Expanded View Figures

Figure EV1. Generation and characteristics of EHT populations of interest.

A Schematic of the hematopoietic differentiation system. Following embryoid body setup, BMP4, Activin A, CHIR99021, VEGF, and hematopoietic cytokines were added sequentially to induce HE cell formation and EHT. Cells of interest were sorted at day 8 of the protocol.

B Sorting strategy for obtaining pure HE, EHT, and HSC-like cell populations. At day 8 of differentiation, representative plots show the level of $CD34^+$ cells following magnetic bead enrichment, separation on the basis of CD43 expression and further gating on $CXCR4^-CD73^-$ and $CD90^+VEcad^+$ for HE and EHT cells; and $CD90^+CD38^-$ for HSC-like cells.

C, D Pseudotime analysis of EHT populations taking a G0 (C) or S/G2M (D) path and corresponding bar graphs showing abundance of populations.

E scCoGAPS mapping of cord blood $CD34^+$ cells (CB HSC) to the EHT dataset and violin plot showing pattern weights.

F scCoGAPS mapping of the human CS 13 dorsal aorta dataset on the EHT dataset, with plot showing colocalization of populations.

G Pie charts showing abundance of each cell type mapping from the human CS 13 dataset onto HE, EHT, or HSC-like cell types, according to the scCoGAPS analysis in Fig EV1F.

