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Cardiopharyngeal progenitor specification: multiple roads to the heart and head muscles

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Short title: Cardiopharyngeal progenitor specification

Abstract

During embryonic development, the heart arise from various sources of undifferentiated mesodermal progenitors, with an additional contribution from ectodermal neural crest cells. Mesodermal cardiac progenitors are plastic and multipotent, but are nevertheless specified to a precise heart region and cell type very early during development. Recent findings have defined both this lineage plasticity and early commitment of cardiac progenitors, using a combination of single-cell and population analyses.

In this review, we discuss several aspects of cardiac progenitor specification. We discuss their markers, fate potential *in vitro* and *in vivo*, early segregation and commitment, and also intrinsic and extrinsic cues regulating lineage restriction from multipotency to a specific cell type of the heart. Finally, we also discuss the sub-divisions of the cardiopharyngeal field, and the shared origins of the heart with other mesodermal derivatives, including head and neck muscles.

Introduction

Throughout the animal kingdom, organogenesis takes place early during embryonic development. This developmental process consists in the formation of organs through the progressive commitment and specification of progenitor cells, their migration towards the final location of the organ and their signaling interactions with surrounding tissues. Organogenesis starts with the commitment of multipotent and plastic progenitors that will progressively acquire the molecular program of more restricted progenitors and finally differentiate into mature functional cells.

The heart is the first organ to form in mammals and arises from the specification of cardiac progenitor cells, which can be identified as early as during gastrulation (Kinder et al. 1999), and which represent the building blocks of cardiac development. Cardiac progenitors are included in a larger mesodermal population termed the cardiopharyngeal field (CPF) that contributes to both the heart and skeletal muscles of the head and neck (Diogo et al. 2015). In vertebrates, the first specified cardiac cells generally express *Nkx2-5* (Harvey 2002), that notably also marks certain skeletal muscle progenitors of the head (Diogo et al. 2015). Nevertheless, cardiopharyngeal progenitor cells (CPCs) can be identified earlier during development, before the onset of *Nkx2-5* expression, and there are now several lines of evidence showing that the fate of CPCs is specified during or prior to gastrulation.

In mammals, cardiac progenitors will build a four-chambered heart with two atria and two ventricles, consisting of different cell types including cardiomyocytes (CMs), endocardial cells (ECs) forming the inner layer, epicardial-derived cells (EPDCs) at the outer layer and smooth muscle cells (SMCs) (Lescroart and Meilhac 2012). It is now well established that vertebrate cardiogenesis occurs through progressive addition of several populations of progenitors to the forming linear heart tube. An early differentiating population of progenitors, termed the first heart field (FHF) gives rise to the left ventricle (LV) and some atrial cells, while a second wave of differentiation occurs from the second heart field (SHF) with a distinct contribution to the four-chambered heart, including the right ventricle (RV), the outflow tract (OFT) and the venous pole (Buckingham et al. 2005). Further subdivisions of the SHF distinguish the contribution of the anterior SHF (aSHF) to the OFT and RV from that of the posterior SHF (pSHF) to the OFT and venous region (Meilhac and Buckingham 2018). Subdivision of the FHF has not been clearly established but based on lineage tracing (Bardot et al. 2017), we could wonder whether the FHF can also be patterned along the antero-posterior axis.

Throughout the evolution of vertebrates, the heart has acquired a complex multi-chambered structure, although conserved FHF and SHF populations can already be identified in tunicates, which are the closest living relatives of an early chordate precursor. Therefore, CPC specification has been studied in different models, ranging from the simple chordate *Ciona robusta*, that presents a simple beating heart tube, to the mammalian mouse model. Moreover, differentiation of mouse and human pluripotent stem cells (m- or h-PSCs) *in vitro* into different cardiac lineages has been shown to recapitulate the first steps of CPC specification, including the molecular pathways involved in this process, and are thus an attractive model to determine the minimal cues necessary for the formation of specific cardiac lineages.

In this review, we present currently known markers of early CPCs and their differentiation potential as studied by single-cell approaches. We also discuss insights into the regulation of CPC specification toward specific cardiac or pharyngeal lineages, which have recently been discovered through the multiplication of genetic techniques and reduction in costs of next-generation sequencing. Although cardiac neural crest cells also make important contributions to the OFT and valves (Kirby et al. 1983; Kirby and Waldo 1995), we choose here to focus only on the mesodermal contribution to the forming heart.

1. Markers of early CPCs

Several key studies have isolated and characterized CPCs and more specifically cardiac progenitors from mouse embryos and differentiating PSCs, which are capable of proliferating and giving rise to multiple cell types of the heart.

As the majority of cardiac and pharyngeal muscle cells derive from the mesoderm, genes that are expressed in the nascent mesoderm such as *Brachyury*, also called *T*, encoding a T-box transcription factor (TF) (Kispert and Herrmann 1993; Showell et al. 2004), and *Mesp1* (*mesoderm posterior 1*), encoding for a bHLH TF (Saga et al. 1996), are characteristic of early CPCs. *Mesp1* is thereafter rapidly downregulated once the mesoderm is formed. During differentiation of both mouse and human PSCs, the expression of *Mesp1* is also transient and precedes the expression of other cardiac markers (Liu et al. 2007; Ueno et al. 2007; Bondue et al. 2008; Lindsley et al. 2008; Den Hartogh et al. 2015). *Mesp1* has spurred great interest in the last two decades as a potential marker of early cardiac progenitor specification from both heart fields (Bondue and Blanpain 2010). Moreover, in the chordate *Ciona*, *Mesp*, the unique homolog of *Mesp1*, is expressed transiently in the B7.5 cells pair which will give rise to the *Ciona* heart, supporting a conserved role of *Mesp* genes in CPC specification throughout chordates (Satou et al. 2004).

Specific combinations of surface markers expressed in CPCs can now facilitate their isolation, purification and characterization. Flk1 (or Vegfr2) and Pdgfra are both expressed in nascent mesoderm (Kataoka et al. 1997; Motoike et al. 2003; Ema et al. 2006). Interestingly, cells co-expressing Flk1 and Pdgfra during differentiation of PSCs are enriched for cardiac potential (Hirata et al. 2007). Cxcr4 has also been used in combination with Flk1 (Nelson et al. 2008) or both Flk1 and Pdgfra (Bondue et al. 2011) to isolate CPCs with cardiac potential. The expression of these three signaling receptors coincides with that of *Mesp1*, and overexpression of *Mesp1* directly activates their transcription (Bondue et al. 2011). Flk1/Pdgfra/Cxcr4 also mark early *Mesp1*⁺ CPCs *in vivo* (Lescroart et al. 2014). Human PSCs undergoing differentiation into cardiac cell types also necessarily pass through an Flk1⁺ mesodermal stage (Yang et al. 2008). Expression of Flk1 and Pdgfra together is generally predictive of CM potential in hPSCs (Kattman et al. 2011). Several other markers have recently been proposed for the isolation of hPSC-derived cardiac progenitors, such as Ror2 and Cd13 (Drukker et al. 2012; Den Hartogh et al. 2015; Skelton et al. 2016), or Fzd4 (Yoon et al. 2018).

One subunit of a chromatin-remodelling complex, Smarcd3, has cardiac-specific expression. *Smarcd3* is expressed in a subset of *Mesp1*⁺ CPCs, and a smaller 2.5 kb regulatory region of this gene can activate reporter gene expression in progenitors for both heart fields in mouse embryos (Devine et al. 2014). Interestingly, the same regulatory region marks zebrafish CPCs, even though this particular sequence was not conserved between both species (Yuan et al. 2018). This suggests that the same core transcriptional regulators are active in CPCs throughout vertebrates and can induce the expression of CPC-specific regulatory regions from other species. As regulatory regions of genes have readily been used to activate reporter gene expression in specific populations of CPCs for the whole heart (Wu et al. 2006) or of the SHF (Verzi et al. 2005), the advent of whole-genome predictions of cardiac enhancers and validation of their activity *in vivo* (He et al. 2011; Yuan et al. 2018) may accelerate the identification of cardiac-specific enhancers expressed as early as during gastrulation in the FHF, SHF, or whole CPF. It would then be interesting to investigate whether these enhancers are specifically active in multipotent pan-cardiac CPCs or rather independently activated in different lineages.

