Blood Advance

Title: Angiocrine factors from HUVECs amplify erythroid cells

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Abstract

Erythropoiesis is regulated by microenvironmental factors from the vasculature. Enhanced erythropoiesis, which occurs under stress or during development, amplifies erythroid cells to meet the demand of red blood cells. This process uncouples cell division and differentiation, thus the accumulated erythroid cells remain undifferentiated in the vasculature. However, little is known about how vascular endothelial cells (ECs) regulate erythropoiesis. Here we identified that human umbilical vein endothelial cells (HUVECs) keep erythroid cells undifferentiated and amplify their number. We determined that HUVECs amplify erythroid cells via secreted angiocrine factors. The expression profile of these factors suggested that they resemble macrophage-crines for enhanced erythropoiesis. Molecularly, HUVECs mediate the activation of ERK signaling. These data indicate that angiocrine factors from HUVECs enhance erythropoiesis via the amplification of undifferentiated erythroid cells. Our study contributes to the ultimate goal of harnessing erythropoiesis to replace blood transfusions.

Introduction

Every year, approximately 36,000 units of red blood cell transfusions are used for patients suffering from diseases, undergoing surgical operations, or some other medical need every day in the US (https://www.redcrossblood.org/donate-blood/how-to-donate/how-blood-donations-help/blood-needs-blood-supply.html). The source of these transfusions has depended on blood donors, but more stable sources are required for the anticipated growth in demand (Batta, 2016). One alternative is stem cells, which can be proliferated to high numbers that produce the needed volume (Chung, 2017; Doulatov, 2013; Kinney, 2019; Orkin, 2008). However, limited understanding of erythropoiesis has prevented the production of clinically relevant quantities of erythrocytes (Fang, 2016; Wei, 2019).

Erythropoiesis is the process through which red blood cells are produced (An, 2015). Erythropoiesis begins with the commitment of hematopoietic progenitor cells to erythroid cells that takes places in both embryos and

adults (Nandakumar, 2016; Pimkin, 2014). Under stress erythropoiesis, glucocorticoids uncouple cell division and differentiation, thus maintaining and amplifying undifferentiated erythroid cells (Li, 2019).

The amplification of erythroid cells takes place in the vascularized regions of tissues, such as yolk sac, fetal liver, placenta, and adult bone marrow (BM) (Baron, 2012; Van Handel, 2010). The role of vascular endothelial cells (ECs) has been documented in the maintenance of hematopoietic stem cells, the differentiation to both myeloid and lymphoid lineage types, and the production of platelets (Morrison, 2014; Pinho, 2019). However, the role of vascular ECs in erythropoiesis is unclear. Elucidating the contribution of vascular ECs and their effector molecules is expected to achieve the clinically relevant number of red blood cells from stem cells (Butler, 2010; Ziyad, 2018).

Herein we demonstrate the role of human umbilical vein endothelial cells (HUVECs) in enhanced erythropoiesis. We show that angiocrinie factors secreted from HUVECs maintain and amplify undifferentiated erythroid cells. We profiled these angiocrine factors and found that they shared features

with macrophage-crines known to enhance erythropoiesis (Lopez-Yrigoyen, 2019). The prospective downstream target of the angiocrine factors is ERK signaling (Eblen, 2018; Grasman, 2017; Rezaei, 2019; Smalley, 2018), whose suppression terminated the HUVEC-mediated amplification of erythroid cells.

Results

HUVECs amplify erythroid cells

To address whether embryonic ECs have a role in erythropoiesis, we co-cultured human BM-CD34+ hematopoietic progenitor cells with either HUVECs or human pluripotent stem cell (hPSC)-derived ECs seeded on fibronectin. Co-culture with HUVECs resulted in the amplification of Pro-erythroblasts (EB)s (CD71+GLY-A-) and EBs (CD71+GLY-A+), so that overall total of erythroid cells increased (fig. 1A-B). HUVECs on fibronectin stalled the waterfall pattern of erythroid differentiation, particularly before the entry to Late-EB (CD71-GLY-A+) stage (fig. 1A). In contrast to HUVECs, co-culture with human PSC-derived ECs fully differentiated to Late-EBs (fig. 1A). We measured the differentiation potential of the amplified erythroid cells with HUVEC co-culture. We isolated and cytospun the EB population to identify basophilic erythroids (fig. 2A). To examine if the amplified erythroid cells could undergo further differentiation, we cultured them for an additional week. We found that 32% of them exited from EB to become Late-EB and differentiated to orthochromatic erythroid (fig. 2B), indicating

that differentiation capacity could be restored. These data demonstrate that HUVECs amplify erythroid cells by keeping them undifferentiated.

Angiocrine factors from HUVECs amplify erythroblasts

To assess whether cell-cell interactions are required for the enhanced amplification of erythroid cells, we used a transwell assay, where the media and secreted factors could be exchanged but direct HUVEC contact was prevented. We found an increase in erythroid cells in the transwell setting (fig. 3), suggesting that secreted angiocrine factors are involved in the erythroid amplification. To determine the angiocrine factors produced by HUVECs, we conducted an RNA-seq analysis for the genes that encode secreted factors in HUVECs and hPSC-ECs (fig. 4A). Based on literature search, we classified the secreted factors into three categories (fig. 4B). Erythropoiesis enhancing factors (EEFs) include classical hematopoietic cytokines and morphogens whose roles are validated in erythropoiesis (Paulson, 2011). Recent studies proved that both OP9 (Trakarnsanga, 2018) and macrophages (Lopez-Yrigoyen, 2019) enhance erythropoiesis via secreted factors, which are termed OP9-crines and macrophage-crines respectively in this study. EPO, EGF ligands, and glucocorticoid synthases

were included among EEFs, whose expressions were not detected in HUVECs (fig. 4B). Additionally, OP9-crines were not enriched in HUVECs (fig. 4B). On the other hand, NRG1 and IGFBP6, which are included among macrophage-crines, were exclusively expressed in HUVECs (fig. 4B, table 1). These data demonstrate that HUVECs express and share their profile of angiocrine factors with macrophages.

ERK is involved in amplification of erythroid cells

NRG1 and IGFBP6 regulate ERK signaling (fig. 5A; Kataria, 2019; Zinn, 2013). We confirmed that HUVECs activated the ERK signal in erythroid cell line K562 (fig. 5B). Accordingly, we observed that the chemical inhibition of ERK diminished the HUVEC-mediated amplification of erythroid cells (fig. 5C-D). Consistent with this reduced amplification, the inhibition of ERK moved the scatter profile of the GLY-A+ population toward differentiation (fig. 5C). Of note, we made consistent observations in both BM- and cord blood (CB)-CD34+ cells, suggesting the mechanisms of erythroid cell amplification is common between the cell source type: CB from fetus and BM from adult (fig. 5D). These data demonstrate that ERK activation amplifies erythroid cells through angiocrine factors from HUVECs.

