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Hey2 enhancer activity defines unipotent progenitors for left ventricular cardiomyocytes in juxta-cardiac field of early mouse embryo

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The cardiac crescent is the first structure of the heart and contains progenitor cells of the first heart field, which primarily differentiate into left ventricular cardiomyocytes. The interface between the forming cardiac crescent and extraembryonic tissue is known as the juxta-cardiac field (JCF), and progenitor cells in this heart field contribute to the myocardium of the left ventricle and atrioventricular canal as well as the epicardium. However, it is unclear whether there are progenitor cells that differentiate specifically into left ventricular cardiomyocytes. We have previously demonstrated that an enhancer of the gene encoding the Hey2 bHLH transcriptional repressor is activated in the ventricular myocardium during mouse embryonic development. In this study, we aimed to investigate the characteristics of cardiomyocyte progenitor cells and their cell lineages by analyzing *Hey2* enhancer activity at the earliest stages of heart formation. We found that the *Hey2* enhancer initiated its activity prior to cardiomyocyte differentiation within the JCF. *Hey2* enhancer-active cells were present rostrally to the *Tbx5*-expressing region at the early phase of cardiac crescent formation and differentiated exclusively into left ventricular cardiomyocytes in a lineage distinct from the *Tbx5*-positive lineage. By the late phase of cardiac crescent formation, *Hey2* enhancer activity became significantly overlapped with *Tbx5* expression in cells that contribute to the left ventricular myocardium. Our study reveals that a population of unipotent progenitor cells for left ventricular cardiomyocytes emerge in the JCF, providing further insight into the mode of cell type diversification during early cardiac development.

ventricular cardiomyocytes | progenitor cells | juxta-cardiac field | *Hey2* enhancer | cell lineage

The heart is composed of cells derived from progenitors that reside in different regions called heart fields during early developmental stages. In mouse embryos, mesodermal cells ingress through the primitive streak and migrate anteriorly during gastrulation. The first cells that reach the anterior-proximal part of the embryo form the cardiac crescent, and progenitor cells within this structure constitute the first heart field (FHF), which mainly gives rise to the left ventricle and atria. Subsequently migrating mesodermal cells lie medial to the cardiac crescent, and these second heart field (SHF) cells contribute to the development of the right ventricle, outflow tract, and a part of atria (1–5). Another distinct cardiac progenitor at this stage is identified at the interface of the cardiac crescent and extra-embryonic tissue, named the juxta-cardiac field (JCF); progenitor cells in the JCF contribute to the myocardium of the left ventricle and atrioventricular canal and the epicardium (6, 7).

Several genes are specifically expressed in each heart field during cardiac crescent formation. *Tbx5* is expressed in the FHF, posterior SHF (pSHF), and a subpopulation of cells in the JCF. *Tbx5*-expressing cells differentiate into cardiomyocytes in the left ventricle and atria, and loss of *Tbx5* leads to hypoplastic structures in the left ventricle and atria (6, 8–12). *Hcn4* is a FHF marker, and the *Hcn4*-expressing cell lineage contributes to the development of the left ventricle and a part of the atria (13, 14). The expression of *Tbx1* and its target genes *Fgf8* and *Fgf10* in the anterior SHF (aSHF) is indispensable for the formation of the outflow tract and right ventricle, whereas pSHF development is regulated by *Hoxa1* and *Hoxb1,* which are expressed in this region (15–23). JCF cells are characterized by the expression of *Mab21l2* and *Hand1* but not *Nkx2-5*, and both *Mab21l2*- and *Hand1*-knockout embryos show abnormal ventricular myocardial differentiation (6, 7, 24–26). These studies have revealed genes that specifically define each cardiac field; however, no specific markers have been reported that identify only left ventricular progenitor cells.

A member of the Hey family of basic helix-loop-helix transcription factors, *Hey2*, is expressed in the ventricular myocardium of the developing heart (27, 28), and *Hey2*-knockout

Significance

It is largely unknown how fate determination and cell type diversification of cardiomyocytes occurs during the development of a mutichambered heart. We identified unipotent progenitor cells for left ventricular cardiomyocytes in the juxta-cardiac field during cardiac crescent formation, which were marked by *Hey2* enhancer activity. At the early phase of cardiac crescent formation, *Hey2* enhancer-active cells were distinct from *Tbx5*-expressing cells, suggesting initial heterogeneity of progenitor cells for left ventricular cardiomyocytes. Subsequently, the *Hey2* enhancer activity significantly overlapped with *Tbx5* expression in cells that contribute to the left ventricular myocardium. Understanding such cell complexity in building the heart will provide a clue to the mechanisms of phenotype diversity in congenital heart diseases.

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mice die perinatally owing to the ventricular septal defect, dysplastic tricuspid valve, and hypoplastic right ventricle (29–36). *Hey2* is critical for transcriptional repression of atrial and atrioventricular canal genes in the embryonic ventricle, and the lack of *HEY2* significantly increases the number of atrial-like cells during cardiomyocyte differentiation from human iPS cells (37). We previously reported that an enhancer for the *Hey2* gene at the distal upstream region (the *Hey2* enhancer) exhibits specific transcriptional activity in the ventricular myocardium of developing embryos (38), but *Hey2* enhancer activity at the early stages of cardiomyocyte differentiation remained uncharacterized.

In this study, we found that the *Hey2* enhancer was activated in a subset of progenitors within the JCF prior to cardiomyocyte differentiation during cardiac crescent formation and that *Hey2* enhancer-active cells exclusively contributed to the formation of the left ventricular myocardium. At the early phase of cardiac crescent formation, *Hey2* enhancer-active cells were localized rostral to *Tbx5*-expressing cells, which are known to differentiate into left ventricular as well as atrial cardiomyocytes (10, 11). Identification of a novel, unipotent progenitor cell population for left ventricular cardiomyocytes suggests that the emergence and differentiation of progenitors should be finely orchestrated to form each part of a multichambered heart.

Results

***Hey2* Enhancer Activity Was Initiated prior to Cardiomyocyte Differentiation during Cardiac Crescent Formation.** We previously identified an enhancer of the mouse *Hey2* gene, which is essential for its ventricular expression during cardiac development (38). The enhancer activity was highly specific to the ventricular myocardium and not observed in the atria, outflow tract, and SHF at the heart looping stage, embryonic day (E)9.5 (Fig. 1*A*). To evaluate the precise timing of the enhancer activation, we analyzed *Hey2* enhancer-*LacZ* reporter transgenic (Tg) mouse embryos from the gastrulation (E7.0) to heart tube (E8.5) stages (Fig. 1*A*). The enhancer was inactive in the lateral plate mesoderm during gastrulation but induced LacZ expression around the cardiac crescent as it began to form at the early head-fold stage (Fig. 1*A*). The enhancer-active cells increased during cardiac crescent formation from the late head-fold to 5 somite stages (Fig. 1*A*) and subsequently occupied the heart tube structure, which mainly has a primitive left ventricular identity, at E8.5 (Fig. 1*A*) (39).

