Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells

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Compact cardiomyocytes that make up the ventricular wall of the adult heart represent an important therapeutic target population for modeling and treating cardiovascular diseases. Here, we established a differentiation strategy that promotes the specification, proliferation and maturation of compact ventricular cardiomyocytes from human pluripotent stem cells (hPSCs). The cardiomyocytes generated under these conditions display the ability to use fatty acids as an energy source, a high mitochondrial mass, well-defined sarcomere structures and enhanced contraction force. These ventricular cells undergo metabolic changes indicative of those associated with heart failure when challenged in vitro with pathological stimuli and were found to generate grafts consisting of more mature cells than those derived from immature cardiomyocytes following transplantation into infarcted rat hearts. hPSC-derived atrial cardiomyocytes also responded to the maturation cues identified in this study, indicating that the approach is broadly applicable to different subtypes of the heart. Collectively, these findings highlight the power of recapitulating key aspects of embryonic and postnatal development for generating therapeutically relevant cell types from hPSCs.
Human pluripotent stem cell (hPSC)-derived cardiomyocytes represent a promising source of cells for developing in vitro models to study diseases of the human heart and for establishing therapies to treat them. Successful translation of these applications to clinical and/or commercial practice is, however, dependent on our ability to derive the appropriate target cell(s) of the disease of interest from hPSCs in vitro. While diseases can affect different cell types of the heart, those that impair ventricular function such as myocardial infarction (MI) and ventricular arrhythmias are amongst the most severe and debilitating and can lead to organ dysfunction and failure. Given this, hPSC-derived ventricular cardiomyocytes are attractive therapeutic targets for both modeling life threatening arrhythmias and heart failure in vitro and for developing cell therapies to remuscularize ventricular myocardium damaged by MI. However, to produce appropriate ventricular cells for these applications, it is essential to understand how they are specified and develop in the embryo and neonate and apply this knowledge to the generation of comparable cell types from hPSCs.

In the mouse, the cardiomyocytes that make up the ventricular chambers derive from progenitors that are specified at the earliest stages of heart development, prior to the emergence of progenitors that contribute to the atrial and sinoatrial pacemaker cells of the disease of interest from hPSCs in vitro. While hPSCs have investigated different stages of this developmental progression, none have recapitulated the entire developmental path from specification to maturation and function. With respect to the earliest lineage specification step, we have previously shown that the human ventricular lineage develops from a mesodermal population that is distinct from the one that gives rise to atrial cells, recapitulating the finding in the early embryo that these cell types derive from different progenitors. The cardiomyocyte populations generated in our study as well as in most others published to date are immature and display characteristics similar to cardiomyocytes in the early fetal heart. Strategies aimed at maturing hPSC-derived cardiomyocytes have used electromechanical stimulation as well as exposure to pathway agonists and hormones that promote maturation of the metabolic machinery. With respect to the latter approach, a number of different studies have shown that the addition of either lipids, hormones or PPARalpha (PPARa) agonists to the differentiation cultures will induce metabolic changes in the hPSC-derived cardiomyocytes indicating that the in vitro-derived cells can respond to stimuli that promote cardiomyocyte maturation in vivo.

In this study, we followed a staged approach to generate functionally mature compact cardiomyocytes from hPSCs focusing on recapitulating the key events that regulate ventricular development and maturation in vivo. We show that Wnt and IGF signaling specify a compact cardiomyocyte population that in response to transient activation of PPARa signaling and hormonal and FA stimuli undergoes a series of maturation steps that include a switch from glycolysis to FAO. The mature cells are able to use exogenous FAs as an energy source, and show improved sarcolemmal structure, increased contraction force, increased mitochondrial oxidative capacity and changes in global RNA expression patterns. The metabolically mature ventricular cells initiate a reversion to glycolysis and an increase in lipid accumulation and progression to apoptosis following exposure to stimuli that mimic heart failure. Transplantation studies using the nude rat model revealed that the mature cells generated more mature grafts than the immature cells, suggesting that the maturation status of the transplant population can impact the quality of the graft. When exposed to the combination of maturation factors, atrial cardiomyocytes showed similar patterns of maturation, indicating that the strategy is not chamber specific.

Results

Specification of the compact cardiomyocyte lineage. To be able to model diseases that affect the compact ventricular myocardium and to develop cell based therapies to treat them, we focused our initial studies on identifying the signaling pathways that regulate the generation of ventricular compact cardiomyocytes from hPSCs. As a first step, we characterized the trabecular and compact composition of the day 20 cardiomyocyte population generated with the ventricular differentiation protocol previously established in our lab through single cell RNA sequencing (scRNAseq) analyses (Fig. 1a–c, Supplementary Table 1). T-SNE plots of clustered scRNAseq data identified 9 distinct groups within the cTNT+ cardiac population (Fig. 1b). The large majority of the cells in the different clusters expressed MYL2 confirming that they are ventricular cardiomyocytes (Fig. 1c). Most of the MYL2+ cells expressed HEY2 a marker of the compact fate, indicating that a high proportion of the population is compact cardiomyocytes. A small subpopulation (cluster 4) of HEY2 negative cells expressed the trabecular marker NPPA suggesting that the day 20 population also contains some trabecular...
cardiomyocytes (Fig. 1c). More detailed analyses of the HEY2 positive clusters (1 and 2) and the NPPA expressing cluster (4) confirmed this lineage assignment (Supplementary Fig. 1a). HIF1A and CCND2, genes associated with compact myocardium, were expressed at higher levels in clusters 1 and 2 than in cluster 4 whereas SCN5A and IRX3 known to be preferentially expressed in trabecular myocardium (cluster 4) showed the opposite pattern (Supplementary Fig. 1b).

Pathway analyses of clusters 1, 2 and 4 identified a number of signaling pathways that were expressed at higher levels in the compact than in the trabecular cardiomyocytes (Fig. 1d, Supplementary Fig. 1c). Components of the Netrin 1 and Wnt pathways...
were among the most differentially expressed (Supplementary Fig. 1c). Given that Wnt ligands secreted by the epicardium are known to induce proliferation of compact cardiomyocytes in vivo4,23, we focused our efforts on investigating the role of this pathway in compact cardiomyocyte development from hPSCs. Additionally, as it has been reported that epicardial-derived IGF2 also induces compact layer proliferation in the developing heart4, we evaluated the role of this pathway in parallel. Using Ki67 as a measure of proliferation, we found that the highest proportion of Ki67+ cTNT+ cardiomyocytes was detected between days 12 and 14 of culture in the absence of any added cytokines (Supplementary Fig. 1d). Using this timeframe as an indication of the proliferative stage of ventricular development, we investigated the consequences of adding either the small molecule inhibitor of GSK3, CHIR (Wnt pathway agonist), IGF2 or both to the cultures between days 10 and 16 of differentiation (Fig. 1e). For comparison, we also treated cells with Neuregulin 1 (NRG1) to promote a trabecular fate28,29. The addition of either CHIR or IGF2 led to a significant increase in the proportion of Ki67+ cTNT+ cardiomyocytes as well as the total number of cardiomyocytes generated (Fig. 1f, g). The induction of Ki67+ cardiomyocytes and the increase in cell number (2-fold increase) were greatest when both factors were added together. Although the factors were maintained in the cultures until day 16 of differentiation, the proportion of Ki67+ cells dropped sharply between days 14 and 16, suggesting that the population becomes refractory to the proliferative effects of these cytokines. The addition of NRG1 during this timeframe led to a 1.3-fold increase in cell numbers. Inhibition of Wnt (XAV) in the control cultures (without CHIR or IGF2) led to a reduction in the proportion of Ki67+ cells suggesting that proliferation in the absence of exogenous stimuli is mediated through endogenous Wnt signaling (Fig. 1f, g). RT-qPCR analyses revealed that Wnt signaling was the main driver of the compact fate as demonstrated by the upregulation of expression of the compact marker MYCN and downregulation of the trabecular markers NPPA and BMP10 in the CHIR-treated population (Fig. 1h). Other compact markers including HEY2, TBX20 and FZD1 that were expressed at high levels in the pre-treated population were not impacted by the addition of CHIR and IGF2 (Fig. 1h, Supplementary Fig. 1e). In contrast to the effects of CHIR, the addition of NRG1 led to a downregulation of HEY2, TBX20 and FZD1 expression and an upregulation of the trabecular markers NPPA, BMP10, IRX3, NPPB and HAS2. Both populations expressed markers indicative of ventricular cardiomyocytes including cTNT, IRX4 and MYH6 (Fig. 1h, Supplementary Fig. 1e).

Immunostaining analyses confirmed the molecular profiles and showed that the untreated day 16 ventricular population was made up of a majority of HEY+ compact cardiomyocytes and a small fraction (~20%) of ANF (NPPA)+ trabecular cells (Fig. 1i, j, Supplementary Fig. 1f). The proportion of ANF+ cells was significantly reduced following treatment with CHIR/IGF2, indicating that activation of the Wnt/IGF2 pathways specified a population highly enriched in HEY2+ compact cardiomyocytes. By contrast, treatment with NRG1 efficiently promoted a trabecular fate as demonstrated by the presence of ANF+ cells and a lack of HEY+ compact cells (Fig. 1i, j, Supplementary Fig. 1f). Collectively, these findings show that it is possible to efficiently specify compact and trabecular fates from an immature ventricular cardiomyocyte population through staged (days 10–16) manipulation of the Wnt and NRG pathways.

