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Modulation of chromatin modifying factors' gene expression in embryonic and induced pluripotent stem cells

Carlos Luzzani [a,2], Claudia Solari [a,2], Noelia Losino [a,2], Waisman Ariel [a,2], Leonardo Romorini [b,3], Carolina Bluguermann [b,3], Gustavo Sevlever [b,3], Lino Barañao [a,1,2], Santiago Miriuka [b,1], Alejandra Guberman [a,c,*,1]

[a] Laboratorio de Regulación de la Expresión Génica en el Crecimiento, Supervivencia y Diferenciación Celular, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina
[b] Laboratorio de Biología del Desarrollo Celular, Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI), Argentina
[c] Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are a promising source of cells for regenerative medicine because of their potential of self renew and differentiation. Multiple evidences highlight the relationship of chromatin remodeling with stem cell properties, differentiation programs and reprogramming for iPSC obtention.

With the purpose of finding chromatin modifying factors relevant to these processes, and based on ChIP on chip studies, we selected several genes that could be modulated by Oct4, Sox2 and Nanog, critical transcription factors in stem cells, and studied their expression profile along the differentiation in mouse and human ESCs, and in mouse iPSCs. In this work, we analyzed the expression of Gcn5l2, GTF3C3, TAF15, ATF7IP, Myst2, HDAC2, HDAC3, HDAC5, HDAC10, SUV39H2, Jarid2, and Bmi-1. We found some genes from different functional groups that were highly modulated, suggesting that they could be relevant both in the undifferentiated state and during differentiation. These findings could contribute to the comprehension of molecular mechanisms involved in pluripotency, early differentiation and reprogramming. We believe that a deeper knowledge of the epigenetic regulation of ESC will allow improving somatic cell reprogramming for iPSC obtention and differentiation protocols optimization.

1. Introduction

Embryonic stem cells (ESCs) are able to self renew indefinitely *in vitro* while retaining the capability of differentiating into cells of the three germ layers. A small set of transcription factors, essentially Oct4, Nanog, and Sox2 have been identified as responsible for maintaining the undifferentiated state of ESCs [1]. Additionally, forced expression of these factors reprograms terminally differentiated cells into ES-like cells denominated induced pluripotent stem cells (iPSCs) [2,3]. Because of their properties, pluripotent cells are a promising source of cells for regenerative therapy and in particular, iPSC could help to overcome immune rejection after transplantation [4].

During cellular differentiation, mayor changes in cellular morphology and function occur. These changes are mainly determined by the execution of tissue-specific programs that modulate gene expression. However, tissue-specific transcriptional regulatory proteins are not sufficient to initiate differentiation. Changes, both, at the level of higher order chromatin structure and in the

Abbreviations: ESC, embryonic stem cell; MEF, mouse embryonic fibroblasts; mESC, mouse embryonic stem cell; hESC, human embryonic stem cell; iPSC, induced pluripotent stem cell; HAT, histone acetyltransferase; HMT, histone methyltransferase; HDAC, histone deacetylase.

* Corresponding author at: Laboratorio de Regulación de la Expresión Génica en el Crecimiento, Supervivencia y Diferenciación Celular Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71 (C1428EGA), Buenos Aires, Argentina. Fax: +54 11 4576 3342.

E-mail addresses: carlosluzzani@qb.fcen.uba.ar (C. Luzzani), cmsolari@qb.fcen.uba.ar (C. Solari), nlosino@qb.fcen.uba.ar (N. Losino), waisman@qb.fcen.uba.ar (W. Ariel), leoromo@qb.fcen.uba.ar (L. Romorini), carobluguer@gmail.com (C. Bluguermann), gsevlever@fleni.org.ar (G. Sevlever), sbaranao@mincyt.gov.ar (L. Barañao), smiriuka@fleni.org.ar (S. Miriuka), algub@qb.fcen.uba.ar (A. Guberman).

[1] Established investigator from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

[2] Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71 (C1428EGA), Buenos Aires, Argentina. Fax: +54 11 4576 3342.

[3] Montañeses 2325, (1428) Buenos Aires, Argentina.

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chromatin organization at individual genes are also essential [5]. Together, transcriptional and epigenetic processes specify gene expression programs that are critical for the maintenance of ESC properties.

