early cardiac progenitor cell heterogeneity and are consistent with avian fate mapping and transplantation experiments showing that regional contributions to the embryonic heart are prefigured by anterior posterior patterning in the primitive streak and prior medial-lateral cell position in the epiblast. A systematic reconstruction of cell fate trajectories in the mouse, integrating multiple scRNA-seq datasets, has confirmed that the heart fields arise sequentially in splanchnic mesoderm and maintain their specific signatures as FHF and SHF trajectories converge on a common cardiomyocyte transcriptional state.

The juxtacardiac field: progenitor cells of the FHF ventrolateral to the cardiac crescent contribute to growth of the early heart

A number of scRNA-seq studies have focused on cardiac development in the mouse embryo between the cardiac crescent and early heart tube stages. A common motif emerging from these analyses is the convergence of differentiation trajectories along two routes to a myocardial attractor state (Figure 3A). These trajectories appear to reflect FHF and SHF cell addition at the venous and arterial poles of the developing heart from distinct upstream progenitor cell clusters, highlighting the importance of the venous/inflow to arterial/outflow axis during early heart morphogenesis. Tyser and colleagues combined scRNA-seq with spatial analysis of gene expression in a series of embryos separated by two hour intervals and identified two trajectories converging on a myocardial fate with reduced proliferative capacity. Sequencing the transcriptomes of smaller numbers of cells from microdissected reference regions enabled cells along these trajectories to be mapped to locations ventrolateral or dorsomedial to the cardiac crescent, corresponding to FHF and SHF progenitor states (Figure 3B). The ventrolateral population identified an exclusively FHF trajectory from an upstream state demarcated by expression of Hand1 and Mab21L2, encoding the transcription factor Hand1 and Mab21-Like-2 protein. Live tracking with a fluorescent nuclear reporter revealed cell movement from this lateral position towards the cardiac crescent. This is a major advance in our understanding of the FHF, the rapid differentiation of which has largely precluded the identification of FHF progenitor
Genetic fate mapping confirmed that the Mab21l2 lineage contributed to the left ventricle, atrioventricular and atrial myocardium as well as the epicardium, the mesothelial outer lining of the heart that contributes fibroblasts and smooth muscle cells to the fetal heart. This suggests that the early heart tube grows by addition of cells from both sides of the cardiac crescent: medial SHF cells and lateral FHF cells originating in a progenitor population which the authors termed the juxtacardiac field, that maps to the confluence of splanchnic and extraembryonic mesoderm. The position of the juxtacardiac field during early heart tube morphogenesis can be visualized in a recent reconstruction of the dynamic topology of the myocardium and adjacent progenitor lineages. These findings were extended and reinforced in a paper from the Chi and Evans groups. Zhang et al studied the transcriptome of cells in the Mesp1 genetic lineage at similar stages to the Tyser study. Transcriptome based lineage inference identified a Hand1 expressing upstream source of left ventricular cardiomyocytes which was mapped using RNA fluorescent in situ hybridization to the embryonic-extraembryonic mesodermal interface. Analysis using an inducible Cre allele demonstrated that early Hand1 expressing cells in this lateral population are multipotent and also give rise to extraembryonic tissue. The cardiac trajectory from this lateral position passes through a Mab21l2 expressing cell state, similar to that defined by Tyser et al., and was mapped to the ventral side of the venous pole of the early heart. The early Hand1 lineage subsequently gives rise to the dorsolateral, but not septal proximal, region of the left ventricle, as well as epicardium and pericardial tissue (Figure 4). The new data highlight previously unappreciated progressive FHF contributions to growth of the early heart as well as revealing the extreme lateral origin of FHF progenitor cells in splanchnic mesoderm. Indeed, the distinct origin of the juxtacardiac field relative to the SHF points to its correspondence with the first heart lineage identified by retrospective clonal analysis.

