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(54) **GENERATION OF HOXA-EXPRESSING HEMOGENIC ENDOTHELIUM WITH ENHANCED T CELL POTENTIAL FROM HPSCS**

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(52) **U.S. Cl.**

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(57) **ABSTRACT**

The present invention provides methods of creating a population of hemogenic endothelial cells with arterial specification and enhanced T cell potential. The methods involve inducing the expression of a SOX17 transgene in human pluripotent stem cells starting at day 2 of differentiation. Stem cells that express the SOX17 transgene are also provided.

Specification includes a Sequence Listing.

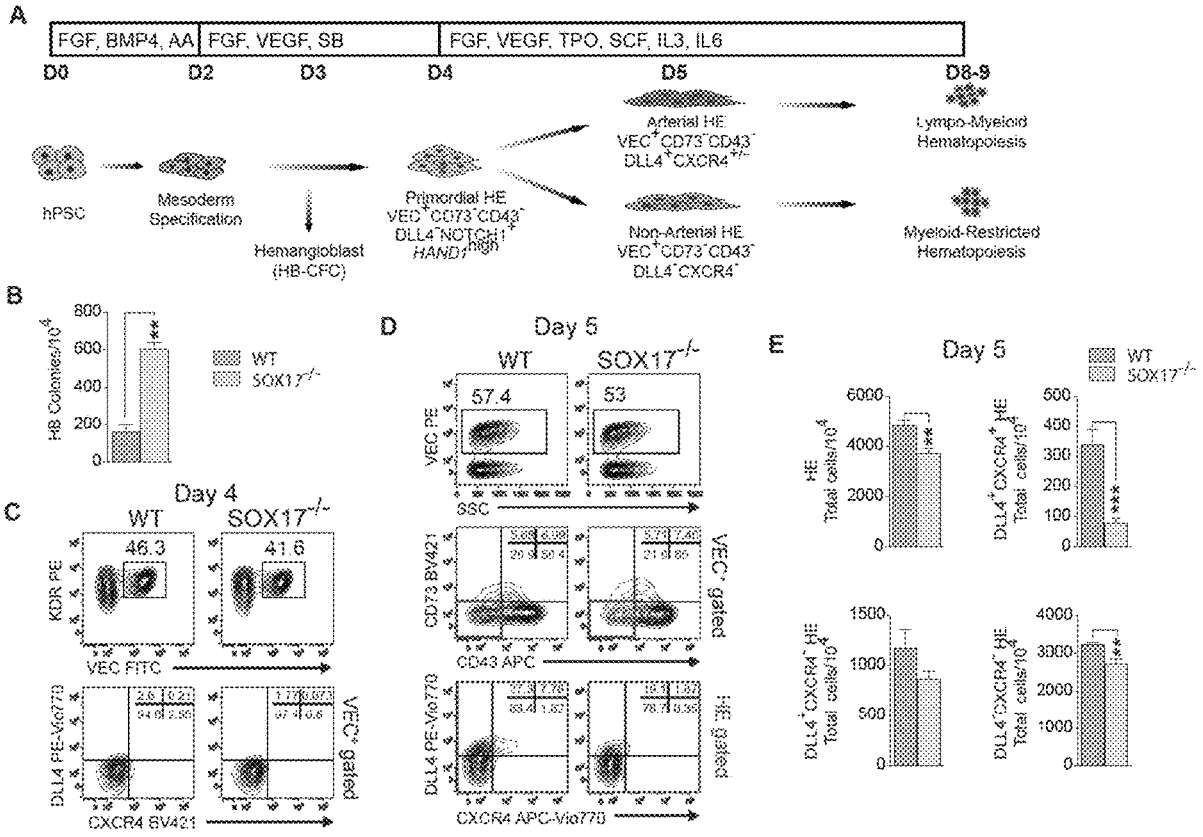


Figure 1

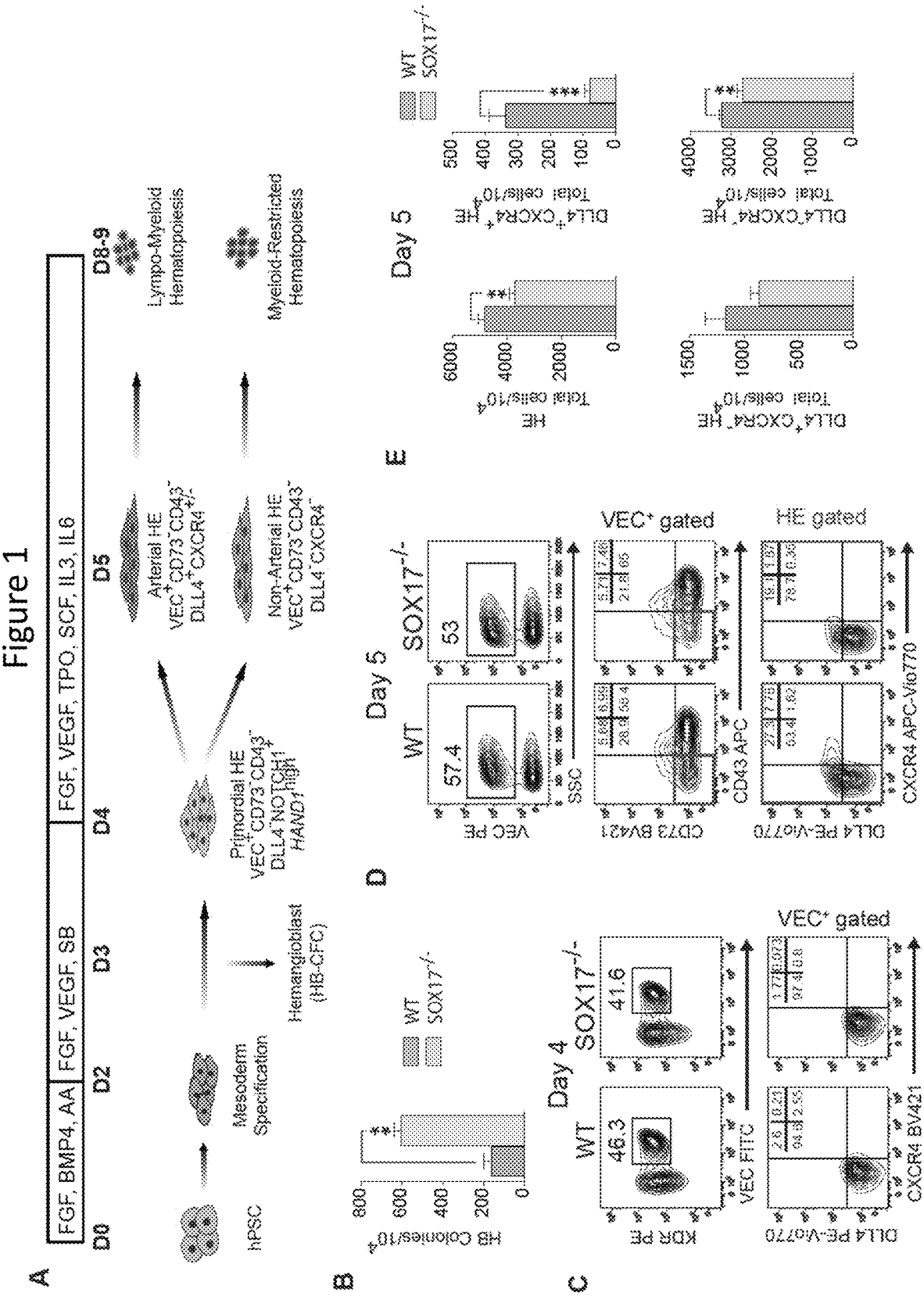

A
FGF, BMP4, AA
FGF, VEGF, SB
FGF, VEGF, TPO, SCF, IL3, IL6
D0
D2
D3
D4
D5
D8-9
hPSC
Mesoderm Specification
Hemangioblast (HB-CFC)
Primordial HE
VEC+CD73-CD43-
DLL4+NOTCH1+
HAND1high
Arterial HE
VEC+CD73-CD43-
DLL4+CXCR4+/-
Non-Arterial HE
VEC+CD73-CD43-
DLL4-CXCR4-
Lympo-Myeloid Hematopoiesis
Myeloid-Restricted Hematopoiesis
B
HB Colonies/10^4
WT
SOX17-/-
C
Day 4
WT
SOX17-/-
KDR PE
VEC FITC
DLL4 PE-Vio770
CXCR4 BV421
VEC+ gated
D
Day 5
VEC PE
SSC
CD73 BV421
CD43 APC
HE gated
CXCR4 APC-Vio770
E
Day 5
HE
Total cells/10^4
DLL4+CXCR4+ HE
DLL4-CXCR4- HE

Figure 1 (continued)

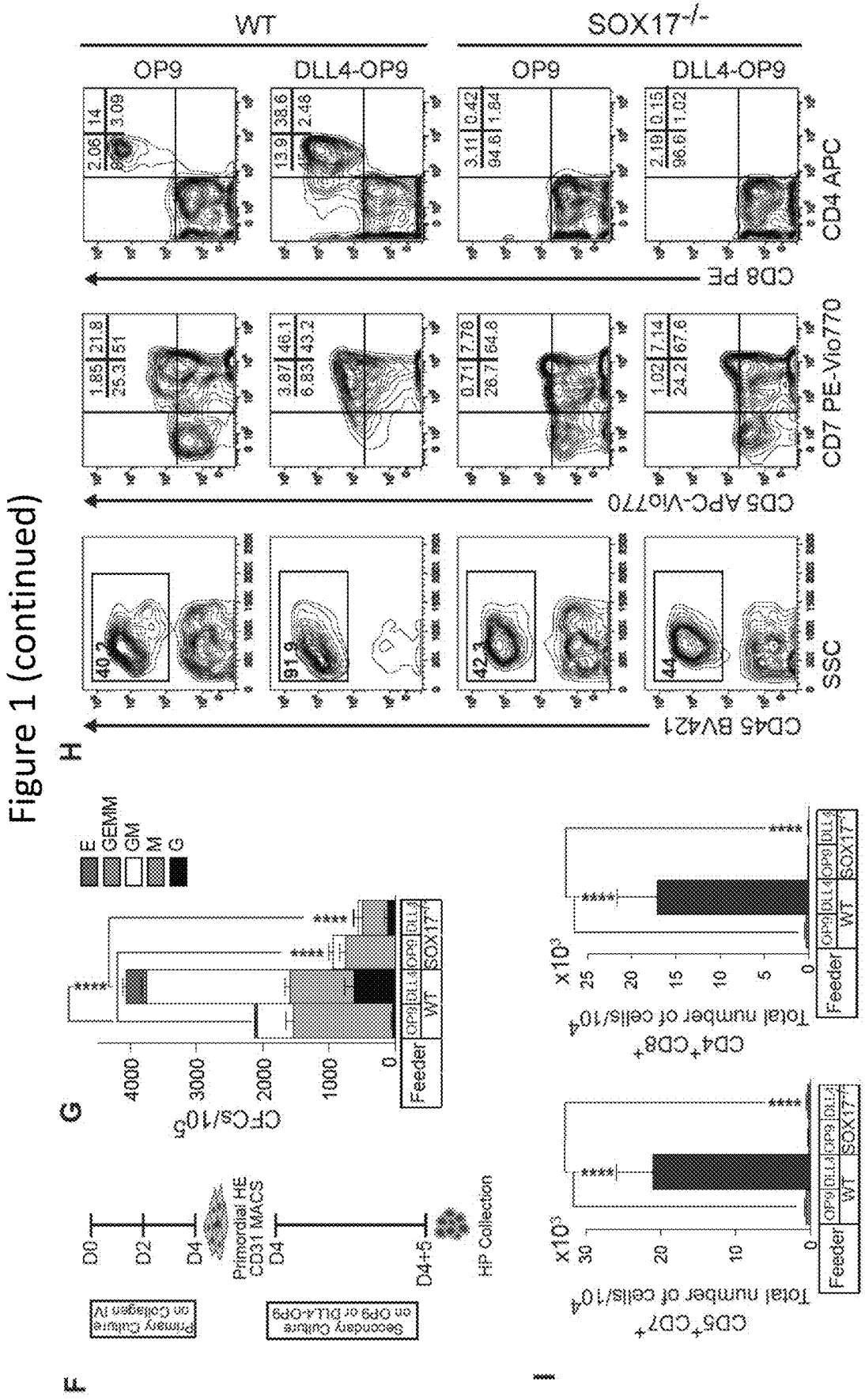


Figure 2

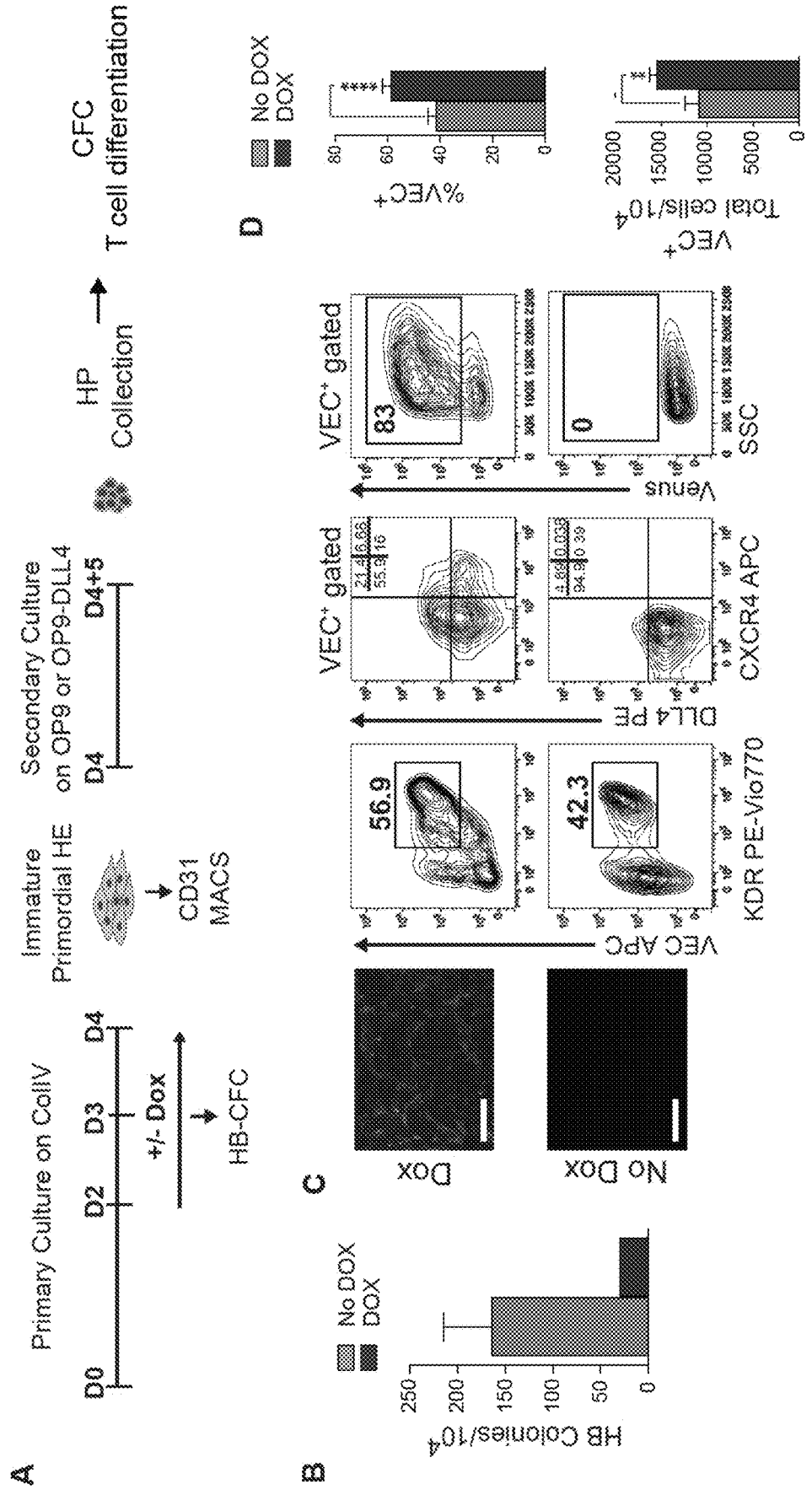

A
Primary Culture on ColIV
D0
D2
D3
D4
+/- Dox
HB-CFC
Immature Primordial HE
CD31 MACS
Secondary Culture on OP9 or OP9-DLL4
D4
D4+5
HP Collection
CFC T cell differentiation
B
No DOX
DOX
HB Colonies/10^4
C
Dox
No Dox
VEC APC
KDR PE-Vio770
56.9
42.3
VEC+ gated
DLL4 PE
CXCR4 APC
21.4
46.6
55.9
16
4.66
0.038
94.9
0.39
Venus
SSC
83
0
D
No DOX
DOX
%VEC+
VEC+ Total cells/10^4

Figure 2 (continued)

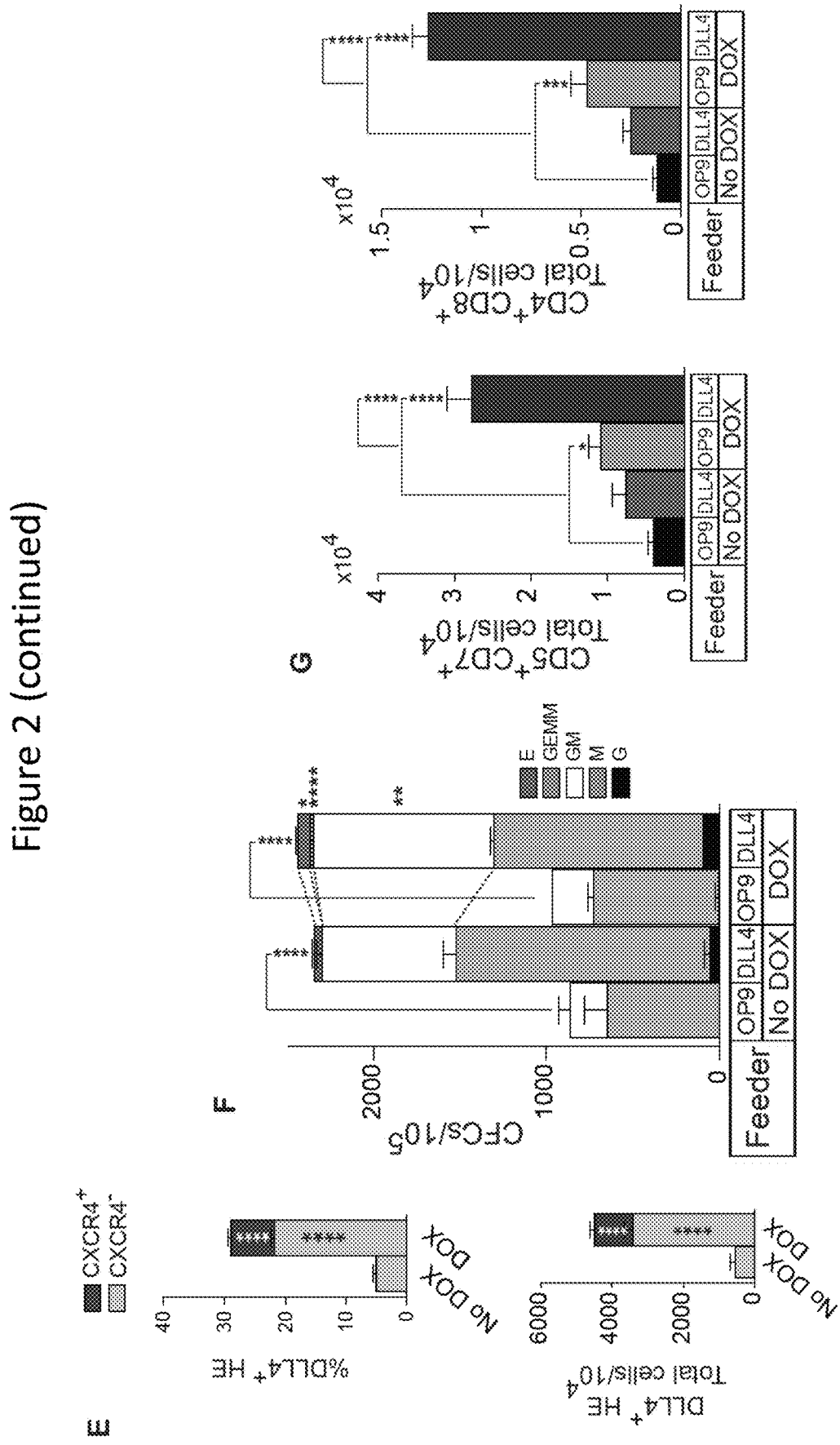


Figure 3

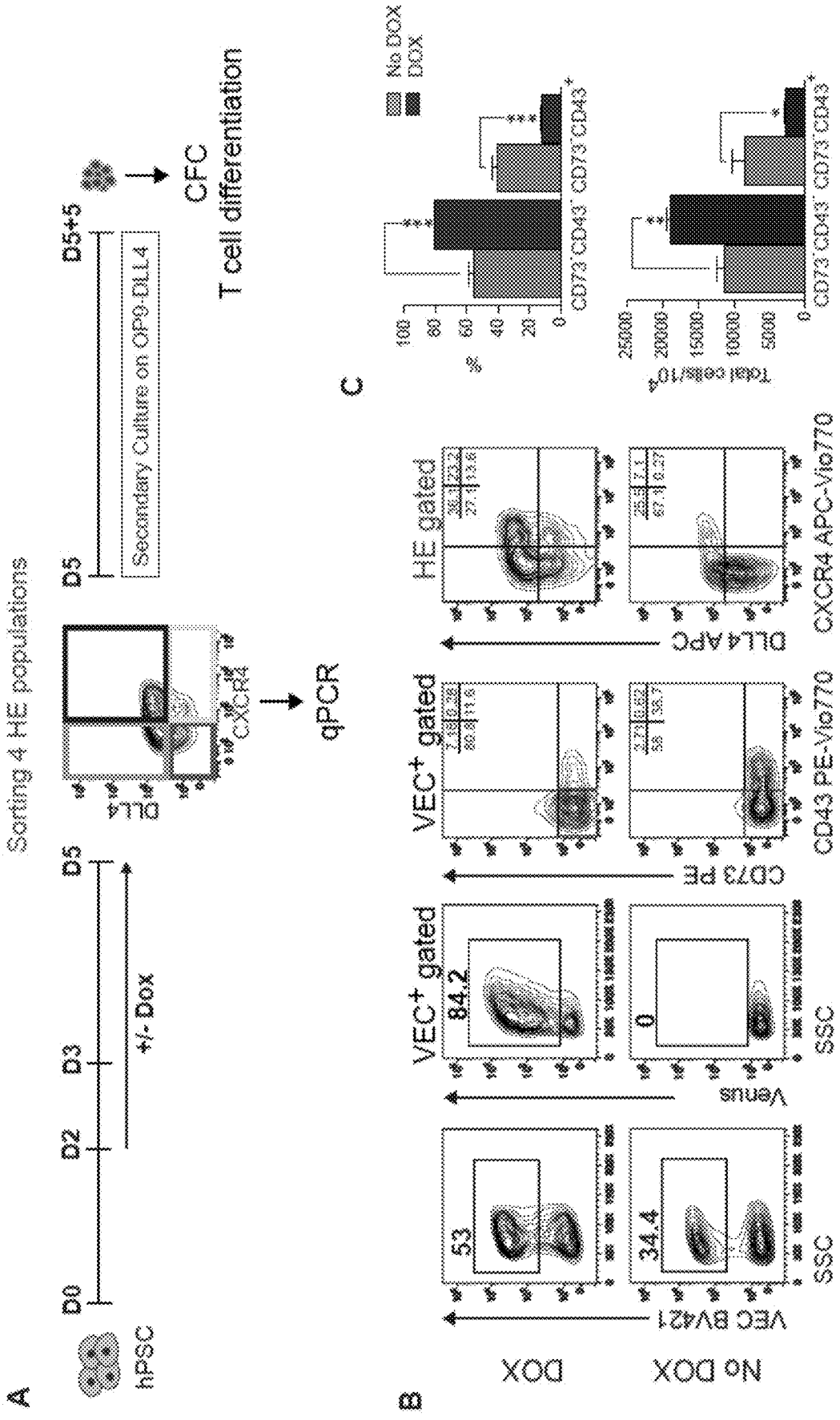

A
hPSC
D0
D2
D3
D5
+/- Dox
Sorting 4 HE populations
DLL4
CXCR4
qPCR
D5
Secondary Culture on OP9-DLL4
D5+5
CFC
T cell differentiation
B
No DOX
DOX
VEC BV421
SSC
53
34.4
VEC+ gated
84.2
0
Venus
SSC
CD73 PE
CD43 PE-Vio770
DLL4 APC
CXCR4 APC-Vio770
HE gated
C
No DOX
DOX
%
CD73+CD43-
CD73+CD43+
Total cells/10^4

Figure 3 (continued)