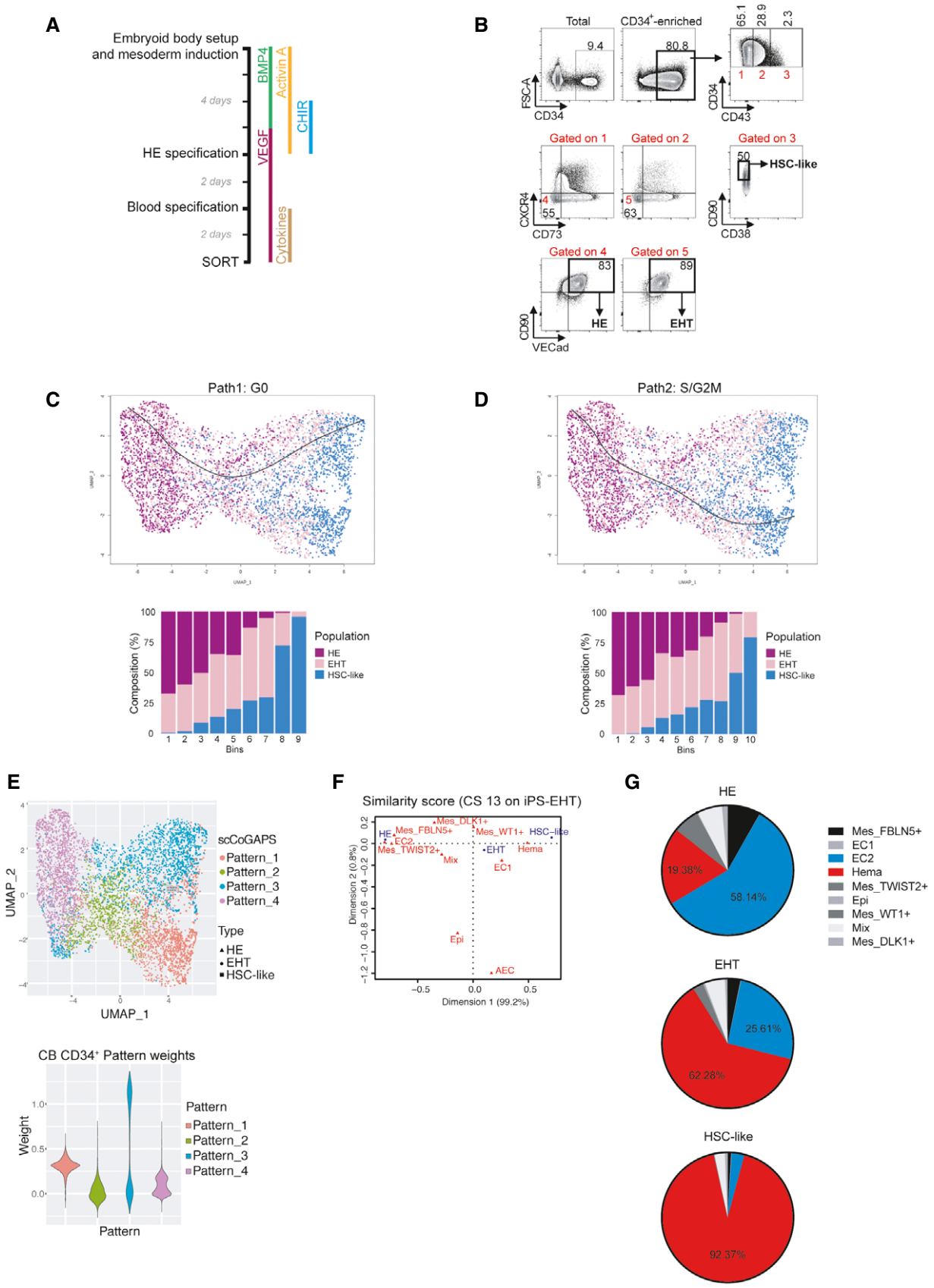

Figure EV1.