Intriguingly, these new results are consistent with a number of prior studies of what appear to be later stages on the Mab21l2/Hand1 FHF trajectory. Experiments carried out by Maria Victoria de la Cruz, who pioneered the concept that the heart grows by addition of extracardiac cells at the poles, had shown that labelled cells at the ventral inlet of the early avian heart contribute to trabeculated regions of the left ventricle, presumably capturing cells on the newly identified trajectory as they enter the heart tube. In another study, cells at the ventral inflow region of the early mouse heart tube were shown to express Endra, encoding Endothelin Receptor Type A, and, by fluorescent dye labeling and transplantation experiments, to be a subset of the FHF that later contributes to atrial, atrioventricular and left ventricular myocardium. Genetic tracing using a Cre allele of the T-box transcription factor 2 (Tbx2) gene revealed that the free wall and base of the left ventricle, but not the left side of the interventricular septum or ventricular apex, are derived from Tbx2 expressing cells, that thus contribute to a similar left ventricular domain as the early Hand1 lineage. Dil labelling experiments suggested that the free wall and base of the left ventricle are progressively added from Tbx2 expressing cells in the atrioventricular canal, coincident with heart tube elongation. These new left ventricular cells were
shown to downregulate Tbx2 expression on leaving the atrioventricular canal where Tbx2 functions to suppresses the chamber program 40. Interestingly, ventricular wall hypoplasia and expanded Tbx2 expression are observed in Endra mutant hearts 39. The juxtacardiac field is distinct from a lateral population of splanchnic mesoderm expressing the T-box transcription factor gene Tbx18. These Tbx18 expressing cells have been shown by genetic tracing experiments to give rise to sinus venosus and pacemaker myocardium 41. Indeed, evidence from clonal analysis indicates that sinus venosus myocardium is derived from the posterior SHF, highlighting the complexity of progenitor cell contributions at the venous pole of the heart 42. Together with the new findings based on scRNA-seq these studies suggest that the FHF is not simply a rapidly differentiating population of cells in the early heart tube; instead FHF cells progressively contribute to the ventral venous pole of the early heart giving rise to atrial, atrioventricular and left ventricular myocardium. This conclusion has important implications for our understanding of the mechanisms driving cardiac looping, ballooning morphogenesis and the etiology of ventricular hypoplasia.

The heart field interface and the origins of left ventricular myocardium

Analysis of the fate of the juxtacardiac field suggests that the left ventricle is derived from at least two progenitor cell populations (Figure 4) 13. Prior genetic tracing experiments provide clues as to the source of left ventricular cardiomyocytes in the early Hand1 and Tbx2 lineage negative region, proximal to the ventricular septum. Genetic tracing of early differentiating cardiomyocytes in the cardiac crescent using an inducible Cre allele at the Acta2 locus, encoding Smooth Muscle Actin, results in labelling of the left side of the muscular septum of fetal hearts (Figure 4) 43. This is consistent with a left ventricular fate of cells in the cardiac crescent and suggests that the primordium of the septal region of the left ventricle is a scaffold on which progenitor cells build during heart tube morphogenesis. In support of this, recent in vivo labelling experiments have shown that a large part of the linear heart tube gives rise to the interventricular region in the avian heart 44. The entire murine left ventricle, including the left side of the septum, has been shown to derive from cells expressing the T-box transcription factor gene Tbx5, a regulator of FHF cells and venous pole progenitors 21,45. Similar experiments using a Cre transgene expressed in the SHF under control of an enhancer from the Mef2c locus, revealed labelling of the future right ventricle that extended into the left side of the interventricular septum, while a lacZ reporter transgene under control of the same enhancer is expressed only in the right ventricle 46. Early expression of the Mef2c enhancer is thus downregulated in Tbx5 expressing cells on the left side of the septum primordium, consistent with emergence of a clonal boundary at the interventricular septum only after initiation of septal morphogenesis 20. Further analysis using a dual recombinase reporter activated by Mef2c enhancer Dre and Tbx5 Cre identified a small population of cells constituting an intersectional lineage 21. The ventricular boundary of Tbx5 expression defines the site of muscular septum formation in avian hearts 47 and conditional mutagenesis experiments in mice have shown that Tbx5 is required
for septation precisely where its expression overlaps with the Mef2c Cre lineage. Moreover, Tbx5 and Mef2c interact genetically during formation of the muscular interventricular septum, where Mef2c coregulates Tbx5 target genes. This ventricular region, that is unlabelled by Tbx2 and Hand1 Cre genetic tracing experiments discussed above, thus appears to be derived from early cardiomyocytes differentiating at the interface between the FHF and SHF.

