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Experimental Hematology

BRIEF COMMUNICATION

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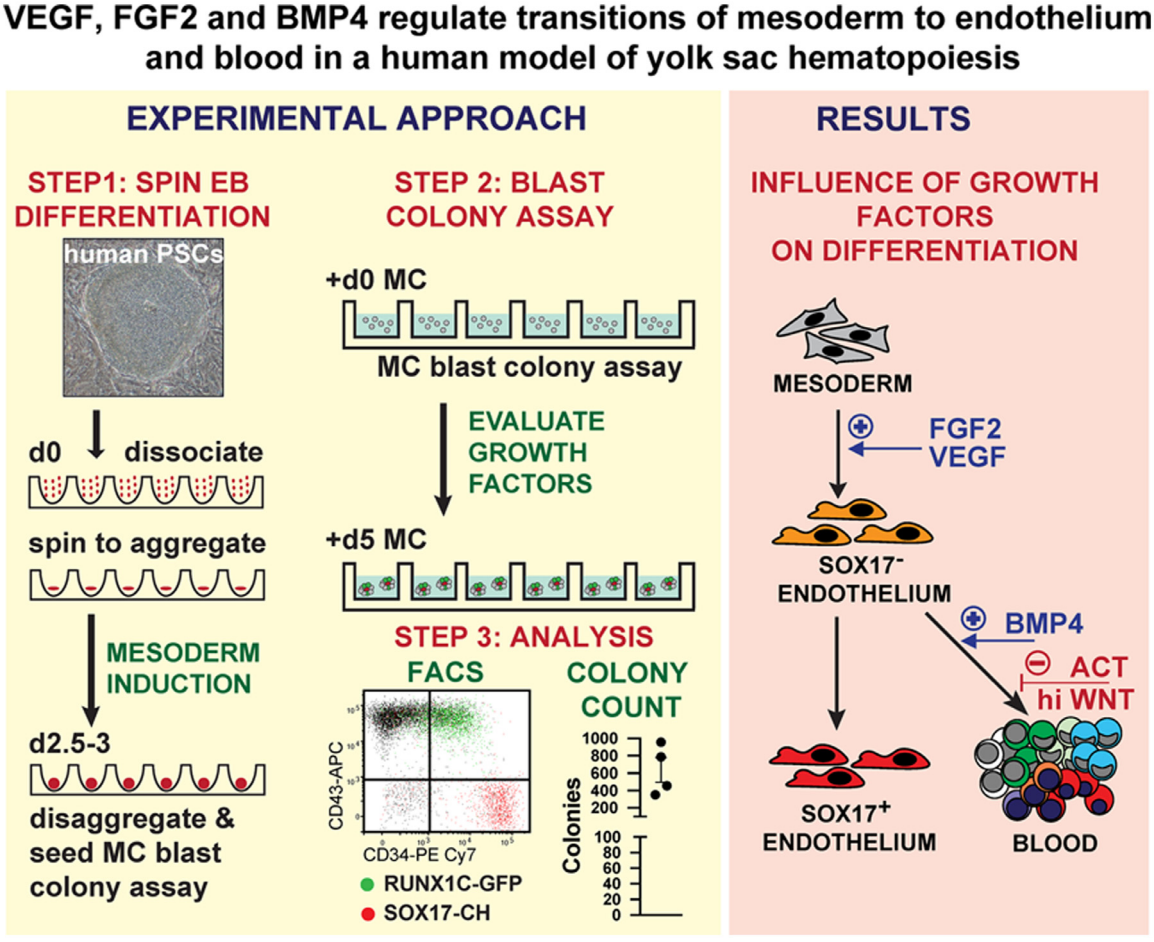

BRIEF COMMUNICATION

VEGF, FGF2, and BMP4 regulate transitions of mesoderm to endothelium and blood cells in a human model of yolk sac hematopoiesis

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Exogenous growth factors play an important role in mediating hematopoietic differentiation of human pluripotent stem cells. We explored the role of different factors in early human blood cell production using blast colony formation in methylcellulose as a surrogate assay for yolk sac hematopoiesis. A reporter cell line that read out endothelial (SOX17$^+$) and hematopoietic (RUNX1C$^+$) progenitors facilitated the identification of basic fibroblast growth and vascular endothelial growth factor as critical signals for the progression of mesoderm into endothelium. Bone morphogenetic protein 4 was needed for the subsequent generation of blood from hemogenic endothelium, and this was antagonized by Activin A or high concentrations of the WNT agonist CHIR-99021. Manipulations of the Hedgehog pathway or inhibition of Notch signaling reduced blast colony frequency but did not perturb cell differentiation. These data help to define distinct roles for prerequisite growth factors that commit mesoderm to hemogenic endothelium and subsequently allocate cells to blood lineages.

HIGHLIGHTS

- Methylcellulose blast colony assay mimics yolk sac hematopoiesis.
- FGF2 and VEGF are critical signals for the progression of mesoderm into endothelium.
- BMP4 is required for subsequent generation of blood from hemogenic endothelium.
- Blocking BMP4 signaling phenocopies aspects of *RUNX1* gene deletion.

The production of blood cells is regulated by multiple signaling pathways. The differentiation of human pluripotent stem cells is an experimentally tractable model allowing analysis of factors influencing early human development in vitro. A number of laboratories have published protocols that direct human pluripotent stem cell differentiation to early hematopoietic cells using the blast colony formation as a surrogate assay for extra-embryonic hematopoiesis from the yolk sac [1–4]. Mesodermal precursors of mammalian hematopoietic cells first arise from the posterior primitive streak [5–7]. This hematopoietically patterned mesoderm then develops into endothelium, from which a hemogenic subset undergoes an endothelial-to-hematopoietic transition to form blood lineages [8–11].

We recently defined the requirements for *RUNX1, GFI1,* and/or *GFI1B* in this human pluripotent stem cell (hPSC) model of yolk sac-like human hematopoiesis [4]. A combination of gene deletion studies and small molecule inhibitors revealed that *RUNX1* was required for all blood formation except an initial wave of *GFI1/1B*-dependent erythropoiesis. To complement this genetic analysis, here we examine the capacity of exogenous growth factors to regulate human blood and endothelial lineage specification in the blast colony model.

METHODS

Ethics

Studies using hPSCs were approved by The Royal Children's Hospital (Reference No. 33001A) and Monash University (Reference No. 2002/225MC) Human Research Ethics Committees.

Cell lines

The dual reporter *SOX17*$^{mCHERRY/w}$ *RUNX1C*$^{GFP/w}$ (SOX-RUNX) H9 and the *RUNX1*$^{-/-}$ (*RUNX1*-KO) PSC lines used in these studies have been previously described [4,12]. Human PSCs were co-

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FFB, ESN, and AGE designed and performed experiments and interpreted data. FFB, EGS, and AGE wrote the article, which all authors edited and approved.