2. Lineage potential of CPCs

While in *Ciona* the cardiac lineage tree is highly ordered and reproducible, starting from only 2 *Mesp*⁺ progenitors at the time of gastrulation (Satou et al. 2004), lineage diversification is more complex and plastic in vertebrates, with about 250 *Mesp1*⁺ progenitors contributing to the heart in the mouse model (Chabab et al. 2016).

The first experiments assessing the lineage potential of CPCs were performed in the chick and the mouse, using dye labeling or grafting experiments (Stalsberg and DeHaan 1969; Tam and Behringer 1997; Kinder et al. 1999; Lawson 1999; Tzahor et al. 2003). Grafting experiments have shown that cardiac progenitors are committed to a cardiac fate only after the formation of the cardiac crescent (Tam and Behringer 1997; Auda-Boucher et al. 2000), suggesting a certain degree of plasticity of CPCs at gastrulation stages.

Lineage tracing experiments, clonal analyses and, more recently, single-cell RNA-sequencing (scRNA-seq) have allowed us to decipher the appearance of different cell lineages that arise from early CPCs.

2.1. Lineage tracing and *in vitro* differentiation demonstrates the multipotency of CPCs

Lineage tracing experiments with a *Flk1-Cre* knock-in mouse model showed that although originally involved in hematopoietic lineages, Flk1+ cells also contribute to the development of CMs and ECs (Motoike et al. 2003; Ema et al. 2006). Furthermore, during differentiation of PSCs, T+Flk1+ cells present the ability to form colonies comprising CMs, SMCs and ECs, suggesting that this early mesodermal cell population is at least tri-potent (Kattman et al. 2006). Tracking the progeny of *Mesp1*-expressing cells revealed that they comprise the majority of cardiac cells including CM, ECs and EPDCs (Saga et al. 1999). In addition, the *Mesp1* lineage also contributes to non-cardiac derivatives, including skeletal muscles of the head and neck (see Table 1) (McBratney-Owen et al. 2008; Yoshida et al. 2008; Asahina et al. 2009; Harel et al. 2009). Interestingly, this observation seems consistent with findings in *Ciona*, where *Mesp+* cells also produce the anterior and tail muscles, indicating their multipotency (Figure 1) (Stolfi et al. 2010). During mouse and human PSC differentiation, isolated *Mesp1*-expressing cells have the potential to become ECs, SMCs, and CMs in a clonal assay (Bondue et al. 2011; Den Hartogh et al. 2015). Furthermore, an *in vitro* clonal assay using mPSCs demonstrated the bi-potential of *Mesp1*+ cells to generate skeletal and cardiac muscles, in agreement with their broad fate potential *in vivo* (Chan et al. 2016).

Additional PSC studies have further shown that cardiac progenitors from slightly later stages, when *Nkx2-5* is co-expressed with other markers such as the transcription factor *Isl1*, are able to generate colonies with potential to give rise to 2 or 3 cardiac cell types (Moretti et al. 2006; Wu et al. 2006). Therefore, early CPCs are multipotent and plastic. Nevertheless, we will discuss below that they are also specified very early towards a certain fate, and that when left in their endogenous environment, they are mostly unproductive and contribute to specific cardiac regions and cell types.

2.2. Single cells analyses reveal heterogeneity among CPCs

Single-cell analyses have considerably changed our vision of CPC specification and have shown that the differential contribution of CPCs in the mouse model is complex, with many branching points and possible outcomes.

The first clonal analyses performed in mouse, using retrospective clonal analysis, demonstrated the existence of two myocardial cell lineages, corresponding to the FHF and SHF. On the basis of the number of β -gal+ cells included in clones covering both lineages, lineage segregation between the first and second myocardial lineages was estimated to occur at the onset of gastrulation (Meilhac et al. 2004). More recent clonal tracing of *Mesp1*+ CPCs using two independent strategies (*Mesp1-rtTA/tetO-Cre/Rosa-Confetti* or a *Mesp1-Cre/MADM* system) established that lineage segregation of the FHF and SHF occurs before the onset of *Mesp1* expression, as shown by the absence of clones in both FHF and SHF derivatives (Devine et al. 2014; Lescroart et al. 2014). Similarly, clonal tracing of *Smarcd3*+ progenitors showed no evidence of a common progenitor for the FHF and SHF (Devine et al. 2014).

Like in tunicates, multipotent progenitor cells giving rise to the SHF in the mouse also generate skeletal muscles of the head and neck, with at least three different populations of bi-potent progenitors found along the antero-posterior axis of the pharyngeal region, contributing to the masticatory muscles and RV, facial expression muscles and OFT or neck muscles and venous pole as well as OFT myocardium (Figure 1) (Lescroart et al. 2010; Lescroart et al. 2015). Furthermore, *Mesp1* lineage tracing also identified clones labeling both skeletal muscles of the head and the myocardium, suggesting that segregation between cranial and cardiac muscles occurs after the onset of *Mesp1* expression (Lescroart et al. 2014). Nevertheless, most *Mesp1*+ CPCs are unproductive and contribute in the heart to either the epicardium, CMs, SMCs or ECs, with only a few bipotent *Mesp1*-derived clones

contributing to CMs and ECs, or SMCs and CMs, and only in SHF derivatives (Devine et al. 2014; Lescroart et al. 2014).

The heterogeneity of CPCs is exemplified by the different genetic lineage tracing experiments that have been performed and that label different compartments of the heart (Table 1) (Meilhac and Buckingham 2018). For example, *Foxa2*⁺ cells, found very early during gastrulation, preferentially contribute to the ventricles and epicardium. Interestingly, *Foxa2*⁺ cells seem to express lower levels of *Mesp1* (Bardot et al. 2017). Tracing with a particular enhancer of *Mef2c*, activated during gastrulation, labels only a subpopulation of cardiac tissue and specifically the aSHF, comprising the OFT and RV, as well as some head muscles (Verzi et al. 2005; Lescroart et al. 2010; Devine et al. 2014). The anterior *Hox* genes (*Hoxa1*, *Hoxa3*, *Hoxb1*) distinguish pSHF progenitors with contributions to the inferior wall of the OFT and atria (Bertrand et al. 2011). *Hand1*⁺ cells only contribute to the CM of the LV (Barnes et al. 2010). Similarly, early *Hcn4*-expressing cells contribute specifically to the cardiac crescent and subsequently to LV CMs (Liang et al. 2013; Spater et al. 2013). Interestingly, *Sfrp5*, which encodes a secreted receptor for Wnt signaling, is expressed in progenitors of all cardiac regions including CMs, ECs and EPDCs, with the exception of the RV (Fujii et al. 2017).

In addition, there is also clearly a dynamic temporal expression of markers during CPC specification such that CPCs expressing *Mesp1* at different time points during gastrulation will have different contributions to the heart and head. Temporally controlled clonal analysis of *Mesp1*⁺ CPCs has thus shown that LV progenitors express *Mesp1* early (induced with doxycycline at E6.25) while the OFT, RV and atria and head muscles derive preferentially from CPCs that express *Mesp1* slightly later (induced at E7.25) (Lescroart et al. 2014). Similarly, clonal analysis using a *Six2-Cre-ERT2* line revealed that *Six2* is expressed in a subset of late CPCs and that *Six2* is successively activated in different populations of CPCs that contribute to the RV (induction with tamoxifen at E6) and then to the OFT (induction at E7.5) together with head muscles (Zhou et al. 2017). Interestingly, inducible lineage tracings of *Hcn4*⁺, *Tbx5*⁺ or *Sma*⁺ (*smooth muscle actin*, expressed in embryonic CMs) show a progressive contribution of cardiac progenitors to the heart with early differentiating cells contributing specifically to the left side of the interventricular septum (Spater et al. 2013; Devine et al. 2014; Choquet et al. 2016).

