**Stem Cell Reports, Volume *11***

## Supplemental Information

# Early Human Hemogenic Endothelium Generates Primitive and Definitive Hematopoiesis *In Vitro*

**Eva Garcia-Alegria, Sara Menegatti, Muhammad Z.H. Fadlullah, Pablo Menendez, Georges Lacaud, and Valerie Kouskoff**

## SUPPLEMENTAL FIGURE AND LEGENDS

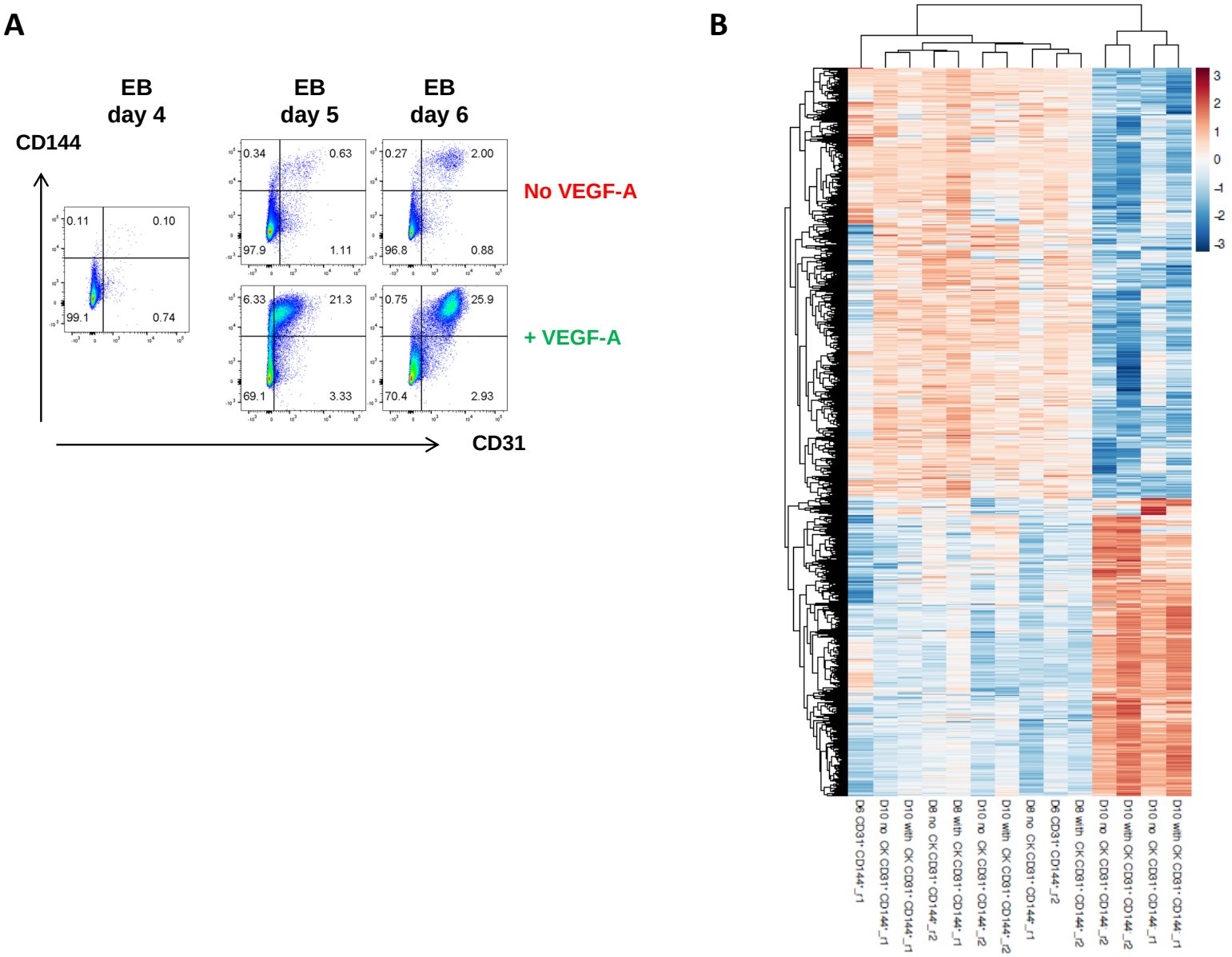


**Figure S1:** related to experimental procedures and Figure 1.

A. Effect of VEGF-A addition at day 4 of EB differentiation on the emergence of human $CD144^{+}CD31^{+}$ cell populations. Data shown are representative of 3 independent experiments.
B. Heat map of all differentially expressed genes (DEG) between the $CD31^{+}CD144^{+}$ population obtained at different times of EB differentiation and the hematopoietic committed population $CD31^{+}CD144^{neg}$ based on transcriptomic RNA-seq data.

