Supplemental Information

A Combined Human Gastruloid Model of Cardiogenesis and Neurogenesis

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Figure S1

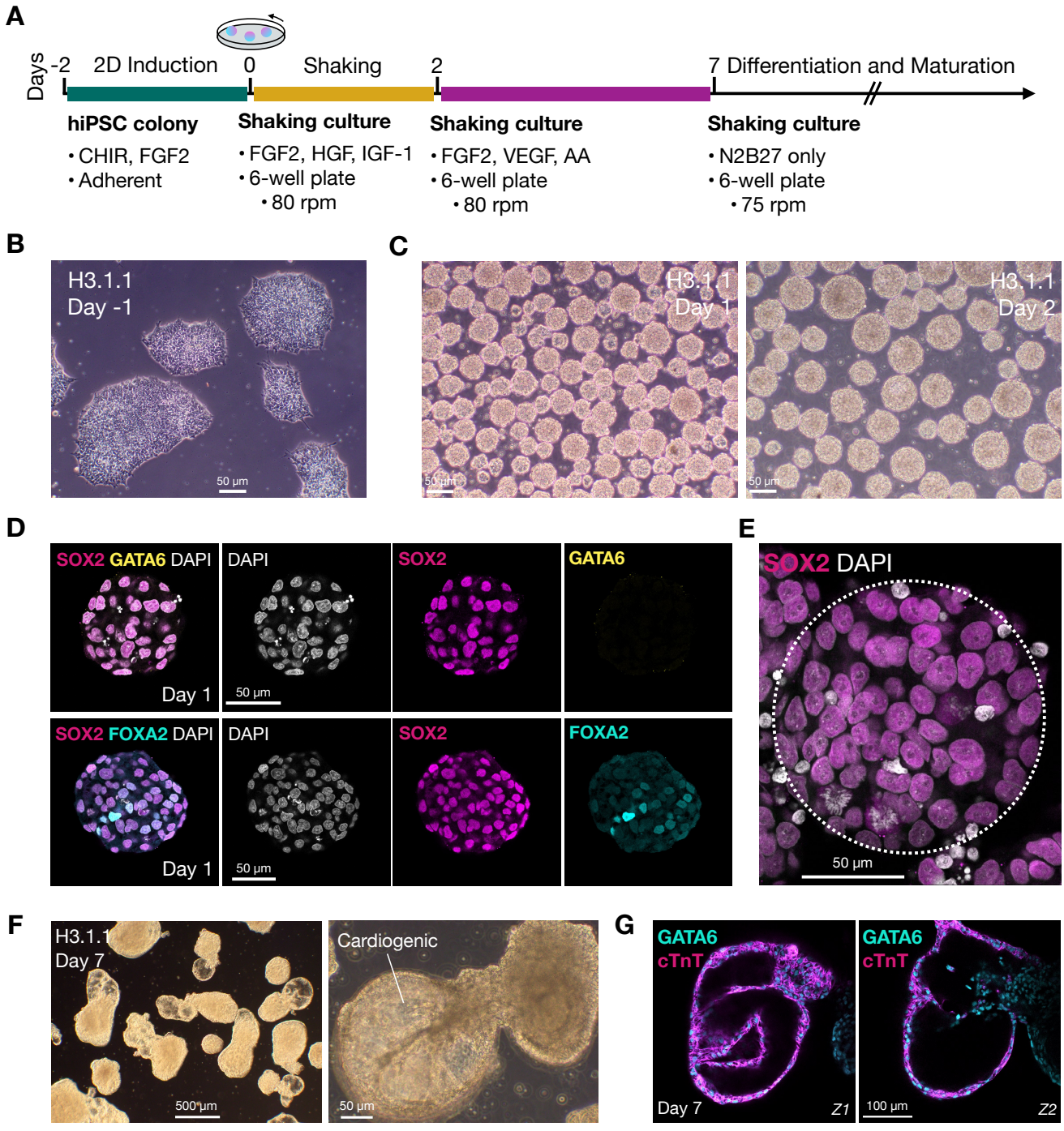
A
Days
-2
2D Induction
0
Shaking
2
7 Differentiation and Maturation
hiPSC colony
• CHIR, FGF2
• Adherent
Shaking culture
• FGF2, HGF, IGF-1
• 6-well plate
• 80 rpm
Shaking culture
• FGF2, VEGF, AA
• 6-well plate
• 80 rpm
Shaking culture
• N2B27 only
• 6-well plate
• 75 rpm
B
H3.1.1
Day -1
50 µm
C
H3.1.1
Day 1
H3.1.1
Day 2
D
SOX2 GATA6 DAPI
DAPI
SOX2
GATA6
Day 1
SOX2 FOXA2 DAPI
FOXA2
E
SOX2 DAPI
F
H3.1.1
Day 7
500 µm
Cardiogenic
G
GATA6
cTnT
Day 7
Z1
Z2
100 µm

