Guided Differentiation of Pluripotent Stem Cells for Cardiac Cell Diversity

Erik McIntire[1], Kenneth A. Barr[2], Natalia M. Gonzales[2], Yoav Gilad[1,2]
1 Department of Human Genetics, University of Chicago, Chicago, Illinois, United States of America
2 Department of Medicine, University of Chicago, Chicago, Illinois, United States of America

SUMMARY

We have developed a guided differentiation protocol for induced pluripotent stem cells (iPSCs) that rapidly generates a temporally and functionally diverse set of cardiac-relevant cell types. By leveraging techniques used in embryoid body and cardiac organoid generation, we produce both progenitor and terminal cardiac cell types concomitantly in just 10 days. Our results show that guided differentiation generates functionally relevant cardiac cell types that closely align with the transcriptional profiles of cells from differentiation time-course collections, mature cardiac organoids, and *in vivo* heart tissue. Guided differentiation prioritizes simplicity by minimizing the number of reagents and steps required, thereby enabling rapid and cost-effective experimental throughput. We expect this approach will provide a scalable cardiac model for population-level studies of gene regulatory variation and gene-by-environment interactions.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) have redefined *in vitro* cellular experimentation, offering a transformative platform for many areas of research,[1–3] including the study of dynamic gene regulation.[4–7] The capability to derive various cell types rapidly and in a controlled manner through directed differentiation protocols has been particularly instrumental for population-level studies of gene regulation.[8–11]

In humans, population-level studies of dynamic gene regulation are only possible using *in vitro* models. Such studies have been proven quite insightful. *In vitro* studies of gene-by-environment (GxE) interactions in humans, on a population level, can reveal why some individuals are at higher risk for disease when they are exposed to certain environments[7,11] and allow us to determine who is more at risk for suffering from negative side effects of a drug.[6] Another type of study of dynamic gene regulation are time-course experiments, where one characterizes gene regulation through repeated temporal measurement during a biological process, e.g., differentiation.[4,5] In all these examples of population-level gene regulatory studies, access to the most relevant cell type is critical,[12] and directed differentiation of iPSCs has facilitated such access.

Amid these advancements, a critical question arises: How can we extend these studies to encompass multiple cell types concurrently? Must each cell type demand a distinct directed differentiation protocol, resulting in a laborious and time-intensive process?

One possible answer would be to use *in vitro* tissue organoids instead of directing iPSCs to a single specific cell type. Organoids include multiple cell types and are quite useful for studying specific features of tissue function or for modeling diseases.[13] However, organoids are currently impractical for population-level studies because they are difficult and time-consuming to establish. Because the optimal culture conditions for different cell types within the organoid are often conflicting, it can be challenging to maintain multiple cell types in the same medium.[14,15] As a result, organoid culture tends to be inefficient and takes weeks to months to establish. Further, organoids can be difficult to dissociate to the single-cell level for sequencing and characterization of regulatory phenotypes.[16,17]

These challenges propel our exploration of ‘guided’ differentiation, an approach that aims to balance protocol efficiency and cellular diversity. We specifically focused on cardiac cell types. We adapted methods used in embryoid body and cardiac organoid generation to create a model that harbors a diversity of cardiac-relevant cell types for population-level studies. It is designed for efficiency, requiring minimal protocol steps and reagents, and allows for fast experimental throughput. We call our approach 'guided' differentiation, because as opposed to ‘directed’ differentiation, we gently bias iPSCs towards the cardiac mesoderm lineage, while maintaining the greatest degree of cellular diversity possible. Within guided cultures, individual cells differentiate at dissimilar rates, permitting diversity with respect to function (cell type) and time (cell differentiation stage). The inclusion of early progenitor cells, in addition to differentiated cells, provides

developmental trajectories for detecting transient genetic effects that typically can only be captured by using time-course experimental design.[4,5] In what follows, we demonstrate that guided cultures contain functionally diverse cell types, which represent multiple stages of cardiac differentiation, and show that the transcriptional profiles of single cells in our cultures are comparable to that of single cells from mature cardiac organoids. In the methods section, we provide a detailed protocol to establish cardiac cultures using guided differentiation. We anticipate that guided cultures may have applications in population-level studies; examples include associating genetic variants with cardiovascular traits, studying cell-type-specific responses to drugs and treatments, and exploring gene-by-environment interactions.

RESULTS

We performed guided differentiation (Figure 1A) using three iPSC lines to generate three-dimensional cardiac cell cultures. First, we formed iPSCs into three-dimensional aggregates measuring ~300µM in diameter (the optimal size for inducing pluripotent stem cells towards cardiac lineage).[18,19] We cultured iPSC aggregates in the formation plate using Essential 8™ medium (E8) with 10µM CEPT[20] for 24 hours and transferred the aggregates to ultra-low attachment plates for an additional 48 hours with only E8. We then biased iPSC aggregates towards cardiac lineage using temporal Wnt modulation.[21,22] We exchanged E8 for 'heart medium' (RPMI-1640 with a 2% v/v concentration of B-27 supplement, sans insulin) plus the Wnt activator CHIR99021 ('Chiron') at a final concentration of 4µM. After 24 hours, we exchanged Chiron+ heart medium for base heart medium. We refreshed the heart medium 48 hours later, this time adding IWP4, a Wnt inhibitor, at a final concentration of 2µM. After 48 hours, we replaced IWP4+ heart medium with base heart medium for 48 hours before harvest. Contraction became visible 5 days after Wnt activation, or 8 days overall. We collected cells at day 10 (Figure S1) using the 10x Genomics platform for sequencing on an Illumina NovaSeq 6000.

