Supplemental Items

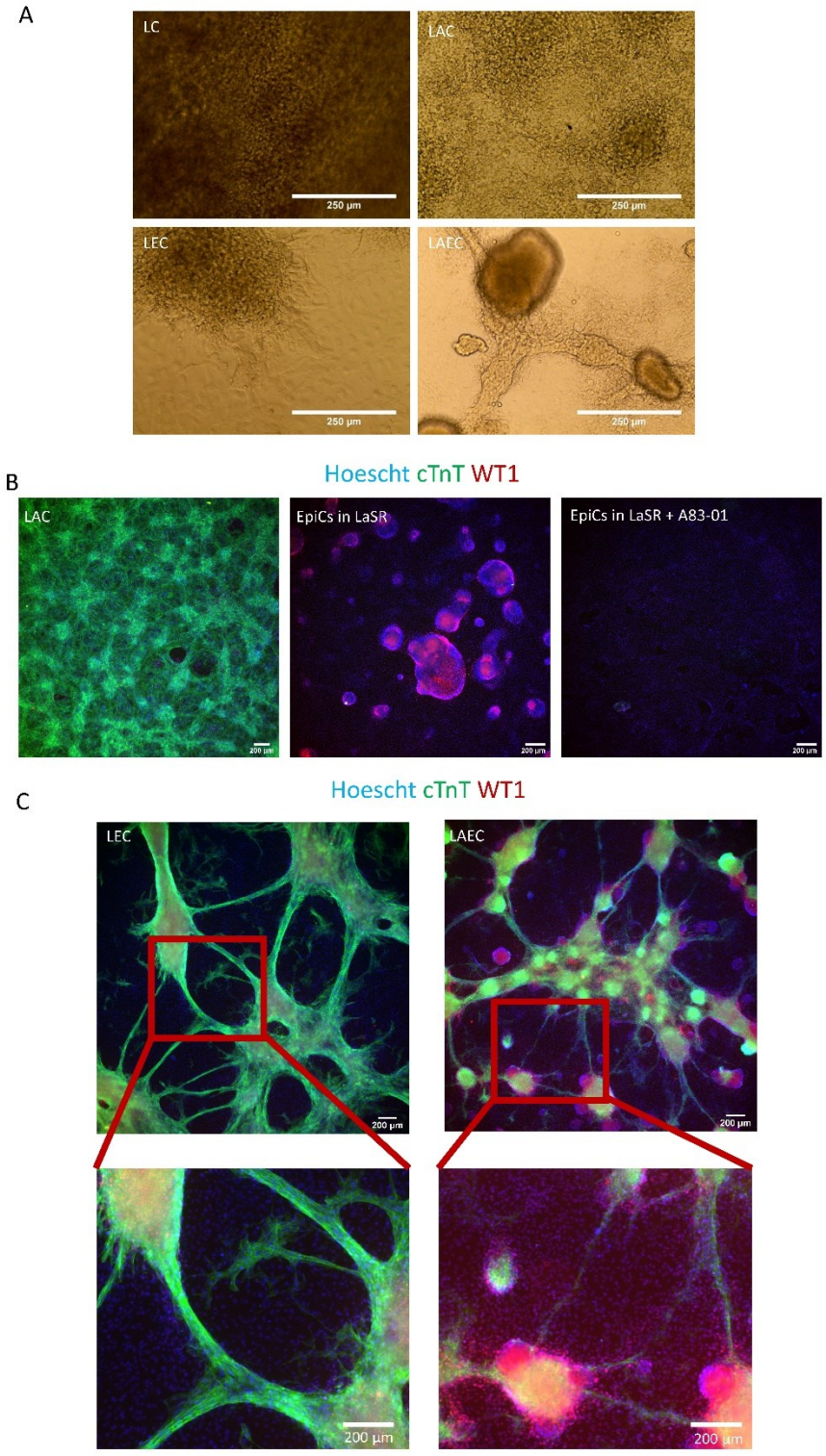
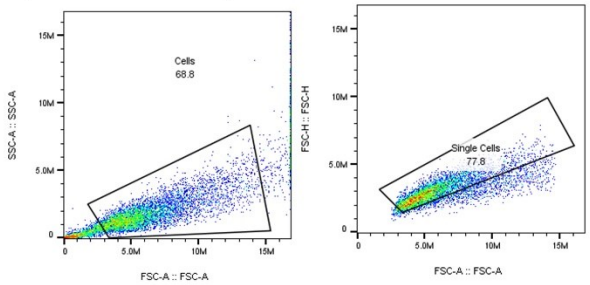
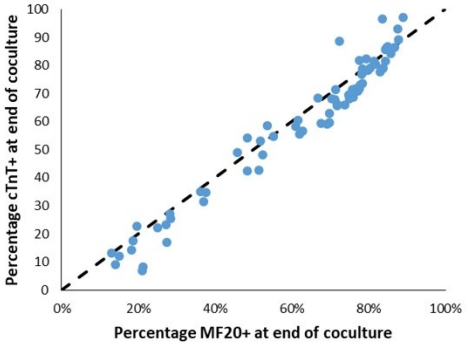


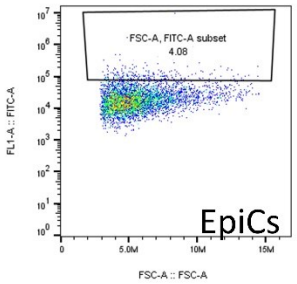
Figure S1, Images of at end of two week EpiC-CPC cocultures, Related to Figure 1. *(A) Brightfield images of representative cocultures. Scale bar is 250µm. (B) Immunocytochemistry of LAC monocultured CPCs, monocultured EpiCs in LaSR medium, and monocultured EpiCs in LaSR + A83-01 medium where blue is Hoescht, green is cTnT, and red is WT1. Scale bar is 200µm. (C) Immunocytochemistry of cocultures where blue is Hoescht, green is cTnT, and red is WT1. Scale bar is 200µm.*

