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Supplemental Information

Retinoic Acid Promotes Endothelial Cell Cycle Early G1 State to Enable Human Hemogenic Endothelial Cell Specification

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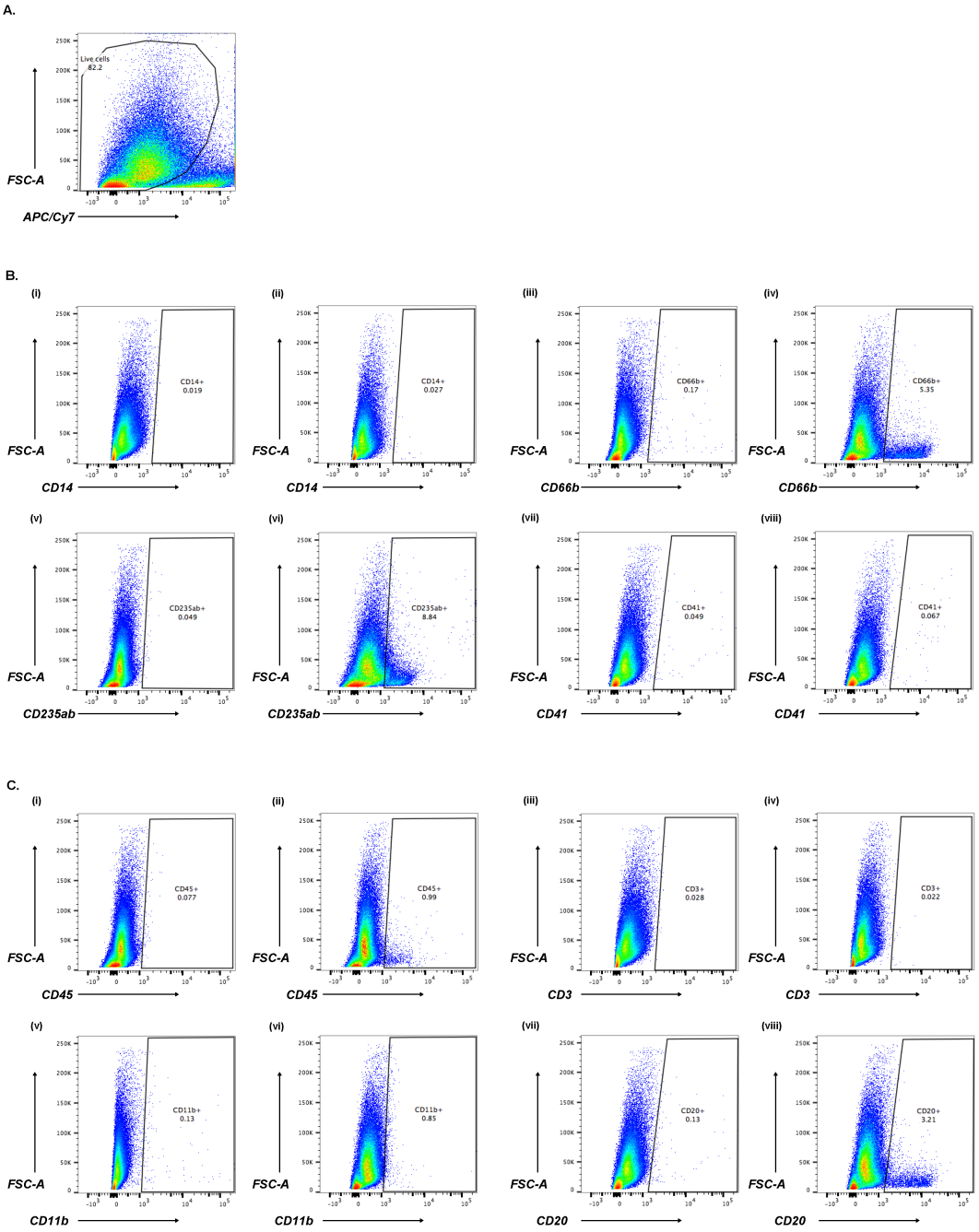
Supplemental Information

