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Epigenetic disruption and glucose homeostasis changes following low-dose maternal bisphenol A exposure

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Abstract

Developmental exposure to bisphenol A (BPA) has been linked to impaired glucose homeostasis and pancreatic function in adulthood, which has been hypothesized to result from the disruption of pancreatic β-cell development at early life. Here we evaluated whether maternal BPA exposure disrupts β-cell development and glucose tolerance and the role of epigenetic modifications of key regulator in this process. We found that maternal exposure to BPA (10 μg/kg/d) reduced the pancreatic β-cell mass and the expression of pancreatic and duodenal homeobox 1(*Pdx1*) at birth, as well as the expression of *Pdx1* at gestational day (GD) 15.5. In parallel with the decreased expression of *Pdx1*, histones H3 and H4 deacetylation, along with demethylation of histone 3 lysine 4 (H3K4) and methylation of histone 3 lysine 9 (H3K9), were found at the promoter of *Pdx1*, while no significant changes in DNA methylation status were detected at this region. Moreover, these alterations were observed in adult life along with impaired glucose tolerance. We conclude that maternal exposure to BPA reduces pancreatic β-cell mass at birth by reducing $PDX1^{+}$ progenitors during fetal development through altering the histone modifications of *Pdx1*, which can be propagated to later life and increase the susceptibility to glucose intolerance.

Keywords: Bisphenol A; pancreatic β-cell; pancreatic and duodenal homeobox 1; histone modification; DNA methylation; glucose homeostasis

Introduction

The rapidly increasing prevalence of obesity and type 2 diabetes (T2D) has posed huge health and economic burden globally in recent years. Data from the World Health Organization revealed that diabetes was responsible for 1.5 million deaths and 89 million disability-adjusted life years (DALYs) in 2012.[1] Although genetic predisposition, lifestyles and socioeconomic are the generally accepted risk factors for developing T2D, it is now recognized that early-life chemical exposure also play a significant role in the growing epidemic of diabetes and obesity in later life.[2] For example, prenatal exposure to nicotine has been proved to be a risk factor for obesity and metabolic disorders.[3] Endocrine disrupting chemicals (EDCs), to which people are widely exposed, have recently drawn much attention because of their associations with energy imbalance, obesity and T2D.[4]

Bisphenol A (BPA) is one of the highest production-volume EDCs used in manufacturing polycarbonate plastics and epoxy resins. Data from more than 80 biomonitoring studies all over the world indicates that human are widespread exposed to BPA.[5] Animal studies have raised the concern that perinatal exposure to BPA may induce obesity and/or metabolic syndrome.[6, 7] However, the underlying mechanisms are still not clarified.[8] Previous studies have suggested that pancreatic β-cell may serve as the target of BPA. Prenatal exposure to BPA has been shown to lead to alterations in Ca^{2+} signaling and insulin secretion in islets of Langerhans in adult male offspring, and reduction in proliferating insulin-secreting β-cells.[9] Exposure to di-2-ethylhexyl phthalate (DEHP), another kind of EDCs, through gestation and lactation reduced pancreatic β-cell mass, pancreatic insulin content and the expression of pancreatic and duodenal homeobox 1 (*Pdx1*) in weaning Wistar rats.[10]

Previous studies have also linked the development of T2D with impaired pancreatic β-cell development in early-life.[3] The development of pancreatic β-cells represents a concert of a hierarchy of transcription factors,[11] which is initiated by the expression of *Pdx1* in endodermal cells.[12] *Pdx1* is a critical regulator of pancreatic development and β-cell ontogeny during fetal development. Both genetic and acquired reduction in expression of *Pdx1* have verified the role of *Pdx1* in pancreatic development in both humans and animal models,[13, 14] while overexpression of *Pdx1* in pancreatic β-cells has been shown to restore cell mass and prevent the development of diabetes.[15, 16]

The fact that maternal exposure to environmental chemicals lead to metabolic disorders even long after the exposure was removed indicates that epigenetic mechanisms may be involved in this process. A number of studies have linked EDCs with the potential role of epigenetics in the development of T2D.[17] Interestingly, it is reported that point mutations of the proximal promoter drastically impaired the *Pdx1* promoter activity, indicating that epigenetic modifications in this region may have huge influences on the *Pdx1* expression.[18] Indeed, altered epigenetic modifications of *Pdx1* has been implicated as the reason of β-cell anomaly and occurrence of T2D in intrauterine growth retarded (IUGR) rats.[19] However, to our knowledge, no evidence has ever linked maternal exposure to BPA with the pancreatic β-cell development and the role of epigenetic modifications of *Pdx1* in this process.

In the present study, we aim to verify the hypothesis that maternal BPA exposure disturbs epigenetic modifications of key regulators in the development of pancreatic β-cell, which may contribute to the development of the T2D in adulthood.

Materials and methods

Animals and treatment

Wistar rats were purchased from Hubei Research Center of Experimental Animals (Wuhan, China) and were housed on a 12:12-h light-dark cycle. Rats were given ad libitum access to water and a standard rat chow. The water bottles and cages were made of BPA-free polypropylene. Two female rats were mated with 1 male rat after acclimatization for one week. The day on which a vaginal plug or a sperm positive vaginal smear was found was defined as gestational day (GD) 0.5. Pregnant rats were randomly assigned to two weight-matched treatment groups: BPA (10 μg/kg/d) or the vehicle corn oil (both were from Sigma-Aldrich, Saint louis, MO). This exposure dose was proved to make the internal exposure level similar to that detected in human.[20] BPA was first dissolved in corn oil to make a 10 μg/mL solution and both reagents were given at a volume of 1mL/kg body weight via gavage throughout gestation and lactation. Neonates were weighed and distributed within each group to balance the litter size (4 males and 4 females per dam), and caged with dams until weaning. For all experiments, both male and female animals were used due to the technical challenge of simultaneously identifying fetal sexes when isolating pancreatic buds. All the procedures were reviewed and approved by the Ethics Committee of Tongji Medical College (Huazhong University of Science and Technology, Wuhan, China).

Isolation of the pancreatic islets

Pancreatic islets of Langerhans from adult rats were isolated as previously described.[21] Briefly, rats were first anesthetized. Then the peritoneal cavity was opened and the hepatopancreatic ampulla of the duodenal was located following the common bile duct and the pancreatic duct.

After the duodenum was clamped off with hemostats at both up and downstream of the ampulla, the common bile duct was injected with collagenase V (1 mg/ml, Sigma-Aldrich, Saint louis, MO) dissolved in Hank's balanced sodium solution (HBSS) plus 1% w/v BSA until the pancreas was fully distended. The pancreas was removed to a 15 ml conical tube and put in 37 °C water bath for 18 min. Following washing for twice, the resulting tissue debris was re-suspended in a discontinuous Ficoll 400 gradient (25%, 23%, 20.5% and 11% w/v; Amresco, Solon, OH, USA) and centrifuged at 800 g for 20 min. The tissue debris at 23/20.5 and 20.5/11 interface was collected and washed twice with cold HBSS.

