Fig. S3

a

Neurl3

Expression level / Cluster: HC, vEC, earlyAEC, lateAEC, HEC, PK44

b

Stage	Region	Exp (n)	Population in EC (CD41⁻CD43⁻CD45⁻CD31⁺)	Cell dose[a]	Clusters[b]
E9.5-E10.0 (30-34 sp[c])	Caudal half	5	CD44⁺Neurl3-EGFP⁺	1.5-2.5 ee[d]	6/6 (100%)
			CD44⁺Neurl3-EGFP⁻	1.5-5.0 ee	3/6 (50%)

[a]cell dose per recipient. [b]hematopoietic cluster positive wells/total wells. [c]somite pairs. [d]embryo equivalents.

c

$Neurl3$-$EGFP$ $\log_2(TPM/10+1)$

NE+ (CD41⁻CD43⁻CD45⁻CD31⁺ CD44⁺Neurl3-EGFP⁺)

d Pearson correlation analysis

R = 0.33 , P = 0.036 — $Neurl3$, $\log_2(TPM/10+1)$ vs $Neurl3$-$EGFP$, $\log_2(TPM/10+1)$

R = 0.35 , P = 0.027 — $Runx1$, $\log_2(TPM/10+1)$ vs $Neurl3$-$EGFP$, $\log_2(TPM/10+1)$

e

Neurl3

Expression level / Cluster: HC, vEC, earlyAEC, lateAEC, tif-HEC, NE+

f

Cell cycle phase: G2/M, S, G1, Quiescent

Proportion (%) — earlyAEC, NE+, tif-HEC

g

Neurl3-EGFP/Runx1/CD44 — E10.0, E10.5

Neurl3-EGFP/Runx1/CD44/CD31 — E11.0, nt, DA

Neurl3-EGFP/CD44 — DA

Runx1/CD44 — DA

h

Frequency of NE+ cells in CD44⁺ ECs (%) — E9.5 CH, E10.0 AGM, E10.5 AGM

i

E10.0 AGM

PE-Cy7-CD31, FSC-A — CD31⁺ 9.2

APC-CD41 CD43 CD45, FSC-A — CD41⁻CD43⁻CD45⁻ 77.9

BV510-CD44, FSC-A — CD44⁺ 23.8

PK44: APC-Cy7-Kit, PE-CD201 — Kit⁺CD201⁺ 11.1

NE+: BV510-CD44, Neurl3-EGFP — Neurl3-EGFP⁺ 44.3

with PK44 mapped on — 23% PK44, 77% PK44

Frequency in CD44⁺ ECs (%): PK44 2.6, HSC-competence 8.5, NE+ 35.8

j

Single-cell co-culture

Stage	Region	Exp (n)	Population in EC (CD41⁻CD43⁻CD45⁻CD31⁺)	Constitution in the region (%)	Constitution in EC (%)	Constitution in NE+[a] (%)	Endothelial only[b]	Endothelial and hematopoietic[c]	Hematopoietic only[d]
E9.5 (26-30 sp[e])	Caudal half	8[f]	CD44⁺Neurl3-EGFP⁺	0.20±0.07	2.70±0.66	100.0	42/301 (1:7)	1/301 (1:301)	25/301 (1:12)
			PK44 within NE+			39.5	28/119 (1:4)	1/119 (1:119)	17/119 (1:7)
E10.0 (31-35 sp)	AGM	5[g]	CD44⁺Neurl3-EGFP⁺	1.20±0.57	11.3±3.51	100.0	49/467 (1:10)	2/467 (1:234)	37/467 (1:13)
			PK44 within NE+			29.3	21/137 (1:7)	2/137 (1:69)	32/137 (1:4)
E10.5 (36-39 sp)	AGM	6[h]	CD44⁺Neurl3-EGFP⁺	0.29±0.10	2.74±0.52	100.0	32/159 (1:5)	1/159 (1:159)	5/159 (1:32)
			PK44 within NE+			51.6	20/82 (1:4)	1/82 (1:82)	5/82 (1:16)

