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Down-regulation of Halr1 during induced differentiation of embryonal carcinoma P19 cells

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Abstract: Maintenance of pluripotency depends to diverse regulatory factors. Studies in embryonic stem cells (ESCs) have indicated that large intergenic non-coding RNAs (lincRNAs) are involved in the regulatory network of pluripotency. However, the presence and function of pluripotency-associated lincRNAs in cancer cells with pluripotency features are unknown. In this study, we used embryonal carcinoma (EC) P19 cell lines to investigate the expression level of Halr1 in pluripotency and retinoic acid (RA)-induced differentiated states. Down-regulation of pluripotency associated factors such as OCT4, NANOG, SSEA1 and alkaline phosphatase at transcript and protein levels were used to confirm the differentiated status of P19 cells. Quantitative measurement of Halr1 transcript levels revealed a 79% decrease during RA-induced differentiation of P19 cells. These results indicate that upon exiting the pluripotency state the expression level of Halr1 similar to core pluripotency factors is remarkably reduced.

Introduction

The state of pluripotency is operationally defined by self-renewal and the capacity to differentiate into all lineages in an organism (Thomson *et al*., 1998; Yu and Thomson, 2008). Different molecular features have been identified that associate with the pluripotency state. Expression of pluripotency-associated transcription factors (including Oct4, Sox2, and Nanog) comprises one of the most-studied and essential features for the maintenance of pluripotency (Avilion *et al*., 2003; Botquin *et al*., 1998; Chambers *et al*., 2003; Mitsui *et al*., 2003). Also, several factors, involved in the epigenetic regulation of pluripotency states, have been identified. These include the dynamics of PML nuclear bodies (Butler *et al*., 2009; Chuang *et al*., 2011; Gupta *et al*., 2009; Gupta *et al*., 2008), alteration of chromatin structure (Ahmed *et al*., 2010; Efroni *et al*., 2008), DNA methylation (Beaujean *et al*., 2004; Bird, 2007), post-translational modifications of histones (Pan *et al*., 2007), cell signaling pathways (Kunath *et al*., 2007), and involvement of non-coding RNAs (Dinger *et al*., 2008; Mohamed *et al*., 2010).

Members of the two classes of short and long non-coding RNAs regulate pluripotency. One subclass of large intergenic non-coding RNAs (lincRNAs) (Gibb *et al*., 2011) is recognized based on intergenic K4-K36 domains, which are chromatin signatures of actively transcribed genes (Guttman *et al*., 2009). LincRNAs possess some general features including levels of expression lower than those of protein-coding transcripts (Loewer *et al*., 2010), evolutionarily conserved sequences and predicted secondary structures (Guttman *et al*., 2009; Khalil *et al*., 2009; Loewer *et al*., 2010). LincRNAs have biological roles in DNA damage response and NFκB signaling (Guttman *et al*., 2009). Several studies have shown that lincRNAs are involved in the pluripotency state of embryonic stem cells (ESCs) (Bergmann *et al*., 2015; Chuang *et al*., 2011; Dinger *et al*., 2008; Guttman *et al*., 2009; Guttman *et al*., 2011; Liu *et al*., 2016; Mohamed *et al*., 2010).

Halr1 lincRNA (Hoxa adjacent long non-coding RNA 1, also known as Haunt, and Gm15055), transcribing from a genomic locus approximately 50 kbp away from Hoxa, represses Hoxa1 transcription in cis (Maamar *et al*., 2013) by recruiting PRC2 and maintaining the H3K27me3 modification on Hoxa promoter (Liu *et al*., 2016). Therefore, the expression of Halr1 lincRNA in mouse ESCs leads to repression of Hoxa1. Moreover, Hox genes (e.g., Hoxb1, Hoxa1, and Hoxa5) and their cofactors (e.g., Meis2) rapidly and sequentially become activated in response to differentiation signals, including retinoic acid treatment (De Kumar *et al*., 2015; Papalopulu *et al*., 1991; Simeone *et al*., 1990). However, Yin *et al*. (2015) reported an opposite role for Halr1 genomic region acting as an enhancer in the

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regulation of Hoxa gene in ESCs.