Discussion

Elucidating a manner to amplify erythroid cells is expected to achieve a clinically relevant number of red blood cells (Koury, 2016). Several studies have shown that HUVECs proliferate hematopoietic stem and progenitor cells (Yildirim, 2005), but the understanding of their role in erythropoiesis remains incomplete. Our work demonstrated that angiocrine factors secreted by HUVECs amplify erythroid cells, suggesting a possible mechanism to produce red blood cells *in vitro*.

ERK signaling mediates erythroid commitment of hematopoietic progenitor cells at the initial phase of erythropoiesis, but its role in the subsequent phases of erythropoiesis is not clear (Guihard, 2010). We demonstrated that ERK signaling amplifies erythroid cells through angiocrine factors from HUVECs. Instead of other erythropoiesis factors, such as EPO (Kuhrt, 2015), SCF (Comazzetto, 2019) or glucocorticoids (Narla, 2011), HUVECs produce angiocrine factors in common with those from macrophages to enhance erythropoiesis (Lopez-Yrigoyen, 2019; Seu, 2017). One study showed the contribution of cell-cell contacts between murine

hemangioma-derived ECs and erythropoiesis. However, we did not find these contacts to be a factor with HUVECs. (Ohneda, 1997). To conclude, our findings suggest that angiocrine factors could enhance erythropoiesis from either donor-derived or hPSC-derived hematopoietic progenitor cells.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

R.S. and R.O. designed the study, conducted the experiments, interpreted the data and wrote the manuscript. C.M., E.S., T.S. T.N., and Y-s..T. conducted the experiments. M.K.S. supervised the study. R.S., R.O., Y-s.T., A.N., and M.K.S. commented on and wrote the paper.

Disclosures

The authors declare they have no competing financial interests.

Methods

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ryohichi Sugimura (ryohichi.sugimura@gmail.com).

Experimental Model and Subject Details

Cell lines

All the experiments of this study were performed with 409B2 iPSC and CBA11 iPSC lines (Ohta, 2019). K562 was obtained through RIKEN Bioresource Center. Human BM-CD34+ cells were purchased from Lonza (Tissue Acquisition Number: 35843, 35845, 32423, 34781, 30968). Human CB-CD34+ cells were purchased from Stemexpress (mixed donor, CB34P3401C). HUVECs were either purchased from Angio-Proteomie (GFP-HUVEC, cAP-01001GFP) or Lonza (issue Acquisition Number: 29000).

Accession number of RNA-sequencing

GSE138104

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138104

Method details

hPSC culture

The maintenance of hPSCs was done using iMatrix-511 (Matrixome) in mTeSR1 media (STEMCELL Technologies). Media were changed every

other day, and the cells were passaged as single cells every 7 days using TrypLE Express (Life technologies).

Endothelial differentiation

hPSC spheroids were formed as described previously (Ohta, 2019). hPSC spheroids were suspended in mTeSR1 (StemCell Technologies) containing 1.25 μg/mL iMatrix-511 (Matrixome) and subsequently plated onto non-coated culture plates. After three days, the medium was replaced with Essential 8 (Life technologies) containing 4 μM CHIR99021 (WAKO), 80 ng/mL BMP4 (R&D systems), and 80 ng/mL VEGF (R&D systems). After two more days, the cells were dissociated to the single-cell level with TrypLE Express (Life technologies) for 20 minutes at 37℃ and subsequently plated onto an iMatrix-411 (Matrixome)-coated plate in Stempro34-SFM (Life technologies) containing 80 ng/mL VEGF. After four more days of culture on iMatrix-411, the cells were passaged onto a Type I collagen-coated plate at a density of 10,000/cm^2 in EGM-2 containing 25 ng/mL VEGF and cultured for 9 days with passage every four days.

Co-culture

20,000 BM or CB CD34+ cells were cultured with 60,000 HUVECs or hPSC-derived ECs seeded on 50 µg/mL of fibronectin-coated plates in alpha-MEM medium supplemented with 15% FBS, 1% insulin-transferrin, 5 ng/mL IL-7, 10 ng/mL FLT-3L, 50 µg/mL L-ascorbic acid, and 1% L-Glut/Pen/Strep. The cells were co-cultured for 7 days. For the transwell analysis, we used a 0.4 µM pore transwell (MCHT12H48 Millipore) and seeded CD34+ cells on the transwell apparatus. ERK inhibitor FR180204 (10 µM) was added to culture where described in the text.

Cytospin

5,000 FACS-sorted CD71+GLY-A+ or CD71-GLY-A+ cells were cytospun onto slides (500 r.p.m. for 5 min), air dried, and stained with MayGrunwald and Giemsa stains, washed with water, air dried, and mounted, followed by examination by light microscopy.

Erythroid differentiation of K562

K562 cells were maintained in Ham F12 with 10% FBS. For erythroid differentiation, sodium butyrate was added to the medium (1 mM) and cultured for 7 days.

Antibody

Anti-pERK (#4370), anti-ERK (#4695) and anti-beta-actin (#4970) were purchased from Cell Signaling Technology.

Western blot

Equal amounts of protein extracted from K562 cells were subjected to SDS-PAGE in Tris-Glycine buffer and transferred to PVDF membranes. The membrane was blocked with 5% Skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature and probed with the appropriate primary antibody (1:1,000, anti-pERK, ERK or beta-actin

antibody) overnight at room temperature. After washing with TBS-T, the membrane was incubated with the appropriate secondary antibody (anti-rabbit IgG HRP-linked antibody (1:1,000, cell signaling)) for 1 hour at room temperature. After washing with TBS-T, the membrane was incubated with West Femto super signal reagent (Thermo scientific), and the specific proteins were visualized with LAS-4000 (GE healthcare).

Flow cytometry

Cells grown in culture or harvested from animal tissues were stained with 4:200-1:200 dilution of each antibody for at least 30 min on ice in the dark with the following antibodies; CD71-PE and GLY-A-PECY7. Unless specifically indicated, all the antibodies used are against human cells. Acquisitions were done on a BD FACSAria II cell sorter or BD LSRFortessa cytometer. Sorting was performed on a BDFACS Aria II cell sorter. Flow cytometry data were analyzed using FlowJo V.10.

Bulk RNA-seq

Sequencing was performed on an Illumina HiSeq 2500 platform in a 75-base

single-end mode. Illumina Casava1.8.2 software was used for basecalling. Sequenced reads were mapped to the human reference genome sequences (hg19) using TopHat v2.0.13 in combination with Bowtie2 ver. 2.2.3 and SAMtools ver. 0.1.19. The fragments per kilobase of exon per million mapped fragments (FPKMs) was calculated using Cufflinks version 2.2.1.