Next, we compared the expression of the LacZ reporter and cardiomyocyte markers, *Tnnt2* mRNA and myosin heavy chain (MyHC) protein, to confirm the differentiation status of enhancer-active cells during cardiac crescent formation (Fig. 1 *B* and *C*). Both *LacZ* mRNA expression and β-galactosidase activity were detected at the early phase of cardiac crescent formation (early CC phase); however, *Tnnt2* and MyHC expression had not yet been initiated. At the late phase of cardiac crescent formation (late CC phase), when the cardiac crescent structure became apparent, *Tnnt2* and MyHC expression started at the caudal side of the enhancer-active region, whereas enhancer-active cells without *Tnnt2* and MyHC expression were present in the rostral region of the cardiac crescent (Fig. 1 *B* and *C*). These results indicate that the *Hey2* enhancer is activated in undifferentiated cardiomyocyte progenitor cells from the early CC phase.

***Hey2* Enhancer-Active Cells during Cardiac Crescent Formation Contributed to the left Ventricular Myocardium.** To determine the fate of *Hey2* enhancer-active undifferentiated cells, we generated *Hey2* enhancer-*CreERT2* Tg mouse lines for genetic lineage tracing experiments (Fig. 2*A*). The expression pattern of CreERT2 in *Hey2* enhancer-*CreERT2* Tg embryos was comparable to that of LacZ in the *Hey2* enhancer-*LacZ* Tg embryos (*SI Appendix*, Fig. S1). Using *Hey2* enhancer-*CreERT2* Tg and *Rosa26R*-enhanced yellow fluorescent protein (*EYFP*) reporter mice, *Hey2* enhancer-active cells in embryos were labeled with low dose of tamoxifen administration to pregnant female mice at different time points, and the contribution of EYFP-labeled cells to heart formation was assessed at E11.5 (Fig. 2*A*). EYFP signals were not observed in E11.5 hearts when tamoxifen was administered at E5.5 (Fig. 2*B*). In contrast, EYFP-labeled cells with tamoxifen administration at E6.5 were detected in the left ventricle at E11.5, while they were rarely present in the right ventricle (Fig. 2*B*). With tamoxifen administration at E7.5, the number of EYFP-expressing cells significantly increased in E11.5 left ventricle. Their number remained small in the right ventricle (Fig. 2*B*). As expected from the enhancer activity after cardiac tube formation (Fig. 1*A*), tamoxifen administration at E8.5 resulted in strong EYFP expression in both ventricles (*SI Appendix*, Fig. S2). Nearly no EYFP-positive cells appeared in the atria and outflow tract after tamoxifen administration at any stage (Fig. 2*B* and *SI Appendix*, Fig. S2 *B*). Section immunohistochemistry analysis further revealed that EYFP expression was restricted to cardiomyocytes (MyHC+) in the left ventricle, and endocardial (CD31+) and epicardial (WT1+) cells were EYFP-negative (Fig. 2*C*). Similar results were obtained using another *Hey2* enhancer-*CreERT2* Tg mouse line (*SI Appendix*, Fig. S3). Taken together, the initial *Hey2* enhancer activity during cardiac crescent formation identifies a subset of cells that specifically differentiate into cardiomyocytes of the left ventricle.

***Hey2* Enhancer-Active Cells Were Identified in the JCF by Single-Cell RNA-seq Analysis.** Specific markers that label only progenitor cells for left ventricular cardiomyocytes have not been reported. We expected *Hey2* enhancer activity to serve for the characterization of cardiomyocyte progenitor cells at the earliest stages of cardiac development. To elucidate gene expression profiles of cell types that activate the *Hey2* enhancer, we performed single-cell RNA-seq analysis using *Hey2* enhancer-*LacZ* Tg embryos at E7.5 (the late CC phase based on morphology) after removing the distal extraembryonic and posterior embryonic tissues (Fig. 3*A*). Cells were divided into 15 clusters based on differentially expressed genes reported in a previous study (40), as shown in the UMAP plot (*SI Appendix*, Fig. S4*A* and Dataset S1). One of them was identified as the cardiac mesoderm cluster with high expression of its typical markers including *Mef2c*, *Gata6*, and *Hand2*, and *LacZ*-expressing cells were present in this cluster (*SI Appendix*, Fig. S4 *B* and *C* and Dataset S1).

The cardiac mesoderm cluster was further divided into the lateral plate mesoderm, aSHF, pSHF, FHF, differentiating cardiomyocytes, and JCF according to their gene expression patterns (Fig. 3 *B*–*D* and Dataset S2). The cell cluster of the lateral plate mesoderm expressing *Mesp1* was the most undifferentiated. aSHF and pSHF cell clusters were characterized by known markers such as *Tbx1* and *Prdm1*, and *Alx1* and *Nr2f2*, respectively. *Mef2c*, *Nkx2-5*, *Bmp5,* and *Amph* were commonly expressed in the FHF, aSHF, and differentiating cardiomyocyte clusters, whereas the differentiation markers *Ankrd1*, *Myh6*, *Ttn,* and *Myl3* were only expressed in the differentiating cardiomyocyte cluster. It is known that *Mab21l2* and *Hand1*, but not *Nkx2-5*, are expressed in the JCF (6, 7). *Tbx5*-expressing cells are abundant in differentiating cardiomyocytes and are significantly present in the FHF and JCF, as shown in a previous report (6). Among these clusters, cells expressing *LacZ* were mostly present in the JCF and differentiating cardiomyocytes; however, few were detected in the FHF (Fig. 3 *C*–*E*). Cells expressing *Hey2* transcripts were widely