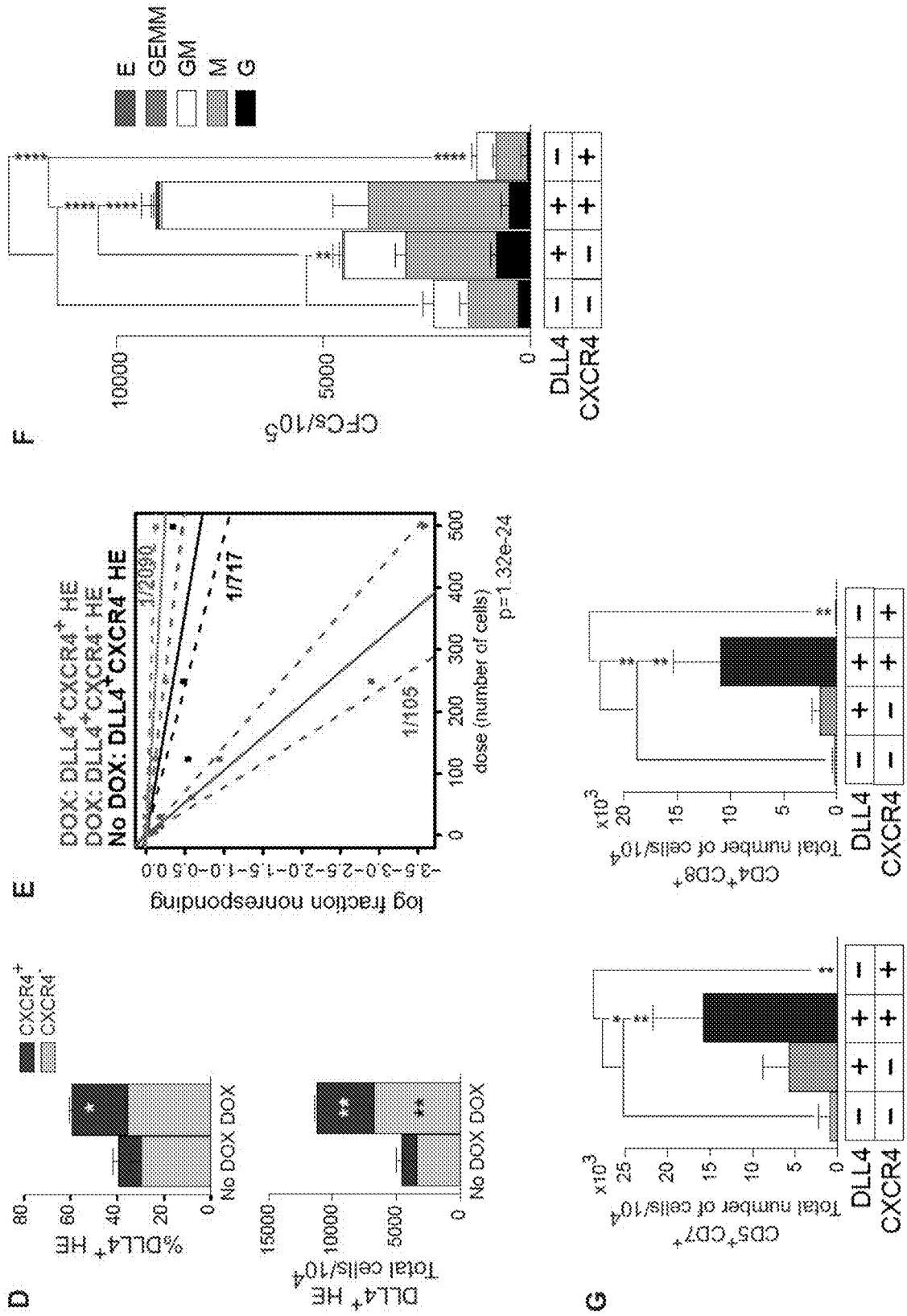


Figure 4

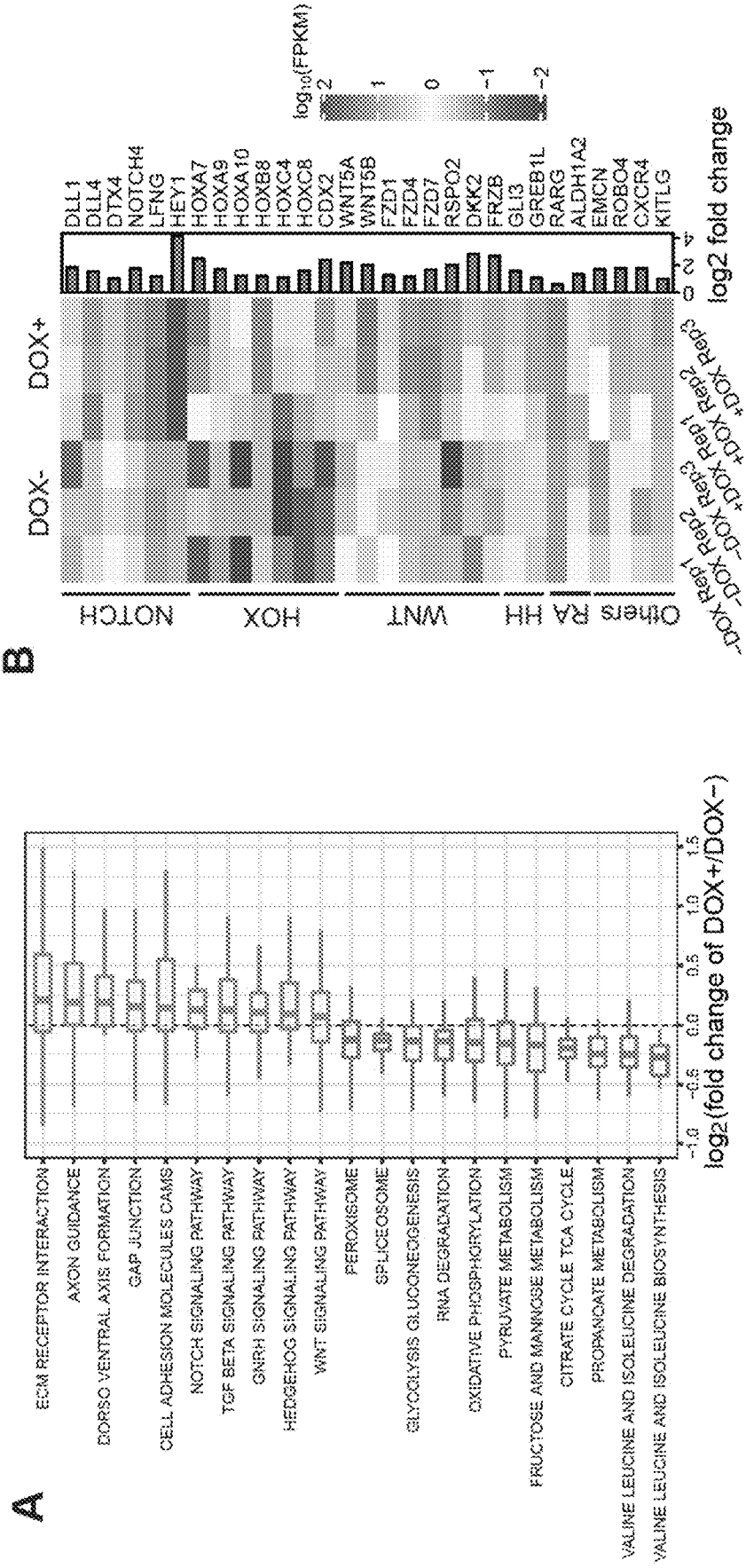

A
ECM RECEPTOR INTERACTION
AXON GUIDANCE
DORSO VENTRAL AXIS FORMATION
GAP JUNCTION
CELL ADHESION MOLECULES CAMS
NOTCH SIGNALING PATHWAY
TGF BETA SIGNALING PATHWAY
GNRH SIGNALING PATHWAY
HEDGEHOG SIGNALING PATHWAY
WNT SIGNALING PATHWAY
PEROXISOME
SPLICEOSOME
GLYCOLYSIS GLUCONEOGENESIS
RNA DEGRADATION
OXIDATIVE PHOSPHORYLATION
PYRUVATE METABOLISM
FRUCTOSE AND MANNOSE METABOLISM
CITRATE CYCLE TCA CYCLE
PROPANOATE METABOLISM
VALINE LEUCINE AND ISOLEUCINE DEGRADATION
VALINE LEUCINE AND ISOLEUCINE BIOSYNTHESIS
−1.0 −0.5 0.0 0.5 1.0 1.5
log2(fold change of DOX+/DOX−)
B
DOX-
DOX+
DLL1
DLL4
DTX4
NOTCH4
LFNG
HEY1
HOXA7
HOXA9
HOXA10
HOXB8
HOXC4
HOXC8
CDX2
WNT5A
WNT5B
FZD1
FZD4
FZD7
RSPO2
DKK2
FRZB
GLI3
GREB1L
RARG
ALDH1A2
EMCN
ROBO4
CXCR4
KITLG
NOTCH
HOX
WNT
HH
RA
Others
log10(FPKM)
2
1
0
−1
−2
log2 fold change
−4
−2
0
2
4
-DOX Rep1
-DOX Rep2
-DOX Rep3
+DOX Rep1
+DOX Rep2
+DOX Rep3

Figure 4 (continued)

Figure 4 (continued)

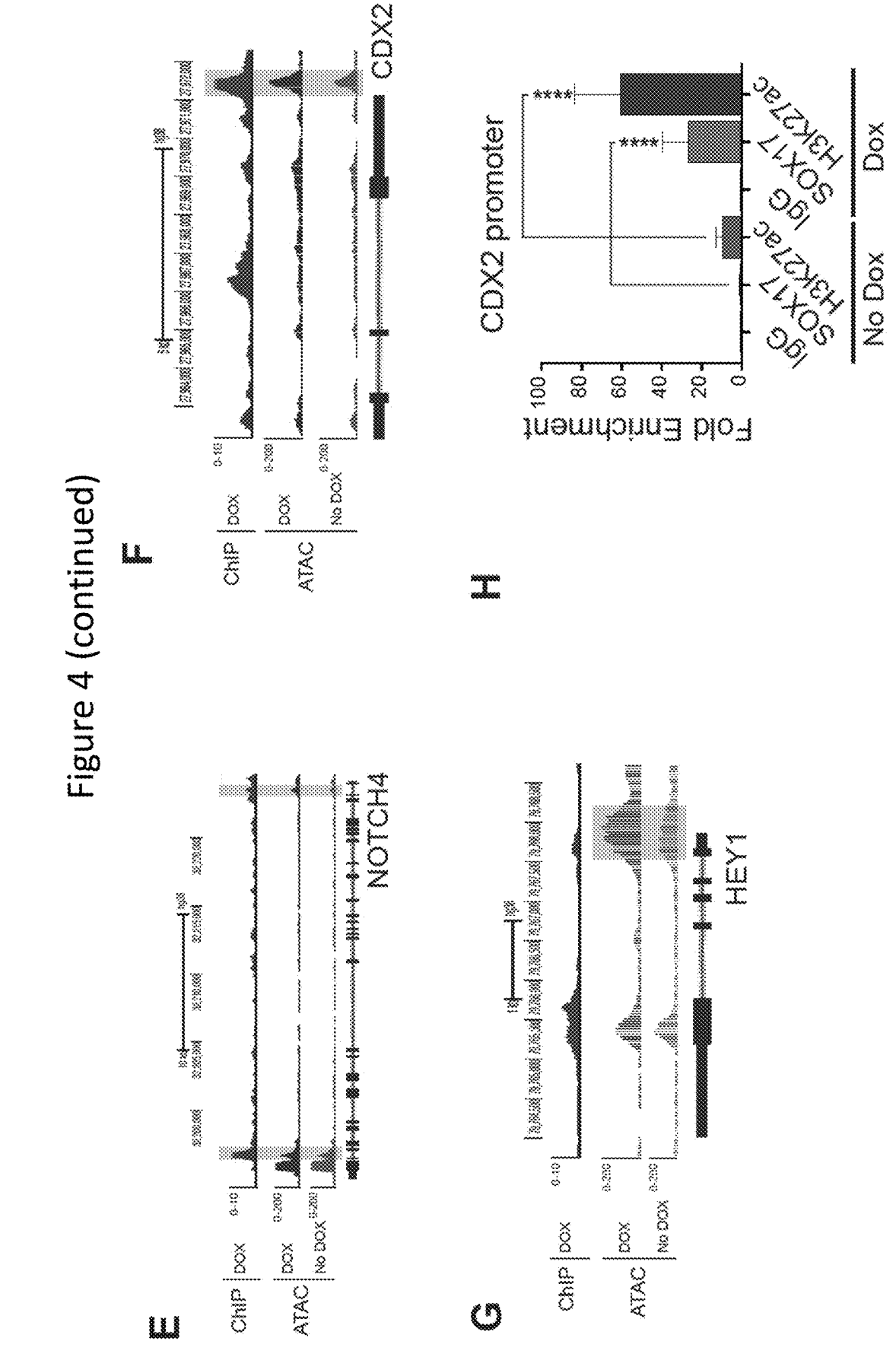


Figure 5

A

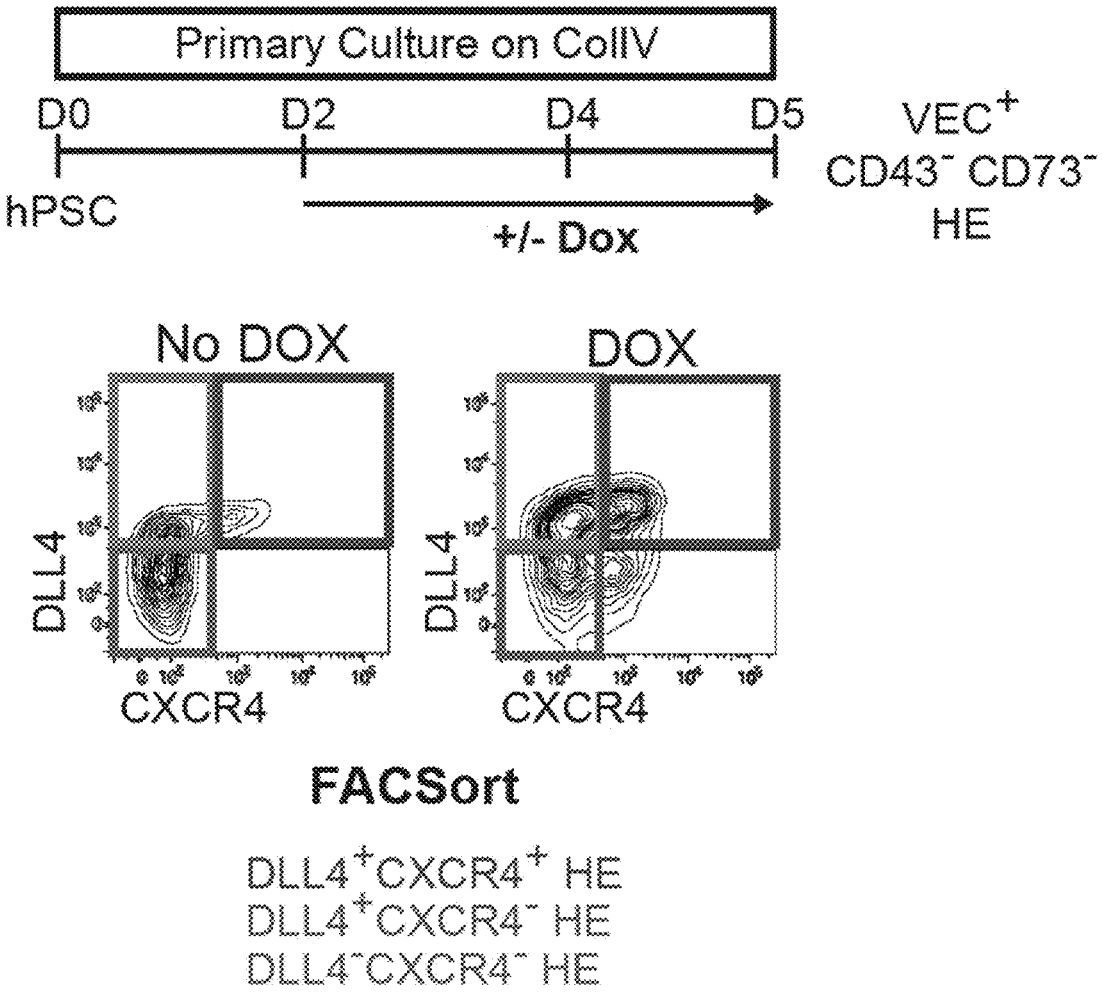

Primary Culture on CollV
D0
D2
D4
D5
hPSC
+/- Dox
VEC+
CD43- CD73-
HE
No DOX
DOX
DLL4
CXCR4
FACSort
DLL4+CXCR4+ HE
DLL4+CXCR4- HE
DLL4-CXCR4- HE

Figure 5 (continued)

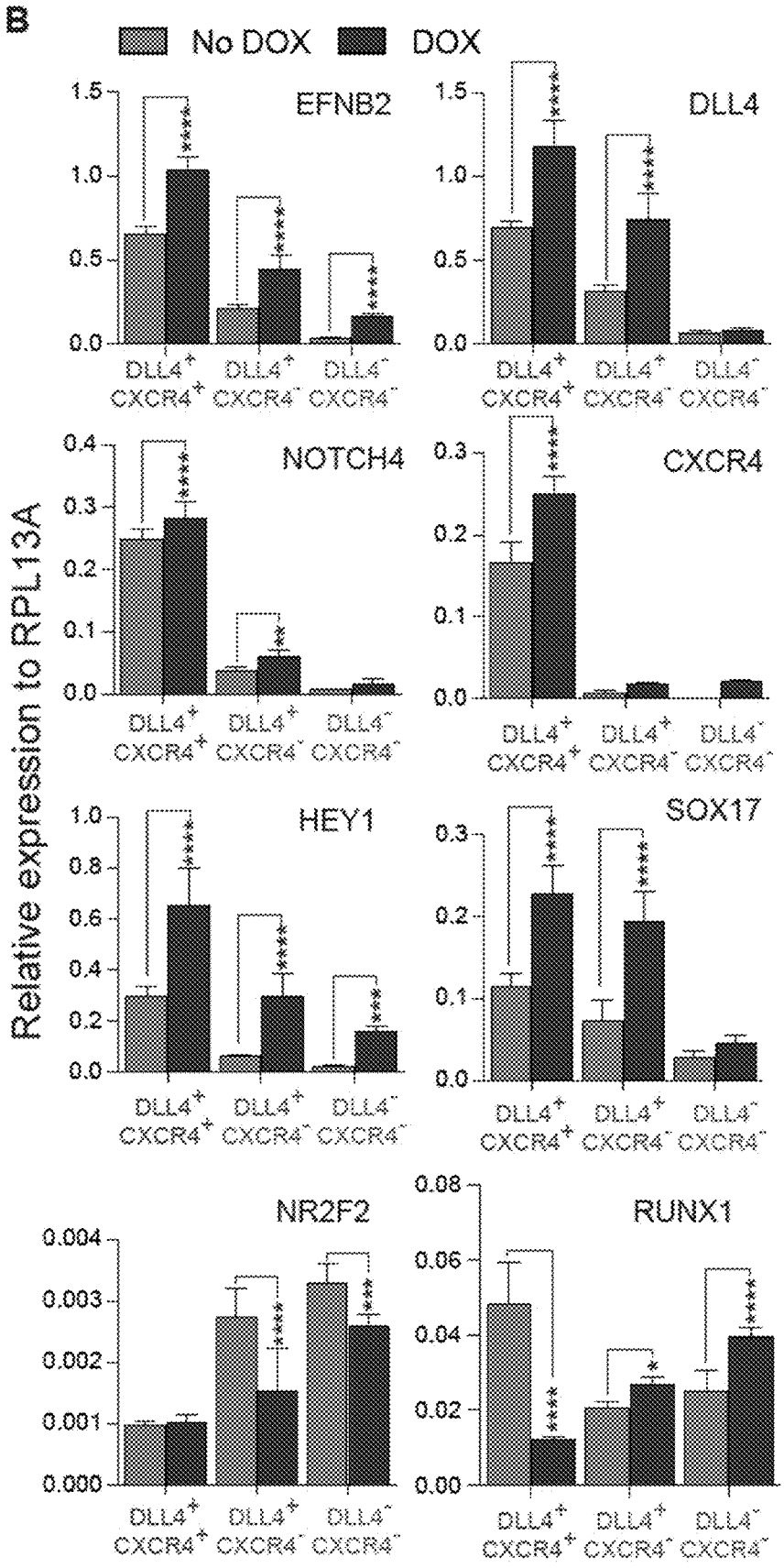


Figure 5 (continued)

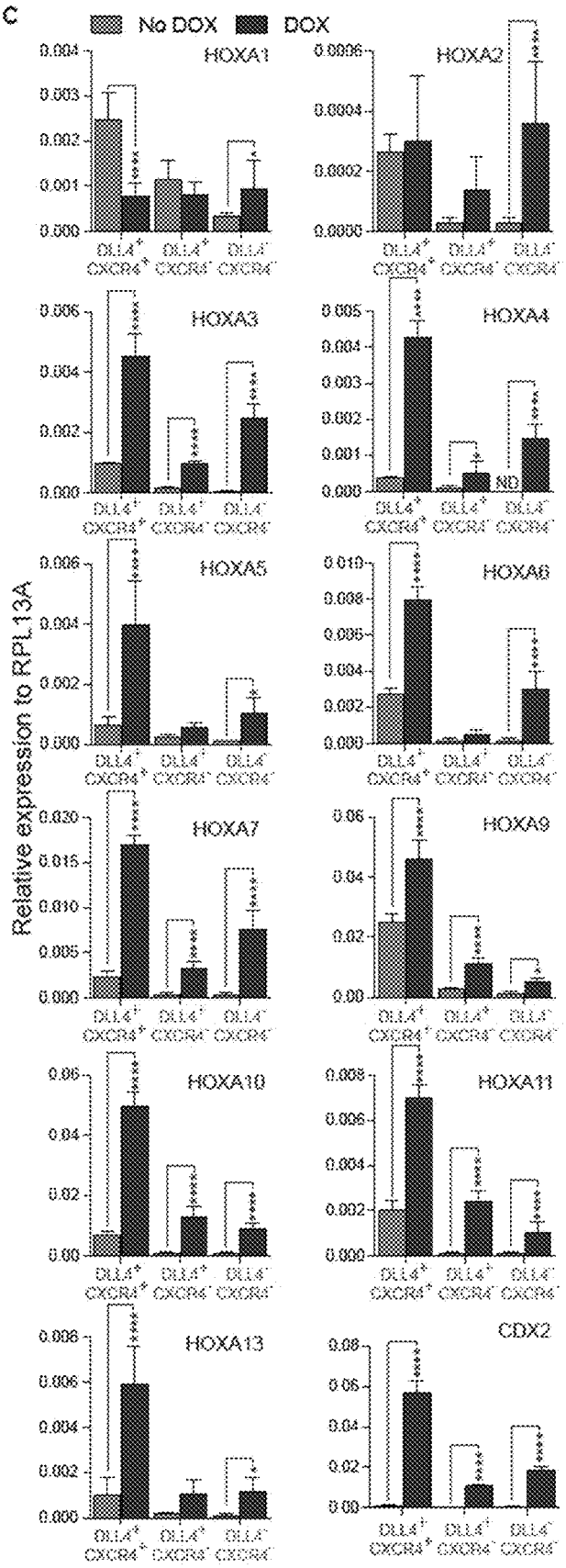


Figure 6

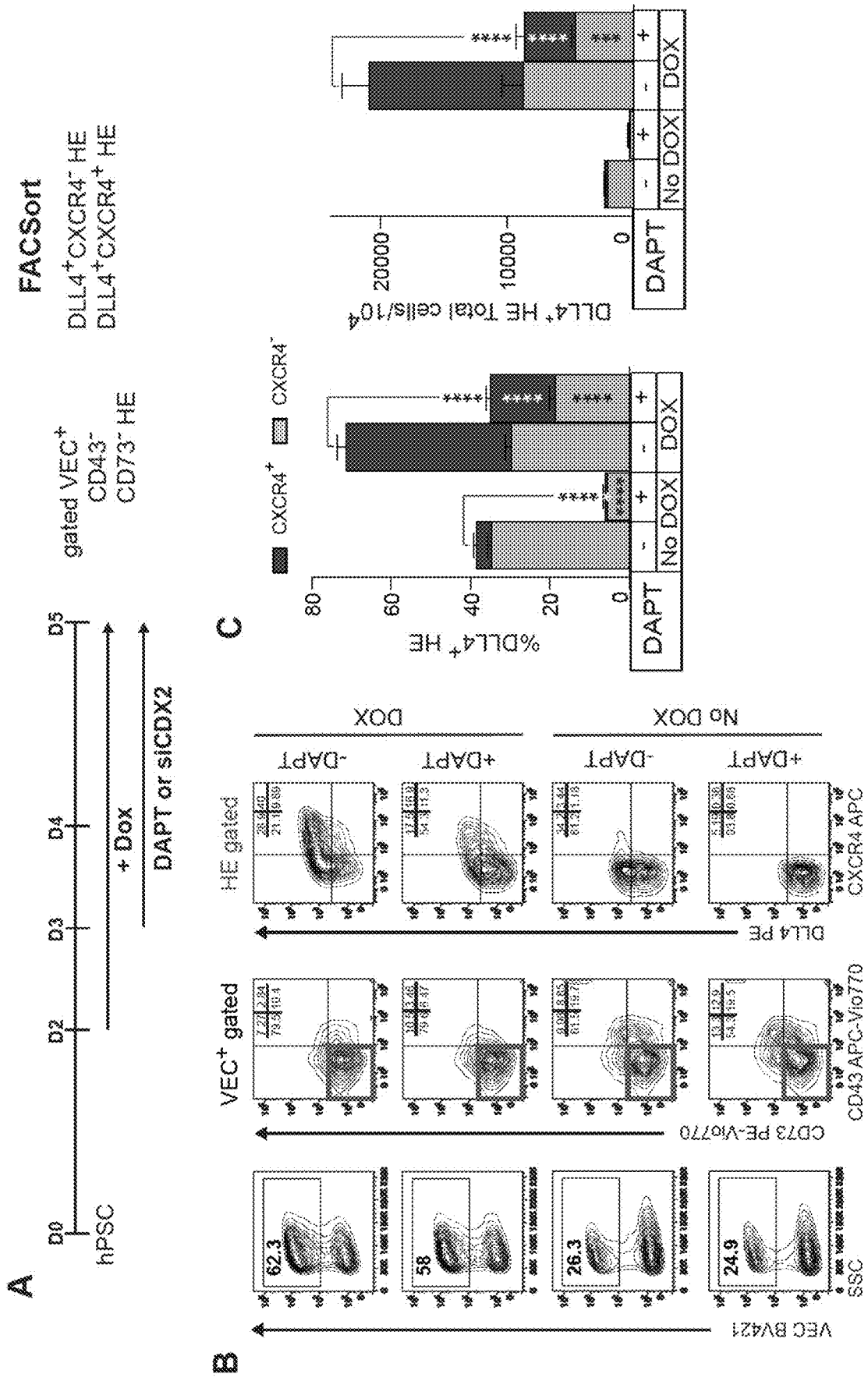

A
hPSC
D0
D2
D3
D4
D5
+ Dox
DAPT or siCDX2
gated VEC+ CD43- CD73- HE
FACSort
DLL4+CXCR4- HE
DLL4+CXCR4+ HE
B
VEC+ gated
HE gated
VEC BV421
SSC
CD73 PE-Vio770
CD43 APC-Vio770
DLL4 PE
CXCR4 APC
62.3
58
26.3
24.9
+DAPT
-DAPT
No DOX
DOX
C
CXCR4+
CXCR4-
%DLL4+ HE
DLL4+ HE Total cells/10^4
DAPT
No DOX
DOX
****
***

Figure 6 (continued)

