**Supplementary information**

**Title: *Regulation of cyclin E1 expression in human pluripotent stem cells and derived neural progeny***

**Authors:** María Soledad Rodríguez Varela1, Sofía Mucci1, Guillermo Agustín Videla Richardson1, Olivia Morris Hanon1, Verónica Alejandra Furmento1, Santiago Gabriel Miriuka1, Gustavo Emilio Sevlever1, María Elida Scassa1 and Leonardo Romorini1,\*.

**Author affiliations:** 1Laboratorios de Investigación Aplicada en Neurociencias (LIAN-CONICET), Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI), Ruta 9, Km 52.5, Belén de Escobar, Provincia de Buenos Aires, B1625XAF, Argentina.

**\* Corresponding authors:** Leonardo Romorini, e-mail: lromorini@fleni.org.ar

**Supplementary methods:**

***Antibodies and primers used:***

|  |  |  |
| --- | --- | --- |
|  | **Primer sequence ( 5'** → **3')** | |
| **Name** | **Forward** | **Reverse** |
| RPL7 | AATGGCGAGGATGGCAAG | TGACGAAGGCGAAGAAGC |
| cyclin A2 | CCTGCAAACTGCAAAGTTGA | AAAGGCAGCTCCAGCAATAA |
| cyclin B1 | CAAGCCCAATGGAAACATCT | GGATCAGCTCCATCTTCTGC |
| cyclin D1 | GATCAAGTGTGACCCGGACT | TCCTCCTCCTCTTCCTCCTC |
| cyclin D2 | TTGTTCCCGAGCGATAGATG | ACCAGAAGCGAAGAGTAACC |
| cyclin D3 | GTGGCCACTAAGCAGAGGAG | TTTGTGAAGGGGGAACAGAC |
| cyclin E1 | AGGGGACTTAAACGCCACTT | AGGGGACTTAAACGCCATT |
| Oct-4 | CTGGGTTGATCCTCGGACCT | CACAGAACTCATACGGCGGG |
| Nanog | AAAGAATCTTCACCTATGCC | GAAGGAAGAGGAGAGACAGT |
| Brachyury | TCCCAGGTGGCTTACAGATGA | GGTGTGCCAAAGTTGCCAAT |
| AFP | TGCTGGATTGTCTGCAGGATG | ACGTTCCAGCGTGGTCAGTTT |
| Nestin | CAGCTGGCGCACCTCAAGATG | AGGGAAGTTGGGCTCAGGACTGG |

**Table 1.** *Primers used for RT-qPCR experiments.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibody** | **Specie** | **Brand** | **N° Catalogue** | **Dilution** |
| α-cyclin E1 | Monoclonal-Mouse | BD Biosciences | BDB-551159 | 1/1000 |
| α-cyclin D1 | Monoclonal-Mouse | Santa Cruz | Sc-8396 | 1/1000 |
| α-cyclin D2 | Monoclonal-Mouse | Santa Cruz | Sc-376676 | 1/1000 |
| α-cyclin A | Monoclonal-Mouse | Santa Cruz | Sc-271682 | 1/1000 |
| α-cyclin B1 | Monoclonal-Mouse | Santa Cruz | Sc-245 | 1/1000 |
| α-Actin | Polyclonal-Goat | Santa Cruz | Sc-1616 | 1/1000 |

**Table 2.** List of *primary antibodies used for Western Blot analysis*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibody** | **Specie** | **Brand** | **N° Catalogue** | **Dilution** |
| α-Nanog | Monoclonal-Rabbit | Cell Signaling | D73G4 | 1/400 |
| α-Oct-4 | Monoclonal-Mouse | Santa Cruz | Sc-5279 | 1/200 |
| α-Vimentin | Monoclonal-Mouse | Dako | M0725 | 1/200 |
| α-Nestin | Polyclonal-Rabbit | Chemicon | AB5922 | 1/400 |
| α-cTnT | Monoclonal-Mouse | Santa Cruz | Sc-20025 | 1/200 |
| α-AFP | Monoclonal-Mouse | Santa Cruz | Sc-166325 | 1/200 |
| α-MAP2 | Monoclonal-Mouse | Sigma | M1406 | 1/400 |
| α-TUJ-1 | Monoclonal-Mouse | Covance | MMS-435P | 1/400 |