**Divergent trajectories from the SHF: progenitor cells dorsomedial to the cardiac crescent contribute to both poles of the heart tube**

Tyser et al identified two trajectories by which SHF cells, located dorsomedially to the cardiac crescent, contribute to myocardium, one of which involves a Tbx5 expressing FHF-like intermediate state (Figure 3A). Similarly, Zhang et al identified a FHF-like population that shared a lateral mesodermal origin with cells in the SHF trajectory. These results can be interpreted in the light of the distinct differentiation programs of anterior and posterior SHF subpopulations that emerge early in Mesp1 expressing mesoderm. While the anterior SHF contributes to the arterial pole of the heart, cells from the posterior SHF converge with FHF cells on a venous pole differentiation trajectory. A caudal proliferative center has been identified in pharyngeal mesoderm, expressing the transcription factor genes Tbx1 and Hoxb1, from which SHF cells contribute to both poles of the heart tube, including late contributions to outflow tract myocardium and atrial septal structures. Indeed, a clonal relationship has been demonstrated between arterial and venous pole myocardium using retrospective lineage analysis. Investigation of the mechanism by which SHF cells segregate to alternate cardiac poles has revealed the importance of the retinoic acid (RA) signaling pathway, a critical regulator of cardiac progenitor cell patterning. RA signaling leads to upregulation of Tbx5 in posterior SHF cells close to the venous pole that subsequently downregulate expression of Tbx1 and the Mef2c SHF enhancer (Figure 5A); recent evidence has emerged for a feedforward loop between Tbx5 and RA signaling in the posterior SHF. The posterior SHF contributes to dorsal venous pole myocardium, in contrast to the venral FHF contributions discussed above, subsequently giving rise to atrial septal structures (Figure 5B). Thus while arterial pole progenitor cells retain a SHF program, posterior SHF cells contributing to the venous pole converge on a common differentiation program with FHF cells, consistent with the bifurcating trajectories defined in the Tyser and Zhang studies and an inflow-outflow patterning model of early heart development. Downregulation of the SHF transcriptional program in cells expressing Tbx5 is strikingly similar to the trajectory of cells contributing to left ventricular myocardium close to the interventricular septum, as discussed above. Furthermore, Tbx5 is required in the Mef2c SHF enhancer inage for atrial, as well as ventricular septation. This raises the intriguing possibility that common transcriptional programs operating at the heart field interface may regulate the sites of both atrial and ventricular septation. This is particularly relevant given that septation defects account for over 50% of CHD.
Multilineage primed progenitor cells in pharyngeal mesoderm

