Hepatocyte nuclear factor (HNF)-4 α -driven epigenetic silencing of the human *PED* gene

P. Ungaro · R. Teperino · P. Mirra · M. Longo ·
M. Ciccarelli · G. A. Raciti · C. Nigro · C. Miele ·
P. Formisano · F. Beguinot

Received: 1 February 2010 / Accepted: 25 February 2010 / Published online: 16 April 2010
© Springer-Verlag 2010

Abstract

Aims/hypothesis Overexpression of *PED* (also known as *PEA15*) determines insulin resistance and impaired insulin secretion and may contribute to progression toward type 2 diabetes. Recently, we found that the transcription factor hepatocyte nuclear factor (HNF)-4 α binds to *PED* promoter and represses its transcription. However, the molecular details responsible for regulation of *PED* gene remain unclear.

Methods Here we used gain and loss of function approaches to investigate the hypothesis that HNF-4 α controls chromatin remodelling at the *PED* promoter in human cell lines.

Results HNF-4 α production and binding induce chromatin remodelling at the -250 to 50 region of *PED*, indicating that remodelling is limited to two nucleosomes located at the proximal promoter. Chromatin immunoprecipitation assays also revealed concomitant HNF-4 α -induced deacetylation of histone H3 at Lys9 and Lys14, and increased dimethylation of histone H3 at Lys9. The latter was followed by reduction of histone H3 Lys4 dimethylation. HNF-4 α was also shown to target the

histone deacetylase complex associated with silencing mediator of retinoic acid and thyroid hormone receptor, both at the *PED* promoter, and at *GRB14* and *USP21* regulatory regions, leading to a reduction of mRNA levels. Moreover, HNF-4 α silencing and *PED* overexpression were accompanied by a significant reduction of hepatic glycogen content.

Conclusions/interpretation These results show that HNF-4 α serves as a scaffold protein for histone deacetylase activities, thereby inhibiting liver expression of genes including *PED*. Dysregulation of these mechanisms may lead to upregulation of the *PED* gene in type 2 diabetes.

Keywords Chromatin remodelling · HNF-4 α · Insulin resistance · *PED* · Type 2 diabetes

Abbreviations

ChIP	Chromatin immunoprecipitation
ECL	Electrochemiluminescence
HeLa _{HNF-4α}	HeLa cells overproducing exogenous HNF-4 α
HepG2 _{HNF-4α-sh}	HepG2 cells subjected to silencing of the endogenous HNF-4 α
H3K4	Histone H3 Lys4
H3K9	Histone H3 Lys9
HNF	Hepatocyte nuclear factor
HRE	HNF-4 α response element
MNase	Micrococcal nuclease
panSMRT	Regions common to SMRT α and SMRT β
PED	Phosphoprotein enriched in diabetes
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor

P. Ungaro and R. Teperino contributed equally to the present study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-010-1732-x) contains supplementary material, which is available to authorised users.

P. Ungaro (✉) · R. Teperino · P. Mirra · M. Longo ·
M. Ciccarelli · G. A. Raciti · C. Nigro · C. Miele · P. Formisano ·
F. Beguinot
Dipartimento di Biologia e Patologia Cellulare e Molecolare “L.
Califano” & Istituto di Endocrinologia ed Oncologia Sperimentale
del CNR, Università di Napoli Federico II,
Via Sergio Pansini, 5,
Naples 80131, Italy
e-mail: pungaro@ieos.cnr.it

Introduction

Recent genetic studies [1–3] have considerably expanded the list of known genes that may cause a predisposition to diabetes. However, it remains largely unclear how these genes determine the development of type 2 diabetes. Phosphoprotein enriched in diabetes (PED, also known as phosphoprotein enriched in astrocytes-15 [PEA15]) is a scaffold cytosolic protein widely produced in human tissues [4, 5]. Early studies indicated that PED has an important role in controlling glucose disposal by impacting on protein kinase C signalling [5, 6]. It was later found that PED is commonly overproduced in individuals with type 2 diabetes as well as in their euglycaemic offspring [5, 7]. In these persons PED overproduction causes insulin resistance in GLUT4-mediated glucose disposal. Studies in tissue-specific transgenics and in null mice have indicated that the upregulation of PED observed in type 2 diabetic patients might also contribute to impaired beta cell function [7]. PED cellular levels are regulated by ubiquitinylation and proteosomal degradation [8], but run-on experiments in cultured cells from type 2 diabetic patients have demonstrated that, at least in part, the overproduction observed in these participants is caused by transcriptional abnormalities [5]. The molecular details responsible for these abnormalities and how the *PED* gene is regulated remain unclear. Moreover, the role of PED in liver glucose metabolism has been less extensively investigated.

We have recently demonstrated that the hepatocyte nuclear factor (HNF)-4 α (NR2A1), a highly conserved member of the nuclear receptor superfamily involved in the control of glucose homeostasis [9, 10], regulates transcription of the *PED* gene by binding to a *cis*-regulatory element of the *PED* promoter and represses its transcription [11].

HNF-4 α is essential for hepatocyte differentiation at the developmental and the functional levels [12], as well as for accumulation of hepatic glycogen stores and generation of normal hepatic epithelium [13]. Point mutations in HNF-4 α impair liver and pancreatic regulation of glucose homeostasis and cause maturity onset diabetes of the young type 1. More recently, genetic and biochemical evidence has been generated indicating that HNF-4 α may also have a role in the development of more common forms of type 2 diabetes [14–16], but understanding of the underlying mechanisms is incomplete.