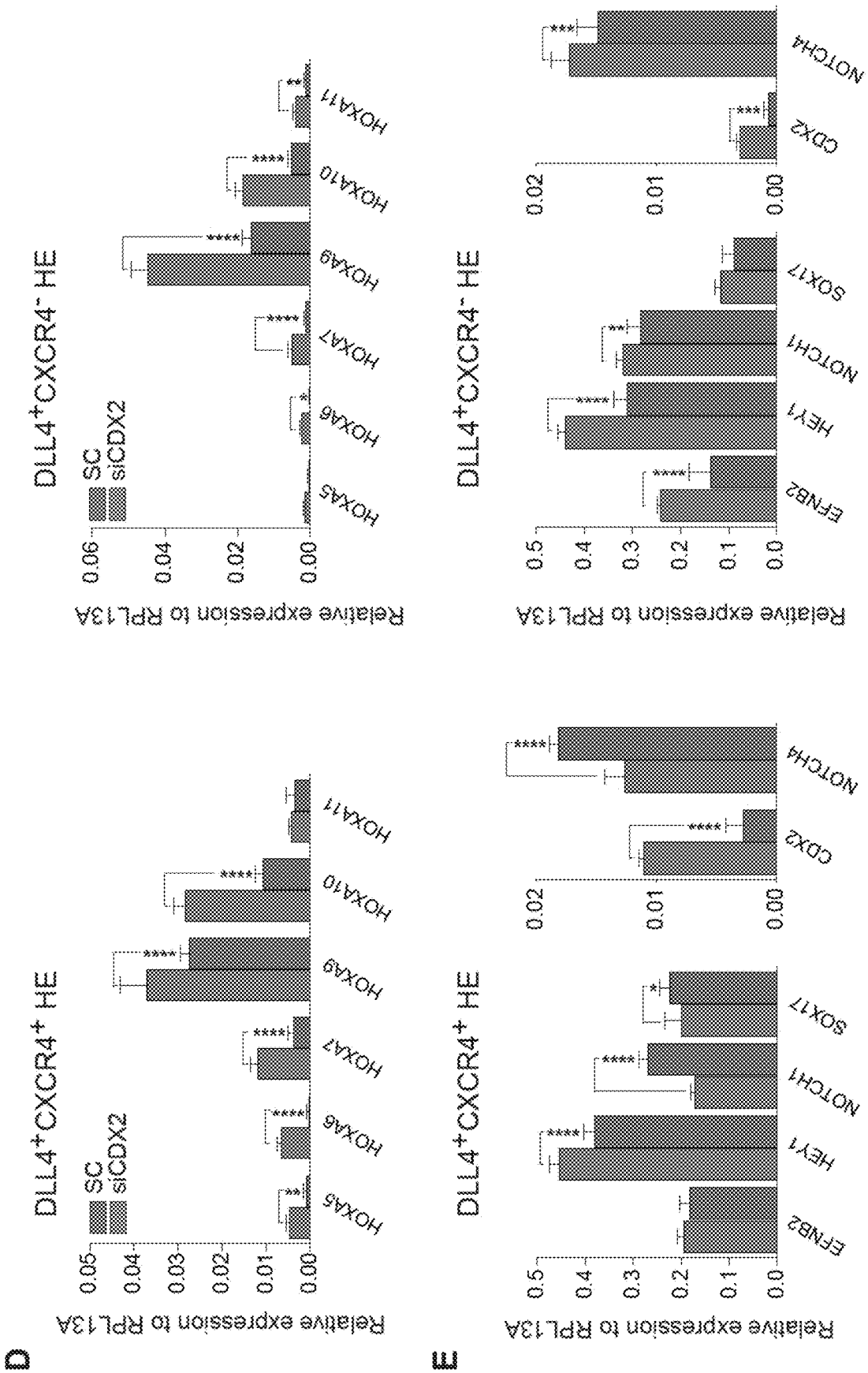


Figure 7

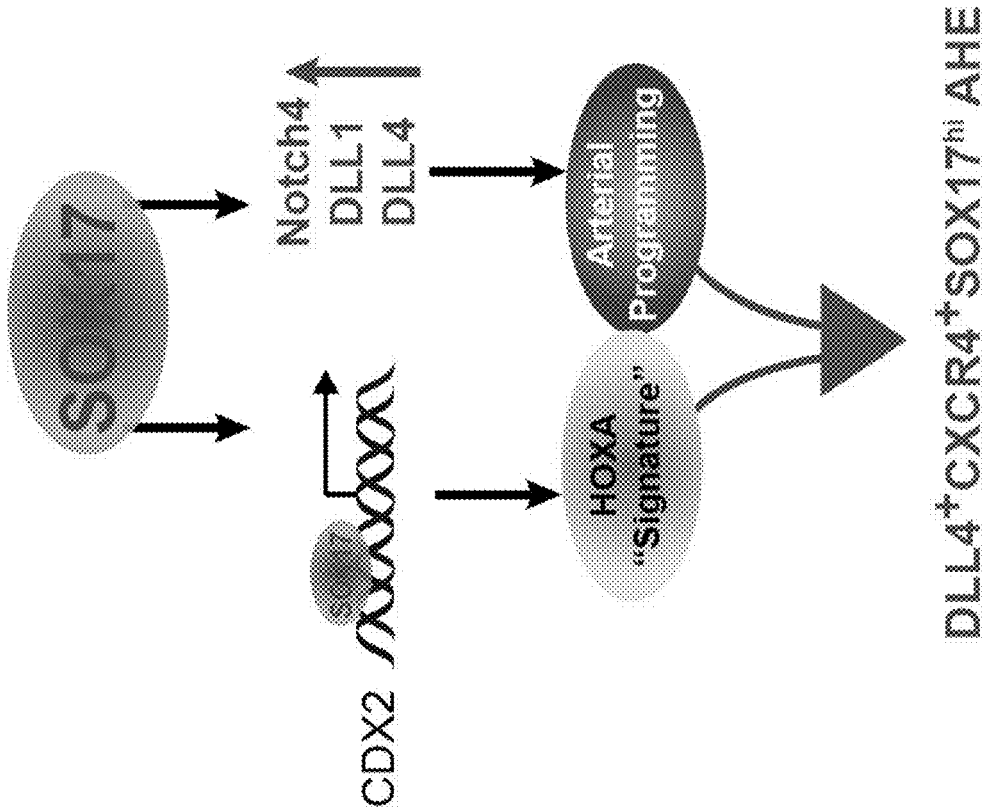

SOX17
Notch4
DLL1
DLL4
CDX2
Arterial Programming
HOXA "Signature"
DLL4+CXCR4+SOX17hi AHE Specification

Figure 8

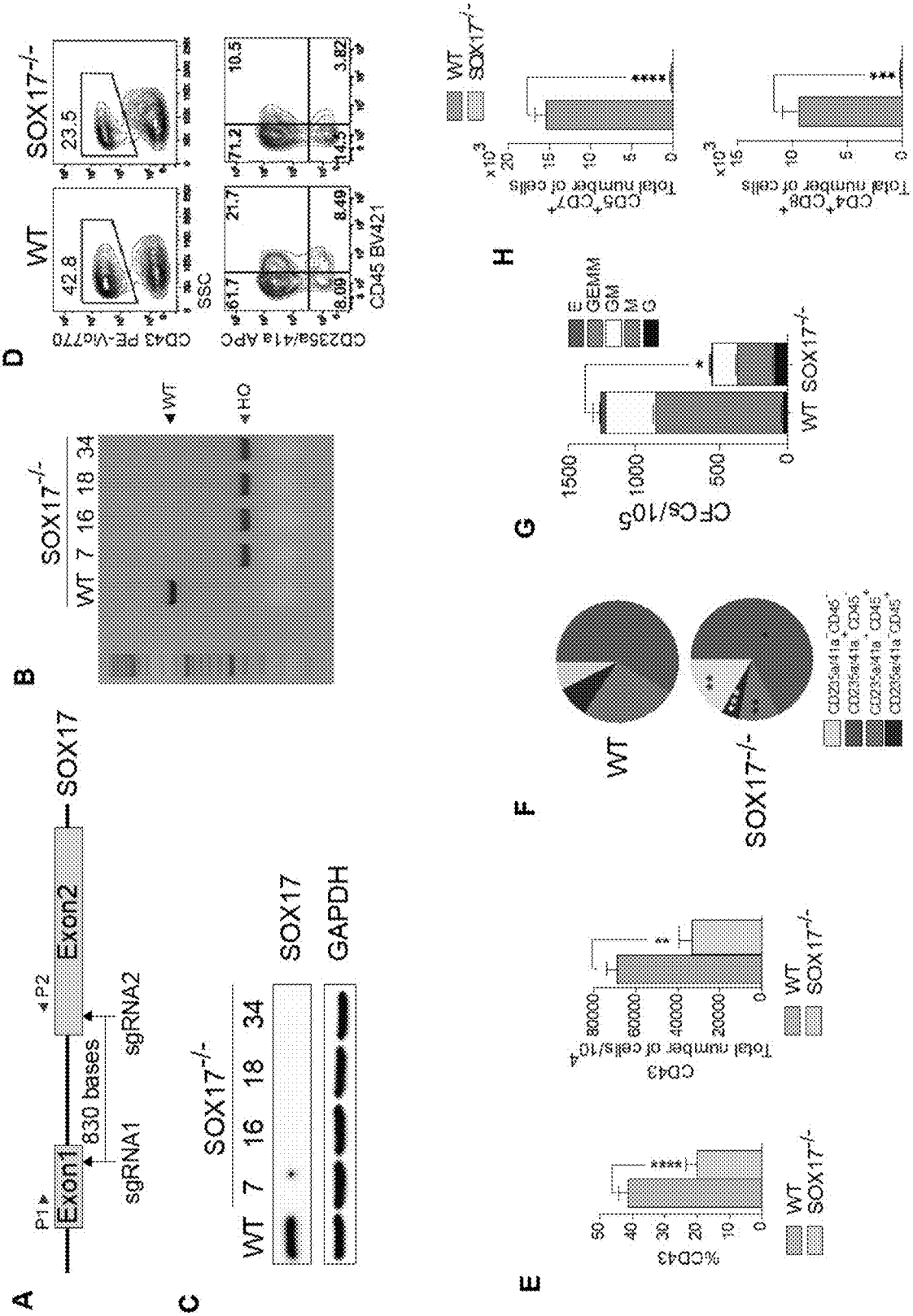

A
P1
Exon1
830 bases
sgRNA1
sgRNA2
P2
Exon2
SOX17
B
SOX17-/-
WT 7 16 18 34
WT
HO
C
SOX17-/-
WT 7 16 18 34
SOX17
GAPDH
D
WT
SOX17-/-
42.8
23.5
SSC
CD43 PE-Vio770
61.7
21.7
8.09
8.49
71.2
10.5
14.5
3.82
CD235a/41a APC
CD45 BV421
E
%CD43
WT
SOX17-/-
****
CD43
Total number of cells/10^4
F
WT
SOX17-/-
CD235a/41a+ CD45-
CD235a/41a+ CD45+
CD235a/41a- CD45+
CD235a/41a- CD45-
G
E
GEMM
GM
M
G
CFCs/10^5
WT SOX17-/-
H
CD3+CD7+
Total number of cells
CD4+CD8+
Total number of cells
WT
SOX17-/-
****
***

Figure 8 (continued)

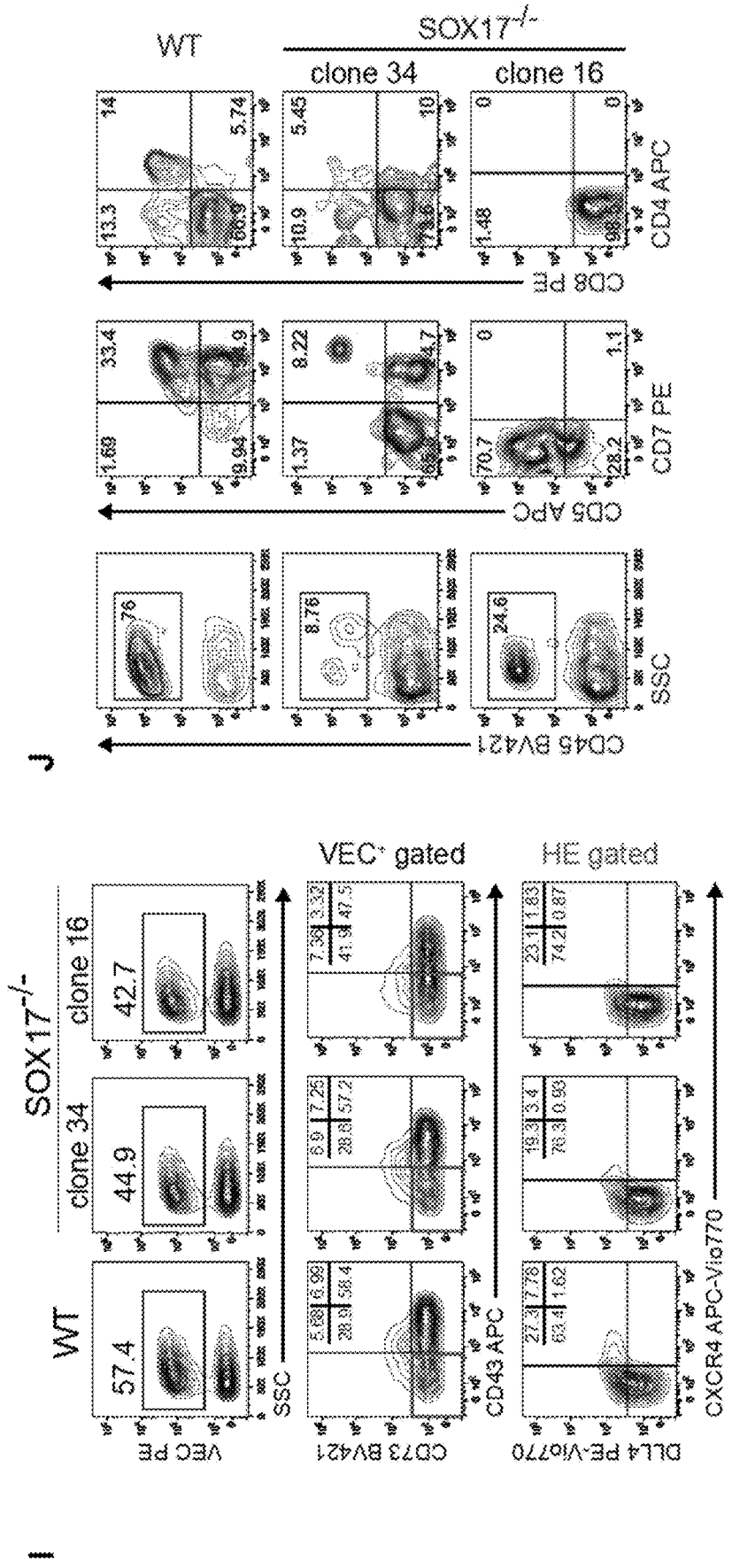


Figure 9

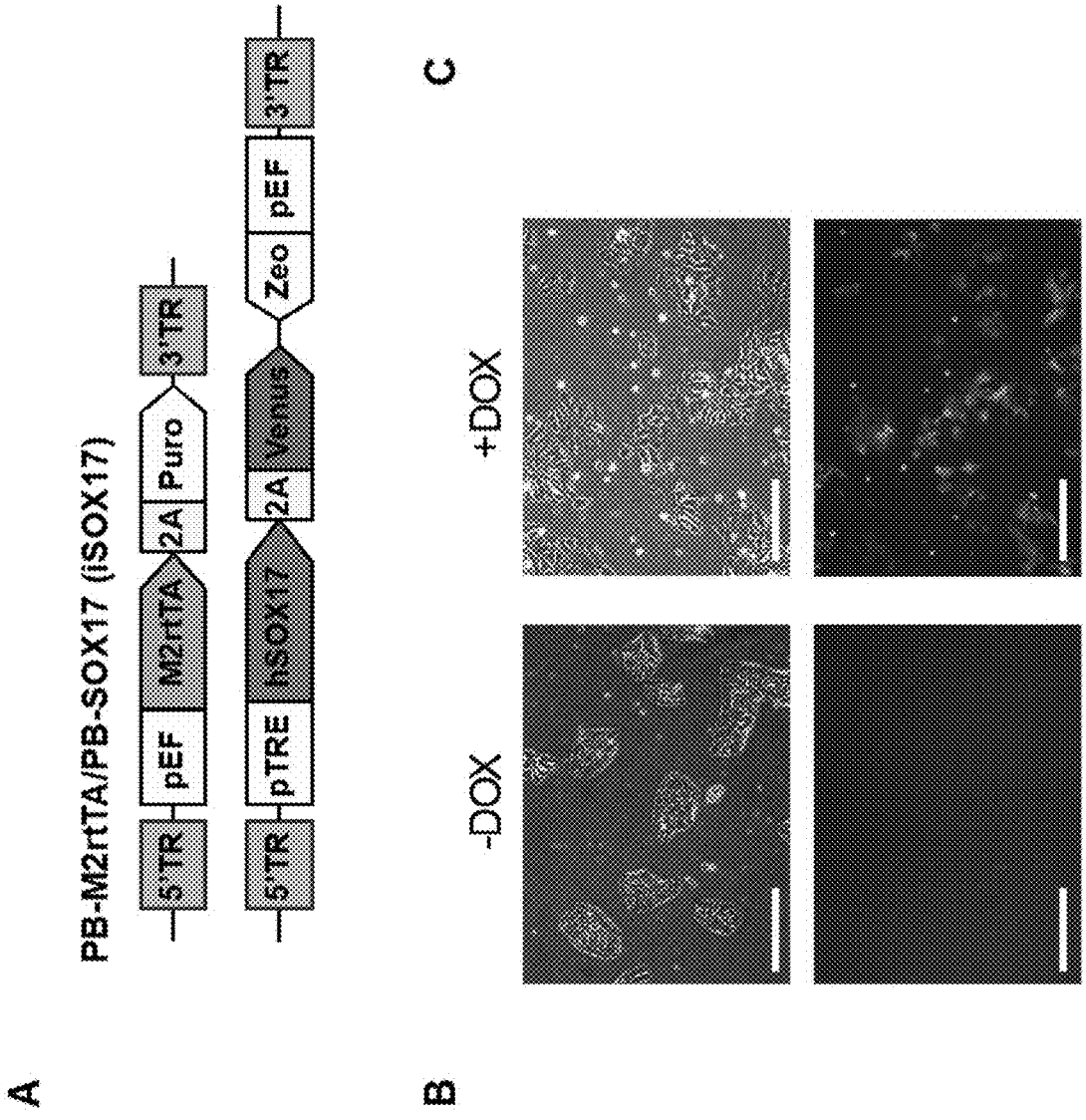

A
PB-M2rtTA/PB-SOX17 (iSOX17)
5' TR
pEF
M2rtTA
2A
Puro
3' TR
5' TR
pTRE
hSOX17
2A
Venus
Zeo
pEF
3' TR
B
-DOX
+DOX
C
No Dox
Dox
SOX17
GAPDH

Figure 10

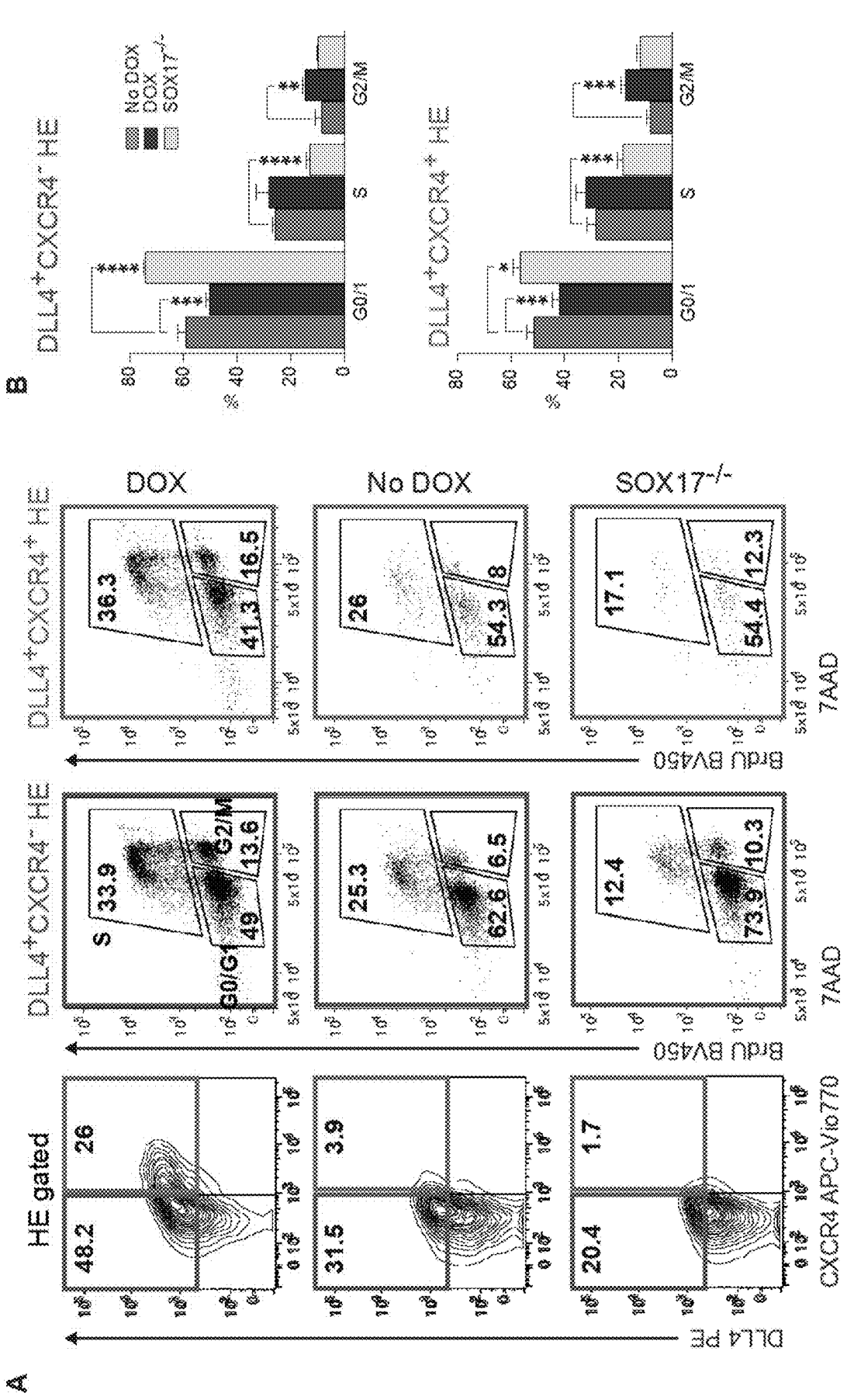

A
HE gated
DLL4+CXCR4- HE
DLL4+CXCR4+ HE
DOX
No DOX
SOX17-/-
DLL4 PE
CXCR4 APC-Vio770
BrdU BV450
7AAD
48.2
26
31.5
3.9
20.4
1.7
33.9
S
G0/G1
49
G2/M
13.6
25.3
62.6
6.5
12.4
73.9
10.3
36.3
41.3
16.5
26
54.3
8
17.1
54.4
12.3
B
DLL4+CXCR4- HE
DLL4+CXCR4+ HE
No DOX
DOX
SOX17-/-
%
G0/1
S
G2/M

Figure 10 (continued)

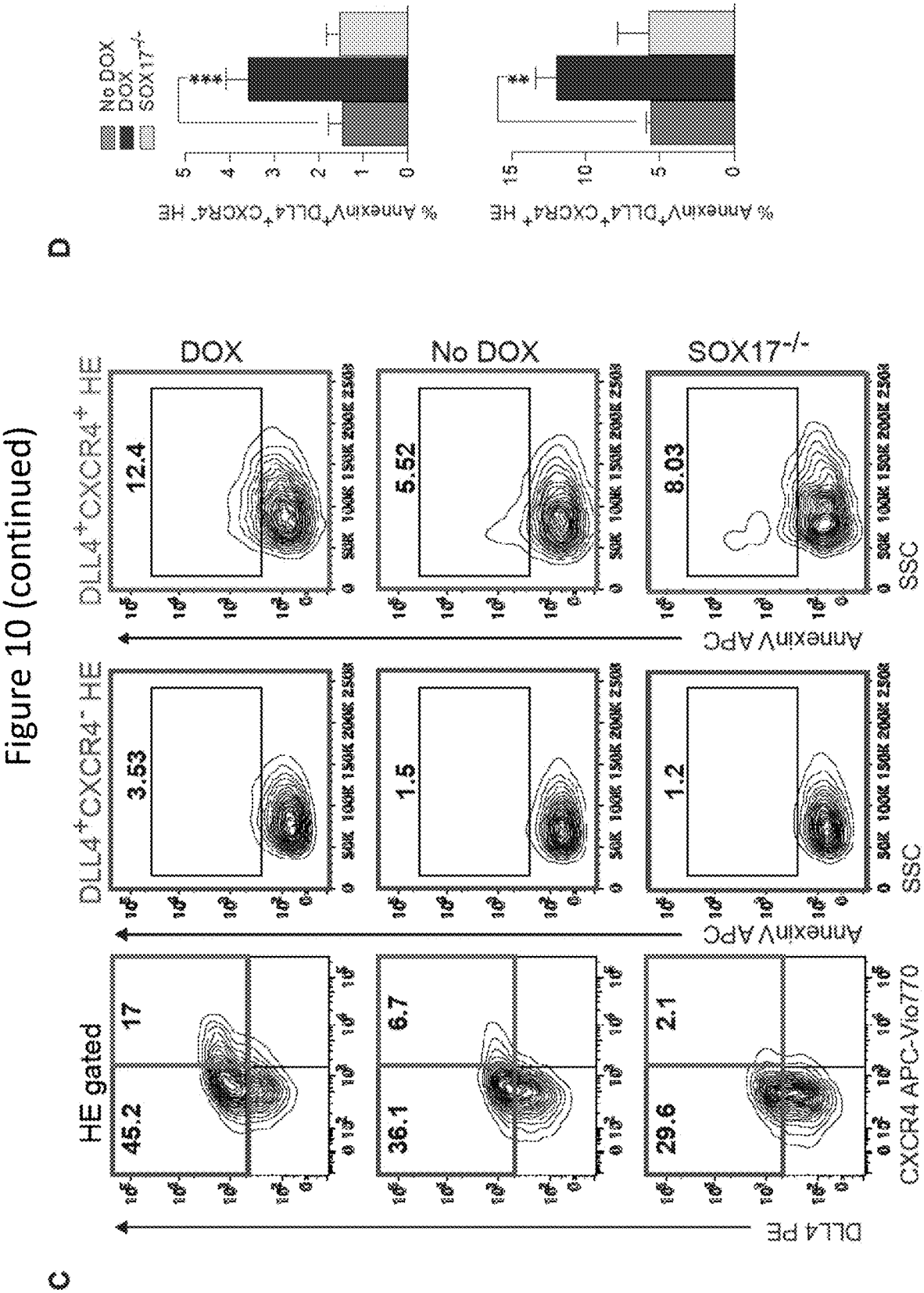


Figure 11

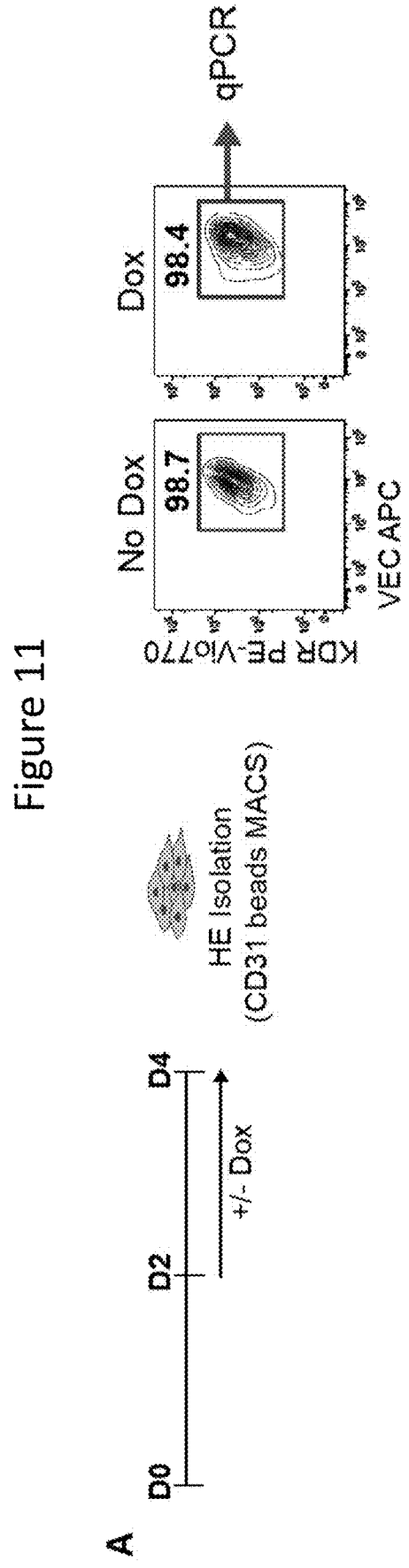

A
D0
D2
D4
+/- Dox
HE Isolation
(CD31 beads MACS)
KDR PE-Vio770
VEC APC
No Dox
98.7
Dox
98.4
qPCR

