MATERNAL EXPOSURE TO BISPHENOL-A DURING PREGNANCY INCREASES PANCREATIC B-CELL GROWTH DURING EARLY LIFE IN MALE MICE OFFSPRING

Marta García-Arévalo*[1,4], Paloma Alonso-Magdalena*[2,4], Joan-Marc Servitja[3,4], Talía Boronat[1,4], Beatriz Merino[1,4], Sabrina Villar[1,4], Gema Medina-Gómez[5], Anna Novials[3,4], Ivan Quesada[1,4] and Angel Nadal[1,4]

[1] Institute of Bioengineering and [2]Department of Applied Biology, Miguel Hernández University of Elche, Elche, Alicante, Spain.
[3]Diabetes and Obesity Research Laboratory, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, Spain
[4]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, CIBERDEM, Spain.
[5] Department of Basic Sciences of Health, Area of Biochemistry and Molecular Biology, Rey Juan Carlos University, Madrid, Spain.

*These authors contributed equally to this work.

Abbreviate title: Maternal exposure to BPA and offspring β-cell mass

Key terms: Endocrine disruptors, β-cells, bisphenol-A
Word count: 5433
Number of figures and tables: 6 figures, 2 tables

Correspondence should be addressed to:

Prof. Angel Nadal
Instituto de Bioingeniería and CIBERDEM
Universidad Miguel Hernández de Elche
Avenida de la Universidad s/n
03202 Elche, Spain.
Phone: (+34) 96 5222002
Fax: (+34) 965222033
Email: nadal@umh.es

Disclosure statement: The authors have nothing to disclose

Alterations during development of metabolic key organs such as the endocrine pancreas affect the phenotype later in life. There is evidence that *in utero* or perinatal exposure to bisphenol-A (BPA), leads to impaired glucose metabolism during adulthood. However, how BPA exposure during pregnancy affects pancreatic β-cell growth and function in offspring during early life has not been explored. We exposed pregnant mice to either vehicle (Control) or BPA (10 and 100 µg/kg/day, BPA10 and BPA100) and examined offspring on postnatal days (P) P0, P21, P30 and P120. BPA10 and BPA100 mice presented lower birth weight than Control and subsequently gained weight until day 30. At that age, concentration of plasma insulin, C-peptide and leptin were increased in BPA-exposed animals in non-fasting state. Insulin secretion and content were diminished in BPA10 and maintained in BPA100 compared to Control. A global gene expression analysis indicated that genes related with cell division were increased in islets from BPA-treated animals. This was associated with an increase in pancreatic β-cell mass at P0, P21 and P30, together with increased β-cell proliferation and decreased apoptosis. On the contrary, at P120, BPA treated animals presented either equal or decreased β-cell mass compared to Control and altered fasting glucose levels. These data suggest that *in utero* exposure to environmentally relevant doses of BPA alters the expression of genes involved in β-cell growth regulation, incrementing β-cell mass/area and β-cell proliferation during early life. An excess of insulin signaling during early life may contribute to impaired glucose tolerance during adulthood.

59 INTRODUCTION

60 Chronic diseases like diabetes and obesity are due to gene-by-environment interactions over time,
61 starting during fetal development**.** The developmental origins of health and disease (DOHaD)
62 hypothesis proposes that "an adverse environment experienced by a developing individual can
63 increase the risk of diseases later in life" (1). This hypothesis was formulated after the work by Barker
64 (2) based on the strong association between poor nutrition during intrauterine life and the increased
65 incidence of metabolic disorders among the offspring. Thus, maternal nutrition during early
66 development is considered a major intrauterine environmental factor influencing the development and
67 progression of obesity and type 2 diabetes later in life. In addition, the metabolic conditions of the
68 mother affect the development of the endocrine pancreas. This is extremely important since fetal life
69 represents a critical period of time in which a correct β-cell function and an appropriate β-cell mass
70 are set in place. A substantial number of animal models have been developed to elucidate the
71 consequences and mechanisms in maternal overnutrition and malnutrition. The former includes
72 animal models of obesity or high fat diet (3-5) and the later include low protein diet (6, 7) or low
73 energy diet (8-10) as well as models of hypoxia (11), gestational diabetes (12), hyperglycaemia (13)
74 and insulin resistance (14). In most of these models β-cell mass, β-cell function or both are altered.
75 Exposure to EDCs during pregnancy has been recognized for decades to cause adverse outcomes in
76 progenies, both in humans and in animal models (15, 16). One early and well-studied example was in
77 utero exposure to diethylstilbestrol (DES), a potent non-steroidal estrogen drug designed by Dodds in
78 1936 (17, 18) and prescribed from 1940 to 1975 as an antiabortive drug. In the 1970s it was proved
79 that exposed daughters presented clear-cell adenocarcinomas at an early age (19, 20). Remarkably,
80 work with animal models reproduced the effects clinically detected in humans (21). Like DES, BPA
81 was demonstrated to have estrogenic activity at about the same time (17, 18), but because DES
82 resulted to have stronger activity than BPA, DES was used in clinical practice.
83 In the 1950s BPA was rediscovered as a compound that could be polymerized to make polycarbonate
84 plastic. From that moment it has been extensively used in the plastic industry with approximately 15
85 billion pounds per year of BPA produced annually in the world (22)

86 In addition to its role as the base component of polycarbonate plastic, BPA is used to produce epoxy
87 resins for the coating of pipes and metal equipment and the lining of food cans (23) as well as a
88 plasticizer in the manufacture of other plastics such as PVC (24). Heat, acid or basic media have been
89 shown to cause the leaching of the monomer to the environment (25).

90

91 It was described that BPA has lower affinity than 17-β-estradiol for the nuclear receptors ERα and
92 ERβ which will act as transcription factors binding to estrogen response elements in the DNA (26,
93 27). More recently we have proposed that it can behave also as a potent estrogen (within the
94 picoMolar-nanoMolar range) in β-cells when binding ERα and ERβ out of the nucleus. In this
95 manner, BPA triggers the activation of different signaling pathways, involving kinases as well as the
96 activation of other transcription factors which could explain many of the low doses effects of BPA
97 (28-30)

98 BPA is a widespread EDC which has been found in the urine of 93% of USA citizens (31). Its
99 concentration ranges within the nanograms per mL reported by some authors (32-34) and the
100 picogram per mL range reported by other authors (35). In any case, exposure of mice and rats to BPA
101 at low doses during pregnancy, or pregnancy and lactation, produced alterations in blood glucose
102 homeostasis and β-cell function in male adult offspring (36-40). The adult phenotype is dependent on
103 gender, age, dose and timing of exposure; yet in the majority of reports there is insulin resistance,
104 glucose intolerance, hyperinsulinemia and alteration in blood adipokine levels. In particular
105 alterations in glucose homeostasis was observed in adult offspring (between 3 and 8 months of life)
106 after BPA exposure through gestation or gestation and lactation in OF-1 mice, CD-1 mice or rats at
107 doses of 3.5, 5, 10, 40, 50 or 100 μg/kg/day (36-40, 42-46). No effect on glucose metabolism was
108 observed when exposure occurred at lower levels 2.5 ng/kg/day (47).

109

110 In the present study, we used pregnant mice exposed to environmentally relevant doses of BPA to
111 determine how BPA exposure affects glucose homeostasis, β-cell function and β-cell mass at an early
112 age in offspring. Based on the United States-Environmental Protection Agency (U.S.-EPA) criterion

113 for low-dose effects of EDCs, we considered levels below the current lowest observed effect level
114 (LOAEL) of 50 μg/kg/day as low doses for *in vivo* studies. Our hypothesis is that exposure during
115 pregnancy to BPA will alter these parameters at the beginning of life. This could be connected with
116 the increased susceptibility to the development of type 2 diabetes observed later in life. We based our
117 hypothesis in published results of undernutrition during pregnancy which showed altered β-cells mass
118 and function as described in the first paragraph. Our results demonstrate that intrauterine exposure to
119 BPA is an important environmental factor that promotes early structural and functional changes in
120 pancreatic β-cells.
121

122 MATERIALS AND METHODS

123

124 **Animals and treatment**

125 Pregnant OF-1 mice were purchased from Charles River (Barcelona, Spain) and individually housed
126 under standard conditions. Mice were maintained on 2014 Teklad Global 14% Protein Rodent
127 Maintenance Diet (Harlan Laboratories, Barcelona, Spain), which does not contain alfalfa or soybean
128 meal. The composition of the diet is as follows: calories from protein, 18%; calories from fat, 11%;
129 and calories from carbohydrate, 71%, with energy of 2.9 kcal/g. Bisphenol-A (MP Biomedicals, cat.
130 No. 155118) and 17-β-estradiol (E2) (Sigma, cat. No. E8875) were dissolved in tocopherol-stripped
131 corn oil (MP Biomedicals, cat. No. 901415, Illkirch, France) and administered subcutaneously on
132 days 9–16 of gestation. The daily dose used was 10 or 100 µg/kg in a constant volume of 100µL,
133 either to vehicle. For BPA experiments 192 pregnant mice were used in the study (control n=73;
134 BPA10 n=63; BPA100 n=56). For E2 experiments 18 pregnant mice were used (control n=10; E10
135 n=8). We selected litters with a number of pups between 10 and 12 only, to avoid pups/litter number
136 as a variable. After weighting at P0, pups from the same treatment were pooled together and then
137 placed in equal number with foster mothers of the same treatment group. The litter size was
138 maintained constant. Animals were sexed and weaned on postnatal day 21. They were housed (7 male
139 mice/group) from weaning through day of sacrifice. After weaning, they were maintained, ad libitum,
140 on diet described above. Experiments were performed when mice were on postnatal days (P) P0, P21,
141 P30 and P120.

142 The ethical committee of Miguel Hernandez University "Comisión de Ética en la Investigación
143 Experimental" specifically reviewed and approved this study (approvals ID: UMH-IB-AN-01-14 and
144 IB-PAM-01-15). Animals were treated humanely and with regard to alleviate suffering.

145 All experiments have been done in non-fasting condition. Only the group of animals used for
146 performing the glucose tolerance test was maintained in fasted state for 12 h (n=6-14 animals from 6-
147 10 litters). In addition, a second group of animals was also fasted (12 h) for taking blood samples and
148 measuring insulin plasma levels (n=9-14 animals from 8-14 litters).

149

150 **Islet cell isolation**

151 Pancreatic islets of Langerhans were isolated by collagenase (Sigma, Madrid, Spain) digestion
152 (modified from (48)) The solution used for the isolation of the islets of Langerhans contained (in
153 mmol/l): 115 NaCl, 10 $NaHCO_3$, 5 KCl, 1.1 $MgCl_2$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 25 HEPES, and 5 D-
154 glucose, pH 7.4, as well as 0.25% BSA. Freshly isolated islets were used for insulin secretion and
155 content measurements after 2 hours of recovery.

156

157 **Glucose and insulin tolerance tests**

158 For intraperitoneal glucose tolerance tests (ipGTT), animals were fasted for 12 h, and blood samples
159 were obtained from the tail vein. Animals were then injected intraperitoneally with 2g/kg body weight
160 of glucose, and blood samples were taken at the indicated intervals.

161 For intraperitoneal insulin tolerance tests (ipITT), fed animals were used. Animals were injected
162 intraperitoneally with 0.75 IU/kg body weight of soluble insulin (Lilly), and blood samples were
163 obtained from the tail vein. Blood glucose was measured in each sample using an Accu-check
164 compact glucometer (Roche, Madrid, Spain). Levels of glycemia after insulin injection are expressed
165 as % of glycemia compared to basal glycemia levels in fed state.

166

167 **Serum analysis**

168 Blood samples were collected for biochemical analysis at decapitation in fed or fasted (12 hours) state
169 animals. Serum samples were obtained by centrifugation for 15 minutes at 1200 rpm at 4ºC. Samples
170 were stored at -80ºC. The serum insulin level was analyzed by Ultra Sensitive Mouse Insulin ELISA
171 Kit (Crystal Chem, Downers Grove, IL). C-peptide level was determined using C-peptide (mouse)
172 ELISA (Alpco immunoassays, Salem, NH). Leptin level was analyzed by Mouse Leptin ELISA kit
173 (Crystal Chem, Downers Grove, IL). Non-esterified fatty acids (NEFAs) were measured using a
174 NEFA-HR(2) kit for serum determination (Wako).

