ARTICLE

Exendin-4 increases histone acetylase activity and reverses epigenetic modifications that silence *Pdx1* in the intrauterine growth retarded rat

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Abstract

Aims/hypothesis The abnormal intrauterine milieu of intrauterine growth retardation (IUGR) permanently alters gene expression and function of pancreatic beta cells leading to the development of diabetes in adulthood. Expression of the pancreatic homeobox transcription factor *Pdx1* is permanently reduced in IUGR islets suggesting an epigenetic mechanism. Exendin-4 (Ex-4), a long-acting glucagon-like peptide-1 (GLP-1) analogue, given in the newborn period increases *Pdx1* expression and prevents the development of diabetes in the IUGR rat.

Methods IUGR was induced by bilateral uterine artery ligation in fetal life. Ex-4 was given on postnatal days 1–6 of life. Islets were isolated at 1 week and at 3–12 months.

Histone modifications, PCAF, USF1 and DNA methyltransferase (Dnmt) 1 binding were assessed by chromatin immunoprecipitation (ChIP) assays and DNA methylation was quantified by pyrosequencing.

Results Phosphorylation of USF1 was markedly increased in IUGR islets in Ex-4 treated animals. This resulted in increased USF1 and PCAF association at the proximal promoter of *Pdx1*, thereby increasing histone acetyl transferase (HAT) activity. Histone H3 acetylation and trimethylation of H3K4 were permanently increased, whereas Dnmt1 binding and subsequent DNA methylation were prevented at the proximal promoter of *Pdx1* in IUGR islets. Normalisation of these epigenetic modifications reversed silencing of *Pdx1* in islets of IUGR animals.

Conclusions/interpretation These studies demonstrate a novel mechanism whereby a short treatment course of Ex-4 in the newborn period permanently increases HAT activity by recruiting USF1 and PCAF to the proximal promoter of *Pdx1* which restores chromatin structure at the *Pdx1* promoter and prevents DNA methylation, thus preserving *Pdx1* transcription.

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Abbreviations

cAMP	Cyclic AMP
CEx	Control Ex-4
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element-binding protein
CV	Control vehicle
Dnmt	DNA methyltransferase
Ex-4	Exendin-4
GLP-1	Glucagon-like peptide-1
HAT	Histone acetyl transferase

H3K4	Histone H3 lysine 4
H3K4me3	Trimethylated histone H3 lysine 4
H3K9	Histone H3 lysine 9
IEx	IUGR Ex-4
IUGR	Intrauterine growth retardation
IV	IUGR vehicle
LiCl	Lithium chloride
PCAF	p300/CBP (CREB binding protein) associated factor
PD 1–6	Postnatal day 1–6

Introduction

Intrauterine growth retardation (IUGR), a common complication of pregnancy, has been linked to the later development of diseases in adulthood such as type 2 diabetes [1]. We have demonstrated that the abnormal intrauterine milieu associated with IUGR limits the supply of critical substrates and hormones and affects the development of the fetus by permanently modifying gene expression and function of susceptible cells, such as the pancreatic beta cell leading to the development of diabetes in adulthood [2–4].

Pdx1 encodes a homeobox transcription factor critically important for pancreatic beta cell function and development and plays a pivotal role in the development of diabetes in humans and rodents. Even a relatively modest decrease in *Pdx1* expression impairs the compensatory response to insulin resistance [2, 4–13]. Expression of *Pdx1* is permanently reduced in IUGR beta cells and in previous studies we demonstrated that aberrant histone modifications and later gain of DNA methylation at the proximal promoter of *Pdx1* are responsible for decreased *Pdx1* transcription [14].

The long-acting GLP-1 analogue, exendin-4 (Ex-4) increases *Pdx1* expression in IUGR beta cells [4, 15–18] and is being used to treat humans with type 2 diabetes. The molecular mechanisms by which Ex-4 increases *Pdx1* transcription in IUGR animals are unknown. In previous studies, we found that administration of Ex-4 during the prediabetic neonatal period (postnatal days 1–6 [PD 1–6]) prevents the development of diabetes in IUGR animals by restoring expression of *Pdx1* to normal levels [4].

One of the earliest molecular events involved in silencing the *Pdx1* promoter in islets of IUGR animals is the loss of binding of the critical activator, USF1 [14]. However, USF1 protein levels are not reduced in IUGR animals, and this leads us to posit that IUGR may induce post-translational modifications (e.g. phosphorylation), which prevent the association of USF1 with the *Pdx1* promoter. Transcriptional activity of USF1 is dependent on phosphorylation at Ser[257] [19–23]; and USF1 purified from rat liver, spleen and kidney is primarily present in the phosphorylated form in the nucleus [20].

In the present study, we hypothesised that normalisation of *Pdx1* transcription by Ex-4 treatment is mediated by phosphorylation of USF1, which in turn mediates epigenetic changes involving histone modifications and DNA methylation at the proximal promoter of *Pdx1*.

Methods

For a complete description of the methods, please see the electronic supplementary material (ESM).

