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

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SUMMARY

Development of blood-forming (hemogenic) endothelial cells that give rise to hematopoietic stem and progenitor cells (HSPCs) is critical during embryogenesis to generate the embryonic and postnatal hematopoietic system. We previously demonstrated that the specification of murine hemogenic endothelial cells is promoted by retinoic acid (RA) signaling and requires downstream endothelial cell cycle control. Whether this mechanism is conserved in human hemogenic endothelial cell specification is unknown. Here, we present a protocol to derive primordial

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AUTHOR CONTRIBUTIONS

This project was funded by grants to K.K.H., E.C.B., and S.N. J.Q. designed and performed most of the experiments, in coordination with K.K.H. S.N. performed the bioinformatic analysis of the RNA-seq data. H.H.V. performed the qPCR analyses. J.Q. drafted the manuscript. K.K.H., S.N., and E.C.B. contributed to subsequent revisions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

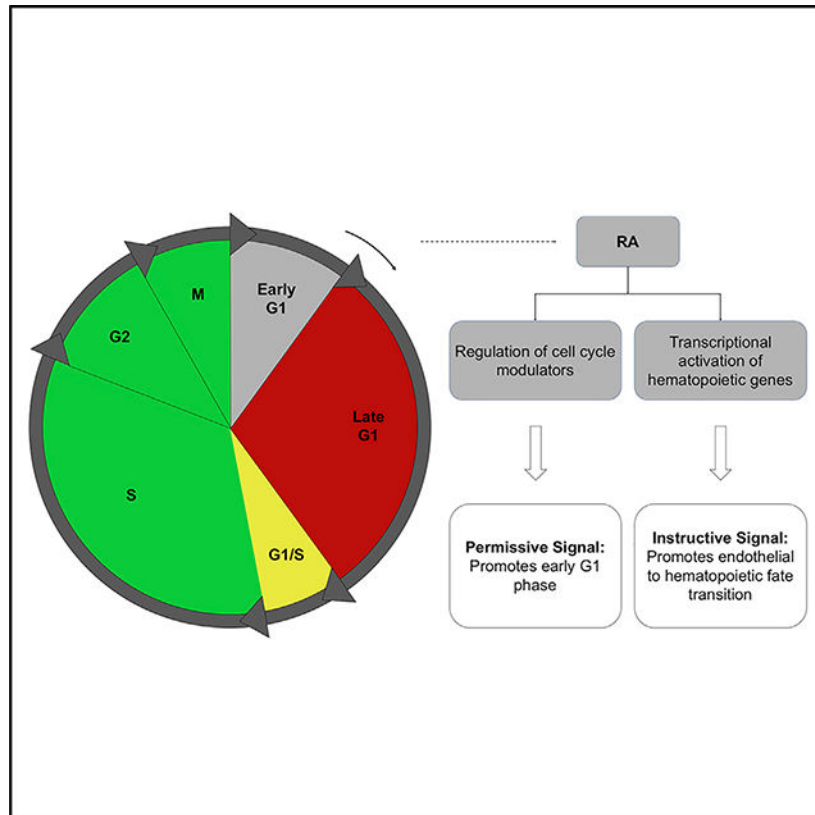
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endothelial cells from human embryonic stem cells and promote their specification toward hemogenic endothelial cells. Furthermore, we demonstrate that RA treatment significantly increases human hemogenic endothelial cell specification. That is, RA promotes endothelial cell cycle arrest to enable RA-induced instructive signals to upregulate the genes needed for hematopoietic transition. These insights provide guidance for the *ex vivo* generation of autologous human hemogenic endothelial cells that are needed to produce human HSPCs for regenerative medicine applications.

In Brief

Retinoic acid signaling and cell-cycle control are known to promote mouse hemogenic endothelial cell specification. Qiu et al. present a protocol to derive endothelial cells from human stem cells and promote their hemogenic specification. These insights provide guidance for generating human hemogenic endothelial cells for regenerative medicine applications.

Graphical Abstract



INTRODUCTION

Mammalian hematopoiesis occurs in two phases during embryogenesis: primitive and definitive hematopoiesis. In the mouse, definitive hematopoiesis takes place mainly within the yolk sac and the aorta-gonad-mesonephros (AGM) region (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Müller et al., 1994), and is marked by the generation of

RESULTS

Simple *In Vitro* System to Derive Human Hemogenic Endothelial Cells from Human Embryonic Stem Cells (hESCs)

A number of protocols were developed to differentiate hESCs toward hematopoietic cells (Krassowska et al., 2006; Lengerke et al., 2009; Niwa et al., 2011); however, these protocols require either usage of feeder cells, formation of embryoid bodies, and/or addition of numerous growth factors, and can take up to 3 weeks. Sriram et al. (2015) previously established a feeder-free protocol to differentiate hESC into endothelial cells. We discovered that treating hESC-derived endothelial cells with RA can promote specification toward hemogenic endothelial cells that give rise to differentiated hematopoietic lineages (Figure 1A). This protocol requires no feeder cells or formation of embryoid bodies. In addition, it requires only 4 growth factors and takes ~1 week from start to finish.

To validate the protocol, we performed qPCR analysis of markers associated with cell fate transitions (Figure 1B). We found that on day 1 (D1) of differentiation, the level of pluripotency marker SOX2 significantly decreased, accompanied by the upregulation of primitive streak marker TBXT. Primitive streak cells were then differentiated into mesoderm cells marked by elevated HAND1 expression on D3. Endothelial cell marker CDH5 peaked on D5, with elevated expression of both arterial endothelial cell marker EFNB2 and venous endothelial cell marker EPHB4. Upon treatment with 0.5 μ M RA, we observed gradually increased expression of RUNX1 mRNA, which marks the transition toward hematopoietic cell fates.

We then performed flow cytometry on D8 to isolate adherent endothelial cells (CD31⁺CD45⁻) and blood cells (CD45⁺), and confirmed their phenotypes on the protein level (Figure 1C). We also observed 40%–45% live cells in the culture supernatant (Figure S2A); using Giemsa staining to better evaluate their phenotype, we identified blood cells of all lineages, including erythrocytes, lymphocytes, macrophages, monocytes, megakaryocytes, neutrophils, and basophils (Figure 1D). Lastly, we confirmed the generation of blood cells of multiple lineages using flow cytometry, using markers of lymphoid cells (CD20 and CD3), myeloid cells (CD14, CD66b, CD41, and CD235ab), and leukocytes (CD11b and CD45) (Figures 1E, S1, and S2). These data demonstrate that our protocol promotes the efficient differentiation of hESCs into endothelial cells and hematopoietic cells.

Characterization of the Phenotype of Human Hemogenic Endothelial Cells on Population and Single-Cell Levels

We next sought to identify the subset of endothelial cells that presumably generated the hematopoietic cells, and define their phenotype. The phenotype of murine hemogenic endothelial cells has been previously defined as Flk-1⁺Kit⁺CD45⁻ Side Population (SP) cells (Goldie et al., 2008; Marcelo et al., 2013b; Nadin et al., 2003; Wulf et al., 2003). Here, we used flow cytometric separation of subpopulations of cells and a methylcellulose-based blood-forming assay to define the phenotype of human hemogenic endothelial cells as

CD31⁺KDR⁺KIT⁺CD34⁺CDH5⁻CD45⁻ cells, as outlined in Figure 2A and described further below.

Initially, we tested the ability of our whole-cell population to generate multiple hematopoietic cell colony types, including multilineage CFU-GEMM (colony-forming unit-granulocyte/erythrocyte/monocyte/megakaryocyte), CFU-GM (colony-forming unit-granulocyte/monocyte), BFU-E (burst-forming unit-erythroid) and CFU-E (colony-forming unit-erythroid) colonies (Figure 2B). To begin to characterize the phenotype of the blood-forming endothelial cells within this population, we excluded other cells with blood-forming potential, specifically CD45⁺ HSPCs, by isolating CD45⁻ cells using fluorescence-activated cell sorting (FACS). We then further separated the CD45⁻ cells into various subgroups, based on the expression of known endothelial- or hematopoietic-enriched cell surface proteins. Among the CD45⁻ cells, hematopoietic potential was enriched within the CD31⁺ population (Figures 2C and S3A).

We then isolated CD31⁺CD45⁻ cells, known to be an enriched population of endothelial cells, and similarly separated them into additional subgroups based on the expression of specific proteins enriched in endothelial or hematopoietic cells. Among the CD31⁺CD45⁻ endothelial cells, hematopoietic potential was enriched within the CDH5⁻ and CD34⁻ populations (Figures 2D and S3B). Since CD34 marks definitive human hematopoietic progenitors but not primitive hematopoietic progenitors (Dege and Sturgeon, 2017), we further isolated CD31⁺CD45⁻CDH5⁻ cells. Among them, hematopoietic potential (colony-forming ability) was enriched within the KIT⁺ and CD34⁺ populations (Figures 2E and S3C). Lastly, we isolated CD31⁺CD45⁻CDH5⁻KIT⁺CD34⁺ cells and found that hematopoietic potential was enriched within the kinase insert domain receptor-positive (KDR⁺) population (Figures 2F and S3D–S3H). Thus, the CD31⁺KDR⁺KIT⁺CD34⁺CDH5⁻CD45⁻ population of hESC-derived cells is highly enriched for human hemogenic endothelial cells (Figure S3H).

Upon characterizing these cells on a population level, we further evaluated their hematopoietic potential on a clonal level (Figure 3A). Single human hemogenic endothelial cells (CD31⁺KDR⁺KIT⁺CD34⁺CDH5⁻CD45⁻) were FACS sorted into 96-well plates on D0 (Figure 3Bi). By D3, small endothelial cell colonies formed. These cells not only demonstrated the typical cobblestone morphology (Figure 3Bii) but also the functionality of acetylated, fluorescently labeled low-density lipoprotein (DiI AcLDL) uptake (Figure 3Biii) and the expression of endothelial nitric oxide synthase (eNOS) (Data S1, Data S2, and Data S3), which are characteristics of endothelial cells. Although some mature blood cells, such as monocytes or macrophages, can also take up DiI AcLDL, they were FACS excluded from the population tested in this assay, which contained only CD45⁻ cells. Thus, these data show that human hemogenic endothelial cells derived from hESCs are able to proliferate in culture and maintain the characteristics of endogenous vascular endothelial cells. By D14, endothelial cells could still be observed among the hematopoietic colonies (Figure 3Biv and v). We further confirmed the identity of various hematopoietic colonies that single hESC-derived hemogenic endothelial cells were capable of giving rise to using immunostaining. These colonies include BFU-E erythroid colonies marked by CD235ab expression (Figure 3Ci and ii), CFU-E erythroid colonies marked by CD235ab expression (Figure 3Ciii and iv),

CFU-GM colonies marked by CD66b and CD14 expression (Figure 3D), and CFU-GEMM multipotent hematopoietic progenitor colonies marked by the expression of CD66b, CD235ab, CD14, and CD41 (Figure 3E). Overall, we demonstrated that these hESC-derived CD31⁺KDR⁺KIT⁺CD34⁺CDH5⁻CD45⁻ human hemogenic endothelial cells exhibit multi-lineage hematopoietic potential on both population and single-cell levels.