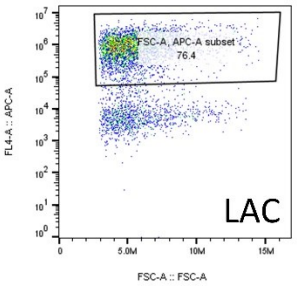
Single Cell Gating



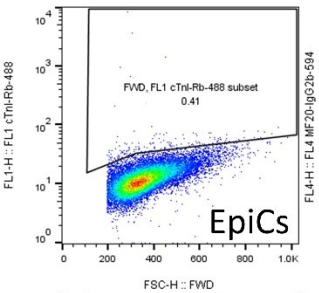


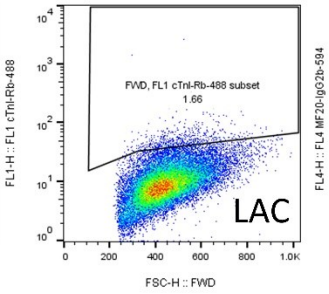
cTnT



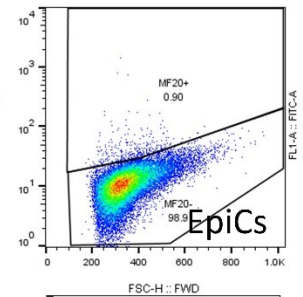


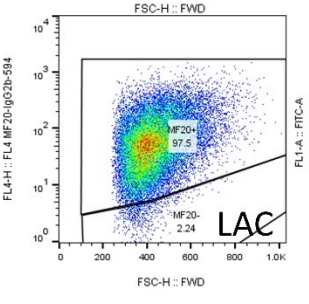
cTnI



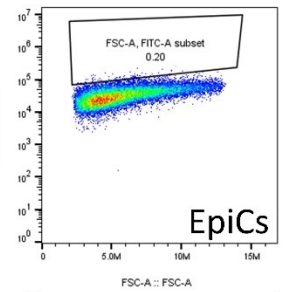


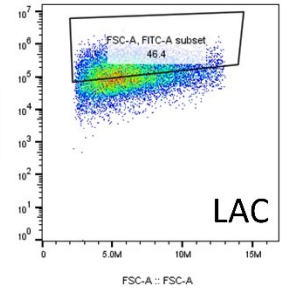
MF20



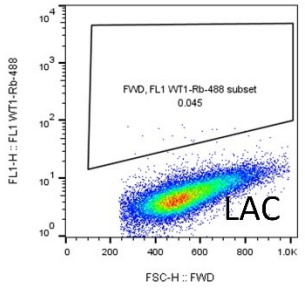


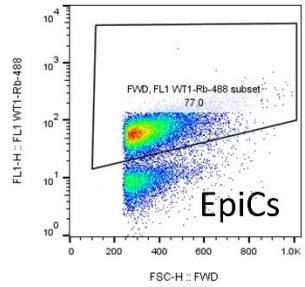
MLC2v



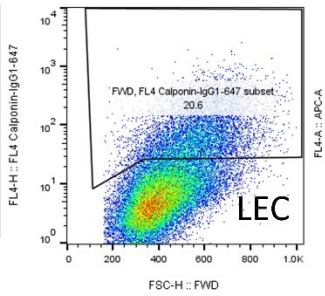


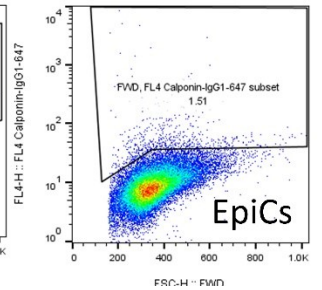
WT1



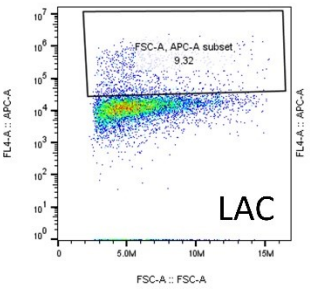


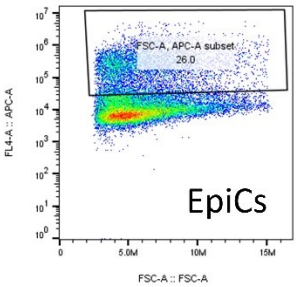
Calponin





Ki67





Ki67

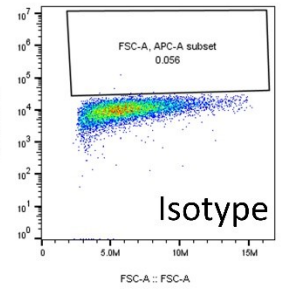


Figure S2, *Example flow cytometry gating, Related to Figure 1.* *Single cell gating was performed by first gating out debris using FSC-A and SSC-A, and then single cells were selected by gating on FSC-A and FSC-H. MF20 and cTnT antibodies identified similar percentages of CMs across all samples. Beneath are flow gating plots with controls for cTnT, cTnI, MF20, MLC2v, WT1, Calponin, and Ki67.*

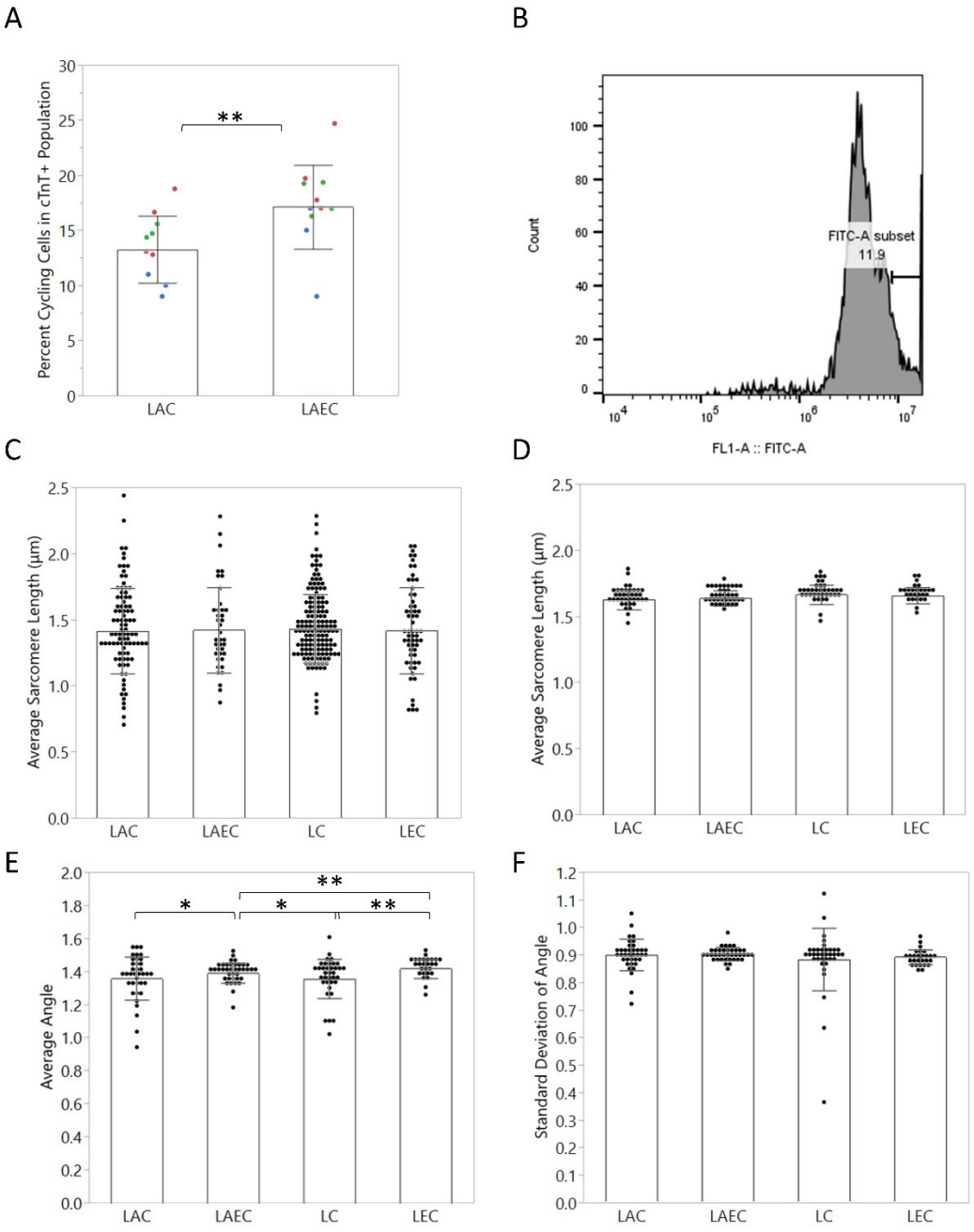

A
Percent Cycling Cells in cTnT+ Population
30
25
20
15
10
5
0
**
LAC
LAEC
B
Count
100
80
60
40
20
0
FITC-A subset
11.9
10^4
10^5
10^6
10^7
FL1-A :: FITC-A
C
Average Sarcomere Length (µm)
2.5
2.0
1.5
1.0
0.5
0.0
LAC
LAEC
LC
LEC
D
Average Sarcomere Length (µm)
2.5
2.0
1.5
1.0
0.5
0.0
LAC
LAEC
LC
LEC
E
Average Angle
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
**
**
LAC
LAEC
LC
LEC
F
Standard Deviation of Angle
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
LAC
LAEC
LC
LEC