In the last few years, much evidence has been gathered about the importance of chromatin remodeling in the maintenance of ESC properties and in the achievement of successful cellular differentiation. Moreover, ESCs have a distinct state of chromatin in a large number of genes that are important for development. This state is characterized both for the presence of H3K27me3 repressive mark and the active gene mark, H3K4me3. Importantly, many of these sites are bound by Oct4, Sox2 and Nanog [6]. Recently, ChIP on chip approaches have unraveled the nature of the regulatory networks that link the pluripotency transcription factors with many chromatin modifiers and chromatin remodelers [7]. However, while some modifiers have been studied extensively, the role of other still remains unknown.

Based on the hypothesis that pluripotency transcription factors such as Oct4, Sox2 and Nanog may be regulating the expression of genes that encode chromatin modifier enzymes, we decided to look for modifiers whose expression was altered in mouse and human ESC compared to that of a terminally differentiated cell type. We have also evaluated their expression in iPSC lines developed by us. Subsequently, we analyzed if candidate genes were modulated during *in vitro* differentiation in both mouse and human ESCs. In this work, we report the expression profile of selected chromatin modifiers in mouse and human pluripotent stem cells. We found genes from different groups that were highly modulated during the differentiation process, suggesting that they could be relevant both in the undifferentiated state and during differentiation processes and showed similar behavior in ESC compared to iPSC and in mESC compared to hESC. These evidences suggest that this screening may lead us to find genes that play a role in maintaining ESCs properties, helping to untangle chromatin regulation contribution to pluripotency maintenance and cellular differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

The E14-derived Ainv15 and R1 mES cell lines were obtained from ATCC, the human embryonic stem cell (hESC) line WAO9 was purchased from WiCell Research Institute (WI). Cell lines were cultured and differentiated as previously described [8,9].

2.2. Lentivirus production and induction of pluripotent stem cells

Lentiviruses were produced using the pHAGE-EF1α-STEMCCA vector as previously described [10], with minor modifications [11]. Induced pluripotent stem cells were developed as previously described [11].

2.3. Real-time quantitative RT–PCR

mESCs were cultured in standard medium plus LIF on 0.1% bovine gelatin-coated tissue culture plates for at least three passages and then set to differentiate as described. hESC were grown in standard medium plus bFGF on MEFs and then set to differentiate as described. Total cellular RNA was isolated from subconfluent cultures or EBs using TRIZOL reagent (Invitrogen). The yield and purity of RNA samples were assessed by the absorbance at 260 nm and 260 nm/280 nm ratio, respectively. 1 μg of total RNA was retro-transcribed using MMLV reverse transcriptase (Promega) and 30 ng/μl Random Primers (Invitrogen). Real time quantitative PCR amplification of DNA was carried out using Real Time Mix (Biodynamics) and specific oligonucleotides (Table S1) in Opticon Real Time DNA engine (Bio-Rad). A melting curve analysis was performed immediately after amplification at a linear temperature transition rate of 0.3 °C/s from 60 °C to 90 °C with continuous fluorescence acquisition. The amplicon size was confirmed by gel electrophoresis. Raw data were analyzed using MJ Opticon Monitor Software 3.1.32 (Bio-Rad). Gene expression was normalized to the housekeeping genes β-Actin or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as indicated. Quantification of relative gene expression was calculated using the ΔΔCt method. At least, two technical replicates of two independent samples were used for each experiment; a no-template blank served as negative controls.

2.4. Immunofluorescence

Primary and secondary antibodies are showed in Table S2. Immunofluorescence was developed as previously described [8]. Images were acquired with a confocal microscope Olympus IX81/Fluoview FV 1000.

3. Results

Previously reported ChIP on chip studies revealed a myriad of genes that could be regulated by transcription factors expressed in ES cells and that are critical for self-renewal and pluripotency [6,7]. Based on these studies and others, we decided to study the expression of selected genes involved in chromatin remodeling, with the purpose of finding chromatin remodeling related genes that were relevant to preserve self-renewal, to promote differentiation, and/or to define the cell fate. We designed oligonucleotides to study gene expression of some selected genes, among them, several histone acetyltransferases (HATs), some histone methyltransferases (HMTs), several histone deacetylases (HDACs), a histone demethylase, and a Polycomb Group member. Afterward, we looked for those that were differentially expressed in undifferentiated compared to differentiated state.

