Single Cell Approaches to Understand the Earliest Steps in Heart Development

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Abstract

Purpose of Review. Cardiac progenitors are the building blocks of the heart. Our knowledge, on how these progenitors build the heart, has considerably increased over the last two decades with the development of single cell approaches. We discuss the lessons learnt from clonal analyses and from single cell sequencing technologies on the understanding of the earliest steps of cardiac specification and lineage segregation.

Recent Findings. While experiments were initially performed at the population level, the development of approaches to investigate heart development at the single cell resolution, has clearly demonstrated that cardiac progenitors are highly heterogeneous, with different progenitors contributing to different cardiac regions and different cardiac cell types. Some critical transcriptional determinants have also been identified for cardiac progenitor specification.

Summary. Single cell approaches have finally provided insights into the spatio-temporal specification of unipotent and multipotent cardiac progenitors and provided a framework for investigating congenital heart defects.

Keywords: cardiac specification; cardiac progenitors; heart development; clonal analysis; single-cell transcriptomics; cardiac lineages; second-heart field.

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Introduction

Building organs, such as the heart, starts with the specification of a pool of progenitor cells and segregation towards the different lineages that will form the tissue. The mammalian heart is a complex organ, with different chambers, that ensure the oxygenation of the blood and transport of oxygen and nutrients throughout the organism during both fetal and post-natal life. The adult heart contains diverse cell types including myocardial and endocardial cells that appear progressively during embryonic development, and which are essential to maintain its function. Heart development is a complex but ordered process that is spatially and temporally regulated [1]. Studies in chick embryos have first defined the cardiogenic mesoderm as the area of the lateral plate mesoderm that has the potential to form myocardium. In the mouse, the earliest cardiac progenitors, originate from the primitive streak during gastrulation, around embryonic day (E) 6.5, and then migrate rostrally (anteriorly) to form the first differentiated structure called the cardiac crescent at E7.75. The earliest molecular regulator of these cardiac progenitor cells (CPCs) is the transcription factor Mesp1 (Mesoderm posterior 1) [2]. *Mesp1* is expressed in gastrulating mesoderm but then is rapidly downregulated. Cells in the cardiac crescent constitute the first heart field (FHF), and mainly form the left ventricle (LV) [3]. Convergence of the bilateral structures results in the formation of the linear heart tube. The heart tube is a transient structure composed of an inner layer, the endocardium, and covered by a myocardial layer. The rapid growth of the heart tube is driven by progressive addition of second heart field (SHF) progenitor cells from adjacent splanchnic or so called pharyngeal mesoderm [4]–[6]. These cells ultimately contribute to the cardiac outflow tract (OFT), right ventricle (RV) and a major part of atrial myocardium [4], [7]. Impaired SHF development has been shown in animal models to result in a spectrum of congenital heart defects (CHD). As the tube forms, it undergoes asymmetric morphogenesis (heart looping), which corresponds to the formation of a rightward helix at E8.5. After rightward looping, the heart is shaped by expansion of the myocardium, which leads to the formation of four cardiac chambers. Separation of the left and right ventricular chambers depends on the interventricular septum, which has dual contribution from the two heart fields [8], [9]. The heart is connected by the OFT to the aortic sac and the pharyngeal arch arteries. At its maximal extension, the OFT is a tensioned myocardial cylinder lined with endocardial cells. Interactions between multiple cell types including myocardial, endocardial and neural crest cells are involved in septation of the OFT. In addition to myocardial (CMs) and endocardial lineages (ECs), the adult heart contains smooth muscle cells (SMCs), fibroblasts and vascular cells that derive from the epicardium, which itself originates from the proepicardium located at the base of the looping heart [10].

Our knowledge on the early steps of heart development has increased considerably since the discovery of the SHF, 20 years ago, and the emergence of new approaches that allow investigation of cardiac specification and differentiation at single cell resolution. We will discuss in this review what these single cell techniques have taught us on how CPCs can build a functional heart, with a special focus on clonal analyses and single-cell transcriptomics.

1. Clonal analyses as tools to investigate cardiac progenitor derived lineages

The first description of the two sources of cardiac progenitors was based on a mouse cell lineage study using β-galactosidase labelling in a retrospective clonal analysis [4], [11]. This approach relies on a rare and random recombination event in the $\alpha_{cardiac}$-*actin-nlaacZ* cassette that reverts the non-functional *nlaacZ* cassette into a

functional *nlacZ* gene that will be transmitted in all descendant cells. The spatial distribution of clonally related β-gal positive cells at E8.5 revealed two categories of clones with distinct patterns of regionalization in the heart tube [11]. One lineage contributes to LV, atrioventricular canal (AVC), and the atria while the other contributes to the OFT and all other heart regions, except the LV. This second lineage corresponds to the contribution of the SHF, in which the transcription factor Isl1 (LIM homeodomain transcription factor Islet1) is expressed [12]. This retrospective clonal analysis demonstrated that the first and second lineages segregate from a common progenitor at the onset of gastrulation. Interestingly, SHF cells also give rise to multiple cell types including CMs, ECs, SMCs and skeletal muscle cells that contribute to the heart, pharyngeal arches and head/neck muscle cells [13], [14]. Indeed, retrospective clonal analysis performed at E14.5, showed that masticatory muscles, which derive from the first pharyngeal arch, share a common progenitor with the RV myocardium, whereas facial muscles deriving from the second pharyngeal arch, are clonally related to myocardium at the base of the great arteries [14]. Moreover, non-somite-derived skeletal muscles of the neck, which derive from the most posterior pharyngeal arches, showed lineage relationship with the myocardium at the venous pole of the heart [13]. These findings revealed a lineage relationship between neck/head muscles and SHF myocardial derivatives with left and right segregation very early during embryogenesis (Fig. 1a).

At E7.5, the first differentiated cells in the cardiac crescent are marked by *Tbx5* and *Hcn4*. Genetic or lineage tracing experiments, based on the use of a recombinase (Cre or Dre for example) under the regulatory sequences of a gene marker and a conditional reporter that will be activated when the marker is expressed, are particularly helpful to trace cells that express a marker of interest [15]. Studies using *Tbx5CreERT2* and *Hcn4CreERT2* transgenic mice confirmed that FHF derivatives contribute to cardiomyocyte in the LV and parts of the atria [16], [17]. Lineage tracing experiments using *Mesp1-Cre* mice showed that all cardiac cells, including derivatives of the FHF and SHF, derive from CPCs expressing *Mesp1* [18], [2]. Similarly, the study of *Flk1-Cre* mouse line, which marks early mesodermal cells, showed that Flk1+ CPCs contribute to both CMs and ECs development [19], [20]. *In vitro* experiments using mouse or human embryonic stem cells (ESCs) have further validated the multipotentiality of CPCs. Flk1+ or Mesp1+ CPCs were able to differentiate into CMs, SMCs and ECs [21]–[24] and even to skeletal muscles [25]. However, all these experiments did not rule out whether a single CPC, in its niche, has the ability to contribute to multiple cardiac lineages. Only clonal analysis can address whether there is collective or individual multipotency and whether FHF and SHF progenitor cells arise from a common Flk1+/Mesp1+ progenitor. Thus, Lescroart *et al*. (2014) used clonal analysis of the earliest prospective CPCs in a temporal controlled manner (using tetracycline-inducible *Mesp1-rtTA* transgenic mouse with low dose induction) during early gastrulation to show that *Mesp1* progenitors consist of two temporally distinct pools of progenitors restricted to either the FHF or the SHF. Indeed, FHF and SHF cells correspond to the early and late Mesp1+ populations respectively [18]. The clonal analysis of single Mesp1+ CPCs revealed that FHF progenitors are unipotent since they are restricted to either CMs or ECs fates at the time of their specification (Fig. 1a). In contrast, *Mesp1*-derived SHF progenitors can be unipotent or bipotent (Fig. 1a). Interestingly, the ultimate fate of the progenitors can be regulated by the environmental factors that the different progenitors encounter during cardiac morphogenesis. Devine et al. (2014) used *in vivo* clonal analysis, by generating mosaic mice in which very few *Mesp1*+ cells were labeled, to confirm that during gastrulation, CPCs are already split into two distinct populations. Results from this study also suggest that these two populations are separated by a boundary that is established very early during development to form the septum that separates the LV and RV in the matured heart.