The presence of lncRNA Halr1 in pluripotent cancer cells has not been investigated (Yin *et al*., 2015). This study was designed to explore the expression dynamics of Halr1 during retinoic acid-induced differentiation of P19 cells. In this study, using quantitative PCR we found that Halr1 is highly expressed in pluripotent P19 cells and upon differentiation by retinoic acid, its expression becomes significantly down-regulated. Our findings indicate that Halr1 expression level, similar to a biomarker, illustrates the pluripotency status in pluripotent cancer cells.

Material and Methods

Cell culture and RA-induced differentiation

P19 cells were purchased from the Pasteur Institute Cell Bank (Tehran, Iran). P19 cells are a line of embryonic carcinoma, derived from a teratocarcinoma formed following transplantation of a 7.5-day embryo into mouse testis (Martin, 1980; Stevens, 1962). Teratocarcinoma cells have been characterized as pluripotent cells that differentiate into all derivatives of the three primary germ layers (McBurney, 1993).

It is known that retinoic acid treatment can induce the differentiation of teratocarcinoma cells. These cells were cultured in Alpha Modification of Minimum Essential Medium Eagle (α-MEM, Sigma-Aldrich, Munich, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, USA). The cultures were maintained at 37°C in a 5% CO_2 atmosphere. To induce differentiation of P19 cells, 10^5 cells were cultured in suspension in 80-mm-diameter bacteriological Petri dishes containing 8 mL of complete α-MEM medium, supplemented with 0.5 μM RA (retinoic acid; Sigma, Munich, Germany). After 4 days in the medium containing RA, colonies of embryoid bodies (hereafter named EB4) were formed.

RNA extraction and cDNA Synthesis

Total RNA was isolated from undifferentiated P19 cells and differentiated EB4 colonies using the Total RNA Isolation kit (DENAzist Asia, Mashhad, Iran) following the manufacturer's instruction. Quality and quantity of RNA were evaluated by gel electrophoresis (data not shown) and a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) (Supplementary Tab. 1). First strand cDNA was generated from 1 μg of total RNA in a 20 μL reaction containing 0.5 μg Oligo $(dT)_{18}$ Primer (Thermo Scientific, Waltham, MA, USA), 1X RT buffer, 4 mM dNTPs (Parstous, Mashhad, Iran), and 200 U of reverse transcriptase (RT; Thermo scientific, Waltham, MA, USA). In the negative control (RT minus) reaction, water was used instead of RT.

TABLE 1

Oligonucleotide sequences of primers and probes used in this study

Gene	Primer and probe sequence (5′–3′)	Product length (bp)	Application
Halr1 (Linc-Hoxa1; Hoxa adjacent long noncoding RNA 1) NC_000072.6 (52102949..52113684)	External primers F:GAT ACA ACC TGA CTG ATG R:GAT GTG TCG TTG AAC TAG	168	RT-PCR
	Internal primer R:GTT GAA CTA GGC TGC CCA TC	160	Nested PCR
	F:GACTGATGATGGCTGACA R:GGCTGCCCATCTTGAATA FAM-TTCCTTTCCTTGGTGACATTTGTGAC-TAMRA	141	RT-qPCR
POU domain, class 5, transcription factor 1 (Oct4) NCBI: NM_013633.3	F:CTCTGAGCCCTGTGCCGACC R:GCTGAACACCTTTCCAAAGAGAACGC	202	RT-qPCR
Nanog homeobox (Nanog) NCBI: NM_028016.2	F:GAACTCTCCTCCATTCTGAACCTG R:GGTGCTGAGCCCTTCTGAATC	137	RT-qPCR
Ribosomal protein L37 (Rpl37) NCBI: NM_026069.3	F:GCAGATTCAGACATGGATTC R:GGAAGAAGCGTAGGATCC HEX-TCATATAACCGAACTCTGAACCGATGT-BHQ1	200	RT-qPCR

