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Supplemental Information

Cell-Type-Specific Gene Regulatory Networks Underlying Murine Neonatal Heart Regeneration at Single-Cell Resolution

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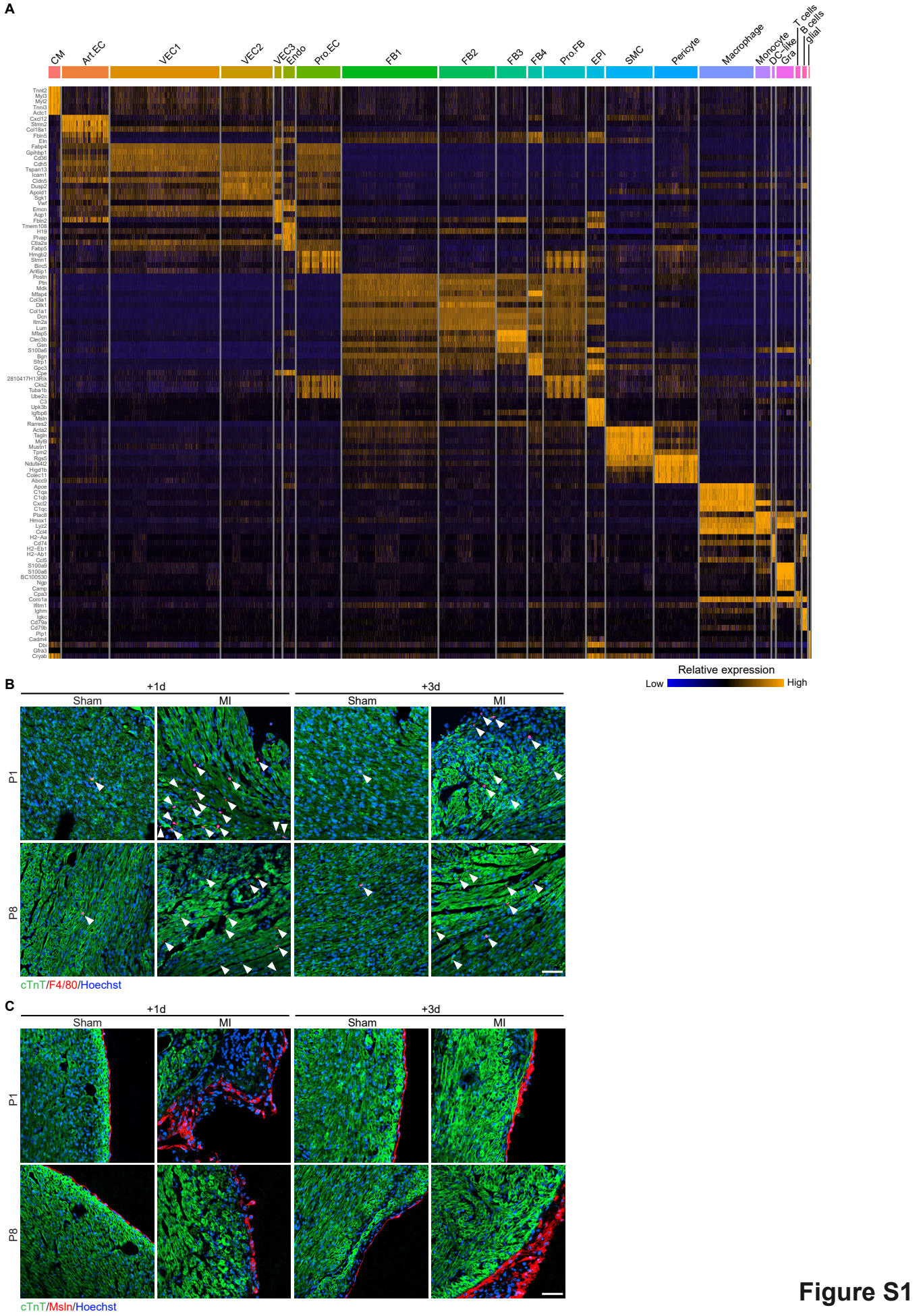

A
CM
Art.EC
VEC1
VEC2
VEC3
Endo
Pro.EC
FB1
FB2
FB3
FB4
Pro.FB
EPI
SMC
Pericyte
Macrophage
Monocyte
DC-like
Gra
T cells
B cells
Glial
Relative expression
Low
High
B
+1d
+3d
Sham
MI
P1
P8
cTnT/F4/80/Hoechst
C
cTnT/Msln/Hoechst

Figure S1

Figure S1. Single cell RNA-seq (scRNA-seq) identifies composition and transcription landscapes of different cardiac cells, Related to Figure 1.

A. Heatmap showing the expression of top enriched genes for each cell cluster identified in the scRNA-seq data.

B. Macrophage infiltration in regenerating and non-regenerating neonatal hearts. Sections from 1d- and 3d- post MI/Sham hearts were stained for F4/80 (macrophage marker, red), cardiac troponin T (cTnT, CM marker, green) and nuclei were counterstained with Hoechst (blue). Representative images were shown from n=4 individual sections collected from n=2 animals for each timepoint and condition. Compared with age-matched sham controls, more macrophages (indicated by arrowheads) can be detected in infarct and border zones of MI hearts at all timepoints examined. Scale bar, 50 μm.

C. Expansion of epicardial cells in regenerating and non-regenerating neonatal hearts. Sections from 1d- and 3d- post MI/Sham hearts were stained for Msln (epicardial cell marker, red), cardiac troponin T (cTnT, CM marker, green) and nuclei were counterstained with Hoechst (blue). Representative images were shown from n=4 individual sections collected from n=2 animals for each timepoint and condition. The expansion of epicardial cells and their morphological changes were observed adjacent to the infarct region of post-MI hearts at all timepoints examined. Scale bar, 50 μm.