Statistics and source data

Statistical analyses were done with t-test. We used Microsoft Excel for calculations and expressed the results as the means ± s.d. The source data for each graph is available in the supplementary tables.

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FIGURE LEGENDS

Fig. 1. Co-culture with HUVECs amplified erythroid cells from BM-CD34+ cells. (A) Flow cytometry plots of CD71 and GLY-A from BM-CD34+ cells cultured without ECs (left), with HUVECs (middle), and with hPSC-ECs (right). (B) Bar graphs show the number of Pro-EBs (CD71+GLY-A-), EBs (CD71+GLY-A+) and Late-EBs (CD71-GLY-A+). The right panel shows a stack of cell numbers for each population. Each dot represents the result from a biologically independent experiment (left three panels). N=4. The data shown as mean ± s.d. * $p<0.05$ (comparison between w/o EC samples).

Fig. 2. Amplified erythroid cells can differentiate. (A) CD71+ GLY-A+ EBs from BM-CD34+ cells were amplified in the presence of HUVECs, sorted, and then cultured an additional 7 days. Flow cytometry plots of CD71 and GLY-A from BM-CD34+ cells (middle). Cytospinning shows basophilic erythroids (right). (B) Flow cytometry plots of CD71 and GLY-A from cultured EBs (left). Cytospinning shows orthochromatic erythroids (right). Scale bar = 10 um.

Fig. 3. Angiocrine factors from HUVECs amplify erythroid cells from

BM-CD34+ cells. (A) Flow cytometry plots of CD71 and GLY-A from BM-CD34+ cells cultured without ECs (left), with HUVECs (middle), and with Transwell (TW)-HUVECs (right). (B) Bar graphs show the number of Pro-EBs (CD71+GLY-A-), EBs (CD71+GLY-A+), and Late-EBs (CD71-GLY-A+). The right panel shows a stack of cell numbers for each population. Each dot represents the result from a biologically independent experiment (left three panels). N=3. The data shown as mean ± s.d. * $p<0.05$ (comparison between w/o EC samples).

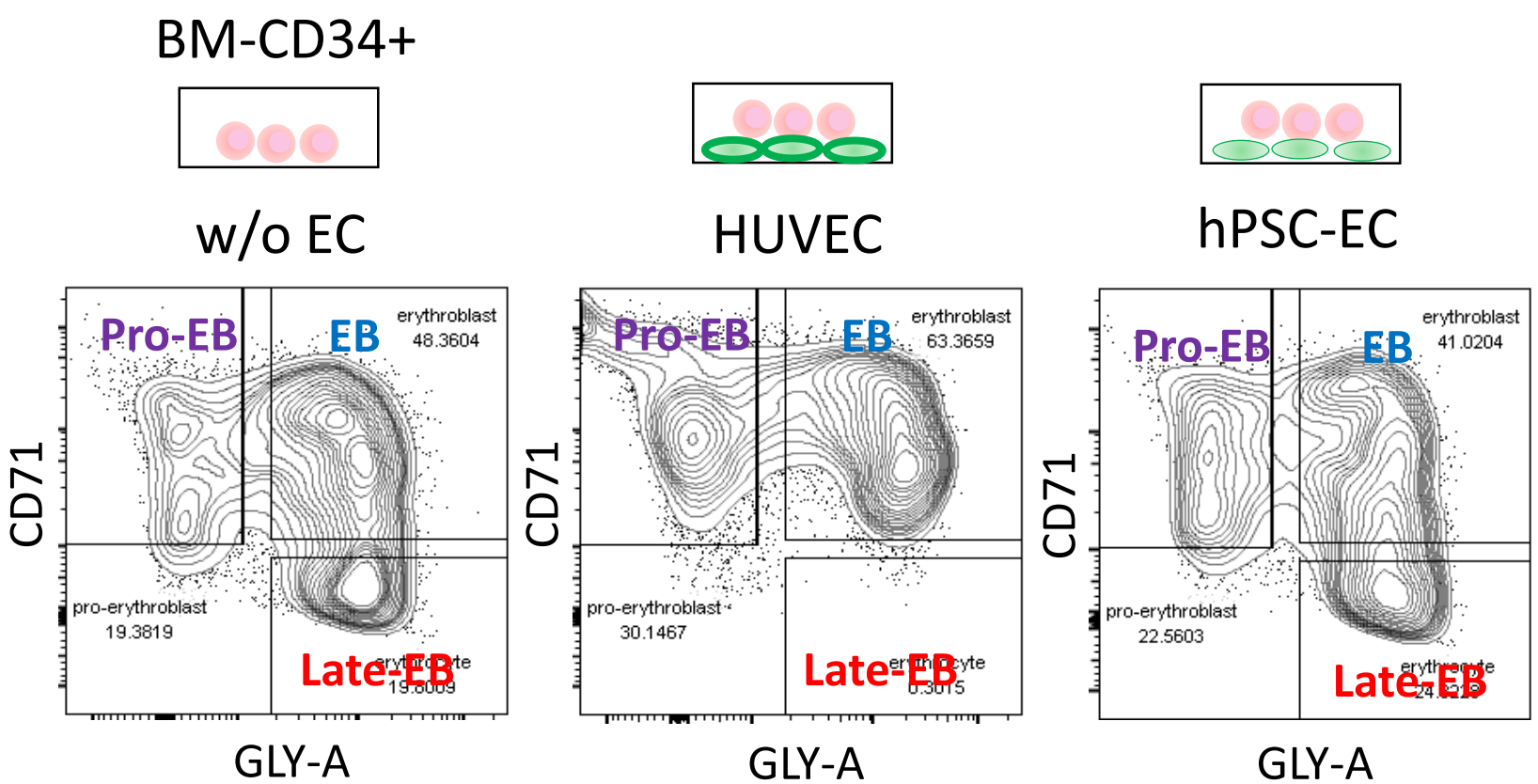
Fig. 4. Profiling the angiocrine factors secreted from HUVECs. (A) Bulk RNA-seq analysis of HUVECs and hPSC-ECs was conducted and profiled for erythropoiesis enhancing factors (EEFs) including glucocorticoid (GCC) synthases, OP9-crines and macrophage-crines that are known to promote erythropoiesis (B) Expression of angiocrine factors enriched in HUVECs compared with hPSC-ECs. Refer to Table 1 for the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of each gene.

Fig. 5. ERK activation amplifies erythroid cells. (A) Scheme of the ERK signal in erythropoiesis. (B) Western blot analysis of the ERK and AKT

pathways in K562 cocultured with HUVECs. N=1. (C) Flowcytometry plots of CD71 and GLY-A from BM-CD34+ cells cultured without ECs (left), with HUVECs (middle), and HUVECs + ERK inhibitor (right). (D) A stack of cell numbers for Pro-EBs, EBs, and Late-EBs from the experiments from BM-CD34+ and CB-CD34+ cells. N=3. The data shown as mean ± s.d. * $p<0.05$ (comparison between w/o EC samples).