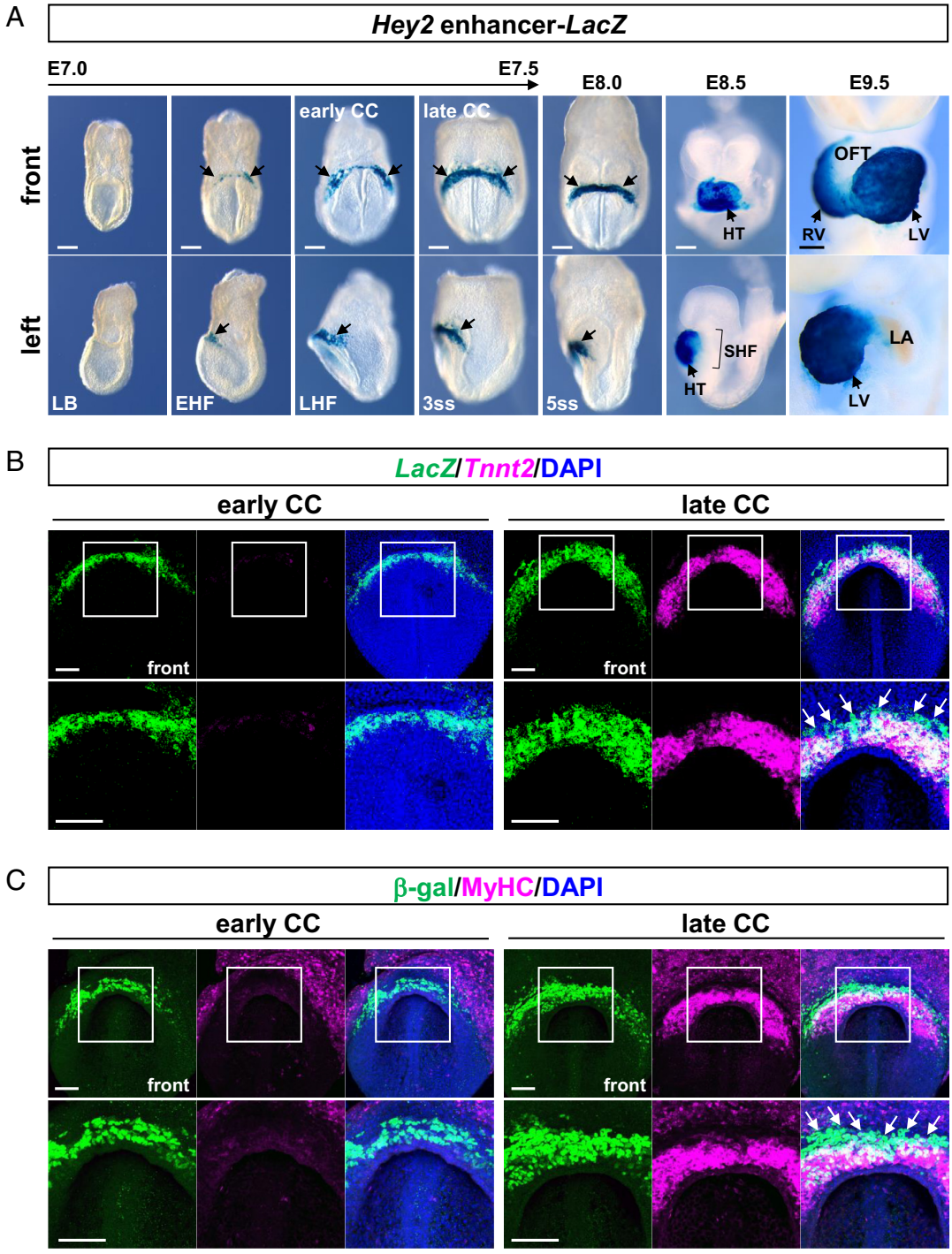

Fig. 1. Transcriptional activity of the *Hey2* enhancer during early cardiac development. (*A*) β-galactosidase activity in *Hey2* enhancer-*LacZ* transgenic (Tg) embryos was evaluated from the gastrulation to heart tube stages. Arrows indicate the enhancer-active regions. HT, heart tube; SHF, second heart field; LV and RV, left and right ventricles; OFT, outflow tract; LB, late bud stage; EHF, early head-fold stage; LHF, late head-fold stage; ss, somite stage. (Scale bars, 200 μm.) (*B* and *C*) *LacZ* transcript and β-galactosidase expression in *Hey2* enhancer-*LacZ* Tg embryos at early and late phases of cardiac crescent formation (early and late CC) was compared with the expression of myocardial markers *Tnnt2* and myosin heavy chain (MyHC) using RNAscope analysis and immunohistochemistry, respectively. *LacZ*-expressing cells emerged prior to the expression of myocardial markers at the early CC phase, and undifferentiated *LacZ*-expressing cells were present rostral to the differentiating myocardium at the late CC phase (arrows). (Scale bars in *B* and *C*, 100 μm.)

distributed in those clusters (Fig. 3*C*). As suggested in our previous study (38), other enhancers may likely control *Hey2* expression in these cardiac cells.

The JCF cluster was divided into two subgroups of cells (*SI Appendix*, Fig. S5 and Dataset S3). *LacZ*-expressing cells were enriched in one of them (*SI Appendix*, Fig. S5), although the expression of typical JCF markers (*Mab21l2* and *Hand1*) was comparable between the two subgroups. The *LacZ*-dominant subgroup had higher expression of *Myl4* and *Tnnc1* (*SI Appendix*, Fig. S5), which are sarcomere genes, and *Myl4* is expressed from the cardiac progenitor state during differentiation from human ES cells (41). The other subgroup with few *LacZ*-expressing cells showed the expression profile of the pSHF, which is known to be continuously located at the caudal side of the JCF (Fig. 3*D* and *SI Appendix*, Fig. S5). Even within the JCF, the cellular state slightly toward differentiation may be accompanied by