We first analyzed the expression of the HATs Gcn5l2 (KAT2A), GTF3C3, ATF7IP and Myst2 (HBO1), the HDACs HDAC2, HDAC3 and HDAC5, and the TATA Box Associated Factor, TAF15 in undifferentiated mouse E14 derived ESC line, Ainv15, compared to that of a terminally differentiated cell type, mouse embryonic fibroblasts (Fig. S1A). Some of these genes were also analyzed in a different ESC line, R1, and similar results were obtained (data not shown). We also studied the expression levels of the same chromatin remodelers in mouse induced pluripotent stem cells (iPSCs) generated by us. The iPS cell lines obtained were validated evaluating self-renewal and pluripotency by specific gene markers expression, along with *in vitro* and *in vivo* differentiation capability (Figs. S2–S4). As we expected, the evaluated chromatin remodelers showed a similar trend when comparing either mESC or iPSC to MEF (Figs. S1A and B). Again, when we analyzed some of the chromatin remodelers in other obtained iPS cell line, we observed akin behavior (data not shown). Interestingly, most of the studied genes showed different expression levels when comparing pluripotent cells with respect to terminally differentiated cells.

We then sought to extend our analysis to a human system. To achieve this, we studied the expression of the HATs, Myst 2 and 3, GTF3C3 and ATF7IP; the HDACs, HDAC2, HDAC3, and HDAC10; the HMT SUV39H2 (KMT1A/B); the histone demethylase Jarid2; and the Polycomb Group member, Bmi-1, in WA09 human ESCs. In this case, we compared gene expression between undifferentiated hESCs and human skin fibroblasts, as a representative cell type of terminally differentiated cells (Fig. S5). As in their mouse counterpart, hESC showed some genes that were differentially ex-

pressed in undifferentiated stem cells with respect to terminally differentiated cells. The substantial difference of expression levels found in the analyzed systems strongly suggests that many of these genes may be important for maintaining stem cell basic properties.

Based on the aforementioned results and on gene promoters features, we delimited our selection and analyzed the expression of some selected genes along *in vitro* differentiation protocols, with the purpose of having a broad view of chromatin modifier genes' modulation during the differentiation process. We conducted this study, both in mouse (Fig. 1) and human (Fig. 2) ESCs. Concomitantly, we measured the expression of specific gene markers to corroborate the undifferentiated or differentiated state of the cells validating the differentiation protocols. We detected Oct4 and Nanog down-regulation and up-regulation of differentiation markers, along the protocol (Figs. 1B and 2B). Moreover, during the differentiation process, contracting areas were observed in the outgrowth of the EBs (Supplementary video).

We found that the HAT Myst2 was highly repressed early in the differentiation process in mESC and late in hESC. GTF3C3 was also repressed early in both systems and later, while in mESC declined, in hESC raised along the process, to similar levels as undifferentiated cells'. The HAT ATF7IP behaved similar in human and mouse ESC showing high repression since day 2 of the differentiation process. Concerning the HDACs, we found that the three genes evaluated in mESC were downregulated since the beginning of the differentiation process, while in their human counterpart they didn't show any modulation. It is worth mentioning that in hESC, Jarid2 and Bmi1 showed clear progressively modulation along the differentiation process; while the first showed strong repression, the other one was induced. To our knowledge, this is the first report about Jarid2 and Bmi1 modulation in hESC.

4. Discussion

Pluripotent cells have a unique epigenetic signature that reflects their broad potential [12]. Histone modifications influence genes' chromatin structure and consequently, their transcriptional activity [13]. Multiple evidences link chromatin remodeling to stem cells pluripotency [14–17]. Conversely, there are evidences that indicate that chromatin remodeling occurs during differentiation as well [18].

Although these and a myriad of studies have clearly established that histone modifications are essential for development, many questions remain unanswered regarding the role of individual factors [12] and the mechanisms by which the activities of the different chromatin remodeling enzymes are integrated in ES cells [19].