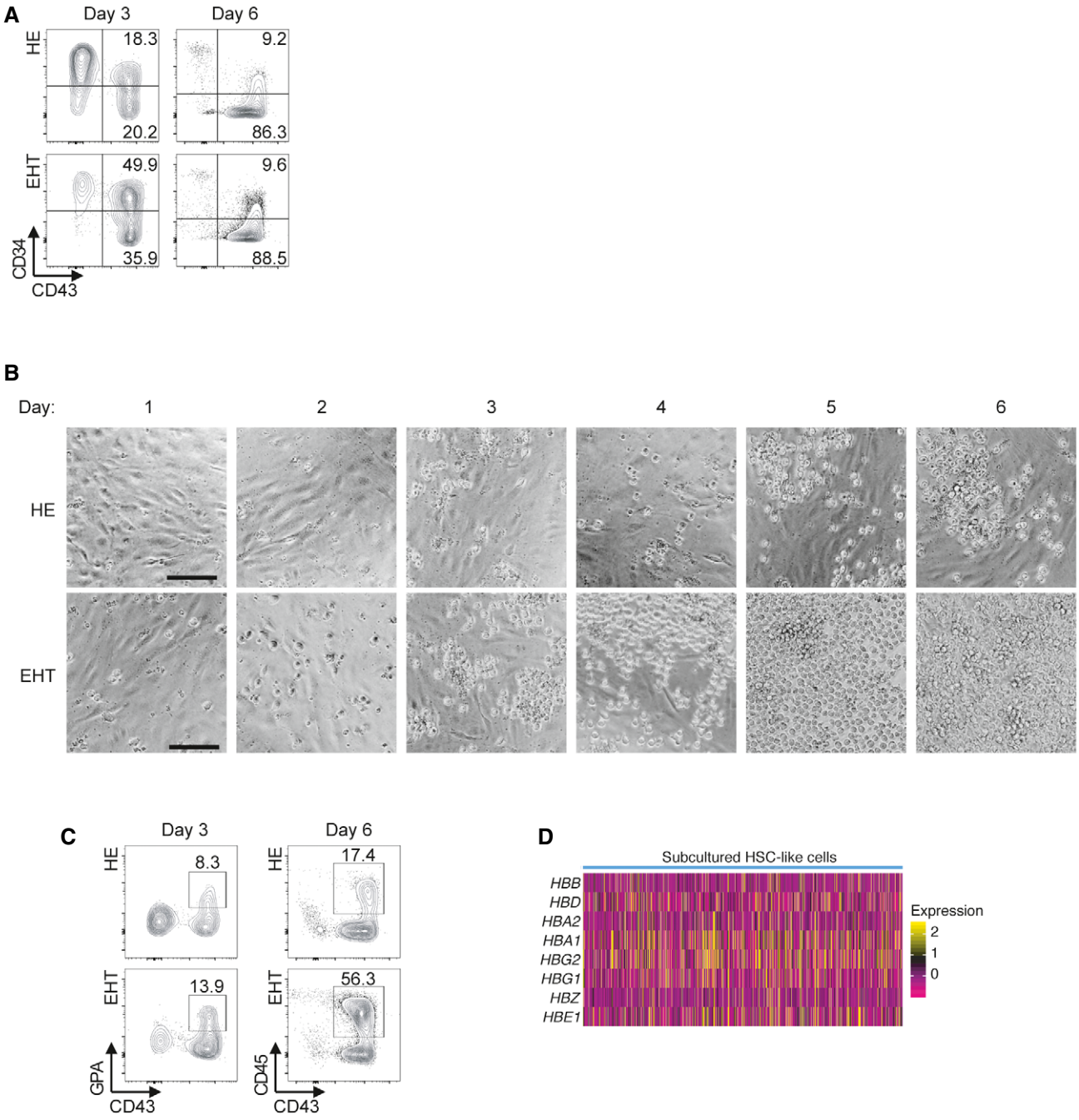

Figure EV2. Validation of the hematopoietic potential of HE and EHT cells.

A–C Sorted HE and EHT cells were subcultured for 6 days (representative of $n = 5$). The levels of CD43 and CD34 markers (A) and the levels of CD43, GPA, and CD45 markers (C) were assessed at subculture days 3 and 6. (B) Representative pictures of the wells were taken every day during HE and EHT subculture. Scale bars, 100 μm.

D Expression of globin genes in HSC-like cells assessed by scRNAseq.