Supplementary Table 1

CDH5-F	GCAGCAGCAGGTGCTAACC
CDH5-R	TTGCCCACATATTCTCCTTTG
EFNB2-F	TATGCAGAACTGCGATTTCCAA
EFNB2-R	TGGGTATAGTACCAGTCCTTGTC
EPHB4-F	TGAAGAGGTGATTGGTGCAG
EPHB4-R	AGGCCTCGCTCAGAAACTCAC
GAPDH-F	ACAACTTTGGTATCGTGGAAGG
GAPDH-R	GCCATCACGCCACAGTTTC
HAND1-F	GAGAGCATTAACAGCGCATTCG
HAND1-R	CGCAGAGTCTTGATCTTGGAGAG
RUNX1-F	TGAGCTGAGAAATGCTACCGC
RUNX1-R	ACTTCGACCGACAAACCTGAG
SOX2-F	GCCGAGTGGAAACTTTTGTCG
SOX2-R	GGCAGCGTGTACTTATCCTTCT
TBXT-F	TATGAGCCTCGAATCCACATAGT
TBXT-R	CCTCGTTCTGATAAGCAGTCAC

Supplementary Table 1: Sequences of primers used for qPCR. Related to Figure 1.
All sequences are listed in 5’ to 3’ orientation.

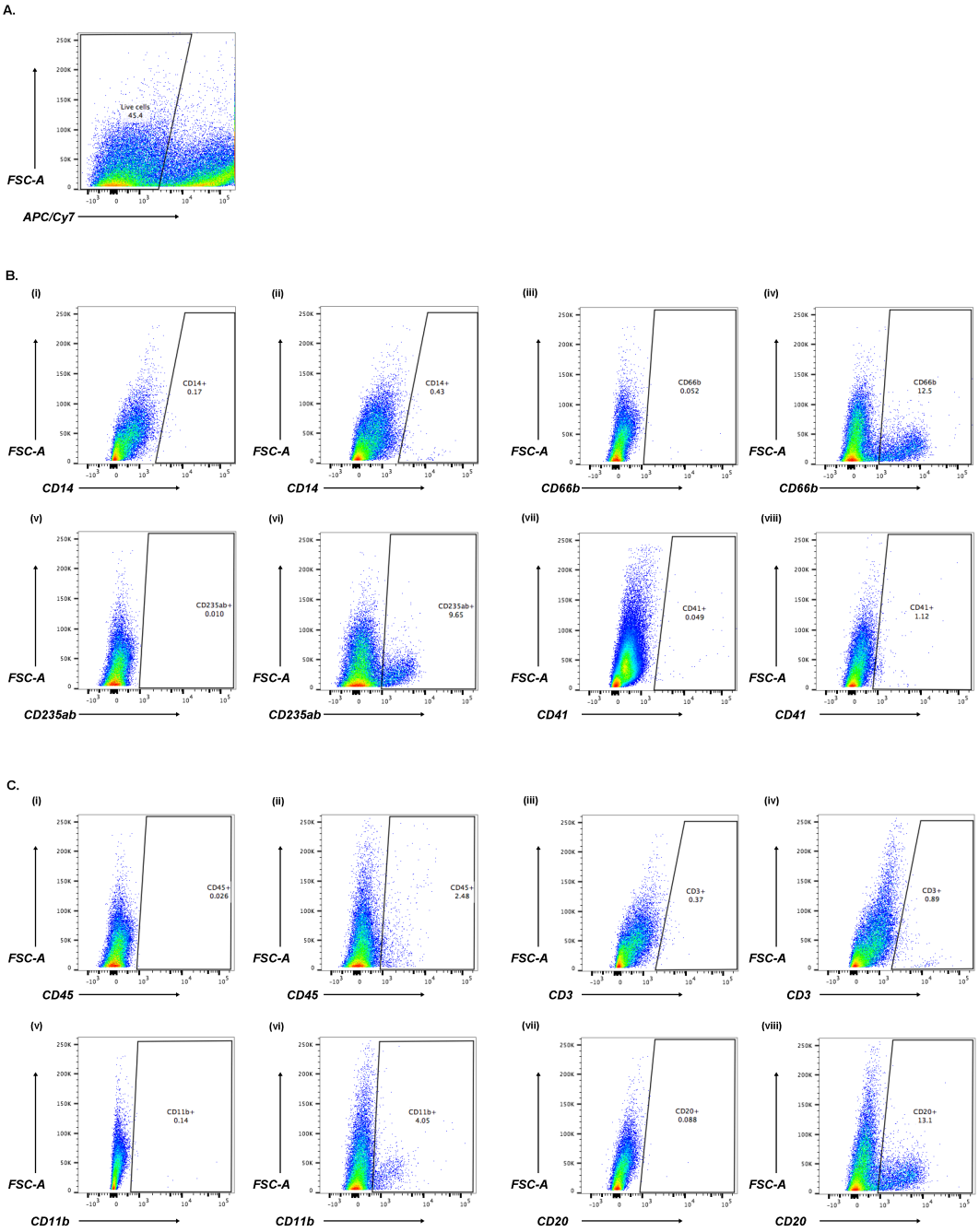
Supplementary Figure 1



Supplementary Figure 1: Blood cells of multiple lineages were identified within adherent cell population on Day8 of differentiation. Related to Figure 1. Expression of various blood cell markers were evaluated on Day8 of differentiation for adherent cells. Flow cytometry analyses were done using two panels of markers. n=3 for all groups. A) Live vs. dead cells were distinguished using a viability dye that is non-permeable to live cells. B) Expression of blood cell markers within the adherent cell population (panel 1). (i)

Expression of CD14 in adherent cells (unstained control). (ii) Expression of CD14 in adherent cells. (iii) Expression of CD66b in adherent cells (unstained control). (iv) Expression of CD66b in adherent cells. (v) Expression of CD235ab in adherent cells (unstained control). (vi) Expression of CD235ab in adherent cells. (vii) Expression of CD41 in adherent cells (unstained control). (viii) Expression of CD41 in adherent cells. C) Expression of blood cell markers within the adherent cell population (panel 2). (i) Expression of CD45 in adherent cells (unstained control). (ii) Expression of CD45 in adherent cells. (iii) Expression of CD3 in adherent cells (unstained control). (iv) Expression of CD3 in adherent cells. (v) Expression of CD11b in adherent cells (unstained control). (vi) Expression of CD11b in adherent cells. (vii) Expression of CD20 in adherent cells (unstained control). (viii) Expression of CD20 in adherent cells.