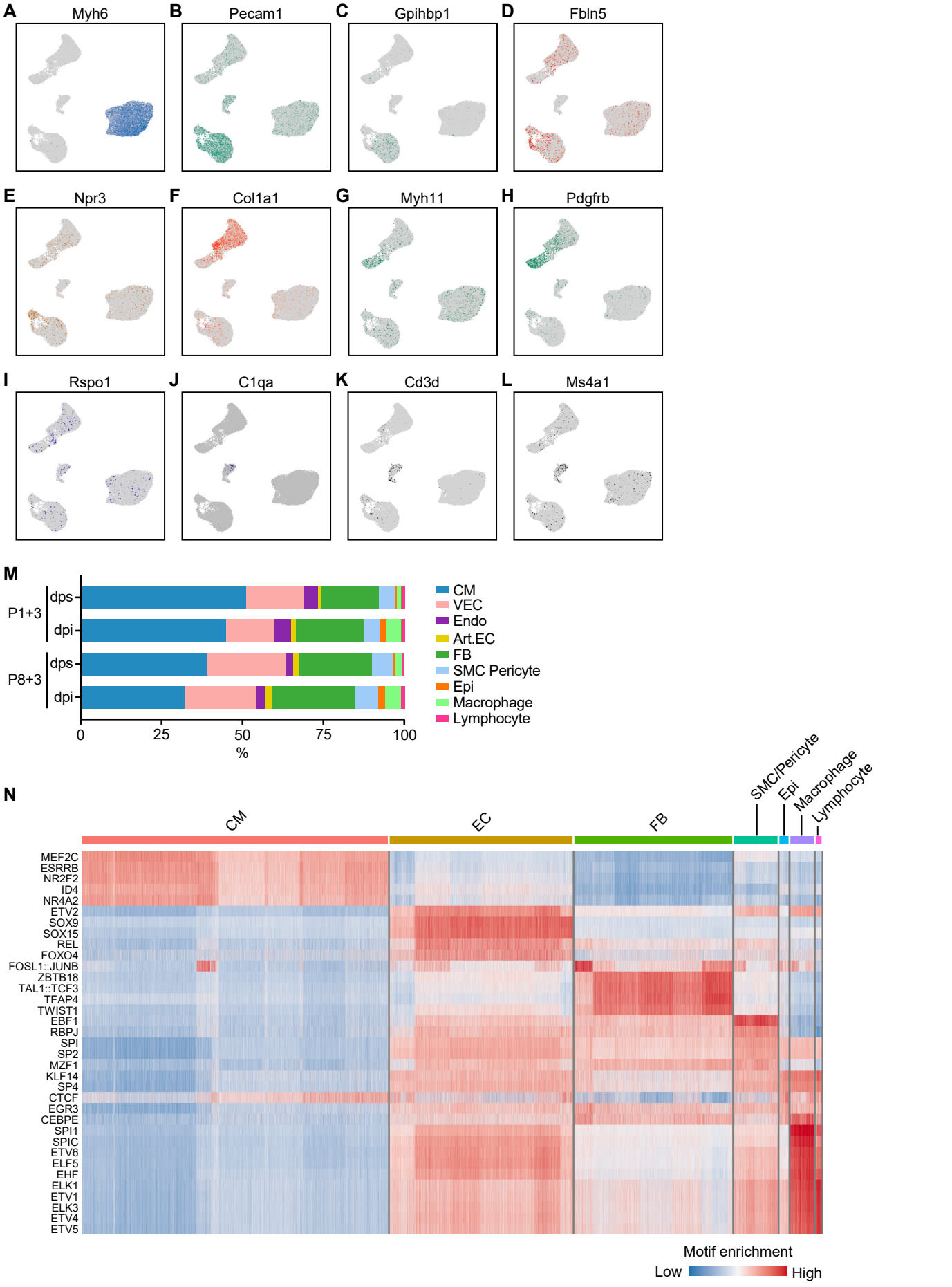
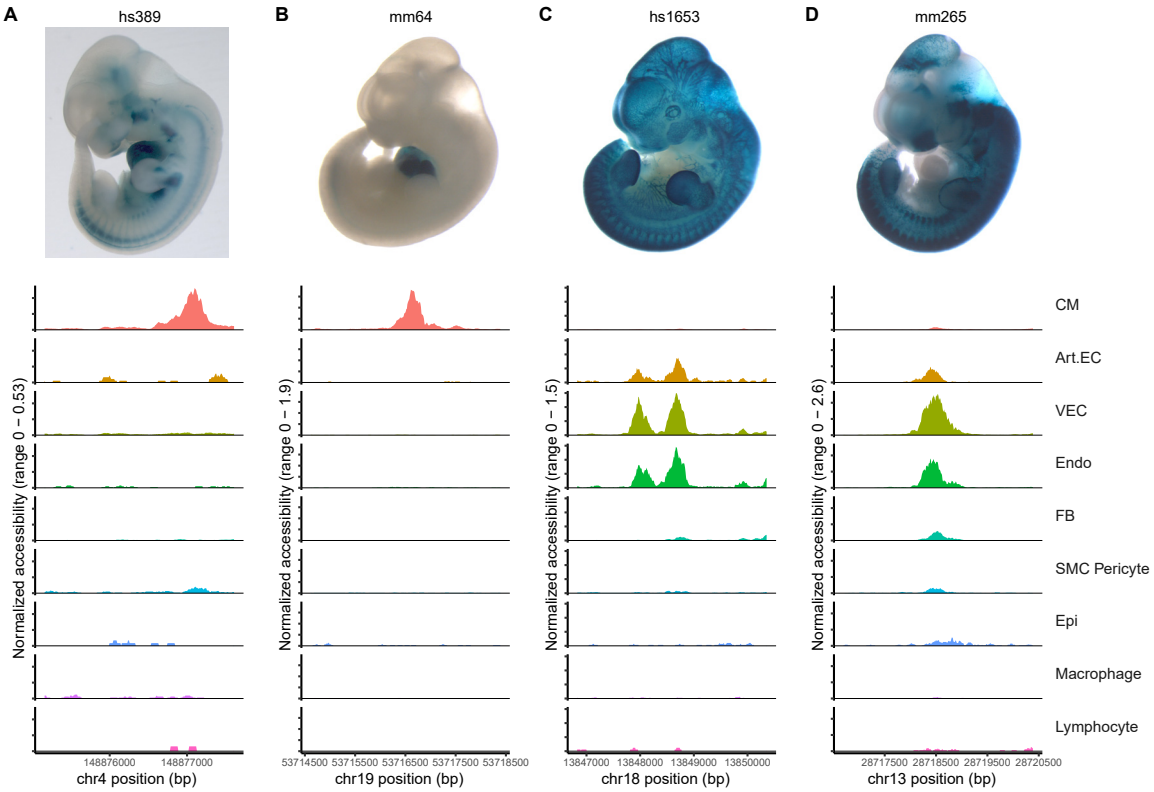
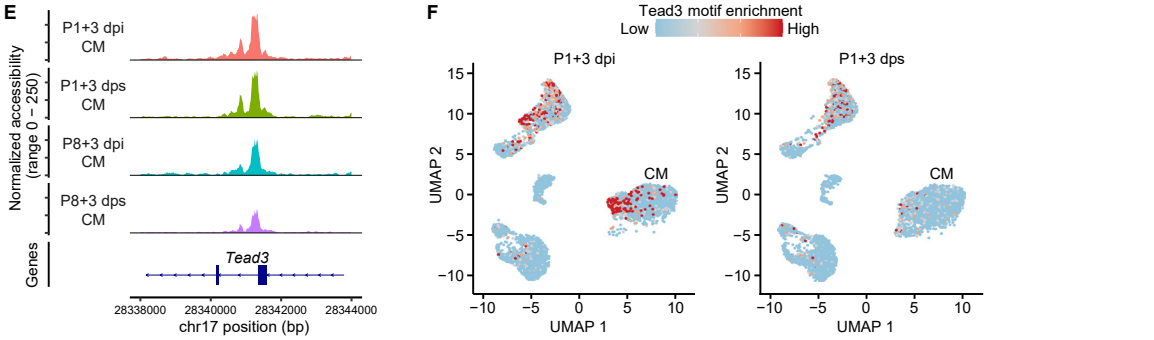