A recent study addressed the location of distinct FHF and SHF progenitors in the early gastrulating embryos. This study used *Hand1CreERT2* mice to perform a lineage tracing analysis and revealed a *Hand1*-expressing population at the boundary of the extraembryonic and intraembryonic regions of the gastrulating embryo that contributes to FHF derivatives [26]. Interestingly, these *Hand1*-labeled cardiomyocytes reside largely within the dorsal region of the LV and AVC. Since LV cardiomyocytes have been shown to derive from the FHF, these results suggest that cells marked by *Hand1* represent a subset of the FHF. Zhang et al. (2021) used *Hand1CreERT2* and *Rosa26-Confetti* mice to perform clonal analysis and showed that this subset of FHF-derived cardiomyocytes derive from a common multipotent *Hand1*+ progenitor, which also contributes to the proepicardium (Fig. 1a).

Clonal analysis was also used to trace other cardiac cell lineages during early heart development. Red-Horse et al. (2010) used clonal analysis to investigate the origins and early development of coronary arteries. Clonal analysis showed that coronary arteries develop from endothelial sprouts of the venous inflow tract of the forming heart [27]. Clonal analysis was also used to study the lineage relationships between non-venous SHF-derived cells at the base of the great arteries and the cardiac lymphatic vasculature. Lioux *et al*. performed a random, lineage-unrestricted clonal analysis of the developing heart to explore all lineage relationships in an unbiased manner. This study characterized the fate of the SHF progenitors at the base of the great arteries and found a contribution of SHF cells to the coronary lymphatic vasculature. The clonal analysis identified a shared lineage for arterial mesothelial and sub-mesothelial cells and cells derived from the SHF like right ventricular cardiomyocytes, valve mesenchyme and coronary ECs [28].

The cardiac conduction system (CCS) coordinates atrial and ventricular contraction via generation and propagation of electrical impulses. The ventricular conduction system (VCS) is composed of central components (the atrioventricular node, bundle, and right and left bundle branches) and a peripheral Purkinje fiber network. Retrospective clonal analysis combined with genetic tracing experiments using *Connexin40*-GFP (*Cx40*-GFP) mice, that delineate the VCS, revealed a dual contribution of FHF and SHF progenitor cells during the formation of the VCS [29]. Clonal analysis using *SmaCreERT2* and *Rosa26-Confetti* mice highlighted the early segregation of the VCS lineage within SMA+ cardiomyocytes [30]. This study using SMA prospective clonal analysis suggested also that the atrioventricular bundle is specified earlier than the bundle branches or during a more restricted time window. These findings are in accordance with the retrospective clonal analysis indicating the existence of early common progenitors for conductive and working cardiomyocytes [29]. These studies suggested that clonal analysis is a strategy more appropriate to distinguish lineage segregation. More recently the same group used *Cx40CreERT2* and *Rosa26-Confetti* mice as a temporal clonal analysis of CPCs to identify distinct phases of peripheral Purkinje fiber network formation [31]. In addition, this study demonstrated the existence of bipotent progenitors that participate in the early and late phase of CSS development.

Photoconvertible assay was used in zebrafish as a clonal analysis to quantify the dynamics and regulation of myocardial cell addition during heart development. Similar to murine heart growth, Lazic and Scott used photoconvertible fluorescent protein to reveal gradual myocardial addition at the arterial pole of the zebrafish heart between 24 hours post-fertilization (hpf) and 34hpf [32]. This study showed that the cells contributing to the early heart tube, and the later addition to the arterial pole, are derived from the same pre-gastrula region of the embryo, suggesting a shared progenitor. This result was confirmed by a lineage tracing analysis showing that latent TGF-β binding protein 3 (*ltbp3*) transcripts mark a field of CPCs with defining characteristics of the SHF in mammals [33].

In addition to their contribution to lineage inference, clonal analyses have also been determinant for investigating cell behavior, such as CPC's mode of growth [34]–[37]. Ivanovitch and colleagues have also elegantly tracked single CPCs during heart morphogenesis and showed the dynamics of differentiation of FHF and SHF progenitors [38]. Using live imaging of *ex-vivo* cultured mouse embryos, they could demonstrate three phases during heart morphogenesis: First, FHF progenitors rapidly differentiate to form the cardiac crescent, then cardiac differentiation slows down while important morphogenesis processes occur, and finally SHF progenitors differentiate and participate in the heart tube closure.

All these studies have contributed to a better understanding of CPC specification and deployment. Clonal analyses have thus been really determinant to reconstruct the lineage history of CPCs and their contribution to the heart (Fig. 1a). These findings also revealed a high heterogeneity in CPCs. However, the molecular characteristics, such as gene expression and gene regulatory networks, cannot be addressed with classical clonal analysis. The recent development of single-cell transcriptomics has opened new avenues to address these questions.

2. Single-cell transcriptomics as a novel platform to investigate CPC specification and differentiation at a single cell resolution

While bulk transcriptomic analyses provide an average gene expression, ignoring cell heterogeneity and stochasticity of gene expression, transcriptomics at the single cell level is a powerful approach for characterizing individual cells. Analyzing the transcriptome in single cells, indeed allows the description of tissue cell heterogeneity, the identification of rare cell types and comparison of cell heterogeneity in healthy and pathological contexts. In addition, new tools now also allow elucidation of dynamics transition states during development [39], to some extent to infer lineage trajectories [40] and to hypothesize upon cell-cell communications via ligand-receptor binding [41], [42]. Protocols are detailed in a review by Kolodziejczyk et al. [44].

Single cell transcriptomics has started to be used to investigate early cardiac specification in the early 2010s. To explore the potential of Mesp1+ early CPCs *in vitro*, Bondue et al. have performed single-cell PCR using key marker genes of the different cardiovascular lineages (*Pecam1* for ECs, *Acta2* for SMCs, *Tnnt2* for CMs, *Isl1* as a pan SHF marker and *Tbx5* for the FHF) and showed the pluripotency of single Mesp1+ ESC-derived CPCs [21]. Similarly, *in vivo*, cell sorting of *Mesp1*+ CPCs at gastrulation, followed by single-cell PCR, have demonstrated the molecular heterogeneity of CPCs and supported the results of uni- and bipotency revealed by clonal analysis [18]. With the development of techniques for single cell encapsulation, Li et al. have then further investigated and compared *in vitro* and *in vivo* cardiac specification using a microfluidic-enabled multiplex PCR platform. They showed the utility of single cell transcriptome profiling to study cardiac specification and cell fate choices [46]. These experiments showed, in particular, that ESC-derived cardiac lineages are close to cells isolated from the embryonic heart. This approach has been also used to describe the molecular heterogeneity of cells in the heart tube and has allowed the definition of the different gene expression profiles of different subpopulations in the early heart tube [47]. In a parallel set of experiments, Delaughter and colleagues have used the fluidigm platform to perform single cell RNA sequencing (scRNAseq) of cardiac cells from E9.5 to the neonatal stage [48]. scRNAseq has, this time, allowed a non-biased study of the whole transcriptome. These studies have provided an

atlas of the transcription expression profile of all major embryonic cardiac cell types, which is fundamental and represent the basis for further studies on progenitor cell fate decisions toward these different characterized cardiac cell types. In addition, atlases have been generated for different embryonic stages in the mouse, focusing on different cardiac cell types [49], [50], [51, p. 201], and for other species such as tunicates or even human [52], [53] (Table 1).