**Table 3.** List of *primary antibodies used for immunofluorescence studies.*

**Supplementary figures:**

***hPSCs, NP and HF characterization: expression of stem cell and differentiation markers.***

Suppl Fig1.tif

**Supplementary Figure 1. *hPSCs, NP and HF characterization: expression of stem cell and lineage-specific markers.*** **(a)** Representative micrographs of immunofluorescent staining of WA09 (H9) hESCs, FN2.1 hiPSCs and HF cultured as described in Methods and then fixed and stained with primary antibodies recognizing Octamer 4 (Oct-4) and Nanog stem cell markers and Vimentin mesenchymal marker. The scale bars represent 100 µm. **(b)** RT-qPCR analyses of stem cell-like transcripts *nanog* and *oct-4* were performed on undifferentiated H9 hESCs and FN2.1 hiPSCs, HF and H9-derived NP. *rpl7* expression was used as normalizer. Graph shows mRNA fold induction relative to HF. The mean + SEM from three independent experiments are shown. (\*) p<0.05, (\*\*) p<0.01, (\*\*\*) p<0.001 vs. HF. (&&) p<0.01 vs. H9. **(c)** Representative micrographs of immunofluorescent staining of H9-derived NP fixed and stained with primary antibodies recognizing Oct-4 and Nanog stem cell markers and Nestin neural progenitor cell marker. The scale bars represent 100 µm. **(d)** H9 and FN2.1 cells were differentiated using an embryoid body- (EB) based protocol, grown on gelatin coated plates from day 7 to 14 of differentiation and then ectoderm (neural rosettes), cardiac mesoderm (contractile EB) and endoderm structures were stained with primary antibodies that recognize Nestin (ectoderm), Cardiac troponin T (cTnT) (cardiac mesoderm) and Alpha-feto protein (AFP) (endoderm) markers. Figure shows representative images. The scale bars represent 100 µm. **(e)** Representative images of neuronal-like cells differentiated from H9-derived NP stained with primary antibodies against MAP2 and TUJ-1. The scale bars represent 100 μm. BF, bright field.

***Comparison of mRNAs expression levels for cyclins in synchronous hPSCs, NP and HF.***

***Suppl Fig2.tif***

**Supplementary Figure 2. *Comparison of mRNAs expression levels for cyclins in synchronous hPSCs, NP and HF.*** mRNA expression levels quantified by RT-qPCR of *cyclins D1, D2, D3, E1, A2* and *B1* in HF, hiPSCs (FN2.1), hESCs (H9) and NP cells arrested in G1 with PD0332991 (PD) or in G2/M with Nocodazole (NOCO) under the same experimental conditions described in Figure 1A. *rpl7* expression was used as normalizer. Graph shows mean + SEM mRNA fold induction relative to HF control cells arbitrarily set as 1 from five independent experiments. \* *p*<0.05 vs. HF.

***Cell cycle profile of asynchronous and pharmacologically arrested hPSCs cultured with a fully defined medium***

***Suppl Fig3.tif***

**Supplementary Figure 3. *Cell cycle profile of asynchronous and pharmacologically arrested hPSCs cultured with E8 medium.*** Validation of stemness phenotype **(a)** Representative micrographs ofimmunofluorescent staining of H9 hESCs and FN2.1 hiPSCs cultured in fully defined conditions (Vitronectin coated dishes and E8 medium) and then fixed and stained with primary antibodies recognizing Oct-4 and Nanog stem cell markers. The scale bars represent 100 µm. **(b)** RT-qPCR analyses of stem cell-associated transcripts *nanog* and *oct-4* were performed on undifferentiated H9 hESCs and FN2.1 hiPSCs cultured in Vitronectin coated dishes and E8 medium. *rpl7* expression was used as normalizer. Graph shows mRNA fold induction relative to HF. The mean + SEM from three independent experiments are shown. (\*) p<0.05, (\*\*) p<0.01, (\*\*\*) p<0.001 vs. HF. **(c)** Indirect intracellular flow cytometry analysis of fixed and permeabilized H9 hESCs and FN2.1 hiPSCs cultured in Vitronectin coated dishes and E8 medium stained with Oct-4, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (blue line histogram). Red line histogram represents isotype control. N=3. **(d)** hiPSCs (FN2.1) and hESCs (H9) grown and maintained in fully defined conditions (Vitronectin coated dishes and E8 medium) were arrested in G1 with PD0332991 (PD) (5μM for 30h) and in G2/M with Nocodazole (100ng/ml for 24h) or with Vincristine (250nM for 24h). Cell cycle profile of asynchronous and pharmacologically arrested cells was analyzed after cells were fixed with cold 70% ethanol. DNA content was measured with propidium iodide (PI) and its fluorescence was determined with a flow cytometer. A representative DNA content frequency histogram plot is shown for each condition. The percentage of cells in each cell cycle phase was calculated by the FlowJo v10.0.7’s univariate platform. N=3.