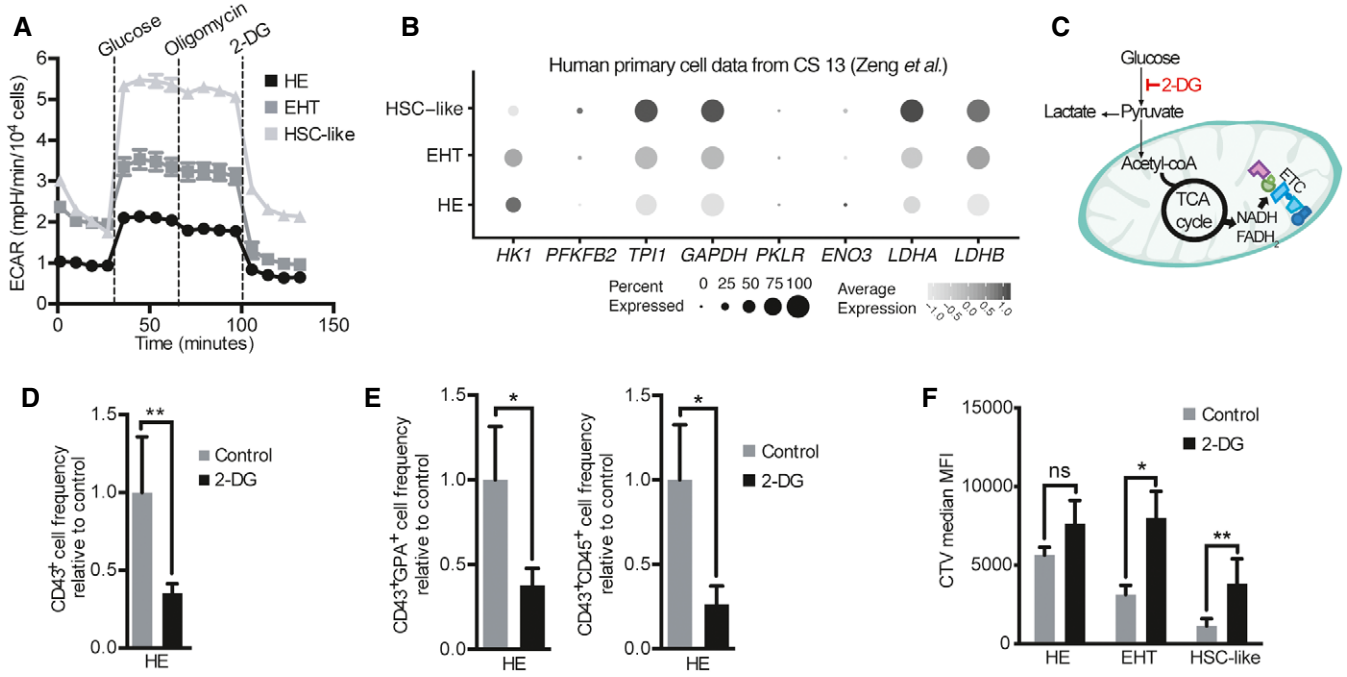

Figure EV3. Glycolysis is essential for hematopoietic specification.

A Representative assay data show the extracellular acidification rate (ECAR) measured in HE and EHT cells under basal conditions as well as after the addition of the indicated compounds. Bar graphs are shown in Fig 2A.

B Dot plots showing gene expression levels of glycolytic enzymes detected in human CS 13 AGM region (data from Zeng *et al*, 2019), by mapping of their scRNAseq data onto our dataset (as shown in Fig 1C) and based on percent expressed (size of the dots) and average level of expression (color intensity).

C Glucose is broken down through glycolysis and the resulting pyruvate gives rise to lactate or converts to acetyl-coA for integration into the TCA cycle. 2-Deoxy-D-glucose (2-DG) blocks the glycolytic flux.

D Subculture day 3 $CD43^+$ cell frequencies ± SEM relative to the control are shown (n = 7 biological replicates, paired t-test).

E Subculture day 3 $CD43^+GPA^+$ and subculture day 6 $CD43^+CD45^+$ cell frequencies ± SEM relative to the control are presented (n = 6 and n = 5 biological replicates, respectively, paired t-tests).

F Subculture day 3 CellTrace Violet (CTV) fluorescence was assessed by flow cytometry and median MFI values ± SEM are shown (n = 4 biological replicates, paired t-tests).

Data information: ns, not significant, $*P < 0.05$, $**P < 0.01$.

Figure EV4. OXPHOS is increased during EHT even in the absence of glucose.

A Representative assay data show oxygen consumption rate (OCR) measured in HE and EHT cells under basal conditions as well as after the addition of the indicated compounds. Bar graphs are shown in Fig 2G.

B Heatmap showing scRNAseq data of OXPHOS-related genes expressed in HE, EHT, and HSC-like populations.

C Heatmap showing scRNAseq data of OXPHOS-related genes expressed in human CS 13 AGM region (data from Zeng *et al*, 2019), by mapping of their scRNAseq data onto our dataset (as shown in Fig 1C). Note that more OXPHOS-related genes are detectable in this primary cell dataset.

D Dot plot showing scRNAseq data of TCA cycle enzymes expressed in human CS 13 AGM region (data from Zeng *et al*, 2019), by mapping of their scRNAseq data onto our dataset (as shown in Fig 1C).