RA Promotes Early G1 Cell Cycle State of hESC-Derived Endothelial Cells and the Enrichment of Hemogenic Endothelial Cells

To determine whether RA could enhance the specification of human hemogenic endothelial cells, we compared untreated hESC-derived endothelial cells (control group) to similar cells treated at D5 with vehicle (DMSO group) or RA (RA group), and quantified hemogenic endothelial cell generation on D8 of differentiation (Figure 4A). We used both control and DMSO groups because DMSO was previously shown to increase pluripotent stem cell differentiation (Chetty et al., 2013). We found that after 72 h of treatment, RA significantly increased the differentiation of hESC-derived human hemogenic endothelial cells, whereas DMSO had no effect (Figures 4B and S4A). When cells were treated with both RA and a RA receptor (RAR) pan-antagonist (RAi), RA-induced human hemogenic endothelial cell specification was largely blocked (Figures 4B and S4A).

We previously found that cell cycle state plays a critical role in murine arterial and hemogenic endothelial cell specification *in vivo* (Fang et al., 2017; Marcelo et al., 2013b). Here, we investigated the effect of RA on cell cycle distribution *in vitro* using H9-hESCs carrying the fluorescent ubiquitination-based cell cycle indicator (FUCCI) (H9-FUCCI-hESC reporter cell line) (Pauklin and Vallier, 2013). The FUCCI system uses antiphase oscillating proteins to mark distinct cell cycle states with fluorescent indicators, and cell cycle distribution can be analyzed via flow cytometry (Figure 4C) (Sakaue-Sawano et al., 2008). We found that RA treatment significantly increased the proportion of hESC-derived endothelial cells in early G1 phase and decreased the proportion transitioning from G1 to S phase (Figures 4D, S4B, and S4C). We also found that DMSO significantly increased the proportion of cells in late G1 phase (data not shown), which is consistent with previous findings (Chetty et al., 2013). When cells were treated with both RA and RAi, RA-induced changes in the cell cycle state were suppressed. These data show that RA promotes the differentiation and enrichment of hemogenic endothelial cells from hESCs, and this is accompanied by an increased proportion of cells in early G1 phase and a decreased proportion transitioning from G1 to S phase.

Mouse and Human Hemogenic Endothelial Cells Are Predominantly in G1 Cell Cycle State *In Vivo* and *In Vitro*

Our previous studies of murine yolk sac and AGM endothelial cells (Marcelo et al., 2013b), and the studies above in hESC-derived endothelial cells, demonstrate that in both model systems, RA promotes hemogenic specification, as well as cell cycle arrest. To further define the cell cycle state of murine hemogenic endothelial cells versus non-blood-forming endothelial cells *in vivo* during definitive hematopoiesis, we used FUCCI-red mice, carrying mKO fluorescent protein that marks cells in G1 phase (Sakaue-Sawano et al., 2008). Using flow cytometry, we isolated murine E10.5 AGM endothelial cells (CD31⁺CD45⁻SP⁻) and

hemogenic endothelial cells (Flk-1⁺Kit⁺CD45⁻SP) and found that the proportion of hemogenic endothelial cells in G1 phase was significantly higher than in non-blood-forming endothelial cells in the AGM (Figures 4E and S5A). To further define the cell cycle state of hESC-derived human hemogenic endothelial cells, we used the H9-FUCCI-hESCs described above (shown in Figure 4F) (Pauklin and Vallier, 2013), which can clearly delineate cells in early G1, late G1, G1-to-S, and S/G/M phases (depicted in Figure 4C). We found that compared to total human endothelial cells (CD31⁺CD45⁻), a significantly greater proportion of human hemogenic endothelial cells (CD31⁺KDR⁺KIT⁺CD34⁺CDH5⁻CD45⁻) were also in G1 phase and a significantly lower proportion were actively cycling (Figures 4G and S5B). Overall, these studies implicate a role for cell cycle state in human hemogenic endothelial cell specification and highlight a potentially conserved developmental paradigm in mice and humans.

Early G1 Cell Cycle State Is Necessary but Not Sufficient to Enable Hemogenic Endothelial Cell Specification

We found that hemogenic endothelial cells *in vivo* and *in vitro* were predominantly in G1 phase, and G1 arrest is required for murine hemogenic endothelial cell specification (Marcelo et al., 2013b). In addition, RA treatment, which promoted human hemogenic endothelial cell development, specifically increased the proportion of hESC-derived endothelial cells in early G1 phase, and concomitantly decreased the proportion of cells exiting G1 phase. Therefore, we specifically focused on the role of G1 state in hemogenic specification, and tested whether early G1 versus late G1 cell cycle state is necessary for hemogenic specification. To do so, we treated endothelial cells with a CDK4/6 inhibitor (CDK4/6i), which causes an accumulation of cells specifically in late G1 phase (Schmidt and Sebastian, 2018), and tested their response to RA (Figure 5A). As expected, RARACDK4/6i treatment increased the proportion of endothelial cells in late G1 phase (Figures 5Bii and S6Aii). In contrast, RA treatment alone increased the proportion of endothelial cells in early G1 phase (Figures 5Bi and S6Ai), consistent with the studies described above. The effects of RA and CDK4/6i on G1/S and S/G2/M phases were comparable (Figures 5Biii, 5Biv, S6Aiii, and S6Aiv). When hESC-derived endothelial cells were arrested in late G1 phase, as opposed to early G1 phase, hemogenic endothelial cell specification was suppressed even in the presence of RA (Figures 5C and S6B). Thus, the early G1 cell cycle state appears to be necessary for RA-induced hemogenic endothelial cell specification. Of note, we did not test the potential role of S/G2/M states for several reasons: a low proportion (<10%, data not shown) of hemogenic endothelial cells reside in this state *in vivo* and *in vitro*, we cannot verify arrest in S versus G2 versus M using this FUCCI reporter cell line, and the drugs available to arrest cells in these phases are associated with confounding effects such as cytotoxicity, increased apoptosis, and chromosome damage.

To test whether the early G1 cell cycle state in endothelial cells is sufficient for hemogenic specification, hESC-derived endothelial cells were treated with either RA, CDK4/6i, or CDK2 inhibitor (CDK2i), which arrests cells specifically in early G1 phase of the cell cycle (Chao et al., 2019) (Figure 5D). The CDK2i (CVT-313) that we used is a cell-permeable purine analog that acts as a potent and selective ATP-competitive inhibitor of CDK2, which inhibits other kinases only at much higher concentrations. As expected, and similar to RA,

CDK2i increased the proportion of endothelial cells in early G1 phase (Figures 5Ei and S7Ai); in contrast, CDK4/6i increased the proportion of endothelial cells in late G1 phase (Figures 5Eii and S7Aii). We found that arresting endothelial cells in either early G1 or late G1 alone (in the absence of RA treatment) was not sufficient to promote hemogenic specification (Figures 5F and S7B). In addition, when hESC-derived endothelial cells were treated with both RA and CDK2i, there was a trend toward an increase in hemogenic endothelial cell specification (Figures 5G and S7C). Thus, early G1 cell cycle arrest of human endothelial cells is necessary but not sufficient to promote hemogenic specification.

Bulk RNA Sequencing (RNA-Seq) Reveals Candidates of Additional Signals That RA Provides to Promote Hemogenic Endothelial Cell Specification

To gain further mechanistic insight into the role of RA during human hemogenic endothelial cell specification, we performed bulk RNA-seq of H1-hESC-derived endothelial cells (CD31⁺CD45⁻) treated with or without RA (Figure 6A). Both control (Figure 6Bi) and RA-treated (Figure 6Bii) endothelial cells (CD31⁺CD45⁻) were FACS isolated on D8 and subjected to bulk RNA-seq.

We identified 2,547 genes significantly downregulated and 2,208 genes significantly upregulated in RA-treated endothelial cells, and performed gene set enrichment analysis of the datasets, respectively. We found that genes downregulated by RA were strongly associated with the vascular endothelial growth factor (VEGF) signaling pathway, which plays an essential role in endothelial cell proliferation and arterial specification (Bernatchez et al., 1999; Lawson et al., 2002) (Figure 6C). Transcription factors associated with these genes include ETV2, which is necessary for endothelial cell differentiation (De Val and Black, 2009), and KLF5, which promotes endothelial cell proliferation via the regulation of VEGF (Gao et al., 2015; Nagai et al., 2005). In addition, our analysis revealed a shift away from an endothelial phenotype upon RA treatment, demonstrated by the downregulation of genes associated with KDR expression and a human umbilical vein endothelial cell (HUVEC) phenotype. Lastly, the gene set enrichment analysis results further confirmed the role of RA in cell cycle regulation. Genes downregulated by RA are linked to transcription factor E2F1, which promotes the G1-to-S phase transition (Poppy Roworth et al., 2015).

In contrast, we found that genes upregulated by RA treatment were strongly associated with RA signaling (as expected), as well as Notch, Wnt and Hedgehog (HH) signaling. Notch is known to promote murine hemogenic specification downstream of RA (Marcelo et al., 2013b), and these results suggest conserved molecular regulation of mouse and human hemogenic endothelial cell specification. Wnt signaling has long been recognized as an important regulator of hematopoiesis. More specifically, canonical Wnt signaling plays an important role in maintaining hematopoietic stem cell (HSC) self-renewal, and has also been shown to interact with other signaling pathways, including HH and Notch (Staal and Luis, 2010) (Figure 6D). SMAD factors are also significantly upregulated with RA treatment. They play a critical role in regulating endothelial cell growth suppression (Bohnsack et al., 2004), as well as the fate of HSPCs during development and postnatally, by mediating transforming growth factor β (TGF- β) signaling (Larsson and Karlsson, 2005). Lastly, genes upregulated by RA are also associated with SOX2 and TRIM28 activities, and both have

been shown to support the early stages of blood cell development (Hosoya et al., 2013; Sarkar and Hochedlinger, 2013).