After filtering and normalizing the data, we performed principal component analysis with 5,000 highly variable features and used the top 50 principal components for graph-based unsupervised clustering. Guided differentiation cultures include a diversity of cell types from all three germ layers. We annotated cells using marker gene expression and differential expression analysis (Figures 1B and 1C) and identified a high diversity of cell types across all three cell lines (Figure S2). Primitive cell types included pluripotent cells marked by *POU5F1* and *L1TD1* expression, as well as neural ectoderm expressing marker genes *SOX2* and *CRABP1*. We classified two *FOXA2*-expressing endoderm populations: foregut endoderm based on expression of *HHEX*, and a hepatic endoderm population wherein *HHEX* was downregulated, but the liver-specific marker *AFP* showed high expression. Subcluster analysis of foregut endoderm revealed posterior and anterior foregut populations (Figure S3), which have been reported previously in cardiac organoids.[23] We annotated an endothelial cell cluster using expression of *KDR*, *CDH5*, and *PECAM1*, and further specified this population as endocardial cells based on expression of the endocardial cell-specific marker *NFATC1*. We also identified a cluster of epicardial cells based on expression of *BMP4*, *WT1*, and *TBX18*. We classified cardiomyocytes per expression of sarcomere genes (*TNNT2* and *ACTN2*) and ion channel genes (*RYR2* and *CACNA1C*). Although the guided cell culture was only given 7 days to differentiate, cardiomyocytes showed robust expression of *TNNI3*, indicative of maturing myofibrils. In addition to cardiomyocytes, we identified two progenitor populations consistent with the first heart field (FHF) and second heart field (SHF). We classified the FHF based on expression of *TBX5* and *TBX20*. We annotated the SHF based expression of *EYA1*, *ISL1*, and *FGF10*.

Guided differentiation permits individual cells within the culture to differentiate asynchronously; therefore, as demonstrated by our manual cell type classification, the culture can harbor coexisting cell populations from multiple stages of cardiomyogenesis. As guided differentiation yields both terminal and progenitor cell types, it can potentially recapitulate data from a more standard differentiation time-course experiment. To demonstrate this, we performed Seurat integration[24] of our scRNA-seq data with data from a previous 16-day time-course study of iPSC-CM differentiation performed by Elorbany *et al.* (2022).[5] Elorbany *et al.* collected cells undergoing directed differentiation at 7 discrete timepoints; specifically, cells were collected on days 0 (as iPSCs, before initiating differentiation), 1, 3, 5, 7, 11, and 15. Sub-setting the time-course study cells by their collection day illustrates progression through iPSC-CM differentiation. While, as expected, cell states were rather specific to certain days in the time-course study, the 'single time-point' guided differentiation culture harbored cells throughout the entire UMAP space (Figure 2), suggesting that guided differentiation produced cells that are transcriptionally similar to those collected during the entire time-course study. Further,

comparative analysis of gene expression showed conservation of marker genes during cardiomyogenesis (Figure S4).

We next investigated similarity between the transcriptional profiles of cells from guided cultures and their *in vivo* counterparts; to that end, we used automated cell classification. For a cell reference, we leveraged a dataset of annotated normal fetal heart cells published by Miao *et al.* (2020),[25] which consisted of 11 different cell types (Figure 3A). We utilized this reference to train the scPred[26] prediction model for automated cell annotation (Figure 3B). Individual cells from our guided differentiation culture were assigned probabilities of belonging to each of the 11 reference cell types. As the guided differentiation culture harbors cell types that are not present in fetal heart tissue (e.g., foregut endoderm), we set the probability threshold to 0.9; cells below the threshold were classified as unassigned and not included in the UMAP plots. As a negative control, we applied the prediction model to a non-cardiac dataset from peripheral blood mononuclear cells[27] (Figure S5). Based on the prediction model analysis, our guided differentiation culture putatively harbored all cell types except red blood cells and pericytes (Figure 3C). These included cardiomyocytes, fibroblasts, endothelial and endocardial cells, epicardial cells, and nervous system cells, consistent with the manual cell annotation. Additionally, scPred cell type predictions included immune cells and conduction system cells, which were not detected by manual annotation. ScPred classifies cells individually, without clustering, and is capable of identifying small cell populations that would otherwise be masked by clustering. To benchmark our guided differentiation culture against more established *in vitro* models, we applied the scPred prediction model to scRNA-seq data from mature multi-lineage organoids (100-day culture) published by Silva, *et al.* (2021)[16] (Figure 3D). Both iPSC-based cultures showed comparable levels of cell diversity and transcriptional similarity relative to the fetal heart tissue, demonstrating that our guided differentiation culture method can generate diverse, transcriptionally relevant cell types in just 10 days.

DISCUSSION

We present a model that combines cardiac cell diversity with labor and reagent efficiency to fill a gap in population-level studies not currently addressed by standard embryoid body and cardiac organoid approaches. The guided differentiation of iPSCs generates a broad spectrum of cardiac-relevant cell types within the short period of 10 days, while recapitulating the cellular complexity seen in mature cardiac organoids. Guided differentiation gently nudges iPSCs towards a cardiac lineage while preserving a high degree of cellular diversity. This method facilitates differentiation of individual cells at varying (asynchronous) rates, thereby producing a broad range of cardiac cell types and stages.

Guided differentiation cultures produce the differentiated cardiac cell types of cardiomyocytes, fibroblasts, epicardial cells, endocardial cells, along with endoderm, which is supportive of cardiac cell differentiation.[28] Although existing cardiac differentiation protocols can produce these cell types,[16,23,29] guided differentiation also generates concomitant progenitor cells. Previously, inclusion of progenitor cardiac cell types with more mature cell types required a time-course approach and several collections,[5,30] whereas guided differentiation captures all types in a single collection.

