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Revised version

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ABSTRACT (150 words)

With the aim to analyze whether bisphenol A (BPA) modifies β-Casein (β-Cas) synthesis and transcriptional regulation in perinatally exposed animals, here, pregnant F0 rats were orally exposed to 0, 0.6 or 52 µg BPA/kg/day from gestation day 9 until weaning. Then, F1 females were bred and mammary glands were obtained on lactation day 2**. Perinatal** BPA exposure decreased β-Cas expression without modifying the activation of prolactin receptor. It also decreased the expression of glucocorticoid receptor in BPA52-exposed dams and β1 and α6 integrins as well as dystroglycan in both BPA groups. In addition, BPA exposure altered the expression of histone-modifying enzymes and induced histone modifications and DNA methylation in the promoter, enhancer and exon VII of the β-Cas gene. An impaired crosstalk between the extracellular matrix and lactogenic hormone signaling pathways and epigenetic modifications of the β-Cas gene could be the molecular mechanisms by which BPA decreased β-Cas expression.

Key words: Bisphenol A; mammary gland differentiation; β-Casein; epigenetic modifications; lactation.

Abbreviations: β-Casein: β-Cas; BPA: bisphenol A; ChIP: chromatin immunoprecipitation; DAG1: dystroglycan; DNAme: DNA methylation; ECM: cell-extracellular matrix; EDCs: endocrine-disrupting chemicals; EZH2: enhancer of zeste homolog 2; F0: dams directly exposed to xenoestrogens; F1: F0 offspring; F2: F1 offspring; GD: gestation day; GR: glucocorticoid receptor; IHC: immunohistochemistry; IPs: immunoprecipitated complexes; ITGα6: integrin α6; ITβ1: integrin β1; H3Ac: acetyl histone 3; H3K27me3: trimethyl-histone H3 (Lys27); HDAC: histone deacetylase; LD: lactation day; MG: mammary gland; PRL: prolactin; PRL-R: prolactin receptor; pStat5a/b: phosphorylated Stat5a/b; qRT-PCR: real time RT-PCR ; SDS: sodium dodecyl sulfate; Stat5: signal transducer and activator of transcription 5.

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29 **1 Introduction**

30 Milk protein gene expression is influenced by lactogenic hormones, which, together
31 with the cooperation of local growth factors and cell-cell and cell-extracellular matrix
32 (ECM) interactions, activate specific transcription factors, alter cytoskeletal
33 organization, and change the chromatin state and nuclear structures (Kabotyanski et al.,
34 2009; Qian and Zhao, 2014). β-Casein (β-Cas) is one of the main milk proteins secreted
35 during lactation and is regarded as a functional differentiation marker of the mammary
36 gland (Rijnkels et al., 2010). β-Cas gene expression is transcriptionally regulated by
37 several signal transduction pathways. The lactogenic hormones prolactin (PRL) and
38 glucocorticoids modulate the activity of the signal transducer and activator of
39 transcription 5 (Stat5) and the glucocorticoid receptor (GR), respectively (Groner,
40 2002), which in turn bind to composite response elements present in both the β-Cas
41 proximal promoter and distal enhancer. The latter is also known as ECM-responsive
42 element and is located -3.5 kb 5' to the start site for transcription in the rat (Kabotyanski
43 et al., 2006; Rijnkels et al., 2003). In addition to PRL and glucocorticoids, adhesion to
44 basement membrane proteins (especially laminin-1) is also necessary to regulate β-Cas
45 gene expression (Alcaraz et al., 2008; Streuli et al., 1995). Laminin transduces its
46 signals by binding to integrin receptors (e.g. α6β1 and α6β4) and other cell surface
47 molecules like dystroglycan (Muschler et al., 1999), modulating the enzyme-signaling
48 pathway driven by PRL through activating Stat5-DNA interactions (Edwards et al.,
49 1998; Streuli et al., 1995).

50 Epigenetic changes can lead to alterations in chromatin conformation by DNA
51 methylation (DNAme), histone modifications and noncoding RNAs which are
52 associated with an active/open chromatin or an inactive/closed chromatin (Li, 2002).
53 Taking into consideration that epigenetic mechanisms are also considered transcription

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54 regulators of milk protein gene expression (Qian and Zhao, 2014), β-Cas gene
55 expression could be controlled at the transcriptional level by modifying the accessibility
56 of the DNA to transcription factors. In this regard, it has been suggested that the
57 environment during mammary gland development, from fetal life to lactation, could
58 influence the lactation performance of the animal through epigenetic alterations of the
59 genome (Rijnkels et al., 2010). Bisphenol A (BPA), an endocrine-disrupting chemical
60 (EDC) and one of the highest-volume chemicals produced worldwide (Osborne et al.,
61 2015; Vandenberg et al., 2009), is one of the environmental factors that has been shown
62 to disrupt mammary gland development (Gore et al., 2015; Soto et al., 2013) and
63 functional differentiation in rodents (Altamirano et al., 2015; Kass et al., 2012).
64 Moreover, several studies have demonstrated that maternal BPA exposure induces
65 postnatal effects on the DNAme status and histone modification that alter the expression
66 of specific genes in the offspring (Kundakovic and Champagne, 2011).

67 Previously, we have shown that perinatal exposure to low doses of BPA delays
68 mammary gland differentiation, modifies milk yield, and alters both milk protein and
69 lipid composition in F1 lactating rats (Altamirano et al., 2015; Kass et al., 2012). In the
70 present study, we examined whether perinatal exposure to low doses of BPA alters the
71 transcriptional regulation of the β-Cas gene through either changes in the lactogenic
72 hormones/ECM-derived signaling pathways or epigenetic modifications in the
73 mammary glands of F1 dams during early lactation.

74 **2 Materials and methods**

75 **2.1 Animals**

76 Sexually mature female rats (90 days old) of a Wistar-derived strain bred at the
77 Department of Human Physiology [Facultad de Bioquímica y Ciencias Biológicas,

78 Universidad Nacional del Litoral (UNL), Santa Fe, Argentina] were used. The animals
79 were maintained in a controlled environment (22 ± 2°C; 14 h of light) and had free
80 access to pellet laboratory chow (16-014007 Rat-Mouse Diet, Nutrición Animal, Santa
81 Fe, Argentina). For more information regarding the food composition, see Altamirano et
82 al. (2015). To minimize additional exposure to EDCs, the rats were housed in stainless
83 steel cages with sterile pine wood shavings as bedding, and glass bottles with rubber
84 stoppers were used to supply drinking water and oral treatments. All the experimental
85 protocols were approved by the Ethical Committee of the Facultad de Bioquímica y
86 Ciencias Biológicas, UNL. Animals were treated humanely and with regard for
87 alleviation of suffering.

88 **2.2 Experimental design**

89 The experimental procedures have been previously described in Kass et al. (2012) and
90 Altamirano et al. (2015). Briefly, females in proestrus were caged overnight with males
91 of proven fertility. The day on which the sperm was found in the vagina was designated
92 day 1 of gestation (GD1). On GD9, corresponding to the beginning of organogenesis in
93 the fetus, pregnant rats (F0) were weighed, and randomly divided into three groups (8-
94 10 dams/group) as follows: a) Control (0.002 % ethanol), b) BPA0.6 (0.6 μg
95 BPA/kg/day) and c) BPA52 (52 μg BPA/kg/day). BPA was administered in the drinking
96 water of the F0 dams from GD9 to weaning (Fig. 1). BPA solutions (99 % purity,
97 Sigma-Aldrich, Buenos Aires, Argentina) were prepared according to Kass et al. (2012).
98 The doses were calculated on the basis of their average body weight and water
99 consumption during pregnancy and lactation, as previously reported in Altamirano et al.
100 (2015).

