

Estradiol differentially induces progesterone receptor isoforms expression through alternative promoter regulation in a mouse embryonic hypothalamic cell line

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Abstract Progesterone receptor (PR) presents two main isoforms (PR-A and PR-B) that are regulated by two specific promoters and transcribed from alternative transcriptional start sites. The molecular regulation of PR isoforms expression in embryonic hypothalamus is poorly understood. The aim of the present study was to assess estradiol regulation of PR isoforms in a mouse embryonic hypothalamic cell line (mHypoE-N42), as well as the transcriptional status of their promoters. MHypoE-N42 cells were treated with estradiol for 6 and 12 h. Then, Western blot, real-time quantitative reverse transcription polymerase chain reaction, and chromatin and DNA immunoprecipitation experiments were performed. PR-B expression was transiently induced by estradiol after 6 h of treatment in an estrogen receptor alpha (ER α)-dependent manner. This induction was associated with an increase in ER α phosphorylation (serine 118) and its recruitment to PR-B promoter. After 12 h of estradiol exposure, a

downregulation of this PR isoform was associated with a decrease of specific protein 1, histone 3 lysine 4 trimethylation, and RNA polymerase II occupancy on PR-B promoter, without changes in DNA methylation and hydroxymethylation. In contrast, there were no estradiol-dependent changes in PR-A expression that could be related with the epigenetic marks or the transcription factors evaluated. We demonstrate that PR isoforms are differentially regulated by estradiol and that the induction of PR-B expression is associated to specific transcription factors interactions and epigenetic changes in its promoter in embryonic hypothalamic cells.

Keywords Progesterone receptor isoforms · Estradiol · Epigenetic marks · Transcription factors · Hypothalamus · Histone acetylation

Introduction

Progesterone receptor (PR) is a member of the nuclear receptor superfamily that regulates several reproductive and nonreproductive functions [1]. PR gene encodes two main isoforms, PR-A and PR-B, whose transcription is regulated by two specific promoters and alternative transcription start sites (TSS) [2]. PR-A differs from PR-B by lacking 164 amino acids at the amino terminus of the protein. PR-A usually functions as a transcriptional inhibitor of PR-B-regulated genes [3]. However, it has been also demonstrated that both PR isoforms act as transcriptional activators of different target genes in the same cell line [4]. Therefore, it has been proposed that PR function results from the counterbalancing proportion of both isoforms when they are expressed in the same cell type [5, 6]. It has been reported that there is a specific variation in PR

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isoforms expression during the estrous cycle and under estradiol and progesterone treatments in the rodent hypothalamus. Particularly, PR-B isoform is induced by estradiol to a greater extent than PR-A in the hypothalamus and preoptic area of the female rat, and this differential regulation has been associated with the display of sexual behavior [7–12]. It has been reported that the expression of PR-A is higher than that of PR-B in the immortalized rat embryonic hypothalamic cell line, D12, and that the expression of both PR isoforms was induced by estradiol [13]. Moreover, it has been demonstrated that PR expression regulation by estradiol is sexually dimorphic during development in several brain regions, particularly in those that control sexual differentiation and reproductive behavior during adulthood, such as medial preoptic nucleus and the ventromedial nucleus of hypothalamus [14–16].

Expression of PR isoforms is mainly regulated by estrogen receptor alpha (ER α), despite the lack of consensus estrogen responsive elements (ERE) in the PR gene promoter [17]. ER α is recruited to the PR gene promoter through interactions with specific protein 1 (SP1) and activator protein-1 (AP1) in MCF7 cells [18, 19]. In turn, ER α recruits transcriptional coregulators that induce changes in chromatin in order to activate transcription [20]. Moreover, both DNA methylation and chromatin basal states in the PR promoter region also influence the expression of PR isoforms, highlighting the importance of epigenetic processes in the regulation of this gene [21, 22]. We recently reported a transient and differential DNA methylation pattern of PR gene isoform promoters during the evening of proestrus in rat hypothalamus [10]. It has also been reported that estradiol induced a differential DNA methylation pattern of PR-A promoter in the mediobasal hypothalamus of developing rats [23].

It has been described a specific and a transient induction pattern of transcriptionally active and repressive histone marks in mouse hypothalamus after 6 h of estradiol treatment [24]. Furthermore, the authors found a specific enrichment of H3Ac and H3K4me3 histone marks on four different regions upstream of the TSS of PR-A which included PR-A promoter and other three regions near PR-A and PR-B promoters. Interestingly, this histone marks enrichment was dependent on the estradiol exposure time and the hypothalamic region. The authors also reported that ER α was not recruited on any of the four PR gene studied regions [24].

Although the regulation of PR isoforms expression by estradiol has been extensively reported in adult hypothalamic cells, there is lacking information about their regulation in embryonic hypothalamic cells, as well as the molecular mechanisms involved in such regulation. In this study, we used the estradiol responsive mHypoE-N42 mouse embryonic hypothalamic cell line as a model for

exploring PR isoforms regulation [25]. This cell line expresses PR-A and PR-B, and it has been previously used for analyzing the transcriptional regulation of the aromatase gene by estradiol [26].

The aim of this study was to assess the regulation of PR isoforms expression by estradiol in the mHypoE-N42 cell line, as well as the epigenetic changes and interaction of transcription factors on PR-A and PR-B promoters associated to such regulation.

Materials and methods

Cell culture and treatments

Mouse hypothalamic mHypoE-N42 cells were purchased from Cellutions Biosystems[®] (Toronto, Canada) and are derived from mouse embryonic hypothalamic primary cultures (from days 15, 17, and 18) although the sex of embryos was not determined. Cells were maintained in high-glucose DMEM medium (Life Technologies[®], California, USA) supplemented with 10 % of fetal bovine serum (FBS). Cells were used for experiments at passages 24–26. Before treatments, 2×10^6 cells were maintained in DMEM medium without phenol red and charcoal-stripped FBS until they reached a confluence of 80–90 %, and then cells were serum starved in the same medium overnight. Cells were treated with different concentrations of estradiol (Sigma[®], Missouri, USA) or 0.002 % of ethanol (vehicle) for 6 and 12 h. In order to confirm that the estradiol regulation of gene expression was mediated by ER α and not by other estrogen receptor subtypes, the effect of a highly selective ER α antagonist, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP, Tocris Bioscience, Bristol, UK), was evaluated. 5 nM of MPP was administered alone or in combination with 100 nM of estradiol. Subsequent methodological approaches and the corresponding experiments were performed from whole cell preparations.