Primer and probe design, and PCR reactions

Conventional PCR reaction was performed in a total volume of 25 µL containing 1 µL cDNA, 1X PCR buffer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 0.04 U of Taq DNA polymerase (Genet Bio, Daejeon, Korea), and 200 nM of each primer (Tab. 1) in a Peqlab thermocyler. Touchdown PCR reaction was used for initial amplification of lincRNA fragment, then the product of touchdown PCR reaction was subsequently subjected to a nested PCR amplification. Amplification conditions for touchdown PCR reaction were: initial denaturation at 95°C for 3 min, 20 cycles of melting at 95°C for 1 min, decreasing the annealing temperature 1°C every second cycle from 60°C to 50°C, followed by 15 additional cycles at 50°C, and elongation at 72°C for 1 min. Nested PCR conditions consisted of: 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. Ten µL of PCR product was analyzed by electrophoresis on a 1% agarose gel. Sequencing of PCR products was performed by Macrogen Inc. (South Korea).

Primers and hydrolysis probes for quantitative (real-time) reverse transcription polymerase chain reaction (RT-qPCR) were designed using "Beacon designer" software version 8 (PREMIER Biosoft). Hydrolysis probes were labeled with either 6-carboxyfluorescein (FAM) or hexachloro-6-carboxy-fluorescein (HEX) as reporter dyes at the 5' end and either carboxytetramethylrhodamine (TAMRA) or black hole quencher 1 (BHQ1) as fluorescent quenchers at the 3' end (Tab. 1; Supplementary Fig. 1).

Real-time PCR reaction for quantification of lincRNA and L37 consisted of 200 nM probe, 1 µL cDNA, 1X PCR buffer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 0.04 U of Taq DNA polymerase (Genet Bio, Daejeon, Korea) and 200 nM each of forward and reverse primers in a total volume of 25 µL. An initial step of 94°C (3 min), followed by 40 cycles of 94°C (30 s), 58°C for L37 (30 s) and 62°C for Halr1 (30 s), and 72°C (30 s), was used. Real-time PCR for Oct4 and Nanog were performed in the presence of 1X SYBR-green Real-time PCR Master Mix (ParsTous, Mashhad, Iran), 1 µL cDNA template and each primer at 250 nM in a 20 µL reaction volume. The qPCR condition comprised the following steps: 94°C, 10 min; 94°C, 62°C, and 72°C, 30 s each of 40 cycles, and a final extension of 72°C for 10 min. This profile was followed by a melting curve ramping from 60°C to 95°C rising by 1°C each step, and fluorescence collection for 5 s at each degree.

Data analysis

To quantify the level of each transcript, the quantitative RT-PCR was performed. In order to make standard curves (Supplementary Fig. 2), PCR reactions at each serial dilution were carried out three times, each time in triplicate, and the copy number of transcripts plotted against Ct number. To perform qPCR reactions for each experimental group, all RNA samples (undifferentiated P19 cells and differentiated EB4 colonies) were prepared in triplicate and used for cDNA synthesis. For each sample, qPCR reactions and readings were performed in triplicate in a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany). The value of Ct for each triplicate was extrapolated onto the standard curve to calculate the copy number of transcripts in each group. The calculated copy numbers for Oct4, Nanog, and Halr1 were normalized by the copy number of L37 transcript in each experimental group.

Statistical analysis

All data were compiled from a minimum of three replicate experiments. The statistical differences between the control and EB4 groups were analyzed using Mann-Whitney *U*-test. A $p < 0.05$ was considered statistically significant.

Alkaline phosphatase staining

To show alkaline phosphatase (AP) activity in P19 cells and its decrease after RA-induced differentiation, P19 cells were grown on coverslips. For AP activity assay on aggregates, EB4 aggregates were transferred onto glass coverslips placed in each well of a six-well tissue culture plate. A few hours incubation leads to the attaching the aggregates to the glass surface. Cells cultured on coverslips were fixed with 4% paraformaldehyde for 10 min. After fixation, each coverslip was washed three times with AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween-20, pH 9.5; all from Merck, Ballerup, Denmark), and then was covered with 1 mL of AP solution (120 mL of 1% 5-Bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide, 120 mL of 1.5% Nitro Blue Tetrazolium in 70% dimethylformamide, and 5 mL of AP buffer). After 15 min, coverslips were washed with AP buffer and were observed under a microscope.

