



Lab Resource: Multiple Cell Lines

## Establishment of 2 control and 2 hPSC cell lines constitutively expressing the Notch ligand DLL4



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## ABSTRACT

The Notch ligand DLL4 has key roles during embryonic development of different tissues, but most of the data comes from animal models. Here we describe the generation and characterization of 2 human Pluripotent Stem Cell (hPSC) lines that overexpress DLL4, as well as the two corresponding control hPSC lines. DLL4 expression can be detected at the mRNA and protein level, and does not affect the pluripotency of the cells. These hPSC lines can be used to study the role of DLL4 during human embryonic development.

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## Resource table.

Unique stem cell lines identifier	- WAe009-A-5 - WAe009-A-6 - GENYOi001-A-1 - GENYOi001-A-2
Alternative names of stem cell lines	- H9 NEO (WAe009-A-5) - H9 DLL4 (WAe009-A-6) - PBMC1-iPS4F1 NEO (GENYOi001-A-1) - PBMC1-iPS4F1 DLL4 (GENYOi001-A-2)
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Type of cell lines	iPSCs and hESC
Origin	Human
Cell Source	The original hESC line is the H9 (WA09) from Wicell. The original iPSCs PBMC1-iPS4F1 has been derived from Peripheral Blood Mononuclear Cells
Method of reprogramming	The original iPSCs PBMC1-iPS4F1 was generated using non-integrative Sendai virus
Multiline rationale	For each cell type, we present the data for the control cells transduced with the empty vector plasmid (NEO) and the cells transduced with a vector containing the human DLL4 gene.
Gene modification	YES
Type of modification	Transgene expression, selected using Neomycin resistance
Associated disease	NA

(continued)

Gene/locus	If applicable, use appropriate standard nomenclature of locus/gene of mutation/insertion
Method of modification	Transduction with lentiviruses
Name of transgene or resistance	Cells expressing the transgene DLL4 and Neomycin Resistance
Inducible/constitutive system	Constitutive expression under the promoter EF1alpha
Date archived/stock date	In progress
Cell line repository/bank	In progress
Ethical approval	Ethical Committee from the Regional Andalusian Health Board (Spain). Approval number PR_04_2016.

## Resource utility

The Notch ligand DLL4 participates in embryonic development of cardiovascular and neural systems (Duarte et al., 2004; Rocha et al., 2009). Most of this knowledge comes from animal models, so these hPSCs overexpressing DLL4 are useful tools to study the role of DLL4 during human development.