RNA isolation and real-time RT-qPCR

Total RNA was isolated with TRIzol[®] reagent (Life Technologies[®], California, USA) and RT-qPCR was carried out using the SuperScript[®] III First-Strand Synthesis SuperMix[®] (Life Technologies[®], California, USA), as specified by the distributor. Total RNA isolated was quantified by the Quant-iT[®] RiboGreen[®] RNA Assay Kit (Life Technologies[®], California, USA). 100 ng of cDNA were amplified using the 7500 real-time PCR system (Life Technologies[®], California, USA). 1 μ l of RT reaction was subjected to PCR in order to simultaneously amplify a gene

fragment of PR-B isoform, total PR (PR-B + PR-A), and GAPDH. This was used as an internal control and was obtained from Life Technologies[®], California, USA (Assay ID: Mm99999915_g1). The sequences of the specific primers and probes for PR-B and total PR amplifications are depicted in Table 1. Negative controls without cDNA and with non-retrotranscribed RNA were included in all the experiments. All PCR products were always studied and analyzed together throughout the experiments. In order to obtain the relative quantification by the $\Delta\Delta C_t$ method, TaqMan[®] probes (Life Technologies[®], California, USA) were used as the detection system. Master Mix and TaqMan[®] assays were used at 1X. Cycling conditions were followed as specified by the distributor, with the exception of PR-B (50 °C for 2 min; 95 °C for 10 min; 50 cycles of 95 °C for 30 s and 64 °C for 3 min). PR-A expression levels were calculated by subtracting total PR minus PR-B expression levels. All assays produced a single PCR product at the molecular weight expected, as confirmed using 2 % agarose gels stained with GelRed[®] (Biotium[®], USA).

Western blot

Cells were homogenized in RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 % v/v NP40, 0.25 % w/v sodium deoxycholate) containing protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). Proteins were obtained by centrifugation at 12,500 rpm at 4 °C for 30 min and quantified by the Bradford method (BioRad, California, USA). Proteins (40 µg) were separated by denaturing electrophoresis in a 7.5 % polyacrylamide gel at 95 V. Gels were transferred to a polyvinylidene fluoride membrane (Millipore, Massachusetts, USA) by semidry transfer (BioRad, California, USA). Membranes were blocked with 10 % w/v nonfat dry milk and 0.1 % v/v Tween-20 at room temperature for 30 min. Membranes

were incubated with primary antibodies against PR, S118 phosphorylation of ER α and SP1 (Santa Cruz, Texas, USA) at 4 °C overnight. The respective secondary antibodies conjugated to horseradish peroxidase were incubated at room temperature for 1 h. Membranes were stripped with glycine (25 mM, pH 2.5; 1 % w/v SDS) at room temperature for 30 min and reprobed with the ER α and GAPDH primary antibodies (Santa Cruz, Texas, USA) as previously mentioned. Proteins were detected by chemiluminescence with the ECL kit (Amersham Piscataway, USA) and analyzed by densitometry using the Vision Works LS Software (UVP[®], USA).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was carried out as previously described [26], with minor modifications. Briefly, treated cells were incubated with 1 % w/v of formaldehyde for 10 min and with 125 mM of glycine for 5 min. Cells were harvested and homogenized in a lysis buffer (1 % w/v SDS, 10 mM EDTA, 50 mM Tris pH 8) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Chromatin shearing was performed in a Vibra Cell[®] sonicator (Sonics, Connecticut, USA) by 6 pulses of 10 s ON, 60 s OFF at 75 % amplitude, to obtain DNA fragments with a modal size of 1000 bp, which was confirmed by a 2 % w/v agarose gel stained with GelRed[®] (Biotium[®], USA). 25 µg of chromatin were diluted 1:10 with buffer containing protease inhibitor cocktail. Precleared chromatin was incubated at 4 °C overnight with 2–5 µg of antibodies against H3K9ac, H3K4me3, H3K9me2, and RNA Pol II (Abcam[®], Cambridge, UK), ER α , and SP1 (Santa Cruz, TX, USA). The immune complexes were incubated with protein A/G agarose (Santa Cruz, TX, USA) in agitation at 4 °C for 1 h. The immunoprecipitated products were washed with low salt wash buffer, high

Table 1 Primers and probes used for real-time PCR assays

Target	Sequence (5'–3')	Detection	Application
PR-B	Forward-CAGCACCGCCACACCAGTT	TaqMan [®]	Gene expression
	Reverse-CCAAGCGTGCAAGCAAGGGG		
	Probe-ACACGTCTGGCGCTTCGCCCTCCCC		
Total PR	Forward-ATTCTACTCGCTGTGCCTTACC	TaqMan [®]	Gene expression
	Reverse-CATGGGTACCTGGAGTTTGA		
	Probe-ATGTGGCAAATCCC		
PR-B promoter	Forward-GGTGGGCTGGCATGCTTCT	TaqMan [®]	ChIP
	Reverse-ACTCCCGCTATCTCCGACTTCT		
	Probe-TGGGCGGGCCTTCTAGAGCGCCA		
PR-A promoter	Forward-CAGCACCGCCACACCAGTT	TaqMan [®]	ChIP
	Reverse-CCAAGCGTGCAAGCAAGGGG		
	Probe-ACACGTCTGGCGCTTCGCCCTCCCC		

salt wash buffer, lithium chloride wash buffer, and TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The complexes were eluted twice with elution buffer (1 % w/v SDS, 100 mM NaHCO₃) for 15 min at room temperature. Samples obtained were incubated with RNase A solution (Promega, Wisconsin, USA) for 30 min at 37 °C. Reverse crosslinking was performed by incubating with Proteinase K (Roche, Basel, Switzerland) at 55 °C overnight and then with NaCl (5 M) for 6 h at 65 °C. The enriched genomic DNA was purified with the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). The purified products were amplified using the 7500 real-time PCR system (Life Technologies[®], California, USA) to obtain the fold enrichment of each immunoprecipitated sample. TaqMan[®] probes (Life Technologies[®], California, USA) were used for detection. TaqMan[®] assays were designed using Primer3 software in order to amplify the promoter regions of both PR isoforms according to previous studies and databases [2, 17, 27, 28] (Fig. 1). Cycling conditions were the following: 50 °C for 2 min; 95 °C for 10 min; 50 cycles of 95 °C for 30 s and 64 °C for 3 min. Normalization was carried out using the input sample (1 % v/v of diluted chromatin). Primers and probe used for PR isoform promoters are shown in Table 1. Both assays produced a single PCR product at the molecular weight expected, as confirmed using a 2 % w/v agarose gel stained with GelRed[®] (Biotium[®], USA).