Further scRNAseq studies performed on CPCs have revealed the transcriptional heterogeneity of these cells during early heart development, as well as, at later time points within SHF progenitors. The analysis of early *Mesp1* expressing cells at E6.25 and E7.25 revealed a relative heterogeneity among CPCs, at the time of gastrulation, with a continuum of cells to at least 4 distinct cardiac related trajectories [54]. These 4 final subpopulations likely correspond to ECs (marked by the expression of *Sox17* and *Etv2*), CMs (*Hand1*+, *Bmp4*+, *Tnnt2*+), and to anterior (*Tbx1*+, *Foxc2*+) and posterior SHF progenitors (*Wnt2b*+, *Hoxb1*+, *Hoxa1*+) (Fig. 1b). These results have further demonstrated the heterogeneity of the CPCs, reflecting their different lineage and regional contributions to the adult heart and suggested that CPCs segregate early in the different lineages. Furthermore, high resolution single molecule fluorescent *in situ* hybridization (smFISH) has shown that markers of the 4 Mesp1+ subpopulations were expressed in specific locations in the embryo. CMs progenitors are, for example, located close to the epiblast, while ECs progenitors are located in the vicinity of the visceral endoderm (Fig. 1b). These findings were further confirmed by Ivanovitch et al., with recovery of clusters for FHF, anterior and posterior SHF from scRNAseq of E7.5 embryos, which could be further subdivided into subclusters that likely correspond to different cardiac regions [55]. The combination of lineage tracing with scRNAseq has allowed to conclude that FHF, anterior and posterior SHF derive from distinct groups of cells with different transcriptional programs, that are found in particular spatial and temporal regions of the primitive streak at gastrulation [55]. At slightly later time points, de Soysa et al. have shown that the *Isl1*+ CPCs could be divided into at least 3 subclusters with distinct transcriptional profiles: the anterior and posterior SHF, as well as, the head muscle progenitors [56]. This conclusion is consistent with findings obtained by clonal analysis experiments. Cells from the heart tube could be also subdivided into different transcriptional clusters, representative of the LV and RV, atrial, sinus venosus, AVC and OFT myocardium, confirming previous reports on the significant heterogeneity in the transcriptional profile of cardiomyocytes in the heart depending on their location [47], [48], [56].

As single-cell transcriptomics allow the analysis of pseudotime and the reconstruction of lineage trajectories, lessons on cardiac lineage contribution could be learnt from scRNAseq of CPCs. With such approaches, two recent scRNAseq studies have recently highlighted the contribution of a population of CPCs that express *Mesp1* early and is marked by the expression of *Hand1* and *Mab2l12.* This population contributes specifically to the left ventricle and was called the JuxtaCardiac Field (JCF), likely a subdivision of the FHF [26], [57]. In their study, Tyser and colleagues showed that, at early stages of heart development (between E7.75 and E8.5), heart derivatives could be divided into 5 clusters with specific transcriptional signatures. Analysis of the transcriptional trajectories showed that differentiated cardiomyocytes could be produced though transition states corresponding to the SHF and FHF progenitors, as well as, through a population expressing a newly identified marker, *Mab2l21* [57] (Fig. 1c). Zhang and colleagues have applied lineage inference (URD) to reconstruct lineages from scRNAseq of Mesp1-derived cells between E7.25 and E8.25. This analysis revealed a close

relationship between clusters defined as late extraembryonic mesoderm (LEM) and lateral plate mesoderm and a common contribution to a cluster of CMs. The LEM cluster is characterized by high *Hand1* expression and *Mab2l12* is expressed along the trajectory from this cluster toward differentiated CMs [26] (Fig. 1d). Lineage tracing of *Hand1* and *Mab2l12* derived cells showed that this population contributes to the epicardium as well as to the CMs of the LV and AVC. Once again, with high resolution smFISH, the clusters defined by scRNAseq were mapped to the embryo, showing that the JCF or *Hand1*-derived *Mab2l12*+ population is found at the interface between the cardiac crescent and the extraembryonic tissue [26], [57] (Fig. 1c-d). These results are in total agreement with temporal *Mesp1* clonal analyses, which showed that early *Mesp1* CPCs contribute to the epicardium and CMs of the LV [18]. To investigate the multipotency of CPCs, other groups have analyzed the transcriptome of single Nkx2-5+ vs Isl1+ progenitors between E7.75 and E9.5. Trajectory inferrence suggested that Nkx2-5 expressing cells were committed to a unidirectional CM fate whereas *Isl1* expressing CPCs could diverge into multiple developmental branches toward a CM or EC fate, with evidences for an intermediate cell state [58]. However, in another study, transcriptional profiling of *Nkx2-5*-derived CPCs has showed that the Nkx2-5 lineage seems to have a lineage trajectory toward both CM and ECs [59]. The discrepancies between these analyses could be due to the difference between *Nkx2-5* derived CPCs and *Nkx2-5* expressing CPCs or might exemplify the limits of lineage inference using single-cell transcriptomics, that will be further discussed later in this review.

Single cell transcriptomics of control versus mutant cells also offers a way to identify genes involved in cardiac specification or in lineage bifurcation. Those analyses have confirmed, for example, that Mesp1/Mesp2 are critical for the exit of pluripotency and for early cardiac specification as cells with decreased level of *Mesp1/Mesp2* showed a transcriptional profile similar to epiblast cells [54]. Computational methods can also be used to predict lineage-specifiers from scRNAseq. With such approach, De Soysa et al. have identified Hand2 as a potential lineage-specifier with important role for OFT but not RV development, despite the failure of RV formation in *Hand2* null mutant mice [60]. Upon loss of *Hand2*, the cluster of OFT myocardial cells disappeared showing the critical role of Hand2 for OFT specification [56]. Using scRNAseq during human ESC differentiation and in human fetal hearts, *LGR5* has been identified as a human specific OFT myocardium marker and is expressed together with *MESP1* in some clusters. Further analysis in hESC have showed that LGR5 is required for cardiac specification through expansion of the ISL1+TNNT2+ intermediates [61]. Different labs have similarly shown that Nkx2-5 is essential for progenitor specification/maturation toward a CM fate [47], [48], [58] but also that *Nkx2-5* is required for the proper expression of Cxcr4 and Cxcr2, thus regulating SHF progenitor migration [59]. Using scRNAseq, Isl1 has been shown to be critical for cardiac specification, as *Isl1*-knock-out cells appeared to be stuck in a transition state, unable to differentiate into the CM or EC lineages *in vivo* [58]. However, profiling of ISL1 knock-out hESC-derived cells showed that ISL1 KO cells are redirected toward a neural program but that CMs specification is not affected in such model [62]. This can potentially reveal discrepancies between mouse and human or between the *in vivo* and *in vitro* situation. *In silico* analysis of scRNAseq data can also allow the identification of transcription factors that are involved in CPCs specification. Friedman et al. have used a probabilistic method for constructing regulatory networks from scRNAseq during iPSC (induced pluripotent stem cell) cardiac differentiation. They showed different bifurcation points with a segregation between definitive endoderm and mesendoderm lineages and another bifurcation from a CPC state toward a CM lineage and a non-

muscular lineage. Their *in silico* analyses notably highlighted a role for YY1 in the transition toward a CM fate and PBX1 for non-muscular cardiac fate [63].

