Stem Cell Research 16 (2016) 735–739

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Stem Cell Research: Lab Resource

Generation of human pluripotent stem cell lines with suppressed expression of the Notch ligand DLL4 using short hairpin RNAs

Federico González-Pozas [1], Rosa Montes [1], Lourdes López-Onieva, Tamara Romero, Joan Domingo-Reinés, Pedro J. Real, Verónica Ramos-Mejía, Verónica Ayllón *

Gene Regulation, Stem Cells and Development Group, Department of Genomic Oncology, GENYO — Centre for Genomics and Oncological Research — Pfizer/University of Granada/Junta de Andalucía, PTS Granada. 18016. Granada, Spain

ARTICLE INFO

Article history:
Received 1 April 2016
Accepted 5 April 2016
Available online 16 April 2016

ABSTRACT

Studies with different animal models have shown that the Notch ligand DLL4 has a key role in the development of the embryonic vasculature. Here we describe the generation and characterization of a human embryonic stem cell line and an induced pluripotent stem cell line that constitutively express short hairpin RNAs targeting DLL4 mRNA. These cells present reduced DLL4 expression at both protein and mRNA level, as well as a reduced induction of DLL4 target genes. They represent an ideal tool to study the role of DLL4 in human embryonic vascular and hematopoietic development.

Resource Table

Name of Stem Cell line	H9 shSCR H9 shDLL4 PBMC1-iPS4F1 shSCR PBMC1-iPS4F1 shDLL4
Institution	GENYO — Centre for Genomics and Oncological Research - Pfizer/University of Granada/Junta de Andalucía, PTS Granada. 18,016. Granada, Spain.
Person who created resource	Federico González-Pozas, Rosa Montes, Verónica Ayllón
Contact person and email	Verónica Ayllón veronica.ayllon@genyo.es
Date archived/stock date	Stock created in September 2015
Origin	The H9 cell line was derived from the inner cell mass of a human blastocyst (Thomson et al., 1998). The PBMC1-iPS4F1 cell line originates from the reprogramming of healthy human female peripheral blood mononuclear cells (Montes et al., 2015)
Type of resource	Two human embryonic stem cell lines (H9) and two human induced pluripotent stem cell lines (PBMC1-iPS4F1) genetically modified to constitutively express short hairpin RNA constructs.
Sub-type	Cell line
Key transcription factors	
Authentication	Identity and purity of cell line confirmed
Link to related literature	http://science.sciencemag.org/content/282/5391/1145.long http://www.sciencedirect.com/science/article/pii/S1873506115001440 http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/learning-center/mission-application-data/shrna-lentiviral-vectors.html
Information in public databases	The cell lines are pending their deposit in the Spanish National Stem Cell Biobank
Ethics	Ethics Review Board-competent authority approval obtained

Resource Details

The Notch ligand DLL4 is essential for the correct formation of the embryonic vasculature in mouse. DLL4 knock-out is embryonic lethal, and heterozygous animals present severe vascular defects, such as reduced dorsal aorta caliber, abnormal branching of vessels and vascular remodeling (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). In order to investigate if DLL4 has a similar function in human embryonic development we have generated human pluripotent stem cell lines (hPSCs) that stably express a combination of short hairpin RNAs (shRNA) targeting DLL4 mRNA.

We transduced the iPSC cell line PBMC1-iPS4F1 (Montes et al., 2015) and the hESC cell line H9 (Thomson et al., 1998) with lentiviruses expressing either a control, non-targeting shRNA with a scrambled sequence (shSCR), or a cocktail of 5 different shRNAs targeting the human DLL4 mRNA (shDLL4). After selection with Puromycin for 10 days we obtained resistant colonies that were expanded, leading to the establishment of the following four cell lines: PBMC1-iPS4F1

* Corresponding author at: Gene Regulation, Stem Cells and Development Group, Department of Genomic Oncology, GENYO — Centre for Genomics and Oncological Research Pfizer/University of Granada/Junta de Andalucía, PTS Granada, Avenida Ilustración, 114, 18016 Granada, Spain.
E-mail address: veronica.ayllon@genyo.es (V. Ayllón).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.scr.2016.04.005

shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4. We confirmed the identity of the transgenic cell lines by Short Tandem Repeat (STR) profiling (Table 1). They are routinely tested for mycoplasm and remain mycoplasm-free.