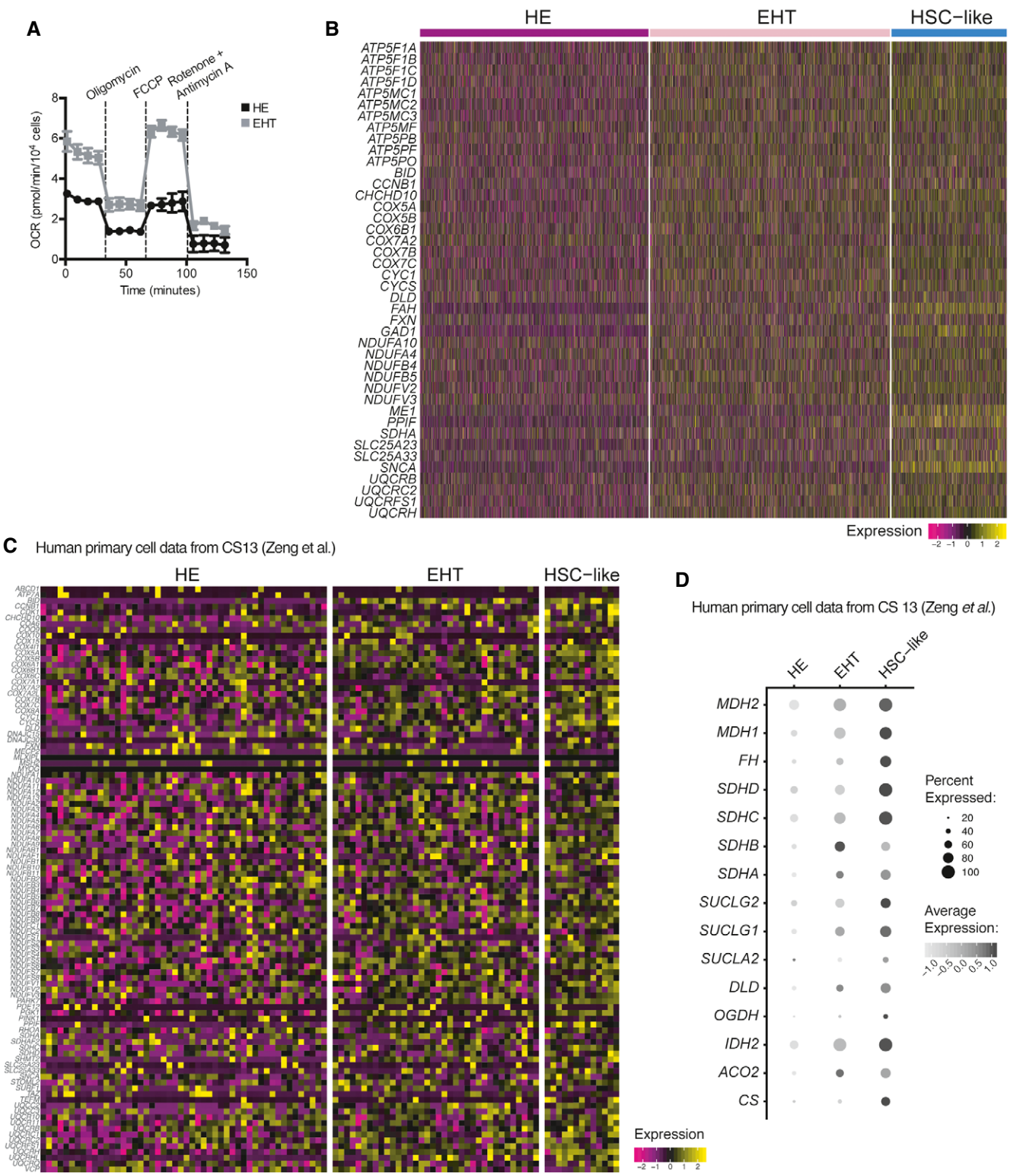
A
Oligomycin
FCCP
Rotenone + Antimycin A
HE
EHT
OCR (pmol/min/10^4 cells)
Time (minutes)
B
HE
EHT
HSC−like
ATP5F1A
ATP5F1B
ATP5F1C
ATP5F1D
ATP5MC1
ATP5MC2
ATP5MC3
ATP5MF
ATP5PB
ATP5PF
ATP5PO
BID
CCNB1
CHCHD10
COX5A
COX5B
COX6B1
COX7A2
COX7B
COX7C
CYC1
CYCS
DLD
FAH
FXN
GAD1
NDUFA10
NDUFA4
NDUFB4
NDUFB5
NDUFV2
NDUFV3
ME1
PPIF
SDHA
SLC25A23
SLC25A33
SNCA
UQCRB
UQCRC2
UQCRFS1
UQCRH
Expression
C
Human primary cell data from CS13 (Zeng et al.)
HE
EHT
HSC−like
Expression
D
Human primary cell data from CS 13 (Zeng et al.)
HE
EHT
HSC−like
MDH2
MDH1
FH
SDHD
SDHC
SDHB
SDHA
SUCLG2
SUCLG1
SUCLA2
DLD
OGDH
IDH2
ACO2
CS
Percent Expressed:
20
40
60
80
100
Average Expression:

Figure EV4.

Figure EV5. Pyruvate catabolism directs hematopoietic lineage specification.

A FACS-sorted HE, EHT, or HSC-like cells were subcultured with or without UK5099 (10 μM). Subculture day 3 $CD43^+/GPA^+$ cell frequencies ± SEM relative to the controls for all populations are presented (HE, $n = 5$ biological replicates; EHT, $n = 6$ biological replicates; HSC-like, $n = 4$ biological replicates; paired *t*-tests).

B FACS-sorted HE cells were subcultured with or without 1-AA (4 mM). Subculture day 3 $CD43^+GPA^+$ cell frequencies ± SEM relative to the control are shown ($n = 4$ biological replicates, paired *t*-test).

C Fold change of expression of *MPC1* and *MPC2* ± SEM relative to *HPRT1* in shRNA-transduced cells compared to shScrambled (shScr) is shown ($n = 3$ biological replicates, unpaired *t*-tests). Untr, untransduced.

D HE cells were transduced with shScrambled (shScr), shMPC1, shMPC2, or both the day after the sort and day 3 $CD43^+/GPA^+$ cell frequencies ± SEM relative to shScr are presented ($n = 4$ biological replicates; one-way ANOVA test). Untr, untransduced.

E FACS-sorted HE cells were stained with CTV and fluorescence was assessed by flow cytometry for GPA^+ cells at day 3 of subculture with or without UK5099 (10 μM). Representative of $n = 3$.

F, G FACS-sorted HE, EHT, or HSC-like cells were subcultured with or without UK5099 (10 μM). Subculture day 6 $CD43^+$ (F) and $CD43^+CD45^+$ (G) cell frequencies ± SEM relative to the control for all populations are presented (HE, $n = 7$ biological replicates; EHT, $n = 7$ biological replicates; HSC-like, $n = 4$ biological replicates; paired *t*-tests).

H FACS-sorted HE cells were subcultured with or without 1-AA (4 mM). Subculture day 6 $CD43^+$ and $CD43^+CD45^+$ cell frequencies ± SEM relative to the control are shown ($n = 3$ biological replicates, paired *t*-test).

I, J FACS-sorted HE cells were subcultured for 3 days with or without UK5099 (10 μM). CTV for HE-derived $CD45^+$ cells (I) and HE-derived HSC-like cell frequencies ± SEM relative to the control ($n = 7$ biological replicates, paired *t*-test) (J) are shown.

K–N FACS-sorted HE and EHT cells were subcultured with or without DCA (3 mM). Subculture day 3 (K, $n = 3$ biological replicates) and day 6 (L, HE, $n = 5$ biological replicates; EHT, $n = 4$ biological replicates) $CD43^+GPA^+$ cell frequencies ± SEM relative to the controls are shown (paired *t*-test). (M) CTV for HE-derived GPA^+ cells at subculture day 3 is shown. Representative of $n = 3$. (N) Subculture day 6 $CD43^+CD45^+$ cell frequencies ± SEM relative to the controls for both populations are shown (HE, $n = 5$ biological replicates; EHT, $n = 4$ biological replicates; paired *t*-tests).