Other single cell sequencing approaches are available and can be used to investigate CPCs specification at the single cell level. Single-cell ATACseq (scATAC-seq), for example, is designed to profile chromatin accessibility and represents an alternative way to investigate progenitor cell heterogeneity. Jia et al. have shown that scATAC-seq is indeed a powerful tool which is equal or superior to scRNAseq for revealing cell heterogeneity [58]. Additionally, profiling chromatin accessibility can allow the investigation of potential molecular mechanisms for lineage segregation. With such analysis, Jia et al. could find the enrichment of particular transcription factor motif accessibility along the trajectories from an Isl1+ CPC state toward a CM or EC fate. The FOX motif seems, for example, more accessible in the CPC state whereas TBX5/GATA/HOX/HAND motifs are closely associated with a CM fate and SOX motifs with an EC fate, thus indicating some potential transcription factors critical for cardiac cell fate decisions.

It is important to note that scRNAseq approaches alone are not sufficient to draw conclusions. Most of the studies published and discussed in this review, have required additional validations, using lineage tracing [26], [54], [55], [57], clonal analysis [26] and single cell resolution smFISH [26], [54], [56], [57] (see also Table 1). One main caveat of scRNAseq is the loss of spatial information, ultimately linked to the cell dissociation protocols that disrupt cell-cell communication. *In silico* tools have been developed to compute and hypothetize potential receptor-ligand pairs between cell populations. Xiong et al. have performed such kind of *in silico* single-cell receptor-ligand pairing screen and found an interesting ligand-receptor paring between CM, expressing the ligand *Mif,* and SHF progenitors that express its receptor *Cxcr2*. These results, further validated by *in situ* hybridization and functional assays, have showed a role for FHF CM in the guidance of SHF progenitor migration through the MIF/Cxcr2 chemotaxis [59]. While Li et al. have identified, with scRNAseq, the AVC and CM trabeculae region as cardiac regions with reduced proliferation activity, they also applied ligand-receptor pairing to find signal pathways that could drive activation or repression of proliferation and found *in silico* that the expression of Tgfß1 from the endocardium and Rspo1 from the epicardium might control the proliferation of cardiomyocytes [64]. Spatial transcriptomics are alternative approaches that are now emerging quite fast with different degree of resolution [65]. So far, this technique has not been used for early stages of heart development but only at fetal human stages [66]. This approach opens new avenues and could be really important to resolve questions regarding the spatial molecular heterogeneity of CPCs. While the antero-posterior heterogeneity has been well described [56], [67], it would be really interesting to further investigate, for example, the differences that are found along the left-right axis of the embryo.

Single cell sequencing approaches have thus significantly increased our understanding of CPCs specification. It has demonstrated the high transcriptomic heterogeneity of CPCs and has provided some insights into the mechanisms that lead to cardiac specification and lineage specification.

Conclusion and perspectives

Clonal analyses and single cell transcriptomic approaches have redefined the field and have clearly provided a deeper understanding of CPCs specification at higher resolution. In some studies, some discrepancies could however be found between clonal analysis and scRNAseq or between the trajectories inferred from different scRNAseq studies. These discrepancies highlight some caveats of trajectories directly deduced from scRNAseq analyses. For example, Weinreb and colleagues have already discussed the limitations of inferring trajectories from snapshots of scRNAseq [68]. However, new tools have been recently developed to reconstruct lineage history from single cell sequencing, using CRISPR/Cas9 genomic editing or recombination for generation of lineage barcodes [40], [69]. If applied to CPCs, these approaches could help reconstruct the full lineage history of CPCs to investigate if early CPCs, at gastrulation, have a broader contribution than what is accepted, and to investigate the time of lineage segregation, which is still not completely resolved. Recent techniques allow recording of both the transcriptome and lineage history of single cells, as it has been performed for example in the hematopoietic system [70], [71].

Single cell multi-omics approaches in general are emerging very fast [72] and these will allow, in combination, future investigations of the transcriptome (scRNAseq), chromatin accessibility (scATCseq), protein expression (CITE-seq) or the methylome (Me-seq) of single CPCs. This should help uncover the molecular mechanisms driving CPC specification and lineage segregation and ultimately to better understand CHDs.

References

[1] R. E. Poelmann and A. C. Gittenberger-de Groot, "Cardiac Development," *The Scientific World JOURNAL*, vol. 8, pp. 855–858, 2008, doi: 10.1100/tsw.2008.102.

[2] Y. Saga, S. Miyagawa-Tomita, A. Tagaki, S. Kitajima, J. Miyazaki, and T. Inoue, "MesP1 function on heart tube formation," *Development*, vol. 126, p. 11, 1999.

[3] S. Zaffran, R. G. Kelly, S. M. Meilhac, M. E. Buckingham, and N. A. Brown, "Right Ventricular Myocardium Derives From the Anterior Heart Field," *Circulation Research*, p. 8, 2004.

[4] M. Buckingham, S. Meilhac, and S. Zaffran, "Building the mammalian heart from two sources of myocardial cells," *Nat Rev Genet*, vol. 6, no. 11, pp. 826–835, Nov. 2005, doi: 10.1038/nrg1710.

[5] F. Rochais, K. Mesbah, and R. G. Kelly, "Signaling Pathways Controlling Second Heart Field Development," *Circulation Research*, vol. 104, no. 8, pp. 933–942, Apr. 2009, doi: 10.1161/CIRCRESAHA.109.194464.

[6] S. Zaffran and R. G. Kelly, "New developments in the second heart field," *Differentiation*, vol. 84, no. 1, pp. 17–24, Jul. 2012, doi: 10.1016/j.diff.2012.03.003.

[7] L. A. Dyer and M. L. Kirby, "The role of secondary heart field in cardiac development," *Developmental Biology*, vol. 336, no. 2, pp. 137–144, Dec. 2009, doi: 10.1016/j.ydbio.2009.10.009.

[8] D. Franco, S. M. Meilhac, V. M. Christoffels, A. Kispert, M. Buckingham, and R. G. Kelly, "Left and right ventricular contributions to the formation of the interventricular septum in the mouse heart," *Developmental Biology*, vol. 294, no. 2, pp. 366–375, Jun. 2006, doi: 10.1016/j.ydbio.2006.02.045.

[9] J. K. Takeuchi *et al.*, "*Tbx5* specifies the left/right ventricles and ventricular septum position during cardiogenesis," *Development*, vol. 130, no. 24, pp. 5953–5964, Dec. 2003, doi: 10.1242/dev.00797.

[10] J. Männer, "The development of pericardial villi in the chick embryo," *Anat Embryol (Berl)*, vol. 186, no. 4, pp. 379–385, Sep. 1992, doi: 10.1007/BF00185988.

[11] S. M. Meilhac, M. Esner, R. G. Kelly, J.-F. Nicolas, and M. E. Buckingham, "The Clonal Origin of Myocardial Cells in Different Regions of the Embryonic Mouse Heart," *Developmental Cell*, vol. 6, no. 5, pp. 685–698, May 2004, doi: 10.1016/S1534-5807(04)00133-9.