Animal model Four experimental groups from our previously reported rat model of IUGR were studied [2, 3]: (1) control pups treated with vehicle (PBS); (2) control pups treated with Ex-4 (Bachem, King of Prussia, PA, USA); 1 nmol/kg body weight injected subcutaneously for 6 days starting on day of life 1; (3) IUGR pups treated with vehicle; and (4) IUGR pups treated with Ex-4. On day of life 7, islets were harvested for neonatal experiments. For neonatal experiments, islets were harvested from 16 litters of IUGR and 16 litters of control animals and pooled from one litter for each experiment. Islets were used from 22 male adult animals from different litters for each treatment group. These studies were approved by the Animal Care Committee of The Children's Hospital of Philadelphia.

Pdx1 mRNA Total RNA was isolated from islets ($n=5$, all from different litters, per group) using RNAzol B (Tel-Test, Friendswood, TX, USA). Quantitative PCR was performed as previously described [4]. *Pdx1* expression was normalised to β-actin expression after assessment of a panel of housekeeping genes found this to be the most stable.

Chromatin immunoprecipitation (ChIP) assay ChIP was performed as previously described [14] using approximately 1,000 neonatal and 500 adult islets per experiment per group. Quantitative PCR was used to measure binding of USF1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), acetylated H3 (Millipore, Billerica, MA, USA), H3K9me2 (Millipore), p300 (Millipore), DNA methyltransferase (Dnmt) 1 (Millipore) and H3K4me3 (AbCAM, Cambridge, MA, USA) at the promoters of *Pdx1* and β-actin. Results were expressed as IP per total input for each experimental group and each experiment was normalised to the IP per total input value for the control vehicle experimental group for each antibody. Control vehicle groups were not compared across age groups. Standard methods of ChIP data normalisation were used to normalise individual sample immunoprecipitated DNA to both total input DNA for each experimental group and to a control sequence [24].
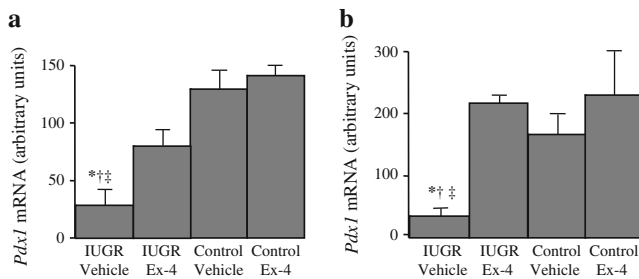
Fig. 1 *Pdx1* expression is normalised by neonatal Ex-4 treatment. **a** *Pdx1* mRNA levels at 2 weeks of age were measured by quantitative PCR as described in Methods (*n*=5 per treatment group). **b** *Pdx1* mRNA levels in adult animals (*n*=5 per treatment group). *$p<0.05$ IV vs IEx; †$p<0.05$ IV vs CV; ‡$p<0.05$ IV vs CEx treatment groups; $p>0.10$ CV vs CEx and IEx vs CV

For the adult experiments, ChIP was performed at 3–4 months for PCAF, 6 months for Dnmt1 and 9–12 months for AcH3, H3K4me3 and H3K9me2. For the histone modifications, data were independently analysed for the 9- and 12-month ChIP experiments (ESM Fig. 1, ESM Tables 1–3). The individual histone modification profiles were consistent at 9 and 12 months and thus these results were combined for the manuscript.

HAT activity Islet HAT activity was measured from pooled islet samples from 1-week-old animals, approximately 1,000 islets per group, via colorimetric assay using cofactor acetyl CoA (Kamiya, Seattle, WA, USA). Measurements were made at baseline and at 1, 2 and 3 h. Total HAT activity was computed as area under the curve and normalised to control vehicle.

USF1 phosphorylation and PCAF coimmunoprecipitation Islets were harvested from animals at 1 week of age and nuclear protein extracts were then prepared from islets. Nuclear extracts (10 μg of protein/reaction) were first immunoprecipitated with normal rabbit serum (negative control) or anti-USF1. Membranes were incubated with anti-phosphoserine (1:500 dilution) or PCAF (1:500 dilution) antibody, and immunoreactive proteins were visualised by incubation with horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:5,000 dilution) and the ECL Plus western blotting system (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Densitometric analyses were then performed and values were normalised to control samples treated with vehicle.

Methylation analysis of the Pdx1 CpG island Genomic DNA was extracted from islets from 6–9 month old animals. Bisulfite modification was done using the Zymo Research EZ Methylation Gold kit (Irvine, CA, USA). Pyrosequencing analysis was performed by EpigenDx (Worchester, MA, USA).

Statistical analyses Statistical analyses were performed using analysis of variance and Student's unpaired *t* test not requiring equal variance between groups. A *p* value less than 0.05 was considered significant.

Results

Ex-4 treatment restores Pdx1 mRNA levels in IUGR islets Similar to our previous findings, Ex-4 treatment of newborn IUGR rats significantly increased *Pdx1* expression [4]. At 14 days of age, *Pdx1* mRNA levels were reduced by 75% in IUGR vehicle-treated rats compared with controls ($p<0.05$ vs control vehicle, Fig. 1a). We have previously shown that beta cell mass remains normal at 14 days in the IUGR rat [4], indicating that the reduction in *Pdx1* mRNA level at this age is not due to decreased beta cell mass. Neonatal Ex-4 treatment led to a restoration of *Pdx1* mRNA levels in IUGR rats at 14 days (Fig. 1a), an effect that persisted into adulthood (Fig. 1b).