Immunostaining

For immunofluorescence, fetuses from 6 dams were delivered by caesarean at GD 15.5 and fixed in 4% w/v paraformaldehyde overnight, followed by being embedded in paraffin. Four-micrometer sagittal sections were cut, of which contained the pancreas were stained with rabbit antiserum (Millipore, Temecula, CA), and visualized with goat anti-rabbit secondary antibody conjugated to Alexa Fluo 555 (CST, CA). $PDX1^+$ cell fraction was calculated by dividing $PDX1^+$ cell number with the whole cell number in the pancreas area of the section. Given the regional variation in islet distribution and cell composition, every 30th section was analyzed, which yielded 5 to 7 sections from each pancreas. For immunohistochemistry, pancreas was excised from neonates from six randomly chosen dams. Pancreas were sectioned and immunostained with a mouse anti-insulin + proinsulin monoclonal antibody (Abcam, Cambridge, MA) at a dilution of 1:1250. Sections were detected by a secondary anti-mouse macromolecule ligated to horse radish peroxidase (Vector Laboratories, Burlingame, CA) and visualized in brown with 3,3 -Diaminobenzidine. As

described above, every 30th section was analyzed and 5 to 7 sections were yielded from each pancreas. All images were taken by Olympus IX71 (Olympus, Tokyo, Japan) equipped with Image-Pro Plus software (version 5.0; Media Cybernetics, Inc., Rockville, MD USA). Pancreatic β-cell mass was calculated by multiplying the total weight of the unfixed pancreas by the β-cell fraction, which was calculated as the ratio of insulin-positive area to the total tissue area of the section.

Real-time PCR

At GD 15.5 and birth, total RNA and DNA were extracted from pancreas using an AllPrep DNA/RNA Micro Kit (QIAGEN, Hilden, Germany). The mRNA was first reversely transcribed to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Carlsbad, CA, USA). The resulting cDNA was subjected to real-time PCR using FastStart Universal SYBR Green Master (Rox) (Roche, Germany) on a 7900HT Fast Real-Time PCR system. The primers for *Pdx1* amplification were as follows: forward, 5′-CGGACATCTCCCCATACG-3′; reverse, 5′-AAAGGGAGATGAACGCGG-3′. Primers for insulin amplification were: forward, 5′-TCTTCTACACACCCATGTCCC-3′; reverse, 5′-GGTGCAGCACTGATCCAC-3′. The PCR conditions were as follows: initial denaturing at 95 °C for 10 min; followed by 40 cycles of denaturing at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Data was analyzed using the $2^{-\Delta\Delta Ct}$ method,[22] with cyclophilin used as the internal control.

DNA methylation analysis of the proximal promoter of *Pdx1*

DNA extracted from fetal and neonatal pancreas, as well as from islets isolated from 8 week-old offspring were treated with bisulphite and subjected to methyl-specific PCR amplification. Pancreas from three fetuses at GD 15.5 and pancreas from two neonates were

pooled together to get 1 tube of DNA. The primer pair used in this amplification was as follows: forward, 5′-GGGGGATTAGTATTGAATTTTGGTA-3′; reverse, 5′-AAACCTCCTTCTTAAAACAAAACCA-3′. The resulting PCR products were transcribed to RNA using the T7 RNA polymerase in vitro, followed by RNase A cleavage into fragments containing the CpG sites. These RNA fragments were finally analyzed by the Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometry.

Chromatin immunoprecipitation assay

Pancreas from fetuses and neonates, and islets from 8 week-old offspring were cut into pieces and cross-linked in PBS containing 1% w/v formaldehyde (Sigma-Aldrich, Saint louis, MO) and protease inhibitor cocktail for 10 min on a rocking platform. Cells were lysed using SDS lysis buffer and the resulting chromatin was sheared into fragments ranging from 200 to 1000 bp. DNA shearing was conducted using a VCX 750 ultrasonic processor with a time-course of six 15 s pulses at 50% output with 60 s ice-rest between pulses. Supernatants were precleared with protein A-sepharose (Millipore, Temecula, CA). An aliquot of supernatant was 1:100 diluted and used as the input control. The precleared supernatants were incubated with rabbit anti-acetyl-histone H3/H4, rabbit anti-dimethyl-histone H3 (Lys 4) or mouse anti-trimethyl-histone H3 (Lys 9) (all were purchased from Millipore, Temecula, CA) overnight at 4 °C on a rocking platform. DNA-protein-antibody complexes were precipitated by protein A-sepharose and washed serially with low-salt buffer, high-salt buffer, IP wash buffer and TE buffer. Precipitated complexes were eluted from protein A with an elution buffer (1% w/v $NaHCO_3$, 1% w/v SDS), followed by reverse cross-linking using 5M NaOH

and thoroughly digestion by proteinase K. The resulting DNA was purified with QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and subjected to PCR amplification. The primer set used in this study was learned from previous research (15), which was as follows: forward, 5′-GCAGGACAGGAGAGATCAGC-3′; reverse, 5′-CCCAGATCGCTTTGACAGTT-3′.

Intraperitoneal glucose or insulin tolerance tests

Rat offspring randomly chosen at 4 weeks, 8 weeks and 20 weeks were subjected to intraperitoneal glucose tolerance test (ipGTT) or intraperitoneal insulin tolerance test (ipITT) to evaluate the glucose homeostasis and insulin sensitivity. For ipGTT, rats were fasted overnight and injected with 2 g/kg glucose dissolved in saline intraperitoneally. Blood glucose was measured at 0, 15, 30, 60, and 120 min later using an Accu-chek glucometer and test strips (Roche, Mannheim, Germany). For ipITT, rats were fasted for 6 h and given an i.p. injection of 0.75 IU/kg human insulin (Novo Nordisk, Bagsvaerd, Denmark) and the time points for blood glucose measurement was 0, 15, 30, 45, and 60 min. Rats were excluded from data analysis if they did not exhibit a rise in blood glucose greater than 20 mmol/L (360 mg/dl) in the first 15 min or if they exhibited diarrhea.

Statistics

Statistical analysis was carried out with SPSS 13.0. A repeated measure analysis of variances (ANOVA) was used in the analysis of data from ipGTT and ipITT. Other statistical analyses were performed using two-tailed Student's t-test. Data were presented as mean ± SEM, $p < 0.05$ was considered significant in all the analyses.

Results

Maternal exposure to BPA reduces pancreatic β-cell mass at birth.

The results of birth weight, pancreas weight, and organ coefficient are shown in Table 1. Birth weight and pancreas weight were significantly reduced ($p < 0.001$) due to maternal exposure to BPA, while no significant variation in the pancreas organ coefficient (organ weight/body weight) was observed. Fig. 1 shows that BPA exposure significantly reduced ($p < 0.001$) the pancreatic β-cell mass (BCM) at birth (Fig. 1A, B, and C). However, this reduction in cell mass did not alter the content of mRNA for insulin in isolated pancreas (Fig. 1D).

Maternal exposure to BPA disrupts the expression and epigenetic modifications of *Pdx1* at birth

We also examined the expression of mRNA for some key regulators which are important for the pancreatic β-cell development. The expression of mRNA for *Pdx1* was decreased in the BPA group, whereas no changes were observed in the expression of pancreas transcription factor 1 subunit alpha (*Ptf1a*), glucagon (*Gcg*), and amylase (*Amy*) (Fig. 2A).