101 After parturition [lactation day 0 (LD0)], F1 pups were weighed and sexed according to

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102 the anogenital distance, and litters of eight pups (preferably four males and four
103 females) were left with F0 lactating mothers until weaning on LD21. At weaning, the
104 female F1 offspring (exposed to BPA transplacentally and through the milk) were
105 transferred to new stainless steel cages with new glass drinking water bottles until the
106 end of the experiment. To evaluate whether perinatal exposure to BPA altered β-Cas
107 protein expression and gene regulation during early lactation, randomly chosen 90-day-
108 old F1 females were bred to unexposed males of proven fertility on the night of
109 proestrus (as determined by vaginal smears). After pregnancy confirmation, one F1
110 female per litter from each treatment group was assigned to the present research. The
111 remaining females and all males were used for other experiments. Mammary gland
112 samples from F1 dams were obtained on LD2 (n = 8-10 dams/group). The left fourth
113 pair of abdominal mammary glands was fixed in 10% (v/v) buffered formalin and
114 embedded in paraffin. Portions of the contralateral gland excluding the lymph nodes
115 were snap-frozen in liquid nitrogen and kept at -80°C for real-time RT-PCR (qRT-
116 PCR), DNAme and chromatin immunoprecipitation (ChIP) analysis.

117 **2.2 Immunofluorescence and immunoperoxidase assays**

118 The protein expression of β-Cas and phosphorylated Stat5a/b (pStat5a/b) was analyzed
119 by immunofluorescence and immunohistochemistry (IHC) assays, respectively, as
120 described previously in Kass et al. (2012). Briefly, mammary gland sections (5 μm in
121 thickness) were deparaffinized and dehydrated in graded ethanol. For
122 immunofluorescence assays, sections were blocked with 10 mg/ml sodium borohydride
123 (Sigma-Aldrich) to minimize autofluorescence. Incubation with the primary antibody
124 against β-Cas (1:400, rabbit; kindly provided by Dr. Bussmann, IBYME-CONICET,
125 Argentina) was performed overnight at 4°C. Sections were incubated with the
126 secondary antibody anti-rabbit Alexa Fluor 488 (Invitrogen) for 1 h, mounted in

127 ProLong Gold (Molecular Probes, Thermo Fisher Scientific, Buenos Aires, Argentina)
128 with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Fluka, Sigma-Aldrich) and
129 stored in the dark at 4°C until evaluated according to Kass et al. (2012).

130 For IHC assays, primary antibody against pStat5a/b (1:100, rabbit; Tyr 694, Santa Cruz
131 Biotechnology, Santa Cruz, CA, USA) was incubated overnight at 4°C. The reactions
132 were developed using the streptavidin-biotin peroxidase method and diaminobenzidine
133 (Sigma-Aldrich). Each run included negative controls in which the primary antibody
134 was replaced with non-immune rabbit serum (Sigma-Aldrich). pStat5a/b protein
135 expression and localization were evaluated in two mammary tissue sections per animal.

136 **2.3 qRT-PCR**

137 The qRT-PCR assay was carried out as previously described (Altamirano et al., 2015).
138 Briefly, mammary glands from eight animals from each experimental group were
139 individually homogenized in TRIzol (Invitrogen, Buenos Aires, Argentina), and RNA
140 was prepared according to the manufacturer's protocol. Equal quantities of total RNA
141 were reverse-transcribed into cDNA according to Ramos et al. (2003). The primer
142 sequences used for PCR are shown in Table 1. cDNA levels were detected using RT-
143 PCR with a Rotor-Gene Q cycler (Qiagen Instruments AG, Hombrechtikon,
144 Switzerland). After initial denaturation at 95°C for 15 min, the reaction mixture was
145 subjected to successive cycles of denaturation at 95°C for 15 s, annealing at 52-60°C for
146 15 s, and extension at 72°C for 15 s. Product purity was confirmed by dissociation
147 curves, and random samples were subjected to agarose gel electrophoresis. The
148 calculation of the relative expression level of each target was conducted using the
149 standard curve method (Cikos et al., 2007), and L19 and the control group were used to

150 normalize the C_T values. No significant differences in C_T values were observed in L19
151 between the different experimental groups.

152 **2.4 Bioinformatics**

153 The β-Cas rat gene (accession number NM_017120) was analyzed for CpG islands
154 using Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA,
155 USA). In addition, the β-Cas promoter, enhancer and exon VII regions were also
156 checked for restriction sites for *BstU*I or *Mae II*. To recognize the putative binding sites
157 for transcription factors, we used the TFSEARCH program
158 (http://www.cbrc.jp/research/db/TFSEARCH.html). PCR primers were designed with
159 Vector NTI Suite Version 6.0 software (Infomax Inc., North Bethesda, MD, USA).

160 **2.5 Methylation-sensitive analysis**

161 The DNAme status of the β-Cas distal enhancer, proximal promoter and exon VII was
162 analyzed using a combination of single digestions with methylation-sensitive restriction
163 enzymes and subsequently performing real-time PCR analysis (Rossetti et al., 2015).
164 Genomic DNA was isolated from mammary gland samples using
165 phenol/chloroform/isoamyl alcohol extraction. The total concentration of DNA was
166 quantified by A_{280} and stored at 2-8°C until needed. Equal quantities of DNA (1 µg)
167 were digested with 7.5 units of *EcoRI* (Promega, Madison, WI, USA) to reduce the size
168 of the DNA fragments and then purified with phenol/chloroform extraction and ethanol
169 precipitation. Then, 1 µg of *EcoRI*-cleaved DNA was incubated for 1 h with 1 unit of
170 *MaeII* (Roche Applied Science, Indianapolis, IN, USA) or 10 units of *BstUI* (New
171 England BioLab, Beverly, MA, USA) and 1X enzyme buffer at 50°C or 60°C
172 respectively, following the manufacturer's instructions. The digestion products were
173 purified with the phenol/chloroform method. The relative expression level of the

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174 different DNA regions was analyzed by real-time PCR (as mentioned above). The
175 primer sequences are shown in Table 2. Each sample was quantified in duplicate or
176 triplicate. The methylation restriction enzymes *MaeII* or *BstUI* are unable to cut at
177 methylated sites, allowing amplification of the fragment. A region without *Mae II* or
178 *BstU*I restriction sites was used as an internal control. The relative degree of
179 methylation was determined by plotting C_T values against the log input (internal
180 control), which yielded standard curves for the quantification of unknown samples, and
181 finally dividing by the normalized target value of the control sample (Cikos et al.,
182 2007).

183 **2.6 ChIP assay**

184 ChIP analysis was performed according to a modified protocol (Kazi and Koos, 2007).
185 Briefly, 40 mg of frozen mammary gland was dissociated into small pieces and
186 immersed in 1% formaldehyde solution for 15 min. Cross-linking was stopped by
187 adding 1.5 ml of 1M glycine for 5 min. The tissue pellet was homogenized in 600 µl of
188 RIPA lysis buffer [composed of 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1%
189 sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM sodium fluoride (Sigma-Aldrich),
190 1X protease inhibitor (Complete Mini, Protease Inhibitor Cocktail Tablet, Roche
191 Diagnostics GMBH, Germany) and phosphatase inhibitor (PhosSTOP, Phosphatase
192 Inhibitor Cocktail Tablets, Roche Diagnostics GMBH) in PBS, pH 7]. Homogenates
193 were centrifuged at 12000 rpm for 5 min at 4°C and the supernatants removed and
194 discarded. The separated nuclei were lysed in SDS lysis buffer [50mM Tris-HCl (pH
195 8.1), 5 mM EDTA, 0.1% SDS] containing proteases and phosphatase inhibitors (as
196 above), and incubated on ice for 10 min. Then, samples were sonicated on ice for 40 x 2
197 sec cycles, followed by 1 min cooling interval between each cycle, using a Sonic Vibra-
198 Cell™ VCX750 (Sonics & Materials, Newtown, CT, USA) at 30% of power. The