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cultured with mouse embryonic fibroblasts [13], isolated from murine embryos according to Mouse Animal Ethics Approval A774 (Murdoch Children's Research Institute).

Culture and differentiation of hPSCs

The H9 hPSCs used in these studies were provided by ES Cell International and WiCell. Cell lines were regularly tested to exclude mycoplasma contamination and confirm genomic integrity. Human PSC culture and enzymatic passaging of lines were performed as previously described [14].

To identify blast colonies, cells were differentiated toward extraembryonic yolk sac hematopoietic lineages using the spin embryoid body (EB) method in STEMdiff APEL medium (StemCell Technologies, Vancouver, BC, Canada) [14]. Cultures were supplemented for 2.5–3 days with 10–20 ng/mL recombinant human (rh) Activin A (R&D Systems, Minneapolis, MN), 20 ng/mL rh bone morphogenetic protein 4 (BMP4, R&D Systems), 30 ng/mL rh vascular endothelial growth factor (VEGF, PeproTech, Rocky Hill, NJ), and 40 ng/mL stem cell factor (SCF, PeproTech) before dissociation and input into methylcellulose assays.

For analysis, EBs and methylcellulose cultures were harvested and dissociated into a single-cell suspension using TrypLE Select (Invitrogen, Waltham, MA) and passed through a 40-μm cell strainer.

Blast colony-forming cell assay

Blast colony-forming cells were detected by culturing 3×10^3 dissociated cells from day 2.5–3 EBs in a formulation designated MC-APEL (1% methylcellulose in APEL medium) supplemented with 100 ng/mL rh SCF, 2 U/mL rh erythropoietin (EPO, PeproTech), 50 ng/mL rh VEGF, 50 ng/mL rh interleukin (IL)-3 (PeproTech), 50 ng/mL rh IL-6, 50 ng/mL rh thrombopoietin (TPO, PeproTech), 20 ng/mL rh BMP4, and 10 ng/mL rh fibroblast growth factor 2 (FGF2, PeproTech). Colony formation was scored after 9–11 days of differentiation. Where indicated, methylcellulose cultures also contained 5–10 mmol/L SU5402 (Sigma-Aldrich, St. Louis, MO), 0.7 mmol/L DMH-1 (Sigma-Aldrich), 10 ng/mL Activin A, 4 mmol/L SB431542 (Cayman Chemical Co., Ann Arbor, MI), 3–6 mmol/L CHIR-99021 (Tocris Bioscience, Bristol, UK), 5–10 mmol/L IWR-1 (Sigma-Aldrich), 5–10 mmol/L XAV939 (Sigma-Aldrich), 0.25-1 mmol/L SANT-1 (Sigma-Aldrich), 1–3 mmol/L purmorphamine (Sigma-Aldrich), and 10–20 mmol/L *tert*-butyl (2*S*)-2-[[(2*S*)-2-[[2-(3,5-difluorophenyl)acetyl]-amino]propanoyl]amino]-2-phenylacetate (DAPT) (Sigma-Aldrich).

Flow cytometry and cell sorting

Antibodies directed against the following cell surface antigens, listed in Table 1, were used to stain dissociated cells for flow cytometric analysis. Platelet-derived growth factor receptor α (PDGFRα) and TIE2/TEK were detected with secondary antibodies conjugated with allophycocyanin (APC) or phycoerythrin-cyanine 7 (PE-Cy7). Flow cytometric analysis was performed using a BD Biosciences (Franklin Lakes, NJ) LSR Fortessa analyzer. Flow sorting used a BD Biosciences Influx or BD Biosciences FACSAria Fusion cell sorter. Samples were gated using forward scatter area (FSC-A) and forward scatter height (FSC-H) to exclude doublets. Live cells were selected by FSC and propidium iodide exclusion. Positive gates were determined by comparing stained samples with unstained samples.

Transcriptional profiling using RNA sequencing

The RNA sequencing data presented in this article were described previously [4]. Briefly, differentiated SOX-RUNX cultures were harvested from spin EBs at day 2 and from methylcellulose blast colony

Table 1 Flow cytometry antibodies

Antigen	Producer	Fluorochrome	Catalogue no.	Antibody clone	Dilution
Platelet-derived growth factor receptor α (PDGFRα)	BD Pharmingen		556001	aR1	1:100
TIE2/TEK	BD Pharmingen		557039	c33	1:100
CD31	BioLegend	APC	303115	WM59	1:50
	BioLegend	BV421	303123	WM59	1:30
CD34	BioLegend	PE-Cy7	43515	581	1:100
CD43	BioLegend	APC	343206	10G7	1:50
	BD Pharmingen	BV421	562916	1G10	1:30
CD45	BioLegend	BV421	304032	H130	1:30
C-X-C chemokine receptor 4 (CXCR4)	BioLegend	BV421	306518	12G5	1:30
	BioLegend	PE-Cy7	306513	12G5	1:100
Glycophorin A (GYPA)	BD Pharmingen	APC	551336	GA-R2 (HIR2)	1:2,500
Vascular endothelial growth factor receptor 2 (VEGFR2/KDR)	BioLegend	Alexa Fluor-647	338909	HKDR-1	1:10
APC goat anti-mouse IgG	BD Pharmingen	APC	550826	Poly 1270	1:100
	BioLegend	APC	405308	Poly 4053	1:100
PE-Cy7 goat anti-mouse IgG	BioLegend	PE-Cy7	405315	Poly 4053	1:100