Figure 11 (continued)

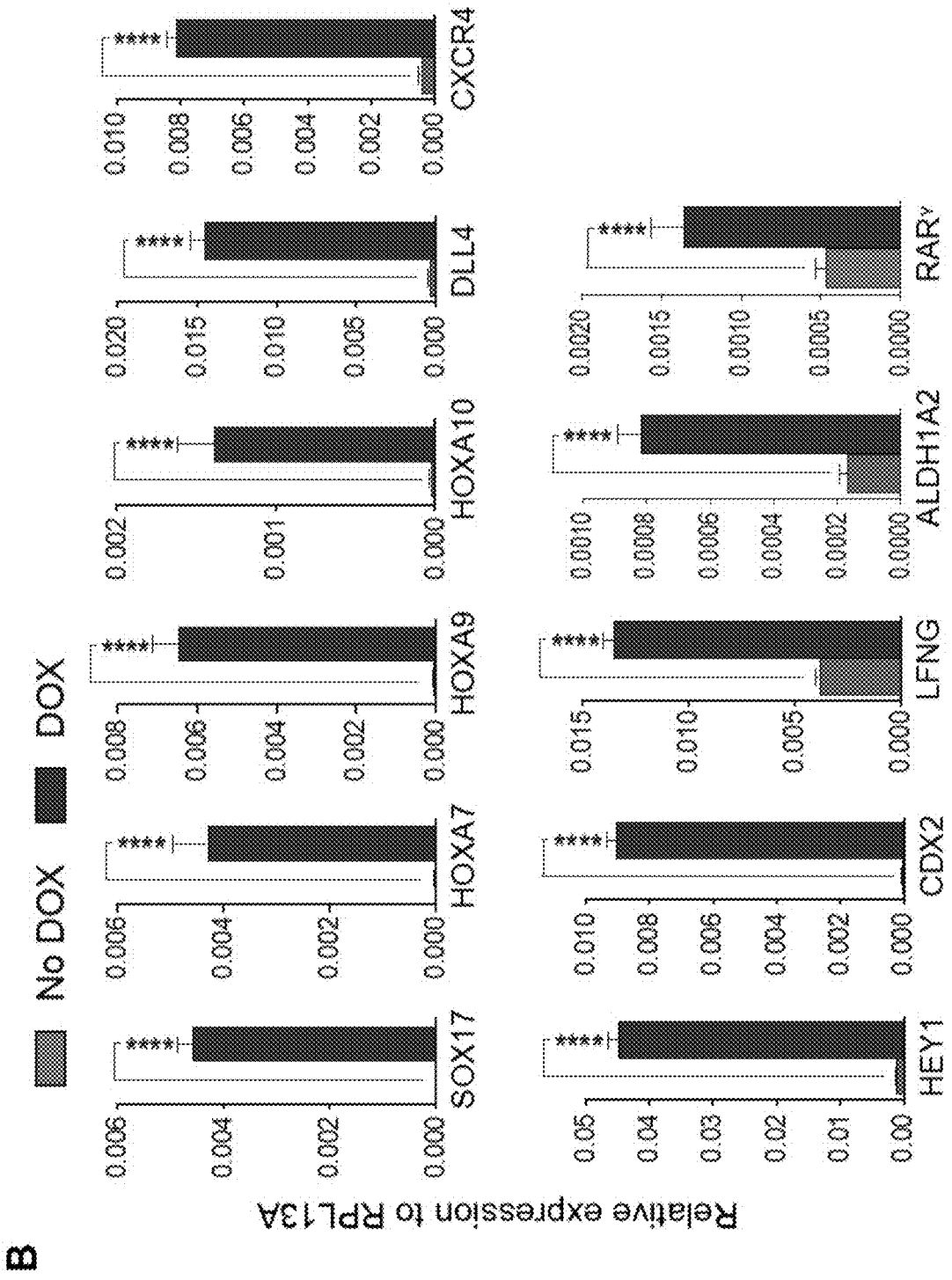


Figure 12

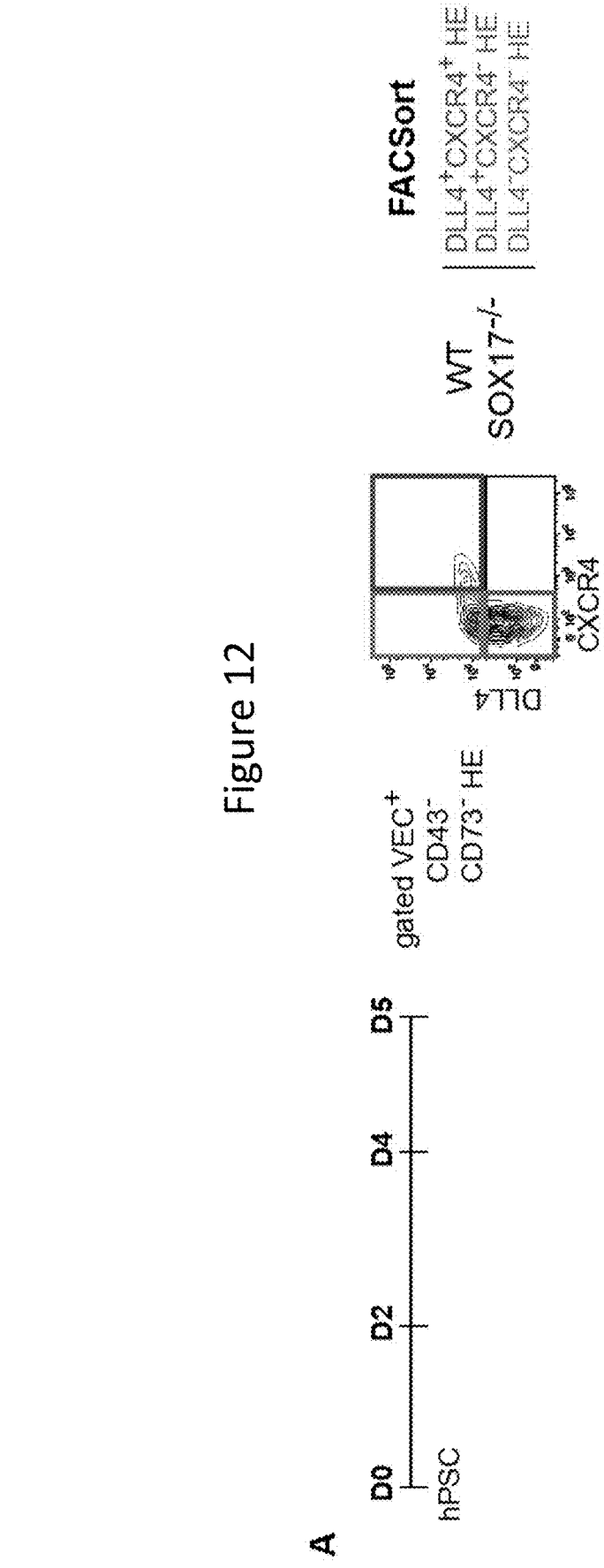

A
D0
D2
D4
D5
hPSC
gated VEC+
CD43-
CD73- HE
DLL4
CXCR4
WT
SOX17-/-
FACSort
DLL4+CXCR4+ HE
DLL4+CXCR4- HE
DLL4-CXCR4- HE

Figure 12 (continued)

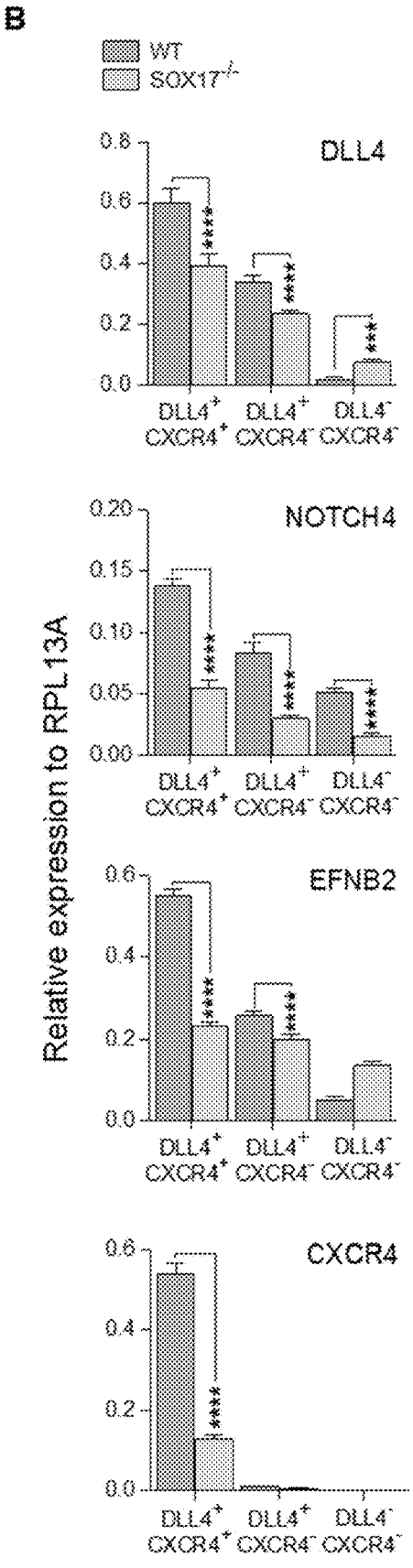


Figure 12 (continued)

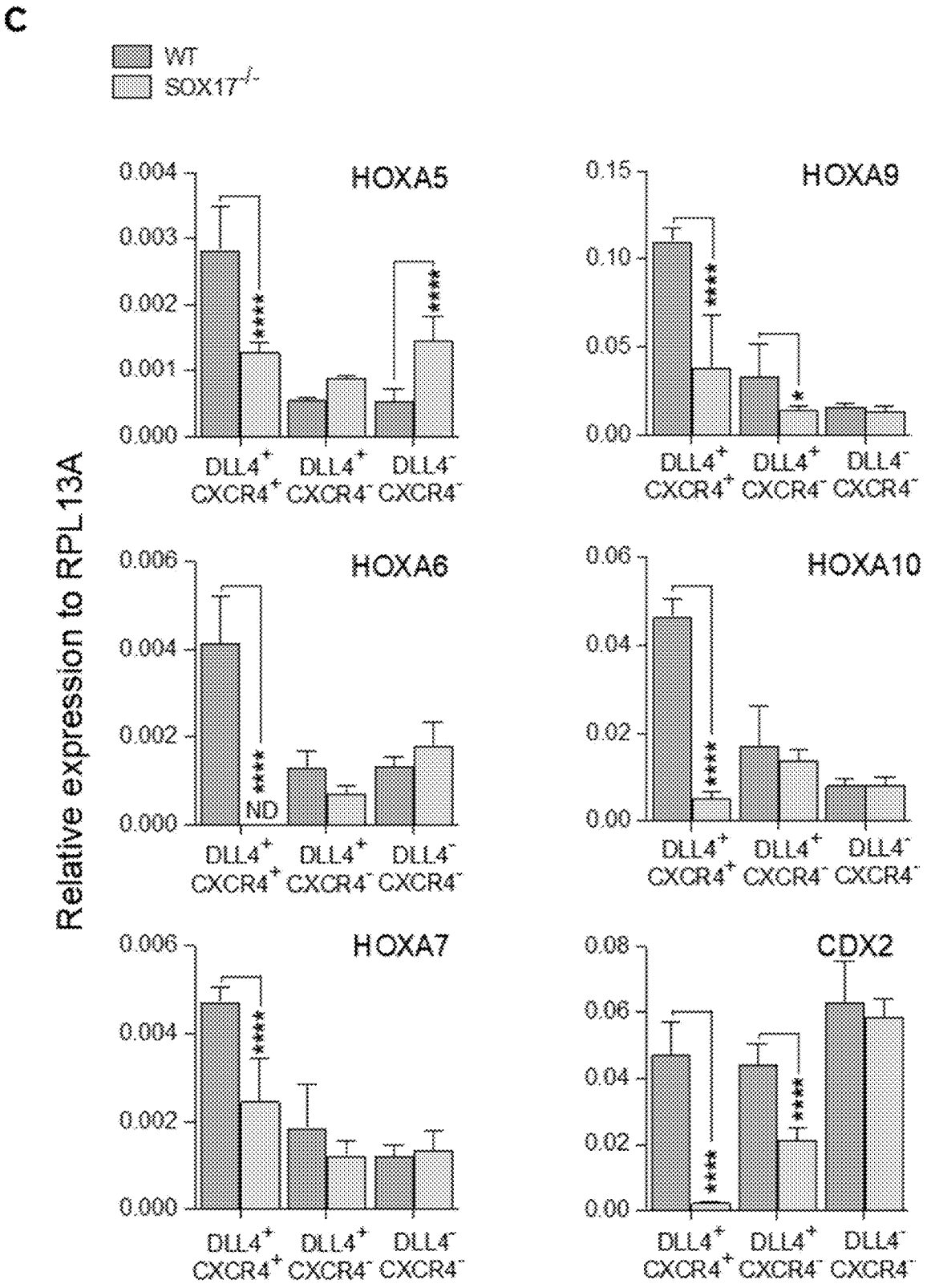


Figure 13

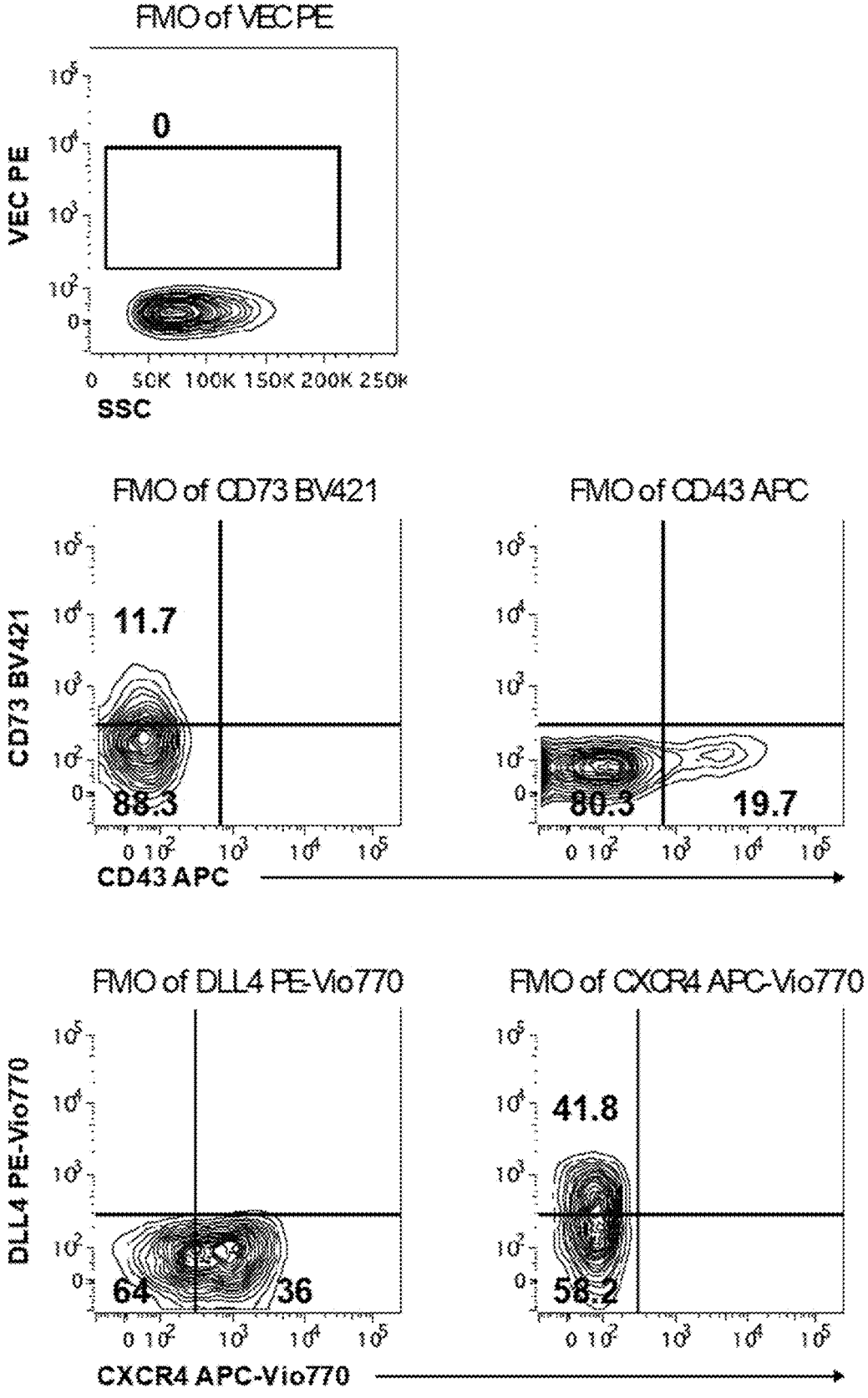
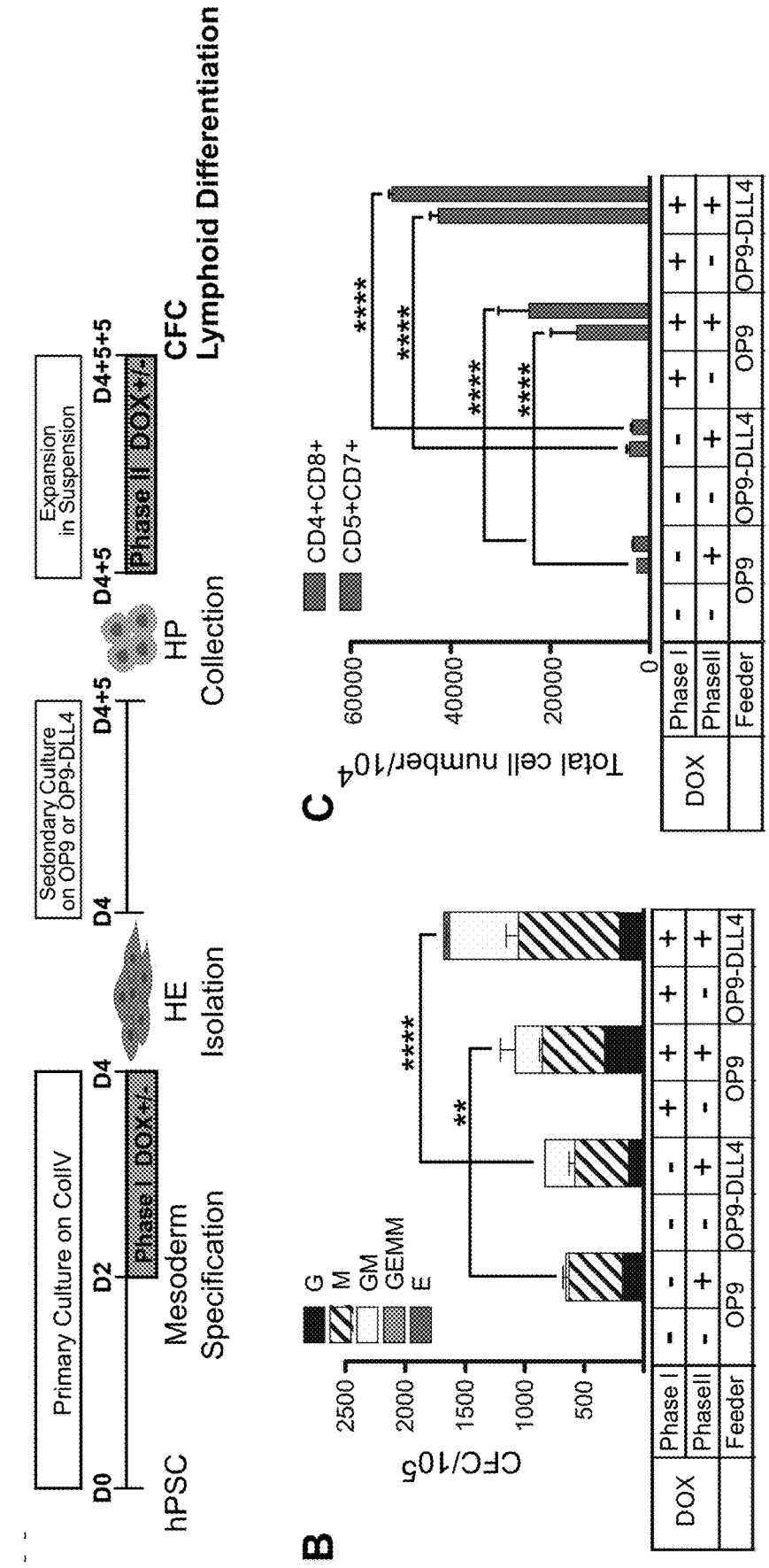

FMO of VEC PE
0
VEC PE
SSC
FMO of CD73 BV421
11.7
88.3
CD73 BV421
FMO of CD43 APC
80.3
19.7
CD43 APC
FMO of DLL4 PE-Vio770
64
36
DLL4 PE-Vio770
FMO of CXCR4 APC-Vio770
41.8
58.2
CXCR4 APC-Vio770

Figure 14


hPSC
D0
Primary Culture on ColIV
D2
Phase I DOX+/-
D4
Mesoderm Specification
HE
Isolation
D4
Sedondary Culture on OP9 or OP9-DLL4
D4+5
HP
Collection
D4+5
Expansion in Suspension
D4+5+5
Phase II DOX+/-
CFC
Lymphoid Differentiation
B
CFC/10^5
2500
2000
1500
1000
500
G
M
GM
GEMM
E
**
****
DOX
Phase I
PhaseII
Feeder
OP9
OP9-DLL4
OP9
OP9-DLL4
C
Total cell number/10^4
60000
40000
20000
0
CD4+CD8+
CD5+CD7+
****
****
****
****
DOX
Phase I
PhaseII
Feeder
OP9
OP9-DLL4
OP9
OP9-DLL4

GENERATION OF HOXA-EXPRESSING HEMOGENIC ENDOTHELIUM WITH ENHANCED T CELL POTENTIAL FROM HPSCS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/026,494 filed on May 18, 2020, the contents of which are incorporated by reference in their entireties.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under HL142665, OD011106, CA014520, and HL134655 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] A Sequence Listing accompanies this application and is submitted as an ASCII text file of the sequence listing named "960296_04147_ST25.txt" which is 18.5 KB in size and was created on Apr. 30, 2021. The sequence listing is electronically submitted via EFS-Web with the application and is incorporated herein by reference in its entirety.

BACKGROUND

[0004] De novo production of hematopoietic stem cells (HSCs) from in vitro expandable human cells, such as pluripotent stem cells (hPSCs), represents a promising approach for stem cell-based therapies and modeling of hematologic diseases. However, generation of HSCs and lymphoid cells from hPSCs remains a significant challenge. During development, blood cells and HSCs arise from hemogenic endothelium (HE) via a definitive hematopoiesis program that produces the entire spectrum of adult-type erythro-myeloid progenitors (EMP) and lymphoid cells. Importantly, these in vivo-produced cells have the capacity to provide long-term cell repopulation in an adult recipient following engraftment. Although previous studies have successfully generated hematopoietic progenitors (HPs) with a HSC phenotype and limited engraftment potential from pluripotent stem cells (PSCs), cells with the capacity for robust and consistent engraftment with recapitulation of the full spectrum of terminally differentiated hematopoietic cells, including lymphoid cells, has not been achieved. Thus, identifying key cellular and molecular programs required for proper lymphoid and HSC specification in vitro is essential to overcome the current roadblocks to advance the lymphoid cell and HSC manufacturing technology.

SUMMARY

[0005] The present invention provides methods of enhancing HOXA gene expression and arterial specification of hemogenic endothelium with superior lymphomyeloid potential in differentiating human pluripotent stem cells (hPSCs). The methods comprise (a) introducing an inducible SOX17 transgene into a population of hPSCs; (b) culturing the hPSCs for at least two days under conditions to differentiate the hPSC into KDR$^+$ mesoderm cells; and (c) inducing expression of the SOX17 transgene in the KDR$^+$mesoderm cells and culturing for at least two days, such that DLL4$^+$CXCR4$^+$ arterial hemogenic endothelium (AHE) cells are obtained.

[0006] In some embodiments, the methods involve transducing the hPSCs with a vector comprising an inducible promoter operably linked to the SOX17 transgene.

[0007] In another aspect, the present invention provides cell populations produced by the methods disclosed herein.

[0008] In another aspect, the present invention provides isolated in vitro populations of DLL4$^+$CXCR4$^+$ AHE cells differentiated from an hPSC population comprising a SOX17 transgene.

[0009] In another aspect, the present invention provide a method of expansion of hematopoietic progenitors comprising a) generating hemogenic endothelium (HE) cells in presence of SOX17 upregulation; b) culturing the HE cells on OP9 or OP9-DLL4 cells in medium comprising FLT3L, TPO SCF, IL6, and IL3 for at least an additional 5 days; c) collecting the floating hematopoietic progenitor cells (HP); and d) culturing the HPs of step c in medium comprising FLT3L, TPO SCF, IL6, and IL3 for at least an additional 5 days to expand HPs with myeloid and lymphoid potential. In some aspects, the method further comprises (e) passaging the cells of step (d) for at least two weeks in medium comprising SCF, FLT3L and IL-7 for at least two weeks to produce CD4+CD8+ T cells.

[0010] In some embodiments, the cells are further differentiated to form an isolated in vitro T cell population that may be used for several downstream applications, such as the generation of exogenous chimeric antigen receptors (CARs).

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. **1** demonstrates that SOX17 knockout impairs arterial specification and definitive hematopoiesis. (A) Schematic diagram of hematopoietic development and in defined conditions. D is day of differentiation. (B) HB-CFC potential of wild-type or SOX17$^{-/-}$ H19 cells (mean±SD, for 2 independent experiments performed in duplicates). **p<0.01, t-test. (C) Flow cytometric analysis of day 4 HE. (D) and (E) flow cytometric analysis of day 5 HE. Graphs show the percentages and total number of cells generated from 10^4 hESCs (mean±SD, n=3 experiments). **p<0.01 and ***p<0.001, t-test. (F) Schematic diagram of experiments. Wild and SOX17$^{-/-}$ cells were purified using CD31 MACS on day 4 and plated on OP9 or OP9-DLL4 for 5 days. (G) Hematopoietic colony-forming potential of day 4 HE after 5 days of culture on OP9 or OP9-DLL4 (mean±SD, n=2 experiments). ****p<0.0001, two-way ANOVA, Tukey's multiple comparisons test. (H) Flow cytometric analysis of T cell differentiation. (I) Graphs show the total number of T cells generated from 10^4 CD43$^+$ cells (mean±SD, n=3 experiments). ****p<0.0001, two-way ANOVA Tukey's multiple comparisons test. See also FIG. **8**.