199 resulting DNA fragment size was about 0.5-1.0 kb. After sonication, the samples were
200 centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were then collected and
201 stored at -80°C. Sonicated sample aliquots were thawed on ice and diluted 1:10 with
202 dilution buffer [20 mM Tris-HCl (pH. 8.1), 150 mM NaCl, 2 mM EDTA, 1% triton X-
203 100, and protease inhibitors (as above)]. Then, 50 µl of Dynabeads® Protein A
204 (Invitrogen) was incubated with 2.5 µl of rabbit polyclonal antibody Anti-Acetyl-
205 Histone H3 (H3Ac; Upstate Biotechnology, Lake Placid, NY, USA) or Anti-trimethyl-
206 Histone H3 (Lys27) (H3K27me3; EMD Millipore, Darmstadt, Germany) for 10 min.
207 The samples previously diluted were incubated with the Dynabeads® Protein A-
208 antibody complex overnight at 4°C with rotation. For the nonspecific antibody control,
209 an equal volume of normal rabbit serum was substituted for the specific antibody. The
210 immunoprecipitated complexes (IPs) were washed sequentially three times with PBS
211 solution and once with TE buffer (pH 8.0). Protein-DNA complexes were eluted from
212 the Dynabeads® Protein A by incubation in 100 µl of elution buffer [50 mM NaHCO3
213 and 1% SDS], and 0.5 µl of proteinase K (Sigma-Aldrich) at 65°C for 2 h to remove
214 protein. The cross-linking was reversed with incubation at 95°C for 10 min. Ten percent
215 of total supernatant was saved as a total input control and processed with the eluted IPs
216 beginning with the cross-linking reversal step. DNA was purified with a PureLink™
217 Quick Gel Extraction & PCR Purification Combo kit (Invitrogen). The recovered DNA
218 was then quantified by real-time PCR (as above). Primers were used to amplify genomic
219 sequences at the enhancer, promoter and exon VII regions of the β-Cas gene (Table 2).
220 The relative amounts of IPs and input DNA were determined by comparison to a
221 standard curve generated by serial dilutions of input DNA. Both experimental IPs and
222 input DNA were run in triplicate. IPs with specific antibodies was normalized by first
223 subtracting the signals obtained with the nonspecific antibody control, and then

224 expressing the normalized value as a ratio to input DNA and to the normalized value of
225 the control samples.

226 **2.7 Statistical analysis**

227 All data are expressed as the mean ± SE. ANOVA was performed to obtain the overall
228 significance, and Dunnett's ($p<0.05$) post-hoc test was used to compare the
229 experimental and control groups. The Mann-Whitney U test was used for qRT-PCR,
230 DNAme and ChIP analysis. Values with a $p<0.05$ were accepted as significant.

231 **3 Results**

232 As found in our previous work (Altamirano et al., 2015; Bosquiazzo et al., 2013; Kass
233 et al., 2012; Vigezzi et al., 2015), exposure to BPA through the drinking water produced
234 no signs of embryo toxicity. In addition, the rate of pregnancy of the F1 BPA-exposed
235 females did not differ from that of the F0 or F1 unexposed animals, and both the
236 average number of live F2 pups per litter and body weight of F2 pups were not
237 significantly different from those of the control group (Kass et al., 2012).

238 **3.1 Perinatal exposure to BPA impaired β-Cas expression independently of**
239 **Prolactin receptor PRL-R activation.**

240 In a previous work (Kass et al., 2012), we demonstrated that perinatal exposure to BPA
241 alters β-Cas milk protein synthesis during mammary gland differentiation in pregnant
242 F1 rats. To define BPA actions on β-Cas synthesis during secretory activation, its
243 mRNA and protein expression were analyzed on LD2. β-Cas mRNA level was reduced
244 in both BPA-treated groups during early lactation (Fig. 2A; $p<0.05$) and, as shown in
245 Fig. 2B, this lower mRNA expression was associated with a decreased level of β-Cas
246 protein expression.

247 To evaluate whether the decreased β-Cas expression observed in BPA-exposed F1 dams
248 was related to changes in PRL or glucocorticoid actions in the mammary epithelial cells,
249 the expression of PRL-R mRNA and its downstream transcription factor pStat5a/b as
250 well as GR mRNA were analyzed in the lactating mammary gland. BPA exposure
251 modified neither PRL-R mRNA nor pStat5a/b protein expression in the mammary gland
252 (Fig. 3A,C). Interestingly, IHC assays showed that pStat5a/b expression was nuclear in
253 all experimental groups, suggesting the activation of PRL signaling in the mammary
254 gland (Fig. 3C). On the other hand, GR mRNA expression was lower in BPA52-
255 exposed dams than in control F1 dams (Fig. 3B; $p<0.05$).

256 **3.2 Laminin-cell interactions were modified by perinatal exposure to BPA.**

257 It has been shown that laminin cooperates with PRL to regulate the binding of pStat5a/b
258 to the β-Cas promoter in mammary epithelial cells (Xu et al., 2007). To examine
259 whether cell surface receptors for laminin were affected by perinatal exposure to BPA,
260 the mRNA expression of two integrin subunits (ITGα6 and ITβ1) of laminin receptors
261 and one of the most important cell surface molecules related to its signaling (DAG1)
262 were evaluated in the mammary gland. On LD2, the transcriptional levels of ITGα6,
263 ITβ1 and DAG1 were decreased in all BPA-exposed animals compared with control rats
264 (Fig. 4; $p<0.05$).

265 **3.3 Histone-modifying enzymes were altered by perinatal exposure to BPA.**

266 Histone modifiers like p300 and histone deacetylase 1 (HDAC1) are recruited and
267 involved in the activation of β-Cas gene expression after hormonal lactogenic
268 stimulation (Kabotyanski et al., 2009). In contrast, HDAC3, together with YY-1 and
269 C/EBP, is part of a repress complex that binds to the β-Cas promoter in the absence of
270 lactogenic hormones (Kabotyanski et al., 2009). To define whether perinatal oral

271 exposure to BPA altered the histone modifiers HDAC1 and HDAC3, their mRNA
272 expression levels were evaluated. On LD2, HDAC1 mRNA level was up-regulated (Fig.
273 5A; $p<0.05$), whereas HDAC3 was down-regulated (Fig. 5B; $p<0.05$) in BPA-exposed
274 F1 females.

275 Another histone-modifying enzyme of interest is the enhancer of zeste homolog 2
276 (EZH2), a methyltransferase specific to histone 3 lysine 27, which is involved in gene
277 silencing and tumorigenesis (Kuzmichev et al., 2002; Rea et al., 2000). To evaluate
278 whether EZH2 expression was affected by BPA perinatal oral treatment, we analyzed its
279 mRNA expression. On LD2, both doses of BPA increased its expression in comparison
280 with control dams (Fig. 5C; $p<0.05$).

281 **3.4 Perinatal exposure to BPA induced alterations in DNAme and histone**
282 **modification in both the β-Cas promoter and the β-Cas enhancer.**

283 Epigenetic mechanisms like DNAme and histone modification play a key role in
284 transcriptional regulation of the casein genes during mammary gland development
285 (Qian and Zhao, 2014; Rijnkels et al., 2013; Rijnkels et al., 2010). To assess whether
286 perinatal exposure to BPA induced changes in DNAme and histone modifications in the
287 β-Cas proximal promoter and distal enhancer, methylation-sensitive and ChIP analyses
288 were performed on LD2.

289 First, the β-Cas gene was studied and searched for candidate sites for DNAme by a
290 Bioinformatic software. No CpG islands were observed in the β-Cas gene; however,
291 two DNAme-sensitive restriction sites for *MaeII* were found: one in the β-Cas promoter
292 and the other in the β-Cas enhancer (Fig. 6A and 6B). Both BPA-exposed groups
293 exhibited a decreased methylation state in the β-Cas promoter (Fig. 6C; $p<0.05$). In

294 contrast, the β-Cas enhancer showed an increased methylation state in BPA52 animals
295 and a decreased one in BPA0.6 ones (Fig. 6D; $p<0.05$).

296 Additionally, the levels of H3Ac and H3K27me3 in different regions of the β-Cas
297 promoter and enhancer were measured by ChIP assays followed by PCR analysis (Fig.
298 6E-H). In most of the regions analyzed, ChIP data showed a decreased level of H3Ac in
299 both BPA-exposed groups (Fig. 6E and 6F; $p<0.05$). In conjunction with histone
300 methylation analysis, BPA0.6-exposed dams revealed an increased H3K27me3 level in
301 the β-Cas promoter compared with control animals (Fig. 7E; $p<0.05$). Also, the level of
302 H3K27me3 was increased in the β-Cas enhancer of both BPA-exposed groups (Fig. 6H;
303 $p<0.05$).