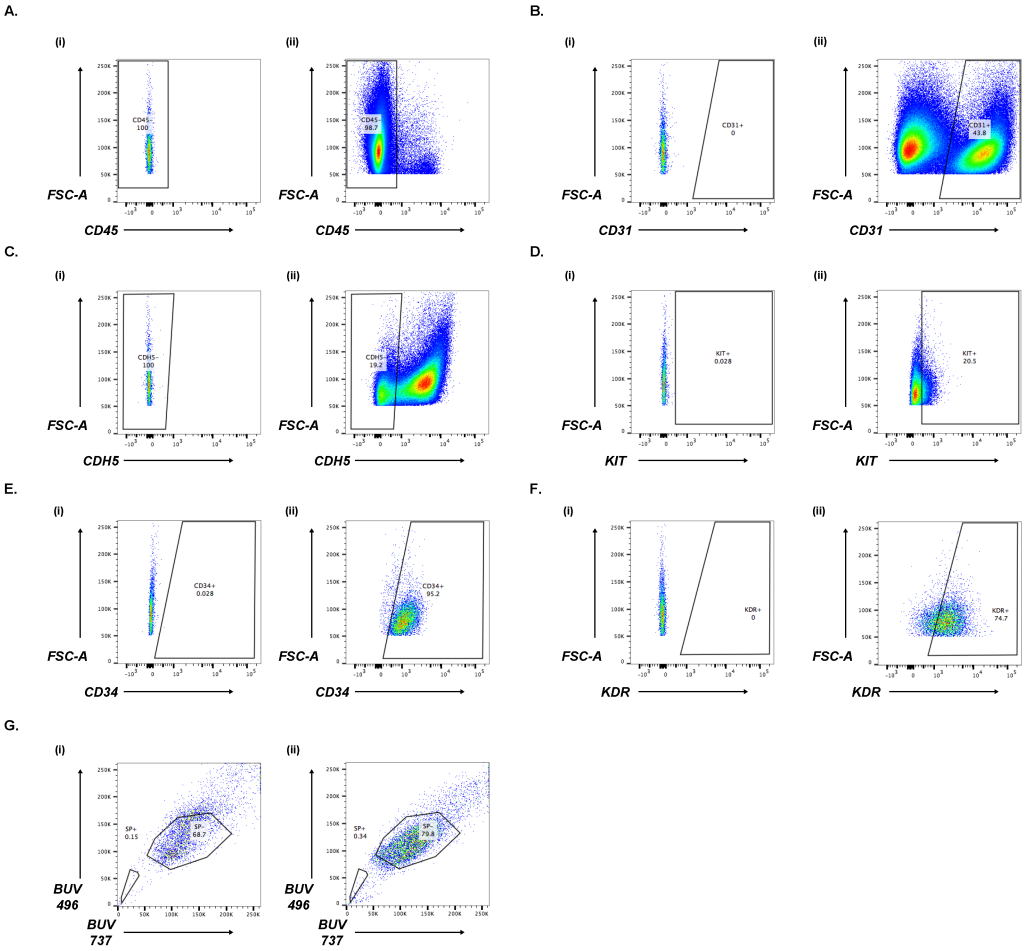
Supplementary Figure 2



Supplementary Figure 2: Blood cells of multiple lineages were identified within suspended cell population on Day8 of differentiation. Related to Figure 1. Expression of various blood cell markers were evaluated on Day8 of differentiation for suspended cells. Flow cytometry analyses were done using two panels of markers. n=3 for all groups. A) Live vs. dead cells were distinguished using a viability dye that is non-permeable to live cells. B) Expression of blood cell markers within the suspended cell

population (panel 1). (i) Expression of CD14 in suspended cells (unstained control). (ii) Expression of CD14 in suspended cells. (iii) Expression of CD66b in suspended cells (unstained control). (iv) Expression of CD66b in suspended cells. (v) Expression of CD235ab in suspended cells (unstained control). (vi) Expression of CD235ab in suspended cells. (vii) Expression of CD41 in suspended cells (unstained control). (viii) Expression of CD41 in suspended cells. C) Expression of blood cell markers within the suspended cell population (panel 2). (i) Expression of CD45 in suspended cells (unstained control). (ii) Expression of CD45 in suspended cells. (iii) Expression of CD3 in suspended cells (unstained control). (iv) Expression of CD3 in suspended cells. (v) Expression of CD11b in suspended cells (unstained control). (vi) Expression of CD11b in suspended cells. (vii) Expression of CD20 in suspended cells (unstained control). (viii) Expression of CD20 in suspended cells.