Figure S3, LAEC CMs have increased cell cycling and have increased sarcomere angle, Related to Figure 2. *(A) Percent cycling cells in the cTnT$^+$ population. Cycling determined using Vybrant DyeCycle Green DNA dye. Dots represent well replicates (n=3-4) and colors represent 3 independent differentiations. Statistics are a two-way ANOVA where * is p<0.05 and ** is p<0.01. (B) Example gating of cycling cTnT$^+$ cells. (C) Average sarcomere length across four bands, determined by image analysis of at least three sarcomeres in images with sarcomere ratings of 3-4. Images from 3 different differentiations with more than 40 cells per condition. Statistics are a two-way ANOVA with Tukey's post hoc test where * is p<0.05 and ** is p<0.01. (D-F) Average sarcomere length, average angle, and standard deviation of angle using automated SarcTrack2 software. Dots represent the average across a single image. Bars represent the average across all differentiations and error bars represent the standard deviation. Statistics are two-way ANOVA with Tukey's post-hoc test, where * is p<0.05 and ** is p<0.01.*

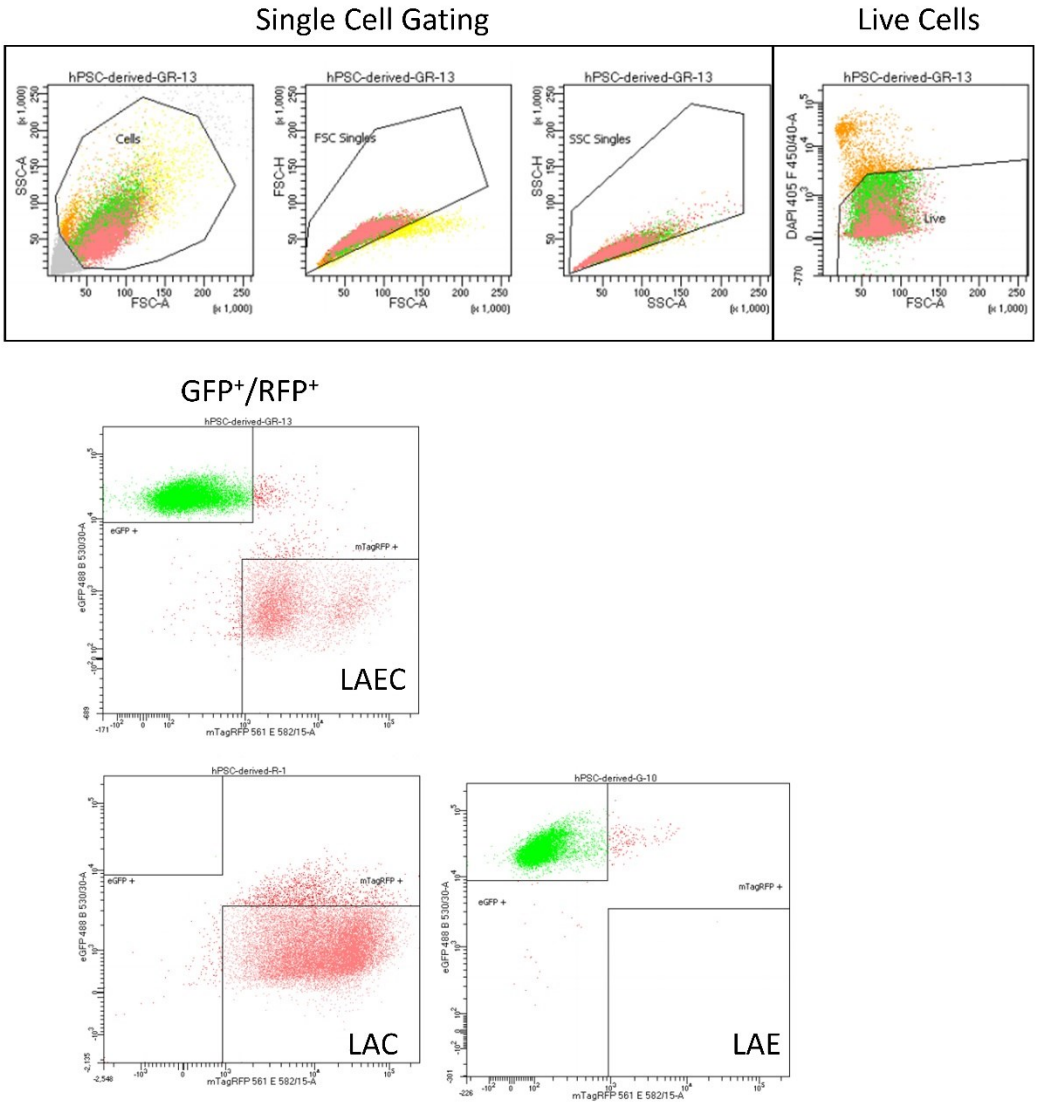


Figure S4, Example flow cytometry gating prior to single cell RNA sequencing, RNA extractions, and analysis of cell fusion, Related to Figure 2 and 4. *Single cell gating was performed by first removing debris then selecting single cells. Live cells were sorted out based on DAPI staining. Finally, GFP and RFP expression were used to detect cells from the CM and EpiC differentiations.*