Figure 1

A



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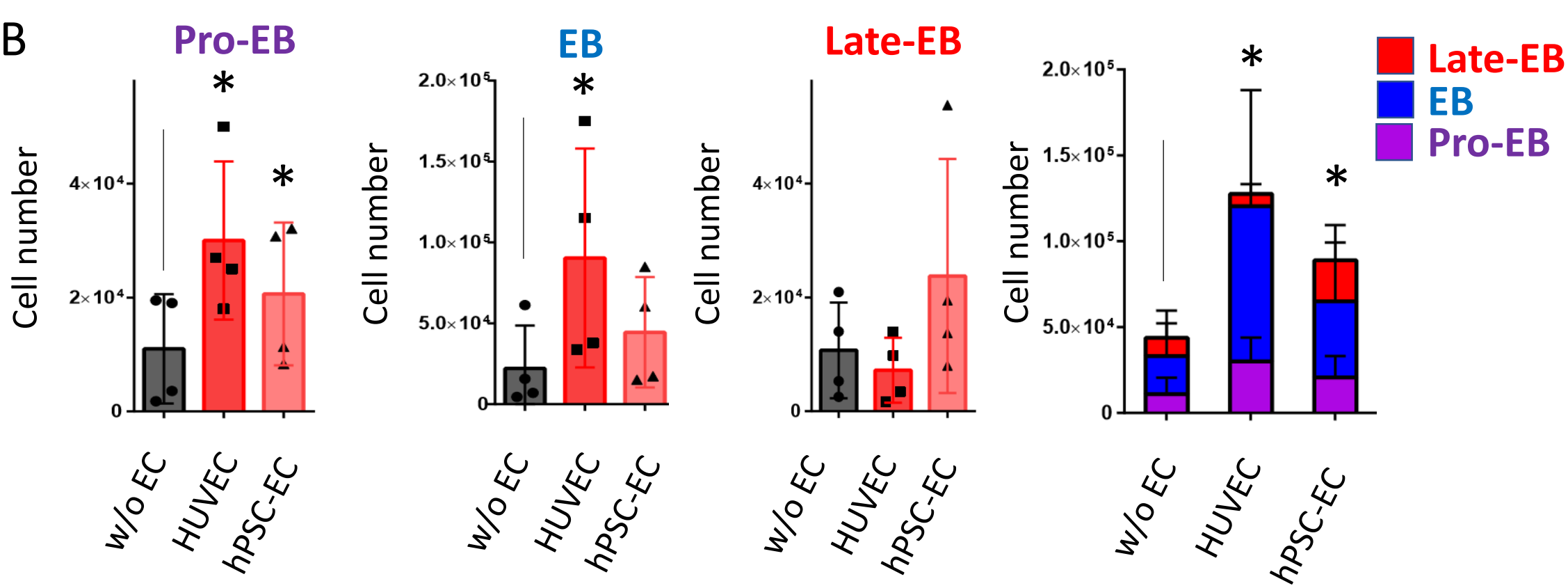
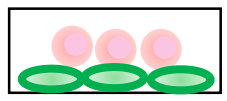


Figure 2

A

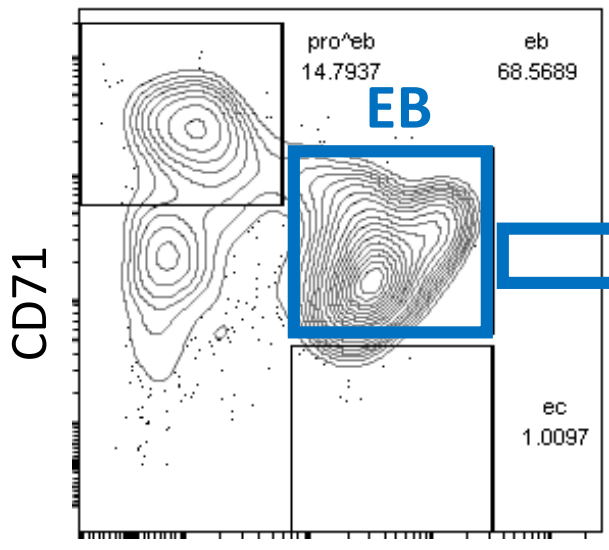
BM-CD34+

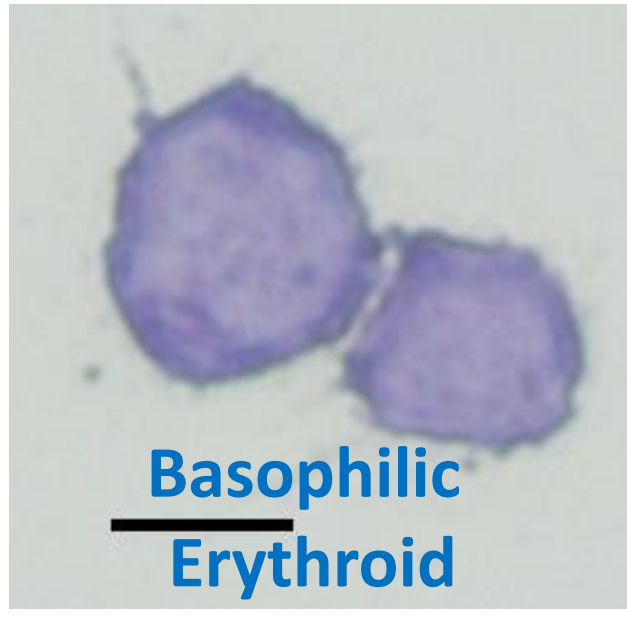


HUVEC

Sort **EB**

+7 days





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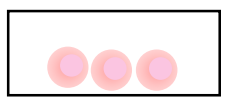

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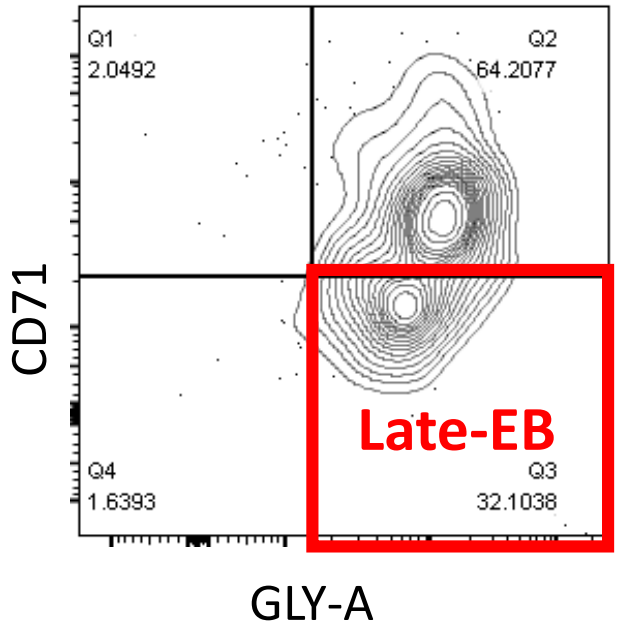