Ex-4 treatment restores histone acetylation at the Pdx1 promoter Histone acetylation is associated with active chromatin structure, whereas histone deacetylation is associated with a repressed chromatin state [25]. To determine whether the restoration of *Pdx1* transcription in IUGR Ex-4 treated animals was due to increased acetylation of H3 at the proximal promoter of *Pdx1*, we performed ChIP analyses on pancreatic islets using primers that encompass the region of the CpG island shown in Fig. 2. This area is obligate for *Pdx1* transcription. At 1 week of age, there was a significant reduction in histone H3 acetylation at the proximal promoter of *Pdx1* in IUGR islets compared with control animals (Fig. 3a). Treatment with Ex-4 increased histone H3 acetylation by approximately ninefold (IUGR vehicle (IV) vs IUGR Ex-4 (IEx); $p=0.042$) in IUGR animals at 1 week of age, but had no significant effect in control animals (control vehicle (CV) vs control Ex-4 (CEx): $p=0.46$; Fig. 3a) consistent with the lack of change in expression of *Pdx1* in response to Ex-4 treatment in control animals. The effects of Ex-4 on H3 acetylation persisted into adulthood in IUGR animals, resulting in a fourfold increase in H3 acetylation (IV vs IEx: $p=0.026$; Fig. 3b). Similar to 1-week-old animals, there was no effect of Ex-4 in controls (CV vs CEx; $p=0.177$).
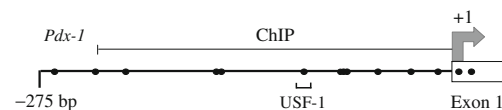

Fig. 2 Map of promoter region of *Pdx1*. The proximal promoter of *Pdx1* is part of a more extensive CpG island. Filled circles represent CpG dinucleotides and numbers indicate positions relative to the transcriptional start site. The position of the E-box bound by USF1 is indicated
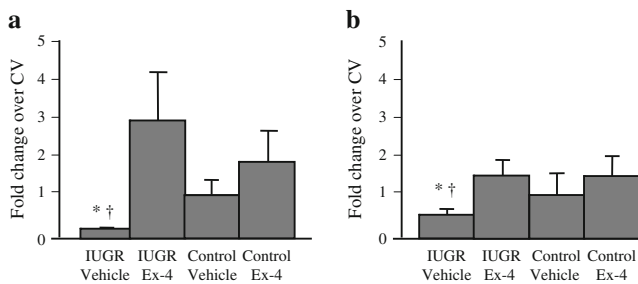
Fig. 3 Histone H3 acetylation at the proximal promoter of *Pdx1*. ChIP analysis of cross-linked chromatin from islets of IUGR and control animals with and without Ex-4 treatment at (**a**) 1 week of age, and (**b**) adult, immunoprecipitated with antibody to acetylated H3. The IgG and no antibody immunoprecipitate showed negligible PCR product, indicating little or no immunoprecipitation in the absence of primary antibody (see ESM). The relative amount of acetylated H3 bound at the *Pdx1* promoter was measured by QPCR and normalised to total input DNA for each experimental group. Data are represented as fold change relative to control vehicle values. *n*=6 experiments, data are ±SEM. **a** *p=0.004 IV vs CV; †p=0.042 IV vs IEx; p>0.05, IEx vs CV and CV vs CEx. **b** *p=0.004 IV vs CV; †p=0.026 IV vs IEx; p>0.10 IEx vs CV and CV vs CEx

Effects of Ex-4 on trimethylation of H3K4 Trimethylation of lysine 4 at H3 (H3K4me3) is associated preferentially at the promoters of active genes and is influenced by the density of acetylated histone H3 [26–28]. In islets from controls at all ages, there was a robust association of H3K4me3 at the *Pdx1* promoter. At 1 week, the abundance of H3K4me3 was markedly reduced at the proximal promoter of *Pdx1* in IUGR islets, and this reduction was completely prevented by neonatal Ex-4 as shown by the 27-fold increase (IV vs IEx: p=0.04) in Fig. 4a. This effect persisted with a sixfold increase of H3K4me3 in adulthood (IV vs IEx: p=0.033; Fig. 4b). Ex-4 had no significant effect on trimethylation of H3K4 in controls at 1 week of age (Fig. 4a; CV vs CEx: p=0.244). In adults, Ex-4 controls showed an approximate twofold increase in enrichment of H3K4 trimethylation compared with controls (p=0.035; Fig. 4b).

In previous studies, we observed increased abundance of H3K9me2 at the *Pdx1* proximal promoter in islets from adult IUGR rats. Thus, to determine whether Ex4 prevented H3K9 dimethylation, ChIP assays were performed in both 1 week and adult islets. There was very little association of H3K9me2 at the *Pdx1* promoter in all groups at 1 week of age (Fig. 4c). However, in adults, H3K9me2 was abundant at the *Pdx1* promoter in IUGR islets and Ex-4 prevented this silencing histone modification (Fig. 4d; IV vs IEx: p=0.044).