We next investigated whether this change in expression of *Pdx1* was correlated with epigenetic modifications of the proximal promoter of *Pdx1*. The acetylation status of core histones H3 and H4, trimethylation of lysine 4 at H3 (H3K4me3) and dimethylation of lysine 9 at H3 (H3K9me2) were determined by chromatin immunoprecipitation (ChIP). Maternal exposure to BPA significantly reduced the abundance of histones H3 ($p < 0.001$) and H4 ($p < 0.05$) acetylation and H3K4me3 ($p < 0.01$), whereas increased the abundance of H3K9me2 (p

< 0.05), leading to repressed chromatin structure (Fig. 2B). In addition to histone modifications, we also determined the DNA methylation status of the proximal promoter of *Pdx1*. The CpG sites examined in the present study were illustrated in Fig. 2C. However, no substantial changes in the DNA methylation status were observed at birth. Two sites (2 and 12) were consistently methylated across the study.

Maternal exposure to BPA disrupts the expression and epigenetic modifications of *Pdx1* at GD 15.5

Since the development of pancreatic β-cell initiates and develops under the control of *Pdx1* at fetal stages before birth, we also examined the expression and epigenetic modifications of *Pdx1* at gestational day (GD) 15.5. The expression of mRNA for *Pdx1* and *Ptf1a* were reduced by 30.9% and 63.6% respectively, indicating a potential decrease in the abundance of pancreatic progenitor cells, while the expression of mRNA for insulin (*Ins*), *Gcg, Amy*, and neurogenin 3 (*Ngn3*) remained unchanged (Fig. 3A). In addition to the reduction in expression of mRNA for *Pdx1*, morphological results also showed that BPA exposure significantly reduced the $PDX1^+$ cell fraction ($p < 0.01$) at GD 15.5 compared to the control group (Fig. 3B and C).

At this life stage, BPA also significantly reduced the acetylation of histones H3 and H4, as well as the H3K4me3 (($p < 0.05$), while H3K9me2 was not detected in control group. Still, we did not observe any changes in DNA methylation status at the promoter of *Pdx1*.

Altered epigenetic modifications of *Pdx1* propagates to 8 weeks of age

To verify the continuity and progressiveness of the epigenetic modifications, we also investigated the epigenetic modifications of *Pdx1* at 8 weeks of age. The difference in histone

code between two groups was strengthened at this life stage. Acetylated histones H3 and H4 were barely detected by 8 weeks in BPA exposed group whereas the H3K9me2 was significantly increased compared to the control (Fig. 4A). Surprisingly, DNA methylation status still did not show any changes between two groups (Fig. 4B).

Maternal exposure to BPA induces glucose intolerance and insulin resistance in adulthood

We also tried to verify if the disrupted pancreatic β-cell development at birth correlates with the glucose homeostasis at adulthood. There was no significant difference in both ipGTT and ipITT at 4 weeks (Fig. 5A, G). At 8 weeks, the results of ipGTT and ipITT showed slight differences after the injection (Fig. 5B, H), with the area under curve (AUC) of ipGTT showed a marginal difference (p = 0.088; Fig. 5E). At 20 weeks, repeated measures ANOVA showed significant differences in interaction between treatment and time (p = 0.001 and 0.026 for ipGTT and ipITT respectively). The BPA group exhibited higher blood glucose levels at 15, 30 and 60 min after glucose injection (Fig. 5C). There were significant differences in blood glucose at 0 and 15 min after the injection during the ipITT (Fig. 5I). The AUC also showed significant differences at this stage (Fig. 5F, L).

Separated analyses of data for males and females revealed no differences for both sexes at 4 weeks of age (supplementary Fig. S1A-H). At 8 weeks, BPA exposed males first exhibited insulin resistance (supplementary Fig. S2C), which was confirmed by the increased AUC in BPA rats (supplementary Fig. S2D), whereas females showed no difference between control and BPA group (supplementary Fig. S2E-H). At 20 weeks, BPA exposed males showed both glucose intolerance and insulin resistance compared to control males (supplementary Fig.

S3A-D). Glucose intolerance was also detected in BPA exposed females at this age (supplementary Fig. S3E, F).

Discussion

Previous studies have demonstrated that in utero exposure to low-dose BPA disrupts glucose homeostasis and induces diabetes [6, 9, 17]. However, the underlying molecular mechanisms remain unclear. In this study, we found that perinatal exposure to BPA impaired the fetal development of pancreatic β-cell, characterized by decreased $PDX1^+$ cell fraction at GD 15.5, and reduced pancreatic β-cell mass at birth. Furthermore, alterations in histone modification of *Pdx1* promoter towards an inactivated chromatin were observed in parallel with reduction in $PDX1^+$ cell fraction at GD 15.5. These alterations propagated to 8 weeks of age, when glucose intolerance and insulin resistance were observed. To our knowledge, this is the first study to report the correlation between pancreatic β-cell development and the histone modification of *Pdx1* gene in a BPA exposure model. We believe this will help understand the underlying mechanisms by which early life exposure to BPA may impact on the development of diabetes in adulthood.

This research further verifies that perinatal period is the critical time window for BPA exposure. Rodent models and human studies have shown that BPA can be transferred across the placenta,[23, 24] and through the milk,[25] which makes the developing organ systems particularly susceptible to the potential adverse effects of BPA, including brain and behavior,[26, 27] reproductive system,[28] and metabolism.[29] The perinatal period being one of the most crucial time windows is due to not only directly exposure but also the effects of BPA on maternal behaviors.[30]

Both *in vivo* and *in vitro* studies have suggested that pancreatic β-cell as the potential target, by which BPA may exert its effects on blood glucose homeostasis and diabetes.[31, 32] However, to our knowledge, no evidence has ever linked BPA exposure with the development of endocrine pancreas *in vivo*, of which impairment has been proposed to greatly increase the susceptibility to the development of T2D.[33] In our present study, we found that perinatal exposure to BPA significantly reduced the pancreatic β-cell mass at birth, which, at least in part, account for the association between prenatal exposure to BPA and the onset of T2D. The involvement of endocrine pancreas in the development of T2D has been verified by various previous studies involving developmental malnutrition and/or developmental exposure to environmental chemicals. Both maternal low-protein and low-energy diet during pregnancy significantly decreased the rat fetal β-cell mass.[34] Similar results have been demonstrated in vitamin A deficiency and exposure to DEHP, nicotine, and dexamethasone models during development.[10, 35-37] Furthermore, studies indicate that even a 37% increase in pancreatic β-cell proliferation in IUGR animals at 3 months of age, the pancreatic β-cell mass remained less than that of the normally nourished ones, indicating a persistent reduction in β-cell mass in adulthood.[38] This may in turn strengthen the reduction in β-cell mass, because adult pancreatic β-cells are maintained by self-duplication of existing cells rather than stem-cell differentiation.[39] When this persistent reduction in pancreatic β-cell mass encounters aging, glucose intolerance was trigged.[40] Intriguingly, the reduced beta cell mass by BPA was not mirrored by a reduction of insulin mRNA, suggesting a higher insulin content per beta cell.[34]

Pdx1 is a key regulator in determining cell lineage in pancreatic development and maintaining β-cell function in adulthood. Increasing evidence suggests that decreased expression level of