175 Triglycerides and Cholesterol levels were measured using sample provide from the tail vein and were
176 analyzed using Accutrend Plus (Roche, Madrid, Spain).

Insulin secretion and content.

Freshly isolated islets were left to recover in the isolation medium for 2h in the incubator at 37ºC and 0.5% CO_2. After recovery, groups of 5 islets were transferred to 400µl of a buffer solution containing 140 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 20mM HEPES and the corresponding glucose concentration (3, 8 or 16mM) with final pH at 7.4. Afterwards, 100µl of the buffer solution with the corresponding glucose concentration with 5% BSA was added. Then, the medium was collected and insulin was measured in duplicate samples by radioimmunoassay using a Coat-a-Count kit (Siemens, Los Angeles, CA, USA). Protein concentration was measured by the Bradford dye method (49).

To obtain the insulin content, groups of 5 islets had incubated overnight in an ethanol/HCl buffer (75 % Ethanol ($^{v}/_{v}$); 0.4 % HCl (stock 37%) ($^{v}/_{v}$) and 24.6 % distilled water ($^{v}/_{v}$)) at 4°C. At the end of the incubation period, the buffer was removed and studied for insulin content using radioimmunoassay with a Coat-a-Count kit. Protein determination was performed using the Bradford dye method.

Global gene-expression profiling.

RNA from mouse pancreatic islets was hybridized onto GeneChip® Mouse Genome 430 2.0 Array (Affymetrix). Expression data were normalized with RMA, and the LIMMA package was used for statistical analysis to identify differentially expressed genes, as described elsewhere (50). To generate gene cluster representations, expression levels of each gene were normalized across all samples analyzed and then clustered based on their similarity according to the Euclidian distance using Cluster3.0. Clusters were represented using Treeview1.1.1. Data have been deposited in Gene Expression Omnibus (51), accession number GSE82175. The DAVID Functional Annotation Tool (52) was used to identify enriched functional categories in differentially expressed genes.

Real-time PCR

Quantitative PCR assays were performed using CFX96 Real Time System (Bio-Rad, Hercules, CA) and 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Groups from 150 isolated

205 islets were used for RNA extraction. RNA extraction was made with RNeasy Micro Kit (Qiagen,
206 USA), and 0.5µg of RNA was used for retrotranscription reaction (HighCapacity cDNA Reverse
207 transcription, Applied Biosystems). Reactions were carried out in a final volume of 10 µl, containing
208 200 nM of each primer, 1 µl of cDNA, and 1× IQ SYBR Green Supermix (Bio-Rad). Samples were
209 subjected to the following conditions: 10 min at 95ºC, 40 cycles (10 s at 95ºC, 7 s at 60ºC, and 12 s at
210 72º C), and a melting curve of 63–95ºC with a slope of 0.1 C/s. Expression levels were normalized to
211 the expression of Hprt1. The resulting values were analyzed with CFX Manager Version 1.6 (Bio-
212 Rad), and values were expressed as the relative expression respect to control levels ($2^{-\Delta\Delta CT}$) (53).
213 Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta
214 C(T)). Primer sequences are listed in supplementary material (Table 1).
215

216 **Immunohistochemistry and β-cell mass**

217 Pancreas samples from 5-8 different mice per experimental condition from 5-8 different litters (see
218 figure legend), were removed and fixed overnight in 4% paraformaldehyde. Subsequently, pancreatic
219 tissue was embedded in paraffin and sections were prepared. After dehydration, sections were heated
220 to 100°C in the presence of citrate buffer (10 mM; pH 6.0) for 20 min. Endogenous peroxidase was
221 blocked by incubation for 30 min with a solution of 3% hydrogen peroxidase in 50% methanol. To
222 block nonspecific binding, sections were incubated in 3% BSA in PBS for 1 h at room temperature.
223 Tissue sections were then stained for β-cells with a rabbit antihuman insulin antibody (1:100; Santa
224 Cruz Biotechnology, Inc., Santa Cruz, CA) (table 1), overnight at 4°C. After washing, sections were
225 incubated with the secondary antibody biotinylated anti-rabbit IgG (H+L) (Vector laboratories, CA)
226 for 1 h at room temperature. The Vectastain ABC kit (Vector Laboratories, CA) was used for the
227 avidin-biotin complex (ABC) method according the manufacturer′s instructions. Peroxidase activity
228 was visualized with 3, 3′-diaminobenzidine (DAKO, CA). The sections were lightly counterstained
229 with hematoxylin, dehydrated through an ethanol series to xylene, and mounted. For morphometric
230 analysis, 2-4 sections of each pancreas per animal, separated by 200 µm, were completely covered
231 systematically by capturing images from non-overlapping fields with a digital camera (Kappa ACC1).
232 The islet cross-sectional area and total pancreatic area were measured using the analysis program

233 Metamorph Software. Beta cell mass (mg per pancreas) was calculated by multiplying relative
234 insulin-positive area (the ratio of insulin positive area over total pancreas area) by pancreas weight.
235 For quantification of the number of islets per area, only islets with more than 5 positive-stained cells
236 were scored.

237

238 **β-cell replication and apoptosis**

239 The same mice used for pancreatic β-cell area (5-8 different mice per experimental condition from 5-8
240 different litters (see figure legend)), were given intraperitoneal injections of BrdU (100 µg/g) 6 hr
241 before sacrifice. Pancreatic tissue was collected, fixed, and processed as described above. After
242 dehydration, sections were heated to 100°C in the presence of citrate buffer (10 mM) for 20 min and
243 immersed in 2 N HCl for 5 min, followed by incubation in a 0.1 M borax solution for 10 min at RT
244 and washed with phosphate-buffered saline. Slides were then blocked by incubating for 1h in 3%
245 bovine serum albumin in phosphate-buffered saline. Samples were then incubated with antibodies for
246 insulin (1:100, rabbit polyclonal; Santa Cruz Biotechnology, Madrid, Spain) (table 1) and BrdU
247 (1:100, mono-clonal; DAKO, Barcelona, Spain) (table 1) overnight at 4°C. After incubation with
248 secondary anti-bodies (Alexa Fluor, Molecular Probes, Barcelona, Spain), sections were incubated
249 with Hoechst 33342 (Alexa Fluor, Molecular Probes, Barcelona, Spain) and then mounted using
250 ProLong Gold Antifade Reagent (Invitrogen, Barcelona, Spain). Images were acquired for triple-
251 stained sections. BrdU-positive nuclei were scored only in cells that were also positive for insulin. At
252 least 1200 cells per pancreas were counted. To identify apoptosis, TUNEL was performed by using an
253 *in situ* cell death detection kit (Roche, Madrid, Spain) according to the manufacturer's specifications
254 for paraffin-embedded tissues. Sections were then washed and stained for insulin as previously
255 described.

256

257 **Statistical analysis**

258 SigmaStat 3.1 software (Systat Software, Inc., Chicago, IL, USA) was used for all statistical analyses.
259 To assess differences between treatment groups for each exposure paradigm, we used the one-way
260 analysis of variance (ANOVA). We used a post hoc test only when ANOVA gave a statistically

significant difference. When data did not pass the parametric test, we used Kruskal-Wallis ANOVA on ranks followed by Dunn's test. We used student t- test when comparing two groups. Results were considered significant at $p < 0.05$. Data are shown as mean ± SEM. We specified statistic tests used in each experiment in figure legends.

272 RESULTS

273 To examine the effects of BPA on the glucose metabolism of offspring, we treated pregnant mice with
274 either vehicle or BPA at doses of 10 or 100 µg/kg/day on GD9–GD16. In total, we had 3 different
275 groups that have been represented in the figures in the following manner: vehicle treated animals
276 (Control, white bars), animals exposed to 10 µg/kg/day of BPA (BPA10, grey bars) and animals
277 exposed to 100 µg/kg/day of BPA (BPA100, black bars).

278

279 *Low birth weight and weight changes during P0 and P30*

280 Body weights (BWs) from the different groups were measured periodically starting on postnatal day 0
281 (P0). Pups born from BPA10 and BPA100 mothers presented a reduced weight compared to Control
282 (control: 1.76±0.02g; BPA10: 1.47±0.03g; BPA100: 1.59±0.04g) (Figure 1A). The BPA10 offspring
283 rapidly gained weight to the same levels as Control, while BPA100 maintained a reduced weight until
284 weaning (P21) (Figure 1A). Remarkably, those in the BPA100 group started to gain weight during the
285 period between P21 and P30, reaching a higher body weight than control and BPA10 at P30 (control:
286 22.9±0.5g; BPA10: 23.3±0.6g; BPA100: 25.5±0.9g) (Figure 1B).

287

288 *Insulinemia, glucose tolerance and insulin sensitivity*

289 To evaluate the effect of BPA exposure on glucose homeostasis at P30, intraperitoneal glucose
290 tolerance and insulin tolerance tests were performed. In both BPA10 and BPA100 glucose tolerance
291 and insulin sensitivity were similar to Control (Figure 1C,D). Plasma insulin and glucose levels in the
292 fasted state were not significantly changed (Table 2). Contrarily, plasma insulin in non-fasting state
293 was significantly elevated in BPA10 and BPA100 compared to control (Table2). To determine
294 whether the increase in plasma insulin was a consequence of enhanced insulin release we measured
295 plasma C-peptide levels which is a manner of evaluating pancreatic β-cell insulin secretion (54). C-
296 peptide levels were significantly higher in both cases BPA10 and BPA100 indicating that insulin
297 release is increased in BPA exposed animal compared to Control (table2).

298 Leptin plasma levels, which are a marker of adiposity, were elevated more than two fold in BPA
299 animals vs control (table2); particularly in BPA100 mice which presented the highest weight (Figure
300 1B). Levels of cholesterol, triglycerides and NEFA were not significantly changed (Table2).

301

302 *Insulin release and insulin content in isolated islets*

303 To determine whether the hyperinsulinemia in the non-fasting state was because an enhanced glucose
304 stimulated insulin secretion (GSIS), we isolated whole islet of Langerhans from Control, BPA10 and
305 BPA100 treated mice and we exposed them to increasing glucose concentrations. Figure 1E shows
306 that GSIS was decreased in BPA10 and unchanged in BPA100 compared to Control. Pancreatic
307 insulin content followed the same pattern, it decreased in BPA10 and was similar in BPA100
308 compared to Control (Figure 1F). These experiments suggest that hyperinsulinemia in non-fasting
309 state must be related to factors other than enhanced GSIS.

