Biochemical and Biophysical Research Communications 473 (2016) 194–199

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Protein arginine Methyltransferase 8 gene is expressed in pluripotent stem cells and its expression is modulated by the transcription factor Sox2

Claudia Solari [a, b, 1, 3], Camila Vázquez Echegaray [a, b, 1, 3], Carlos Luzzani [a, b, 2, 3], María Soledad Cosentino [a, b, 3], Ariel Waisman [a, b, 3], María Victoria Petrone [a, b, 3], Marcos Francia [a, b, 3], Alina Sassone [a, b, 3], Jésica Canizo [a, b, 3], Gustavo Sevlever [c, d, 4], Lino Barañao [a, b, d, 3], Santiago Miriuka [c, d, 4], Alejandra Guberman [a, b, d, e, *, 3]

[a] Laboratorio de Regulación Génica en Células Madre, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Instituto de Química Biológica – Ciencias Exactas y Naturales, (IQUIBICEN), UBA/CONICET, Buenos Aires, Argentina
[c] Laboratorio de Investigación Aplicada a las Neurociencias (LIAN)-CONICET, Fundación para la Lucha Contra las Enfermedades Neurológicas de la Infancia (FLENI), Buenos Aires, Argentina
[d] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[e] Departamento de Fisiología y Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 10 March 2016
Accepted 17 March 2016
Available online 21 March 2016

Keywords:
Prmt8
Embryonic stem cells
Induced pluripotent stem cells
Oct4
Sox2
Nanog

ABSTRACT

Addition of methyl groups to arginine residues is catalyzed by a group of enzymes called Protein Arginine Methyltransferases (Prmt). Although Prmt1 is essential in development, its paralogue Prmt8 has been poorly studied. This gene was reported to be expressed in nervous system and involved in neurogenesis. In this work, we found that Prmt8 is expressed in mouse embryonic stem cells (ESC) and in induced pluripotent stem cells, and modulated along differentiation to neural precursor cells. We found that Prmt8 promoter activity is induced by the pluripotency transcription factors Oct4, Sox2 and Nanog. Moreover, endogenous Prmt8 mRNA levels were reduced in ESC transfected with Sox2 shRNA vector. As a whole, our results indicate that Prmt8 is expressed in pluripotent stem cells and its transcription is modulated by pluripotency transcription factors. These findings suggest that besides its known function in nervous system, Prmt8 could play a role in pluripotent stem cells.

1. Introduction

Embryonic Stem Cells (ESC) are derived from the inner cell mass of blastocysts and are able to self-renew indefinitely in culture and to give rise to cells from the three germ layers, property known as pluripotency. There are three main transcription factors that govern the pluripotent state: Oct4, Sox2 and Nanog. They are simultaneously recruited to promoter regions of multiple genes throughout the genome inducing the expression of those that promote pluripotency and inhibiting the ones that lead to differentiation [1,2]. Moreover, forced expression of these factors reprograms terminally differentiated cells into pluripotent cells named induced pluripotent stem cells (iPSC) [3–5]. ESC can be maintained in undifferentiated state indefinitely, whereas *in vivo*, as development progresses, these cells leave their pluripotent state to follow diverse differentiation programs. The beginning of such

Abbreviations: Embryonic stem cells, ESC; induced pluripotent stem cells, iPSC; Mouse embyonic fibroblasts, MEFs; Protein arginine methyltransferase, Prmt; short hairpin RNA, shRNA.

* Corresponding author. Laboratorio de Regulación Génica en Células Madre, 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.

E-mail addresses: cmsolari@qb.fcen.uba.ar (C. Solari), camivazqueze@yahoo.com.ar (C.V. Echegaray), carlosluzzani@gmail.com (C. Luzzani), solecosen@qb.fcen.uba.ar (M.S. Cosentino), waisman@qb.fcen.uba.ar (A. Waisman), mavipetrone@gmail.com (M.V. Petrone), marcosfrancia@hotmail.com (M. Francia), alina.sassone@gmail.com (A. Sassone), jesicanizo@gmail.com (J. Canizo), 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] These authors contributed equally to this work.
[2] Current address: LIAN – CONICET, Buenos Aires, Argentina.
[3] Intendente Guiraldes 2160, Ciudad Universitaria, Pab. 2, 4to piso, QB-71, (C1428EGA), Buenos Aires, Argentina.
[4] Ruta 9 Km. 52.5, Escobar, (B1625XAF) Buenos Aires, Argentina.