We further examined RA regulation of genes known to be involved in vascular development and hemogenic specification (Figure 6E). Similar to our findings above, RA treatment led to a shift away from endothelial phenotype and toward a hematopoietic phenotype. More specifically, RA suppressed the expression of VEGF receptors KDR and Fms-related receptor tyrosine kinase 1 (FLT1), which are important for endothelial cell proliferation and maintenance (Melincovici et al., 2018). In contrast, RA upregulated the expression of a number of genes associated with murine hemogenic endothelial cells and HSPCs, including RAR, KIT, NOTCH, CDKN1B, GATA2 and RUNX1. In addition, the expression of a number of SMAD family members was upregulated, as were ligands of the HH and Wnt signaling pathways, and homeobox A1 (HOXA1) genes that are known to play a role in hematopoietic development (Alsayegh et al., 2019). These results are consistent with the gene set enrichment analyses, and suggest that there is potential crosstalk among the RA/HOX/SMAD/HH/Wnt signaling pathways that instructs the hemogenic specification of endothelial cells and concomitant downregulation of endothelial cell identity.

RNA-Seq Analysis Provides Insights That Link Endothelial Cell Cycle State to Hemogenic Specification

To gain further mechanistic insight into the role of cell cycle regulation in human hemogenic endothelial cell specification, we performed bulk RNA-seq of H9-FUCCI-hESC-derived endothelial cells (CD31⁺CD45⁻) in distinct cell cycle states (Figure 7A). Cells in early G1, late G1, G1/S, and S/G2/M phases were FACS isolated and subjected to bulk RNA-seq (Figure 7B). We identified genes exclusively upregulated in early G1 phase (1,854 genes) and late G1 phase (2,387 genes) and performed gene set enrichment analysis. Genes exclusively upregulated in early G1 were predominantly associated with hematopoietic development, and hESC-derived endothelial cells in early G1 phase showed high resemblance to cell types such as MOLT-4 cell (T lymphoblast), erythroid cell, B lymphoblast, and cord blood (Figure 7C). In contrast, genes exclusively upregulated in late G1 were predominantly linked to vascular development and angiogenesis, while some genes were associated with heart, ventricle, atrium, placenta, whole blood, and monocytes (Figure 7C).

To further investigate the link between endothelial cell cycle state and cell fate decisions, we examined the differential expression of specific genes associated with vascular development, hemogenic specification, and arterial specification in endothelial cells in distinct cell-cycle states (Figure 7D). We found an enrichment of genes associated with hemogenic specification in early G1 phase (e.g., CDKN1B, ESAM, HES1, GATA2, TAL1, LMO2) (Figure 7E). In contrast, we observed an enrichment of genes associated with arterial specification in late G1 phase (e.g., GJA4, GJA5, NRP1, EFNB2, DLL4, TGFBR2) (Figure 7F), which is consistent with our previous findings in murine arterial endothelial cell specification (Fang et al., 2017).

Our studies suggest that RA plays a dual role in human hemogenic endothelial cell specification. That is, it promotes endothelial cell arrest in early G1 phase, which is

necessary to enable hemogenic specification (Figure 7G), and also induces signaling pathways that are necessary for altering gene expression, specifically in early G1 state, to promote a hemogenic phenotype and suppress endothelial cell identity.

DISCUSSION

Previous studies have demonstrated the endothelial origin of blood cells *in vivo* (Marcelo et al., 2013a; Medvinsky and Dzierzak, 1996; Taoudi and Medvinsky, 2007). Further mechanistic studies have shed light on how a subset of murine endothelial cells acquires hematopoietic potential, highlighting the critical role of the RA/Kit/Notch/p27 signaling pathway and cell cycle regulation (Goldie et al., 2008; Marcelo et al., 2013b). However, whether a specific cell cycle state is necessary for hemogenic specification of endothelial cells or whether this mechanism is conserved in humans was unknown. In these studies, we established a simple *in vitro* system to derive human endothelial and hemogenic endothelial cells and delineate their mechanism(s) of development.

Compared to existing systems that can be used to derive endothelial and hematopoietic cells from hESCs, our system has several advancements. For example, it does not require the use of mouse feeder cells or the formation of embryoid bodies, and is therefore less technically challenging to adopt. In addition, our system reduces the presence of other cell types and/or the development of other organ systems to provide a more controlled environment and consistency for molecular mechanistic studies. Our system also requires fewer growth factors and less time and is therefore more economical. Using this system, we showed that RA plays a critical role in the specification of human hemogenic endothelial cells, which we defined on a clonal level.

We propose that RA has two roles during hemogenic specification—promoting cell cycle arrest of endothelial cells in early G1 phase (permissive signal) and activating transcriptional programs (instructive signal) that enable the transition from endothelial to hemogenic endothelial cells, specifically in early G1. Thus, we hypothesize that arrest in early G1 state provides a distinct “window of opportunity” for endothelial cells to respond to external stimuli that promotes their specification toward a hemogenic endothelial cell phenotype. In addition, RA extends the window of time for hemogenic specification by enriching endothelial cells in early G1; RA also activates signaling pathways and transcriptional regulators that promote hemogenic specification in early G1. Consistent with this idea, others have shown that the cell cycle state determines the propensity for hESCs to commit to mesodermal versus endodermal and ectodermal lineages (Pauklin and Vallier, 2013).

We demonstrated that both of these functions of RA are required for hemogenic specification. To test the cell-cycle-regulation role of RA, we showed that chemically enriching endothelial cells in a different cell cycle phase (late G1 versus early G1 that is induced by RA) prevented hemogenic specification despite the presence of RA. To test the transcriptional role of RA, we demonstrated that chemically enriching endothelial cells in early G1 phase with CDK2i was not capable of inducing hemogenic specification in the absence of RA. These data show that both the cell cycle regulation and the transcriptional role of RA are necessary for hemogenic specification. Although hemogenic specification

occurs while endothelial cell cycle progression is arrested, after specification, hemogenic endothelial cells must progress into S/G2/M states to divide and give rise to HSPCs. Thus, further understanding of cell cycle control throughout this endothelial-to-hematopoietic transition process is needed.

Our RNA-seq analyses revealed signaling pathway(s) and transcriptional regulators that RA may activate to enable cell cycle arrest and an endothelial to hemogenic endothelial cell fate transition. Interestingly, some of the same RA-induced pathways that regulate murine hemogenic specification, such as Notch and KIT, appear to be conserved in this process in human endothelial cells. Our analyses also revealed crosstalk with other pathways, including SMAD, Wnt, and HH signaling. Thus, it will be important in future studies to elucidate the exact roles of and potential interactions among these factors that may function downstream of RA to regulate the human endothelial cell cycle state and hemogenic specification.

It is not clear why hemogenic specification occurs in early G1 state of the endothelial cell cycle. Our RNA-seq data revealed enrichment of genes associated with hemogenic specification in early G1 phase and genes associated with arterial specification in late G1 phase, consistent with our previous studies (Fang et al., 2017). However, we also observed an enrichment of genes associated with hemogenic endothelial cells (e.g., NOTCH1, DLL4, HEY1, KIT, RUNX1) in late G1 phase. Thus, we propose that hemogenic specification initiates in early G1 and continues into late G1. Similarly, our data also suggest that arterial specification initiates in late G1 and continues into G1/S; that is, an enrichment of genes associated with arterial specification (e.g., BMP2, BMP4, BMP7, WNT2, WNT5B, WNT8A) was observed in G1/S phase in addition to late G1 phase. Other studies showed that expression of developmental regulators such as HOXD1, TBX5, and SOX11 is specifically upregulated in both early and late G1 phases (Singh et al., 2013). In contrast, human stem cells in S and G2 phases have been shown to possess an intrinsic tendency toward the pluripotent state (Gonzales et al., 2015). Despite recent discoveries linking cell cycle state to cell fate decisions in stem cells, understanding the molecular role of the cell cycle state during endothelial cell differentiation and hemogenic specification requires further investigation.

Finally, although our study focused on the process of hemogenic endothelial cell specification, cell cycle control has also been previously implicated in murine arterial endothelial cell specification (Fang et al., 2017), and our RNA-seq data suggest that this is conserved in human endothelial cell development. The use of emerging technologies such as single-cell RNA-seq will enable us to further delineate the heterogeneity within and phenotypic continuum among endothelial cell subtypes and their molecular regulators during their development. Such insights will improve our ability to apply developmental advances to the generation of distinct endothelial cell subtypes for tissue engineering and regenerative medicine. In addition, our system could likely be developed further to optimize the generation of transplantable HSPCs from human hemogenic endothelial cells for clinical therapies.

STAR★METHODS

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Karen K. Hirschi (kkh4yy@virginia.edu).

Materials Availability—This study did not generate new unique reagents.

Data and Code Availability—The accession number for the RNA-seq data reported in this paper is Gene Expression Omnibus (GEO): GSE159694.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture—H1-hESC were obtained from Yale Stem Cell Center and used up to passage 90. H9-Fucci-hESC were obtained from Dr. Ludovic Vallier's lab at Cambridge Stem Cell Institute and used up to passage 90 (Pauklin and Vallier, 2013). All hESC were cultured in mTeSR™ medium (STEMCELL Technologies cat# 85850) on Matrigel-coated plates (Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free, 5 mL, Corning cat# 356230) as described in Fu et al. (2011). Importantly, 50 µg/ml G-418 solution (Sigma cat# 4727878001) was added to the culture medium for H9-Fucci-hESC to eliminate non-transduced cells. For passage, 160 µl/ml Dispase (STEMCELL Technologies cat# 07913) was added to cell culture. Cells were incubated with Dispase at 37°C for 45min. Afterward, cells were transferred to Falcon tubes containing DMEM:F12 medium (GIBCO cat# 11320–033) for washing. 12ml of DMEM:F12 medium was used per 1ml of cell culture medium. Let tubes stand at room temperature for 5–10min or until all cell pellets settle to the bottom of the tubes. Remove supernatant and cells were resuspended in mTeSR™ medium, dispersed into small clumps, and re-plated onto Matrigel-coated plates.