[0012] FIG. **2** demonstrates that SOX17 enhances arterial specification and definitive lympho-myeloid potential of HE at day 4 of differentiation. (A) Schematic diagram of experiments. (B) Effect of SOX17 overexpression during D2-3 on HB-CFCs (mean±SD, n=2 experiments). Graph shows HB-CFCs per 10^4 cells collected on day 3 of differentiation. (C) Expression of arterial markers and Venus-reporter in iSOX17 cells on day 4 differentiation with or without DOX. Scale bars are 200 μm. (D) and (E) SOX17 overexpression increases the percentages and total numbers of VEC$^+$ cells

and AHE on day 4 of differentiation from 10^4 hESCs. Results are mean±SD, n=3 experiments; **p<0.01, and ****p<0.0001, two-way ANOVA Sidak's multiple comparisons test. (F) CFC potential of HP collected following culture of day 4 HE on OP9 or OP9-DLL4 for 5 days (mean±SD, n=2 experiments performed in duplicates). *p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA, Tukey's multiple comparisons test. (G) Graphs show the total number of T cells from produced from 10^4 HPs collected following culture of D4 HE on OP9 or OP9-DLL4 for 5 days (mean±SD, n=3 experiments). *p<0.05, ***p<0.001, ****p<0.0001, two-way ANOVA Sidak's multiple comparisons test. See also FIG. **9**.

[0013] FIG. **3** demonstrates that SOX17 induction promotes specification of $DLL4^+CXCR4^+$ AHE with superior lympho-myeloid potential on day 5 of differentiation. (A) Schematic diagram of experiments. (B) Flow cytometric analysis of HE on day 5 of differentiation in DOX+ and DOX− conditions. (C) DOX effect on HE formation on day 5 of differentiation (D5 HE). (D) DOX treatment enhances specification of $DLL4^+CXCR4^+$ arterial type HE. (C and D) Graphs show the percentages and total number of cells generated from 10^4 hESCs (mean±SD and triplicate independent experiments). *p<0.05 and **p<0.01, ***p<0.001 two-way ANOVA, Sidak's multiple comparisons test. (E) Limiting dilution assay to determine the frequency of hemogenic progenitors in $DLL4^+CXCR4^-$ and $DLL4^+CXCR4^+$ HE cultures with or without DOX. (F) CFC potential of HPs collected after 5 day of culture of indicated day 5 HE subset (mean±SD, n=2 experiments performed in duplicates). **p<0.01, ***p<0.001, and **** p<0.0001 two-way ANOVA, Tukey's multiple comparisons test. (G) Graphs show the total number of T cells produced from 10^4 HPs collected after 5 day of culture of indicated day 5 HE subset (mean±SD, n=2-3 experiments). *p<0.05 and **p<0.01, one-way ANOVA Tukey's multiple comparisons test. See also FIG. **10**.

[0014] FIG. **4** shows molecular profiling of day 4 HE generated with and without DOX. (A) Expression fold changes between DOX+ and DOX− conditions for genes in selected KEGG pathways. GENCODE genes that have symbols mapping to the same KEGG pathway genes were removed. (B) Heat map represents expression of selected genes. (C) GO terms enriched for the top 5% of genes with ATAC-seq signals increased at promoters upon DOX induction. Selected enriched terms were required to have adjusted p-value<0.05. (D) Venn diagram showing enriched motifs in ATAC-seq peaks at gene's promoters. (E-G) SOX17 ChIP-seq fold enrichment over IgG control (DOX+: dark red) and ATAC-seq signals (DOX+: dark magenta; DOX−: light magenta) around NOTCH4, CDX2 and HEY1 genes. ATAC-seq signals are from one of the two replicates. (H) Quantitative ChIP analysis of SOX17 and H3K27ac at the CDX2 promoter (mean±SD, n=3 experiments). ****p<0.0001, two-way ANOVA, Tukey's multiple comparisons test. See also FIG. **11** and FIG. **12**.

[0015] FIG. **5** demonstrates that SOX17 induces expression of arterial and HOXA genes in day 5 HE. (A) Schematic diagram of experiments. (B and C) qPCR analysis of arterial markers (EFNB2, DLL4, Notch4, HEY1, CXCR4, SOX17), venous (NR2F2), RUNX1, and HOX (HOXA and CDX2) genes in day 5 HE subpopulations. Results are mean±SD for three independent experiments; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, two-way ANOVA, Sidak's multiple comparisons test.

[0016] FIG. **6** demonstrates that SOX17 regulates AHE formation through NOTCH signaling activation and HOXA genes expression through CDX2. (A) Schematic diagram of experiments. SOX17 cells were differentiated with DOX, and treated with DAPT or transfected with siCDX2. After 2 days (day 5 of differentiation), two major HE subsets were assessed by flow cytometry and isolated for RT-PCR analysis. (B) and (C) Flow cytometric analysis of AHE formation in DOX or No DOX cultures with or without DAPT. Plots in (C) show the percentages and total number of cells generated from 10^4 hESCs. (mean±SD and triplicated independent experiments). *p<0.05, ***p<0.001, and ****p<0.0001, two-way ANOVA, Sidak's and Tukey's multiple comparisons test. qPCR analysis of HOXA genes (D) and arterial markers (EFNB2, NOTCH1, NOTCH4, HEY1, SOX17), and CDX2 (C) in day 5 HE subpopulations treated with scramble siRNA (SC) or siCDX2. The silencing efficiency of CDX2 with siRNA is more than 75% as shown in (E), right graph. Results are mean±SD for three independent experiments; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, two-way ANOVA, Sidak's multiple comparisons test and t-test.

[0017] FIG. **7** shows a model of SOX17 action in HE. SOX17 upregulates expression of genes associated with NOTCH signaling and binds directly to the CDX2 promoter, upregulating CDX2 expression. These molecular events lead to the upregulation of HOXA gene expression and establishment of AHE with robust lympho-myeloid potential and $DLL4^+CXCR4^+SOX17^{hi}$ phenotype resembling AHE at AGM region.

[0018] FIG. **8** depicts the generation and characterization of SOX17 knockout H9 ESCs by CRISPR (related to FIG. **1**). (A) Schematic diagram of SOX17 knockout strategy. P1 and P2 are screening primers. (B) SOX17 deletion was confirmed by genomic PCR using P1/P2 primers, WT (wild type; 1,233 bp) and HO (Homozygote; 403 bp). (C) SOX17 knockout in day 5 differentiated cells confirmed by western blot. (D and E) Flow cytometric analysis of $CD43^+$ HPs generated on day 9 of differentiation. Graphs show the percentages and total number of cells generated from 10^4 hESCs. (mean±SD and triplicated independent experiments) **p<0.01 and ****p<0.0001, t-test. (F) Pie charts displaying composition of $CD43^+$ subsets on day 9 of differentiation. Results are mean±SD and triplicated independent experiments. *p<0.05, **p<0.01, and ***p<0.001, t-test. (G) CFC potential on day 9 of differentiation. (H) T cell output from 10^4 $CD43^+$ cells collected on day 9 of differentiation (mean±SD, n=3 experiments). ***p<0.001 and ****p<0.0001, t-test. (I) and (J) Two different SOX17 knockout H9 hESC clones confirms reduced arterial specification and T cell potential in setting of SOX17 loss.

[0019] FIG. **9** depicts the generation of DOX-inducible SOX17 H9 hESC line (related to FIG. **2**). (A) Schematic diagram of PiggyBac system used to generate iSOX17 H9 cells. (B) Expression of Venus reporter in undifferentiated iSOX17 cells with or without DOX. Scale bars are 200 μm. (C) Western blot confirms upregulation of SOX17 expression in undifferentiated cell with or without dox.

[0020] FIG. **10** demonstrates the effects of SOX17 on proliferation and apoptosis of AHE at day 5 of differentiation (related to FIG. **3**). (A) and (B) Flow cytometric analysis of

cell cycle in the AHE in DOX, No Dox, and SOX17 knockout cells. Representative dot plots (A) and mean±SD (B) of triplicated independent experiments are shown. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, one-way ANOVA Dunnett's multiple comparisons test. (C) and (D) flow cytometric analysis of cell apoptosis using annexin V staining in the AHE in DOX, No Dox, and SOX17 knockout cells. Representative dot plots (C) and mean±SD (D) of triplicated independent experiments are shown. **p<0.01 and ***p<0.001, one-way ANOVA Dunnett's multiple comparisons test.

[0021] FIG. **11** shows a qPCR analysis that confirms the upregulation of HOXA, arterial and retinoic acid signaling associated genes in day 4 HE following SOX17 overexpression (related to FIG. **4**). (A) Schematic diagram of experiments. (B) qRT-PCR analysis. Results are mean±SD and triplicated independent experiments. ****p<0.0001, t-test.

[0022] FIG. **12** demonstrates that SOX17 knockout suppresses the expression of HOXA genes and arterial markers in day 5 HE subsets (related to FIG. **4**). (A) Schematic diagram of experiments. (B and C) qRT-PCR analysis of arterial (EFNB2, DLL4, NOTCH4, CXCR4) (B) and HOXA genes (HOXA5, HOXA6, HOXA7, HOXA9, HOXA10) and CDX2 (C) in D5 HE subpopulations. Results are mean±SD for three independent experiments; *p<0.05, ***p<0.001, and ****p<0.0001, 2-way ANOVA, Sidak's multiple comparisons test.

[0023] FIG. **13** shows the FMO controls for flow cytometric analysis of AHE at Day 5 of differentiation. Representative flow cytometry contour plots of AHE specification on day 5 of differentiation. FMO control for VEC, CD73, CD43, DLL4 and CXCR4 were used to determine the background.

[0024] FIG. **14** demonstrates that overexpression of SOX17 in hematopoietic progenitors generated from day 4 hemogenic endothelium enhances their myeloid and T lymphoid potentials. (A) Schematic diagram of experiments. (B) CFC potential of HP collected following culture of day 4+5+5. (C) Graphs show the total number of T cells produced from 10^4 hematopoietic progenitors (HPs) collected following culture of day 4+5+5. **p<0.01, ***p<0.001, and ****p<0.0001

DETAILED DESCRIPTION

[0025] The generation of functional hematopoietic stem cell (HSC)-like cells from pluripotent stem cells (PSCs) has been a long-sought goal in hematology research. Previous efforts to generate cells with myeloid and T cell hematopoietic potential from human pluripotent stem cells (hPSCs) have produced few, if any, cells capable of engrafting in irradiated mice.

[0026] Recent advances in understanding the major bottlenecks in the derivation of engraftable hematopoietic cells and definitive lympho-myeloid progenitors from PSCs have identified deficiencies in NOTCH and HOXA signaling as major contributing factors to the observed functional deficiencies. However, the hierarchy of molecular events that are critical to establishing these programs is still poorly understood. In the present application, the inventors identified the transcription factor SOX17 as a critical upstream activator of these pathways. As is described in the Examples, the inventors generated SOX17-knockout and SOX17-inducible human hPSCs, and using cell biology and molecular profiling, they show that SOX17 activates HOXA and arterial programs in hemogenic endothelium (HE) and establishes definitive lympho-myeloid hematopoiesis. The inventors further show that SOX17 produces these effects through activation of NOTCH and CDX2 signaling.

[0027] Based on these findings, the inventors have devised a novel strategy for producing superior HE that expresses HOXA cluster genes and has enhanced T lymphoid potential. Importantly, these methods enable enhanced T cell production, which could facilitate the development of off-the-shelf immunotherapies derived from hPSCs.

Methods:

[0028] The present invention provides methods of enhancing arterial specification of hemogenic endothelium in differentiating human pluripotent stem cells (hPSCs). The methods involve (a) introducing an inducible SOX17 transgene into a population of hPSCs; (b) culturing the hPSCs for at least two days under conditions to differentiate the hPSC into mesoderm cells; and (c) inducing expression of the SOX17 transgene in the mesoderm cells on at least about day two of differentiation, such that $DLL4^+CXCR4^+$ arterial hemogenic endothelium (AHE) cells are obtained.

[0029] As is described in the Examples, the inventors discovered that they could promote the arterial program and HOXA gene expression in hemogenic endothelium derived from hPSCs in vitro by overexpressing SOX17 at a specific time (i.e., starting at day 2) and for a defined period (i.e., 2-4 days) during differentiation. SOX17 is a member of the Sry-related high mobility group domain (SOX) family of transcription factors, and is key developmental regulator of endothelial and hematopoietic lineages. In the methods of the present invention, differentiating hPSCs are forced to overexpress SOX17 during the mesoderm differentiation by introducing an inducible SOX17 transgene into the population of mesodermal cells to facilitate formation of AHE with robust lymphomyeloid potential. In addition, inventors discovered that an SOX17 expands in suspension cultures lymphomyeloid progenitors generated from hemogenic endothelium. This method provided increased amounts of progenitors which could differentiate into lymphoid cells.

[0030] The SOX17 transgene used with the present invention may comprise any nucleic acid sequence encoding the SOX17 protein. For Example, the SOX17 transgene may be obtained by amplifying the SOX17 gene sequence from the genomic locus in human cells or by amplifying SOX17 mRNA from hPSCs differentiated into endothelial and blood cells and converting it into cDNA. Alternatively, genomic DNA or cDNA clones can be obtained commercially (e.g., from Sino Biological, Origene, IDT, etc.). In some embodiments, the transgene comprises SEQ ID NO:58, a cDNA sequence encoding the human SOX17 protein.

[0031] In some embodiments, the SOX17 transgene further comprises a vector sequence that can be used to drive the expression of the SOX17 transgene within the cells. In these embodiments, the transgene is introduced by into the population of hPSCs by transducing the cells with said vector. As used herein, the term "vector" refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression

vectors". Vectors suitable for use with the present invention comprise a nucleotide sequence encoding a SOX17 transgene and a heterogeneous sequence necessary for proper propagation of the vector and expression of the encoded polypeptide. The heterogeneous sequence (i.e., sequence from a different species than the transgene) can comprise a heterologous promoter or heterologous transcriptional regulatory region that allows for expression of the polypeptide. Suitable vectors for the expression of the SOX17 transgene include plasmids and viral vectors. In a preferred embodiment, the vector comprises heterologous sequence that allows the transient and/or inducible expression of the encoded SOX17 protein.

[0032] In some embodiments, the vector includes a transposase system, such as the PiggyBac transposon system (see Examples). The PiggyBac transposon is a TTAA-specific mobile genetic element that efficiently transposes between vectors and chromosomes via a "cut and paste" mechanism. PiggyBac transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) and moves the intervening contents to a TTAA insertion site in a chromosome or another vector. Thus, inserting a gene of interest between two ITRs in a transposon vector allows one to efficiently insert the gene into a target genome. Other suitable transposase systems for use with the present invention include, for example, Sleeping Beauty.

[0033] In other embodiments, the vector is a plasmid, a viral vector, a cosmids, or an artificial chromosome. Suitable plasmids include, for example, *E. coli* cloning vectors. Many suitable viral vectors are known in the art and include, but are not limited to, an adenovirus vector; an adeno-associated virus vector; a pox virus vector, such as a fowlpox virus vector; an alpha virus vector; a baculoviral vector; a herpes virus vector; a retrovirus vector, such as a lentivirus vector; a Modified Vaccinia virus Ankara vector; a Ross River virus vector; a Sindbis virus vector; a Semliki Forest virus vector; and a Venezuelan Equine Encephalitis virus vector. In one particular embodiment, the vector comprises SEQ ID NO:1.

[0034] In some embodiments, the vector is an expression vector that comprises a promoter that drives the expression of the SOX17 transgene, preferably transient or inducible expression of the SOX17 transgene. As used herein, the term "promoter" refers to a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a coding sequence. Although promoters are most commonly found immediately upstream of a coding sequence, they may also be found downstream of or within the coding sequence. Promoters may be derived in their entirety from a native gene or may be composed of multiple elements, including elements derived from promoters found in nature or elements comprising synthetic DNA sequences. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, at different stages of development, or in response to different environmental conditions. Preferably, the promoters used with the present invention are inducible promoters. An "inducible promoter" is a promoter that is activated (i.e., initiates transcription) only in the presence of a particular molecule. Inducible promoters allow tight control the expression of a transgene within cells. Many suitable inducible expression systems are known in the art and include, for example, Tet-On gene expression systems that allow one to induce the expression of a gene by administering tetracycline (Tc) or tetracycline-derivatives like doxycycline (DOX). Suitable Tet-On systems for use with the present invention include, without limitation, Tet-On Advanced and Tet-On 3G. Tet-On systems utilize several promoters, including both minimal promoters (e.g., CMV) flanked by a tetracycline response element (TRE) and engineered Tet-inducible promoters (e.g., TRE2 and TREtight). For instance, in the Examples, the SOX17 transgene is inserted (i.e., via conventional cloning methods) downstream of the doxycycline-inducible TREtight promoter within a vector. This vector was introduced into the hPSCs, allowing the inventors to induce expression of the SOX17 transgene at the desired stage of differentiation by adding doxycycline to the cell culture to activate expression from the TREtight promoter. Those of skill in the art are aware of many additional inducible gene expression systems, including both chemical-inducible and temperature-inducible systems. Other suitable inducible gene expression systems for use with the present invention include, without limitation, the glucocorticoid-responsive mouse mammary tumor virus promoter (MMTVprom), the tamoxifen-responsive hormone-binding domain of the estrogen receptor (ER^{TAM}), the ecdysone-inducible promoter (EcP), heat shock inducible promoters (e.g., Hsp70 or Hsp90-derived promoters), and the T7 promoter/T7 RNA polymerase system (T7P). The SOX17 transgene may be introduced into the hPSCs using any suitable method, for example by transfection or transduction. In one embodiment, the transgene is introduced by transducing the hPSCs with a vector comprising the SOX17 transgene. In another embodiment, the hPSCs are transduced with an exogenous SOX17 mRNA. In yet another embodiment, the hPSCs are transduced with the SOX17 protein.

[0035] In the present methods, the hPSCs are cultured under conditions for at least two days to differentiate these cells into mesoderm cells. Notably, mesoderm cells can be identified by their KDR^+ phenotype. Methods of differentiating hPSCs into progenitor mesoderm cells are known in the art. In one embodiment, mesoderm cells are obtained by culturing hPSCs in a chemically defined culture medium for about 2 days to about 4 days, whereby a cell population comprising mesoderm cells is obtained. For example, the hPSCs may be cultured in xenogen-free, serum-albumin free chemically defined medium comprising BMP4, activin A, LiCl, and FGF2, as described in Uenishi et al. (Stem cell reports (2014): 1073-1084) and U.S. Pat. No. 9,938,499, which is incorporated by reference in its entirety. For example, the chemically defined medium comprises about 10 ng/ml to about 50 ng/ml FGF2, about 50 ng/ml to about 250 mg/ml of BMP4 (e.g., 50 ng/ml to about 500 ng/ml BMP4), about 10 ng/ml to about 15 ng/ml Activin A, and about 1 to 2 mM LiCl under hypoxic conditions. In other embodiments, the cells are attached to a culture plate via extracellular matrix proteins. For example, in one embodiment, the cells are attached via collagena, fibronectin, matrigel™ or Tenascin C (TenC). In a preferred embodiment, the cells are cultured on plates coated with Collagen IV, as described in Uenishi et al. and U.S. Pat. No. 9,938.499.

[0036] The term "defined culture medium" is used herein to indicate that the identity and quantity of each medium ingredient is known. As used herein, the terms "chemically-defined culture conditions," "fully defined, growth factor free culture conditions," and "fully-defined conditions" indicate that the identity and quantity of each medium ingredient is known and the identity and quantity of supportive surface is known. As used herein the term "xenogen-free" refers to

medium that does not contain any products obtained from a non-human animal source. As used herein, the term "serum albumin-free" indicates that the culture medium used contains no added serum albumin in any form, including without limitation bovine serum albumin (BSA) or any form of recombinant albumin. Standardizing culture conditions by using a chemically defined culture medium minimizes the potential for lot-to-lot or batch-to-batch variations in materials to which the cells are exposed during cell culture. Accordingly, the effects of various differentiation factors are more predictable when added to cells and tissues cultured under chemically defined conditions. As used herein, the term "serum-free" refers to cell culture materials that do not contain serum or serum replacement, or that contain essentially no serum or serum replacement. For example, an essentially serum-free medium can contain less than about 1%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1% serum. "Serum free" also refers to culture components free of serum obtained from animal blood and of animal-derived materials, which reduces or eliminates the potential for cross-species viral or prion transmission. Further, serum-containing medium is not chemically defined, producing a degree of variability in the culture conditions. Suitable defined media include, but are not limited to, E8 medium.

[0037] Expression of the SOX17 transgene may be induced using any suitable methods known in the art. For example, in embodiments in which the transgene is introduced as a vector comprising an inducible promoter operably linked to the SOX17 transgene, transgene expression can be induced by contacting the cells with the signaling molecule required for activation of the chosen promoter. In embodiments in which the transgene is introduced as SOX17 mRNA or protein, induction is accomplished by transducing with cells with the mRNA or protein at the appropriate stage of hPSC differentiation.

[0038] In a preferred embodiment, expression of the SOX17 transgene is induced at about day 2 of hPSC differentiation, i.e., in the mesoderm stage of development. In embodiments in which the transgene is provided as a vector, induction may involve treating the cells with the signaling molecule required to activate an inducible promoter (e.g., DOX). Alternatively, one can induce SOX17 expression by transfecting the hPSCs with mRNA encoding SOX17 or with the SOX17 protein at about day 2 of differentiation. The inventors discovered that inducing SOX17 expression for two to four days (i.e., from day 2 to day 4-6 of differentiation) generated the greatest number of $CD43^+/CD45^+$ cells (FIG. **12**B-D), and produced cells with the greatest hematopoietic colony-forming potential. Thus, in preferred embodiments, the population of hPSCs expresses the SOX17 transgene for two to four days. In specific embodiments, the SOX17 transgene is expressed for at least two days, for at least three days, or for at least four days.

[0039] In another embodiment, SOX17 was upregulated in $CD43^+$ hematopoietic progenitors generated from hemogenic endothelium on day 9 of hematopoietic differentiation to further expand lymphomyeloid progenitors. This additional SOX17 upregulation resulted in an greater expansion of lymphomyeloid progenitors when compared to the method without such upregulation.

[0040] The human pluripotent stem cells (hPSCs) used with the present methods may be embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Many stem cells lines are commercially available. For example, the inventors utilized a H9 hESC (WA09) line from WiCell.

[0041] The goal of the present invention is to produce a population of arterial type hemogenic endothelial cells (AHEs) with broad lymphoid potential that are able to give rise to engraftable hematopoietic cells. These superior AHE cells can be identified as having the $DLL4^+CXCR4^+$ phenotype. CXCR4 is a HSC homing receptor that is not present in hematopoietic progenitors produced by traditional hESC differentiation methods, and DLL4 (Delta Like Canonical Notch Ligand 4) is a NOTCH-signaling ligand expressed by HE in vivo. In the Examples, the inventors show that by inducing SOX17 expression in differentiating hPSCs they produce a greater number of AHE cells with these desirable characteristics. Specifically, they found that SOX17 expression increases the production of AHE cells by about 50%.

[0042] As is demonstrated in the Examples, inducing the expression of SOX17 causes upregulation of genes encoding HOXA family members and arterial markers, which facilitates arterial specification of HE from mesoderm cells. In the present methods, hPSCs are cultured under conditions to differentiate the hPSC into mesoderm cells for about 4 days, and 24 hours later (on day 5) the HE begin to specify into $DLL4^+CXCR4^{+/-}$ arterial HE and non-arterial $DLL4^-CXCR4^-$ HE. Enhanced arterial specification may be detected at day 4 and 5 as (1) an increase in the percentage of $VEC^+CD73^-CD43^-$ HE cells generated, (2) an increase in the percentage of cells expressing DLL4 and CXCR4, which express increased levels of arterial marker genes (EFNB2, NOTCH4, HEY1, CXCR4, DLL4) and produce higher numbers of blood cells with colony forming cell (CFC) potential and/or T cell potential, as compared to the cells generated when SOX17 expression is not induced.

[0043] The inventors discovered that SOX17 expression enhances arterial specification via activation of several signaling pathways, including the Notch signaling pathways. Additionally, the inventors demonstrated that SOX17 is essential for the expression of HOXA genes in AHE. Thus, in some embodiments, the AHE cells produced by the methods disclosed herein express one or more HOXA genes selected from the group consisting of HOXA5, HOXA7, HOXA9, HOXA10, and HOX11. HOX genes have been implicated in hematopoiesis across species. For example, HOXA9 is the key homeotic gene that defines HSC identity, supporting HSC renewal during embryogenesis and stress hematopoiesis. HOXA5, HOXA9, and HOXA10 are transcriptional targets of Notch signaling in T cell progenitors, suggesting a role for these protein in T-lymphopoiesis. While SOX17 expression was previously known to be correlated with HOXA expression, it was not known to be causative. Thus, the inventors' discovery that SOX17 activates HOXA expression represents a significant advance in understanding the mechanisms of hematopoietic specification.

[0044] Further, the inventors have determined that SOX17 activates HOXA expression by binding to the promoter of CDX2 (a master regulator of HOX gene expression) and upregulating its expression. Thus, in some embodiments, the AHE cells produced by the methods disclosed herein express CDX2.

[0045] The AHE cells produced by the methods of the present invention have definitive lympho-myeloid potential and are, thus, useful for the production of myeloid and lymphoid progenitor cells. Those of skill in the art may

consult a standard cell differentiation protocol (e.g., Stem cell reports (2014): 1073-1084; Nature protocols (2011): 296-313; Stem cells and development (2011): 1639-1647; and Blood (2005): 617-626) to obtain cell populations of the desired hematopoietic cell type. For example, the AHE of the present invention may be further differentiated into T cells, beta-hemoglobin-producing red blood cells, megakaryocytic cells, and multipotential myeloid progenitors, including granulocyte, erythrocyte, megakaryocyte, macrophage (GEMM) and granulocyte-macrophage (GM) colony forming cells (CFCs), and mature myelomonocytic cells. In some embodiments of the present methods, the cells are further differentiated into lymphoid cell lines.

[0046] In the Examples, the inventors demonstrate that $DLL4^+CXCR4^+$ AHE cells produced by the present methods have increased T cell potential, i.e., they give rise to an increased number of T cells. Specifically, in DOX-treated conditions, $DLL4^+CXCR4^+$ AHE cells generate 54-fold more $CD4^+CD8^+$ T cells as compared to $DLL4^-CXCR4^-$ HE cells. Scalable T cell production is essential to advance iPSC-based immunotherapies into the clinic. Thus, in preferred embodiments, the cells are further differentiated into T cells. T cells may be differentiated using known methods, including the method disclosed in the Examples (see the Materials and Methods section titled "T cell differentiation"). The T cells can be identified as CD4+CD8+. In some embodiments, the T cells are identified as CD7+CD5+, CD8+CD4+, or a combination thereof (CD7+CD5+ and CD8+/CD4+).