Methylated and hydroxymethylated DNA immunoprecipitation

Immunoprecipitation of methylated and hydroxymethylated DNA was performed as previously reported [29, 30]

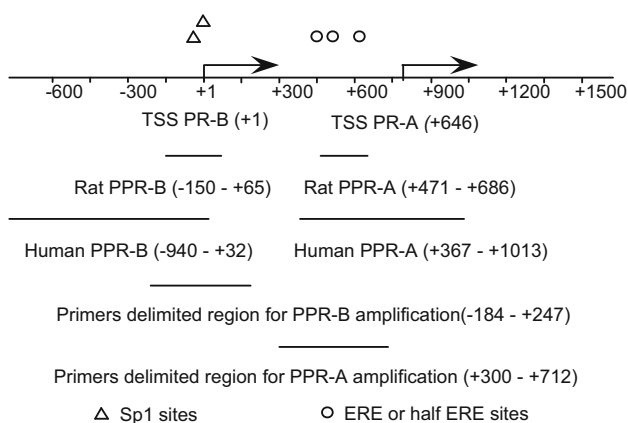


Fig. 1 Schematic representation of PR gene promoters of *Mus musculus*. Promoter regions and primers delimited regions for PCR amplification are represented. TSS transcription start site, PPR-B PR-B isoform promoter region, PPR-A PR-A isoform promoter region, ERE estrogen responsive element. SP1 sites are represented by triangles, ERE or half ERE sites are represented by circles

with minor modifications. Briefly, genomic DNA was extracted from mHypoE-N42 cells using the Wizard[®] Genomic DNA purification kit (Promega, Wisconsin, USA). 15 µg of genomic DNA were fragmented in a Vibra Cell[®] sonicator by 6 pulses of 10 s ON, 60 s OFF at 40 % amplitude, to obtain DNA fragments with a modal size of 1000 bp, confirmed by a 1.5 % w/v agarose gel stained with GelRed[®] (Biotium[®], USA). 2.5 µg of sonicated DNA was diluted in TE buffer and denatured for 10 min at 100 °C, followed by snap-chilling samples on wet ice. 10 % v/v of sample was transferred on a clean tube for the input. Diluted DNA was incubated with immunoprecipitation buffer (10 mM Na-phosphate, 140 mM NaCl, 0.05 % v/v Triton X-100) and 1 µg of anti-methylcytosine or anti-hydroxymethylcytosine antibodies (Zymo[®], California, USA) at 4 °C overnight on a rotating wheel. Collection and washing of the immune complexes were performed as described for ChIP. To release the DNA from the beads, samples were incubated with digestion buffer (50 mM Tris, 10 mM EDTA, 0.5 % w/v SDS) and Proteinase K overnight at 55 °C in agitation. DNA purification and real-time PCR were performed as described for ChIP.

Statistical analysis

Statistical analysis was performed using the Graph Pad Prism[®] 6 software (Graph Pad software, USA). Experimental data are presented as mean with standard deviation from three or more independent experiments. One way ANOVA tests were performed in all data followed by Tukey post hoc test. Statistical differences were considered when $P < 0.05$.

Results

Regulation of PR isoforms expression in mHypoE-N42 cells

A series of experiments was performed to assess the effects of estradiol in both PR-A and PR-B expression in mHypoE-N42 cells. An estradiol dose–response curve was performed after 6 h of treatment, and a significant induction of PR-B protein content with 100 nM of estradiol was observed by Western blot (Fig. 2a, b). PR-A was identified as the predominant isoform (Fig. 2a). The regulation of PR isoforms expression by estradiol at mRNA level was assessed by RT-qPCR. A significant induction of PR-B mRNA expression after 6 h of treatment was followed by a marked decrease after 12 h (Fig. 2c). No significant changes in PR-A mRNA expression after estradiol

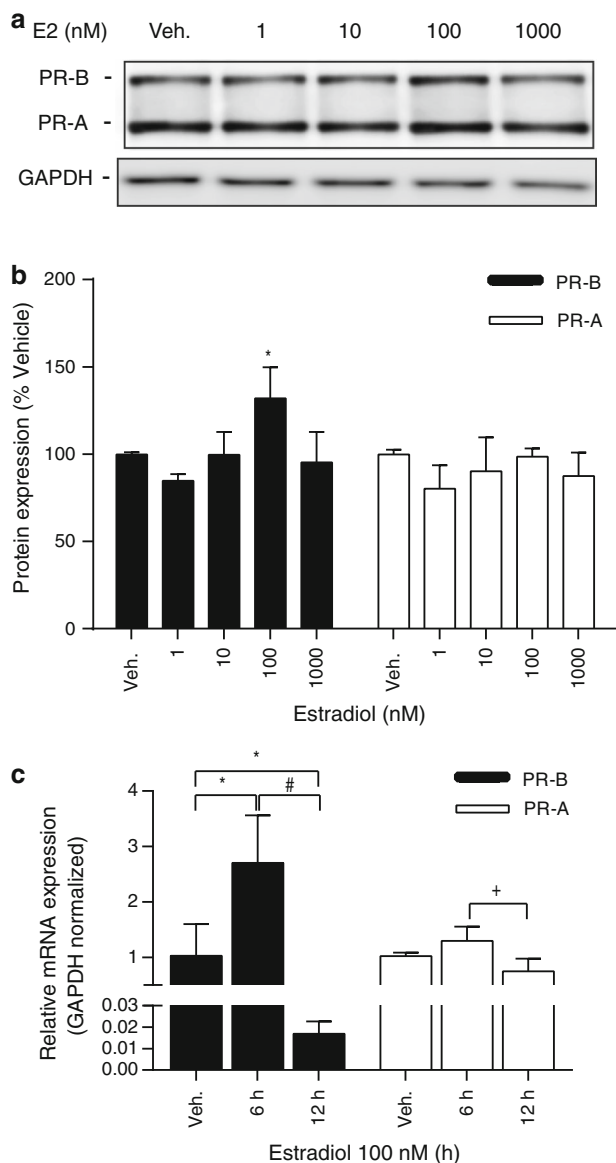


Fig. 2 Estradiol differentially regulates PR isoforms expression in the mHypoE-N42 cell line. Cells were treated as specified in the “Materials and methods” section and then total protein or RNA were extracted. PR isoforms expression was normalized using GAPDH. Transcripts relative expression of PR-B and PR-A was calculated by the $\Delta\Delta Ct$ method, and data were normalized using GAPDH gene as endogenous control. **a** and **b** Western blot analysis of PR isoforms content after 6 h of estradiol treatment. **c** PR isoforms relative mRNA expression after 6 and 12 h of estradiol (100 nM) and vehicle (ethanol 0.02 %) treatments. Significant differences between vehicle treatments at 6 and 12 h were not observed (data not shown). Data are expressed as mean \pm SD of four independent replicates. Veh vehicle, E2 estradiol. * $P < 0.05$ versus Veh.; # $P < 0.01$ versus 6 h; + $P < 0.05$ versus 6 h

treatments were detected as compared to vehicle, although a decrease in PR-A mRNA expression after 12 h of estradiol treatment as compared with hormone treatment after 6 h was observed.

