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HCN4 charges up the first heart field

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The concept of cardiac progenitors that persist late into heart development and potentially into postnatal life has ignited the interest of cardiac biologists. Little more than a decade ago, the prevailing notion of heart development centered on a cardiac tube containing immature cardiomyocytes that were committed to form specific segments of the mature heart[1]. Over the past 15 years, this textbook view was overturned by the modern rediscovery of the pioneering work of Viragh and Chalice and others, which showed that the heart tube grows by addition of new cardiomyocytes to the arterial and venous poles by differentiation of non-cardiomyocyte progenitors[2–5]. Thus, at the time that the initial heart tube is first visible in the developing embryo through heart looping and the initiation of septation, cardiac progenitors present at both poles of the heart differentiate into cardiomyocytes and thereby significantly contribute to heart growth (Figure panel A).

These later differentiating progenitors have been referred to as "second heart field progenitors", and reside in the "second heart field" (SHF) located at the arterial and venous pole sof the developing heart. Evans, Cai, and colleagues made the seminal discovery that these SHF progenitors express the transcription factor Islet 1 (ISL1)[6]. *Isl1* is required in the progenitor cells for their normal activity, but is shut off as the progenitors differentiate. $ISL1^{+}$ SHF progenitors are multipotent and differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells, the major lineages of the heart[7]. Development of Cre-based reagents allowed investigators to selectively label, isolate, and genetically manipulate these progenitors and their descendants in developing embryos. *Isl1*-based genetic reagents also proved valuable in isolating these progenitor cells and their descendants from differentiating ES cells, facilitating their in vitro analysis[7, 8]. Cre-based genetic lineage tracing using most Cre-activated reporters indicated that $Isl1^{Cre}$-marked SHF progenitors contribute to the right ventricle, the outflow tract, and much of the atria, but little of the left ventricle[6]. These data suggested that most of these regions of the heart are generated by SHF progenitors following specification of the initial linear heart tube, although an important

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caveat is that the precise boundaries of the Isl1^{Cre} domain depend on both the specific Isl1^{Cre} allele and the specific Cre-dependent reporter used[9, 10].

Relatively less is known about the first differentiating cardiomyocytes of the linear heart tube. By analogy to the terminology used for the SHF, it would be logical to define the "first heart field" as the region of the embryo that houses the "first heart field progenitors", i.e., those cardiac progenitors which first differentiate into the cardiomyocytes that populate the cardiac crescent and linear heart tube (Fig. 1b). By inference based on the Isl1^{Cre} genetic fate map, descendants of the FHF generate most of the left ventricle and a subset of the atria. However, a lack of appropriate markers and genetic reagents has hindered studies of FHF progenitors and their descendants. This gap in knowledge has been particularly acute because these cells most closely match the needs of regenerative applications for acquired heart disease, which primarily affect the left ventricle.

In this issue of Circulation Research, Evans, Liang and colleagues report that the pacemaker channel gene *Hcn4* (hyperpolarization activated nucleotide gated cation channel 4) marks the FHF[11]. Using a suite of *Hcn4* knockin alleles (Hcn4^{LacZ}, Hcn4^{H2BGFP}, and Hcn4^{CreERT2}), Liang et al. show that *Hcn4* is expressed throughout the cardiac crescent at mouse embryonic day 7.5, in a population distinct from Isl1^{+} SHF progenitors. Subsequently, *Hcn4* is dynamically expressed in portions of the conduction system. Activation of Hcn4^{CreERT2} with tamoxifen at E7.5 pulse-labeled allowed the fate of their descendants to be traced. As anticipated by inference from Isl1^{+} SHF progenitor genetic lineage tracing, the Hcn4^{CreERT2}-labeled descendants were found primarily in the left ventricle. Additional descendants were found in the atria, coronary sinus, venous valves, and atrioventricular (AV) and sinoatrial (SA) nodes.

What types of cells in the E7.5 cardiac crescent express *Hcn4* and are labeled by Hcn4^{CreERT2}? Are they cardiomyocyte progenitors and if so are they multipotent like Isl1^{+} progenitors[7]? Or have they already committed to the cardiomyocyte lineage by the time that they express *Hcn4* and are labeled at E7.5? Unfortunately, the study of Liang et al. does not directly address these points. After E9.5 Hcn4^{H2BGFP} was expressed primarily in cardiomyocytes, but whether or not *Hcn4* expression is similarly confined to differentiated cardiomyocytes at E7.5 is unclear. Analysis of *Hcn4* or *Hcn4*-driven labels (e.g. H2BGFP or preferably CreERT2) with cardiac progenitor (e.g. Mesp1; Nkx2-5) or cardiomyocyte (e.g. Mlc2a) lineage markers at E7.5 would have been helpful to address this point. Similarly, more detailed analysis of the cell lineages derived from E7.5 Hcn4^{CreERT2}-labeled cells would have provided information about their fate and multipotency. As it is, we can unequivocally say that Hcn4^{CreERT2} activated at E7.5 marks FHF derivatives (probably cardiomyocytes), but whether or not it marks FHF progenitors is uncertain based on the available data.

Broad expression of *Hcn4* in myocardial cells is rapidly extinguished after the linear heart tube stage, so that by E16.5 it is confined to the conduction system, including the SA and AV nodes, the His bundle, the bundle branches, and the Purkinje fibers[11]. These data, consistent with findings based on a previously reported Hcn4^{CreERT2} allele[12], suggest that *Hcn4*-driven alleles will be useful tools to label and isolate conduction system cells at later

stages of heart development. As pointed out by Liang et al., Tam pulse labeling at these later stages should not be confused with descent from FHF but rather reflects the dynamic nature of *Hcn4* expression in different myocardial compartments.