To demonstrate that cardiac differentiation can generate a continuous cardiac developmental trajectory, we integrated our data with an scRNA-seq dataset produced by a 16-day iPSC-CM differentiation time-course study.[5] Results demonstrate that a single collection from a guided differentiation culture can effectively capture cell states collected from several timepoints over the more extended period. Despite the inclusion of primitive cell types, automated cell classification performed by scPred showed that guided differentiation culture also harbors cells transcriptionally akin to those from *in vivo* heart tissue. Further, based on an 83-day normal fetal heart reference,[25] ScPred classified cells from 10-day guided differentiation and 100-day multi-lineage organoids[16] at comparable levels of cell type diversity.

The ability to grow multiple cardiovascular cell types in the same dish obviates the need for complex differentiation protocols and allows for greater control over confounding variables that might mask genetic effects on gene expression. Guided differentiation therefore circumvents many of the challenges associated with directed iPSC differentiation, while providing a multi-cellular model for high-throughput testing. We expect the reduced time and labor needed for guided differentiation (relative to standard organoids and differentiation time-course studies) will enable dynamic population-level studies at scale. For example, guided differentiation

may facilitate the identification of genetic variants that regulate gene expression levels (expression quantitative trait loci, or eQTLs) at cell type resolution. Additionally, guided differentiation harbors continuous developmental trajectories for detecting transient genetic effects in a single collection, which simplifies study design, eases logistical challenges, and reduces cost. Transient genetic effects exclusive to early development can go undetected in terminal cell types,[4,5] which may partially explain the lack of disease-associated QTLs.[31] Given the inclusion of both progenitor and differentiated cells, we anticipate that guided differentiation will be well-suited to investigate this hypothesis. Moreover, the transcriptional similarity between guided cardiac cell types and their *in vivo* counterparts suggests that dynamic patterns identified using guided cultures will be functionally relevant to human tissues.

Limitations of the study

We focus on demonstrating guided differentiation as a methodological advancement using exploratory analyses; data generated herein is insufficient to infer causality. Guided differentiation does not recapitulate the structure or physiology of the heart and lacks *in vivo* fidelity in those respects. Finally, the *in vivo* reference used in automated cell classification was derived from human fetal heart at a gestational age of 83 days;[25] heart cells from this *in vivo* reference are more mature than those generated by guided differentiation.