The ability of nuclear receptors to induce specific transcription events depends on their recruitment of chromatin remodelling cofactors and enzymes, and on the assembly of the basal transcription machinery [17]. In the absence of ligand, nuclear receptors recruit co-repressors such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor. These, in turn, bind repressive enzymes such as

the histone deacetylases and histone methyltransferases, specifically controlling the methylation state of lysine 9 at histone H3 and certain chromatin remodelling events.

Increasing evidence now indicates that chromatin remodelling is an important mechanism enabling transcription regulation [18]. Chromatin remodelling occurs through different mechanisms. One is the covalent modification of histone tails, including acetylation, methylation and phosphorylation [19]. These changes also affect the ability of chromatin to interact with transcription factors and the basal transcription machinery [20]. Acetylation and methylation of lysine residues at H3 and H4 amino termini represent the most common modifications. Indeed, increased acetylation induces transcriptional activation [21], while reduced acetylation usually signals transcriptional repression [22, 23]. Methylation of H3 lysine 9 is also associated with transcriptional repression [24].

In this work, we have investigated the molecular mechanisms responsible for HNF-4 α -dependent silencing of *PED* expression in liver. We show that by causing epigenetic changes at the *PED* gene, HNF-4 α controls transcriptional activity of *PED* and may affect glucose metabolism in liver.

Methods

Materials Media, sera and antibiotics for cell culture, and the lipofectamine reagent were purchased from Invitrogen (Paisley, UK). Goat polyclonal HNF-4 α and acetyl-histone H3 (K9/K14) and rabbit polyclonal SMRTe antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal histone H3 Lys9 (H3K9) and rabbit monoclonal histone H3 Lys4 (H3K4) antibodies were from Abcam (Cambridge, UK). The PED antibody, the pCDNA3/HNF-4 α expression vector and the HNF-4 α -specific short hairpin RNA (shRNA) plasmid have been previously described [11]. All short interfering RNAs (siRNAs) were chemically synthesised by Ambion (Austin, TX, USA) as oligonucleotide duplexes. siRNA target sequences for silencing mediator for retinoid and thyroid hormone receptors (SMRT) were directed at regions common to SMRT α and SMRT β (panSMRT) [25]. As non-specific siRNA controls, the Ambion Silencer 2 negative control was used.

Western blot and electrochemiluminescence (ECL) reagents were from Thermo Scientific (Rockford, Illinois, USA). All other reagents were from Sigma (St Louis, MO, USA).

Animal studies, cell culture, transfections, RT-PCR and western blot assay The PED transgenic mice and cellular models generation have been previously described [11, 26]. Total RNA extraction, cDNA synthesis, real-time PCR and

western blot analysis were performed as described in [11]. Antibodies against SMRT, HNF-4 α , PED and actin were used for detection of proteins. All the experiments involving animals were approved by the Local Ethics Committee and conducted in accordance with the Principles of Laboratory Care.

Formaldehyde-assisted isolation of regulatory elements Whole cells were fixed in growth medium by addition of 37% (vol./vol.) formaldehyde to a final concentration of 1% (vol./vol.) formaldehyde for 10 min. The fixed cells were resuspended in nuclear lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), 10 mmol/l EDTA, 0.8% (wt/vol.) sodium dodecyl sulphate, 1 mmol/l phenyl methyl sulfonyl fluoride and inhibitors cocktail (Sigma), and then incubated on ice for 10 min. The extracts were then sonicated (Misonix 3000) and microcentrifuged for 10 min at 16,000 g and 4°C. Protein-free DNA was then analysed by SYBR Green real-time PCR [27]. The primer sets used are shown in Electronic supplementary material (ESM) Table 1. The amount of PCR product (representing nucleosome-free DNA) was plotted as a percentage of the input DNA representing total cellular DNA.

Micrococcal nuclease protection assay Nuclei were isolated from 1×10^8 of the following cells: HeLa wild-type, HeLa overproducing exogenous HNF-4 α (HeLa_{HNF-4 α}) HepG2 and HepG2 subjected to silencing of the endogenous HNF-4 α (HepG2_{HNF-4 α -sh}). Isolated nuclei were suspended in 1 ml of wash buffer (10 mmol/l Tris-HCl, pH 7.4, 15 mmol/l NaCl, 50 mmol/l KCl, 0.15 mmol/l spermine, 0.5 mmol/l spermidine and 8.5% [wt/vol.] sucrose) and digested with 120 U of micrococcal nuclease (MNase) for 30 min at 37°C. The purified DNA was quantified and identified on an agarose gel, and subsequently amplified by PCR using the C, D and F primer sets. An aliquot of undigested DNA was obtained as a control of total cellular DNA quality.

Chromatin immunoprecipitation and re-chromatin immunoprecipitation assay procedures Chromatin immunoprecipitation (ChIP) assay was performed as previously described [11]. Sheared chromatin samples were taken as input control or used for immunoprecipitation with the following antibodies: acetyl-histone H3 (sc-8655), dimethylated histone H3 at lysine 9 (H3K9me2; ab-1220), H3K4me2 (ab-7766), HNF-4 α (sc-6556), SMRTe (sc-1612) and normal rabbit IgG as a negative control. DNA fragments were recovered and subjected to real-time PCR amplification using primer sequences described in Table 1.

For re-chromatin immunoprecipitation (Re-ChIP) assay, immunoprecipitates with the first antibody were eluted in 50 μ l of dithiothreitol 10 mmol/l, diluted tenfold in ChIP

dilution buffer supplemented with protease inhibitors and immunoprecipitated with the second antibody. Following immunoprecipitation, samples were processed as described for ChIP assay [11] and eluted DNA was amplified by real-time PCR with specific oligos.

Trichostatin A treatment of cells HepG2 cells were seeded in 10 cm dishes at a density of 1×10^6 cells 1 day before drug treatment. The cells were treated with 330 nmol/l trichostatin A (Sigma-) for 1 day, and total RNA and chromatin were prepared as described before.