The four cell lines present the typical morphology of pluripotent stem cells, growing in compact colonies, with tightly packed cells (Fig. 1A). They express the pluripotency markers SSEA4, SSEA3, TRA-1-81, TRA-1-60 and OCT3/4, assessed by flow cytometry (Fig. 1B), and OCT4, REX1, NANOG and SOX2, assessed by PCR (Fig. 1C). They are also positive for alkaline phosphatase activity (Fig. 1D). Therefore, neither the expression of shSCR nor shDLL4 constructs has affected the pluripotency of the cell lines.

The transgenic cell lines were capable of forming embryoid bodies (EBs) (Fig. 2A). We harvested EBs after 21 days of spontaneous differentiation, and analyzed by PCR the expression of makers of the three germ layers: PAX6 for ectoderm, HNF3A for endoderm, and KDR for mesoderm. The four cell lines expressed the three markers (Fig. 2B), demonstrating that the expression of shSCR and shDLL4 does not affect the ability of these pluripotent stem cells to differentiate to the three germ layers.

DLL4 is expressed at very low levels in human pluripotent stem cells, and its expression increases as the cells are differentiated towards the hematopoietic lineage (Ayllón et al., 2015). In fact, the DLL4 protein located at the plasma membrane only becomes detectable by flow cytometry in a subpopulation of hemato-endothelial progenitors (HEPs) (Ayllón et al., 2015) (Fig. 2C). These HEPs are bipotent progenitors that can give rise to either hematopoietic or endothelial cells, and they express the surface markers CD34, KDR, VE-cadherin and CD31 (Ayllón et al., 2015; Uenishi et al., 2014) (Fig. 2C). To confirm that the expression of DLL4 was indeed suppressed in our transgenic cell lines we differentiated them towards hemato-endothelial progenitors and assessed the levels of DLL4 expression by flow cytometry. At day 8 of differentiation DLL4 is only expressed in CD34 + KDR + VE-CAD + CD31 + cells (HEPs) in control shSCR cell lines, and its expression is reduced down by 75–90% in shDLL4 cell lines (Fig. 2D).

By qPCR we confirmed that DLL4 mRNA levels increase dramatically as the control shSCR cells undergo differentiation, but in shDLL4 cells the DLL4 mRNA is efficiently suppressed (Fig. 2E, left panel). To confirm that DLL4 mRNA suppression had a functional effect we analyzed the mRNA levels of two well known targets of DLL4-mediated Notch activation, HEY1 and HEY2 (Benedito et al., 2008; Gale et al., 2004). We saw that in control shSCR cells both HEY1 and HEY2 mRNAs are induced during differentiation following a similar pattern as DLL4 mRNA. However, in shDLL4 cells both genes are induced at a lower level, especially at day 8 of differentiation, when DLL4 suppression is more acute (Fig. 2E, center and right panels).

In summary, we have generated human pluripotent stem cell lines that cannot induce DLL4 expression when they are differentiated towards hemato-endothelial progenitors. They are very useful tools to study the role of DLL4 in the early stages of human embryonic endothelial and hematopoietic differentiation. We are currently characterizing how the suppression of DLL4 affects theses processes, and the molecular pathways involved.

Table 1
Short Tandem Repeat (STR) profiling of PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 cell lines with the original cell lines.

	PBMC1-iPS4F1			H9		
MARKER	WT[a]	shSCR	shDLL4	WT[b]	shSCR	shDLL4
D8S1179	13, 16	13, 16	13, 16		8, 14	8, 14
D21S11	30, 34	30, 34	30, 34		30, 30	30, 30
D7S820	9, 10	9, 10	9, 10	9, 11	9, 11	9, 11
CSF1PO	11, 12	11, 12	11, 12	11, 11	11, 11	11, 11
D3S1358	16, 17	16, 17	16, 17		13, 16	13, 16
THO1	7, 9	7, 9	7, 9	9.3, 9.3	9.3, 9.3	9.3, 9.3
D13S317	11, 12	11, 12	11, 12	9, 9	9, 9	9, 9
D16S539	9, 13	9, 13	9, 13	12, 13	12, 13	12, 13
D2S1338	17, 17	17, 17	17, 17		18, 24	18, 24
D19S433	12, 14	12, 14	12, 14		12, 15	12, 15
vWA	15, 19	15, 19	15, 19	17, 17	17, 17	17, 17
TPOX	8, 11	8, 11	8, 11	10, 11	10, 11	10, 11
D18S51	10, 16	10, 16	10, 16		13, 13	13, 13
AMEL	X, X	X, X	X, X	X, X	X, X	X, X
D5S818	11, 12	11, 12	11, 12	11, 12	11, 12	11, 12
FGA	18, 22	18, 22	18, 22		26, 28	26, 28

[a] Original cell line reported by Montes R et al.
[b] STR data obtained from WiCell (http://www.wicell.org/home/stem-cell-lines/order-stem-cell-lines/wa09-feeder-dependent.cmsx).