**Induction of fatty acid oxidation in compact cardiomyocytes.** With access to enriched populations of compact cardiomyocytes, we were next interested in identifying the regulatory pathways that promote their maturation, reasoning that more mature cells may function better following transplantation into the adult infarcted heart and provide the appropriate target population for modeling cardiovascular disease in vitro. Following their proliferative stage, compact cardiomyocytes undergo a series of maturation steps that include a reduction in proliferation and a shift in energy metabolism from glycolysis to fatty acid oxidation (FAO). To promote the exit from cell cycle, we treated the developing cardiomyocyte population with the Wnt inhibitor XAV for 2 days given that our previous analyses showed that endogenous Wnt signaling can promote proliferation. As shown in Supplementary Fig. 2a, the addition of XAV following the CHIR/IGF2-induced proliferative stage significantly reduced the proportion of Ki67+ cells in the population.

To measure the competence of the XAV-treated population to undergo FAO, we first analyzed the day 18 compact cardiomyocytes for expression of the FA transporter CD36, essential for the efficient transport of FAs into cells30 (Supplementary Fig. 2b). As shown in Fig. 2a, very few cells within the day 18 population expressed CD36 consistent with the interpretation that they are metabolically immature. A small CD36+ population emerged following an additional 2 weeks of culture (day 32), suggesting that the cells are undergoing the initial stages of the metabolic transition to FAO in the absence of additional manipulations (Fig. 2a). To promote the metabolic switch in cardiomyocytes, we evaluated the effects of PPAR-related signaling, the response to steroid (dexamethasone) and thyroid (T3) hormones and the response to FA (palmitate), all of which have been shown to regulate FAO and mitochondrial function12,15,31,32. Palmitate is a common dietary fatty acid that can be incorporated into multiple fatty acid synthesis and oxidation pathways. RT-qPCR analyses showed that the day 10 and day 18 cardiomyocytes expressed PPARα as well as the thyroid hormone (THRA) and glucocorticoid receptors.
(NR3C1) suggesting that they should respond to these stimuli (Supplementary Fig. 2c). Addition of the PPARa agonist (GW7647, 1 μM) to day 18 cardiomyocytes led to a significant increase in the size of CD36+ population detected at day 32 of culture (Fig. 2a, Supplementary Fig. 2d). The size of CD36+ population increased further following the addition of palmitate (Pal) to the PPARa agonist-treated cultures (Fig. 2a, Supplementary Fig. 2d). Neither dexamethasone (Dex) nor thyroid hormone (T3) added alone with the PPARa agonist and palmitate impacted the size of the CD36+ population. However, the addition of both hormones with the PPARa agonist and palmitate did promote the development of a significantly larger CD36+ subpopulation, which represented ~50% of the total day 32 SIRPA+ cardiomyocyte population (Fig. 2a, Supplementary Fig. 2e).
The apparent use of endogenous FA as an energy source may be reflective of the pattern of FA usage in the neonatal heart, as the cells are transitioning from glycolysis to FAO. Immediately after birth, lipid droplets accumulate within the cardiomyocytes in the heart, likely serving as an endogenous lipid reserve as the cells undergo the switch to FAO. Transmission electron microscopy (TEM) analyses of the PPDT- and DT-treated cells showed that both populations contained structures resembling lipid droplets. Quantification of the lipid droplet size for each treatment group was performed using ImageJ software. The data were analyzed using one-way ANOVA with Tukey’s multiple comparisons test. All statistical analyses were performed using GraphPad Prism software. The results are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All experiments were performed in triplicate with three biological replicates. For all experiments, data are representative of at least three independent experiments.
membrane area measured by Nile Red staining revealed that these populations contained significantly larger positive areas than the untreated or Pal-treated cells (Fig. 2g). The presence of lipid droplets in these cells would support the interpretation that they have access to internal stores of FA and as such would provide an explanation as to why we did not observe differences in the OCR in the presence of palmitate (blue line) or BSA (green line).

Collectively, these findings indicate that the hPSC-derived mature compact cardiomyocytes undergo changes associated with the switch to FAO observed in the newborn heart, including the metabolism of long chain FAs, the storage of lipids and the upregulation of anti-oxidative stress genes to protect against the effects of oxidative stress.

**Transient activation of FAO improves metabolic profiles.** The lack of spare capacity in the PPDT-treated population suggests that the prolonged maturation stimulus may be inducing an abnormal hyperactive stressed phenotype in these cells. To determine if manipulation of the duration of treatment could impact metabolic function, we shortened the induction time from 2 weeks to 9 days and then maintained the cells in either Pal and PPARα, Pal alone or no factors for the remaining 5 days (Fig. 3a). These conditions were designed to mimic transient activation of the FAO pathway and the hormonal surge observed in the neonatal heart during the early postnatal adaptation period.16,38,39

Seahorse analyses revealed that cells maintained in Pal alone for the final 5 days of culture showed significant increase in maximal respiration and spare capacity (Fig. 3b3). Notably, we observed a large difference in OCR between cells in the presence of palmitate (blue line) and those in the presence of BSA (green line), indicating that the cells are competent to use an exogenous source of lipids for energy. The increase in spare capacity and the ability to use exogenous lipids was lost with the removal of palmitate, suggesting that the presence of long chain FAs is essential for maintaining the FAO state of the cells (Fig. 3b4). Quantification of the OCR curve parameters showed that cells treated for 9 days with PPDT and then cultured in Pal alone showed significantly higher maximal respiration and spare capacity compared to cells treated with the other combinations of factors (Fig. 3c). Nile Red staining revealed that the shortened induction time led to a reduction in the endogenous lipid stores in the cells compared to those subjected to continuous activation (day 18–32) (Fig. 3d). RT-qPCR analyses showed some differences between the populations subjected to continuous stimulus and those induced for 9 days followed by culture in Pal (transient) including a reduction in the expression levels of CD36, CPT1B, MLYCD and UCP2, genes involved in FAO (Supplementary Fig. 3). In contrast, the levels of mitochondrial related genes, such as ATP5A1, COX7A1, and CKMT2, were similar in the two populations (Supplementary Fig. 3). Together, these findings demonstrate that induction of hPSC-derived cardiomyocytes for 9 days with PPDT followed by 5 days of culture in Pal (PPDT/PAL) (Fig. 3e) promotes metabolic maturation of the cells yielding a population that displays a metabolic phenotype similar to that of postnatal cardiomyocytes including the ability to oxidize exogenous FAs. We will refer to these cardiomyocytes as ‘mature’ in the following studies.

**Structural and functional changes in mature cardiomyocytes.** Along with changes in metabolism, the mature cells showed differences in structural and functional properties compared to the immature cells. The mature cells were larger than those in the other groups and the population contained more bi-nucleated cells than the control or Pal treated populations (Fig. 4a–c). Transmission electron microscope (TEM) analyses showed that the mature cardiomyocytes had longer sarcomeres with more organized structure including detectable Z lines, I bands, and A bands and larger mitochondria with more defined cristae matrix than the control cells or those treated with the other combinations of factors (Fig. 4d–g, Supplementary Fig. 4a). Ca2+ transient analyses in the monolayer format using Fluo4 dye revealed that the mature cardiomyocytes as well as those treated with DT displayed improved Ca2+ handling capacity compared to the untreated control and the Pal-treated cardiomyocytes (Fig. 4h, i). As mature cardiomyocytes are quiescent, we next measured the proportion of Ki67+ cells in each population as an additional indication of maturation status. These analyses showed that the percentage of Ki67+ cells in each population as an additional indication of maturation status. These analyses showed that the percentage of Ki67+ cells in each population was significantly lower in mature and DT-treated populations than in the control or Pal-treated populations (Supplementary Fig. 4b). To determine if the day 32 populations contained cells that could still respond to proliferative signals, each was treated with CHIR and IGF2 for 2 days and then analyzed for the presence of Ki67+ cells (Supplementary Fig. 4c, d). As shown in Supplementary Fig. 4d, the untreated control, as well as the Pal- and DT-treated populations contained CHIR/IGF2 responsive cells. In contrast, no significant response was detected in the mature population, suggesting that these cells have lost their capacity to respond to these proliferative stimuli (Supplementary Fig. 4d).

Given the observed structural differences between the cells in the various populations, we next evaluated their contraction force using engineered ‘biowire’ cardiac tissues.40 For these studies, tissues generated with day 18 compact cardiomyocytes were treated with the different combinations of factors (no factors, Pal, DT or PPDT/PAL) for two weeks and then analyzed for contraction force as previously described. As shown in Fig. 4j, the contraction force of the PPDT/PAL-treated (mature) tissue was significantly higher than the non-treated control tissues or those treated with Pal or DT (Fig. 4j). TEM analyses showed that the cells in the mature tissues had more distinct, mature sarcomere structures than the cells in the tissues of the other groups (Supplementary Fig. 4e). Immunocytochemical analyses revealed the presence of comparable proportions of cTNT+ cardiomyocytes and CD90+ fibroblasts in the tissue constructs indicating that the differences in force are not due to dramatic differences in the proportion of these cell types (Supplementary Fig. 4f, g).

Collectively, the findings from this set of analyses show that the PPDT/PAL-treated cells display features of maturation that extend beyond a switch in metabolism and include changes in cell morphology, calcium handling capacity and force contraction.