Estradiol regulates the expression and phosphorylation of ER α and SP1 transcription factors

The expression and phosphorylation of ER α and SP1, two main transcription factors that regulate PR gene expression [17, 19, 31], were assessed by Western blot (Fig. 3a). An increase in ER α expression was observed after 12 h of estradiol treatment, while SP1 expression was increased at 6 h. In order to determine the functional status of these transcription factors, ER α (Ser118) and SP1 (total) phosphorylation was analyzed. Higher levels of Ser118 phosphorylation were found after 6 h of estradiol treatment as compared to vehicle and 12 h of estradiol treatment (Fig. 3a, b). An increase in the total phosphorylation levels of SP1 was found after 12 h of estradiol treatment (Fig. 3a, c).

As expected, treatment with the highly selective ER α antagonist (MPP) blocked the estradiol-dependent induction of PR-B protein content, without affecting PR-A protein content (Fig. 3d, e).

Estradiol induces a differential occupancy of estrogen-related transcription factors and RNA pol II on PR isoform promoters

The local estradiol effects on both PR isoform promoters were assessed by ChIP. The recruitment of specific transcription factors of estrogen signaling (ER α and SP1) and RNA pol II was assessed after estradiol treatments (Fig. 4). RNA pol II was differentially recruited at PR-B promoter after 6 and 12 h of estradiol treatments (Fig. 4a). However, a significant difference between the 6 h of estradiol and vehicle treatments was not observed, but a significant decrease occurred at 12 h (Fig. 4a). In contrast, RNA pol II did not show a differential occupancy on PR-A promoter at any of the studied conditions (Fig. 4b).

SP1 occupancy was detected on PR-B promoter after vehicle and 6 h of estradiol treatment, but it was not detected after 12 h of estradiol treatment (Fig. 4a). On the other hand, SP1 and ER α occupancy on PR-A promoter was very low at all the studied conditions, and it was not statistically different from the negative control of ChIP assay (Fig. 4b and data not shown). Interestingly, ER α was only recruited on PR-B promoter after 6 h of estradiol treatment (Fig. 4a).

Estradiol induces a specific enrichment of active and repressive histone marks on PR isoform promoters

In order to determine the epigenetic status of both PR isoform promoters after estradiol treatments, the analysis of