***Differentiation status of pharmacologically G1- and G1/S arrested hPSCs after inhibitors treatment.***

Figure Oct y Nanog y Dif.tif

**Supplementary Figure 4. *Differentiation status of pharmacologically G1- and G1/S-arrested hPSCs after treatment.* (a)**and **(b)** hESCs (H9) and hiPSCs (FN2.1) grown and maintained in fully defined conditions (Vitronectin coated dishes and E8 medium) were arrested in G1 with PD0332991 (PD) (5μM for 30h) and in G1/S with Aphidicolin (Aph.) (10μg/ml for 20h) or treated with U0126 (20µM for 24h), 10058-F4 (100µM for 24h) and HLM006474 (40µM for 7 days), and then RT-qPCR analyses of **(a)** stem cell-associated (*nanog* and *oct-4*) and **(b)** early differentiation (*brachyury*, *alpha-fetoprotein* or *afp* and *nestin* for mesoderm, endoderm and ectoderm, respectively) transcripts were performed. hPSCs differentiated using an embryoid body- (EB) based protocol, at days 4 (4d) and 7 (7d) of differentiation and NP were used as differentiated controls. *rpl7* expression was used as normalizer. Graph shows mRNA fold induction relative to Control (Cont.) (asynchronous growing cells) or day 4 (4d) of the EB differentiation protocol. The mean + SEM from three independent experiments are shown. Und: undeterminated. (\*) p<0.05, (\*\*) p<0.01, (\*\*\*) p<0.001 vs. Control. **(c)** hESCs (H9) and hiPSCs (FN2.1) grown and maintained in fully defined conditions (Vitronectin coated dishes and E8 medium) were treated with Nocodazole (NOCO, 100ng/ml for 24h), GSK690693 (10µM for 24h), U0126 (20µM for 24h), 10058-F4 (100µM for 24h), HLM006474 (40µM for 7 days), MG-132 (MG132, 62.5nM for 24 h), CDK2 inhibitor II (CKD2i, 5µM for 24h) and CHIR99021 (CHIR, 10µM for 24h) and then RT-qPCR analyses of stem cell-associated (*nanog* and *oct-4*) markers were performed. *rpl7* expression was used as normalizer. Graph shows mean + SEM mRNA fold induction relative to Vehicle (DMSO treated) control cells (arbitrarily set as 1) of three independent experiments.

***Changes in cyclins mRNA expression levels throughout hPSCs cell cycle progression after Aphidicolin block and release.***

arresto y liberación qPCR otras ciclinas.tif

**Supplementary Figure 5. *Changes in cyclins mRNA expression levels throughout hPSCs cell cycle progression after Aphidicolin block and release.*** H9 hESCs and FN2.1 hiPSCs grown in Vitronectin coated dishes with E8 medium were synchronized at G1/S boundary by Aphidicolin (Aph.) block (10μg/ml for 20h) and released to enter the cell cycle, and then*cyclins A2, B1, D1, D2* and *D3* mRNA expression levels were quantified by RT-qPCR inasynchronous or at different time points after Aphidicolin (Aph.) removal. *rpl7* expression was used as normalizer. Graphs show mean + SEM mRNA fold induction relative to asynchronous control cells (arbitrarily set as 1) of three independent experiments. h: hours. (\*) *p*<0.05 and (\*\*) *p*<0.01 vs. Control (asynchronous cells).

***Representative images (whole membranes) of Western blots***

***Figure ciclinas completas + E.tif***

**Supplementary Figure 6. *Representative images of Western blot analyses.*** Representative images of Western blot analyses (whole membranes) of cyclins (D1, D2, E1, A and B1) expression levels in HF, FN2.1 (hiPSCs), H9 (hESCs) and NP cells asynchronously growing (Asynchronous) or arrested in G1 with PD0332991 (PD) (1µΜ for 48h for HF, 5μM for 30h for hPSCs and 1μM for 24h for NP). Actin was used as loading control. Positive control: HeLa cells lysate provided by manufacturer.