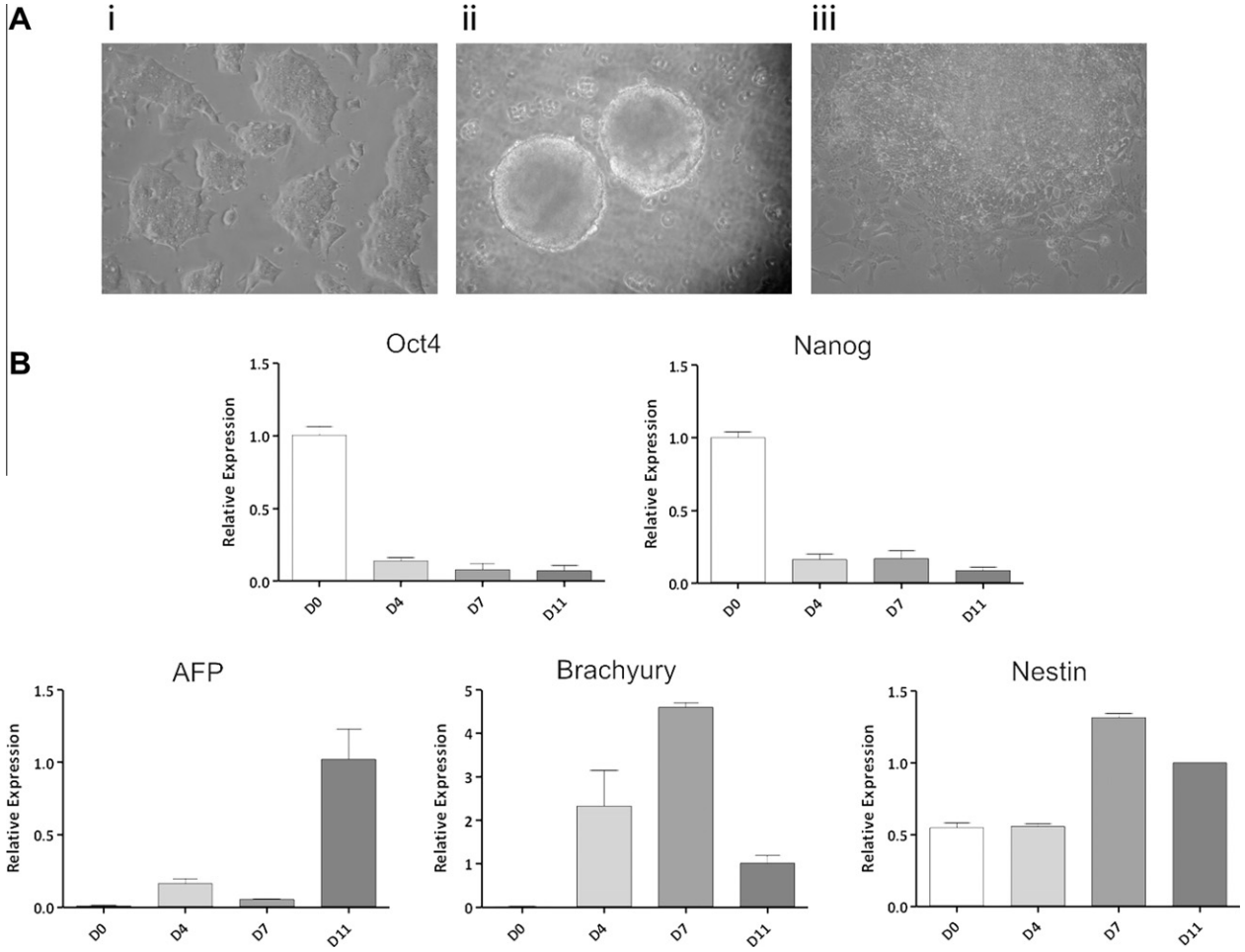

Fig. 1. Expression levels of chromatin remodelers along *in vitro* differentiation of mESC. Ainv15 mESCs were subjected to the hanging drop protocol. RNA was extracted at days 0, 4, 7 and 11 after the induction of differentiation. (A) Representative pictures of (i) Ainv15 mESC line, (ii) embryoid bodies growing under suspension and (iii) embryoid bodies growing in adherence. (B) Undifferentiated state and lineage specific gene markers and (C) chromatin remodelers levels, as indicated, were measured by Real Time qPCR and normalized to the undifferentiated state (D0) or day 11 (D11), using the ΔΔCT method. Gene expression was normalized to GAPDH. Results are shown as mean and standard deviation of at least two technical replicates of two independent samples.