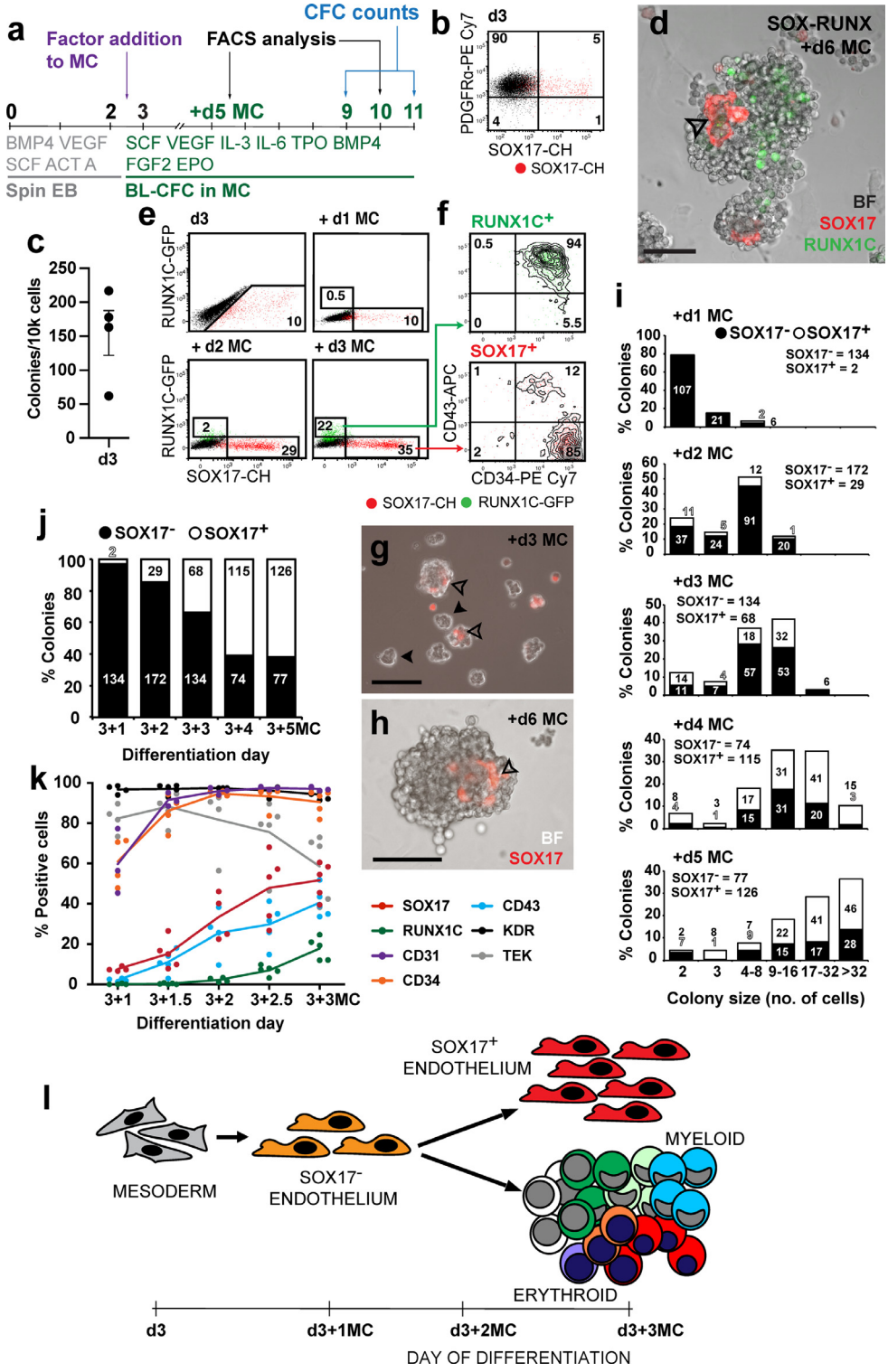

Figure 1 Blast colony development from SOX-RUNX cells. **(A)** Schematic of blast colony differentiation protocol. Differentiation method, time points (day 0−day 11) for factor addition to methylcellulose, growth factor combinations, and analyses (fluorescence-activated cell sorting [FACS] and colony-forming unit [CFC] counts) are indicated. +d11 MC indicates 11 days of methylcellulose (MC) culture after EB differentiation. **(B)** Flow cytometry profile of day 3 (d3) embryoid bodies, with most cells expressing PDGFRα (89.8 ±4 .2%, n = 5 independent experiments). **(C)** Scatter dotplot of blast colonies per 10^4 plated d3 differentiated cells (mean ± SEM, n = 4 experiments). **(D)** After 6 days in methylcellulose culture (+6 MC), the blast colony SOX17$^+$ vascular/stromal core (*black*

assays at days 2+2 and 2+3, and fractioned by flow cytometry on expression of PDGFRα, CD34, CD43, and mCHERRY (SOX17) using a BD Biosciences Influx or BD Biosciences FASCAria Fusion cell sorter. Total RNA was isolated from the sorted fractions using the RNA Isolate II Mini or Micro Kits (Bioline, London, UK) as specified by the manufacturer. cDNA was reverse transcribed using random hexamer priming and the Tetro cDNA synthesis (Bioline) kit in accordance with the manufacturers' instructions. Total RNA was sequenced and analyzed at the Murdoch Children's Research Institute as described [4]. These data have been deposited with Gene Expression Omnibus under Accession No. GSE124086 SuperSeries, GSE124085 SubSeries.

Gene expression analysis

Total RNA was isolated from differentiated hPSCs using the RNA Isolate II Mini or Micro Kits (Bioline) as specified by the manufacturer. cDNA was reverse transcribed using random hexamer priming and the Tetro cDNA synthesis (Bioline) kit in accordance with the manufacturer's instructions. TaqMan gene expression probes (Applied Biosystems, Waltham, MA) and Bioline reagents were used for quantitative real-time polymerase chain reaction using *GAPDH* as the reference gene to normalize the data. TaqMan assays directed toward the following target sequences were used to detect gene expression: *RUNX1* (Hs00231079_m1).

RESULTS AND DISCUSSION

To dissect the early segregation of human blood and endothelial lineages, we used a *SOX17*$^{mCHERRY/w}$ *RUNX1C*$^{GFP/w}$ dual-reporter hPSC line (termed SOX-RUNX cells) [4,12] to track the emergence of SOX17$^+$ endothelial and RUNX1C$^+$ blood progenitors in combination with cell surface antigens indicative of lineage assignment.

Blast colonies generate SOX17-positive endothelium and RUNX1C-positive blood cells

We differentiated SOX-RUNX cells for 2.5–3 days as spin EBs [14] and subsequently transferred the dissociated cells to a methylcellulose-based blast colony-forming cell (BL-CFC) assay (Figure 1A and Methods). At day 3 most differentiated cells expressed the mesendodermal marker PDGFRα (89.8 ± 4.2%, $n = 5$) (Figure 1B), from which BL-CFCs (155 ± 38 colonies per 10^4 cells, $n = 4$) arose (Figure 1C) [2]. Maturing blast colonies comprised RUNX1C-positive and -negative blood cells, SOX17-positive and -negative endothelium, and stromal cells (Figure 1D) [3,4].

In tracking reporter expression, small numbers of SOX17$^+$ cells were evident at day 3 when the cells were seeded into methylcellulose (12.9 ± 4.9%, $n = 5$), but RUNX1C$^+$ cells were not observed until 2 days of methylcellulose culture (denoted +d2 MC) (2.23 ± 0.52%, n = 4) (Figure 1E). As previously described [4], RUNX1C$^+$CD34$^+$CD43$^+$ blood progenitors and a small population of SOX17$^+$CD34$^+$CD43$^+$ hematopoietic cells were observed after 3 days of methylcellulose culture (Figure 1F).