304 **3.5 Perinatal exposure to BPA induced changes in DNAme and histone**
305 **modification in exon VII of the β-Cas gene.**

306 The β-Cas hydrophobic domain is encoded in exon VII and is involved in micelle
307 formation (Jones et al., 1985; Rijnkels, 2002). In the present study, we used this exon to
308 quantify β-Cas mRNA expression, as done by other researchers (Buser et al., 2011;
309 Kabotyanski et al., 2006; Kabotyanski et al., 2009; Winklehner-Jennewein et al., 1998).
310 As shown in Fig. 2A, its expression was decreased by perinatal exposure to BPA. To
311 identify whether this lower expression was due to epigenetic alterations, changes in
312 DNAme and histone modification were analyzed in this exon. DNAme changes in the
313 mammary gland were evaluated on the methylation sensitive site *BstUI* (Fig. 7A). This
314 site had a higher methylation state in BPA-exposed dams than in control animals (Fig.
315 7B; $p<0.05$). Then, the levels of H3Ac and H3K27me3 in different regions of β-Cas
316 exon VII were analyzed by ChIP assays (Fig. 7A). Although H3Ac level was increased
317 in the exon VII(a) region in both BPA-exposed groups, only BPA52-exposed F1 dams

318 revealed an increased expression of H3Ac in the exon VII(b) region and a decreased
319 expression in the exon VII(c) region of the β-Cas gene (Fig. 7C; $p<0.05$). In addition,
320 both BPA-exposed groups showed higher levels of H3K27me3 in all the regions of β-
321 Cas exon VII analyzed than in control animals (Fig. 7D; $p<0.05$).

322 **4 Discussion**

323 For decades, the β-Cas gene has been one of the milk protein genes most widely
324 studied, and its promoter extensively investigated as a model for hormone signaling
325 control of milk protein gene expression (Qian and Zhao, 2014). In previous research, we
326 have shown that perinatal exposure to low doses of BPA delays alveolar maturation
327 during gestation and early lactation, and impairs β-Cas synthesis and/or secretion during
328 late pregnancy (Altamirano et al., 2015; Kass et al., 2012). In the current study, we
329 demonstrated that β-Cas synthesis is also diminished in BPA-exposed F1 dams during
330 secretory activation of the mammary gland, and that different molecular mechanisms
331 could be responsible for this down-regulation.

332 A multistep process involving different signaling pathways that are led principally by
333 lactogenic hormones is required to activate β-Cas gene expression in mammary
334 epithelial cells (Anderson et al., 2007). During secretory activation, PRL increases the
335 transcription of milk protein genes through the activation of PRL-R and
336 phosphorylation and dimerization of Stat5 (Anderson et al., 2007) (Fig. 8A). Perinatal
337 exposure to BPA did not modify the expression of PRL-R or pStat5a/b on LD2 (Fig. 8
338 B,C). Interestingly, pStat5a/b expression in BPA-exposed F1 females was nuclear,
339 indicating that the PRL signaling pathway was active in these animals. pStat5a/b
340 interaction with the DNA is also influenced by the co-operation with GR. Ligand
341 activation of GR causes the formation of a GR-pStat5a/b complex and the subsequent

342 deviation to the Stat5-DNA binding site (Groner, 2002) (Fig. 8A). In our experiment,
343 the mRNA expression level of GR was lower in BPA52-exposed F1 dams and this
344 could be related to the decreased expression of β-Cas observed in these animals (Fig.
345 8C). In addition, it has been established that mammary epithelial cells, in the presence
346 of PRL, are able to induce DNA binding activity of pStat5a/b only when they are
347 cultured on laminin-rich ECM (Streuli et al., 1995). The structural organization of the
348 cell is an essential component of laminin signaling and this is mediated by α6β4 and β1
349 integrins as well as by E3 laminin receptors (Muschler et al., 1999). DAG1, considered
350 a co-receptor for laminin, is also required for the correct signaling through α6β4 and β1
351 integrins to allow efficient β-Cas production in mammary epithelial cells (Muschler et
352 al., 1999; Weir et al., 2006) (Fig. 8A). Our data showed that not only the mRNA
353 expression of ITGα6 and ITβ1 but also that of DAG1 were decreased in the lactating
354 mammary gland of BPA-exposed rats (Fig. 8B,C). Therefore, the diminished β-Cas
355 expression observed in BPA-exposed F1dams could be explained in part by a disruption
356 in the anchoring of laminin to the cell surface and, in consequence, a decreased
357 signaling to stimulate β-Cas expression. Furthermore, several studies have demonstrated
358 that deletion of the ITβ1 or DAG1 gene results in defective gland outgrowth, lactation
359 and Stat5 activity (Muschler and Streuli, 2010). Thus, the decrease in DAG1 and ITβ1
360 mRNA levels exhibited in BPA-exposed groups could be also associated with the delay
361 of alveolar maturation during secretory activation of the mammary gland observed in
362 our previous research (Altamirano et al., 2015; Kass et al., 2012).
363
364 Milk protein gene transcription requires not only the activation of transcription factors
365 but also chromatin remodeling during mammary gland development (Rijnkels et al.,
366 2010; Xu et al., 2007). Steroid hormones can induce permanent effects on gene activity

367 and program target genes to respond to secondary hormonal cues later in life
368 (Kundakovic and Champagne, 2011). There is evidence that exposure to BPA alters
369 enzymes involved in the epigenetic regulatory machinery such as DNA
370 methyltransferases, HDACs and EZH2 in different sensitive organs (Bhan et al., 2014;
371 Dhimolea et al., 2014; Doherty et al., 2010; Kundakovic and Champagne, 2011; Vigezzi
372 et al., 2016). In the present study, BPA-exposed F1 dams exhibited changes in mRNA
373 expression of histone-modifying enzymes during early lactation. We found that HDAC1
374 mRNA level was increased whereas HDAC3 was decreased in BPA-exposed groups
375 compared with control animals (Fig. 8B and C). It is known that HDAC enzymes
376 reverse acetylation and stabilize the local chromatin architecture, which is consistent
377 with HDACs being predominantly transcriptional repressors (Bannister and Kouzarides,
378 2011). However, in the β-Cas gene, HDAC1 and HDAC3 play a role in the sequential
379 formation of complexes that leads to its activation after hormonal stimulation
380 (Kabotyanski et al., 2009). YY-1 binds to the β-Cas promoter, interacting with C/EBP
381 and HDAC3, and represses its expression in the absence of lactogenic hormones. In
382 contrast, PRL signaling pathway activation induces the dimerization, translocation and
383 DNA binding of Stat5, which promotes both the displacement of YY-1 and HDAC3,
384 and recruits HDAC1 to activate β-Cas gene transcription (Kabotyanski et al., 2009). If
385 we relate our results with this activation pathway, β-Cas gene expression should be up-
386 regulated in BPA-exposed F1 females; however, we analyzed HDAC1 and HDAC3
387 expression in the whole mammary gland and not on the β-Cas gene itself. Therefore, the
388 BPA-induced changes observed in the present work could be modifying HDAC
389 transcriptional repressor activity in genes not studied herein. Also, BPA-induced
390 DNAme of exon VII or histone modifications could be more important regarding down-
391 regulating β-Cas expression than the increase in HDAC3 mRNA. Additionally, many *in*

392 *vitro* and *in vivo* studies have evidenced that exposure to EDCs such as BPA and
393 diethylstilbestrol enhances EZH2 expression in mammary epithelial cells and that EZH2
394 is an estradiol-regulated gene (Bhan et al., 2014; Doherty et al., 2010). In agreement, in
395 our experiment, BPA perinatal exposure stimulated the expression of EZH2 mRNA
396 levels in the mammary gland of early lactating F1 dams (Fig. 8B and C). It is thought
397 that EZH2 is critical in gene silencing and chromatin condensation (Bhan et al., 2014).
398 Therefore, in BPA-exposed dams, a more condensed chromatin conformation could be
399 found in the β-Cas gene region, down-regulating its expression.

