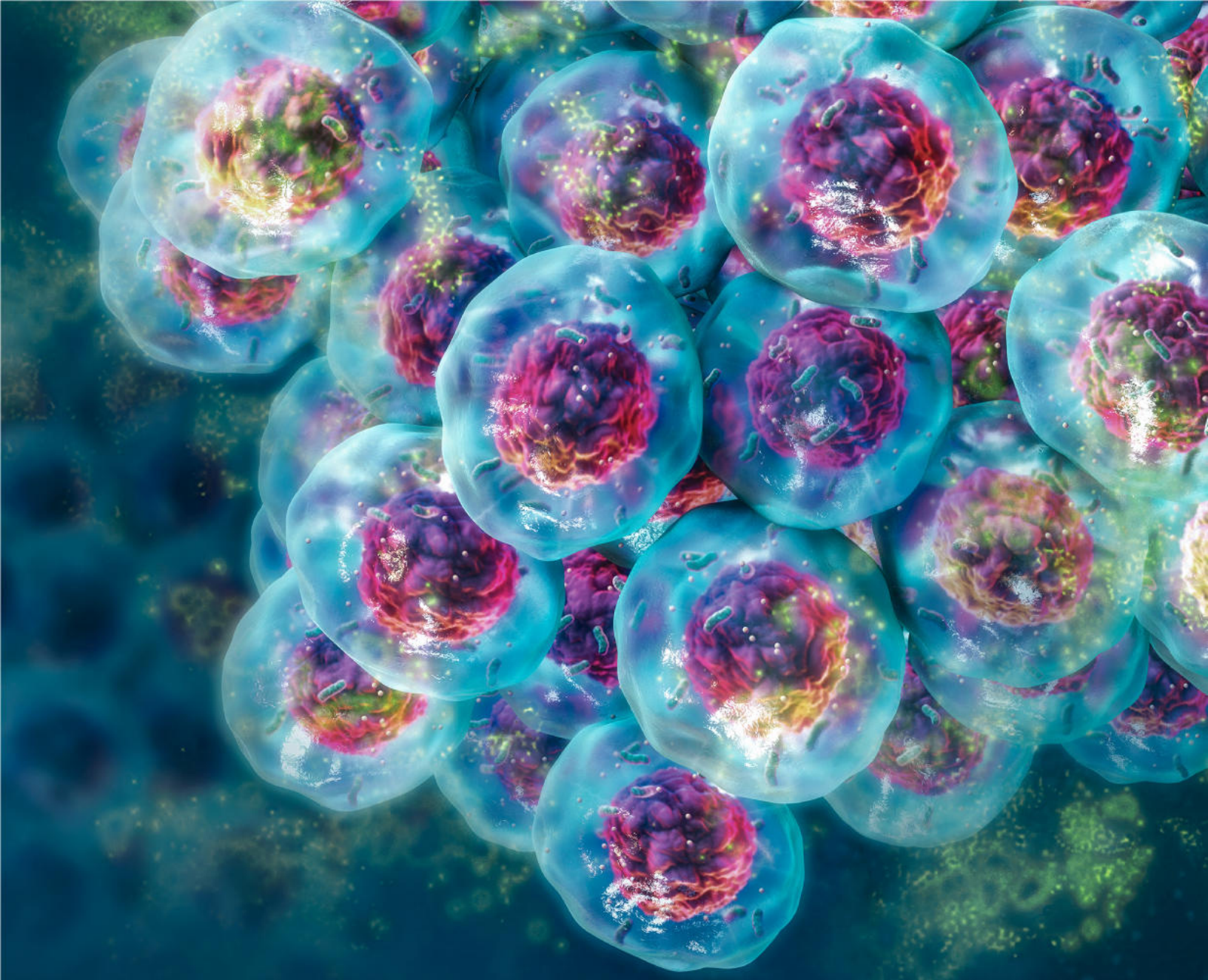

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# *RUNX1c* Regulates Hematopoietic Differentiation of Human Pluripotent Stem Cells Possibly in Cooperation with Proinflammatory Signaling

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## ABSTRACT

**Runt-related transcription factor 1 (Runx1) is a master hematopoietic transcription factor essential for hematopoietic stem cell (HSC) emergence. *Runx1*-deficient mice die during early embryogenesis due to the inability to establish definitive hematopoiesis. Here, we have used human pluripotent stem cells (hPSCs) as model to study the role of RUNX1 in human embryonic hematopoiesis. Although the three *RUNX1* isoforms *a, b*, and *c* were induced in CD45+ hematopoietic cells, *RUNX1c* was the only isoform induced in hematoendothelial progenitors (HEPs)/hemogenic endothelium. Constitutive expression of *RUNX1c* in human embryonic stem cells enhanced the appearance of HEPs, including hemogenic (CD43+) HEPs and promoted subsequent differentiation into blood cells. Conversely, specific deletion of *RUNX1c* dramatically reduced the generation of hematopoietic cells from HEPs, indicating that *RUNX1c* is a master regulator of human hematopoietic development. Gene expression profiling of HEPs revealed a RUNX1c-induced proinflammatory molecular signature, supporting previous studies demonstrating proinflammatory signaling as a regulator of HSC emergence. Collectively, *RUNX1c* orchestrates hematopoietic specification of hPSCs, possibly in cooperation with proinflammatory signaling.** STEM CELLS *2017;35:2253–2266*

## SIGNIFICANCE STATEMENT

This study aimed to elucidate the role of Runt-related transcription factor 1 (*RUNX1*) isoforms throughout hematopoietic specification of human embryonic stem cells (hESCs). Results show that *RUNX1c* isoform is the first to be induced in hematoendothelial progenitors (HEPs), while the other main RUNX1 isoforms are upregulated later in hESC-derived CD45+ hematopoietic cells. Constitutive expression of *RUNX1c* in hESCs improved the generation of HEPs and CD45+ hematopoietic cells, and specific deletion of this isoform exclusively affected hematopoietic differentiation from HEPs. Furthermore, evidence that RUNX1c-overexpression in HEPs activates a proinflammatory signature in accordance with recent in vivo results is presented. The results of this study advance knowledge of how human embryonic hematopoiesis occurs and could improve current protocols for hematopoietic differentiation from human pluripotent stem cells.

## INTRODUCTION

Runt-related transcription factor 1 (RUNX1) also known as acute myeloid leukemia 1 is a member of the core-binding factor family with a pivotal role in hematopoiesis. *Runx1*-deficient mice die during embryonic development between embryonic days (E) E11.5 and E12.5 due to massive hemorrhages in the central nervous system and the inability to establish definitive hematopoiesis [1, 2]. Conditional *Runx1* knockout mice revealed its requirement for endothelial-to-hematopoietic transition (EHT) allowing the formation of intra-arterial clusters and the generation of hematopoietic stem cells (HSCs) from hemogenic endothelium (HE) but not thereafter [3]. Furthermore, studies using live-imaging to monitor in vivo the emergence of hematopoietic cells from HE have revealed that *Runx1* is necessary for the EHT [4–6]. Several *RUNX1* transcripts can be transcribed from two different promoters—*P1* or distal promoter and *P2* or proximal promoter—by alternative splicing [7, 8]. The isoforms *RUNX1a, b*, and *c* are the most frequently expressed in

hematopoietic tissues [7–10]. *RUNX1a* and *RUNX1b* expression is driven from the *P2* promoter while *RUNX1c* is transcribed from the *P1* promoter [7, 8]. RUNX1b and c contain a transactivation domain and act as transcription factors, whereas RUNX1a lacks this region and has long been considered a natural dominant negative [11–13].

There are several discrepancies about the temporal expression and the relevance of each *RUNX1* isoform during embryonic and adult hematopoiesis. In mice, *Runx1a* and *Runx1b* are dominant in primitive erythrocytes at E7.5 [14]. Subsequently, both *P1* and *P2 Runx1* isoforms are concurrently expressed in hematopoietic stem and progenitors cells (HSPCs) from yolk sac and Aorta-Gonad-Mesonephros (AGM), whereas *P1*-driven *Runx1* isoforms become predominant in fetal liver and throughout adulthood [10, 14–16]. However, in zebrafish enhanced green fluorescence protein (EGFP) expression driven by *runx1 P1* occurs in erythromyeloid progenitors and precedes *runx1 P2* activation in HSCs from AGM [17]. Lie-A-Ling et al. used mouse ESCs to show *Runx1b* regulates the formation of cell clusters in the HE with hematopoietic potential [18].

Despite considerable advances in animal models, the knowledge about the specific effect of the different *RUNX1* isoforms in human hematopoietic development is still scarce. Studying human embryonic development is challenging due to logistic difficulties for accessing embryonic and fetal human tissues. Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), are the only nontransformed human cellular model with unlimited cell growth, self-renewal capacity, and pluripotency potential [19, 20]. Directed differentiation of hPSCs toward hematopoiesis provides a unique cellular system for studying the molecular pathways regulating human early hematopoiesis.