A
Myh6
B
Pecam1
C
Gpihbp1
D
Fbln5
E
Npr3
F
Col1a1
G
Myh11
H
Pdgfrb
I
Rspo1
J
C1qa
K
Cd3d
L
Ms4a1
M
P1+3
dps
dpi
P8+3
dps
dpi
0
25
50
75
100
%
CM
VEC
Endo
Art.EC
FB
SMC Pericyte
Epi
Macrophage
Lymphocyte
N
CM
EC
FB
SMC/Pericyte
Epi
Macrophage
Lymphocyte
MEF2C
ESRRB
NR2F2
ID4
NR4A2
ETV2
SOX9
SOX15
REL
FOXO4
FOSL1::JUNB
ZBTB18
TAL1::TCF3
TFAP4
TWIST1
EBF1
RBPJ
SPI
SP2
MZF1
KLF14
SP4
CTCF
EGR3
CEBPE
SPI1
SPIC
ETV6
ELF5
EHF
ELK1
ETV1
ELK3
ETV4
ETV5
Motif enrichment
Low
High

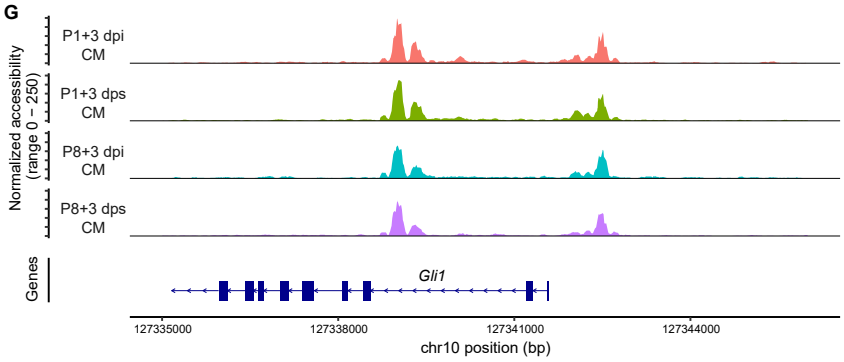
Figure S2

Figure S2. A single cell atlas of open chromatin landscapes of neonatal hearts, Related to Figure 2.

A. UMAP plot showing the gene activity (chromatin accessibility at promoter and gene body regions) of *Myh6*, a CM marker gene.
B. UMAP plot showing the gene activity of *Pecam1*, a pan-EC marker gene.
C. UMAP plot showing the gene activity of *Gpihbp1*, a capillary EC marker gene
D. UMAP plots showing the gene activity of *Fbln5*, an artery EC marker gene.
E. UMAP plot showing the gene activity of *Npr3*, an endocardial cell marker gene.
F. UMAP plot showing the gene activity of *Col1a1*, a FB marker gene.
G. UMAP plot showing the gene activity of *Myh11*, a SMC marker gene.
H. UMAP plot showing the gene activity of *Pdgfrb*, a pericyte marker gene.
I. UMAP plot showing the gene activity of *Rspo1*, an epicardial cell marker gene.
J. UMAP plot showing the gene activity of *C1qa*, a macrophage marker gene.
K. UMAP plot showing the gene activity of *Cd3d*, a T cell marker gene.
L. UMAP plot showing the gene activity of *Ms4a1*, a B cell marker gene.
M. Percentage of each cell type within each scATAC-seq sample.
N. Heatmap showing the relative enrichment of selected top enriched TF binding motifs for each cell cluster.