Materials and methods

Generation of PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 cell lines.

We used the lentiviral vectors pLKO.1 MISSION shRNA (Sigma-Aldrich) containing either a non-targeting sequence (shSCR; clone SHC0016), or a combination of five different shRNAs targeting the human DLL4 mRNA (shDLL4; clones TRCN0000033414, TRCN0000033415, TRCN0000033416, TRCN0000033417, TRCN0000033418). Viral particles pseudotyped with VSV-G were generated on 293T cells by calcium-phosphate transfection protocol and concentrated by ultracentrifugation (Montes et al., 2011; Ramos-Mejía et al., 2014). The parental cell lines PBMC1-iPS4F1 (Montes et al., 2015) and H9 (Thomson et al., 1998) were infected overnight on the day of passage with concentrated virus in the presence of 8 μg/ml Polybrene and 10 μM Y-27632 (Sigma-Aldrich). After 48 h, the transduced cells were selected with 0.5 μg/ml Puromycin (Sigma-Aldrich) for 10 days.

Flow cytometry analysis

We dissociated PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 colonies using Tryple Express (Life Technologies). Then, the cells were resuspended in FACS buffer (5% FBS 2 mM EDTA in PBS) and incubated with the specific primary antibody for 15 min at room temperature. An irrelevant isotype-match antibody was always used. To analyze intracellular Oct3/4, sequential incubations with fixation and permeabilization solutions were necessary (A and B Fix & Perm Solutions, Invitrogen). After Oct3/4 primary antibody incubation, the cells were incubated with FITC-conjugated secondary antibody (BD BioScience). Then, the cells were washed with FACS buffer and stained with 7-aminoactinomycin D (7-AAD) (BD Bioscience) for 5 min at RT. Stained cells were analyzed using a FACS Verse flow cytometer using FACS Diva™ software program (BD Bioscience). The antibodies used are listed on Table 2.

RT-PCR

RNA was extracted using High Pure RNA Isolation Kit (Roche) following manufacturer's instructions. cDNA from total RNA was synthesized using the First-Strand cDNA Synthesis Kit (GE Healthcare) following manufacturer's instructions. We perform the RT-PCRs using the primers listed on Table 3.

Alkaline phosphatase activity assay

We seeded PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR cell lines on 12-well plates coated with Matrigel™ (BD). After 5 days in culture, we tested the phosphatase alkaline enzymatic activity using the Alkaline Phosphatase Detection Kit (Merck-Millipore) following manufacturer's instructions.

