



Unveiling Complexity and Multipotentiality of Early Heart Fields

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RATIONALE: Extraembryonic tissues, including the yolk sac and placenta, and the heart within the embryo, work to provide crucial nutrients to the embryo. The association of congenital heart defects with extraembryonic tissue defects further supports the potential developmental relationship between the heart and extraembryonic tissues. Although the development of early cardiac lineages has been well-studied, the developmental relationship between cardiac lineages, including epicardium, and extraembryonic mesoderm remains to be defined.

OBJECTIVE: To explore the developmental relationships between cardiac and extraembryonic lineages.

METHODS AND RESULTS: Through high-resolution single-cell and genetic lineage/clonal analyses, we show an unsuspected clonal relationship between extraembryonic mesoderm and cardiac lineages. Single-cell transcriptomics and trajectory analyses uncovered 2 mesodermal progenitor sources contributing to left ventricular cardiomyocytes, 1 embryonic and the other with an extraembryonic gene expression signature. Additional lineage-tracing studies revealed that the extraembryonic-related progenitors reside at the embryonic/extraembryonic interface in gastrulating embryos and produce distinct cell types forming the pericardium, septum transversum, epicardium, dorsolateral regions of the left ventricle and atrioventricular canal myocardium, and extraembryonic mesoderm. Clonal analyses demonstrated that these progenitors are multipotent, giving rise to not only cardiomyocytes and serosal mesothelial cell types but also, remarkably, extraembryonic mesoderm.

CONCLUSIONS: Overall, our results reveal the location of previously unknown multipotent cardiovascular progenitors at the embryonic/extraembryonic interface and define the earliest embryonic origins of serosal mesothelial lineages, including the epicardium, which contributes fibroblasts and vascular support cells to the heart. The shared lineage relationship between embryonic cardiovascular lineages and extraembryonic mesoderm revealed by our studies underscores an underappreciated blurring of boundaries between embryonic and extraembryonic mesoderm. Our findings suggest unexpected underpinnings of the association between congenital heart disease and placental insufficiency anomalies and the potential utility of extraembryonic cells for generating cardiovascular cell types for heart repair.

GRAPHIC ABSTRACT: An online [graphic abstract](#) is available for this article.

Key Words: congenital heart defects ■ embryonic development ■ genomics ■ myocardium ■ stem cells

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A developmental field is a transient collection of cells with a shared potential to produce a restricted subset of embryonic structures, which is present only at specific developmental stages. Past studies of heart development have defined a first heart field (FHF) and second heart field (SHF), according to their potential to produce specific myocardial lineages within the developing heart.

The FHF and SHF were initially inferred by clonal analyses in the mouse embryo, which revealed two clonally distinct myocardial lineages, the first and second heart lineages.^{1–3} At E8.5, clones of the first heart lineage were observed to be excluded from the outflow tract and to populate the left ventricle (LV) and left atrioventricular canal (AVC), whereas clones of the second heart lineage

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Novelty and Significance

What Is Known?

- Two developmental heart fields, the first heart field and the second heart field, have been defined as having the potential to produce specific myocardial lineages within the developing heart; however, whether all myocardial progenitors within these lineages differentiate in a similar manner remains unknown.
- The second heart field has been shown to supply other cardiac cell types to the heart, pharyngeal arches, and head/neck including endothelial, endocardial, and smooth/skeletal muscle cells, but whether first heart field progenitors are similarly multipotent is unknown.
- The proepicardium produces epicardial cells that cover the heart, but the origins of the cells remain to be defined.

What New Information Does This Article Contribute?

- Hand1 (heart and neural crest-derived transcript-1)-expressing progenitors at the extraembryonic/intraembryonic boundary of the gastrulating embryo create a subset of first heart field cardiomyocytes that contribute to the dorsolateral regions of the left ventricle and atrioventricular canal.
- These Hand1-expressing progenitors are multipotent and give rise to not only cardiomyocytes but also serosal mesothelial lineages (including proepicardium/epicardium and pericardium) and extraembryonic mesoderm.

Mammalian organs, including the heart, have evolved a broad spectrum of specialized cell types that organize into intricate structures critical for their function. How early multipotent germ layer progenitors diversify to create these cell lineages, especially those deriving from mesoderm such as the heart, remains to be fully illuminated. In the following study, exploring the developmental cellular constituents during the ontogeny of mammalian mesoderm, we reveal an unexpected complexity of the contribution and multipotentiality of mesodermal progenitors to cardiac lineages creating specific cell types such as myocardial, proepicardial/epicardial, and pericardial cells in distinct regions of the heart.

Nonstandard Abbreviations and Acronyms

AVC	atrioventricular canal
CM	cardiomyocyte
CP	cardiac progenitor
DC	developing cardiomyocyte
FHF	first heart field
Hand1	heart and neural crest-derived transcript-1
LEM	late extraembryonic mesoderm
LPM	lateral plate mesoderm
LV	left ventricle
scRNA-seq	single-cell RNA sequencing
SHF	second heart field
ST	septum transversum
tSNE	t-distributed stochastic neighbor embedding

factor *Isl1*) around E7.75.⁴ SHF cells expressing *Isl1* will also supply multiple other cell types that contribute to the heart, pharyngeal arches, and head/neck including endothelial, endocardial, and smooth/skeletal muscle cells.^{4–6} At E7.5, the first differentiating cells in the cardiac crescent are marked by *Tbx5* and *Hcn4*. As *Tbx5-CreERT2* and *Hcn4-CreERT2* labeled cells in the crescent mainly contribute to cardiomyocyte lineages in the LV and parts of the atria, they are thought to represent more differentiated precursors of first heart lineage cardiomyocytes and the FHF at crescent stages.²⁷ Since these original studies, lineage studies, clonal analyses and single-cell studies have added to our knowledge of cardiac progenitors (CPs) and affirmed the presence of two heart fields.^{2,3,8} However, the location of distinct FHF and SHF progenitors in the early gastrulating embryo remains to be explored.

In addition to myocardial and endocardial lineages, the fully formed heart includes fibroblasts and vascular support cells derived from the epicardium. The proepicardium—a transient cluster of cells that forms at the base of the looping heart from the septum transversum (ST) during early heart development—produces cells that cover the heart to form the epicardium. Yet, the developmental origin of the proepicardium and its relationship to previously described heart fields have been elusive.

were found to be excluded from the LV.¹ Of the heart fields predicted by this model, the SHF has been visualized and defined as a population of cells medial to differentiating cardiomyocytes of the cardiac crescent that expresses the transcription factor *Isl1* (LIM homeodomain transcription

To address the developmental origins, of cell lineages creating the heart, we performed single-cell transcriptomic analyses on *Mesp1-cre; Rosa26-tdTomato* (*Rosa26-tdT*) mouse embryos across key developmental stages of cardiac development. Computational trajectory analyses of these data predicted a group of *Hand1* (heart and neural crest-derived transcript-1)-expressing progenitors that may create a subset of first heart lineage cardiomyocytes. In situ and lineage-tracing analyses utilizing *Hand1-CreERT2* revealed a *Hand1*-expressing population at the extraembryonic/intraembryonic boundary of the gastrulating embryo that supplies first heart lineage cardiomyocytes residing largely within dorso-lateral regions of the LV and AVC. Notably, the *Hand1-CreERT2* lineage created a subset of (rather than all) LV cardiomyocytes. As the second heart lineage does not populate the LV,¹ this finding implies an unexpected complexity of the FHF in which the FHF is not a single developmental heart field but rather composed of at least 2 distinct developmental sources, one of which, identified here, is marked by *Hand1*. Utilizing *Hand1-CreERT2* and *Rosa26-Confetti* clonal analyses, we furthermore discovered that this subset of FHF-derived cardiomyocytes derive from a common multipotent *Hand1*+ progenitor that also creates serosal mesothelial lineages (including proepicardium/epicardium and pericardium) and extra-embryonic mesoderm.

METHODS

Data Availability

Detailed Methods are provided in the [Data Supplement](#). Please see the Major Resources Table in the [Data Supplement](#). Raw data used in this study are available upon request. The raw single-cell RNA sequencing (scRNA-seq) data can be found in GEO (Gene Expression Omnibus Database) (GSE176306). Visualization of gene expression of the scRNA-seq is available on the University of California, Santa Cruz (UCSC) cell browser at <https://chi-10x-mouse-cardiomyocytes.cells.ucsc.edu>.