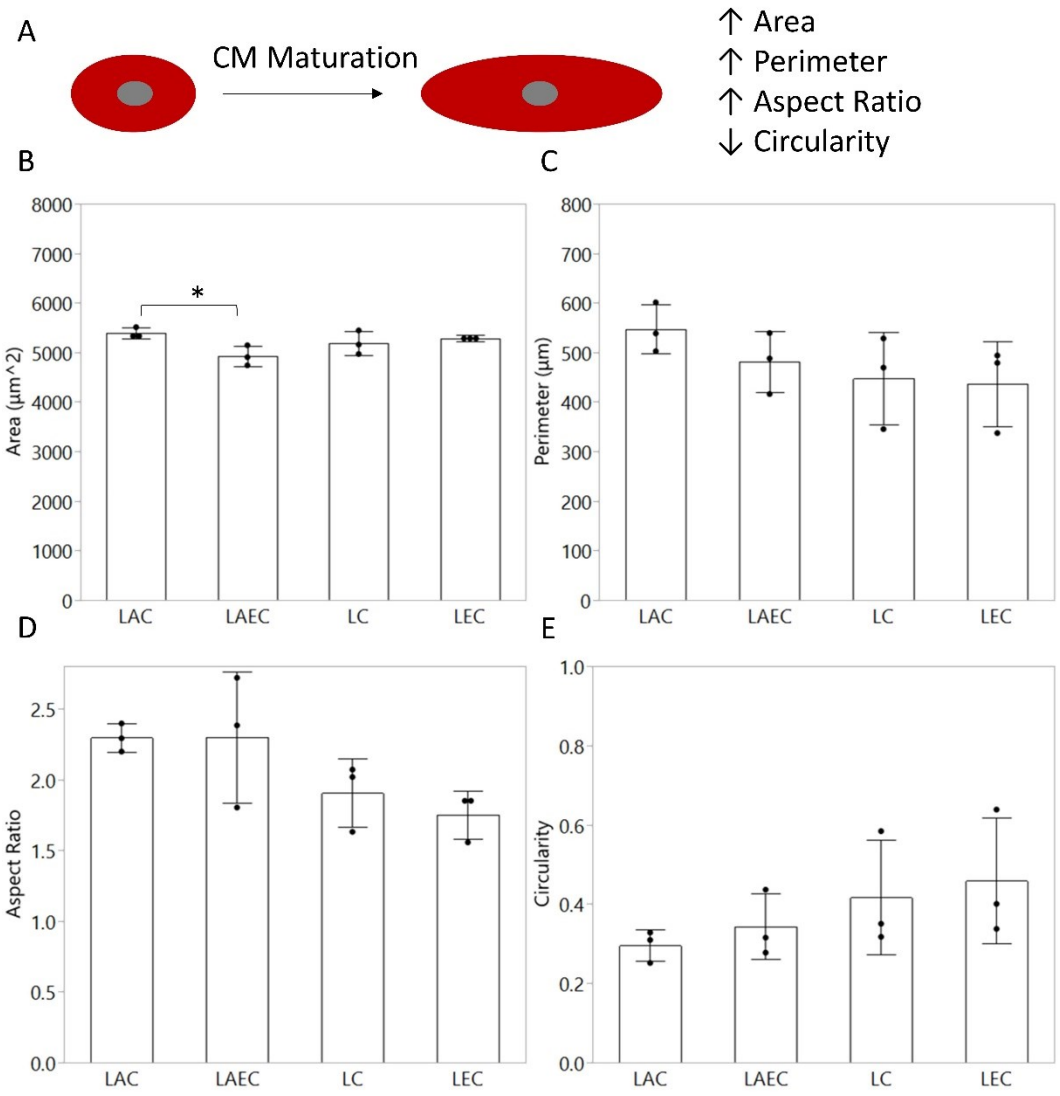


Figure S5, LAEC cocultures cause reduced CM structural maturation, Related to Figures 1 and 2. *(A) Experimental schematic of cell shape parameters measured by image analysis of cTnT⁺ CMs using thresholding in ImageJ. Images were of two week LAEC and LEC cocultures and LAC and LC monocultures. (B-E) Image based analysis of cell size, perimeter, aspect ratio, and circularity. Cells from 3 independent differentiations with at least 200 cells per well, with 3 wells per differentiation were analyzed. Dots represent the average for each differentiation. Bars represent the average across 3 differentiations and error bars represent the standard deviation. Statistical tests are one-way ANOVA on the averages of each differentiation with Tukey's post-hoc where ** is p<0.01 and * is p<0.05.*

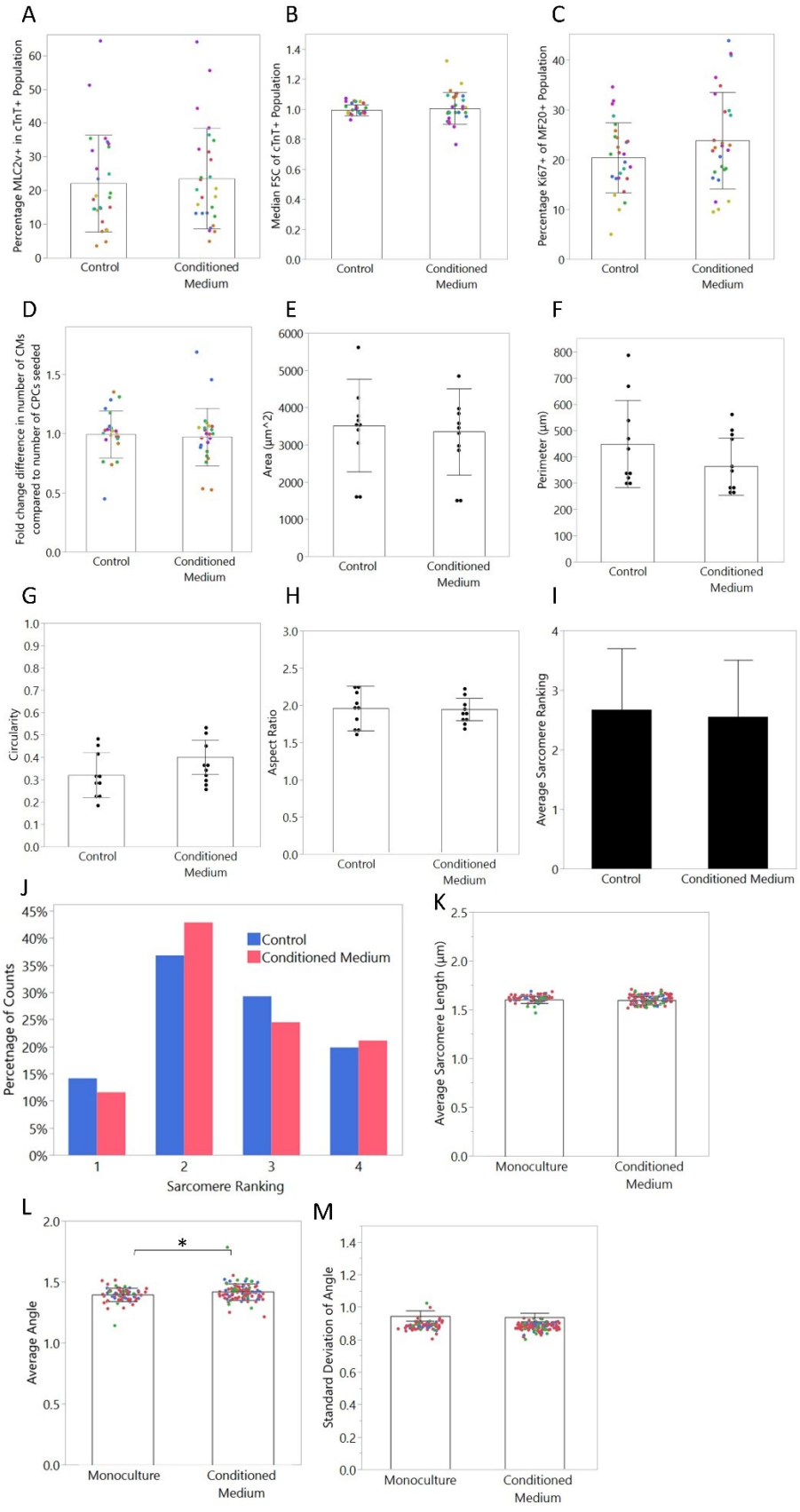

A
Percentage MLC2v+ in cTnT+ Population
Control
Conditioned Medium
B
Median FSC of cTnT+ Population
Control
Conditioned Medium
C
Percentage Ki67+ of MF20+ Population
Control
Conditioned Medium
D
Fold change difference in number of CMs compared to number of CPCs seeded
Control
Conditioned Medium
E
Area (µm^2)
Control
Conditioned Medium
F
Perimeter (µm)
Control
Conditioned Medium
G
Circularity
Control
Conditioned Medium
H
Aspect Ratio
Control
Conditioned Medium
I
Average Sarcomere Ranking
Control
Conditioned Medium
J
Percetnage of Counts
Control
Conditioned Medium
Sarcomere Ranking
K
Average Sarcomere Length (µm)
Monoculture
Conditioned Medium
L
Average Angle
*
Monoculture
Conditioned Medium
M
Standard Deviation of Angle
Monoculture
Conditioned Medium

Figure S6, EpiC-conditioned medium does not alter CM proliferation or structural maturation, Related to Figure 2 and 3. *CMs were treated for two weeks with conditioned media (1:1 fresh LA medium to EpiC-conditioned medium) or unconditioned medium (fresh LA medium) prior to flow cytometry analysis. (A) Percentage MLC2v$^+$ cells of the cTnT$^+$ population. (B) Normalized median forward scatter of cTnT$^+$ population. (C) Percentage Ki67$^+$ cells of MF20$^+$ population as measured by flow cytometry. (D) Fold change difference in number of cardiomyocytes compared to seeding measured by number of cTnT$^+$ cells divided by number of CPCs seeded. Dots represent well replicates (n=3-4) and colors represent 8 independent differentiations. Bars represent the average across all differentiations and error bars represent the standard deviation. Statistics are two-way ANOVA with Tukey's post-hoc test using raw data where * is p<0.05 and ** is p<0.01. (E-H) Image based analysis of cell size, perimeter, aspect ratio, and circularity. Cells were from 10 different differentiations with at least 200 cells per well, 3-4 wells per differentiation with 10 independent differentiations. Dots represent the average from each differentiation. Bars represent the average across all differentiations and error bars represent the standard deviation. Statistics are a Student's t-test using the averages from each differentiation where ** is p<0.01 and * is p<0.05. (I) Average sarcomere rating of cardiomyocytes. Images from 5 independent differentiations with a total of at least 40 images from 2-3 wells per differentiation. Statistics are Student's t-test on the averages of each differentiation, where * is p<0.05 and ** is p<0.01. (J) Histogram of sarcomere ranking scores across 5 independent differentiations. (K-M) Average sarcomere length, average angle, and standard deviation of angle using automated SarcTrack2 software. Dots represent the average across a single image. Bars represent the average across all differentiations and error bars represent the standard deviation. Statistics are two-way ANOVA with Tukey's post-hoc test, where * is p<0.05 and ** is p<0.01.*