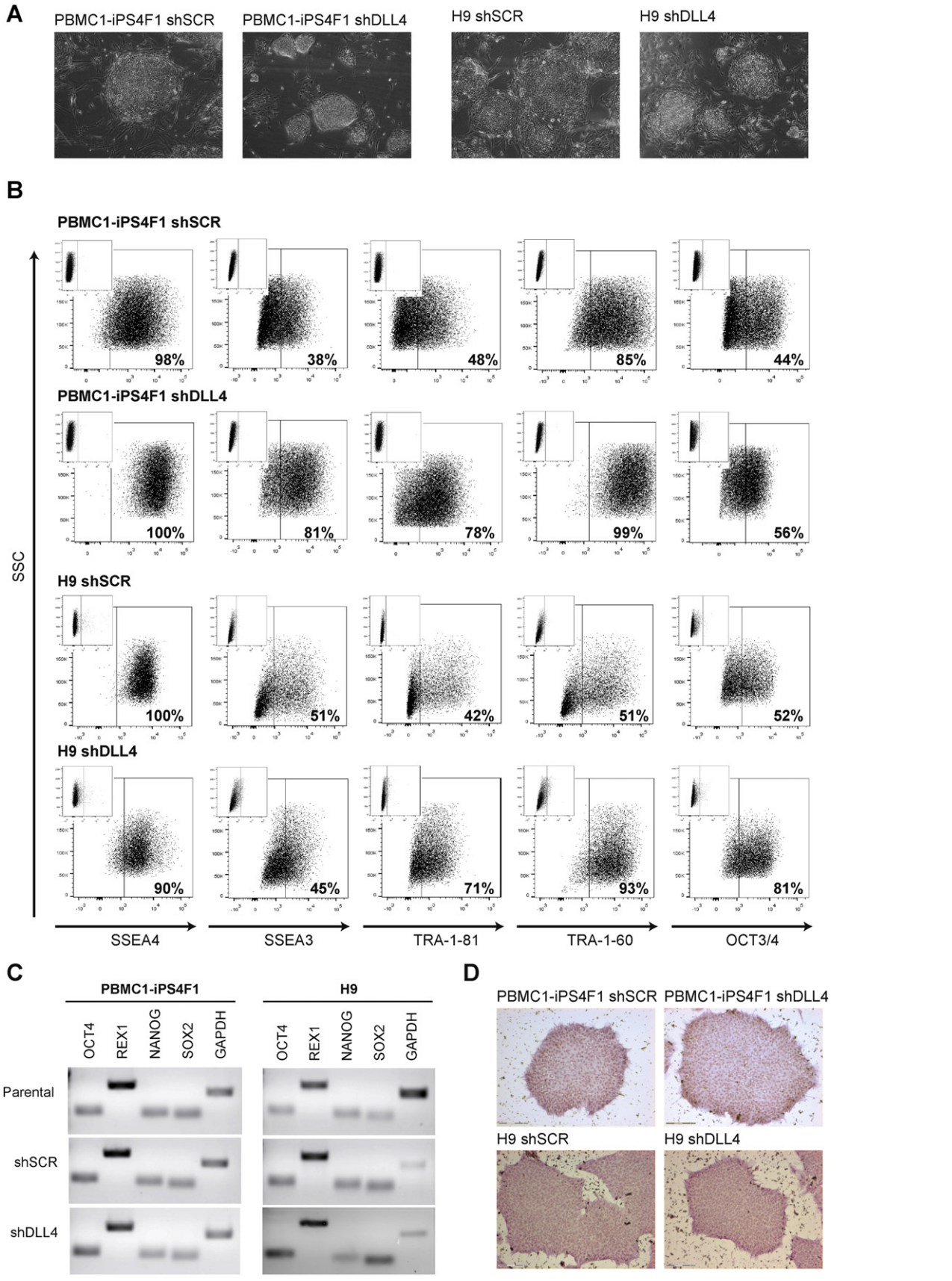

Fig. 1. Characterization and expression of pluripotency-associated markers in PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 cell lines. (A) Micrographs showing the morphology of the colonies, which display a typical round shape with small, tightly packed cells. (B) Expression of pluripotency-associated markers SSEA3, SSEA4, TRA1-81, TRA 1-60 and OCT3/4 at protein level by flow cytometry. The inset shows the staining using the corresponding irrelevant isotype-matched antibody. (C) Expression of the endogenous pluripotency markers OCT4, Sox2, Rex1 y NANOG by RT-PCR. (D) Alkaline phosphatase enzymatic staining.