Figure S1. Directed developmental cardiogenesis and spontaneous contractility in modified neuro-gastro-cardiac EMLOs (EMLOCs), related to Figure 1. (**A**) Overview of protocol for EMLOC gastruloid generation. Cardiogenesis was induced at 48 h post-aggregation by addition of VEGF and ascorbic acid (AA). (**B**) Phase contrast image of 2D hiPSC colonies 24 h after induction with N2B27 + CHIR/FGF2. (**C**) EMLOC suspension cultures 24 h and 48 h after dissociation and spontaneous aggregation on the orbital shaker. (**D**) Day 1 aggregates do not express GATA6 (yellow) and exhibit non-uniform FOXA2 expression (cyan) by immunofluorescence. (**E**) Immunofluorescence in a single day 1 aggregate (white dotted line) demonstrates uniform, non-polarized expression of SOX2 (magenta). Cells are counterstained with DAPI (grey). (**F**) Phase contrast images of polarized gastruloids with spontaneously contracting cardiogenic chambers (day 7). Annotated EMLOC is shown (right, see also **Movie S2**). (**G**) Two immunofluorescence Z-slices with cardiac biomarkers GATA6 (blue) and cardiac Troponin-T (cTnT, magenta) in a day 7 gastruloid demonstrating chamber cytoarchitecture. Images are representative of the general EMLOC population at these stages. Individual scale bars provided.

Figure S2

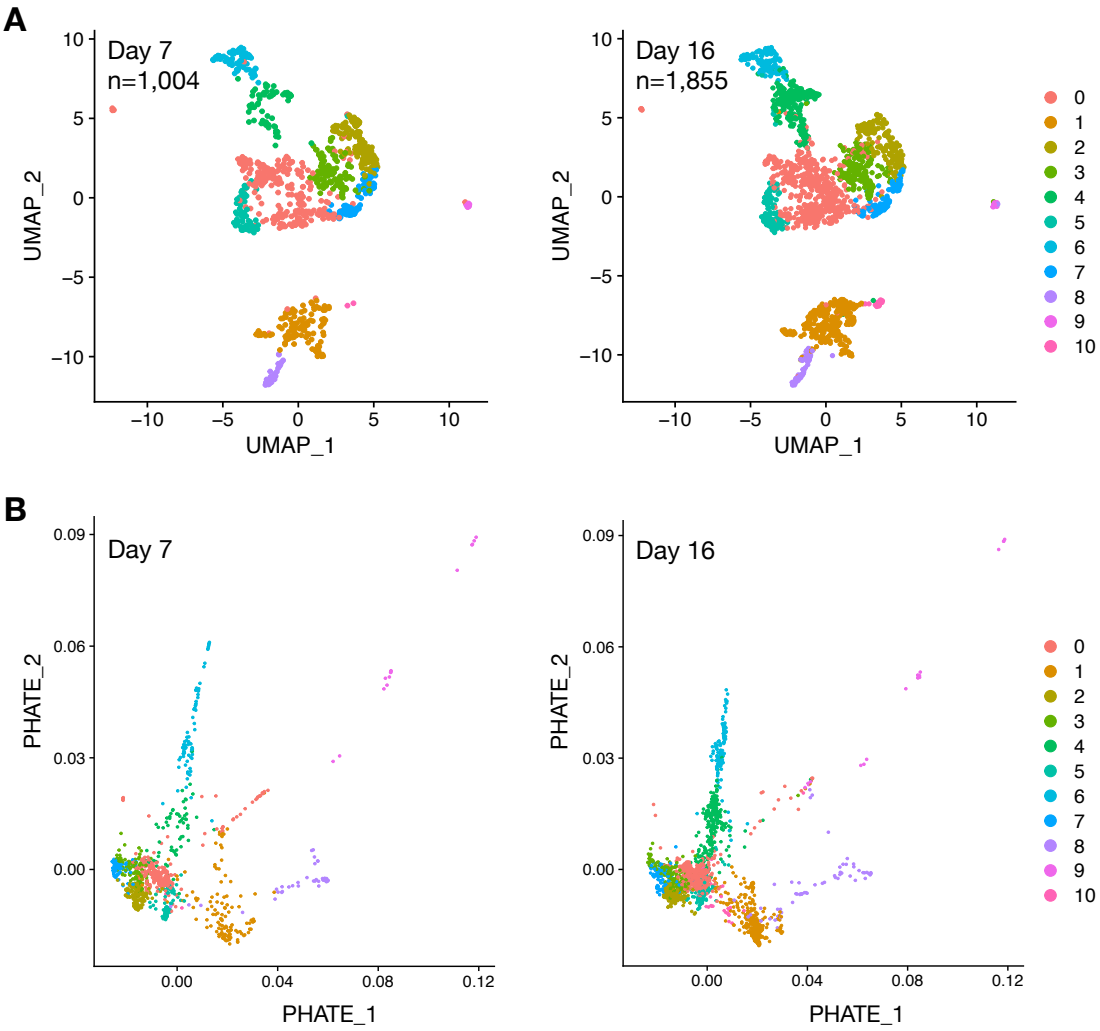
A
Day 7
n=1,004
Day 16
n=1,855
UMAP_2
UMAP_1
10
5
0
−5
−10
0
1
2
3
4
5
6
7
8
9
10
B
Day 7
Day 16
PHATE_2
PHATE_1
0.09
0.06
0.03
0.00
0.04
0.08
0.12