To understand the role of FAO in cardiomyocyte maturation, we next tested the effect of inhibiting this metabolic pathway through the addition of etomoxir (ETO) to the cultures during the maturation process (from day 18 to day 32). RT-qPCR analyses of treated populations showed that the expression levels of the mitochondrial genes COXMT2, COX7A1, and COX6A2, the sarcomere genes MYL2 and MYOZ2 and the Ca2+ handling related gene ATP2A2 were significantly lower in the ETO-treated cells than in the control population (Fig. 4k), suggesting that FAO does promote aspects of cardiomyocyte maturation. However, not all the molecular changes associated with maturation were inhibited by the addition of ETO as the expression levels of other sarcomere (TCAP, DES, MYOM3), mitochondria (ATP5A1, ATP1A3), Ca2+ and ion channel (KCNJ2, HRC, CALM1)-related genes were not affected, indicating that the combination of PPDT promotes aspects of maturation independent of the switch to FAO.
scRNAseq analysis on the day 32 mature population and compared these profiles to those from the untreated age matched immature population (Supplementary Table 1). The mature and immature populations used for these analyses consisted of 92% and 85% cTNT+/MLC2V+ cells respectively (Supplementary Fig. 5a). UMAP analyses of the combined data set identified 5 distinct clusters, three of which expressed high levels of TNNT2 (cTNT) (clusters 0, 1, and 3), one enriched for extracellular matrix (ECM)-related genes (cluster 2) and one that expressed endoderm-related genes (cluster 4) (Fig. 5a, b, Supplementary Table 2). In addition to TNNT2 (cTNT), Cluster 3 also expressed smooth muscle-related genes (Fig. 5b, Supplementary Table 2). Cluster 0 expressed higher levels of genes associated with FAO (CD36, FABP3, ACSL1) and mitochondrial (CKMT2, COX6A2) and muscle function (DES, MYBPC3, ACTN2) than cluster 1 (Fig. 5c), confirming that cluster 0 represents the mature cardiomyocytes while cluster 1 contains the immature cardiomyocytes (Fig. 5d).
To elucidate differences between the cardiomyocytes in cluster 0 and 1, we performed Gene Ontology (GO) enrichment analysis using genes differentially expressed between these 2 clusters. This analyses showed that the mature cells (cluster 0) expressed higher levels of genes associated with FAO, mitochondrial function, muscle contraction and sarcomere organization than the immature cardiomyocytes (cluster 1), further confirming that the PPDT/PAL treatment induces broad maturation changes within the cells (Fig. 5e). Analyses of a curated list of well-characterized genes from these different groups showed higher levels of expression of most in the mature compare to the immature cells (Supplementary Fig. 5b). Several notable exceptions were observed. The first is in the switch in the TNNI1/3 isoforms known to occur during cardiomyocyte maturation. While our mature cells showed lower levels of the fetal form TNNI1 than the immature cells, the levels of the adult isoform, TNNI3, were comparable in the 2 populations. The reason for this is currently not clear, as the mature cells have upregulated many other sarcomere genes. The second is in the patterns of MYH6 and MYH7 expression that are opposite of what one would expect in mature and immature cells. This reverse pattern may be due to the presence of T3 in our maturation cocktail as this hormone has been shown to induce MYH6 and inhibit MYH7 expression.

To define a molecular signature for the metabolically mature cells, we identified genes whose expression patterns are highly discriminatory (on/off) between the populations. This list of genes includes CD36 as well as several lipid metabolism genes (NMRK2, ACSL1, SCD, MASP1, TP53INP2) and adipogenesis-related transcription factors (KLF9, CEBPB) (Fig. 5f, asterisk*). Gene Set Enrichment Analysis (GSEA) between the two populations revealed that the TGACCCTG regulatory motif, which is reported to be a binding site of ESRRA (Estrogen-related receptor A)45, was highly enriched and overlapped with FA metabolism and oxidative phosphorylation genes in the mature cells (Fig. 5g, Supplementary Fig. 5c). Consistent with these findings, expression analysis showed that the mature cardiomyocytes had higher levels of ESRRA than the immature cells (Fig. 5h). In contrast, the expression levels of ESRRG, another member of this receptor family were lower in the mature cells (Supplementary Fig. 5d).

More detailed analyses of the mature cardiomyocyte population (cluster 0) revealed heterogeneity that resolved into 4 distinct clusters (Fig. 6a, Supplementary Table 3). The major distinguishing features of these subpopulations were the expression of stress-related genes, ATF5 and TRIB3 in cluster B, genes indicative of proliferation (MKI67 and FOXM1) in cluster C and extracellular matrix related genes such as FN1 and COL3A1 in cluster D (Supplementary Fig. 6a). These findings indicate that the mature population consists of a large subpopulation of mature, non-proliferating, non-stressed cardiomyocytes (cluster A), along with a subpopulation of proliferating cells (cluster C), a small subpopulation of contaminating fibroblasts (cluster D) and a subpopulation of stressed cells (cluster B). GO analysis using differentially expressed genes in each cluster revealed that muscle stress fiber-related genes and cholesterol import-related genes were upregulated in cluster A, while ER stress-related genes including those of the CHOP-C/EBP complex and the CHOP-ATF4 complex were upregulated in cluster B (Fig. 6b). Further analyses identified a number of genes that were expressed at higher levels in cluster A than in the other clusters including the surface markers CD36 and LDLR (Fig. 6c), the cytokine FGF12, the enzyme ASB2, as well as those annotated to contraction, Ca2+ handling, extracellular matrix production, metabolism, and several with unknown function in the context of cardiomyocytes (Supplementary Fig. 6b, Supplementary Table 3). To confirm the differences in LDLR at the protein level, we used flow cytometric analyses to monitor its cell surface expression and compared it to that of CD36 (Fig. 6d, Supplementary Fig. 6c). Neither marker was detected at day 18 and only small subpopulations of positive cells were present in the day 32 immature population. In contrast, more than 50% of the mature population expressed both markers at day 32. CD36+LDLR+ cardiomyocytes were already detected at day 25 at which point they represented ~35% of the maturing population. Taken together, the findings from these flow cytometric analyses show that expression of CD36 and LDLR are upregulated as the ventricular cardiomyocytes undergo metabolic maturation and as such are ideal markers to monitor these changes.

Our observation that ESRRA expression is upregulated in the mature cardiomyocytes is in line with a recent study showing that this receptor along with ESRRG functions to regulate postnatal cardiac maturation in the mouse46. Given this, we were next interested in determining if ESRRA plays a role in the maturation of the hPSC-derived cardiomyocytes under the conditions used here. To address this, we first tested the different components of our maturation cocktail to determine which was responsible for inducing ESRRA expression. As shown in Fig. 6e, the combination of Dex and T3 appears to be the main driver of ESRRA expression in the mature cardiomyocytes. PPARa agonist did not induce ESRRA expression, highlighting important differences in the effect of these stimuli. We next performed siRNA-mediated knockdown of ESRRA to determine if it plays any role in the maturation of the human cardiomyocytes. For these studies, a mixture of three different siRNAs specific for ESRRA (siESRRA) or a non-targeting siRNA were introduced into day 18 cells. The treated cells were aggregated for 2 days and the aggregates were then cultured for 14 days in our maturation cocktail. Treatment with the siESRRA reduced the levels of ESRRA expression to those observed in the immature population (Fig. 6f). This inhibition of ESRRA expression resulted in lower levels of expression of a subset of FAO (FABP3, ACSL1) and sarcomere (MYL2, TCAP)-related genes compared to the control cells (Fig. 6g), indicating that ESRRA is involved in aspects of metabolic and structural maturation. We also observed an
increase in Nile red staining and a downregulation of expression of the hormone sensitive lipase \( HSL \) that functions to release FAs from lipid droplets in the ESRRA KD cells (Fig. 6h, i). These findings suggest an increase in lipid storage indicating that ESRRA also plays a role in the utilization of endogenous lipids. While these differences were detected, the levels of many other genes including \( ESSRG \) and \( PPARA \) (Supplementary Table 4) were not impacted by the downregulation of ESRRA suggesting that this pathway is only part of regulatory machinery that controls cardiomyocyte maturation.

To demonstrate that the protocol described here is applicable across hPSC lines, we used it to promote maturation of cardiomyocytes generated from the hESC line ESI-17. As the efficiency of cardiomyocyte differentiation was not as high as that...
of the HES2 line, we isolated the SIRPA⁺ cells from the day 32 mature and immature populations for the molecular analyses (Supplementary Fig. 7a, b). Flow cytometric analysis showed that the ESI-17 derived mature cardiomyocytes showed a significant upregulation of CD36 and LDLR compared to the immature population (Supplementary Fig. 7c, d). Consistent with these changes, our RT-qPCR analyses revealed that the mature cells expressed significantly higher levels of sarcomere, FAO, mitochondria, Ca²⁺ handling, and ion channel-related genes compared to the immature control cells, while the expression level of HCN4, the pacemaker current gene, was lower in the mature cells (Supplementary Fig. 7e). Notably, we found that the expression levels of our maturation signature genes (including CD36) were also upregulated in the ESI17-derived mature cardiomyocytes verifying their utility as markers of cardiomyocyte maturation (Supplementary Fig. 7e). These findings clearly show that our maturation protocol can be applied to cardiomyocytes from different cell lines.