Immunocytochemistry

Expression of pluripotency-associated proteins was detected by immunocytochemistry (ICC). At first, cells were fixed by 4% PFA and washed three times with PBS (5 min each time). Cells were permeabilized at the presence of Tris-buffered saline containing 0.5% Triton X-100 for 10 min at room temperature and then washed three times with PBS (for 5 min each time). Endogenous peroxidase was blocked by incubating in 3% H_2O_2 (Sigma) in PBS for 30 min. To block non-specific binding, cells were incubated in 4% (v/v) bovine serum albumin (BSA; Invitrogen, Grand Island, USA) for 45 min and then were incubated with the specific primary antibodies: mouse anti-OCT4 (1:100; Cat. No. sc-5279, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), mouse anti-NANOG (1:500; Cat. No. sc-293121, Santa Cruz Biotechnology, Inc.), mouse anti-SSEA1 (1:100; Cat. No. sc-101462, Santa Cruz Biotechnology, Inc.) in blocking buffer (1% BSA) overnight at 4°C. After washing with PBS, cells were incubated for 1 h at room temperature with secondary antibodies specific for each primary antibody: goat anti mouse IgG2b-HRP (1:500; Cat. No. ab97250, Abcam, Cambridge, UK), goat anti-mouse IgG1-HRP (1:1000; Cat. No. ab97240, Abcam), and rat anti-mouse IgM-HRP (1:1500; Cat. No. 04-6820, Invirogen). Finally, cells were washed for several times with PBS and stained with 3,3'-diaminobenzidine (Cat. No. D8001; Sigma-Aldrich) at room temperature for 2 min. After washing with PBS, cells were observed under a microscope. Negative controls were stained with only the secondary antibodies.

Results

Reduced expression of pluripotency-associated genes during RA-induced differentiation of P19 cells

Culture of EC P19 cells in the presence of RA for 4 days in bacterial Petri dishes resulted in the formation of rounded aggregates (EB4). Then, to demonstrate differentiation induction of P19 cells, expression of some pluripotency-related markers was investigated. OCT4 and NANOG are core transcription factors in human embryonic stem cells that are responsible for pluripotency and its maintenance (Boyer *et al*., 2005; Chambers *et al*., 2003; Shakya *et al*., 2015; Silva *et al*., 2017). Other markers of pluripotency that have been identified in EC cells are stage specific embryonic antigen 1 (SSEA1) and AP (Brambrink *et al*., 2008). Therefore, the expression of four markers of OCT4, NANOG, SSEA1, and AP before and during differentiation, was evaluated. Down-regulation of OCT4, NANOG, SSEA1, and AP were assessed at the protein level by ICC during RA-induced differentiation of P19 cells (Fig. 1). Furthermore, similar to the previously published reports (Rhee and Bao, 2009; Soltanian *et al*., 2014; Tan *et al*., 2010; Xie *et al*., 2010) transcript levels of Oct4 and Nanog showed more than 90% decrease in the differentiated P19 cells (Fig. 2).

Halr1 is down-regulated during RA-induced differentiation of P19 cells

To quantify mRNA levels of Halr1 before and after RA-induced differentiation of P19 cells, quantitative real-time qPCR was performed. Since there was no significant variation in the level of L37 transcripts in response to RA treatment in P19 cells (Supplementary Fig. 3), this transcript was selected as the reference gene. In RA-treated cells, Halr1 transcript levels showed around 80% decrease (Fig. 2).