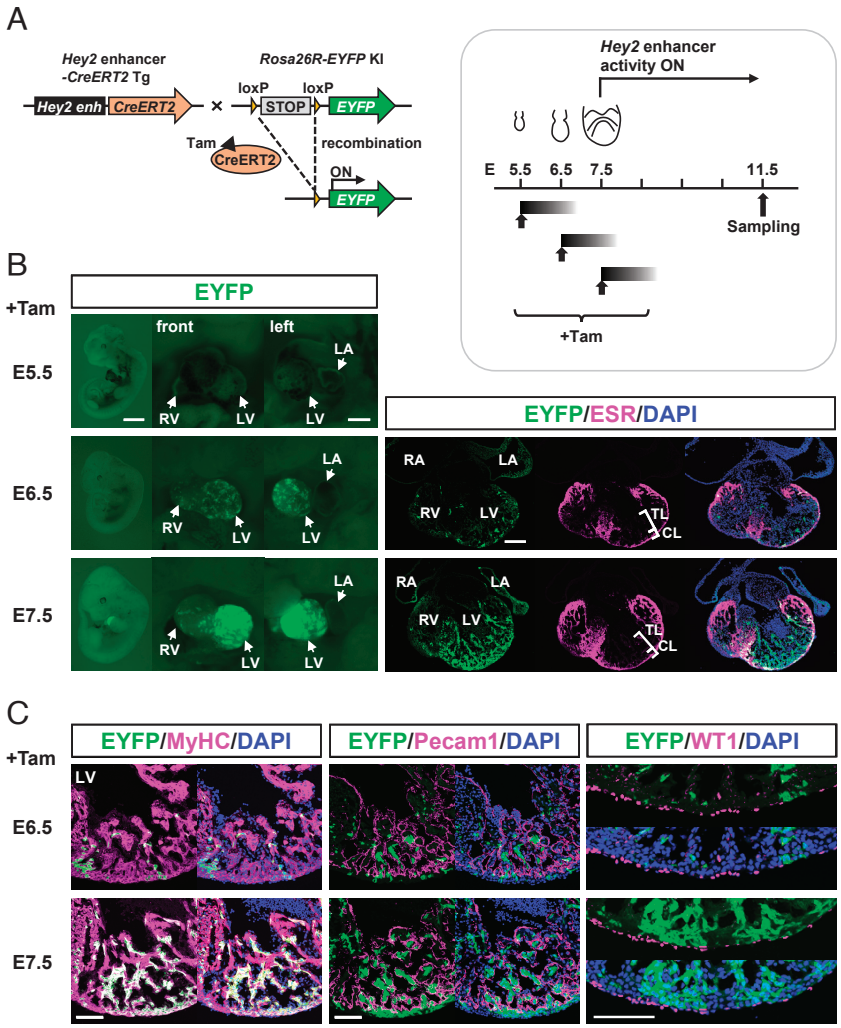

Fig. 2. The lineage of *Hey2* enhancer-active cells from the gastrulation to the cardiac crescent stages. (*A*) *Hey2* enhancer-*CreERT2* Tg and *Rosa26R-EYFP* knock-in (KI) mice were used for the cell lineage analysis. Tamoxifen triggers Cre-mediated loxP recombination to induce EYFP expression. Tamoxifen was administered to pregnant female mice at E5.5, E6.5, or E7.5, and embryos were collected at E11.5 to observe EYFP expression (n = 4, 6, 16 for E5.5, E6.5, and E7.5 administration, respectively). Tamoxifen administration at E6.5 and E7.5 was able to label enhancer-active cells in early and late CC phases, respectively. (*B*) EYFP expression was observed in whole embryos or using section immunohistochemistry. The ESR antibody detected CreERT2 expression driven by the *Hey2* enhancer, and EYFP expression represented the contribution of the *Hey2* enhancer-active cells following tamoxifen administration. The initially EYFP-labeled cells contributed primarily to the left ventricle (LV) formation. LA and RA, left and right atria; RV, right ventricle; TL, trabecular layer; CL, compact layer. Arrows point to each chamber (LA, LV, and RV). (Scale bar, 2 mm in whole embryos, 500 μm in the hearts, and 200 μm in the sections.) (*C*) EYFP expression was compared with the expression of markers for the myocardium (MyHC), endothelium (Pecam1), and epicardium (WT1) in the LV. EYFP was specifically expressed in the myocardium. (Scale bars, 100 μm.)

activation of the *Hey2* enhancer at the rostral side of the cardiac crescent (Fig. 1 *B* and *C*).

***Hey2* Enhancer-Active Cells Were Localized in the JCF Rostral to the *Tbx5*-Expressing Region at the Early CC Phase.** Next, we performed RNAscope analysis to determine the location of *Hey2* enhancer-active cells in relation to cells expressing other region-specific marker genes. *Hey2* enhancer activity was shown by detecting *LacZ* mRNA in *Hey2* enhancer-*LacZ* Tg mouse embryos. Previous studies have identified *Mab21l2* and *Hand1* as JCF markers; and *Nkx2.5* as a FHF, SHF, and cardiomyocyte marker (6, 7). At the early CC phase, costaining of *LacZ*, *Nkx2-5*, and *Hand1* showed that *LacZ* expression was not present in the *Nkx2-5*-expressing region, while it was located in the *Hand1*-expressing JCF region (Fig. 4*A* and Movie S1). *Tbx5* is expressed in the FHF and cardiomyocytes as well as the JCF during cardiac crescent formation (Fig. 3 *C* and *D*) (6, 10, 11). Most of the *LacZ*-expressing cells were observed in the more rostral region where *Tbx5* staining was negative but a JCF marker *Mab21l2* staining was positive (Fig. 4*A* and Movies S2 and S3). At the late CC phase, caudal cells in the cardiac crescent began to differentiate, whereas rostral cells did not (Fig. 1*B* and *C*). The number of *LacZ*-expressing cells increased in the *Nkx2-5*- and *Tbx5*-positive differentiating cardiomyocytes in the cardiac crescent (Fig. 4*B*, *SI Appendix*, Fig. S6, and Movies S4 and S5), while the rostral portion of the *LacZ*-expressing region overlapped with the *Mab21l2*- and *Hand1*-expressing JCF (Fig. 4*B*). These RNAscope data reveal that *Hey2* enhancer-active progenitor cells are present in the JCF, particularly rostral to the *Tbx5*-expressing region at the early CC phase.

***Hey2* Enhancer-Active Cells and *Tbx5*-Expressing Cells Initially Mark Distinct Lineages for Left Ventricular Cardiomyocytes at the Early CC Phase.** *Tbx5*-expressing cells during cardiac crescent formation have been characterized as myocardial progenitors that contribute to the left ventricle and atria (10, 11). While the *Hey2* enhancer-active cells also contributed to the left ventricle formation (Fig. 2), those cells were predominantly localized in the *Tbx5*-negative region at the early CC phase (Fig. 4*A*), suggesting that the left ventricular myocardium is derived from at least two progenitor cell populations. To precisely characterize the cell