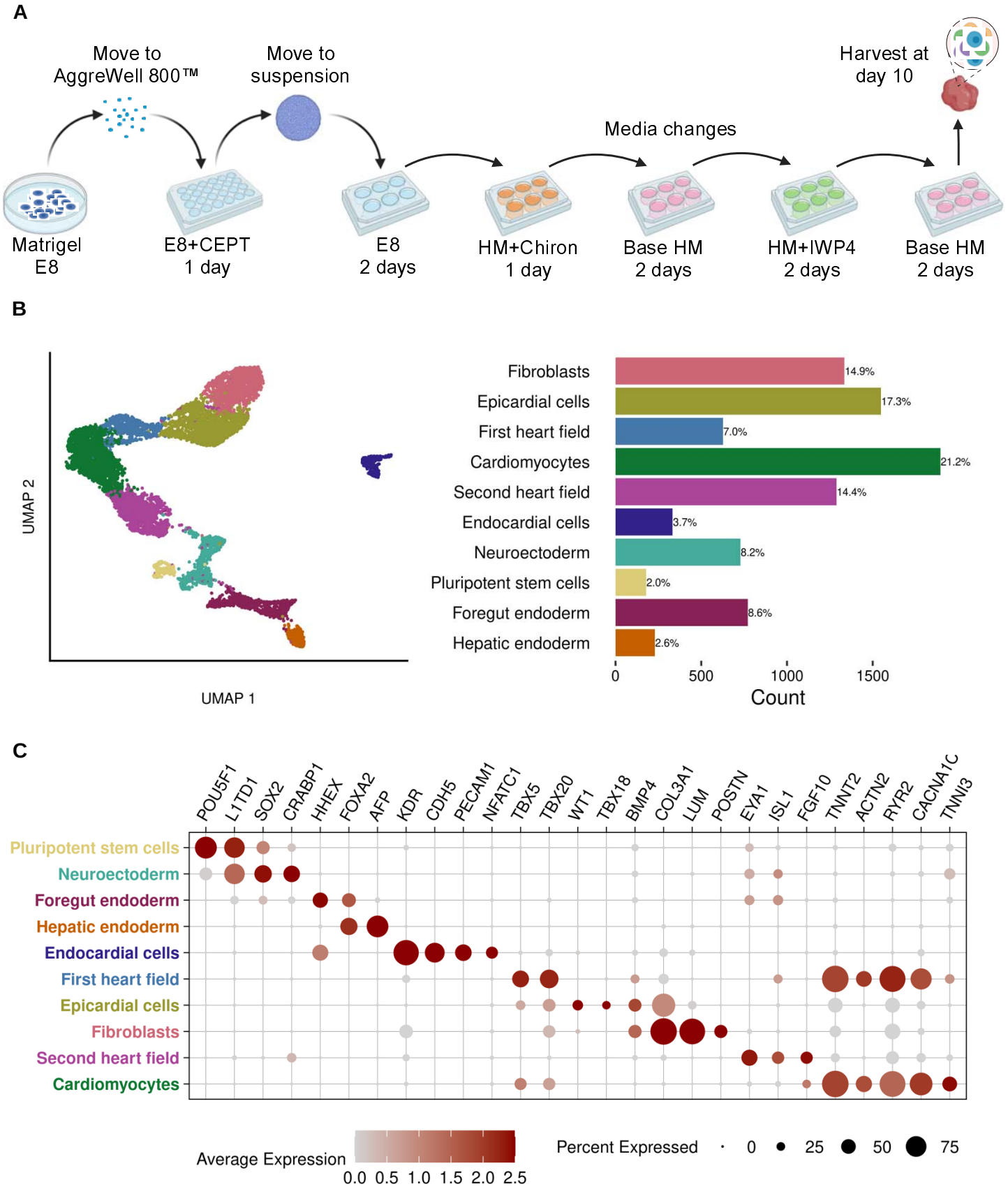

Figure 1. Guided differentiation and characterization.
(A) Overview of the 10-day guided differentiation protocol. E8: Essential 8™ medium. CEPT: Chroman 1, emricasan, polyamines, and trans-ISRIB. HM: heart medium. Chiron: CHIR99021.
(B) UMAP of 8,936 sequenced cells, manually annotated, alongside cell type proportions.
(C) Expression of canonical marker genes for each cell cluster.

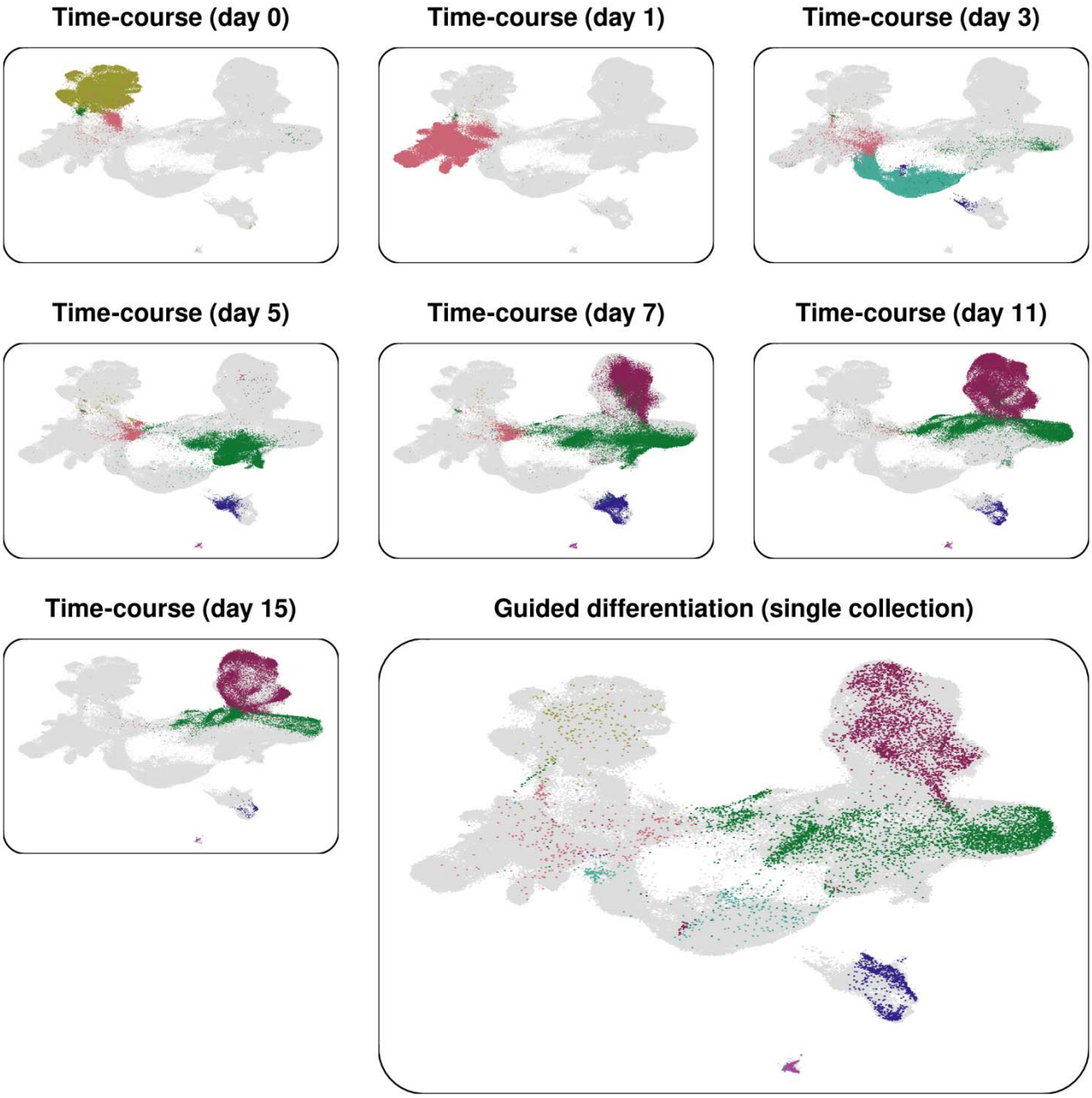

Figure 2. UMAP plots of cells from Seurat integration of a 16-day iPSC-CM differentiation time-course dataset[5] and the guided differentiation dataset. Cells are colored according to cluster and dataset; grey cells are those present in the entire integrated dataset but not present in the selected dataset. The small plots represent cells from the differentiation time-course (n = 230,849), plotted according to their day of collection. The large plot represents cells from a single collection of guided differentiation culture cells (n = 8,936).