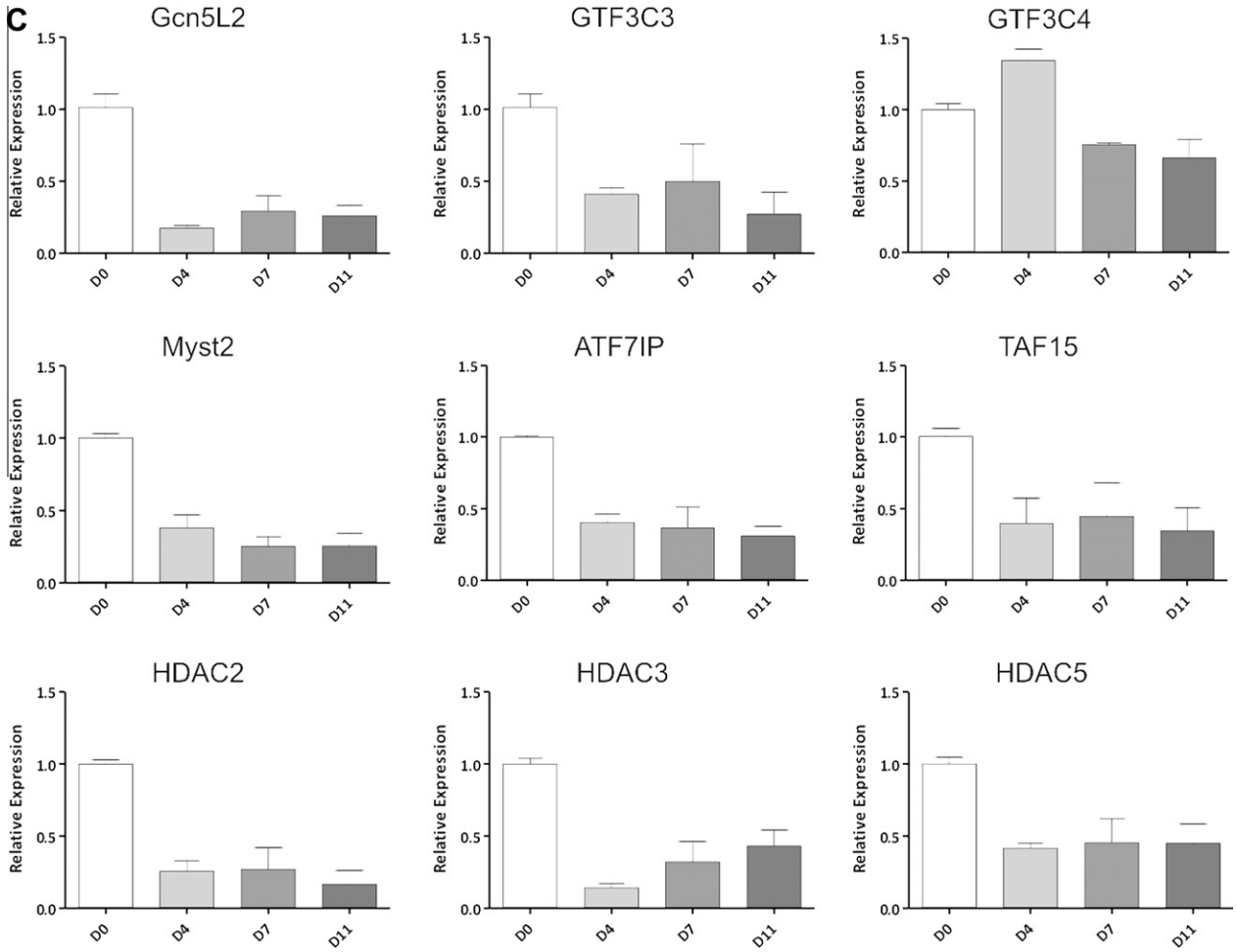

Fig. 1 (*continued*)

In this work we have studied the expression of selected chromatin modifying enzymes. We first found some genes that were differentially expressed in undifferentiated ESC compared to a differentiated cell type. Although we did not find a general pattern of modulation of gene expression, we could detect some resemblance between ES cells and iPS cells, and between mouse and human ES cells.