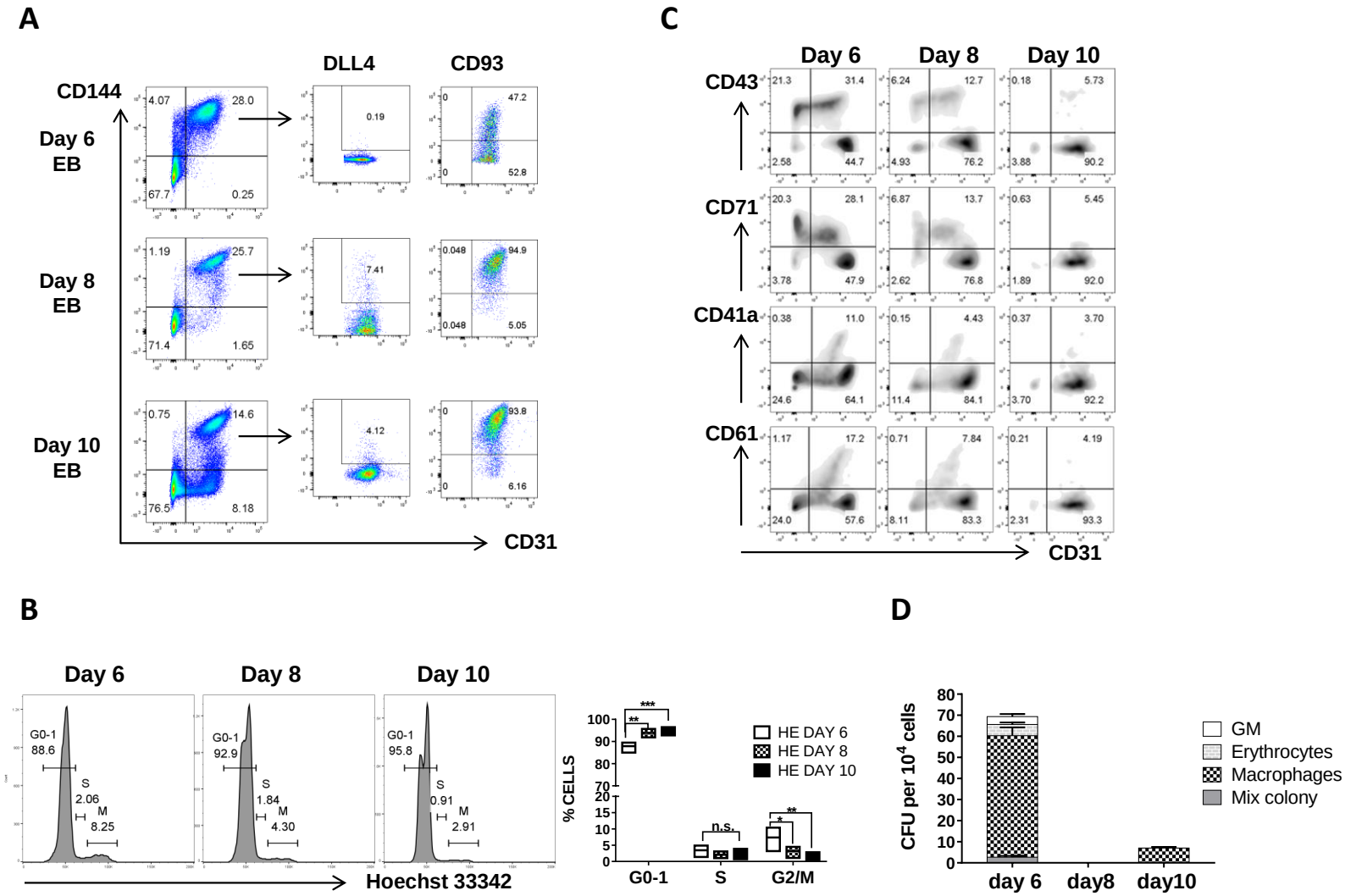


**Figure S2: HE at different time points of the EB differentiation,** related to figure 2

A. Flow cytometric analysis of DLL4 and CD93 expression within the $CD31^{+}CD144^{+}$ cell populations at day 6, 8 and 10 of embryoid body (EB) differentiation. Data shown are representative of 3 independent experiments.

B. Representative plot and statistical analysis of cell cycle of $CD31^{+}CD144^{+}$ cell populations isolated at the indicated times. Error bars indicate the SEM of data from 3 independent experiments. The significance of the difference between samples was confirmed using 2-way ANOVA; p-values *p< 0.05 ** p<0.001 ***p=0.0005.

C. Flow cytometric analysis of the hematopoietic profile obtained after 4 days of culture of $CD31^{+}CD144^{+}CD43^{neg}$ isolated along the EB differentiation at the indicated times. Data shown are representative of 3 independent experiments.

D. Quantification of colony forming unit (CFU) potential of 104 cells obtained after 7 days of culture on gelatine-coated plates of $CD31^{+}CD144^{+}$ isolated from EBs at the indicated times. Error bars indicate the SEM of data from 3 independent experiments.