http://dx.doi.org/10.1016/j.bbrc.2016.03.077
0006-291X/

programs depends largely on the regulation of the expression of tissue-specific genes and changes in global chromatin organization and in specific *loci*. Hence, understanding what genes are involved and how they are regulated is crucial to unravel the process of cell differentiation. There is also increasing evidence that links the chromatin structure of marker genes of the undifferentiated state with the maintenance of pluripotency.

In addition to modulation of gene expression, posttranslational modification of proteins constitutes a major mechanism of cell identity specification. Although phosphorylation remains as the best studied and understood modification [6], methylation of proteins is emerging as a key controlling element in protein function. In particular, the modification of arginine side chain guanidino groups is quantitatively one of the most extensive protein methylation reactions in mammalian cells [7]. Arginine is a positively charged aminoacid known to mediate hydrogen bonding and amino–aromatic interactions. The nitrogen atoms of this aminoacid can be post-translationally modified to contain methyl groups. Protein arginine methylation results in the addition of one or two methyl groups to the guanidine nitrogens of arginine [8]. In mammalian cells there are three forms of methylated arginine residues: ω-NG, monomethylarginines (MMA); ω-NG,NG-asymmetric dimethylarginines (ADMA); and ω-NG,N'G-symmetric dimethylarginines (SDMA) being ADMA the most prevalent form [9].

Addition of methyl groups to arginine residues is catalyzed by a group of enzymes called Protein Arginine Methyltransferases (Prmt). Prmt family consists of nine members, classified as type I, type II or type III according to their capacity of catalyzing the formation of ADMA, SDMA or MMA, respectively. Prmt1, 2, 3, 4, 6 and 8 belong to type I, whereas Prmt5 and 9 belong to type II. In addition, type II and III activities have been observed for Prmt7. Prmt1 is responsible for at least 85% of all arginine methylation reactions in the cell and is ubiquitously expressed in mammalian cells [10]. It has been proven to be an essential enzyme during development, since embryos from Prmt1−/− knock-out mice die shortly after implantation. However, it is dispensable for basic cellular reactions such as gene expression and DNA replication, since ESC from such embryos are viable under cell culture conditions [11]. Prmt8 shares over 80% of aminoacid sequence homology with Prmt1. Mainly, Prmt8 is 33 aminoacids longer in its amino-terminal region, and harbors a glycine that can be myristoylated. Whether this modification occurs and targets this protein to plasmatic membrane [12] or not [13] is still a matter of debate. All type-I PRMTs adopt head-to-tail homodimeric architecture, essential for PRMT activity [14,15]. However, the reported crystal structure of Prmt8 showed a tetrameric structure in which two PRMT8 dimers are held together [16]. The authors proposed both a homo-tetrameric architecture and a hetero-tetramer model for inter-member interactions [16]. Recently, it was demonstrated that human PRMT8 forms an octamer in solution, and that this structure is necessary for plasma membrane localization and efficient methyltransferase activity [17]. It was also reported that the N-terminal domain may regulate Prmt8 activity [18,19].

Although regulation and cellular substrates of Prmt8 are poorly understood, it was reported that Prmt8 expression is restricted to the adult brain neurons of mice [13] and that it plays a critical role in embryonic and neural development in zebrafish [20]. Recently, it was reported that Prmt8, along with Prmt1, have key roles in neuronal differentiation as co-activators of retinoic acid in a differentiation model of mouse ESC to neurons [21].

Since the role and gene regulation of Prmts in ESC is still weakly studied, and Prmt1 seems to be necessary for embryonic development but not for ESC survival, we sought to study its paralogue Prmt8. Here we show that Prmt8 is expressed in mouse ESC and iPSC and is modulated along a neural progenitor differentiation protocol. Moreover, we found that Prmt8 promoter activity is induced by pluripotency transcription factors Oct4, Sox2 and Nanog and its mRNA was downregulated in ESC transfected with shRNA vector targeting Sox2.