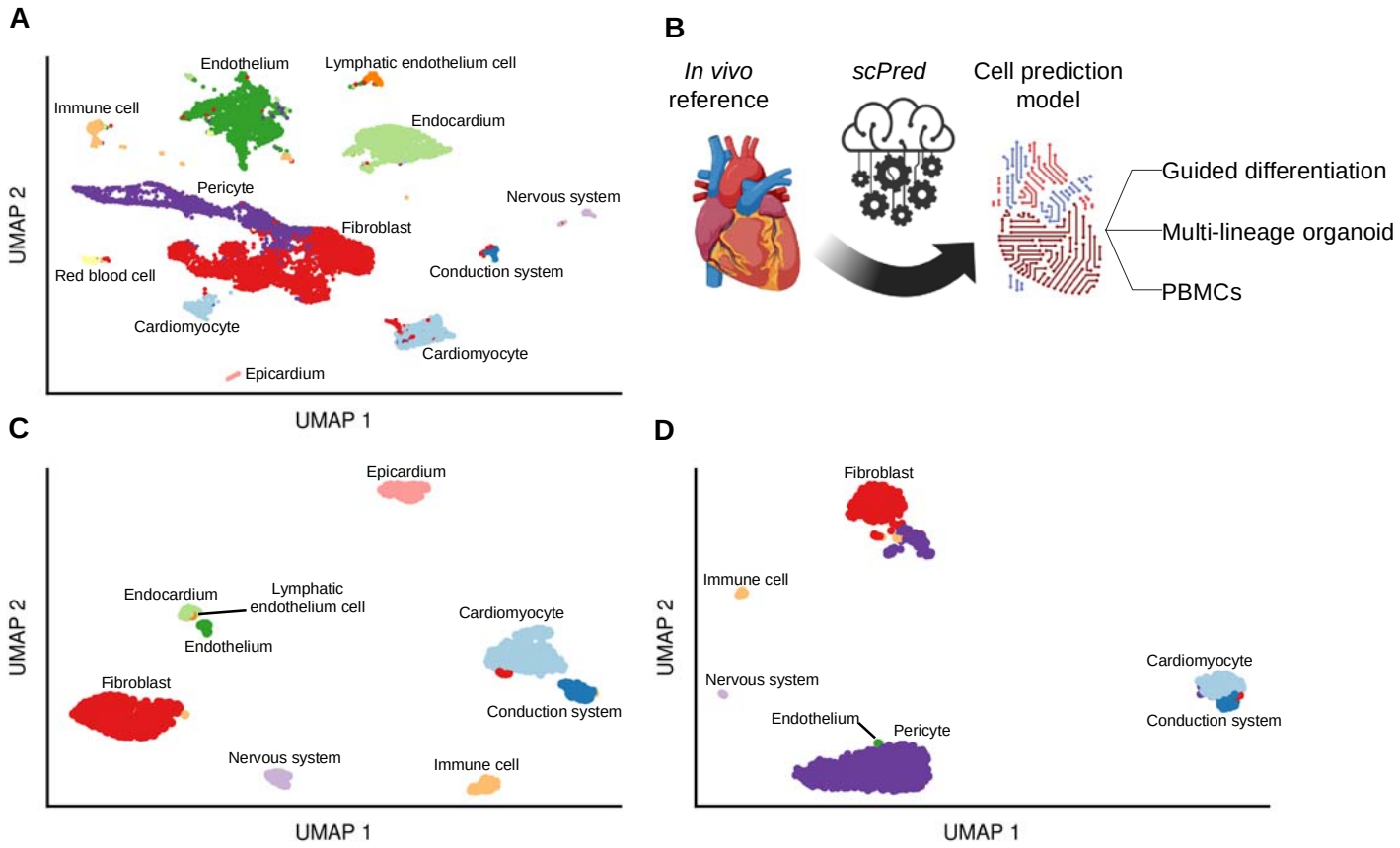

Figure 3. Automated annotation using scPred trained on a normal fetal heart reference.
(A) UMAP of fetal heart cells (n = 13,569) with 11 cell types annotated by Miao *et al.* (2020).[25]
(B) Overview of automated annotation process using scPred. Model was trained using the cell reference and applied to three datasets, including peripheral blood mononuclear cells (PBMCs),[27] used here as a non-cardiac control (Figure S5).
(C) UMAP of classified cell types from the guided differentiation culture (n = 1,114).
(D) UMAP of classified cell types from multi-lineage organoid published by Silva *et al.* (2021)[16] (n = 2,016).

Acknowledgements. We thank all members of the Gilad lab for their support. This work was completed in part with resources provided by the University of Chicago's Research Computing Center. We thank the University of Chicago Functional Genomics Core Facility for their assistance with sequencing the libraries. Figures 1A and 3B were created using BioRender.com.

Author contributions. Y.G. conceived the study and supervised the project. E.M. developed the differentiation method and performed the differentiation experiments. E.M. and K.A.B performed single cell RNA-seq library preparation. E.M. analyzed and interpreted the data with support from K.A.B. E.M. drafted the manuscript with review and editing provided by Y.G and N.M.G.

Competing interests. Y.G. and E.M. are named as inventors with the University of Chicago on a patent application related to guided differentiation.

Funding. This work was funded by a MIRA award (R35GM131726) to Y.G. E.M. was supported by grants from the NIH (T32GM139782, T32HL007381, and F31HL168912).

REFERENCES

1. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**, 861–872 (2007).
2. Shi, Y., Inoue, H., Wu, J. C. & Yamanaka, S. Induced pluripotent stem cell technology: a decade of progress. *Nat. Rev. Drug Discov.* **16**, 115–130 (2017).