Animals—All animals used were on a C57BL/6 background (female, 8 – 12 weeks old) and all studies were conducted in compliance with Yale's Institutional Animal Care and Usage Committee guidelines. FUCCI-red animals (B6.Cg-Tg(FucciG1)#596Bsi, Riken, cat# RBRC02707) were crossed with either FUCCI-red animals or wild-type C57BL/6J animals (JAX, cat# 000664).

METHOD DETAILS

Differentiation protocol—H1-hESC or H9-Fucci-hESC were passaged using the method described above and re-plated onto Matrigel-coated plates. These hESC colonies were maintained in mTeSR for 24 hours, after which the cells were gently washed with DMEM:F12 and differentiated in chemically defined, serum-free, animal component-free medium STEMdiffAPEL2 (STEMCELL Technologies cat# 05270) supplemented with Protein-Free Hybridoma Medium (PFHM-II, GIBCO, cat# 12040077) by adding 5 mL of PFHM to 100 mL of STEMdiffAPEL2 medium. Growth factors depicted in Figure 1A were added sequentially (Stemolecule CHIR99021, 5 µM, Stemgent cat# 04–0004; Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein, 50 ng/ml, R&D systems cat# 233-FB-025; Recombinant Human BMP-4 Protein, 25 ng/ml, R&D systems cat# 314-BP-010;

Recombinant Human VEGF165, 50 ng/ml, PeproTech, cat# 100–20) as described in Sriram et al. (2015). For certain experiments, cells were treated with RA (0.5 μ M, Sigma-Aldrich, cat# R2625–50MG), RAi (AGN 194310, 6nM, MedChemExpress, cat# HY-16681), CDK4/6i (PD 0332991 isethionate, 2nM, Sigma-Aldrich, cat# PZ0199–5MG), and/or CDK2i (CVT-313, 0.5 μ M, Santa Cruz Biotechnology, cat# 199986–75-9) every 24hrs starting Day5.

qPCR—Transcriptomes were prepared from cultured hESC/hESC-derived cells using RNeasy Plus Micro Kit (QIAGEN, cat# 74034) and 5X All-In-One RT MasterMix (abm, cat# G490). Resulting cDNA libraries were probed by qPCR using published qPCR primers (PrimerBank, sequences listed in Table S1) and miScript SYBR® Green PCR Kit (QIAGEN, cat# 218073).

Giemsa stain—1ml of supernatant was taken on Day8 of differentiation and dispersed onto a microscope slide using a slide centrifuge. Slides were then fixed in methanol for 5min. Upon air drying the slides, Giemsa Stain (Sigma-Aldrich, cat# GS500–500ml) was diluted 1:20 with deionized water and slides were stained for 60min at room temperature. Afterward slides were briefly rinsed with deionized water, air-dried, and mounted to be evaluated under a microscope.

Flow cytometry analysis for cell culture—Buffer list: sorting buffer (HBSS, GIBCO, cat# 14175–095; 1%FBS, Gemini Bio, cat# 100–106; supplemented with 1:500 diluted Normocin - Antimicrobial Reagent, InvivoGen, cat# ant-nr-1); antibody staining buffer (HBSS; 10%FBS; supplemented with 1:500 diluted Normocin); Hoechst staining buffer (DMEM:F12-high glucose, GIBCO, cat# 11965–092; 10%FBS; supplemented with 1:500 diluted Normocin).

Antibody list: Pacific Blue anti-human CD31 Antibody (BioLegend, cat# 303113), FITC anti-human CD31 Antibody (BioLegend, cat# 303104), Alexa Fluor® 700 anti-human CD45 Antibody (BioLegend, cat# 304024), APC/Cyanine7 anti-human CD45 Antibody (BioLegend, cat# 304014), PE/Cy7 anti-human CD309 (VEGFR2) Antibody (BioLegend, cat# 359911), APC anti-human CD117 (c-kit) Antibody (BioLegend, cat# 313206), PE anti-human CD144 (VE-Cadherin) Antibody (BioLegend, cat# 348506), Pacific Blue anti-human CD34 Antibody (BioLegend, cat# 343512), FITC anti-human CD20 Antibody (BioLegend, cat# 302303), Brilliant Violet 421 anti-human CD3 Antibody (BioLegend, cat# 317343), Brilliant Violet 421 anti-human CD14 Antibody (BioLegend, cat# 325627), Alexa Fluor® 594 anti-human CD66b Antibody (BioLegend, cat# 392907), Alexa Fluor® 488 anti-human CD41 Antibody (BioLegend, cat# 303723), APC anti-human CD235ab Antibody (BioLegend, cat# 306607), PE anti-human CD11b Antibody (BioLegend, cat# 301305), Alexa Fluor® 700 anti-human CD45 Antibody (BioLegend, cat# 304024), Zombie NIR Fixable Viability Kit (BioLegend, cat# 423105).

If side population staining was needed, prewarmed Hoechst staining buffer in 37°C water bath for 15min. Differentiated cells were harvested with Accutase (STEMCELL Technologies, cat# 7920) and spun 5min at 1,000 \times rcf. at 4°C to pellet. Cells were then washed once with DMEM:F12 and spun 5min at 1,000 \times rcf. at 4°C to pellet. Cells were

resuspended in prewarmed Hoechst staining buffer such that cells were at a final concentration of 1×10^6 cells/ml. At least 100 μ L of sample (1×10^5 cells) was aliquoted into fresh 1.5 mL tubes for control. The following sample and necessary control tubes were included: Unstained; Hoechst 33342 only; Hoechst 33342 only + Verapamil; all single-color antibody controls; Sample (received all colors, except for Verapamil). Verapamil diluted in 95% ethanol was added to the “Hoechst 33342 only + Verapamil” control tube to a final concentration of 50 μ M. All tubes were incubated for 5 minutes at 37°C. (CAUTION: Verapamil is a potent calcium-channel blocking agent and is extremely toxic. Handle with gloves). Hoechst 33342 was then added to the “Hoechst 33342 only” control, to the Hoechst 33342 + Verapamil only control, and to the “Sample” tube to a final concentration of 5 μ g/ml. All tubes were incubated for 1 hour at 37°C, protected from light. Tubes were gently mixed through inversion every 15 minutes. (CAUTION: Hoechst 33342 is a toxic nuclear dye and should be handled with gloves). Tubes were spun 5min at 1,000 \times rcf. at 4°C to pellet. Supernatant was removed and cell pellets were resuspended at a concentration of 1×10^5 cells/ml in cold antibody staining buffer. Fluorescently-conjugated antibodies were added to appropriate antibody-only control tubes, and to the “Sample” tube. Dilutions need to be optimized for different antibodies and FACS machines. Tubes were incubated on ice for 30 minutes, protected from light. Afterward tubes were spun 5min at 1,000 \times rcf. at 4°C to pellet. Supernatant was removed and cell pellets were resuspended in 600 μ L ice-cold sorting buffer. Samples were strained through mesh filter cap of 5 mL round bottom polystyrene FACS tube (Corning, cat# 352235) and stored on ice, protected from light, for immediate FACS.

Methylcellulose-based blood forming assay—For population level assay, cells were sorted using FACS and seeded onto fibronectin coated dishes (Fibronectin Human Protein, Plasma, 4 μ g/cm², GIBCO, cat# 33016015) as described in Dege and Sturgeon (2017), with MethoCult H4435 Enriched (STEMCELL Technologies, cat# 4435). For single-cell level assay, cells were directly sorted into fibronectin coated 96-well plates using FACS. Afterward, a P1000 tip with the tip trimmed to widen its bore was used to gently resuspend each well of the seeded methylcellulose media in a tissue culture hood to ensure optimal growth.

CFU-E erythroid colony and BFU-E erythroid colony were analyzed 8 days after plating. CFU-GM granulocyte-monocyte colony and CFU-GEMM multipotent hematopoietic progenitor colony were analyzed 14 days after plating.

Dil AcLDL uptake—To demonstrate endothelial phenotypic function of hESC-derived hemogenic endothelial cells in culture, active uptake of DiI AcLDL was measured. On Day3 of culturing of hESC-derived hemogenic endothelial cells in MethoCult H4435 Enriched medium (STEMCELL Technologies, cat# 4435), medium was removed and cells were washed 3 times with 1xPBS (GIBCO, cat# 10010031) to remove the residual methylcellulose medium. 1ml of endothelial cell medium (PromoCell, cat# C-22011) was added to the dish and cells were stained with 10 μ g/ml DiI AcLDL (DiI-Ac-LDL Kit, Cell Applications, cat# 022K) for 4hrs at 37°C and then imaging with fluorescence microscope.

Immunostaining of hematopoietic colonies—Antibody list: Alexa Fluor® 488 anti-human CD41 Antibody (Biolegend, cat# 303723), APC anti-human CD235ab Antibody (Biolegend, cat# 306607), CD66b-Alexa594 (Biolegend, cat# 392907), CD14-BV421 (Biolegend, cat# 325627).

Single hemogenic endothelial cell was seeded in fibronectin coated 96-well plates (Cellvis, cat# P96–1.5P) as described above. Cells were cultured in 100 μ L of methylcellulose medium (STEMCELL Technologies, cat# 4435) per well for 8 days before staining for CFU-E and BFU-E, for 14 days before staining for CFU-GM and CFU-GEMM. For staining, antibodies were 1:100 diluted in IMDM (GIBCO, cat# 12440046). 30 μ L of diluted antibodies was added to each well on top of the methylcellulose medium (pipette slowly so as not to disturb the hematopoietic colonies). Incubate plates at 37°C overnight before imaging with fluorescence microscope.

Flow cytometry analysis for animal tissue—Flow cytometry was performed as described in Fang et al. (2016). The list of antibodies used can also be found in Fang et al. (2016).