2. Materials and methods

2.1. Cell culture and differentiation

R1 (ATCC) ESC line and 46C Sox1-GFP ESC line (a kind gift from Austin Smith) were cultured and differentiated as previously described [22–25] and [26], respectively. The iPSC-20 line was derived and validated previously by us and cultured as previously described [27]. NIH/3T3 cell line (ATCC) and mouse embryonic fibroblasts (MEFs) from embryonic day 13.5 were cultured in DMEM supplemented with 10% FBS (GIBCO) and antibiotics.

2.2. Qualitative and quantitative reverse transcription–polymerase chain reaction (RT-PCR and RT-qPCR)

RT-PCR and RT-qPCR were performed and analyzed as previously described [22,25]. Primers sequences are detailed in Ref. [25] except for Prmt8 (S1 Table).

2.3. Reporter vector construction, transfection and luciferase activity assay

To construct the reporter vector pPrmt8-Luc, a 1794 kpb fragment of the promoter region of Prmt8 was amplified by PCR from R1 ESC genomic DNA, and cloned into NheI and XhoI cloning sites in the pGL3-Basic vector (Promega) upstream of the Luciferase gene. The oligonucleotides are listed in S1 Table. Restriction enzymes were obtained from Promega. The construction was verified by sequencing. NIH/3T3 cells were co-transfected in 24-well plate with 100 ng of pPrmt8-Luc reporter and 0 (basal), 100, 200 or 400 ng of pMXs-Nanog, pMXs-Oct4, pMXs-Sox2 (Addgene) or the three pMXs vectors simultaneously, as indicated. Transfection and luciferase assay were carried out as previously described [25].

2.4. Downregulation of transcription factors by shRNA approach

R1 ESC cultured in standard medium on gelatin coated p60 plates, were transfected with 3 μg pLKO.1-puro derived vectors (Sigma), expressing shRNA targeting Nanog (SHCLND-XM_132755), Oct4 (SHCLND-NM_013633), Sox2 (SHCLND-NM_011443) or eGFP (SHC005), which was used as control vector. Transfection, selection and mRNA expression analyses were carried out as previously described [25].

2.5. Statistics and data analysis

Experimental results are presented as mean ± SEM. In all cases, statistical analysis were performed using randomized block design ANOVA for at least three biological replicates on *Infostat* software [28]. When necessary, data was transformed with $\log_{10}$. Residuals fitted normal distribution, assessed by the Shapiro–Wilks test, and homogeneity of variance, using the Levene test. Tukey-test was used for comparisons between means.