Hepatic glycogen measurement Cells and tissues were solubilised in 0.1% (wt/vol.) sodium dodecyl sulphate and then further incubated with 1:1 saturated Na₂SO₄ and 95% (vol./vol.) ethanol. The pellets were rehydrated, and 5% (vol./vol.) phenol and H₂SO₄ were added. Finally, absorbance at 490 nm was measured. Results are expressed as micrograms of glycogen per milligram of protein or tissues.

Statistical procedures Statistical analysis was performed with a software package (StatView 5.0; Abacus Concepts, Berkley, CA, USA) using the Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

HNF-4 α induces DNA remodelling and nucleosome positioning at the PED promoter As previously reported, HeLa cells feature high expression of the *PED* gene, while very low expression was found in the HepG2 cells [11]. Expression of the *PED* silencer HNF-4 α is inversely related to that of *PED* in these cells [11]. Thus, these cell lines represent an attractive model to investigate the molecular mechanisms responsible for HNF-4 α regulation of *PED* gene transcription. To investigate changes induced by HNF-4 α in chromatin structure across the *PED* promoter, we isolated nucleosome-free chromatin DNA from HeLa cells (either wild-type or expressing an HNF-4 α [also known as *HNF4A*] cDNA) and from HepG2 cells (wild-type and expressing an HNF-4 α shRNA) by performing a formaldehyde-assisted isolation of regulatory elements assay [28]. The DNA fragments were amplified using seven sets of PCR primers, covering consecutive 100 bp regions positioned at -500 to 300 in the *PED* gene (Fig. 1a, ESM Table 1) and PCR amplification was monitored by SYBR green incorporation [29]. The amount of DNA amplified (nucleosome-free DNA) was plotted as percentage of the input DNA representing total cellular DNA.

Fig. 1 Enrichment of regulatory DNA across 0.8 kb of *PED* gene using formaldehyde-assisted isolation of regulatory elements. **a** Schematic representation of *PED* showing the HRE, the transcription start site, the exon 2 position and the DNA fragments amplified by primer sets A to F. **b** Protein-free DNA was extracted from HeLa (black bars) and HeLa_{HNF-4α} (grey bars), and **(c)** from HepG2 (white bars) and HepG2_{HNF-4α-sh} (striped bars) cells as described above and analysed by real-time PCR using the indicated primers. The mean and standard errors of at least three independent experiments each performed in triplicate are shown. Statistical significance was determined by *t* test (two-tailed) analysis; ****p*<0.001

In HeLa_{HNF-4α}, the amount of nucleosome-free DNA across the examined regions ranged from 25 to 65% compared with untransfected HeLa cells showing higher free DNA amounts (Fig. 1b). HepG2_{HNF-4α-sh} featured high levels and low reduction of nucleosome-free DNA, compared with untransfected HepG2 cells exhibiting high expression levels of HNF-4α and reduced free DNA across the -500 to 300 region of the *PED* gene (Fig. 1c). In HeLa_{HNF-4α} and HepG2 cells, three regions were found to be associated with reduced levels of free DNA. These corresponded to the HNF-4α response element (HRE; at -350, region B), its immediate proximity (-200, region C)

Fig. 2 Accessibility of *PED* gene regions to MNase digestion. HeLa, HeLa_{HNF-4α}, HepG2 and HepG2_{HNF-4α-sh} nuclei were digested with 120 U of MNase for 30 min, which extensively digests the linker and nucleosome-free regions. **a** Representative image of digested nucleosomes separated on a 2% agarose gel before purification. First lane contains a DNA marker ladder. The arrow indicates mononucleosomes. **b** PCR amplification with primer sets from Fig. 1a as indicated using mononucleosomal DNA (bottom rows) and genomic DNA as positive control (top rows) for C region and **(c)** F region. The PCR products are shown for each primer set after agarose gel electrophoresis. Photographs are representative of three **(a)** and four **(b, c)** independent experiments

and the proximity of the *PED* transcription start site (-50, region D).

To further investigate nucleosome positioning across the *PED* promoter, mononucleosomal DNA averaging 150 bp in size was obtained by nuclear MNase digestion of HeLa cells (either transfected with HNF-4α or untransfected) and HepG2 cells (transfected with HNF-4α-sh or untransfected; Fig. 2a). This DNA was subsequently amplified using the PCR primer sets for the C and D regions shown in Fig. 1b. Figure 2b shows the results obtained with primer set C; similar results were obtained with primer set D (data not shown). In agarose gel electrophoresis, the amplification products were barely detectable when using the mononucleosomal DNA from HeLa cells, but were significantly increased with the DNA from the HeLa_{HNF-4α} cells (C region). Consistently, only a weak band was obtained in HNF-4α-silenced (HepG2_{HNF-4α-sh}) cells compared with the untransfected HepG2 cells (C region). The use of undigested DNA in control PCR assays led to the amplification of a 120 bp band with all of the cell lines (C region). Also, upon MNase treatment, the nucleosome-free exon 2 allowed no PCR amplification with F region primers (Fig. 2c), although clear amplification bands were obtained with the undigested DNA (F region). These data