In human ES cells, we found that a Jumonji–ARID domain containing protein, Jarid2, gene expression was progressively downregulated along the differentiation protocol, and their mRNA levels were also lower in terminally differentiated fibroblasts compared to undifferentiated ESCs. To our knowledge, this is the first report of Jarid2 gene modulation in hESC. Otherwise, our results agree with those reported in mESC [20]. Although it has been suggested that this protein is probably not acting as a histone demethylase, it has been shown that Jarid2 is critical to modulate Polycomb repressive complex (PRC) 2 activity [21] both in ES cells and during cell reprogramming for iPSC obtention [20]. SetDB1 (KMT1E) and Suv39H2 (KMT1A/B) are other histone methyltransferases that have been studied in mESC. SetDB1 showed to be essential for stem cell maintenance [22] and depletion of Suv39H2 led to important changes in gene expression [23]. Here, we found in hESC that SUV39H2 was repressed progressively along the differentiation and later on recovered, reaching similar levels to the undifferentiated state.

Regarding the cell cycle modulator, Bmi1, we found that its expression increases progressively along differentiation in hESC. These results are similar to those recently reported in mESC [24]. The authors did not detect Bmi1 RNA in mESC, so they proposed that this protein should not have an obvious effect on mESC self-renewal. They also proposed that Bmi1 enhanced the propensity of ESC to differentiate towards the hematopoietic lineage and identified genes regulated by Bmi1 during ESC differentiation [24]. In addition, it is known that this factor is critical to hematopoietic stem cells function [25–27]. Bmi1 null mice showed hematopoyetic and neurological abnormalities [28]. Besides, it was recently shown that this factor contributes to DNA repair, suggesting its involvement in chromosome integrity maintenance [29]. In addition, we found that the HAT Myst2 is highly repressed in the evaluated terminally differentiated cells and was early or late repressed in the differentiation protocol in mouse or human ESC, respectively. Although it has been recently reported that this HAT is essential for mouse development [30] and there are multiple studies on Bmi1 function, to our knowledge, neither Myst2 nor Bmi1 modulation were reported in hESCs, yet.

Some of the evaluated genes showed different behavior in the terminally differentiated analyzed cells compared to the ESC-derived embryoid bodies. This could be explained by the fact that the obtained EBs are composed by multiple cell populations that have different patterns of gene expression, instead of the homogeneous primary cultures analyzed as an example of a terminally differentiated cell type. We have also analyzed the expression of these modifiers in a different somatic cell type, the human embryonic kidney cell line, HEK 293, and found that some of them have