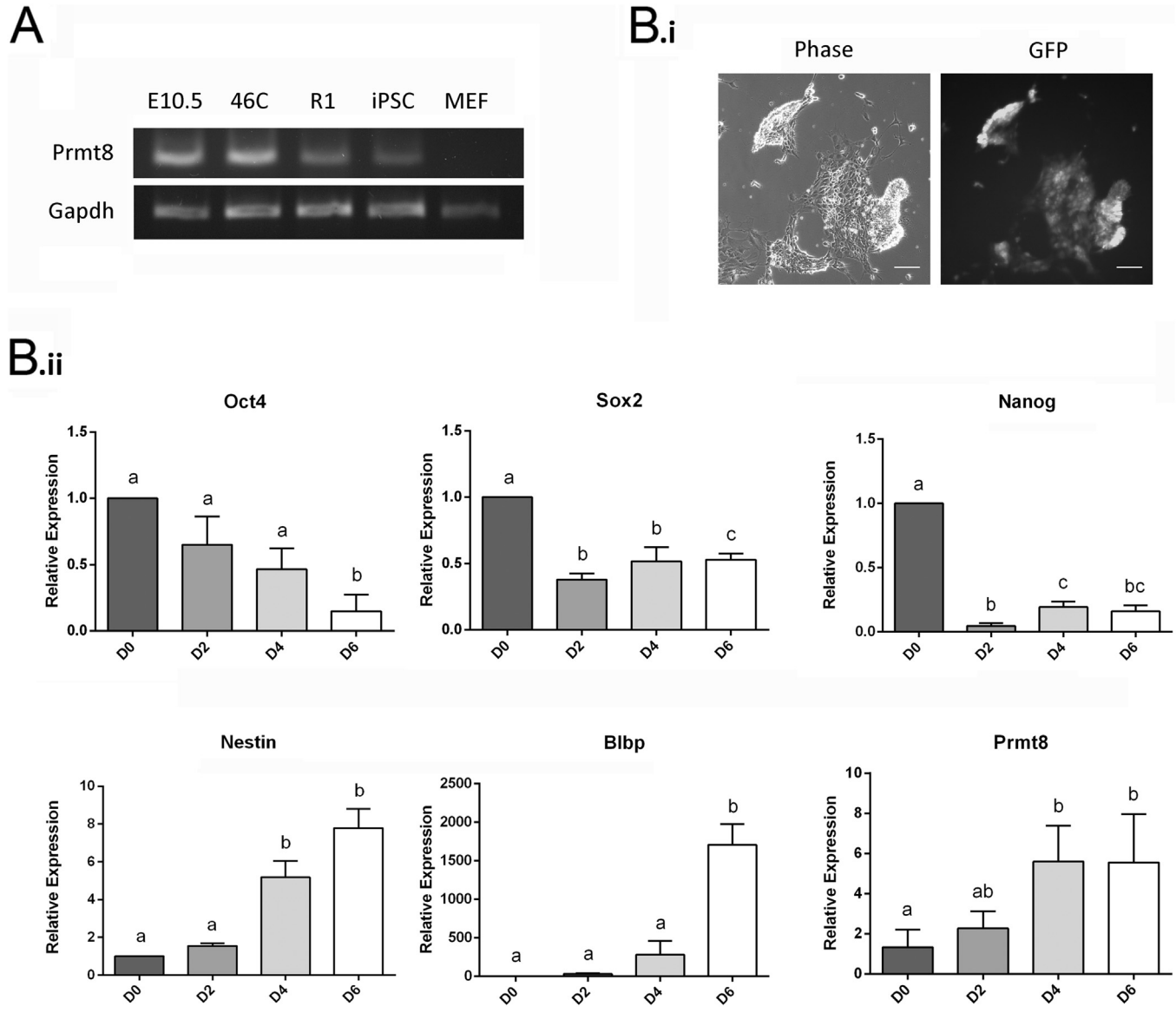

Fig. 1. Prmt8 is expressed in ESC and iPSC and modulated during neural progenitor differentiation protocol. (A) Prmt8 and Gapdh mRNA were detected by RT-PCR in brain lysate of a 10.5 days old mouse embryo, 46C and R1 ESC lines, iPSC 20 line and MEF. (B) 46C ESC were subjected to neural precursor differentiation protocol for 6 days **(i)** Representative pictures of day 6 of differentiation showing expression of GFP driven by Sox1 promoter, marker of neuroectoderm. Phase contrast, left panel; GFP, right panel. Scale bars: 100 μm. **(ii)** RNA was extracted at days 0 (D0), 2 (D2), 4 (D4) and 6 (D6) after the induction of differentiation and mRNA levels of the indicated genes were measured by RT-qPCR. Gene expression was normalized to the geometrical mean of Gapdh and Pgk1 expression and referred to D0. Results are shown as mean ± SEM of three independent experiments. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

3. Results

3.1. Prmt8 is expressed in pluripotent stem cells and upregulated during neural progenitor differentiation

Based on the increasing evidence on the high relevance of epigenetic regulation on pluripotency maintenance and under the hypothesis that genes that are regulated by pluripotency transcription factors are relevant to preserve stemness or to promote differentiation, we looked for epigenetic regulators that could be target of such transcription factors.

Some years ago, a global mapping of promoters bound by the transcription factors critical for pluripotent cells was delineated by a genome wide chromatin immunoprecipitation approach [29]. In a previous work, we reported chromatin remodelers that were modulated during differentiation in pluripotent stem cells [23]. After that first screening, we decided to focus on Prmt8 gene. Even though this gene was included in the vast list of putative target genes in the mentioned wide range ChIP, it was only reported to be restricted to nervous system in mouse [13] and to have a role during neuronal differentiation [21].