310

311 *Microarray analysis reveals differences in mRNA expression patterns between Control and BPA*
312 *groups*

313 BPA exposure during pregnancy may modify the gene expression profile of the islet of Langerhans
314 and, as a consequence, pancreatic β-cell function and/or mass. In order to test this hypothesis, we
315 performed a microarray analysis to compare the transcriptional profiles of islets of Langerhans from
316 control, BPA10 and BPA100 mice. Hierarchical clustering analysis of differentially expressed genes
317 in islets of Langerhans at P30 shows a clear separation between Control and BPA-exposed mice
318 (Figure 2). Down regulated genes (~330 genes) were especially abundant in the BPA10 group and
319 were related to different functional categories. Among the ~325 genes that were upregulated, gene
320 ontology analysis revealed that the most enriched categories were those related with cell cycle,
321 mitosis and, in general, with cell division. These changes were more prominent in islets from the
322 BPA10 group and, although to a lower extent, were also observed in the BPA100 group (Figure 2).
323 Interestingly, the two most upregulated genes in BPA10 islets, *Prss3* (also known as Mesotrypsin)
324 and *Agr2*, although not directly involved in the cell cycle machinery, have been described to act as
325 potent inducers of cell proliferation and tumor progression in several types of cells and cancers

326 (PRSS3 promotes tumor growth and metastasis of human pancreatic cancer) (55). The
327 adenocarcinoma-associated antigen, AGR2, promotes tumor growth, cell migration, and cellular
328 transformation (56).
329
330 Differential expression of genes identified from the microarray data (Figure 2) was validated by qPCR
331 using RNA samples from Control, BPA10 and BPA100 islets at P30. This analysis confirmed that
332 BPA treatment increased the expression of *Ccnb1, Cdk1, Mt1, Procr* and *Idi1*, (Figure 3A-E).
333 Although no significant differences for Mt*2, Spa17* and *Birc5* were found when performing an
334 ANOVA test, these genes were significantly deregulated in BPA10 samples compared to Control by
335 Student's t-test (Figure 3F-H). Expression of *Pdx-1* was significantly increased in BPA10 by qPCR
336 analysis, although significant changes were not observed in the microarray (Figure 3I).
337
338 *BPA treatment increases β-cell mass at P0, P21 and P30 and decreases β-cell mass in P120 offspring*
339 Because the expression of many genes involved in cell cycle was increased in P30 islets after BPA
340 exposure, we decided to examine pancreatic β-cell mass at P30. We found an increase in the
341 percentage of β-cell area relative to the total pancreas area which was significant in the case of BPA10
342 (Figure 4A), according to the microarray data. Pancreatic β-cell mass was also increased in BPA10
343 and BPA100 (Figure 4B). To know whether the increase in β-cell mass was an effect caused during
344 fetal development, lactation or the post-weaning week, we decided to measure β-cell mass at P0 and
345 P21. Notably, both BPA10 and BPA100 offspring at P0 presented a higher relative β-cell mass
346 compared to Control (Figure 4C). Increased β-cell mass was also observed at the day of weaning
347 (P21) (Figure 4D).
348 To assess whether the augmented β-cell mass was maintained during adult life, we analyzed the
349 pancreas from mice at four months of age (P120). BPA 100 mice showed a decrease in pancreatic β-
350 cell mass that was statistically significant when comparing to control by Student's t-test but not
351 significant using ANOVA (Figure 4E). Moreover, these animals presented a higher fasted glucose and

352 a tendency to be glucose intolerant (Figure 4F). Genes upregulated at P30 were equally expressed
353 than Controls at P120 (Supplemental Material Figure 1).
354
355 *BPA treatment increases β-cell proliferation and decreases β-cell apoptosis at P30*
356 To study the contribution of β-cell proliferation in the observed increase in pancreatic β-cell mass, we
357 measured incorporation of BrdU as an indicator of cell proliferation. The percentage of BrdU positive
358 nuclei augmented in BPA10 and BPA100 (Figure 5A and B), indicating that under this conditions cell
359 proliferation was increased. Apoptosis is another important factor in determining β-cell mass. In
360 BPA10 and BPA100 apoptosis measured by TUNEL staining decreased when compared to Control
361 (Figure 5C). These experiments indicate that the elevated β-cell mass in BPA treated animals maybe a
362 consequence of increased β-cell division and decreased apoptosis.
363
364 *E2 treatment partially imitates BPA action on β-cell mass at P30*
365 BPA can exert its effects through different modes of action, although it is mainly considered a
366 xenoestrogen (57). Therefore, we thought in a possible mimetic action of the natural hormone, 17-β
367 estradiol (E2). To evaluate this possibility, we treated pregnant dams with 10μg/kg/day E2 and
368 pancreas were analyzed to analyze β-cell mass, β-cell division and apoptosis. When animals were
369 treated with a higher concentration of E2 (100 μg/kg/day), the offspring died during gestation.
370 At P30, the offspring of E2-treated mice presented increased β-cell mass compared to control (Figure
371 6 A,B), yet nuclei labeled with BrdU (Figure 6C) was not different. In addition, the gene profile
372 observed in E2 treated animals indicated that only some of the genes, increased by BPA (Cdc20 and
373 Ube2c) was elevated by E2 (Supplemental Material Figure 2). Apoptosis, however, was highly
374 reduced by E2 exposure (Figure 6D). These experiments suggest that BPA partially imitates E2 action
375 under these experimental conditions.

376 DISCUSSION

377 Exposure to EDCs is now considered a risk factor for type-2 diabetes, obesity and other metabolic
378 disorders (1, 58, 59). Bisphenol-A is one of the most studied EDCs, including its link with T2D and
379 obesity in animal models and humans (41, 60, 61). Here we have treated pregnant mice from days 9 to
380 16 of gestation with BPA at doses of, 10 and 100 µg/kg/day. We focused our study on male offspring
381 at P0, P21, P30 and P120. We only studied males because in a previous study from our group, using
382 the same treatment, we did not find any change in female phenotype (36). As explained in the
383 Introduction, we considered that the dose of 10 µg/kg/day was low because it is below the current
384 lowest observed effect level (LOAEL) (50µg/kg/day) established by the U.S.-EPA, and similar to the
385 temporary tolerable daily intake by the European Food and Safety Authority (4µg/kg/day). In any
386 case, it must be noted that this study was designed to test a mechanistically-driven hypothesis not to
387 specifically address human risk. At P30, microarray analysis showed that a large amount of genes
388 related to cell division were upregulated in pancreatic islets from offspring mice indicating that
389 pancreatic β-cell mass could be affected by BPA exposure during pregnancy. Accordingly, pancreatic
390 β-cell mass was increased in the offspring of pregnant females exposed to BPA, even in response to
391 the lowest exposure dose of 10 µg/kg/day. This augmented β-cell mass was likely because a rise in
392 cell division, as manifested by BrdU incorporation, together with a decrease in apoptosis.

393 Analysis of blood parameters showed hyperinsulinemia but equal glucose levels together with
394 hyperleptinemia in the non-fasting state (eating *ad libitum)*. Hyperinsulinemia means excessive
395 insulin secretion, which is manifested in this study by an increase in plasma C-peptide in BPA treated
396 animals. Because GSIS measured *ex vivo* was either decreased (BPA10) or unchanged (BPA100), it is
397 plausible that the hyperinsulinemia detected in the non-fasting state was due to the incremented β-cell
398 mass. This hyperinsulinemic state may be a reaction to counteract insulin resistance or a direct action
399 of BPA on pancreas growth. Unaffected glucose tolerance and insulin sensitivity indicate that the
400 increase in β-cell mass was unlikely a consequence of any of these two factors. Remarkably, the fact
401 that β-cell mass was already increased at birth it is inconsistent with an adaptive response to decreased
402 insulin sensitivity. In rodents, the fastest expansion of β-cell mass occurs during late fetal gestation,
403 increasing at a rate of 100% per day (62). An 80% or more is attributed to neogenesis while a 20% or

404 less to cell division (63). Pancreas development in mice stars about embryonic days E9 and E10, with
405 the formation of pancreatic buds. Endocrine cells appear between days E10 and E13.5, but it is mostly
406 at 13.5 when all hormone secreting cells are apparent and at E15 cells are differentiated into exocrine
407 and endocrine cells. By E18 pancreatic islet cells are already visible (64, 65). Based in the
408 experiments showed here we propose that BPA exposure between E9 and E16, altered β-cell mass
409 during fetal development. During the neonatal period there is still growth of β-cell mass but at a lower
410 rate than during late fetal growth. Neogenesis is still occurring during the first week of age yet, after
411 that period, the β-cell mass expands by replication (66). The results showed here, demonstrate that β-
412 cell replication is increased at weaning and P30 and consequently, it suggests that β-cell mass is
413 augmented by β-cell division. This may occur as a consequence of the overexpression of genes related
414 to cell division as demonstrated in the microarray's data.
415 About the time of weaning a "wave" of apoptosis occurs, decreasing the growth of β-cell mass (67,
416 68). The fact that apoptosis is highly decreased in BPA10 and BPA100 animals compared to Control
417 indicates that BPA exposure increased β-cell mass not only by incrementing cell division, but by
418 diminishing apoptosis as well.
419 During life, it is essential to regulate β-cell mass growth in response to different physiological
420 circumstances, including increased body mass and pregnancy (69-72). In addition, metabolic stress
421 during pregnancy such as intrauterine growth restriction disrupts pancreatic β-cell mass growth as
422 well as β-cell function, producing serious consequences in offspring later in life (73, 74). It is
423 plausible that the changes in β-cell mass from birth to the first month of life described in this work
424 affect the phenotype later in life. Studies using mice treated with BPA during the same window of
425 time as here, show a phenotype of altered glucose and lipid homeostasis later in life (from 3 to 6
426 months old). The phenotype includes: glucose intolerance, altered insulin sensitivity,
427 hyperinsulinemia, increase in body weight, adiposity, alterations in adipokines, NEFA and
428 triglyceride levels in blood as well as triglyceride accumulation in the liver (36, 39, 40). Here, the
429 augmented growth of β-cell mass observed during the first month of age it is not maintained.
430 Moreover, at P120 mice presented a great tendency to a decreased β-cell mass together with altered
431 glucose tolerance, particularly in BPA100. In the present work, increased β-cell mass at P30 is

432 associated with hyperinsulinemia in *at libitum* fed animals, which is the regular situation in mice. As a
433 consequence, they will have a constant hyperinsulinemia compared with vehicle treated animals.
434 Could this excess of insulin signaling disrupt glucose homeostasis later in life? It is widely accepted
435 that hyperinsulinemia is simply a compensatory mechanism to counteract insulin resistance. However,
436 hyperinsulinemia may precede insulin resistance in T2D (75-78) and it has been demonstrated that it
437 may contribute to obesity and insulin resistance in ob/ob mice (79). Hyperinsulinemia drives to
438 obesity in genetically designed mice, in which it is possible to control the amount of insulin available
439 (80). In adult mice, it was proposed that EDCs, including BPA, induce insulin resistance and
440 hyperinsulinemia in the non-fasting state (81, 82). It has been demonstrated *ex vivo* and *in vitro* that
441 pollutants, including EDCs, directly stimulate insulin secretion and/or insulin content generating an
442 increase in β cell function in response to nutrients (28, 78, 83). This hyperinsulinemia may be, at least
443 in part, responsible of the insulin resistance caused by some EDCs such as Bisphenol-A (78, 83).
444 It is always difficult to demonstrate whether hyperinsulinemia is a consequence of insulin resistance
445 or the opposite. We propose that alterations in β-cell mass at birth and early life provokes an
446 hyperinsulimemia in the non-fasting state that may influence the phenotype later in life favoring
447 insulin resistance, hyperinsulinemia, hyperleptinemia, increase in body weight and other factors
448 related to metabolic syndrome (36, 37, 39, 40).
449
450 BPA passes the placental barrier (84) and therefore it may act directly in the fetus. It is known that
451 BPA acts as a potent xenoestrogen in β-cells via binding to extranuclearly located estrogen receptor
452 ERα and ERβ (29, 30), yet it is a weak estrogen when acting via the classical ERs pathways working
453 as transcription factors (26). In addition, BPA may act trough other mechanisms of action (57). Here
454 we show that the natural hormone E2 partially mimicked BPA actions at 1 month of age. Both,
455 BPA10 and E10 increased β-cell mass at P30 and decreased apoptosis, however, BrdU incorporation
456 only augmented in BPA treated mice and gene related to cell cycle were activated to a less extent in
457 E10 than BPA10 mice. Therefore, it is possible that BPA acts as a potent xenoestrogen for some of
458 the effects seeing here such as β-cell mass regulation but we cannot discard the involvement of
459 mechanisms other than a direct action in fetal cell mediated by estrogen receptors. In addition to the

460 effect that BPA exposure in utero exerts in offspring, BPA exposure during days 9 to 16 of pregnancy
461 alters blood glucose homeostasis in the mothers at the end of pregnancy. These alterations include:
462 glucose intolerance, insulin resistance, hyperinsulinemia and hyperleptinemia, higher levels of
463 triglycerides and glycerol compared to Controls (36). Therefore, the final phenotype of offspring may
464 not only be influenced by a direct action of BPA on fetal development but also by the abnormal
465 glucose homeostasis of the mothers, as it occurs in the LIRKO mouse model of insulin resistance
466 (14). In the later model, nonetheless, the effect of maternal hyperinsulinemia and transient
467 hyperglycemia decreases β-cell proliferation and islet number.