However, most of the SOX17-expressing cells in +d3 MC represented SOX17$^+$CD34$^+$CD43$^-$endothelium with little hemogenic activity [4] (Figure 1F–H). Tracking the emergence and persistence of SOX17-expressing cells revealed that blast colonies of all sizes developed a SOX17$^+$ component after 2–5 days in methylcellulose (Figure 1I). After 5 days of methylcellulose culture, approximately 60% of colonies contained SOX17$^+$ cells (Figure 1J).

Kinetic analysis of surface marker expression revealed the initial SOX17$^-$ endothelial profile of nascent blast colonies with high proportions KDR-, TIE2 (TEK)-, CD31-, and CD34-expressing cells at +d1 MC, followed by expression of SOX17 and CD43 in predominantly mutually exclusive populations at +d1.5 MC (Figure 1K). RUNX1C expression was observed from +d2 MC (Figure 1K). This progression reflected the separation of hematopoietic from endothelial subsets after the first day of methylcellulose culture (Figure 1L). The results from this set of experiments are consistent with the stepwise progression of mesoderm to endothelium and blood cells in the blast colony formation assay [4].

Signaling pathways modulating blast colony formation and differentiation

SOX-RUNX cells were differentiated to hematopoietic mesoderm for 2.5–3 days in medium supplemented with BMP4, Activin A, VEGF, and SCF. Embryoid bodies were dissociated, and cells were seeded in methylcellulose supplemented with cytokines supporting hematopoietic differentiation (Figure 1A). We included or excluded growth factors and inhibitors affecting FGF, VEGF, BMP/Activin, WNT, Hedgehog, and Notch pathways to determine their influence on blast colony formation and differentiation. Blast colonies were enumerated, and differentiation was analyzed by flow cytometry (Figure 1A and Methods).

Analysis of control SOX-RUNX cultures revealed robust blast colony generation (638 ± 140 blast colonies per 10^4 cells, $n = 4$), with

arrowhead) surrounded by RUNX1C$^+$ and RUNX1C$^-$ blood cells can be visualized. Scale bar = 100 μm. Image representative of eight independent experiments. **(E)** Flow cytometric analysis of SOX17 and RUNX1C expression in day 3 (d3) developing blast colonies after 1 day (+d1 MC) (SOX17 7.6 ± 1.6%, RUNX1C 0.1 ± 0.3%), 2 (+d2 MC) (SOX17 33.4 ± 11.5%, RUNX1C 2.2 ± 1.0%) and 3 days (+d3 MC) (SOX17 51.7 ± 8.9%, RUNX1C 17.8 ± 5.6%) of methylcellulose differentiation. Data are means ± SEM; $n = 5$ independent experiments. **(F)** Three-day blast colony cultures (+d3 MC) identify RUNX1C$^+$CD34$^+$CD43$^+$ progenitor and SOX17$^+$CD34$^+$CD43$^-$ endothelial subsets and hemogenic cells (SOX17$^+$CD34$^+$CD43$^+$). Data from $n = 3$ independent experiments. **(G, H)** Images of d3 blast colonies at **(G)** day 3 (+d3 MC) and **(H)** day 6 (+d6 MC) of MC culture, vascular/stromal core (*black arrowhead*), and SOX17$^+$ cells (*hollow black arrowhead*) are indicated. Scale bar = 50 μm **(G)** and 100 μm **(H)**. Images represent four to five independent experiments. **(I)** Percentage of colonies containing SOX17$^+$ and SOX17$^-$ cells from d3 blast colony differentiation from day 1 (+d1 MC) to day 5 (+d5 MC) in methylcellulose shown relative to colony sizes (number of cells). Data represent the sum of the values at each day from four experiments. **(J)** Total percentage of d3 blast colonies containing SOX17$^+$ cells for the indicated colony sizes from d3+1 (3+1) to d3+5 (3+5) in MC culture (mean ± SEM, $n = 4$ experiments). **(K)** Timecourse analysis of d3 differentiating blast colonies revealing expression of endothelial (KDR, CD31, CD34, TEK[TIE2], SOX17) and hematopoietic (CD43, RUNX1C) cell surface markers and reporters from d3+1 (3+1) to d3+5 (3+5) in MC culture ($n = 3$–5 experiments per marker). **(L)** Model of lineage commitment in blast colony differentiation Adapted, with permission, from Bruveris et al. [4].