3. Rowe, R. G. & Daley, G. Q. Induced pluripotent stem cells in disease modelling and drug discovery. *Nat. Rev. Genet.* **20**, 377–388 (2019).
4. Strober, B. J. *et al.* Dynamic genetic regulation of gene expression during cellular differentiation. 5 (2019).
5. Elorbany, R. *et al.* Single-cell sequencing reveals lineage-specific dynamic genetic regulation of gene expression during human cardiomyocyte differentiation. *PLOS Genet.* **18**, e1009666 (2022).
6. Knowles, D. A. *et al.* Determining the genetic basis of anthracycline-cardiotoxicity by molecular response QTL mapping in induced cardiomyocytes. *eLife* **7**, e33480 (2018).
7. Ward, M. C., Banovich, N. E., Abhishek Sarkar, Stephens, M. & Gilad, Y. Dynamic effects of genetic variation on gene expression revealed following hypoxic stress in cardiomyocytes. *eLife* **10**, e57345 (2021).
8. Cuomo, A. S. E. Single-cell RNA-sequencing of differentiating iPS cells reveals dynamic genetic effects on gene expression. 14 (2020).
9. Pashos, E. E. *et al.* Large, Diverse Population Cohorts of hiPSCs and Derived Hepatocyte-like Cells Reveal Functional Genetic Variation at Blood Lipid-Associated Loci. *Cell Stem Cell* **20**, 558-570.e10 (2017).
10. Banovich, N. E. *et al.* Impact of regulatory variation across human iPSCs and differentiated cells. *Genome Res.* **28**, 122–131 (2018).
11. Hung, A., Housman, G., Briscoe, E. A., Cuevas, C. & Gilad, Y. Characterizing gene expression in an in vitro biomechanical strain model of joint health. *F1000Research* **11**, 296 (2022).
12. Kim-Hellmuth, S. *et al.* Cell type–specific genetic regulation of gene expression across human tissues. *Science* **369**, eaaz8528 (2020).
13. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* **345**, 1247125 (2014).
14. Kim, H., Kamm, R. D., Vunjak-Novakovic, G. & Wu, J. C. Progress in multicellular human cardiac organoids for clinical applications. *Cell Stem Cell* **29**, 503–514 (2022).
15. Thomas, D., Choi, S., Alamana, C., Parker, K. K. & Wu, J. C. Cellular and Engineered Organoids for Cardiovascular Models. *Circ. Res.* **130**, 1780–1802 (2022).
16. Silva, A. C. *et al.* Co-emergence of cardiac and gut tissues promotes cardiomyocyte maturation within human iPSC-derived organoids. *Cell Stem Cell* **28**, 2137-2152.e6 (2021).
17. Voges, H. K. *et al.* Vascular cells improve functionality of human cardiac organoids. *Cell Rep.* **42**, 112322 (2023).
18. Bauwens, C. L. *et al.* Geometric Control of Cardiomyogenic Induction in Human Pluripotent Stem Cells. *Tissue Eng. Part A* **17**, 1901–1909 (2011).
19. Branco, M. A. *et al.* Transcriptomic analysis of 3D Cardiac Differentiation of Human Induced Pluripotent Stem Cells Reveals Faster Cardiomyocyte Maturation Compared to 2D Culture. *Sci. Rep.* **9**, 9229 (2019).
20. Chen, Y. *et al.* A versatile polypharmacology platform promotes cytoprotection and viability of human pluripotent and differentiated cells. *Nat. Methods* **18**, 528–541 (2021).
21. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nat. Protoc.* **8**, 162–175 (2012).
22. Burridge, P. W. *et al.* Chemically defined generation of human cardiomyocytes. *Nat. Methods* **11**, 855–860 (2014).
23. Drakhlis, L. *et al.* Human heart-forming organoids recapitulate early heart and foregut development. *Nat. Biotechnol.* **39**, 737–746 (2021).
24. Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-1902.e21 (2019).
25. Miao, Y. *et al.* Intrinsic Endocardial Defects Contribute to Hypoplastic Left Heart Syndrome. *Cell Stem Cell* **27**, 574-589.e8 (2020).
26. Alquicira-Hernandez, J., Sathe, A., Ji, H. P., Nguyen, Q. & Powell, J. E. scPred: accurate supervised method for cell-type classification from single-cell RNA-seq data. *Genome Biol.* **20**, 264 (2019).
27. 20k Human PBMCs, 3’ HT v3.1, Chromium X Single Cell Gene Expression Dataset by Cell Ranger (2021).
28. Mummery, C. *et al.* Differentiation of Human Embryonic Stem Cells to Cardiomyocytes: Role of Coculture With Visceral Endoderm-Like Cells. *Circulation* **107**, 2733–2740 (2003).
29. Lewis-Israeli, Y. R. *et al.* Self-assembling human heart organoids for the modeling of cardiac development and congenital heart disease. *Nat. Commun.* **12**, 5142 (2021).
30. Galdos, F. X. *et al.* Combined lineage tracing and scRNA-seq reveals unexpected first heart field predominance of human iPSC differentiation. *eLife* **12**, e80075 (2023).

31. Umans, B. D., Battle, A. & Gilad, Y. Where Are the Disease-Associated eQTLs? *Trends Genet. TIG* **37**, 109–124 (2021).
32. The International HapMap Consortium. The International HapMap Project. *Nature* **426**, 789–796 (2003).
33. Zheng, G. X. Y. *et al.* Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* **8**, 14049 (2017).
34. Huang, Y., McCarthy, D. J. & Stegle, O. Vireo: Bayesian demultiplexing of pooled single-cell RNA-seq data without genotype reference. *Genome Biol.* **20**, 273 (2019).
35. Wickham, H. *et al.* Welcome to the Tidyverse. *J. Open Source Softw.* **4**, 1686 (2019).
36. Hafemeister, C. & Satija, R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* **20**, 296 (2019).
37. Tol, P. *Colour Schemes*. https://personal.sron.nl/~pault/ (2021).
38. Neuwirth, E. RColorBrewer: ColorBrewer Palettes. (2014).