In hESCs, *RUNX1c* expression remarkably parallels the expression of hematopoietic markers during differentiation and is restricted to a subpopulation of CD34+ cells [10, 21]. Importantly, the overexpression of RUNX1 in combination with other transcription factors facilitates the conversion of both hPSC-derived HE and mature endothelial cells into HSPCs with engraftment potential [22, 23], indicating a master role of RUNX1 in the regulation of early human hematopoiesis. Here, we have used hPSCs as a cellular model to study the kinetics of expression of *RUNX1* isoforms in human early/embryonic hematopoiesis. We show that *RUNX1c* is the earliest isoform upregulated throughout hematopoietic specification of hPSCs. Importantly, *RUNX1c* is the only isoform enriched in hematoendothelial progenitors (HEPs), while all three *RUNX1* isoforms are expressed in $CD45^+$ hematopoietic cells. Both gain- and loss-of-function experiments demonstrate that *RUNX1c* regulates the emergence of both hemogenic HEPs and hematopoietic cells. Finally, transcriptomic analysis of HEPs identified a proinflammatory signature linked to RUNX1c-overexpression. Our study provides new insights into the expression pattern of *RUNX1* isoforms during early human hematopoietic development and the contribution of *RUNX1c* isoform in regulating hematopoietic specification.

## Materials and Methods

### hESC Culture

The hESCs lines AND1, H9, HS181, and HES3 and the iPSC cell line PBMC1-iPS4F1 were maintained undifferentiated in a feeder-free culture as described previously [24–26]. HES3 *RUNX1c* knock-out cells (*RUNX1c*$^{-/-}$) were generated by conventional homologous recombination [27, 28]. Approval to work with hPSCs was obtained from the Spanish National Stem Cell Steering Committee.

### Cloning Strategy, Lentiviral Vectors, and Transduction

Human *RUNX1c* cDNA (Addgene #12426, http://www.addgene.org) was directionally subcloned into the intermediate vector KJ-EGFP-2A (kindly provided by Dr. García-Perez; GENyO, Granada, Spain). Then, the full cassette EGFP-2A-Flag-*RUNX1c* was removed and cloned into the PmeI site in the pRRL-EF1α-PGK-NEO vector (kindly provided by Prof. Trono, Lausanne, Switzerland). HEK-293T cells were transfected with pRRL-EF1α-PGK-NEO (empty lentivector [EV]) or pRRL-EF1α-EGFP-2A-Flag-RUNX1c-PGK-NEO (RUNX1c) together with packaging plasmids (psPAX and pMD2.G, from Addgene, http://www.addgene.org) by standard calcium-phosphate transfection [25]. Supernatants were collected 48 hours after transfection and concentrated by ultracentrifugation. AND1 and HS181 were infected overnight on the day of passage with concentrated virus supplemented with polybrene (8 μg/ml; Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com). The viral supernatants were removed on the next day and infected hESCs were washed and maintained in culture. Transduced cells were selected with G418 (150 μg/ml; Invitrogen, Edinburgh, Scotland, https://www.thermofisher.com) from day 3 to day 15. *RUNX1c* expression was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting.

### Characterization of Pluripotency Markers in hPSCs by Flow Cytometry

hPSC colonies were dissociated with TrypLE Express (Invitrogen, https://www.thermofisher.com) and the cell suspension was stained with polyethylene (PE)-conjugated TRA-1–60 and stage-specific embryonic antigen (SSEA)-3, allophycocyanin (APC)-conjugated TRA-1–81 and SSEA-4, and fluorescein (FITC)-conjugated OCT-4 antibodies (BD Bioscience, San José, CA, http://www.bdbiosciences.com) for 30 minutes. Cells were washed and stained with 7-aminoactinomycin D (7AAD) (BD Bioscience, http://www.bdbiosciences.com) and live cells identified by 7AAD exclusion were analyzed using a fluorescence-activated cell sorting (FACS) Canto II flow cytometer [25].

### RNA Isolation, RT-PCR, and qRT-PCR Analysis

Total RNA was isolated using Trizol (Invitrogen, https://www.thermofisher.com) [25]. cDNA was generated using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and analyzed by qRT-PCR using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, La Jolla, CA, http://www.agilent.com/home) on the Mx3005P QPCR System (Agilent Technologies, http://www.agilent.com/home). Gene expression levels were calculated using the ($2^{-\Delta CT}$ method), and *GAPDH* was used to normalize data [29]. Where indicated, the relative gene expression was also calculated using the ΔΔCT method and GAPDH as reference gene. Primer sequences are listed in Supporting Information Table S1.

### Western Blotting

hPSCs were dissociated with TrypLE Express and cells lysed in RIPA buffer (Sigma-Aldrich, https://www.sigmaaldrich.com) containing complete protease inhibitors cocktail (Roche, Basel,

Switzerland, http://www.roche.com) and phosphatase inhibitors (Sigma-Aldrich, https://www.sigmaaldrich.com). Cell lysates were electrophoresed on 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. RUNX1c protein was detected with the Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, NE, https://www.licor.com) with an anti-RUNX1 antibody (Abcam, Cambridge, MA, http://www.abcam.com). An anti-β-actin antibody (Sigma-Aldrich, https://www.sigmaaldrich.com) was used as loading control.

### In Vivo Teratoma Formation

Animal protocols were approved by the Animal Care Committee of the University of Granada. In vivo pluripotency was analyzed as described [30]. Briefly, 1–2 $\times$ $10^5$ hPSCs were subcutaneously implanted into the flank of 6- to 8-week-old non-obese diabetic/severe combined immunodeficient interleukin 2 receptor γ (IL2Rγ)$^{-/-}$ mice (NSG) (The Jackson Laboratory, Bar Harbor, MA, https://www.jax.org). Teratoma growth was monitored weekly. Mice were killed at 8–10 weeks post implantation and teratomas were removed, fixed in formaldehyde, embedded in paraffin, sectioned and stained with H&E or by immunocytochemistry [31].

### OP9 Hematopoietic Differentiation System

hESC-OP9 cocultures were performed as described [25, 32, 33]. Briefly, OP9 stromal cells were plated in gelatin-coated 10-cm dishes in minimum essential media α (αMEM) basal medium supplemented with 20% non-heat-inactivated fetal bovine serum (FBS) for 8 days. hESCs grown in Matrigel-coated flasks were prepared as a suspension of small aggregates using collagenase IV followed by gentle scraping in differentiation medium (DM: αMEM basal medium, 10% non-heat-inactivated FBS, 100 μM monothioglycerol, and 50 μg/ml ascorbic acid). One fifth of the cell suspension was plated on top of the OP9 stroma in 10 ml of DM and cells were fed with fresh DM the following day. From day 4 to 10 of coculture, a half-volume medium change was performed every other day. Hematopoietic specification was analyzed by flow cytometry (at days 6, 8, and 10 of coculture) and colony-forming unit (CFU) assays (at day 8 of coculture). OP9 cells were stained with anti-mouse CD29-FITC (AbDSerotec, Düsseldorf, Germany) to exclude mouse cells. The percentage of human HEPs ($CD31^+CD34^+CD45^-$), primitive blood cells ($CD34^+CD45^+$), and total blood cells ($CD45^+$) was analyzed as described [25].

### Feeder-Free Hematopoietic Differentiation System

Feeder-free hematopoietic differentiation was performed as described previously [26]. Confluent hPSC cultures were disaggregated into single cells with TryPLE and plated (0.5–3 $\times$ $10^5$ hPSCs) onto six-well plates coated with 0.5 μg/cm$^2$ ColIV in E8 media supplemented with 10 μM Y27632. Next day, the medium was changed to IF9S medium containing 50 ng/ml bone morphogenic protein-4, 50 ng/ml fibroblast growth factor (FGF)-2, 15 ng/ml Activin A, and 2 mM LiCl. On day 2, media were changed to IF9S containing 50 ng/ml FGF2 and 50 ng/ml vascular endothelial growth factor (VEGF), and on day 4, the media were changed to DM 3 (IF9S containing 50 ng/ml FGF2, 50 ng/ml VEGF, 50 ng/ml stem cell factor (SCF), 50 ng/ml thrombopoietin (TPO), 50 ng/ml IL6 and 10 ng/ml IL3 [all cytokines from Preprotech, London, U.K.]). Cell cultures were maintained in hypoxia (5% $O_2$, 5% $CO_2$) for the first 6 days. From day 6 onwards, cells were maintained in DM 3 in normoxia. The frequency of hemogenic (CD43+) and non-hemogenic (CD43–) HEPs (CD34 + CD31 + KDR + CD45–) were analyzed at day 6 and 8 of development.

### CFU Assay

Human clonogenic progenitor assays were performed by plating 5 $\times$ $10^4$ cells from OP9-hESC cocultures into methylcellulose H4436 (Stem Cell Technologies, Vancouver, Canada). Cells were incubated at 37°C in a humidified atmosphere and colonies were counted between days 10 and 12 using standard morphological criteria [25, 34].

### Cell Cycle Analysis of HEPs

OP9 cocultures were harvested at day 8 of development, fixed in 70% ice-cold ethanol, and stored overnight at −20°C. The next day, cells were washed and incubated with anti-CD31-FITC and anti-CD34-FITC (MiltenyiBiotec) for 15 minutes. After washing, the cells were resuspended in propidium iodide (PI) buffer containing 50 μg/ml PI and 100 μg/ml RNAase in phosphate-buffered saline. Cell cycle distribution was analyzed using a FACSCantoII cytometer equipped with Modfit software (Verity Software House, Topsham, ME) [25, 35].