RESULTS

scRNA-Seq Analysis of *Mesp1* Lineage-Traced Cells Reveals Developmental Cell Types Participating in Mesoderm-Related Organogenesis

To track cell lineages contributing to the development of mesoderm-derived organs including the heart, we used a mouse *Mesp1-Cre⁹; Rosa26-tdTomato* (*R26R-tdT*)¹⁰ genetic fate mapping system (Figure 1A). Utilizing scRNA-seq, we interrogated the transcriptomes of *Mesp1-Cre; Rosa26-tdT* genetically labeled cells at E7.25 (no bud stage), E7.5 (early bud stage), E7.75 (late head fold stage), and E8.25 (somite stage; Figure 1A; Figure I in the [Data Supplement](#)). Unsupervised

k-means clustering¹¹ of these single-cell data revealed a broad array of cell types, which were identified based on gene expression (Figure 1B through 1F; Figures I and II in the [Data Supplement](#); Data Set I in the [Data Supplement](#)). As mesodermal progenitors differentiated into organ-specific cell types during mouse embryogenesis, we observed that the number of identified cell types increased with developmental age. For example, nascent, early extraembryonic, and hemangiogenic mesoderm cell types were detected at E7.25 as described¹²; however, many more intermediate and differentiated organ-specific cell types were identified by E7.75 and E8.25, including 2 cardiomyocyte clusters: developing cardiomyocytes (DCs) and cardiomyocytes (Figure 1B through 1E), which appear to represent developing and more established cardiomyocytes based on their differential expression of sarcomeric (*Tnnt2*, *Ttn*, and *Myl3*) and CP genes (*Tbx5*, *Sfrp1/5*, and *Meis1*; Figure 1F).

Trajectory Analysis Elucidates Developmental Pathways During Mesoderm Organogenesis

To illuminate the developmental origins and cell fate decisions of organ-specific cell types arising from mesodermal progenitor cells, we organized cells from our single-cell data along developmental trajectories using the lineage inference analysis, URD.¹³ These reconstructed developmental trajectories not only ordered cells by a pseudotime, which correlate with the developmental age of analyzed cells, but also revealed new and known developmental cell fate decisions (Figure 2A; Figure IIIA in the [Data Supplement](#)). In particular, we observed developmental trajectories that identified previously described mechanisms of development for some cell types including endothelial, blood, muscle, and cranial-pharyngeal cells^{6,14,15} (Figure 2A). On the other hand, examination of the cardiomyocyte developmental trajectory uncovered 2 potential developmental sources that may contribute to DCs: a known intraembryonic progenitor from the lateral plate mesoderm (LPM) and a previously undescribed CP from the late extraembryonic mesoderm (LEM; Figure 2A, box). A 3-dimensional force-directed URD representation revealed how these two progenitor sources originate and converge to independently contribute to DCs (Figure 2B; Figure IIIB in the [Data Supplement](#)). Consistent with these findings, a URD lineage inference analysis of previously published mouse embryonic scRNA-seq data¹² showed that analogous LPM and LEM cells could be identified forming similar developmental trajectories contributing to DCs (Figure IIIC through IIIE in the [Data Supplement](#)). Thus, our bioinformatic analyses support that cells with an extraembryonic signature (LEM) may contribute to the heart that is separate from that of the embryonic (LPM) lineage.

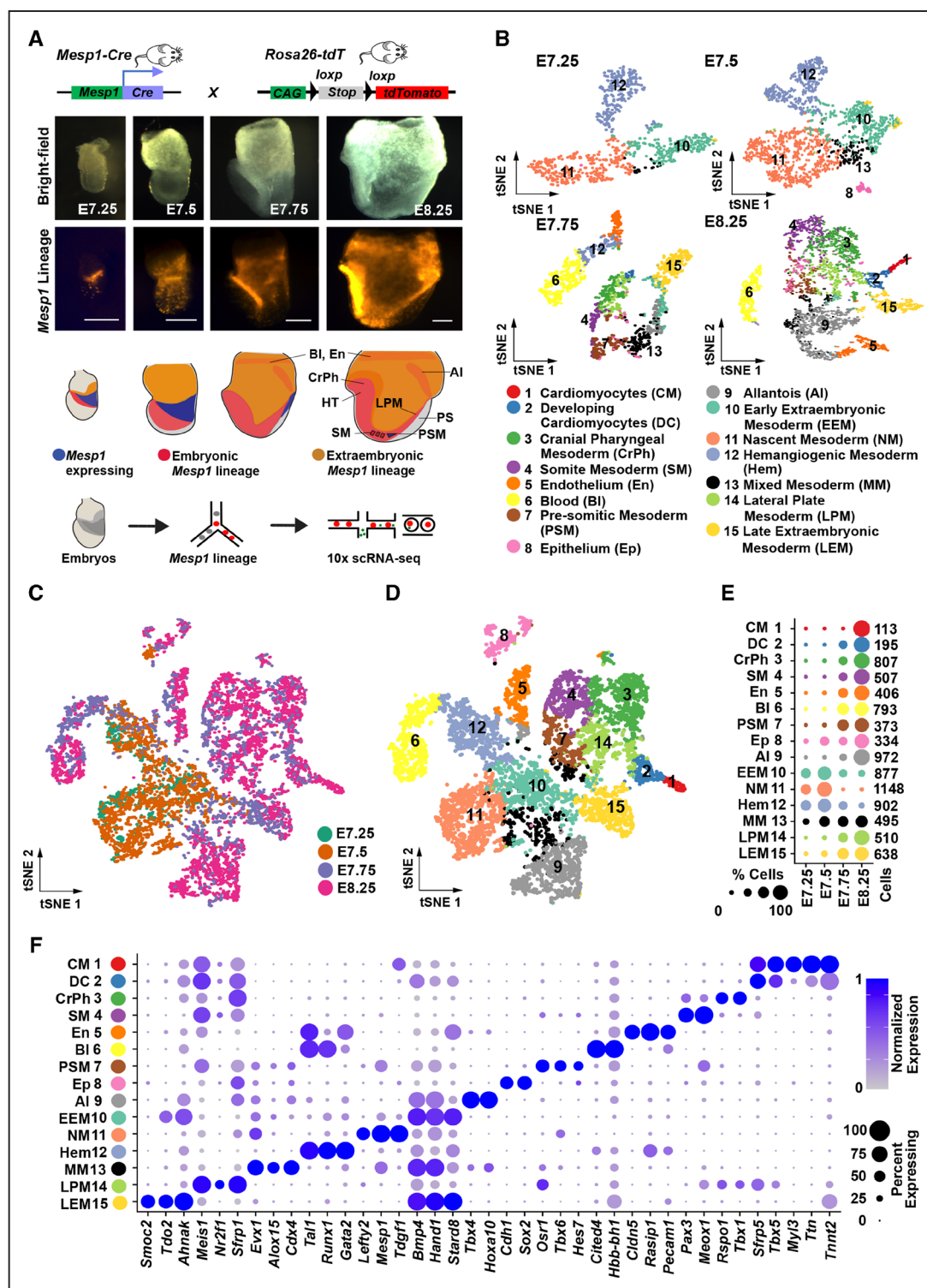


Figure 1. *Mesp1*-Cre single-cell maps reveal diverse cell types participating in early mouse mesoderm development.

A, *Mesp1*-Cre single-cell RNA sequencing (scRNA-seq) experimental design. *Mesp1*-Cre; *Rosa26-tdT* embryos were harvested for scRNA-seq at E7.25 (no bud stage), E7.5 (early bud stage), E7.75 (early head fold stage), and E8.25 (somite stage) as shown in representative bright-field and *Mesp1*-Cre; tdT+ (*Mesp1* lineage) micrographs. Illustration below these micrographs shows tissues genetically labeled by *Mesp1*-Cre in embryos and workflow for capturing these labeled single cells for RNA sequencing. Scale bars, 150 μ m. **B**, scRNA-seq data are displayed by tSNE (t-distributed stochastic neighbor embedding) plots at each developmental stage. Cells are colored according to their cell identities in **D**, **E**, and **F**. **C** and **D**, tSNE plot of scRNA-seq data across all examined stages displays individual cells (single dots) by **(C)** developmental stages or **(D)** cell types. **E**, Dot plot shows distribution of each cell type across different embryonic stages. **F**, Dot plot of key marker genes identifies each cell cluster. Al indicates allantois; Bl, blood; CM, cardiomyocyte; CrPh, cranial-pharyngeal mesoderm; DC, developing cardiomyocyte; EEM, early extraembryonic mesoderm; En, endothelium; Ep, epithelium; Hem, hemangiogenic mesoderm; HT, heart tube; LEM, late extraembryonic mesoderm; LPM, lateral plate mesoderm; MM, mixed mesoderm; NM, nascent mesoderm; PSM, presomitic mesoderm; PS, primitive streak; and SM, somite mesoderm.