We first qualitatively analyzed Prmt8 mRNA levels in different undifferentiated pluripotent stem cell lines. We studied two ESC lines, 46C and R1, and an iPSC line previously generated in our lab [27]. We successfully detected Prmt8 transcripts in all the undifferentiated pluripotent stem cells lines analyzed (Fig. 1A). Moreover, we did not detect Prmt8 mRNA in MEFs, the cells from which our iPSC line was derived, suggesting that this gene was induced during the reprogramming process. Brain lysates of a 10.5 days old mouse embryo were used as positive control.

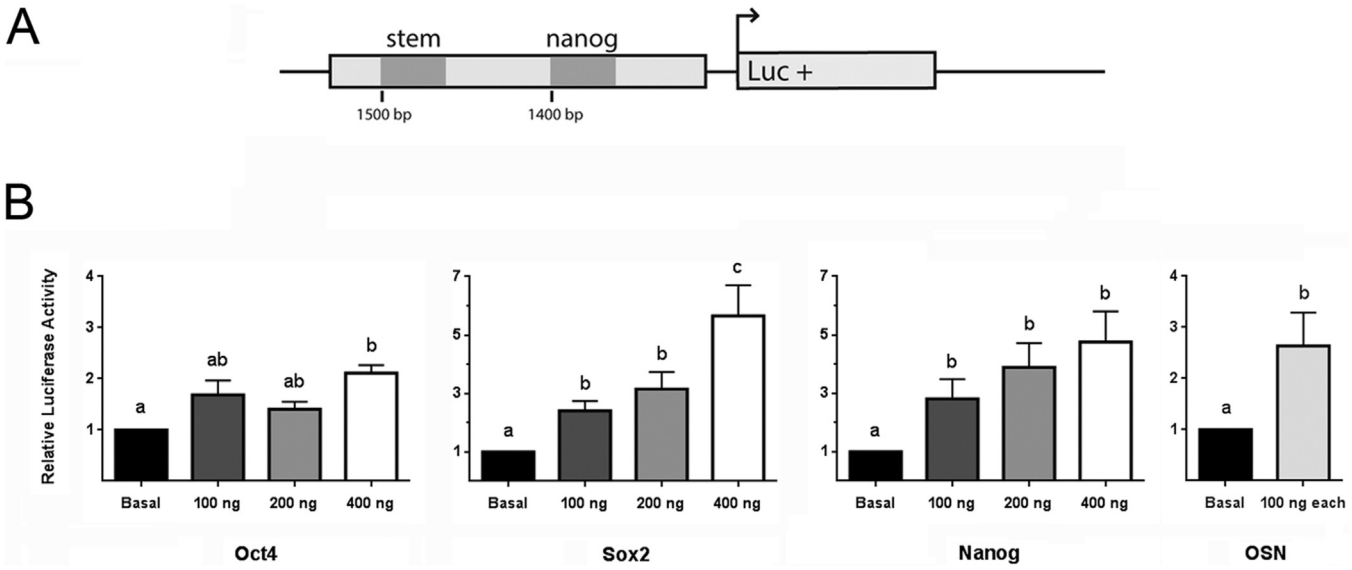

Fig. 2. Oct4, Sox2 and Nanog induced pPrmt8-Luc. (**A**) Scheme of pPrmt8-Luc construction. The putative binding sites for Nanog and for complexes composed by Oct4, Sox2, Nanog, Tcf3 (Tcf7l1) and/or Sall4b ("Stem") are shown. (**B**) NIH/3T3 cells were transfected with pPrmt8-Luc and with pMXs-Oct4, pMXs-Sox2, pMXs-Nanog, the three vectors simultaneously or none (basal), as indicated. Values were normalized to *Renilla*'s Luciferase and referred to the basal condition (without the addition of any transcription factor). Results are shown as mean ± SEM of at least three independent experiments. Different letters indicate statistically significant differences between treatments ($p < 0.01$).

Next, we studied the expression profile of Prmt8 as ESC differentiate to neural precursor cells. For this, we used the 46C cell line, which expresses GFP under the control of Sox1 promoter, a specific neural stem cell marker [26]. Successful differentiation was assessed by GFP fluorescence detection (Fig 1B, i) and by RT-qPCR of pluripotency and neural markers at day 0, 2, 4 and 6 (Fig 1B, ii). In agreement with the previously reported rise during neuronal differentiation [21], we found that Prmt8 mRNA levels increased along this neural precursor differentiation protocol (Fig 1B, ii).