USF1 binding is restored in IUGR Ex-4 treated animals USF1 is a homeobox transcription factor that binds to and functionally regulates the *Pdx1* gene promoter and its binding is obligate for *Pdx1* transcription [29, 30]. The USF1 binding site at *Pdx1* is highly conserved and is located at a CpG site within the CpG island at the proximal region of the promoter (Fig. 1) [31, 32]. USF1 binding at *Pdx1* was markedly reduced in IUGR islets from 1-week-old animals compared with control islets (Fig. 5). Ex-4 significantly increased association of USF1 at *Pdx1* in IUGR islets from 1-week-old animals compared with vehicle-treated animals (Fig. 5: IEx vs IV: 32-fold increase, p=0.032). Control animals treated with Ex-4 showed no change in USF1 binding (CEx vs CV, p=0.374).

USF1 phosphorylation PKA-dependent serine phosphorylation of USF1 has been implicated in modulating its DNA binding and transcriptional activation [19–23]. We found no differences in USF1 protein levels between the four study groups; however, endogenous levels of serine phosphorylated USF1 were very low in islets from 1-week-old IUGR animals compared with CV-treated animals (Fig. 6). Ex-4 treatment significantly increased phosphorylated USF1 in IUGR islets, but not controls.
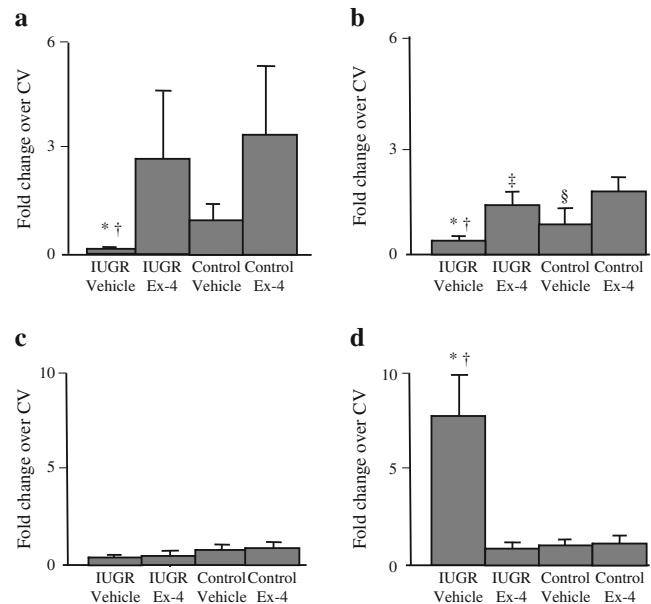

Fig. 4 H3K4 trimethylation and H3K9 dimethylation at the proximal promoter of *Pdx1*. ChIP analysis of cross-linked chromatin from islets of IUGR and control animals with and without Ex-4 treatment immunoprecipitated with antibody to H3K4me3 and H3K9me2: (**a**) H3K4me3 at 1 week of age and (**b**) H3K4me3 adult; (**c**) H3K9me2 at 1 week of age and (**d**) H3K9me2 adult. The IgG and no antibody immunoprecipitate showed negligible PCR product, indicating little or no immunoprecipitation in the absence of primary antibody. The relative amount of H3K4me3 and H3K9me2 bound at the *Pdx1* promoter was measured by QPCR and normalised to total input DNA for each experimental group. Data are represented as fold change relative to CV values. *n*=6 experiments, data are ±SEM. **a** *p=0.04 IV vs IEx; †p=0.001 IV vs CV; p>0.05 IEx vs CV and CV vs CEx. **b** *p=0.033 IV vs IEx; †p=0.002 IV vs CV; ‡p=0.035 IEx vs CV; §p=0.035 CV vs CEx. **c** There was minimal binding of H3K9me2 for all groups at 1 week of age. **d** *p=0.044 IV vs IE; †p=0.043 IV vs CV; p>0.10 IEx vs CV and CV vs CEx
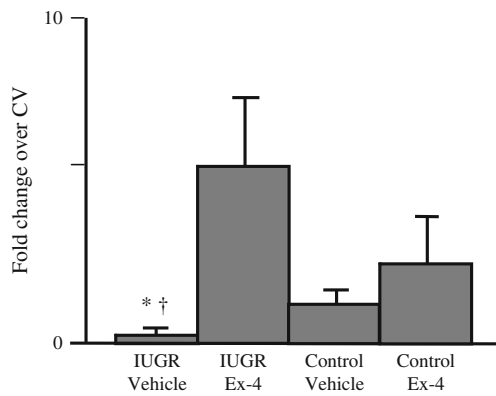
Fig. 5 USF1 binding at the proximal promoter of *Pdx1* in 1-week-old islets. ChIP analysis of cross-linked chromatin from islets of IUGR and control animals with and without Ex-4 treatment at 1 week of age immunoprecipitated with antibody to USF1. The IgG immunoprecipitate showed negligible PCR product, indicating little or no immunoprecipitation in the absence of primary antibody. The relative amount of USF1 bound at the *Pdx1* promoter was measured by QPCR and normalised to total input DNA. Data are represented as fold change relative to control vehicle values. $n=6$ experiments, data are ±SEM. *$p=0.032$ IV vs IEx; †$p=0.034$ IV vs CV; $p>0.10$ IEx vs CV and CV vs CEx