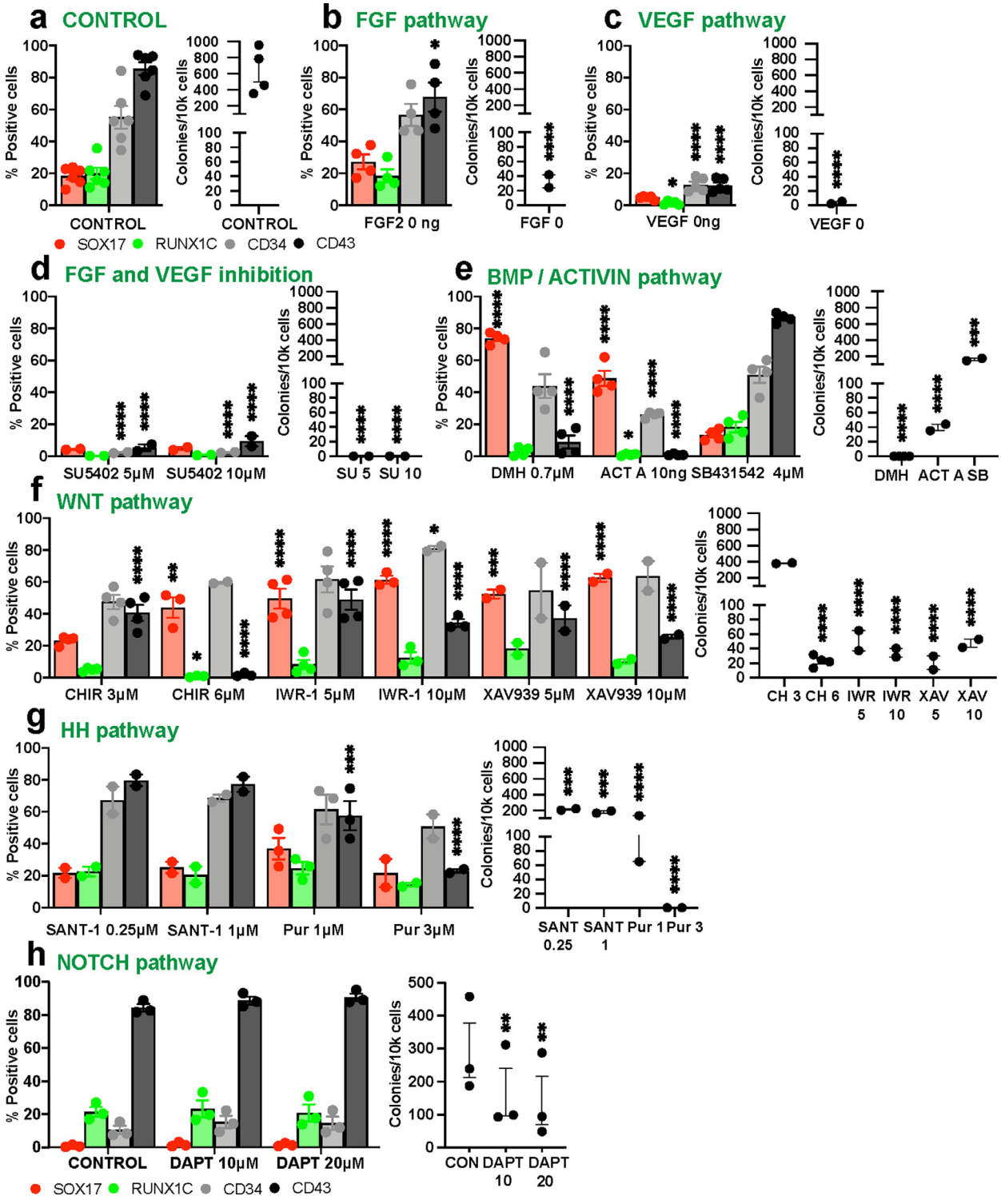

Figure 2 Signaling pathways modulating blast colony formation and differentiation. **(A–H)** Bar graphs illustrating the proportion of cells expressing the indicated reporter or surface marker in blast colonies after 5–6 days of differentiation in methylcellulose and complementary scatter plots illustrating the frequency of blast colonies formed in methylcellulose culture after 9–11 days. Each panel gives the results of stimulation and/or inhibition of the indicated pathway. **(A)** CONTROL differentiation conditions (see Figure 1A). **(B)** FGF pathway. **(C)** VEGF pathway. **(D)** Dual FGF and VEGF inhibition. **(E)** BMP/Activin pathway. **(F)** WNT pathway. **(G)**

$CD34^+$ (55.1 ± 7.1%, $n = 6$) hematovascular precursors, comprising predominantly $SOX17^+$ endothelium (18.4 ± 2.2%, $n = 6$) and $RUNX1C^+$ (20.0 ± 3.6%, $n = 6$) blood cells. The blast colonies included a large proportion of $CD43^+$ (85.7 ± 4.0%, $n = 6$) blood cells (Figure 2A; Supplementary Figure E1A, online only, available at www.exphem.org).

FGFs are required for murine mesoderm formation, with *fgfr-1* deficient mouse embryonic stem cells (ESCs) exhibiting impaired hematopoietic colony formation [15,16]. VEGF signaling is essential for angiogenesis and hematopoiesis, with heterozygous mice embryonic lethal in midgestation from severe defects in vascular and hematopoietic development [17,18]. By removal of exogenous FGF2 and VEGF from our methylcellulose assays, blast colony frequency was significantly reduced for both FGF2 (<19-fold) and VEGF (<150-fold) (Figure 2B, C), consistent with the results of an earlier study [1]. Interestingly, the proportions of blood and endothelial populations were not greatly altered in the absence of FGF2 (Supplementary Figure E1B, online only, available at www.exphem.org), suggesting that FGF2 is required for commitment to colony formation rather than for further differentiation. However, transcriptional profiling studies of blast colony sorted fractions [4] raise the possibility that the effects of FGF2 exclusion are mitigated by autocrine or paracrine production of FGF family members by mesodermal blast colony precursors and their progeny (Supplementary Figure E1C, D, online only, available at www.exphem.org). In the absence of VEGF (Figure 2C; Supplementary Figure E1E, online only, available at www.exphem.org) or with dual inhibition of FGF and VEGF signaling by the small molecule SU5402, colony formation was ablated, and hematopoietic and endothelial marker expression was lost (Figure 2D; Supplementary Figure E1F, online only, available at www.exphem.org), with only stromal cells remaining in the cultures (Supplementary Figure E1, online only, available at www.exphem.org). Although VEGFs and the VEGF receptor, KDR, are expressed during blast colony development, the level of endogenous VEGF is apparently insufficient for blast colony formation, underscoring the requirement for exogenous VEGF at the mesodermal precursor stage for endothelium and blood development (Supplementary Figure E1H, I, online only, available at www.exphem.org).

Next, we investigated the TGF-β family members Activin A and BMP4, two key drivers of early mesoderm differentiation. BMP4 is essential for human blast colony formation [1], and Nodal signaling, provided by Activin A, is required for primitive hematopoietic development in mouse and human PSC cultures [19–22]. To block BMP4 signaling, we omitted exogenous BMP4 from the methylcellulose matrix and substituted it with a BMP pathway inhibitor, dorsomorphin homologue 1 (DMH-1). This promoted $SOX17^+$ endothelium (73.8 ± 1.7%, $n = 4$; control 18.4 ± 2.2%, $n = 6$) at the expense of hematopoietic cells and colonies (Figure 2E; Supplementary Figure E1J, online only, available at www.exphem.org). To manipulate the Nodal pathway, we used Activin A as a surrogate ligand for Nodal, whereas inhibition was achieved via the type I Activin receptor-like kinase (ALK) 5 small molecule antagonist SB431542 (SB). Methylcellulose cultures supplemented with Activin A exhibited a similar decrease in hematopoietic differentiation and colony formation as seen with loss of BMP4 signaling. Inhibition with SB did not change the distribution of endothelial and hematopoietic lineages, although it slightly decreased colony frequency (Figure 2E; Supplementary Figure E1J, online only, available at www.exphem.org). This contrasts with the requirement for Activin A for the formation of $GYPA^+$ erythroid precursors at day 2 of yolk sac-type differentiation from hPSCs [22]. At the equivalent differentiation stage, prior to seeding in methylcellulose, our cultures also included Activin A (Figure 1A). Our experiments revealed little effect of SB on hematopoietic cell generation, although prior work suggests that SB inhibited or augmented blood cell formation depending on the time of addition [23,24]. These reports are consistent with the concept that a "temporal window" for Activin A signaling exists in hematopoiesis [25]. Specifically, if added prior to mesoderm formation (between days 0 and 3), SB inhibited the generation of primitive hematopoietic progenitors [22], and if added later to cultures (day 4 and after), the molecule had little effect or enhanced hematopoietic cell proliferation [23,24].