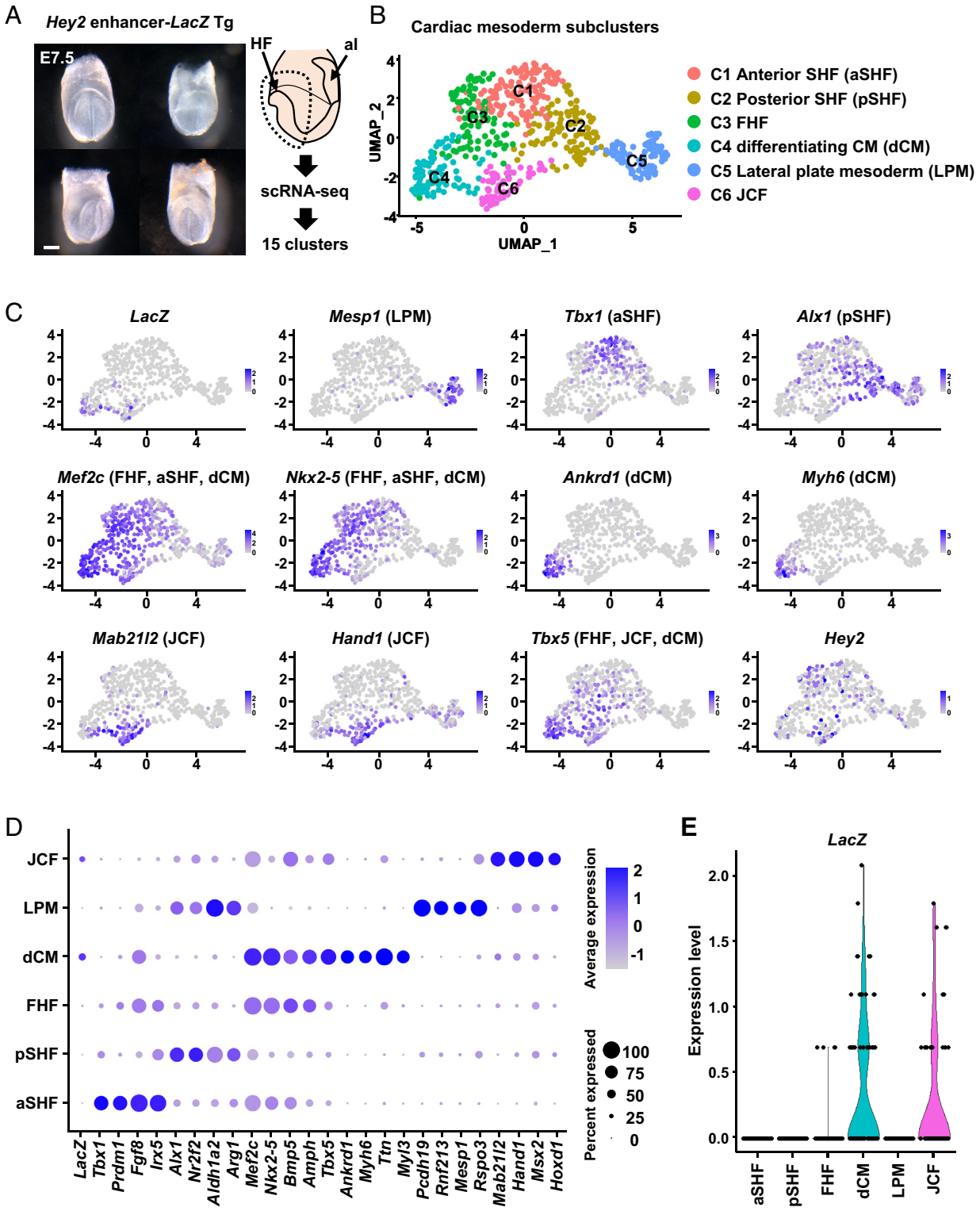

Fig. 3. Single-cell RNA-seq analysis of *Hey2*-enhancer-*LacZ* Tg embryos. (*A*) Four E7.5 *Hey2*-enhancer-*LacZ* Tg embryos were selected, and the anterior portions of the embryos indicated in the illustration were used for the single-cell RNA-seq analysis. The analyzed tissue was separated into 15 cell clusters (*SI Appendix*, Fig. S4). HF, head fold; al, allantois. (*B*) Only the cardiac mesoderm cluster was used for further analyses, and it was divided into six cell subclusters (C1 to C6), as shown by the UMAP plot. Each cluster was annotated as the anterior or posterior second heart field (aSHF, pSHF), first heart field (FHF), lateral plate mesoderm (LPM), differentiating cardiomyocytes (dCM), and juxta-cardiac field (JCF). (*C* and *D*) UMAP and dot plots showing the distribution of cells expressing *LacZ, Hey2,* and selected genes in particular clusters. The expression level of each gene is indicated by the color intensity (*C* and *D*), and the proportion of expressing cells is depicted by the circle size (*D*). (*E*) The violin plot shows *LacZ*-expressing cells and their expression levels in each cluster. *LacZ*-expressing cells were abundant in the JCF and dCM.

lineages for the formation of the left ventricular myocardium, we performed double genetic lineage tracing of the *Hey2* enhancer-active and *Tbx5*-expressing cells in the same embryos using Dre-rox and Cre-loxP recombination systems (Fig. 5*A*). DreERT2, driven by *Hey2* enhancer activity, induced mRFP reporter expression in the presence of tamoxifen (Fig. 5 *B* and *C*). As indicated by mRFP expression, the contribution of *Hey2* enhancer-active cells to the left ventricular myocardium was comparable to that observed in

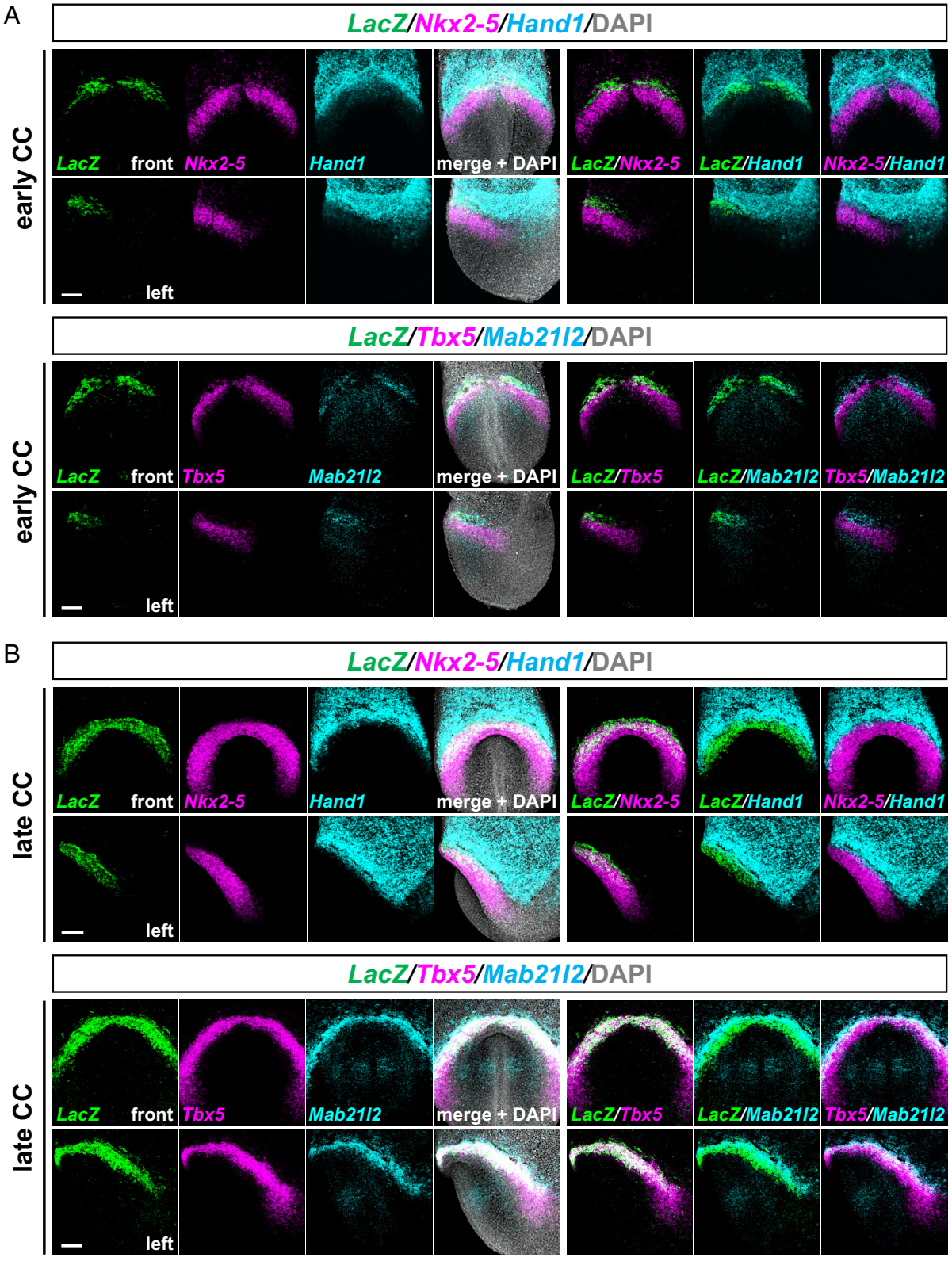

Fig. 4. Distribution of *Hey2* enhancer-active cells during cardiac crescent formation. (*A* and *B*) *LacZ* and marker gene (*Nkx2-5, Hand1*, *Tbx5*, and *Mab21l2*) expression was detected in *Hey2* enhancer-*LacZ* Tg embryos at early and late CC phases using RNAscope analysis. The maximum projections of the z-stack images are shown. *LacZ*-expressing cells were predominant in the JCF (*Mab21l2/Hand1*-positive and *Nkx2-5*-negative) at the early CC phase and in differentiating cardiomyocytes (*Nkx2-5/Tbx5/Tnnt2*-positive and *Mab21l2/Hand1*-negative) (Fig. 1*B*) in addition to the JCF at the late CC phase. (Scale bars, 100 μm.)

the analysis using the *Hey2* enhancer-*CreERT2* mouse line (Fig. 2). The lineage of *Tbx5*-expressing cells was analyzed using *BAC-Tbx5-CreERT2* and *Rosa26R-EYFP* mouse lines (11).