Finally, with the emergence of new technologies such as high-throughput scRNA-seq, it is now possible to study the heterogeneity of CPCs in detail, as they arise and specify to a cardiac or muscular fate during gastrulation. A first study by Chan et al. applied scRNA-seq to *Mesp1*⁺ mPSCs during differentiation and revealed the existence of at least 6 subpopulations of *Mesp1*⁺ progenitors, with some primed for hematopoietic lineages while others were primed toward the cardiac lineages (Chan et al. 2016). Single cell expression profiling of nascent mesoderm at the late stage of mouse gastrulation demonstrated the existence of distinct populations of cardiac progenitors with different states of expression of *Mesp1*, *Tbx5*, *Nkx2-5* and *Isl1* (Kokkinopoulos et al. 2015). scRNA-seq of *Mesp1*⁺ CPCs *in vivo* (from E6.75 to E7.25) has showed a continuum of cell states along distinct cardiopharyngeal-related trajectories towards 4 destination cell types. These distinct trajectories likely correspond to CMs, ECs, anterior CPF (including aSHF) or posterior CPF (including pSHF) lineages, with different markers enriched in these different populations. RNA-FISH using specific markers of these four types further demonstrated that these populations of pre-specified CPCs have distinct spatial localization in the gastrulating embryo (Figure 2). EC-primed *Mesp1*⁺ progenitors are found in the outer layer of mesodermal cells, in the vicinity of the visceral endoderm, while CM-primed *Mesp1*⁺ progenitors are found closer to the epiblast. aSHF and pSHF-*Mesp1*⁺ markers were largely non-overlapping, with *Wnt2b* (pSHF marker) being expressed close to the PS while *FoxC2* (aSHF marker) was expressed more anterolaterally (Figure 2) (Lescroart et al. 2018). *Tcf21*, which labels cardiopharyngeal progenitors that contribute to head muscle formation, is expressed in the anterior CPF population.

In *Ciona*, the two *Mesp*⁺ progenitors divide once in the early gastrula to give rise to four equivalent multipotent progenitors, which then divide asymmetrically at the end of gastrulation to generate anterior tail muscle progenitors (ATMs) and trunk ventral cells (TVCs). Cardiac cells (CMs and pericardial cells) derive from TVCs (Davidson et al. 2005; Davidson et al. 2006; Beh et al. 2007; Christiaen et al. 2008). TVCs are multipotent progenitors that divide asymmetrically a second time to

form first heart precursors (FHPs) and second TVCs (STVCs) (Stolfi et al. 2010). STVCs divide asymmetrically again to form lateral atrial siphon muscle founder cells (ASMFs) and second heart precursors (SHPs) (Stolfi et al. 2010; Wang et al. 2013) (Figure 1). scRNA-seq of progenitor cells covering the early branches of the cardiopharyngeal lineage tree in *Ciona intestinalis* identified 3 distinct transcriptional clusters that correspond to the cardiac FHP and SHP populations as well as an Ebf+ pharyngeal muscle population. Interestingly, this analysis showed that specific transcriptional programs are activated in fate-committed progenitors. In addition, this single cell transcriptomic study identified new specific-lineage markers, including *Mmp21* for the FHP and *Dach* as a SHP specific marker. Remarkably, in the mouse, *Dach1* is first broadly expressed in pharyngeal mesoderm and then also restricted later to cardiac SHF progenitors (Wang et al. 2019).

While single-cell approaches have helped us investigate in more detail the heterogeneity of the CPC population at different stages of development mostly through gene expression, the combination of these techniques with rapidly advancing systems approaches to functional genomic analysis (single-cell CITE-seq, single-cell ATAC-seq) will further improve our understanding of the molecular mechanisms implicated in early cell fate decisions.

3. Regulation of CPC Specification

3.1. Early induction of cardiogenic mesoderm

The extrinsic signals given to cells during development *in vivo* can be used as guidance cues during PSC differentiation from pluripotency towards specific cell types. It is therefore important to understand the stage-specific requirements of signaling pathways and their downstream transcription factors during early differentiation (Nosedá and Schneider 2010; Loh et al. 2016).

CPCs arise from epiblast cells that form the primitive streak (PS). Their specification therefore depends on correct positioning and formation of the PS: this process relies on several signaling pathways, including canonical Wnt/ β -catenin and Tgfb β /Nodal pathways (Conlon et al. 1994; Haegel et al. 1995; Nosedá and Schneider 2010). The same pathways are required to induce formation of PS-like cells and subsequent cardiogenic mesoderm from PSCs (Xu et al. 1999; Gadue et al. 2006). Canonical Wnt signaling has a biphasic role during cardiogenesis, as it promotes early steps of cardiogenesis but then inhibits CM differentiation (Marvin et al. 2001; Schneider and Mercola 2001; Tzahor and Lassar 2001; Naito et al. 2006; Liu et al. 2007; Qyang et al. 2007; Ueno et al. 2007; Paige et al. 2010). BMP signaling is also required for subsequent gastrulation and formation of the cardiac crescent (Mishina et al. 1995; Miura et al. 2006). The requirement of these signaling pathways for cardiac development is well conserved throughout chordates (Nosedá and Schneider 2010). Modulation of these pathways and others has guided most protocols of differentiation of PSCs towards cardiac lineages (Laflamme et al. 2007; Yang et al. 2008; Kattman et al. 2011). Briefly, formation of cardiac mesoderm can be induced from PSCs by BMP4, Vegf, Activin A and Fgf2, and subsequent differentiation is enhanced by Wnt inhibition. These are the minimal requirements for the formation of cardiac lineages *in vitro* in serum-free conditions. These extrinsic signals induce an intrinsic cascade of molecular events, where the activation of core transcriptional regulators thereafter progressively promotes cardiac cell fates during differentiation. We will now discuss these intrinsic events.

In *Drosophila*, *tinman*, the ortholog of *Nkx2-5*, is considered to be the master regulator of cardiogenesis (Azpiázu and Frasch 1993; Bodmer 1993; Frasch 1995). While mutations in *Nkx2-5* are associated with congenital heart disease (Schott et al. 1998), *Nkx2-5* mouse mutants form an early heart tube (Lyons et al. 1995; Tanaka et al. 1999) showing that during evolution, *Nkx2-5* lost its central role in cardiac specification, likely due to expansion of the vertebrate *Nkx* gene family. A key role for *Mesp1* has clearly been established for cardiac specification in chordates (Saga et al. 2000; Bondué and Blanpain 2010; Liu 2017). *Mesp1* mutant mouse embryos present *cardia bifida*, with differentiated CMs and ECs in separated heart fields (Saga et al., 1999). Double mutants for *Mesp1* and its closest homologue, *Mesp2*, on the other hand, have severe defects of gastrulation. Chimeras with *Mesp1/2* null and wild type cells showed contributions of mutant cells to most of the embryo excluding the heart, indicating a cell autonomous requirement of *Mesp1/2* for CPC specification (Kitajima et al. 2000).

Mesp1^{Cre/Cre} knock-out cells present a developmental block similar to that of double mutant embryos, with mesodermal precursors being stuck in the gene program of the epiblast, as shown by scRNA-seq. Transcripts of several regulators of pluripotency such as *Nanog*, *Oct4* and *Eras* or markers of the epiblast including *E-cadherin* and *Epcam*, were thus upregulated in *Mesp1*-null cells (Lescroart et al. 2018).