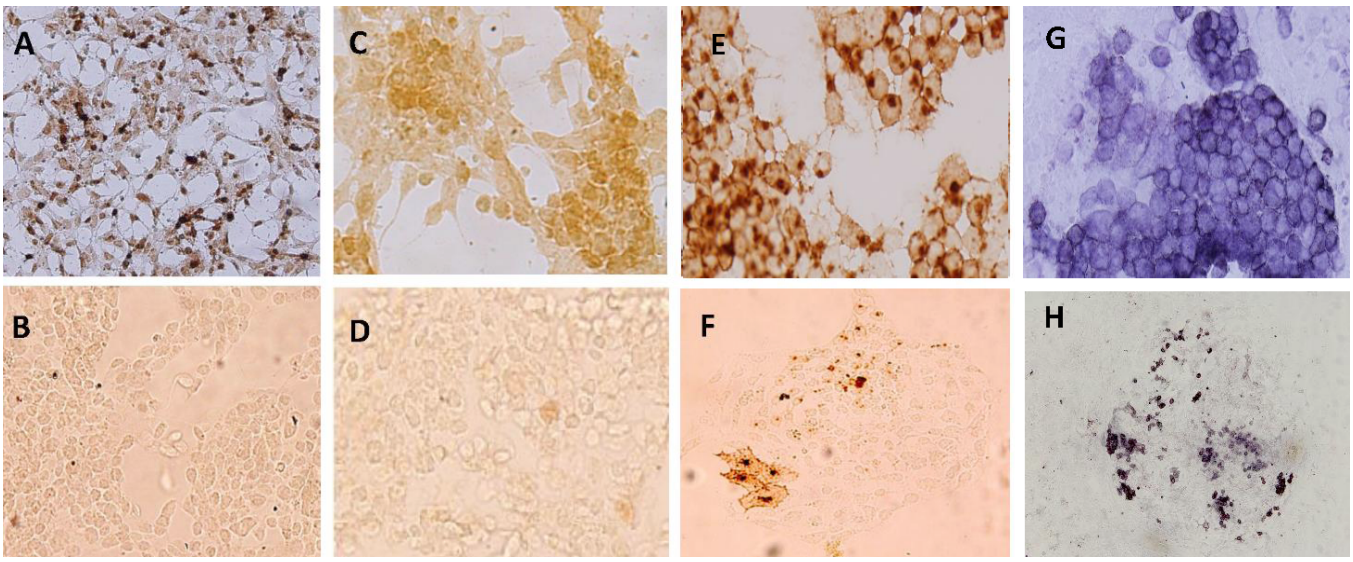

FIGURE 1. Expression analysis of Oct4, Nanog, SSEA1 and AP during RA-induced P19 cell differentiation. Undifferentiated P19 cells express OCT4 (A), NANOG (C), SSEA1 (E) and AP (G). The expression of OCT4 (B), NANOG (D), SSEA1 (F) and AP (H) is downregulated after differentiation (EB4). Scale bars represent 100 mm.