When tamoxifen was administered at E6.5 to label the enhancer-active and *Tbx5*-expressing cells, both mRFP and EYFP fluorescence was observed in the left ventricular myocardium at E11.5, indicating that both *Hey2* enhancer-active and *Tbx5*-expressing cells are progenitor cells that differentiate into the left ventricular cardiomyocytes (Fig. 5*B*). The EYFP-positive cardiomyocytes were more abundant than the mRFP-positive cardiomyocytes. Importantly,

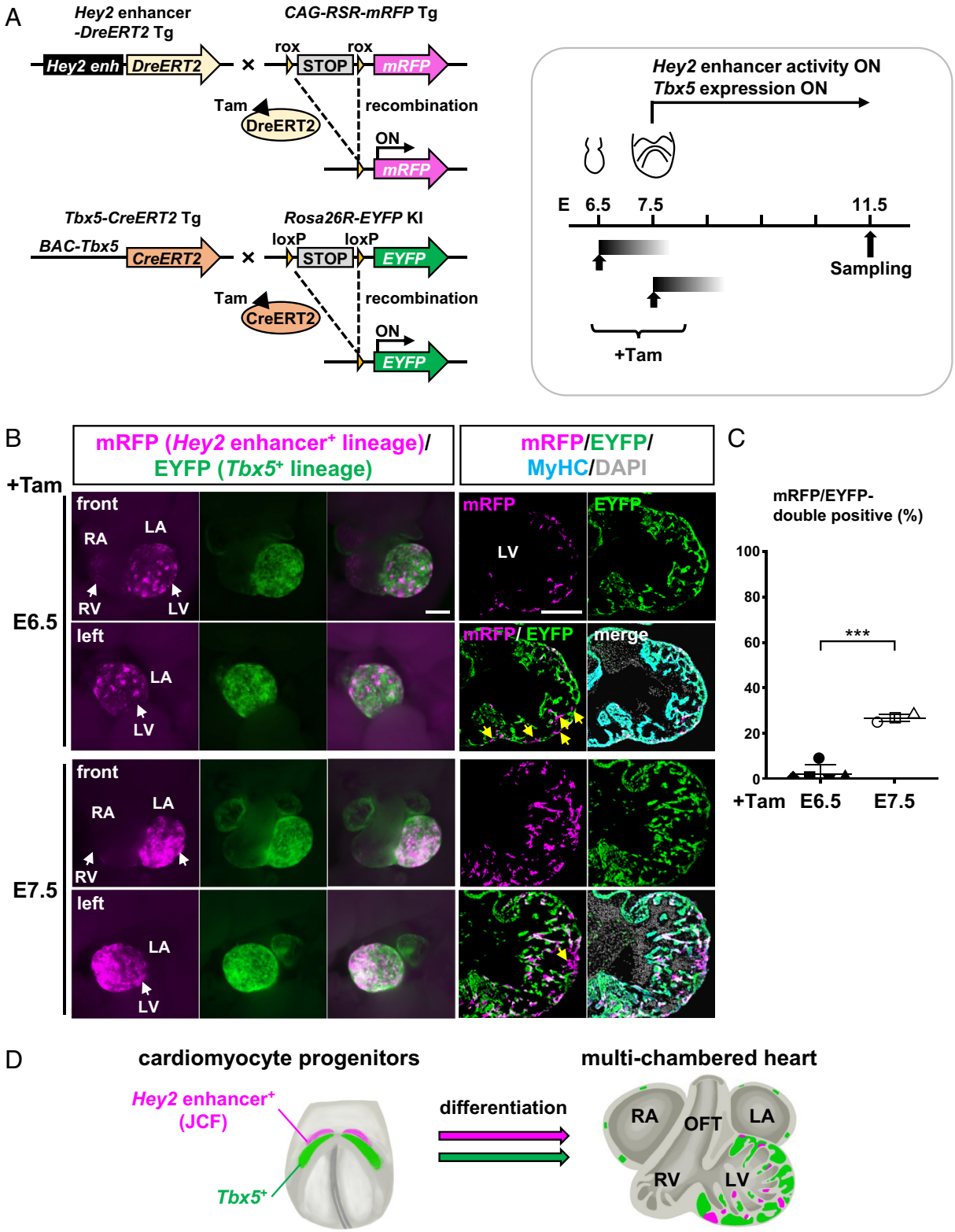

Fig. 5. Double lineage analysis of *Hey2* enhancer-active and *Tbx5*-expressing cells at early and late CC phases. (*A*) *Hey2* enhancer-*DreERT2* Tg;*BAC-Tbx5-CreERT2* Tg mice were mated with *CAG-rox-STOP-rox-mRFP* (*CAG-RSR-mRFP*) Tg;*Rosa26R-EYFP* KI reporter mice for the double cell lineage analysis. Tamoxifen triggers Dre-mediated rox and Cre-mediated loxP recombinations, which induce mRFP and EYFP expression, respectively. Tamoxifen was administered to pregnant female mice at E6.5 or E7.5, and embryos were collected at E11.5 to observe mRFP and EYFP expression. (*B*) mRFP and EYFP expression was observed in whole hearts or using section immunohistochemistry. Both mRFP- and EYFP-labeled cells contributed to the MyHC-positive myocardium in the left ventricle (LV). White arrows point to each ventricle (LV and RV), and yellow arrows point to cardiomyocytes that are positive only for mRFP in the LV. (Scale bars, 200 μm.) (*C*) The percentage of mRFP/EYFP double-positive area in the LV myocardium positive for mRFP and/or EYFP was calculated and statistically analyzed. The contribution of double-positive cells to the LV myocardium was higher with tamoxifen administration at E7.5 compared with that at E6.5. The mRFP/EYFP double-positive area was only marginal when tamoxifen was administered at E6.5, while it occupied 30% of the LV myocardium positive for mRFP and/or EYFP in the experiments with tamoxifen administration at E7.5 (n = 5 for E6.5, n = 3 for E7.5). ***$P < 0.001$. (*D*) *Hey2* enhancer-active cells were present in the JCF rostral to the *Tbx5*-expressing region at the early phase of cardiac crescent formation. These enhancer-active cells contributed primarily to the LV myocardium in a multi-chambered heart, and this lineage was temporally distinct from the *Tbx5*-expressing cell lineage. LA and RA, left and right atria; RV, right ventricle; OFT, outflow tract.

there were only a few mRFP/EYFP double-positive cells (Fig. 5*B*), reflecting the small number of cells that were positive for both *Hey2* enhancer activity and *Tbx5* expression at the early CC phase (Fig. 4*A*). Tamoxifen administration at E7.5 resulted in increased numbers of mRFP- and EYFP-positive cells at E11.5 (Fig. 5*B* and *SI Appendix*, Fig. S7), and a significant portion of the left ventricular myocardium became mRFP/EYFP double-positive (Fig. 5*B*), as expected from overlapping distributions of *Hey2* enhancer activity and *Tbx5* expression at the late CC phase (Fig. 4*B*). EYFP-positive cells derived from *Tbx5*-expressing cells were observed in the atria, as shown by previous reports (10, 11). However, the *Hey2* enhancer-active cell lineage remained detectable solely in the left ventricle (Fig. 5*B*). Quantitative analysis also revealed that the percentage of mRFP/EYFP double-positive area was very low when tamoxifen was administered at E6.5, in contrast to the higher value with tamoxifen administration at E7.5 (Fig. 5*C*). Taken together, the observations in this study provide evidence that *Hey2* enhancer activity defines a distinct progenitor cell population in the JCF at the onset of cardiac crescent formation, which specifically differentiates into left ventricular cardiomyocytes (Fig. 5*D*).