*TBX1* is the major gene implicated in 22q11.2 deletion or DiGeorge syndrome, patients being characterized by a spectrum of CHD. *Tbx1* is required for SHF contributions to both cardiac poles and has been shown by scRNA-seq analysis to be expressed in a multilineage primed progenitor cell population upstream in the SHF trajectory. These cells were mapped using RNA FISH to the posterior pharyngeal region, from where they progressively give rise to anterior and posterior SHF cells, as well as to progenitor cells of skeletal muscles of the head and neck (Figure 5C). Multilineage primed cells giving rise to the anterior and posterior SHF, first observed in nascent *Mesp1*-expressing mesoderm, are thus present throughout the process of heart tube elongation. Multilineage primed progenitors represent a transition state within which extrinsic signaling events influence lineage outcomes. Multilineage priming has also been documented in cardiac and pharyngeal muscle progenitor cells in *Ciona*, followed by de novo gene activation and downregulation of alternate programs on differentiation. scRNA-seq analysis of the *Mesp1* and *Tbx1* lineages in *Tbx1* mutant mouse embryos, together with RNA FISH experiments, have shown that *Tbx1* regulates progressive differentiation of multilineage primed cells and blocks expression of alternative transcriptional programs. Failure of SHF and branchiomeric muscle differentiation is likely to underlie the cardiac and skeletal muscle defects in *Tbx1* mutant embryos and *TBX1* haploinsufficient 22q11.2 deletion syndrome patients. These experiments identified new markers of upstream SHF cells, including genes encoding the Apelin receptor Aplnr and signaling molecule Nrg1, both shown by ATAC-seq to be direct Tbx1 target genes. The deployment of multilineage primed SHF cells in the posterior pharyngeal region to both cardiac poles is dependent on another *Tbx1* target gene, *Wnt5a*, encoding a non-canonical Wnt signaling ligand. De Soysa et al took a network-based prediction approach to identify specifiers of different myocardial fates in a scRNA-seq study of cardiac tissue during early heart tube development. This led to the identification of the transcription factor *Hand2*, required for development of the right ventricle, as a specifier of outflow tract fate. While right ventricular cells were specified in *Hand2* mutant embryos, they failed to migrate to the arterial pole or differentiate. Moreover, RA signaling was ectopically activated, leading to posteriorization of cells in the anterior SHF. This work thus implicates *Hand2*, as well as *Tbx1*, in normal segregation of anterior and posterior fate in the SHF. Intriguingly, both factors have been shown to control epithelial integrity in the anterior SHF, potentially impacting on progenitor cell deployment to the arterial pole. Together the Nomaru and De Soysa studies demonstrate the power of scRNA-seq approaches for molecular phenotyping and uncovering the pathological mechanisms leading to CHD. Further work is necessary to dissect the regulatory networks underlying the maintenance of multilineage primed cells in pharyngeal mesoderm, as well as the progressive emergence of different cell fates.
The existence of temporally distinct progenitor populations that sequentially give rise to the right ventricle and subdomains of the outflow tract has been confirmed by pulsed genetic tracing experiments using inducible Cre recombinase expressed in the SHF. Distinct SHF populations have also been defined on the basis of clonal links with different groups of head and neck skeletal muscles. SHF cells giving rise to the right ventricle are clonally related to mastication muscles from the first pharyngeal arch, outflow tract progenitors to second arch derived muscles of facial expression and atrial and outflow tract myocardium at the base of the pulmonary trunk to neck muscles from posterior pharyngeal arches. These links arise from caudal displacement of the elongating heart during pharyngeal morphogenesis. SHF cells at the level of different pharyngeal arches are thus likely to be characterized by as yet unresolved temporal, as well as spatial, changes in the transcriptional program of the multilineage primed progenitor population. The posterior SHF program is shared by pulmonary mesodermal progenitor cells and scRNA-seq experiments point to complex subpopulations of cells with posterior SHF transcriptional profiles, that remain to be fully characterized, both with respect to their location in the early embryo and their contributions to the heart.

scRNA-seq has provided further insights into the regulatory steps controlling SHF deployment at the arterial pole of the heart. Progressive differentiation of SHF cells in a transition zone from progenitor to myocardial fate is coordinated by BMP and Wnt signaling from cells of the early heart tube. scRNA-seq of pan-cardiac (Nkx2-5) and SHF (Isl1) progenitor cell lineages identified additional cross talk involving the cytokine MIF (Macrophage Migration Inhibitory Factor) in FHF cells and the receptor Cxcr4 in the SHF that promotes progenitor cell migration. Genetic analysis revealed that Cxcr4 responds to MIF signaling together with Cxcr2 during SHF addition. This work also identified a series of intermediate SHF differentiation states in contrast to rapid wave like differentiation observed in FHF cells. A second study of Nkx2-5 and Isl1 expressing progenitor cells using scRNA-seq showed that Isl1 expression in the SHF is associated with an attractor state, exit from which requires Isl1. ScRNA-seq analysis has also revealed that the SHF differentiation trajectory is interrupted in the absence of the transcription factor Pitx2, leading to failure to activate Tgfb1 and Hand1 in the outflow tract. Finally, a specific subpopulation of proximal outflow tract progenitor cells expressing the stem cell regulator LGR5 has been identified in human but not mouse hearts using scRNA-seq; this may promote amplification of a transitional cell population for correct arterial pole alignment.