Figure S2. UMAP and PHATE visualization of scRNAseq for day 7 and day 16 EMLOC time points, related to Figure 1. (**A**) UMAP representation of day 7 (1,004 cells) and day 16 (1,855 cells) according to the integrated dataset with ten clusters. (**B**) PHATE representation of samples in (**A**).

Figure S3

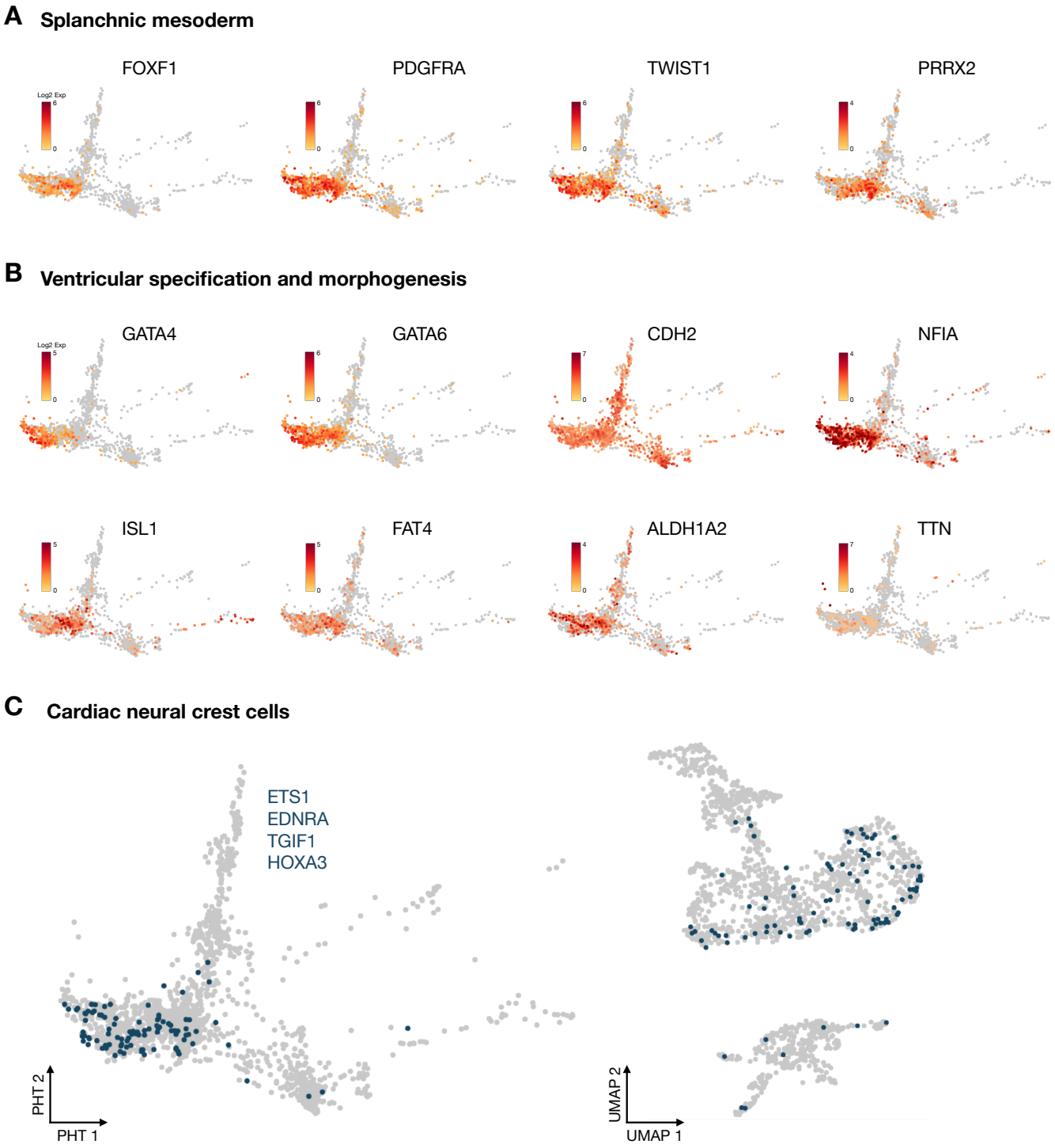
A
Splanchnic mesoderm
FOXF1
PDGFRA
TWIST1
PRRX2
Log2 Exp
B
Ventricular specification and morphogenesis
GATA4
GATA6
CDH2
NFIA
ISL1
FAT4
ALDH1A2
TTN
C
Cardiac neural crest cells
ETS1
EDNRA
TGIF1
HOXA3
PHT 2
PHT 1
UMAP 2
UMAP 1