●● The first clonal analysis of CPCs revealed the existence of two myocardial cell lineages.

[12] C.-L. Cai *et al.*, "Isl1 Identifies a Cardiac Progenitor Population that Proliferates Prior to Differentiation and Contributes a Majority of Cells to the Heart," *Developmental Cell*, vol. 5, no. 6, pp. 877–889, Dec. 2003, doi: 10.1016/S1534-5807(03)00363-0.

[13] F. Lescroart, W. Hamou, A. Francou, M. Théveniau-Ruissy, R. G. Kelly, and M. Buckingham, "Clonal analysis reveals a common origin between nonsomite-derived neck muscles and heart myocardium," *Proc Natl Acad Sci USA*, vol. 112, no. 5, pp. 1446–1451, Feb. 2015, doi: 10.1073/pnas.1424538112.

[14] F. Lescroart, R. G. Kelly, J.-F. Le Garrec, J.-F. Nicolas, S. M. Meilhac, and M. Buckingham, "Clonal analysis reveals common lineage relationships between head muscles and second heart field derivatives in the mouse embryo," *Development*, vol. 137, no. 19, pp. 3269–3279, Oct. 2010, doi: 10.1242/dev.050674.
[15] M. E. Buckingham and S. M. Meilhac, "Tracing Cells for Tracking Cell Lineage and Clonal Behavior," *Developmental Cell*, vol. 21, no. 3, pp. 394–409, Sep. 2011, doi: 10.1016/j.devcel.2011.07.019.
[16] W. P. Devine, J. D. Wythe, M. George, K. Koshiba-Takeuchi, and B. G. Bruneau, "Early patterning and specification of cardiac progenitors in gastrulating mesoderm," *eLife*, vol. 3, p. e03848, Oct. 2014, doi: 10.7554/eLife.03848.
[17] X. Liang *et al.*, "HCN4 Dynamically Marks the First Heart Field and Conduction System Precursors," *Circ Res*, vol. 113, no. 4, pp. 399–407, Aug. 2013, doi: 10.1161/CIRCRESAHA.113.301588.
[18] F. Lescroart *et al.*, "Early lineage restriction in temporally distinct populations of Mesp1 progenitors during mammalian heart development," *Nat Cell Biol*, vol. 16, no. 9, pp. 829–840, Sep. 2014, doi: 10.1038/ncb3024.

•• Clonal analysis of Mesp1+ CPCs revealed early regional and lineage contributions.

[19] M. Ema, S. Takahashi, and J. Rossant, "Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors," *Blood*, vol. 107, no. 1, pp. 111–117, Jan. 2006, doi: 10.1182/blood-2005-05-1970.
[20] T. Motoike, D. W. Markham, J. Rossant, and T. N. Sato, "Evidence for novel fate of Flk1+ progenitor: Contribution to muscle lineage," *genesis*, vol. 35, no. 3, pp. 153–159, 2003, doi: 10.1002/gene.10175.
[21] A. Bondue *et al.*, "Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation," *Journal of Cell Biology*, vol. 192, no. 5, pp. 751–765, Mar. 2011, doi: 10.1083/jcb.201007063.
[22] S. C. Den Hartogh *et al.*, "Dual Reporter MESP1mCherry/w-NKX2-5eGFP/w hESCs Enable Studying Early Human Cardiac Differentiation," *STEM CELLS*, vol. 33, no. 1, pp. 56–67, 2015, doi: 10.1002/stem.1842.
[23] S. J. Kattman, T. L. Huber, and G. M. Keller, "Multipotent Flk-1+ Cardiovascular Progenitor Cells Give Rise to the Cardiomyocyte, Endothelial, and Vascular Smooth Muscle Lineages," *Developmental Cell*, vol. 11, no. 5, pp. 723–732, Nov. 2006, doi: 10.1016/j.devcel.2006.10.002.
[24] S. M. Wu *et al.*, "Developmental Origin of a Bipotential Myocardial and Smooth Muscle Cell Precursor in the Mammalian Heart," *Cell*, vol. 127, no. 6, pp. 1137–1150, Dec. 2006, doi: 10.1016/j.cell.2006.10.028.
[25] S. S.-K. Chan *et al.*, "Development of Bipotent Cardiac/Skeletal Myogenic Progenitors from MESP1+ Mesoderm," *Stem Cell Reports*, vol. 6, no. 1, pp. 26–34, Jan. 2016, doi: 10.1016/j.stemcr.2015.12.003.
[26] Q. Zhang *et al.*, "Unveiling Complexity and Multipotentiality of Early Heart Fields," *Circ Res*, vol. 129, no. 4, pp. 474–487, Aug. 2021, doi: 10.1161/CIRCRESAHA.121.318943.

•• scRNAseq and clonal analysis of early CPCs revealed the contribution of a Hand1+ CPCs to CMs of the LV and AVCs as well as to the epicardium.

[27] K. Red-Horse, H. Ueno, I. L. Weissman, and M. A. Krasnow, "Coronary arteries form by developmental reprogramming of venous cells," *Nature*, vol. 464, no. 7288, pp. 549–553, Mar. 2010, doi: 10.1038/nature08873.
[28] G. Lioux *et al.*, "A Second Heart Field-Derived Vasculogenic Niche Contributes to Cardiac Lymphatics," *Developmental Cell*, vol. 52, no. 3, pp. 350-363.e6, Feb. 2020, doi: 10.1016/j.devcel.2019.12.006.

• Unbiased clonal analysis revealed additional contribution from SHF CPCs to cardiac lymphatics.

[29] L. Miquerol *et al.*, "Resolving cell lineage contributions to the ventricular conduction system with a Cx40-GFP allele: A dual contribution of the first and second heart fields," *Developmental Dynamics*, vol. 242, no. 6, pp. 665–677, 2013, doi: 10.1002/dvdy.23964.
[30] C. Choquet, L. Marcadet, S. Beyer, R. G. Kelly, and L. Miquerol, "Segregation of Central Ventricular Conduction System Lineages in Early SMA+ Cardiomyocytes Occurs Prior to Heart Tube Formation," *Journal of Cardiovascular Development and Disease*, vol. 3, no. 1, Art. no. 1, Mar. 2016, doi: 10.3390/jcdd3010002.
[31] C. Choquet, R. G. Kelly, and L. Miquerol, "Nkx2-5 defines distinct scaffold and recruitment phases during formation of the murine cardiac Purkinje fiber network," *Nat Commun*, vol. 11, no. 1, p. 5300, Dec. 2020, doi: 10.1038/s41467-020-19150-9.
[32] S. Lazic and I. C. Scott, "Mef2cb regulates late myocardial cell addition from a second heart field-like population of progenitors in zebrafish," *Developmental Biology*, vol. 354, no. 1, pp. 123–133, Jun. 2011, doi: 10.1016/j.ydbio.2011.03.028.
[33] Y. Zhou *et al.*, "Latent TGF-β binding protein 3 identifies a second heart field in zebrafish," *Nature*, vol. 474, no. 7353, pp. 645–648, Jun. 2011, doi: 10.1038/nature10094.
[34] S. Chabab, F. Lescroart, S. Rulands, N. Mathiah, B. D. Simons, and C. Blanpain, "Uncovering the Number and Clonal Dynamics of Mesp1 Progenitors during Heart Morphogenesis," *Cell Reports*, vol. 14, no. 1, pp. 1–10, Jan. 2016, doi: 10.1016/j.celrep.2015.12.013.