Metabolic maturation of atrial cardiomyocytes. To determine if the above strategy could be used to promote maturation of other cardiomyocyte subtypes, we treated day 18 atrial cells generated with our previously published protocol17 with the combination of PPDT/PAL for 14 days. Both the untreated and treated day 32 populations expressed atrial specific genes, including KCNA5, KCNJ3, GJA5 (CX40) and NR2F2 (COUPTF2), but no MYL2 (MLC2V), indicating that they consisted predominantly of atrial cells with few, if any, contaminating ventricular cardiomyocytes (Supplementary Fig. 8a, b). TEM analyses showed improved sarcomere structure and increased sarcomere length in the PPDT/PAL-treated (mature) atrial cells compared to the non-treated controls (Fig. 7a, b). PPDT/PAL treatment also led to an increase in mitochondrial size in the atrial cardiomyocytes, although the overall size did not reach that found in the mature ventricular cells (p < 0.01 compared to the size in Fig. 4g) (Fig. 7c, d). As observed with the ventricular lineage cells, the PPDT/PAL-treated mature atrial cells also upregulated CD36, however the proportion of positive cells was somewhat lower than observed in the ventricular cardiomyocyte population (Fig. 7c, f). Molecular analyses revealed that PPDT/PAL treatment led to an upregulation of expression of genes associated with the metabolic switch (CD36, FABP3, CPT1B, MLYCD) and mitochondrial activity (ATP5A1, COX7A1, CKMT2) (Fig. 7g, Supplementary Fig. 8c). The treated cells also showed higher levels of KCNJ2, an ion channel gene, and TCAP, while HCN4 showed an opposite pattern and was found at lower levels in the mature than in the immature cells (Supplementary Fig. 8d). OCR measurements using the Seahorse assay showed that basal respiration, ATP production, proton leak, and maximal respiration were enhanced by the PPDT/PAL treatment of the atrial cardiomyocytes, although the levels of most of these parameters were significantly lower than those in mature ventricular cells (compared to the value in Fig. 3c) (Fig. 7h, i). As observed with the ventricular cells, ETO (red line) blocked the mitochondrial oxidation, indicating that these atrial cardiomyocytes are also dependent on FAO. Together, these findings demonstrate that the factors that regulate maturation of the ventricular lineage cells also promote maturation of hPSC-derived atrial cells.

Modeling pathological changes in mature cardiomyocytes. It is well established that heart failure is characterized by distinct changes in myocardial metabolism, including a switch from primarily FA oxidation to glycolysis47. These metabolic changes are associated with increased concentrations of FA and lipid accumulation in the form of lipid droplets that, when excessive can lead to cardiomyocyte death48. Given that the mature cardiomyocytes induced with PPDT/PAL have acquired the capacity to undergo FAO, they should provide a platform for modeling some of the above metabolic (and lipid accumulation) changes associated with heart failure. As hyperstimulation of the sympathetic adrenergic system is a characteristic of heart failure and activation of the pathway is a well establish pathological stimuli, we analyzed our mature cardiomyocytes for expression of adrenergic receptor B1 (ADRB1), known to bind adrenaline and mediate responses in the adult heart. RT-qPCR analysis showed that the mature cardiomyocytes express significantly higher levels of ADRB1 than those in the immature hPSC-derived cells (Supplementary Fig. 9a) indicating that they should be able to respond to appropriate stimuli.

To induce a pathological response in the mature cardiomyocytes we cultured them in the presence of isoproterenol (100 uM), a small molecule adrenergic agonist in a hypoxic (5% O2) environment, in media containing low glucose (2.0 g/L) and Palmitate (200 uM) for 6 days (Supplementary Fig. 9b). Immature control cells were treated under the same conditions. This treatment induced the upregulation of expression of the glycolysis-related genes GLUT1, GAPDH, and LDHA in the mature cardiomyocytes suggesting that they are undergoing a switch in metabolism (Fig. 8a). The immature cells showed similar changes in GLUT1 and LDHA expression (Fig. 8a). To assess the glycolytic flux in these cells, we measured the extracellular acidification rate (ECAR) as an index of glycolytic activity using the Seahorse XF assay. Mature cardiomyocytes exposed to the pathological stimuli showed a rapid increase in
Fig. 5 Single cell RNA sequencing analyses of mature compact cardiomyocytes. a UMAP plot of the combined data set of day 32 immature and mature cardiomyocytes showing 5 different clusters. UMAP plots of the expression patterns of representative genes in the different clusters (b) and of the expression patterns of representative genes found at higher levels in cluster 0 than the other clusters (c). d UMAP plot indicating the lineage composition of the 5 different populations. e Violin plots of the Gene Ontology (GO) analyses comparing the mature (cluster 0, red) to the immature cardiomyocytes (cluster 1, green). f Heatmap visualization of marker genes (left) and transcription factors (right) using a binary enrichment search. *maturation signature genes. g Enrichment score plot of the TGACCTTG_SF1_Q6 gene set. h Violin plot of ESRRα expression in the immature (cluster 1) and mature (cluster 0) cardiomyocytes.
**Fig. 6** Detailed molecular analyses of the mature cardiomyocyte population.  

**a** UMAP plot of mature cardiomyocytes showing 4 different clusters.  
**b** Violin plots of the Gene Ontology (GO) analysis comparing expression patterns in the indicated clusters.  
**c** UMAP plot showing expression patterns of CD36 and LDLR.  
**d** Representative flow cytometric analyses of CD36 and LDLR expression in the immature and mature cardiomyocytes on the indicated days of differentiation.  
**e** RT-qPCR expression analyses of ESRRA in day 32 cardiomyocytes cultured in the indicated conditions (N = 4).  
**f** RT-qPCR expression analyses of ESRRA in the immature, mature, and mature ESRRA knock down cardiomyocytes (KD mature) (N = 10).  
**g** RT-qPCR expression analyses of FAO (FABP3, ACSL1) and sarcomere (MYL2, TCAP)-related genes in the immature, mature, and KD mature cardiomyocytes (N = 10).  
**h** Flow cytometric analyses of Nile Red staining in the indicated cell populations (N = 4).  
**i** RT-qPCR expression analyses of HSL in the immature, mature, and KD mature cardiomyocytes (N = 10).  

All statistical analyses were performed by one-way ANOVA with Tukey’s multiple comparisons (**p < 0.0001). Data are presented as mean values ± SEM. Fetal LV and adult LV tissues were included as a reference for in vivo expression in the RT-qPCR analyses. Fetal LV: Fetal left ventricular tissue (N = 3–4), Adult LV: Adult left ventricular tissue (N = 4).
ECAR after the injection of glucose, and a rapid decrease in ECAR following the injection of 2-deoxy-glucose (2-DG), an inhibitor of glycolysis (Fig. 8b). This response was not detected in the untreated mature cardiomyocytes indicating that glycolytic activity was significantly upregulated by the pathological stimuli. Exposure to the pathological stimuli also enhanced glycolysis in the immature cells (Fig. 8b), which might be expected as these cells are dependent on glucose metabolism.

In addition to changes in metabolism, we also detected increases in lipid accumulation in the treated populations as demonstrated by Nile red staining (Fig. 8c). Expression of genes associated with triacylglycerol synthesis, CD36 and GPD1 as well as Perilipin 2 (PLIN2) that plays a role in abnormal lipid
Fig. 7 Maturation of atrial cardiomyocytes. a Representative TEM images of sarcomere structures in the control (immature) and PDPT/PAL treated (mature) atrial cardiomyocytes. Scale bar; 1 µm. b Quantification of sarcomere length (based on TEM analyses) in immature and mature atrial cardiomyocytes (N = 49 cells from control atrial and 74 cells from mature atrial examined over 3 independent experiments). c Representative TEM images of mitochondria structure in control and mature atrial cardiomyocytes. Scale bar; 1 µm. Correspondingly, expression of cytotes cultured under the pathological stimuli (Fig. 8a, d). Analyses of the treated mature cardiomyocytes (Fig. 8d). Analyses of CASP9 expression were performed by two-sided unpaired t-test (**p < 0.001). Data are presented as mean values ± SEM. Fetal RA: Fetal right atrial tissue (N = 2).

Fig. 8 Modeling pathological adaptation using mature compact cardiomyocytes. a RT-qPCR expression analyses of glycolysis-related and TAG synthesis-related genes in untreated and hypoxia+ISO-treated immature and mature ventricular cardiomyocytes (N = 5). b Left: Representative kinetics of the oxygen consumption rate (OCR) measured with the FAO Cell Mito stress test assay in control and mature atrial cardiomyocytes. Right: Quantification of glycolysis based on ECAR measurement with the seahorse XF assay in the indicated cardiomyocyte populations (N = 3 biologically independent experiments). c Flow cytometric analyses of Nile Red staining in the indicated populations (N = 7). d RT-qPCR expression analyses of PLIN2 and HSL and e CASP9 in the indicated populations (N = 5). f Flow cytometric-based quantification of the proportion of Annexin V + cells in untreated and hypoxia+ISO-treated immature and mature ventricular cardiomyocyte populations (N = 7). All statistical analyses were performed by two-sided unpaired t-test (****p < 0.0001). Data are presented as mean values ± SEM. ns not significant.

accumulation were only upregulated in the mature cardiomyocytes cultured under the pathological stimuli (Fig. 8a, d). Correspondingly, expression of HSL was downregulated only in the treated mature cardiomyocytes (Fig. 8d). Analyses of expression of the apoptosis-related gene CASP9 and the proportion of Annexin V + cells revealed that isoproterenol/hypoxia treatment induced apoptosis in the mature but not in the immature population (Fig. 8e, f, Supplementary Fig. 9c). Taken together, these findings show that it is possible to model pathological responses in the mature cardiomyocytes and that
these cells recapitulate changes associated with heart failure including activation of glycolysis, lipid accumulation and apoptosis, as summarized in Supplementary Fig. 9d.