USF1 binding and histone modifications at the β-actin promoter Given the dramatic changes in USF1 binding, H3 acetylation and H3K4me3 binding in islet chromatin at the proximal *Pdx1* promoter, we considered the possibility that these changes may have represented non-specific transcription factor/protein binding to chromatin induced by tissue preparation technique. To exclude this possibility, we measured binding of USF1 and enrichment of acetyl H3 and H3K4me3 at the β-actin promoter, a gene whose expression does not change in IUGR islets. In 1-week-old islets there was no change in USF1 binding at the *β actin* promoter in any experimental group (ESM Fig. 2; $p>0.10$ for IV vs IEx, CV and CEx). In addition, we did not detect any differences in acetyl H3 or H3K4me3 enrichment at the β-actin promoter in adult animals in any of the four
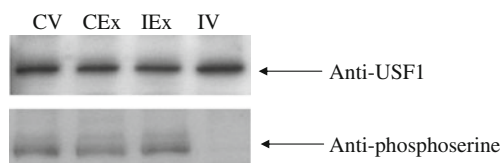

Fig. 6 Immunoprecipitation/western blot analyses of phosphorylated USF proteins in islets. Nuclear extracts were prepared from islets at 1 week of age from CV-, CEx-, IV- and IEx-treated animals. Extracts (10 μg of protein/reaction) were first immunoprecipitated with anti-USF1 (top panel) and then subjected to western blot analyses using anti-phosphoserine antibodies (anti-PS; bottom panel). Densitometric analyses of the anti-USF1 and anti-phosphoserine coimmunoprecipitation were performed. Values were measured as densitometry units and normalised to CV; $n=3$ experiments, data are ±SEM. IV=0.147± 0.06; IEx=1.22±0.73; CV=1.00±0.35; CEx=1.11±0.02. *$p<0.05$ between IV and all other groups

experimental groups (ESM Fig. 2: $p>0.10$ for IV vs IEx, CV and CEx for both acetyl H3 and H3K4Me3 in adult islets). In order to ensure that the transcription factor/protein binding alterations that we observed at the *Pdx1* promoter were not due to non-specific antibody binding, we measured IgG and no antibody binding in experiments using antibodies to acetyl H3, H3K4me3, USF1 and PCAF. There was a negligible amount of immunoprecipitate when the ChIP experiments were run without antibody or with IgG antibody, indicating that we were successfully measuring the proteins of interest compared with non-specific protein binding at the *Pdx1* promoter (ESM Table 4).

Ex-4 treatment increases histone acetyl transferase activity in IUGR islets Given the increased levels of histone acetylation at the proximal *Pdx1* promoter induced by Ex-4 treatment, we hypothesised that Ex-4 increased HAT activity and thus we measured generalised HAT activity in islet chromatin from 1-week-old animals. Figure 7a shows
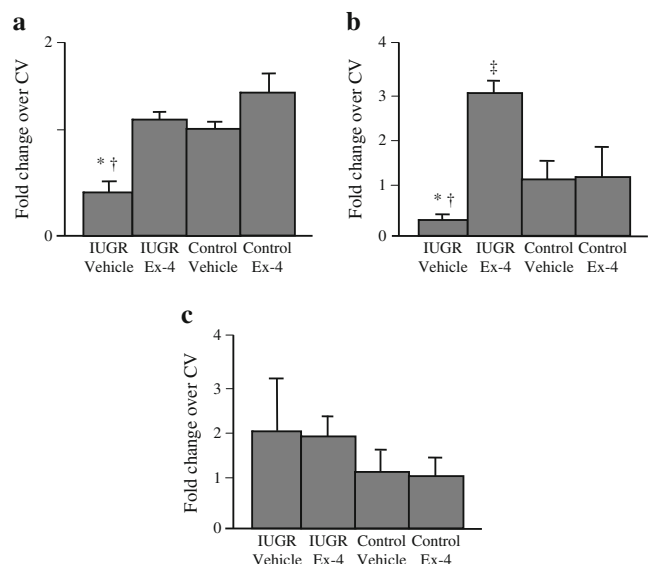

Fig. 7 a HAT activity: islets from IUGR and control animals with and without treatment with Ex4 were isolated. HAT activity was measured via colorimetric assay with the cofactor acetyl CoA at baseline, 1, 2, and 3 h. Integrated HAT activity was computed as area under the curve and represented as per cent of CV values. $n=3$ experiments, data are ±SEM. *$p=0.003$ IV vs IEx; †$p=0.002$ IV vs CV; $p>0.10$ IEx vs CV and $p>0.05$ CV vs CEx. **b, c** PCAF binding at the proximal promoter of *Pdx1*. ChIP analysis of cross-linked chromatin from islets of IUGR and control animals with and without Ex-4 treatment at (**b**) 1 week of age and (**c**) 4 months of age, immunoprecipitated with antibody to PCAF. The IgG immunoprecipitate showed negligible PCR product, indicating little or no immunoprecipitation in the absence of primary antibody. The relative amount of PCAF bound at the *Pdx1* promoter was measured by QPCR and normalised to total input DNA. Data are represented as fold change relative to control vehicle values. $n=6$ experiments, data are ±SEM. **b** *$p=0.037$ IV vs IEx; †$p=0.015$ IV vs CV; ‡$p=0.018$ IEx vs CV; $p>0.05$ CV vs CEx. **c** IV vs IEx: $p=0.97$; IV vs CV: $p=0.461$; IEx vs CV: $p=0.148$; CV vs CEx: $p=0.781$