299 *Pdx1* in development may be responsible for the reduction in pancreatic β-cell mass induced
300 by developmental deficiency in nutrition.[19, 34] Restoration of expression of *Pdx1* by neonatal
301 administration of exendin-4 counteracted the progressive reduction in pancreatic β-cell mass
302 after intrauterine growth retardation.[16] In consistent with the reduction in pancreatic β-cell
303 mass, our data suggest that the expression of *Pdx1* was significantly reduced by BPA
304 exposure at birth. We also found that the expression level of *Pdx1* and *Ptf1a* were
305 significantly reduced compared to that of the control group at GD 15.5. Genetic tracing
306 experiments have demonstrated that both endocrine and exocrine cells of the pancreas derive
307 from a pool of progenitor cells that express the transcription factors *Pdx1* [41] and *Ptf1a*.[42]
308 These results indicate that reduction in the $PDX1^+$ progenitor cells at an early developmental
309 stage may be responsible for the impairment of pancreatic β-cell development in our
310 experiment. Conditional progenitor cell ablation revealed that pancreas size was limited by
311 the size of the progenitor cell pool that was set aside in early development and was not subject
312 to growth compensation.[43] The same study also suggested that early ablation of the $PDX1^+$
313 progenitor cells decreased $insulin^+$ cell area at E18.5 while $amylase^+$ (exocrine) and DBA^+
314 (duct) cell area showed small non-significant changes, and adult mice derived from early
315 ablation of pancreatic progenitor cells exhibited glucose intolerance at 11 weeks.[43] These
316 results indicate that BPA induced reduction in $PDX1^+$ progenitor cells may have profound and
317 permanent effects on pancreatic β-cell development and glucose homeostasis in adulthood.
318 Indeed, in addition to the reduced pancreatic β-cell mass at birth, we also observed slight
319 changes in glucose metabolism and insulin response in BPA exposed offspring at as early as 8
320 weeks, and this propagated to insulin resistance at 20 weeks of age. Although we believe that

perinatal exposure to BPA may possibly decrease the pancreatic β-cell mass by reducing the $PDX1^+$ progenitor cell pool at early stage, studies are still needed to elucidate the exact events mediating the effects. It is worth noting that previous studies indicate that females are more resistant to the toxicity of BPA on glucose homeostasis.[9] However, identifying the fetal sexes when isolating the pancreatic buds at GD 15.5 turned out to be technically challenging for us. In this case, following experiments were conducted on rats irrespective of the sexes. However, we still tried to analyze the GTT and ITT data separately by sex (see supplementary materials), and found that the emerging time of glucose intolerance for females was later than that for males, which is in consistent with previous studies.[6]

Epigenetic modifications provide a mechanism, by which the activity of gene expression can be reprogrammed and propagated to later life stage. The dynamic epigenome is sensitive to environmental signals, which makes it an interface between the dynamic environment and the static genome.[44] Moreover, pancreas development and T2D have both been implicated to be associated with epigenetic modifications.[45, 46] In mammals, epigenetic modifications are mainly mediated by DNA methylation and chromatin modifications. In vitro studies suggest that the proximal promotor region of *Pdx1* contains a highly conserved CpG island and is heavily acetylated at histone H3 and H4.[18, 47] We first observed a decrease in the acetylated H3 and H4, and H3K4me3 at GD 15.5 in BPA exposed fetuses, followed by an increase in H3K9me2 at birth. These results indicate that BPA significantly altered the chromatin modifications of *Pdx1* promotor towards an inactive direction, through which the expression level of *Pdx1* was significantly decreased. Moreover, these epigenetic modifications were progressively propagated to 8 weeks of age, indicating permanent impairments of expression

level of *Pdx1*.

We also detected the DNA methylation status of the proximal promotor region in parallel with histone modifications in the same region. Surprisingly, we did not detect any significant changes at all the 19 CpG sites at different time points. Emerging evidence supports the view that DNA methylation and histone modifications may be mutually reinforcing and interdependent, and histone modifications are readily reversible whereas DNA methylation is stable.[48] Given that methylation of H3K9 precedes DNA methylation,[49] it is possible that the histone modifications of the *Pdx1* promoter in BPA exposed group may represent an early stage of DNA methylation in this study. However, to our knowledge, no previous studies have ever identified the DNA methylation status of the promoter of *Pdx1* in primary islet tissue when exposed to BPA during fetal development. Future studies are required to fully verify the long-term effects of BPA on DNA methylation of the *Pdx1* promoter and the molecular interactions in this process.

Conclusions

In conclusion, our study demonstrates that perinatal exposure to BPA induces altered histone modifications towards an inactive status in the proximal promotor of *Pdx1*. This leads to the reduction in $PDX1^+$ progenitor cells during development, which in turn impairs the development of the pancreatic β-cell. And these finally disrupt glucose homeostasis at adulthood. We believe that our research provides new insights of the mechanisms by which perinatal exposure to BPA may impair pancreatic β-cell development and induce T2D.

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371 **Conflicts of interest**

372 There are no conflicts of interest to declare.

References

1. WHO, Global status report on noncommunicable diseases 2014, http://www.who.int/nmh/publications/ncd-status-report-2014/en/, (accessed 20th May, 2015).
2. H. Inadera, Developmental origins of obesity and type 2 diabetes: molecular aspects and role of chemicals, *Environmental health and preventive medicine*, 2013, **18**, 185-197.
3. M. Behl, D. Rao, K. Aagaard, T. L. Davidson, E. D. Levin, T. A. Slotkin, S. Srinivasan, D. Wallinga, M. F. White, V. R. Walker, K. A. Thayer and A. C. Holloway, Evaluation of the Association between Maternal Smoking, Childhood Obesity, and Metabolic Disorders: A National Toxicology Program Workshop Review, *Environmental Health Perspectives (Online)*, 2013, **121**, 170.
4. P. Alonso-Magdalena, I. Quesada and A. Nadal, Endocrine disruptors in the etiology of type 2 diabetes mellitus, *Nat Rev Endocrinol*, 2011, **7**, 346-353.
5. L. N. Vandenberg, I. Chahoud, J. J. Heindel, V. Padmanabhan, F. J. Paumgartten and G. Schoenfelder, Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A, *Environmental health perspectives*, 2010, 1055-1070.
6. J. Wei, Y. Lin, Y. Li, C. Ying, J. Chen, L. Song, Z. Zhou, Z. Lv, W. Xia, X. Chen and S. Xu, Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet, *Endocrinology*, 2011, **152**, 3049-3061.
7. M. Susiarjo, F. Xin, A. Bansal, M. Stefaniak, C. Li, R. A. Simmons and M. S. Bartolomei, Bisphenol a exposure disrupts metabolic health across multiple generations in the mouse, *Endocrinology*, 2015, **156**, 2049-2058.
8. R. Rezg, S. El-Fazaa, N. Gharbi and B. Mornagui, Bisphenol A and human chronic diseases: Current evidences, possible mechanisms, and future perspectives, *Environment International*,

2014, **64**, 83-90.

9. P. Alonso-Magdalena, E. Vieira, S. Soriano, L. Menes, D. Burks, I. Quesada and A. Nadal, Bisphenol A exposure during pregnancy disrupts glucose homeostasis in mothers and adult male offspring, *Environ Health Perspect*, 2010, **118**, 1243-1250.