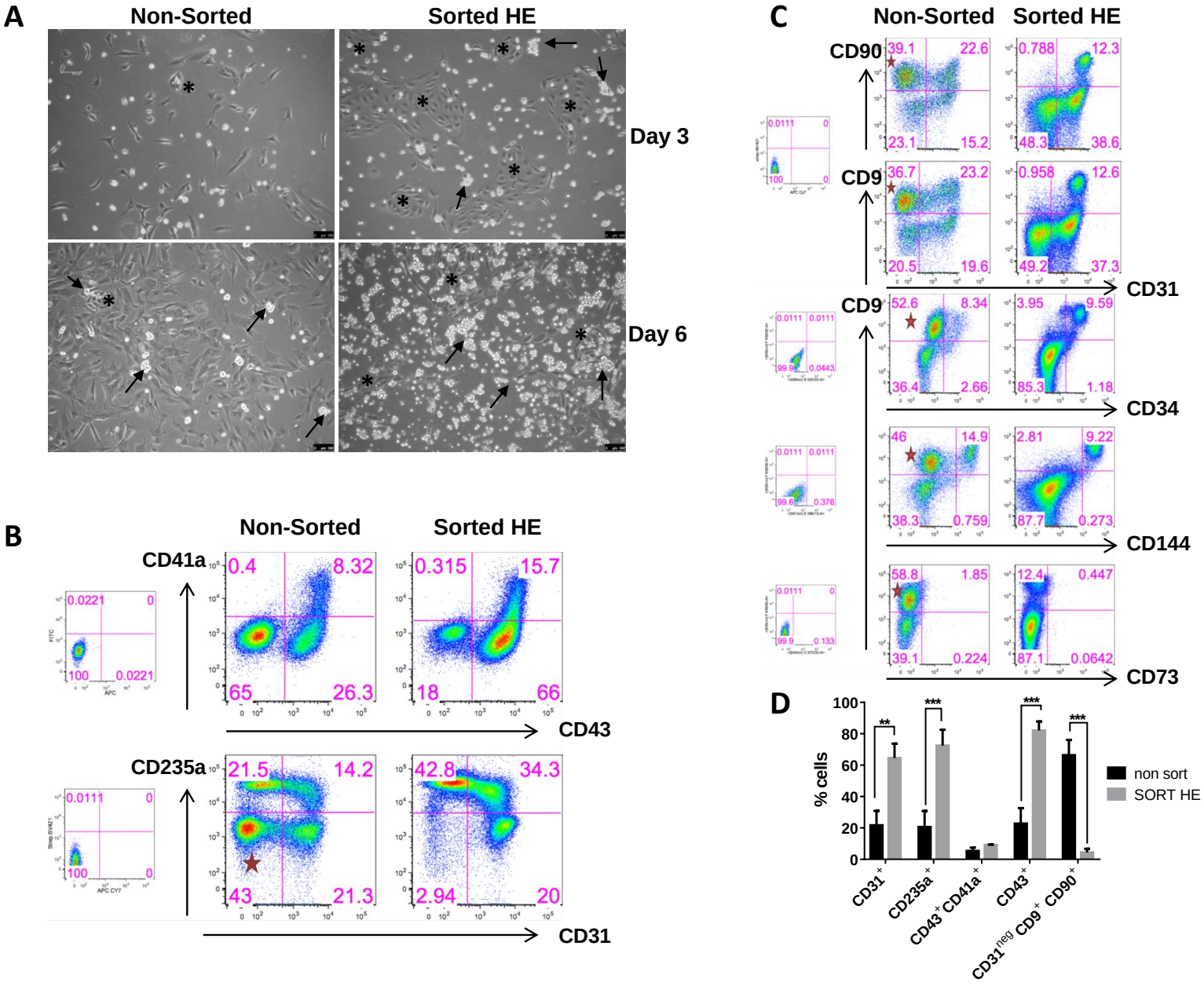


**Figure S3: Enrichment of the HE,** related to experimental procedures

A. Representative photographs of cell morphologies obtained after 3 and 6 days of culture on gelatin-coated dishes in hematopoietic-inducing condition from non-sorted day 6 embryoid body (EB) or from purified $CD31^{+}CD144^{+}$ cells at the same time of differentiation (scale bars 100µm). Progression and emergence of the hematopoietic round cells can be observed. Asterisks indicate endothelial clusters with hematopoietic cells emerging from them. Arrows indicate some of the emerging clusters of blood cells.

B. Flow cytometric analysis of the hematopoietic markers CD43, CD235a and CD41a and endothelial CD31 marker after 4 days of culture. Red star marks the presence of a cell population only present in the non-sorted culture (B-C).

C. Immuno-phenotypic characterization of the cell populations found in the non-sorted and sorted cultures after 4 days in gelatine coated culture. Data are representative of 3 independent experiments.

D. Quantification and statistical analysis of the flow cytometry data obtained after 4 days of culture. Error bars indicate the SEM of data from 3 independent experiments. The significance of the difference between samples was confirmed using 2-way ANOVA; p-value **p=0.004 and ***p<0.001.

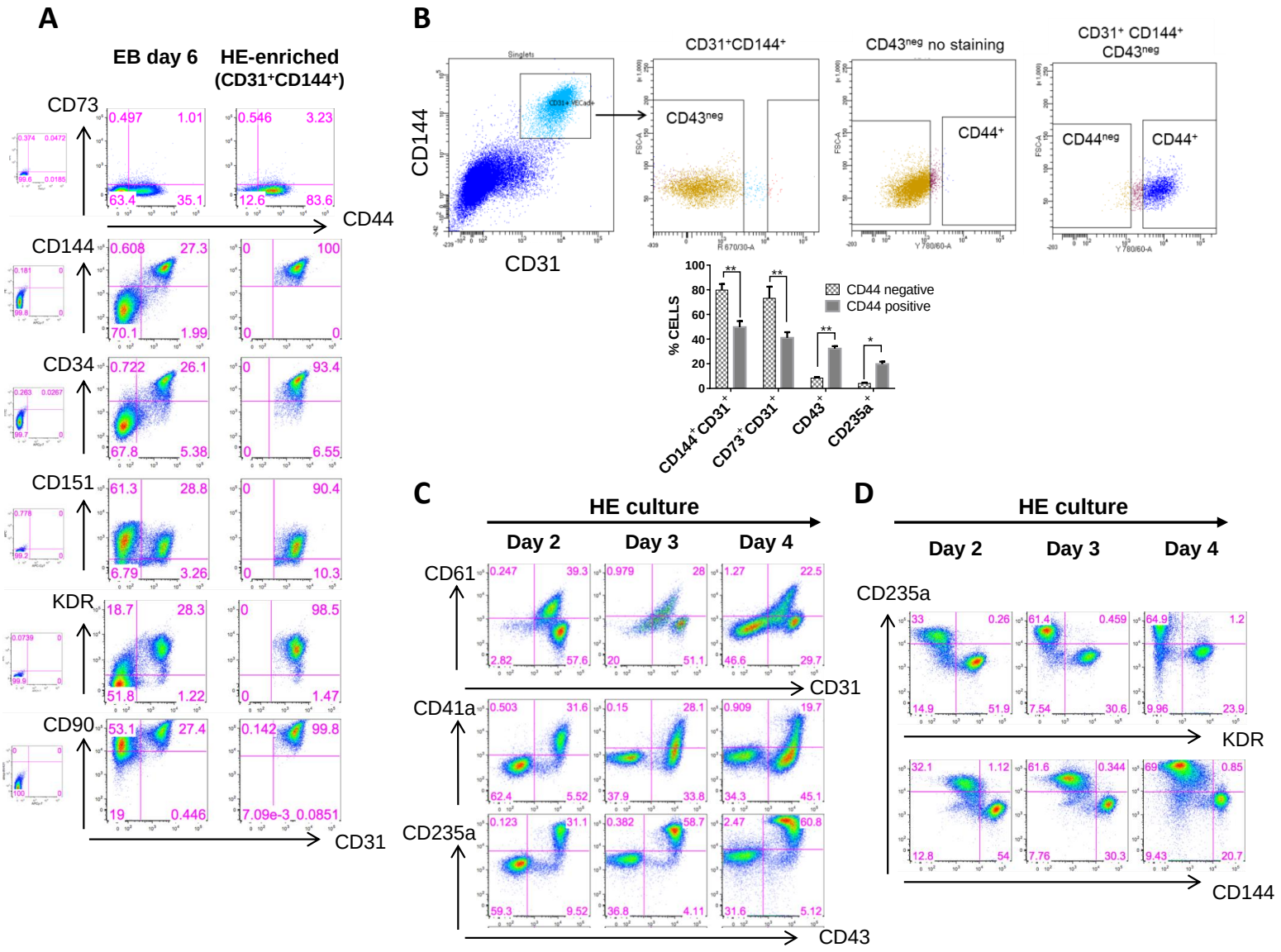


**Figure S4: Purified HE of Man-5 cell line and hematopoietic development in gelatine,** related to figure 3

A. Immuno-phenotypic characterization of $CD31^{+}CD144^{+}$ cells at day 6 of EB differentiation. Data shown are representative of 5 independent experiments.

B. Sorting strategy of $CD31^{+}CD144^{+}$ cells at day 6 of EB differentiation to better characterization of CD44 expressing population. Statistical analysis shows an increase of hematopoietic cells after 7 days in culture from the $CD44^{+}CD31^{+}CD144^{+}$ cell population. Error bars indicate the SEM of data from 3 independent experiments. The significance of the difference between samples was confirmed using 2-way ANOVA; p value *$p<0.05$ and **$p< 0.005$.