### Apoptosis Analysis of HEPs

hESCs cultured over OP9 stromal cells were harvested at day 8, washed with Annexin V-binding buffer, and incubated with anti-human CD31-FITC and anti-CD34-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) and Annexin V-APC (Becton Dickinson) antibodies for 20 minutes. After washing, the cells were resuspended in Annexin V-binding buffer with 7AAD and apoptotic cell death was detected in the $CD31^+CD34^+CD45^-$ cell population by flow cytometry using a FACSCanto II flow cytometer.

### Mouse Transplantation and Analysis of Engraftment

NSG mice were housed under sterile conditions. Cord blood (CB)-derived $CD34^+$ HSPCs (3 $\times$ $10^4$ cells in 50 μl) as well as EV (1–2.5 $\times$ $10^5$ cells in 50 μl) or RUNX1c hESC derivatives (1–2.5 $\times$ $10^5$ cells in 50 μl) purified from day 8 differentiating OP9-co-cultures were transplanted intrahepatically into newborn NSG mice [25, 36]. Mouse health was monitored throughout the entire experiment. Mice were killed 6–8 weeks after transplantation and bone marrow (BM), spleen, liver, and peripheral blood were collected and analyzed for human chimerism. Cells were stained with anti-HLA-ABC-PE and anti-CD45-APC (BD Bioscience, http://www.bdbiosciences.com) antibodies to analyze human chimerism by flow cytometry.

### Gene Expression Profiling and Analysis

Undifferentiated hPSCs and 6, 8, and 10-day-old purified HEPs from hESC-EV and hESC-RUNX1c cells were sorted using a FACS Aria sorter (BD Bioscience, http://www.bdbiosciences.com) and total RNA was isolated using Trizol. High quality RNA was confirmed using the Agilent 2100 Bionalyzer (Agilent Technologies, http://www.agilent.com/home). RNA samples were labeled (Agilent Low Input Quick Amp Labeling kit, Agilent Technologies, http://www.agilent.com/home) with Cy3 following the manufacturer's instructions. The hybridization procedure was accomplished using the Agilent Gene

 

Expression Hybridization kit and Agilent Whole Human Genome Oligo Microarray, 8 × 60 K (Agilent Technologies, http://www.agilent.com/home). Two independent samples per condition and cell line were labeled and hybridized. Candidate genes with a fold change >2 and a $p < .01$ were considered differentially expressed. Functional analysis and canonical pathway studies were performed using the Panther public web tool (http://www.pantherdb.org) and ingenuity pathway analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com) as described [37, 38].

### Statistical Analysis

All data are expressed as mean ± SEM. Statistical comparisons were performed with a paired Student's *t* test with the exception of the CFU score analysis, where Wald's test was applied. Values were considered statistically significant at $p < .05$.

## Results

### Expression of *RUNX1* Isoforms During Hematopoietic Differentiation of hPSCs

We first differentiated hPSCs toward the hematopoietic lineage [25, 32, 33] and analyzed the expression pattern of *RUNX1a*, *b*, and *c* isoforms by qRT-PCR. HEPs emerged at day 6 of OP9-coculture and then differentiate into CD45+ cells from day 8 onwards [25, 37]. *RUNX1a* and *RUNX1a/b* isoforms expression did not increase until day 10 of hematopoietic differentiation, whereas the *RUNX1c* isoform was readily induced at day 6, paralleling the appearance of HEPs, and its expression progressively increased throughout differentiation (Fig. 1A). We then analyzed the expression pattern of the key hematopoietic transcription factors *SCL/TAL1*, *GATA1*, and *PU.1/SPI1* by qRT-PCR (Fig.1A; Supporting Information Fig. S1A). *SCL*, a master factor for HEPs specification [25] and a direct activator of *RUNX1* [39, 40], was the earliest induced gene. *RUNX1*c either preceded or paralleled the induction of *GATA1* and *PU.1,* both RUNX1 targets [41, 42].

To further characterize the expression pattern of the *RUNX1* isoforms, we FACS-purified HEPs (CD34 + CD31 + CD45–) and CD45+ blood cells at day 8 and 10 of differentiation, respectively, and non-hematopoietic cells ($CD31^{-}CD34^{-}CD45^{-}$) were also isolated as a control (Fig. 1B; Supporting Information Fig. S1B). qRT-PCR analysis revealed that all *RUNX1* isoforms are highly expressed in blood cells, while *RUNX1c* was the only isoform enriched in HEPs in comparison with non-hematopoietic cells (Fig. 1C; Supporting Information Fig. S1C). Importantly, when HEPs were identified more exhaustively as CD45-CD43-CD41-CD34 + CD31 + CD73-CD184-) [16, 43] an identical trend was observed, confirming our initial sorting strategy (Fig. 1D). Collectively, *RUNX1c* is the isoform whose expression best parallels early human hematopoietic specification in vitro, being the most enriched in HEPs.

### *RUNX1c* Enhances Hematoendothelial Specification of hPSCs and Hematopoietic Commitment of HEPs

To further assess the contribution of *RUNX1c* during hematopoietic specification of hPSCs, we over-expressed *RUNX1c* in AND1 and HS181 hESCs lines which have good and poor intrinsic hematopoietic differentiation potential, respectively [24, 25], and one iPSC cell line [31] with either an EV or a RUNX1c-expressing lentivector (RUNX1c) (Fig. 2A). After selection with G418, the transgenic hPSCs showed stable expression of RUNX1c at the mRNA and protein level (Fig. 2B, 2C). Six-to-eight weeks after selection, EV- and RUNX1c-hESCs were analyzed for expression of pluripotency markers and in vivo differentiation potential. RUNX1c-overexpressing hESCs expressed the pluripotency-associated transcription factors *OCT4*, *NANOG*, and *SOX-2* (Supporting Information Fig. S2A, S2B) and the pluripotency-associated antigens Tra-1-60, Tra-1-81, SSEA-3, and SSEA-4 (Supporting Information Fig. S2B). Both EV and RUNX1c-hESC lines formed teratomas with identical efficiency, latency, and histological composition with differentiation into tissues representing the three germ layers [25] (Supporting Information Fig. S2C). Thus, *RUNX1c* overexpression is compatible with pluripotency.

Irrespective of the hematopoietic potential of the hPSC line, hematopoietic differentiation on OP9 cells demonstrated that *RUNX1c* over-expression significantly increased (approximately 2- to 12-fold) the generation of HEPs (CD31 + CD34 + CD45) (Fig 2D; Supporting Information Fig. S3A, left panels, S3B). We next analyzed in more detail the composition of HEPs, and we found that *RUNX1c* over-expression significantly increased the frequency of CD43+ hemogenic HEPs at day 5, while the CD43– HEPs were not significantly altered all over differentiation (Fig. 2E), suggesting that constitutive expression of RUNX1c accelerated the emergence of CD43+ hemogenic HEPs at early stages of hematopoietic differentiation. To rule out an effect of RUNX1c in either the proliferation or apoptosis of the emerging HEPs, we analyzed HEPs for cell cycle distribution and apoptosis and found no differences in the proportion of cycling cells (33% ± 0.3% vs. 34% ± 2.4%) or apoptotic cells (0.3% vs. 0.9%) between EV and RUNX1c HEPs (Supporting Information Fig. S4), suggesting that ectopic *RUNX1c* likely promotes specification of HEPs rather than proliferation or survival.

The RUNX1c-mediated increase in HEPs specification resulted in an approximately 6- to 10-fold increase in CD34 + CD45 + hematopoietic progenitors and total CD45+ hematopoietic cells (Fig. 2F; Supporting Information Fig. S3A, middle and right panels, S3B, S3C). As an indirect measure of the hematopoietic output of HEPs, we calculated the number of total CD45+ blood cells produced in culture by HEPs. As shown in Supporting Information Figure S3D, by day 10 of differentiation RUNX1c-HEPs produced three- to fourfold higher numbers of CD45+ cells than EV-HEPs. We next analyzed the clonogenic capacity of EV- and RUNX1c-hematopoietic derivatives at day 8 of OP9 coculture and found that clonogenic potential was approximately three- to eightfold higher in RUNX1c-expressing hematopoietic derivatives than in EV control cells (Fig. 2G). CFU scoring revealed a slight skew toward macrophage commitment (CFU-M) in RUNX1c-expressing hematopoietic progenitors at the expense of granulocyte (CFU-G)/granulocyte-macrophage (CFU-GM) differentiation (Fig. 2G). Supporting the RUNX1c hematopoietic-promoting effects, the hematopoietic transcription factors *SCL*, *GATA1*, and *PU.1* were also significantly upregulated throughout hematopoietic differentiation (Fig. 2H; Supporting Information Fig. S5A). The ectopic levels/expression of *RUNX1c* was maintained throughout differentiation (Supporting Information Fig. S5B). Collectively, our data indicates that *RUNX1c* over-expression increases the generation of CD43+ hemogenic HEPs, resulting in an increased hematopoietic (CD45+) differentiation of hPSCs.