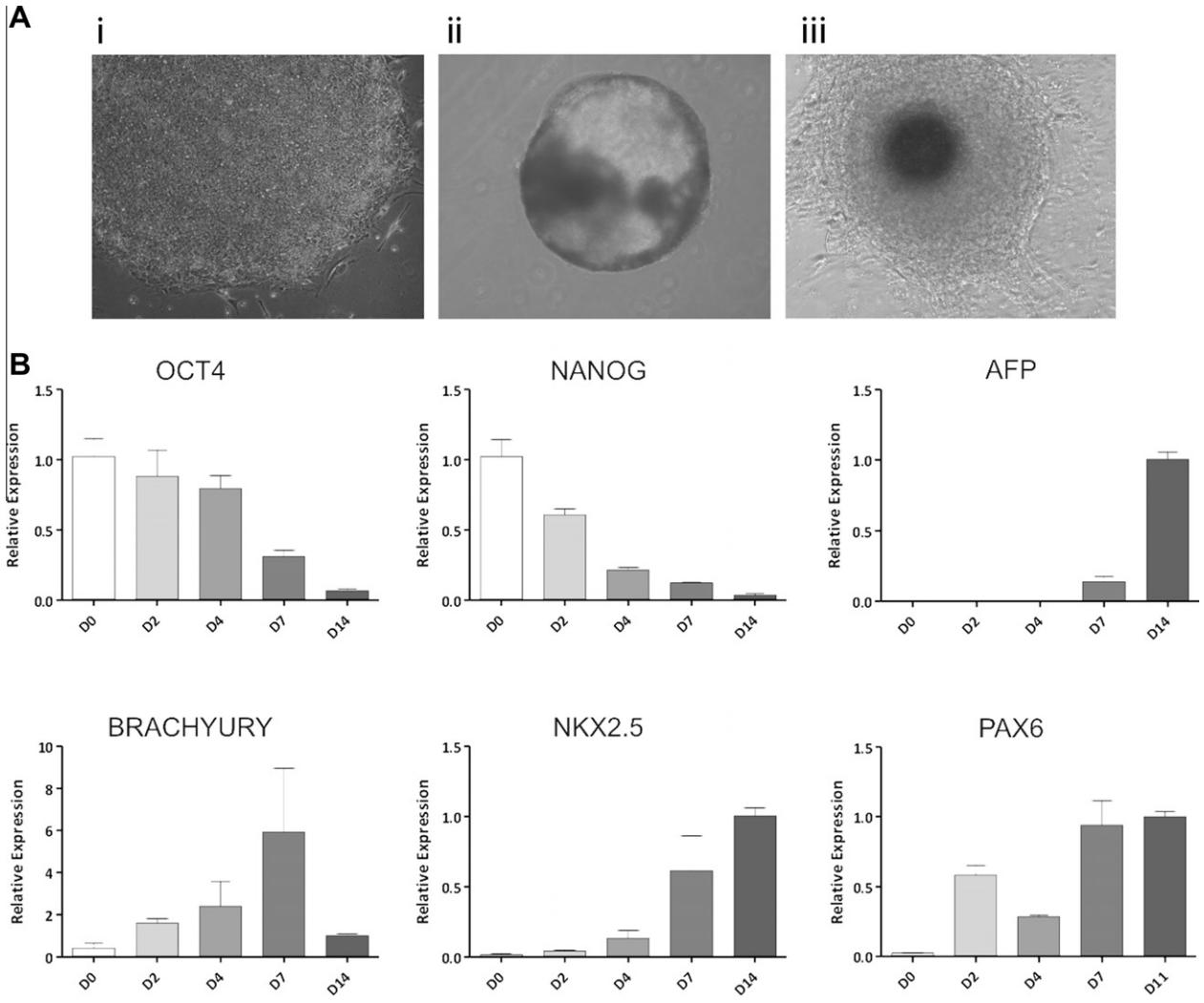

Fig. 2. Expression levels of chromatin remodelers along i*n vitro* differentiation of hESC. WA09 hESCs were *in vitro* differentiated as described in material and methods. RNA was extracted at the indicated days after the induction of differentiation. (A) Representative pictures of (i) WA09 hESC line, (ii) embryoid body growing under suspension and (iii) embryoid bodies growing in adherence. (B) Undifferentiated state and lineage specific gene markers and (C) chromatin remodelers levels, as indicated, were measured by Real Time qPCR and normalized to the undifferentiated state (D0) or day 11 (D11), using the ΔΔCT method. Gene expression was normalized to GAPDH. Results are shown as mean and standard deviation of at least two technical replicates of two independent samples.

similar pattern compared to MEF, and others were expressed at higher or lower levels (data not shown). These evidences suggest that each terminally differentiated cell type should have their own pattern of chromatin modifying factors expression.

On the other hand, as iPSC are reported to be similar, but not identical to ESC [31], we expected some discrepancy between them. Nevertheless, we didn't find evident differences in the expression of the evaluated genes. We found that some of the genes were differentially modulated comparing mouse to human ESC. This could be explained by the fact that even though high similitude may exist, the regulatory sequences of the gene promoters diverge among the different species. Moreover, ChIP on chip studies predict different targets for the same factor comparing human (unpublished results from Young's lab) and mouse ES cells [7]. From a different viewpoint, it is expected to find a different modulation for the same genes in the different species. As an example of this, we found that HDAC2 was almost unchanged in hESC, and aversely, we found an important modulation in mouse systems, both mESC and iPSC. It must be noted, that we could not find any modulation along the differentiation in the HDACs studied in hESC. The expression of HDAC2 was stoutly downregulated along the differentiation and was higher in undifferentiated ESC or iPSC compared to MEF. It has been reported that HDAC2 would be dispensable both for mESC self-renewal and embryoid bodies' differentiation as HDAC2 knock out ESC preserved self-renewal and pluripotency [32]. Since it has been suggested that HDAC1 is required to moderate the differentiation process [32] and that HDAC1 and HDAC2 interact to form the catalytic core of repressive complexes [33], we propose that HDAC2 could collaborate with HDAC1 in controlling differentiation pathways, based on the modulation that we found. Further experiments should be done to test this hypothesis.