WNT family members have well-characterized roles in regulating hematopoiesis in mouse and human PSCs [19,25–27]. Canonical WNT pathway activation through methylcellulose supplementation with the glycogen synthase kinase (GSK)-3β inhibitor CHIR-99021 (CHIR) significantly reduced hematopoietic cell generation and blast colony numbers in a concentration-dependent manner (Figure 2F; Supplementary Figure E1M, online only, available at www.exphem.org). These results were consistent with previously reported results from our laboratory [28] and others [22], whereby WNT pathway activation reduced blast colony formation and inhibited primitive hematopoietic differentiation. Conversely, WNT inhibition via the antagonists IWR-1 and XAV939 also significantly decreased blast colony frequency and blood cell production, while favoring $SOX17^+$ endothelial marker expression (Figure 2F; Supplementary Figure E1M, online only, available at www.exphem.org), implying the requirement for low, but not high, levels of WNT signaling.

The highly conserved Hedgehog pathway is required for primitive and definitive hematopoiesis [29]. Addition of the SMOOTHENED antagonist SANT-1 or the agonist purmorphamine reduced colony formation two- to four-fold (Figure 2G; Supplementary Figure E1J, online only, available at www.exphem.org). SANT-1 did not

HH (Hedgehog) pathway (mean ± SEM, n = 2–6 experiments for flow cytometry analysis and n = 2–4 experiments for blast colony frequency). **(H)** Notch pathway. Bar graphs and scatter plots of flow cytometry and colony frequency data from an independent series of experiments analyzed at day 10 of methylcellulose culture. For all panels, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared with control values, using two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test for flow cytometry data and one-way ANOVA with Dunnett's multiple comparison test for colony frequency data. Growth factors: fibroblast growth factor (FGF), Activin A (ACT A), vascular endothelial growth factor (VEGF). Concentrations are indicated in nanograms per milliliter. Small molecules: Dual FGF and VEGF inhibitor, SU5402 (SU), FGF receptor antagonist; DMH-1 (DMH), inhibitor of the bone morphogenic protein (BMP) type I receptor, activin receptor-like kinase 2 (ALK2); SB431542 (SB), inhibitor of the transforming growth factor-β (TGF-β) type I receptor, activin receptor-like kinase 5 (ALK5); CHIR-99021 (CH), GSK-3β inhibitor leading to WNT activation; IWR-1 (IWR), WNT inhibitor; XAV939 (XAV), WNT inhibitor; SANT-1 (SANT), Hedgehog/Smoothened antagonist; purmorphamine (Pur), Hedgehog/Smoothened agonist; DAPT, γ-secretase inhibitor of Notch signaling. Concentrations are indicated in millimolar.

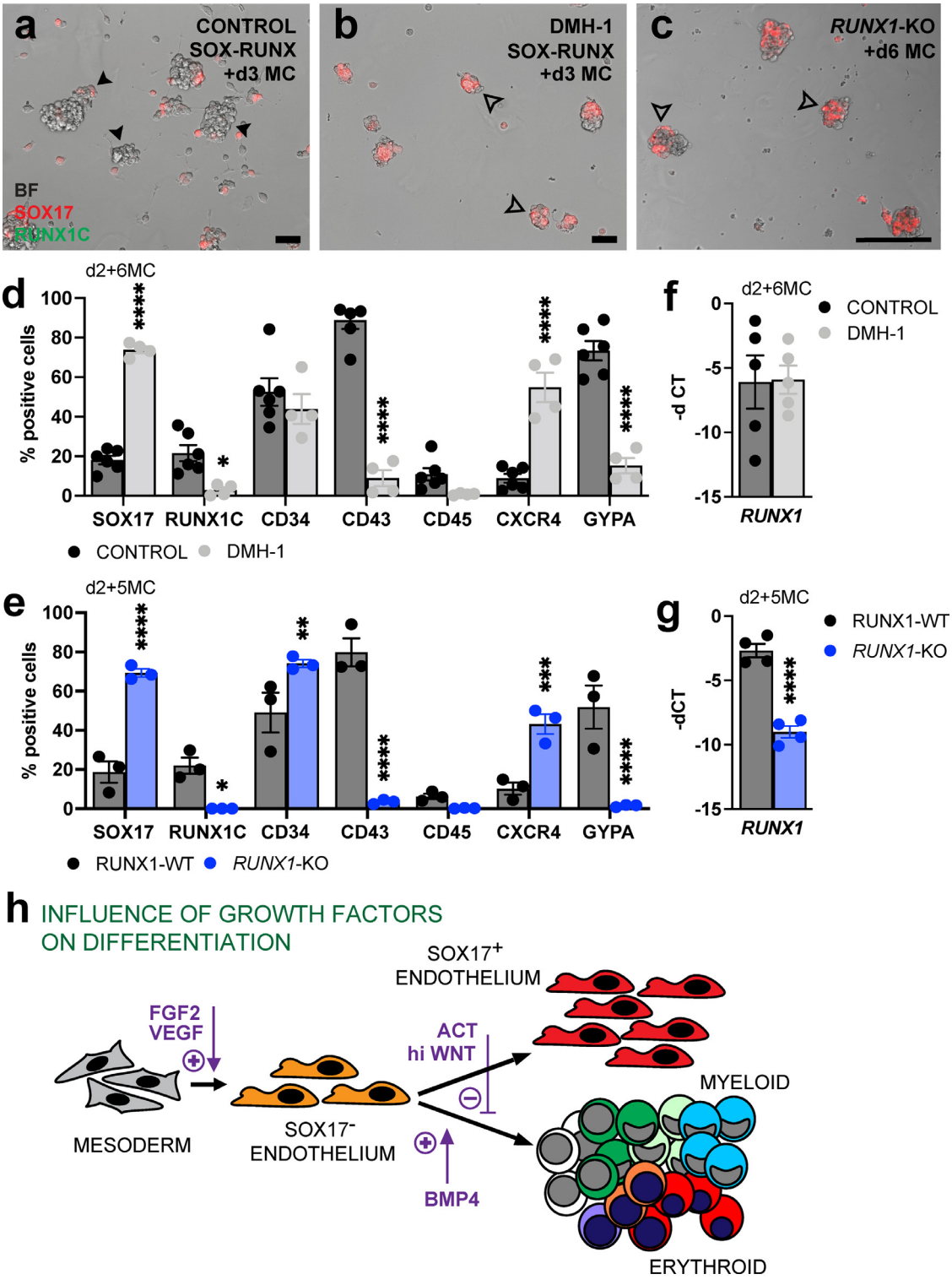

Figure 3 Inhibiting BMP signaling or activating WNT activity perturbs blast colony hematopoietic capacity. **(A–C)** Images of hematopoietic colonies (*black arrowheads*) generated from **(A)** control SOX-RUNX cultures after 3 days of MC cultures (+d3 MC) and

significantly alter hematopoietic or endothelial surface marker expression, but purmophamine reduced CD43$^+$ cells and, at high concentrations, ablated colony formation, perhaps because of nonspecific toxicity (Figure 2G and Supplementary Figure E1N, online only, available at www.exphem.org). The reduction in colony formation with SANT-1 was consistent with prior reports of a block in erythroid proliferation and differentiation by the SMOOTHENED antagonist, cyclopamine [30].