468 In the present study, we evaluated the early effects of maternal exposure to BPA on glucose
469 homeostasis, pancreatic β-cell mass and function. We found that offspring mice presented an
470 augmented β-cell mass associated with hyperinsulinemia in the absence of insulin resistance and
471 insulin oversecretion. The change in β-cell mass was associated with an increase in the expression of
472 genes related to cell division and cell cycle regulation. In addition, BPA treated animals presented
473 elevated β-cell division and decreased apoptosis. This early changes may affect the phenotype later in
474 life and may be responsible of the alterations in glucose homeostasis already described.

475 Further research is needed to fully understand the mechanisms underlying the increase in β-cell mass
476 and β-cell proliferation at birth and during the first weeks of life, and whether this predisposes to type
477 2 diabetes with aging in animal models and humans.

478

479 **Acknowledgments**

480 We thank Ms. M. Luisa Navarro and Salomé Ramón for their excellent technical assistance

481 This work was supported by Generalitat Valenciana PROMETEOII/2015/016, Ministerio de
482 Economia y Competitividad (SAF2014-58335-P, BFU2013-42789-P), EFSD/Lilly Fellowship
483 Program ref 94224, Sociedad Española de Diabetes (SED) CY1002IL.

484 CIBERDEM is an initiative of IS Carlos III

485

486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503

504 FIGURE LEGENDS

505

506 FIGURE 1. A) Body weight evolution from P0 to P21 (body weight data on P0: ANOVA followed by
507 Holm-Sidak post hoc test, P (maternal treatment), P (Control vs. BPA10) < 0.001; P (Control vs. BPA
508 100) < 0.001; P (BPA10 vs. BPA100) < 0.01; body weight data on P5: ANOVA followed by Holm-
509 Sidak post hoc test, P (maternal treatment), P (Control vs. BPA10) < 0.01; P (BPA10 vs. BPA100) <
510 0.05); body weight data on P12: Kruskal-Wallis ANOVA on ranks followed by Dunn´s post hoc test,
511 P (maternal treatment), P (Control vs. BPA100) < 0.05, P (BPA10 vs. BPA100) < 0.05); body weight
512 data on P16: (Kruskal-Wallis ANOVA on ranks followed by Dunn´s post hoc test, P (maternal
513 treatment), P (Control vs. BPA100) < 0.05, P (BPA10 vs. BPA100) < 0.05) (n = 42-77 animals from
514 10-12 litters). B) Weight comparison at P30. BPA100 was significantly different compared to Control
515 and BPA10. ANOVA followed by Holm-Sidak post hoc test, P (maternal treatment), P (Control vs.
516 BPA100) < 0.05, P (BPA10 vs. BPA100) < 0.05) (n=32-43 animals from 7-10 litters). C)
517 Intraperitoneal glucose tolerance test were performed on the three groups at P30 (n=6-14 animals
518 from 6-10 litters). D) Intraperitoneal insulin tolerance test were performed on the three groups at P30
519 (n=15-17 animals 15-17 litters). E) Insulin secretion from islets exposed to 3, 8 and 16 mM glucose
520 for 1 hour, in animals from the three different groups at P30. Kruskal-Wallis ANOVA on Ranks
521 followed by Dunn´s post hoc test, P (maternal treatment) P (Control vs. BPA10) <0.05 (n=10-15
522 groups of five islets per condition from 6-8 animals from 6-7 litters) F) Insulin content from isolated
523 islets at P30 ANOVA followed by Holm-Sidak´s post hoc test, P (maternal treatment), P (Control vs.
524 BPA10) *<0.05* (n=31-35 groups of five islets per condition from 6-8 animals from 6-7 litters).
525 Data are expressed as mean ± SEM.; *Control vs. BPA10 or BPA 100; *, P < 0.05, **, P < 0.01, ***,
526 P < 0.001; # BPA10 vs. BPA100, #, P < 0.05, ##, P < 0.01.

527

528 FIGURE 2. BPA treatment of pregnant females affects the transcriptome of the offspring's pancreatic
529 islets. The gene cluster representations illustrate the changes in gene expression in pancreatic islets
530 from control, BPA10 and BPA100 mice (intense blue indicates the lowest expression, and intense red,
531 the highest expression). Genes were clustered according to their pattern of expression across the

532 different samples analyzed. The arrows indicate if genes were upregulated (up) or downregulated
533 (down) in the BPA10 and BPA100 samples respect to the control ones.

534

535 FIGURE 3. mRNA gene expression assessed by real-time RT-PCR of representative genes that
536 increased expression in the microarray analysis. Data are expressed as mean± SEM.; *Control vs.
537 BPA10 or BPA 100; *, P < 0.05; $, P < 0.05, Student´s t-test compared to Control. N=4-6 from 15
538 mice/group from 6-9 litters. Details on statistics used: *Ccnb1* (Kruskal-Wallis ANOVA on Ranks
539 followed by Dunn´s post hoc test, P (maternal treatment), P (Control vs. BPA100) <0.05). *Cdk1*
540 (ANOVA followed by Dunnett´s post hoc test, P (maternal treatment), P (Control vs. BPA100) <
541 0.05). *Mt1* (Kruskal-Wallis ANOVA on Ranks followed by Dunn´s post hoc test, P (maternal
542 treatment), P (Control vs. BPA100) <0.05). *Procr* (ANOVA followed by Dunnet´s post hoc test, P
543 (maternal treatment), P (Control vs. BPA10) <0.05; P (Control vs. BPA 100) < 0.05). *Idi1* (ANOVA
544 followed by Dunnet´s post hoc test, P (maternal treatment), P (Control vs. BPA10) <0.05; (n=4-6
545 samples from 15 mice/group from 6-7 litters)). *Pdx-1*, ANOVA followed by Dunnet´s post hoc test, P
546 (maternal treatment), P (Control vs. BPA10) <0.05; (n=4-6 samples from 15 mice/group from 6-9
547 litters). Mt*2, Spa17* and *Birc5* were not statistically significant by ANOVA, yet these genes were
548 significantly down-regulated in BPA10 samples compared to Control by Student's t-test (P (maternal
549 treatment), P (Control vs. BPA10) <0.05; (n=4-6 samples from 15 mice/group from 6-7 litters)).

550

551 FIGURE 4. A) Relative β-cell mass calculated as the percentage of the insulin-positive area over the
552 total pancreas area. Pancreas were obtained from P30 animals. ANOVA followed by Holm-Sidak´s
553 post hoc test, P (maternal treatment), P (Control vs. BPA10) <0.05; (n=5 mice/group from 5 litters).
554 B) Analysis of pancreatic β-cell mass (milligrams per pancreas), calculated as the ratio of the insulin-
555 positive area over the total pancreas area, multiplied by pancreas weight at the same age as in A.
556 ANOVA followed by Holm-Sidak´s post hoc test, P (maternal treatment), P (Control vs. BPA10)
557 <0.05, P (Control vs. BPA100) <0.05*;* (n=5 mice/group from 5 litters). C) Relative β-cell mass
558 calculated as the percentage of the insulin-positive area over the total pancreas area. Pancreas were
559 obtained from P0 animals. Kruskal-Wallis ANOVA on Ranks followed by Dunn´s post hoc test, P

560 (maternal treatment) P (Control vs. BPA10) <0.05; P (Control vs. BPA100) <0.05*;* (n=8 mice/group
561 from 7-8 litters). D) β-cell mass calculated as the ratio of the insulin-positive area over the total
562 pancreas area multiplied by pancreas weight. Pancreas were obtained from P21 animals. ANOVA
563 followed by Dunnett´s post hoc test, P (maternal treatment) P (Control vs. BPA10) <0.01; P (Control
564 vs. BPA100) <0.001; (n=8 mice/group from 7-8 litters). E) β-cell mass calculated as the ratio of the
565 insulin-positive area over the total pancreas area multiplied by pancreas weight. Pancreas were
566 obtained from P120 animals. Significant using Student's t-test (P (maternal treatment), P (Control vs.
567 BPA100) <0.05. No statistically significant by ANOVA (n= 5 mice/group from 5 litters). F)
568 Intraperitoneal glucose tolerance test performed in the three groups. Open circles for Control, filled
569 circles for BPA10, filled squares for BPA100 (n= 5 mice/group from 5 litters). Data are expressed as
570 the mean ± SEM. *Control vs. BPA10 or BPA 100, # BPA10 vs. BPA100; *, P <0.05, **, P <0.01,
571 ***, P < 0.001. $, P <0.05, Student´s t-test compared to Control.
572
573 FIGURE 5. A) Representative images of pancreas sections stained with antibodies against BrdU
574 (green) and insulin (red) and counterstained with Hoechst (blue). Scale bar, 25 μm. White arrows
575 indicate some positive BrdU cells. B) Percentage of BrdU-positive β-cells in control, BPA10, and
576 BPA100 mice at P30.. ANOVA followed by Holm-Sidak´s post hoc test, P (maternal treatment) P
577 (Control vs. BPA10) <0.05, P (Control vs. BPA100) <0.05; (n=5 mice/group from 5 litters). C)
578 Analysis of apoptotic β-cells quantified in pancreas sections using a fluorescein in situ cell death
579 detection assay (TUNEL) at P30. Kruskal-Wallis ANOVA on Ranks followed by Dunn´s post hoc
580 test, P (maternal treatment), P (Control vs. BPA10) <0.05, P (Control vs. BPA100) <0.05; (n=5
581 mice/group from 5 litters). Data are expressed as the mean ± SEM. *Control vs. BPA10 or BPA 100;
582 *, P < 0.05
583
584 FIGURE 6. A) Relative β-cell mass calculated as the percentage of the insulin-positive area over the
585 total pancreas area. Pancreas were obtained from P30 animals treated *in utero* with vehicle (Control)
586 or E210μg/kg/day (E10). B) Analysis of pancreatic β-cell mass (milligrams per pancreas), calculated
587 as the ratio of the insulin-positive area over the total pancreas area, multiplied by pancreas weight in

the same conditions as in A (n=8 mice/group from 8 litters). C) Percentage of BrdU-positive β-cells in control and E2 mice at P30 (n = 6 mice/group from 6 litters). B) Analysis of apoptotic β-cells quantified in pancreas sections using a fluorescein in situ cell death detection assay (TUNEL) in control and E10 (n=7-8 mice/group from 7 litters). Data are expressed as the mean ± SEM, and statistical significance was determined using Student´s t-test compared to Control. *Control vs. BPA10 or BPA 100; *, $P < 0.05$.