[0047] Suitably, in one embodiment, the day 4 HE or day 5 AHE cells were cultured on OP9-DLL4 in presence of TPO (about 20-70 ng/ml, e.g., 50 ng/ml), SCF (about 20-70 ng/ml, e.g., 50 ng/ml), IL-6 (about 10-40 ng/ml, e.g., 20 ng/ml), IL-3 (about 5-30 ng/ml, e.g. 10 ng/ml) and FLT3L (3-20 ng/ml, e.g., 10 ng/ml) for about 4-5 days to produce hematopoietic progenitors enriched in T cell potential which were differentiated into T cells by culturing in the presence of delta-like ligand 4, e.g., cells overexpressing DLL4 (DLL4-expressing cells, e.g., OP9-DLL4 cells) for a sufficient time to produce T cells (e.g., at least 2 weeks, preferably at least three weeks or more). The media further comprise about 5-20 ng/ml SCF, about 3-20 ng/ml FLT3L and about 3-20 ng/ml IL-7 to augment T cell production. For example, in one embodiment, the AHE cells are passaged weekly onto fresh OP9-DLL4 cells in media comprising about 10 ng/ml SCF, about 5 ng/ml FLT3L and about 5 ng/ml IL-7 for about 3 weeks. Cells can be analyzed by flow cytometry for T cell surface markers.

[0048] In another embodiment, the hematopoietic progenitors obtained from AHE cells are cultured in media comprising 5-50 ng/ml SCF, about 3-20 ng/ml FLT3L and about 3-20 ng/ml IL7 in the presence of DLL4-Fc (commercially available), DLL4-Fc ligands or on DLL4-OP9 cells for a sufficient time to differentiate into T cells.

[0049] It was also surprisingly found that expansion of hemogenic progenitors (HP) which are subject to a second increased expression of SOX17 at day 4+5 through day 4+5+5 led to an increased expansion of myeloid and T lymphoid progenitors, as demonstrated in FIG. **14**, which allows for the differentiation of a significant increase in the production of CD4+CD8+ T cells.

[0050] Thus, in another embodiment, CD43+ hematopoietic progenitors generated from day 4 hemogenic endothelium in coculture with OP9 or OP9-DLL4 in medium comprising 20-70 ug/ml TPO and SCF (e.g., 50 ug/ml), 10-30 ng/ml IL6 (e.g., 20 ng/ml), and 5-25 ng/ml IL3 and FLT3L (e.g., 10 ng/ml) for 5 days, were collected and cultured in low attachment plate with IF9S media with 50-150 ng/ml FLT3L (e.g. 100 ng/ml) FLT3L, TPO and SCF, and about 10-40 ng/ml (e.g., 20 ng/ml) IL6, 3-20 ng/ml, e.g. 10 ng/ml IL3, with 2 μM Doxycyclin to induce expression of SOX17. As demonstrated in FIG. **14**, after 5 days, the cells were collected and demonstrated an enhanced myeloid CFC and T cell potentials. As demonstrated in Example 2, a second SOX17 overexpression in day 4+5 hematopoietic progenitors expanded the myeloid and T lymphoid progenitors and this expansion was more profound when day4 hemogenic endothelial cells were generated in presence of doxycycline (day 2-4 doxycycline treatment) and cultured on OP9-DLL4 (FIG. **14**).

[0051] T cells produced by the methods disclosed herein may be used for the production of various therapeutics, including chimeric antigen receptors (CAR) T cells. CAR T cells are T cells that have been genetically engineered to produce an artificial T cell receptor (i.e., a CAR) that allows them to target a specific protein of choice. CARs comprise an antigen-specific recognition domain that binds to specific target antigen or cell and a transmembrane domain linking the extracellular domain to an intracellular signaling domain. CAR T cells are commonly designed to recognize cancer cells (e.g., via recognition of an antigen that is present on the tumor surface) for use in cancer immunotherapies. Thus, in one embodiment, the methods of the present invention further comprise using the AHE to generate CAR expressing T cells that can be used to kill tumor cells. Methods of designing and producing CART cells are known in the art.

Compositions:

[0052] The present invention also encompasses cell populations produced by the methods disclosed herein. In some embodiments, the AHE cells are sorted from the cell culture, e.g., based on expression of DLL4 and CXCR4. The resulting hemogenic cell population will contain a SOX17 transgene, and may be at least 90%, 95% or 99% pure.

[0053] In another aspect, the present invention provides hPSC populations that comprise a SOX17 transgene and are capable of differentiating into $DLL4^+CXCR4^+$ arterial hemogenic endothelium (AHE) cells. In some embodiments, the hPSC cells comprise a vector comprising the SOX17 transgene. In some embodiments, said vector comprises an inducible promoter operably linked to the SOX17 transgene (e.g., the vector of SEQ ID NO:1 or a vector comprising SEQ ID NO:58), allowing for induction of SOX17 expression within the cells. Suitable vectors are discussed in the previous section.

[0054] Additionally, the present invention provides isolated in vitro populations of $DLL4^+CXCR4^+$ arterial hemogenic endothelium (AHE) cells differentiated from an hPSC population comprising a SOX17 transgene. As used herein, the phrase "isolated in vitro population" refers to a population of cells that is grown outside of a living organism (e.g., in a test tube, flask, or culture dish) under defined conditions.

[0055] These $DLL4^+CXCR4^+$ AHE cells may be further differentiated into T cells, forming an isolated in vitro T cell population. T cell differentiation may be accomplished using known methods, as discussed above. In some embodiments,

the T cell population comprises more than 90% $CD4^+CD8^+$ T cells, alternatively at least 95% $CD4^+CD8^+$ T cells.

[0056] In some embodiments, the AHE derived herein can produce hematopoietic progenitors (HP) with a high T cells potential. As used herein, the phrase "hematopoietic progenitors with high T cell potential" refers to a population of HE cells that is able to differentiate into T cells at least 10 fold more efficiently.

[0057] Further, in some embodiments, the present invention provides hematopoietic progenitors (HP) derived from the AHE that have high myeloid and T cell potential, as demonstrated in Example 2. These cells are differentiated by a method comprising at least two induction of SOX17 expression, leading to a more robust production of cells with myeloid and T cell potential as compared to without SOX17 expression.

[0058] As is discussed in the previous section, T cells are useful for the production of therapeutics, including, for example, chimeric antigen receptors (CAR) T cells. Thus, in some embodiments, the T cells are engineered to express an exogenous chimeric antigen receptor (CAR).

[0059] The following non-limiting examples are included for purposes of illustration only, and are not intended to limit the scope of the range of techniques and protocols in which the compositions and methods of the present invention may find utility, as will be appreciated by one of skill in the art and can be readily implemented. The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention.

EXAMPLES

Example 1

[0060] SOX17 has been implicated in arterial specification and the maintenance of hematopoietic stem cells (HSCs) in the murine embryo. However, knowledge about molecular pathways and stage-specific effects of SOX17 in humans remains limited. Here, using SOX17-knockout and SOX17-inducible human pluripotent stem cells (hPSCs), paired with molecular profiling studies, we reveal that SOX17 is a master regulator of HOXA and arterial programs in hemogenic endothelium (HE), and is required for the specification of HE with robust lympho-myeloid potential and $DLL4^+$ $CXCR4^+$ phenotype resembling arterial HE at sites of HSC emergence. Along with activation of NOTCH signaling, SOX17 directly activates CDX2 expression leading to the upregulation of the HOXA cluster genes. Since deficiencies in NOTCH signaling and HOXA regulation were identified as major contributing factors to the impaired engraftment potential of hPSC-derived hematopoietic cells, identification of SOX17 as a key regulator linking arterial and HOXA programs in HE may help to program the HSC fate from hPSCs.

Background:

[0061] Sox17 has been found to be expressed in the arterial vasculature (Liao et al., 2009) and the hemogenic endothelium (HE) in aorta-gonad-mesonephros (AGM) region (Clarke et al., 2013; Corada et al., 2013), in which it is required for arterial specification (Corada et al., 2013) and essential for establishing the definitive, but not primitive, hematopoietic program (Clarke et al., 2013) within the murine embryo. Although Sox17 actively prevents endothelial-to-hematopoietic transition (EHT) by repressing Runx1 (Lizama et al., 2015), Sox17 remains critical for maintaining intra-aortic hematopoietic clusters (IAHC) and fetal liver HSCs (Kim et al., 2007; Nobuhisa et al., 2014; Saito et al., 2018). Transduction of human embryonic stem cell (hESC)-derived $CD34^+$ HE/OP9 cocultures with a tamoxifen-inducible murine Sox17 transgene revealed that tamoxifen treatment expands $CD34^+CD43^+CD45^{-/low}$ cells co-expressing the endothelial marker VE-cadherin (VEC) (Nakajima-Takagi et al., 2013). Although these expanded cells possessed the capacity to form compact colonies in hematopoietic CFC medium with SCF, TPO and IL3, they were interpreted as HE cells. In mouse studies, the effects of Sox17 were attributed to the activation of the NOTCH signaling pathway by its direct binding to Dll4, Notch1 and Notch4 loci (Clarke et al., 2013; Corada et al., 2013). However, no activation of NOTCH pathway following Sox17 overexpression was observed during hESC differentiation (Nakajima-Takagi et al., 2013). While these studies established an important role of SOX17 in specification of definitive hematopoiesis and its diverse effects on EHT and HSCs, the molecular program induced by SOX17 at distinct stages of hematopoietic development, especially in humans, remains poorly understood.

[0062] To define the mechanisms of SOX17 action during specification and diversification of HE, we established SOX17-knockout and SOX17-inducible hESC lines and assessed their differentiation in a 2D chemically defined, feeder- and xeno-free human pluripotent stem cell (hPSC) differentiation system in which all stages of hematopoietic development are temporally, phenotypically, and functionally defined (Uenishi et al., 2014). In this study we specifically focused on the earliest stages of HE emergence and its arterial specification which have not been previously assessed. We reveal that SOX17 is required for the activation of HOXA expression and establishing arterial-type HE (AHE) with robust lympho-myeloid potential that can be identified by $DLL4^+CXCR4^+$ phenotype resembling AHE at sites of HSC emergence in vivo. Furthermore, the SOX17 effects are mediated by CDX2. These findings are important for understanding the molecular mechanisms controlling HE and definitive blood lineage development and designing strategies for specifying HSC fate from hPSCs.

Materials and Methods:

Generation Inducible SOX17 H9 ESC Line and SOX17 Knockout H9 ESC Line

[0063] We generated an inducible SOX17 cell line using the PiggyBac system (Park et al., 2018a). Human SOX17 CDS was cloned into PiggyBac transposon vector (Transposagen) downstream of TREtight promoter of pTRE-P2A-Venus-rpEF1a-Zeo plasmid, and cotransfected with pEF1α-M2rtTA-T2A-Puro and transposase plasmid into H9 hESCs using human stem cell nucleofector kit 2 (Lonza). Cells were selected in Zeocin (0.5 μg/ml, Thermofisher) and Puromycin (0.5 μg/ml, Sigma) for 10 days and resistant clones screened for Venus expression following DOX (Sigma) treatment. To generate $SOX17^{-/-}$ knockout H9 ESC line, two single guide RNAs were designed in CRISPR design tool (Synthego). The two sgRNA sequences are provided as SEQ ID NO:2

and SEQ ID NO:3. H9 ESCs were electroporated with the two sgRNAs and Cas9 protein (PNA Bio), and then plated at a low density on 6 well plate. After 7 days, individual colonies were picked and further expanded. After expansion, individual clones were screened by genomic PCR for the acquisition of 830 bp deletion in wildtype SOX17 allele using primers P1 and P2.

hESC Lines Maintenance and Hematopoietic Differentiation

[0064] hPSCs (H9 hESC (WA09) line from WiCell), iSOX17 H9 line and knockout SOX17 H9 line were maintained and passaged on Matrigel in mTeSR1 media (WiCell). The cell lines were differentiated on collagen IV (ColIV)-coated plate (Uenishi et al., 2014). Cell lines were plated at a density of 5,000 cells/cm^2 onto 6 well plates with E8 media containing 10 μM Rock inhibitor (Y-27632, Cayman Chemicals). The following day, the media was changed to IF9S media with 50 ng/ml FGF2 (PeproTech), 50 ng/ml BMP4 (PeproTech), 15 ng/ml Activin A (PeproTech), and 2 mM LiCl (Sigma), and cultured in hypoxia (5% CO_2, 5% O_2). On day 2, the media was changed to IF9S media with 50 ng/ml FGF2, 50 ng/ml VEGF (PeproTech), and 2.5 μM TGF-β inhibitor (SB-431542, Cayman), and cultured in hypoxia (5% CO_2, 5% O_2). On days 4 and 6, the media was changed to IF9S media with 50 ng/ml FGF2, 50 ng/ml VEGF, 50 ng/ml TPO (PeproTech), 50 ng/ml IL-6 (PeproTech), 20 ng/ml SCF (PeproTech), and 10 ng/ml IL-3 (PeproTech), and cultured in normoxia (20% CO_2, 5% O_2). DOX (Sigma) was added to cultures on day 2 of differentiation at concentration of 2 μg/ml.

Hemangioblast (HB)-CFC and Hematopoietic CFC Assay

[0065] HB-CFCs were detected using a semisolid colony-forming serum-free medium (CF-SFM) containing 40% ES-Cult M3120 methylcellulose (2.5% solution in IMDM, Stem Cell Technologies), 25% StemSpan serum-free expansion medium (SFEM, Stem Cell Technologies), 25% human endothelial serum-free medium (ESFM, ThermoFisher), 10% BIT 9500 supplement (Stem Cell Technologies), GlutaMAX (1/100 dilution, ThermoFisher), Ex-Cyte (1/1000 dilution, Millipore), 100 μM MTG, 50 μg/ml ascorbic acid and 20 ng/ml FGF (Peprotech) (Vodyanik et al., 2010). Hematopoietic CFCs were detected using serum containing H4435 MethoCult with FGF, SCF, IL-3, IL-6 and EPO (Stem Cell Technologies) following plating 1000 $CD43^+$ cells/dish in duplicates. CFCs numbers recalculated per 10^5 cells.

Isolation and Culture of D4 HE

[0066] Immature/primordial HE cells were isolated from knockout SOX17 or DOX+ and DOX− iSOX17 differentiation cultures by CD31 MACS (Miltenyi Biotec) at D4. Isolated cells were plated on OP9 or OP9-DLL4 in α-MEM (Gibco) with 10% FBS (Hyclone) with TPO, SCF (50 ng/ml), IL-6 (20 ng/ml), IL-3 and FLT3L (10 ng/ml; all from Peprotech). The media was changed 24 hours later, and extra media was added another 2 days later. After 5 days in secondary culture, cells were collected and assessed for CFC and T cell potential.

Isolation and Culture of D5 HE and Limiting Dilution Assay (LDA)

[0067] H9, iSOX17 and $SOX17^{-/-}$ ESCs were collected on day 5 of differentiation, singularized by 1× TrypLE, and stained for VEC (CD144), CD73, CD43, DLL4, CXCR4 with dead cells excluded using Ghost Dye Violet 540 (Tonbo Biosciences). FMO controls for flow cytometric analysis are shown in FIG. **13**. $VEC^+CD73^-CD43^-DLL4^-CXCR4^-$, $VEC^+CD73^-CD43^-$ $DLL4^+CXCR4^-$, $VEC^+CD73^-CD43^-$ $DLL4^+CXCR4^+$ and $VEC^+CD73^-CD43^-DLL4^-CXCR4^+$ subsets were isolated using a FACSAria II cell sorter (BD Biosciences) and MA900 cell sorter (Sony Biotechnology) and were plated on OP9 or OP9-DLL4 at 20,000 cells/well of a 12-well plate in α-MEM media with 10% FBS (Hyclone) with TPO, SCF (50 ng/ml), IL-6 (20 ng/ml), IL-3 and FLT3L (10 ng/ml). On the next day, the media was changed and extra media was added 2 days later. The floating $CD43^+$ cells were collected after 5 days of secondary culture and used for T cell and CFC assay. LDAs were conducted with sorted cells from day 5 differentiation cultures (no dox: $DLL4^+CXCR4^-$ HE and dox: $DLL4^+CXCR4^-$ HE or $DLL4^+CXCR4^+$ HE) following culture on DLL4-OP9 for 8 days. Row A of a 96-well plate received 3 cells/well, and each subsequent row afterward had twice the previous row (Row B contained 7, Row C contained 15 . . . Row H contained 500 cells). Eight days later, the cells were fixed and stained for immunofluorescent staining with anti-CD43 PE and DAPI in order to score the hematopoietic colonies using immunofluorescence microscopy. Extreme limiting dilution analysis was conducted using a previously established algorithm (Hu and Smyth, 2009).

[0068] Expansion of Hematopoietic Progenitors.

[0069] On day 4, HE cells were isolated from DOX+ or DOX− cultures by CD31 MACS. Isolated cells were plated on OP9 or OP9-DLL4 in α-MEM with 10% FBS with 50 ug/ml TPO and SCF, 20 ng/ml IL6, and 10 ng/ml IL3 and FLT3L. The media was changed 24 hours later, and extra media was added another 2 days later. After 5 days in in secondary culture, floating cell were collected and plated lin ow attachment 24 well plate. Cell were cultured with IF9S media with 100 ng/ml FLT3L, TPO and SCF, 20 ng/ml IL6, 10 ng/ml IL3, and with or without 2 μM Doxycyclin, and extra media was added another 2 days later. After 5 days, cells were collected and assessed for CFC and T cell potential.

T Cell Differentiation

[0070] Floating hematopoietic cells were collected from day 9 differentiation cultures or day 5 secondary OP9 or OP9-DLL4 cocultures (D4 HE+5 or D5 HE +5), and were cultured on OP9-DLL4 in α-MEM with 20% FBS, 10 ng/ml SCF, 5 ng/ml FLT3L and IL-7 (PeproTech) on OP9-DLL4 for 3 weeks. Cells were passaged weekly onto fresh OP9-DLL4 cells. Cells were analyzed by flow cytometry for T cell surface markers after 21 days.

DAPT Treatment and CDX2 Knockdown in Differentiation Cultures Using siRNA

[0071] Notch signaling was blocked by DAPT (?-secretase inhibitor, 10 μM, Cayman Chemical) added on day 3 of differentiation. For knockdown of CDX2 expression, DOX-treated iSOX17 cells were transfected with 100 nM CDX2 siRNA SMARTpool (Dharmacon) or Scramble negative control siRNA (Dharmacon) on day 3 of differentiation using Lipofectamine RNAiMAX (ThermoFisher). Next day, differentiation media was replaced with fresh media and cells were harvested at day 5 of differentiation.

Apoptosis and Cell Cycle Analysis

[0072] Apoptosis was detected by flow cytometry using Annexin V (BD). For cell-cycle analysis, D5 cells were incubated in culture medium with BrdU (10 μM, BD Pharmingen) for 2 hours and stained with antibodies. For BrdU detection, the BrdU flow kit with 7 AAD was used and performed per the manufacturer's instructions. Fluorescent reagents used for analysis, cell viability, apoptosis, and proliferation are listed in Table 1.

TABLE 1

Key resource information

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Annexin V-APC	BD Biosciences	Cat# 550474; RRID: AB_2868885
BrdU BV450 (clone; 3D4)	BD Biosciences	Cat# 560810; RRID: AB_2033930
CD4 APC (clone: RPA-T4)	BD Biosciences	Cat# 555349; RRID: AB_398593
CD5 APC (clone: UCHT2)	BD Biosciences	Cat# 555355; RRID: AB_398594
CD5 APC-Vio770 (clone: REA782)	Miltenyi Biotec	Cat# 130-111-110; RRID: AB_2658602
CD5 PE-Vio770 (clone: REA782)	Miltenyi Biotec	Cat# 130-111-109; RRID: AB_2658600
CD7 FITC (clone: M-T701)	BD Biosceinces	Cat# 555360; RRID: AB_395763
CD7 PE (clone: CD7-6B7)	Miltenyi Biotec	Cat# 130-123-247; RRID: AB_2802013
CD7 PE-Vio770 (clone: CD7-6B7)	Miltenyi Biotec	Cat# 130-105-901; RRID: AB_2659106
CD8 PE (clone: HIT8a)	BD Biosceinces	Cat# 555635; RRID: AB_395997
CD31 MicroBeads	Miltenyi Biotec	Cat# 130-091-935
CD41a APC (clone: HIP8)	BD Biosceinces	Cat# 559777; RRID: AB_398671
CD43 BV510 (clone: 1G10)	BD Biosceinces	Cat# 563377; RRID: AB_2722767
CD43 PE (clone: 1G10)	BD Biosceinces	Cat# 560199; RRID: AB_1645655
CD43 PE-Vio770 (clone: DF-T1)	Miltenyi Biotec	Cat# 130-099-763; RRID: AB_2658133
CD43 APC (clone: DF-T1)	Miltenyi Biotec	Cat# 130-097-367; RRID: AB_2658128
CD43 APC-Vio770 (clone: DF-T1)	Miltenyi Biotec	Cat# 130-101-174; RRID: AB_2658135
CD45 BV421 (clone: HI30)	BD Biosceinces	Cat# 563879; RRID: AB_2744402
CD73 APC (clone: AD2)	BD Biosceinces	Cat# 560847; RRID: AB_10612019
CD73 BV421 (clone: AD2)	BD Biosceinces	Cat# 562430, RRID: AB_11153119
CD73 PE (clone: AD2)	BD Biosceinces	Cat# 550257; RRID: AB_393561
CD73 PE-Vio770 (clone: AD2)	Miltenyi Biotec	Cat# 130-120-795; RRID: AB_2752200
CD144 APC (clone: REA199)	Miltenyi Biotec	Cat# 130-100-708; RRID: AB_2655155
CD144 BV421 (clone: 55-7H1)	BD Biosceinces	Cat# 565670; RRID: AB_2744284
CD144 BV605 (clone: 55-7H1)	BD Biosceinces	Cat# 743705; RRID: AB_2741685
CD144 FITC (clone: REA199)	Miltenyi Biotec	Cat# 130-100-742; RRID: AB_2655151
CD144 PE (clone: REA199)	Miltenyi Biotec	Cat# 130-118-495; RRID: AB_2751528
CD144 PE-Vio770 (clone: 12G5)	Miltenyi Biotec	Cat# 130-100-720; RRID: AB_2655158
CD184 APC (clone: 12G5)	BD Biosceinces	Cat# 555976; RRID: AB_398616
CD184 APC-Vio770 (clone: REA649)	Miltenyi Biotec	Cat# 130-116-521; RRID: AB_2727587
CD184 BV421 (clone: 12G5)	BD Biosceinces	Cat# 562448; RRID: AB_11153865

TABLE 1-continued

Key resource information		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD235a APC (clone: GA-R2 (HIR2))	BD Biosceinces	Cat# 551336; RRID: AB_398499
CD309 PE (clone: 89106)	BD Biosceinces	Cat# 560494; RRID: AB_1645503
CD309 PE-Vio770 (clone: REA1046)	Miltenyi Biotec	Cat# 130-117-986; RRID: AB_2733181
DLL4 APC (clone: MHD4-46)	Miltenyi Biotec	Cat# 130-096-560; RRID: AB_10827749
DLL4 PE (clone: MHD4-46)	Miltenyi Biotec	Cat# 130-096-567; RRID: AB_10831209
DLL4 PE-Vio770 (clone: MHD4-46)	Miltenyi Biotec	Cat# 130-101-587; RRID: AB_2651569
GAPDH	Santa Cruz Biotechnology	Cat# SC-25778; RRID: AB_10167668
Goat IgG	R&D	Cat# AB-108-C; RRID: AB_354267
Goat IgG HRP	Santa Cruz Biotechnology	Cat# SC-2354; RRID: AB_628490
H3K27ac	Millipore Sigma	Cat# 07-360; RRID: AB_310550
Mouse IgG HRP	Santa Cruz Biotechnology	Cat# SC-2005; RRID: AB_631736
SOX17	R&D	Cat# AF1924; RRID: AB_355060
7AAD	BD PharMingen	Cat# 559925; RRID: AB_2869266
Chemicals, peptides, And recombinant proteins		
Recombinant Human/Murine/Rat Activin A	PeproTech	Cat# 120-14E
Recombinant Human BMP4	PeproTech	Cat# 120-05ET
Recombinant Human FGF-basic	PeproTech	Cat# 100-18B
Recombinant Human VEGF165	PeproTech	Cat# 100-20
Recombinant Human IL-6	PeproTech	Cat# 200-06
Recombinant Human IL-3	PeproTech	Cat# 200-03
Recombinant Human SCF	PeproTech	Cat# 300-07
Recombinant Human TPO	PeproTech	Cat# 300-18
Recombinant Human Flt3-Ligand	PeproTech	Cat# 300-19
Recombinant Human IL-7	PeproTech	Cat# 200-07
LiC1	Sigma	Cat# L9659
SB431542 (TGF-13 inhibitor)	Cayman Chemical	Cat# 13031
Collagen IV	Sigma	Cat# C5533
Y-27632 (Rock inhibitor)	Cayman Chemical	Cat# 10005583
Doxycycline hyclate	Sigma	Cat# D9891
DAPI	Sigma	Cat# D8417
DAPT (γ-secretase inhibitor)	Cayman Chemical	Cat# 13197
Cas9 protein	PNA Bio	Cat# CP01
Lipofectamine RNAiMAX	ThermoFisher	Cat# 13778150

TABLE 1-continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Key resource information		
Critical commercial assays		
BrdU KIT	BD PharMingen	Cat# 559619; RRID: AB_2617060
Human stem cell nucleofector kit2	Lonza	Cat# VPH-5022
EZ-Magna ChIP AIG Chromatin Immunoprecipitation kit	Sigma	Cat# 17-10086
ATAC-SEQ	Active Motif	Cat# 25079
Deposited data		
RNA-seq, ATAC-seq, ChIP-seq	This study	GEO: GSE140341
Experimental models: cc/Hines		
WA09 (H9) human ES cell	WiCell	Cat# RB66492
pTRE-SOX17-P2A-Venus-rpEF1a-Zeo and pEF1α-M2rtTA-T2A-Puro (PiggyBac) H9 hESC line	This paper	N/A
SOX17-/- H9 hESC line	This paper	N/A
Oligonucleotides		
Primers of RT-qPCR, gPCR, and ChIP-PCR, See Table S1	This paper	N/A
sgRNA targeting seq. for SOX17 #1 GTTCATCGGCCGCCGGATAC (SEQ ID NO: 2)	Synthego	N/A
sgRNA targeting seq. for SOX17 #2 TTCACCTGCTTGCGCCGCCG (SEQ ID NO: 3)	Synthego	N/A
ON-TARGETplus human CDX2 siRNA SMARTpool	Dharmacon	Cat# L-015636-00-0010
ON-TARGETplus Non-targeting Control pool	Dharmacon	Cat# D-001810-10-05
Recombinant DNA		
pTRE-SOX17-P2A-Venus-rpEF1a-Zeo	This paper	N/A
pEF1α-M2rtTA-T2A-Puro	This paper	N/A
Super piggyBac transposase expression vector	Transposagen	Cat# SPB-DNA
Software and algorithms		
Prism versions 8	GraphPad Software Inc.	www.graphpad.com/scientific-software/prism/
FlowJo 8.8.6	FlowJo	www.flowjo.com
STAR (version 2.5.2b)	PMID: 23104886 (Dobin et al., 2013)	github.com/alexdobin/STAR
RSEM (version 1.3.0)	PMID: 21816040 (Li and Dewey, 2011)	deweylab.github.io/RSEM/
DESeq2 (version 1.22.2)	DESeq2 (version 1.22.2)	bioconductor.org/packages/release/bioc/html/DESeq2.html

TABLE 1-continued

Key resource information

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MEME (version 5.0.4)	PMID: 22610855 (Bailey and Machanick, 2012)	meme-suite.org
BWA (version 0.7.15)	PMID: 19451168 (Li and Durbin, 2009)	github.com/lh3/bwa
MACS2 (version 2.1.0)	PMID: 18798982 (Zhang et al., 2008)	github.com/macs3-project/MACS

Western Blot Assay

[0073] Cell extracts were prepared by adding IP Lysis buffer (ThermoFisher) with protease inhibitor cocktail (Sigma). Cell lysates (10 μg) were separated by Mini-protean TGX gels (Bio-rad). The separated proteins were transferred to a PVDF membrane, and were stained with antibodies for SOX17 (R&D) and GAPDH (Santa Cruz). Immunoblots were visualized using the ECL PLUS detection kit (Amersham Pharmacia) and analyzed using Chemi-Dox XRS+ Image Lab Software Version 5.2.1 (Bio-Rad).