Supplementary Figure 3



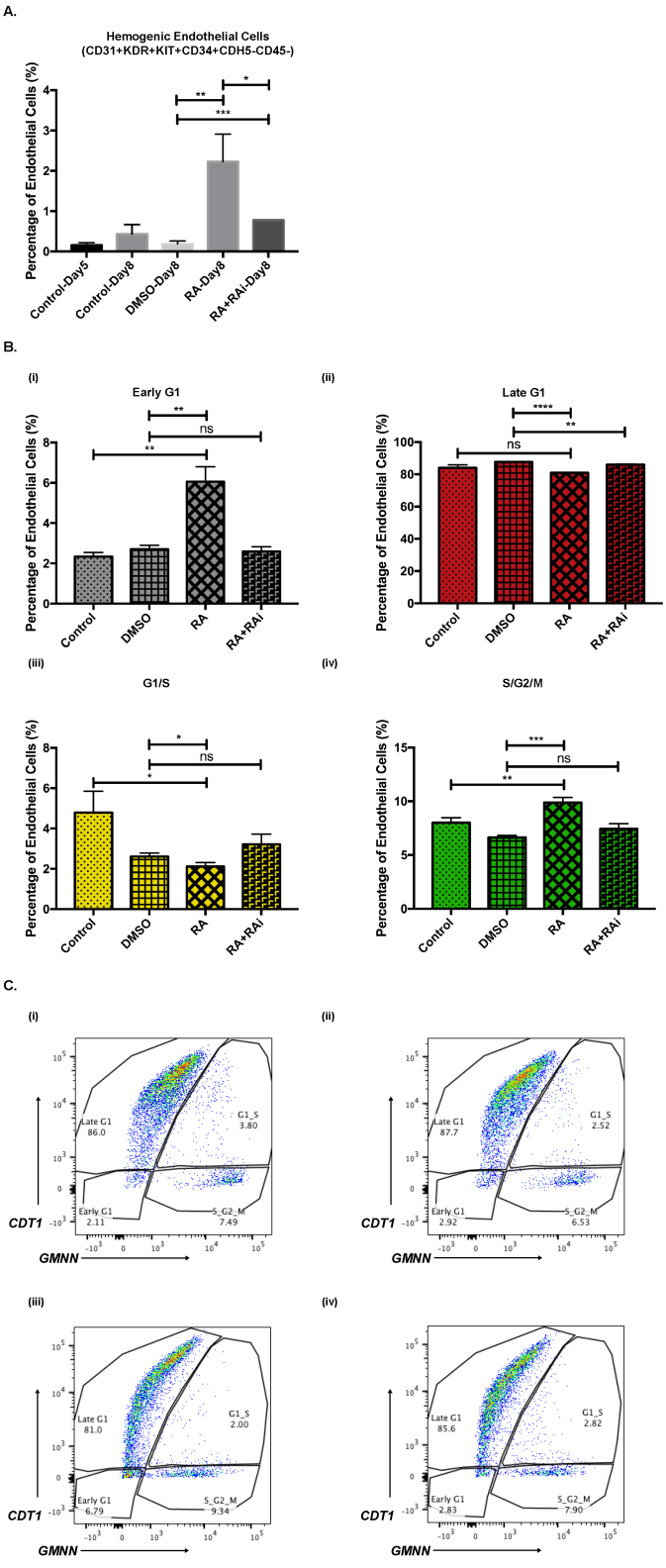
H.

Surface Marker	Percentage of Total Cells (%)	Cumulative Fold-Enrichment for Hemogenic Endothelial Cells
CD45-	98.7	-
CD31+CD45-	43.2	2.3
CD31+CDH5-CD45-	8.3	11.9
CD31+KIT+CDH5-CD45-	1.7	58.1
CD31+KIT+CD34+CDH5-CD45-	1.6	61.7
CD31+KDR+KIT+CD34+CDH5-CD45-	1.2	82.3

Supplementary Figure 3: Characterizing the phenotype of human hemogenic endothelial cells. Related to Figure 2. Expression of various potential markers of human hemogenic endothelial cells were evaluated by flow cytometry on Day8 of differentiation. A) Expression of CD45 within all cultured cells. (i) Unstained control. (ii) Sample. B) Expression of CD31 within CD45- cells. (i) Unstained control. (ii) Sample. C) Expression of CDH5 within CD31+CD45- cells. (i) Unstained control. (ii) Sample. D) Expression of KIT within CD31+CD45-CDH5- cells. (i) Unstained control. (ii) Sample. E) Expression of CD34 within CD31+CD45-CDH5-KIT+ cells. (i) Unstained control. (ii) Sample. F) Expression of KDR within CD31+CD45-CDH5-KIT+CD34+ cells. (i) Unstained control. (ii) Sample. G) Expression of SP within CD31+CD45-CDH5-KIT+CD34+ cells.