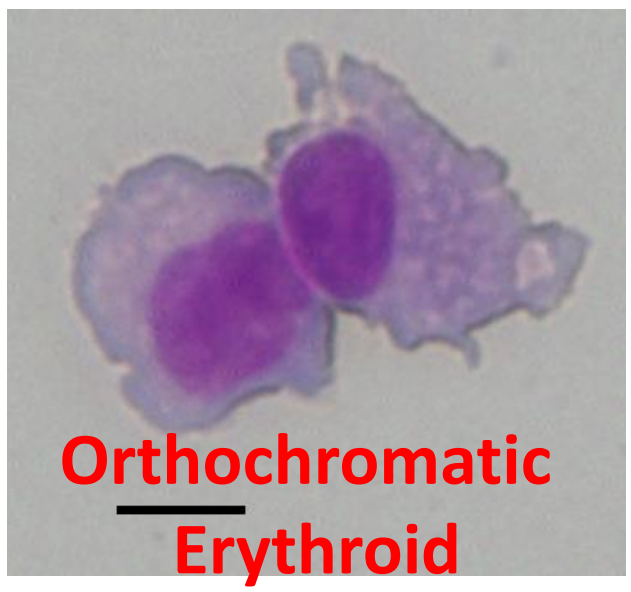

Figure 3

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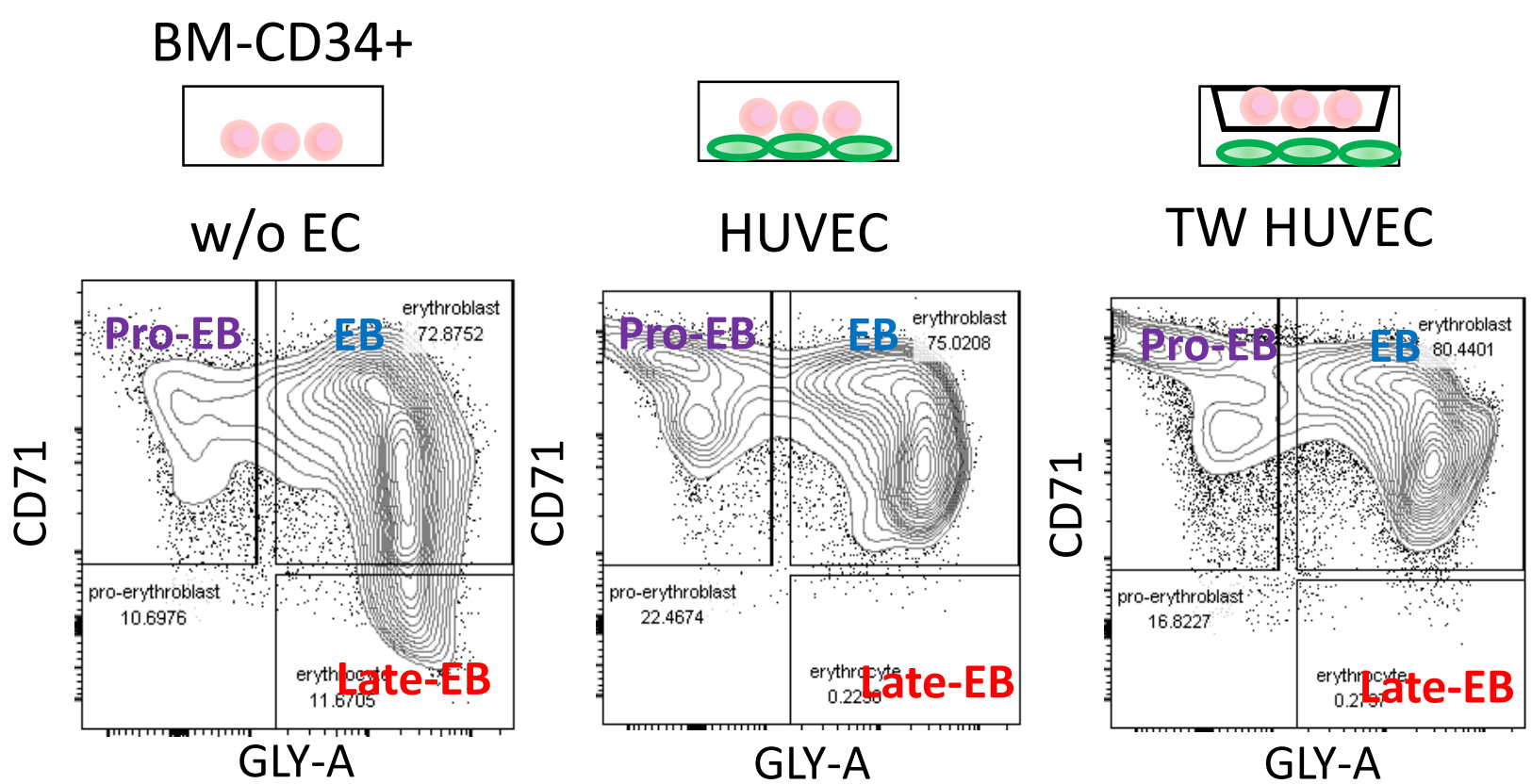