Interestingly, during mPSC differentiation, overexpression of human or murine *Mesp1* accelerates and increases cardiac differentiation, as shown by the appearance of beating areas and enhanced troponin T expression (Bondue et al. 2008; David et al. 2008; Lindsley et al. 2008). However, continuous expression of *Mesp1* inhibits cardiac differentiation (Bondue et al. 2008). *Mesp1* has been shown to directly bind to the promoter of key cardiac TFs, such as *Mef2c*, *Tbx20*, *Gata4* and *Nkx2-5*, thereby upregulating their expression and promoting further upregulation of downstream cardiac genes (Figure 3). Nevertheless, in the context of reprogramming, forced *Mesp1* expression in cardiac fibroblasts is not sufficient to induce cardiac specification. In contrast, *Smarcd3*, *Tbx5* and *Gata4* have been shown to be sufficient for cardiac reprogramming (Takeuchi and Bruneau 2009; Ieda et al. 2010).

The key role of *Mesp* genes during cardiac progenitor specification appears to be highly conserved in chordates, as shown by the induction of ectopic cardiac differentiation upon expression of a constitutive form of *Mesp* ortholog in *Ciona* and human *Mesp1* in *Xenopus* (Davidson et al. 2005; David et al. 2008). Knock-down experiments using morpholinos also demonstrated the importance of *Mesp* for cardiac specification in ascidians (Satou et al. 2004). In fish, the master regulator role of *Mesp* is not clear. While there are four *mesp* genes, only *mespaa* has a pro-cardiogenic role after animal cap transplants with *mespaa* over-expressing cells. Surprisingly, *mespaa* or even quadruple *mespaa/ab/ba/bb* CRISPR/Cas9 mutants did not show any cardiac defects (Deshwar et al. 2016).

Mesp1 is activated in nascent mesoderm by key regulators of endoderm and mesoderm development Eomesodermin (*Tbr2*) and Brachyury (*T*) (Costello et al. 2011; David et al. 2011; van den Aamele et al. 2012). In *Ciona* embryos, *Mesp* is activated by *Tbx6* and *Lhx3*, only in B7.5 cells co-expressing these factors (Davidson et al. 2005; Christiaen et al. 2009). Consistent with these findings, a recent report searching for mesoderm-inducing factors in mouse embryonic fibroblasts found that *Tbx6* was sufficient to activate *Mesp1* and subsequent CPC gene expression. During PSC differentiation, *Tbx6* promoted mesoderm and endoderm induction in the absence of serum and growth factors in both a cell-autonomous and non-cell-autonomous manner (Sadahiro et al. 2018). Further studies in mouse will be required to determine how the precise domain of *Mesp1* expression is defined within the nascent mesoderm, although other factors are also implicated in early specification of cardiac progenitors, such as long non-coding RNAs (Klattenhoff et al. 2013; Ounzain et al. 2015; Guo et al. 2018), which have even been used to promote trans-differentiation to cardiac cell types (Hou et al. 2017) (Figure 3).

Finally, the dynamic gene expression changes involved in transitions from pluripotency to cardiogenic mesoderm are accompanied by modifications of the epigenetic landscape or chromatin accessibility, with regulatory regions of genes being first primed then activated, and other regions involved in alternative fates being de-commissioned (Paige et al. 2012; Wamstad et al. 2012; Racioppi et al. 2019). Transcription factors cooperate to induce these changes, through interactions with each other and specific chromatin modifiers (Chang and Bruneau 2012; Luna-Zurita et al. 2016). Combining data from single-cell expression and broad epigenetic studies, such as repressive histone mark ChIP-seq data from many tissues, may help us to identify new genetic drivers of cell fate specification (Shim et al. 2019).

3.2. CPC specification to the different cardiac lineages

In vitro, mPSCs cultured in serum conditions can form three populations with differential expression of T and Flk1 (Kouskoff et al. 2005; Kattman et al. 2006). Hemangioblast specification usually precedes cardiac specification, as the former derives from a first wave of Flk1-expressing Brachyury+ cells while the cardiogenic mesoderm arises from a Brachyury+ population, with later expression of Flk1 (Kattman et al. 2006). Interestingly, if *Mesp1* is induced earlier during mPSC differentiation, there is an increase in the hematopoietic progenitor population through direct activation of *Tall1*, encoding a

vascular TF, at the expense of cardiac fates (Chan et al. 2013). Conversely, in the absence of *Tall*, extra-embryonic tissue (also expressing *Mesp1*) can differentiate into CM (Van Handel et al. 2012).

CM and EC lineages of the FHF segregate early during gastrulation (Mikawa and Fischman 1996; Wei and Mikawa 2000; Lescroart et al. 2014). scRNA-seq of *Mesp1*⁺ CPCs showed that several Notch signaling actors, including Notch1, are specifically downregulated in CM-fated cells and conversely upregulated in EC-fated cells (Lescroart et al. 2018). The Notch pathway has been implicated in several key processes of cardiac development, including trabeculation and valve formation (MacGrogan et al. 2010; MacGrogan et al. 2018). *In vitro* activation of Notch reduces CM differentiation (Nemir et al. 2006) and repression of Notch increases CM differentiation (Schroeder et al. 2003). Lineage tracing of early Notch1⁺ cells preferentially marks the endocardium, suggesting a potential role for the Notch pathway in early CPC fate decisions between CMs and ECs (Lescroart et al. 2018). Moreover, further crosstalk between these two lineages happens *in vitro* and *in vivo*, suggesting paracrine mechanisms governing CPC specification and differentiation (Saba et al. 2019; Saint-Jean et al. 2019). Interestingly, endocardial cells with “valve-like” properties can be derived from hPSCs through the activation of other signaling pathways. VEGF, together with FGF2 and FGF8, can induce EC differentiation at the expense of CMs, thus favoring the specification of a pre-valvular cell type (Neri et al. 2019).

The PSC model has also been used to specify PSCs into other cardiac cell lineages (Figure 4). Epicardial cells, for example, can be produced from hPSCs. Indeed, stage-specific activation of BMP, retinoic acid (RA) and Wnt/ β -catenin signaling pathways after induction of CPCs induces the specification of epicardial-like progenitors expressing *TBX18* and *WT1*, two markers of the pro-epicardial organ (Iyer et al. 2015; Bao et al. 2016; Guadix et al. 2017). In line with these findings, mouse embryos lacking the Wnt inhibitors *Dkk1* and *Dkk2* have a thicker epicardium and thinner myocardium (Phillips et al. 2011), showing again the necessity and usefulness of translating our current *in vivo* knowledge for *in vitro* generation of specific cell types. Epicardial cells can differentiate into cardiac fibroblasts or vascular SMCs through epithelial-to-mesenchymal transition, which can be induced *in vitro* by addition of Tgf- β 1 to the cells. Nevertheless, as the epicardium gives rise to multiple cell lineages, the resulting cell population is heterogeneous. In order to obtain a pure population of cardiac fibroblasts without going through an epicardial-like stage, another protocol has recently been developed where activation of the FGF pathway in hPSC-derived CPCs induces formation of Periostin- and Vimentin-expressing cardiac fibroblasts, presenting many functional similarities with their *in vivo* counterparts (Zhang et al. 2019).

Pacemaker CMs can also be generated from mPSC by induction of *Tbx3* (Jung et al. 2014) or *Shox2* (Ionta et al. 2015). Sino-atrial node (SAN)-like pacemaker cells arise from *Nkx2.5*⁻, *Tbx18*⁺ CPCs (Mommersteeg et al. 2010). As a first demonstration of generating SAN-like pacemaker cells from hPSCs without recurring to overexpression, inhibition of the FGF pathway with activation of BMP and RA signaling generated a *NKX2-5*⁻ but *SIRPA*⁺ cardiac cell type, presenting pacemaker properties similar to SAN cells. These cells were able to display pacemaker activity when grafted into rat hearts (Protze et al. 2017).