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA sequencing—All mRNA samples were isolated using RNeasy Plus Micro Kit (QIAGEN, cat# 74034). Next-generation whole transcriptome Illumina sequencing was performed with the Yale Center for Genome Analysis. Fastq-files were generated with bcl2fastq2_v2.19.0 and then uploaded to the usegalaxy.org platform (Afgan et al., 2018) for quality control and trimming (Galaxy ToolShed v 1.0.2; FASTQ/A short-reads pre-processing tools: http://hannonlab.cshl.edu/fastx_toolkit/) (Blankenberg et al., 2010). Sequences were aligned to the hg38 genome using HISAT2 (v 2.1.0+galaxy4) (Kim et al., 2015) and aligned sequences were quantified with featureCounts (v 1.6.3+galaxy2) (Liao et al., 2014). Differential gene expression analysis was performed with DESeq2 (R package v 1.24.0) (Love et al., 2014) using unmanipulated count data. Heatmaps were generated with ComplexHeatmap (R package v 2.0.0) using data transformed with DESeq2 and batch-corrected with limma (R package v 3.40.6) (Phipson et al., 2016).

Statistical analysis—Two-tailed Student's t tests were performed using Prism 7 for MacOSX software for all analyses. p values < 0.05 were considered as significant: *, p 0.05; **, p 0.01; ***, p 0.001; ****, p 0.0001. Standard deviations are depicted in all graphs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A simple and efficient protocol to derive human hemogenic endothelial cells is described
- Functional human hemogenic endothelial cells are characterized on a clonal level
- Retinoic acid signaling is required for human hemogenic endothelial specification
- Cell-cycle state is a critical determinant of human endothelial cell specification

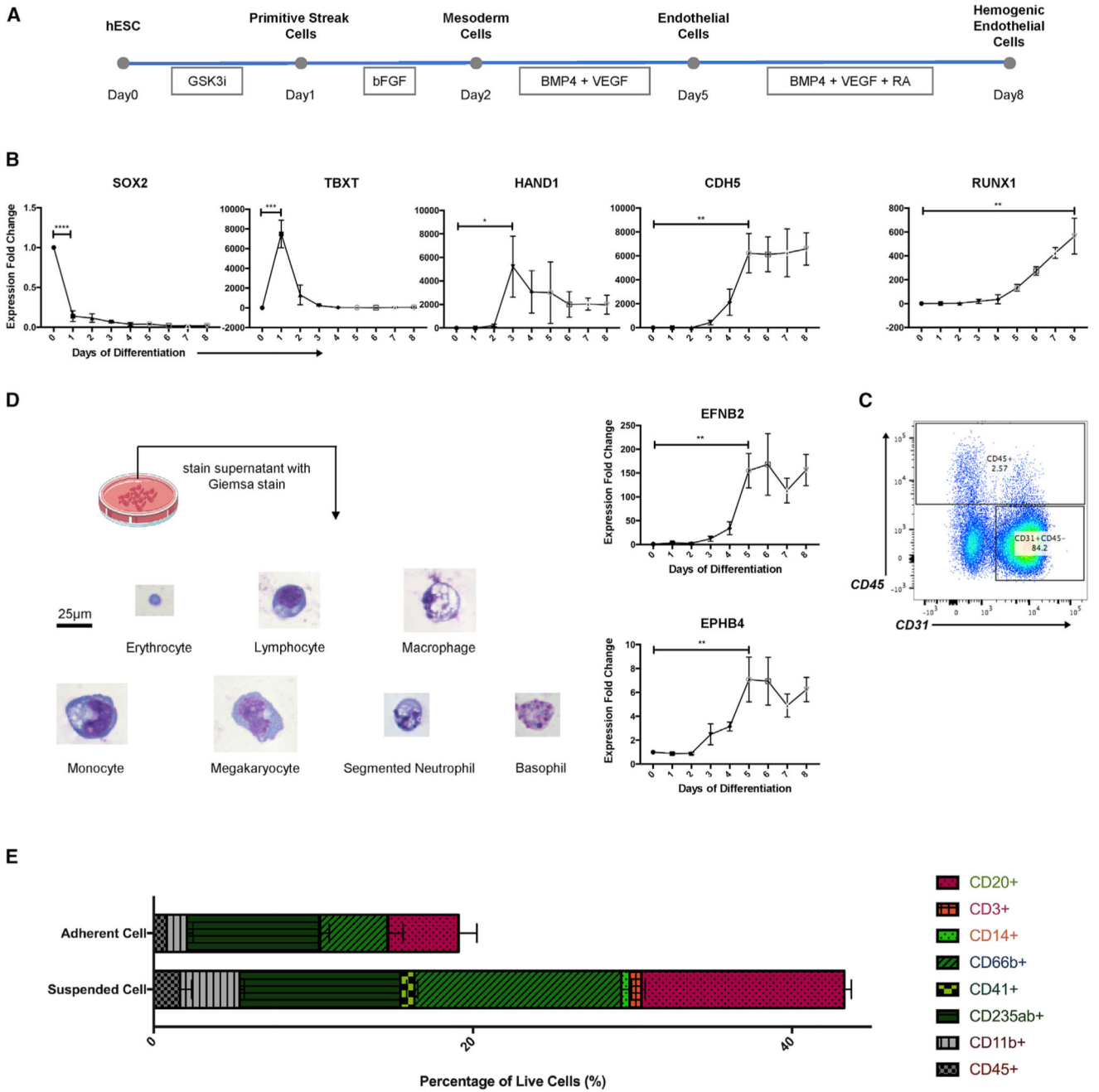


Figure 1. Simple *In Vitro* System to Derive Human Hemogenic Endothelial Cells from Human Embryonic Stem Cells (hESCs)

(A) Schematic representation of differentiation protocol, partially adapted from Sriram et al. (2015).

(B) qPCR analysis of markers associated with pluripotency (SOX2), primitive streak/early mesoderm (TBXT), mesoderm (HAND1), endothelium (CDH5), arterial endothelium (EFNB2), venous endothelium (EPHB4), and hematopoiesis (RUNX1); n = 3 for all groups. Student's t test: p < 0.0001 (SOX2), p = 0.0008 (TBXT), p = 0.0253 (HAND1), p = 0.0028 (CDH5), p = 0.0018 (EFNB2), p = 0.0049 (EPHB4), p = 0.0029 (RUNX1).

(C) Flow cytometry result showed generation of endothelial cells (CD31⁺CD45⁻) and blood cells (CD45⁺) on day 8 (D8).

(D) Giemsa staining revealed generation of all blood cell lineages. Original magnification, 40×. Scale bar, 25 μm.

(E) Flow cytometry result showed generation of multi-lineage blood cells.

Data are represented as means ± SDs.

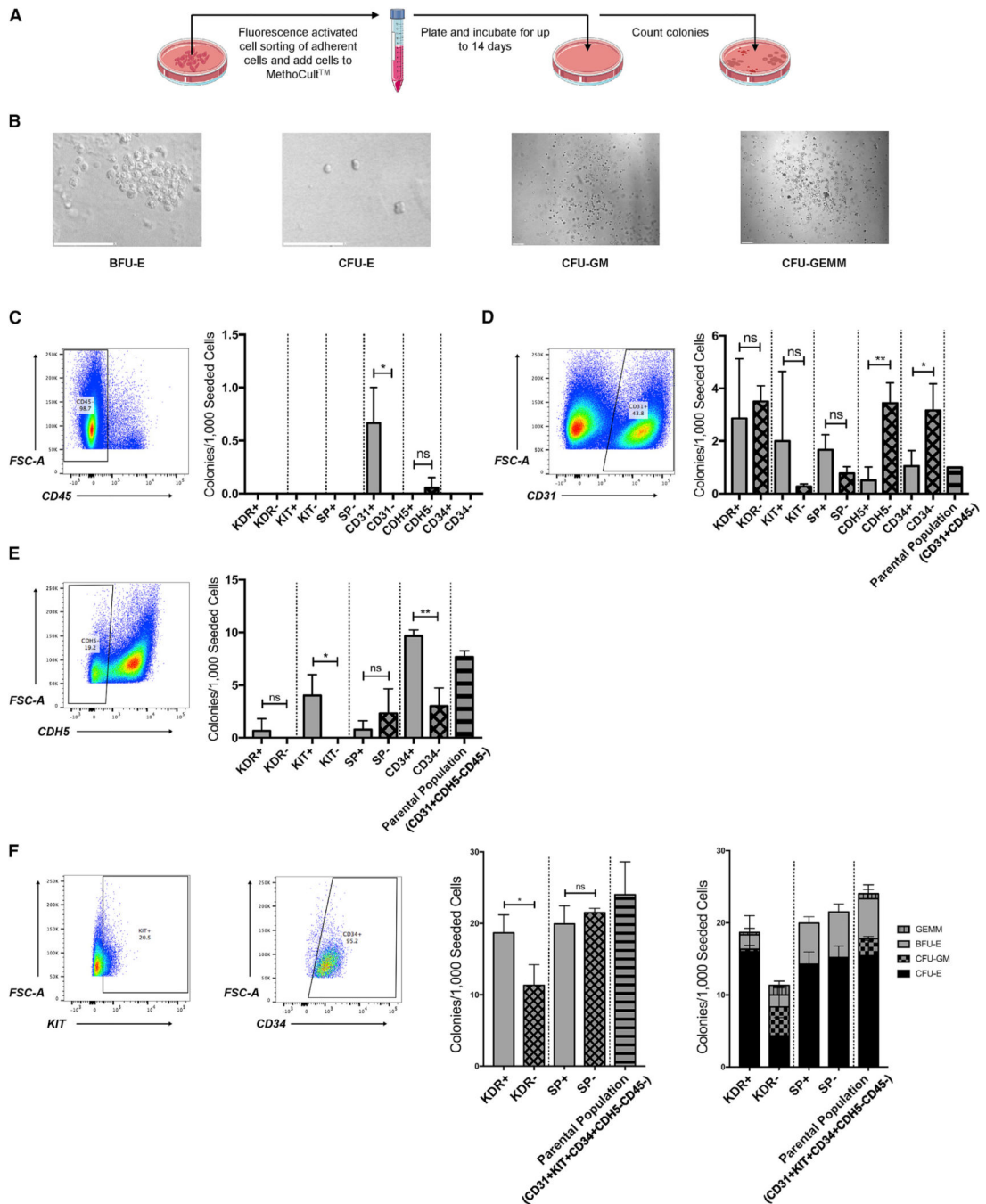


Figure 2. Characterization of the Phenotype of Human Hemogenic Endothelial Cells on a Population Level

(A) Schematic representation of blood-forming assay.