Fig. 3 HNF-4 α production determines repressive histone modifications at the *PED* promoter. **a, b** HeLa and HepG2 cells were transfected with 1 μ g of the pCDNA3/HNF-4 α expression vector and of the HNF-4 α -specific shRNA clone, respectively. At 48 h after transfection, total RNAs were extracted from transfected and non-transfected cells. *PED* mRNA levels were then quantified by RT-PCR. Data were normalised to β -actin mRNA and are expressed as per cent decrease or increase vs control (untransfected cells). ChIP experiments were performed using antibodies against active histone marks, i.e. (**c–f**) acetyl-histone H3 and (**g–j**) H3K4me2, in the *PED* gene in HeLa (black bars) and HeLa_{HNF-4 α} cells (grey bars), and in HepG2 (white

bars) and HepG2_{HNF-4 α -sh} (striped bars) cells. ChIP was also performed against the repressive histone mark H3K9me2 (**k–n**) in HeLa and HeLa_{HNF-4 α} cells, and in HepG2 and HepG2_{HNF-4 α -sh} cells. ChIPs were followed by quantitative PCR amplification with primer set for the C region of the *PED* promoter. Representative gels (**d, f, h, j, l, n**) are shown. Levels in bar graphs (**c, e, g, i, k, m**) are presented as per cent enrichment relative to input DNA and corrected for IgG control levels as analysed by quantitative PCR. Bars represent the mean $\pm$ SE of three independent experiments each performed in triplicate. Statistical significance was assessed by *t* test analysis. ****p* < 0.001

suggest that HNF-4 α production and binding to the HRE causes nucleosome positioning at C and D regions in the proximal *PED* gene promoter.

*HNF-4 α induces histone deacetylation and methylation at the *PED* promoter* We next focused on the significance of HNF-4 α cellular levels for the epigenetic state of the *PED* promoter region. To this end, we looked for typical transcriptional activation and repression marks in the cells with high (HeLa_{HNF-4 α} , HepG2) and low (HeLa, HepG2_{HNF-4 α -sh}) HNF-4 α levels, in which *PED* transcription is suppressed and active, respectively (Fig. 3a, b). ChIP assays were performed using antibodies recognising histone modifications, as well as normal rabbit IgG as a negative control, with the purified DNA from the immunoprecipitates being used for real-time PCR analysis with primer sets amplifying the C region nucleosome. In HeLa_{HNF-4 α} and HepG2 cells we saw an almost twofold reduction in histone H3 acetylation at lysines 9 and 14 (a mark of active transcription) compared with HeLa and the HepG2_{HNF-4 α -sh} cells (Fig. 3c, e). A further mark of active transcription [30], lysine 4 dimethylated histone H3, was similarly reduced in these cells (Fig. 3g, i). Also, the dimethylation of histone H3 at lysine 9 (H3K9me2), a mark typical of repressed gene promoters [31], was almost threefold higher at the C region nucleosome from HeLa_{HNF-4 α} and HepG2 as compared with HeLa or HepG2_{HNF-4 α -sh} cells (Fig. 3k, m). Similar results were obtained with the D region nucleosome (data not shown). Thus, HNF-4 α production and response element binding is accompanied by enrichment in repressive histone modifications together with reduced rate of *PED* expression.

In addition, the HepG2_{HNF-4 α -sh} cells, which feature high levels of *PED* mRNA, exhibited reduced hepatic glycogen content compared with control cells. Similar results were obtained in stably transfected HepG2 cells overexpressing human *PED* (Fig. 4a).

We also analysed glycogen content in livers from *PED* transgenic mice. These animals have been previously characterised and described [26]. As shown in Fig. 4b, the abundance of hepatic glycogen content was lower in transgenic mice than in control non-transgenic littermates (Fig. 4b).

*HNF-4 α recruits repressive enzymes at the *PED* promoter and silences expression* Since HNF-4 α production results in decreased histone acetylation, we investigated the presence of histone-modifying enzymes at the HRE region in the *PED* promoter in the presence and absence of HNF-4 α . Using ChIP assays, we demonstrated the presence of the SMRT co-repressor complex at the HNF-4 α in HepG2 cells but not in HeLa cells (Fig. 5a). Importantly, HeLa and HepG2 cells feature very comparable levels of SMRT, indicating that

Fig. 4 Effect of *PED* overproduction on hepatocyte glycogen content. **a** HepG2, HepG2_{HNF-4 α -sh} and HepG2/*PED* cells were assayed for glycogen content after incubation with 100 nmol/l insulin (black bars) as described above. White bars, without insulin. **b** Glycogen content was also compared in liver tissue from transgenic mice overexpressing *PED* (Tg-*PED*) and from their non-transgenic littermates (BDF) maintained under random-feeding conditions. Bars represent the means $\pm$ SE of three (**a**) and four (**b**) independent measurements each in triplicate. * p <0.05, ** p <0.01 and *** p <0.001

the presence of SMRT at the HRE observed in the HepG2 cells is not dependent on SMRT levels (ESM Fig. 1a). The assembly of HNF-4 α /SMRT complexes on the *PED* promoter region was then investigated by Re-ChIP experiments using HNF-4 α antibody in the first ChIP and a SMRT antibody in the second ChIP. As shown in Fig. 5b, c, we detected HNF-4 α together with SMRT at the HRE in HeLa_{HNF-4 α} and HepG2 cells, while the complex was barely detectable in cells producing lower amounts of HNF-4 α (HeLa and the HepG2_{HNF-4 α -sh}). Nuclear extracts from each cell type were immunoprecipitated with the SMRT antibody and the immunocomplexes blotted with the HNF-4 α antibody. Again, we detected an HNF-4 α /SMRT interaction only in the HNF-4 α -producing cells, HeLa_{HNF-4 α} and HepG2, compared with HeLa and HepG2_{HNF-4 α -sh} cells, respectively (Fig. 5d), indicating that recruitment of the co-repressor SMRT was entirely dependent on the presence of HNF-4 α .