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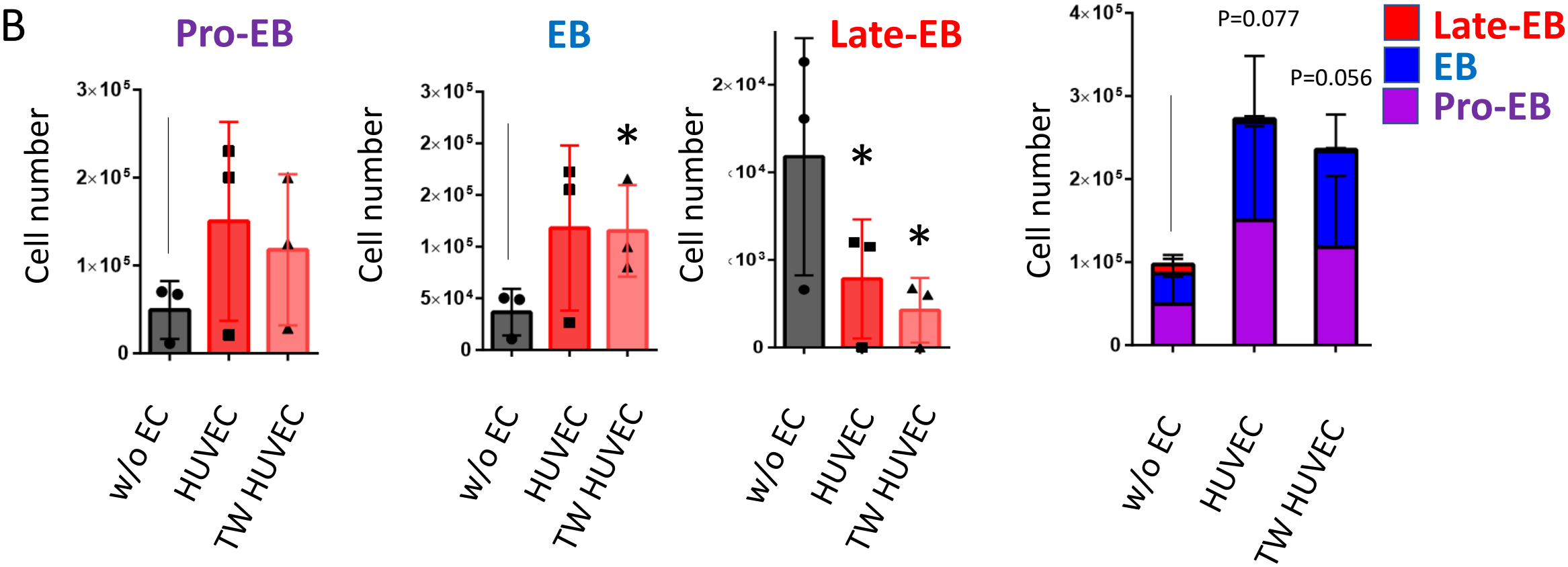

Figure 4

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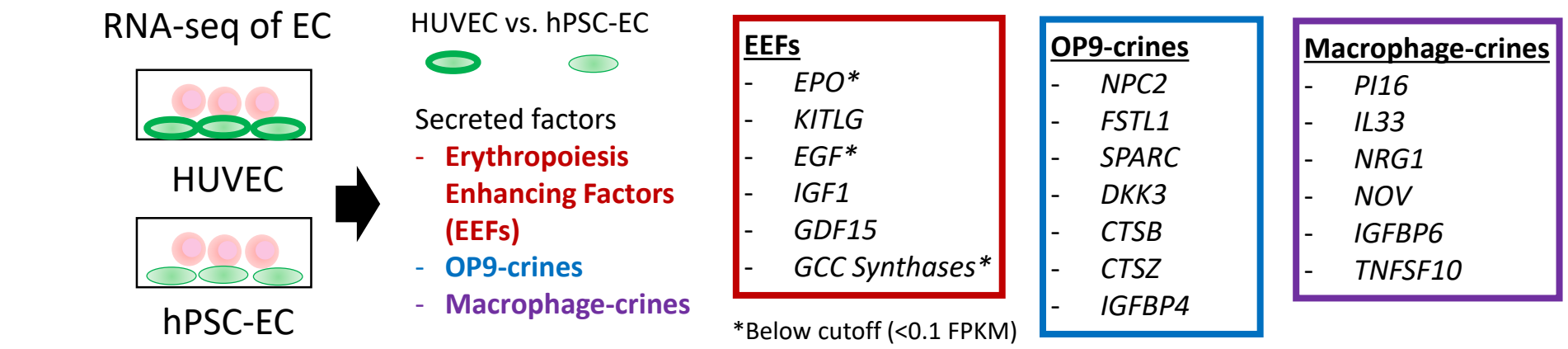