[a]NE+: CD41⁻CD43⁻CD45⁻CD31⁺CD44⁺Neurl3-EGFP⁺ cells. [b]only endothelial tube positive wells/total wells. [c]both endothelial and hematopoietic progeny positive wells/total wells. [d]only haematopoietic progeny positive wells/total wells. [e]somite pairs. [f]8 independent expreiments, totally 32 embryos were used. [g]5 independent expreiments, totally 37 embryos were used. [h]6 independent expreiments, totally 23 embryos were used.
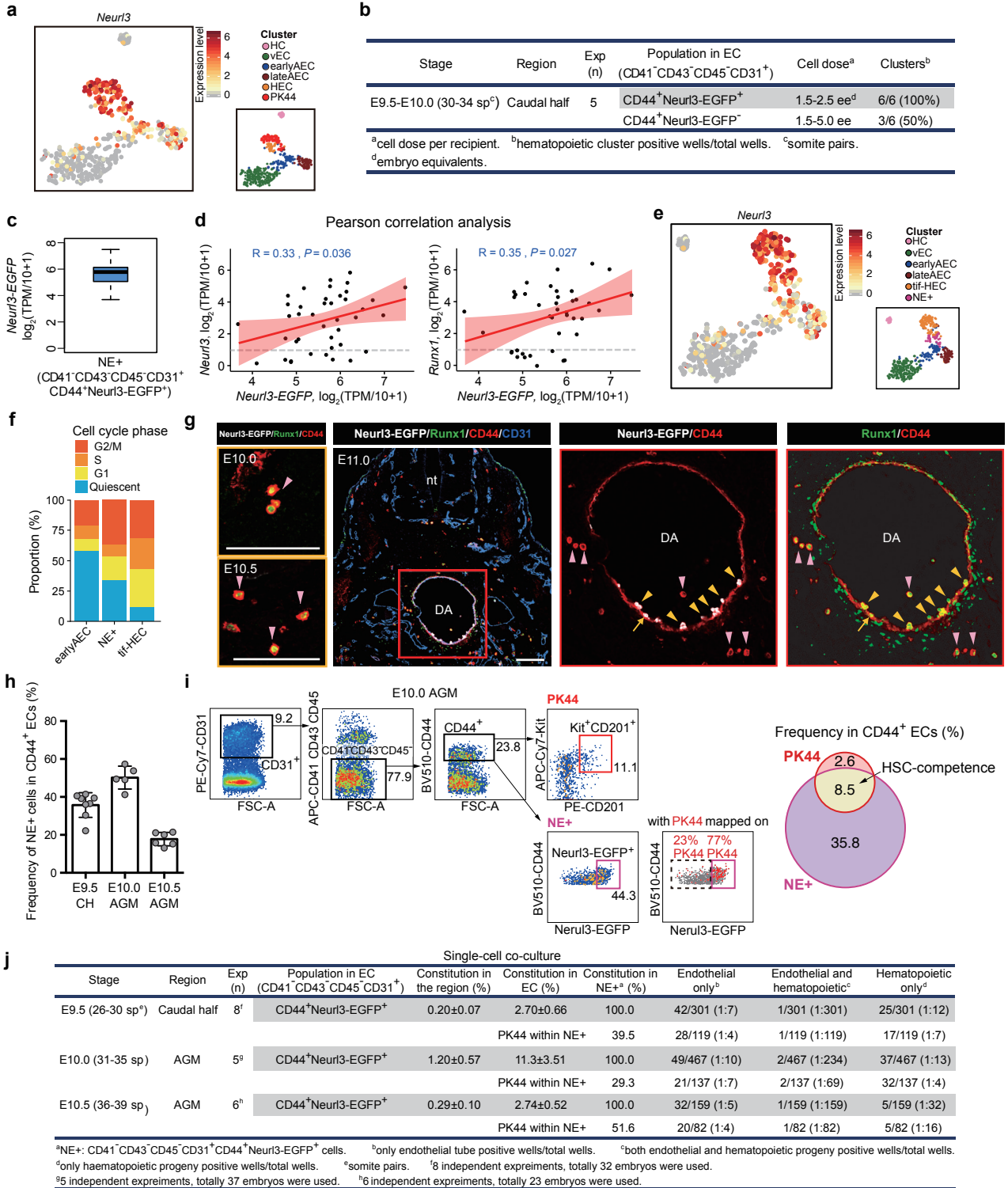
Supplementary Figure 3. Identification of the HSC-competent HECs marked by Neurl3-EGFP reporter. **a.** Expression of *Neurl3* exhibited on t-SNE plot. The same t-SNE plot with the clusters of interest (HC, vEC, earlyAEC, lateAEC, HEC, and PK44) mapped on it is shown to the right. **b.** Detailed information of the co-culture/transplantation assays performed with the caudal half cells from E9.5-E10.0 *Neurl3$^{EGFP/+}$* embryos. **c.** Boxplot showing the transcriptional expression level of *EGFP* in NE+ cell population, NE+, CD41$^-$CD43$^-$CD45$^-$CD31$^+$CD44$^+$Neurl3-EGFP$^+$ population from AGM region of E10.0 *Neurl3$^{EGFP/+}$* embryos. **d.** Scatter plots showing correlation of the expression of *EGFP* with that of *Neurl3* and *Runx1*, respectively. Fitted line and 95% confidence interval are shown in red. Pearson correlation coefficients and *P* values are also shown in blue text. **e.** Expression of *Neurl3* exhibited on t-SNE plot. The same t-SNE plot with the clusters of interest (HC, vEC, earlyAEC, lateAEC, tif-HEC, and NE+) mapped on it is shown to the right. **f.** Stacked bar chart showing the constitution of different cell cycle phases in the indicated clusters. **g.** Representative immunostaining on cross sections at AGM region of E10.0-E10.5 (left columm) and E11.0 (right three panels) *Neurl3$^{EGFP/+}$* embryos. Arrow indicates Neurl3-EGFP+ aortic ECs; Yellow arrowheads indicate Neurl3-EGFP+ bulging and bulged cells and also IAHCs. White pink arrowheads indicate CD44$^+$Runx1$^+$Neurl3-EGFP$^-$ hematopoietic cells distributed outside the aorta. Images in yellow boxes show the corresponding high magnification views of those in Fig. 5a. nt, neural tube; DA, dorsal aorta. Scale bars, 100 μm. **h.** Graph showing the frequencies of the NE+ (CD41$^-$CD43$^-$CD45$^-$CD31$^+$CD44$^+$Neurl3-EGFP$^+$) cells in CD44$^+$ ECs in E9.5 caudal half (CH) or E10.0-E10.5 AGM region of *Neurl3$^{EGFP/+}$* embryos. Data are means ± s.d.. Data are from 8, 5, and 6 independent experiments for E9.5, E10.0, and E10.5 embryos, respectively. **i.** Representative FACS plots and the corresponding diagram to the right showing the relationship between PK44 and NE+ populations in E10.0 AGM of *Neurl3$^{EGFP/+}$* embryos. **j.** Detailed information of endothelial-hematopoietic dual-potential induction assays performed with cells from E9.5 caudal half and E10.0-E10.5 AGM region of *Neurl3$^{EGFP/+}$* embryos.