10. Y. Lin, J. Wei, Y. Li, J. Chen, Z. Zhou, L. Song, Z. Wei, Z. Lv, X. Chen, W. Xia and S. Xu, Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat, *American journal of physiology. Endocrinology and metabolism*, 2011, **301**, E527-538.

11. J. M. Oliver-Krasinski and D. A. Stoffers, On the origin of the beta cell, *Genes & development*, 2008, **22**, 1998-2021.

12. C. McKinnon and K. Docherty, Pancreatic duodenal homeobox–1, PDX-1, a major regulator of beta cell identity and function, *Diabetologia*, 2001, **44**, 1203-1214.

13. J. Jonsson, L. Carlsson, T. Edlund and H. Edlund, Insulin-promoter-factor 1 is required for pancreas development in mice, *Nature*, 1994, **371**, 606-609.

14. D. A. Stoffers, N. T. Zinkin, V. Stanojevic, W. L. Clarke and J. F. Habener, Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence, *Nature genetics*, 1997, **15**, 106-110.

15. J. A. Kushner, J. Ye, M. Schubert, D. J. Burks, M. A. Dow, C. L. Flint, S. Dutta, C. V. E. Wright, M. R. Montminy and M. F. White, Pdx1 restores β cell function in Irs2 knockout mice, *The Journal of Clinical Investigation*, 2002, **109**, 1193-1201.

16. D. A. Stoffers, B. M. Desai, D. D. DeLeon and R. A. Simmons, Neonatal Exendin-4 Prevents the Development of Diabetes in the Intrauterine Growth Retarded Rat, *Diabetes*, 2003, **52**,

734-740.

17. Y. Ma, W. Xia, D. Wang, Y. Wan, B. Xu, X. Chen, Y. Li and S. Xu, Hepatic DNA methylation modifications in early development of rats resulting from perinatal BPA exposure contribute to insulin resistance in adulthood, *Diabetologia*, 2013, **56**, 2059-2067.

18. S. Sharma, J. Leonard, S. Lee, H. D. Chapman, E. H. Leiter and M. R. Montminy, Pancreatic islet expression of the homeobox factor STF-1 relies on an E-box motif that binds USF, *Journal of Biological Chemistry*, 1996, **271**, 2294-2299.

19. J. H. Park, D. A. Stoffers, R. D. Nicholls and R. A. Simmons, Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1, *The Journal of clinical investigation*, 2008, **118**, 2316-2324.

20. G. S. Prins, S.-H. Ye, L. Birch, S.-m. Ho and K. Kannan, Serum bisphenol A pharmacokinetics and prostate neoplastic responses following oral and subcutaneous exposures in neonatal Sprague–Dawley rats, *Reproductive Toxicology*, 2011, **31**, 1-9.

21. J. D. Carter, S. B. Dula, K. L. Corbin, R. Wu and C. S. Nunemaker, A practical guide to rodent islet isolation and assessment, *Biological procedures online*, 2009, **11**, 3-31.

22. K. J. Livak and T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods*, 2001, **25**, 402-408.

23. B. Balakrishnan, K. Henare, E. B. Thorstensen, A. P. Ponnampalam and M. D. Mitchell, Transfer of bisphenol A across the human placenta, *Am J Obstet Gynecol*, 2010, **202**, 393.e391-397.

24. M. Nishikawa, H. Iwano, R. Yanagisawa, N. Koike, H. Inoue and H. Yokota, Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus, *Environ Health*

Perspect, 2010, **118**, 1196-1203.

25. B. F. Healy, K. R. English, P. Jagals and P. D. Sly, Bisphenol A exposure pathways in early childhood: Reviewing the need for improved risk assessment models, *J Expos Sci Environ Epidemiol*, 2015, **25**, 544-556.

26. H. Chang, M. Wang, W. Xia, T. Chen, W. Huo, Z. Mao, Y. Zhu, Y. Li and S. Xu, Perinatal exposure to low-dose bisphenol A disrupts learning/memory and DNA methylation of estrogen receptor alpha in the hippocampus, *Toxicology Research*, 2016.

27. Z.-H. Liu, Y. Yang, M.-M. Ge, L. Xu, Y. Tang, F. Hu, Y. Xu and H.-L. Wang, Bisphenol-A exposure alters memory consolidation and hippocampal CA1 spine formation through Wnt signaling in vivo and in vitro, *Toxicology Research*, 2015, **4**, 686-694.

28. J. Peretz, L. Vrooman, W. A. Ricke, P. A. Hunt, S. Ehrlich, R. Hauser, V. Padmanabhan, H. S. Taylor, S. H. Swan, C. A. VandeVoort and J. A. Flaws, Bisphenol a and reproductive health: update of experimental and human evidence, 2007-2013, *Environ Health Perspect*, 2014, **122**, 775-786.

29. K. A. Thayer, J. J. Heindel, J. R. Bucher and M. A. Gallo, Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review, *Environmental health perspectives*, 2012, **120**, 779.

30. M. C. Catanese, A. Suvorov and L. N. Vandenberg, Beyond a means of exposure: a new view of the mother in toxicology research, *Toxicol. Res.*, 2015, **4**, 592-612.

31. P. Alonso-Magdalena, S. Morimoto, C. Ripoll, E. Fuentes and A. Nadal, The Estrogenic Effect of Bisphenol A Disrupts Pancreatic β-Cell Function In Vivo and Induces Insulin Resistance, *Environmental Health Perspectives*, 2005, **114**, 106-112.

32. A. Nadal, P. Alonso-Magdalena, S. Soriano, I. Quesada and A. B. Ropero, The pancreatic beta-cell as a target of estrogens and xenoestrogens: Implications for blood glucose homeostasis and diabetes, *Molecular and cellular endocrinology*, 2009, **304**, 63-68.

33. C. N. Hales and D. J. Barker, Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis, *Diabetologia*, 1992, **35**, 595-601.

34. O. Dumortier, B. Blondeau, B. Duvillie, B. Reusens, B. Breant and C. Remacle, Different mechanisms operating during different critical time-windows reduce rat fetal beta cell mass due to a maternal low-protein or low-energy diet, *Diabetologia*, 2007, **50**, 2495-2503.

35. K. A. Matthews, W. B. Rhoten, H. K. Driscoll and B. S. Chertow, Vitamin A deficiency impairs fetal islet development and causes subsequent glucose intolerance in adult rats, *The Journal of nutrition*, 2004, **134**, 1958-1963.

36. E. Somm, V. M. Schwitzgebel, D. M. Vauthay, E. J. Camm, C. Y. Chen, J. P. Giacobino, S. V. Sizonenko, M. L. Aubert and P. S. Huppi, Prenatal nicotine exposure alters early pancreatic islet and adipose tissue development with consequences on the control of body weight and glucose metabolism later in life, *Endocrinology*, 2008, **149**, 6289-6299.

37. O. Dumortier, N. Theys, M. T. Ahn, C. Remacle and B. Reusens, Impairment of rat fetal beta-cell development by maternal exposure to dexamethasone during different time-windows, *PloS one*, 2011, **6**, e25576.

38. A. Garofano, P. Czernichow and B. Breant, Beta-cell mass and proliferation following late fetal and early postnatal malnutrition in the rat, *Diabetologia*, 1998, **41**, 1114-1120.