400 Among epigenetic modifications, DNA methylation and histone modifications have
401 been most intensively studied in the context of gene transcription and abnormal events
402 (Vaissiere et al., 2008). In the mammary gland, the extensive cell proliferation during
403 pregnancy and early lactation lead to passive demethylation of milk protein gene
404 regulatory elements, which correlates with the induction of gene expression (Rijnkels et
405 al., 2010). Moreover, certain restriction sites in the rat β-Cas gene from the lactating
406 mammary gland are readily digested by the methylation-sensitive restriction enzymes,
407 resulting in a hypomethylated state specific to the gland (Johnson et al., 1983) (Fig. 8A).
408 By using a similar strategy, we found alterations in the methylation status of DNAme
409 sensitive restriction sites of the β-Cas promoter and enhancer in BPA-exposed groups
410 (Fig. 8B,C). In the β-Cas promoter, we identified one *MaeII* site next to a ½ GRE-
411 biding site that was hypomethylated in both BPA-exposed groups (Fig. 8B,C).
412 Vanselow et al. (2006) characterized a novel Stat5-binding site in the enhancer of the
413 bovine α-S1-casein-encoding gene and proved that the CpG methylation of this area is
414 associated with a down-regulation of αS1-casein synthesis. Thereby, we decided to
415 evaluate the *MaeII* site located near a Stat5-binding site in the β-Cas enhancer. This
416 *MaeII* site was hypomethylated in BPA0.6-exposed dams (Fig. 8B) and

417 hypermethylated in BPA52-exposed dams (Fig. 8C). Based on these findings, β-Cas
418 synthesis should be increased in BPA0.6-exposed dams and decreased in BPA52-
419 exposed F1 dams. Additionally, the enrichment of H3Ac in the regulatory region of
420 casein genes has been shown to enhance their transcription rate due to a relaxed
421 chromatin structure (Rijnkels et al., 2013) (Fig. 8A). Here, we showed a lower presence
422 of H3Ac in the β-Cas promoter and enhancer in both BPA-exposed groups compared
423 with control animals (Fig. 8B,C). The deacetylation of H3 histone results in the
424 compaction of the chromatin structure and, therefore, can reduce the transcription of the
425 β-Cas gene. Moreover, the histone H3 hypoacetylation observed in these animals could
426 be related to the disruption of the anchoring of laminin to the cell surface of the
427 epithelial cells suggested in the BPA-exposed animals, taking into consideration that the
428 cooperation between laminin and PRL is necessary to induce histone acetylation in the
429 β-Cas promoter (Xu et al., 2007). Also, histone H3 hypoacetylation could be related to
430 laminin-ECM-induced changes in the actin cytoskeleton, as suggested by Le Beyec et
431 al. (2007); however, no differences in morphology, size or cytoskeletal organization
432 were noticed in mammary epithelial cells between experimental groups. Another histone
433 modification associated with gene silencing is histone H3K27 methylation (Bhan et al.,
434 2014). In our experiment, in the β-Cas promoter, H3K27me3 level was increased only
435 in BPA0.6-exposed lactating rats whereas in the β-Cas enhancer it was increased in both
436 BPA-exposed groups (Fig. 8B and C). The increase in H3K27me3 in the β-Cas
437 promoter or enhancer could be related to the higher EZH2 expression found in BPA-
438 exposed animals, considering that EZH2 possesses histone H3K27-specific
439 methyltransferase activity and is a key epigenetic regulator (Kuzmichev et al., 2002).
440 Our results indicate that the modification of the chromatin conformation in the
441 regulatory regions of the β-Cas gene is an important mechanism of BPA action on β-

442 Cas gene transcription during secretory activation of the mammary gland. Additionally, 443 β-Cas exon VII was also used to evaluate the DNAme state and histone modification. In 444 both BPA-exposed groups, DNAme and histone methylation of H3K27me3 were 445 increased in this exon. Conversely, the recruitment of H3Ac to β-Cas exon VII was 446 higher in some of the regions evaluated by ChIP, indicating an open chromatin structure 447 in these regions (Fig. 8B,C).

448 Taken together, our results show that perinatal exposure to BPA reduces the expression 449 of β-Cas through different molecular mechanisms during secretory activation in the rat 450 mammary gland (Fig. 8). Epigenetic modifications and an impaired crosstalk between 451 ECM and lactogenic hormone signaling pathways seem to act synergistically to down-452 regulate β-Cas expression. These findings underline the high sensitivity to BPA 453 exposure during the *in utero* and lactation period of the offspring, and the lasting 454 consequences on milk protein synthesis during secretory activation of the mammary 455 gland.

456 **Declaration of interest**

457 The authors declare that there are no conflicts of interest that could be perceived as 458 prejudicing the impartiality of the research reported.

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621 **FIGURE LEGENDS**

622 **Figure 1.** Schematic representation of the experimental protocol used to study the
623 effects of perinatal (gestation + lactation) exposure to low doses of BPA on the
624 mammary glands of F1 females during early lactation. GD: gestation day; LD: lactation
625 day; PND: postnatal day.

626 **Figure 2.** β-Cas expression during early lactation. **(A)** qRT-PCR quantification of β-Cas
627 mRNA expression in F1 dams on LD2. The bars represent the mean value ± SE of 8
628 animals/group. The samples were normalized to L19 expression and to the control
629 animals, and a value of 1 was assigned to the control group (* $p<0.05$, Mann-Whitney
630 test). **(B)** Representative IF images of β-Cas expression in mammary alveoli on LD2.
631 The cytoplasm and the apical lumen of the alveolar cell were positive for β-Cas (green)
632 while the nuclei (blue, DAPI +) were negative. On LD2, both BPA-exposed groups had
633 a lower intensity of β-Cas staining than the Control group, in accordance with the
634 results from the mRNA expression. Scale bar = 40 μm.

635 **Figure 3.** PRL signaling pathway and GR expression in the mammary glands of BPA-
636 exposed F1 dams. The mRNA expression level of PRL-R **(A)** and GR **(B)** were
637 quantified during secretory activation. The bars represent the mean value ± SE of 8
638 animals/group. The samples were normalized to L19 expression and to the control
639 animals, and a value of 1 was assigned to the control group (* $p<0.05$, Mann-Whitney
640 test). **(C)** Representative IHC images of pStat5a/b expression in the mammary gland.
641 Nuclear pStat5a/b expression was observed in all experimental groups. Scale bar = 20
642 μm.

643 **Figure 4.** Expression of laminin receptors in the lactating mammary gland of BPA-
644 exposed F1 dams. The mRNA expression levels of ITGα6 **(A)**, ITβ1 **(B)** and DAG1 **(C)**
645 were evaluated on LD2. The bars represent the mean value ± SE of 8 animals/group.
646 The samples were normalized to L19 expression and to the control animals, and a value
647 of 1 was assigned to the control group (* p<0.05, Mann-Whitney test).

648 **Figure 5.** Expression of histone modifiers enzymes involved in the chromatin
649 conformation of the mammary gland during early lactation. **(A)** HDAC1**, (B)** HDAC3
650 and **(C)** EZH2 mRNA expression was quantified on LD2. The bars represent the mean
651 value ± SE of 8 animals/group. The samples were normalized to L19 expression and to
652 the control animals, and a value of 1 was assigned to the control group (* p<0.05,
653 Mann-Whitney test).

654 **Figure 6.** DNAme and histone modifications analysis in the regulatory regions of β-Cas
655 gene during early lactation. Schematic representation of the β-Cas promoter **(A)** and
656 enhancer **(B)**, their binding proteins, methylation targeted CG areas and of the
657 amplicons used in ChIP assays. The positions of the TATA box are indicated. Predicted
658 binding sites for Signal transducer and activator of transcription 5 (Stat5),
659 CCAAT/enhancer binding protein (C/EBP), and Yin Yang 1 (YY-1) binding sites, half
660 glucocorticoid response elements (½ GREs) for glucocorticoid receptor, octamer
661 binding factor-1 (Oct-1), the E26 transformation-specific (Ets) site and nuclear factor 1
662 (NF-1) binding site are shown as different shapes. CG target sites for digestion by the
663 methylation-sensitive restriction enzyme *Mae II* (ACGT) are indicated. Methylation-
664 sensitive analysis of β-Cas promoter **(C)** and enhancer **(D)** was carried out on LD2. The
665 bars represent the mean value ± SE of 8 animals/group. The samples were normalized to
666 an internal control expression and to the control animals, and a value of 1 was assigned
667 to the control group (* p<0.05, Mann-Whitney test). Histone acetylation (H3Ac) was

668 determined in different regions of the β-Cas promoter **(E)** and β-Cas enhancer **(F)** by
669 ChIP assays. Also, histone methylation (H3K27me3) was evaluated in different regions
670 of the β-Cas promoter **(G)** and β-Cas enhancer **(H)** by ChIP analysis. The bars represent
671 the mean value ± SE of 8 animals/group. The samples were normalized to INPUT
672 expression and to the control animals, and a value of 1 was assigned to the control
673 group (* $p<0.05$, Mann-Whitney test).