 

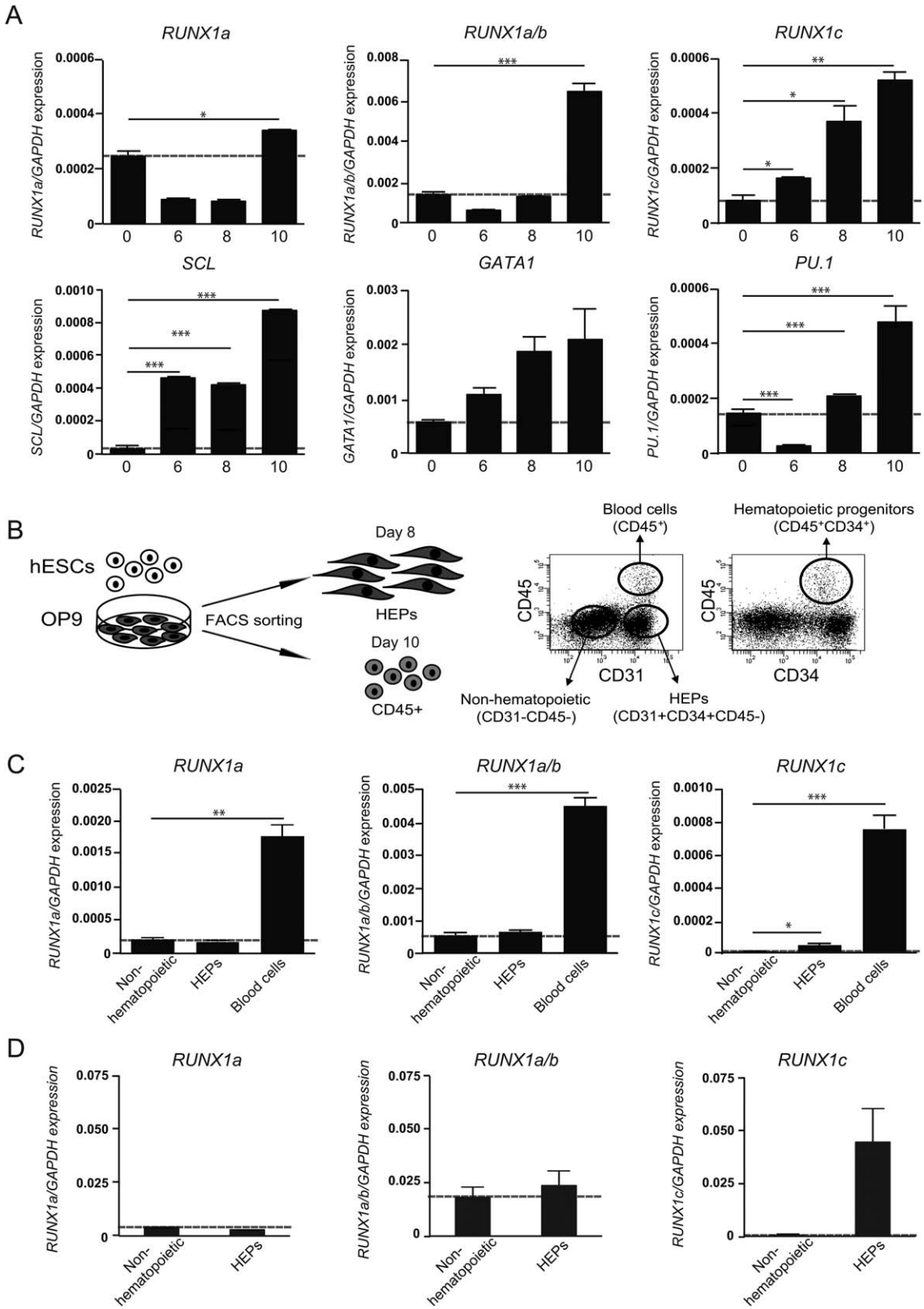


**Figure 1.** Expression of Runt-related transcription factor 1 (*RUNX1*) isoforms in hematoendothelial progenitors (HEPs) and blood cells derived from human embryonic stem cells. **(A)**: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showing expression levels of endogenous *RUNX1* isoforms (*RUNX1a, RUNX1a/b*, and *RUNX1c*), *SCL*, *GATA1*, and *PU.1* throughout hematopoietic differentiation. **(B)**: Schematic representation of fluorescence-activated cell sorting (FACS) of HEPs and hematopoietic cells (left panel), and representative flow cytometry dot plots showing how HEPs ($CD31^+CD34^+CD45^-$), hematopoietic progenitors ($CD34^+CD45^+$) and total blood cells ($CD45^+$) are identified (right panel). **(C)**: qRT-PCR showing expression levels of endogenous *RUNX1a, RUNX1a/b*, and *RUNX1c* isoforms in isolated cell populations in one representative human pluripotent stem cells (hPSC) line. **(D)**: qRT-PCR analysis of endogenous RUNX1 isoforms in highly-purified FACS-sorted HEPs (CD45-CD43-CD41-CD34+CD31+CD73-CD184-) in one representative hPSC line. Data represent mean ± SEM of three independent experiments. Statistical significance was assessed with Student's *t* test. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: FACS, fluorescence-activated cell sorting; HEP, hematoendothelial progenitor; hESC, human embryonic stem cell.

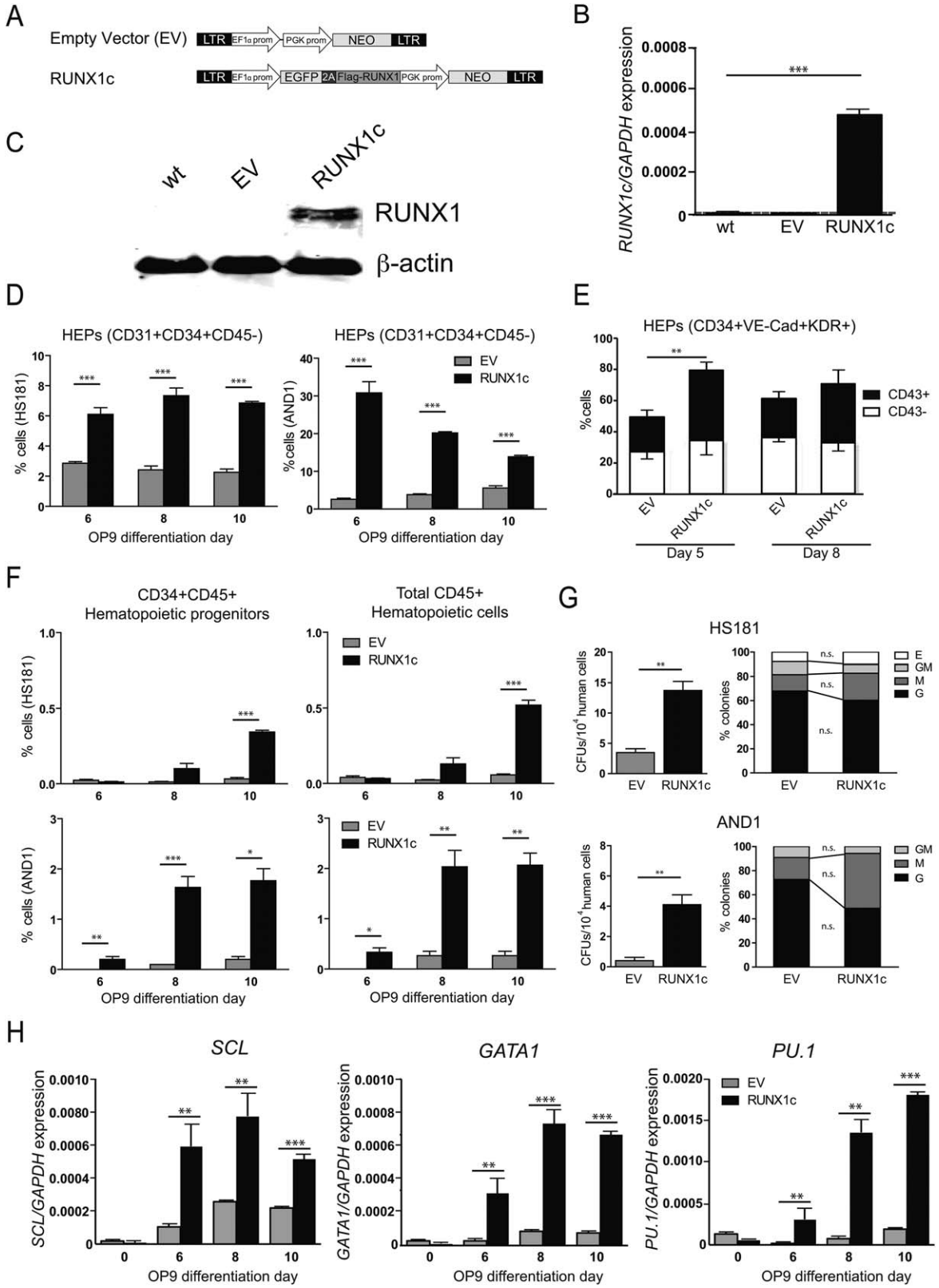


**Figure 2.** Enforced expression of Runt-related transcription factor 1 (*RUNX1c*) augments hematopoietic specification from human pluripotent stem cells (hPSCs). **(A)**: Schematic representation of the lentiviral vectors used to overexpress RUNX1c and the empty lentivector (EV) control. **(B)**: Representative quantitative real-time polymerase chain reaction analysis of *RUNX1c*. Expression levels are shown normalized to *GAPDH*, used as reference gene. Data represent mean ± SEM of three independent experiments. **(C)**: Representative Western blot detection of RUNX1c protein in hPSCs. β-Actin is used as a loading control.**(D)**: Kinetics of hematoendothelial progenitors (HEPs) specification in two different hPSCs lines (HS181 and AND1) transduced with the EV or RUNX1c-expressing vector (RUNX1c). **(E)**: Kinetics at days 5 and 8 of hematopoietic differentiation for CD43+ (hemogenic) and CD43– (non-hemogenic) HEPs in EV or RUNX1c-overexpressing (RUNX1c) hPSCs. **(F)**: Emergence of hematopoietic progenitors (CD34+CD45+) and total blood cells (CD45+) from two different human embryonic stem cell lines. **(G)**: Number and colony distribution of colony-forming units (CFUs) per 10,000 human cells and colony type distribution. **(H)**: Expression levels of endogenous *SCL, GATA1,* and *PU.1* in hPSC-derived cells. Data represent mean ± SEM of three independent experiments. Statistical significance was assessed with Student's *t* test except for the CFU scoring data where the Wald's test was used. n.s. *, $p < .05$, **, $p < .01$ and ***, $p < .001$. Abbreviations: 2A, 2A self-cleaving peptide; EF1α, elongation factor 1α; EGFP, enhanced green fluorescent protein; EV, empty lentivector; Flag, Flag epitope; HEP, hematoendothelial progenitor; LTR, long terminal repeat; NEO, Neomycin resistance cassette; PGK, phosphoglycerate kinase; wt, wild-type.