Summing up, in this work we have presented a modest landscape of chromatin modifiers' modulation in mouse and human pluripotent stem cells. Currently, we are studying the relevance of some genes presented here, inhibiting their expression by a shRNA approach. We believe these findings may contribute to understand the epigenetic mechanisms involved in pluripotency, early differentiation and reprogramming. A better comprehension of these processes will allow improving somatic cell reprogramming for iPSC obtention and differentiation protocols optimization.

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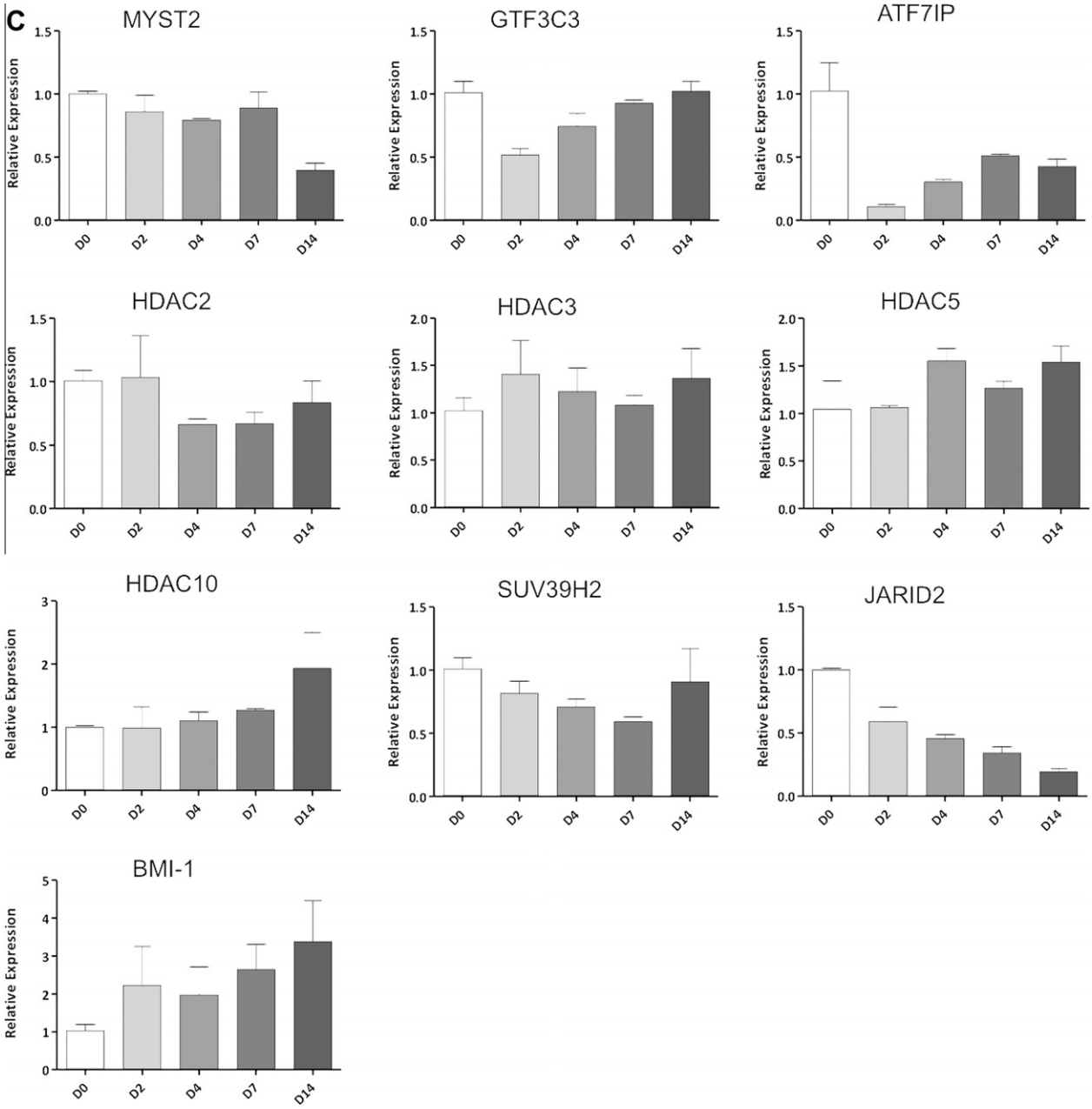

Fig. 2 (*continued*)

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.070.

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