A
hs389
B
mm64
C
hs1653
D
mm265
Normalized accessibility (range 0 – 0.53)
Normalized accessibility (range 0 – 1.9)
Normalized accessibility (range 0 – 1.5)
Normalized accessibility (range 0 – 2.6)
CM
Art.EC
VEC
Endo
FB
SMC Pericyte
Epi
Macrophage
Lymphocyte
148876000 148877000
chr4 position (bp)
53714500 53715500 53716500 53717500 53718500
chr19 position (bp)
13847000 13848000 13849000 13850000
chr18 position (bp)
28717500 28718500 28719500 28720500
chr13 position (bp)


E
Normalized accessibility (range 0 – 250)
P1+3 dpi CM
P1+3 dps CM
P8+3 dpi CM
P8+3 dps CM
Genes
Tead3
28338000 28340000 28342000 28344000
chr17 position (bp)
F
Tead3 motif enrichment
Low
High
P1+3 dpi
P1+3 dps
UMAP 2
UMAP 1
CM


G
Normalized accessibility (range 0 – 250)
P1+3 dpi CM
P1+3 dps CM
P8+3 dpi CM
P8+3 dps CM
Genes
Gli1
127335000 127338000 127341000 127344000
chr10 position (bp)

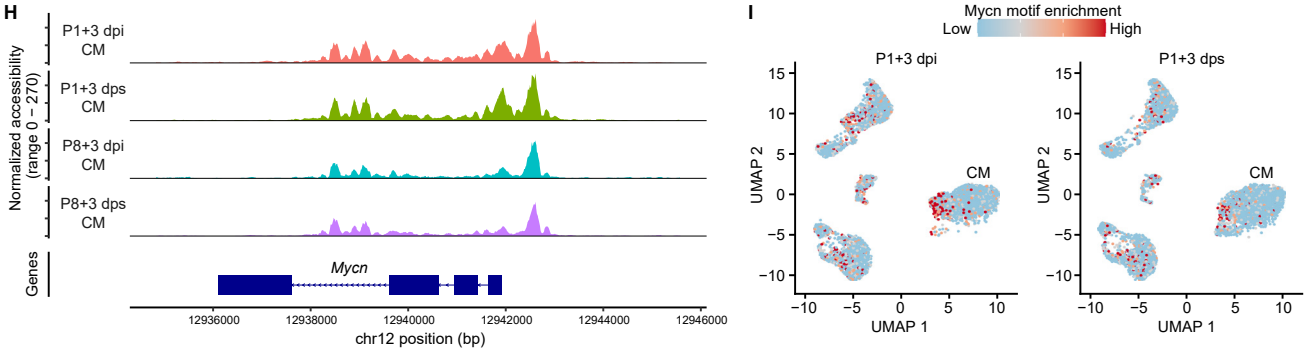

H
Normalized accessibility (range 0 – 270)
P1+3 dpi CM
P1+3 dps CM
P8+3 dpi CM
P8+3 dps CM
Genes
Mycn
12936000 12938000 12940000 12942000 12944000 12946000
chr12 position (bp)
I
Mycn motif enrichment
Low
High
P1+3 dpi
P1+3 dps
UMAP 2
UMAP 1
CM

Figure S3

Figure S3. Validation of scATAC-seq with VISTA enhancers and known transcription factors involved in CM proliferation, Related to Figure 2 and Figure 3.

A-D. Upper, representative transgenic embryo carrying VISTA enhancer elements hs389 (A, n=3), mm64 (B, n=6), hs1653 (C, n=5), and mm265 (D, n=3) with LacZ reporter staining indicating enhancer activity in the heart or blood vessels; lower, scATAC-seq tracks of genomic regions surrounding candidate VISTA enhancer elements. Embryo images were used with permission from VISTA Enhancer Browser Program of Lawrence Berkeley National Laboratory (https://enhancer.lbl.gov/).

E. scATAC-seq tracks from CMs of different samples showing the chromatin accessibility of a *Tead3* enhancer in the gene body region.

F. chromVAR analysis showing the enrichment of Tead3 motif (MA0808.1) in open chromatin regions of single cells from P1+3 dpi and P1+3 dps samples. There are more CMs with high Tead3 motif enrichment from P1+3 dpi sample than P1+3 dps sample.

G. scATAC-seq tracks from CMs of different samples showing the chromatin accessibility in the promoter region of *Gli1*.

H. scATAC-seq tracks from CMs of different samples showing the chromatin accessibility in *Mycn* gene body.

I. chromVAR analysis showing the enrichment of Mycn motif (MA0104.4) in open chromatin regions of single cells from P1+3 dpi and P1+3 dps samples. There are more CMs with high Mycn motif enrichment from P1+3 dpi sample than P1+3 dps sample.