3.3. Regional specification

Multiplication of the cardiac chambers has significantly increased the efficiency but also complexity of the heart during evolution. The specification of early CPCs toward a particular cardiac region is not yet fully understood and there may be a lot to glean from *in vitro* PSC models and *in vivo* studies.

One of the main regional distinctions is between atrial and ventricular cardiac tissue. As discussed previously, lineage-tracing experiments in the mouse have shown the existence of distinct CPCs that contribute to different cardiac regions. For example, *Foxa2*⁺ CPCs specifically label ventricular derivatives. Formation of chimeric embryos from wild type and *Foxa2*^{-/-} mutant mPSCs have shown that *Foxa2* is cell-autonomously required for ventricular specification such that there is no contribution of mutant cells to the ventricles. Similarly, *Foxa2*^{-/-} mPSCs have a decreased ability to form ventricular cardiomyocytes (Bardot et al. 2017). Retinoic acid (RA) signaling is also a known player for regional specification, such that exogenous RA treatment patterns the CPCs along the antero-posterior axis and

promotes atrial identity (Xavier-Neto et al. 1999; Hochgreb et al. 2003; Huang et al. 2011; Stefanovic and Zaffran 2017; De Bono et al. 2018). The specific addition of RA at the cardiac mesoderm-CPC stage during hPSC differentiation favors specification toward an atrial-like phenotype (KCNJ3+) at the expense of ventricular fate (MYL2+) (Zhang et al. 2011; Lee et al. 2017). A study has shown that ventricular hPSC-derived CPCs are marked by the expression of CYP26A1, which encodes an enzyme responsible for RA degradation. In contrast, atrial hPSC-derived CPCs express RALDH2 (an enzyme required for the synthesis of RA). RALDH2+ CPCs will preferentially generate atrial CMs (Lee et al. 2017). Consistently, in the mouse model, early *Mesp1*-expressing CPCs, which contribute preferentially to the LV, express *Cyp26a1*, while atrial *Mesp1*+ CPCs that express *Mesp1* at later stages preferentially express *Raldh2* (Lescroart et al. 2014). Interestingly, ISL1 knock-out hPSCs resemble PSC cultures exposed to RA signaling and lead to the preferential specification of atrial CMs. Further investigation showed that ISL1 suppresses the atrial specifier NR2F1. Specification of hPSCs into atrial CMs through RA signaling appears to be mediated by activation of the TALE transcription factor MEIS2, which antagonizes ISL1 expression (Quaranta et al. 2018).

In fish, RA signaling also indirectly counteracts the activity of *Isl1* through *Ajuba*, a LIM domain protein (Witzel et al. 2012). In addition, *in vitro* studies have demonstrated that the proportion of atrial versus ventricular progenitors can be modulated by different concentrations of Activin A and *Bmp4* early during hPSC differentiation (Lee et al. 2017) (Figure 4). It would be very informative to evaluate the levels of BMP4 and Activin A *in vivo* to see whether this reflects a distinct environmental context for atrial and ventricular progenitors. Furthermore, differentiation protocols established so far do not yet allow us to differentiate between LV versus RV or between atrial cardiac cell types, such as right versus left atrial CMs or pacemaker cells. These might be achieved by understanding the specification of the FHF and anterior and posterior SHF as they contribute to different regions of the heart.

The establishment of spheroids from *HCN4-GFP/Tbx1-Cre; Rosa-tdTomato* mPSCs has allowed study of FHF versus SHF specification, despite the fact that *Tbx1* is only expressed in a subpopulation of the SHF. Transcriptomic analysis demonstrated that *Bmp* signaling is enriched in HCN4+ FHF progenitors while *Wnt* signaling appears to be specific to *Tbx1*-derived SHF cells. Modulating levels of *Bmp4* or *Wnt3A* demonstrated that *Bmp4* can promote FHF or *Tbx1*+ SHF specification depending on its concentration, while *Wnt3a* specifically promotes the appearance of the *Tbx1*-derived SHF population. Interestingly, the same study showed that *Cxcr4*, which initially marks early CPCs, is not expressed later in the FHF and specifically labels the *Tbx1*+ SHF population (Andersen et al. 2018).

Finally, scRNA-seq has enabled prediction of lineage specifiers for specific cardiac regions. For example, *Hopx*, *Chd3*, *Ncor1* and *Shox2*, *Sox4* and *Hoxb4* were among the major genes predicted to play a role in the specification of the atrial or sinus venosus lineages, respectively. Similarly, *Nr1d1* could be a cell fate determinant for the RV while *Hand2*, *Msx1* may specify the OFT (de Soysa et al. 2018). Indeed, scRNA-seq of *Hand2*-null cells shows dysregulation of the RV and OFT gene program with abnormal activation of posterior genes, but these data suggest a failure of RV cells to migrate to their final location rather than a defect in their specification (de Soysa et al. 2018). Similarly, ectopic expression of *Hoxb1* in aSHF CPCs causes deregulation of the aSHF gene program, suggesting that homeodomain genes play a key role in the antero-posterior patterning and cell identity of CPCs (Stefanovic et al. 2019). Relatedly, in tunicates, knock-out of the SHP-specific gene *Dach* in multipotent CPCs led to ectopic expression of the FHP marker *Mmp21*, suggesting a change in cell fate identity (Wang et al. 2019).

3.4. Cardiomyocyte versus skeletal muscle fate

As discussed above, the CPF is able to differentiate into both cardiac tissue and skeletal muscles of the head and neck. Several studies in mice, chick and fish have shown that there is a conserved regulatory network (including *Tbx1*, *Pitx2*, *Nkx2-5* and *Isl1*) that governs cardiac and skeletal muscle progenitor specification (Tirosh-Finkel et al. 2006; Nathan et al. 2008; Sambasivan et al. 2009; Nevis et al. 2013; Paffett-Lugassy et al. 2017; Guner-Ataman et al. 2018). Interestingly, fundamental insights on how the CPF is able to differentiate into cardiac or skeletal muscle lineages arise from studies in *Ciona robusta*. It is now clear that in tunicates, there is an antagonism between *Nk4* (homolog of *Nkx2-5*) and *Tbx1/10* to regulate cardiac versus skeletal muscle specification. Thus, in committed cardiac

progenitors, NK4 inhibits Tbx1/10-dependent activation of Ebf, while in ASM progenitors, NK4 is repressed, thereby allowing Tbx1/10 to activate the skeletal muscle program (Wang et al. 2013). Ebf is then required for differentiation of ASMs by promoting the pharyngeal myogenic program (Razy-Krajka et al. 2014).

Fgf/MAPK signaling also plays a critical role in Ciona in skeletal vs cardiac specification. Activated in multipotent progenitors, Fgf signaling is then maintained only in the lateral-most daughter cells during cell division within the CPF lineage. Fgf signaling is thereafter restricted to ASM progenitors, thus promoting skeletal muscle identity (Razy-Krajka et al. 2018). Even if a role for Fgf signaling in cardiac versus skeletal muscle specification has not yet been shown in vertebrates, it is required for development of the CPF (Abu-Issa et al. 2002; Vitelli et al. 2002; Brown et al. 2004; Hu et al. 2004; Aggarwal et al. 2006; Ilagan et al. 2006; Park et al. 2006; Kelly and Papaioannou 2007; Park et al. 2008; de Pater et al. 2009; Watanabe et al. 2010; Lazic and Scott 2011; Watanabe et al. 2012).

sc-RNAseq in Ciona has further enabled precise investigation of temporal CPC specification. It led to the characterization of ten discrete regulatory states along specification pseudotime, with identification of “primed” and “*de novo*” markers that are respectively expressed either throughout these states or only in more committed progenitors. About 50% of cardiac or skeletal muscle genes are “primed” and therefore already expressed in multipotent progenitors. Cross-comparison with other published sc-RNAseq of developing mouse hearts showed high conservation of the transcriptional network governing cardiac versus skeletal muscle specification (Wang et al. 2019).