O Fold change of expression of *PDK1*, *PDK2*, *PDK3*, and *PDK4* ± SEM relative to *HPRT1* in shRNA-transduced cells compared to shScrambled (shScr) is shown ($n = 3$ biological replicates, paired *t*-tests).

P HE cells were transduced with shScrambled (shScr) or shPDK1, shPDK2, shPDK3, or shPDK4 the day after the sort and day 6 $CD43^+/CD45^+$ cell frequencies ± SEM relative to shScr are presented ($n = 3$ biological replicates; one-way ANOVA tests).

Q CTV for HE-derived $CD45^+$ cells are shown. Representative of $n = 3$.

R HE-derived HSC-like cell frequencies ± SEM relative to the control ($n = 4$ biological replicates, paired *t*-test) are shown.

S EdU incorporation ± SEM into HE cells was assessed by flow cytometry after a 24-h pulse at days 1 and 2 of subculture with or without UK5099 (10 μM) or DCA (3 mM) ($n = 3$ biological replicates).

T, U Percentages of CFU assay colony types ± SEM obtained from HE cells subcultured with the indicated compounds for 3 days (U) ($n = 3$ biological replicates, two-way ANOVA test) or 6 days (T) ($n = 5$ biological replicates, two-way ANOVA test). CFU, colony-forming unit; E, erythroid; M, macrophage; G, granulocyte; GEMM, mixed.

V EryD and EryP CFU-Es obtained from HE cells subcultured with the indicated compounds for 3 days. Scale bars, 100 μm.

W Fold change in the expression of *HBA1-2* transcripts ± SEM normalized to *KLF1* in CFUs obtained from HE cells treated with UK5099 (10 μM) or DCA (3 mM) relative to non-treated cells ($n = 3$ biological replicates, paired *t*-test).

X Plots showing percentages of $CD45^+CD56^+$ cells obtained following 35-day co-culture of 3-day subcultured HE cells with OP9-DL1 stroma. During the 3-day subculture, HE cells were treated with the indicated compounds.