Figure S3. Developmental features of cardiogenesis in EMLOCs, related to Figure 2. (**A**) Characteristic gene biomarkers for splanchnic mesoderm (*FOXF1, PDGFRA, TWIST1, PRRX2*). (**B**) Gene biomarkers of ventricular cell specification and morphogenesis (*GATA4, GATA6, CDH2, NFIA, ISL1, FAT, ALDH1A2, TTN*). (**C**) PHATE (left) and UMAP (right) visualization of cells co-expressing *ETS1, EDNRA, TGIF1, HOXA2* consistent with a cardiac neural crest cell phenotype.

Figure S4

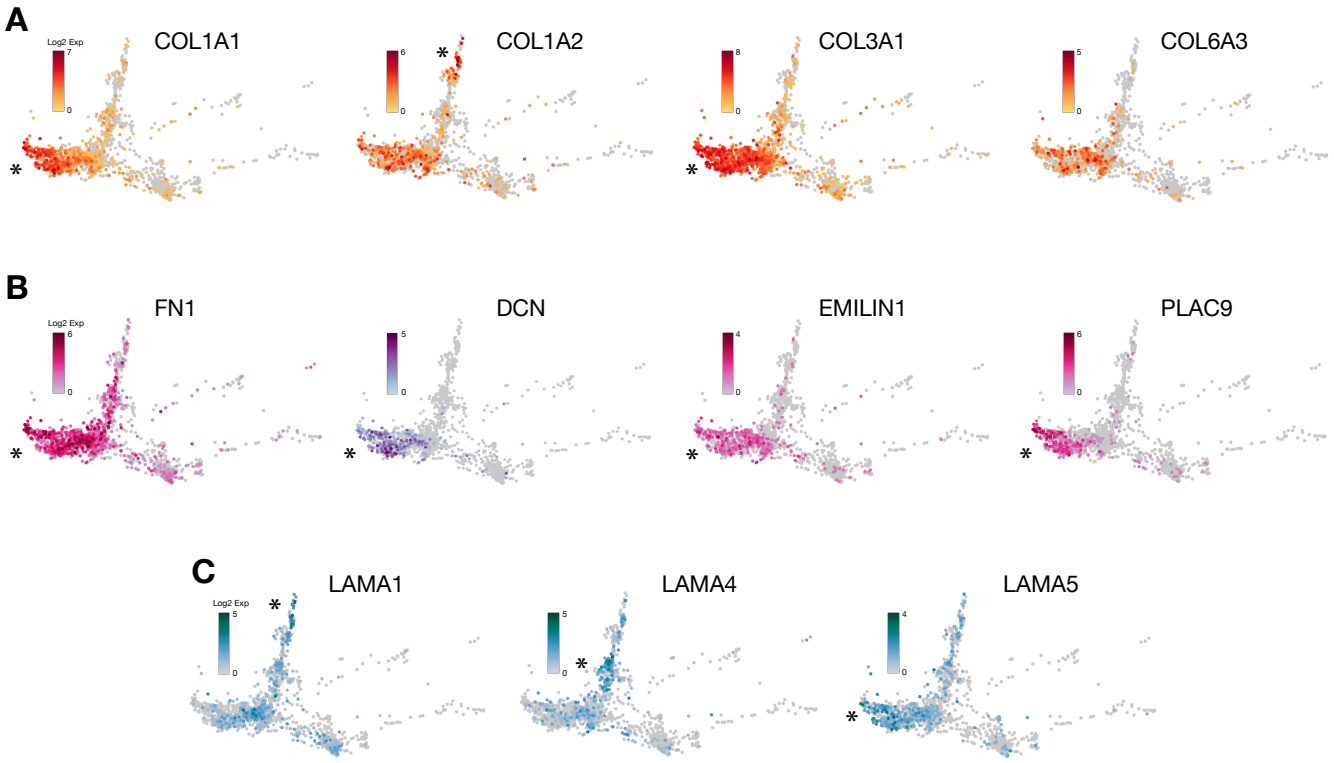
A
COL1A1
COL1A2
COL3A1
COL6A3
B
FN1
DCN
EMILIN1
PLAC9
C
LAMA1
LAMA4
LAMA5
Log2 Exp

Figure S4. EMLOCs generate the appropriate cardiac ECM milieu, related to Figure 2. (**A**) Four collagen genes (*COL1A1, COL1A2, COL3A1, COL6A3*). (**B**) Glycoprotein (*FN1*) and proteoglycan (*DCN*) genes along with *EMILIN1, PLAC9*. (**C**) Three laminin genes (*LAMA1, LAMA4, LAMA5*) show distinct distributions by PHATE. Asterisk (*) indicates region of highest expression (Cui et al., 2019).