**Engraftment of mature and immature cardiomyocytes.** To determine if the maturation status of the cells can influence their ability to engraft heart tissue in vivo, we transplanted both the mature and immature populations into infarcted nude rat hearts and analyzed the graft quality by immunohistology at 2 and 8 weeks post-transplantation. Freshly prepared mature and immature cells were used for these studies as the mature cardiomyocytes did not survive the cryopreservation process well. Grafts containing cTNT+ cardiomyocytes were detected in most of the transplanted rats at both time points (5 out of 6 rats at 2 weeks, 8 out of 9 rats at 8 weeks in mature cell transplantation group and 8 out of 8 rats at 2 weeks, 8 out of 8 rats at 8 weeks in immature cell transplantation group, respectively) (Fig. 9a, Supplementary Fig. 10a). At 2 weeks post transplantation, the size of the grafts generated by the two cardiomyocyte populations was comparable (Fig. 9a, b). Detailed analyses showed that the cardiomyocytes in the grafts from the mature cells had significantly
longer sarcomeres than those in the immature grafts (Fig. 9c, d). Additionally, the grafts from the mature cells contained fewer proliferating cells as measured by Ki67 and phosphorylated histone H3 (pH3; a mitotic marker) and more connexin 43 (CX43) protein than those derived from the immature cells (Fig. 9e–h, Supplementary Fig. 10b, c). Differences in CX43 were already detected in the populations prior to transplantation as the day 32 mature embryoid bodies (EBs) showed higher levels of CX43 message and contained more CX43 protein than immature EBs (Supplementary Fig. 10d, e). The presence of CX43 is important as it is essential for the formation of gap junctions and electrical integration in the heart. One of the commonly used measures of cardiomyocyte maturation is the switch from the fetal to the adult isoform of the sarcomeric protein TNNI42. Analyses of TNNI expression showed that the cells in the 2-week grafts from both cell populations expressed only the fetal isoform (TNNI1) indicating that this aspect of cardiac development was not fully mature at this time point (Supplementary Fig. 10f). At 8 weeks following transplantation, the grafts from the immature cells were modestly larger than those generated from the mature populations (Fig. 9b). The cells in both the mature and immature grafts had further matured between 2 and 8 weeks as demonstrated by changes in most of the above parameters (Fig. 9a–h). Although the changes were most pronounced in the immature cell-derives grafts, the cells in the mature grafts still showed a more mature profile at the 8-week time point as demonstrated by the presence of longer sarcomeres, a lower proliferative index and higher levels of CX43 than those in the immature grafts (Fig. 9a–h, Supplementary Fig. 10b, c). Despite the increased levels of CX43 protein, it remained distributed throughout the graft and was not aligned at the cell-cell junctions as observed in the host rat myocardium indicating that human tissue was not yet fully mature (Supplementary Fig. 10g). The 8-week immature grafts contained a higher percentage of Ki67 and pH3-positive cardiomyocytes than the mature grafts, an observation that could account for their larger size. Cells in both the mature and immature 8-week grafts upregulated expression of TNNI3 the adult isoform of TNNI. However, both populations also continued to express the fetal isoform (TNNI1) demonstrating that this aspect of maturation was not yet complete (Supplementary Fig. 10f).

Collectively, these findings show that the mature cardiomyocytes generated more mature grafts than the immature cardiomyocytes, providing evidence that the maturation status of the transplanted cells can impact the quality of the graft.

Discussion
In this study, we used insights from our understanding of cardiomyocyte development and maturation in the fetus and neonate to design a staged protocol to generate mature compact cardiomyocytes from hPSCs. Using flow cytometric analysis of CD36 expression as a primary screen for maturation, we found that the combination of a PPARα agonist, dexamethasone, T3 and palmitate in media containing low glucose was most effective at inducing a mature phenotype in the hPSC-derived ventricular cardiomyocytes. These regulators were selected, as the levels of PPAR signaling as well as the concentrations of T3 and corticosteroids increase dramatically in different model organisms and in humans during the perinatal period. Additionally, genetic studies have shown that signaling through the PPAR pathway as well as responsiveness to hormones and lipids regulate cardiomyocyte maturation in vivo12–16,38,39. A number of previous studies have addressed the question of metabolic maturation of hPSC-derived cardiomyocytes and have shown that treatment with different combinations of the above stimuli did promote some degree of maturation in vitro19–22,33. While changes were well documented, comparisons of the effect of the manipulations and the degree of maturation between studies is difficult as quantitative analyses were not used to evaluate entire populations. Through our use of flow cytometric analyses, we were able to compare different combinations of stimuli and show that PPDT was significantly more efficient than DT which in turn was more efficient than Pal alone at generating CD36+ cells. Beyond upregulation of CD36, the PPDT-treated cells expressed higher levels of key metabolic genes, were larger, had longer sarcomeres, greater mitochondrial mass and showed better contraction force than those treated with either DT or Pal alone. A summary of the differences observed in the cardiomyocytes induced under these different conditions is shown in Table 1. The correlation of CD36 expression with other parameters of maturation validates the use of this approach for quantifying maturation in populations of cardiomyocytes. This notion is supported by findings from a recent study which showed that CD36+ cardiomyocytes isolated from long-term cultured hPSC-derived cardiovascular populations displayed more mature properties than the CD36− cells51.

For maturation protocols to be most useful, they need to be evaluated and optimized on defined cardiomyocyte populations. Previous studies with hPSCs have not used well characterized populations and as a consequence it is not known if the protocols described to date promote maturation in different subsets of cardiomyocytes. Here, we developed and optimized our PPDT/PAL protocol using compact ventricular cardiomyocytes that were specified through activation of the Wnt and IGF pathways known to be required for proliferation and expansion of the compact lineage in vivo5,7. When tested on atrial cardiomyocytes, PPDT/PAL also promoted their maturation, albeit to a lesser extent than observed in the ventricular cells. The smaller changes...
observed in the atrial cells may reflect the fact that atrial cardiomyocytes display lower metabolic activity than ventricular cardiomyocytes.

Our Seahorse analyses showed that although the cells treated with either PPDT or DT for 2 weeks were capable of undergoing FAO, they were not dependent on exogenous FAs, indicating they were using an endogenous source of lipids. The distinction between exogenous and endogenous lipid use is important as the ability to oxidize exogenously supplied lipids can be considered as the final step in metabolic maturation. The endogenous lipid source in the cells treated with either PPDT or DT in our study is likely the lipid droplets. Similar droplets have been detected in neonatal cardiomyocytes suggesting that cells in the heart rely on an endogenous source of lipids as they undergo the transition from glycolysis to FAO. Our findings strongly suggest that the hyperactive phenotype observed in the 14-day PPDT/PAL-treated cells is likely due to prolonged hormonal exposure as restriction of the duration of PPARα/T3/Dex treatment led to the development of a population that displayed spare capacity and the ability to oxidize exogenous FAs. This restriction in the timing of treatment more accurately recapitulates the transient perinatal spikes in the levels of T3 and corticosteroids observed in vivo during the first several weeks of life. Increases in FAO, mitochondrial biogenesis and ROS collectively have been shown to inhibit cardiomyocyte proliferation. Similar inhibition may be occurring in vitro, as the PPDT/PAL treated population contained very few proliferating cells and was refractory to the proliferative stimuli provided by CHIR and IGF2. Combined with their increase in size, these characteristics suggest that these cells are transitioning from the hyperplasia stage (fetal) of cardiovascular development when increases in heart size result from proliferation to the hypertrophy stage (postnatal) when increases in size are due to increases in the size of the cardiomyocytes.

The findings from our scRNAseq analyses showed that the majority of cells in the population upregulated genes indicative of metabolic and structural maturation supporting the utility of CD36 analysis as a method to track population-wide responses to the stimuli. The demonstration that the mature cells upregulate LDLR and that its expression can be measured by flow cytometry provides a second surface marker for monitoring cardiomyocyte maturation in the differentiation cultures. Although broad changes in maturation were observed in the PPDT/PAL-treated cells they failed to undergo the switch in expression of the TNNI isoforms in vitro and only upregulated expression of the adult form following transplantation in vivo. One interpretation of these findings is that the upregulation of TNNI3 expression requires mechanical force and/or other factors that are not be provided in the EBs in vitro. The observation that the mature cells show increased expression of ESRRA is consistent with previous studies that have shown an upregulation of TGGACCTGT/ESRRA during the fetal to postnatal transition and with the recent findings of Sakamoto et al. that demonstrated that this receptor, together ESSRG is required for the maturation of mouse cardiomyocytes in vivo. Although the function of these receptors is not completely understood, evidence suggests that ESSRR interacts with PGC1α/PPARA to induce global metabolic changes and structural maturation. Our KD studies showed that ESRRA plays some role in maturation of the human cardiomyocytes but also indicated that other pathways are involved in this process. The finding that PPARα signaling failed to induce ESRRA expression and that the levels of PPARα were not reduced in the KD cells, suggests that under the conditions used in our study, these receptors are not regulating each other, but are likely functioning in parallel. Our global analyses of HES2-derived cells identify a set of genes that can be used as a molecular signature of metabolically mature ventricular cardiomyocytes. The demonstration that the ESI-17-derived mature cardiomyocytes upregulated the genes in this signature verifies their utility for monitoring the maturation status of cardiomyocytes produced from different hPSC lines as well as for comparing populations generated with different protocols in different labs.