C. Flow cytometric analysis of the hematopoietic development obtained from sorted $CD31^{+}CD144^{+}CD43^{neg}$ on gelatine-coated plates at the times indicated. Data shown are representative of 3 independent experiments.

D. Downregulation of CD144 endothelial and KDR mesodermal marker in the hematopoietic population obtained from sorted $CD31^{+}CD144^{+}CD43^{neg}$ cultured on gelatine-coated plates and analysed at the indicated day of culture. Data shown are representative of 3 independent experiments.

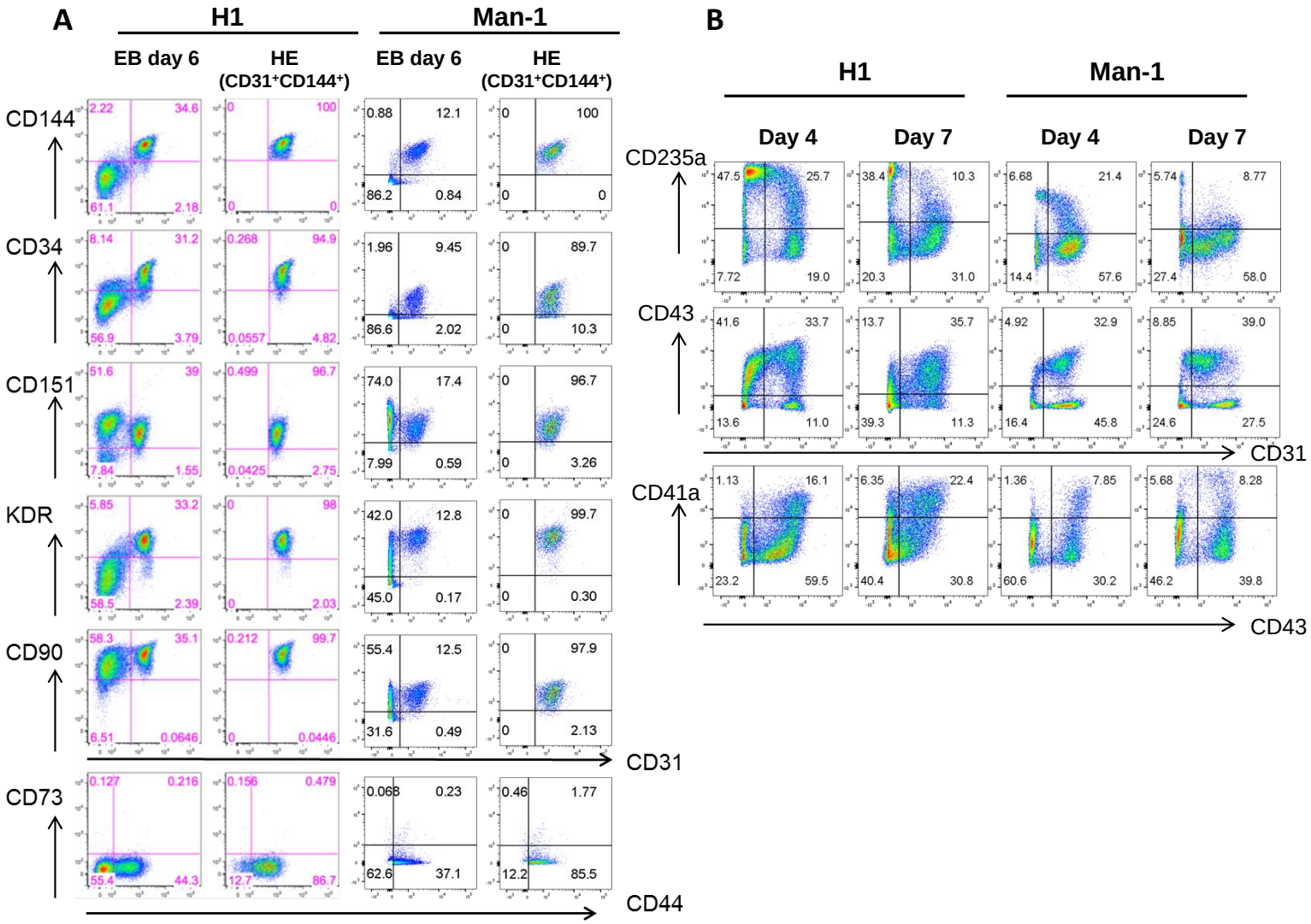


**Figure S5: Characterization of HE and hematopoietic differentiation obtained from H1 and Man1 cell lines**, related to experimental procedures.

A. Phenotypic characterization of $CD31^{+}CD144^{+}$ at day 6 of EB differentiation for H1 and Man1 hESC lines. Representative plots from 3 different experiments per cell line, showing reproducible marker characterization of the human HE-enriched populations.

B. Flow cytometric analysis of the hematopoietic development obtained from sorted $CD31^{+}CD144^{+}CD43^{neg}$ cells on gelatine-coated plates at day 4 and 7 for the H1 and Man1 hESC lines. Data shown are representative of 3 independent experiments per cell line.