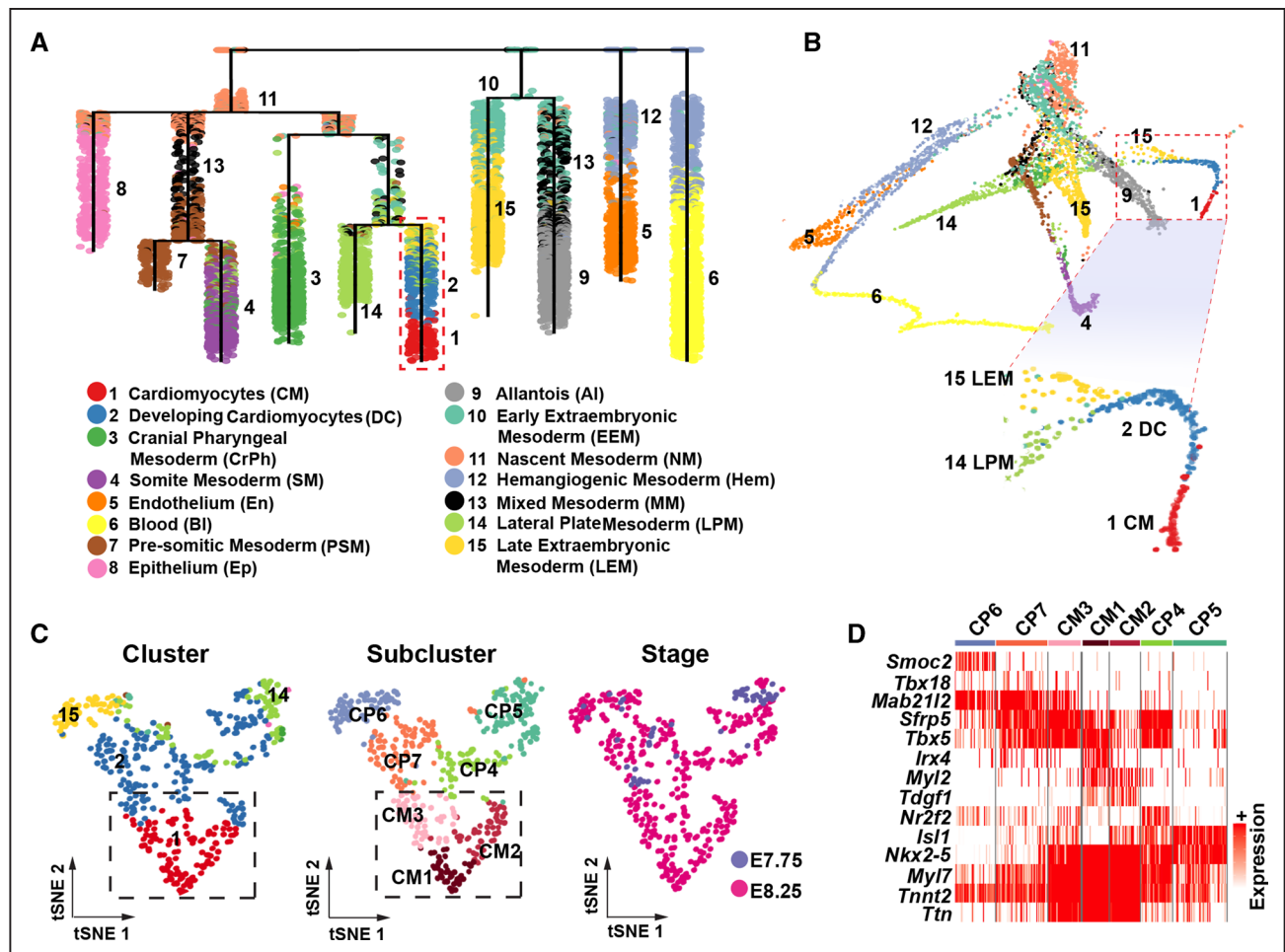


Figure 2. *Mesp1*-Cre single-cell RNA sequencing trajectory analysis reconstructs developmental cell lineage trees during mesoderm/heart organogenesis.

A and **B**, URD inferred lineage tree, as displayed by **(A)** dendrogram or **(B)** force-directed layout, reveals the developmental history of *Mesp1* mesoderm-derived organs. Red dashed box in **A** and **B** outlines cardiomyocyte (CM) branch, which is further magnified in **B**. The magnified CM branch shows that CMs may derive from both late extraembryonic mesoderm (LEM) and lateral plate mesoderm (LPM) progenitor cells. **C**, tSNE (t-distributed stochastic neighbor embedding) layout of cells from only the CM branch (boxed area in **A** and **B**) reveals 7 cardiac subclusters composing the CM branch including 3 distinct CM populations (CM1–3) and 4 specific cardiac progenitor cell types (CP4–7). **D**, Heat map of differentially expressed marker genes identifies each cardiac subcluster. AI indicates allantois; CrPh, cranial-pharyngeal mesoderm; DC, developing cardiomyocyte; EEM, early extraembryonic mesoderm; En, endothelium; Hem, hemangiogenic mesoderm; MM, mixed mesoderm; NM, nascent mesoderm; and SM, somite mesoderm.

Multiple Developmental Pathways Create Distinct Cardiomyocyte Populations

Previous studies have reported distinct populations of cardiomyocytes during heart development, which arise from specific heart fields.^{1–3} Thus, we investigated whether these cardiomyocyte populations could be detected as subclusters within our identified DC/cardiomyocyte clusters (Figure 1D) and how LPM and LEM cells in our cardiomyocyte trajectories may contribute to these subclusters (Figure 2B, magnification). Subclustering analysis of cells comprising the initial DC/cardiomyocyte branches (Figure 2A and 2B, boxed area, magnification: LEM, LPM, DC, and cardiomyocyte) uncovered 7 distinct subpopulations (Figure 2C and 2D; Figure IVA and IVB in the [Data Supplement](#); Data Set

II in the [Data Supplement](#)). Three of these subclusters exhibited increased expression of cardiomyocyte sarcomeric genes including *Ttn*, *Tnnt2*, and *Myl7* (Figure 2C and 2D, cardiomyocyte/CM1–3; Figure IVA, IVC, and IVE in the [Data Supplement](#)) and correlated with the cardiomyocyte cluster (Figure 2C, boxed area), whereas the other 4 subclusters displayed relatively low expression of these sarcomeric genes but high expression of CP markers including *Isl1*, *Sfrp5*, and *Tbx5* (Figure 2C and 2D, CP/CP4–7; Figure IVA, IVD, and IVE in the [Data Supplement](#)) and associated closely with the DC cluster and specific portions of LPM and LEM clusters (Figure 2C, unboxed area). Differential gene marker analyses of the cardiomyocyte subclusters revealed that CM1, CM2, and CM3 cells displayed a combinatorial enrichment of *Irx4/Tbx5*, *TdGF1/Isl1*, and *Mab21l2/Tbx5*, respectively, and

that CM1 cells exhibited increased expression of mature cardiomyocyte gene markers including *Actc1*, *Actn2*, *Myh6*, *Myh7*, and *Myl1* (Figure IVA and IVF in the [Data Supplement](#)). These findings indicate that CM1 and CM2 subclusters may represent cardiomyocytes arising from the FHF and SHF,^{2–4,7,16,17} whereas the developmental source of the CM3 subcluster remains to be identified. Additional gene marker analyses of CP subclusters support that cell types from these subclusters represent CPs for cardiomyocytes and potentially other differentiated cardiac cell types (Figure 2D; Figure IVA, IVD, and IVE in the [Data Supplement](#)). For instance, CP6 and CP7 expressed genes overlapping with those in CM3 and proepicardial cells (*Upk3b*, *Ccbe1*, *Sfrp5*, *Mab21l2*, and *Tbx18*^{18–23}; Figure IVA in the [Data Supplement](#)), suggesting that CP6/CP7 subclusters contain progenitors for CM3 and proepicardial cells. Additionally, CP5 and a small region of CP4 are enriched for genes expressed within the anterior and posterior SHF including *Isl1*, *Fgf10*, *Hoxb1*, *Hoxa1*, and *Osr1* (Figures IVA, IVD, IVE, and V in the [Data Supplement](#)).