An important use of cardiac lineage markers to be permit isolation of cardiac cell types from pluripotent stem cell differentiation systems, where there is a paucity of anatomical information to facilitate the segregation of populations. It would be desirable, for instance, to differentiate ES cells and isolate FHF progenitors or their derivatives. Whether or not this will be possible with *Hcn4* remains to be determined. The dynamic pattern of *Hcn4* expression suggests that precisely staged differentiation systems and/or a collection of multiple markers will be needed to permit isolation of the equivalent of the labeled cardiac crescent cells and their derivatives. Interestingly, because *Hcn4* is a transmembrane channel, it is possible that its extracellular epitopes could be used as a cell surface marker to permit isolation of *Hcn4*$^+$ cells without the need for genetic modification.

Over the past decade, considerable advances have been made in elucidating the lineage map of heart development (Figure panel B). Retrospective lineage mapping approaches have defined two distinct cardiac lineages that bifurcate from a common cardiac progenitor probably at or soon after gastrulation[13]. This common cardiac progenitor, likely marked by *Mesp1*[14, 15], differentiates into Isl1$^+$ SHF progenitors, which subsequently differentiate to yield the right ventricle, outflow tract, and portions of the atria and conduction system. Conceptually, the common Mesp1$^+$ progenitor also gives rise to a FHF progenitor, which then differentiates to yield the left ventricle and other portions of the atria and conduction system. Evans, Liang, and colleagues have advanced the field by providing a means to label and isolate these FHF derivatives from embryos. In the future, it will be important to determine if the *Hcn4*-expressing cells are already committed cardiomyocytes, and if so it will be important to develop means to isolate the hypothetical FHF progenitors. Alternatively, it is possible that Mesp1$^+$ progenitors differentiate directly into committed *Hcn4*$^+$ cardiomyocytes without a defined progenitor intermediate. It will also be crucial to determine whether and how *Hcn4* might be used to isolate bona fide left ventricular cardiomyocytes or their precursors from pluripotent stem cell cultures, and whether the kinetics of human cardiogenesis also permit *HCN4* to be used for this purpose in human pluripotent stem cell differentiation.

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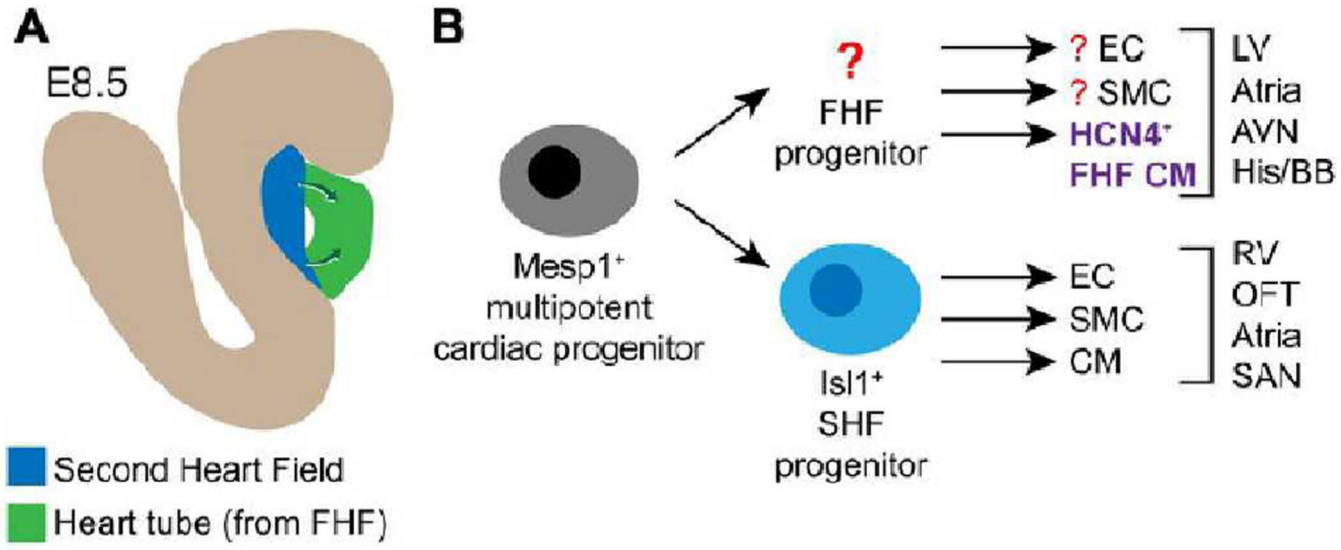

Figure. Cardiac progenitor populations in the developing heart

A. Illustration of an E8.5 embryo highlighting the heart tube, containing differentiated cardiomyocytes and originating from the first heart field (FHF), and the second heart field (SHF), containing SHF progenitors. SHF progenitors differentiate into cardiac lineages to contribute to the ends of the heart tube (arrows). **B.** Lineage map of the developing heart. Around the time of gastrulation, a multipotent cardiac progenitor (likely $Mesp1^{+}$) gives rise to $Isl1^{+}$ SHF progenitors and possibly a FHF progenitor (yet to be identified). SHF progenitors yield most of the right ventricle (RV), outflow tract (OFT), atria, and sinoatrial node (SAN). FHF cardiomyocyte derivatives, arising from the hypothetical FHF progenitor or directly form $Mesp1^{+}$ progenitors, express HCN4 at the cardiac crescent stage. These cells ultimately yield most of the left ventricle (LV) and portions of the atria and the cardiac conduction system including the atrioventricular note, bundle of His, and the bundle branches.