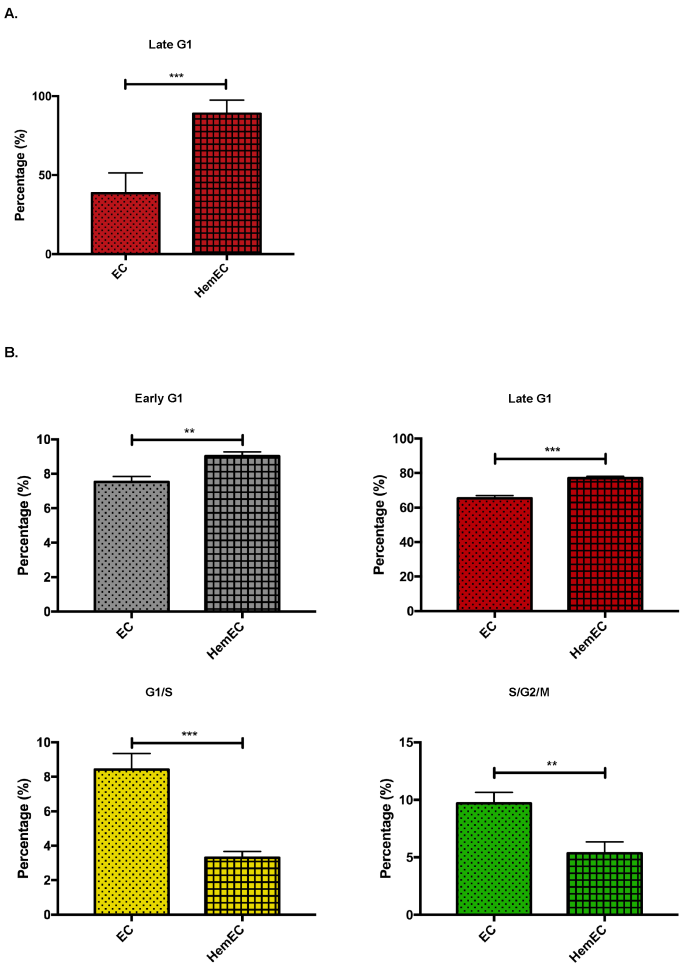
SP events were identified using a differential linear-scale plot of Hoechst blue (BUV 496) vs. Hoechst red (BUV 737) fluorescence. (i) Verapamil control. (ii) Sample. H) Enrichment of CD31+KDR+KIT+CD34+CDH5-CD45- hemogenic endothelial cells is shown in the table. Cumulative fold-enrichment for hemogenic endothelial cells was calculated by comparing each group to the original CD45- group.

Supplementary Figure 4



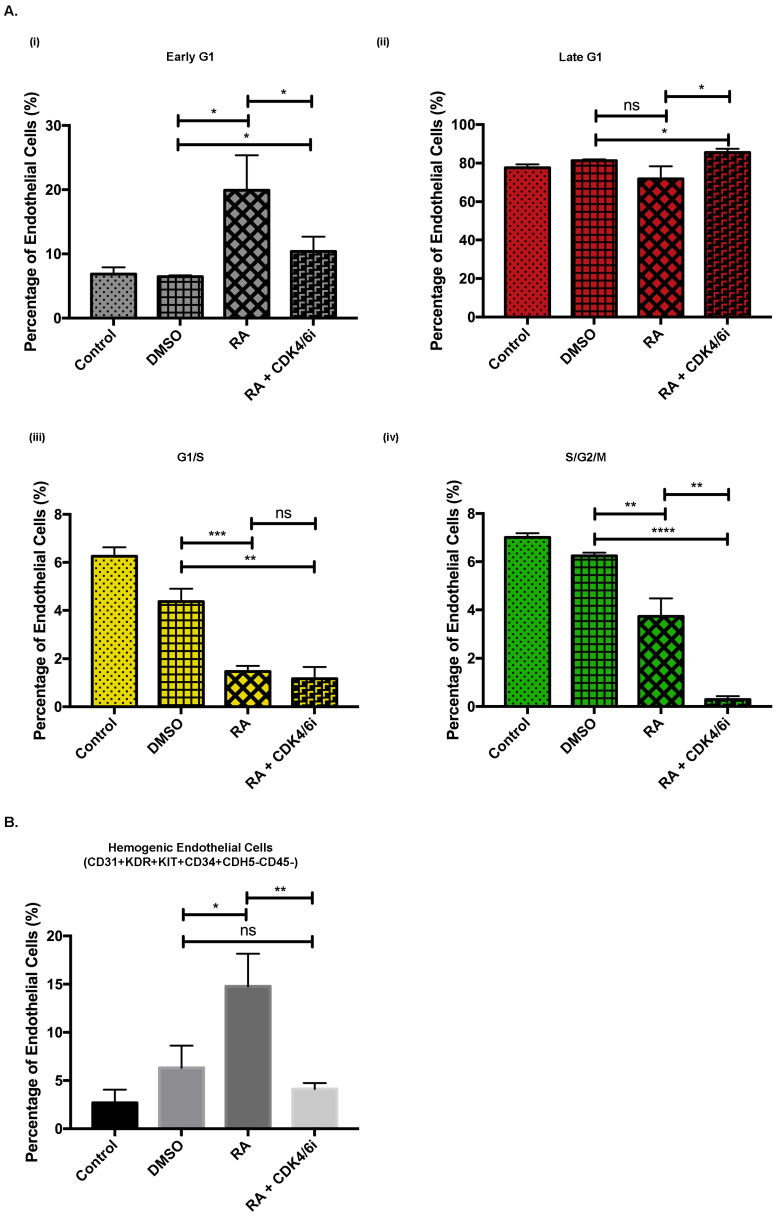
Supplementary Figure 4: RA promotes early G1 cell cycle state of hESC-derived endothelial cells and their specification toward hemogenic endothelial cells (percentage shown). Related to Figure 4. A) RA induces differentiation of hemogenic endothelial cells. n=3 for all groups. Students' t-test: $p = 0.0068$ (DMSO vs. RA), $p = 0.0005$ (DMSO vs. RA+RAi), $p = 0.0218$ (RA vs. RA+RAi). B) RA induces early G1 cell cycle enrichment in endothelial cells. Cell cycle distribution was measured by expression of the FUCCI reporter construct using flow cytometry. n=3 for all groups. (i) Students' t-test: $p = 0.0011$ (control vs. RA), $p = 0.0016$ (DMSO vs. RA). (ii) Students' t-test: $p < 0.0001$ (DMSO vs. RA), $p = 0.0026$ (DMSO vs. RA+RAi). (iii) Students' t-test: $p = 0.0127$ (control vs. RA), $p = 0.0311$ (DMSO vs. RA). (iv) Students' t-test: $p = 0.0084$ (control vs. RA), $p = 0.0004$ (DMSO vs. RA). C) Representative flow cytometry dot plot of endothelial cell cycle distribution on Day 8 upon treatment of (i) control on Day5. (ii) DMSO on Day5. (iii) RA on Day5. (iv)RA+RAi on Day5.