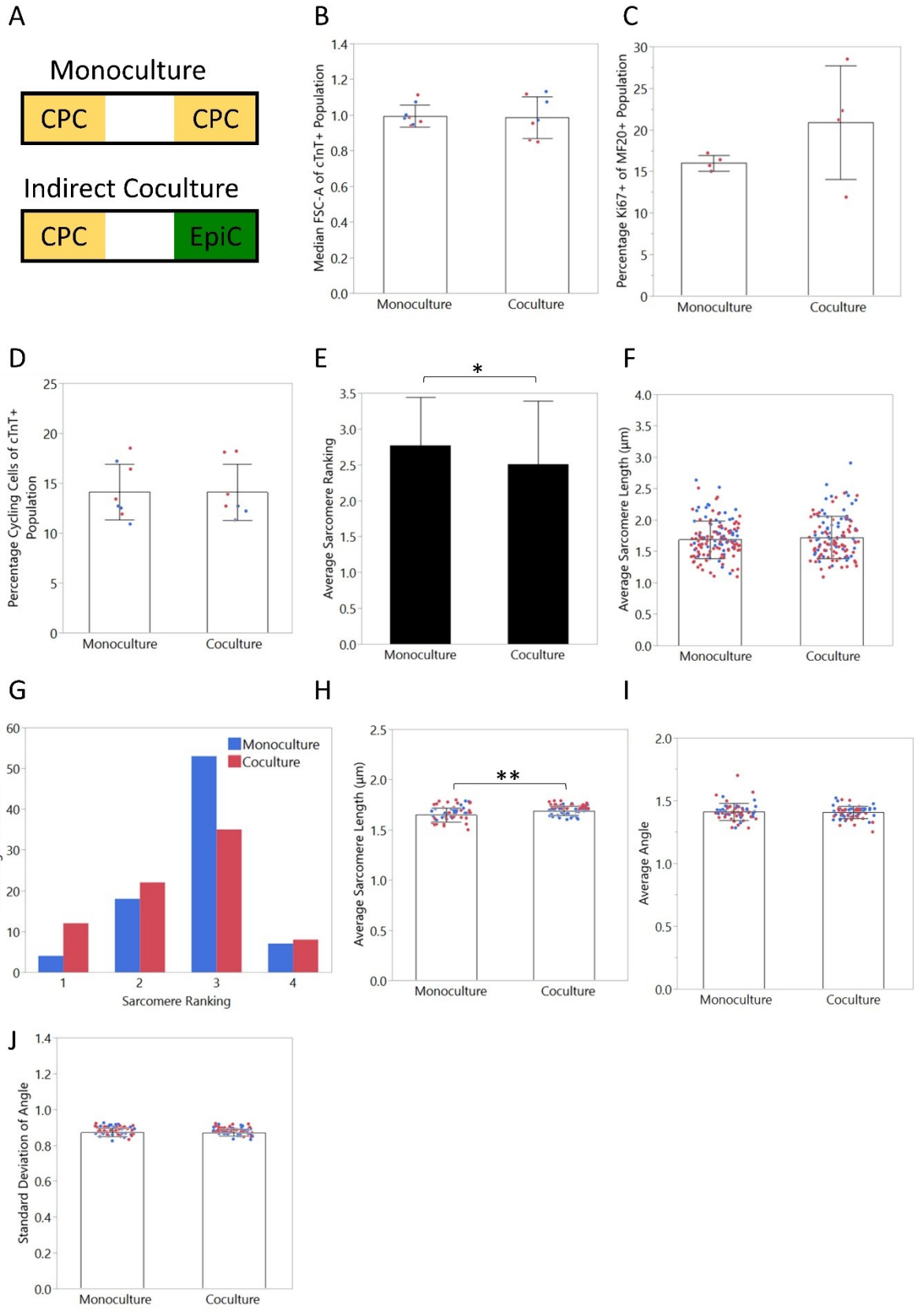

A
Monoculture
CPC
CPC
Indirect Coculture
CPC
EpiC
B
Median FSC-A of cTnT+ Population
Monoculture
Coculture
C
Percentage Ki67+ of MF20+ Population
Monoculture
Coculture
D
Percentage Cycling Cells of cTnT+ Population
Monoculture
Coculture
E
*
Average Sarcomere Ranking
Monoculture
Coculture
F
Average Sarcomere Length (µm)
Monoculture
Coculture
G
Monoculture
Coculture
Percentage of Counts
Sarcomere Ranking
H
**
Average Sarcomere Length (µm)
Monoculture
Coculture
I
Average Angle
Monoculture
Coculture
J
Standard Deviation of Angle
Monoculture
Coculture

Figure S7, Indirect EpiC coculture does not alter CM proliferation or structural maturation, Related to Figure 2 and 3. *CMs were cultured across from CMs or EpiCs on slides for two weeks prior to flow cytometry analysis. (A) Schematic of coculture system. (B) Normalized median forward scatter of $cTnT^+$ population. Dots represent well replicates (n=3-4) and colors represent 2 independent differentiations. Statistics are a two-way ANOVA where * is $p<0.05$ and ** is $p<0.01$. (C) Percentage $Ki67^+$ cells of $MF20^+$ population as measured by flow cytometry. Dots represent well replicates (n=3-4) samples are from one differentiation. Statistics are a student's t-test where * is $p<0.05$ and ** is $p<0.01$. (D) Percent cycling cells in the $cTnT^+$ population. Cycling determined using Vybrant DyeCycle Green DNA dye. Dots represent well replicates (n=3-4) and colors represent 2 independent differentiations. Statistics are a two-way ANOVA where * is $p<0.05$ and ** is $p<0.01$. (E) Average sarcomere rating of cardiomyocytes. Images from 2 independent differentiations with a total of at least 40 images from 3-4 wells per differentiation. Statistics are Student's t-test on the averages of each differentiation with Tukey's post-hoc test, where * is $p<0.05$ and ** is $p<0.01$. (F) Average sarcomere length across four bands, determined by image analysis of at least three sarcomeres in images with sarcomere ratings of 3-4. Images from 2 different differentiations with more than 40 cells per condition. Statistics are a two-way ANOVA where * is $p<0.05$ and ** is $p<0.01$. (G) Histogram of sarcomere ranking scores across 2 independent differentiations. (H-J) Average sarcomere length, average angle, and standard deviation of angle using automated SarcTrack2 software. Dots represent the average across a single image. Bars represent the average across all differentiations and error bars represent the standard deviation. Statistics are two-way ANOVA on the averages of each differentiation with Tukey's post-hoc test, where * is $p<0.05$ and ** is $p<0.01$.*