## Resource details

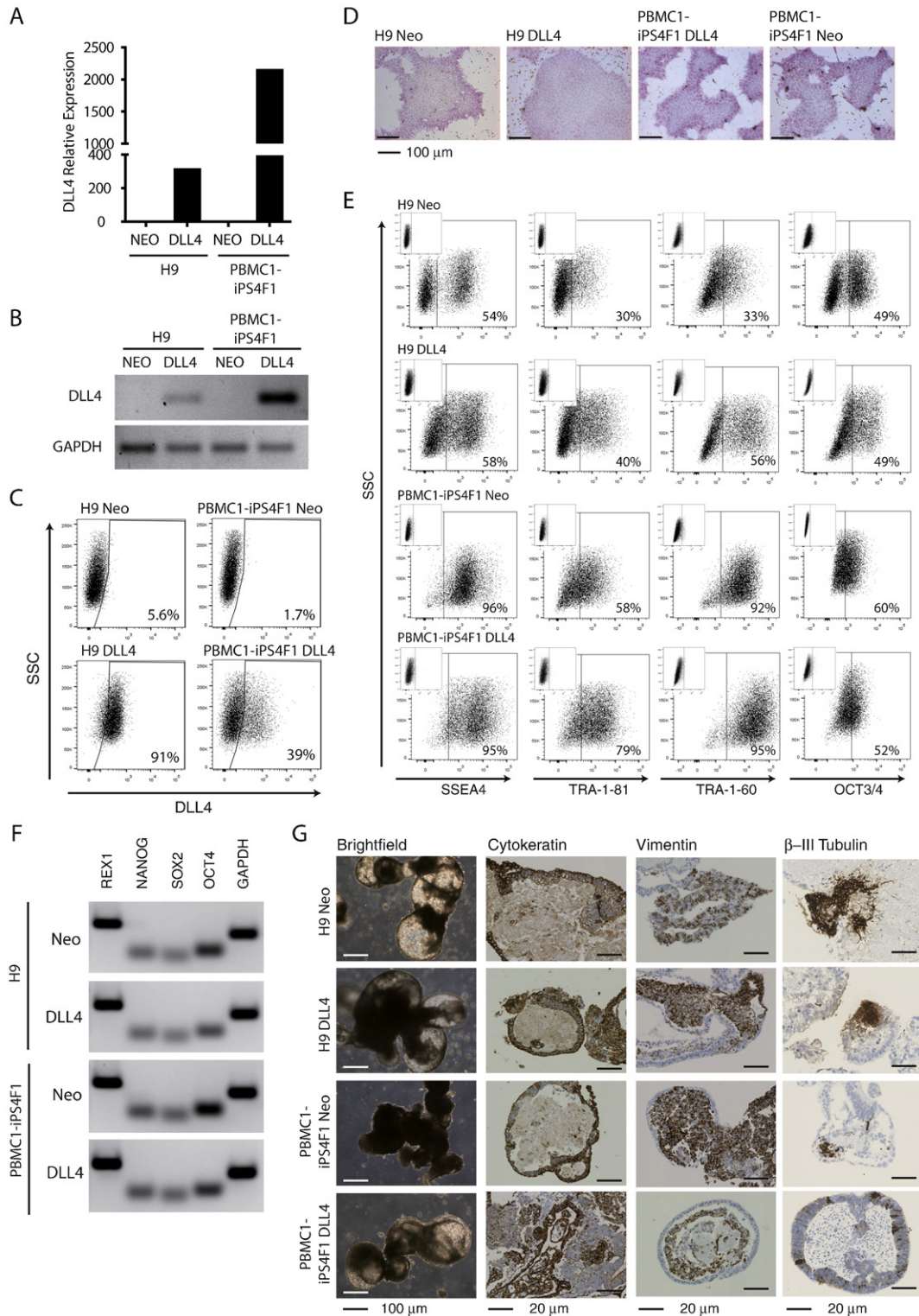
We transduced the iPSC line PBMC1-iPS4F1 and the hESC line H9 (Thomson et al., 1998) with lentiviruses expressing either a control vector (containing only the Neomycin resistance) or a vector containing the cDNA of the human DLL4 gene (Supplementary Fig. S1A). After selection with Neomycin for 10 days we obtained resistant colonies that were grown and expanded, resulting in the generation of four cell lines, all them representing a mixed population or pools: H9 NEO (WAe009-A-5), H9 DLL4 (WAe009-A-6), PBMC1-iPS4F1 NEO (GENYOi001-A-1)

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**Table 1**  
Summary of lines.

hPSC line	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
H9 NEO (WAe009-A-5)	H9 NEO	Female	Embryo			
H9 DLL4 (WAe009-A-6)	H9 DLL4	Female	Embryo			
PBMC1-iPS4F1 NEO (GENYOi001-A-1)	PBMC1-iPS4F1 NEO	Female	39 years	Spanish Caucasian		
PBMC1-iPS4F1 DLL4 (GENYOi001-A-4)	PBMC1-iPS4F1 DLL4	Female	39 years	Spanish Caucasian		



**Fig. 1.** The specific instructions for a Lab Resource: Multiple Stem Cell Lines say "No figure legend. Reference to images included in Resource details section".

and PBMC1-iPS4F1 DLL4 (GENYOi001-A-2) (Table 1). The identity of the cell lines was confirmed by Short Tandem Repeat (STR) profiling. The cells are routinely tested and are mycoplasma free.

We confirmed that the DLL4 hPSC lines expressed higher amounts of DLL4 mRNA than control NEO cells by quantitative PCR (Fig. 1A) and semi-quantitative PCR (Fig. 1B). We detected cell surface expression of DLL4 protein in our DLL4 overexpressing hPSC by flow cytometry (Fig. 1C), while it is barely expressed in undifferentiated hPSCs (Ayllón et al., 2015). H9 DLL4 cells show a homogenous DLL4 expression at the membrane in most cells, while ~40% of PBMC1-iPS4F1 cells express DLL4 at the membrane, reflecting the mix population nature of the cell lines. Another reason for this heterogeneity could be that DLL4 can localize at either the plasma membrane or intracellularly. By immunohistochemistry, that detects total DLL4 expression, we confirmed that both DLL4 over-expressing cell lines show a rather homogenous staining for DLL4 after EB differentiation (Supplementary Fig. S1C). The four cell lines present the typical morphology of pluripotent stem cells, growing in compact colonies, with tightly packed cells (Supplementary Fig. S1B) and are positive for alkaline phosphatase activity (Fig. 1D). They express the pluripotency markers SSEA4, TRA-1-81, TRA-1-60 and OCT3/4, assessed by flow cytometry (Fig. 1E; the insets depict the isotype controls), and OCT4, REX1, NANOG and SOX2, assessed by PCR (Fig. 1F).