To confirm the identity of subcluster cell types and investigate their relationship during embryogenesis, we spatially mapped these cell types in E8.25 embryos using RNAscope in situ hybridization analysis of markers that are specific to these subclusters (Figure VI in the [Data Supplement](#)). *Irx4*, *Tdgf1*, and *Mab21l2*, markers of CM1, CM2, and CM3 subclusters, were expressed in distinct regions of the heart tube as labeled by *Myl7* and *Nkx2-5*: the middle segment (primitive LV), arterial pole (primitive outflow tract and right ventricle), and venous pole of the heart tube (Figure VIA and VIB in the [Data Supplement](#)), indicating that CM1 and CM2 cells correspond to cardiomyocytes derived from the FHF and SHF,^{2–4,7,16,17} whereas the source of progenitors giving rise to CM3 cardiomyocytes remains to be determined. Using differentially expressed genes in the CP subclusters, we investigated the location of CP subcluster cell types during embryogenesis (Figure 2D; Figures IVA, IVD, IVE, and VIC through VIK in the [Data Supplement](#)) and discovered that CP4 markers *Sfrp5*, *Nr2f2*, and *Arg1* were coexpressed in regions posterior to the venous pole and contiguous with CM1 (Figure VIC, VIE, and VIJ in the [Data Supplement](#)). The CP5 marker *Isl1* was enriched in regions anterior and dorsal to the arterial pole and contiguous with CM2 (Figure VID and VIF in the [Data Supplement](#)). The combined CP6 markers *Smoc2* and *Mab21l2* were expressed at the interface between the forming heart and extraembryonic tissues, near the ventral venous pole, and contiguous with CM3 (Figure VIH in the [Data Supplement](#)), and the CP7 markers *Twist1*, *Sfrp5*, and *Mab21l2* were located in regions connected to the ventral side of the venous pole and contiguous with CP6 (Figure VIE, VIG, and VII in the [Data Supplement](#)). The adjacent locations of CP6, CP7, and CM3 and overlapping genes between them (*Cpa2*,

Mab21l2, *Bmp4*, and *Hand1*; Figures IVA, IVD, and VI in the [Data Supplement](#)), suggest that these cell types may be developmentally related.

We further investigated the developmental relationship of the CM1–3 subpopulations and how LPM and LEM progenitors contribute to them. Using the three cardiomyocyte subcluster populations CM1–3 (Figure 2C) as end points for URD trajectory analysis,¹³ we reconstructed our developmental trajectories (Figure 3A and 3B; Figure VII in the [Data Supplement](#)), which created 3 new cardiomyocyte trajectory branches (Figure 3A and 3B, box). The CM1 and CM2 trajectory branches, whose cells expressed genes associated with FHF (*Tbx5*) and SHF (*Isl1/Tdgf1*), respectively (Figure 2D; Figure IVA, IVC, and IVE in the [Data Supplement](#)), shared a common intraembryonic cellular origin associated with LPM and nascent mesoderm cells, whereas the CM3 lineage branch was distinct from the CM1 and CM2 branches and shared an origin with early extraembryonic mesoderm and LEM cells (Figure 3A and 3B; Figure VII in the [Data Supplement](#)). The CM2 and cranial-pharyngeal branches expressed the SHF marker, *Isl1*, and appeared along a developmentally related trajectory consistent with previous studies of SHF development (Figure 3A through 3C).^{4–6}

Interrogating Transcriptional Profiles of CM1–3 Lineage Branches Uncovers Distinct Cell Fate Programs for Each Cardiomyocyte Population

To identify gene programs that regulate the cell fate decisions creating these distinct cardiomyocyte lineages, we interrogated transcriptional profiles of cells along each of the cardiomyocyte developmental trajectories. We analyzed differentially expressed genes, including transcription factors, at each branch point, and found that *Hand1* appeared important for the branch-point decision between embryonic nascent mesoderm and early extraembryonic mesoderm (branch point 1; Figure 3C; Figure VIIIA through VIIIC in the [Data Supplement](#)), which coincides with *Hand1*'s role in extraembryonic mesoderm development.^{24,25} Supporting previous cardiac developmental studies,^{24,26,27} the transcription factors *Tbx5*, *Isl1*, *Hand2* (Heart- and neural crest derivatives-expressed protein 2), and *Tbx1* (T-box transcription factor 1) exhibited differential expression in FHF-related CM1, SHF-related CM2, and SHF-related cranial-pharyngeal cell types at branch point 3 (Figure 3C; Figure VIIIA, VIIIB, and VIIIE in the [Data Supplement](#); Data Set III in the [Data Supplement](#)). In the extraembryonic branch, *Cdx2/Cdx4* and *Tsc22d1* were expressed at branch point 2 where allantois and LEM cells arise from early extraembryonic mesoderm cells (Figure VIIIA, VIIIB, and VIIID in the [Data Supplement](#); Data Set III in the [Data Supplement](#)). *Mef2c*, *Id2*, and *Cited2* in CM3 cells,^{28–30} and *Hoxb6* and *Hand1* in LEM cells, were predicted to regulate cell fate decisions

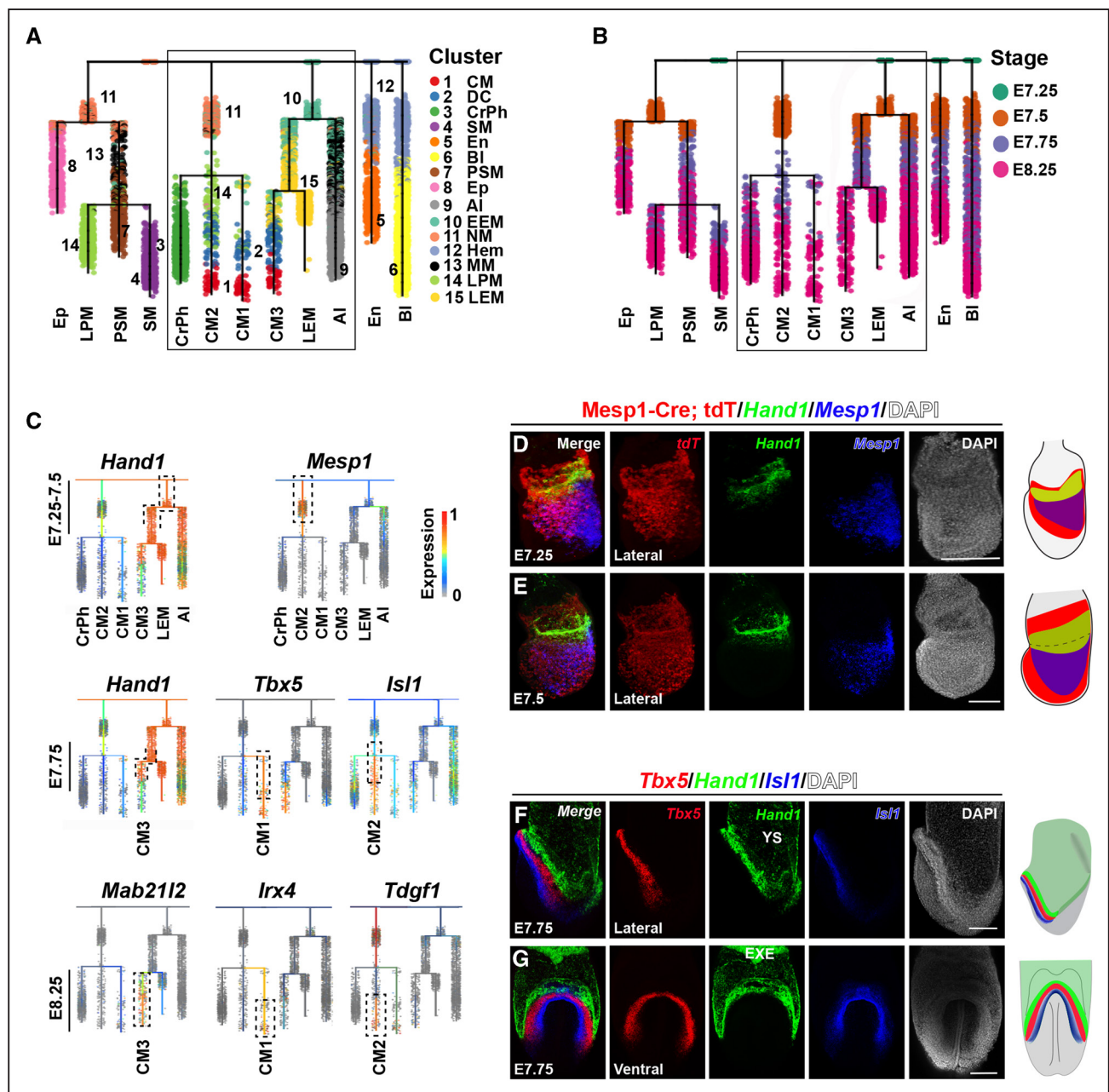


Figure 3. Distinct cardiomyocyte lineages derive from intraembryonic- and extraembryonic-related developmental origins.