Figure S5

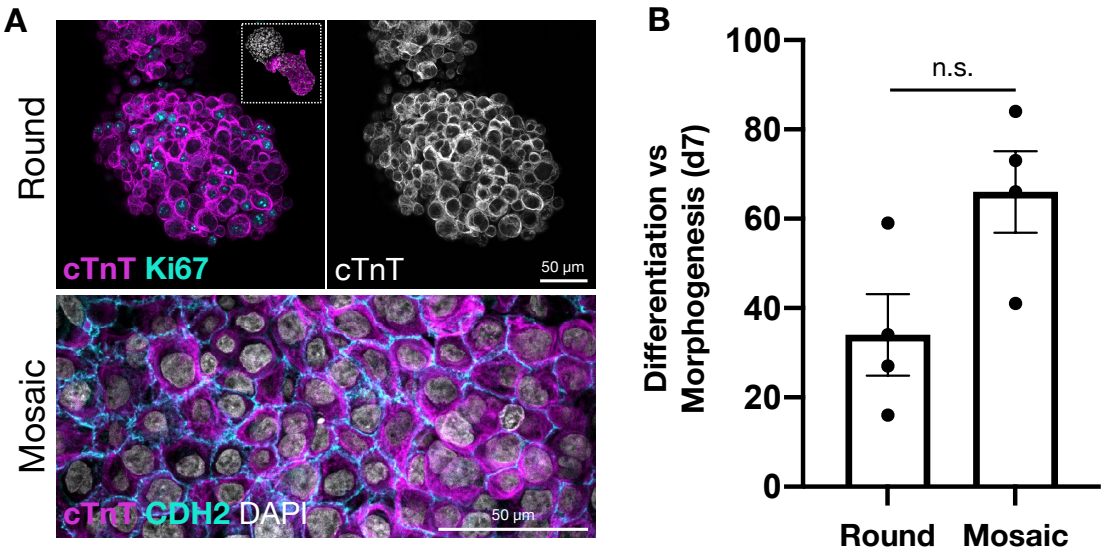
A
Round
cTnT Ki67
cTnT
50 µm
Mosaic
cTnT CDH2 DAPI
50 µm
B
Differentiation vs Morphogenesis (d7)
100
80
60
40
20
0
n.s.
Round
Mosaic

Figure S5. Assessment of cardiomyocyte morphology with differentiation and morphogenesis states, related to Figure 5. (**A**) Relative proportions of day 7 EMLOCs with rounded, proliferating cardiomyocyte progenitors versus flattened, mosaic cardiomyocyte progenitors suggests co-existing phases of differentiation and morphogenesis as previously described for mouse cardiogenesis (N = 4 replicate experiments; n.s. $p = 0.1776$, $t = 1.754$, $df = 3$ by paired two-tailed t-test). (**B**) Immunofluorescence examples of rounded, proliferating progenitors (top: cTnT, Ki67) versus flat, mosaic progenitors (bottom: cTnT, CDH2, DAPI). Left inset is example of whole EMLOC with rounded cardiomyocytes. Individual scale bars provided.