[35] F. Lescroart, T. Mohun, S. M. Meilhac, M. Bennett, and M. Buckingham, "Lineage Tree for the Venous Pole of the Heart: Clonal Analysis Clarifies Controversial Genealogy Based on Genetic Tracing," *Circ Res*, vol. 111, no. 10, pp. 1313–1322, Oct. 2012, doi: 10.1161/CIRCRESAHA.112.271064.

[36] S. M. Meilhac, R. G. Kelly, D. Rocancourt, S. Eloy-Trinquet, J.-F. Nicolas, and M. E. Buckingham, "A retrospective clonal analysis of the myocardium reveals two phases of clonal growth in the developing mouse heart," *Development*, vol. 130, no. 16, pp. 3877–3889, Aug. 2003, doi: 10.1242/dev.00580.

[37] S. M. Meilhac, M. Esner, M. Kerszberg, J. E. Moss, and M. E. Buckingham, "Oriented clonal cell growth in the developing mouse myocardium underlies cardiac morphogenesis," *Journal of Cell Biology*, vol. 164, no. 1, pp. 97–109, Jan. 2004, doi: 10.1083/jcb.200309160.

[38] K. Ivanovitch, S. Temiño, and M. Torres, "Live imaging of heart tube development in mouse reveals alternating phases of cardiac differentiation and morphogenesis," *eLife*, vol. 6, p. e30668, Dec. 2017, doi: 10.7554/eLife.30668.

•• Single cell tracking of CPCs in real-time with live imaging revealed different phases of CPCs differentiation.

[39] S. S. Potter, "Single-cell RNA sequencing for the study of development, physiology and disease," *Nat Rev Nephrol*, vol. 14, no. 8, pp. 479–492, Aug. 2018, doi: 10.1038/s41581-018-0021-7.

[40] D. E. Wagner and A. M. Klein, "Lineage tracing meets single-cell omics: opportunities and challenges," *Nat Rev Genet*, vol. 21, no. 7, pp. 410–427, Jul. 2020, doi: 10.1038/s41576-020-0223-2.

[41] R. Hou, E. Denisenko, H. T. Ong, J. A. Ramilowski, and A. R. R. Forrest, "Predicting cell-to-cell communication networks using NATMI," *Nat Commun*, vol. 11, no. 1, p. 5011, Dec. 2020, doi: 10.1038/s41467-020-18873-z.

[42] J. A. Ramilowski *et al.*, "A draft network of ligand–receptor-mediated multicellular signalling in human," *Nat Commun*, vol. 6, no. 1, p. 7866, Nov. 2015, doi: 10.1038/ncomms8866.

[43] S. Picelli, O. R. Faridani, Å. K. Björklund, G. Winberg, S. Sagasser, and R. Sandberg, "Full-length RNA-seq from single cells using Smart-seq2," *Nat Protoc*, vol. 9, no. 1, pp. 171–181, Jan. 2014, doi: 10.1038/nprot.2014.006.

[44] A. A. Kolodziejczyk, J. K. Kim, V. Svensson, J. C. Marioni, and S. A. Teichmann, "The Technology and Biology of Single-Cell RNA Sequencing," *Molecular Cell*, vol. 58, no. 4, pp. 610–620, May 2015, doi: 10.1016/j.molcel.2015.04.005.

[45] V. Y. Kiselev, T. S. Andrews, and M. Hemberg, "Challenges in unsupervised clustering of single-cell RNA-seq data," *Nat Rev Genet*, vol. 20, no. 5, pp. 273–282, May 2019, doi: 10.1038/s41576-018-0088-9.

[46] G. Li, K. Plonowska, R. Kuppusamy, A. Sturzu, and S. M. Wu, "Identification of cardiovascular lineage descendants at single-cell resolution," *Development*, p. dev.116897, Jan. 2015, doi: 10.1242/dev.116897.

• One of the first single-cell transcriptomic analysis of CPCs.

[47] G. Li *et al.*, "Transcriptomic Profiling Maps Anatomically Patterned Subpopulations among Single Embryonic Cardiac Cells," *Developmental Cell*, vol. 39, no. 4, pp. 491–507, Nov. 2016, doi: 10.1016/j.devcel.2016.10.014.

[48] D. M. DeLaughter *et al.*, "Single-Cell Resolution of Temporal Gene Expression during Heart Development," *Developmental Cell*, vol. 39, no. 4, pp. 480–490, Nov. 2016, doi: 10.1016/j.devcel.2016.10.001.

[49] W. R. Goodyer *et al.*, "Transcriptomic Profiling of the Developing Cardiac Conduction System at Single-Cell Resolution," *Circ Res*, vol. 125, no. 4, pp. 379–397, Aug. 2019, doi: 10.1161/CIRCRESAHA.118.314578.

[50] B. Pijuan-Sala *et al.*, "A single-cell molecular map of mouse gastrulation and early organogenesis," *Nature*, vol. 566, no. 7745, pp. 490–495, Feb. 2019, doi: 10.1038/s41586-019-0933-9.

[51] T. Su *et al.*, "Single-cell analysis of early progenitor cells that build coronary arteries," *Nature*, vol. 559, no. 7714, pp. 356–362, Jul. 2018, doi: 10.1038/s41586-018-0288-7.

[52] Y. Cui *et al.*, "Single-Cell Transcriptome Analysis Maps the Developmental Track of the Human Heart," *Cell Reports*, vol. 26, no. 7, pp. 1934-1950.e5, Feb. 2019, doi: 10.1016/j.celrep.2019.01.079.

[53] W. Wang *et al.*, "A single-cell transcriptional roadmap for cardiopharyngeal fate diversification," *Nat Cell Biol*, vol. 21, no. 6, pp. 674–686, Jun. 2019, doi: 10.1038/s41556-019-0336-z.

[54] F. Lescroart *et al.*, "Defining the earliest step of cardiovascular lineage segregation by single-cell RNA-seq," *Science*, vol. 359, no. 6380, pp. 1177–1181, Mar. 2018, doi: 10.1126/science.aao4174.

•• scRNAseq of early CPCs at the time of gastrulation revealed CPCs molecular heterogeneity and early lineage segregation.

[55] K. Ivanovitch *et al.*, "Ventricular, atrial, and outflow tract heart progenitors arise from spatially and molecularly distinct regions of the primitive streak," *PLOS Biology*, vol. 19, no. 5, p. e3001200, May 2021, doi: 10.1371/journal.pbio.3001200.

•• Elegant combination of lineage tracing experiments with scRNAseq of early CPCs at gastrulation.

[56] T. Y. de Soysa *et al.*, "Single-cell analysis of cardiogenesis reveals basis for organ-level developmental defects," *Nature*, vol. 572, no. 7767, pp. 120–124, Aug. 2019, doi: 10.1038/s41586-019-1414-x.

●● scRNAseq analysis of CPCs and investigation of the role of Hand2 as an OFT specifier.

[57] R. C. V. Tyser *et al.*, "Characterization of a common progenitor pool of the epicardium and myocardium," *Science*, vol. 371, no. 6533, p. eabb2986, Mar. 2021, doi: 10.1126/science.abb2986.

●● scRNAseq and lineage tracing of early CPCs revealed the contribution of a newly identified population, the JCF, to the LV and epicardium.

[58] G. Jia *et al.*, "Single cell RNA-seq and ATAC-seq analysis of cardiac progenitor cell transition states and lineage settlement," *Nat Commun*, vol. 9, no. 1, p. 4877, Dec. 2018, doi: 10.1038/s41467-018-07307-6.