674 **Figure 7.** DNAme and histone modifications analysis in the exon VII of the β-Cas gene.
675 **(A)** Map of the β-Cas exon VII. CG target sites for digestion by the methylation-
676 sensitive restriction enzyme *BstUI* (CGCG) is indicated as well as the amplicons used in
677 ChIP assay. **(B)** Methylation state level of *BstUI* site was determined in β-Cas exon VII
678 on LD2. The bars represent the mean value ± SE of 8 animals/group. The samples were
679 normalized to internal control expression and to the control animals, and a value of 1
680 was assigned to the control group (* $p<0.05$, Mann-Whitney test). ChIP assays to
681 investigate **(C)** histone acetylation (H3Ac) and **(D)** histone methylation (H3K27me3) in
682 β-Cas exon VII were done on LD2. The bars represent the mean value ± SE of 8
683 animals/group. The samples were normalized to INPUT expression and to the control
684 animals, and a value of 1 was assigned to the control group (* $p<0.05$, Mann-Whitney
685 test).

686 **Figure 8.** Bisphenol A regulation of β-Cas gene expression. **(A)** Integration signals
687 from laminin, PRL and glucocorticoids to induce β-Cas expression in Control F1 dams.
688 One of the most important signaling pathways that regulate β-Cas gene expression is the
689 activation of PRL-R, that together with laminin allows the sustained activation of Stat5
690 that it is necessary for chromatin remodeling of mammary-specific gene loci and histone
691 acetylation in β-Cas promoter. Perinatal exposure to BPA **(B and C)** induced a
692 reduction in the expression of laminin-binding receptors leading possibly to a transient

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693 Stat5 activation which is not sufficient to fully activate β-Cas gene expression. In
694 addition, the reduced presence of H3Ac in β-Cas promoter and enhancer, indicating a
695 deacetylation of histones, and the increase in EZH2 expression could be related to the β-
696 Cas gene decline in BPA-exposed F1 dams. Beside the deacetylation of histones,
697 BPA0.6 increased H3K27me3 levels in both β-Cas promoter and enhancer, resulting in
698 chromatin compaction and, in consequence a disrupted β-Cas gene expression **(B)**. The
699 lower GR expression and the increased DNAme and H3K27me3 levels in β-Cas
700 enhancer could also be related with the decrease expression of β-Cas in BPA52-exposed
701 dams **(C)**. The dash arrows show the signal transduction pathways altered by perinatal
702 exposure to BPA. α-Dyst: α-dystroglycan; β-Dyst: β-dystroglycan; β-Cas: β-Cas; Ac:
703 histone acetylation; EZH2: enhancer of zeste homolog 2; GR: glucocorticoids receptor;
704 HDAC: histone deacetylase; Me: histone methylation; me: DNA methylation; Stat5:
705 signal transducer and activator of transcription 5; PRL: prolactin; RPLR: prolactin
706 receptor.

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Table 1: Primers used for qRT-PCR

Gene	Primer sense (5'→ 3')	Primer antisense (5'→ 3')
β-Cas	AAACATCCAGCCTATTGCTC	CATCTGTTTGTGCTTGGGAA
PRLR	AAAGTATCTTGTCCAGACTCGCTG	AAACAGATGACAGCAGAGAGAATG
GR	ACAGCATCCCTTTCTCAGCA	CTCATTCCAGGGCTTGAGTAC
ITGA6	CTCGTGAGGGCGTCCATAGA	TCCACCACGCTATCCCTGA
ITB1	GGCTGAAGACTACCCTATTG	GCGGAAGTCTGAAGTAATCC
DAG1	AACCAGCTTGAGGCGTCCAT	CTGTTGGAATGCTCACTCGG
HDAC1	CAATGAAGCCTCACCGAATC	TTGGTCATCTCCTCAGCGTT
HDAC3	CAACTGGGCTGGTGGTCTAC	CGAGGGTGGTACTTGAGCAG
EZH2	GATTTTCCAGCACAAGTCAT	AACAGTTTCATCTTCCACCA
L19	AGCCTGTGACTGTCCATTCC	TGGCAGTACCCTTCCTCTTC

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Table 2: Primers used for methylation-sensitive and ChIP analyses

Gene	Primer sense (5'→ 3')	Primer antisense (5'→ 3')
Analysis of DNA methylation		
Internal Control Enhancer	GACTTAGAATGCCCACAGACTTAA	CGAGTCTGTTTTACAGCAATGG
MaeII Enhancer	AAAAGGAAATGTTGCCTGGG	TTGGCTGCCTTGTGTATCCT
Internal Control Promoter	TGTCCCCCAGAATTTCTTGG	GGATGATGGTCTATCAGACTCTGTG
MaeII (a) Promoter	TAGGTGAGGTTAAAGCCCCC	GGTGAAGGAACTTGTCCAGG
MaeII (b) Promoter	TCTTGTCCTCCGCTAAAGGT	CTGACTTTCAAAAGCCTGTCC
Internal Control Exon VII	ATTCCACAAAACATCCAGCC	CATCTGTTTGTGCTTGGGAA
BstUI Exon VII	ACCTTCCTCAGTCTCCAGCC	GGAAGAGGGGTGCTAGTTGC
Analysis of histone modification		
Enhancer (a)	TTTTTTTTTGGCTCAGACCA	AACACAGAAGTAAGCAAGCCTTA
Enhancer (b)	TTTCTTAGAAGTCACGTATTGTTGC	TGAATTAAGTCTGTGGGCATTCT
Promoter (a)	TGTGTACTAGGCTGGAGAGA	TCTGGGGGACATTAAACAAG
Promoter (b)	TGTCCCCCAGAATTTCTTGG	GGATGATGGTCTATCAGACTCTGTG
Exon VII (a)	ATTCCACAAAACATCCAGCC	CATCTGTTTGTGCTTGGGAA
Exon VII (b)	ACCTTCCTCAGTCTCCAGCC	GGAAGAGGGGTGCTAGTTGC
Exon VII (c)	AACTAGCACCCCTCTTCCAA	GGAATGAAGTTGTTGAGTGGC

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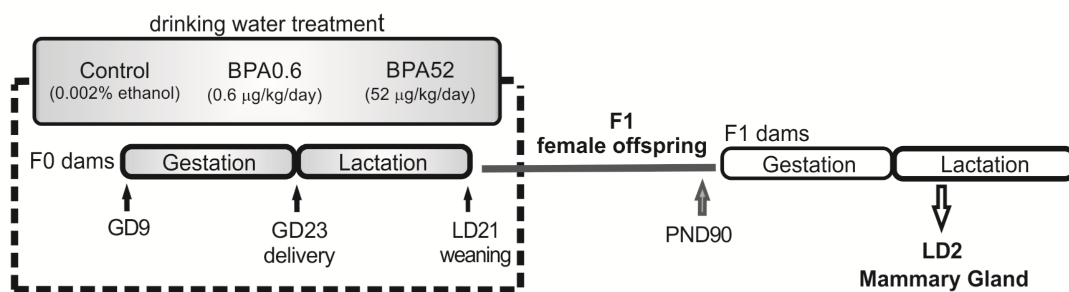

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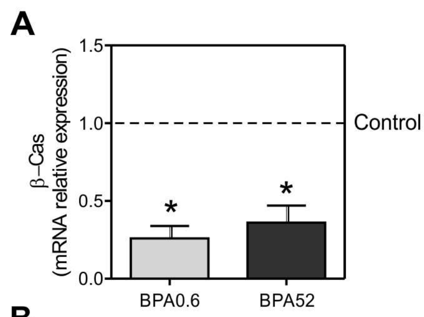