Table 1 PCAF association with USF1 at the proximal promoter of *Pdx1*

IV	IEx	CV	CEx
0.13±0.004	1.29±0.042	1.00±0.068	1.13±0.004

Nuclear extracts were prepared from islets at 1 week of age from CV-, CEx-, IV- and IEx-treated animals. Extracts (10 μg of protein/reaction) were first immunoprecipitated with anti-USF1and then subjected to coimmunoprecipitation and western blot analyses using anti-PCAF antibodies. Densitometric analyses of anti-PCAF and anti-USF1 coimmunoprecipitation were performed and expressed as densitometry units±SEM; *n*=3 experiments

an approximate threefold increase in HAT activity in IUGR Ex-4 islets compared with IUGR vehicle islets (*p*= 0.003). There was no significant effect of Ex-4 on HAT activity in control animals (CV vs CEx; *p*=0.074) at 1 week of age.

GLP-1 and its long-acting agonist, Ex-4, increase cAMP levels [33, 34]. The transcriptional effects of cAMP are mediated by the binding of the cAMP response element-binding protein (CREB), which is facilitated by the coactivators CBP and p300. p300/CBP have intrinsic acetyltransferase activity on histones [34]. Surprisingly, we found little association of p300 at the proximal promoter of *Pdx1* in control or IUGR islets and there were no significant differences in binding of p300 to *Pdx1* between any of the treatment groups. We then examined PCAF binding to the proximal promoter of *Pdx1* by ChIP assay. PCAF acetylates free histones or nucleosomes, primarily on histone H3 through a mechanism that can be independent of the CBP/p300 complex-mediated pathway [35]. In contrast to p300/CBP, we found a dramatic increase in PCAF binding at *Pdx1* in Ex-4 IUGR islets compared with IUGR vehicles at 1 week of age (Fig. 7b; IV vs IEx: tenfold increase, *p*=0.037). There was no significant effect of Ex-4 on PCAF binding in controls (CV vs CEx; *p*=0.94). Similar to USF1, Ex-4 had no effect on PCAF protein levels in controls or IUGR animals at 1 week of age. After establishing that Ex-4 treatment increased binding of PCAF at *Pdx1*, we investigated whether Ex-4 treatment enhanced PCAF and USF1 interaction in 1-week-old islets from IUGR animals treated with Ex-4 using coimmunoprecipitation experiments (Table 1). As measured by densitometry, neonatal Ex-4 treatment increased USF1 and PCAF interaction in 1-week-old islets (Table 1), whereas there was no change in interaction of USF1 and PCAF in control groups. Finally, we sought to determine whether neonatal Ex-4 treatment permanently increased the association of PCAF at the *Pdx1* promoter or whether the effects of Ex-4 were transient. In ChIP experiments performed in islets harvested from adult rats, we found no change in PCAF binding among experimental groups (Fig. 7c; *p*>0.05 in IV vs IEx, CV and CEx).

Methylation status of the Pdx1 gene promoter in IUGR and control islets The proximal 5′ flanking region of the *Pdx1* gene and its first exon include a highly conserved CpG island that encompasses nucleotides −275 to +200 relative to the transcriptional start site (ESM Fig. 3). Using pyrosequencing, we evaluated the DNA methylation status of the portion of the CpG island located in the *Pdx1* promoter in islets of IUGR and control animals. At 1 week of age, none of the 11 CpG sites in the *Pdx1* promoter were methylated in either group [14]. However, in adulthood, *Pdx1* promoter DNA methylation levels across the CpG island in IUGR vehicle-treated animals averaged 51.3±10.3% compared with an absence of CpG methylation in control vehicles (Fig. 8a; IV vs CV; *p*<0.05). No single CpG site on *Pdx1* was consistently methylated in IUGR vehicle-treated islets. Neonatal Ex-4 treatment completely prevented DNA methylation at all CpG sites in *Pdx1* in adult IUGR animals. *Pdx1* DNA methylation levels across the CpG island in adult IUGR Ex-4 treated animals averaged 3.9±0.64% and this was not significantly different from adult control animals treated with Ex-4, averaging 3.46±0.52% (ESM Fig. 3 and Fig. 8a; IEx vs CEx; *p*=0.63).
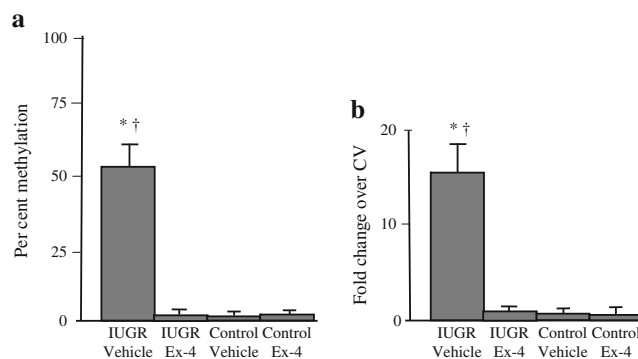