Figure S6

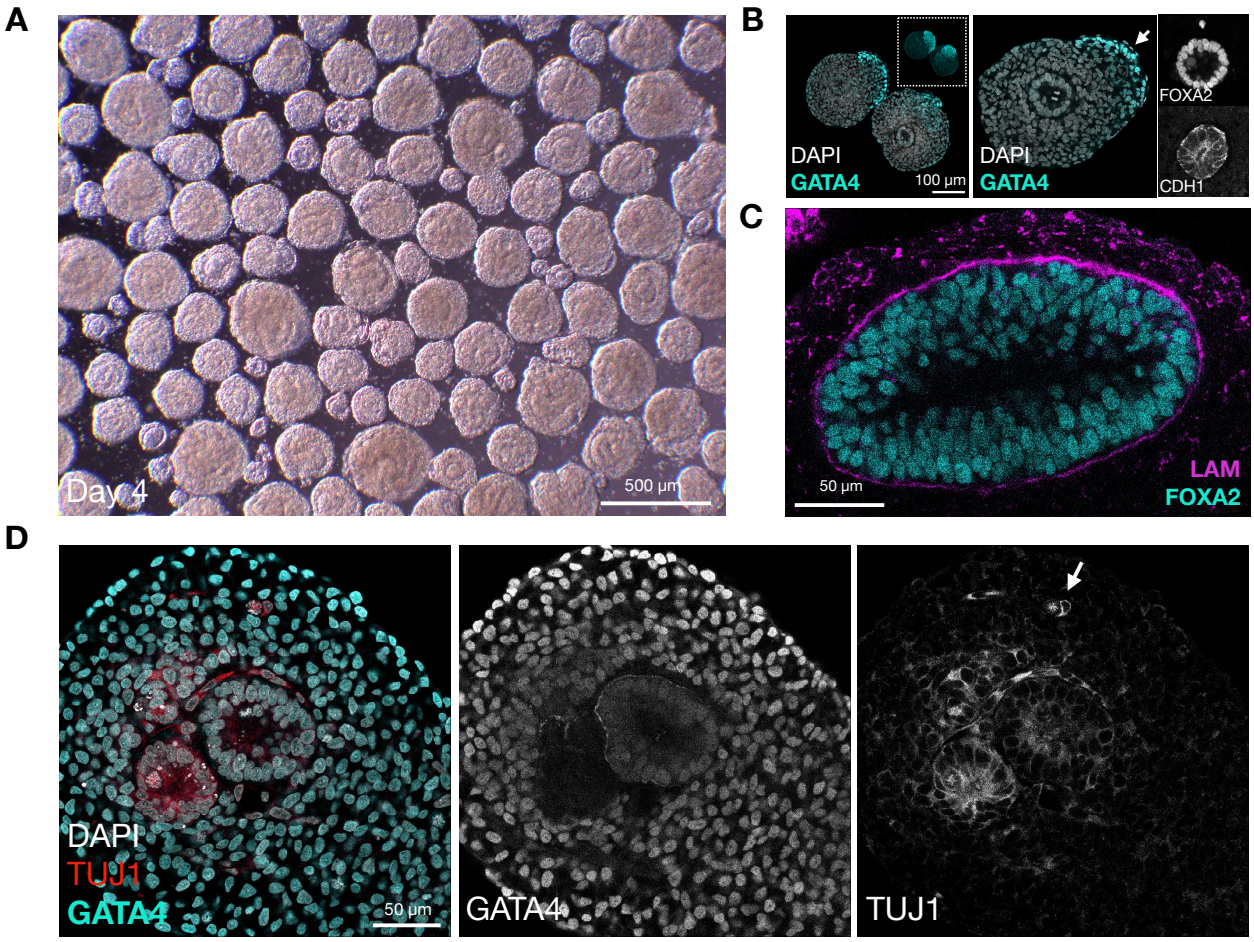
A
Day 4
500 µm
B
DAPI
GATA4
100 µm
DAPI
GATA4
FOXA2
CDH1
C
50 µm
LAM
FOXA2
D
DAPI
TUJ1
GATA4
50 µm
GATA4
TUJ1

Figure S6. Additional characterization of the gut tube in EMLOCs, related to Figure 5. (**A**) Day 4 EMLOCs with visible gut tube endoderm. (**B**) FOXA2+/CDH1+ gut tube endoderm is self-organized posterior to the GATA4+ cardiac crescent. White arrow points to early serous lining of the cardiac crescent. (**C**) FOXA2+ gut tube endoderm is laminated, where has TUJ1+/GATA4- neural rosettes are more continuous with surrounding cells, labeled in (**D**). TUJ1 was also observed in mitotic spindle MTs (white arrow). Individual scale bars provided.