The cell lines H9 Neo and H9 DLL4 have a normal karyotype 46,XX, but 20% of H9 DLL4 cells present a trisomy of chromosome 12 (47,XX,+12). This trisomy has already been described as a common event in hESCs (Catalina et al., 2008). PBMC1-iPS4F1 NEO and PBMC1-iPS4F1 DLL4 present the abnormal karyotype 46,XX,+der(1)t(1;14)(q11;q11),-14, suggesting that the alteration was already present in the parental cell line (Supplementary Fig. S1D). We generated embryoid bodies (EBs) with all four cell lines and allowed them to differentiate spontaneously for 20–23 days. As it is shown in Fig. 1G, all 4 cell lines were capable of differentiation to the three germ layers, with all samples showing positive staining for the markers Cytokeratin (endoderm), Vimentin (mesoderm) and  $\beta$ -III tubulin (ectoderm), assessed by immunohistochemistry (Table 2).

In summary, we have successfully established hPSC lines that stably overexpress the Notch ligand DLL4. Neither the expression of Neomycin

resistance or DLL4 gene has affected the pluripotency of the cell lines. Therefore, these cell lines are now available to study how DLL4 regulates human embryonic development, with a special interest on its role during cardiovascular development and specification and commitment of neural progenitors.

## Materials and methods

### Generation of H9 Neo, H9 DLL4, PBMC1-iPS4F1 NEO and PBMC1-iPS4F1 DLL4 cell lines

We used the lentiviral vector pRRL-EF1 $\alpha$ -PGK-Neo to clone the cDNA of the human DLL4 gene under the control of the EF1 $\alpha$  promoter (Supplementary Fig. S1A). Viral particles pseudotyped with VSV-G were generated on 293 T cells by calcium-phosphate transfection protocol. The parental cell lines PBMC1-iPS4F1 and H9 were infected overnight on the day of passage with the lentiviruses in the presence of 8  $\mu$ g/ml polybrene and 10  $\mu$ M Y-27632 (Sigma-Aldrich). After 48 h, the transduced cells were selected with 100  $\mu$ g/ml Neomycin (Calbiochem) for 10 days. The cells are routinely grown on Matrigel-coated flasks using E8 medium.

### Flow cytometry analysis

We dissociated the colonies using Tryple Express (Life Technologies). 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, using an isotype-match antibody as negative control. For Oct3/4 staining, we used the 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 the life probe 7-aminoactinomycin D (7-AAD) (BD Bioscience) for 5 min at RT. We analyzed the cells in a FACS Verse flow cytometer using FACS Diva™ software program (BD Bioscience). The antibodies used are listed on Table 3.

**Table 2**  
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal appearance of packed colonies	Supplementary Fig. S1 panel B
Phenotype	PCR	All cell lines express OCT4, REX, NANOG and SOX2	Fig. 1 panel F
	Alkaline phosphatase activity	All cell lines are positive	Fig. 1 panel D
	Flow cytometry	All cell lines express the pluripotency markers SSEA4, TRA-1-81, TRA-1-60 and OCT3/4 at levels that range between 30%–96%	Fig. 1 panel E
Genotype	PCR	DLL4 cell lines overexpress DLL4 mRNA.	Fig. 1 panels A and B
	Flow cytometry	DLL4 cell lines express the protein at the cell surface	Fig. 1 panel C
	Karyotype (G-banding) and resolution	- H9 NEO (WAe009-A-5): 46,XX	Supplementary Fig. S1 panel C
		- H9 DLL4 (WAe009-A-6): 46,XX, with a 20% mosaic of 47,XX,+12	
- PBMC1-iPS4F1 NEO (GENYOi001-A-1): 46,XX,+der(1)t(1;14)(q11;q11),-14			
		- PBMC1-iPS4F1 DLL4 (GENYOi001-A-2): 46,XX,+der(1)t(1;14)(q11;q11),-14	
		All karyotypes have a resolution of 400–550	
Identity	Microsatellite PCR (mPCR)	NA	NA
	STR analysis	All cell lines were tested for 16 loci, and they perfectly matched those of the parental cell lines	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	NA	NA
	Southern Blot OR WGS	NA	NA
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT quantitative PCR: Negative	Submitted in archive with journal
Differentiation potential	Embryoid body formation	Proof of three germ layers formation by immunohistochemistry against markers vimentin, cytokeratin and $\beta$ -III tubulin	Fig. 1 panel G
Donor screening (OPTIONAL)	NA	NA	NA
Genotype additional info (OPTIONAL)	NA	NA	NA
	NA	NA	NA