A and **B**, Reconstructed URD developmental cell lineage trees using the 3 distinct subclustered cardiomyocyte (CM) populations predict that CM1/CM2 and CM3 CMs derive, respectively, from intraembryonic- and extraembryonic-related progenitor sources, as displayed by **(A)** cell type and **(B)** developmental stages. The CM-related branches of the URD developmental tree are outlined with box. **C**, Marker genes differentially expressed among the lineages for each CM subcluster are plotted on the URD CM-related branches. *Hand1* (heart and neural crest-derived transcript-1) and *Mab21l2* mark early and late regions of the CM3 lineage, respectively. *Mesp1*, *Tbx5*, *Isl1* (LIM homeodomain transcription factor Islet1), *Irx4*, and *Tdgf1* label different regions of the CM1 and CM2 lineage branches. **D** and **E**, RNAscope in situ hybridization (ISH) of *Mesp1* and *Hand1* was performed in **(D)** E7.25 and **(E)** E7.5 *Mesp1*-Cre; *Rosa26*-tdT embryos. The diagram illustrates both the gene expression pattern of *Hand1* and *Mesp1* and *Mesp1*-Cre lineage-traced cells in these embryos. **F** and **G**, RNAscope ISH of *Hand1*, *Tbx5*, and *Isl1* was performed in E7.75 embryos. The diagram illustrates the expression pattern of *Hand1*, *Tbx5*, and *Isl1* in these embryos. n=3 per panel. Scale bars, 100 μ m. AI indicates allantois; BI, blood; CrPh, cranial-pharyngeal mesoderm; DC, developing cardiomyocyte; DAPI indicates 4',6-diamidino-2-phenylindole; EEM, early extraembryonic mesoderm; En, endothelium; Ep, epithelium; EXE, extraembryonic ectoderm; Hem, hemangiogenic mesoderm; LEM, late extraembryonic mesoderm; LPM, lateral plate mesoderm; MM, mixed mesoderm; NM, nascent mesoderm; PSM, presomitic mesoderm; SM, somite mesoderm; and YS, Yolk Sac.

at branch point 4 (Figure VIIIA, VIIIB, and VIIIF in the [Data Supplement](#); Data Set III in the [Data Supplement](#)).

To illuminate the dynamics of cell fate choices in these cardiomyocyte lineages, we examined genes

differentially expressed in each cardiomyocyte lineage trajectory along a pseudotime, which revealed 3 major differentiation states for each trajectory: an early state, intermediate state, and late state (Figure IX in the [Data](#)

Supplement). Consistent with our branch-point analyses (Figure VIII in the [Data Supplement](#)), CM1 and CM2 early-state genes were similar to each other but distinct from CM3 early-state genes; however, genes across these pseudotime analyses converged as each intermediate-state CP differentiated into its corresponding late-state cardiomyocyte population (Figure IX in the [Data Supplement](#)). Specifically, *Mesp1* was expressed in the CM1 and CM2 early states, but *Tbx5* and *Isl1* were activated in these lineages at intermediate states (Figure 3C; Figure IXA, IXB, IXD, IXE, IXG, and IXH in the [Data Supplement](#)), suggesting that CM1 and CM2 may derive from a common developmental trajectory but *Tbx5* and *Isl1* may direct their specification in more distinct cardiomyocyte populations. In contrast, *Hand1* and BMP signaling-related genes *Bmp4* and *Msx2* were expressed in CM3 early states, and *Mab21l2* and *Cpa2* were activated in CM3 intermediate states (Figure 3C; Figure IXC, IXF, and IXI in the [Data Supplement](#)). Finally, *Mef2c* and sarcomeric genes were expressed at CM1-3 late stages; however, some genes appeared specific for each cardiomyocyte population including *Irx4* (CM1) and *Tdgf1* (CM2; Figure IXD through IXI in the [Data Supplement](#)). Confirming these analyses, RNAscope in situ hybridization revealed that *Hand1* was expressed at the embryonic and extraembryonic boundary, whereas *Mesp1* was expressed in the intraembryonic migrating mesoderm at E7.25 and E7.5 (Figure 3D and 3E). Furthermore, *Hand1*, *Tbx5*, and *Isl1* marked different locations in the crescent region at E7.75 where *Hand1* labeled regions anterolateral to the cardiac crescent, which was marked by *Tbx5*², whereas *Isl1* labeled regions posteromedial to the cardiac crescent (Figure 3F and 3G; Figure X in the [Data Supplement](#)). These bioinformatic and spatial gene expression analyses reveal a potentially unexplored developmental source of cardiomyocytes along the proximal extraembryonic/embryonic boundary that appears distinct from FHF and SHF progenitors.

Hand1 Lineage Tracing Reveals an Unexpected Heart Field That Contributes to Specific Subsets of the First Heart Lineage and Serosal Mesothelial Lineages

To examine this predicted extraembryonic-related heart field, we used an inducible Cre recombinase fate mapping strategy to lineage trace cells from this potential heart field. Because *Hand1* was expressed in early extraembryonic-related CM3 progenitors but not CM1 and CM2 progenitors, we examined whether *Hand1* could be used to genetically label progenitors from the CM3 heart field (Figure 3C). To explore this possibility, we performed RNAscope in situ hybridization analyses to examine *Hand1* expression in developing embryos. At E6.75, *Hand1*+ cells coexpressed *Mesp1* at the extraembryonic/embryonic boundary (Figure XIA in the [Data Supplement](#))

but downregulated *Mesp1* after E6.75 (Figure 3D and 3E). *Hand1* was expressed in the extraembryonic mesoderm at E7.75, E8.25, and pericardium at E8.25 but not in *Hcn4*+ or *Myl7*+ cardiomyocytes (Figure XIB through XIE in the [Data Supplement](#)). However, at E8.5 and E9.0, *Hand1* was expressed in a portion of cardiomyocytes in the LV and AVC, as well as the pericardium and ST (Figure XIF and XIG in the [Data Supplement](#)).

Based on these *Hand1* expression studies, which reveal a developmental time window (E6.25–E8.25) in which the contributions of early gastrulating *Hand1*+ CM3 progenitors to the heart can be investigated, we generated a *Hand1-CreERT2* mouse that was confirmed by RNAscope in situ hybridization of *CreERT2* and analysis of *Hand1-CreERT2*; *Rosa26-tdT* embryos with and without tamoxifen induction (Figure 4A and 4B; Figure XII in the [Data Supplement](#)). Because the half-life of tamoxifen in mice is ≈ 12 hours and persists over an ≈ 24 - to 36-hour time period³¹ (Figure XIII in the [Data Supplement](#)), we studied *Hand1-CreERT2*; *Rosa26-tdT* embryos from pregnant mice given tamoxifen at E5.75 (Figure 4B) to avoid *CreERT2* activation in differentiated cardiomyocytes expressing *Hand1* at E8.5. Consistent with our cardiomyocyte trajectory branches (Figure 3C), examining these embryos at E7.75, E8.25, E8.5, E9.5, and E12.5 revealed that *Hand1* lineage-traced cells contributed to the heart and extraembryonic tissues (Figure 4C through 4F; Figure XIV in the [Data Supplement](#)). Within extraembryonic tissues including the yolk sac (Figure 4C through 4E; Figure XIB, XIVE, and XIVF in the [Data Supplement](#)), *Hand1* lineage-traced tdT+ cells produced *Pecam*+ endothelial cells, α -SMA (Alpha Smooth Muscle Actin) + smooth muscle cells, and *Pdgfr β* (platelet-derived growth factor receptor beta)+ mesothelial cells (Figure XIVE and XIVF in the [Data Supplement](#)). In the developing embryo, *Hand1* lineage-traced tdT+ cells contributed in a more spatiotemporal restricted manner (Figure 4C through 4F). Specifically, *Hand1* lineage-traced tdT+ cells supplied *Hcn4*+ cardiomyocytes in the cardiac crescent at E7.75 and then cardiomyocytes (*Myl7*+) on the ventral side of the venous pole and medial regions of the heart tube at E8.25 (Figure 4C and 4D). Between E8.5 and E12.5, tdT+ cardiomyocytes were restricted spatially to the primitive AVC region and LV at E8.5 and then further to the AVC/sinus venosus, dorsolateral LV, and atrial regions of E12.5 hearts (Figure 4E and 4F; Figure XIB and XIBD in the [Data Supplement](#)). Furthermore, tdT+ cells appeared in nonmyocardial heart tissue including the pericardium, proepicardium/ST, epicardium, and occasionally endocardium from E8.25 to E12.5 (Figure 4D through 4F; Figure XIVA through XIVD in the [Data Supplement](#)). Supporting these lineage studies, the CM3 URD trajectory, including CP6 and CP7 subclusters, comprised cells that express AVC (*Msx1/2*, *Twist1*, and *Tbx2*^{23, 32}) and proepicardial/pericardial markers