Table 2. Serum hormone and metabolite levels in animals exposed to BPA in utero. n= insulin fasted state 9-14 animals from 8-14 litters; insulin non-fasting state 41-51 animals from 39-51 litters; c-peptide 20-24 animals from 20-24 litters; leptin 18-24 animals from 18-23 litters; cholesterol 12-22 animals from 8-22 litters; triglyceride 9-11 animals from 8-9 litters and NEFA 15 animals from 8-9 litters**.** Data are expressed as mean±SEM. Significance was determined using ANOVA one way followed by Holm-Sidak post hoc test. When data did not pass the parametric test, we used Kruskal-Wallis ANOVA on ranks followed by Dunn's test. See below for further details. *Control vs. BPA10 or BPA 100; *, $P < 0.05$; # BPA10 vs. BPA100, #, $P < 0.05$. Insulin non-fasting, Kruskal-Wallis ANOVA on ranks followed by Dunn´s method, P (maternal treatment), P (Control vs. BPA10) < 0.05, P (Control vs. BPA100) < 0.05; (n=41-51 animals from 39-51 litters). C-Peptide, ANOVA followed by Holm-Sidak post hoc test, P(maternal treatment), P (Control vs. BPA10) < 0.05, P (Control vs. BPA100) < 0.05; (n=20-24 animals from 20-24 litters. Leptin, Kruskal-Wallis ANOVA on ranks followed by Dunn´s post hoc test, P (maternal treatment), P (Control vs. BPA10) < 0.05, P (Control vs. BPA100) < 0.05, P (BPA10 vs. BPA100) < 0.05; (n=18-24 animals from 18-23 litters).

e,

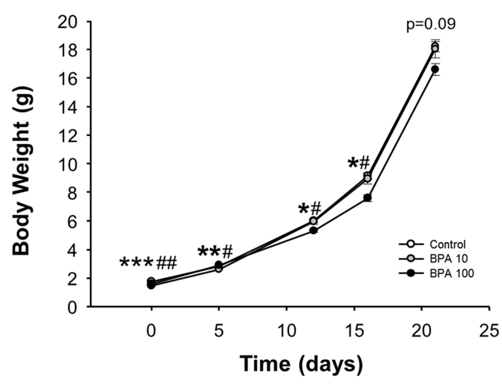
612 1. **Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT** 2015
613 EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting
614 Chemicals. Endocr Rev 36:E1-E150
615 2. **Barker DJ** 1990 The fetal and infant origins of adult disease. BMJ 301:1111
616 3. **Portha B, Chavey A, Movassat J** 2011 Early-life origins of type 2 diabetes: fetal programming
617 of the beta-cell mass. Exp Diabetes Res 2011:105076
618 4. **Williams L, Seki Y, Vuguin PM, Charron MJ** 2014 Animal models of in utero exposure to a
619 high fat diet: a review. Biochim Biophys Acta 1842:507-519
620 5. **Patel N, Pasupathy D, Poston L** 2015 Determining the consequences of maternal obesity for
621 offspring health. Exp Physiol 100:1421-1428
622 6. **Tarry-Adkins JL, Chen JH, Jones RH, Smith NH, Ozanne SE** 2010 Poor maternal nutrition
623 leads to alterations in oxidative stress, antioxidant defense capacity, and markers of fibrosis
624 in rat islets: potential underlying mechanisms for development of the diabetic phenotype in
625 later life. FASEB J 24:2762-2771
626 7. **Feres NH, Reis SR, Veloso RV, Arantes VC, Souza LM, Carneiro EM, Boschero AC, Reis MA,**
627 **Latorraca MQ** 2010 Soybean diet alters the insulin-signaling pathway in the liver of rats
628 recovering from early-life malnutrition. Nutrition 26:441-448
629 8. **Alheiros-Lira MC, Araujo LL, Trindade NG, da Silva EM, Cavalcante TC, de Santana Muniz G,**
630 **Nascimento E, Leandro CG** 2015 Short- and long-term effects of a maternal low-energy diet
631 ad libitum during gestation and/or lactation on physiological parameters of mothers and
632 male offspring. Eur J Nutr 54:793-802
633 9. **Fernandez E, Martin MA, Fajardo S, Escriva F, Alvarez C** 2007 Increased IRS-2 content and
634 activation of IGF-I pathway contribute to enhance beta-cell mass in fetuses from
635 undernourished pregnant rats. Am J Physiol Endocrinol Metab 292:E187-195
636 10. **Jimenez-Chillaron JC, Isganaitis E, Charalambous M, Gesta S, Pentinat-Pelegrin T, Faucette**
637 **RR, Otis JP, Chow A, Diaz R, Ferguson-Smith A, Patti ME** 2009 Intergenerational
638 transmission of glucose intolerance and obesity by in utero undernutrition in mice. Diabetes
639 58:460-468
640 11. **Giussani DA, Niu Y, Herrera EA, Richter HG, Camm EJ, Thakor AS, Kane AD, Hansell JA,**
641 **Brain KL, Skeffington KL, Itani N, Wooding FB, Cross CM, Allison BJ** 2014 Heart disease link
642 to fetal hypoxia and oxidative stress. Adv Exp Med Biol 814:77-87
643 12. **Friedman JE** 2015 Obesity and Gestational Diabetes Mellitus Pathways for Programming in
644 Mouse, Monkey, and Man-Where Do We Go Next? The 2014 Norbert Freinkel Award
645 Lecture. Diabetes Care 38:1402-1411
646 13. **Donovan LE, Cundy T** 2015 Does exposure to hyperglycaemia in utero increase the risk of
647 obesity and diabetes in the offspring? A critical reappraisal. Diabet Med 32:295-304
648 14. **Kahraman S, Dirice E, De Jesus DF, Hu J, Kulkarni RN** 2014 Maternal insulin resistance and
649 transient hyperglycemia impact the metabolic and endocrine phenotypes of offspring. Am J
650 Physiol Endocrinol Metab 307:E906-918
651 15. **Grandjean P, Barouki R, Bellinger DC, Casteleyn L, Chadwick LH, Cordier S, Etzel RA, Gray**
652 **KA, Ha EH, Junien C, Karagas M, Kawamoto T, Paige Lawrence B, Perera FP, Prins GS, Puga**
653 **A, Rosenfeld CS, Sherr DH, Sly PD, Suk W, Sun Q, Toppari J, van den Hazel P, Walker CL,**
654 **Heindel JJ** 2015 Life-Long Implications of Developmental Exposure to Environmental
655 Stressors: New Perspectives. Endocrinology 156:3408-3415
656 16. **Heindel JJ, Balbus J, Birnbaum L, Brune-Drisse MN, Grandjean P, Gray K, Landrigan PJ, Sly**
657 **PD, Suk W, Cory Slechta D, Thompson C, Hanson M** 2015 Developmental Origins of Health
658 and Disease: Integrating Environmental Influences. Endocrinology 156:3416-3421
659 17. **Dodds, Lawson** 1936 The phenanthrene condensed-ring structure is not necessary for
660 estrogenic activity. A table of 14 synthetic substances is given, all except one showing 100%
661 positive estrus responses. . Nature (London, United Kingdom)
662 137:996

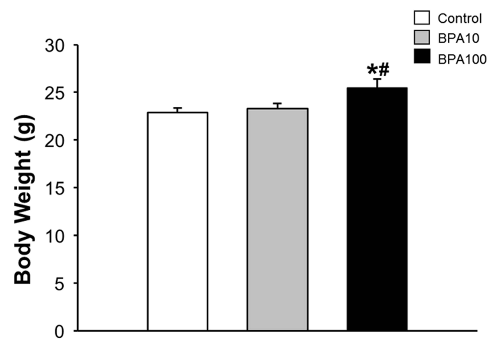
663 18. **Dodds E, Lawson W** 1938 Molecular structure in relation to oestrogenic activity. Compounds
664 without a phenanthrene nucleus. Proceedings of the Royal Society London B 125:222-232
665 19. **Herbst AL, Ulfelder H, Poskanzer DC** 1971 Adenocarcinoma of the vagina. Association of
666 maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284:878-
667 881
668 20. **Troisi R, Hatch EE, Titus-Ernstoff L, Hyer M, Palmer JR, Robboy SJ, Strohsnitter WC,**
669 **Kaufman R, Herbst AL, Hoover RN** 2007 Cancer risk in women prenatally exposed to
670 diethylstilbestrol. Int J Cancer 121:356-360
671 21. **McLachlan JA, Dixon RL** 1977 Toxicologic comparisons of experimental and clinical exposure
672 to diethylstilbestrol during gestation. Adv Sex Horm Res 3:309-336
673 22. **vom Saal FS, Welshons WV** 2014 Evidence that bisphenol A (BPA) can be accurately
674 measured without contamination in human serum and urine, and that BPA causes numerous
675 hazards from multiple routes of exposure. Mol Cell Endocrinol 398:101-113
676 23. **Schecter A, Malik N, Haffner D, Smith S, Harris TR, Paepke O, Birnbaum L** 2010 Bisphenol A
677 (BPA) in U.S. food. Environ Sci Technol 44:9425-9430
678 24. **Lopez-Cervantes J, Paseiro-Losada P** 2003 Determination of bisphenol A in, and its migration
679 from, PVC stretch film used for food packaging. Food Addit Contam 20:596-606
680 25. **Welshons WV, Nagel SC, vom Saal FS** 2006 Large effects from small exposures. III. Endocrine
681 mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology
682 147:S56-69
683 26. **Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B,**
684 **Gustafsson JA** 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen
685 receptor beta. Endocrinology 139:4252-4263
686 27. **Matthews JB, Twomey K, Zacharewski TR** 2001 In vitro and in vivo interactions of bisphenol
687 A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta.
688 Chem Res Toxicol 14:149-157
689 28. **Alonso-Magdalena P, Ropero AB, Carrera MP, Cederroth CR, Baquie M, Gauthier BR, Nef S,**
690 **Stefani E, Nadal A** 2008 Pancreatic insulin content regulation by the estrogen receptor ER
691 alpha. PLoS One 3:e2069
692 29. **Soriano S, Alonso-Magdalena P, Garcia-Arevalo M, Novials A, Muhammed SJ, Salehi A,**
693 **Gustafsson JA, Quesada I, Nadal A** 2012 Rapid insulinotropic action of low doses of
694 bisphenol-A on mouse and human islets of Langerhans: role of estrogen receptor beta. PLoS
695 One 7:e31109
696 30. **Alonso-Magdalena P, Ropero AB, Soriano S, Garcia-Arevalo M, Ripoll C, Fuentes E,**
697 **Quesada I, Nadal A** 2012 Bisphenol-A acts as a potent estrogen via non-classical estrogen
698 triggered pathways. Mol Cell Endocrinol 355:201-207
699 31. **Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL** 2008 Exposure of the U.S. population to
700 bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 116:39-44
701 32. **Gerona RR, Woodruff TJ, Dickenson CA, Pan J, Schwartz JM, Sen S, Friesen MW, Fujimoto**
702 **VY, Hunt PA** 2013 Bisphenol-A (BPA), BPA glucuronide, and BPA sulfate in midgestation
703 umbilical cord serum in a northern and central California population. Environ Sci Technol
704 47:12477-12485
705 33. **Liao C, Kannan K** 2012 Determination of free and conjugated forms of bisphenol A in human
706 urine and serum by liquid chromatography-tandem mass spectrometry. Environ Sci Technol
707 46:5003-5009
708 34. **Veiga-Lopez A, Pennathur S, Kannan K, Patisaul HB, Dolinoy DC, Zeng L, Padmanabhan V**
709 2015 Impact of gestational bisphenol A on oxidative stress and free fatty acids: Human
710 association and interspecies animal testing studies. Endocrinology 156:911-922
711 35. **Teeguarden JG, Hanson-Drury S** 2013 A systematic review of Bisphenol A "low dose" studies
712 in the context of human exposure: a case for establishing standards for reporting "low-dose"
713 effects of chemicals. Food Chem Toxicol 62:935-948