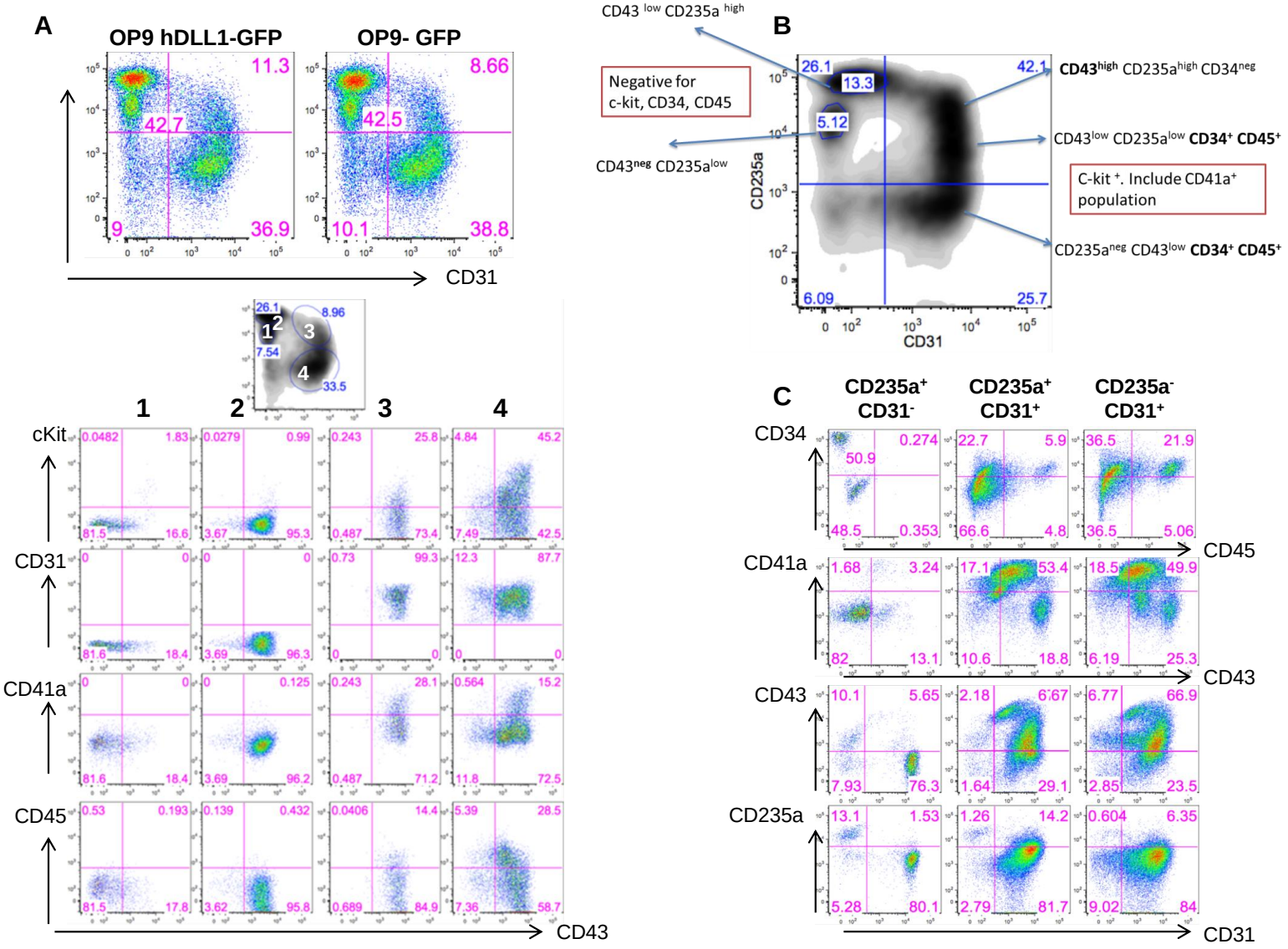


**Figure S6: CD235a/CD31 kinetics in stromal co-culture,** related to figure 3 and 5

A. Representative flow cytometric analysis of CD235a/CD31 populations obtained from $CD31^{+}CD144^{+}CD43^{neg}$ co-cultured for 7 days with irradiated OP9 and OP9-hDLL1 (upper panels). Characterization of the cell surface markers expressed or not in each population following 7 days of co-culture with stromal cells OP9-hDLL1, (lower panels). Data shown are representative of 3 independent experiments.

B. Summary of the cell surface markers expressed by each population after 7 days of $CD31^{+}CD144^{+}CD43^{neg}$ co-culture with stromal cells.

C. Flow cytometric analysis of hematopoietic development from the different CD235a/CD31 fractions after 7 day of culture on irradiated OP9 GFP stromal cells.