**Table 3.**  
Reagents details.

	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	Mouse anti-SSEA4 Alexa647	1:100	BD Biosciences Cat# 560796, RRID:AB_2033991
Pluripotency marker	Mouse anti-TRA-1-81 Alexa647	1:100	BD Biosciences Cat# 560793, RRID:AB_10550550
Pluripotency marker	Mouse anti-TRA-1-60 PE	1:100	Thermo Fisher Scientific Cat# 12-8863-82, RRID:AB_891602
Pluripotency marker	Mouse anti-OCT3/4	1:100	BD Biosciences Cat# 611203, RRID:AB_398737
Differentiation marker	Mouse anti-CK-AE1-3	1:50	Dako Cat# M3515, RRID:AB_2132885
Differentiation marker	Rabbit anti-Vimentin	1:100	Roche Cat# 05499917001
Differentiation marker	Rabbit anti- $\beta$ -III tubulin	1:100	Abcam Cat# ab7751, RRID:AB_306045
Secondary antibody	Goat Anti-Mouse IgG FITC	1:200	BD Biosciences Cat# 554001, RRID:AB_395197
Isotype control	Mouse IgM PE	1:100	Miltenyi Biotec Cat# 130-093-177, RRID:AB_871723
Isotype control	Mouse IgG2b APC	1:100	Miltenyi Biotec Cat# 130-092-217, RRID:AB_871715
DLL4	Anti-DLL4 APC	1:100	Miltenyi Biotec Cat# 130-096-560, RRID:AB_10827749
DLL4	Anti-DLL4	1:200	Abcam Cat# ab7280, RRID:AB_449562
Primers	Target	Forward/reverse primer (5'-3')	
Pluripotency marker (PCR)	OCT4	AGTGAGAGGCAACTGGAGA/ACACTCGGACCACATCCTTC	
Pluripotency marker (PCR)	SOX2	TCAGGAGTTGTCAAGGCAGAGAAG/TCAGGAGTTGTCAAGGCAGAGAAG	
Pluripotency marker (PCR)	REX1	CAGATCCTAAACAGCTCGCAGAAT/GCGTACGCAAATTAAGTCCAGA	
Pluripotency marker (PCR)	NANOG	TGCAGTTCACGCCAAATTCTC/CCTAGTGGTCTGCTGATTACATTAAGG	
House-keeping genes (qPCR and PCR)	GAPDH	GAAGGTGAAGGTCCGAGT/GAAGATGGTATGGGATTC	
Genotyping	DLL4	TTGGATGAGCAAACCAGCACCC/TGACAGCCCGAAAGACAGATAGG	

### RT-PCR and quantitative 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. The quantitative PCR was performed using SYBR green (BioRad) in an ABI 7900HT instrument. We performed the PCRs using the primers listed on Table 3.

### Alkaline Phosphatase activity assay

The cells were seeded on 12-well plates coated with Matrigel. After 5 days in culture, we tested the alkaline phosphatase enzymatic activity using the Alkaline Phosphatase Detection Kit (Merck-Millipore) following manufacturer's instructions.

### 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 20–23 days with E6 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, fixed and embedded in paraffin for histological examination by immunohistochemistry. The antibodies used are listed on Table 3.

### Karyotype analysis

Chromosomal analysis was performed by GTG-banding analysis at the Andalusian Public Health System Biobank, Spain, according to the International System Cytogenetics Nomenclature recommendations.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scr.2017.07.008>.

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Depósito de la línea celular PBMC1-iPS4F1 DLL4.

Figura Suplementaria:  
Expresión del marcador de pluripotencia SSEA3 por citometría de flujo.