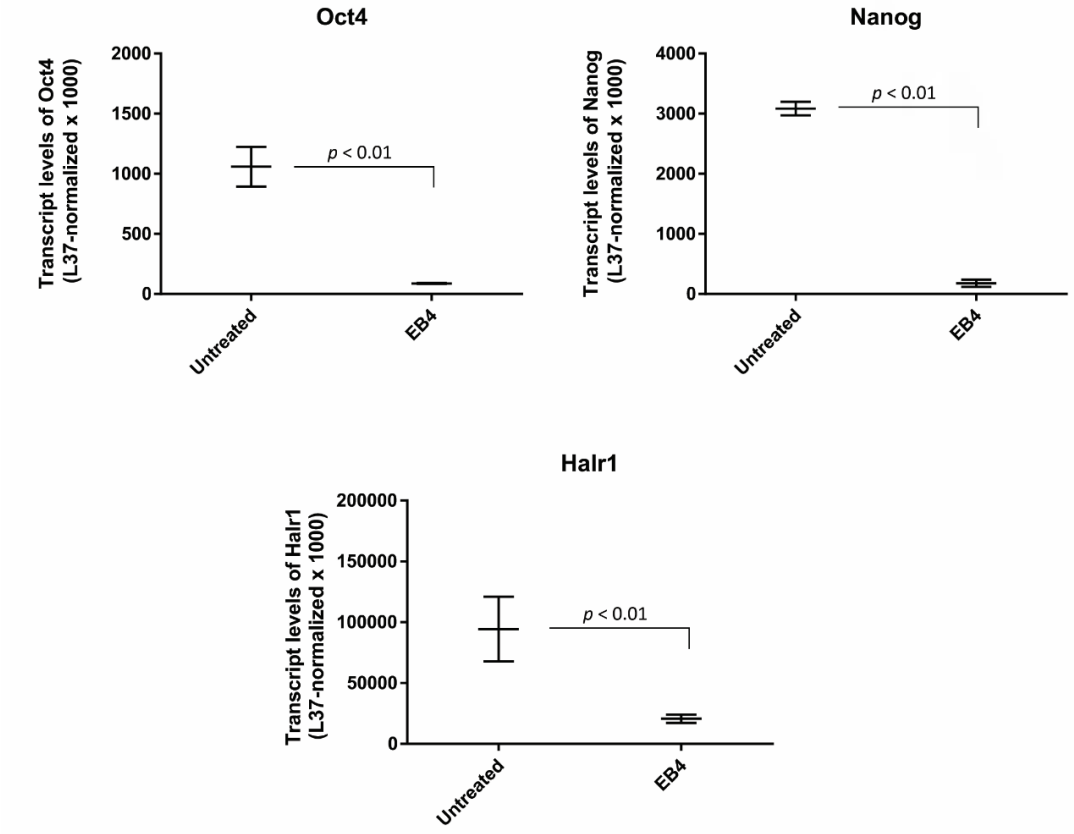

FIGURE 2. Down-regulation of Halr1 along with Oct4 and Nanog during RA-induced P19 cell differentiation. The RNA copy numbers measured using quantitative RT-PCR during RA-induced P19 differentiation has been shown for Oct4, Nanog and Halr1.

Discussion

Our findings reveal significant decrease of Halr1 during RA-induced differentiation of P19 cells. These results are in agreement with the previously published results that have shown that RA-induced differentiation of mouse ESCs results in down-regulation of several lncRNAs that are required for the proliferation and maintenance of self-renewal capacity (Dinger *et al.*, 2008; Guttman *et al.*, 2011; Jain *et al.*, 2016; Rosa and Ballarino, 2015; Ulitsky *et al.*, 2011; Winzi *et al.*, 2018). It has been demonstrated that lncRNAs act as molecular scaffolds to attract chromatin modification complexes to specific genomic loci to play the essential role of epigenetic regulation of pluripotency and differentiation (Chakraborty *et al.*, 2017; Guttman *et al.*, 2011; Li and Belmonte, 2017; Morlando *et al.*, 2014; Rinn *et al.*, 2007; Tsai *et al.*, 2010; Wang *et al.*, 2011). As an instance, the involvement of some lncRNAs has been indicated in the maintenance of active chromatin and embryonic stem cell pluripotency by interaction with the MLL family of H3K4 methylases and PRC2 H2K27 demethylases (Kaneko *et al.*, 2014; Subhash *et al.*, 2018; Yang *et al.*, 2014).

A more detailed characterization has shown that some lncRNAs could be under the direct control of core pluripotency-associated transcription factors (Guttman *et al.*, 2011; Jen *et al.*, 2017; Lin *et al.*, 2014; Loewer *et al.*, 2010). In detail, Guttman *et al.* (2011) showed that at least one of 9 transcription factors related to pluripotency (including OCT4, SOX2, NANOG, cMyc, nMyc, Klf4, Zfx, Smad, and Tcf3) binds to many of lincRNA promoters. In addition, lncRNAs are also able to regulate the expression of pluripotency-associated transcription factors. For example, AK028326 and AK141205 lncRNAs can activate transcription of OCT4 (Mohamed *et al.*, 2010), and lncRNA TUNA binds NANOG and SOX2 promoters and upregulates their expression (Lin *et al.*, 2014) Furthermore, the interaction of lncRNAs and pluripotency transcription factors is able to regulate the pluripotency and cancer states. For example, the interaction of rhabdomyosarcoma 2-associated transcript (RMST) lncRNA and SOX2 has been demonstrated in ESCs (Ng *et al.*, 2013). Two transcription factors TP63 and SOX2 activate CCAT1 and form a complex with this lncRNA to promote squamous cell carcinomas (SCCs) through EGFR expression (Jiang *et al.*, 2018). Taken together, these examples indicate that some lncRNAs are involved in the maintenance of pluripotency and repression of differentiation states (Guttman *et al.*, 2011).

Our study indicates that during RA induction of embryonal carcinoma P19 cells, the expression of Halr1 is reduced similar to core pluripotency-associated factors. According to the interplay between some lncRNAs and pivotal regulators of pluripotency, simultaneous repression of some lncRNAs such as Halr1 along with the essential pluripotency factors can be regarded as an evidence for their role and relevance to pluripotency state in P19 embryonal carcinoma cells.