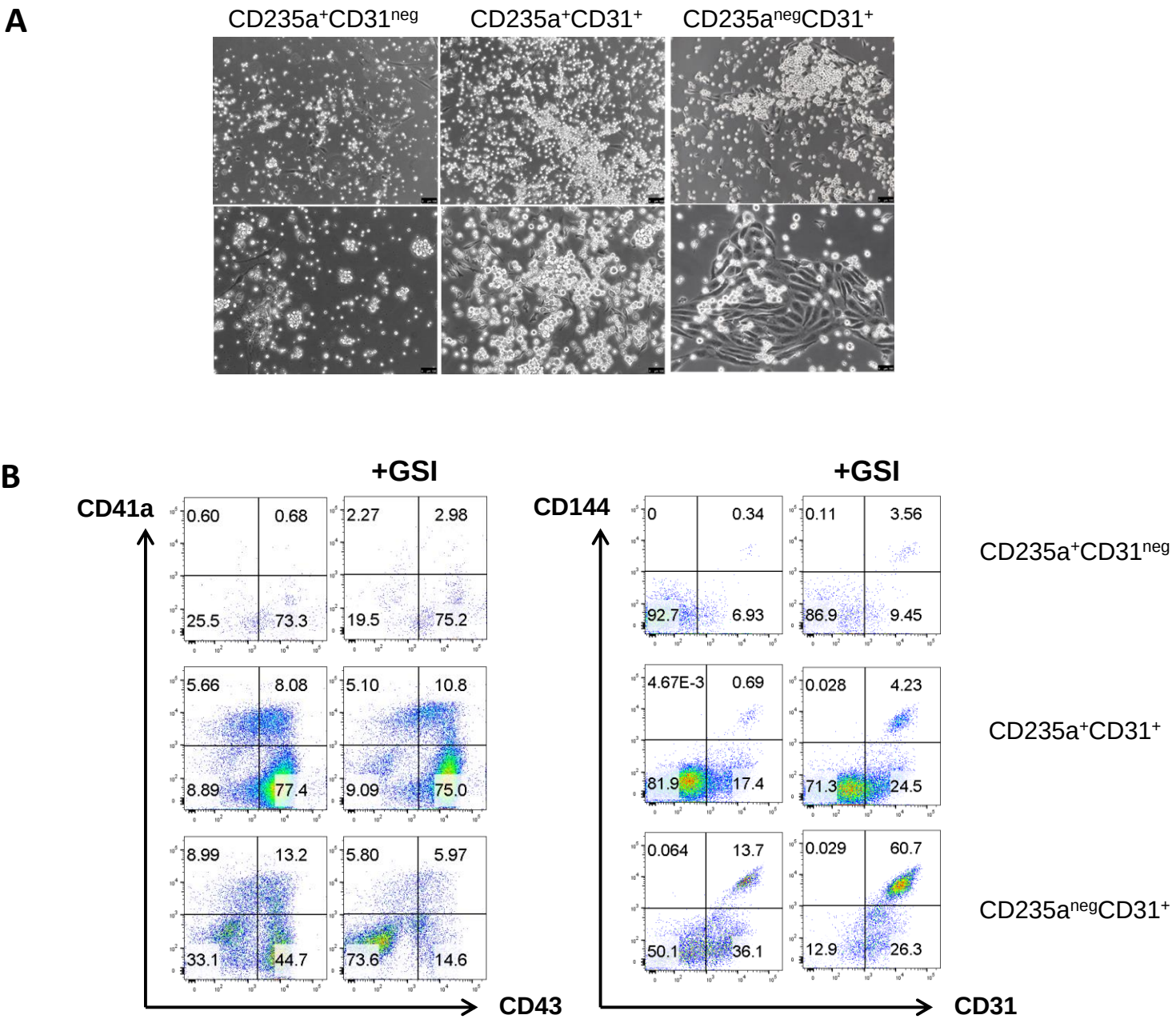


**Figure S7: Culture of CD235a/CD31 populations in gelatine and effect of Notch inhibition in co-culture**, related to figures 5 and 7.

A. Representative pictures of cell cultures obtained from sorted CD235a/CD31 fractions after 7 days of culture on gelatine-coated plates magnification (10X (upper row) and 20X (lower row)). Scale bars 100µm.
B. Effect of Notch inhibition as measured by the addition of 10 µM gamma secretase inhibitor (GSI) RO4929097 or DMSO control in the culture of the indicated populations after 7 days in co-culture with OP9h-DLL1. Data are representative of 3 independent experiments.

**Table S1**

| EnsembleGeneID | symbol | Fold change | EnsembleGeneID | symbol | Fold change |
|---|---|---|---|---|---|
| ENSG00000135638 | *EMX1* | 0.032 | ENSG00000139618 | *BRCA2* | 0.370 |
| ENSG00000171388 | *APLN* | 0.038 | ENSG00000161681 | *SHANK1* | 0.371 |
| ENSG00000170373 | *CST1* | 0.052 | ENSG00000163520 | *FBLN2* | 0.371 |
| ENSG00000139438 | *FAM222A* | 0.070 | ENSG00000139734 | *DIAPH3* | 0.372 |
| ENSG00000115085 | *ZAP70* | 0.073 | ENSG00000138821 | *SLC39A8* | 0.377 |
| ENSG00000101441 | *CST4* | 0.076 | ENSG00000134333 | *LDHA* | 0.377 |
| ENSG00000109255 | *NMU* | 0.076 | ENSG00000198838 | *RYR3* | 0.377 |
| ENSG00000188803 | *SHISA6* | 0.096 | ENSG00000184661 | *CDCA2* | 0.379 |
| ENSG00000105672 | *ETV2* | 0.100 | ENSG00000128683 | *GAD1* | 0.387 |
| ENSG00000148704 | *VAX1* | 0.101 | ENSG00000090530 | *LEPREL1* | 0.387 |
| ENSG00000163492 | *CCDC141* | 0.119 | ENSG00000115687 | *PASK* | 0.389 |
| ENSG00000185666 | *SYN3* | 0.132 | ENSG00000011426 | *ANLN* | 0.391 |
| ENSG00000203805 | *PPAPDC1A* | 0.134 | ENSG00000198826 | *ARHGAP11A* | 0.392 |
| ENSG00000102385 | *DRP2* | 0.139 | ENSG00000075218 | *GTSE1* | 0.395 |
| ENSG00000155657 | *TTN* | 0.139 | ENSG00000109805 | *NCAPG* | 0.398 |
| ENSG00000075340 | *ADD2* | 0.142 | ENSG00000186638 | *KIF24* | 0.400 |
| ENSG00000197046 | *SIGLEC15* | 0.156 | ENSG00000122966 | *CIT* | 0.405 |
| ENSG00000143369 | *ECM1* | 0.164 | ENSG00000131747 | *TOP2A* | 0.405 |
| ENSG00000168280 | *KIF5C* | 0.164 | ENSG00000050555 | *LAMC3* | 0.406 |
| ENSG00000244588 | *RAD21L1* | 0.165 | ENSG00000142945 | *KIF2C* | 0.414 |
| ENSG00000222033 | *LINC01124* | 0.171 | ENSG00000141526 | *SLC16A3* | 0.414 |
| ENSG00000171643 | *S100Z* | 0.174 | ENSG00000187164 | *KIAA1598* | 0.417 |
| ENSG00000110092 | *CCND1* | 0.177 | ENSG00000138778 | *CENPE* | 0.421 |
| ENSG00000100351 | *GRAP2* | 0.182 | ENSG00000166845 | *C18orf54* | 0.421 |
| ENSG00000130477 | *UNC13A* | 0.193 | ENSG00000139946 | *PELI2* | 0.424 |
| ENSG00000166342 | *NETO1* | 0.197 | ENSG00000101447 | *FAM83D* | 0.425 |
| ENSG00000171587 | *DSCAM* | 0.198 | ENSG00000178295 | *GEN1* | 0.427 |
| ENSG00000158764 | *ITLN2* | 0.205 | ENSG00000119969 | *HELLS* | 0.434 |
| ENSG00000178568 | *ERBB4* | 0.235 | ENSG00000079739 | *PGM1* | 0.436 |
| ENSG00000159212 | *CLIC6* | 0.238 | ENSG00000148773 | *MKI67* | 0.436 |
| ENSG00000179715 | *PCED1B* | 0.249 | ENSG00000126787 | *DLGAP5* | 0.440 |
| ENSG00000105464 | *GRIN2D* | 0.262 | ENSG00000123124 | *WWP1* | 0.441 |
| ENSG00000159307 | *SCUBE1* | 0.263 | ENSG00000162367 | *TAL1* | 0.442 |
| ENSG00000095777 | *MYO3A* | 0.266 | ENSG00000095637 | *SORBS1* | 0.444 |
| ENSG00000189120 | *SP6* | 0.270 | ENSG00000140534 | *TICRR* | 0.446 |
| ENSG00000179403 | *VWA1* | 0.273 | ENSG00000183850 | *ZNF730* | 0.447 |
| ENSG00000111981 | *ULBP1* | 0.285 | ENSG00000051341 | *POLQ* | 0.448 |
| ENSG00000144136 | *SLC20A1* | 0.308 | ENSG00000117724 | *CENPF* | 0.450 |
| ENSG00000185565 | *LSAMP* | 0.310 | ENSG00000180773 | *SLC36A4* | 0.450 |
| ENSG00000186777 | *ZNF732* | 0.322 | ENSG00000105227 | *PRX* | 0.450 |
| ENSG00000166450 | *PRTG* | 0.334 | ENSG00000137642 | *SORL1* | 0.451 |
| ENSG00000155760 | *FZD7* | 0.337 | ENSG00000162063 | *CCNF* | 0.454 |