### RUNX1c Expression Does Not Confer In Vivo Engraftment Potential to hPSC-Derived Hematopoietic Derivatives

The RUNX1c-mediated enhancement of in vitro hematopoietic differentiation prompted us to examine whether enforced expression of *RUNX1c* was sufficient to confer in vivo engraftment ability to the hPSC-derived RUNX1c-expressing hematopoietic derivatives. We transplanted $3 \times 10^4$ CB-derived CD34+ HSPCs as a positive control, or $1.5–2.5 \times 10^5$ hESC-derived EV and RUNX1c hematopoietic derivatives intrahepatically into newborn NGS mice [25, 36]. After 8 weeks, we analyzed the presence of human hematopoietic cells in the transplanted animals. *RUNX1c* expression by itself was not enough to confer in vivo engraftment potential to hPSC blood derivatives (Supporting Information Fig. S6), in line with the so far reported marginal repopulating ability of hPSC-blood derivatives, and with the idea that a defined set of factors seems necessary to endow in vivo repopulating ability [22, 23, 25, 37, 44–47].

### *RUNX1c* Targeted Deletion Reduces EHT

To further investigate the specific contribution of *RUNX1c* isoform to the hematopoietic specification of hPSCs, we harnessed a *RUNX1c* knock-out hESC line (*RUNX1c*$^{-/-}$) previously generated by conventional homologous recombination [28]. Slight decrease was observed in HEP specification in *RUNX1c*$^{-/-}$ hESCs (Fig. 3A; Supporting Information Fig. S7A, left panel, S7B); however, the generation of hematopoietic cells in vitro from these HEPs was significantly reduced in *RUNX1c*$^{-/-}$ cells (Fig. 3A, middle and right panel; Supporting Information Fig. S7A, S7B). Importantly, *RUNX1c* ablation significantly impaired the number of CD45+ blood cells per HEP, confirming a role of RUNX1c in regulating EHT (Supporting Information Fig. S7C). The transcription factors *SCL*, *GATA1*, and *PU.1* were also robustly downregulated in *RUNX1c*$^{-/-}$ hESC-blood derivatives throughout hematopoietic differentiation (Supporting Information Fig. S7D). qRT-PCR confirmed the complete absence of *RUNX1c* expression throughout blood differentiation in *RUNX1c*$^{-/-}$ cells (Fig. 3B), while *RUNX1a* and *RUNX1a/b* isoforms were similarly induced in both *RUNX1c*$^{-/-}$ and *RUNX1c*$^{+/+}$ cell lines (Fig. 3C, 3D). When we assessed the clonogenic potential of the hematopoietic progenitors in CFU assays, we found that *RUNX1c*$^{-/-}$ and *RUNX1c*$^{+/+}$ hESC-hematopoietic derivatives produced similar total number of colonies; however, *RUNX1c*$^{-/-}$ hESC derivatives had an increase in E, a decrease in M, and disappearance of G/GM colonies (Fig. 3E), suggestive of a more primitive hematopoiesis in the absence of *RUNX1c*. Moreover, *SCL*, *GATA1*, and *PU.1* were robustly downregulated in *RUNX1c*$^{-/-}$ hESC-derived CD45+ cells throughout hematopoietic differentiation (Supporting Information Fig. S7C). Taken together, *RUNX1c* is necessary for the generation of CD45+ cells from HEPs.

### *RUNX1c*-HEPs Show an Activated Proinflammatory Transcriptional Signature

To explore the potential mechanisms by which *RUNX1c* regulates EHT, we performed gene expression profiling using microarrays in HEPs from EV- and RUNX1c-hESCs at different time points of hematopoietic differentiation. We confirmed the expression levels of 20 selected genes by qRT-PCR and found a significant concordance correlation coefficient (0.76) with the microarray data, validating the global gene expression data (Supporting Information Fig. S8). To explore the biological meaning of the changes in gene expression imposed by *RUNX1c* overexpression, we used first the web tool Panther (www.pantherdb.org) and applied a statistical enrichment test to find the most significant gene ontology biological processes altered in RUNX1c-HEPs versus EV-HEPs. Both at day 8 and day 10 of hematopoietic differentiation, the most relevant biological processes, ranked by *p* value, were associated with immune system process and immune/defense responses (Fig. 4A). To confirm this prediction, we used the software IPA and found that the category "Hematological System Development and Function" was consistently the most significant biological process affected in RUNX1c-HEPs and EV-HEPs (Fig. 4B). Using IPA, we analyzed in detail the biofunctions altered within this category and found many functions related to the migration, adhesion, and differentiation of myeloid and lymphoid cells predicted to be activated (*z*-score $>2$) in RUNX1c-over-expressing HEPs (Fig. 4C).

Next, we used IPA to identify which upstream regulators could be responsible for the gene expression changes associated to *RUNX1c* over-expression in HEPs. We could distinguish five different gene clusters of upstream regulators predicted to be activated in *RUNX1c* over-expressing cells (Fig. 4D): (a) regulators of macrophage differentiation; (b) megakaryocytic/erythroid regulators; (c) RUNX1 partners and RUNX1-regulated T-cell development; (d) regulators of hematopoietic differentiation from hPSCs [48, 49]; and (e) components of inflammatory signaling, which represent the biggest cluster and agrees with the Gene Ontology data presented in Figure 4A.

IPA predicted that *RUNX1c* over-expression activated CSF1 and CSF2 from day 6 to 10 and CSF1R at day 8 in HEPs (Fig. 5D). CSF1 (M-CSF), CSF2 (GM-CSF), and their receptor CSF1R are necessary for the production, differentiation, expansion, and function of macrophages, granulocytes, and their progenitors [50]. Therefore, the activation of the CSF1R signaling pathway could be responsible for the CFU-M/CFU-G skew observed in the clonogenic assays upon *RUNX1c* over-expression (Fig. 2G).

Proinflammatory signals including tumor necrosis factor (TNF)-$\alpha$, interferon (IFN)-$\alpha$, and IFN$\gamma$ have been recently proposed as key in vivo regulators of definitive hematopoiesis in zebrafish and mouse AGM [51–54]. We used IPA to visualize the transcriptional network formed by the key inflammatory regulators and their known targets in RUNX1c HEPs over the differentiation period. At day 6 of differentiation, the inflammatory regulators are predicted to be inactive (Supporting Information Fig. S9), but at day 8, the network of inflammatory regulators has expanded and it is now predominantly activated, with a complex network including many genes coregulated by different factors. IFN$\gamma$ appears as the most important factor, based on quantity of target genes and coregulations (Supporting Information Fig. S10). At day 10, the inflammatory regulators remain activated; the relative importance of IFN$\gamma$ now balanced by a high level of TNF target genes and coregulations (Supporting Information Fig. S11). Our results suggest that *RUNX1c* over-expression induces a gene signature that recapitulates an activation of different inflammatory signals that converge and form a complex network at the differentiation time when HEPs are undergoing EHT.