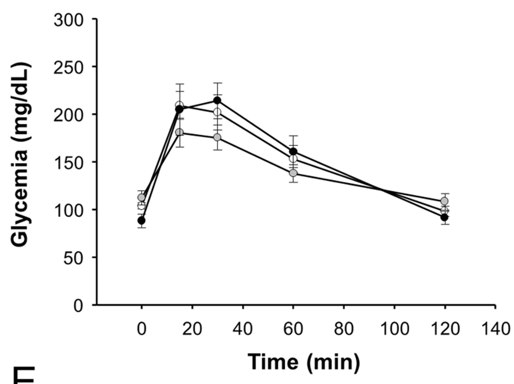
714 36. **Alonso-Magdalena P, Vieira E, Soriano S, Menes L, Burks D, Quesada I, Nadal A** 2010
715 Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult
716 male offspring. Environ Health Perspect 118:1243-1250
717 37. **Wei J, Lin Y, Li Y, Ying C, Chen J, Song L, Zhou Z, Lv Z, Xia W, Chen X, Xu S** 2011 Perinatal
718 exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in
719 adult rats on a high-fat diet. Endocrinology 152:3049-3061
720 38. **Liu J, Yu P, Qian W, Li Y, Zhao J, Huan F, Wang J, Xiao H** 2013 Perinatal bisphenol A exposure
721 and adult glucose homeostasis: identifying critical windows of exposure. PLoS One 8:e64143
722 39. **Angle BM, Do RP, Ponzi D, Stahlhut RW, Drury BE, Nagel SC, Welshons WV, Besch-Williford
723 CL, Palanza P, Parmigiani S, vom Saal FS, Taylor JA** 2013 Metabolic disruption in male mice
724 due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on
725 body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation.
726 Reprod Toxicol 42:256-268
727 40. **Garcia-Arevalo M, Alonso-Magdalena P, Rebelo Dos Santos J, Quesada I, Carneiro EM,
728 Nadal A** 2014 Exposure to bisphenol-A during pregnancy partially mimics the effects of a
729 high-fat diet altering glucose homeostasis and gene expression in adult male mice. PLoS One
730 9:e100214
731 41. **Alonso-Magdalena P, Garcia-Arevalo M, Quesada I, Nadal A** 2015 Bisphenol-A treatment
732 during pregnancy in mice: a new window of susceptibility for the development of diabetes in
733 mothers later in life. Endocrinology 156:1659-1670
734 42. **Mackay H, Patterson ZR, Khazall R, Patel S, Tsirlin D, Abizaid A** 2013 Organizational effects
735 of perinatal exposure to bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry
736 controlling food intake and energy expenditure in male and female CD-1 mice.
737 Endocrinology 154:1465-1475
738 43. **Ma Y, Xia W, Wang DQ, Wan YJ, Xu B, Chen X, Li YY, Xu SQ** 2013 Hepatic DNA methylation
739 modifications in early development of rats resulting from perinatal BPA exposure contribute
740 to insulin resistance in adulthood. Diabetologia 56:2059-2067
741 44. **Ding S, Fan Y, Zhao N, Yang H, Ye X, He D, Jin X, Liu J, Tian C, Li H, Xu S, Ying C** 2014 High-fat
742 diet aggravates glucose homeostasis disorder caused by chronic exposure to bisphenol A. J
743 Endocrinol 221:167-179
744 45. **Li G, Chang H, Xia W, Mao Z, Li Y, Xu S** 2014 F0 maternal BPA exposure induced glucose
745 intolerance of F2 generation through DNA methylation change in Gck. Toxicol Lett 228:192-
746 199
747 46. **Susiarjo M, Xin F, Bansal A, Stefaniak M, Li C, Simmons RA, Bartolomei MS** 2015 Bisphenol
748 a exposure disrupts metabolic health across multiple generations in the mouse.
749 Endocrinology 156:2049-2058
750 47. **Ryan KK, Haller AM, Sorrell JE, Woods SC, Jandacek RJ, Seeley RJ** 2010 Perinatal exposure
751 to bisphenol-a and the development of metabolic syndrome in CD-1 mice. Endocrinology
752 151:2603-2612
753 48. **Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ** 2009 A protocol for islet isolation from mouse
754 pancreas. Nat Protoc 4:1649-1652
755 49. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram
756 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254
757 50. **Moreno-Asso A, Castano C, Grilli A, Novials A, Servitja JM** 2013 Glucose regulation of a cell
758 cycle gene module is selectively lost in mouse pancreatic islets during ageing. Diabetologia
759 56:1761-1772
760 51. **www.ncbi.nlm.nih.gov/geo** In:
761 52. **http://david.abcc.ncifcrf.gov** In:
762 53. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time
763 quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

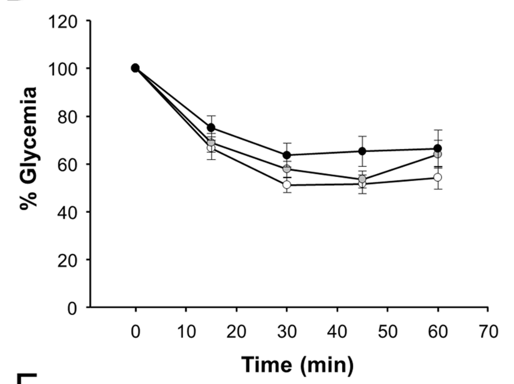
764 54. **Tura A, Ludvik B, Nolan JJ, Pacini G, Thomaseth K** 2001 Insulin and C-peptide secretion and
765 kinetics in humans: direct and model-based measurements during OGTT. Am J Physiol
766 Endocrinol Metab 281:E966-974
767 55. **Jiang G, Cao F, Ren G, Gao D, Bhakta V, Zhang Y, Cao H, Dong Z, Zang W, Zhang S, Wong**
768 **HH, Hiley C, Crnogorac-Jurcevic T, Lemoine NR, Wang Y** 2010 PRSS3 promotes tumour
769 growth and metastasis of human pancreatic cancer. Gut 59:1535-1544
770 56. **Wang Z, Hao Y, Lowe AW** 2008 The adenocarcinoma-associated antigen, AGR2, promotes
771 tumor growth, cell migration, and cellular transformation. Cancer Res 68:492-497
772 57. **Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS,**
773 **Zoeller RT, Belcher SM** 2007 In vitro molecular mechanisms of bisphenol A action. Reprod
774 Toxicol 24:178-198
775 58. **Alonso-Magdalena P, Quesada I, Nadal A** 2011 Endocrine disruptors in the etiology of type
776 2 diabetes mellitus. Nat Rev Endocrinol 7:346-353
777 59. **Neel BA, Sargis RM** 2011 The paradox of progress: environmental disruption of metabolism
778 and the diabetes epidemic. Diabetes 60:1838-1848
779 60. **Nadal A, Alonso-Magdalena P, Soriano S, Quesada I, Ropero AB** 2009 The pancreatic beta-
780 cell as a target of estrogens and xenoestrogens: Implications for blood glucose homeostasis
781 and diabetes. Mol Cell Endocrinol 304:63-68
782 61. **Vom Saal FS, Nagel SC, Coe BL, Angle BM, Taylor JA** 2012 The estrogenic endocrine
783 disrupting chemical bisphenol A (BPA) and obesity. Mol Cell Endocrinol 354:74-84
784 62. **McEvoy RC, Madson KL** 1980 Pancreatic insulin-, glucagon-, and somatostatin-positive islet
785 cell populations during the perinatal development of the rat. II. Changes in hormone content
786 and concentration. Biol Neonate 38:255-259
787 63. **Bouwens L, Rooman I** 2005 Regulation of pancreatic beta-cell mass. Physiol Rev 85:1255-
788 1270
789 64. **Rojas A, Khoo A, Tejedo JR, Bedoya FJ, Soria B, Martin F** 2010 Islet cell development. Adv
790 Exp Med Biol 654:59-75
791 65. **Cano DA, Soria B, Martin F, Rojas A** 2014 Transcriptional control of mammalian pancreas
792 organogenesis. Cell Mol Life Sci 71:2383-2402
793 66. **Georgia S, Bhushan A** 2004 Beta cell replication is the primary mechanism for maintaining
794 postnatal beta cell mass. J Clin Invest 114:963-968
795 67. **Scaglia L, Cahill CJ, Finegood DT, Bonner-Weir S** 1997 Apoptosis participates in the
796 remodeling of the endocrine pancreas in the neonatal rat. Endocrinology 138:1736-1741
797 68. **Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B** 2000 Beta-cell proliferation and
798 apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy.
799 Diabetes 49:1325-1333
800 69. **Sorenson RL, Brelje TC** 1997 Adaptation of islets of Langerhans to pregnancy: beta-cell
801 growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res
802 29:301-307
803 70. **Nadal A, Alonso-Magdalena P, Soriano S, Ropero AB, Quesada I** 2009 The role of
804 oestrogens in the adaptation of islets to insulin resistance. J Physiol 587:5031-5037
805 71. **Bergman RN, Finegood DT, Kahn SE** 2002 The evolution of beta-cell dysfunction and insulin
806 resistance in type 2 diabetes. Eur J Clin Invest 32 Suppl 3:35-45
807 72. **Mauvais-Jarvis F, Clegg DJ, Hevener AL** 2013 The role of estrogens in control of energy
808 balance and glucose homeostasis. Endocr Rev 34:309-338
809 73. **Sandovici I, Smith NH, Nitert MD, Ackers-Johnson M, Uribe-Lewis S, Ito Y, Jones RH,**
810 **Marquez VE, Cairns W, Tadayyon M, O'Neill LP, Murrell A, Ling C, Constancia M, Ozanne SE**
811 2011 Maternal diet and aging alter the epigenetic control of a promoter-enhancer
812 interaction at the Hnf4a gene in rat pancreatic islets. Proc Natl Acad Sci U S A 108:5449-5454
813 74. **Ozanne SE, Sandovici I, Constancia M** 2011 Maternal diet, aging and diabetes meet at a
814 chromatin loop. Aging (Albany NY) 3:548-554

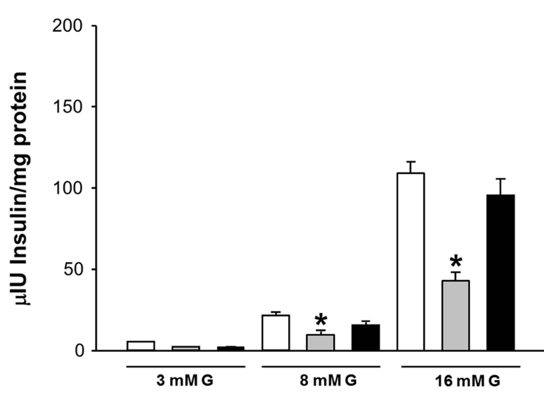
815 75. **McGarry JD** 1992 What if Minkowski had been ageusic? An alternative angle on diabetes.
816 Science 258:766-770
817 76. **Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y, Roth J** 2008 Insulin resistance and
818 hyperinsulinemia: is hyperinsulinemia the cart or the horse? Diabetes Care 31 Suppl 2:S262-
819 268
820 77. **Gray SL, Donald C, Jetha A, Covey SD, Kieffer TJ** 2010 Hyperinsulinemia precedes insulin
821 resistance in mice lacking pancreatic beta-cell leptin signaling. Endocrinology 151:4178-4186
822 78. **Corkey BE** 2011 Banting lecture 2011: hyperinsulinemia: cause or consequence? Diabetes
823 61:4-13
824 79. **Levi J, Gray SL, Speck M, Huynh FK, Babich SL, Gibson WT, Kieffer TJ** 2011 Acute disruption
825 of leptin signaling in vivo leads to increased insulin levels and insulin resistance.
826 Endocrinology 152:3385-3395
827 80. **Mehran AE, Templeman NM, Brigidi GS, Lim GE, Chu KY, Hu X, Botezelli JD, Asadi A,**
828 **Hoffman BG, Kieffer TJ, Bamji SX, Clee SM, Johnson JD** 2012 Hyperinsulinemia drives diet-
829 induced obesity independently of brain insulin production. Cell Metab 16:723-737
830 81. **Alonso-Magdalena P, Morimoto S, Ripoll C, Fuentes E, Nadal A** 2006 The estrogenic effect
831 of bisphenol A disrupts pancreatic beta-cell function in vivo and induces insulin resistance.
832 Environ Health Perspect 114:106-112
833 82. **Ropero AB, Alonso-Magdalena P, Garcia-Garcia E, Ripoll C, Fuentes E, Nadal A** 2008
834 Bisphenol-A disruption of the endocrine pancreas and blood glucose homeostasis. Int J
835 Androl 31:194-200
836 83. **Corkey BE** 2012 Diabetes: have we got it all wrong? Insulin hypersecretion and food
837 additives: cause of obesity and diabetes? Diabetes Care 35:2432-2437
838 84. **Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV** 2007 Human exposure to
839 bisphenol A (BPA). Reprod Toxicol 24:139-177
840
841
842