Re-ChIP experiments using antibodies toward the repressive histone mark H3K9me2 followed by HNF-4 α or SMRT antibodies revealed that levels of these complexes were reduced in the cells with low HNF-4 α levels

Fig. 5 Recruitment of repressive enzymes at the *PED* promoter is dependent on HNF-4α. ChIP experiments were performed using antibodies against SMRT (**a**) and HNF-4α (**b**, **c**). The binding of SMRT to the HRE of *PED* was analysed by quantitative PCR in (**a**) HeLa and HepG2 wild-type cells. HNF-4α binding was analysed by quantitative PCR in the HRE of *PED* in HeLa (black bar) and HeLa_{HNF-4α} (grey bar) (**b**), and (**c**) in HepG2 (white bar) and HepG2_{HNF-4α-sh} (striped bar) cells. Re-ChIP experiments were performed using antibodies against SMRT (**b**, **c**) to identify protein complexes at the HRE of *PED*. The amount of precipitated DNA from the first ChIP was used as input. Results are expressed as enrichment relative to input (%) and corrected for IgG control levels. **d** HeLa, HeLa_{HNF-4α}, HepG2 and HepG2_{HNF-4α-sh} cells were grown as described and total lysates of the cells immunoprecipitated with antibodies against SMRT and then analysed by immunoblot with antibodies against HNF-4α and SMRT. Filters were revealed by ECL. The autoradiograph shown is representative of four independent experiments. **e** HepG2 cells were treated with 330 nmol/l trichostatin A (black bars) for 24 h. White bar, vehicle. *PED* and *HNF-4α* expression were determined by real-time PCR. Data were normalised to β-actin levels and expressed as relative to vehicle treatments. Bars (**a**–**e**) represent the mean ± SE of at least three independent experiments each performed in triplicate. Statistical significance was assessed by *t* test analysis. ****p* < 0.001

(HeLa and the HepG2_{HNF-4α-sh}), in which *PED* transcription is active. This finding indicates that HNF-4α/SMRT assembly to the *PED* gene is accompanied by histone deacetylation and methylation at its proximal promoter (ESM Fig. 1b).

Since HNF-4α production results in changes of the epigenetic state of chromatin, we investigated the possibility of activating the *PED* gene by pharmacological inhibition of histone deacetylation. Incubation of HepG2 cells with the deacetylase inhibitor trichostatin A revealed an increase in *PED* expression in these cells (Fig. 5e). Since HNF-4α levels also significantly increased upon trichostatin A treatment, we sought to verify whether hyperacetylation induced by trichostatin A treatment impairs HNF-4α binding to its response element, as this effect may have been sufficient to enhance *PED* transcription. We found no difference in HNF-4α at its B region-amplified response element in HepG2 cells, whether preincubated with trichostatin A or not (ESM Fig. 2). This supports the role of histone deacetylation in restraining *PED* activity. We conclude that histone deacetylation is required for HNF-4α-mediated *PED* repression.

SMRT silencing rescues *PED* expression in HepG2 cells To further address the role of SMRT in HNF-4α-dependent repression of *PED* function, HepG2 cells were transiently transfected with siRNAs for *SMRT* (also known as *NCOR2*)-α and *SMRT*β (panSMRT) or a control siRNA [25]. Real-time PCR assays on total RNA from panSMRT-silenced HepG2 cells showed about twofold increased *PED* RNA levels compared with control siRNA and wild-type cells (Fig. 6a). Western blot analysis of extracts from the different cell types also revealed significantly increased *PED* and decreased SMRT protein levels upon the panSMRT transfection (Fig. 6b). In addition, ChIP assays in panSMRT-treated HepG2 cells using acetyl-histone H3 (K9/K14) and H3K9me2 antibodies showed significantly increased acetylation and decreased methylation of the histone H3 lysine 9 compared with controls (Fig. 6c).

The role of SMRT in HNF-4α regulation of *PED* expression was further addressed in HepG2 cells transfected with a *PED* promoter luciferase construct featuring the HRE (pPED477). Co-transfection of the panSMRT siRNA in these cells increased the pPED477 reporter gene activity by twofold. Importantly, the luciferase activity of a vector containing the mutagenised HNF-4α binding sequence (pPED477mut) abolishing its transcriptional activity was increased at similar levels in control and panSMRT transfected HepG2 cells (Fig. 6d) compared with HepG2 wild-type cells transfected with the pPED477 vector alone (Fig. 6d). This indicates that the recruitment of HNF-4α and SMRT to the HRE at the *PED* promoter is necessary for *PED* transcriptional inhibition.

SMRT enables HNF-4α inhibition of *GRB14* and *USP21* genes To verify whether SMRT action on HNF-4α silencing is unique to the *PED* gene, we analysed expression of the two other genes repressed by HNF-4α, *GRB14* and

Fig. 6 Effect of SMRT depletion on *PED* expression in HepG2 cells. HepG2 cells were transfected with Silencer negative control siRNA (grey bars) or siRNAs for panSMRT (black bars). **a** *SMRT* and *PED* mRNA levels were quantified by real-time PCR. Data were normalised to β-actin mRNA and are expressed as fold over control (untransfected cells [NT], white bars). **b** Lysates prepared from HepG2 cells transfected with siRNAs were western-blotted with PED and SMRT antibodies and further analysed by ECL and autoradiography. The autoradiograph shown is representative of four independent experiments. **c** ChIP experiments were performed using antibodies against active (acetyl-histone H3 [AcH3]) or repressive (H3K9me2) histone marks. Their binding was analysed by quantitative PCR in the nucleosome C region of *PED* promoter in control or (panSMRT) HepG2 cells and expressed as fold over control (untransfected cells). **d** HepG2 cells were co-transfected with 3 μg of the indicated *PED* promoter–luciferase constructs (or 3 μg of the promoterless pGL3 basic vector) alone or in combination with SMRTα and SMRTβ siRNAs or the Silencer negative control siRNA and 1 μg of the pRSVβ-gal vector DNA. Luciferase activity was assayed as described and is presented as the increase above the activity measured with the control pGL3 basic vector. Bars represent the mean ± SE of three independent experiments each performed in triplicate. Statistical significance was assessed by *t* test analysis. ****p* < 0.001