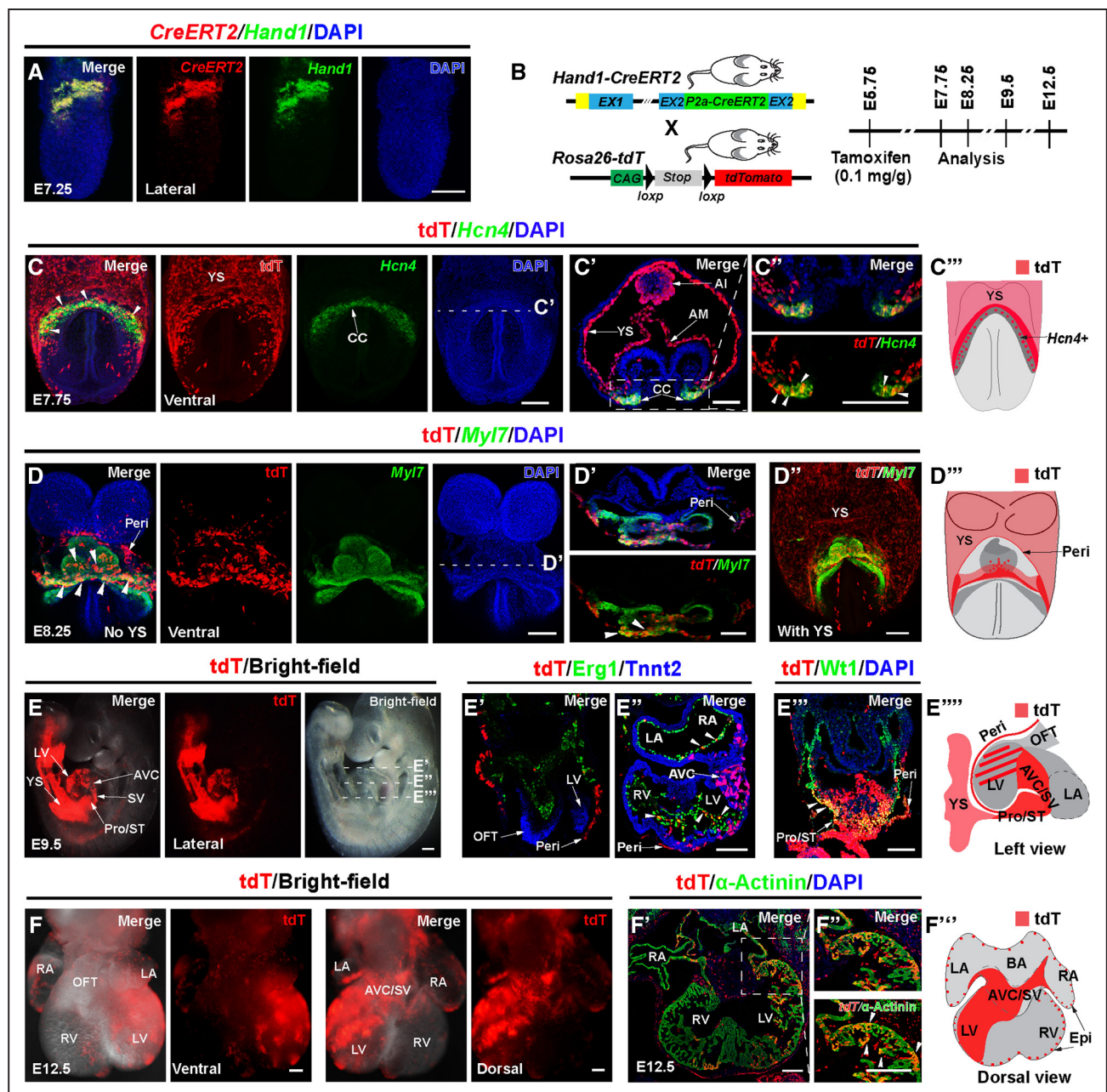


Figure 4. Lineage-tracing studies reveal that early gastrulating *Hand1*+ cells contribute to not only a distinct subpopulation of first heart lineage cardiomyocytes (CMs) but also serosal mesothelial lineages (pericardial, epicardial cells) in the heart.

A, RNAscope in situ hybridization (ISH) in *Hand1* (heart and neural crest-derived transcript-1)-*CreERT2* embryos shows that expression of *CreERT2* precisely recapitulates the expression of *Hand1*. **B**, Lineage-tracing studies using *Hand1-CreERT2* and *Rosa26-tdT* mice (shown in left) map the fate of early gastrulating *Hand1*+ cells. Schematic outlines the experimental strategy for *Hand1-CreERT2* genetic fate mapping studies shown in right. Tamoxifen was given at E5.75, and embryos were examined for *Hand1-CreERT2* genetically labeled tdT+ cells at E7.75, E8.25, E9.5, and E12.5. **C-F**, RNAscope ISH and immunohistochemistry of whole mount and cross sections of these embryos reveal the contribution of *Hand1-CreERT2* genetically labeled tdT+ cells at (**C**) E7.75, (**D**) E8.25, (**E**) E9.5, and (**F**) E12.5. **C', D', E', E'', and E'''**, Insets show transverse sections of **C**, **D**, and **E** at indicated dashed lines, respectively. **F'**, Inset shows coronal section of **F**. **C'' and F''**, Insets are magnification of **C'** and **F'** boxed area. Arrowheads point to tdT+ cells expressing (**C** and **C''**) *Hcn4*, (**D** and **D'**) *Myl7*, (**E''**) *Erg1*, (**E'''**) *Wt1*, and (**F''**) α -actinin (**C'''**, **D'''**, **E'''**, and **F'''**). Diagrams summarize the anatomic location of *Hand1-CreERT2* genetically labeled tdT+ cells at the embryonic stages analyzed. n=3 embryos for each stage. Scale bars, 100 μ m. AI indicates allantois; AM, amnion; AVC, atrioventricular canal; BA, base of the atrium; CC, cardiac crescent; CPC, cardiac progenitor cell; DAPI, 4',6-diamidino-2-phenylindole; Epi, epicardium; FHF, first heart field; LA, left atrium; LV, left ventricle; OFT, outflow tract; Peri, pericardium; Pro, proepicardium; RA, right atrium; RV, right ventricle; SHF, second heart field; ST, septum transversum; SV, sinus venosus; and YS, yolk sac.

(*Upk3b*, *Ccbe1*, *Sfrp5*, *Mab21l2*, and *Tbx18*¹⁸⁻²³; Figure IVA in the Data Supplement), while the lateral location

of E8.25 tdT+ cells coincides with *Mab21l2* expression (Figure VIH in the Data Supplement) and the previously reported location of *Tbx18*+ venous pole progenitors.³³

To confirm these findings, we examined *Hand1-CreERT2; Rosa26-tdT* embryos from pregnant mice given tamoxifen at E6.25 (Figure XV in the [Data Supplement](#)). In addition to recapitulating results in E5.75 tamoxifen-induced *Hand1-CreERT2; Rosa26-tdT* embryos (Figure XVA through XVF in the [Data Supplement](#), compared with Figure 4C through 4F), *Hand1* tdT+ cells genetically labeled at E6.25 also contributed cardiomyocytes and epicardial-derived cell types including fibroblasts and vascular support cells to E17.5 embryos (Figure XVG in the [Data Supplement](#)). Altogether, these data suggest that during early gastrulation, *Hand1* marks a progenitor population that produces not only cardiomyocytes within the AVC and LV before *Hand1* is expressed in differentiated cardiomyocytes but also pericardial, epicardial, and extraembryonic-derived mesoderm cell types. As these *Hand1*+ cardiomyocyte progenitors contribute to cardiomyocytes within the developing AVC and dorsolateral LV, they likely represent a distinct subset of the reported first heart lineage cardiomyocytes,¹ suggesting that FHF is not a single heart field, but is rather composed of at least 2 distinct sources, including a *Hand1*-derived source.