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Conflict of Interest

The authors declare that they have no competing interests.

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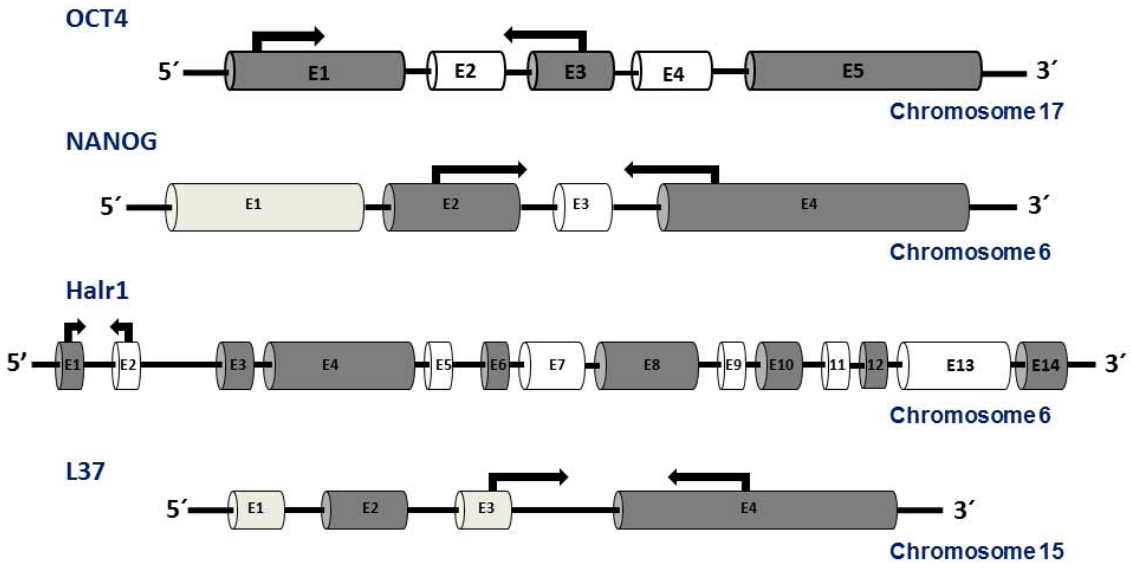
Supplementary File