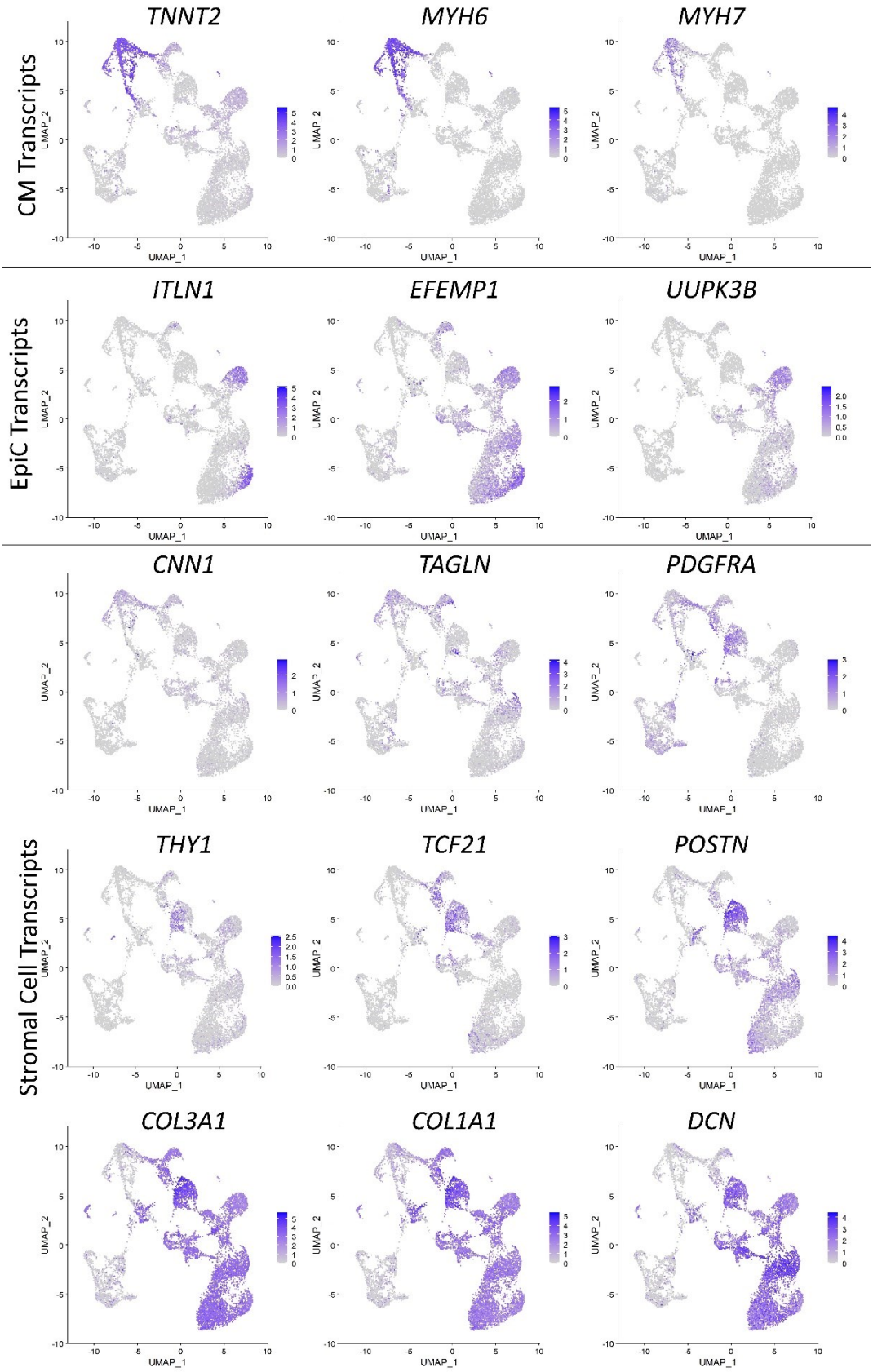
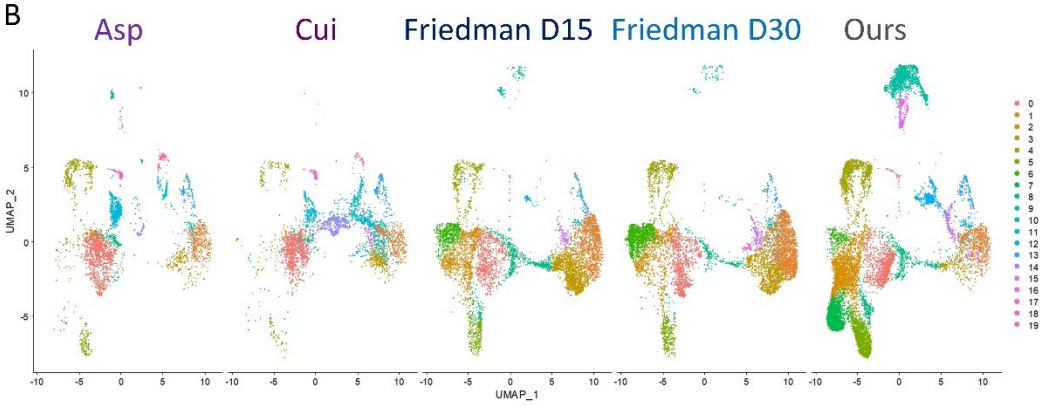

CM Transcripts
TNNT2
MYH6
MYH7
EpiC Transcripts
ITLN1
EFEMP1
UUPK3B
CNN1
TAGLN
PDGFRA
Stromal Cell Transcripts
THY1
TCF21
POSTN
COL3A1
COL1A1
DCN
UMAP_1
UMAP_2

Figure S8, Single cluster identification, Related to Figure 4. *Feature plots of CM transcripts, EpiC transcripts, and stromal cell transcripts.*

A

Dataset	Sample	Number of cells	Number of features
Cui et al.	Human heart PCW 5-26	3885	24140
Asp et al.	Human heart PCW 6	3777	15323
Friedman et al.	hPSC CM D15	6440	33020
Friedman et al.	hPSC CM D30	7233	33020
Ours	hPSC Cocultures D20	12145	34403

B



C

Cluster 0: FB
Cluster 1: CMs
Cluster 2: Stromal Cells
Cluster 3: CMs
Cluster 4: Stromal Cells
Cluster 5: EpiCs
Cluster 6: Stromal Cells
Cluster 7: Stromal Cells
Cluster 8: Stromal Cells
Cluster 9: Stromal Cells
Cluster 10: CMs
Cluster 11: Stromal Cells
Cluster 12: Stromal Cells
Cluster 13: Stromal Cells
Cluster 14: Immune cells
Cluster 15: CMs
Cluster 16: Stromal Cells
Cluster 17: CMs
Cluster 18: Endothelial Cells
Cluster 19: Stromal Cells

D

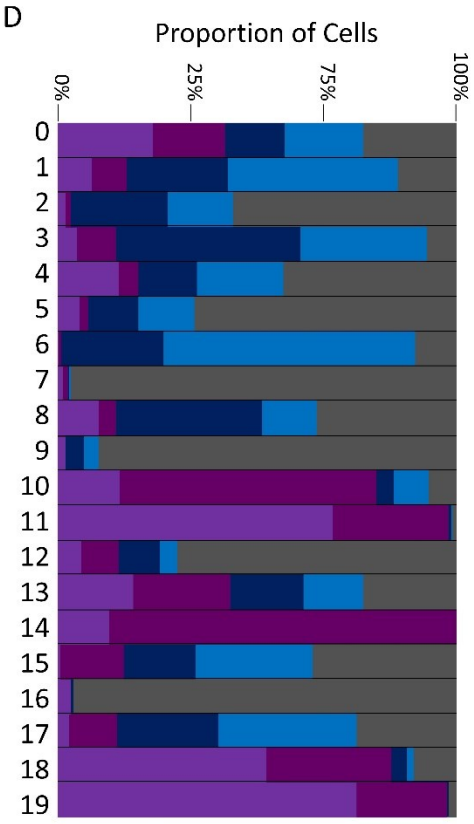


Figure S9, Validation of single cell clusters with other single cell datasets, Related to Figure 4. *(A) Table denoting available developing human heart datasets and hPSC-CM datasets. (B) UMAP integration of all five datasets colored by unbiased clustering. (C) Identification of clusters. (D) Proportion of cells from each dataset in a given cluster where dark purple represents Asp et al, bright purple represents Cui et al., dark blue represents Friedman et al day 15 samples, bright blue represents Friedman et al day 30 samples, and gray represents our dataset.*