To validate these predictions, we performed qRT-PCR analysis of key genes of these inflammatory pathways in purified EV- and RUNX1c-HEPs at days 6, 8, and 10 of hematopoietic

 

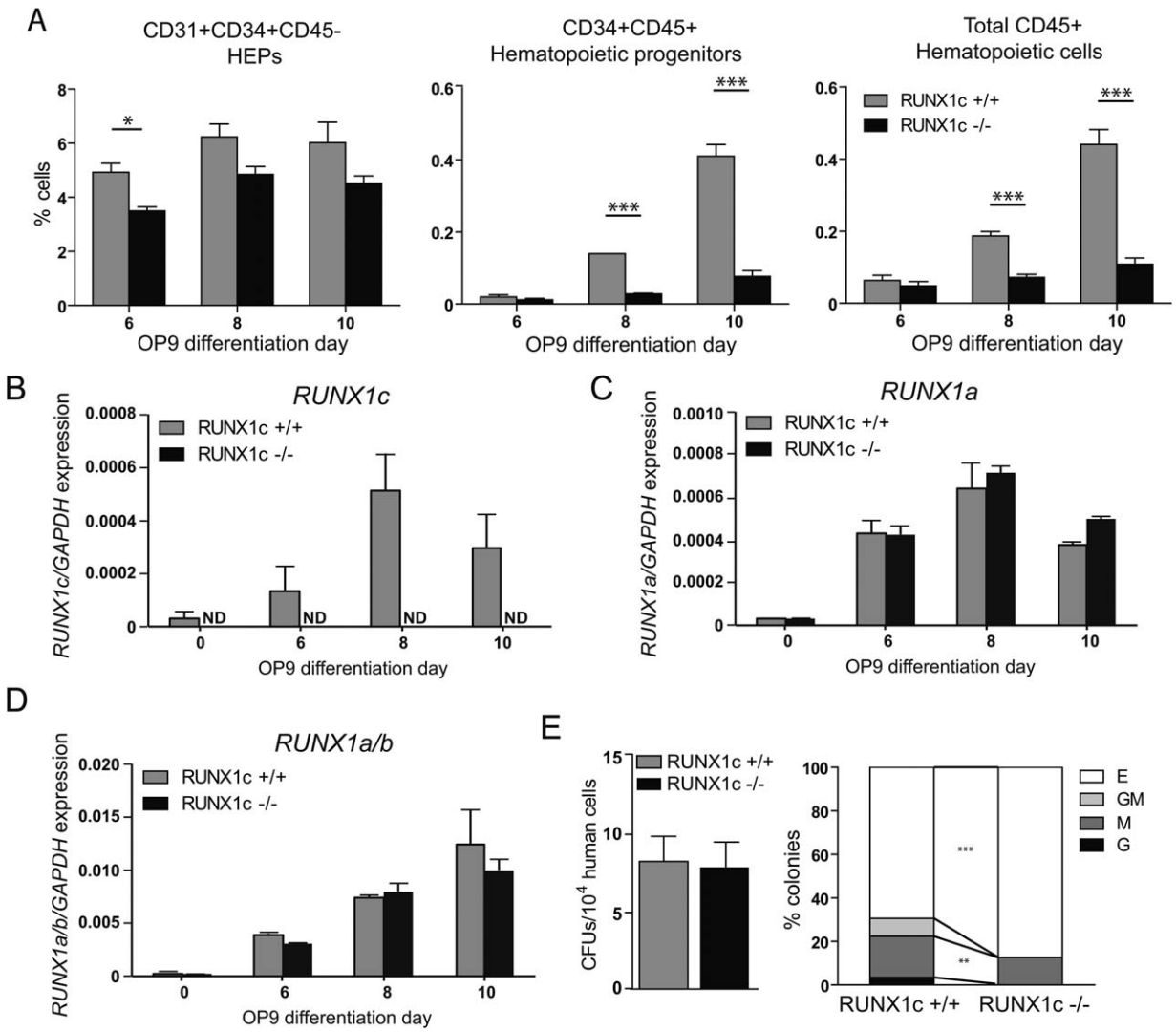


**Figure 3.** Runt-related transcription factor 1 (RUNX1c) specific deletion drastically diminishes endothelial-to-hematopoietic transition. **(A)**: Kinetics of hematopoietic specification from *RUNX1c*$^{+/+}$ and *RUNX1c*$^{-/-}$ human pluripotent stem cells (hPSCs). **(B)**: Expression levels of endogenous *RUNX1c* in *RUNX1c*$^{-/-}$ hematopoietic cells confirming *RUNX1c* specific deletion in *RUNX1c*$^{-/-}$ human embryonic stem cells. **(C, D)**: Quantitative real-time polymerase chain reaction analysis showing similar induction of *RUNX1a* (C) and *RUNX1a/b* (D) endogenous expression in *RUNX1c*$^{+/+}$ and *RUNX1c*$^{-/-}$ hPSCs. **(E)**: Number of colony-forming units (CFUs) per 10,000 human cells and colony type distribution. Data represent mean ± SEM of three to six independent experiments. Statistical significance was assessed with Student's *t* test except for the CFU scoring data where the Wald's test was used. *, $p < .05$; **, $p < .01$; and ***, $p < .001$. Abbreviations: E, erythrocyte; G, granulocyte; GM, granulocyte–macrophage; HEP, hematoendothelial progenitor; M, macrophage; ND, non-detected.

differentiation. Among the members of the interferon pathway, we found *IFNB1, IFNA2*, and interferon regulatory factors (IRFs) *IRF7* and *IRF1* upregulated (Fig. 5A, 5B; Supporting Information Table S2). Type I interferon signaling is mediated by Janus Kinase proteins that recruit and activate signal transducers and activators of transcription (STATs) [55]. All *STAT* genes analyzed were significantly upregulated in late (day 10) RUNX1c-HEPs (Fig. 5C; Supporting Information Table S2). In addition, RUNX1c over-expression also increased the expression of the nuclear factor (NF)-κB members *NFKB1* and *RELA* (Fig. 5D; Supporting Information Table S2) and *TLR4* by day 10, while did not upregulate *TNF* expression in HEPs (Fig. 5E; Supporting Information Table S2). Interestingly, BloodChiP analysis reveals that most of these genes are direct targets of RUNX1 in human HSCs and we found a good correlation between the genes modulated by *RUNX1c* over-expression in hESC-derived HEPs and promoter occupancy in HSCs (Supporting Information Table S4) [56]. We also sorted HEPs from *RUNX1c*$^{+/+}$ and *RUNX1c*$^{-/-}$ hPSCs and analyzed all proinflammatory signaling genes by qRT-PCR. Remarkably, all were downregulated or not expressed in *RUNX1c*$^{-/-}$ purified HEPs (Fig. 5F; Supporting Information Table S3). These data reinforce the hypothesis that *RUNX1c* over-expression imposes a gene expression profile that reflects an activation of proinflammatory signaling networks which may contribute to in vitro hematopoietic differentiation of hPSCs.

## Discussion

The elucidation of the molecular determinants inducing hematopoietic specification of hPSCs would facilitate the generation of bona fide HSCs for developmental biology and cell therapy. Our

 