Genetic Clonal Analysis Reveals the Multipotentiality of *Hand1*+ Cardiac Precursor Cells

To examine the lineage potential of single *Hand1*-expressing cells during early gastrulation, we crossed *Hand1-CreERT2* mice with *Rosa26-Confetti* multicolor reporter mice³⁴ to genetically fate map early *Hand1*+ individual clones expressing a specific fluorescent protein (Figure 5A). Tamoxifen doses were initially titrated to ensure low levels of *Hand1*+ labeling (Figure 5B). The observed frequency of each confetti color upon addition of tamoxifen was consistent with those reported previously³ (Figure XVIB in the [Data Supplement](#)). At the lowest dose of tamoxifen (0.0025 mg/g) given at E6.75, only 8.6% of exposed embryos (n=73 of 849) were labeled, which was less than the expected *Hand1-CreERT2; Rosa26-confetti* genotype positivity rate (50%; Figure 5B). At this dose, unicolor embryos occurred in the highest proportion followed by bicolor and tricolor embryos (Figure XVID in the [Data Supplement](#)). To reduce the probability of observing a multirecombinant clone, only the unicolor embryos were further analyzed. At E9.5, the majority of the clones (n=23) from the unicolor embryos were present in only the extraembryonic tissue. However, 12 clones did fluorescently label both extraembryonic and cardiac tissues including the proepicardium/ST, pericardium, LV, and AVC (Figure 5C and 5D), which was consistent with the distribution of genetically labeled *Hand1-CreERT2; Rosa26-tdT* cells at a similar stage, thus supporting the multipotentiality of these clones (Figure 4E, compared with Figure 5C and 5D). To confirm this observation on a larger sample size,

clones induced with a higher dose of tamoxifen (0.005 mg/g) given at E6.25 or E7.25 were analyzed (Figure XVIA and XVIIE in the [Data Supplement](#)). We used a rigorous statistical analysis to reduce the chance of analyzing clones generated from multirecombinant events at this higher dose. The number of cells in each clone (an individual color) that contributes to cardiac tissues or to both extraembryonic and cardiac tissues was used to create a model with a mixture of 2 gaussian distributions: one for cell counts that would be expected for a single recombination event and the other for cell counts that would be expected for ≥ 2 recombination events (Figure XVIIIP and XVIIIR in the [Data Supplement](#)). Based on this model, we excluded 35 (of 88) clones, which likely derive from >1 progenitor (Figure XVIIQ and XVIIIS in the [Data Supplement](#)). The majority of these remaining 53 clones analyzed contributed to 2 or 3 distinct lineages, including combinations of extraembryonic mesoderm, pericardium, proepicardium/ST, and AVC or LV myocardium (Figure XVIIQ and XVIIIS in the [Data Supplement](#)), thus supporting the multipotentiality of *Hand1*+ progenitors. Immunofluorescence staining shows that these *Hand1*+ clones contributed to not only α -actinin+ cardiomyocytes in the AVC and LV but also *Wt1*+ proepicardial and pericardial cells; however, *Hand1*+ clones were not observed in the endocardium (Figure XVIIH through XVIIK in the [Data Supplement](#)). Finally, *Hand1*+ clones supplied α -SMA+ smooth muscle cells and *Pdgfr β* + mesothelial cells to the extraembryonic mesoderm (Figure XVIIJ and XVIIIM in the [Data Supplement](#)).

To investigate the clonal relationships among *Hand1*+ progenitor-derived cardiac cell types and their location in later stage hearts, we also examined E6.75 or E7.25 tamoxifen-induced *Hand1-CreERT2; Rosa26-Confetti* clones at E12.5 when most cardiac structures and cell types have been determined (Figure XVIIF in the [Data Supplement](#)). In unicolor embryos, we identified clones marking the epicardium and cardiomyocytes in the AVC or LV (Figure 5E through 5H; Figure XVIIIN and XVIIIO in the [Data Supplement](#)), supporting that multipotential *Hand1*+ CP cells can give rise to both cardiomyocytes and noncardiomyocytes cardiac cells. Altogether, these results reveal multipotential *Hand1* CPs in the early ingressing mesoderm that can create extraembryonic mesoderm, mesothelial cardiac lineages (epicardium and pericardium), and LV and AVC myocardium (Figure 5I).

DISCUSSION

Our single-cell transcriptomic, genetic fate mapping, and clonal analyses reveal not only a developmental cardiac-extraembryonic tissue connection but also delineate the progenitors creating these lineages and their specific contributions to the developing heart and extraembryonic structures (Figure 5I). Highlighting the complexity of organogenesis, we show how similar cell

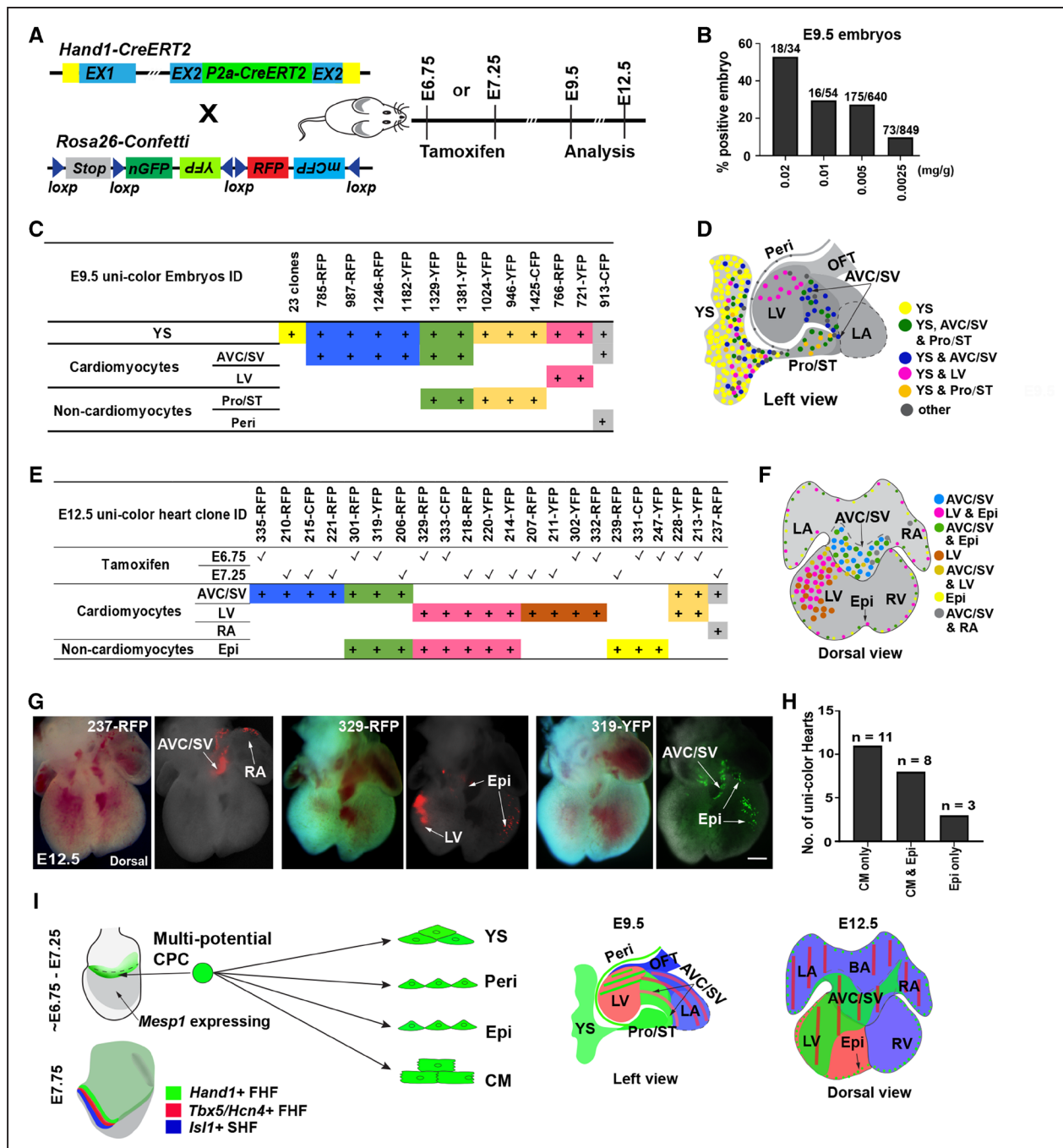


Figure 5. Clonal analysis reveals multipotentiality in early *Hand1*+ progenitors.