Figure S7

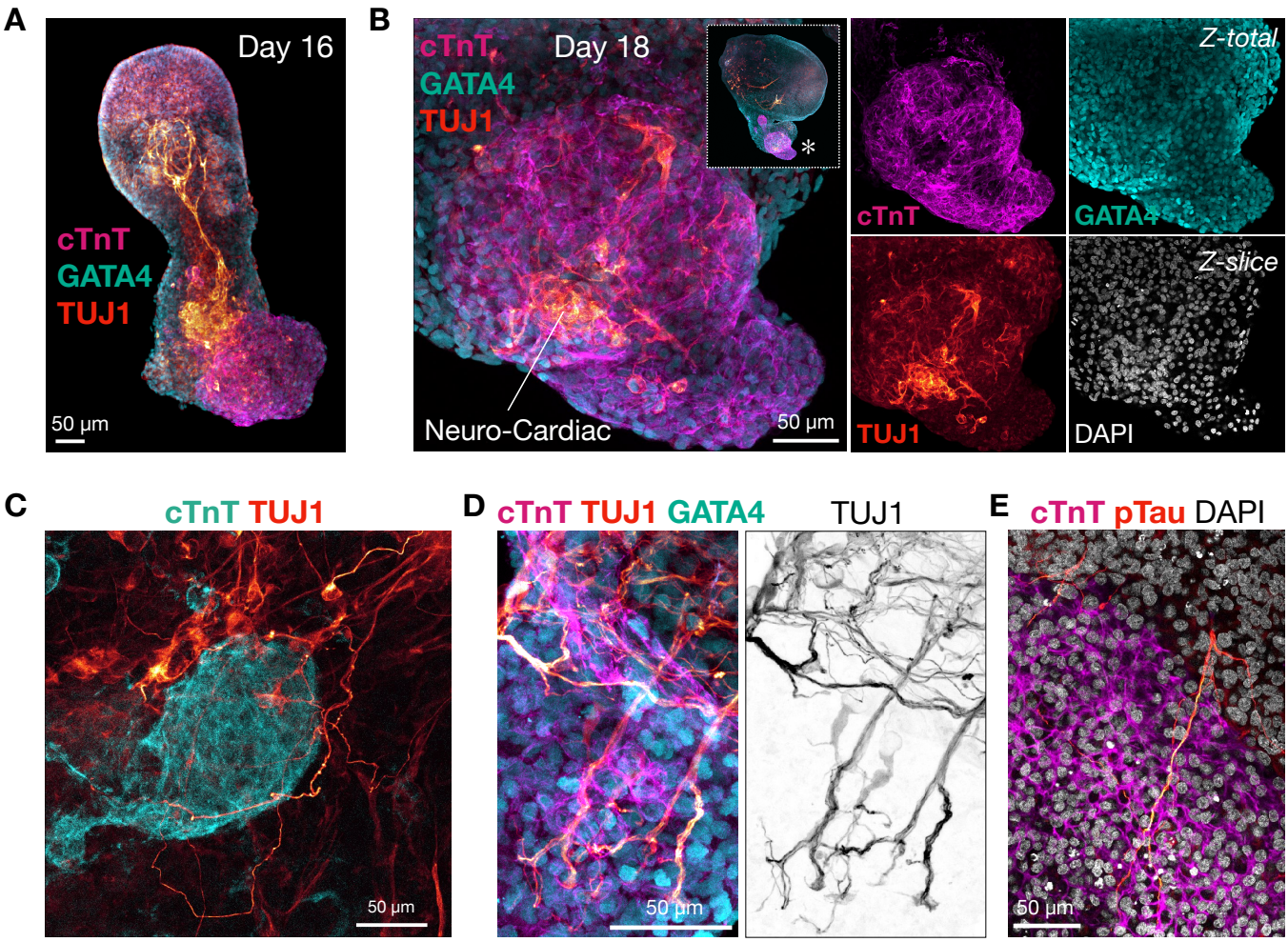
A
Day 16
cTnT
GATA4
TUJ1
50 µm
B
cTnT,
GATA4
TUJ1
Day 18
*
Neuro-Cardiac
50 µm
cTnT
Z-total
GATA4
TUJ1
Z-slice
DAPI
C
cTnT TUJ1
50 µm
D
cTnT TUJ1 GATA4
TUJ1
50 µm
E
cTnT pTau DAPI
50 µm

Figure S7. Additional characterization of neuronal fibers in EMLOCs, related to Figure 8. (**A**) Immunofluorescence of cardiac biomarkers cTnT (magenta) and GATA4 (cyan) along with TUJ1 (red) in day 16 H3.1.1 EMLOCs demonstrate the emergence of neurons proximal to the cardiac region. (**B**) Immunofluorescence of cTnT (magenta) and TUJ1 (red) at day 18 depicts ganglionated neuronal plexus-like structures in the cardiac region. Merge is shown with individual cTnT, GATA4 (cyan), TUJ1 channels. Left inset is low magnification EMLOC image. (**C**) TUJ1+ neuronal fiber termination (red) onto cTnT+ cells (cyan) reminiscent of nodal innervation. (**D**) Cardiac biomarkers cTnT (magenta) and GATA6 (cyan) with TUJ1. (**E**) Axons identifiable by pTau within the cTnT+ myocardium (right). Individual scale bars provided.

Table S1. Summary of scRNAseq biomarkers reported in Results, related to Supplementary data file*

Cell type, tissue type or process	Biomarker reported
Cell proliferation	MKI67
First heart field	TBX5
First heart field	NKX2-5
First heart field	HAND1
Second heart field	HAND2
Second heart field	MEF2C
Second heart field	TBX18
Second heart field	ISL1
Cardiac sarcomere	TNNT2
Cardiac sarcomere	TNNT1
Cardiac sarcomere	MYL7
Fetal heart development	KRT8
Fetal heart development	KRT18
Fetal heart development	APOE
Fetal heart development	PLAC9
Fetal heart development	S100A10
Splanchnic mesoderm	FOXF1
Splanchnic mesoderm	PDGFRA
Splanchnic mesoderm	TWIST1
Splanchnic mesoderm	PRRX2
Ventricular specification	GATA4
Ventricular specification	GATA6
Ventricular specification	CDH2
Ventricular specification	NFIA
Ventricular specification	ISL1
Ventricular specification	FAT4
Ventricular specification	ALDH1A2
Ventricular specification	TTN
Left-right asymmetry	IRX3
Left-right asymmetry	HAND1