● scRNAseq and scATACseq of early CPCs.

[59] H. Xiong *et al.*, "Single-Cell Transcriptomics Reveals Chemotaxis-Mediated Intraorgan Crosstalk During Cardiogenesis," *Circ Res*, vol. 125, no. 4, pp. 398–410, Aug. 2019, doi: 10.1161/CIRCRESAHA.119.315243.

[60] D. Srivastava, T. Thomas, Q. Lin, M. L. Kirby, D. Brown, and E. N. Olson, "Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND," *Nat Genet*, vol. 16, no. 2, pp. 154–160, Jun. 1997, doi: 10.1038/ng0697-154.

[61] M. Sahara *et al.*, "Population and Single-Cell Analysis of Human Cardiogenesis Reveals Unique LGR5 Ventricular Progenitors in Embryonic Outflow Tract," *Developmental Cell*, vol. 48, no. 4, pp. 475-490.e7, Feb. 2019, doi: 10.1016/j.devcel.2019.01.005.

[62] M. M. Mononen, C. Y. Leung, J. Xu, and K. R. Chien, "Trajectory mapping of human embryonic stem cell cardiogenesis reveals lineage branch points and an ISL1 progenitor-derived cardiac fibroblast lineage," *STEM CELLS*, vol. 38, no. 10, pp. 1267–1278, 2020, doi: 10.1002/stem.3236.

[63] C. E. Friedman *et al.*, "Single-Cell Transcriptomic Analysis of Cardiac Differentiation from Human PSCs Reveals HOPX-Dependent Cardiomyocyte Maturation," *Cell Stem Cell*, vol. 23, no. 4, pp. 586-598.e8, Oct. 2018, doi: 10.1016/j.stem.2018.09.009.

[64] G. Li *et al.*, "Single cell expression analysis reveals anatomical and cell cycle-dependent transcriptional shifts during heart development," *Development*, p. dev.173476, Jan. 2019, doi: 10.1242/dev.173476.

[65] M. Asp, J. Bergenstråhle, and J. Lundeberg, "Spatially Resolved Transcriptomes—Next Generation Tools for Tissue Exploration," *BioEssays*, vol. 42, no. 10, p. 1900221, 2020, doi: 10.1002/bies.201900221.

[66] M. Asp *et al.*, "A Spatiotemporal Organ-Wide Gene Expression and Cell Atlas of the Developing Human Heart," *Cell*, vol. 179, no. 7, pp. 1647-1660.e19, Dec. 2019, doi: 10.1016/j.cell.2019.11.025.

● First report of application of spatial transcriptomic for heart development.

[67] S. Stefanovic *et al.*, "Hox-dependent coordination of mouse cardiac progenitor cell patterning and differentiation," *eLife*, vol. 9, p. e55124, Aug. 2020, doi: 10.7554/eLife.55124.

[68] C. Weinreb, S. Wolock, B. K. Tusi, M. Socolovsky, and A. M. Klein, "Fundamental limits on dynamic inference from single-cell snapshots," *Proc Natl Acad Sci USA*, vol. 115, no. 10, pp. E2467–E2476, Mar. 2018, doi: 10.1073/pnas.1714723115.

[69] C. Weinreb, A. Rodriguez-Fraticelli, F. D. Camargo, and A. M. Klein, "Lineage tracing on transcriptional landscapes links state to fate during differentiation," *Science*, vol. 367, no. 6479, p. eaaw3381, Feb. 2020, doi: 10.1126/science.aaw3381.

[70] S. Bowling *et al.*, "An Engineered CRISPR-Cas9 Mouse Line for Simultaneous Readout of Lineage Histories and Gene Expression Profiles in Single Cells," *Cell*, vol. 181, no. 6, pp. 1410-1422.e27, Jun. 2020, doi: 10.1016/j.cell.2020.04.048.

[71] W. Pei *et al.*, "Resolving Fates and Single-Cell Transcriptomes of Hematopoietic Stem Cell Clones by PolyloxExpress Barcoding," *Cell Stem Cell*, vol. 27, no. 3, pp. 383-395.e8, Sep. 2020, doi: 10.1016/j.stem.2020.07.018.

[72] I. Kucinski and B. Gottgens, "Advancing Stem Cell Research through Multimodal Single-Cell Analysis," *Cold Spring Harb Perspect Biol*, vol. 12, no. 7, p. a035725, Jan. 2020, doi: 10.1101/cshperspect.a035725.

[73] H. Ruan et al., "Single-cell reconstruction of differentiation trajectory reveals a critical role of ETS1 in human cardiac lineage commitment," *BMC Biol*, vol. 17, no. 1, p. 89, Dec. 2019, doi: 10.1186/s12915-019-0709-6.

[74] J. M. Churko et al., "Defining human cardiac transcription hierarchies using integrated single-cell heterogeneity analysis," *Nat Commun*, vol. 9, no. 1, p. 4906, Dec. 2018, doi: 10.1038/s41467-018-0733-4.

Figure legend

Fig. 1: Clonal analyses and scRNAseq in the early stages of mouse heart development. a. Model of the early steps of CPC specification and lineage commitment during mouse development as shown by clonal analyses. Clonal analysis of *Mesp1*+ and *Hand1*+ progenitors showed the existence of temporally distinct *Mesp1* progenitors that contribute to different regions (top) and to different cell lineages (bottom). Early CPCs are either bi/multipotent (dark black circles) or unipotent. Early *Mesp1* progenitors (labelled at E6.75 – red) contribute preferentially to the LV, RV while late *Mesp1* progenitors contribute to the RV, RA, LA, inflow region (RSCV, LSCV and PV) and to some skeletal muscles of the head or neck. LV, left ventricle ; AVC, atrioventricular canal ; RV, right ventricle ; pt, pulmonary trunk ; ao, aorta ; LSCV, left superior caval vein ; RSCV, right superior caval vein ; PV, pulmonay vein ; fac. Exp. Muscles, facial expression muscles ; Epi, epicardium ; Peri, pericardium ; CM, cardiomyocyte ; EC, endothelium/endocardium ; SMC, smooth muscle cell ; skM, skeletal muscle. **b**. In scRNA-seq experiments of Mesp1+ cells at E6.75 and E7.25, four distinct *Mesp1*+ subpopulations have been identified that emerge from epiblast cells and that correspond to the endothelial (EC –green), cardiomyocyte (CM – red), anterior SHF (aSHF, pink) and posterior SHF (pSHF, purple) progenitors. These populations are enriched for some transcripts (in brackets). On the right panel, map of the gastrulating embryo, where the different CPC subpopulations are found. *Mesp1*+ EC population (green) is found in the outer layer of the mesodermal wing while *Mesp1*+ CM populations are found close to the epiblast. Populations of the FHF (red), aSHF (pink) and pSHF (purple) are represented along the antero-medial axis of the embryo. This summarizes the results from Lescroart et al. and Ivanovitch et al. [54], [55]. PS, primitive streak; A, anterior, P, posterior. **c**. The left panel represents the scRNA-seq experiments of CPCs from E7.75 to E8 by Tyser et al. [57]. Six distinct cardiac subpopulations have been identified (Me3-Me8). These populations are enriched for different transcripts (in brackets). JCF, juxta-cardiac field; CrM, cranial mesoderm. The trajectories identified among these cell clusters are shown in the middle. These subpopulations have been mapped to the developing embryo at the cardiac crescent stage (left panel). L, left; R, right. **d**. The left panel represents the scRNA-seq experiment of *Mesp1*-derived cardiac cells from E7.25 to E8.25 by Zhang et al. [26]. Seven distinct cardiac subpopulations have been identified (cardiomyocytes or CM1-3 and cardiac progenitors or CP4-7). Lineage inference (in the middle) can link the different clusters in an inferred lineage tree. LEM, late extra-embryonic mesoderm; Al, allantois. These subpopulations have been mapped to the embryo at an early heart tube stage (right panel). D, dorsal; V, ventral.