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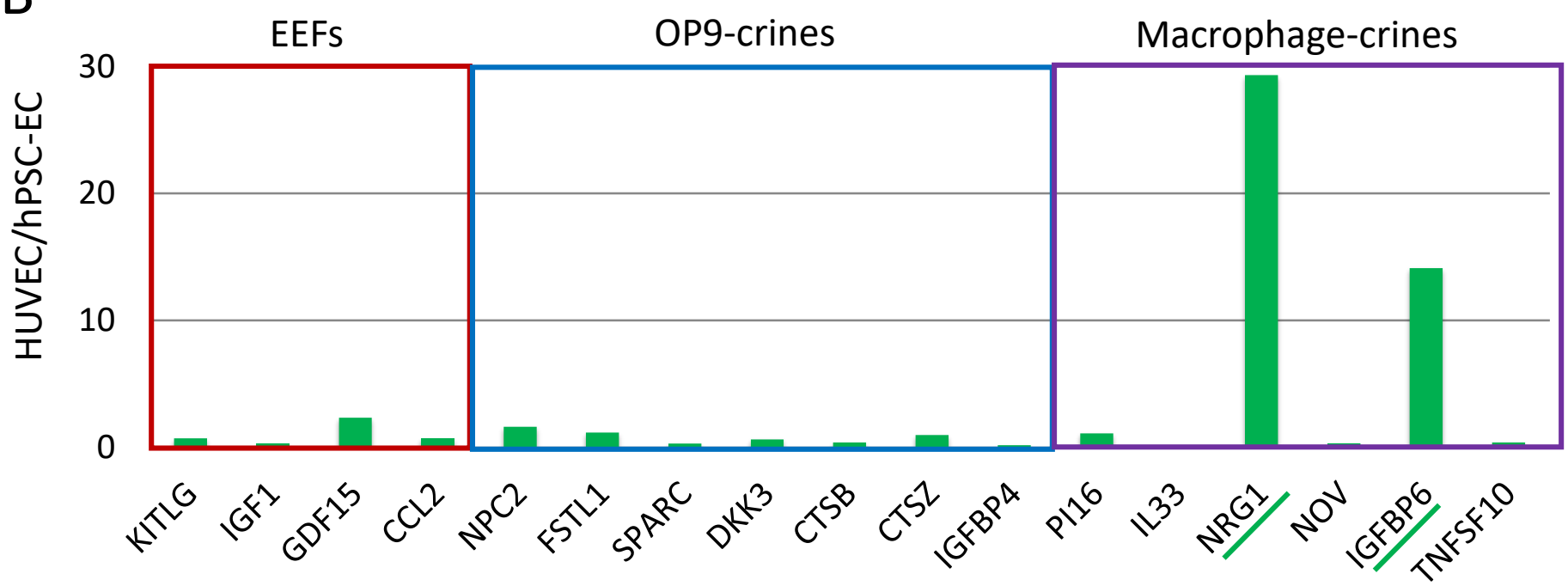
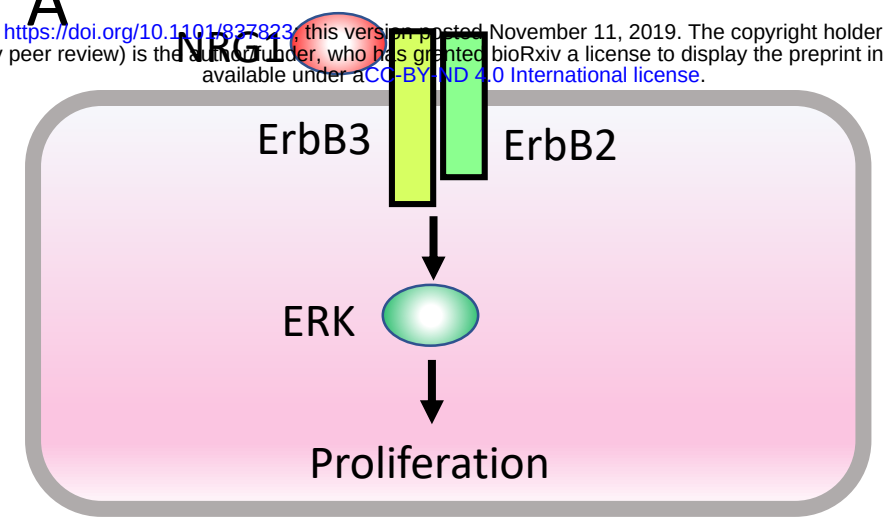
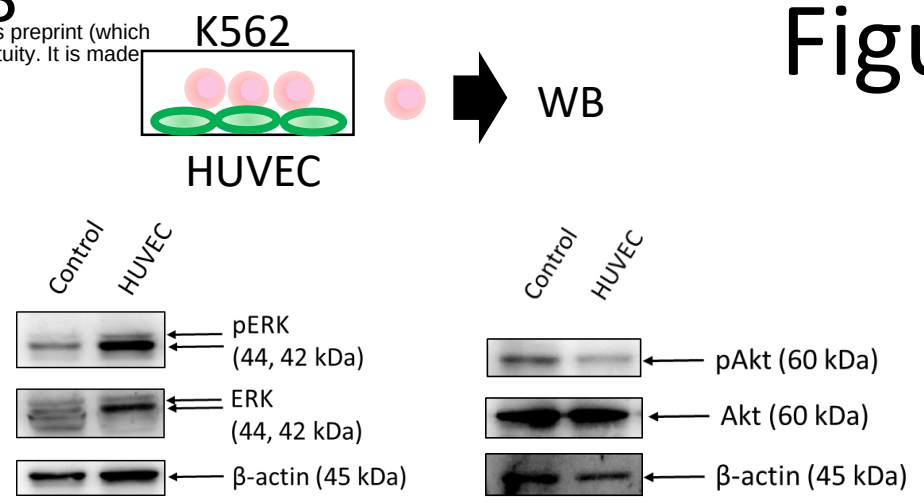