Conclusions and perspectives

Single cell RNA-seq has refined the contours of the heart field transcriptional landscape and brought the complexity of early heart development into focus. The studies discussed here, together with genetic tracing experiments, have revealed that the early cardiac primordium grows by addition of FHF as well as SHF progenitor cells from the ventrolateral and dorsomedial sides of the cardiac crescent,
respectively. While the juxtacardiac FHF lineage contributes to the ventral venous pole of the heart, SHF cells segregate to the arterial and dorsal venous poles from a multilineage primed progenitor population. These observations highlight the dominance of venous/inflow and arterial/outflow transcriptional trajectories through which FHF and SHF progenitor populations deploy during early heart morphogenesis. Two progenitor populations make complementary contributions, that remain to be precisely defined, to the left ventricle: the juxtacardiac FHF field gives rise to most of the left ventricle while cells at the heart field interface, that share a developmental trajectory with atrial septal progenitors, contribute to the septal region. In considering these results, the limitations of transcriptome-based trajectory inference, the challenge of precisely mapping the spatiotemporal location of single cells in the embryo, and caveats associated with genetic lineage interpretation, need to be taken into account. Nevertheless, the new findings significantly extend our understanding of progenitor cell contributions to the heart, reinforcing a two heart field model and the importance of venous/inflow to arterial/outflow patterning. Further mining of scRNA-seq datasets, together with ongoing advances in single cell omics approaches, including deeper sequencing to detect genes transcribed at low levels, and insights from ATAC-seq and ChIP-seq will clarify understanding of the heart field landscape as well as dissecting the intrinsic regulatory hierarchies and extrinsic signals driving progenitor cell trajectories during early heart development. Open questions include, for example, how embryonic laterality impacts on progenitor cell deployment during looping morphogenesis. The dominance of the differentiation trajectory in defining cell position in low dimensional representation of single cardiac progenitor cell transcriptome datasets has not yet allowed potentially more subtle information, for example concerning embryonic laterality, to be resolved. Combining single cell RNA-seq with rapidly emerging spatial transcriptomic technologies will be essential to fully map the progenitor cell landscape. Similarly, downstream effector genes driving regional cardiac development, including the establishment of septal primordia, remain largely unidentified. Additional advances already underway combining scRNA-seq with lineage recording, spatial transcriptomics and live imaging, as well as proteomic approaches, will allow construction of a more complete roadmap of early heart development. Establishing such a roadmap is essential to address outstanding challenges in cardiac biology and disease. For example, integrating scRNA-seq datasets with genetic analysis of CHD patients can provide insights into the critical processes and pathways underlying distinct sets of defects; this strategy has been used to identify OFT progenitor cells as a cellular hotspot in the pathogenesis of tetralogy of Fallot. Finally, development of in vitro organoid systems to study heart fields using pluripotent stem cells is a promising approach to study cardiogenesis and disease mechanisms at high throughput. FHF and SHF trajectories have been documented by scRNA-seq during cardiogenesis in a self-organizing gastruloid system that recapitulates steps of early heart development in vitro with in vivo-like fidelity. Human induced pluripotent stem cells can be directed along heart field-specific differentiation trajectories to generate defined cardiomyocyte subpopulations for disease modelling and development of therapeutic strategies. Recent studies have highlighted the power of such in vitro approaches, revealing
conservation of the cardiac progenitor cell transcriptional landscape in mice and humans and the ability of different populations of cardiac progenitors to sort and co-develop as multichambered cardioids\textsuperscript{89,90}.