Discussion

In this study, we demonstrated that *Hey2* enhancer-active cells in the JCF specifically differentiated into left ventricular cardiomyocytes. Those enhancer-active cells at the early CC phase were localized rostral to known *Tbx5*-expressing progenitors for the left ventricular myocardium before the overlap of *Hey2* enhancer activity and *Tbx5* expression by the late CC phase. These results provide evidence for the presence of previously undefined unipotent progenitor cells in the JCF and indicate that at least two different types of progenitor cells for left ventricular cardiomyocytes reside at the earliest stage of the cardiac crescent formation.

The heart is formed by mesodermal cells that arise through the primitive streak during gastrulation. The transcription factors *Mesp1*, *T* (*Brachyury*), and *Foxa2* are expressed in these mesodermal cells (1, 3, 42). Lineage analysis of mesodermal cells positive for these factors or the *Smarcd3*-F6 enhancer during this period showed that they contributed first to the myocardium of the left ventricle and atrioventricular canal, as well as the proepicardium. Subsequently migrating mesodermal cells differentiate into the myocardium of the right ventricle and later to that of the outflow tract and atria (1, 3–5). When the first group of mesodermal cells reaches the anterior-proximal region of the embryo, they undergo the mesenchymal-epithelial transition to form the cardiac crescent (5). JCF cells rostral to the cardiac crescent are motile at this stage, and they are progenitors for both myocardium and epicardium (5–7). Since early *Hey2* enhancer-active progenitor cells exclusively differentiated into the left ventricular myocardium, they are most likely derived from the initially invaginated mesodermal cells, and a subset of those mesodermal cells are defined by *Hey2* enhancer activity as progenitors for left ventricular cardiomyocytes.

Genetic tracing experiments using the *Hey2* enhancer provided additional support for our notion concerning the differentiation trajectory from the JCF to the myocardium (6). Previous studies have identified *Mab21l2* and *Hand1* as JCF markers (6, 7), and cells expressing these genes contribute to the myocardium of the left ventricle and atrioventricular canal, and also to the epicardium (6, 7). Since iPS-derived cells expressing these JCF markers can give rise to cells with both cardiomyocyte and epicardial markers (43), it is possible that *Hey2* enhancer-active unipotent progenitor cells are derived from such bipotent cells in the JCF of mouse embryos. *Tbx5* is regarded as a marker gene for the cells fated to the left ventricle and atria (10, 11), but a majority of *Hey2* enhancer-active progenitors did not show *Tbx5* expression at the early CC phase (Fig. 4*A*). *Hey2* enhancer-active cells, however, may have an undetectable level of *Tbx5* expression, and the possibility cannot be ruled out that *Hey2* enhancer activity and *Tbx5* expression have opposing gradients from the cardiac crescent to its rostral region, making progenitor cell populations with these characteristics not completely separable.

By the late CC phase, *Hey2* enhancer activity and *Tbx5* expression became significantly overlapped, which is probably due to a reshaping of the cardiac progenitor fields during cardiac crescent formation (5). Single-cell RNA-seq analysis at the late CC phase showed *Tbx5* expression in the FHF cluster with few *Hey2* enhancer-active cells, in addition to its expression in the JCF and differentiating cardiomyocytes (Fig. 3*D*). This observation suggests that the mRFP-negative;EYFP-positive myocardium in the left ventricle is mainly composed of the lineage derived from *Tbx5*-expressing FHF cells. It has been shown that the left side of the ventricular septum is derived from *Tbx5*- and *Acta2*-expressing cells but not from *Hand1*-expressing cells (7, 10, 11, 44). Consistently, the septal myocardium mainly contains the *Tbx5*-positive;*Hey2* enhancer-negative lineage labeled at the early CC phase (Figs. 2*B* and 5*B*), suggesting that progenitor cells in the JCF preferentially differentiate into cardiomyocytes in the ventricular free wall.

It will be intriguing to understand how *Hey2* enhancer activity is controlled by upstream signaling and transcriptional regulators in progenitor cells. We previously demonstrated that the *Hey2* enhancer is activated by the Tbx20 and Gata family transcription factors in the ventricular myocardium at the looping stage (38). Both Tbx20 and Gata4 are broadly expressed in cardiac progenitors including the JCF during cardiac crescent formation (*SI Appendix*, Fig. S5*C*) (45, 46) and may be important for *Hey2* enhancer activity. The loss of *Tbx5* abolishes *Hey2* expression in the embryonic ventricle (8). It is possible that Tbx5 activates the *Hey2* enhancer in the region where *Tbx5* expression and *Hey2* enhancer activity overlap at the late CC phase, although *Tbx5* expression extends posteriorly to the area where atrial progenitor cells reside (47). The specificity of *Hey2* enhancer activity may also rely on the repression in cells other than the progenitors of the left ventricular myocardium. One candidate repressor is retinoic acid signaling, which negatively regulates ventricular cell differentiation (48, 49). In addition, iPS-derived cardiovascular progenitor cells cultured with retinoic acid show preferential integration into the left ventricular myocardium after the transplantation into the heart-forming region of mouse embryos (43). As shown in our single-cell RNA-seq analysis, a subgroup of the JCF with fewer enhancer-active cells showed higher expression of *Aldh1a2*, which encodes an essential enzyme for retinoic acid synthesis, and *Nr2f2* and *Hoxb1,* downstream genes of retinoic acid signaling. Future work should address the regulatory machinery of the *Hey2* enhancer to elucidate how progenitor cells for left ventricular cardiomyocytes emerge.

Materials and Methods

Mouse Strains. *Hey2* enhancer-nls-*LacZ* and BAC-*Tbx5*-*CreERT2* Tg mouse lines have been previously described (11, 38). *Hey2* enhancer-*CreERT2* and -*DreERT2* and *CAG-rox-Stop-rox-lyn-mRFP* Tg mice were generated by injecting linear DNA fragments into the pronuclei of BDF1 fertilized eggs, and independent lines were established that showed reproducible activity and expression. The *Rosa26R-EYFP* mouse line (Jax #006148) was obtained from Jackson Laboratory. For the genetic lineage tracing analysis, tamoxifen (Sigma #T5648, 50 μg/g body weight) was administered to pregnant female mice, and CreERT2 and DreERT2-expressing cells were labeled with EYFP and RFP, respectively, and the contribution of the labeled cells to heart formation was assessed at E11.5. All the animal experiments were

approved by the Institutional Animal Care and Use Committee of the National Cerebral and Cardiovascular Center and followed the ARRIVE guidelines. The institutional approval numbers for this study were as follows: animal experiments, #20018, 21019, and 22057; recombinant DNA experiments, #20-4, 21-4, and 22-4.