| | | | | | |
|---|---|---|---|---|---|
| ENSG00000123485 | *HJURP* | 0.338 | ENSG00000166851 | *PLK1* | 0.456 |
| ENSG00000233224 | *HIST1H2AM* | 0.339 | ENSG00000138182 | *KIF20B* | 0.460 |
| ENSG00000066279 | *ASPM* | 0.344 | ENSG00000161800 | *RACGAP1* | 0.461 |
| ENSG00000159399 | *HK2* | 0.345 | ENSG00000100629 | *CEP128* | 0.463 |
| ENSG00000135476 | *ESPL1* | 0.355 | ENSG00000146263 | *MMS22L* | 0.464 |
| ENSG00000167703 | *SLC43A2* | 0.357 | ENSG00000131389 | *SLC6A6* | 0.472 |
| ENSG00000088826 | *SMOX* | 0.358 | ENSG00000060982 | *BCAT1* | 0.472 |
| ENSG00000168421 | *RHOH* | 0.363 | ENSG00000068489 | *PRR11* | 0.473 |
| ENSG00000137812 | *CASC5* | 0.369 | | | |

| EnsembleGeneID | symbol | Fold change | EnsembleGeneID | symbol | Fold change |
|---|---|---|---|---|---|
| ENSG00000150681 | *RGS18* | 489.068 | ENSG00000168938 | *PPIC* | 3.821 |
| ENSG00000176956 | *LY6H* | 234.933 | ENSG00000143603 | *KCNN3* | 3.819 |
| ENSG00000166979 | *EVA1C* | 109.390 | ENSG00000112149 | *CD83* | 3.817 |
| ENSG00000133800 | *LYVE1* | 109.316 | ENSG00000005108 | *THSD7A* | 3.772 |
| ENSG00000188536 | *HBA2* | 86.679 | ENSG00000137266 | *SLC22A23* | 3.763 |
| ENSG00000145192 | *AHSG* | 66.773 | ENSG00000132357 | *CARD6* | 3.630 |
| ENSG00000000971 | *CFH* | 39.367 | ENSG00000111261 | *MANSC1* | 3.629 |
| ENSG00000079385 | *CEACAM1* | 35.535 | ENSG00000205336 | *GPR56* | 3.596 |
| ENSG00000099260 | *PALMD* | 17.622 | ENSG00000182240 | *BACE2* | 3.491 |
| ENSG00000150630 | *VEGFC* | 17.299 | ENSG00000169594 | *BNC1* | 3.332 |
| ENSG00000118777 | *ABCG2* | 15.342 | ENSG00000071242 | *RPS6KA2* | 3.201 |
| ENSG00000176435 | *CLEC14A* | 11.762 | ENSG00000075426 | *FOSL2* | 3.184 |
| ENSG00000182851 | *GPIHBP1* | 11.583 | ENSG00000104870 | *FCGRT* | 3.165 |
| ENSG00000113389 | *NPR3* | 10.919 | ENSG00000173598 | *NUDT4* | 3.108 |
| ENSG00000081051 | *AFP* | 10.575 | ENSG00000240583 | *AQP1* | 3.107 |
| ENSG00000101542 | *CDH20* | 10.468 | ENSG00000163171 | *CDC42EP3* | 3.088 |
| ENSG00000146674 | *IGFBP3* | 10.344 | ENSG00000001561 | *ENPP4* | 3.049 |
| ENSG00000172031 | *EPHX4* | 9.580 | ENSG00000145623 | *OSMR* | 3.027 |
| ENSG00000129422 | *MTUS1* | 9.079 | ENSG00000164930 | *FZD6* | 2.892 |
| ENSG00000132872 | *SYT4* | 7.611 | ENSG00000185112 | *FAM43A* | 2.876 |
| ENSG00000175874 | *CREG2* | 7.029 | ENSG00000164035 | *EMCN* | 2.871 |
| ENSG00000154133 | *ROBO4* | 6.793 | ENSG00000147862 | *NFIB* | 2.863 |
| ENSG00000171864 | *PRND* | 6.676 | ENSG00000143801 | *PSEN2* | 2.840 |
| ENSG00000136960 | *ENPP2* | 6.575 | ENSG00000166750 | *SLFN5* | 2.707 |
| ENSG00000184113 | *CLDN5* | 6.308 | ENSG00000197461 | *PDGFA* | 2.679 |
| ENSG00000116016 | *EPAS1* | 6.018 | ENSG00000176597 | *B3GNT5* | 2.539 |
| ENSG00000143140 | *GJA5* | 5.109 | ENSG00000069122 | *GPR116* | 2.476 |
| ENSG00000137393 | *RNF144B* | 4.982 | ENSG00000198168 | *SVIP* | 2.428 |
| ENSG00000127241 | *MASP1* | 4.728 | ENSG00000078269 | *SYNJ2* | 2.401 |
| ENSG00000213949 | *ITGA1* | 4.712 | ENSG00000164929 | *BAALC* | 2.311 |
| ENSG00000179144 | *GIMAP7* | 4.678 | ENSG00000125810 | *CD93* | 2.254 |
| ENSG00000165702 | *GFI1B* | 4.430 | ENSG00000123240 | *OPTN* | 2.249 |
| ENSG00000167680 | *SEMA6B* | 4.085 | ENSG00000126785 | *RHOJ* | 2.134 |
| ENSG00000133574 | *GIMAP4* | 4.063 | ENSG00000101974 | *ATP11C* | 2.108 |
| ENSG00000165507 | *C10orf10* | 4.035 | ENSG00000170989 | *S1PR1* | 2.096 |
| ENSG00000124772 | *CPNE5* | 3.929 | | | |