(B) Representative photographs showing morphology of BFU-E erythroid colony, CFU-E erythroid colony, CFU-GM granulocyte-monocyte colony, and CFU-GEMM multipotent hematopoietic progenitor colony. Scale bars, 100 μm.

(C) Among non-HSPC (CD45⁻), hematopoietic potential is enriched within the CD31⁺ population; n = 3 for all groups. Student's t test: p = 0.03. Solid bars represent positive groups. Checkered bars represent negative groups. Striped bars represent parental groups.

(D) Among CD31⁺CD45⁻ cells, hematopoietic potential is enriched within the CDH5⁻ and CD34⁻ populations; n = 3 for all groups. Student's t test: p = 0.005 (CDH5), p = 0.04 (CD34). Solid bars represent positive groups. Checkered bars represent negative groups. Striped bars represent parental groups.

(E) Among CD31⁺CDH5⁻CD45⁻ cells, hematopoietic potential is enriched within the KIT⁺ and CD34⁺ populations; n = 3 for all groups. Student's t test: p = 0.03 (KIT), p = 0.003 (CD34). Solid bars represent positive groups. Checkered bars represent negative groups. Striped bars represent parental groups.

(F) Among CD31⁺KIT⁺CD34⁺CDH5⁻CD45⁻ cells, hematopoietic potential is enriched within the KDR⁺ population (~85% CFU-E, 2% BFU-E, 11% CFU-GM, 2% CFU-GEMM); n = 3 for all groups. Student's t test: p = 0.03. Unless labeled otherwise, solid bars represent positive groups. Checkered bars represent negative groups. Striped bars represent parental groups.

Data are represented as means ± SDs.

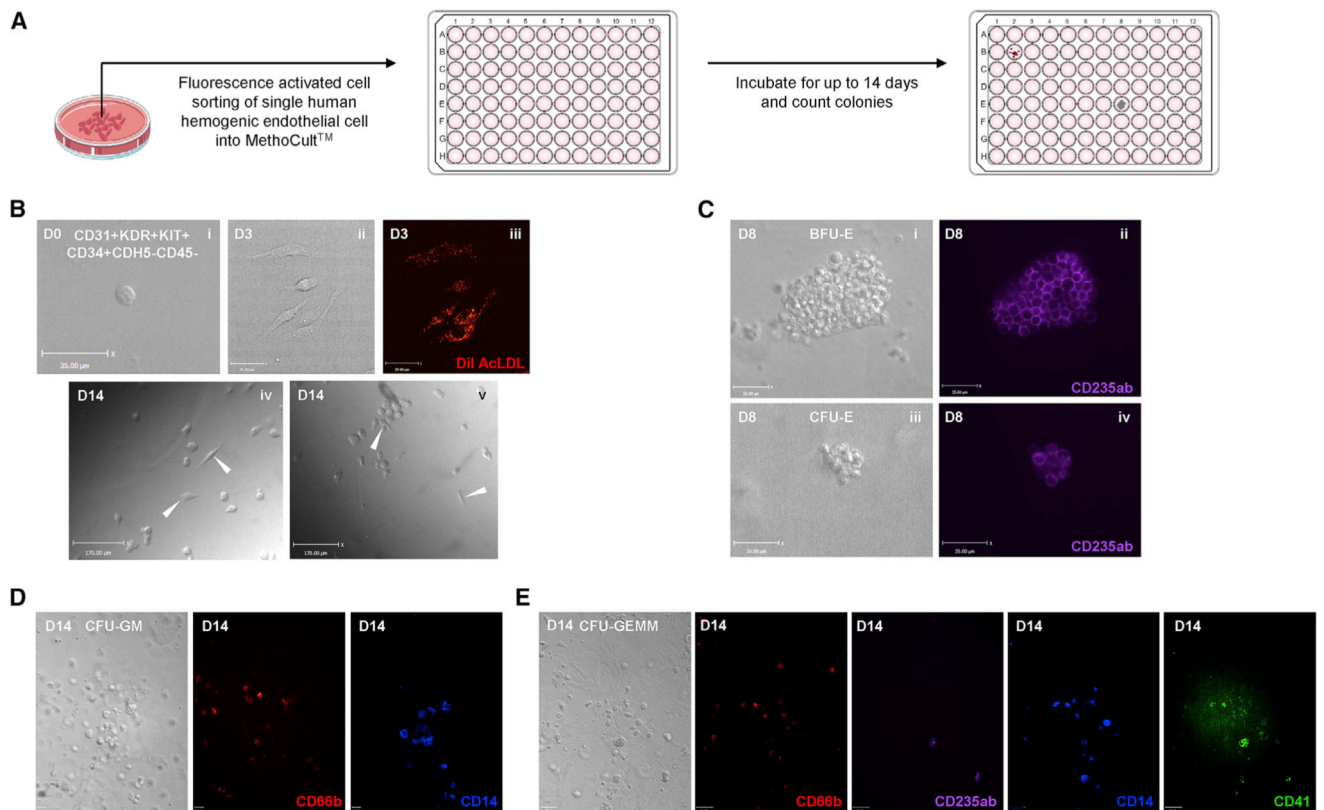


Figure 3. Characterization of the Phenotype of Human Hemogenic Endothelial Cells on a Single-Cell Level

(A) Schematic representation of single-cell blood-forming assay.

(B) Representative photographs showing single human hemogenic endothelial cell on D0 (i), hemogenic endothelial cell colony on D3 (ii), DiI AcLDL uptake of hemogenic endothelial cells on D3 (iii), and adherent hemogenic endothelial cells among blood cells on D14 (iv and v). Scale bars, 35 μ m for (i), (ii), and (iii). Scale bars, 170 μ m for (iv) and (v).

(C) Representative photographs showing BFU-E (i and ii) and CFU-E (iii and iv) on D8, marked by CD235ab. Scale bars, 35 μ m.

(D) Representative photographs showing CFU-GM on D14, marked by CD66b and CD14. Scale bars, 35 μ m.

(E) Representative photographs showing CFU-GEMM on D14, marked by CD66b, CD235ab, CD14, and CD41. Scale bars, 70 μ m.

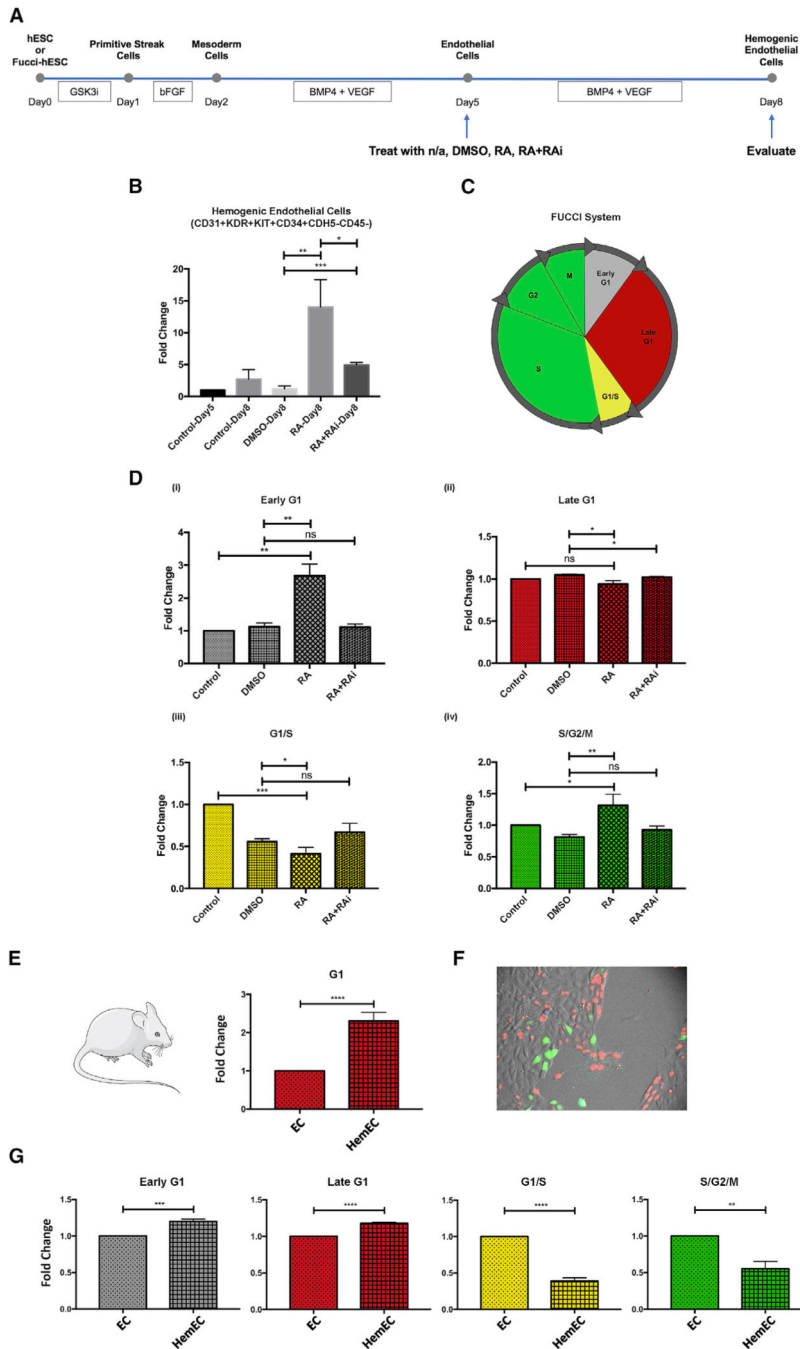


Figure 4. RA Promotes Early G1 Cell Cycle State of hESC-Derived Endothelial Cells and Their Specification toward Hemogenic Endothelial Cells

(A) Schematic representation of experimental design.

(B) RA induces differentiation of hemogenic endothelial cells; $n = 3$ for all groups.

Student's t test: $p = 0.0068$ (DMSO versus RA), $p = 0.0005$ (DMSO versus RA+RAi), $p = 0.0218$ (RA versus RA+RAi).

(C) Schematic representation of the cell cycle states observable using the Fucci system.