USP21. Interestingly, *GRB14* and *USP21* were expressed at reduced levels in the HeLa_{HNF-4α} compared with untransfected cells (Fig. 7a). In addition, expression of these genes was rescued by HNF-4α silencing in HepG2_{HNF-4α-sh} cells, further supporting the repressor function of HNF-4α (Fig. 7b). The regulatory region of *GRB14* and *USP21* has not been reported yet. By in silico analysis, we therefore identified their HREs at -700 and -450 bp upstream of their transcriptional start sites, respectively (Fig. 7c). ChIP and Re-ChIP assays with specific antibodies and HRE primer sets revealed largely increased HNF-4α and HNF-4α/SMRT complex abundance at the HRE in the HeLa_{HNF-4α} compared with untransfected cells (Fig. 7d). Consistently, the presence of HNF-4α and HNF-4α/SMRT complex at the response element was significantly reduced

in HepG2 cells transfected with the HNF-4α-sh (Fig. 7e). We then examined the acetylation state of the *GRB14* and *USP21* proximal regulatory regions and performed ChIP assays using acetyl-histone H3 (K9/K14) antibodies and

Fig. 7 HNF-4α-dependent inhibition of *GRB14* and *USP21* expression. Total RNA preparations were obtained (**a**) from HeLa (black bars) and HeLa_{HNF-4α} (grey bars) cells, and (**b**) from HepG2 (white bars) and HepG2_{HNF-4α-sh} (striped bars) cells. Levels of *GRB14* and *USP21* mRNA were then determined by real-time PCR and normalised to β-actin mRNA. mRNA levels are relative to those in control (HeLa, HepG2) cells. **c** Schematic representation of the *GRB14* and *USP21* genes, with arrows indicating ChIP primers against the HNF-4α binding site (HRE) and the region downstream the HRE. ChIP experiments were performed using antibodies against HNF-4α (**d**, **e**) and (**f**, **g**) active histone marks (acetyl-histone H3). HNF-4α binding was analysed by quantitative PCR in the HRE of *GRB14* and *USP21* in HeLa and HeLa_{HNF-4α} (**d**) and HepG2 and HepG2_{HNF-4α-sh} (**e**) cells. Acetyl-histone H3 enrichment at the *GRB14* and *USP21* regulatory regions was analysed by quantitative PCR in HeLa and HeLa_{HNF-4α} (**f**), and HepG2 and HepG2_{HNF-4α-sh} (**g**) cells. Re-ChIP experiments were performed using antibodies against SMRT (**d**, **e**) to identify protein complexes at the HRE of *GRB14* and *USP21* genes. The amount of precipitated DNA from the first ChIP was used as input. Results are expressed as per cent enrichment relative to input DNA and corrected for IgG control levels. Bars represent the mean ± SE of three independent experiments each performed in triplicate. Statistical significance was assessed by *t* test analysis. ***p* < 0.01, ****p* < 0.001

primer sets amplifying a 300 bp region downstream of the HREs. Histone H3 (K9/K14) acetylation was significantly reduced in the HeLa_{HNF-4 α} , but rescued in the HepG2_{HNF-4 α -sh} cells (Fig. 7f, g), indicating common mechanisms in the silencing of the *PED*, *GRB14* and *USP21* genes by HNF-4 α .

Discussion

Previous work has established the significance of the nuclear receptor superfamily in regulating a broad range of cellular processes by activating or repressing different sets of genes that harbour nuclear receptor-recognising DNA motifs [32]. Several mechanisms leading to transcriptional activation by the nuclear receptors have been elucidated in considerable detail [33]. Against this, the molecular bases of transcriptional repression remain more unclear [34, 35].

In the present study, we show that HNF-4 α , a member of the steroid receptor class of nuclear receptors [36, 37], triggers SMRT recruitment to the *PED* proximal promoter and causes histone tail hypoacetylation at H3-K9/K14, inducing a transcriptionally non-permissive state of the gene. These changes are followed by the appearance of H3K9 hypermethylation and H3K4 hypomethylation, two marks of heterochromatin (Fig. 8). These findings indicate for the first time that, in intact cells, HNF-4 α represses gene transcription by directly recruiting SMRT to the promoter region, leading to histone deacetylation-associated remodelling of chromatin.

Previous studies in HNF-4 α -producing cells and in cells with no HNF-4 α production have shown that SMRT represses HNF-4 α -mediated transcription of reporter constructs containing heterologous, as well as native promoters [38]. In addition, using glutathione *S*-transferase pull-down assays, the same team of authors showed that HNF-4 α directly binds the receptor interacting domain 2 of SMRT in vitro [38]. These observations suggest that SMRT repres-

sion might represent a physiologically relevant mechanism responsible for HNF-4 α regulation and that functional cooperation requires physical interaction of SMRT and HNF-4 α . The finding we now report, namely that the HNF-4 α /SMRT complex induces *GRB14* and *USP21* repression in addition to *PED* gene silencing, further underlines the significance of these mechanisms to the in vivo transcriptional repressor activity of HNF-4 α .

HNF-4 α has long been known to serve as a transcriptional activator [39]. Indeed, HNF-4 α can: (1) activate transcription in the absence of exogenously added ligands [40]; and (2), in mammalian cells, in yeasts and in vitro, respond to several coactivators including glucocorticoid receptor interacting protein 1 (GRIP1), steroid receptor coactivator 1 (SRC-1) and CREB binding protein (CBP)-p300 [41, 42]. However, Ruse et al. reported that HNF-4 α regulation by these coactivators can be competitively abolished by interaction of the histone deacetylase-associated co-repressor SMRT with the F domain of HNF-4 α [38]. Thus, the nature of the recruited co-regulator and of its association with direct effectors of histone modifications such as histone deacetylases appears to determine the transcriptional activity of HNF-4 α .