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Declaration of interests

The author declares no competing interests.
Figure legends

Figure 1. Investigating cardiac progenitor cells and early mouse heart development by single cell RNA-seq. (A) Table of the major scRNA-seq studies discussed in this review showing the relevant embryonic days (E) of mouse development. Together these datasets include the transcriptomes of over 10^5 cells. Where relevant, the genetic tools used to select cells for scRNA-seq are indicated on the right. (B) Cartoon showing cardiac structure at the stages in (A) in left lateral (gastrulation) and ventral views. Transverse sections are shown in (C) equivalent to the level of the broken lines at the cardiac crescent (left) and heart tube stages (right). SHF cells and derivatives are shown in green. PS, primitive streak; AP, arterial pole; VO, venous pole; OFT, outflow tract; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Embryonic axes are indicated: A, anterior; P, posterior; D/M, dorsomedial; V/L, ventrolateral; M, medial; L, lateral.

Figure 2. Early transcriptional diversity in cardiac progenitor cells. Cartoons showing the emergence of SHF and FHF progenitor populations in nascent mesoderm in (A) a two dimensional representation of Mesp1-expressing scRNA-seq data and (B) a left lateral view of a mouse embryo at embryonic day 7 showing progenitor cells migrating from the primitive streak to the heart forming region; future cardiac fate is indicated: LV, left ventricle; RV, right ventricle; A, atrium; OFT, outflow tract. Nascent mesoderm is indicated in A by light grey and endothelial progenitors by blue. Cells in B are color-coded based on interpretation of transcriptional states from Figure 3. EEM, extraembryonic mesoderm; A, anterior; P, posterior; L, lateral; M, medial.

Figure 3. Cardiac progenitor cell trajectories to the heart. Cartoons showing (A) two dimensional representation of scRNA-seq data based on the Tyser and Zhang studies showing inferred Juxtacardiac JCF/FHF and SHF trajectories to myocardium through intermediate venous and arterial pole differentiation states; (B) the location of lateral JCF/FHF and medial SHF cells on either side of the cardiac crescent at E7.5.

Figure 4. Genetic lineage contributions to left ventricular myocardium. Schema of ventral (left) and dorsal (right) views of a fetal heart, showing the contributions of genetic lineages derived from cells expressing Acta2 in early differentiating cardiomyocytes (orange) and Hand1 during gastrulation (red), to the lateral wall and septal regions of the left ventricle. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

Figure 5. Divergent differentiation trajectories from the SHF. (A) Schema showing the segregation of SHF cells to anterior (aSHF) and posterior (pSHF) SHF transcriptional states; (B) sagittal section showing the addition of SHF and FHF progenitor cells to the elongating arterial and venous poles at
E8.5; (C) right lateral view of an E9.5 embryo highlighting the contribution of multilineage primed progenitor cells (MLP) to alternate cardiac poles and branchiomeric muscle progenitor fates (blue) from caudal pharyngeal mesoderm. 1 and 2 indicate the first and second pharyngeal arch; A, anterior; P, posterior; D, dorsal; V, ventral.
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Figure 1

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Mesp1-rtTA; TetO-GFP
Microdissection with T-Cre
Is11-Cre, Nkx2.5-Cre
Mesp1-Cre
Nkx2.5 and Is11-GFP
Microdissection
Microdissection
Mesp1-Cre
Tbx1-Cre
Microdissection with Foxa2-Cre

B

Gastrulation
Cardiac crescent formation
Heart tube extension and looping
Midgestation

C

Neural tube
Ectoderm
Somatic mesoderm
Splanchnic mesoderm
Endoderm
Cranial mesoderm
Cardiac crescent
Extraembryonic tissue
Somatic mesoderm
Pericardial cavity
Neural tube
Dorsal mesocardium
Heart tube
Pericardium

Kelly, Figure 1
Figure 2

A

Epiblast

Hoxb1

Tbx1

SHF

FHF

\(\text{FHF cardiomyocytes}\)

\(\text{Anterior SHF}\)

\(\text{Posterior SHF}\)

B

EEM

RV

OFT

L

M

A

P

\(\text{Primitive streak}\)

Kelly, Figure 2
Kelly, Figure 4

- Hand$1^{CreERT2}$ (induced at E5.75)
- Acta$2^{CreERT2}$ (induced at E7.5)
Figure 5

A

Tbx1 → SHF → Hand2 → RA → Tbx5 → pSHF → Arterial pole

B

Arterial pole

C

Venous pole

Kelly, Figure 5