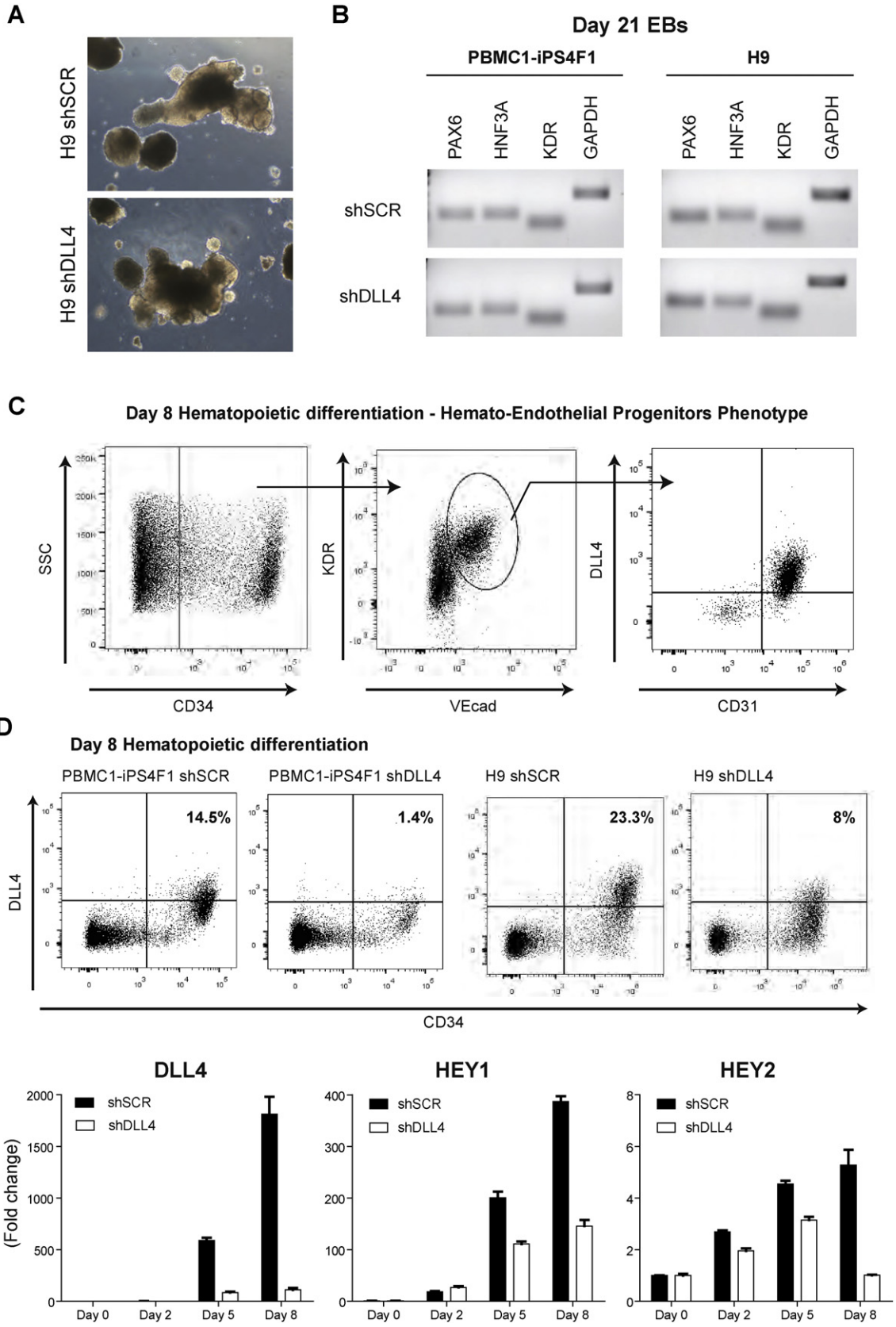

Fig. 2. Differentiation of PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 cell lines to the three germ cell layers and analysis of DLL4 and associated target genes expression. (A) Micrographs depicting the morphology of representative embryoid bodies (EBs) at day 21. (B) Expression of germ layers markers PAX6 (Ectoderm), HNF3A (Endoderm) and KDR (Mesoderm) in day 21 EBs assessed by RT-PCR. (C) Flow cytometry dot plots depicting the phenotype of Hemato-Endothelial Progenitors (HEPs). HEPs co-express CD34, KDR, VE-Cadherin and CD31; a subpopulation of HEPs also expresses DLL4. (D) DLL4 plasma membrane levels at day 8 of hematopoietic differentiation in PBMC1-iPS4F1 shSCR, PBMC1-iPS4F1 shDLL4, H9 shSCR and H9 shDLL4 cell lines assessed by flow cytometry. (E) DLL4, HEY1 and HEY2 mRNA levels during hematopoietic differentiation assessed by quantitative RT-PCR.

Table 2
Flow cytometry antibodies used in this work.

Antigen	Fluorophore	Brand
SSEA3	PE	eBioscience
Isotype SSEA3	PE	Stemgen
TRA-1-60	PE	eBioscience
Iso TRA-1-60	PE	Miltenyi
SSEA4	Alexa Fluor® 647	BD Biosciences
TRA-1-81	Alexa Fluor® 647	BD Biosciences
Iso SSEA4	Alexa Fluor® 647	BD Biosciences
OCT3/4	–	BD Biosciences
Anti-mouse IgG	FITC	BD Biosciences
CD31	FITC	BD Biosciences
CD34	PE-Cy™7	BD Biosciences
CD144/VE-Cadherin	V450	BD Biosciences
CD309 VEGFR-2; KDR	PE	BD Biosciences
DLL4	APC	Miltenyi

Table 3
Primers sets used for RT-PCR.