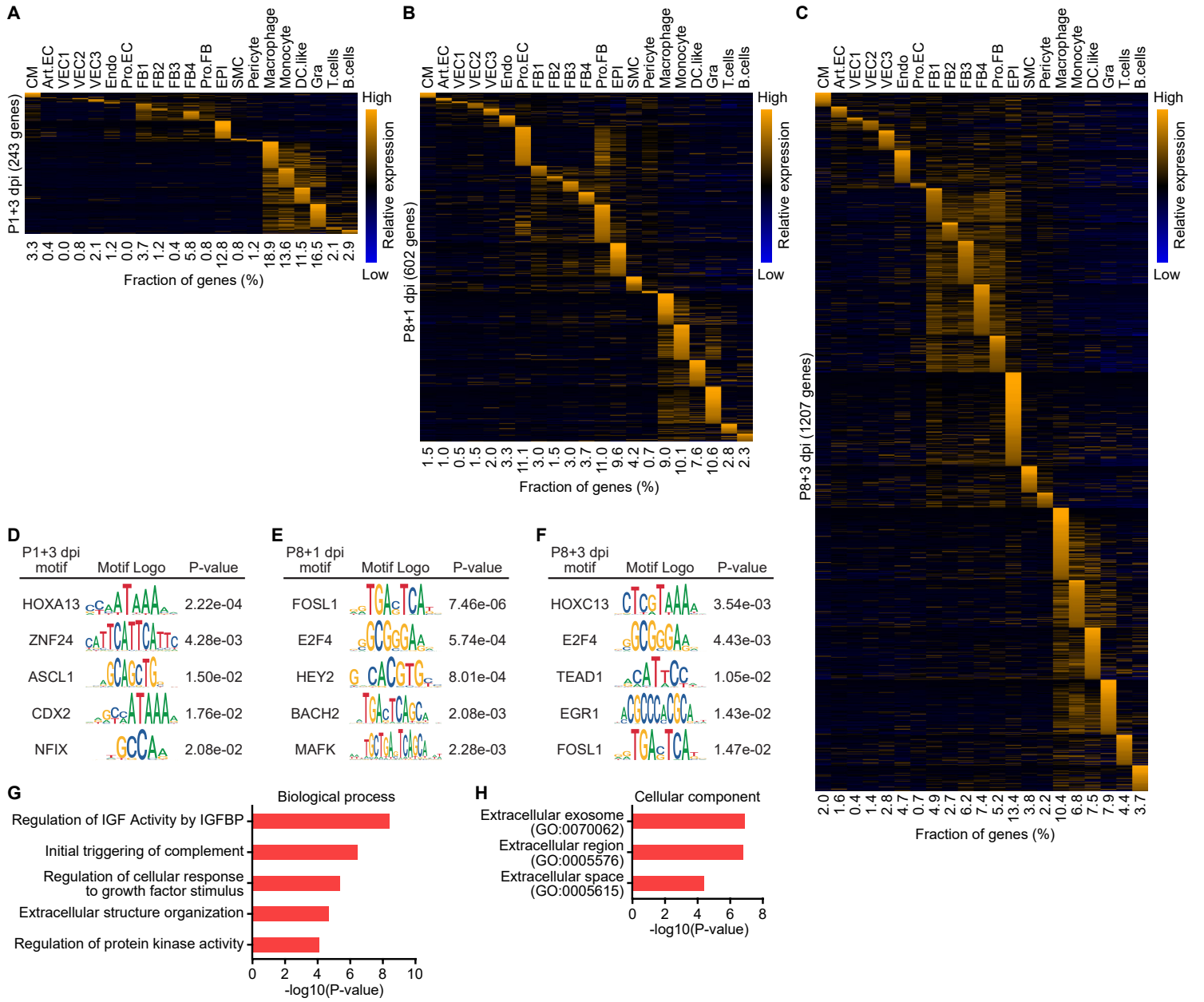

A
CM
Art.EC
VEC1
VEC2
VEC3
Endo
Pro.EC
FB1
FB2
FB3
FB4
Pro.FB
EPI
SMC
Pericyte
Macrophage
Monocyte
DC.like
Gra
T.cells
B.cells
P1+3 dpi (243 genes)
3.3 0.4 0.0 0.8 2.1 1.2 0.0 3.7 1.2 0.4 5.8 0.8 12.8 0.8 1.2 18.9 13.6 11.5 16.5 2.1 2.9
Fraction of genes (%)
High
Relative expression
Low
B
P8+1 dpi (602 genes)
1.5 1.0 0.5 1.5 2.0 3.3 11.1 3.0 1.5 3.0 3.7 11.0 9.6 4.2 0.7 9.0 10.1 7.6 10.6 2.8 2.3
Fraction of genes (%)
C
P8+3 dpi (1207 genes)
2.0 1.6 0.4 1.4 2.8 4.7 0.7 4.9 2.7 6.2 7.4 5.2 13.4 3.8 2.2 10.4 6.8 7.5 7.9 4.4 3.7
Fraction of genes (%)
D
P1+3 dpi motif
Motif Logo
P-value
HOXA13 2.22e-04
ZNF24 4.28e-03
ASCL1 1.50e-02
CDX2 1.76e-02
NFIX 2.08e-02
E
P8+1 dpi motif
Motif Logo
P-value
FOSL1 7.46e-06
E2F4 5.74e-04
HEY2 8.01e-04
BACH2 2.08e-03
MAFK 2.28e-03
F
P8+3 dpi motif
Motif Logo
P-value
HOXC13 3.54e-03
E2F4 4.43e-03
TEAD1 1.05e-02
EGR1 1.43e-02
FOSL1 1.47e-02
G
Biological process
Regulation of IGF Activity by IGFBP
Initial triggering of complement
Regulation of cellular response to growth factor stimulus
Extracellular structure organization
Regulation of protein kinase activity
0 2 4 6 8 10
-log10(P-value)
H
Cellular component
Extracellular exosome (GO:0070062)
Extracellular region (GO:0005576)
Extracellular space (GO:0005615)
0 2 4 6 8
-log10(P-value)

Figure S4

Figure S4. Unique epicardial features in the post-MI responses of P1 and P8 hearts, Related to Figure 4.

A-C. Heatmaps showing expression of cell type-specific, injury-induced genes across different cell types at P1+3 dpi (A), P8+1 dpi (B), and P8+3 dpi (C).

D-F. Tables summarizing TF motifs enriched in open chromatin regions that are associated with epicardial-specific, MI-induced genes at P1+3 dpi (D), P8+1 dpi (E), and P8+3 dpi (F).