(D) 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) Student's t test: $p = 0.0012$ (control versus RA), $p = 0.0019$ (DMSO versus RA). (ii) Student's t test: $p = 0.0122$ (DMSO versus RA), $p = 0.0102$ (DMSO versus RA +RAi). (iii) Student's t test: $p = 0.0002$ (control versus RA), $p = 0.0389$ (DMSO versus RA). (iv) Student's t test: $p = 0.0355$ (control versus RA), $p = 0.0084$ (DMSO versus RA).

(E) 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. Student's t test: $p < 0.0001$.

(F) Picture of H9-FUCCI-hESC-derived endothelial cells. Original magnification, 20 \times .

(G) In hESC culture on D8, significantly more hemogenic endothelial cells were in early and late G1 compared to non-blood-forming endothelial cells; $n = 3$ for all groups. Student's t test: $p = 0.0005$ (early G1), $p < 0.0001$ (late G1), $p < 0.0001$ (G1/S), $p = 0.0016$ (S/G2/M). Data are represented as means \pm SDs.

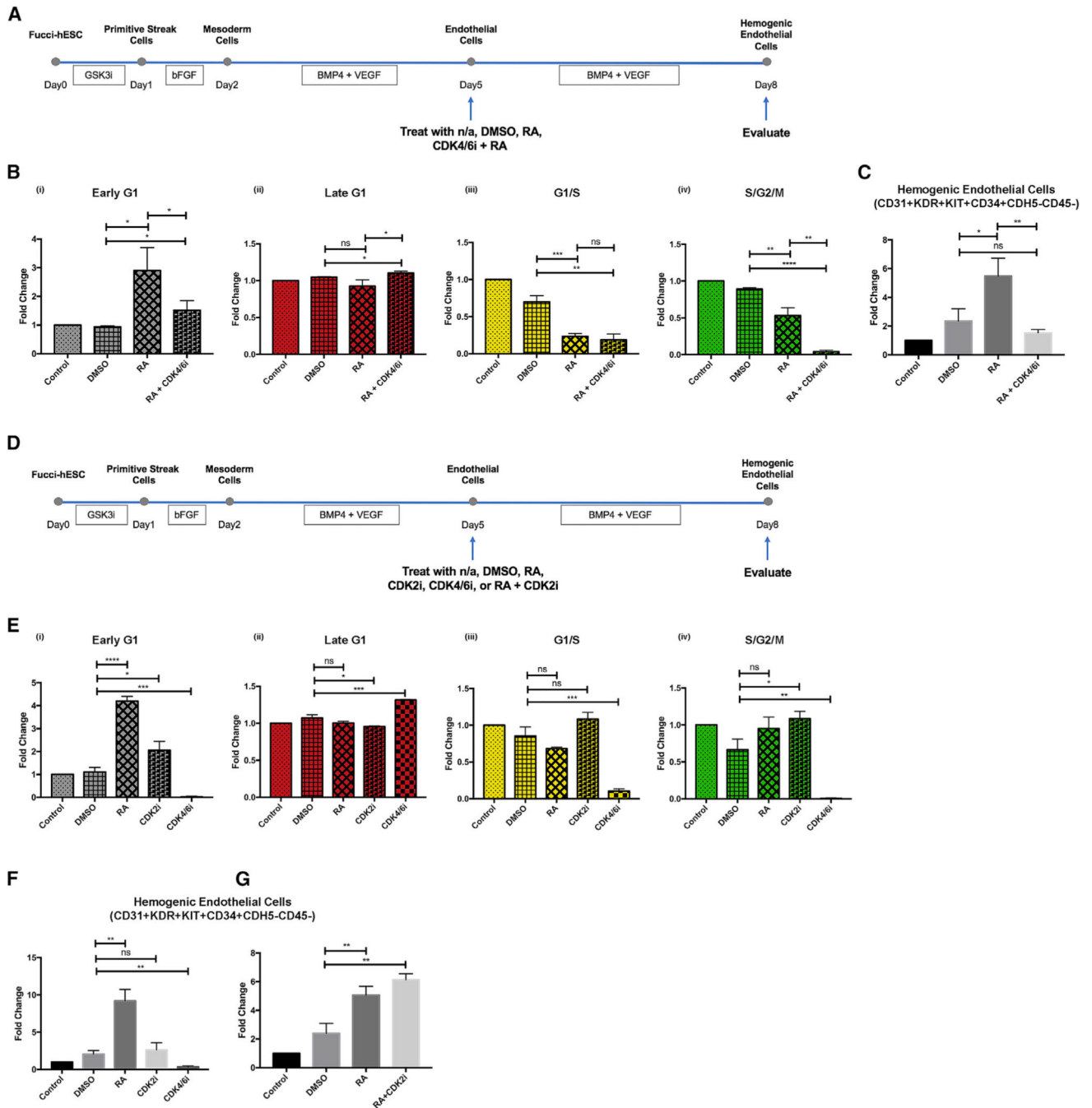


Figure 5. Early G1 Cell Cycle State of Endothelial Cells Is Necessary but Not Sufficient to Enable Hemogenic Specification

(A) Schematic representation of experimental design.

(B) When cells were treated with both RA and CDK4/6i, CDK4/6i overrides effect of RA 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) Student's t test: p = 0.0130 (DMSO versus RA), p = 0.0423 (DMSO versus RA + CDK4/6i), p = 0.0497 (RA versus RA + CDK4/6i). (ii) Student's t test: p = 0.0205 (DMSO versus RA + CDK4/6i), p = 0.0253 (RA versus RA + CDK4/6i). (iii) Student's t test: p =

0.0010 (DMSO versus RA), $p = 0.0016$ (DMSO versus RA⁺CDK4/6i). (iv) Student's t test: $p = 0.0046$ (DMSO versus RA), $p < 0.0001$ (DMSO versus RA⁺CDK4/6i), $p = 0.0015$ (RA versus RA⁺CDK4/6i).

(C) 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. Student's t test: $p = 0.0233$ (DMSO versus RA), $p = 0.0059$ (RA versus RA⁺CDK4/6i).

(D) Schematic representation of experimental design.

(E) 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) Student's t test: $p < 0.0001$ (DMSO versus RA), $p = 0.0196$ (DMSO versus CDK2i), $p = 0.0007$ (DMSO versus CDK4/6i). (ii) Student's t test: $p = 0.0115$ (DMSO versus CDK2i), $p = 0.0007$ (DMSO versus CDK4/6i). (iii) Student's t test: $p = 0.0005$ (DMSO versus CDK4/6i). (iv) Student's t test: $p = 0.0148$ (DMSO versus CDK2i), $p = 0.0014$ (DMSO versus CDK4/6i).

(F) Unlike RA, CDK2i could not induce hemogenic endothelial cell specification; $n = 3$ for all groups. Student's t test: $p = 0.0016$ (DMSO versus RA), $p = 0.0051$ (DMSO versus CDK4/6i).

(G) CDK2i did not artificially suppress cell growth or cell differentiation in culture.

Student's t test: $p = 0.0077$ (DMSO versus RA), $p = 0.0015$ (DMSO versus RA⁺CDK2i).

Data are represented as means \pm SDs

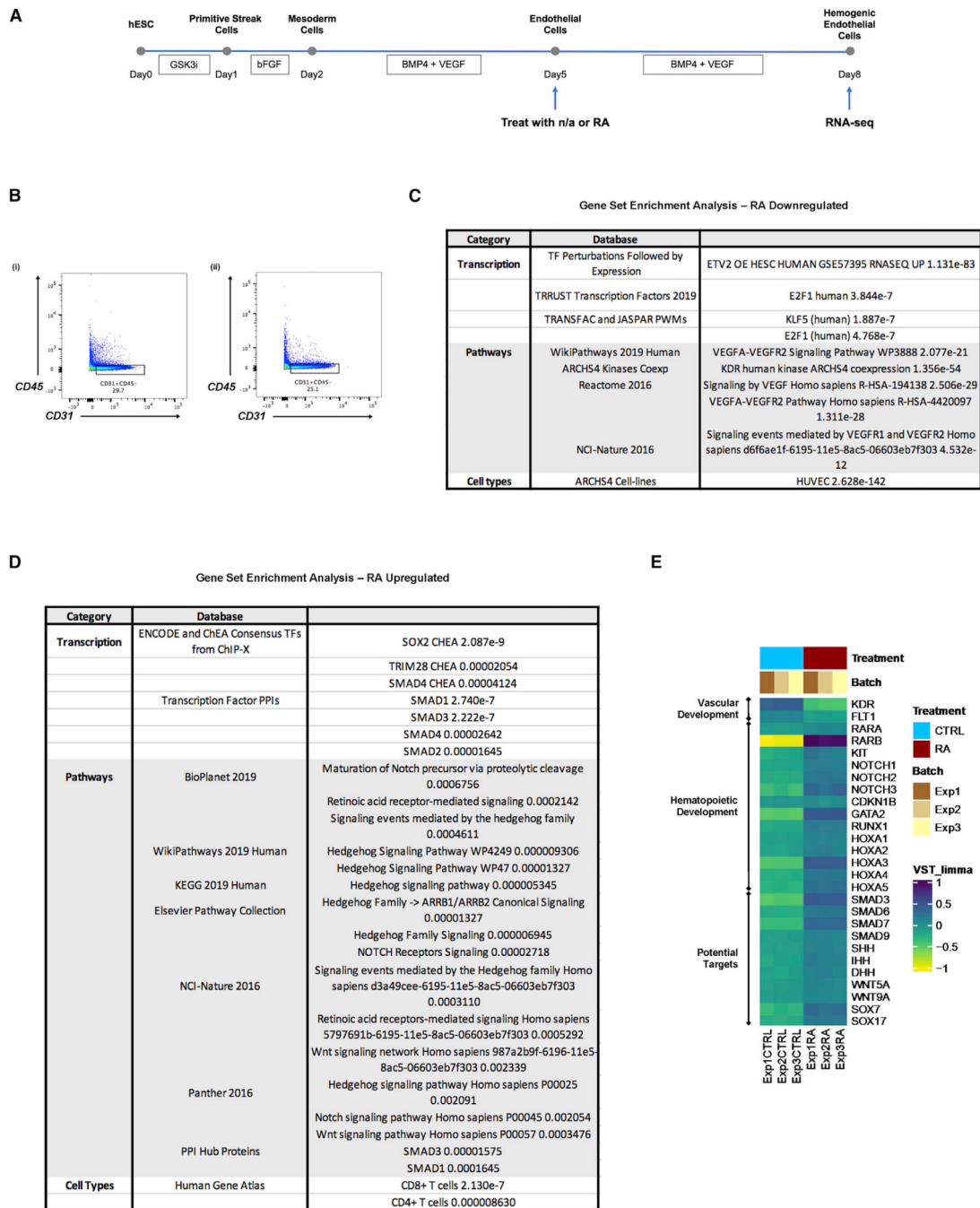


Figure 6. Bulk RNA Sequencing Reveals Candidate Mediators of RA Regulation of Human Endothelial Cell Cycle Control and Hemogenic Specification

(A) Schematic representation of experimental design.
 (B) Endothelial cells (CD31⁺CD45⁻) treated with (i) n/a or (ii) RA were collected on D8 for RNA sequencing using FACS sorting.
 (C) Gene set enrichment analysis was performed for genes exclusively downregulated in RA-treated endothelial cells. Relevant and significant ($p < 0.05$) terms are shown.
 (D) Gene set enrichment analysis was performed for genes exclusively upregulated in RA-treated endothelial cells. Relevant and significant ($p < 0.05$) terms are shown.