Supplementary Figure 5



Supplementary Figure 5: Mouse and human hemogenic endothelial cells are predominantly in G1 cell cycle state in vivo and in vitro. Related to Figure 4. A) In E10.5 mouse AGM, significantly more hemogenic endothelial cells were in late G1 phase compared to non-blood forming endothelial cells. n=4 for all groups. Students' t-test: p = 0.0006. B) In hESC culture on Day8, significantly more hemogenic endothelial cells were in early and late G1 compared to non-blood-forming endothelial cells. n=3 for all groups. Students' t-test: p = 0.0031 (Early G1), p = 0.0005 (Late G1), p = 0.0009 (G1/S), p = 0.0054 (S/G2/M).

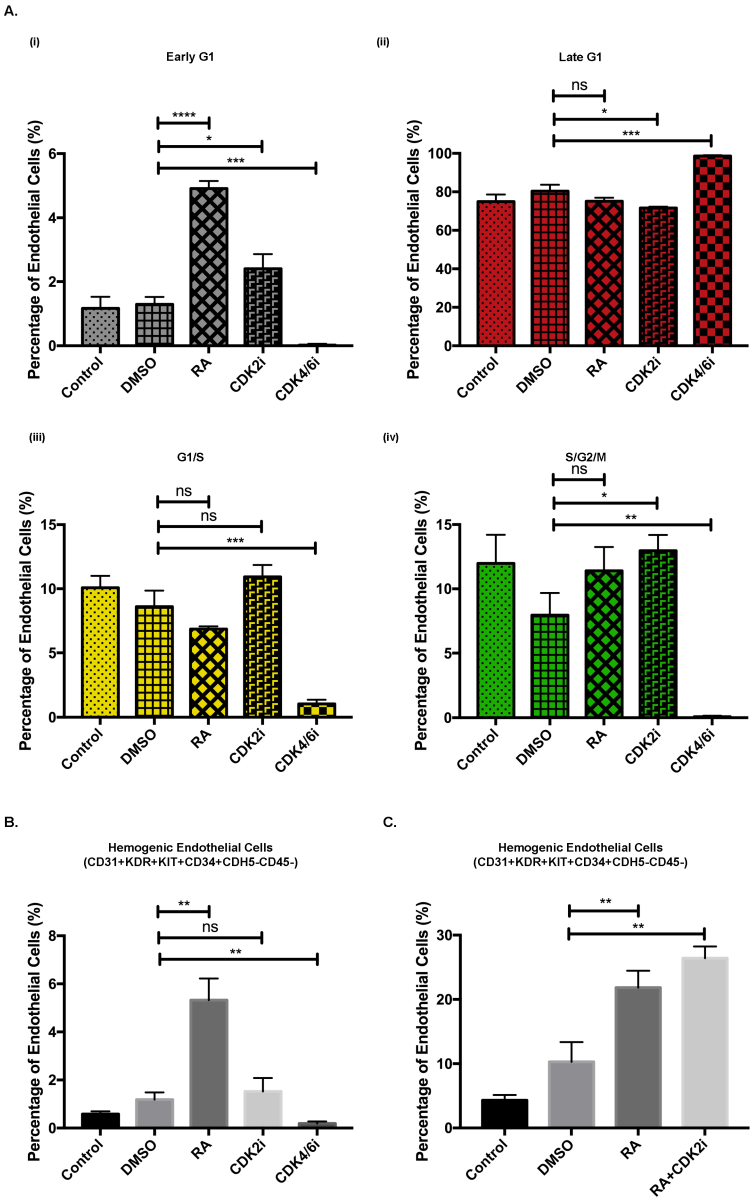
Supplementary Figure 6



Supplementary Figure 6: Early G1 cell cycle state of endothelial cells is necessary to enable hemogenic specification (percentage shown). Related to Figure 5. A) When cells were treated with both RA and CDK4/6i, CDK4/6i overrides RA's effect on cell cycle state and enriches endothelial cells in late G1 phase. Cell cycle distribution was measured by expression of the FUCCI reporter construct using flow cytometry. n=3 for all groups. (i) Students' t-test: p = 0.0130 (DMSO vs. RA), p = 0.0423 (DMSO vs. RA+CDK4/6i), p = 0.0497 (RA vs. RA+CDK4/6i). (ii) Students' t-test: p = 0.0205 (DMSO vs. RA+CDK4/6i), p = 0.0253 (RA vs. RA+CDK4/6i). (iii) Students' t-test: p = 0.0010 (DMSO vs. RA), p = 0.0016 (DMSO vs. RA+CDK4/6i). (iv) Students' t-test: p = 0.0046

(DMSO vs. RA), $p < 0.0001$ (DMSO vs. RA+CDK4/6i), $p = 0.0015$ (RA vs. RA+CDK4/6i). B) In the absence of early G1 cell cycle arrest in endothelial cells, RA + CDK4/6i could not induce hemogenic endothelial cell specification. n=3 for all groups. Students' t-test: $p = 0.0233$ (DMSO vs. RA), $p = 0.0059$ (RA vs. RA+CDK4/6i).

Supplementary Figure 7



Supplementary Figure 7: Early G1 cell cycle state of endothelial cells is not sufficient, to enable hemogenic specification (percentage shown). Related to Figure 5. A) Similar to RA, CDK2i induces early G1 cell cycle arrest in endothelial cells. Cell cycle distribution was measured by expression of the FUCCI reporter construct using flow cytometry. n=3 for all groups. (i) Students' t-test: $p < 0.0001$ (DMSO vs. RA), $p = 0.0196$ (DMSO vs. CDK2i), $p = 0.0007$ (DMSO vs. CDK4/6i). (ii) Students' t-test: $p = 0.0115$ (DMSO vs. CDK2i), $p = 0.0007$ (DMSO vs. CDK4/6i). (iii) Students' t-test: $p = 0.0005$ (DMSO vs. CDK4/6i). (iv) Students' t-test: $p = 0.0148$ (DMSO vs. CDK2i), $p = 0.0014$ (DMSO vs. CDK4/6i). B) Unlike RA, CDK2i could not induce hemogenic

endothelial cell specification. n=3 for all groups. Students' t-test: p = 0.0016 (DMSO vs. RA), p = 0.0051 (DMSO vs. CDK4/6i). C) CDK2i did not artificially suppress cell growth or cell differentiation in culture. Students' t-test: p = 0.0077 (DMSO vs. RA), p = 0.0015 (DMSO vs. RA+CDK2i).