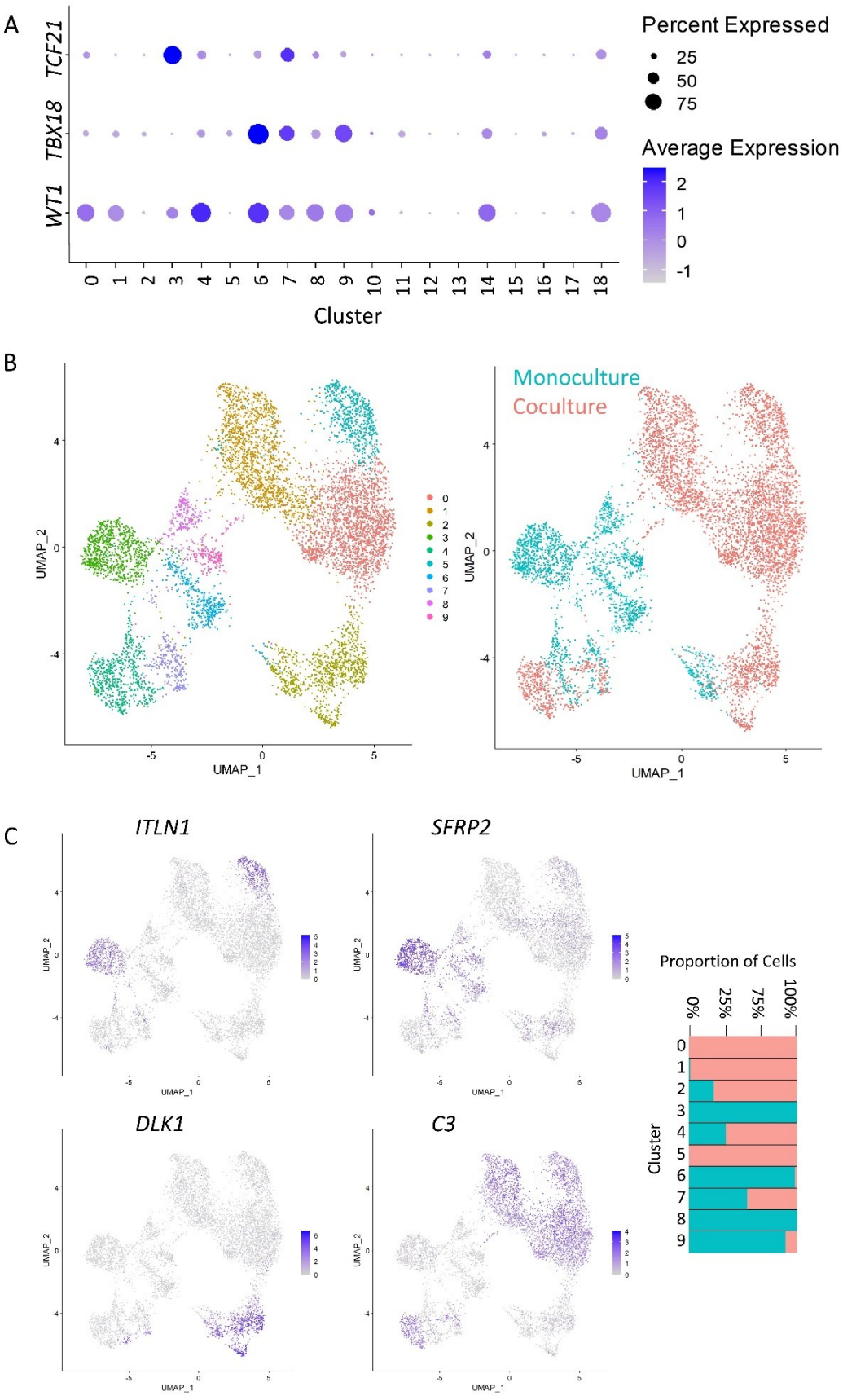
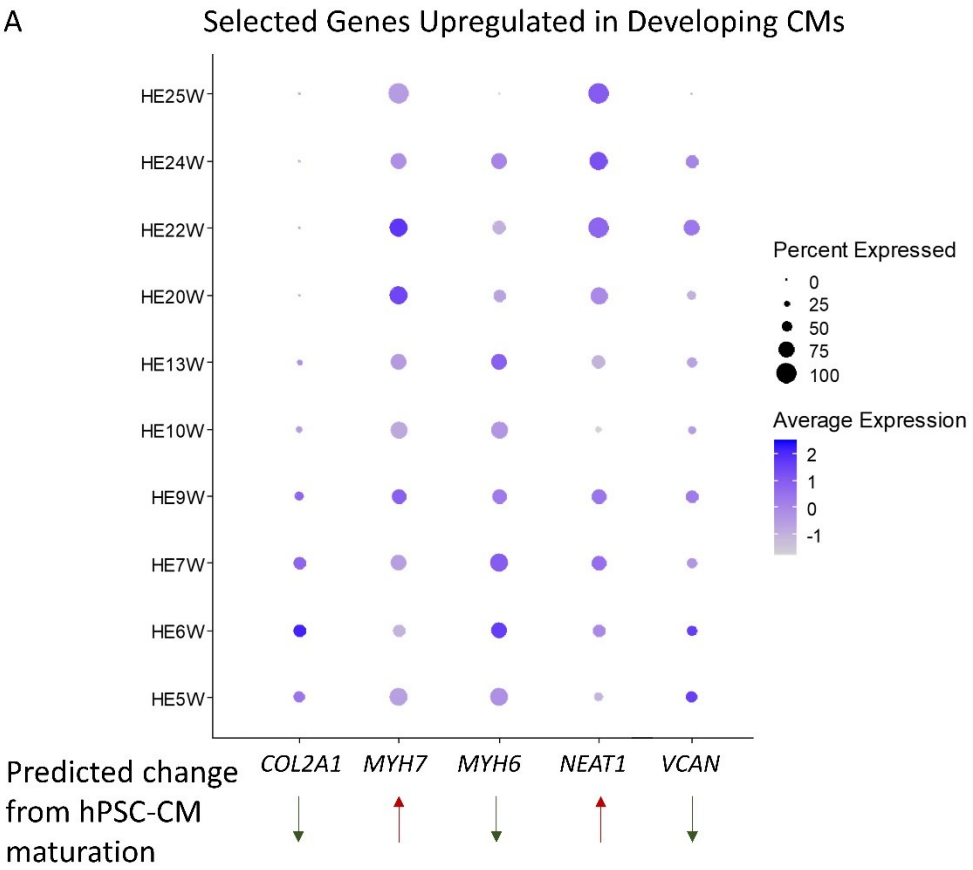

A
TCF21
TBX18
WT1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Cluster
Percent Expressed
25
50
75
Average Expression
2
1
0
-1
B
UMAP_2
UMAP_1
0 1 2 3 4 5 6 7 8 9
Monoculture
Coculture
C
ITLN1
SFRP2
DLK1
C3
Proportion of Cells
0%
25%
75%
100%
Cluster
0 1 2 3 4 5 6 7 8 9

Figure S10, Single cell analysis of EpiC fate, Related to Figure 4. *(A) Dot plot of EpiC lineage markers WT1, TBX18, and TCF21. (B) Subclustering of epicardial lineages* (*Clusters 0-1, 3-4, 6, 8-9, and 14). (C) Feature plots of ITLN1 (epicardial marker), and SFRP2, DLK1, and C3 and proportion of cells in each cluster from monoculture and coculture conditions.*


A
Selected Genes Upregulated in Developing CMs
HE25W
HE24W
HE22W
HE20W
HE13W
HE10W
HE9W
HE7W
HE6W
HE5W
COL2A1
MYH7
MYH6
NEAT1
VCAN
Percent Expressed
0
25
50
75
100
Average Expression
2
1
0
-1
Predicted change from hPSC-CM maturation