Figure 5

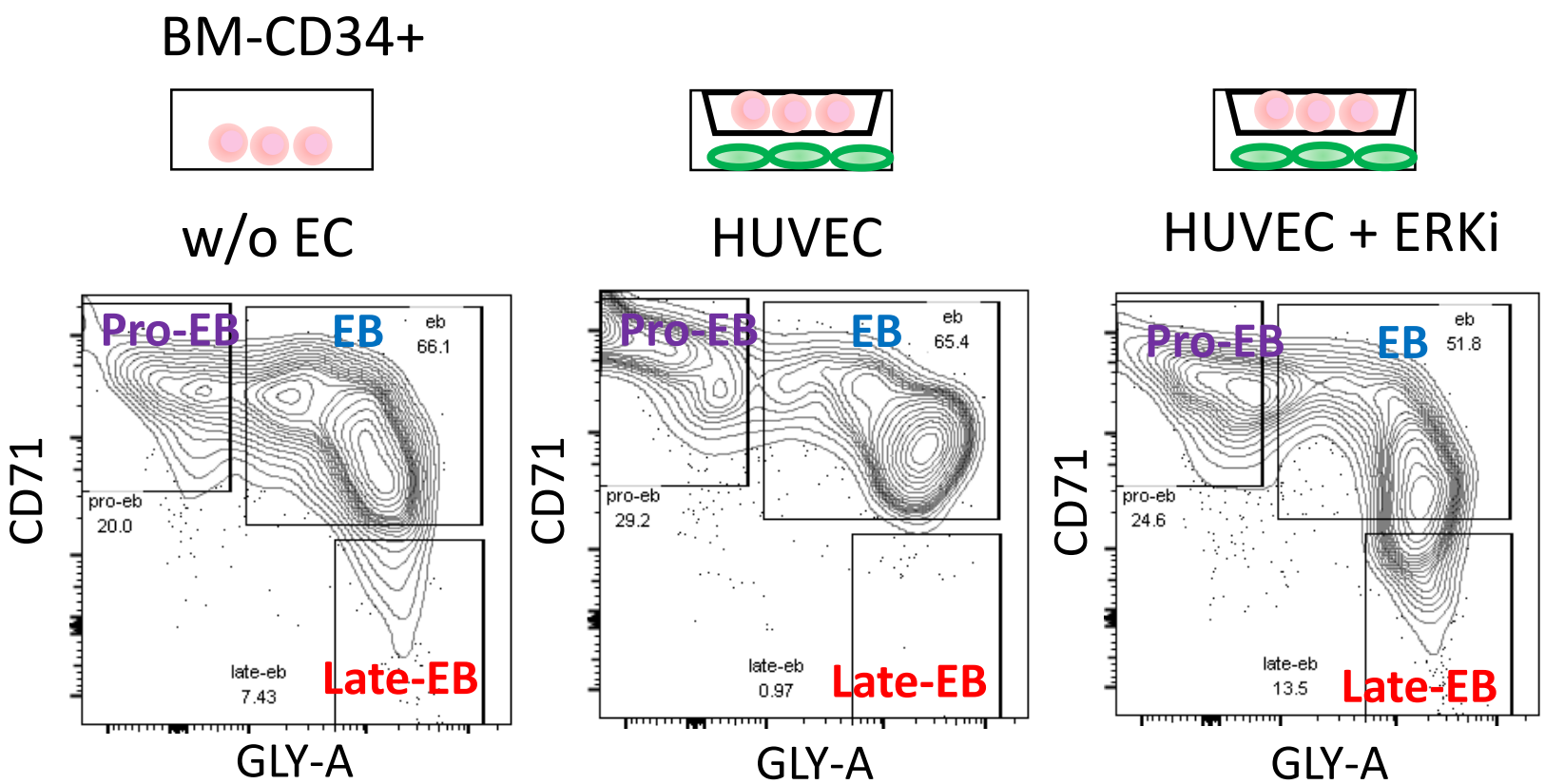
A



B



C



D

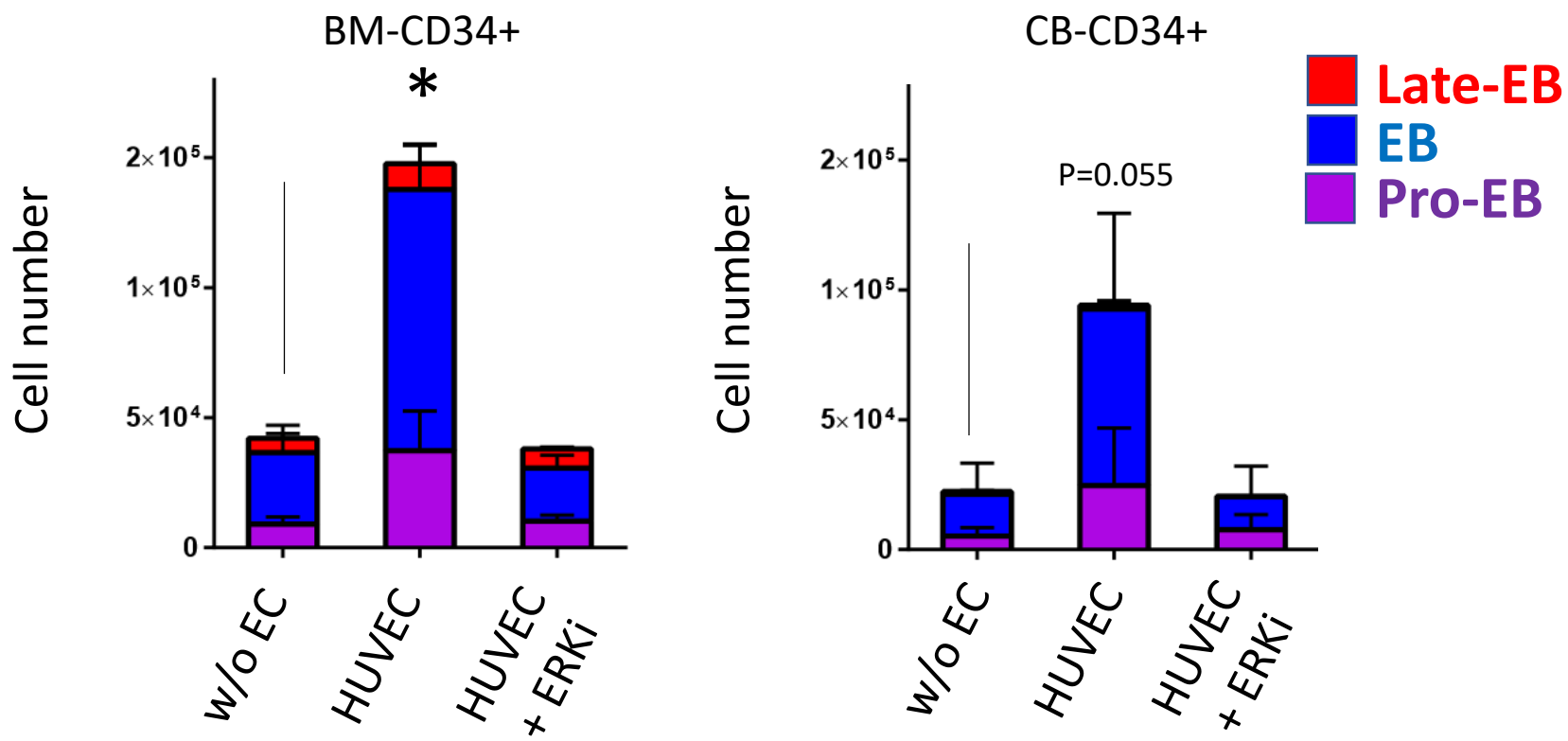


Table.1 FPKM of erythropoiesis enhancing factors, OP9-crines and macrophage-crines in each EC

		FPKM	
Gene name	Description	hPSC-EC	HUVEC
KITLG	KIT ligand	14.416	10.347
IGF1	insulin like growth factor 1	1.547	0.490
GDF15	growth differentiation factor 15	40.987	96.004
CCL2	C-C motif chemokine ligand 2	522.452	369.121
NPC2	NPC intracellular cholesterol transporter 2	109.549	179.006
FSTL1	follistatin like 1	284.146	336.623
SPARC	secreted protein acidic and cysteine rich	1861.880	604.277
DKK3	dickkopf WNT signaling pathway inhibitor 3	116.195	73.664
CTSB	cathepsin B	464.186	185.249
CTSZ	cathepsin Z	232.578	230.470
IGFBP4	insulin like growth factor binding protein 4	546.884	103.969
PI16	peptidase inhibitor 16	0.132	0.148
IL33	interleukin 33	190.364	3.527
NRG1	neuregulin 1	1.169	34.266
NOV	nephroblastoma overexpressed	0.344	0.116
IGFBP6	insulin like growth factor binding protein 6	0.434	6.131
TNFSF10	TNF superfamily member 10	65.905	26.614

Fig1B

Pro-EB

w/o EC	HUVEC	hPSC-EC
1805	18000	8347.2
19500	27000	30750
3603	25000	11333
19027	50000	32085

EB

w/o EC	HUVEC	hPSC-EC
4560	37800	15170
7000	33750	17250
15666	115000	85000
61275	175000	60450

Late-EB

w/o EC	HUVEC	hPSC-EC
5287	1666	13741
20962	14000	53785
2527	3420	8004
14000	9750	19500

Fig3B

Pro-EB

w/o EC	HUVEC	TW HUVEC
70000	200000	125000
67000	230000	200000
11400	20760	28600

EB

w/o EC	HUVEC	TW HUVEC
50400	155400	100000
48910	172500	165400
10680	26400	80600

Late-EB

w/o EC	HUVEC	TW HUVEC
16310	6000	3375
13065	5750	3000
3300	0	0

Fig5D

BM

	Pro-EB	Pro-EB	Pro-EB	EB	EB	EB	Late EB	Late EB	Late EB
w/o EC	6300	12000	8670	20362	39660	22950	7612	4458	4080
HUVEC	20280	49640	42000	80340	111180	110000	13910	1649	14000
HUVEC + ERKi	7720	12300	10750	18640	25900	16500	8040	6750	7500

CB

	Pro-EB	Pro-EB	Pro-EB	EB	EB	EB	Late EB	Late EB	Late EB
w/o EC	3250	8920	3420	17394	27480	3420	1456	1048	720
HUVEC	8775	49980	15200	52050	110160	41600	3427	1190	0
HUVEC + ERKi	3060	14233	5760	10782	25284	2080	0	107	0