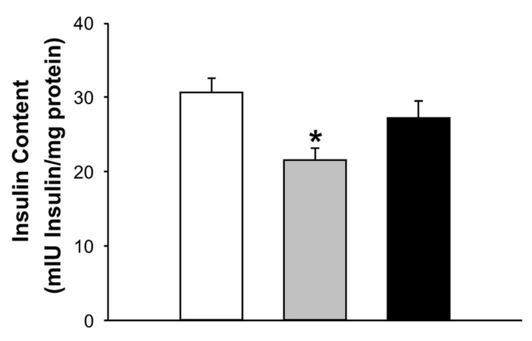


Figure 1

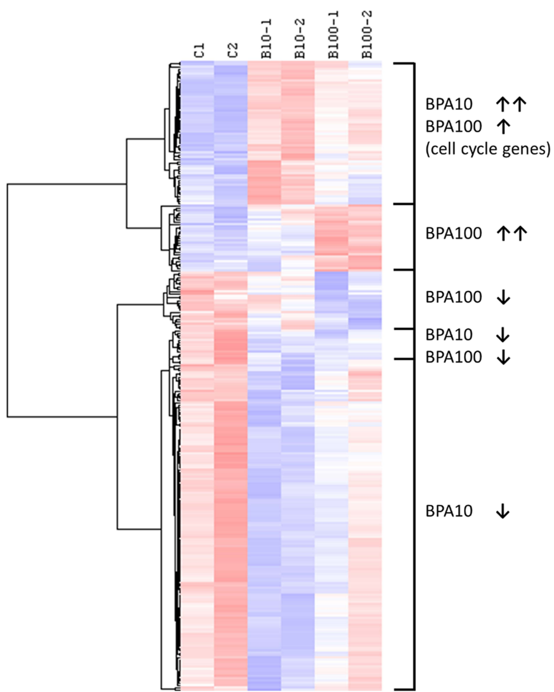


Figure 2

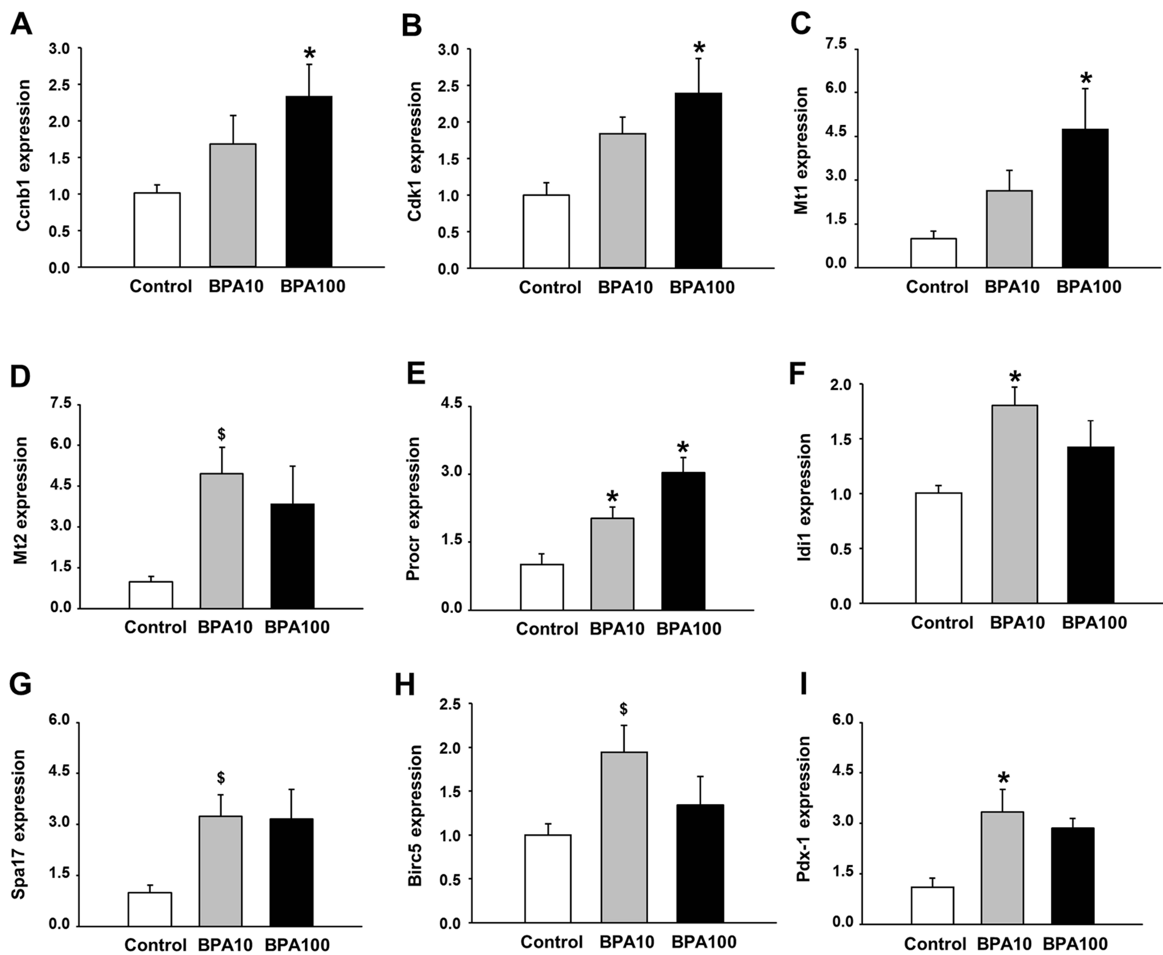


Figure 3

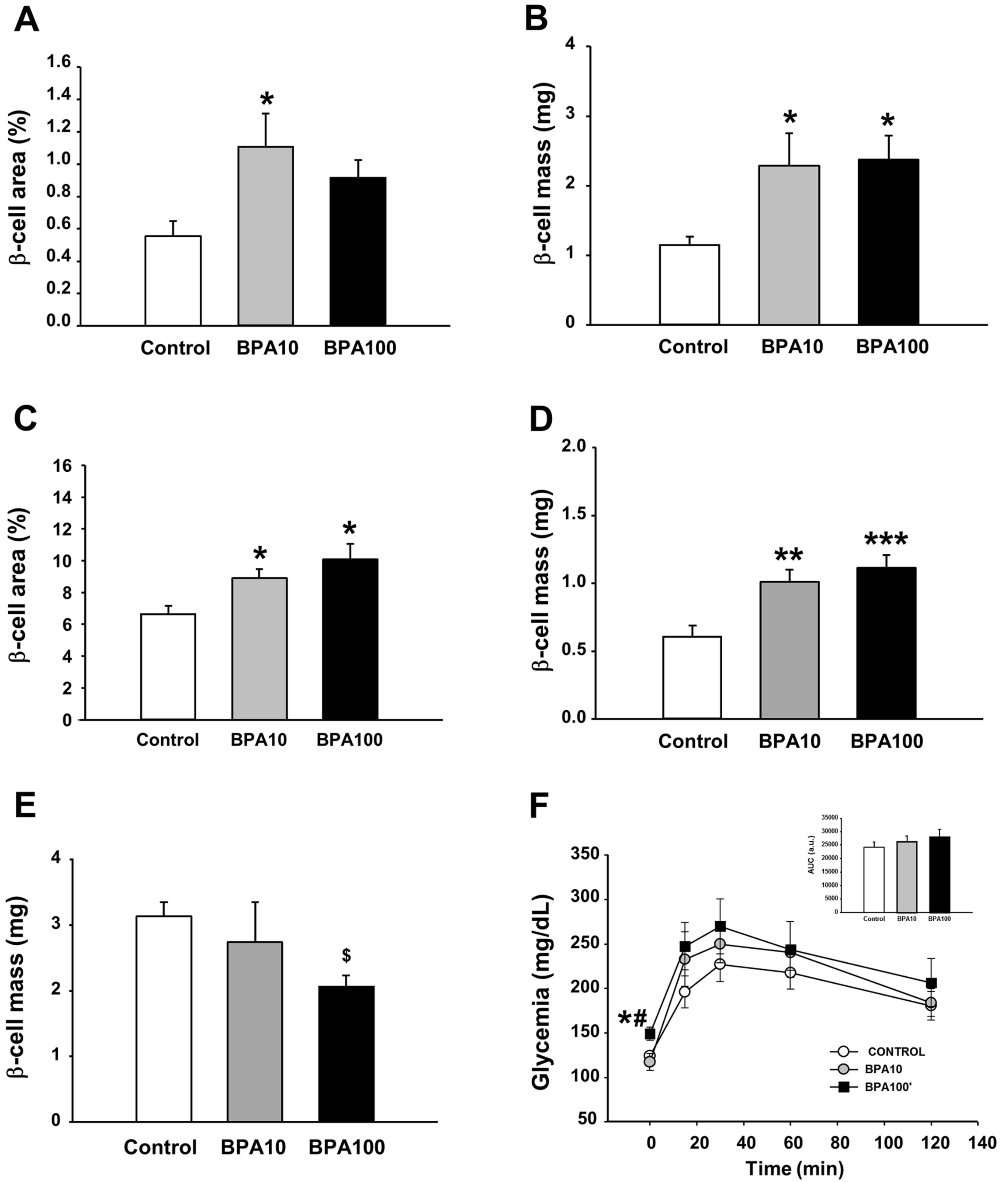
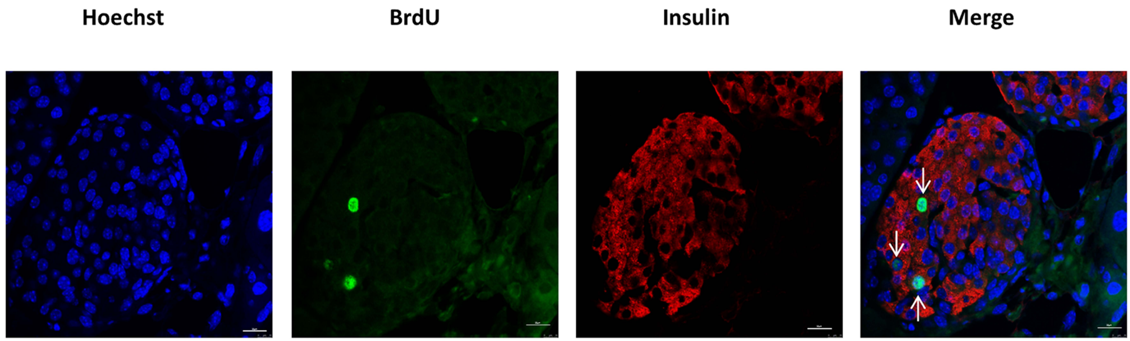
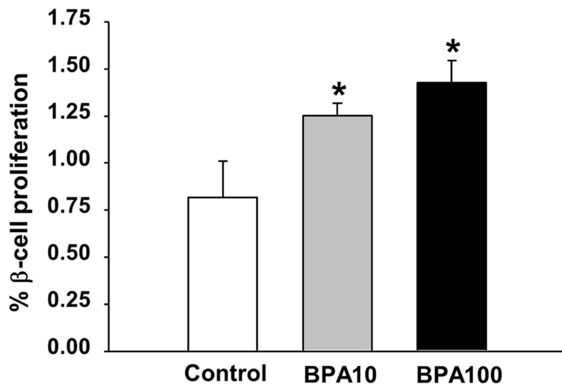


Figure 4

A



B



C

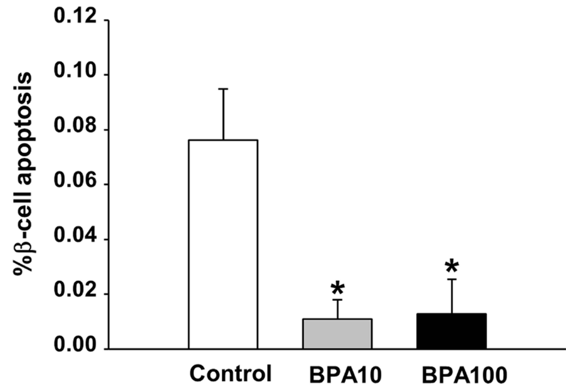
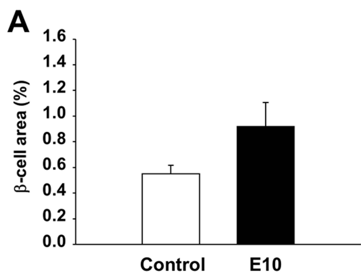
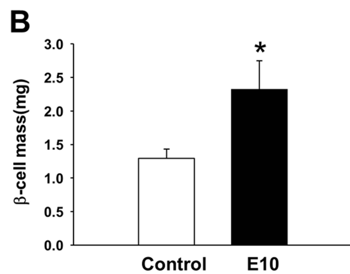
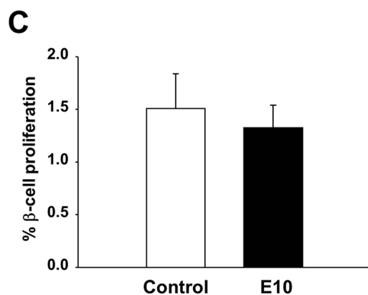