39. Y. Dor, J. Brown, O. I. Martinez and D. A. Melton, Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation, *Nature*, 2004, **429**, 41-46.

40. A. Garofano, P. Czernichow and B. Breant, Effect of ageing on beta-cell mass and function in rats malnourished during the perinatal period, *Diabetologia*, 1999, **42**, 711-718.

41. G. Gu, J. Dubauskaite and D. A. Melton, Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors, *Development*, 2002, **129**, 2447-2457.

42. Y. Kawaguchi, B. Cooper, M. Gannon, M. Ray, R. J. MacDonald and C. V. Wright, The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors, *Nature genetics*, 2002, **32**, 128-134.

43. B. Z. Stanger, A. J. Tanaka and D. A. Melton, Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver, *Nature*, 2007, **445**, 886-891.

44. R. Feil and M. F. Fraga, Epigenetics and the environment: emerging patterns and implications, *Nature Reviews Genetics*, 2012, **13**, 97-109.

45. D. Avrahami and K. H. Kaestner, Epigenetic regulation of pancreas development and function, *Seminars in cell & developmental biology*, 2012, **23**, 693-700.

46. E. R. Gilbert and D. Liu, Epigenetics: The missing link to understanding β-cell dysfunction in the pathogenesis of type 2 diabetes, *Epigenetics*, 2012, **7**, 841-852.

47. K. Gerrish, J. C. Van Velkinburgh and R. Stein, Conserved transcriptional regulatory domains of the pdx-1 gene, *Molecular Endocrinology*, 2004, **18**, 533-548.

48. H. Cedar and Y. Bergman, Linking DNA methylation and histone modification: patterns and paradigms, *Nat Rev Genet*, 2009, **10**, 295-304.

49. K. E. Bachman, B. H. Park, I. Rhee, H. Rajagopalan, J. G. Herman, S. B. Baylin, K. W. Kinzler and B. Vogelstein, Histone modifications and silencing prior to DNA methylation of a

506 tumor suppressor gene, *Cancer cell*, 2003, **3**, 89-95.

507

508 **Table 1. General parameters of neonatal rats.**

	Body weight (g)	Pancreas weight (g)	Organ coefficient
Control (*n* = 61)	4.638 ± 0.011	0.027 ± 6.557E-5	0.006 ±5.882E-6
BPA (*n* = 73)	3.954 ± 0.010*	0.022 ± 6.849E-5*	0.006 ± 1.127E-5

509 Data are represented as mean ± SEM; *$p < 0.001$ by two-tailed Student's t-test.

Figure captions

Fig. 1 Pancreatic β-cell mass and insulin expression at birth. (A, B) Representative images of pancreatic β-cells (brown) in pancreas isolated from the control (A) and BPA (B) group at birth (n = 6). Bars = 50 μm. (C) The pancreatic β-cell mass at birth. Mean pancreatic β-cell fraction was used to calculate the absolute pancreatic β-cell mass as described in the Materials and Methods section (n = 6). (D) The relative mRNA expression level of insulin at birth (n = 6). Data are represented as mean ± SEM, $^{***}p < 0.001$ by two-tailed Student's t-test.

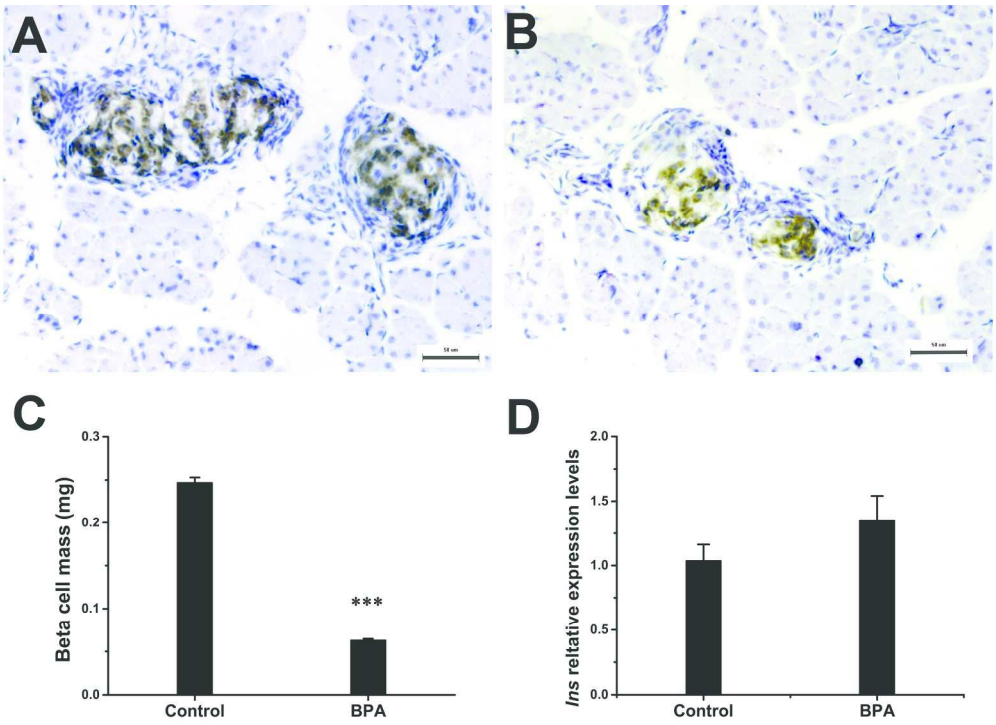
Fig. 2 The expression and epigenetic profiling of *Pdx1* at birth. (A) The expression level of mRNA for *Pdx1* was determined at birth (n = 6). (B) The abundance of acetylated H3 (AceH3), acetylated H4 (AceH4), H3K4me3, and H3K9me2 at the *Pdx1* promoter (n = 3). Data are represented as percent of input control, which went through the analyses exactly as the test samples except for the IP step. (C) A schematic illustration of the analyzed CpG sites at *Pdx1* proximal promoter. CpG sites are marked with open circles relative to translational start site (+1 bp). (D) DNA methylation status of the *Pdx1* promoter (n = 3). Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$ by two-tailed Student's t-test.

Fig. 3 The expression and epigenetic profiling of *Pdx1* at GD 15.5. (A) The expression level of mRNA for *Pdx1* was determined at GD 15.5 (n = 6). (B) $PDX1^{+}$ cell was stained red with anti-PDX1 antibody and the pancreas at GD 15.5 was outlined (n = 6). Bars = 50 μm. (C) $PDX1^{+}$ cell fraction at GD 15.5 (n = 6). (D) The abundance of AceH3, AceH4, H3K4me3, and H3K9me2 at the *Pdx1* promoter (n = 3). Data are represented as percent of input control, which went through the analyses exactly as the test samples except for the IP step. (E) DNA methylation status at the *Pdx1* promoter (n = 3). Data are represented as mean ± SEM. $^{*}p <$

0.05, $^{**}p < 0.01$ by two-tailed Student's t-test.