G. Biological processes enriched for epicardial-specific, MI-induced genes at P1+1 dpi.

H. Cellular components enriched for epicardial-specific, MI-induced genes at P1+1 dpi.

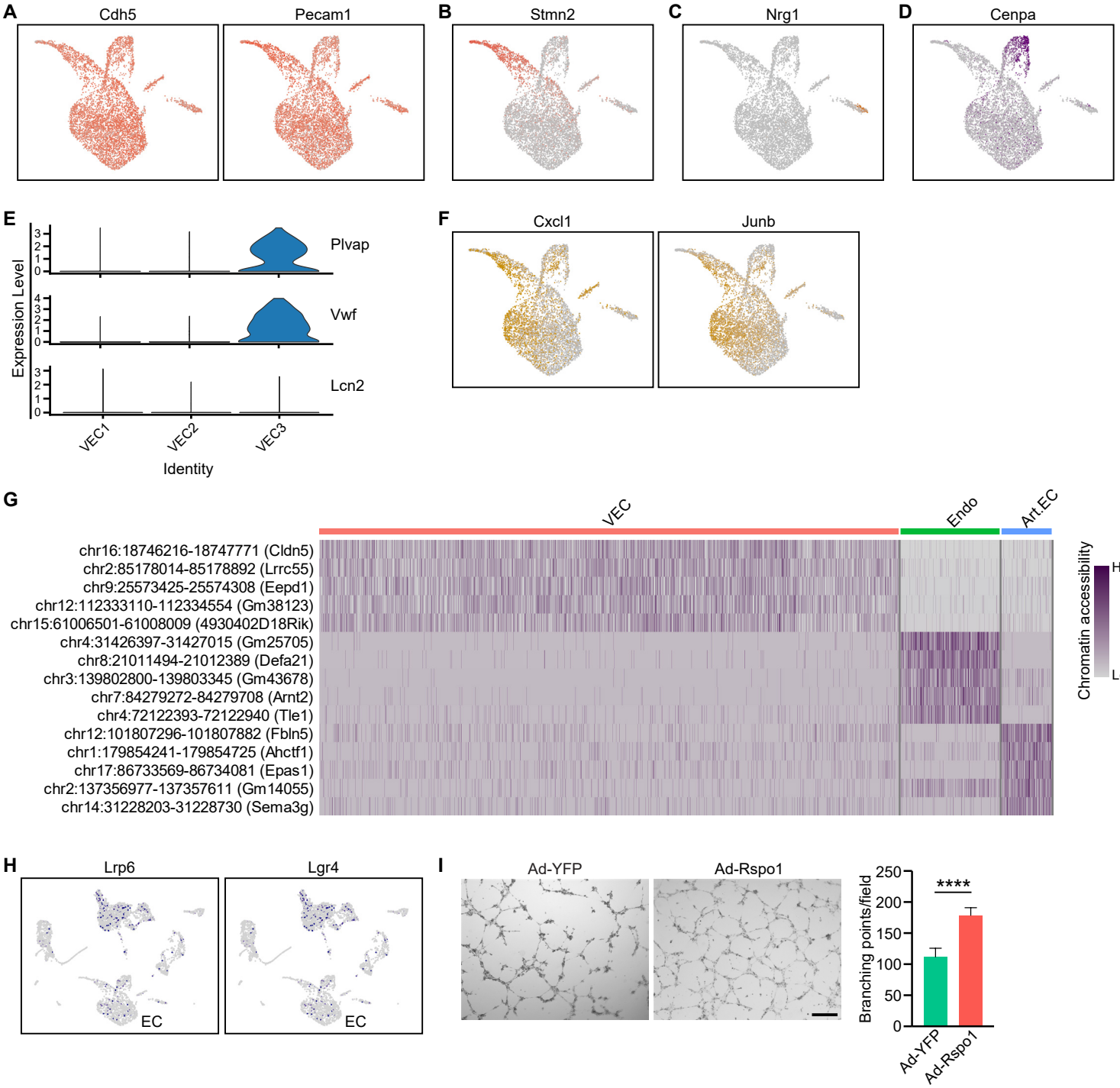

A
Cdh5
Pecam1
B
Stmn2
C
Nrg1
D
Cenpa
E
Plvap
Vwf
Lcn2
Expression Level
VEC1
VEC2
VEC3
Identity
F
Cxcl1
Junb
G
VEC
Endo
Art.EC
chr16:18746216-18747771 (Cldn5)
chr2:85178014-85178892 (Lrrc55)
chr9:25573425-25574308 (Eepd1)
chr12:112333110-112334554 (Gm38123)
chr15:61006501-61008009 (4930402D18Rik)
chr4:31426397-31427015 (Gm25705)
chr8:21011494-21012389 (Defa21)
chr3:139802800-139803345 (Gm43678)
chr7:84279272-84279708 (Arnt2)
chr4:72122393-72122940 (Tle1)
chr12:101807296-101807882 (Fbln5)
chr1:179854241-179854725 (Ahctf1)
chr17:86733569-86734081 (Epas1)
chr2:137356977-137357611 (Gm14055)
chr14:31228203-31228730 (Sema3g)
Chromatin accessibility
High
Low
H
Lrp6
Lgr4
EC
EC
I
Ad-YFP
Ad-Rspo1
Branching points/field
****
Ad-YFP
Ad-Rspo1

Figure S5

Figure S5. Heterogenous ECs during neonatal heart regeneration, Related to Figure 5.

A. UMAP plots showing the expression of *Cdh5* (left), and *Pecam1* (right), two canonical marker genes for ECs, in all ECs analyzed.

B-D. UMAP plots showing the expression of artery EC marker gene *Stmn5* (B), endocardial cell marker gene *Nrg1* (C) and cell cycle-related gene *Cenpa* (D) in ECs.

E. Stacked violin plots showing expression of venous EC enriched genes *Plvap*, *Vwf* and *Lcn2* across VEC1, VEC2 and VEC3 sub-populations. Note that *Lcn2* is a large venous marker identified in (Kalucka et al., 2020).

F. UMAP plots showing expression of VEC2 enriched genes *Cxcl1* (left) and *Junb* (right) in ECs.

G. Heatmap showing accessibility of open chromatin regions enriched in VEC, Endo, and Art.EC clusters, revealed by scATAC-seq.

H. UMAP plots showing the expression of RSPO1 receptor genes *Lrp6* (left) and *Lgr4* (right) among different cell types present in the P1+1 dpi and dps scRNA-seq data.

I. Representative images showing the *in vitro* HUVEC tube formation assay using HUVEC cells pre-infected with YFP (left) or Rspo1 (middle) adenovirus, after 8 h culture in the Matrigel, with quantifications (right) showing the number of branching points per field under each treatment (n=16 per each group; ****, $p<0.0001$). Scale bar, 500 μm.