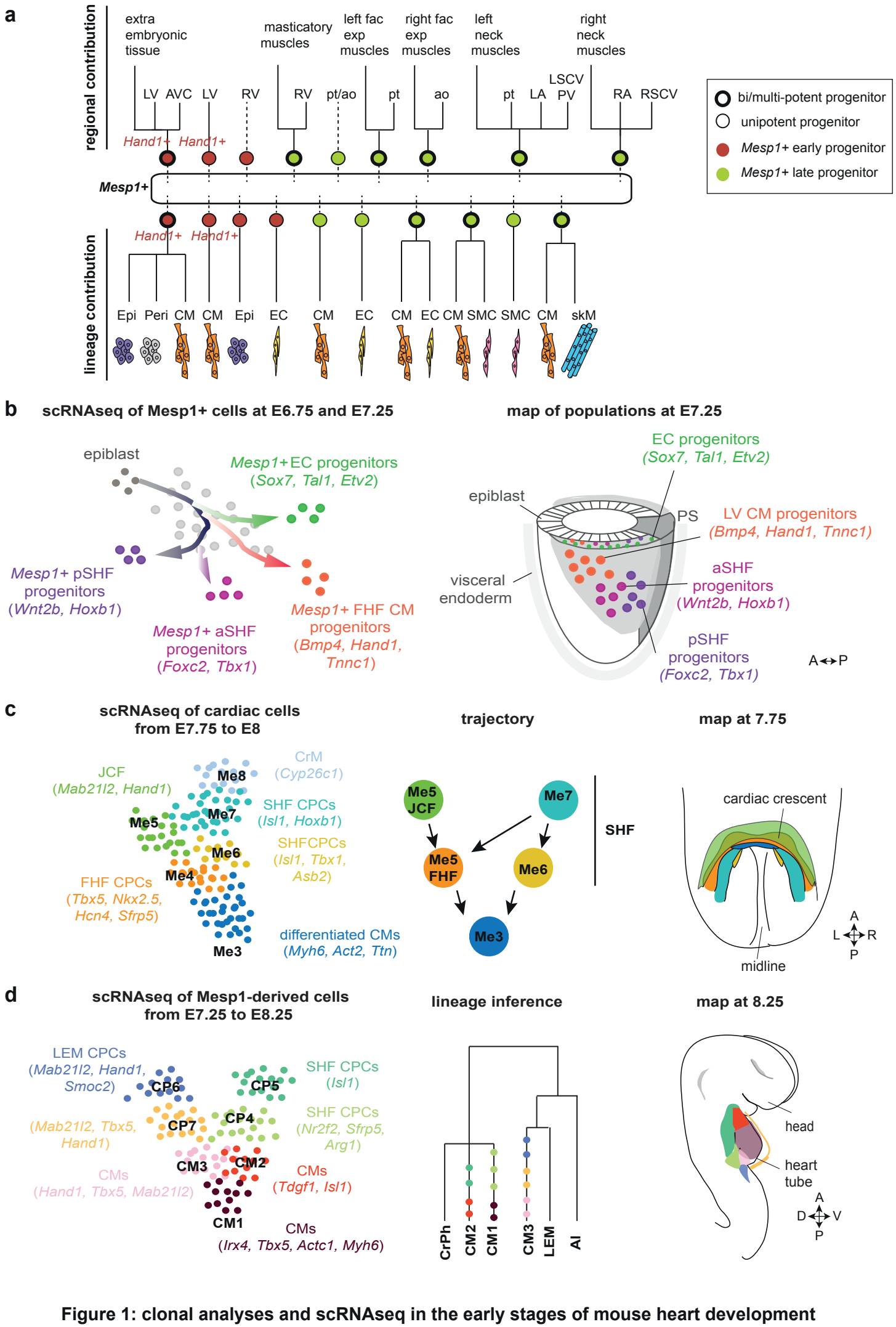

Figure 1: clonal analyses and scRNAseq in the early stages of mouse heart development

Table 1: scRNAseq analyses of CPCs specification

Targeted cell population	Stages	Number of time points	Number of total cells	Capture platform	Single-cell approach	Mutant cells	Validations / Additional analyses	Model / Organism	Reference
Mesp1+	E6.75-E7.25	2	892	facs/plates	full length	*Mesp1Cre/Cre*	RNA-FISH, *Notch1* lineage tracing	mouse	[54]
Mesp1-Cre lineage	E7.25-E8.25	4	9072	facs/droplet	3' end	-	RNA-FISH and *Hand1* lineage tracing, clonal analysis	mouse	[26]
heart and SHF	E7.75-E8.25	6	3105	droplet	3' end	-	RNA-FISH, *Mab21l2* lineage tracing	mouse	[57]
all embryo	E7.75	1	3494	droplet	3' end	-	*T* and *Foxa2* lineage tracing	mouse	[55]
heart and SHF	E7.75-E9.25	3	21366	droplet	3' end	*Hand2-/-*	RNA-FISH	mouse	[56]
Nkx2-5+ or *Isl1+*	E7.75-E9.5	3	421	fluidigm	full length	*Isl1GFP/GFP and Nkx2-5OE*	single-cell ATC-seq	mouse	[58]
Nkx2-5-Cre or *Isl1-Cre* lineages	E7.75-E9.5	4	1231	facs/manual	full length	-	*in silico* single-cell receptor-ligand pairing screen	mouse	[59]
heart	E10.5	1	>10000	droplet	3' end	-	*RNA-FISH, in silico* single-cell receptor-ligand pairing screen	mouse	[64]
conduction system	E16.5	1	22462	droplet	3' end	-	RNA-FISH, immunofluorescence	mouse	[49]
Apj-Cre lineage (coronary arteries)	E12.5-E14.5	2	2067	facs/plate	full length	*Coup-tf2OE*	RNA-FISH, immunofluorescence, lineage tracing	mouse	[51]
Mesp lineage	12-20hpf	5	848	plates	full length	-	RNA-FISH, immunofluorescence	Ciona	[53]
human fetal hearts	5-25 weeks	20	3842	manual	full length	-	immunofluorescence (human)	human fetal heart	[52]
hESC-derived	Day3-15 / 4.5-10 weeks	3	366 / 458	facs/plates	full length	-	immunofluorescence (human) and lineage tracing (mouse)	human ESC / human fetal heart	[61]
hESC-derived	Day0-60	6	6879	ICELL8	full length	-	in silico analyses	human ESC	[73]
hESC-derived	Day3-15	8	1028	manual	full length	*Isl1KO*	comparison with human fetal heart, immunofluorescence	human ESC	[62]
human-iPSC-derived	Day0-45	4	10376	droplet	3' end	*NR2F2-GE, TBX5-GE, HEY2-GE*	time-of-flight mass cytometry	human iPSC	[74]
human-iPSC-derived	Day0-30	5	43168	droplet	3' end	-	probabilistic method for contructing regulatory network	human iPSC	[63]