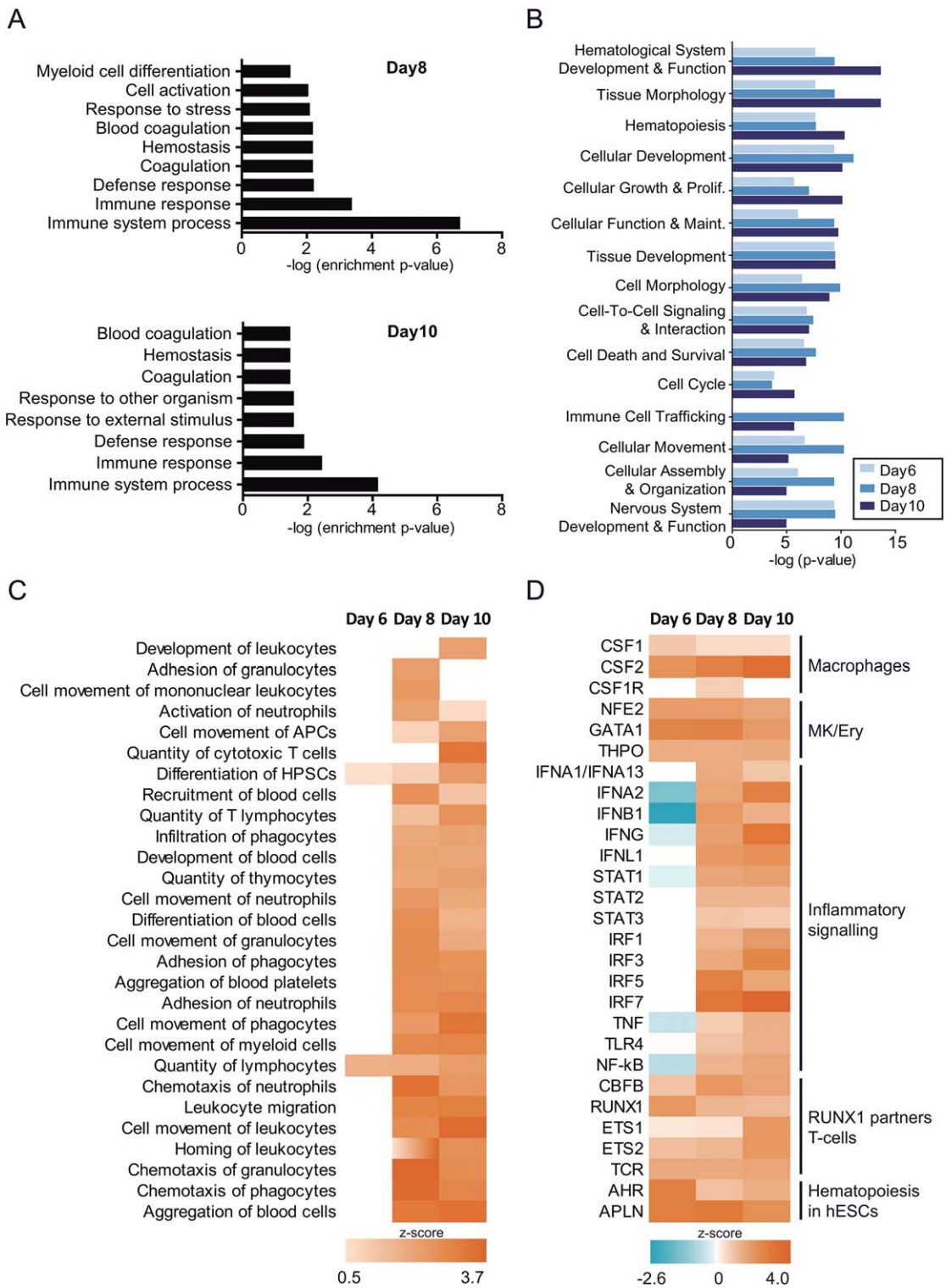


**Figure 4.** Enforced expression of Runt-related transcription factor 1 (*RUNX1c*) in hematoendothelial progenitors (HEPs) induces a proinflammatory signaling signature. **(A)**: Top gene ontology biological processes (obtained by Panther) enriched in genes differentially expressed in RUNX1c-HEPs versus empty lentivector (EV)-HEPs at days 8 and 10 of development. **(B)**: Top 15 biological functions of genes differentially expressed in RUNX1c-HEPs as compared to EV-HEPs at days 6, 8, and 10 using ingenuity pathway analysis. **(C)**: Predicted biofunctions within the hematological system development and function activated in RUNX1-HEPs at days 6, 8, and 10. **(D)**: Highlight of the upstream regulators predicted to be activated (orange) or repressed (blue) by RUNX1-expressing HEPs at days 6, 8, and 10 of OP9-human embryonic stem cells coculture. Abbreviations: AHR, aryl hydrocarbon receptor; APCs, antingen presenting cells; APLN, apelin; CBF, core-binding factor; CSF, colony stimulating factor; Ery, erythroid lineage; GATA, GATA binding protein; hESC, human embryonic stem cell; IFN, interferon; IRF, interferon regulatory factor; MK, megakaryocytic lineage; NFE2, nuclear factor erythroid 2; NF-kB, nuclear factor-kB; STAT, signal transducers and activators of transcription; TCR, T-cell Receptor; THPO, thrombopoietin; TLR, toll like receptor; TNF, tumor necrosis factor.

 

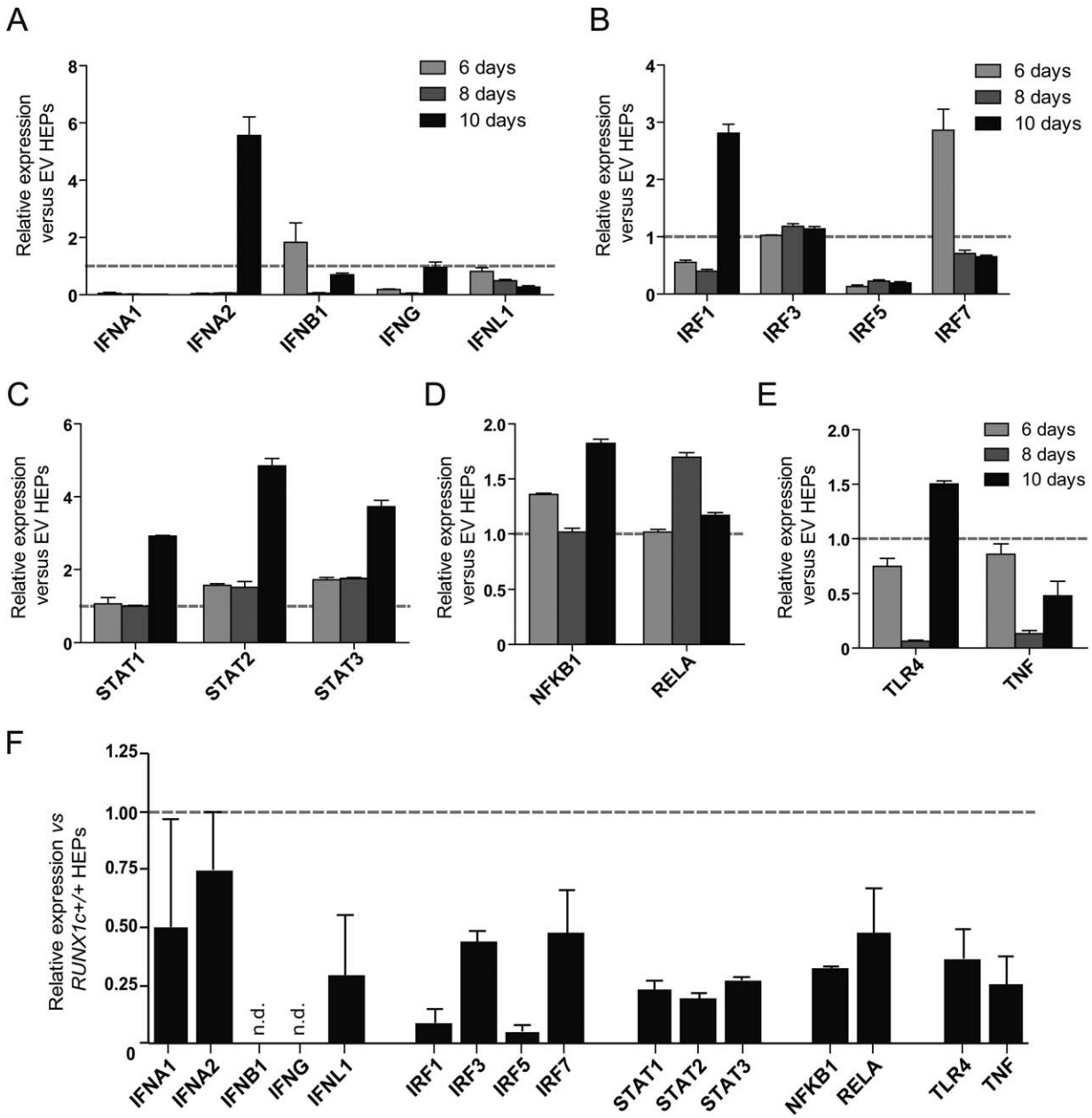


**Figure 5.** Enforced expression of Runt-related transcription factor 1 (*RUNX1c*) in hematoendothelial progenitors (HEPs) regulates the expression of proinflammatory mediators. **(A–E)**: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of several genes in RUNX1c-overexpressing HEPs at days 6, 8, and 10. Relative expression is shown normalized to empty lentivector (EV)-HEPs. *GAPDH* is used as an internal control. qRT-PCR analysis of a set of *IFNs* (A), interferon regulatory factors (*IRFs*) (B), signal transducers and activators of transcription (*STATs*) (C), *NFKB1* and *RELA* (D), and *TLR4* and *TNF* (E) predicted to be activated by RUNX1c over-expression in HEPs at days 6, 8, and 10. **(F)**: qRT-PCR analysis of a set of *IFNs*, *IRFs*, *STATs, NFKB1*, *RELA, TNF*, and *TLR4* in *RUNX1c*$^{-/-}$ sorted HEPs at day 6 of hematopoietic differentiation. Relative expression is shown normalized to *RUNX1c*$^{+/+}$ sorted HEPs. *GAPDH* is used as an internal control. Data represent mean ± SEM of three independent experiments. Abbreviations: EV, empty lentivector; HEP, hematoendothelial progenitor; IFN, interferon; IRF, interferon regulatory factor; NF-kB, nuclear factor kB; STAT, signal transducers and activators of transcription; TLR, toll like receptor; TNF, tumor necrosis factor.

studies demonstrate that *RUNX1c* promotes hematopoietic specification, possibly in cooperation with proinflammatory signals. The study of the expression kinetics of the three main *RUNX1* isoforms during hPSCs hematopoietic differentiation revealed that *RUNX1c* is the first isoform induced upon hematopoietic differentiation, while *RUNX1a/b* isoforms are upregulated later on (day 10). In line with our results, the induction of *RUNX1c* has been reported to precede *RUNX1a/b* expression during hematopoietic specification of hESCs using embryoid bodies (EBs) differentiation protocols [10], although Ran et al. showed the opposite pattern of *RUNX1* induction following the spin-EB differentiation system [57]. Similarly, during mouse ESC hematopoietic differentiation, *P2*-transcribed *Runx1* isoforms emerged earlier that *P1*-transcribed *Runx1c* [14, 15]. Thus, regulation of *RUNX1* isoforms appears subject to species-specific differences, and peculiarities associated with hematopoietic differentiation methods and hPSC lines used. In addition, our qPCR results also show that *SCL* is upregulated before *RUNX1c*, *GATA1* emerges

 

concomitantly to *RUNX1c*; while *PU.1* is induced later on after blood cells appearance.

In our differentiation system, *RUNX1c* is the only isoform enriched in HEPs and the highest up-regulated isoform in hESC-derived CD45+ cells in comparison with non-hematopoietic derivatives, confirming previous results from our and other laboratories [10, 58]. In genetically modified mESCs containing reporter genes controlled by *P1* and *P2* promoters, Sroczynska et al. showed that the HE expresses *RUNX1b* while definitive hematopoietic cells mainly expressed *RUNX1c* [15]. Very recently, Elefanty's laboratory, using spin-EB differentiation protocols showed that induction of *RUNX1c* occurs after emergence of HE [28, 43]. These discrepancies can likely be attributed to species-specific differences, different differentiation strategies, and the read-out assays for measuring *RUNX1c* upregulation [10, 15, 57]. For example, in contrast to our OP9 coculture, EB-based blood differentiation protocols require the addition of hematopoietic cytokines such as IL3, which might bypass RUNX1-mediated transcription regulators [59]. Furthermore, early acting hematopoietic cytokines might modify the finely tuned regulation of *P1* and *P2* promoters, leading to abnormal expression of *RUNX1* transcripts.