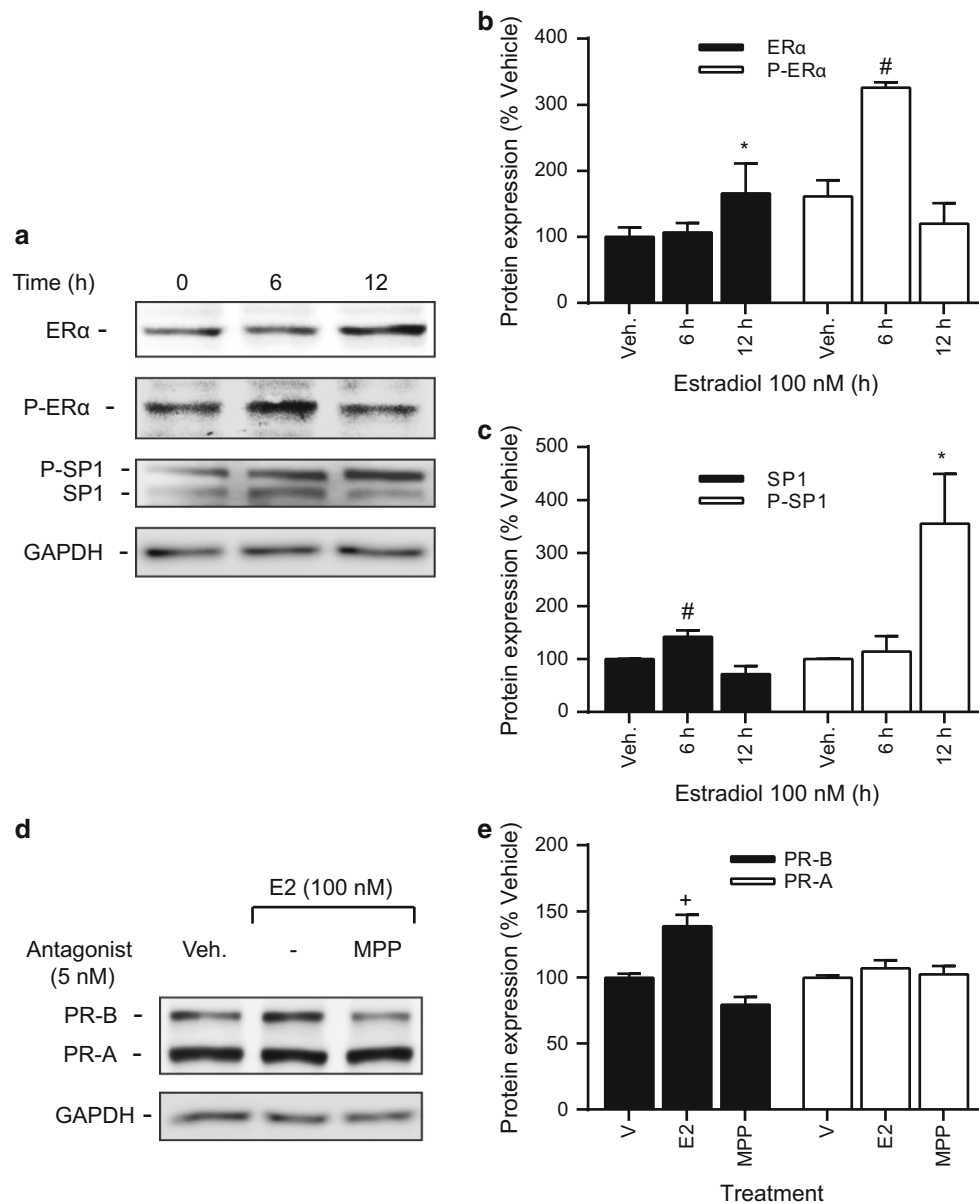


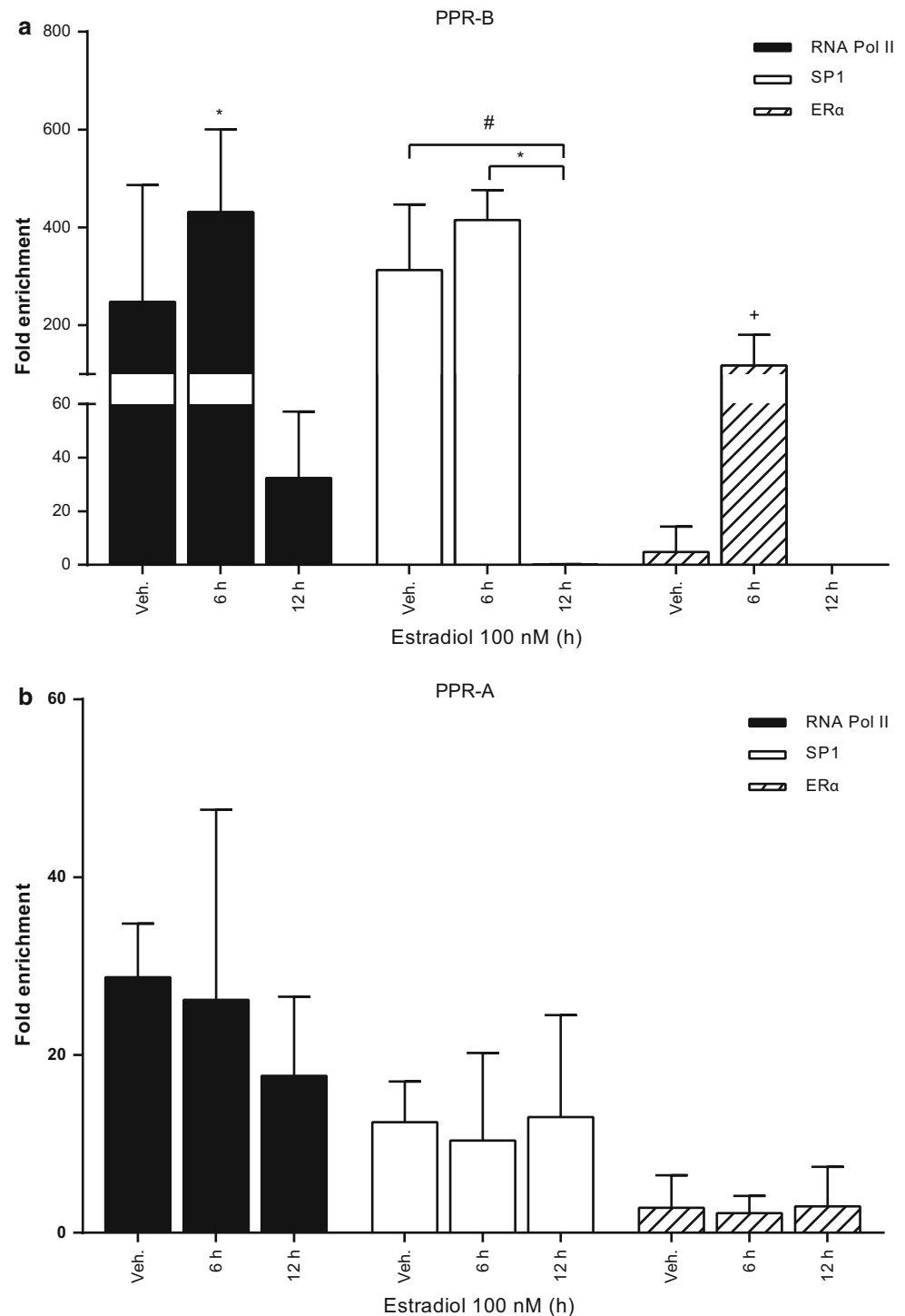
Fig. 3 Regulation of estrogen-related transcription factors expression by estradiol in the mHypoE-N42 cell line. Cells were treated with estradiol (100 nM) or vehicle (ethanol 0.02 %) for 6 and 12 h. Proteins obtained were analyzed by Western blot. Data were normalized using GAPDH as loading control. Significant differences between vehicle treatments at 6 and 12 h were not observed in any of the studied proteins (data not shown). **a** Representative Western blots of ER α , phosphorylated ER α (Ser 118), SP1 and phosphorylated SP1 (total), and GAPDH at the studied conditions. Densitometric analysis of **b** ER α and phosphorylated ER α and **c** SP1 and phosphorylated

SP1. Levels are expressed as percent of the vehicle (mean \pm SD of three to six independent replicates). Phosphorylated ER α and SP1 expression levels were normalized using the total levels of each protein. **d** and **e** Blocking of the estradiol-dependent induction of PR-B by the ER α selective antagonist, MPP, expressed as percent of the vehicle (mean \pm SD of three to six independent replicates). *Veh* vehicle, *E2* estradiol, *P-ER α* phosphorylated ER α at Ser118; *P-SP1* total phosphorylated SP1. * P < 0.05 versus Veh. and 6 h; # P < 0.05 versus Veh. and 12 h; + P < 0.05 versus Veh. and MPP

histone marks related with active gene expression (H3K9Ac and H3K4me3) and gene repression (H3K9me2) was performed by ChIP (Fig. 5). The H3K9Ac histone mark was not altered at any of the studied conditions (Fig. 5a, b), although a higher enrichment of this histone mark on PR-B promoter was observed, as compared with

PR-A promoter. A differential enrichment of the H3K4me3 histone mark on PR promoters was observed (Fig. 5). A higher enrichment of the H3K4me3 mark on PR-B promoter as compared to PR-A promoter was found after vehicle and 6 h of estradiol treatment. Interestingly, H3K4me3 enrichment on PR-B promoter was not detected

Fig. 4 Transcription factors and RNA pol II occupancy on PR isoform promoters regulation by estradiol. mHypoE-N42 cells were treated with estradiol (100 nM) and vehicle (ethanol 0.02 %) for 6 and 12 h. RNA pol II, SP1, and ER α occupancy on **a** PR-B and **b** PR-A promoters was analyzed by ChIP coupled to real-time PCR. Significant differences between vehicle treatments at 6 and 12 h were not observed in any of the immunoprecipitated studied proteins (data not shown). Data are expressed as fold enrichment (mean \pm SD of three to six independent replicates). Veh vehicle, PPR-B PR-B promoter, PPR-A PR-A promoter. * $P < 0.01$ versus 12 h; # $P < 0.05$ versus Veh.; + $P < 0.05$ versus Veh. and 12 h