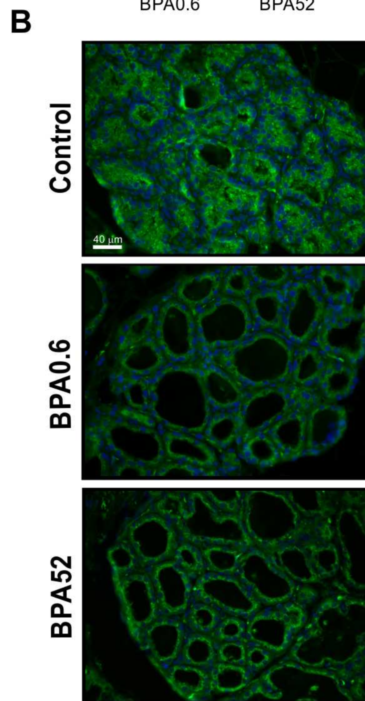

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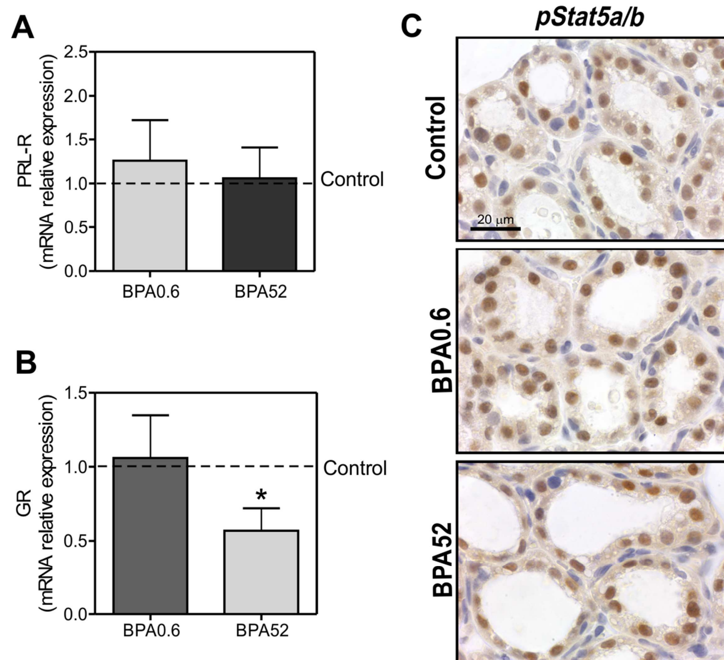

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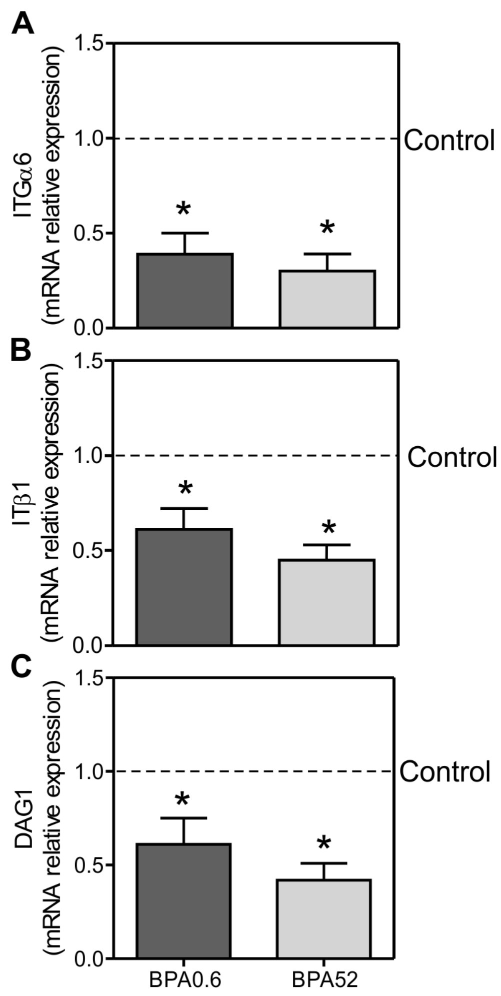

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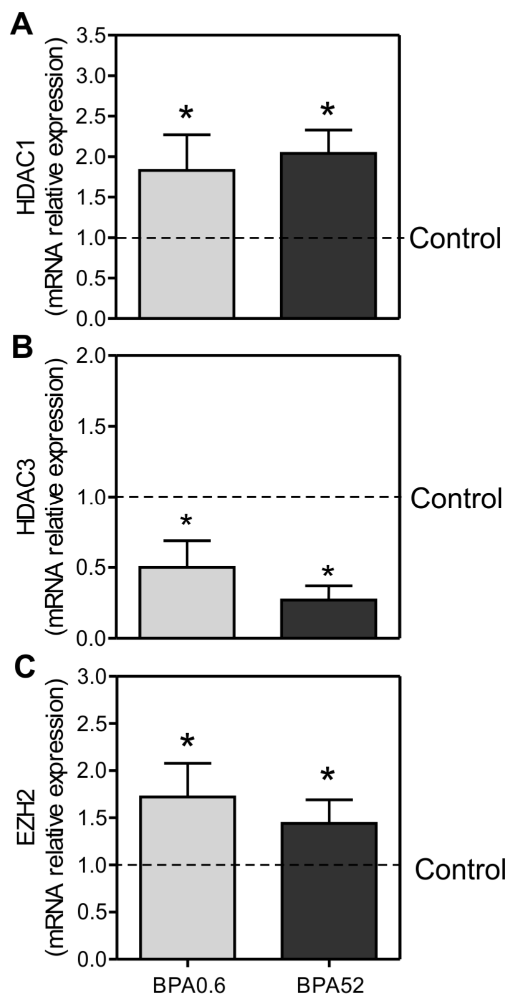

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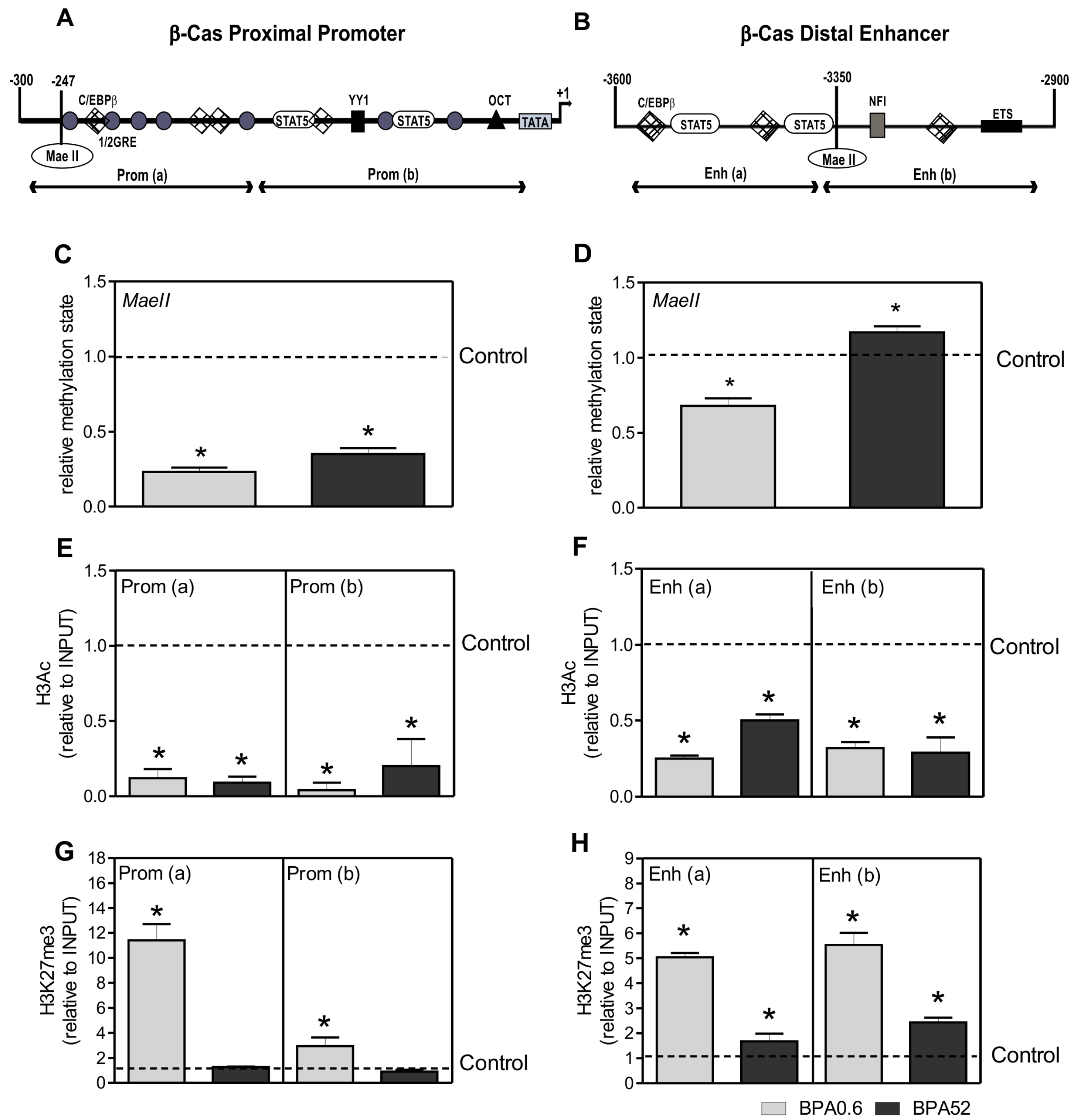