STAR METHODS

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	Cat.# 354230
Essential 8™ (E8) medium	Thermo Fisher Scientific	Cat.# A1517001
Penicillin-Streptomycin	Lonza	Cat.# 17-602F
10µM ROCK inhibitor Y-27632	Abcam	Cat.# ab120129
Chroman 1	Torcis	Cat.# 7163
Emricasan	MedKoo Biosciences	Cat.# 510230
Polyamines	Sigma-Aldrich	Cat.# P8483
Trans-ISRIB	Torcis	Cat.# 5284
CHIR99021	STEMCELL Technologies	Cat.# 72052
IWP4	STEMCELL Technologies	Cat.# 72552
RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES	Thermo Fisher Scientific	Cat.# 72400047
B-27 Supplement, minus insulin	Thermo Fisher Scientific	Cat.# A1895601
AccuMax™	STEMCELL Technologies	Cat.# 07921
Bovine Serum Albumin (BSA) solution	Sigma-Aldrich	Cat.# A8412
Critical commercial assays		
10x Genomics Chromium Next GEM Single Cell 3 Reagent Kits v3.1 (Dual Index)	10x Genomics	Single Cell 3 v3.1
Deposited data		
Guided differentiation scRNA-seq	This paper	GEO: GSE230587
16-day differentiation time-course scRNA-seq	Elorbany, et al., 2022	GEO: GSE175634
Normal fetal heart tissue scRNA-seq dataset	Miao et al., 2020	GEO: GSE138979
Day-100 multi-lineage organoid scRNA-seq	Silva et al., 2021	GEO: GSE153075
Human peripheral blood mononuclear cells (PBMCs)	10x Genomics	20k Human PBMCs, 3' HT v3.1, Chromium X
Experimental models: Cell lines		
Human iPSC line	Banovich, et al., 2018	19114
Human iPSC line	Banovich, et al., 2018	19130
Human iPSC line	Banovich, et al., 2018	19152

Software and algorithms		
Cell Ranger v6.1.2	Zheng, et al., 2017	https://support.10xgenomics.com/single-cell-gene-expression/software/overview/welcome
Vireo v0.5.6	Huang, et al., 2019	https://github.com/single-cell-genetics/vireo
Seurat v4.3.0	Stuart, et al., 2019	https://satijalab.org/seurat/
Tidyverse v2.0.0	Wickham, et al., 2019	https://www.tidyverse.org/
scPred v1.9.2	Alquicira-Hernandez, et al., 2019	https://github.com/powellgenomicslab/scPred
RColorBrewer v1.1-3	Neuwirth, 2022	https://cran.r-project.org/web/packages/RColorBrewer/index.html
Color schemes	Tol, 2021	https://personal.sron.nl/~pault/
Other		
AggreWell™800 24-well plate	STEMCELL Technologies	Cat.# 34811
Anti-Adherence Rinsing Solution	STEMCELL Technologies	Cat.# 07010
Ultra-low adherent plate	STEMCELL Technologies	Cat.# 100-0083
Wide-bore pipette tip	Thermo Fisher Scientific	Cat.# 2079GPK
40µm cell strainer	Bel-Art	Cat.# H136800040

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Yoav Gilad (gilad@uchicago.edu).

Materials availability
This study did not generate any new reagents.

Data and code availability
Code for data analysis available at:
https://github.com/erikmcintire/guided_differentiation
Sequencing data have been deposited in GEO and will be made public concurrent with publication.

METHOD DETAILS

Samples
We used 3 iPSC lines from unrelated Yoruba individuals from Ibadan, Nigeria (YRI): 19114 (female), 19130 (male), and 19152 (female). These iPSC lines were reprogrammed from lymphoblastoid cell lines (LCLs) and were characterized and validated previously.[10] We confirmed cell line identities using genotype data generated by the HapMap project from the original LCL lines.[32]

IPSC maintenance
We maintained the 3 iPSC lines using Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix with Essential 8™ (E8) medium and Penicillin-Streptomycin in an incubator at 37°C and 5% CO_2. At roughly 80% confluency (approximately every 3-5 days), we passaged cell cultures using a dissociation reagent (0.5 mM EDTA, 300 mM NaCl in PBS) and seeded iPSCs with 10µM ROCK inhibitor Y-27632.

Guided differentiation

Similar to directed differentiation, guided differentiation primarily generates cardiac-relevant cell types. However, it also yields cell types representative of all three germ layers and includes cell stages throughout cardiomyogenic lineage, resulting in a greater diversity of cell types. We performed guided differentiation of the 3 iPSC lines by forming three-dimensional aggregates and performing temporal Wnt modulation.[21,22] We formed iPSC aggregates using an AggreWell™800 24-well plate. We coated each well with Anti-Adherence Rinsing Solution. On day 0, we dissociated iPSCs seeded them onto the plate using E8 medium with 10µM CEPT,[20] i.e., chroman 1, emricasan, polyamines, and trans-ISRIB. Cell aggregates were formed at approximately 300µm diameter using 2 million cells per well (i.e., 1 million cells per mL) of the AggreWell™800 plate, as described previously by Branco *et al.* (2019)[19]. We centrifuged the plate at 100*g* for 3 min to aggregate cells together. Cells remained in the plate for 24h. After 24h, we transferred fully formed iPSC aggregates to a suspension culture using an ultra-low adherent plate with E8 medium for 48h; note that the cells were maintained in the initial E8 medium throughout the entire 48-hour duration with no replenishment. On day 3, we changed the cell medium to 'heart medium' plus 4µM CHIR99021. Heart medium is comprised of RPMI 1640 Medium, GlutaMAX™ Supplement, HEPES, 2% v/v B-27 Supplement, minus insulin, and Penicillin-Streptomycin. After 24h, on day 4, we changed medium to base heart medium for 48h. On day 6, we changed medium to heart medium plus 2µM IWP4 for 48h. Contraction became visible on day 8 for all three lines. Lastly, on day 8, we changed medium to base heart medium for 48h and collected cells on day 10.