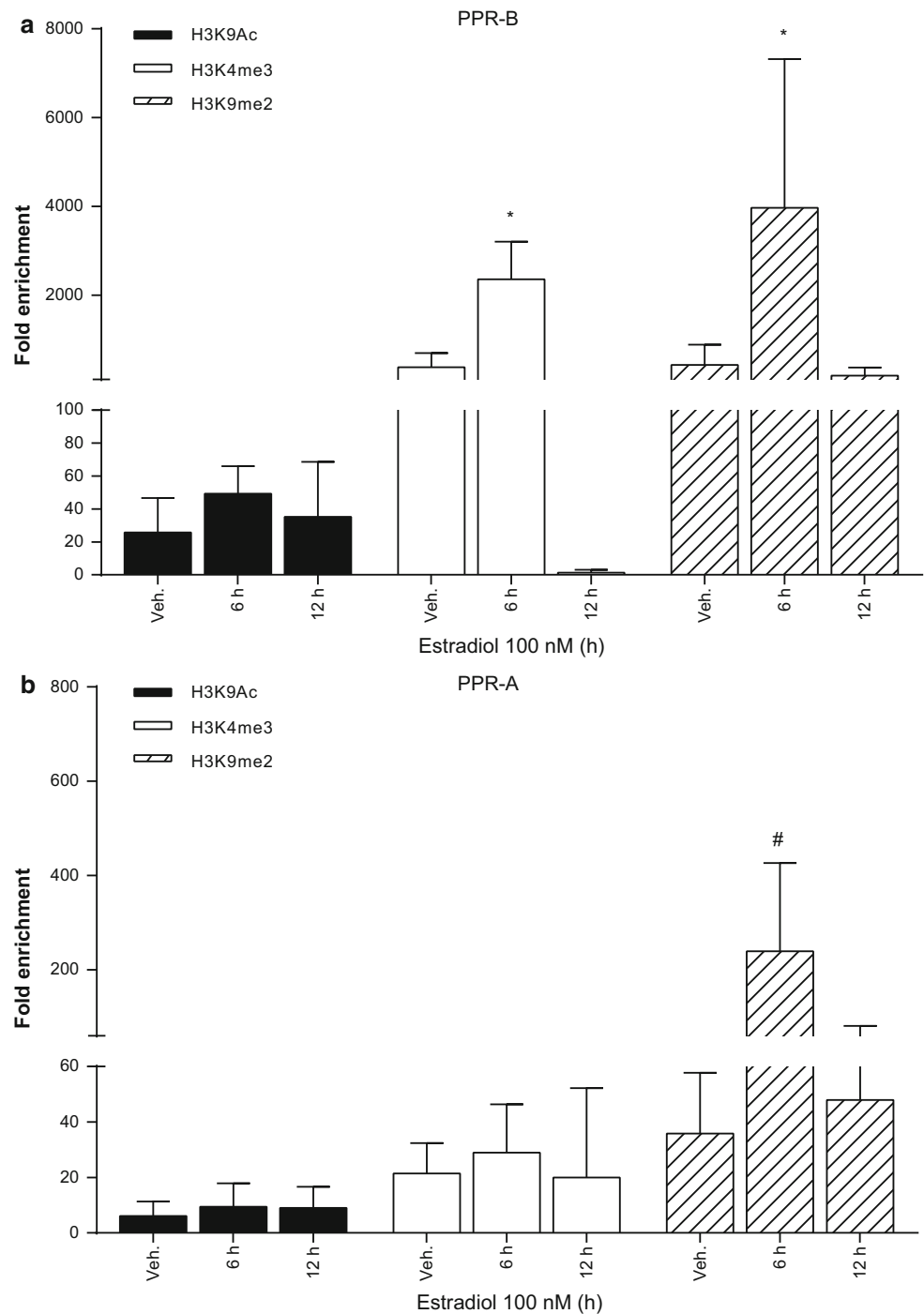


after 12 h of estradiol treatment (Fig. 5a). In contrast, we did not find any estradiol-dependent change in the H3K4me3 histone mark recruitment at PR-A promoter (Fig. 5b). Unexpectedly, estradiol-dependent transient enrichment of H3K9me2 after 6 h was found at both PR isoform promoters (Fig. 5a, b).

DNA methylation and hydroxymethylation marks at PR promoter are not affected by estradiol treatment

Another key component in epigenetic regulation is DNA methylation. In addition, the 5-hydroxymethylcytosine has

Fig. 5 Active and repressive histone marks enrichment on PR isoform promoters is differentially regulated by estradiol. Cells were treated with estradiol (100 nM) and vehicle (ethanol 0.02 %) for 6 and 12 h. H3K9Ac, H3K4me3, and H3K9me2 enrichment on PR-B (a) and PR-A (b) promoters was analyzed by ChIP coupled to real-time PCR. Significant differences between vehicle treatments at 6 and 12 h were not observed in any of the immunoprecipitated studied proteins (data not shown). Data are expressed as fold enrichment (mean \pm SD of three to six independent replicates). Veh vehicle, PPR-B PR-B promoter, PPR-A PR-A promoter. * $P < 0.01$ versus Veh. and 12 h; # $P < 0.001$ versus Veh