Real-Time qPCR

[0074] RNA was extracted using the RNeasy Plus Micro Kit (Qiagen). RNA was reverse-transcribed into cDNA using random hexamer primers (Qiagen) with SMART MMLV reverse transcriptase (TaKaRa). qPCR was conducted using TB Green Advantage qPCR Premix (TaKaRa). RPL13A was used as the reference gene to normalize the data. Primer sequences are listed in Table 2.

TABLE 2

Primers used for RT-qPCR.00

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Purpose
ALDH1A2	TCTGTCCCTCTCTGCTTTCT (SEQ ID NO: 4)	CTGCCTGGCCTACATGTTATC (SEQ ID NO: 5)	qPCR
CDX2	CCGAACAGGGACTTGTTTAGAG (SEQ ID NO: 6)	AGGTTGGCTCTGGCATTTATAG (SEQ ID NO: 7)	qPCR
CXCR4	TCAGTGGCTGACCTCCTCTT (SEQ ID NO: 8)	CTTGGCCTTTGACTGTTGGT (SEQ ID NO: 9)	qPCR
DLL4	CAGTGGGCAGCGAAGCTACA (SEQ ID NO: 10)	ACAGGCAGTGGTAGCCATCCTC (SEQ ID NO: 11)	qPCR
EFNB2	CTCCTCAACTGTGCCAAACCA (SEQ ID NO: 12)	GGTTATCCAGGCCCTCCAAA (SEQ ID NO: 13)	qPCR
HEY1	GGACTATCGGAGTTTGGGATTT (SEQ ID NO: 14)	TGGGAAGCGTAGTTGTTGAG (SEQ ID NO: 15)	qPCR
HEY2	TTCAAGGCAGCTCGGTAACTGAC (SEQ ID NO: 16)	CATACTGATGCACTGCTGGATGG (SEQ ID NO: 17)	qPCR
HOXA1	CCAACAGAAACATGCCAGAAG (SEQ ID NO: 18)	CTCGCCTTTCGCTATATCCTAC (SEQ ID NO: 19)	qPCR
HOXA2	GATGCAGTTTCACCCAGTTTG (SEQ ID NO: 20)	GATTGTGGTGAGTGTGTCTGTA (SEQ ID NO: 21)	qPCR
HOXA3	GATGGAGCCATGGGAAGATTAC (SEQ ID NO: 22)	CATAGGGAGGAGGCTGAGATATAG (SEQ ID NO: 23)	qPCR
HOXA4	GGAGAAGGAGTTCCACTTCAAT (SEQ ID NO: 24)	GGTCTTTCTTCCACTTCATCCT (SEQ ID NO: 25)	qPCR
HOXA5	GTTCCTGTCTCAATAGCTCCAA (SEQ ID NO: 26)	GTGTCTCATCAAGTCACCTCTAC (SEQ ID NO: 27)	qPCR
HOXA6	AAAGCACTCCATGACGAAGG (SEQ ID NO: 28)	GTCTGGTAGCGCGTGTAGGT (SEQ ID NO: 29)	qPCR

TABLE 2-continued

Primers used for RT-qPCR.00

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Purpose
HOXA7	AGGTCCAGGATCAGGGTATT (SEQ ID NO: 30)	CCAGAGAAGGAGGGATTGATTC (SEQ ID NO: 31)	qPCR
HOXA9	GCGCCTTCTCTGAAAACAAT (SEQ ID NO: 32)	CAGTTCCAGGGTCTGGTGTT (SEQ ID NO: 33)	qPCR
HOXA10	GCAAAGAGTGGTCGGAAGAA (SEQ ID NO: 34)	CGCTCTCGAGTAAGGTACATATTG (SEQ ID NO: 35)	qPCR
HOXA11	TGGTCTGGGACTCTCTTGAT (SEQ ID NO: 36)	GGTCCCAAACCTGTCATTCT (SEQ ID NO: 37)	qPCR
HOXA13	CTGGAACGGCCAAATGTACT (SEQ ID NO: 38)	GCTTCTTTCTCCCCCTCCTA (SEQ ID NO: 39)	qPCR
LFNG	GCAACGTGGTCATCACAAAC (SEQ ID NO: 40)	CTCGATGAAGCGGTCATACTC (SEQ ID NO: 41)	qPCR
NOTCH1	CAATGTGGATGCCGCAGTTGTG (SEQ ID NO: 42)	CAGCACCTTGGCGGTCTCGTA (SEQ ID NO: 43)	qPCR
NOTCH4	AGCTCTGGAAAGAGGGTTTAAG (SEQ ID NO: 44)	CTCCTGTGGCCTGTCTTATTT (SEQ ID NO: 45)	qPCR
NR2F2	TGGTTCCAAACCAGTTTATTCTGT (SEQ ID NO: 46)	AAGTGCGTTTCCATCATCTTTGAG (SEQ ID NO: 47)	qPCR
RARγ	ATGACCGGAACAAGAAGAAGAA (SEQ ID NO: 48)	TTGCTGACCTTGGTGATGAG (SEQ ID NO: 49)	qPCR
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA (SEQ ID NO: 50)	TTGAGGACCTCTGTGTATTTGTCAA (SEQ ID NO: 51)	qPCR
SOX17	GCCAAGGGCGAGTCCCGTA (SEQ ID NO: 52)	GCATCTTGCTCAACTCGGCGTTGTGCA (SEQ ID NO: 53)	qPCR
P1/P2	GGGTACGCTGTAGACCAGAC (SEQ ID NO: 54)	TTCAGCCGCTTCACCTGCTT (SEQ ID NO: 55)	Genomic PCR
CDX2 promoter	ACTCCCAAAGCAGTTGGATG (SEQ ID NO: 56)	GCTTTTTTATGGCCCAGGCTG (SEQ ID NO: 57)	ChIP-PCR

RNA-Seq

[0075] One hundred nanograms of total RNA was used to prepare sequencing libraries using the Ligation Mediated Sequencing (LM-Seq) protocol, according to the paper guidelines (Hou et al., 2015) and quantified with the Qubit fluorometer (ThermoFisher). Final cDNA libraries were quantitated with the Qubit Fluorometer (ThermoFisher), multiplexed, loaded at a final concentration of 2.5 nM, and sequenced as single reads on the Illumina HiSeq 3000 (Illumina).

ChIP-seq

[0076] Chromatin immunoprecipitation (ChIP) analysis of day 4 HE was performed, as described in the protocol included in the EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore Sigma). Five nanograms of IP or control DNA was used to prepare sequencing libraries using the TruSeq ChIP Sample Preparation Kit (Illumina) as per the manufacturer instructions and quantified with the Qubit fluorometer (Life Technologies). All six TruSeq ChIP indexed samples were pooled per lane, loaded at a final concentration of 2.5 nM, and sequenced as single reads on the Illumina HiSeq 3000 (Illumina).

ATAC-seq

[0077] Day 4 HE cells were harvested and frozen in culture media containing FBS and 5% DMSO. Cryopreserved cells were sent to Active Motif to perform the ATAC-seq assay. The cells were then thawed in a 37° C. water bath, pelleted, washed with cold PBS, and tagmented as previously described (Buenrostro et al., 2013). Briefly, cell pellets were resuspended in lysis buffer, pelleted, and tagmented using the enzyme and buffer provided in the Nextera Library Prep Kit (Illumina). Tagmented DNA was then purified using the MinElute PCR purification kit (Qiagen), amplified with 10 cycles of PCR, and purified using Agencourt AMPure SPRI beads (Beckman Coulter). Resulting material was quantified using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems), and sequenced with PE42 sequencing on the NextSeq 500 sequencer (Illumina).

RNA-Seq Analysis

[0078] RNA-seq analyses were performed on three biological replicates in DOX− and DOX+ conditions. Sequencing fragments were aligned by STAR (version 2.5.2b) to human genome (hg38) with gene annotations from GEN-

CODE (version 27). Transcript expression levels were quantified by RSEM (version 1.3.0) and differentially expression analysis was performed by DESeq2 (version 1.22.2). KEGG gene sets were defined by MSigDB (version 6.1).

ATAC-Seq Analysis

[0079] ATAC-seq analyses were performed on two biological replicates in DOX− and DOX+ conditions. Sequencing fragments were pre-processed by the company Active Motif, Inc. Briefly, ATAC-seq reads were mapped to the human genome by BWA with default settings. Only reads that passed Illumina's purity filter, aligned with no more than 2 mismatches, and mapped uniquely to human genome were used in the subsequent analysis. Duplicate reads ("PCR duplicates") were removed. To calculate signals, human genome was divided into 32 bp bins and the number of reads in each bin was counted. In order to smooth the data, reads were extended to 200 bp. To normalize signals across ATAC-seq datasets, the number of reads in each dataset was reduced by random sampling to the smallest number of reads present in the datasets.

[0080] ATAC-seq peaks were called by Active Motif, Inc using MACS2. For DOX− or DOX+ condition, we defined condition-specific peaks by selecting those existing in both ATAC-seq replicates of that condition and not overlapping with any peak from the two replicates of the other condition. From condition-specific peaks, we identified 'promoter peaks' by choosing those overlapped with protein-coding transcript's 5 kb upstream region and do not overlap with any intron or exon. DNA sequences for the 250 bp flanking regions to the center of promoter peaks were prepared for motif enrichment analysis. Motif enrichment was performed by MEME (version 5.0.4)'s CentriMo function with default settings based on motifs from HOCOMOCO human database (version 11).

[0081] ATAC-seq signals were calculated for gene's promoter region, which was defined as the 5 kb region upstream of its transcription start site (TSS). If a gene encoded for multiple transcripts, the most upstream TSS will be used as this gene's TSS. A gene's ATAC-seq promoter signal change upon DOX+ activation was computed by taking the difference of averaged signals from the two ATAC-seq replicates under DOX− or DOX+ condition. The top 5% genes that have the largest increase of ATAC-seq promoter signals were collected for GO term analysis by the Bioconductor package limma's function goana. P-values were adjusted by Benjamini & Hochberg method.

ChIP-seq Analysis

[0082] SOX17 and IgG control ChIP-seq fragments from were aligned by BWA (version 0.7.15) with a quality threshold at 5 for read trimming and all the other options in default settings. Normalized SOX17 ChIP-seq signals were calculated by MACS2 by using all the tags at the same loci. SOX17's fold enrichment over IgG control were calculated by MACS2 using all default options.

Statistical Analysis

[0083] Experiments were analyzed using GraphPad Prism versions 8 (GraphPad Software Inc.) and Microsoft Excel (Microsoft Corporation). Tests for statistical significance are listed with each experiment and included two-sided Student's t-test for paired analyses and one-way ANOVA, and two-way ANOVA for experiments with multiple comparisons of or grouped variables, accompanied by Tukey and Sidak post hoc tests indicated as appropriate by the software. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. All error bars represent the mean±SD and duplicated or triplicated independent experiments.

Results:

[0084] SOX17 Knockout Impairs AHE Specification and Definitive Lympho-Myeloid Hematopoiesis from hPSCs

[0085] To assess the effect of SOX17 on hematopoietic development, we generated SOX17 knockout H9 human embryonic stem cell (hESC; $SOX17^{-/-}$) lines using CRISPR/Cas9 (FIG. **8**A-C) and differentiated them into endothelial and hematopoietic cells in a chemically defined culture system (Uenishi et al., 2014). In this differentiation system, the primitive hematopoietic progenitors (HPs) with FGF2-dependent hemangioblast colony-forming cell (HB-CFCs) potential are detected on day 3 (D3) of differentiation (Choi et al., 2012; Uenishi et al., 2014; Vodyanik et al., 2010). The first immature/primordial $VEC^{+}CD43^{-}CD73^{-}NOTCH1^{+}$ HE cells expressing high levels of HAND1 mesodermal gene arise on D4 (D4 HE) and subsequently specify into $DLL4^{+}CXCR4^{+/-}$ arterial-type HE (AHE) with definitive lympho-myeloid potential and $DLL4^{-}$ non-arterial-type HE (non-AHE) with myeloid-restricted potential on D5 (FIG. **1**A) (Choi et al., 2012; Park et al., 2018b; Uenishi et al., 2014; Uenishi et al., 2018). As shown in FIG. **1**B, SOX17 knockout significantly increased frequencies of HB-CFCs on D3, suggesting that SOX17 deficiency promotes primitive hematopoiesis. Evaluation of D4 cultures revealed that SOX17 knockout had minimal effect on D4 HE, however analysis of D5 cultures showed significantly impaired specification of $DLL4^{+}CXCR4^{+}$ AHE when compared to wild-type H9 hESCs (FIG. **1**C-E).

[0086] To assess the hematopoietic potential of $SOX17^{-/-}$ and $SOX17^{+/+}$ HE cells and their dependence on NOTCH signaling, we isolated D4 HE cells and co-cultured them on OP9 or OP9-DLL4 (FIG. **1**F.). We found that $SOX17^{-/-}$ D4 HE had significantly reduced myeloid (FIG. **1**G) and no T lymphoid potential (FIG. **1**H-I). Importantly, there was no change in hematopoietic potential of D4 $SOX17^{-/-}$ HE between coculture on OP9 and OP9-DLL4 (FIG. **1**G), indicating that $SOX17^{-/-}$ cells lack NOTCH-dependent hematopoietic potential. Similarly, hematopoietic cells collected on D9 from $SOX17^{-/-}$ hESCs demonstrated decreased myeloid CFC and T lymphoid potential along with a decreased $CD43^{+}$ cells and proportion of $CD45^{+}$ cells within the $CD43^{+}$ population, as compared to wild-type cells (FIG. **8**D-H). These effects on HE and T cells from SOX17 deficiency were confirmed using two different SOX17 knockout clones (FIG. **8**I-J). The lack of NOTCH sensitivity and abrogation of T lymphoid potential suggests that $SOX17^{-/-}$ HE cells do not possess definitive lympho-myeloid potential (Hadland et al., 2004; Kennedy et al., 2012; Kumano et al., 2003; Robert-Moreno et al., 2005).

[0087] Overall, these results demonstrate the critical role of SOX17 in the specification of definitive lympho-myeloid hematopoiesis and $DLL4^{+}CXCR4^{+}$ AHE from hPSCs. Although both, DLL4 and CXCR4, are considered markers of AHE (Chong et al., 2011; Yamamizu et al., 2010), DLL4 expression has been found in arterial vessels of yolk sac and aorta (Duarte et al., 2004; Herman et al., 2018; Robert-Moreno et al., 2005), while CXCR4 expression was detected

in aorta and vitelline/umbilical arteries (McGrath et al., 1999; Werner et al., 2020), i.e. vasculature harboring precursors capable of maturing into definitive HSCs (Dzierzak and Medvinsky, 1995; Gordon-Keylock et al., 2013), but not in yolk sac (McGrath et al., 1999; Venkatesh et al., 2008; Werner et al., 2020). Thus, SOX17 is the most essential factor for the formation of AHE with the CXCR4$^+$ phenotype typical of HE with HSC potential in vivo.

SOX17 Induction at the Mesodermal Stage Enhances AHE Specification and Definitive Lympho-Myeloid Hematopoiesis

[0088] To further characterize the role of SOX17 during hematoendothelial development, we engineered an H9 hESC line with a transgene cassette that expresses SOX17 upon treatment with doxycycline (DOX; iSOX17-hESCs; FIG. **9**). We then differentiated inducible SOX17 (iSOX17) cells with or without DOX, starting from the mesodermal stage of development (D2) through D4, to analyze the effect on specification of HB-CFCs (D3) and D4 HE specification (FIG. **2**A). As shown in FIG. **2**B, SOX17 induction significantly reduced the numbers of primitive HPs on D3, as evidenced by the decrease in HB-CFCs. This observation is consistent with murine studies that demonstrated suppression of primitive hematopoiesis by Sox17 (Serrano et al., 2010). Concordantly, SOX17 induction increased production of VEC$^+$ HE on D4, along with early upregulation of DLL4 and CXCR4 when HE cells are typically DLL4$^-$ CXCR4$^-$ (Park et al., 2018b; Uenishi et al., 2018), as seen in No DOX cultures (FIG. **2**C). Assessment of hematopoietic potential from D4 HE revealed that blood cells collected from DOX+ conditions had higher T cell potentials from both HE/OP9 and HE/OP9-DLL4 cocultures, as compared to D4 HE collected from DOX− conditions (FIG. **2**F-G). In addition, D4 HE from DOX+ cultures demonstrated more robust multipotent myeloid and lymphoid hematopoiesis following exposure to DLL4 NOTCH ligand (OP9 vs OP9-DLL4 hematopoiesis), while these OP9 vs OP9-DLL4 differences in DOX− D4 HE cocultures were more modest.

[0089] As we previously demonstrated, D4 HE specifies 24 hours later (D5) into DLL4$^+$CXCR4$^{+/-}$ arterial HE and non-arterial DLL4$^-$CXCR4$^-$ HE (Park et al., 2018b; Uenishi et al., 2018). Assessment of HE phenotype on D5 (FIG. **3**A) reveals that SOX17 upregulation significantly increased the percentage of VECCD73$^-$CD43$^-$ HE cells, and expression of arterial markers, DLL4 and CXCR4, within this population, predominantly increasing the DLL4$^+$CXCR4$^+$ HE population (FIG. **3**B-D). In addition, we note that DOX treatment also induced the DLL4$^-$CXCR4$^+$ subpopulation within phenotypical (VECCD73$^-$CD43$^-$) HE subset (FIG. **3**B). Studies of SOX17 in mice have shown that this factor controls proliferation of hematopoietic and endothelial cells (Clarke et al., 2013; Kim et al., 2007; Liu et al., 2019; Nobuhisa et al., 2014; Serrano et al., 2010). Examination of the proliferative potential of HE subsets using BrdU and cell cycle analysis, revealed that SOX17 overexpression led to a significant cell cycle shift from GO/G1 to S and G2/M phases in CXCR4$^-$ and CXCR4$^+$ AHE subsets (FIG. **10**A-B). In contrast, a significant decrease in cycling cells was observed in SOX17$^{-/-}$ DLL4$^+$ populations. Although similar to mouse studies (Serrano et al., 2010), we found that DOX treatment increased apoptosis along with increased proliferation of AHE (FIG. **10**C-D), the total output of viable AHE cells in DOX+ conditions was significantly higher as compared to DOX− conditions (FIG. **3**C-D). Limiting dilution analysis of the hematopoietic potential of AHE subsets revealed that following DOX treatment, the frequency of hemogenic cells was the highest in DLL4$^+$CXCR4$^-$ population while their frequency in the DLL4$^+$CXCR4$^+$ subset was 20-fold lower. In addition, we found that DOX treatment increased the frequency of hemogenic cells in DLL4$^+$ CXCR4$^-$ population by nearly 7-fold as compared to DOX− conditions (FIG. **3**E). To assess the hemogenic potential of four D5 HE subsets (DLL4$^+$CXCR4$^+$, DLL4$^+$CXCR4$^-$, DLL4$^-$CXCR$^-$ DLL4$^-$CXCR4$^+$), each subpopulation was isolated by FACS cell sorting and analyzed for CFC and T cell potential following culture on OP9-DLL4 (FIG. **3**A). In our prior studies, we found that coculture with OP9-DLL4 is essential to induce EHT from AHE (Uenishi et al., 2018). Consistent with our prior observations (Park et al., 2018b), the DLL4$^+$CXCR4$^+$ HE subset had the most robust CFC and T cell potentials (FIG. **3**F-G), despite having the lowest frequency of hemogenic cells as found in the limiting dilution assay (FIG. **3**E). In contrast, CXCR4$^+$DLL4$^-$ HE cells possessed very limited myeloid potential and were completely devoid of T cell potential. Thus, we concluded that SOX17-induced CXCR4$^+$DLL4$^-$ cells are mostly non-hemogenic or primitive hemogenic progenitors and excluded them from further analysis.

[0090] Collectively, these studies suggest that SOX17 upregulation promotes definitive lympho-myeloid hematopoiesis from hPSCs through enhancement of AHE specification with CXCR4$^+$DLL4$^+$ phenotype, typical for HE at sites of HSC emergence.

SOX17 Overexpression Activates NOTCH-Mediated Arterial Program and Expression of HOXA Cluster Genes in HE

[0091] To understand the molecular mechanisms of the effect of SOX17, we performed molecular profiling of D4 HE from DOX+ and DOX− cultures using RNA-seq and ATAC-seq. To analyze chromatin binding of SOX17 by ChIP-seq, we used DOX+ cultures because under DOX− conditions, SOX17 expression was absent in D4 HE, i.e., before AHE was formed (Uenishi et al., 2018) (FIG. **11**). Gene set enrichment analysis (GSEA) of RNA-seq data revealed enrichment in KEGG categories related to dorsoventral axis formation: NOTCH, TGFβ, Hedgehog and WNT signaling pathways with downregulation of multiple metabolic pathways in DOX+ as compared to DOX− (FIG. **4**A and Table 3). The upregulated genes included HOX genes, HOXA genes (HOXA7, HOXA9, HOXA10) and CDX2, as well as key molecules in NOTCH (DLL1, DLL4, NOTCH4) and WNT signaling (WNT5A, WNT5B). In addition, we noted upregulation of molecules associated with retinoic acid signaling (ALDH1A2 and RARG) and molecules associated with HSC development (EMCN, ROBO4, KITLG) (FIG. **4**B). Upregulation of these genes was confirmed by qPCR (FIG. **11**).

TABLE 3

Enriched KEGG terms from day 4 HE RNAseq

Gene set name	Up-regulated in	Adjusted p-value
KEGG_AXON_GUIDANCE	DOX+	2.10837179648431e−8
KEGG_CELL_ADHE_SION_MOLECULES_CAMS	DOX+	2.10837179648431e−8
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE	DOX+	2.10837179648431e−8
KEGG_RIBOSOME	DOX−	6.73770384348161e−8
KEGG_ECM_RECEPTOR_INTERACTION	DOX+	1.00763E−06
KEGG_BASAL_CELL_CARCINOMA	DOX+	4.48645E−06
KEGG_GLYCO_SAMINOGLYCAN_BIOSYNTHESIS_HEPARAN_SULFATE	DOX+	5.33193E−05
KEGG_HEDGEHOG_SIGNALING_PATHWAY	DOX+	0.000133684
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	DOX−	0.000276991
KEGG_FOCAL_ADHESION	DOX+	0.00051598
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	DOX−	0.001401729
KEGG_PROPANOAIL_METABOLISM	DOX−	0.002156859
KEGG_MELANOGENESIS	DOX+	0.002156859
KEGG_GAPJUNCTION	DOX+	0.002241927
KEGG_GLYCO_SPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	DOX+	0.00238555
KEGG_OXIDATIVE_PHOSPHORYLATION	DOX−	0.003148941
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	DOX+	0.003281063
KEGG_RNA_DEGRADATION	DOX−	0.003281063
KEGG_TGF_BETA_SIGNALING_PATHWAY	DOX+	0.003288519
KEGG_SPLICEOSOME	DOX−	0.003288519
KEGG_MELANOMA	DOX+	0.003288519
KEGG_PATHWAYS_IN_CANCER	DOX+	0.00460277
KEGG_RNA_POLYMERASE	DOX−	0.00460277
KEGG_PEROXISOME	DOX−	0.006024659
KEGG_PYRUVAIL_METABOLISM	DOX−	0.006685177
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	DOX−	0.008549323
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	DOX−	0.009118394
KEGG_PROSTATE_CANCER	DOX+	0.010329457
KEGG_NOTCH_SIGNALING_PATHWAY	DOX+	0.010329457
KEGG_ONE_CARBON_POOL_BY_FOLATE	DOX−	0.011068986
KEGG_HUNTINGTONS_DISEASE	DOX−	0.011068986
KEGG_DORSO_VENTRAL_AXIS_FORMATION	DOX+	0.011068986
KEGG_DILATED_CARDIOMYOPATHY	DOX+	0.011676678
KEGG_CITRATE_CYCLEJCA_CYCLE	DOX−	0.012849034
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_BIOSYNTHESIS	DOX−	0.027921813
KEGG_ENDOMETRIAL_CANCER	DOX+	0.031692773
KEGG_GNRH_SIGNALING_PATHWAY	DOX+	0.037610248
KEGG_WNT_SIGNALING_PATHWAY	DOX+	0.038958319
KEGG_GLYCO_SPHINGOLIPID_BIOSYNTHESIS_GANGLIO_SERIES	DOX+	0.039893448
KEGG_RENAL_CELL_CARCINOMA	DOX+	0.047099844

[0092] ATAC-seq analysis of D4 HE isolated from DOX-treated cultures identified 93,615 and 100,036 open chromatin regions in the two ATAC-seq replicates, respectively, of which 5,130 of which were specific to DOX+ conditions. Gene Ontology (GO) analysis of genes with increased ATAC-seq counts at promoters upon DOX treatment revealed enrichment in categories associated with development and morphogenesis, including blood vessel morphogenesis (Table 4), suggesting that SOX17 facilitates the establishment of gene regulatory networks essential for early morphogenesis, including vascular development. Motif-enrichment analysis of ATAC-seq peaks at promoters in DOX+ and DOX− conditions revealed enrichment in ETS-binding motifs for both conditions, consistent with the endothelial nature of the analyzed cells. However, in DOX+ conditions we observed a unique enrichment in retinoic receptor alpha (RARA) and estrogen receptor 2 (ESR2) motifs at open chromatin regions (FIG. **4**C, Table 5).