Notch signaling is required for normal angiogenesis and for HSC development but is reported not to be required for yolk sac hematopoietic colony formation [31–34]. To explore the role of Notch signaling in human blast colony formation, we blocked Notch signaling using the γ-secretase inhibitor DAPT (tert-butyl (2*S*)-2-[[(2*S*)-2-[[2-(3,5-difluorophenyl)acetyllaminol- propanoyllaminol-2-phenylacetate) in a separate series of experiments. The distribution of hematopoietic and endothelial lineages was similar between the control and the DAPT supplemented cultures (Figure 2H; Supplementary Figure E1O, online only, available at www.exphem.org), but colony frequency was halved in the presence of DAPT (Figure 2H). These results are consistent with previous mouse ESC data showing a decline in erythroid EMP-like colonies after the addition of a γ-secretase inhibitor [35]. This supports our argument that the blast colony forming assay largely measures an EMP-like cell during hPSC differentiation that displays a degree of sensitivity to Notch signals [4].

Blocking BMP signaling phenocopies differentiation of *RUNX1*-deleted cells

The addition of the small molecule DMH-1 to the methylcellulose culture strongly selected for SOX17$^+$ endothelial differentiation at the expense of hematopoiesis (Figure 2E; Supplementary Figure E1J, online only, available at www.exphem.org). In DMH-1 cultures, characteristic blast colonies were replaced by SOX17$^+$ endothelial and stromal "core" colonies, reminiscent of structures seen in blast colony assays of *RUNX1* knockout cells [4] (Figure 3A–C).

DMH-1 and *RUNX1*-KO cultures were marked by loss of the hematopoietic markers RUNX1C, CD43, CD45, and GYPA and elevation of endothelial markers SOX17 (DMH-1, 73.8 ± 1.7%, $n = 4$; control 18.0 ± 2.2%, $n = 6$; *RUNX1*-KO, 69.3 ± 2.1%, $n = 3$; control 18.7 ± 5.4%, $n = 3$) and CXCR4 (DMH-1, 54.9 ± 7.4%, $n = 4$; control 8.8 ± 2.2%, $n = 6$; *RUNX1*-KO, 43.2 ± 5.1%, $n = 3$; control 10.2 ± 3.1%, $n = 3$) (Figures 2E and 3D, E). Examination of gene expression revealed similar levels of *RUNX1* in control and DMH-1 treated cells, in contrast to the loss of *RUNX1* expression in the *RUNX1* knockout samples (Figure 3F, G). Examination of RNA sequencing data revealed that mesoderm cells seeding the blast colony assay expressed the closely related *BMP2* and *BMP4* genes and that *RUNX1* was not expressed (Supplementary Figure E1K, online only, available at www.exphem.org). At the SOX17$^-$ endothelial stage 1 day later, *RUNX1* was expressed but endogenous *BMP* expression had waned (Supplementary Figure E1K, online only, available at www.exphem.org). These data suggested that hemogenic endothelium (marked by *RUNX1* expression) formed in the absence of exogenous BMP4, but that the lower levels of endogenous *BMP* in SOX17$^-$ endothelium were insufficient to support the endothelial-to-hematopoietic transition.

In summary, we have elucidated a requirement for VEGF and FGF2 in human blast colony differentiation during the transition of mesoderm to SOX17$^-$ endothelium (Figure 3H). BMP4 was needed for cells to undergo a subsequent step of endothelial-to-hematopoietic transition (Figure 3H). Similarly, Activin A and high concentrations of CHIR inhibited hematopoiesis, leaving residual SOX17$^+$ endothelial and stromal cells (Figure 3H). These studies help to define distinct roles for prerequisite growth factors that commit mesoderm to hemogenic endothelium and subsequently allocate cells to blood lineages.

Conflict of interest disclosure

The authors declare no competing financial interests.

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stromal/vascular colonies (*hollow black arrowheads*) observed after 3 days in **(B)** DMH-1 supplemented cultures, which phenocopied **(C)** *RUNX1*-KO cultures at day 6 of methylcellulose development (+d6 MC) [4]. Scale bars = 50 μm (SOX-RUNX, DMH-1) and 100 μm (*RUNX1*-KO). **(D, E)** Flow cytometry of cell surface marker and reporter expression in day 5–6 blast colony cultures revealed similarly increased endothelial populations (SOX17, CXCR4) and decreased hematopoietic populations (RUNX1C, CD43, CD45 and GYPA) in cultures with **(D)** DMH-1 included in the methylcellulose compared with control cultures (mean ± SEM, $n = 4$–6 experiments) and **(E)** in *RUNX1*-KO compared with *RUNX1*-WT differentiation cultures (mean ± SEM, $n = 3$ experiments) [4]. *$p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, compared with control values, using two-way ANOVA with Dunnett's multiple comparisons test. **(F, G)** Relative gene expression (negative delta [−d] crossing threshold [Ct]) assayed by real-time polymerase chain reaction of *RUNX1* at day 5–6 in methylcellulose culture in supplemented with **(F)** DMH-1 0.7 mmol/L or **(G)** *RUNX1*-KO, compared with *RUNX1*-WT control cultures (mean ± SEM, $n = 4$ or 5 experiments) [4]. ****$p < 0.0001$, compared with control values, using two-way ANOVA with Dunnett's multiple comparisons test. **(H)** Cartoon summarizing the influence of growth factor signaling on the specification of endothelial cells and blood development.