Fig. 8 **a** Methylation status of the *Pdx1* gene promoter in adult IUGR and control islets. The proximal promoter of *Pdx1* is part of a more extensive CpG island. We performed pyrosequencing of bisulfite-treated DNA to determine the DNA methylation status of the portion of the CpG island that is located in the *Pdx1* promoter in islets of IUGR and control animals. Evaluation of islet DNA from IUGR and control animals at 1 week of age revealed that none of the CpG sites in the *Pdx1* promoter were methylated in either group. CpG site DNA methylation levels of DNA isolated from adult islets averaged across the proximal promoter of *Pdx1* in the four treatment groups. *n*=5 experiments. **p*=0.016 IV vs IEx; †*p*=0.016 IV vs CV; *p*>0.10 IEx vs CV and CV vs CEx. **b** Dnmt1 binding at the proximal promoter of *Pdx1* at 6 months. ChIP analysis of cross-linked chromatin from islets of IUGR and control animals with and without Ex-4 treatment at 6 months of age immunoprecipitated with antibody to Dnmt1. The relative amount of Dnmt1 bound at the *Pdx1* promoter was measured by QPCR and normalised to total input DNA. Data are represented as per cent of control vehicle values. *n*=6 experiments, data are ±SEM; **p*=0.016 IV vs IEx; †*p*=0.021 IV vs CV; *p*>0.10 IEx vs CV and CV vs CEx

To determine the underlying mechanisms responsible for prevention of DNA methylation in Ex-4 IUGR islets, we examined the association of Dnmt1 to *Pdx1*. Mammalian CpG methylation is mediated by Dnmts. Dnmt1 mediates replication-coupled maintenance of DNA methylation patterns [36, 37] and previous studies showed marked association of Dnmt1 at *Pdx1* in IUGR islets at 6 months of age [14]. ChIP assays were performed on adult islets when DNA methylation at *Pdx1* was first observed in IUGR islets. Dnmt1 binding was significantly enhanced in IUGR vehicle islets (17.1±2.1-fold change over CV, *p*=0.021; Fig. 8b), and we did not detect any significant association of Dnmt1 at *Pdx1* in IUGR Ex-4, CV or CEx islets (Fig. 8b).

Discussion

The major finding of our study was that a short treatment course of Ex-4 in the newborn period permanently reverses the aberrant epigenetic modifications at the proximal promoter of *Pdx1* in IUGR islets, an animal model of type 2 diabetes. Normalisation of USF1 binding, H3 acetylation and H3K4 trimethylation at the *Pdx1* proximal promoter allows for an open chromatin domain, creating an environment permissive of *Pdx1* transcription that is necessary for proper beta cell function and development. These are the first studies to show that Ex-4, a drug now commonly used for treatment of type 2 diabetes, permanently reverses epigenetic modifications of a key beta cell gene, *Pdx1*, leading to increased transcription.

USF1 is a critical activator of *Pdx1* transcription and decreased binding of USF1 markedly reduces or abolishes *Pdx1* transcription [29, 30]. Other authors and we have shown that loss of USF1 binding leads to an increase in repressive chromatin modifications such as methylation of H3K9 and H3K27 [14, 38]. Furthermore, USF1 binding is important for generating and maintaining adjacent histone modifications associated with active chromatin structure [39]. Here, we show that neonatal treatment with Ex-4 restores binding of USF1 thus allowing for an open chromatin domain and restoration of *Pdx1* transcription in IUGR islets. In the beta cell, Ex-4 activates various signalling pathways in pancreatic beta cells, in particular cAMP, protein kinase A (PKA), Ca^{2+} and protein kinase B (PKB/Akt) leading to phosphorylation of a number of proteins [40]. We found that IUGR markedly decreases serine phosphorylation of nuclear USF1, which is normalised by Ex-4 treatment. Increased phosphorylation in turn is associated with increased USF1 binding at *Pdx1* in IUGR islets. As a number of studies have shown that serine phosphorylation is obligate for USF1 binding to DNA, it is likely that this is the mechanism underlying Ex-4 induced increased association at *Pdx1* in IUGR islets [19–22].

The first epigenetic mark that is modified in pancreatic beta cells of IUGR animals is histone acetylation [14]. Islets isolated from IUGR fetuses show a significant decrease in H3 acetylation at the proximal promoter of *Pdx1* [14]. After birth, histone deacetylation progresses in IUGR animals. In this study, we found that Ex-4 administration during the newborn period normalises H3 acetylation to levels equal to or greater than those of control animals at 1 week of age. Ex-4 mediates this process by increasing HAT activity in IUGR islets to control levels. Our data show that PCAF is the primary histone acetyl transferase that catalyses acetylation at *Pdx1*. Ex-4 treatment of IUGR islets significantly increases PCAF binding at the proximal promoter of *Pdx1*. PCAF, also known as (p300/CBP [CREB-binding protein]-associated factor), was initially described as a member of an activating complex containing CREB and p300. However, in islets, PCAF binds to *Pdx1* in the absence of p300. Other investigators have also shown that PCAF possesses HAT activity independent of CREB and p300 [35].