Fig. 4 Epigenetic profiling of *Pdx1* at 8 weeks of age. (A) The abundance of AceH3, AceH4, H3K4me3, and H3K9me2 at the *Pdx1* promoter. Data are represented as percent of input control, which went through the analyses exactly as the test samples except for the IP step ($n = 3$). (B) DNA methylation status at the *Pdx1* promoter ($n = 3$). Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ by two-tailed Student's t-test.

Fig. 5 Glucose homeostasis and insulin sensitivity in adult rats. ipGTT was performed at 4 weeks (A), 8 weeks (B), and 20 weeks (C), n = 10-19 and 8-17 for control and BPA respectively. Panels D-F show the ipGTT AUC. The ipITT was performed in the same groups at 4 weeks (G), 8 weeks (H) and 20 weeks (I), n = 11-16 and 12-17 for control and BPA respectively. Panels J-L show the ipITT AUC. Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by two-tailed Student's t-test.



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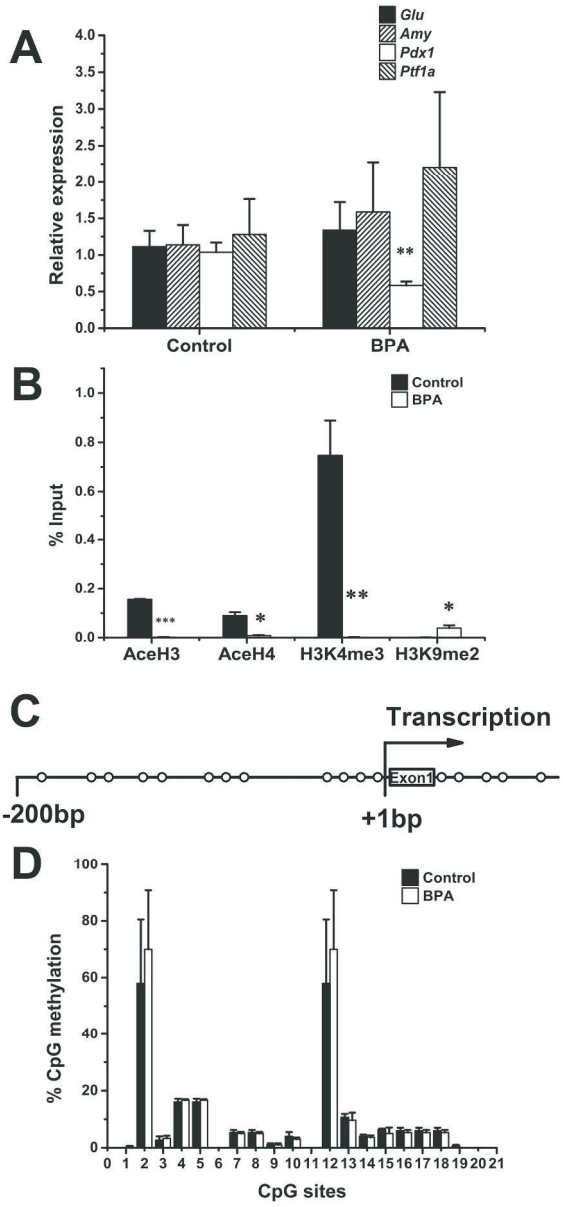

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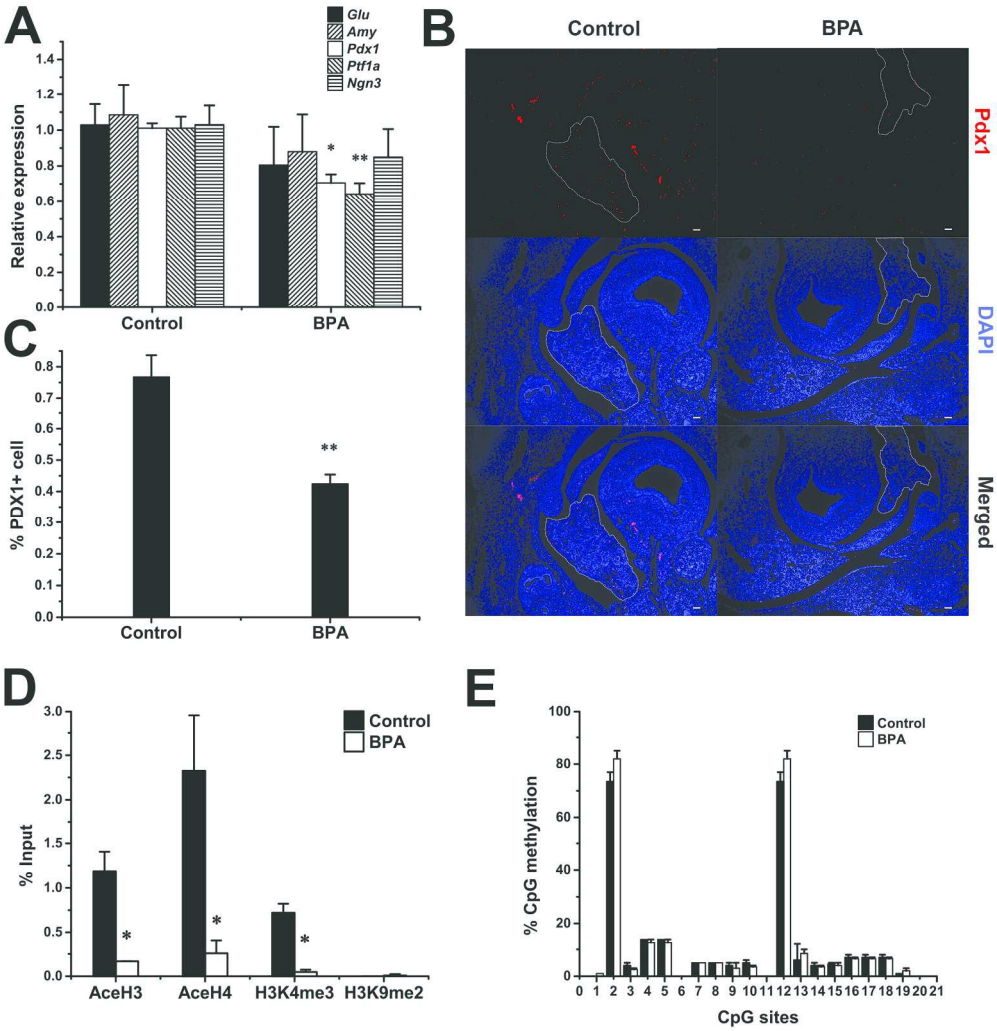

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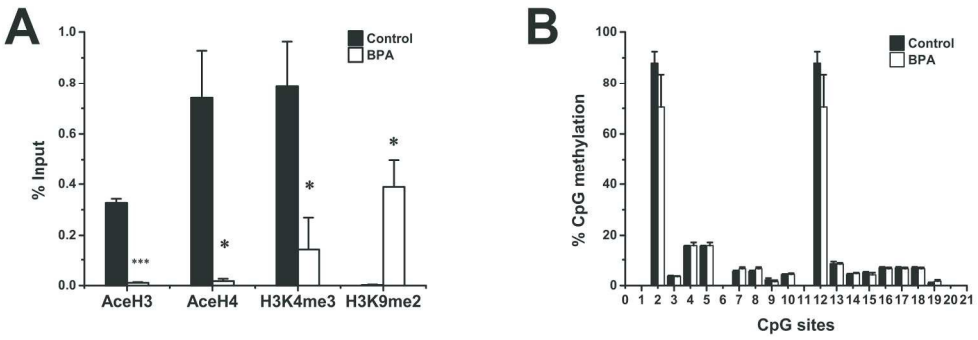