Given its expression pattern during hematopoietic differentiation of hPSCs, we hypothezyed that *RUNX1c* would contribute to HEPs specification and further blood (CD45+) generation. Using a gain-of function approach, we found that *RUNX1c* over-expression was a booster of HEP emergence and blood production, something not described so far. A more detailed flow cytometric analysis within this population confirmed an increase in CD43+ hemogenic HEPs in *RUNX1c* over-expressing cultures. Ectopic over-expression of *RUNX1c* further augmented the production of CD45 + CD34+ hematopoietic progenitors and CD45+ blood cells. Cell cycle and apoptosis assays and the outcome of CD45+ blood cells produced per HEP revealed that *RUNX1c* is involved in hematopoietic specification rather than proliferation and/or survival. We cannot rule out that *RUNX1c* may be favoring the formation of a subpopulation of early hematopoietic progenitors (CD34 + CD43 + CD45–) within our HEP population. Further dissection of the target population of *RUNX1c* could elucidate this hypothesis. CFU assays also revealed higher clonogenic potential for RUNX1c-hematopoietic derivatives. In addition, *RUNX1c* over-expression also alters the distribution of the types of colonies, slightly enhancing the CFU-M at the expense of CFU-G and CFU-GM, suggesting a role in monocytic differentiation. In contrast, *RUNX1a* over-expression in hPSCs provokes an increase in the number of GEMM and erythroid colonies at the expense of the rest [57], that together with the enhanced number of hematopoietic progenitors suggest a role of *RUNX1a* in proliferation of the HSPCs compartment rather than differentiation. Interestingly, *RUNX1c* over-expression induced the expression of the key hematopoietic transcription factors *SCL*, *GATA1*, and *PU.1*. The regulation of *GATA1* [41] and *PU.1* [42] by RUNX1 has been reported previously; however, the especific activation by RUNX1c was unknown so far. More surprising is the induction of *SCL* by RUNX1c, a known upstream regulator of RUNX1, in contrast to previous publications [17, 41]. One posibility will be that RUNX1c upregulates a different isoform of *SCL* not implicated in establishing the HE, as suggested in zebrafish [60]. These results could be reproduced in two hESCs lines with high and low hematopoietic potential and one iPSC cell line. Despite overexpressing different ectopic levels of *RUNX1c*, all lines displayed very similar RUNX1c-mediated hematopoietic phenotype, further validating a role for RUNX1c regardless the cellular background and hematopoietic output.

Similar to many studies using hPSC-derived hematopoietic cells from non-manipulated or genetically modified hPSCs [25, 37, 44–47], RUNX1c-hematopoietic derivatives also failed to engraft into newborn NSG mice. Similarly, murine ESC-derived hematopoietic progenitors transduced with *RUNX1c* failed to confer engraftment in transplantation assays [10]. By contrast, Ran et al. showed short-term myeloid-restricted multilineage engraftment in 33% of mice transplanted with RUNX1a-expressing hESC-derived $CD34^{+}CD45^{+}$ cells [57]. However, RUNX1 was very recently found to be a master factor that in combination with other transcription factors facilitates the conversion of both hPSC-derived HE and mature endothelial cells into HSPCs with engraftment potential [22, 23], further emphasizing the master role of RUNX1 in human hematopoietic formation. Of note, *RUNX1a* over-expression might alter normal hematopoietic development because its expression is significantly upregulated in acute leukemia patients and its constitutive expression in murine BM cells contributes to leukemogenesis [13]. Available evidence suggests that *RUNX1a* and *RUNX1c* isoforms play different roles throughout mammalian hematopoietic development.

To corroborate the relevance of RUNX1c during human embryonic hematopoiesis, we next completed OP9 differentiation with $RUNX1c^{-/-}$ hESCs generated by conventional homologous recombination [28]. Intriguingly, specific deletion of *RUNX1c* did not impact HEP specification, but profoundly impaired the commitment of HEPs into CD45+ hematopoiesis. In contrast, Elefanty's laboratory has recently shown that *RUNX1c* deletion does not affect the emergence of HEPs or CD45+ blood cells using a spin-EB differentiation protocol [28]. The unaffected HEP compartment could be explained whether: (a) *RUNX1c* is not required for its formation or (b) there is a deficiency in a specific subpopulation that we are not able to detect because the limited resolution of the panel of antibodies used. Intriguingly, $RUNX1c^{-/-}$-hematopoietic derivatives displayed similar CFU potential to $RUNX1c^{+/+}$ counterparts but distinct colony type distribution (absence of G/GM, reduced M, and increased E) was observed in $RUNX1c^{-/-}$ hESCs compared with $RUNX1c^{+/+}$ counterparts. Interestingly, Ng et al. did not observe differences in the CFU potential between $RUNX1c^{-/-}$ and $RUNX1c^{+/-}$ hESCs [28]. Our results suggest that *RUNX1c* deletion could block the progression from primitive, where only erythrocytes, macrophages, and megakaryocytes cells can be generated, to definitive hematopoiesis in OP9-based hematopoietic differentiation systems. In line with this hypothesis, $Runx1c^{-/-}$ mice had altered definitive hematopoietic progenitor cell number and only the primitive E, M, and megakaryocyte-CFU were obtained [15, 16]. Alternatively, it cannot be ruled out that cytokine cocktails present in spin-EB differentiation systems and methylcellulose cultures might bypass *RUNX1c* deletion in vitro by activating signaling pathways downstream of RUNX1c. In OP9 coculture system, the absence of those cytokines enhances the relevance of *RUNX1c* in the transition from HEPs to CD45+ blood cells. In addition, *RUNX1c* deficiency caused a drastic reduction in *SCL*, *GATA1* and *PU.1* expression, confirming *RUNX1c* over-expression results, and placing

 

RUNX1c as a putative transcription regulator of these three hematopoietic transcription factors. Collectively, our results suggest that *RUNX1c* is dispensable for the generation of HEPs but is necessary for the generation of CD45+ blood cells from hPSCs [16, 21, 43].

Finally, to decipher the molecular mechanisms triggered by RUNX1c that enhance hematopoietic differentiation of hESCs, we performed gene expression profiling analysis of EV- and RUNX1c-overexpressing HEPs at different time points. As expected, RUNX1c activates a molecular signature related with the "Hematological System Development and Function". A deeper analysis allowed us to discover that *RUNX1c* overexpression predicted the activation of several set of genes with different functionalities. The first cluster included genes controlling macrophage functions, specifically *CSF1*, *CSF2*, and *CSF1R*. This activation could explain the slightly higher number of CFU-M in RUNX1c hematopoietic progeny, a previously unrecognized function of RUNX1c in monocyte/macrophage development.

RUNX1c-mediated hematopoietic specification of hESCs involved a proinflammatory transcriptional signature, consistent with recent studies in zebrafish and mice demonstrating that proinflammatory signaling is a positive regulator of hematopoietic development [51–54]. Our in silico analysis of the gene expression profile imposed by RUNX1c showed that this profile is consistent with an activation of several proinflammatory regulators, but we have no evidence that RUNX1c directly regulates their expression. Expression analysis of RUNX1c-expressing and *RUNX1c*$^{-/-}$ HEPs confirmed that several members of the type I interferon signaling pathway and the *NF-κB* members *NFKB1* and *RELA* were modulated by RUNX1c. Interestingly, BloodChiP analysis reveals that most of these genes are direct targets of RUNX1 in human HSCs [56]. However, Dr. Menendez's laboratory has demonstrated that the addition of individual proinflammatory cytokines throughout hematopoietic differentiation in both EBs and OP9 systems is not sufficient to potentiate definitive hematopoietic specification of hPSCs in vitro [61]. These results indicate that in vitro hPSC hematopoietic differentiation does not recapitulate the microenvironmental signals present in vivo in the embryo. For instance, primitive neutrophils originated in the yolk sac are necessary for the establishment of definitive HSCs in vivo in the AGM [51], but these cells are absent in in vitro experiments. Further work is required to biochemically and functionally confirm that RUNX1c orchestrates hematopoietic specification of hPSCs in cooperation with proinflammatory signaling.

## Conclusion

We show that RUNX1c is an important transcription factor regulating human early blood specification, possibly in cooperation with proinflammatory signals. The resolution of the spatiotemporal expression of these immune regulators and the integration with other known hematopoietic regulators could serve as a platform to efficiently activate a bona fide hematopoietic transcriptional program in HSCs derived from hPSCs.

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## Author Contributions

O.N.-M.: performed most experiments, analyzed the data and wrote the manuscript; V.A. and V.R-M.: contributed the experimental design, data analysis and wrote the manuscript; R.M., M.L., L.L-O., X.G.-C., T.R., and D.R.-M.: contributed to the experiments and data analysis; C.B.: contributed the experimental design and data analysis; E.N., E.S., and A.E.: provided important materials; P.M. and P.J.R.: conceived and supervised the project and wrote the manuscript.

## Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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