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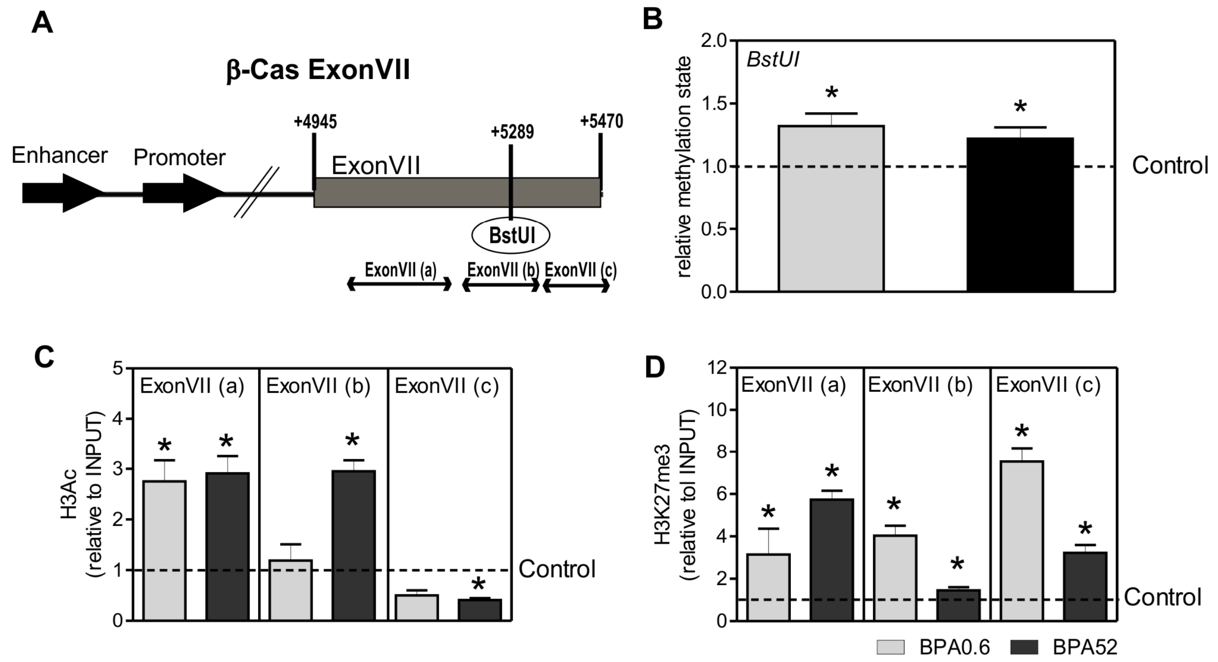

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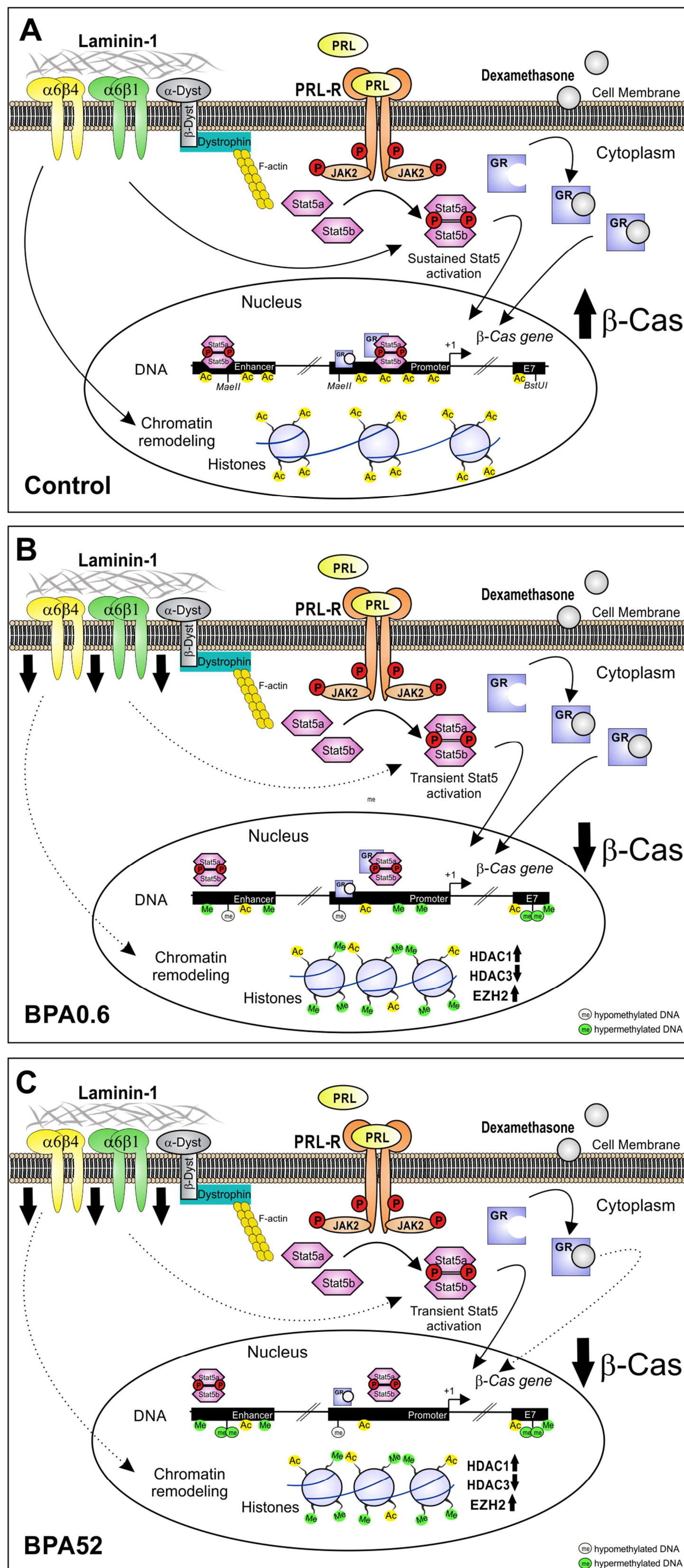
A
Laminin-1
PRL
α6β4
α6β1
α-Dyst
PRL-R
Dexamethasone
Cell Membrane
β-Dyst
Dystrophin
F-actin
JAK2
JAK2
Cytoplasm
GR
Stat5a
Stat5b
Sustained Stat5 activation
Nucleus
β-Cas
β-Cas gene
DNA
Enhancer
Promoter
E7
MaeII
BstUI
Chromatin remodeling
Histones
Control
B
Transient Stat5 activation
HDAC1
HDAC3
EZH2
hypomethylated DNA
hypermethylated DNA
BPA0.6
C
BPA52

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HIGHLIGHTS

- Perinatal BPA exposure decreased β-Cas gene expression independently of PRL signaling
- BPA altered the expression of HDAC1, HDAC3 and EZH2 in the lactating mammary gland
- BPA induced acetylation and methylation of histones in the β-Cas gene
- BPA induced changes in the DNA methylation status of the β-Cas gene

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