One of the goals of generating mature cardiomyocyte populations is to establish platforms to model and treat cardiovascular diseases with target cell populations that approximate those found in the adult heart. To date, most disease modeling studies have used immature cells that may not accurately recapitulate the complex disease processes observed in the adult. The observation that the PPDT/PAL matured cells progressed to a ‘disease phenotype’ in response to culture conditions that mimic the environment of the failing heart indicates that the maturation protocol developed here gives rise to cardiomyocytes that can be used to study cardiac pathologies beyond those resulting from genetic disorders. The ability to induce maturation in engineered biowire tissues demonstrates that this approach can also be used to model disease in 3D cardiovascular tissue; a format that enables the study of the role of different cell types in the disease process. Transplantation of hPSC-derived cardiomyocytes represents a promising cell therapy to remuscularize the human ventricle following MI. Studies with large animals have shown that following transplantation, immature cardiomyocytes will engraft the infarct induced scar and generate myocardium that integrates with the host tissue. However, in all cases, the animals showed ventricular arrhythmias for the first few weeks following transplantation, possibly due to the immature nature of the transplanted cells. The finding that the metabolically mature cardiomyocytes generate grafts that contain higher numbers of connexin 43+ cells, fewer proliferating cells and more structurally mature cells than those from the immature cardiomyocytes suggests that manipulation of the population prior to transplantation may be one approach to mitigate the arrhythmias and develop a safer therapy. As the protocol in this study was designed for maturation of cardiomyocytes in EBs in suspension culture, it is easily scalable for the production of the large number of cells required for transplantation.

### Methods

**Directed differentiation of hPSCs.** For ventricular differentiation, we used a modified version of our embryoid body (EB)-based protocol. hPSC populations (HES2, ESI-17) were dissociated into single cells (TrypLE, ThermoFisher) and
re-aggregated to form EBs in StemPro-34 media (ThermoFisher) containing penicillin/streptomycin (1%, ThermoFisher), L-glutamine (2 mM, ThermoFisher), transferrin (150 mg/ml, ROCHE), ascorbic acid (20 mg/ml, Sigma), and mono-thioglycoller (50 mg/ml, Sigma). ROCK inhibitor Y-27632 (10 µM, TOCRIS) and rhBMPI4 (1 ng/ml, R&D) for 24 h on an orbital shaker (70 rpm). Day 1, the EBs were transferred to mesoderm induction media consisting of StemPro-34 with the above supplements (ROCK inhibitor Y-27632 and rhBMPI4) and stained with unconjugated primary antibodies in FACS buffer overnight using 90% methanol for 20 min at 4 °C. Cells were washed with PBS containing 5% fetal calf serum (FCS) (Wisent) and 0.02% sodium azide. For intracellular staining, cells stained for 30 min at 4 °C in FACS buffer consisting of PBS with 5% fetal calf serum (Jackson ImmunoResearch, 1:500). For cell-surface marker analyses, cells were goat anti-mouse IgG-APC (BD PharMingen, 1:500), or donkey anti-rabbit IgG-PE primary antibodies, the following secondary antibodies were used for detection: mouse anti-mouse IgG-APC (BD PharMingen, 1:500) or donkey anti-rabbit IgG-Alexa 555 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa 488 (ThermoFisher, 1:500), mouse anti-human cTNT (ThermoFisher Scientific, 1:200), rabbit anti-human CD90 (Abcam, 1:100), rabbit anti-human cTNT (Abcam, 1:200), or rabbit anti-human CD90 (Abcam, 1:100). For detecting unconjugated primary antibodies, the following secondary antibodies were used: donkey anti-mouse IgG-Alexa 488 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa 555 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa 488 (ThermoFisher, 1:500), donkey anti-mouse IgG-Alexa 555 (ThermoFisher, 1:200), mouse anti-human cTNT (Abcam, 1:100), rabbit anti-human cTNT (Abcam, 1:200), or rabbit anti-human CD90 (Abcam, 1:200). For detecting unconjugated primary antibodies, the following secondary antibodies were used: donkey anti-mouse IgG-Alexa 488 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa 555 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa 488 (ThermoFisher, 1:500), donkey anti-mouse IgG-Alexa 555 (ThermoFisher, 1:200). Cells were stained with primary antibodies in staining buffer consisting of PBS with 0.1% TritonX, 5% and 0.02% sodium azide. For intracellular staining, cells were fixed for 20 min at 4 °C with 4% PFA in PBS followed by permeabilization using 90% methanol for 20 min at 4 °C. Cells were washed with PBS containing 5% FCS and stained with unconjugated primary antibodies in FACS buffer overnight at 4 °C. Stained cells were washed with PBS with 5% FCS and stained with secondary antibodies in FACS buffer for 30 min at 4 °C. Culture media was changed to glycolysis assay media and the XF Cell Mito Stress Test was run. After the measurement of OCR, EBs were dissociated and the cell number in each well was counted. OCR was normalized per 10,000 cells. For the glycolysis assay, a few EBs were similarly prepared onto a XFe24 cell culture microplate. Glucose (Sigma) and 2-DG (Sigma) were prepared and loaded into the assay cartridge (final concentration: glucose 10 mM, 2-DG 100 mM). Culture media was changed to glycolysis assay media and the XF cell Culture Microplate was immediately inserted into the Seahorse XFe Analyzer and the XF Cell Mito Stress Test was run. After the measurement of OCR, EBs were dissociated and the cell number in each well was counted. ECAR was also normalized per 10,000 cells. Data were analyzed using Wave software (Aglent).

Ca2+ transient measurement. For the Ca2+ transient measurement, the EBs were dissociated into single cell at day 30 and re-plated at a concentration of 2 x 10^6 cells onto 3.5 cm culture dishes coated by Matrigel. We cultured the cells in the monolayer format for 3 days in the different media and then loaded them with Flu-4 (Invitrogen, final concentration; 4 µM) for 30 min at 37 °C on the day of the measurement. Prior to analyses, the cells were washed with culture media and then incubated in the same medium containing additional 30 µM at 37 °C. At the point at which the cells are transferred to Tyrode buffer at 37 °C and then analyzed. For imaging, a Zoom microscope body MXV10 with the objective MVLPLA 0.63X (NA 0.15, WD 87 mm, FN 22, Olympus) for an overall FOV of 10 mm x 10 mm was used with the 10x objective. CA2+ depolarization/repolarization through time. Data were collected from 8 to 20 samples in each condition.

scRNA sequencing and analysis. The EBs were dissociated as described above and the cells were stained with DAPI. DAPI+ live cells were then sorted using FACSAriaRITT (BD Pharamingen) at the Sickkids/UHN flow cytometry facility. After the live cell sorting, scRNA sequencing was performed using the 10x Genomics platform, sequenced on Illumina NovaSeq 6000, and analyzed using GRCh38 (hg38) as follows. Single-cell RNA sequencing of day 20 ventricular cardiomyocytes were first filtered to remove lowly expressed genes (defined as found in less than 3 cells) and damaged cells with high mitochondrial genome transcript content (defined as 12 median absolute deviations above the median to account for the typically high mitochondrial content in cardiomyocytes). The data set was then normalized using the DeNovo method implemented in scran (Robinson et al, 2010) method in the scran R package (Robinson et al, 2010). At this stage, each median standard deviations were similarly minimal and thus would not contribute significantly to resolving variances between cells in downstream analyses. All cells were transcribed into cdNA using oligo (dT) primers and random hexamers and iscript Reverse Transcriptase (ThermoFisher). qRT-PCR was performed on an EP Real- Time Mastercycler (Eppendorf) with a Quantifast SYBR Green PCR kit (QIA- GEN). The copy number of each gene relative to the house keeping gene TBP is shown. Primer sequences are listed in Supplementary Table 5. Fetal heart tissues were included as a reference for in vivo expression. Informed consent was obtained from the participants.

Transmission Electron Microscope (TEM). The samples were fixed in 2.5% glutaraldehyde in PBS, rinsed and post-fixed in 1% O04 (Electron Microscopy Sciences) for 1 h. The tissue was again rinsed with 0.1 M Sorensen’s Phosphate buffer, dehydrated through an ascending ethanol series, then infiltrated with and embedded in modified Spurr’s resin. From the area of interest, identified by thick sectioning, ultrathin sections (90–100 nm) were cut with a Leica UC6 ultra microtome (Leica). Thin sections were stained with Uranyl acetate and lead citrate and then examined using a Hitachi HT7700 transmission electron microscope (Hitachi). Analysis were performed from 3 to 5 independent experiments.