Left-right asymmetry	PITX2
Left-right asymmetry	RTTN
Cardiac neural crest cells	ETS1
Cardiac neural crest cells	EDNRA
Cardiac neural crest cells	TGIF1
Cardiac neural crest cells	HOXA3
Rapid ventricular conduction	IRX3
Cardiac repolarization	IRX5
Calcium flux	ITPR2
Calcium handling	SLC8A1
AV conduction, organization	GJA1
AV conduction, organization	CACNA1H
AV conduction, organization	TBX3
AV conduction, organization	CXCL12
AV conduction, organization	DSP
Atrioventricular valves	POSTN
Atrioventricular valves	NPR3
Atrioventricular valves	TBX3
Atrioventricular valves	NFATC4
Outflow tract smooth muscle	CNN1
Outflow tract smooth muscle	TAGLN
Outflow tract	ISL1
Outflow tract	PDE5A
Outflow tract	CDH11
Outflow tract	FN1
Outflow tract	MEGF6
Outflow tract	MSX2
Outflow tract	SEMA3C
Outflow tract	EMILIN1
Cardiac jelly	VCAN
Cardiac jelly	ADAMTS1
Cardiac jelly	ANGPT1
Vascular endothelial cells	KDR

Vascular endothelial cells	FLT1
Vascular endothelial cells	ESAM
Vascular endothelial cells	CDH5
Vascular endothelial cells	GJA4
Epicardial cells	WT1
Epicardial cells	TCF21
Epicardial cells	TPJ1
Epicardial cells	LHX2
Epicardial cells	LHX9
Epicardial cells	TBX18
Epicardial cells	PLAC9
Cardiac fibroblast	IFI16
Cardiac fibroblast	TBX5
Cardiac fibroblast	IGFBP5
Cardiac fibroblast	BTS2
Extracellular matrix	COL1A1
Extracellular matrix	COL1A2
Extracellular matrix	COL3A1
Extracellular matrix	COL6A3
Extracellular matrix	FN1
Extracellular matrix	DCN
Extracellular matrix	EMILIN1
Extracellular matrix	PLAC9
Extracellular matrix	LAMA1
Extracellular matrix	LAMA4
Extracellular matrix	LAMA5
Foregut endoderm	FOXA2
Foregut endoderm	NKX2-1
Foregut endoderm	EPCAM
Foregut endoderm	CDH1
Foregut endoderm	HHEX
Foregut endoderm	SHH
Trunk neuroectoderm	SOX2

Trunk neuroectoderm	FABP7
Trunk neuroectoderm	ZIC1
Trunk neuroectoderm	RFX4
Trunk neuroectoderm	HES5
Trunk neuroectoderm	EDNRB
Trunk neuroectoderm	NTRK2
Trunk neuroectoderm	OLIG3
Trunk neuroectoderm	MSX1
Trunk neuroectoderm	HOXC6
Trunk neuroectoderm	HOXC9
Neuronal	STMN2
Neuronal	GAP43
Neuronal	TUBB3
Neuronal	ELAVL3
Neuronal	DLG4
Neuronal	CAMK2A
Neuronal	SLC18A3
Neuronal	SLC17A6
Neuronal	CHRNA3
Neuronal	NTRK3
Sensory neuron	POU4F1
Motor neuron	MNX1
Autonomic neuron	PHOX2B
Autonomic neuron	ASCL1
Sympathetic neuron	INSM1
Sympathetic neuron	ISL1
Schwann cell glia	SOX10
Schwann cell glia	PLP1
Schwann cell glia	MPZ
Schwann cell glia	S100B
Schwann cell glia	TFAP2B
Schwann cell glia	NGFR
Neuro-cardiac patterning	NPY

Neuro-cardiac patterning	BDNF
Neuro-cardiac patterning	SEMA3A
Neuro-cardiac patterning	PRPH
Neuro-cardiac patterning	EDNRA
Neuro-cardiac patterning	ISL1
Renal	MAL
Renal	SIM1
Renal	EPCAM
Renal	TFAP2A
Renal	SIX1
Renal	WT1
Renal	ITGA8
Renal	EYA1
Renal	PAX2
Renal	PAX8
Renal	LHX1
Renal	EMX2
BMP signaling	BMP4
BMP signaling	BMP7
WNT signaling	WNT2B
WNT signaling	WNT1
WNT signaling	WNT3A
Hox code	HOXA4
Hox code	HOXC9
Hox code	HOXD8
Cadherin	CDH1
Cadherin	CDH6
Cadherin	CDH11

*An extended version of this table appears as a Supplementary data file