Tbx1 mutant embryos show defects in both craniofacial skeletal muscles and heart (Lindsay et al. 2001; Kelly et al. 2004; Lescroart et al. 2015). Through RNA-sequencing of wild-type versus *tbx1* mutant zebrafish embryos, gdf3-ALK4, a TGF μ superfamily ligand has been identified as a downstream effector of *tbx1* involved in CPC specification (Guner-Ataman et al. 2018). In fish, *tbx1* is also required to specify the *nkx2.5*+ CPF. Moreover, recent studies have also shown a role for Wnt signaling in the balance between cardiac and skeletal muscle lineages, as Wnt activation during or prior to gastrulation inhibits pharyngeal muscle specification at the expense of cardiac lineages (Mandal et al. 2017). Such a role for Wnt has not been found in tunicates (Kaplan et al. 2019). Other factors have been implicated in pharyngeal muscle specification. NR2F or COUP-TF proteins, downstream targets of RA signaling, have been shown, in fish, to promote posterior pharyngeal muscle specification (muscles analogous to the vertebrate neck muscles) while restricting ventricular CM progenitors (Dohn et al. 2019).

BMP signaling, when applied to differentiating mPSCs, leads to the downregulation of skeletal muscle markers while promoting cardiac genes. RNA-seq showed that *Podxl* and *Cdh4* are surface markers that could be used to distinguish between committed skeletal and cardiac progenitors respectively (Chan et al. 2016). After mesoderm induction, dual inhibition of Wnt and Nodal promotes CPC specification, and cells thus express *Tbx1*, *Isl1* and *Nkx2-5*. With specific culture conditions, it is then possible to specify the CPF into skeletal muscle fibers with a cocktail of signaling molecules including a Bmp inhibitor, Hgf, Fgf2 and Igf1 or into beating cardiomyocytes when supplemented with ascorbic acid, Vegf, Fgf2 and Fgf10 (Nandkishore et al. 2018).

Concluding remarks

The specification of CPCs is a complex developmental process that occurs concomitantly with their migration. *Mesp1*, a key transcription master regulator of CPC specification, has also a critical role in CPC migration by controlling the epithelial-to-mesenchymal transition, cell polarity and migration speed (Bondue et al. 2008; Lindsley et al. 2008; Chiapparo et al. 2016). Therefore, it would be important to integrate both CPC migratory trajectories and lineage behavior to investigate the influence of each process on the other. In addition, it is well established that left-right asymmetry is critical for proper heart development (Desgrange et al. 2018) but it is not clear how this affects CPC specification.

With the emergence of single-cell approaches, the model of CPC specification has changed, and it is now clear that CPCs represent a very heterogeneous and dynamic population of progenitors, reflecting distinct physical positions in space and time but also some stochastic factors. In addition, recent research has shown that early CPCs are “primed” toward a particular lineage very early during

embryonic development. The determinants of this early “primed” transcriptional heterogeneity are not yet understood. Comprehending the regulation of CPC specification will help to decipher the developmental mechanisms leading to congenital heart disease, which affect about 1% of newborns world-wide (Srivastava 2006).

In addition, investigating the embryonic regulation of CPC specification has already significantly improved differentiation protocols of PSCs toward a specific cell type. This is particularly relevant in the context of cell therapy after cardiac injury. The identification of multipotent CPCs has brought significant hope to the field of heart repair and further research into the early steps of CPC specification will undoubtedly lead to the design of strategies to specify multipotent CPCs into a specific mature cardiac cell type, that could then be used for cell therapy. As a recent example, hPSC-derived CMs have recently been grafted into infarcted pig hearts and showed significant engraftment, although accompanied by ventricular tachyarrhythmia (Romagnuolo et al. 2019). Further improvements are thus required to successfully repair infarcted hearts in patients.

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Table 1: Lineage tracing in mouse has revealed different early CPCs subpopulations

CPCs subpopulation	Genetic construct used	Labeled cardiac regions	Labeled cardiac cell types	Non-cardiac derivatives	References
<i>Mesp1+</i>	<i>Mesp1-Cre; R26R</i>	OFT, RV, LV, RA, LA, IFT	SMCs, ECs, CMs, EPDCs	Some head bones and head muscles, dorsal aorta, intersomitic and cranial vessels, haematopoietic progenitors, amion, mesothelial cells of the liver	(Saga et al. 1999, Lescroart et al. 2014, Mc-Bratney-Owen et al. 2008, Yoshida et al. 2008, Harel et al. 2009, Asahina et al. 2009)
<i>Smarcd3+</i>	<i>Smarcd3-F6-CreERT2; R26R (Tam E6.5)</i>	OFT, RV, LV, RA, LA	ECs and CMs	Anterior forelimb	(Devine et al. 2014)
<i>Foxa2+</i>	<i>Foxa2-Cre; R26R-tdTomato</i>	RV, LV	CMs, ECs, EPDCs	Endoderm derivatives	(Bardot et al. 2017)
<i>Mef2c-AHF-enh+</i>	<i>Mef2c-AHF-enh-Cre; R26R</i>	OFT, RV, IS	CMs, ECs, SMCs	Head muscles	(Verzi et al. 2005, Devine et al. 2014, Lescroart et al. 2010)
<i>Hcn4+</i>	<i>Hcn4-CreERT2; R26R (Tam E6.-E7.0)</i>	LV	CMs	Cardiac specific	(Liang et al. 2013)
<i>Hand1+</i>	<i>Hand1-Cre; R26R</i>	OFT, LV	CMs, EPDCs	Post-migratory NCCs, Forelimb and Hindlimb mesenchyme	(Barnes et al. 2010)
<i>Sfrp5+</i>	<i>Sfrp5-Cre; R26R</i>	OFT, LV, RA, LA	CMs, ECs, EPDCs	Not determined	(Fujii et al. 2017)
<i>Six2+</i>	<i>Six2-Cre-ERT2; R26R</i>	OFT, RV	SMCs, CMs	Head muscles, Urogenital tissues	(Zhou et al. 2017)
<i>Hoxb1+</i>	<i>Hoxb1-Cre; R26R</i>	OFT, RA, LA	CMs, ECs	Large contribution to the endoderm and mesoderm	(Bertrand et al. 2011)

Summary of the results obtained after lineage tracing of different CPC subpopulations. OFT, outflow tract; RV, right ventricle, LV, left ventricle; RA, right atrium; LA, left atrium, IVS, interventricular septum; CMs, cardiomyocytes, ECs, endothelial/endocardial cells; SMCs, smooth muscle cells; EPDCs, epicardium derived cells

Figure 1: Steps from cardiopharyngeal progenitor cells to cardiac and skeletal muscle cell lineages: comparison between tunicates and mouse