Seahorse OCR / ECAR measurement. For the Seahorse XF FAO assay, a few EBs were plated onto an XFe24 cell culture microplate coated by Matrigel 48 h prior to the assay. 24 h prior to the assay, we replaced the culture media with substrate-limited medium containing 0.5 mM glucose (Sigma), 1.0 mM Glutamine (Life technology), 0.5 mM Carnitine (Sigma), and 1% Fetal Bovine Serum (Wisent) in DMEM no glucose media (ThermoFisher). 45 min prior to the assay, we washed the cells the two times with FAO assay medium, added 375 µl/well FAO assay medium to the cells and incubated for 45 min at 37 °C. We loaded the assay cartridge with XF FAO assay media (3 µM Mito Stress Test cocktail, 5 mM FCCP, 0.5 µM rotenone/0.5 µM antimycin A). 15 min prior to starting the assay, we added 37.5 µl etomoxir (Sigma, 40 µM) or vehicle to each well and then incubated them for 15 min at 37 °C in a non-CO2 incubator. Just prior to starting the assay, we added 87.5 µl XF Palmitate-BSA FAO Substrate or BSA to the appropriate wells. The XF cell Culture Microplate was immediately inserted into the Seahorse XF Analyzer and the XF Cell Mito Stress Test was run. After the measurement of OCR, EBs were dissociated and the cell number in each well was counted. ECAR was also normalized per 10,000 cells. Data were analyzed using Wave software (Aglent).
were then iteratively clustered in Seurat 2.0\textsuperscript{60} at increasing resolutions until the specified enrichment ratio was reached or the maximum resolution was reached. Differentially expressed genes were discovered using the scanpy.tl.findMarkers function with a resolution of 0.2. Highly variable genes detected using the scanpy.preprocessing.highly_variable_genes function were used for further analysis. Enriched gene ontology gene lists were used to generate an enrichment score using the scapy.tl.score_gene function with default settings. Violin plots were used to highlight the difference in gene set enrichment between the previously defined Leiden populations.

Gene signature analysis and network analysis: Gene Ontology Enrichment Analysis (GOEA) was performed using GOATOOLS\textsuperscript{65}. GO ontologies and annotations were downloaded using the goatoools.base.download_go_dict function. The enriched transcript set was then filtered to include only known transcription factor associations. The second heatmap was constructed using a similar scheme save the enrichment analysis. A neighborhood graph was then generated using the scanpy.preprocessing.neighbors function with 10 neighbors specified, otherwise default settings were used. The dataset was normalized sum of the target batch by that of the remainder of the data. The dataset was then filtered to include only genes present in at least 50% of the target batch and less than 25% in the remainder of the data. The normalized transcript set was then used to identify the signaling pathways that are differentially regulated in the HEY2-high versus HEY2-low populations. GOEA results were then visualized using EnrichmentMap in Cytoscape\textsuperscript{63}.

Ki67 flow cytometry. The EBs were dissociated as described above. The following antibodies were used for staining: mouse anti-Ki67 (DAKO, 1:100) and rabbit anti-cardiac isoform of cTNT (ThermoFisher Scientific, 1:500). The following secondary antibodies were used for detection: donkey anti-mouse IgG-APC (BD Pharmingen, 1:500), or donkey anti-rabbit IgG-PE (Jackson ImmunoResearch, 1:500). Cells were fixed for 20 min at 4 °C with 4% PFA followed by permeabilization using 90% methanol for 20 min at 4 °C. Cells were washed with PBS containing 5% FCS (Sigma) and stained with unconjugated primary antibodies in FACS buffer overnight at 4 °C. Stained cells were washed with PBS with 5% FCS and stained with secondary antibodies in FACS buffer for 30 min at 4 °C. Stained cells were analyzed using a LSR II Flow cytometer (BD Pharmingen). Ki67\textsuperscript{67} (%) was measured within the cTNT\textsuperscript{+} populations. Data were analyzed using FlowJo software (Tree Star). The gating strategy is shown in Supplementary Fig. 11b.

Nile red staining by flow cytometry. To quantify the lipid droplets, Cayman’s Lipid Droplets Fluorescence Assay Kit (Cayman) was used. After the EBs were dissociated as described above, cells were fixed with a Fixative Solution for 10 min at room temperature. The cells were then washed with the Assay Buffer and stained with the Nile Red Staining Solution at room temperature for 15 min. The cells were then analyzed with the Assay Buffer and analyzed with filter sets to detect FITC using a LSR II Flow cytometer (BD Pharmingen). Nile Red Staining (MF) was measured using FlowJo software (Tree Star). The gating strategy is shown in Supplementary Fig. 11c.
siRNA-mediated knockdown of ESRRA. siRNA transfection was carried out according to the manufacturer’s instructions. Negative control (non-targeting) and ESRRA (GCTTC-Tca DaRNAi Kit, DVT) siRNAs were transfected at 10 nM into dissociated day 18 cardiomyocytes using Lipofectamine RNAiMax (Invitrogen). The cells were then re-aggregated in low-binding 96 plate wells for 2 days. Following this, the aggregates were collected and cultured in low-binding 10 cm dishes and cultured according to the maturation protocol, shown in Fig. 3e, for 2 weeks. RT-qPCR and Nile red staining were then performed. To maximize the efficiency of the knockdown of ESRRAs, three different specific siRNAs (supplied with the Kit) were transfected simultaneously.

Annexin V apoptosis assay. After the culture in the pathological conditions for 6 days, the EBs were dissociated as described above. To detect apoptosis following treatment with the pathological stimuli, the TACS Annexin V assay (BDE-VIGEN) was performed by flow cytometry. Cells were washed with PBS and stained with Annexin V-FITC for 15 min at room temperature. Binding buffer was then added to samples prior to processing by flow cytometry. Stained cells were analyzed using the LSR II flow cytometer (BD) and data were analyzed using FlowJo software (TreeStar). The gating strategy used is shown in Supplementary Fig. 11e.

Cell transplantation into rat MI models. All animal experimental protocols were approved by the Animal Use and Care Committee at the University Health Network. For the cell transplantation experiments, the HES2-GFP hESCs were previously targeted at the human ROSA locus to enable constitutive eGFP expression. The differentiated EBs were dissociated as described above and the cells directly transplanted without cryopreservation.

Rat myocardial infarction model: A permanent coronary ligation technique was used to generate a myocardial infarction in athymic nude rat hearts. All rats were intubated and positive pressure ventilation was maintained with a Harvard ventilator (Model 683, 100%). The left anterior descending artery was ligated and occluded permanently using a 7–0 silk ligature. The rat heart was exposed through a left anterolateral thoracotomy incision and a 7–0 suture was used to permanently ligate the left anterior descending artery.

Thoracotomy and cell transplantation: Cells were transplanted 3–4 days after the induction of myocardial infarction. For the transplantation, the rats were anesthetized and ventilated as described above. The heart was exposed and 10 × 10⁶ cells (HES2-GFP differentiated cardiomyocytes) diluted by 75 µl Matrigel (100%, BD PharMingen) were injected using a 20 G needle into the infarcted region. Sacrifice and analysis: 2 weeks or 8 weeks following the transplantation, the rats were sacrificed and their hearts were harvested. The hearts were fixed in 10% formaldehyde and embedded, and then sectioned horizontally at 6 levels to cover the entire LV area. After deparaffinization and rehydration, heat-induced epitope retrieval was performed followed by immunostaining. The following antibodies were used for immunostaining: mouse anti-cardiac isoform of cTNT (ThermoFisher Scientific, 1:200), rabbit anti-human cTNT (abcam, 1:200), rabbit anti-GFP (ROCKLAND, 1:200), mouse anti-Ki67 (DAKO, 1:100), rabbit anti-CX43 (abcam, 1:800), rabbit anti-Ph3 (Cell Signaling, 1:200), rabbit anti-TNNI1 (NOVUS, 1:200), rabbit anti-TNNI3 (abcam, 1:200). For detecting unconjugated primary antibodies, the following secondary antibodies were used: donkey anti-mouse IgG-Alexa488 (ThermoFisher, 1:500), donkey anti-rabbit IgG-Alexa488 (ThermoFisher, 1:500), donkey anti-mouse IgG-Alexa555 (ThermoFisher, 1:500), or donkey anti-rabbit IgG-Alexa555 (ThermoFisher, 1:500). Sarcomere length was measured in cTNT+ stained cardiomyocytes randomly selected from 5 to 10 areas. The numbers were averaged for each transplanted heart. Graft size was measured by calculating the ratio of the GFP+ graft area divided by the entire LV area. Imaging was done using either a Zeiss LSM700 confocal microscope or an EVOS Microscope (ThermoFisher) and analyzed with Zen Blue software (Carl Zeiss) and ImageJ (NIH).

Quantification and statistical analysis. All data are represented as mean ± standard error of mean (SEM). Indicated sample sizes (n) represent biological replicates including independent cell culture replicates and individual tissue samples. For single cell data, sample sizes represent the number of cells analyzed from at least three independent experiments. No statistical method was used to pre-determine the samples size. Statistical significance was determined using a Student’s t test (unpaired, two-tailed) or one-way ANOVA with Tukey’s multiple comparisons in GraphPad Prism 6 software (GraphPad Software). All statistical parameters are reported in the respective figures and figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings in this study are available within the article and its Supplementary Information files, and from the corresponding author upon request. Raw scRNAseq data generated in this study (day 20 ventricular cardiomyocytes and day 32 immature and mature cardiomyocytes) have been deposited at the GEO database under accession code: GSE152589.