Recently, it has been shown that a knock-in mouse bearing a mutation in the receptor interacting domain of SMRT (SMRT^{mRID}) that disrupts its interaction with the nuclear hormone receptor [43] develops multiple metabolic defects including altered insulin sensitivity and 70% increased adiposity. These findings indicate that major districts of metabolic regulation such as insulin sensitivity and fat mass depend on the molecular balance of co-repressors and coactivators, which modulate the status of chromatin and nuclear receptor signalling and ultimately regulate gene expression in response to specific physiological situations.

PED expression is upregulated in individuals with type 2 diabetes and in the euglycaemic offspring of these patients, impairing insulin action and insulin secretion [5, 7]. However, the causes for *PED* upregulation required additional investigation. In the present study, we demonstrated that HNF-4 α silencing alters the epigenetic state of the *PED* gene and determines its overexpression in liver cells. We further show that induction of *PED* gene expression in hepatocytes is paralleled by reduced hepatic glycogen content. We have also very recently demonstrated that activation of *PED* expression in HepG2 cells is paralleled by the establishment of a partially dedifferentiated phenotype, accompanied by reduced mRNA levels of genes expressed during normal liver development [11]. Thus HNF-4 α -regulated *PED* gene may have a role in hepatocyte differentiation. Epigenetic changes upregulating its expression might impair development of normal liver function, thereby contributing to progression toward diabetes. Environmental factors have been reported to induce epigenetic changes, leading to the development of

Fig. 8 Epigenetic changes induced by HNF-4 α to *PED* expression. HNF-4 α production and binding to its response element causes chromatin packaging at the *PED* promoter and enrichment of histone H3 methylation at lysine 9 (H3K9me2), a mark typical of repressed gene promoters. HRE binding also causes a decrease in acetylation at lysine 9 and 14 (AcH3K9/K14) and dimethylation of lysine 4 of histone H3 (H3K4me2), two marks of active transcription

abnormal phenotypes [44, 45]. For instance, diet and ageing have been reported to perturb gene expression by inducing DNA methylation and histone modifications [46]. Also, intrauterine growth retardation, a common complication of pregnancy, has been associated with later development of type 2 diabetes, in part through chromatin remodelling effects and epigenetic modulation of the expression of several genes [47]. Type 2 diabetes is a genetically heterogeneous disease resulting from complex interactions of genetic and environmental determinants. Previous studies using different approaches have uncovered a number of type 2 diabetes susceptibility genes, but it remains less clear how these genes determine type 2 diabetes. In particular, the molecular details of gene–environment interplay in diabetes onset have received less attention. Exposure to certain environmental determinants, including maternal obesity [48], intrauterine environment [49, 50] and nutritional factors [51], affects key developmental sequences programming metabolic responsiveness to environmental stimuli during later life. Such concepts are now supported by studies of monozygotic twins, where diabetes risk is discordant and linked to birthweight [52]. Laboratory studies are in progress to assess whether *PED* may serve as an environment gene target contributing to type 2 diabetes progression.

In conclusion, in the present work, we demonstrate that HNF-4 α inhibits *PED* expression by inducing chromatin remodelling and histone modifications at nucleosomes located in the regulatory region of the gene. Dysregulation of these mechanisms might promote pathogenetic sequences leading to type 2 diabetes.

Acknowledgements This work was supported by the European Foundation for the Study of Diabetes (EFSD), the European Community's FP6 EUGENE2 (LSHM-CT-2004-512013) and PRE-POBEDIA (201681) programmes, and grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) and from the Ministero dell'Università e della Ricerca Scientifica (PRIN and FIRB). The financial support of Telethon - Italy is also gratefully acknowledged. We thank F. Blasi (Fondazione Istituto FIRC di Oncologia Molecolare, Milano, Italy) and L. Chiariotti (Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano", Università di Napoli Federico II, Napoli, Italy) for their critical reading this manuscript.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