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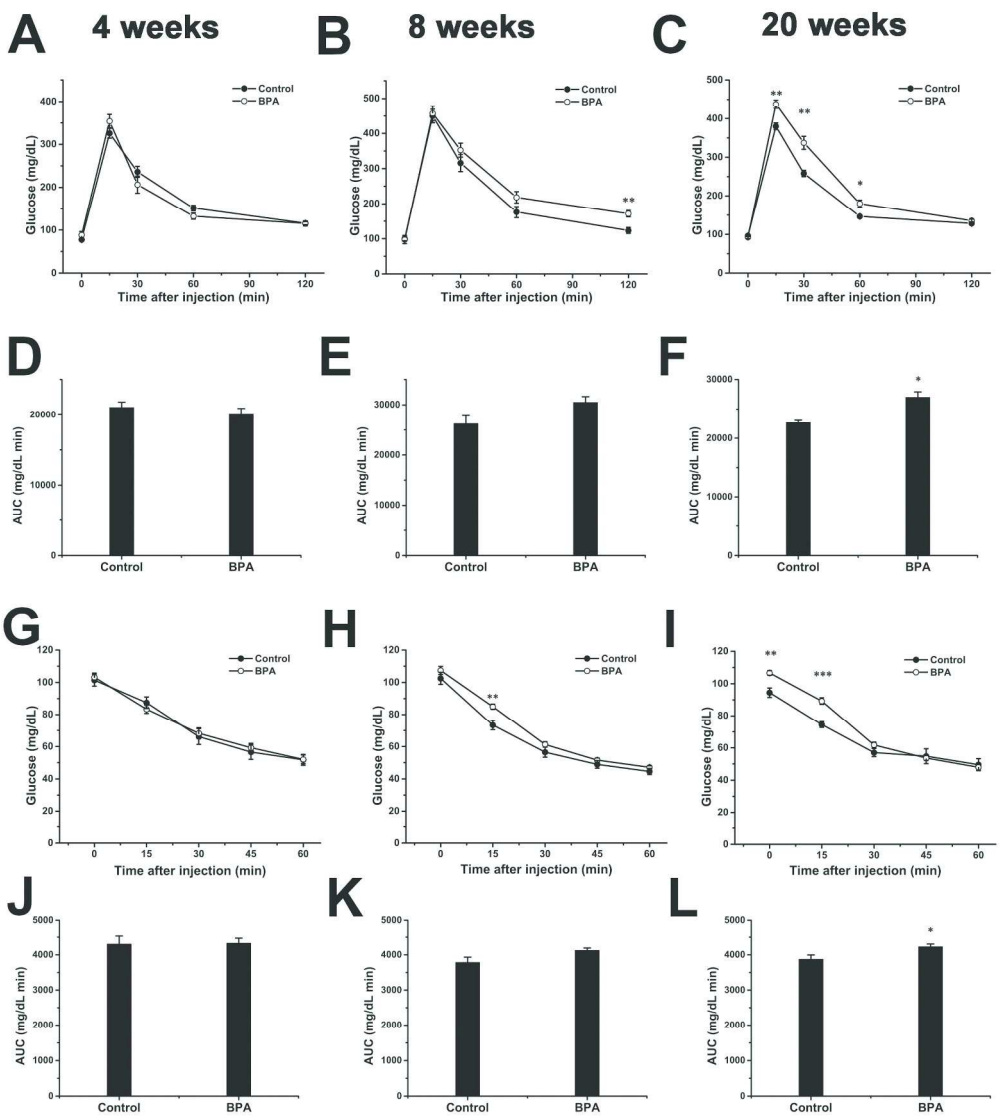

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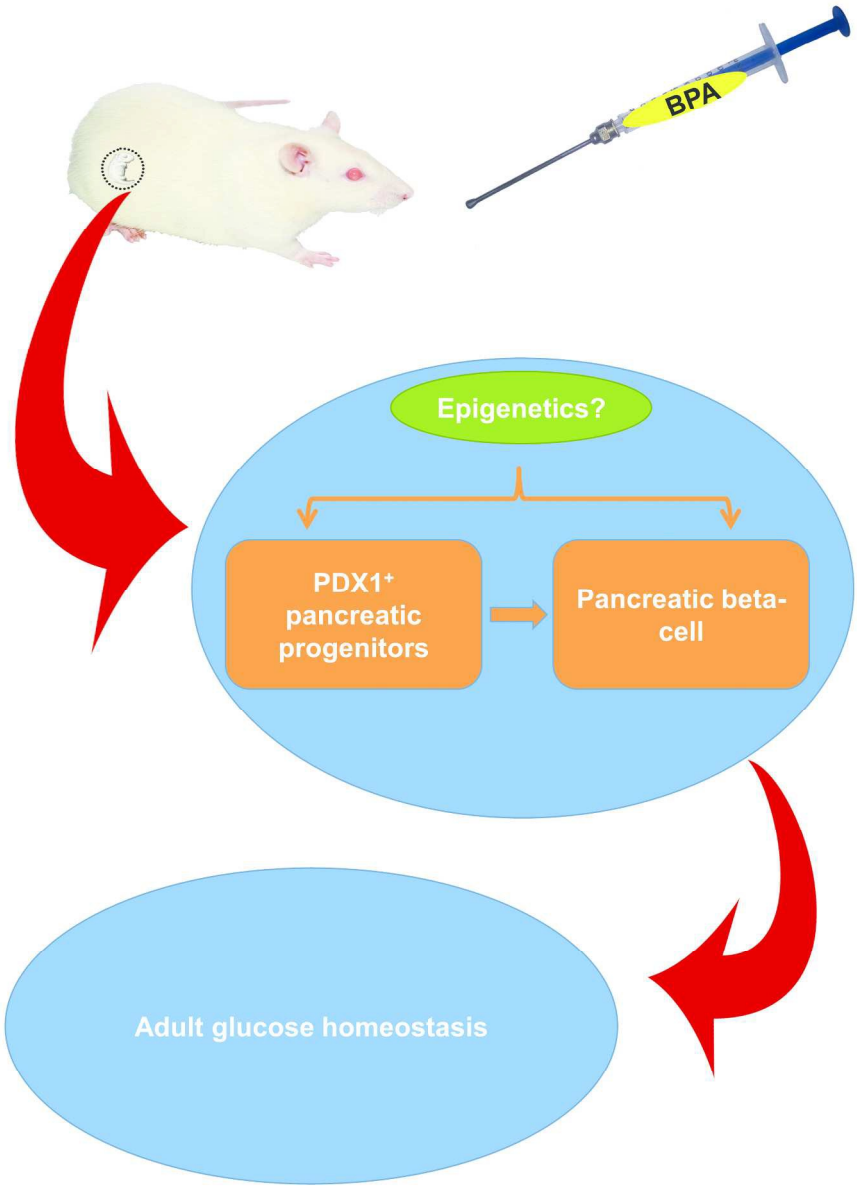

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