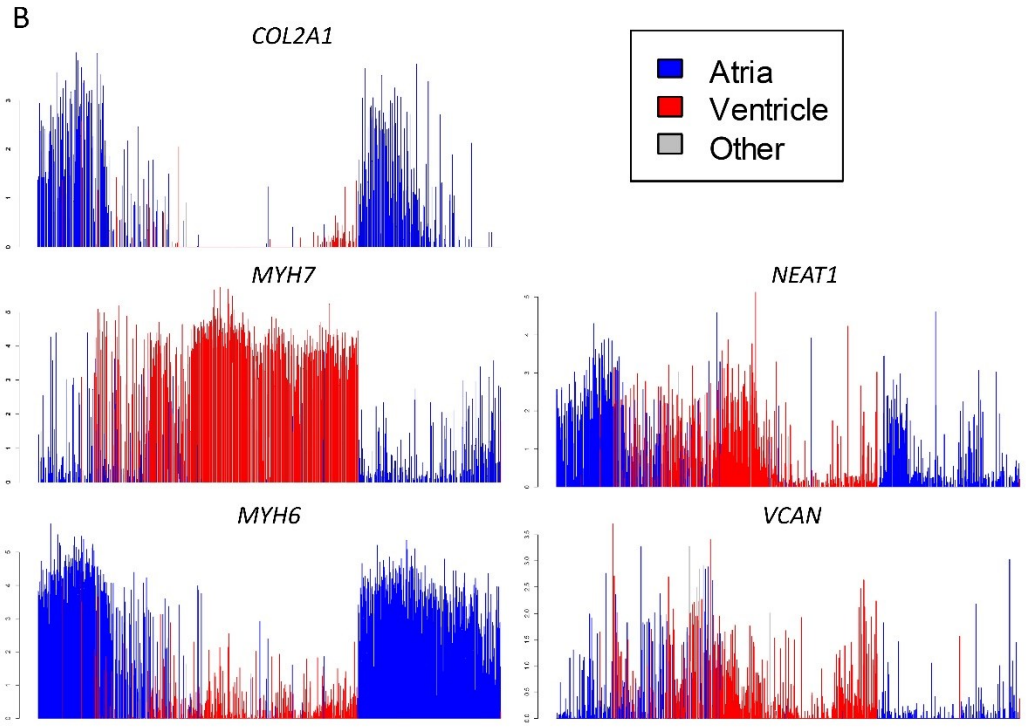

B
COL2A1
Atria
Ventricle
Other
MYH7
NEAT1
MYH6
VCAN

Figure S11, Single cell sequencing analysis of developing human CMs, Related to Figure 5. *(A) Dot plot of COL2A1, MYH7, MYH6, NEAT1, and VCAN. Y-axis represents weeks of human embryo development. Data from GSE106118 [30]. (B) Expression of selected genes by single cells. Blue denotes atrial CMs, red denotes ventricular CMs, and gray denotes CMs from other regions of the heart.*

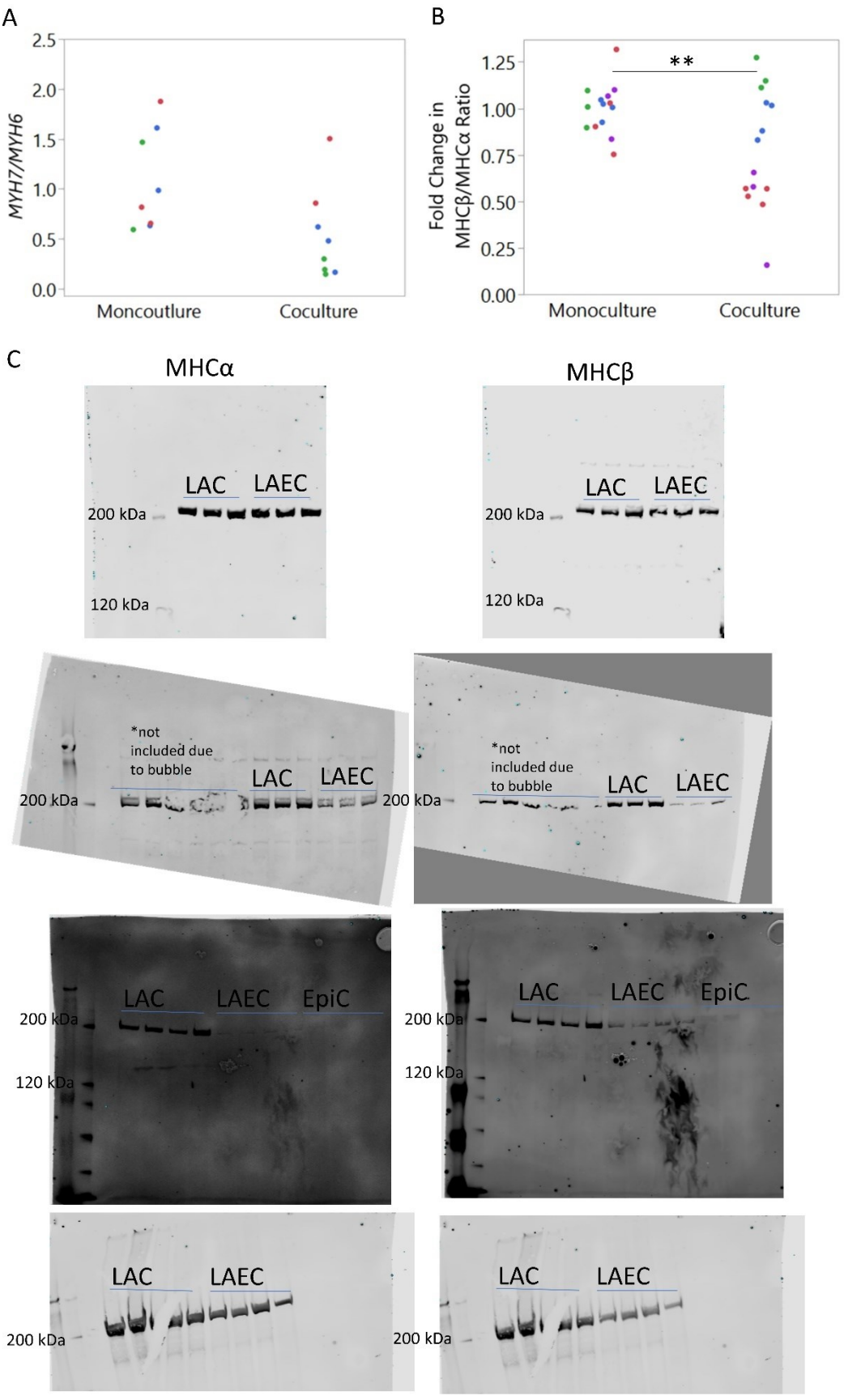

A
MYH7/MYH6
2.5
2.0
1.5
1.0
0.5
0.0
Moncoutlure
Coculture
B
Fold Change in MHCβ/MHCα Ratio
1.25
1.00
0.75
0.50
0.25
0.00
**
Monoculture
Coculture
C
MHCα
LAC
LAEC
200 kDa
120 kDa
MHCβ
LAC
LAEC
200 kDa
120 kDa
*not included due to bubble
LAC
LAEC
200 kDa
*not included due to bubble
LAC
LAEC
200 kDa
LAC
LAEC
EpiC
200 kDa
120 kDa
LAC
LAEC
EpiC
200 kDa
120 kDa
LAC
LAEC
200 kDa
LAC
LAEC
200 kDa

Figure S12, Transcriptomic analysis of *MYH6 and MYH7 and* Western blot analysis of MHCα and β expression, Related to Figure 5. *(A) qPCR analysis of RFP+ populations where the Y-axis is ratio of MYH6/MYH7 expression using the 2^-ΔΔCt method. Each dot represents an independent well (n=2-3) and each color represents three independent differentiations. One outlier was removed based on Grubb's statistical test prior to performing a two-way ANOVA where * is p<0.05. (B) Western blot analysis of LAC monoculture and LAEC coculture across five independent differentiations with 3-4 well replicates per differentiation. Graph indicates fold change in MHCβ/α ratio where each dot indicates an independent well and color indicates independent differentiations. Statistics are a two-way ANOVA where * is p<0.05. (C) Original blot images. One region was excluded from analysis due to presence of a bubble.*

Supplemental Data File 1: Differentially expressed genes in each cluster of our dataset.

Supplemental Data File 2: Differentially expressed genes in each cluster of the integration of our dataset, developing human heart dataset, and hPSC-CM maturation.

Supplemental Data File 3: Differentially expressed genes between monocultured and coculture CMs and differentially expressed genes in CM subclusters.

Supplemental Data File 4. Antibody information.

Supplemental Movies. Example of beating in monoculture and coculture conditions.