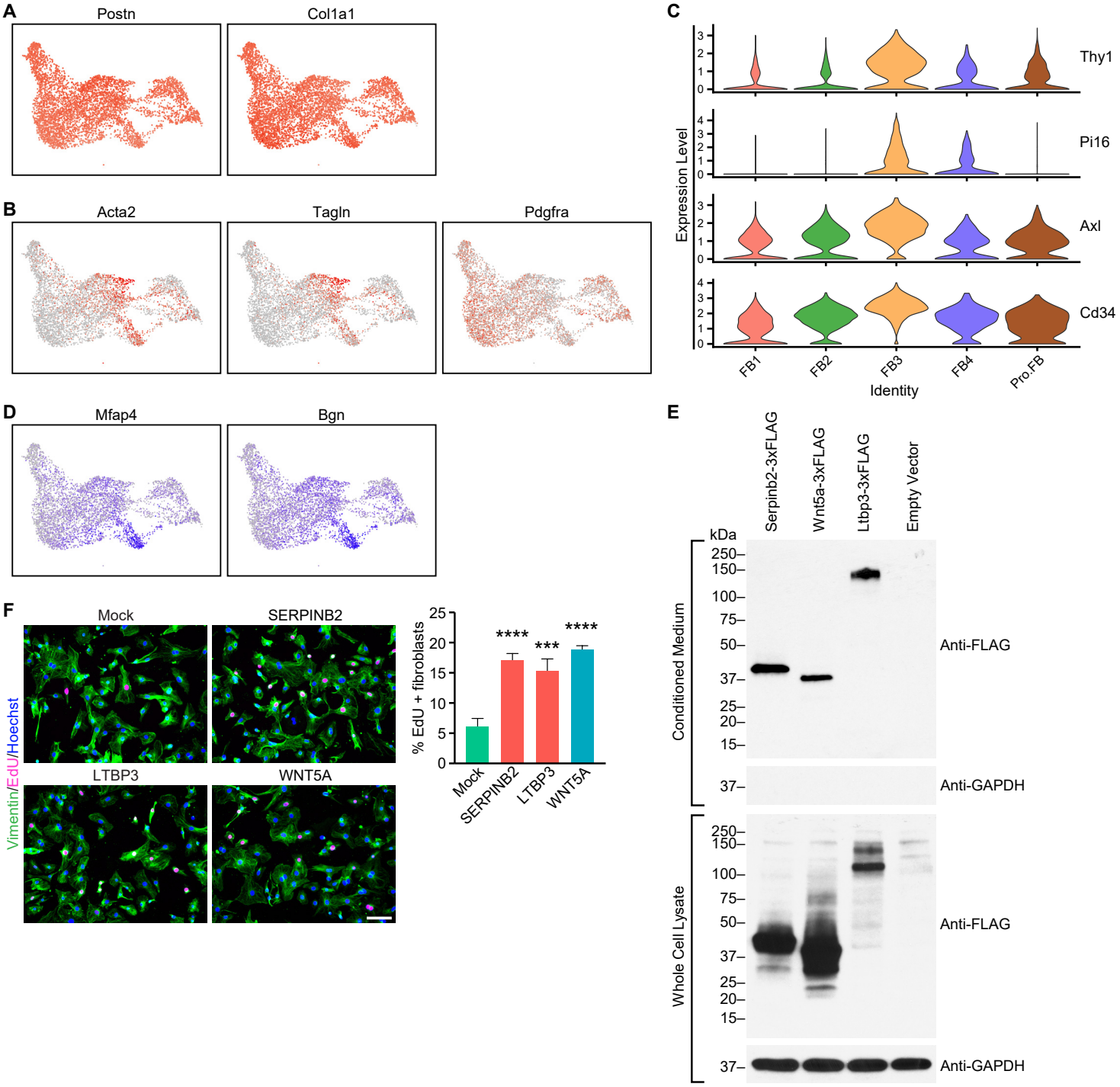

A
Postn
Col1a1
B
Acta2
Tagln
Pdgfra
C
Thy1
Pi16
Axl
Cd34
Expression Level
FB1
FB2
FB3
FB4
Pro.FB
Identity
D
Mfap4
Bgn
E
Serpinb2-3xFLAG
Wnt5a-3xFLAG
Ltbp3-3xFLAG
Empty Vector
kDa
Conditioned Medium
Anti-FLAG
Anti-GAPDH
Whole Cell Lysate
Anti-FLAG
Anti-GAPDH
F
Mock
SERPINB2
LTBP3
WNT5A
Vimentin/EdU/Hoechst
% EdU + fibroblasts
****
***
****
Mock
SERPINB2
LTBP3
WNT5A

Figure S6

Figure S6. Heterogeneous fibroblasts in the neonatal heart, Related to Figure 6.

A. UMAP plots showing the expression of *Postn* (left) and *Col1a1* (right), two marker genes for fibroblasts, in all FBs analyzed.
B. UMAP plots showing the expression of *Acta2* (left), *Tagln* (middle), and *Pdgfra* (right) in FBs.
C. Stacked violin plots showing the expression of *Thy1*, *Pi16*, *Axl* and *Cd34*, across different FB sub-populations.
D. UMAP plots showing the expression of *Mfap4* (left) and *Bgn* (right) in FBs.
E. Western blot analysis showing the expression and secretion of candidate secreted factors. *Serpinb2*, *Wnt5a* and *Ltbp3* were tagged with 3xFLAG at the C' terminus, and transfected into 293A cells. Filtered cell culture medium (conditioned medium) or whole cell lysate was collected for each group for western blot analysis using anti-FLAG antibody, or anti-GAPDH antibody (as negative control for secretion and positive loading control for whole cell lysates).
F. Representative images showing EdU incorporation (magenta) and vimentin immunofluorescent staining (green) of NRCF cells treated with conditioned medium from 293A cells transfected with empty vector (Mock control), or vectors for *Serpinb2* or *Ltbp3*, or vector for *Wnt5a* (positive control), with quantification showing the proportion of EdU positive cells among vimentin positive fibroblasts (n=4 per each group; ****, $p<0.0001$). Scale bar, 100 μm.