3.2. Pluripotent stem cells' transcription factors induce Prmt8 promoter activity

Based on the previously mentioned genome wide analysis [29], and having shown that Prmt8 was expressed in undifferentiated pluripotent stem cells, we decided to study whether Prmt8 expression was modulated by pluripotency transcription factors. We first *in silico* analyzed a 3 kbp region upstream of Prmt8 transcription start site using the Genomatix MatInspector software. We found putative binding sites for Nanog, Oct4 and for stem cells' specific transcription factors complexes ("stem site"), most of them having a high similitude to the consensus sequence (S2 Table). To study the responsiveness of these proximal sites, we constructed a reporter vector containing a fragment of Prmt8 promoter that included one putative binding site for Nanog and one of the stem sites located in the first 1700 bp downstream from the transcription start site, driving the firefly Luciferase reporter gene (Fig. 2A), named pPrmt8-Luc. For performing the transactivation assay, we chose the NIH/3T3 mouse embryonic fibroblast cell line, in which we couldn't detect Oct4, Sox2 or Nanog mRNA levels, so it provides a defined system for our purpose. We co-transfected the reporter vector and different amounts of an expression vector for Nanog, Sox2, Oct4 or the three transcription factors simultaneously. As shown in Fig. 2B, although we didn't find a synergistic effect, the three factors induced luciferase expression in a concentration-dependent manner. These results indicate that the three main pluripotency transcription factors activate the studied Prmt8 promoter fragment.

Then, to study the effect of such transcription factors on the expression of the endogenous Prmt8 gene, we downregulated their mRNA levels by a shRNA approach. We transfected R1 ESC with shRNA vectors targeting each transcription factor or eGFP (shGFP) as a control, and then analyzed Prmt8 gene expression by RT-qPCR. As shown in Fig. 3A, each transcription factor was downregulated by their specific shRNA, showing that the shRNA approach was effective. When analyzing Prmt8 mRNA levels, we found that this gene was highly repressed in R1 ESC transfected with the shRNA targeting Sox2. Contrary to expected, ESC transfected with either shRNA against Oct4 or Nanog didn't show a remarkable effect on Prmt8 mRNA levels (Fig. 3B), indicating that a complex modulation may be involved.

4. Discussion

In previous publications, Prmt8 was reported to be expressed strictly in the adult mouse brain [13] or in human cortex tissue [30,31]. Recently, it was proved to have a role in retinoic acid signaling in a neuronal differentiation model in mouse ESC [21]. In 2008, Kim et al. found more than six thousand genes that bound one of nine pluripotency transcription factors [29]. Prmt8 was included in that vast list but until now, its transcriptional regulation was left unstudied. In this work, we show that Prmt8 contains several putative binding sites for pluripotency transcription factors. We found that this gene is expressed in embryonic stem cells, in agreement with a recent report in human ESC [32] and with the previous finding in pluripotent p19 cell line [33]. Moreover, we did not detect Prmt8 mRNA in MEFs, but we found it in the iPSC line derived from them, suggesting that this gene could be relevant for pluripotency and that it would be switched on during the reprogramming process. We also observed that the mRNA levels increased during an *in vitro* neural precursor differentiation, in concordance with its reported expression in neural development and the previously reported role in retinoic acid-induced multistage neuronal differentiation [21]. These results suggest that Prmt8 is expressed not only in the adult brain but also in early stages of development. To our knowledge, there are few previous reports that evidence this