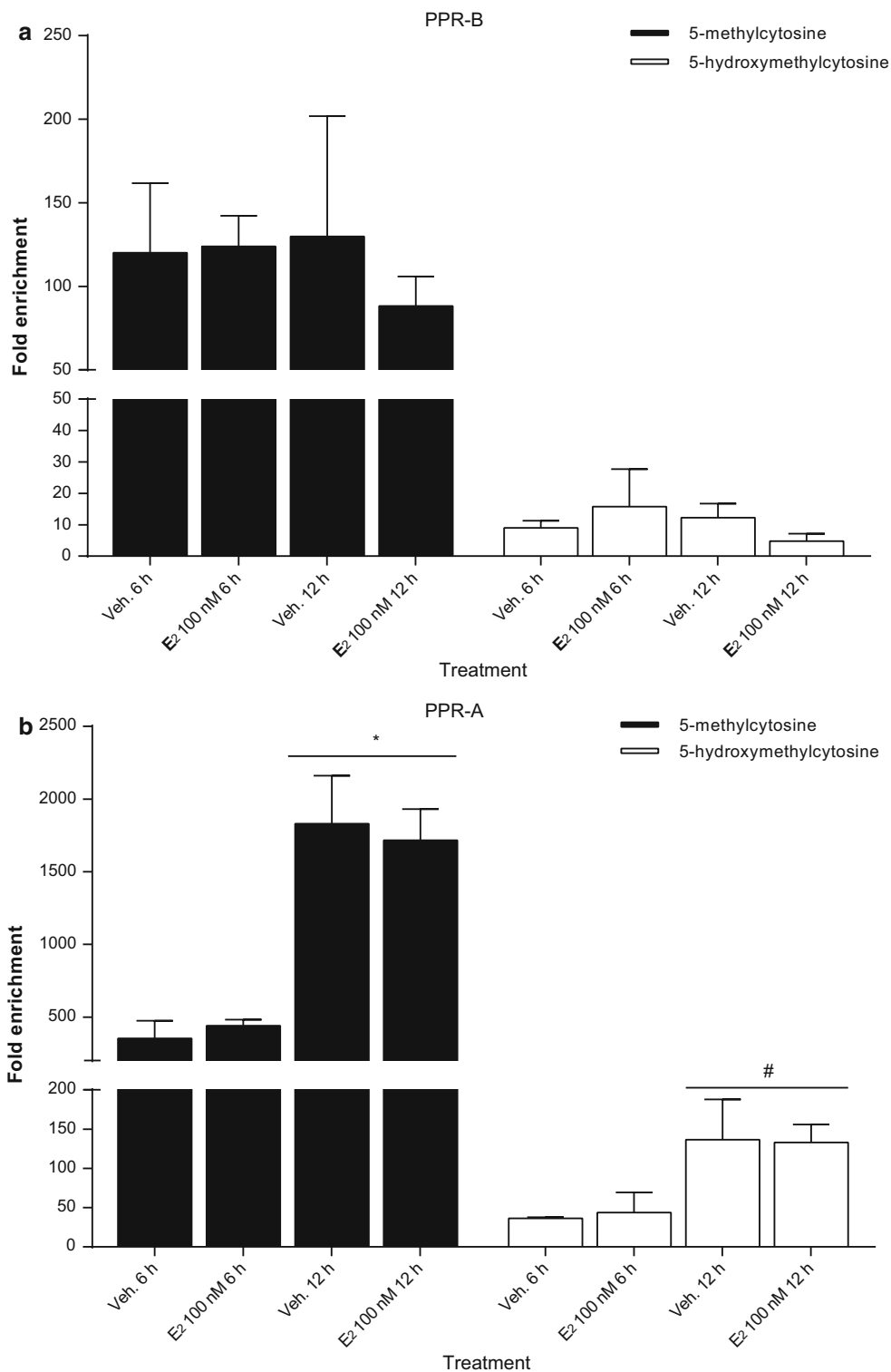


recently emerged as an intermediary of active DNA demethylation [32]. In the present study, estradiol-dependent changes in 5-methylcytosine and 5-hydroxymethylcytosine content at either PR isoform promoters were not observed (Fig. 6). Interestingly, the 5-methylcytosine and 5-hydroxymethylcytosine levels were higher on PR-A promoter than on PR-B promoter. An increase in both hydroxy and methyl cytosine levels on PR-A promoter was observed after 12 h of both vehicle and estradiol treatments (Fig. 6b).

Discussion

In the present study, we demonstrate that PR isoforms are differentially regulated by estradiol at the transcriptional level through changes in promoter occupancy by transcription factors as well as by epigenetic changes in PR-A and PR-B promoters in a mouse embryonic hypothalamic cell line. The transient ER α -dependent induction of PR-B mRNA expression observed after 6 h of estradiol treatment

Fig. 6 DNA methylation and hydroxymethylation levels in PR isoform promoters are not affected by estradiol treatments. mHypoE-N42 cells were treated with estradiol (100 nM) and vehicle (ethanol 0.02 %) for 6 and 12 h. 5-methylcytosine and 5-hydroxymethylcytosine detection in PR-B (a) and PR-A (b) promoters was analyzed by immunoprecipitation coupled to real-time PCR. Data are expressed as fold enrichment (mean \pm SD of three to six independent replicates). *Veh* vehicle, *E2* estradiol, *PPR-B* PR-B promoter, *PPR-A* PR-A promoter. * $P < 0.0001$ versus 6 h; # $P < 0.05$ versus 6 h



was associated with an increase in ER α phosphorylation at Ser118, as well as its recruitment on the PR-B promoter. In line with these data, a down regulation of PR-B mRNA expression after 12 h of estradiol treatment was associated with a decrease of SP1, H3K4me3, and RNA pol II

occupancy on its promoter. In contrast, estradiol modified neither PR-A mRNA expression nor the occupancy of transcription factors and epigenetic markers on its promoter.

We first demonstrated that estradiol (100 nM) produced a transient induction of PR-B mRNA expression in the

mHypoE-N42 cell line (Fig. 2c). Although this estradiol dose is considered higher than those reported in other studies [33], the functional relevance of this estradiol concentration has also been demonstrated in previous reports using this cell line, in terms of aromatase gene expression regulation in an ER α -dependent manner [23]. This differential response to estradiol doses between hypothalamic cultures could be due to the differences in cell type and developmental stage, since it has been proposed that PR expression within the ventromedial nucleus of the hypothalamus becomes more responsive to estradiol as development advances, which supports the idea that higher doses of estradiol are required to induce PR expression during early stages of development than those required in highly estradiol responsive adult cells [34]. Interestingly, the transient induction of PR-B and the absence of changes in PR-A mRNA expression after estradiol treatments observed in the present study were similar to that observed in the hypothalamus of proestrus rats and during development in several hypothalamic nuclei [10, 35]. In contrast to our results, the induction of PR mRNA expression is still observed after 48 h of estradiol administration in the hypothalamus of adult ovariectomized female rats [7, 12]. This suggests that time differences in the regulation of PR mRNA expression by estradiol depend on the developmental stage of the animal. In fact, PR protein content is transiently upregulated in the ventromedial nucleus of rat hypothalamus during embryonic development [35]. On the other hand, the 6 h transient induction of PR expression by estradiol observed in the present study has also been reported in the hypothalamus of adult ovariectomized female mice [24], which suggests that this short-term regulation of PR expression is species dependent. The transient induction of PR-B was also observed at protein level in the present study (data not shown), suggesting that the effects of estradiol on the transcription factors and epigenetic marks studied in the present work are also related with PR isoforms protein content, which in turn indicates that this regulation could have an impact on the functional role of PR in this cell line. Furthermore, PR-A was identified as the predominant isoform in the present study, as previously reported in embryonic rat hypothalamic cells [13].

ER α is the main transcription factor that regulates PR expression in the brain [7, 8, 36]. We therefore, assessed the phosphorylation status of ER α at Ser118, since it has been reported that phosphorylation at this residue is related with the transcriptional activity of ER α in its target genes [37, 38]. Accordingly, higher levels of phosphorylated ER α (Ser118) were found after 6 h of estradiol treatment (Fig. 2a). This finding correlates with the transient induction of PR-B mRNA expression observed after 6 h of estradiol treatment, suggesting that

there is a correlation between the transcriptional activity of ER α and PR-B mRNA expression in rodent embryonic hypothalamic cells.