TABLE 4

Top 5% of genes with ATAC-seq signals increased at promoters upon DOX induction

GO term	Adjusted p-value
anatomical structure morphogenesis	1.78003407755937e−7
nervous system development	2.85807041113842e−7
neurogenesis	4.58194E−06
central nervous system development	8.16944E−06
tube morphogenesis	2.44821E−05
system development	2.44821E−05
generation of neurons	2.92474E−05
tube development	0.000110693
multicellular organism development	0.000110693
regulation of nervous system development	0.000161455
regulation of multicellular organismal development	0.000161455
anatomical structure development	0.000185597
neuron differentiation	0.000185597
locomotion	0.000185597
regulation of anatomical structure morphogenesis	0.000185597
cell migration	0.000246498

TABLE 4-continued

Top 5% of genes with ATAC-seq signals increased at promoters upon DOX induction

GO term	Adjusted p-value
regulation of developmental process	0.000342946
head development	0.000342946
positive regulation of developmental process	0.000465449
blood vessel morphogenesis	0.000758105
neuron development	0.000758105
adenylate cyclase-modulating G protein-coupled receptor signaling pathway	0.000761229
neuron projection development	0.000761229
vasculature development	0.000865103
anatomical structure formation involved in morphogenesis	0.000920372
neuron part	0.001038012
developmental process	0.001038012
angiogenesis	0.00105143
cardiovascular system development	0.00105143
embryonic morphogenesis	0.001067191
basement membrane	0.001075712
brain development	0.001301484
cellular developmental process	0.001476637
animal organ morphogenesis	0.001521223
neuron projection	0.001521223
cell motility	0.001521223
localization of cell	0.001521223
blood vessel development	0.001567922
intrinsic component of plasma membrane	0.001593111
regulation of cell development	0.001593111
regulation of neurogenesis	0.001610939
animal organ development	0.001714315
multicellular organismal process	0.001735658
G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	0.002324971
integral component of plasma membrane	0.002408441
circulatory system development	0.002859264
regulation of multicellular organismal process	0.003115049
cell development	0.003234361
axon development	0.003618464
cell differentiation	0.003626835
embryonic organ development	0.003626835
cell part morphogenesis	0.006182665
embryo development	0.007461113
signaling receptor binding	0.008843617
synapse	0.008843617
movement of cell or subcellular component	0.009048212
cellular component morphogenesis	0.009048212
tumor necrosis factor-activated receptor activity	0.009048212
renal system development	0.009737108
tissue morphogenesis	0.009737108
axonogenesis	0.01004697
regulation of neuron differentiation	0.010178993
positive regulation of cell development	0.010271038
axon	0.010271038
adenylate cyclase-inhibiting G protein-coupled receptor signaling pathway	0.010271038
regulation of cell differentiation	0.011531827
positive regulation of nervous system development	0.012459588
ear development	0.012623805
positive regulation of cell differentiation	0.013451981
metanephros morphogenesis	0.015724879
synaptic membrane adhesion	0.015740799
ear morphogenesis	0.015896
inner ear morphogenesis	0.016315176
cell morphogenesis involved in neuron differentiation	0.017844159
neuron projection morphogenesis	0.020119928
chondrocyte differentiation	0.020119928
plasma membrane bounded cell projection morphogenesis	0.020119928
positive regulation of epithelial cell migration	0.020320479
system process	0.020320479
cell morphogenesis	0.020320479
collagen-containing extracellular matrix	0.020320479
inner ear development	0.02054135

TABLE 4-continued

Top 5% of genes with ATAC-seq signals increased at promoters upon DOX induction

GO term	Adjusted p-value
positive regulation of protein phosphorylation	0.02187698
cell projection morphogenesis	0.02187698
positive regulation of multicellular organismal process	0.02187698
second-messenger-mediated signaling	0.02187698
sensory organ development	0.022685916
positive regulation of neurogenesis	0.022685916
nephron development	0.023439655
chemotaxis	0.023462666
death receptor activity	0.023660162
regulation of system process	0.024178531
cAMP-mediated signaling	0.024615989
taxis	0.024615989
positive regulation of cell proliferation	0.0260098
sensory organ morphogenesis	0.0260098
cell proliferation	0.028080528
positive regulation of cell migration	0.029165326
negative regulation of apoptotic process	0.032275505
cell morphogenesis involved in differentiation	0.03348346
growth factor activity	0.03348346
extracellular matrix	0.033910658
dichotomous subdivision of terminal units involved in lung branching	0.035191974
extracellular matrix organization	0.036705001
extracellular structure organization	0.036705001
intrinsic component of membrane	0.036875421
cell communication	0.038652168
kidney morphogenesis	0.039463928
dopamine transport	0.040553679
regulation of neuron projection development	0.041435175
positive regulation of locomotion	0.042212449
metal ion transmembrane transporter activity	0.042443184
regulation of developmental growth	0.042443184
urogenital system development	0.042443184
regulation of cell migration	0.044587154
regulation of cell proliferation	0.045698414
positive regulation of phosphorylation	0.048504286
receptor tyrosine kinase binding	0.049864381

TABLE 5

Enriched motifs in condition-specific ATAC-seq promoter peaks

Motif	Condition	Adjusted p-value
ETS1	DOX+	0.0000032
ETV1	DOX+	0.00022
ELK4	DOX+	0.00023
ETV2	DOX+	0.00043
ELF1	DOX+	0.0009
ELK1	DOX+	0.002
ERG	DOX+	0.0026
GABPA	DOX+	0.0047
ESR2	DOX+	0.012
ETV4	DOX+	0.018
RARA	DOX+	0.025
GABPA	DOX−	1.4e−13
ELF1	DOX−	1.9e−13
ETV1	DOX−	9.6e−13
ELF2	DOX−	1.2e−12
ELF5	DOX−	2.7e−10
ETS1	DOX−	2.2e−9
ETV2	DOX−	2.6e−9
ERG	DOX−	1.5e−8
ELK4	DOX−	3.2e−8
ETV4	DOX−	9.8e−8
ELK1	DOX−	5.6e−7
EHF	DOX−	9.2e−7
ELF3	DOX−	0.0000071

TABLE 5-continued

Enriched motifs in condition-specific ATAC-seq promoter peaks

Motif	Condition	Adjusted p-value
SPI1	DOX−	0.000012
FOSL1	DOX−	0.00011
ETV5	DOX−	0.0002
CTCF	DOX−	0.00025
ZN341	DOX−	0.00045
FLI1	DOX−	0.00061
SPIB	DOX−	0.0011
ZSC22	DOX−	0.0014
FEV	DOX−	0.0019
ZN449	DOX−	0.0026
CTCFL	DOX−	0.0034
FOSL2	DOX−	0.0071
PBX3	DOX−	0.0077
EGR2	DOX−	0.015
HINFP	DOX−	0.019
KLF12	DOX−	0.023

[0093] To identify direct targets of SOX17 in D4 HE, we analyzed overlapping SOX17 ChIP-seq and ATAC-seq peaks at promoters and intragenic regions of differentially expressed genes (DEGs) between DOX+ and DOX− conditions. The set of DEGs bound by SOX17 at open chromatin regions was enriched in the Hippo, Wnt, TGFβ, and Notch signaling pathways, and included genes important for NOTCH regulation, arterial specification and hematopoietic development such as NOTCH4, CDX2 (FIG. **4**E-F, Table 6), DLL1, LFNG, DTX4, KITLG, HLX, GLI3, EOMES, DKK2, WNT5B, and PRDM16 (Table 6). Selective quantitative ChIP analysis by PCR confirmed SOX17 binding at the CDX2 promoter and demonstrated a prominent increase in levels of activating H3K27ac at this site in DOX+ cultures (FIG. **4**H). However, HOXA genes, except HOXA10, were not found within this set of genes, suggesting that SOX17 regulates the HOXA cluster indirectly. Similarly, we noted a substantial increase in ATAC-seq counts at the HEY1 promoter following DOX treatment without SOX17 binding (FIG. **4**G), reflecting an indirect activation of downstream NOTCH targets following SOX17 overexpression (FIG. **4**F).

TABLE 6

DEGs with overlapping TS and intragenic SOX17 and ATAC peaks

KEGG	Adjusted p-value
Axon guidance	0.000039
Hippo signaling pathway	0.00061
Prostate cancer	0.0025
Parathyroid hormone synthesis, secretion and action	0.0026
Wnt signaling pathway	0.0026
Pathways in cancer	0.0038
EGFR tyrosine kinase inhibitor resistance	0.0047
Cell adhesion molecules (CAMs)	0.0066
TGF-beta signaling pathway	0.0066
Proteoglycans in cancer	0.0066
Breast cancer	0.0066
Gastric cancer	0.0066
Notch signaling pathway	0.0066
MicroRNAs in cancer	0.011
Hippo signaling pathway-multiple species	0.013
ECM-receptor interaction	0.036
Morphine addiction	0.039
Protein digestion and absorption	0.042

TABLE 6-continued

DEGs with overlapping TS and intragenic SOX17 and ATAC peaks

KEGG	Adjusted p-value
Endocrine resistance	0.042
Melanogenesis	0.048
NF-kappa B signaling pathway	0.048

[0094] To confirm the role of SOX17 in establishing the HOXA pattern in HE, we evaluated expression of arterial and HOXA genes in the three major subsets of HE on D5 (FIG. **5**A), excluding the DLL4$^-$CXCR4$^+$ population, which has a very limited hematopoietic potential (FIG. **3**E-F). As shown in FIG. **5**B, in DOX− cultures, the DLL4$^+$CXCR4$^+$ HE subpopulation expressed the highest levels of arterial genes, including SOX17, and lowest levels of NR2F2 venous gene as compared to two other HE subsets. SOX17 overexpression upregulated expression of arterial markers EFNB2, DLL4, NOTCH4, CXCR4 and HEY1 in all three HE subsets, with the highest levels of expression observed in DLL4$^+$CXCR4$^+$ HE subpopulation. Similarly, we observed significant upregulation of CDX2 and HOXA (HOXA7, HOXA9, HOXA10, HOX11) gene expression in all three subsets of HE with the highest levels of these HOXA genes observed in the DLL4$^+$CXCR4$^+$ HE subpopulation, while the lowest level of HOXA gene expression was observed in DLL4$^-$CXCR4$^-$ non-AHE (FIG. **5**C). In addition, upregulation of more anterior HOXA3 through HOXA6 genes was observed in DLL4$^+$CXCR4$^+$ and DLL4$^-$CXCR4$^-$ HE, although their level of expression was much lower as compared to more distal HOXA genes. We also noted inverse correlation between levels of SOX17 and RUNX1 expression (FIG. **5**B), consistent with prior findings of negative regulation of Runx1 by Sox17 in the murine embryo (Clarke et al., 2013; Lizama et al., 2015). However, suppression of RUNX1 following SOX17 upregulation was observed only in the DLL4$^+$CXCR4$^+$ AHE subset, while upregulation of SOX17 in DLL4$^+$CXCR4$^-$ subset was associated with increased RUNX1 expression suggesting stage-specific differences in RUNX1 regulation following AHE formation from hESCs.

[0095] QPCR analysis of phenotypically similar HE populations generated from SOX17$^{-/-}$ and wild type hESCs revealed substantial reduction of arterial genes in both DLL4$^+$CXCR4$^-$ and DLL4$^+$CXCR4$^+$ AHE subsets as compared to wild type cells. However, significant reduction in HOXA5-HOX10 gene expression was observed only in DLL4$^+$CXCR4$^+$ AHE subset (FIG. **12**A-C). This downregulation of HOXA genes in SOX17$^{-/-}$ cells was associated with a significant reduction of CDX2 expression in AHE, while no differences in CDX2 expression were observed in non-AHE (FIG. **12**C). Thus, these findings confirm the essential role of SOX17 in arterial specification of HE and establishing HOXA signature in DLL4$^+$CXCR4$^+$ AHE.

[0096] Taken together, our molecular profiling studies indicate that SOX17 acts as a key factor in activating the arterial program and HOXA expression in HE.

SOX17 Promotes Arterial Program in HE Through Activation of NOTCH Signaling

[0097] To determine whether SOX17 induction promotes arterial specification through activation of NOTCH signal-

ing, we evaluated hematopoiesis following SOX17 upregulation in the presence of the NOTCH signaling inhibitor N—[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (FIG. **6**A). As shown in FIG. **6**B-C, treatment of hESC cultures with DAPT almost completely abrogated formation of AHE in DOX− conditions and markedly reduced the effect of SOX17 on AHE formation in DOX+ conditions confirming the important role of NOTCH activation in SOX17-mediated promotion of the arterial hemogenic program.

SOX17 Mediates HOXA Gene Expression Through Activation of CDX2 Expression in HE

[0098] Despite significant upregulation of HOXA cluster genes in SOX17-expressing HE, our molecular profiling studies lacked of evidence for their direct regulation by SOX17. However, we found that SOX17 binds to and increases ATAC-seq counts of H3K27ac levels at the CDX2 promoter, along with the upregulation of CDX2 gene expression (FIG. **4**H). The CDX2 gene is known to play a critical role in regulating HOX genes, including HOXA genes (Charite et al., 1998; Subramanian et al., 1995; van den Akker et al., 2002), particularly the HOXA genes necessary for the specification of hematopoietic cells during vertebrate embryogenesis (Davidson et al., 2003; Davidson and Zon, 2006). To find out whether the SOX17 effect on HOXA expression is mediated by CDX2, we differentiated the iSOX17-hESC line with DOX treatment from D2-5 and treated the cultures with CDX2 siRNA from D3-5. On D5, $CXCR4^+$ and $CXCR4^-$ AHE subsets were analyzed for HOXA cluster and arterial gene expression (FIG. **6**A). We found that inhibition of CDX2 with siRNA decreased expression of HOXA genes in both AHE subsets, but had variable effects on the expression of arterial markers (FIG. **6**D-E). In the $DLL4^+CXCR4^+$ population, CDX2 siRNA increased NOTCH1 and NOTCH4 expression, while in the $DLL4^+CXCR4^-$ subset, their expression decreased. The effect of siCDX2 on SOX17 expression was minimal (FIG. **6**E).

[0099] Overall, these observations indicate that the effect of SOX17 expression on establishing HOXA signature in HE is mediated through CDX2 signaling.

Discussion:

[0100] The critical role of SOX17 in HSC development has been well-recognized (Clarke et al., 2013; Kim et al., 2007; Lizama et al., 2015; Nobuhisa et al., 2014; Saito et al., 2018). Although SOX17 regulates multiple steps along the HSC developmental path, including HE specification, EHT, and HSC maintenance and expansion, the stage-specific molecular mechanisms of SOX17 are not well understood. Previously, overexpression of Sox17 was demonstrated to decrease cell numbers within the IAHC, while loss of Sox17 had the opposite effect (Lizama et al., 2015). However, the HSC potential of IAHC of manipulated AGM cells has not been characterized. The majority of cells within IAHC are differentiated hematopoietic cells, with only two of those cells possessing HSC potential (Kumaravelu et al., 2002; Solaimani Kartalaei et al., 2015). Thus, increase in IAHC cells following Sox17 downregulation could be associated with increase of lineage-committed progenitors which is accompanied by impaired HSC generation. This hypothesis is supported by observation that Sox17 loss leads to loss of $CD45^+VEC^+$ HSCs in AGM and fetal liver (Clarke et al., 2013; Kim et al., 2007). Demonstration of reduced multilineage CFC potential following knockdown of Sox17 in $CD45^{low}CD117^{high}$ cells from IAHC and expansion of undifferentiated hematopoietic cells following Sox17 overexpression (Nobuhisa et al., 2014) supports this hypothesis. In addition to regulating EHT and HSCs, SOX17 is essential for arterial specification and HE formation in the AGM (Clarke et al., 2013; Corada et al., 2013). Murine studies revealed that Sox17 effects are mediated through NOTCH signaling (Clarke et al., 2013; Lizama et al., 2015), while no effect of SOX17 on NOTCH signaling was observed in hESC cultures (Nakajima-Takagi et al., 2013).

[0101] A prior study revealed that Sox17 overexpression in hESC-derived during EHT expands $VEC^+CD34^+CD43^+CD45^{-/low}$ cells with hematopoietic colony-forming potential (Nakajima-Takagi et al., 2013). In our study, we focused on defining the cellular and molecular pathways by which SOX17 regulates the earliest stages of HE specification and diversification from the mesoderm. Previously, we and others demonstrated that emerging HE cells lack arterial or venous characteristics (Ditadi et al., 2015; Uenishi et al., 2018) and express high levels of the mesodermal gene HAND1 (Uenishi et al., 2018). Therefore, we defined these cells as immature/primordial HE (Uenishi et al., 2018). When primordial HE cells get exposed to NOTCH signaling, they undergo arterial specification and formation of $DLL4^+CXCR4^{+/-}$ AHE (Park et al., 2018b; Uenishi et al., 2018). Here, we show that SOX17 plays a critical role in specifying of AHE by upregulation of NOTCH4, DLL1 and DLL4 eventually leading to the formation of AHE with the $DLL4^+CXCR4^+$ phenotype typical of AHE at sites of HSC emergence, but not yolk sac AHE, which expresses DLL4, but not CXCR4 (McGrath et al., 1999; Venkatesh et al., 2008; Werner et al., 2020). Along with activating the arterial program, SOX17 is essential for expressing HOXA genes in AHE (FIG. **7**). This integrated effect on arterial programming and HOXA expression is unique for SOX17 since it was not observed following overexpression of ETS1, an arterial program specific gene from our prior studies (Park et al., 2018b).

[0102] We found that SOX17 binds directly to the CDX2 promoter and increases chromatin accessibility and levels of H3K27ac activating histone modification leading to upregulated CDX2 expression. CDX2 knockdown with siRNA revokes SOX17-mediated effects on HOXA genes, thus demonstrating the critical role of a SOX17-CDX2 axis in establishing HOXA pattern in AHE. Genes of the CDX family (CDX1, CDX2 and CDX4) are well-known master regulators of HOX genes that mediate anterior-posterior patterning (Charite et al., 1998; Subramanian et al., 1995; van den Akker et al., 2002). In Wnt-activated epiblast stem cells, CDX2 binds to all four HOX cluster genes, including HOXA genes and is required for opening up the HOX cis-sequences (Neijts et al., 2017). Deficiency of cdx1 and cdx4 results in severe blood defects and altered expression of HOX genes in zebrafish (Davidson et al., 2003; Davidson and Zon, 2006). Similarly, impaired hematopoiesis from Cdx1, Cdx2 or Cdx4-deficient ESCs was observed in murine studies (Wang et al., 2008). Although ectopic expression of CDX4 enhanced definitive hematopoiesis from human and murine ESCs (Creamer et al., 2017; Wang et al., 2005), and hematopoietic engraftment in adult mice from murine ESCs (Wang et al., 2005), Cdx1 and Cdx4 double mutant mice

were viable and did not show any hematopoietic defect (van Nes et al., 2006), which could be due to the observed functional redundancy of genes within the Cdx family (Davidson and Zon, 2006; Wang et al., 2008). It has been demonstrated that Cdx2 is the predominant Cdx gene expressed the AGM HE, while the expression of other Cdx genes is substantially lower (Gao et al., 2018). Cdx2 deficiency caused the most significant impairment in blood production from mouse ESCs (Wang et al., 2008) and Cdx1-Cdx2 compound conditional null mice failed to produce any blood at E11.5 (Foley et al., 2019), suggesting that among the Cdx family, Cdx2 is the most critical factor required for establishing hematopoiesis. Our finding of direct regulation of CDX2 expression by SOX17 provides an insight into the mechanisms responsible for establishing a CDX-HOXA pathway required for the formation of definitive AGM-like HE and lympho-myeloid hematopoiesis from hPSCs. Although previous studies with mouse ESCs found that overexpression of Cdx2 inhibits hematopoietic differentiation (McKinney-Freeman et al., 2008), such effect was not observed following upregulation of CDX2 by SOX17 in hESCs. This could be explained by differences in levels of upregulation or molecular programs activated by upregulation of CDX2 alone or in the context of SOX17 overexpression.

[0103] The de novo production of hematopoietic stem cells (HSCs) with robust multilineage reconstitution potential from human pluripotent stem cells (hPSCs) has long been sought after, but remains an elusive goal. Recent advances in understanding the molecular differences between HE and HSCs developed in vivo and their phenotypic counterparts produced from PSCs in vitro, have revealed that deficiencies in NOTCH and HOXA signaling are major factors responsible for aberrant functionality of PSC-derived hemogenic progenitors (Dou et al., 2016; Doulatov et al., 2013; McKinney-Freeman et al., 2012; Ng et al., 2016; Salvagiotto et al., 2008; Sugimura et al., 2017). It is well-established that NOTCH signaling is essential for arterial specification and development of HSCs (Burns et al., 2005; Kumano et al., 2003). Knock-out of the HOXA cluster in adult mice severely compromised HSC activity (Lebert-Ghali et al., 2016). In humans, HOXA5 and HOXA7 were shown to be critical for the expansion of engraftable fetal liver HSCs (Dou et al., 2016). Although overexpression of single or multiple medial HOXA genes in PSC-derived $CD34^+$ cells was insufficient to confer HSC function (Dou et al., 2016; Ramos-Mejia et al., 2014), overexpression of HOXA5, HOXA9, and HOXA10 along with ERG, LCOR, RUNX1 and SPI1 hPSC-derived HE was capable of generating engraftable hematopoietic cells (Sugimura et al., 2017). In the present study, we provided a compelling evidence that SOX17 is a master regulator that integrates HOXA and arterial signature in HE through modulation of CDX2 signaling. This important finding may contribute to the strategic targeting of NOTCH and HOXA pathways to enhance lymphoid and engraftable hematopoietic cell production from hPSCs for the therapies of hematologic and oncologic diseases, including off-the-shelf immunotherapies.

Example 2

Expansion of Hematopoietic Progenitors with Myeloid and T Lymphoid Potentials

[0104] SOX17 expands lymphomyeloid progenitors generated from day 4 hemogenic endothelium.

[0105] To evaluate the role of SOX17 in expansion of hematopoietic progenitors, we isolated day 4 HE from DOX+ and DOX− cultures (phase I DOX treatment) and cultured these cells on OP9 or OP9-DLL4. After 5 days, floating hematopoietic progenitors were collected and cultured with or without DOX for additional 5 days (phase II DOX treatment) and evaluated for CFC and T lymphoid potential (FIG. **14**). We found that hematopoietic progenitors expanded without DOX lost hematopoietic CFC and T cell potential completely. In contrast hematopoietic progenitors treated with DOX in suspension cultures (day 4+5 through day 4+5+5) retained CFC and T cell potential. We also noted a significant increase myeloid CFCs and T cell potential following phase II DOX treatment when HE were isolated from phase I DOX-treated cultures. This effect was especially pronounced in HE cultured on OP9-DLL4 (FIGS. **14**B and C). As compare to phase I only treated cells, T cell production from hematopoietic progenitors produced from phase I and phase II treated cells was increased by 11-16 fold.

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gacgccagct ccgcggtata t 1221

What is claimed:

1. A method of enhancing HOXA gene expression and arterial specification of hemogenic endothelium in differentiating human pluripotent stem cells (hPSCs), the method comprising:

(a) introducing an inducible SOX17 transgene into a population of hPSCs;

(b) culturing the hPSCs for at least two days under conditions to differentiate the hPSC into KDR$^+$ mesoderm cells; and

(c) inducing expression of the SOX17 transgene in the KDR$^+$ mesoderm cells and culturing for at least two days, such that DLL4$^+$CXCR4$^+$ arterial hemogenic endothelium (AHE) cells are obtained.

2. The method of claim **1**, wherein the population of hPSCs expresses the SOX17 transgene for two to four days.

3. The method of claim **1**, wherein step (a) comprises transducing the hPSCs with a vector comprising the SOX17 transgene.

4. The method of claim **3**, wherein the vector comprises an inducible promoter operably linked to the SOX17 transgene.

5. The method of claim **4**, wherein the vector comprises SEQ ID NO:1.

6. The method of claim **1**, wherein the SOX17 transgene comprises SEQ ID NO:58.

7. The method of claim **1**, wherein the AHE cells express one or more arterial markers selected from the group consisting of EFNB2, DLL4, NOTCH4, CXCR4, and HEY1.

8. The method of claim **1**, wherein the AHE cells express one or more HOXA genes selected from the group consisting of HOXA5, HOXA7, HOXA9, HOXA10, and HOX11.

9. The method of claim **1**, wherein the AHE cells express CDX2.

10. The method of claim **1**, wherein the hPSCs are embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs).

11. The method claim **1**, wherein the cells are further differentiated into a myeloid cell line or lymphoid cell line.

12. The method of claim **11**, wherein the method further comprises:

(d) culturing the hematopoietic progenitors produced in SOX17 overexpression cultures in the presence of cells overexpressing DLL4 in media comprising SCF, FLT3L and IL-7 for at least two weeks to produce CD4+CD8+ T cells.

13. The method of claim **11**, wherein the method further comprises:

(d) inducing expression of the SOX17 transgene in the mesodermal cells of step (c) such that $DLL4^+CXCR4^+$ arterial hemogenic endothelium (AHE) cells are obtained;

(e) culturing AHE in the presence of cells overexpressing DLL4 for a sufficient time to differentiate to floating hematopoietic progenitors, and

(f) collecting the floating hematopoietic progenitor cells and culturing in the presence of DLL4 in media comprising SCF, FLT3L and IL-7 for at least two weeks to produce CD4+CD8+ T cells.

14. A cell population produced by the method of claim **1**.

15. The cell population of claim **14**, wherein the cell population is at least 90% $DLL4^+CXCR4^+$ AHE cells.

16. An hPSC population that comprises a SOX17 transgene and is capable of differentiating into $DLL4^+CXCR4^+$ AHE cells.

17. The hPSC population of claim **16**, wherein the cells comprise a vector comprising the SOX17 transgene.

18. The hPSC population of claim **17**, wherein the vector comprises an inducible promoter operably linked to the SOX17 transgene.

19. The hPSC population of claim **18**, wherein the vector comprises SEQ ID NO:1.

20. The hPSC population of any one of claim **16**, wherein the SOX17 transgene comprises SEQ ID NO:58.

21. An isolated in vitro population of $DLL4^+CXCR4^+$ AHE cells differentiated from an hPSC population comprising a SOX17 transgene.

22. An isolated in vitro T cell population differentiated from the AHE cells of claim **21**.

23. The isolated in vitro T cell population of claim **22**, wherein the T cell population comprises more than 90% $CD4^+CD8^+$ T cells.

24. The isolated in vitro T cell population of claim **22**, wherein the T cells are engineered to express an exogenous chimeric antigen receptor (CAR).

25. A method of expansion of hematopoietic progenitors comprising

a) generating hemogenic endothelium (HE) cells in presence of SOX17 upregulation

b) culturing the HE cells on OP9 or OP9-DLL4 cells in medium comprising FLT3L, TPO SCF, IL6, and IL3 for at least an additional 5 days;

c) collecting the floating hematopoietic progenitor cells (HP); and

d) culturing the HPs of step c in medium comprising FLT3L, TPO SCF, IL6, and IL3 for at least an additional 5 days to expand HPs with myeloid and lymphoid potential.

26. The method of claim **25**, further comprising:

(e) passaging the cells of step (d) for at least two weeks in medium comprising SCF, FLT3L and IL-7 for at least two weeks to produce CD4+CD8+ T cells.

* * * * *