Data information: ns, not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

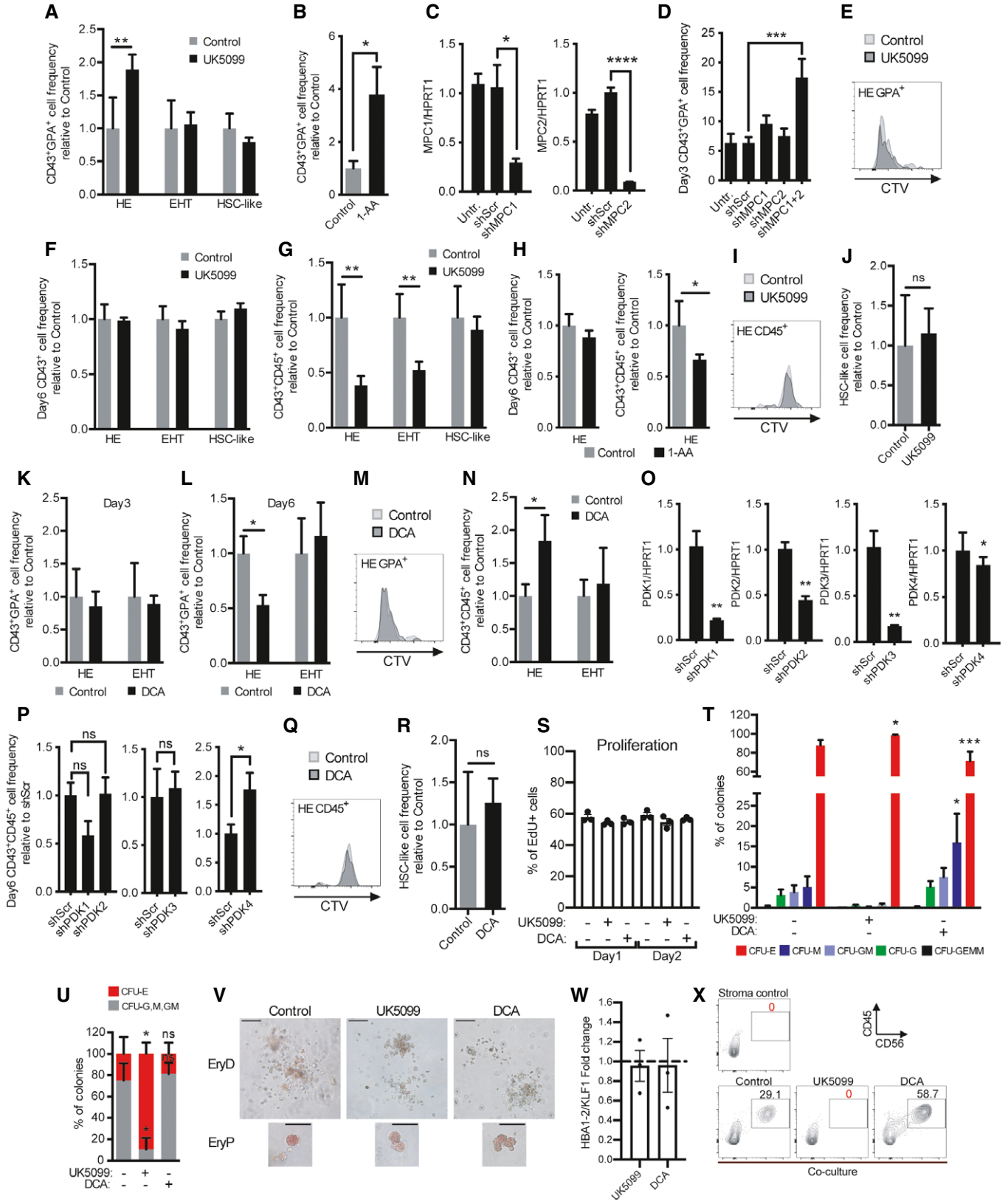
A
CD43+GPA+ cell frequency relative to Control
Control
UK5099
HE
EHT
HSC-like
B
CD43+GPA+ cell frequency relative to Control
Control
1-AA
C
MPC1/HPRT1
MPC2/HPRT1
Untr.
shScr
shMPC1
shMPC2
D
Day3 CD43+GPA+ cell frequency
Untr.
shScr
shMPC1
shMPC2
shMPC1+2
E
Control
UK5099
HE GPA+
CTV
F
Day6 CD43+ cell frequency relative to Control
G
CD43+CD45+ cell frequency relative to Control
H
Day6 CD43+ cell frequency relative to Control
CD43+CD45+ cell frequency relative to Control
I
HE CD45+
J
HSC-like cell frequency relative to Control
ns
K
Day3
L
Day6
M
Control
DCA
HE GPA+
CTV
N
CD43+CD45+ cell frequency relative to Control
O
PDK1/HPRT1
PDK2/HPRT1
PDK3/HPRT1
PDK4/HPRT1
shScr
shPDK1
shPDK2
shPDK3
shPDK4
P
Day6 CD43+CD45+ cell frequency relative to shScr
Q
HE CD45+
R
HSC-like cell frequency relative to Control
S
Proliferation
% of EdU+ cells
Day1
Day2
T
% of colonies
CFU-E
CFU-M
CFU-GM
CFU-G
CFU-GEMM
U
CFU-E
CFU-G,M,GM
% of colonies
V
Control
UK5099
DCA
EryD
EryP
W
HBA1-2/KLF1 Fold change
X
Stroma control
CD45
CD56
Control
UK5099
DCA
29.1
58.7
Co-culture

Figure EV5.