The participation of ER α on PR expression was demonstrated using a selective ER α antagonist (Fig. 2d). Moreover, our ChIP data show that ER α is recruited on PR-B promoter after 6 h of estradiol treatment (Fig. 4a), which is in line with the induction of PR-B mRNA expression. On the other hand, and according to several studies, ER α occupancy on PR-A promoter was not detected at any of the studied conditions (Fig. 4b) [20, 24, 39] despite the presence of a half ERE site in this promoter [27]. The absence of ER α on PR-B promoter after 12 h of estradiol treatment could be due to the lack of SP1 presence on PR-B promoter region at this period of time, since it has been reported that SP1 is required for ER α recruitment [19].

Furthermore, we have recently reported that the dynamic DNA methylation pattern found in PR-B promoter in rat hypothalamus during proestrus transition involves a consensus and highly conserved SP1 site [10]. Our ChIP data suggest that SP1 is present on PR-B promoter after 6 h of estradiol and vehicle treatments, as previously reported [40, 41]. Interestingly, a downregulation of SP1 together with an increase in its phosphorylation after 12 h of estradiol treatment was observed (Fig. 3a), when PR-B mRNA expression was markedly decreased (Fig. 2c). In line with these results, hyperphosphorylation of SP1 has been associated with a decrease of its occupancy on target genes [42, 43]. On the other hand, we did not find SP1 occupancy on PR-A promoter. However, further studies are needed to investigate the role of other SP proteins such as SP3 or SP4 in PR isoforms regulation [44].

Regarding RNA pol II, our results demonstrate that a decrease of its recruitment on PR-B promoter at 12 h of estradiol treatment correlates with a marked decrease on PR-B mRNA expression (Fig. 2c, 4a), suggesting that SP1 is required for the recruitment of RNA pol II to maintain the transcriptional basal levels of PR-B. Interestingly, the transient occupancy of RNA pol II in the promoters of other estrogen-regulated genes has been previously reported in MCF7 human breast cancer cells [45]. In contrast, no differential occupancy of RNA pol II on PR-A promoter at the studied conditions was found (Fig. 4b), correlating with the absence of changes in PR-A mRNA expression after 6 h of estradiol treatments. Interestingly, no significant differences of RNA pol II occupancy on PR-A promoter between the negative control of ChIP assay and the 12 h of estradiol treatment were observed (data not shown), which correlates with the significant decrease on PR-A mRNA expression at this time point as compared with 6 h of estradiol treatment. Further studies are required in order to describe the recruitment of specific phosphorylated forms of RNA pol II that actively participate in ER α -dependent

PR transcription, as we did not find changes in total RNA pol II occupancy after 6 h of estradiol treatment.

Histone acetylation is one of the characteristic features of transcriptionally active chromatin [46]. A higher enrichment of the H3K9Ac histone mark on PR-B promoter as compared to PR-A promoter was observed (Fig. 5). This finding could explain the differential levels of RNA pol II occupancy on the corresponding PR isoform promoters (Fig. 3), which correlate with previous reports [41, 47].

The H3K4me3 histone mark is another of the classical hallmarks of transcriptionally active chromatin. As in the case of H3K9Ac histone mark, a higher enrichment of the H3K4me3 mark on PR-B promoter was found as compared with PR-A promoter, suggesting that PR-B promoter is transcriptionally more permissive than PR-A promoter (Fig. 5). In addition, the increase of the H3K4me3 histone mark after 6 h of estradiol treatment, as well as the marked decrease at 12 h, is in line with the transient induction of PR-B mRNA expression (Fig. 2c) and the corresponding decrease of RNA pol II and estrogen-related transcription factors occupancy on this promoter (Fig. 4a). In contrast, no changes in the H3K4me3 enrichment on PR-A promoter were observed at any of the studied conditions (Fig. 5b).

The H3K9me2 histone mark is a typical hallmark of transcriptional silencing on promoter regions [48]. The enrichment of both transcriptional active (H3K4me3) and repressive (H3K9me2) histone marks was increased after 6 h of estradiol treatment on PR-B promoter, which could be the result of a fine-tune regulation mechanism to avoid ligand-independent expression as previously reported on ER α -regulated genes in MCF7 breast cancer cells [49]. According to our results, it has been reported that both transcriptional active and repressive histone marks are also present on PR isoform promoters in the human term myometrium, in which the upregulated isoform (PR-A) was more extensively marked for transcriptional activation than the non-upregulated one (PR-B) [47]. On the other hand, the enrichment of the H3K9me2 histone mark after 6 h of estradiol treatment was the only estradiol-dependent change on PR-A promoter observed in the present study; however, this modification was not associated with estradiol-dependent changes on PR-A mRNA expression.

The participation of DNA methylation in PR gene expression regulation has been extensively described in pathological and physiological models [10, 50–54]. In the present study, we did not find any estradiol-dependent change in DNA methylation or hydroxymethylation of PR isoform promoters (Fig. 6). It has been reported that DNA methylation on PR-B promoter is observed as a late event after disrupting ER α signaling in MCF7 cells [55]. The higher enrichment of methylated DNA on PR-A promoter as compared with PR-B promoter would confirm the

transcription factor accessibility on the later one (Fig. 6). Interestingly, the increase of both 5-methylcytosine and 5-hydroxymethylcytosine marks on PR-A promoter observed at 12 h was independent of estradiol exposure (Fig. 6b), which could be related with a particular intrinsic regulation mechanism of PR-A mRNA expression.

The fact that PR-B was regulated by estradiol in the present study suggests that the mHypoE-N42 cell line has a male origin, as previous studies have reported that estradiol induces PR expression in the neonatal ventromedial nucleus of hypothalamus in male but not in female rats [16]. However, it has been demonstrated that prenatal exposure to a synthetic estrogen (diethylstilbestrol) induces PR expression in other reproductive relevant brain areas of female rats such as the medial preoptic nucleus and the anteroventral periventricular nucleus [56]. Further studies are required to the complete understanding of molecular mechanisms involved in the differential regulation of PR isoforms mRNA expression by estradiol in embryonic hypothalamic cells, which in turn could be associated with estradiol mediated prenatal programming processes and sexual dimorphism in the rodent brain [15, 16].

The overall results of this study show that estradiol induces a differential regulation of PR isoforms expression in an embryonic hypothalamic cell line, which is associated with specific promoter occupancy of transcription factors and histone marks on PR-A and PR-B promoters.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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