For scRNAseq analysis, the following data sets were used; the Hallmark gene set (h.all. v7.1 symbols) and the TTF_Legacy subset of TTF (c.4tt tf. ttf. legacy.v7.1 symbols); http://www.gecco.mgh.harvard.edu/gega/index.jsp

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References
1. Protze, S. I., Lee, J. H. & Keller, G. M. Human pluripotent stem cell-derived cardiovascular cells: from developmental biology to therapeutic applications. Cell Stem Cell 25, 311–327 (2019).
2. Lescroart, F. et al. Early lineage restriction in temporally distinct populations of Mesp1 progenitors during mammalian heart development. Nat. Cell Biol. 16, 829–840 (2014).
3. Zhang, W., Chen, H., Qu, X., Chang, C. P. & Shou, W. Molecular mechanism of ventricular trabeculation/compaction and the pathogenesis of the left ventricular non-compaction cardiomyopathy (LVNC). Am. J. Med. Genet. C Semin Med Genet 163C, 144–156 (2013).
4. Merki, E. et al. Epicardial retinoic acid receptor alpha is required for myocardial growth and coronary artery formation. Proc. Natl Acad. Sci. USA 102, 18455–18460 (2005).
5. Zamora, M., Tanner, J. & Ruiz-Lozano, P. Epicardium-derived progenitor cells require beta-catenin for coronary artery formation. Proc. Natl Acad. Sci. USA 104, 18109–18114 (2007).
6. Lavine, K. J. et al. Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Dev. Cell 8, 85–95 (2005).
7. Brady, T. E. et al. Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial IGF2. Development 138, 139–148 (2011).
8. Feric, N. T. & Radisic, M. Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. Adv. Drug Deliv. Rev. 96, 110–134 (2016).
9. Jiang, Y., Park, P., Hong, S. M. & Ban, K. Maturation of cardiomyocytes derived from hESCs in 96-well cell stems: current strategies and limitations. Mol. Cells 61, 413–621 (2018).
10. Piquereau, J. et al. Postnatal development of mouse heart: formation of energetic microdomains. J. Physiol. 588, 2443–2454 (2010).
11. Piquereau, J. & Ventura-Clapier, R. Maturation of cardiac energy metabolism during perinatal development. Front Physiol. 9, 395 (2018).
12. Lopashchuk, G. D. & Jaswal, J. S. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. J. Cardiovasc. Pharmac. 56, 130–140 (2010).
13. Rog-Zielinska, E. A. et al. Glucocorticoid receptor is required for foetal heart maturation. Hum. Mol. Genet. 22, 3269–3282 (2013).
14. Rog-Zielinska, E. A., Richardson, R. V., Dervir, M. A. & Chapman, K. E. Glucocorticoids and foetal heart maturation: implications for prematurity and foetal programming. J. Mol. Endocrinol. 52, R125–135 (2014).
15. Hirose, K. et al. Evidence for hormonal control of heart regenerative capacity during endothermy acquisition. Science 364, 184–188 (2019).
16. Buroker, N. E., Ning, X. H. & Portman, M. Cardiac PPARalpha protein expression is constant as alternate nuclear receptors and PGC-1 coordinate increase during the postnatal metabolic transition. PPAR Res. 2008, 279531 (2008).
17. Lee, J. H., Protze, S. I., Laksman, Z., Backs, P. H. & Keller, G. M. Human pluripotent stem cell-derived atrial and ventricular cardiomyocytes develop from distinct mesoderm populations. Cell Stem Cell 21, 179–194 (2017). e174.
18. Rondal-Bouchard, K. & Pras, M. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. Nature 556, 239–243 (2018).
19. Yang, X. et al. Tri-iodo-l-thyronine promotes the maturation of human cardiomyocytes derived from induced pluripotent stem cells. J. Mol. Cell Cardiol. 72, 296–304 (2014).
20. Rogg-Wellinton, C. et al. Targeting HIF-1alpha in combination with PPARalpha activation and postnatal factors promotes the metabolic maturation of human induced pluripotent stem cell-derived cardiomyocytes. J. Mol. Cell Cardiol. 132, 120–135 (2019).
21. Yang, X. et al. Fatty acids enhance the maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cell Rep. 13, 657–668 (2019).
22. Mills, R. J. et al. Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. Proc. Natl Acad. Sci. USA 114, E8372–E8381 (2017).
23. Tian, X. et al. Identification of a hybrid myocardin zone in the mammalian heart after birth. Nat. Commun. 8, 87 (2017).
24. Mesender-Montes, I. et al. Myocardial VHL–HIF signaling controls an embryonic metabolic switch essential for cardiac maturation. Dev. Cell 39, 724–739 (2016).
25. Ye, B. et al. APC controls asymmetric Wnt/beta-catenin signaling and cardiomyocyte proliferation gradient in the heart. J. Mol. Cell Cardiol. 89, 287–296 (2015).
26. Boukens, B. J., Christoffels, V. M., Cornel, R. & Moorman, A. F. Developmental basis for electrophysiological heterogeneity in the ventricular and outflow tract myocardium as a substrate for life-threatening ventricular arrhythmias. Circ. Res. 104, 19–31 (2009).
27. Gaborit, N. et al. Cooperative and antagonistic roles for Irx3 and Irx5 in cardiac morphogenesis and postnatal physiology. Development 139, 4007–4019 (2012).
28. Del Monte-Nieto, G. et al. Control of cardiac jelly dynamics by NOTCH1 and NRGI defines the building plan for trabeculation. Nature 555, 439–445 (2018).
29. Mikryukov, A. A. Peroxisome proliferator-activated receptor (PPAR) signaling in the gene regulatory control of energy metabolism in the normal and diseased heart. J. Mol. Cell Cardiol. 34, 1249–1257 (2002).
30. Rog-Zielinska, E. A. et al. Glucocorticoids promote structural and functional maturation of fetal cardiomyocytes: a role for PGC-1alpha. Cell Death Differ. 20, 1116–1126 (2013).
31. Parikh, S. S. et al. Thyroid and glucocorticoid hormones promote functional T-Tubule development in human-induced pluripotent stem-cell derived cardiomyocytes. Circ. Res. 121, 1323–1330 (2017).
32. Brand, M. D. et al. The basal proton conductance of mitochondria depends on adenine nucleotide translocase context. Biochem J. 392, 353–362 (2005).
33. Jastrow, M., Divakaran, A. S., Mookerjee, S., Treberg, J. R. & Brand, M. D. Mitochondrial proton and electron leaks. Essays Biochem. 47, 53–67 (2010).
34. Puente, B. N. et al. Oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. Cell 157, 565–579 (2014).
35. Van Der Lee, K. A., Willemens, P. H., Van Der Vusse, G. J. & Van Biesen, M. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. FASEB J. 14, 495–502 (2000).
36. Friedrichsen, S. et al. Regulation of isodithyonydeidases in the Pax8/- mouse model of congenital hypothyroidism. Endocrinology 144, 777–784 (2003).
37. Talman, V. et al. Molecular atlas of postnatal mouse heart development. J. Am. Heart Assoc. 7, e010378 (2018).
38. Nunes, S. S. et al. Biowire: a platform for maturation of human pluripotent stem progenitors. Cell Stem Cell 8, 228–240 (2011).
39. Talman, V. et al. Molecular atlas of postnatal mouse heart development. Cell Stem Cell 7, 388–391 (2010).
40. Nunes, S. S. et al. Biowire: a platform for maturation of human pluripotent stem cells—cardiovascular progenitors. Cell Stem Cell 28, 96–111.e7 (2020).
41. Koonen, D. P., Glatz, J. F., Bonen, A. & Luiken, J. J. Long-chain fatty acid uptake and FAT/CMD6 translocation in heart and skeletal muscle. Biochim Biophys. Acta 1736, 163–180 (2005).
42. Fujisawa, K., Naka, K. & Naka, M. Peroxisome proliferator-activated receptor alpha (PPARalpha) signaling in the gene regulatory control of energy metabolism in the normal and diseased heart. J. Mol. Cell Cardiol. 34, 1249–1257 (2002).
43. Wolfs, F. A., Angerer, P. & Theis, F. J. SCANPY: large-scale single-cell gene expression data analysis. Cell 175, 15 (2018).
44. Klopfenstein, D. V. et al. GOATOOLS: a python library for gene ontology biomolecular interaction networks. Cell Stem Cell 13, 2498–2504 (2013).
45. Lambart, S. A. et al. The human transcription factors. Cell 175, 598–599 (2018).
46. Trapp, V. et al. Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat. Biotechnol. 25, 1477–1482 (2007).

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Author contributions
S.F., I.P. conceived the project, performed experiments, analyzed data, and wrote the manuscript. O.M. and S.S.N. performed the measurement of contraction force. D.W., T.T., D.Y., B.B., J.L., S.P., and G.B. designed and analyzed scRNA-seq data. A.M. performed the transplantation. W.D. and M.A.L. performed the measurement of Ca2+ transient and advised on the transplantation experiment. G.M.K. designed the project and wrote the manuscript.

Competing interests
G.M.K. is a scientific co-founder and paid consultant for BlueRock Therapeutics LP, a paid consultant for VisthaGen Therapeutics and a board member of Anagenesis Biotechnologies. M.A.L. is founding investigator and paid consultant for BlueRock Therapeutics. S.P. is a paid consultant for BlueRock Therapeutics. All other authors declare no competing interests.
Additional information

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