A. The *Ciona intestinalis* CPC lineage tree is found on top. Two *Mesp*⁺ CPCs (green) are found at around 4 hours post fertilization (hpf). After two cell divisions, these CPCs form four anterior tail muscle cells (ATMs) and four multipotent trunk ventral cells (TVCs). These TVCs divide asymmetrically to form a second TVC (STVC) and a first heart precursor (FHP - red). The STVC (dark grey) divides to form an atrial siphon muscle progenitor cell (ASM - blue), analogous to the vertebrate head muscles and a second heart precursor (SHP - orange). A subset of the lineage branches is depicted from a single *Mesp* CPC, the other branches being identical in sequence and outcome. Below, layout of the tunicate larva. Black lines indicate sister cells. Time after fertilization is indicated, in hours post-fertilization (hpf). At around 28 hpf, ASM progenitor derivatives form an ASM ring while FHP- and SHP-derived cells form the developing heart, including pericardial cells and cardiomyocytes. **B.** The mouse CPC lineage. On top, early CPCs (in green) are found in the gastrulating embryo and are marked by the expression of *Mesp1*, *Flkl1*, *Pdgfra* and *Cxcr4* as soon as they leave the primitive streak (PS). At embryonic day (E) 8.5, the primary heart tube is mainly formed from first heart field (FHF, red) progenitors, with additional contribution to the arterial and venous pole respectively from anterior and posterior second heart field (aSHF, blue and pSHF, purple) progenitors that lie behind the heart tube, within the cardiopharyngeal mesoderm. On the right, the contribution of FHF and anterior and posterior pharyngeal mesoderm to the head and neck muscles and to the heart; below, the lineage tree deduced from retrospective clonal analysis. Interestingly, there are at least three reiteration of the *Ciona* ontogenetic motif along the antero-posterior axis of the embryo. The expression window of *Mesp1* is indicated by green dotted lines. *Mesp1* is first expressed in the FHF progenitors (red) while expressed later in the anterior (blue) and posterior (purple) progenitors of the cardiopharyngeal field. EOM, extraocular muscles, ao, aorta, pt, pulmonary trunk, RA, right atrium, LA, left atrium, RV, right ventricle, LV, left ventricle.

Figure 2: Heterogeneity of CPC specification as revealed by single-cell analysis

A. In scRNA-seq experiments, four distinct *Mesp1*⁺ CPC subpopulations have been identified that emerge from epiblast cells and that correspond to the endothelial (EC -black), cardiomyocyte (CM - red), anterior cardiopharyngeal field (aCPF, light green) and posterior CPF (pCPF, dark green) progenitors. These populations are enriched for some transcripts (in brackets) and are found in particular locations in the embryo (**B**). **B.** Drawing of the gastrulating embryo, where the different CPC subpopulations are found. The embryo is bilateral: on one side is depicted the localization of the *Mesp1*⁺ EC versus *Mesp1*⁺ CM populations found either in the outer layer or close to the epiblast, while on the other side the localization of aCPF and pCPF progenitors is represented along the antero-medial axis of the embryo. These distinct CPC subpopulations make different regional contributions to the definitive heart as illustrated in **C**. AA, aortic arch, PT, pulmonary trunk, Ao, aorta, PV, pulmonary vein, SCV, superior caval vein, ICV, inferior caval vein, RA, right atrium, LA, left atrium, RV, right ventricle, LV, left ventricle.

Figure 3: Early intrinsic regulation of cardiac specification

Representation of the molecular hallmarks of cardiac progenitor cell commitment. When cardiac progenitors express *Mesp1* in the nascent mesoderm, they turn off the genetic program of pluripotency and turn on the gene regulatory network of cardiac specification and migration. Green arrows represent direct activation, red arrows represent repression. Genes grouped together are regulated in a similar manner by *Mesp1*. Although *Linc1405* was shown to interact with *Eomes*, it was not the case for *Braveheart*, another cardiogenic long non-coding RNA.

Figure 4: Extrinsic regulation of cardiac specification

By differentiating mouse and human pluripotent stem cells (PSCs) under serum-free conditions, the minimal cues necessary for their specification and differentiation to a particular cardiopharyngeal cell lineage were defined. Arrows indicate different steps of differentiation. All protocols pass through a stage where cells present features of multipotent cardiopharyngeal progenitors (CPCs), then diverge in terms of given signaling modulators and cell markers. AA, ascorbic acid; BMP, bone morphogenetic protein; CFs, cardiac fibroblasts; CMs, cardiomyocytes; FGF, fibroblast growth factor; IGF-1, insulin-

like growth factor-1; HGF, hepatocyte growth factor; SAN, sino-atrial node; SHF, second heart field; SMCs, smooth muscle cells; VEGF, vascular endothelial growth factor.