Gen	Forward primer	Reverse primer
OCT4	AGTGAGAGGCAACCTGGAGA	ACACTCGGACCACATCCTTC
SOX2	TCAGGAGTTGTCAAGGCAGAGAAG	TCAGGAGTTGTCAAGGCAGAGAAG
REX1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA
NANOG	TGCAGTTCCAGCCAAATTCTC	CCTAGTGGTCTGCTGTATTACATTAA GG
PAX6	CCGGCAGAAGATTGTAGAGC	CGTTGGACACGTTTTGATTG
HNF3A	GTGGCTCCAGGATGTTAGGA	GCCTGAGTTCATGTTGCTGA
KDR	CCACTGGTATTGGCAGTTGGA	CACAAGGGTATGGGTTTGTCACT
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

Embryoid body (EB) formation

One to two days before reaching confluence, 1 ml of Matrigel 1:6 (diluted in KO-DMEM) was added to the culture. The confluent culture was scrapped and cultured in ultra-low attachment wells (Corning) to induce EB formation. EBs were cultured for 21 days with PSC medium without bFGF2 for spontaneous differentiation to the three germ layers. Medium changes were performed every 2–3 days. At the end of differentiation, EBs were harvested for RNA extraction and analysis by RT-PCR of markers specific for each germ layer.

Hemato-endothelial differentiation

We performed the feeder- and serum-free differentiation protocol described previously (Uenishi et al., 2014). At the indicated times of analysis, we harvested the cells with Tryple and used them for flow cytometry and gene expression analysis by RT-PCR.

Acknowledgments

RM is funded by the Fundacion Publica Andaluza Progreso y Salud (PI-0030-2014) and the Postdoctoral Juan de la Cierva Program founded by the Spanish Ministry of Economy and Competitiveness (JCI_2012_12666). LL-O is a Marie Curie Intra-European Fellow (FP7-MC-IEF-623806). JD-R is supported by the Fundacion Inocente Inocente (FII2015-212). PJR is funded by the FIS/FEDER (CP09/0063, PI12/01596 and CPII15/00018). VR-M is funded by the FIS/FEDER (CP12/03175 and PI14/01412). VA is funded by the L'Oréal-UNESCO for Women in Science 2016 Program.

References

Ayllón, V., Bueno, C., Ramos-Mejía, V., Navarro-Montero, O., Prieto, C., Real, P.J., Romero, T., García-León, M.J., Toribio, M.L., Bigas, A., Menendez, P., 2015. The Notch ligand DLL4 specifically marks human hematoendothelial progenitors and regulates their hematopoietic fate. Leukemia 29, 1741–1753.

Benedito, R., Trindade, A., Hirashima, M., Henrique, D., Costa, L., Rossant, J., Gill, P., Duarte, A., 2008. Loss of Notch signalling induced by Dll4 causes arterial calibre reduction by increasing endothelial cell response to angiogenic stimuli. BMC Dev. Biol. 8, 117.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D., Rossant, J., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 18, 2474–2478.

Gale, N., Dominguez, M., Noguera, I., Pan, L., Hughes, V., Valenzuela, D., Murphy, A., Adams, N., Lin, H., Holash, J., Thurston, G., Yancopoulos, G., 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc. Natl. Acad. Sci. U. S. A. 101, 15949–15954.

Krebs, L.T., Shutter, J.R., Tanigaki, K., Honjo, T., Stark, K.L., Gridley, T., 2004. Haploinsufficient lethality and formation of arteriovenous malformations in notch pathway mutants. Genes Dev. 18, 2469–2473.

Montes, R., Ayllón, V., Gutierrez-Aranda, I., Prat, I., Hernández-Lamas, M.C., Ponce, L., Bresolin, S., Te Kronnie, G., Greaves, M., Bueno, C., Menendez, P., 2011. Enforced expression of MLL-AF4 fusion in cord blood CD34+ cells enhances the hematopoietic repopulating cell function and clonogenic potential but is not sufficient to initiate leukemia. Blood 117, 4746–4758.

Montes, R., Romero, T., Cabrera, S., Ayllon, V., 2015. Generation and characterization of the human iPSC line PBMC1-iPS4F1 from adult peripheral blood mononuclear cells. Stem Cell Res.

Ramos-Mejía, V., Navarro-Montero, O., Ayllón, V., Bueno, C., Romero, T., Real, P.J., Menendez, P., 2014. HOXA9 promotes hematopoietic commitment of human embryonic stem cells. Blood 124, 3065–3075.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

Uenishi, G., Theisen, D., Lee, J.-H.H., Kumar, A., Raymond, M., Vodyanik, M., Swanson, S., Stewart, R., Thomson, J., Slukvin, I., 2014. Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions. Stem Cell Rep. 3, 1073–1084.