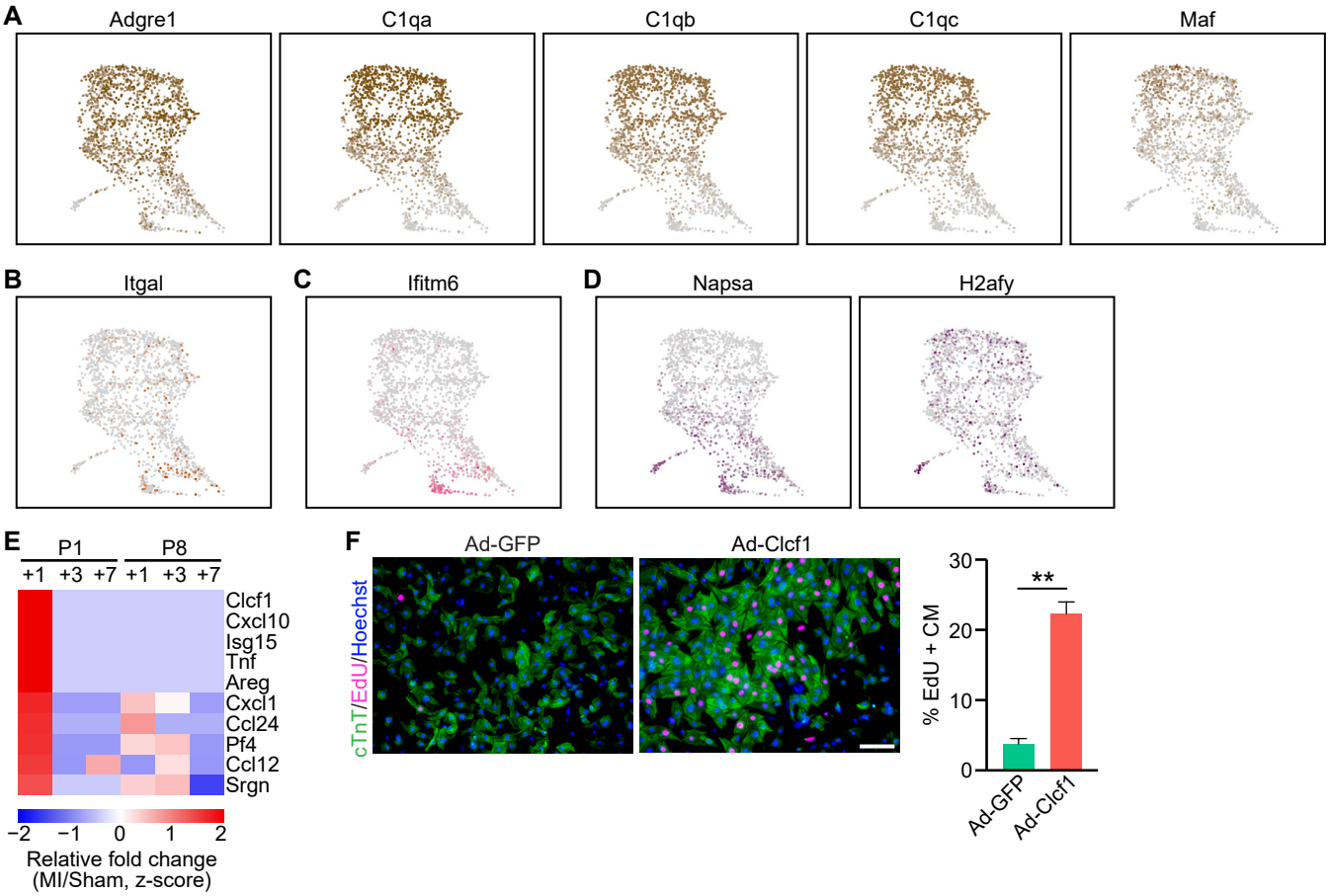

A
Adgre1
C1qa
C1qb
C1qc
Maf
B
Itgal
C
Ifitm6
D
Napsa
H2afy
E
P1
P8
+1 +3 +7 +1 +3 +7
Clcf1
Cxcl10
Isg15
Tnf
Areg
Cxcl1
Ccl24
Pf4
Ccl12
Srgn
−2 −1 0 1 2
Relative fold change
(MI/Sham, z-score)
F
Ad-GFP
Ad-Clcf1
cTnT/EdU/Hoechst
% EdU + CM
30
20
10
0
**
Ad-GFP
Ad-Clcf1

Figure S7

Figure S7. Heterogeneous immune cells and cell-type specific injury response during neonatal heart regeneration, Related to Figure 7.

A. UMAP plots showing the expression of *Adgre1*, *C1qa*, *C1qb*, *C1qc*, and *Maf* in all myeloid cells analyzed.
B. UMAP plot showing the expression of *Itgal*, a monocyte marker gene, in myeloid cells.
C. UMAP plot showing the expression of *Ifitm6*, a gene enriched in M1 monocytes, in myeloid cells.
D. UMAP plots showing the expression of *Napsa* (left) and *H2afy* (right), marker genes for DC-like cells, in myeloid cells.
E. Heatmap showing relative fold change of various P1-unique, macrophage-specific genes at various timepoints after P1 or P8 MI, detected by bulk RNA-seq.
F. EdU incorporation (magenta) and cTnT immunostaining (green) of NRVM cells overexpressing GFP (left) or *Clcf1* (middle) using adenoviral delivery, with quantifications (right) showing the proportion of EdU positive cells among cTnT positive cells (CMs) (n=2 per each group; **, $p<0.01$). Scale bar, 100 μm.