Immunohistochemistry. Mouse embryos were collected in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min. For section immunohistochemistry, PBS-washed embryos were embedded in OCT compound (Tissue-Tek) and sectioned at a thickness of 12 μm. Whole embryos or sections were permeabilized in 0.2% Triton X-100/PBS for 30 min at room temperature and blocked with 1% bovine serum albumin (Wako #017-17846) and 5% lamb serum (Thermo Fischer Scientific #16070-096) in PBS + 0.1% Tween 20 for 1 h at room temperature. The following primary antibodies were diluted 1/500 in the blocking solution and incubated with embryos or sections for 16 to 20 h at 4 °C: α-β-galactosidase (rabbit, kindly provided by Nicolas JF, Pasteur Institute), α-CD31 (rat, BD #550274), α-estrogen receptor (ESR) (rabbit, Abcam #ab16660), α-GFP (rabbit, MBL #598), α-GFP (rat, Nacalai #04404-26), α-myosin heavy chain (mouse, DSHB #MF20), α-RFP (rabbit, MBL #PM005), and α-WT1 (rabbit, Abcam #89901). Alexa Fluor 488-, or 594-conjugated secondary antibodies (Thermo Fischer Scientific #A11029, A21206, A21203, A21207) were diluted 1/1,000 in the blocking solution and incubated with embryos or section for 16 to 24 h at 4 °C. For the dual detection of β-galactosidase activity and ESR, whole embryos were incubated in Spider-βgal solution (Dojin #SG02) for 16 h at 4 °C and then incubated with α-ESR and α-rabbit IgG Alexa Fluor 647-conjugated secondary (Thermo Fischer Scientific #A31573) antibodies. For section immunohistochemistry, the ESR signal was amplified using tyramide-fluorescein (Perkin Elmer #SAT701001KT) for 20 min at room temperature, in combination with the α-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (MBL #458). After staining, E7.5 embryos were embedded in 50% glycerol/PBS. Fluorescent images were captured using the FV3000 confocal microscope (Olympus). Images of whole-mount embryos were captured with a Z-stack every 3 to 5 μm, and the maximum projection images are shown in Figures. In double lineage analysis of *Hey2* enhancer-active and *Tbx5*-expressing cells, fluorescent signals were processed by image thresholding. The areas positive for mRFP, EYFP, and both in MyHC-positive myocardium were measured by the Colocalization Threshold plugin of ImageJ using 3 to 10 slides for each embryo, and statistical analysis was performed using Student's *t* test.

RNAscope Analysis. RNAscope analysis was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics #323100) with a few modifications to the previously described method by Nomaru et al. (50). Briefly, the embryos were fixed in 4% paraformaldehyde at 4 °C overnight. After dehydration and hydration in a graded series of methanol and PBS + 0.1% Tween, the embryos were treated with Protease III (#322102) for 20 min. The following C1, C2, and C3 probes were used for hybridization at 40 °C overnight: *LacZ* (#313451-C1), *Nkx2-5* (#428241-C2), *Tbx5* (#519581-C2), *Tnnt2* (#418681-C3), *Hand1* (#429651-C3), and *Mab21l2* (#456901-C3). Following this, 0.2 × saline sodium citrate buffer (SSC) + 0.01% Tween was used for washing in between steps. The embryos were incubated with AMP1 for 30 min, AMP2 for 30 min, and AMP3 for 15 min at 40 °C. For signal detection, the embryos were incubated in HRP-C1, -C2, or -C3 for 15 min at 40 °C, followed by incubation in Opal 520, 690, and 570 (Akoya Biosciences #FP1487001KT, FP1497001KT, FP1488001KT). HRP was inactivated by HRP-Blocker for 15 min at 40 °C. After staining, the embryos were embedded in 50% glycerol/PBS. Fluorescent images were captured using an FV3000 confocal microscope (Olympus) with a Z-stack every 3 μm.

Single-Cell RNA-Sequencing. E7.5 embryos (the late CC phase based on morphology) were collected in DMEM + 25 mM HEPES and stored on ice during PCR genotyping. Four *Hey2* enhancer-nls-*LacZ* embryos were selected, and the distal part of the extraembryonic tissue and the posterior half of the embryo were removed. The sample was treated with 0.25% Trypsin-EDTA with DNase I (50 U/mL, Millipore #260913-10MU) for 10 min at 25 °C, and the reaction was stopped by adding 1/10 volume of fetal bovine serum (FBS). Dissociated cells were centrifuged at 500 × *g* for 5 min and resuspended in PBS + 10% FBS. The cells were filtered through a cell strainer (70 μm, Falcon #352350) and centrifuged at 300 × *g* for 5 min. The cells were resuspended in PBS + 10% FBS, and the cell count and viability were measured. Single-cell RNA sequencing was performed by KOTAI Biotechnologies, Inc. Briefly, the samples were separated into single cells, barcoded using Chromium Next GEM Single-cell 3′ Reagent Kits on a Chromium controller (10× Genomics), and sequenced using the DNBSEQ-G400 sequencer (MGI).

Single-Cell RNA-Sequencing Data Analysis. Sequence data were processed for read alignment to the mouse mm10 reference genome and the generation of feature-barcode matrices using the Cell Ranger software, version 6.0.2 (10× Genomics). A total of 957,663,228 reads, 64,174 median reads/cell, and 3,854 median genes/cell were obtained. The filtered feature-barcode matrix was analyzed using the R package Seurat version 4.0.4 (51). The cells were filtered by the number of unique feature counts >2,500 and <200 and <3% mitochondrial counts, and their sequence data were normalized using the SCTransform function. A total of 2,000 feature genes that exhibited high cell-to-cell variation in the dataset were selected, and normalization was applied to the dataset using these features. The scaled data were subjected to principal component analysis, and the cells were divided into 15 clusters using FindNeighbors (dims = 24) and FindClusters (resolution = 0.5). The cell clusters were visualized using the nonlinear dimensional reduction technique, UMAP. The cluster identities were assigned based on differentially expressed genes in a previous study (40). The cardiac mesoderm cluster was then subclustered into six clusters with dims = 10, k.param = 12, and resolution = 0.8. The JCF cluster was further subdivided into two clusters with dims = 15, k.param = 12, and resolution = 0.8. *LacZ* was excluded from the clustering and subclustering steps. The expression of genes was visualized by Featureplot, Dotplot, and VlnPlot functions of Seurat (51). Raw/Analyzed data have been deposited in NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo, accession no. GSE227506).

Data, Materials, and Software Availability. Raw/Analyzed data of single-cell RNA-sequencing data have been deposited in NCBI Gene Expression Omnibus (GEO) database (GSE227506) (52).

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