Aggregate dissociation

We collected day 10 aggregates from the 3 iPSC lines and dissociated them by treating with room temperature AccuMax™ followed by incubation at 37°C for 10 min. Following incubation, we pipetted aggregates up-and-down for 30 sec with a p1000 wide-bore pipette tip. Aggregates were then incubated for an additional 5 min at 37°C. We repeated the pipetting every 5 min until aggregates were dissociated, at which point we added 5mL of 4°C Bovine Serum Albumin (BSA) solution and centrifuged cells for 100*g* for 3 min. We resuspended cells in 1mL 4°C BSA solution and strained cells through a 40µm cell strainer. We combined cells from each iPSC line in even proportions (500,000 cells per line) and centrifuged cells at 100*g* for 3 min. We then resuspended cells in 4°C BSA solution at a concentration of approximately 2 million cells per mL. Finally, we strained the cell suspension using a 40µm cell strainer.

Single-cell RNA sequencing analysis

We collected cells from the 3 iPSC lines for scRNA-seq using the 10x Genomics Chromium Next GEM Single Cell 3 Reagent Kits v3.1 (Dual Index). We targeted 10,000 cells total in one lane of a 10x chip and sequenced the library using an Illumina NovaSeq 6000 at the University of Chicago Functional Genomics Core Facility. We obtained 20,755 mean reads per cell. We aligned samples to the human genome (GRCh38) using Cell Ranger[33] and assigned cells to individuals using Vireo.[34] We then analyzed count data in R / RStudio using Seurat[24] with Tidyverse.[35] For cell filtering, we removed cells classified as doublets or unassigned by Vireo. Additionally, we filtered out cells expressing less than 1,500 unique genes. We performed sctransform-based normalization[36] (sctransform function) using 5,000 variable features, performed dimensionality reduction (RunPCA function) and used 50 dimensions for uniform manifold approximation and projection (UMAP) embedding (RunUMAP function). We computed nearest neighbors using 50 dimensions (FindNeighbors function) and performed unsupervised clustering at a resolution of 0.3 (FindClusters function) to yield 10 cell clusters for differential expression analysis using the Wilcoxon Rank Sum test. We annotated cell clusters based on canonical marker gene expression. We shaded cell clusters using color schemes adapted from Paul Tol[37] for all figures unless otherwise noted.

Foregut endoderm subcluster analysis

We subset the foregut endoderm cluster cells as their own Seurat object and re-normalized using the same approach described above. We performed unsupervised clustering at a resolution of 0.1 to yield 2 clusters for differential expression analysis. We annotated cell clusters based on canonical marker gene expression.

Seurat integration

In order to compare cells from our guided differentiation and cells from a 16-day iPSC-CM differentiation time-course published by Elorbany *et al.* (2022),[5] we performed Seurat integration of the two scRNA-seq datasets. First, we filtered the iPSC-CM time-course dataset using the same parameters as in the original study; that is, 1) genes must be detected in at least 10 cells, 2) cells must contain at least 300 unique genes, 3) cells must

have no more than 25% mitochondrial reads, 4) cells must have a doublet probability of 0.3 or less, 5) cell assignment must be unambiguous, 6) cells with feature or read counts more than 4 standard deviations away from the median are excluded. We filtered cells from the guided differentiation dataset using the criteria described in the scRNA-seq analysis section above. Using sctransform, we normalized each dataset individually using 5,000 variable features and selected 5,000 anchor features for integration (SelectIntegrationFeatures function). We then prepared the datasets for integration (PrepSCTIntegration function), determined a set of anchor features (FindIntegrationAnchors function), and integrated the datasets (IntegrateData function). Following integration, we performed dimensionality reduction, UMAP embedding, and computed nearest neighbors as described above. We performed unsupervised clustering at a resolution of 0.1 to yield 7 cell clusters. We subset cells by dataset (and for the time-course dataset, by day of collection) for visualization with UMAP. Prior to comparing gene expression between the two datasets, we corrected counts (PrepSCTFindMarkers).

Automated cell classification

We used scPred[26] to create a prediction classifier model trained on annotated normal fetal heart cells published by Miao *et al.* (2020).[25] The fetal heart dataset comprised 11 annotated cell types: endocardium, endothelium, lymphatic endothelial cells, cardiomyocytes, epicardium, pericytes, fibroblast, immune cells, nervous system, conduction system, and red blood cells. We normalized the fetal heart dataset using the same approach described above and then trained the classifier (getFeatureSpace and trainModel functions) using default parameters. For the multi-lineage organoid dataset, we filtered out cells expressing less than 1,500 unique genes, per the original study.[16] The multi-lineage organoid dataset, guided differentiation dataset, and peripheral blood mononuclear cells (PBMC) dataset[27] were all normalized using the same approach described above. We classified cells from each dataset (scPredict function) using a threshold of 0.9. We shaded cell clusters in figure 3 using RColorBrewer.[38]