Figure 5







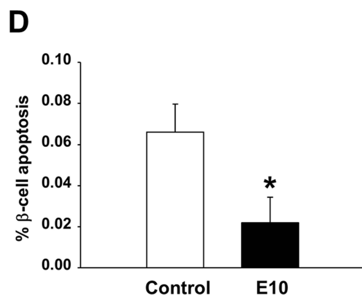


Figure 6

Gen	NM	Forward (5´-3´)	Reverse (5´-3´)
Ccnb1	172301	GTGCGCCTGCAGAAGAGTAT	TGCTCTTCCTCCAGTTGTCGG
Cdk1	7659	ACACGAGGTAGTGACGCTG	CTCTGAGTCGCCGTGGAAAA
Mt1	013602.3	CAGGCTGTCCTCTAAGCGTC	AGGAGCAGCAGCTCTTCTTG
Mt2	008630.2	TGCAAGAAAAGCTGCTGCTCC	GTGGAGAACGAGTCAGGGTTG
Procr	11171	ACGCAAAACATGAAAGGGAGC	ATTAGCAACGCCGTCCACTT
Idi1	145360.2	GCTAGATTGGCAATTGGCTGG	TAGAACACAGAGATTCCGGC
Spa17	011449.2	CGGTTACCCAGCAACGAGAT	TGCCTATATGGTACCTCTTCTTTCT
Birc5	1012273	TGACGCCATCATGGGAGC	AAGGTGGCGATGCGGTAGT
Pdx-1	8814.3	AAGGTGGCGATGCGGTAGT	AAGGTGGCGATGCGGTAGT
Pbk	23209	AGAAGCTTGGCTTTGGGACTG	GGAGAATGAGACAACCCTCTTGG
Cenpa	7681	AGCTCCAGTGTAGGCTCTCA	CACCACGGCTGAACTTCTCA
Cdc20	23223	GCCCACCAAAAAGGAGCATC	ATTCTGAGGTTTGCCGCTGA
Ube2c	26785	GTTCCTCACACCCTGCTACC	CGATGTTGGGTTCTCCTAGC
Hprt	013556.2	GGTTAAGCAGTACAGCCCCA	TCCAACACTTCGAGAGGTCC

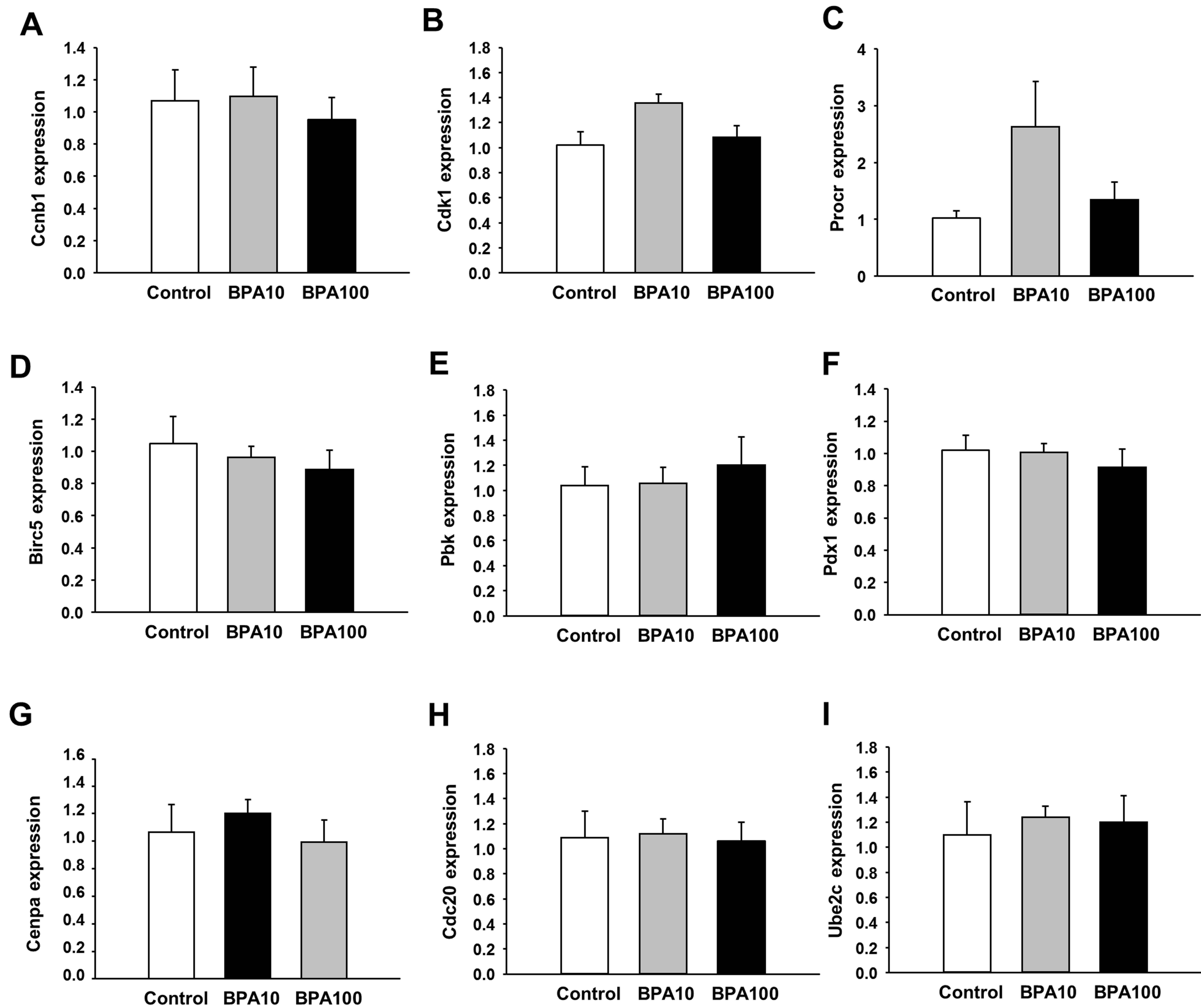
Table 1. Quantitative Real-Time PCR primers.

	Control	BPA10	BPA100
Insulin Fasted (ng/mL)	0.17 ± 0.01	0.17 ± 0.02	0.19 ± 0.02
Insulin Fed (ng/mL)	0.58 ± 0.08	1.17 ± 0.21*	1.35 ± 0.1*
C-peptide Fed (pM)	923 ± 139	1497 ± 171*	1837 ± 178*
Leptin Fed (ng/mL)	1.8 ± 0.3	4.0 ± 0.6*	6.9 ± 0.7*#
Chol Fed (mg/dL)	167 ± 1.7	168 ± 1.4	171 ± 2.7
Tg Fed (mg/dL)	190 ± 18	178 ± 12	148 ± 11
Nefa Fed (mg/dL)	10.2 ± 1.3	7.0 ±1.0	10.3 ± 1.2

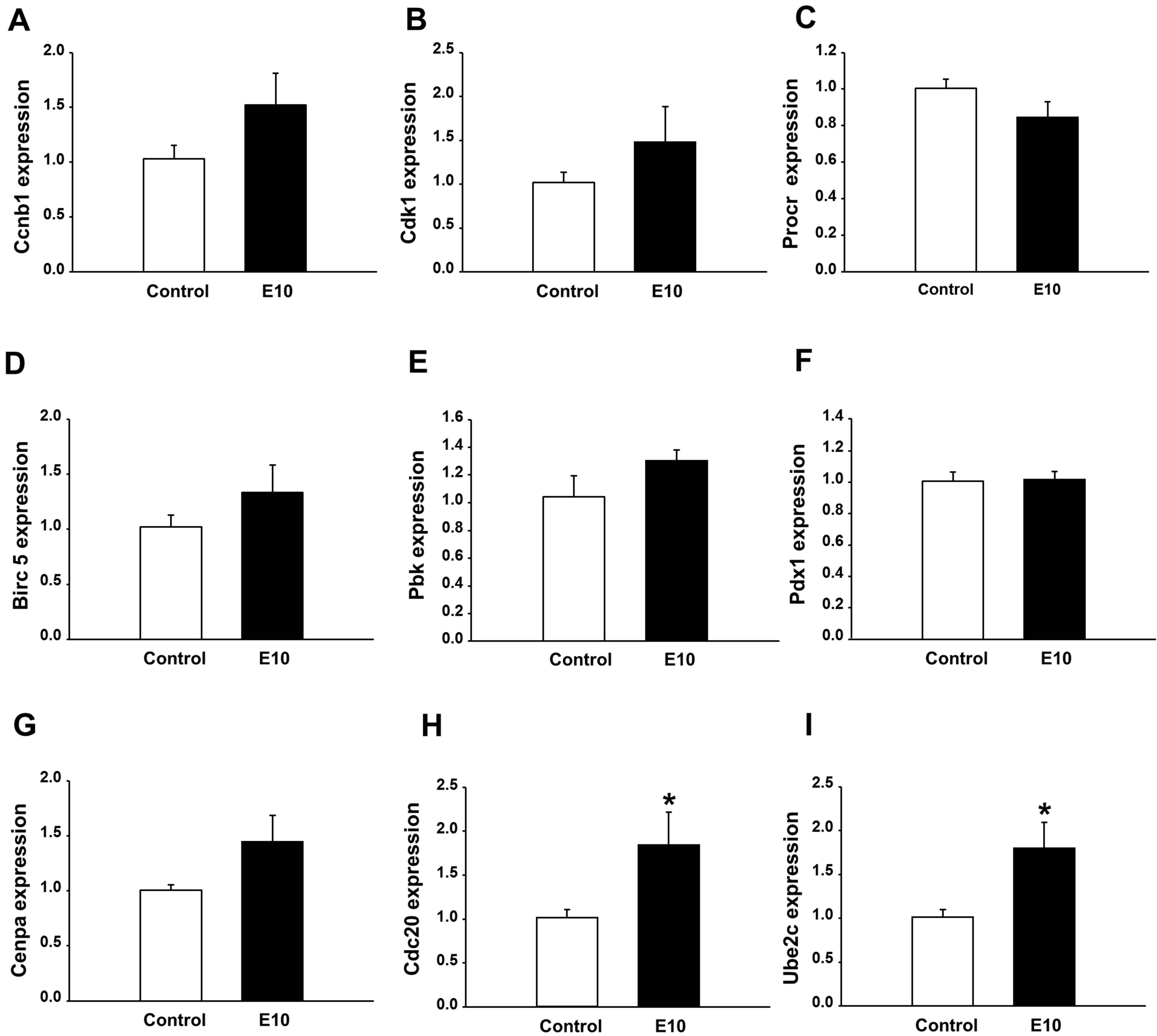
Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
Insulin		Insulin antibody	Santa Cruz Biotechnology	Rabbit; Polyclonal	1:100
BrdU		BrdU antibody	DAKO	Mouse;Monoclonal	1:100

Supplemental Figure 1. mRNA gene expression assessed by real-time RT-PCR of the same genes as in Figure 3 but from islets obtained from P120 mice (n=4-5 from 11-15 mice/group). Data are expressed as mean±SEM.

Supplemental Figure 2. mRNA gene expression assessed by real-time RT-PCR of the same genes as in Figure 3 but in the P30 offspring of mothers treated with vehicle (Control) or E2 10 µg/kg/day (E10) (n=4-5 from 12 mice/group 8-10 litters). Data are expressed as mean±SEM and statistical significance was determined by Student t-test compared to Control;*$p<0.05$.



Supplemental Figure 1



Supplemental Figure 2