(E) Selected markers demonstrate upregulation of genes associated with hematopoietic cell fate in endothelial cells by RA.

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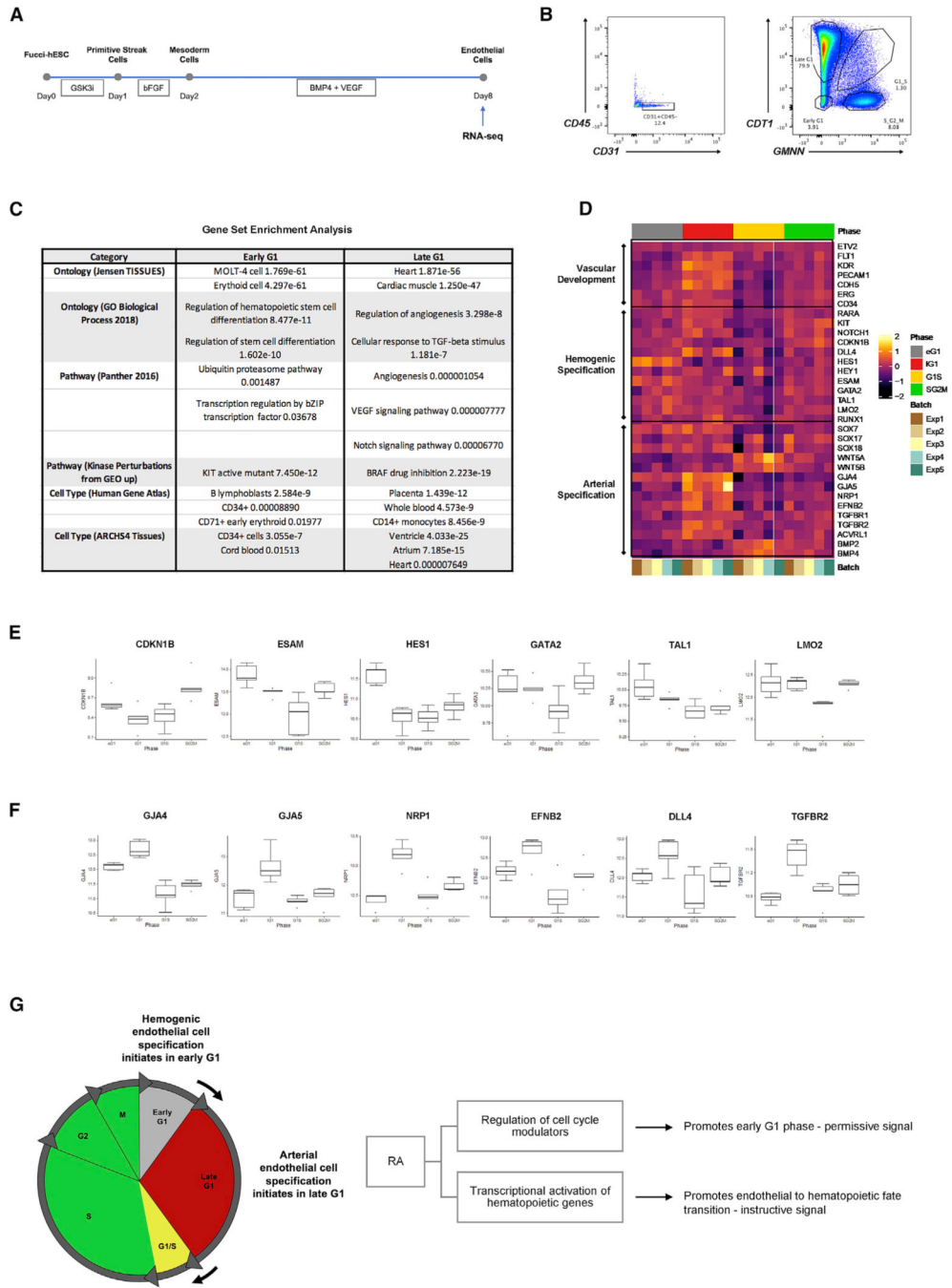


Figure 7. Bulk RNA Sequencing Provides Insights That Link Endothelial Cell Cycle State to Hemogenic Specification

(A) Schematic representation of experimental design.

(B) Endothelial cells (CD31⁺CD45⁻) in different cell cycle phases (early G1, late G1, G1/S, S/G2/M) were collected on D8 for RNA sequencing using FACS sorting.

(C) Gene set enrichment analysis was performed for genes exclusively upregulated in early and late G1 cell cycle phases. Relevant and significant ($p < 0.05$) terms are shown.

(D) Gene marker expression in various cell cycle phases.

(E) Selected markers demonstrate enrichment of genes associated with hematopoiesis in early G1 phase.

(F) Selected markers demonstrate enrichment of genes associated with vascular development and arterial endothelium in late G1 phase.

(G) Schematic representation of working model.

Data are represented as the highest/lowest value within 1.5x interquartile range (IQR) above/below the 75th/25th percentile.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pacific Blue anti-human CD31 Antibody	BioLegend	Cat#303113; RRID:AB_1877196
FITC anti-human CD31 Antibody	BioLegend	Cat#303104; RRID:AB_314330
Alexa Fluor® 700 anti-human CD45 Antibody	BioLegend	Cat#304024; RRID:AB_493761
APC/Cyanine7 anti-human CD45 Antibody	BioLegend	Cat#304014; RRID:AB_314402
PE/Cy7 anti-human CD309 (VEGFR2) Antibody	BioLegend	Cat#359911; RRID:AB_2563551
APC anti-human CD117 (c-kit) Antibody	BioLegend	Cat#313206; RRID:AB_314985
PE anti-human CD144 (VE-Cadherin) Antibody	BioLegend	Cat#348506; RRID:AB_2077978
Pacific Blue anti-human CD34 Antibody	BioLegend	Cat#343512; RRID:AB_1877197
FITC anti-human CD20 Antibody	BioLegend	Cat#302303; RRID:AB_314251
Brilliant Violet 421 anti-human CD3 Antibody	BioLegend	Cat#317343; RRID:AB_2565848
Brilliant Violet 421 anti-human CD14 Antibody	BioLegend	Cat#325627; RRID:AB_2561342
Alexa Fluor® 594 anti-human CD66b Antibody	BioLegend	Cat#392907
Alexa Fluor® 488 anti-human CD41 Antibody	BioLegend	Cat#303723; RRID:AB_2566302
APC anti-human CD235ab Antibody	BioLegend	Cat#306607; RRID:AB_314625
PE anti-human CD11b Antibody	BioLegend	Cat#301305; RRID:AB_314157
Alexa Fluor® 700 anti-human CD45 Antibody	BioLegend	Cat#304024; RRID:AB_493761
Zombie NIR Fixable Viability Kit	BioLegend	Cat#423105
Chemicals, Peptides, and Recombinant Proteins		
Stemolecule CHIR99021	Stemgent	Cat#04-0004
Recombinant Human FGF basic/FGF2/ bFGF (146 aa) Protein	R&D systems	Cat#233-FB-025
Recombinant Human BMP-4 Protein	R&D systems	Cat#314-BP-010
Recombinant Human VEGF165	PeptoTech	Cat#100-20
Retinoic acid	Sigma-Aldrich	Cat#R2625-50MG
AGN 194310	MedChemExpress	Cat#HY-16681
PD 0332991 isethionate	Sigma-Aldrich	Cat#PZ0199-5MG
CVT-313	Santa Cruz Biotechnology	Cat#199986-75-9
Giemsa stain, modified	Sigma-Aldrich	Cat#GS500-500ml
Normocin - Antimicrobial Reagent	InvivoGen	Cat#ant-nr-1
Fibronectin Human Protein, Plasma	GIBCO	Cat#33016015
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, LDEV-free, 5 mL	Corning	Cat#356230
Critical Commercial Assays		
RNeasy Plus Micro Kit	QIAGEN	Cat#74034
5X All-In-One RT MasterMix	abm	Cat#G490
miScript SYBR® Green PCR Kit	QIAGEN	Cat#218073
Dil-Ac-LDL Kit	Cell Applications	Cat#022K
Deposited Data		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNA-seq-1	This paper	GSE159694
RNA-seq-2	This paper	GSE159694
Experimental Models: Cell Lines		
H1-hESC	Yale Stem Cell Center	N/A
H9-Fucci-hESC	Pauklin and Vallier, 2013	N/A
Experimental Models: Organisms/Strains		
Mouse: B6.Cg-Tg(FucciG1)#596Bsi	Riken	Cat#RBRC02707
Mouse: C57BL/6J	JAX	Cat#000664
Oligonucleotides		
See Table S1 for oligonucleotide sequences	N/A	N/A
Software and Algorithms		
Prism 7	Graphpad Software	https://www.graphpad.com/scientific-software/prism/
ImageJ	NIH	https://imagej.nih.gov/ij/
bcl2fastq2_v2.19.0	Illumina	https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html
Galaxy	Afgan et al., 2018	https://usegalaxy.org/
DESeq2	Love et al., 2014	N/A
ComplexHeatmap	Phipson et al., 2016	N/A
limma	Phipson et al., 2016	N/A