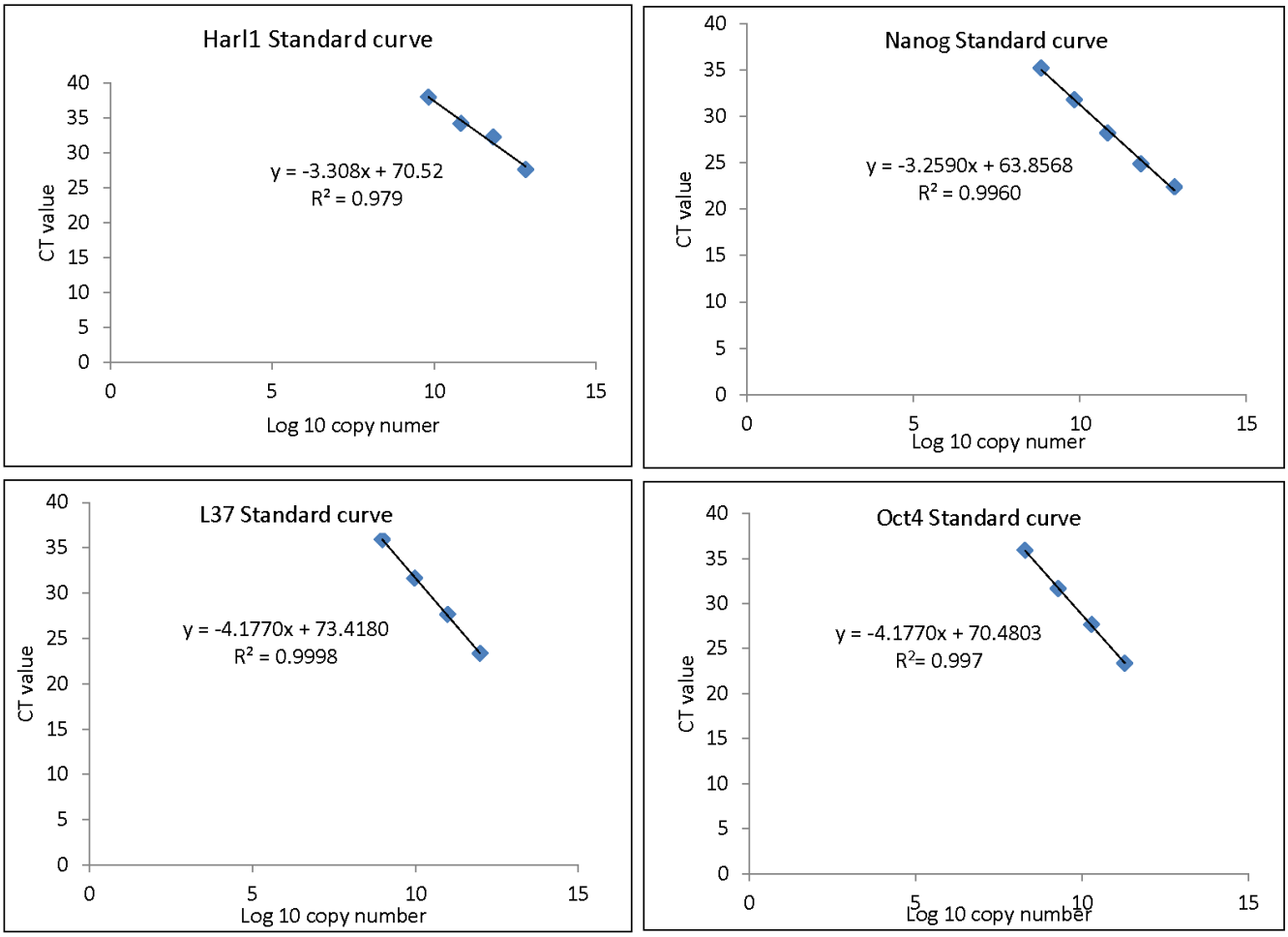
SUPPLEMENTARY TABLE 1

Spectrophotometric analysis of total RNA isolated from undifferentiated and differentiated cells

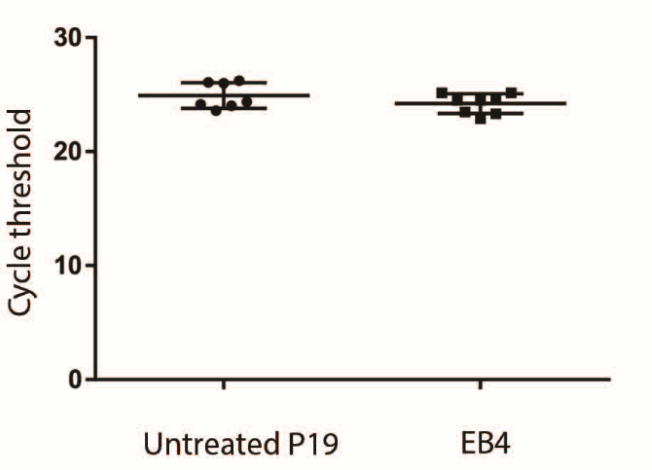
Sample status	Sample	Nucleic acid Conc. (ng/µl)	260/280	280/230
Undifferentiated P19 cells	RNA 1	1075	2.00	2.00
	RNA 2	988.6	2.08	2.04
	RNA 3	1020	2.11	1.91
EB4 P19 cells	RNA 1	977.2	2.00	2.00
	RNA 2	1116	1.80	1.90
	RNA 3	991.0	2.00	1.91



SUPPLEMENTARY FIGURE. 1. Graphical representation of genomic loci and primer binding sites for quantitative RT-PCR of Oct4, Nanog, Halr1, and L37.



SUPPLEMENTARY FIGURE 2. Standard curves for Halr1, Nanog, L37 and Oct4. Y axis shows cycle threshold (Ct) and X axis shows the log10 of copy number.



SUPPLEMENTARY FIGURE 3. Similar cycle thresholds for L37 between untreated and EB4 groups of P19 cells.