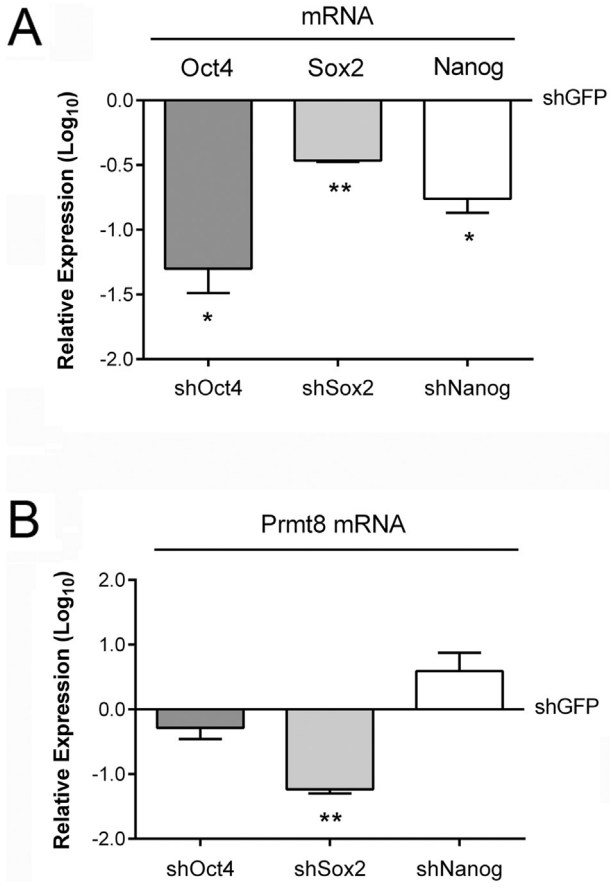

Fig. 3. Prmt8 is repressed in R1 ESC transfected with shRNA targeting Sox2. R1 ESC were transfected with pLKO.1-puro derived vectors targeting stem cells' transcription factors (shOct4, shSox2 or shNanog), as indicated under each bar, or eGFP (shGFP, control, baseline). Then, transfected cells were selected with puromycin for 48 hs and RNA was extracted. The expression levels of Oct4, Sox2, Nanog (A) or Prmt8 (B) were analyzed by RT-qPCR and referred to the control. Gene expression was normalized to the geometrical mean of Gapdh and Pgk1 expression and referred to the control condition. Results are shown as mean ± SEM of at least four independent experiments. Asterisks indicate statistically significant differences between treatments (*$p < 0.05$, **$p < 0.01$).

fact, such as the mentioned work in the neuronal differentiation protocol in ESC, where they found that both Prmt1 and Prmt8 have roles in retinoic acid induced neuronal differentiation [21], the findings in zebrafish [20], and the recent publication that reports Prmt8 expression in hESC [32].

Regarding Prmt8 gene regulation, in our knowledge this is the first report about the effect of pluripotency transcription factors on Prmt8 promoter activity, since it appeared in the mentioned genome wide ChIP-on-chip study [29]. In this work, we constructed a Prmt8 promoter activity reporter vector and found that Oct4, Sox2 and Nanog induced it in a transactivation assay. However, when analyzing Prmt8 mRNA levels in response to pluripotency transcription factors downregulation by shRNA approach, we observed a drastic drop in Prmt8 mRNA levels, only when ESC where transfected with shRNA vector targeting Sox2. The unexpected results obtained in R1 ESC transfected with shOct4 and shNanog vectors suggest that intricate pathways could be involved in Prmt8 gene regulation by pluripotency transcription factors.

As a whole, our results indicate that Sox2 modulates Prmt8 promoter activity, due to binding to the stem consensus sites. Moreover, Sox2 might exert an indirect effect by regulation of other transcription factors, so establishing a complex scenario which may be responsible for Prmt8 regulation. Further research will be required to unravel this conundrum.

In summary, we showed for the first time that Prmt8 is expressed in pluripotent mouse ESC and iPSC and its promoter activity is induced by the pluripotency transcription factors Oct4, Sox2, and Nanog. This evidence suggests that besides its reported function in nervous system, Prmt8 could play a role in pluripotent stem cells.

Acknowledgments

The authors wish to thank to Estefanía Rojas, Marcelo Schultz, Naomi Arakaki, Flavio S. de Souza, Emilly Villodre and Guido Lenz for their helpful assistance. This work was supported by the following grants (to A.G.): ANPCyT, PICT 2011-2713, CONICET, PIP 112-200801-03003 and PIP112-201101-00243. CS, MSC, AW and CL are fellows from CONICET.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.03.077.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.077.

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