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Figure 1

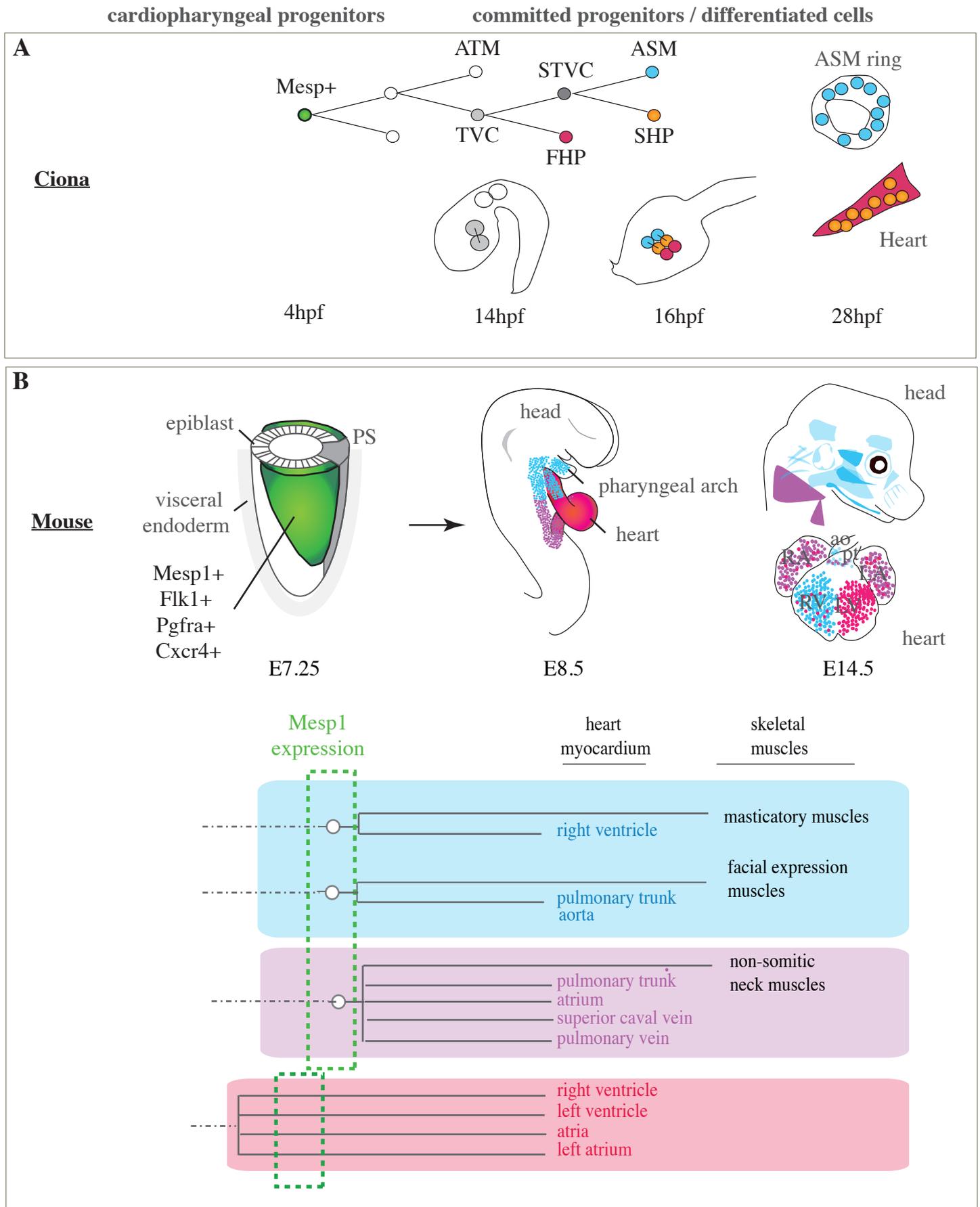


Figure 2

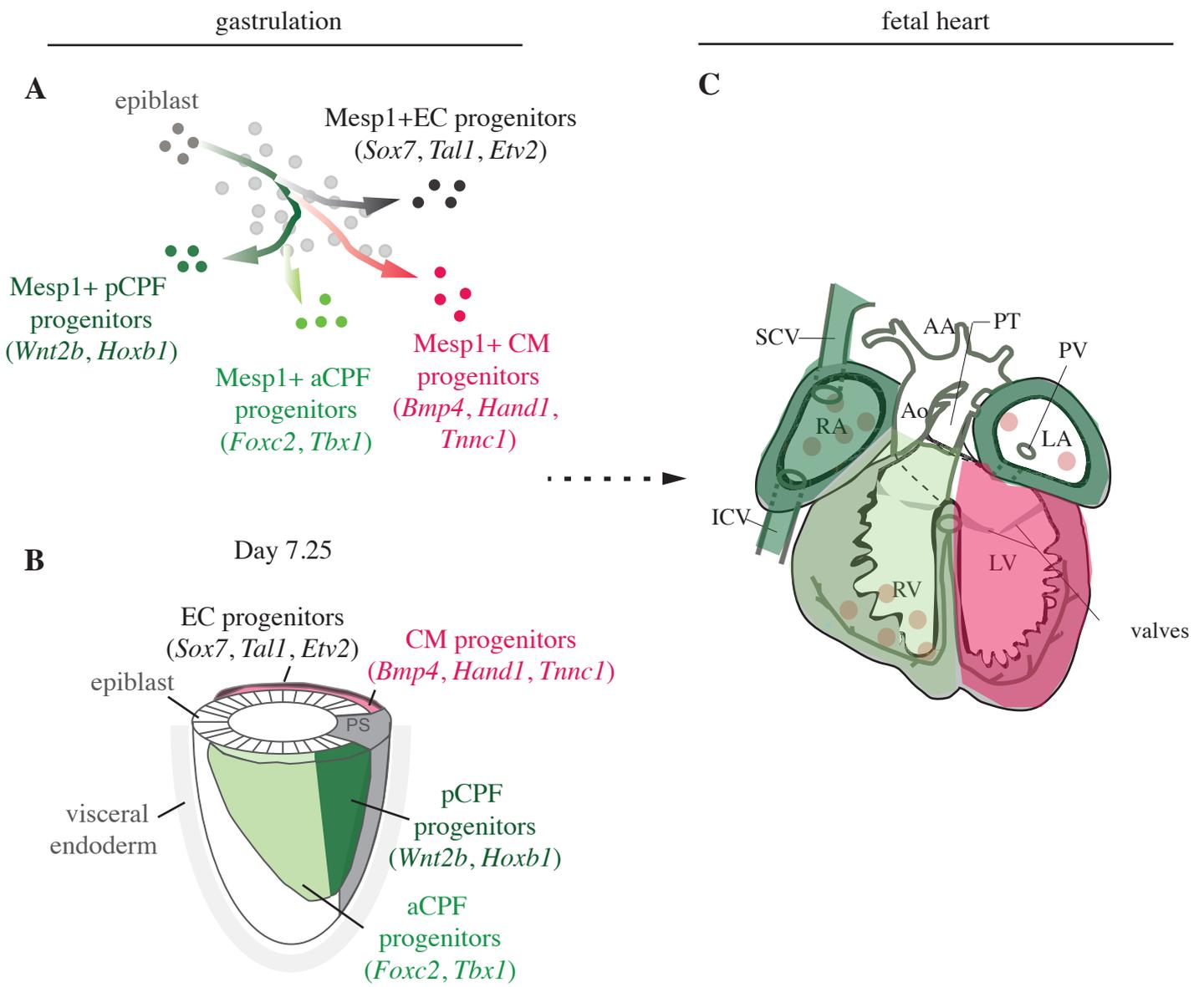


Figure 3

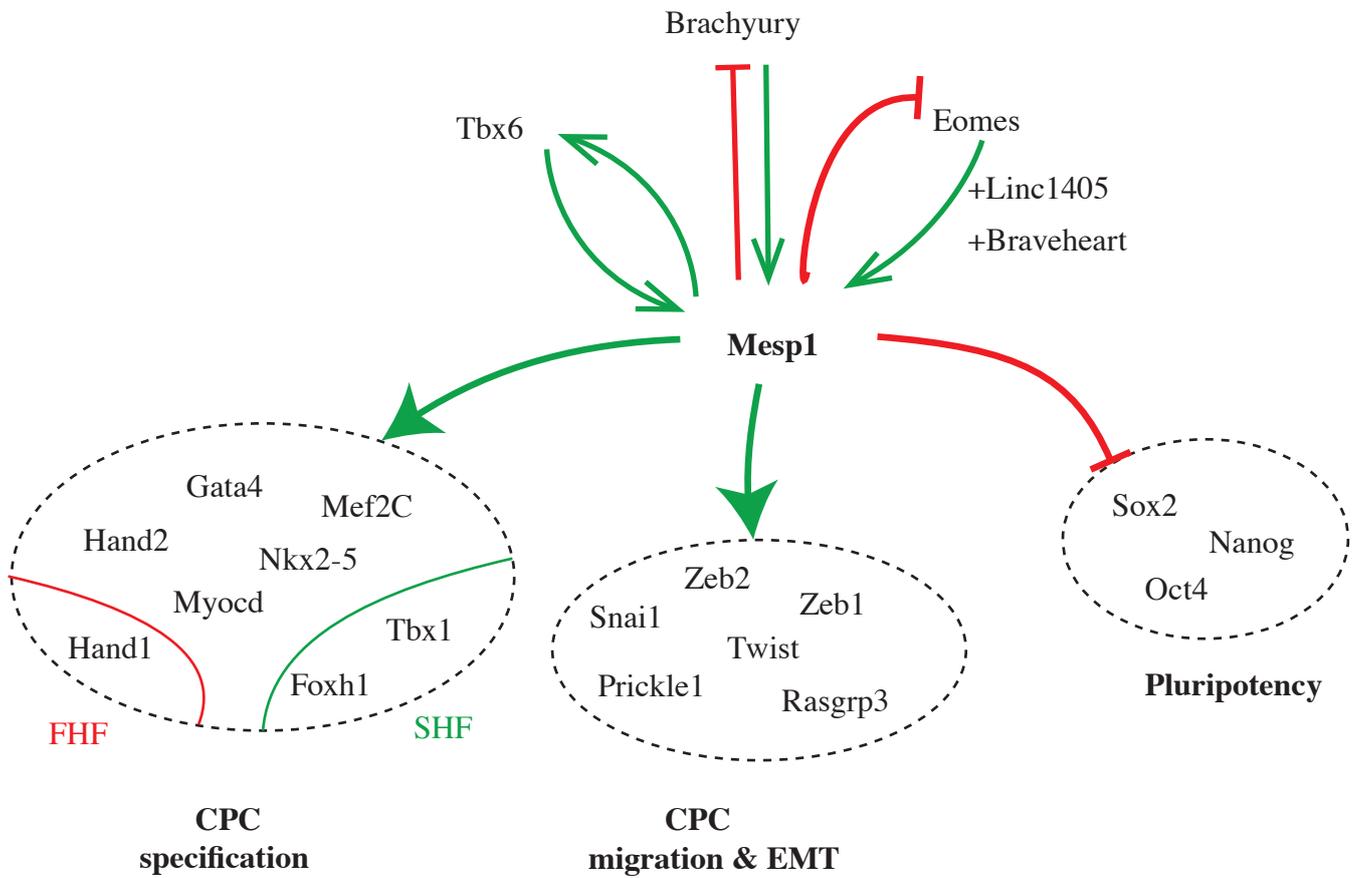


Figure 4