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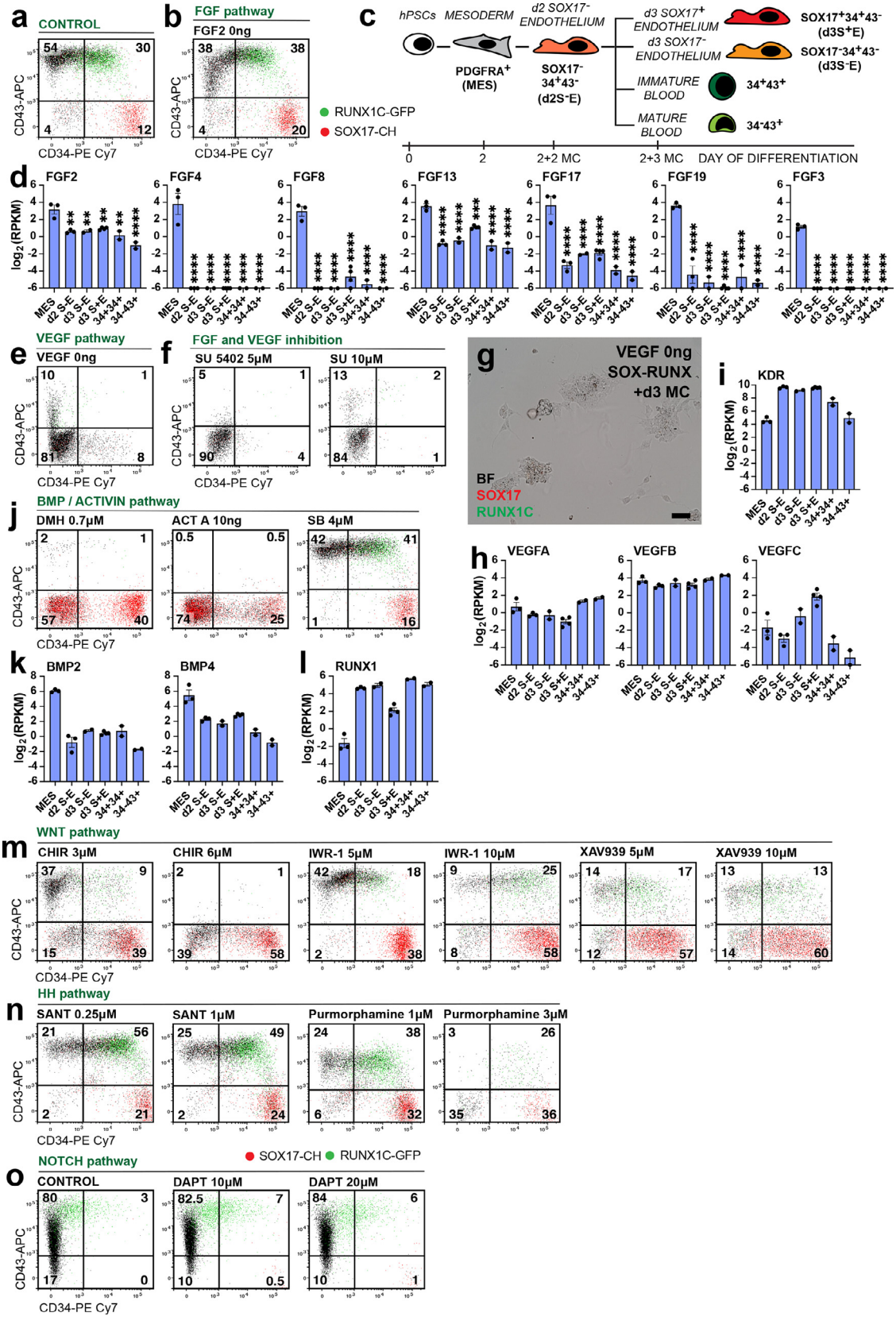

Supplementary Figure E1 Flow cytometry and RNA sequencing profiles of blast colony differentiation.

(a) Representative flow cytometry plot of day 5-6 methylcellulose cultures, differentiated under CONTROL conditions (see Figure 1a) showing CD43 and CD34 expression in combination with RUNX1C-GFP and SOX17-CH reporters. Subsequent panels show results of stimulation and/or inhibition of pathways as indicated. **(b)** FGF pathway. **(c)** Scheme showing sorted fractions collected for RNA-Seq analysis. D2 sorted cells from embryoid bodies: PDGFRa+ mesoderm (MES). D2 MES cells cultured for two days in methylcellulose (2+2 MC): SOX17$^-$ENDO [SOX17$^-$CD34$^+$CD43$^-$] (d2S$^-$E). D2 MES cells cultured for three days in methylcellulose (2+3 MC): SOX17$^+$ENDO [SOX17$^+$CD34$^+$CD43$^-$] (d3S$^+$E), SOX17$^-$ENDO [SOX17$^-$CD34$^+$CD43$^-$] (d3S$^-$E), CD34+CD43+ (34$^+$43$^+$) immature blood and CD34-CD43+ mature blood (34$^-$43$^+$) (adapted from Bruveris et al., 2020). **(d)** FGF family expression in RNA-Seq sorted fractions (as shown in c) illustrating high expression of *FGF* genes in mesoderm. **(e, f)** Flow cytometry plots of **(e)** VEGF pathway and **(f)** FGF and VEGF inhibition. **(g)** Confocal image of cells differentiated under VEGF 0 ng conditions after three days of methylcellulose culture (+d3 MC). Scale bar, 50μm. **(h)** *VEGFA-C* growth factor and **(i)** VEGFR-2 receptor *KDR* expression in RNA-Seq sorted fractions revealing persistent expression of VEGF in the differentiated fractions and upregulation of *KDR* expression after the mesoderm stage. **(j)** Flow cytometry plots of BMP/Activin pathway. **(k)** *BMP 2,4* growth factor and **(l)** *RUNX1* expression in RNA-Seq sorted fractions revealing maximal *BMP* expression in mesoderm and *RUNX1* upregulation after the mesoderm stage. **(m-o)** Flow cytometry plots of **(m)** WNT pathway, **(n)** HH (HEDGEHOG) pathway and **(o)** NOTCH pathway. Flow cytometry plots shown in panels a, b, e, f, j, m, n are representative of 2-6 independent experiments from day 5-6 of methylcellulose cultures, panel o is representative of 3 independent experiments at day 10 of methylcellulose culture. Growth factors: fibroblast growth factor (FGF), ACTIVIN A (ACT A), vascular endothelial growth factor (VEGF). Small molecules: SU5402 (SU), FGF receptor antagonist; DMH-1 (DMH), inhibitor of the bone morphogenic protein (BMP) type-I receptor, activin receptor-like kinase 2 (ALK2); SB431542 (SB), inhibitor of the transforming growth factor-β (TGF-β) type I receptor, activin receptor-like kinase 5 (ALK5); CHIR-99021 (CH), GSK-3β inhibitor leading to WNT activation; IWR-1 (IWR), WNT inhibitor; XAV939 (XAV), WNT inhibitor; SANT-1, Hedgehog/Smoothened antagonist; Purmorphamine, Hedgehog/Smoothened agonist; DAPT, γ-secretase inhibitor of NOTCH signaling.