**Table S1: List of the genes down-regulated (Blue) and up-regulated (red) between day 6 and day 10 in the $CD31^{+}CD144^{+}$ populations sorted from embryoid body EB differentiation**. Related to Figure 1. Differentially expressed genes are derived from RNA-seq data analysis. The data were filtered to include only genes with at least 1 count-per-million reads and genes with a median coverage of more than 10%. Genes with > 2-fold change and false discovery rate < 0.05 were considered as differentially expressed.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Flow cytometry and cell sorting:** For HE-enriched population sorting, cells were stained with hCD144-PE (Miltenyi), hCD43-APC (eBioscience) and hCD31-APC-Cy7 (eBioscience). Further characterization of human HE at day 6 of EB differentiation was performed using hCD90-Biotin (eBioscience), hCD44-PeCy7 (eBioscience), hCD34-FITC (Miltenyi), hCD117-PeCy7 (eBioscience), hKDR-AlexaFluor-647 (eBioscience), hCD151-APC (eBioscience), hCD9-Biotin (eBioscience), hCD73-APC (eBioscience), hDLL4-Biotin (Miltenyi) and hCXCR4-PeCy5.5 (eBioscience). Analysis of hematopoietic differentiation was assessed using hCD41a-FITC (eBioscience), hCD45-PeCy5.5 (eBioscience), hCD61-FITC (Miltenyi) CD71-FITC (eBioscience), and hCD235a-Biotin (eBioscience). BV421-Streptavidin (Biolegend) was used for all biotinylated antibodies. Non-viable cells were excluded by Hoechst 33258 staining. When co-culture was used the stromal cells were excluded based on their size and on their high expression level of GFP expression. Compensations were performed with beads controls. Acquisition was performed on LSRII or Fortessa (BD Biosciences) and data were analysed using FlowJo software (Treestar). Cell sorting was performed on Aria III or Influx sorters (BD Biosciences).

**Clonogenic assay:** Hematopoietic potential was assessed in semi-solid methylcellulose culture. As specified on each graphs, 7 to 10 $x10^3$ cells were seeded into IMDM media (Thermo Fisher) containing 1% methylcellulose and human cytokines as previously described (Kennedy et al., 2012). Counting was performed after 14 days of culture at 37ºC in 5%$CO^2$. All assays were performed in triplicate.

**Cell cycle:** CD31+CD144+CD43neg cells were isolated at day 6, 8 and 10 of EB differentiation. 30,000 unfixed sorted cells maintained in 500 µl of Stemspan medium (Stem Cell Technologies) were stained by incubation with 5µl of 50µM Hoechst 33342 (Thermo Fisher) for 30 min at 37°C. For live discrimination 5 µl 7-amino actinomycin-D (7-AAD) (Thermo Fisher) was added 5 min before analysis at room temperature.

**Globin expression**: RNA was extracted from hematopoietic colonies obtained after 2 weeks in methylcellulose culture from each population identified by CD235a/CD31 markers. Assessment of globin expression was performed by real time PCR using the StepOne plus system (Thermo Fisher) and the human assays Hs00362216_m1 HBE1 Hs00361131_g1 HBG1/2 and the Taqman universal master mix II (Thermo Fisher). Comparative Ct Method was used for relative quantification of the RNA expression using as housekeeping gene the human assay Hs00187842_m1 B2M (Thermo Fisher). Quantification and statistical analysis were performed from 3 independent experiments containing 3 replicates of the CFU culture per experiment and population. Statistical significance was assessed by 2-way ANOVA.

**Notch pathway inhibition:** The three CD235a/CD31 populations were isolated from day 4 of HE culture and 30,000 cells of each population per well of a 12-well plate were further cultured on OP9-hDLL1 stroma cells with the addition of 10µM of gamma secretase inhibitor RO4929097 or control DMSO for 7 days prior to analysis.

**RNA extraction and cDNA synthesis:** Total RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was obtained with GoScript™ Reverse Transcriptase mix (Promega).

**RNA-Seq Libraries**: Indexed total RNA libraries were prepared using 10ng of Total RNA, a 4-minute fragmentation time, and 12 cycles of amplification in the SMARTer Stranded Total RNA-Seq Kit-Pico input (Clontech). Libraries were quantified by qPCR using a Kapa Library Quantification Kit for Illumina sequencing platform (Kapa Biosystems Inc. Cat No: KK4835). Single end 75bp sequencing was carried out by clustering 1.8 pM of the pooled libraries on a NextSeq 500 sequencer (Illumina Inc.).

**RNA-sequencing data analysis:** Basecall files generated from HiSeq sequencing run were converted to FASTQ format with Illumina's bcl2fastq. Lane-wise alignment was performed by bowtie2 (version 2.2.1) (Langmead and Salzberg, 2012) to human reference genome (GRCh37.75) with default parameters. Generated SAM files from bowtie2 alignment were converted to BAM files by samtools v0.1.19. Parameters for samtools SAM to BAM conversion: -q 10 -f 2 -F 260. Resulting lane-wise BAM files from the same sequence library was merged into one BAM file used for downstream analysis. The expression levels of 57,773 annotated features were determined by using the featureCounts (Liao et al., 2014) function from the Bioconductor package Rsubread (version 1.13.13). The Bioconductor package edgeR (Robinson et al., 2010) (version 3.8.5) was used to identify genes that showed statistically significant variation in expression levels. The data was filtered to include only genes with at least 1 count-per-million reads and genes with a median coverage of more than 10%. Differential expression analysis was performed using the function exactTest in edgeR (Robinson et al., 2010). Genes with > 2 fold change and false discovery rate < 0.05 were considered as differentially expressed. Gene ontology analysis of the DEG was performed using DAVID on line tool.

**Statistical Analyses:** Statistical analyses were performed using Graph Pad Prism Version7.0 (Graph Software). Error bars indicate the SEM of data from independent experiments. Statistical differences were assessed by 2-way ANOVA using SIDAK´s or Turkey multiple comparison test to obtain the multiplicity adjusted p-values as showed in the graphs and legends.

**Mitotic inactivation of OP9 stroma cells by Mitomycin C.** Stroma cells thawed in a 162 cm2 flask were maintained in alpha MEM 20% FBS (as described in experimental procedures) until 90% confluence is reached. The cells were then split in 4 flasks and once they reached 90% confluence they were treated with fresh media containing 10µg/ml of Mitomycin C (Bio Techne) for 3 hours. Cultures were then washed twice with media and twice with PBS to remove any trace of the drug. After the treatment cells were trypsinized, counted and frozen at -80C in a density of 1 million cells per vial, normally used within 2 months.

## SUPPLEMENTAL REFERENCES

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods 9, 357-359.
Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923-930.
Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139-140.