The mechanism by which Ex-4 increases PCAF binding at *Pdx1* in IUGR islets is unclear. It is likely that during the period of increased beta cell replication that takes place in the neonatal period, Ex-4 increases association of PCAF and USF1 at the *Pdx1* promoter and that these proteins together are responsible for increasing HAT activity and recruiting histone activating marks to the chromatin. This is in keeping with other studies that have recently shown that USF1 interacts directly with and recruits PCAF to the proximal promoter regions of a number of genes [22, 38, 41]. Our data indicate that in adulthood, the relative increase in PCAF binding in IUGR vehicle-treated islets is no longer present. However, the resulting increase in histone activating marks at the *Pdx1* promoter is permanent thus leading to the permanent increase in *Pdx1* expression. This implies that once histone acetylation is established, a mediator such as PCAF no longer needs to be present at the promoter.

After birth, in addition to persistent histone deacetylation, the IUGR state is further characterised by a marked decrease in H3K4me3 and an increase in H3K9me2, additional marks of repressed chromatin structure [14]. In our studies of IUGR animals treated with Ex-4, by the adult age H3K4me3 at *Pdx1* is restored to control levels and dimethylation of H3K9 is inhibited. Histone acetylation is the initial step in chromatin remodelling leading to increased gene transcription, which is followed by histone methylation (specifically H3K4 trimethylation) [27]. We have previously shown that H3K4 methylation precludes methylation at lysine 9. Our in vivo findings support several in vitro studies showing that active chromatin states are maintained by H3K4 methylation, which opposes the lysine methylations that characterise inactive chromatin [42, 43].

The prevention of the aberrant chromatin structure observed in IUGR animals treated with Ex-4 is permanent.

Increased acetylation of H3 and trimethylation of H3K4 in islets isolated from adult IUGR animals receiving a neonatal course of Ex-4 was maintained well into adulthood. In the normal rat, replication of existing beta cells and formation of new beta cells are higher during the newborn period than at any other time in postnatal life [44–46]. Thus, these histone modifications are somehow maintained during active beta cell replication. It is not known how histone modifications are propagated in daughter cells and it remains to be determined if histone modifications can serve as templates for reproducing these same structures on newly incorporated nucleosomes following cell replication.

Of major significance was our finding that neonatal Ex-4 treatment prevented DNA methylation at the CpG island within the proximal promoter of *Pdx1*, the final step in IUGR induced chromatin remodelling. In IUGR newborn islets, there is no DNA methylation at *Pdx1*; however, by 6 months of age, high levels of DNA methylation are found at the proximal promoter of *Pdx1* in IUGR but the *Pdx1* promoter remains unmethylated in control animals. DNA methylation is thought to be an essential step in 'locking in' the aberrant chromatin changes characteristic of IUGR animals.

The mechanisms by which Ex-4 prevents DNA methylation in IUGR islets are likely to be related to the prevention of H3K9 dimethylation, which in turn prevents recruitment of Dnmt1, the primary DNA methyltransferase that is associated with *Pdx1* in IUGR islets [14]. A number of studies have shown that methylation of H3K9 precedes DNA methylation [37, 47]. It has also been suggested that DNA methyltransferases may act only on chromatin that is methylated at lysine 9 on histone H3 (H3K9) [48]. Histone methyltransferases bind to DNA methylases thereby initiating DNA methylation [37].

Of particular interest is the finding that neonatal Ex-4 treatment increases H3K4 trimethylation at the *Pdx1* promoter in adult islets, but that this was not associated with an increase in *Pdx1* expression. This apparent dissociation between the epigenetic mark and gene expression indicates that there are other yet unidentified mechanisms that are in place to prevent overexpression of any particular gene thus preventing changes in beta cell mass and preserving glucose homeostasis. Such braking mechanisms could include additional epigenetic regulators at the chromatin level or could function through the saturation of GLP-1 or other receptors. Further studies are needed to explore these possible regulatory mechanisms.

In conclusion, our results demonstrate that Ex-4 interrupts the self-propagating aberrant epigenetic cycle in IUGR islets by inducing PCAF and USF1 binding to the *Pdx1* promoter, which in turn restores histone acetylation and H3K4 methylation, thereby preventing H3K9 and DNA methylation. At the neonatal stage, this epigenetic malprogramming is reversible and may define an important developmental window for innovative therapeutic approaches to prevent the development of adult onset diseases. Our studies indicate novel mechanisms by which Ex-4 functions to regulate gene expression in vivo. By increasing HAT activity, Ex-4 is able to reverse epigenetic modifications induced by IUGR and normalises *Pdx1* expression.

Contribution statement SEP designed the experiment, collected data, analysed data, wrote and revised the manuscript. LJJS collected data, analysed data and revised the manuscript. YH collected data, revised the manuscript and analysed data. DAS designed experiments, interpreted data and revised the manuscript. RAS designed experiments, analysed data, and wrote and revised the manuscript. All authors approved the manuscript in the final version.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript except for D.A.S. Stoffers who is a co-inventor on a patent entitled 'Differentiation of non-insulin producing cells into insulin-producing cells by GLP-1 or exendin-4 or uses thereof'.

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