A, Schematic outlines experimental strategy for *Hand1-CreERT2*; *Rosa26-Confetti* clonal analyses. **B**, Bar graph reveals the percentage of E9.5 embryos that displayed fluorescence at titrated doses of tamoxifen. The numerator is the number of fluorescence-positive embryos, and the denominator is the number of total embryos examined. **C**, Clonal analyses of unicolor E9.5 embryos reveal that individual *Hand1-CreERT2*; *Rosa26-Confetti* clones labeled at E6.75 (0.0025 mg/g tamoxifen) have the capacity to generate multiple cell types that can contribute to the yolk sac and heart. **D**, Diagram summarizes the contribution of *Hand1-CreERT2*; *Rosa26-Confetti* genetically labeled clones in the heart and yolk sac at E9.5. **E**, Clonal analyses of unicolor E12.5 hearts reveal that individual *Hand1-CreERT2*; *Rosa26-Confetti* clones labeled at E6.75 or E7.25 (0.005 mg/g tamoxifen) have the capacity to generate multiple cell types within the heart. **F**, Diagram summarizes the contribution of *Hand1-CreERT2*; *Rosa26-Confetti* genetically labeled clones in the heart at E12.5. **G**, Representative E12.5 unicolor hearts show individual *Hand1-CreERT2*; *Rosa26-Confetti* genetically labeled clones contributing to different combinations of tissues and cell types: atrioventricular canal (AVC)/sinus venosus (SV) and right atrium (RA) (clone No. 237-RFP); left ventricle (LV) and epicardium (Epi) (clone No. 329-RFP); the AVC/SV and Epi (clone No. 319-YFP). Scale bars, 200 μ m. **H**, Bar graph displays the number of unicolor E12.5 hearts with clones contributing to cardiomyocytes (CMs) only, epicardial cells and CMs, or only epicardial cells. **I**, Model summarizes the multipotentiality of *Hand1*+ cardiac progenitor cells (CPCs) between E6.75 and E7.25 in relation to the contribution of reported first heart field (FHF)/second heart field (SHF) progenitors. AVC indicates atrioventricular canal; BA, base of the atrium; LA, left atrium; LV, left ventricle; OFT, outflow tract; Peri, pericardium; Pro, proepicardium; RV, right ventricle; RFP, red fluorescent protein; ST, septum transversum; SV, sinus venosus; YFP, yellow fluorescent protein; and YS, yolk sac.

types, such as cardiomyocytes, can derive from multiple developmental origins/progenitors that have potential to produce not only other cell types but also multiple organs/tissue structures. Single-cell subcluster analyses of isolated cardiomyocytes identified at least 3 distinct myocardial lineages including a heart lineage whose progenitor shares a gene signature with extraembryonic mesodermal progenitors including *Hand1*. Trajectory analyses predicted that 2 of these heart lineages derive from a common embryonic source before E7.25, with marker expression at E8.25 suggesting their correspondence to first and second heart lineages, whereas the *Hand1*+ extraembryonic-related heart lineage originates from a distinct developmental source. Expression analyses revealed that at early gastrula stages, *Hand1*+ progenitors reside at the intra/extraembryonic boundary, with genetic fate mapping demonstrating that *Hand1*+ progenitors create cardiomyocytes localized to the dorsal regions of the LV and AVC at E12.5. As LV cardiomyocytes have been shown to derive from the FHF,¹ our results support that this *Hand1*+ CP field represents a distinct subset of the FHF, suggesting that the FHF consists of at least 2 distinct progenitor sources. This finding is supported by the complementary nature of the *Hand1* lineage to previous studies examining the contribution of the lineages labeled by *Tbx5-CreERT2*, *Mef2c-AHF-Cre*, and *SMA-CreERT2*.^{2,35,36} Notably, these *Hand1*+ FHF subpopulation findings are also consistent with a mathematically inferred retrospective myocardial clonal analyses¹ that predicted 2 separate FHF lineages.

One limitation for understanding the lineage potential of the FHF or SHF from previous retrospective clonal studies¹ is that only myocardial clones were studied. However, when *Isl1* was identified as an SHF marker, studies with *Isl1-Cre* or inducible *Isl1-CreERT2* revealed that SHF produces both myocardial lineages and multiple other cardiac lineages.^{4,37} Utilizing *Hand1-CreERT2* with a confetti clonal indicator,³⁴ we uncovered an unsuspected multipotentiality of *Hand1* mesodermal cells, which produce not only a specific subset of FHF-derived LV cardiomyocytes but also extraembryonic mesoderm, ST/epicardial, and pericardial cells. Although previous findings suggest that *Mesp1*+ clones may contribute to LV and epicardial cell types,³ our results reveal a closer lineage relationship between cardiomyocytes of the AVC and dorsolateral LV, and extraembryonic mesodermal and serosal mesothelial cells than expected previously, and furthermore expand upon previous studies³⁸ investigating the elusive origins of the proepicardium/epicardium.

The existence of a *Hand1*+ progenitor that supplies cells to extraembryonic and intraembryonic tissues provides further examples of blurred boundaries between extra/intraembryonic tissues.³⁹ These findings may also account for previous observations that epicardial

progenitors can adopt cardiomyocyte fates⁴⁰ and that loss of *Scf* can result in transdifferentiation of yolk sac hematopoietic cells to beating cardiomyocytes.⁴¹ However, whether extraembryonic mesoderm cells can differentiate into cardiomyocytes in vivo as suggested by our scRNA-seq studies remains to be further examined, but the close developmental relationship between mesothelial lineages of both extraembryonic and intraembryonic tissues and *Hand1*+ cardiac lineages, coupled to the high plasticity of mesothelial cells,⁴² suggests the possibility of transforming extraembryonic and serosal mesothelial tissues into cardiomyocytes to treat heart failure.

The role of *Hand1* in early specification of LV cardiomyocytes and FHF progenitors will be of interest to examine in future studies. Global *Hand1* knockout embryos exhibit placental, yolk sac, and heart defects,^{24,25} resulting in embryonic lethality at E8.5. As placenta and yolk sac defects can secondarily impact the heart, direct requirements for *Hand1* in early heart progenitors remain unclear. Although experiments, including cardiac-specific conditional knockout and tetraploid rescue studies,^{24,25} confirmed heart defects in *Hand1* mutant embryos, these studies could not rule out requirements for *Hand1* in differentiated cardiomyocytes, rather than undifferentiated progenitors. Our findings suggest that these heart defects may be due to abnormalities in undifferentiated progenitors, which can produce cardiomyocytes. Thus, future studies utilizing scRNA-seq of *Hand1* knockout embryos will be important to investigate whether *Hand1* is important for the early stages of CM3 differentiation and the branch-point decisions between embryonic and extraembryonic mesoderm. Additionally, due to the limited number of cells examined at the earliest stages in our study, interrogating these early decisions will also require more robust data sets.

Overall, our studies reveal a multipotential *Hand1*+ progenitor that produces not only cardiovascular lineages contributing to distinct regions of the LV and pericardium but also extraembryonic cell types within the yolk sac. Consistent with our findings, Tyser et al⁴³ also recently reported *Mab21l2* expressing progenitors present at cardiac crescent stages that contain precursors of heart and epicardium. Based on comparison between our studies (Figure XVII in the [Data Supplement](#)), their population likely represents a similar but later subset of the *Hand1* progenitors that we describe at the embryonic/extraembryonic boundary at E6.25 to E7.5. Although clonal relationships were not performed by Tyser et al, our results suggest that these progenitors are likely multipotent and contribute to distinct regions of the heart. Because these *Hand1*+ progenitors can also contribute to extraembryonic tissue, our studies also raise the possibility that congenital heart diseases thought to be caused by placental anomalies⁴⁴ may be due to perturbations of complex interplays between genetic pathways shared between extraembryonic and cardiac lineages.

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Author Contributions

Q. Zhang, D. Carlin, P. Cattaneo, S.M. Evans, J. Bloomekatz, T. Ideker, and N.C. Chi conceived the project and experimental strategy. Q. Zhang performed mouse experimental studies; D. Carlin performed bioinformatic analysis; J. Bloomekatz performed single-cell RNA sequencing studies; and F. Zhu generated Hand1-CreERT2 knock in mice. Q. Zhang, D. Carlin, S.M. Evans, J. Bloomekatz, and N.C. Chi prepared the manuscript.

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Disclosures

None.

Supplemental Materials

Expanded Materials & Methods
Data Supplement Figures I–XVII
Data Supplement Data Set I–IV
References^{9,10,12,13,34,43}

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