- Sladek R, Rocheleau G, Rung J et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 445:881–885
- Saxena R, Voight B, Lyssenko V et al (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 316:1331–1336
- Prokopenko I, McCarthy M, Lindgren C (2008) Type 2 diabetes: new genes, new understanding. *Trends Genet* 24:613–621
- Danziger N, Yokoyama M, Jay T, Cordier J, Glowinski J, Chneiweiss H (1995) Cellular expression, developmental regulation, and phylogenetic conservation of PEA-15, the astrocytic major phosphoprotein and protein kinase C substrate. *J Neurochem* 64:1016–1025
- Condorelli G, Vigliotta G, Iavarone C et al (1998) PED/PEA-15 gene controls glucose transport and is overexpressed in type 2 diabetes mellitus. *EMBO J* 17:3858–3866
- Condorelli G, Vigliotta G, Trencia A et al (2001) Protein kinase C (PKC)- α activation inhibits PKC- ζ and mediates the action of PED/PEA-15 on glucose transport in the L6 skeletal muscle cells. *Diabetes* 50:1244–1252
- Valentino R, Lupoli GA, Raciti GA et al (2006) In healthy first-degree relatives of type 2 diabetics, ped/pea-15 gene is overexpressed and related to insulin resistance. *Diabetologia* 49:3058–3066
- Perfetti A, Oriente F, Iovino S et al (2007) Phorbol esters induce intracellular accumulation of the anti-apoptotic protein ped/pea-15 by preventing ubiquitinylation and proteasomal degradation. *J Biol Chem* 282:8648–8657
- Hayhurst G, Lee Y, Lambert G, Ward J, Gonzalez F (2001) Hepatocyte nuclear factor 4 α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 21:1393–1403
- Pereira F, Tsai M, Tsai S (2000) COUP-TF orphan nuclear receptors in development and differentiation. *Cell Mol Life Sci* 57:1388–1398
- Ungaro P, Teperino R, Mirra P et al (2008) Molecular cloning and characterization of the human PED/PEA-15 gene promoter reveal antagonistic regulation by hepatocyte nuclear factor 4 α and chicken ovalbumin upstream promoter transcription factor II. *J Biol Chem* 283:30970–30979
- Li J, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4a. *Genes Dev* 14:464–474
- Parviz F, Matullo C, Garrison W et al (2003) Hepatocyte nuclear factor 4 α controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet* 34:292–296
- Black MH, Fingerlin TE, Allayee H et al (2008) Evidence of interaction between PPARG2 and HNF4A contributing to variation in insulin sensitivity in Mexican Americans. *Diabetes* 57:1048–1056
- Menjivar M, Granados-Silvestre MA, Montúfar-Robles I et al (2008) High frequency of T130I mutation of HNF4A gene in Mexican patients with early-onset type 2 diabetes. *Clin Genet* 73:185–187
- Johansson S, Raeder H, Eide SA et al (2007) Studies in 3,523 Norwegians and meta-analysis in 11,571 subjects indicate that variants in the hepatocyte nuclear factor 4 α (HNF4A) P2 region are associated with type 2 diabetes in Scandinavians. *Diabetes* 56:3112–3117
- Rosenfeld MG, Lunyak VV, Glass CK (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 20:1405–1428
- Strahl BD, Allis CD (2000) The language of covalent histone modifications. *Nat Genet* 40:41–45
- Kouzarides T (2007) Chromatin modifications and their function. *Cell Mol Life Sci* 128:693–705
- Saha A, Wittmeyer J, Cairns BR (2006) Chromatin remodelling: the industrial revolution of DNA around histones. *Nat Rev Mol Cell Biol* 7:437–447
- Roth TY, Cuddapah S, Cui K, Zhao K (2006) The genomic landscape of histone modifications in human T cells. *Proc Natl Acad Sci USA* 103:15782–15787
- Kurdistani SK, Tavazoie S, Grunstein M (2004) Mapping global histone acetylation patterns to gene expression. *Cell* 117:721–733

23. Schubeler D, MacAlpine DM, Scalzo D et al (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev* 18:1263–1271
24. Fischle W, Wang Y, Allis CD (2003) Histone and chromatin cross-talk. *Curr Opin Cell Biol* 15:172–183
25. Peterson TJ, Karmakar S, Pace MC, Gao T, Smith CL (2007) The silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressor is required for full estrogen receptor alpha transcriptional activity. *Mol Cell Biol* 27:5933–5948
26. Vigliotta G, Miele C, Santopietro S et al (2004) Overexpression of the *ped/pea-15* gene causes diabetes by impairing glucose-stimulated insulin secretion in addition to insulin action. *Mol Cell Biol* 24:5005–5015
27. Rao S, Procko E, Shannon MF (2001) Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the *IL-2* gene. *J Immunol* 167:4494–4503
28. Hogan GJ, Lee C, Lieb JD (2006) Cell cycle-specified fluctuation of nucleosome occupancy at gene promoters. *PLoS Genetics* 2:1433–1450
29. Overbergh L, Valckx D, Waer M, Mathieu C (1999) Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 11:305–312
30. Santos-Rosa H, Schneider R, Bannister AJ et al (2002) Active genes are tri-methylated at K4 of histone H3. *Nature* 419:407–411
31. Lachner M, O'Sullivan RJ, Jenuwein T (2003) An epigenetic road map for histone lysine methylation. *J Cell Sci* 116:2117–2124
32. Chen J, Kinyamu HK, Archer TK (2009) Changes in attitude, changes in latitude: nuclear receptors remodeling chromatin to regulate transcription. *Mol Endocrinol* 20:1–13
33. McKenna NJ, O'Malley BW (2002) Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 108:465–474
34. Grignani F, de Matteis S, Nervi C et al (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature* 391:815–818
35. Guenther MG, Lane WS, Fischle W, Verdin E, Lazar MA, Shiekhattar R (2000) A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes Dev* 14:1048–1057
36. Gupta R, Vatamaniuk M, Lee C et al (2005) The *MODY* gene *HNF4alpha* regulates selected genes involved in insulin secretion. *J Clin Invest* 115:1006–1015
37. Hohmeier H, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard C (2000) Isolation of *INS-1* derived cell lines with robust ATP-sensitive K^+ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49:424–430
38. Ruse MDJ, Privalsky ML, Sladek FM (2002) Competitive cofactor recruitment by orphan receptor hepatocyte nuclear factor 4 alpha 1: modulation by the F domain. *Mol Cell Biol* 22:1626–1638
39. Odom DT, Zizlsperger N, Gordon DB et al (2004) Control of pancreas and liver gene expression by HNF transcription factors. *Science* 303:1378–1381
40. Sladek F, Zhong WM, Lai E, Darnell JE (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev* 4:2353–2365
41. Wang JC, Stafford JM, Granner DK (1998) SRC-1 and GRIP1 coactivate transcription with hepatocyte nuclear factor 4. *J Biol Chem* 273:30847–30850
42. Yoshida E, Aratani S, Itou H et al (1997) Functional association between CBP and HNF-4 in trans-activation. *Biochem Biophys Res Commun* 241:664–669
43. Nofsinger RR, Li P, Hong SH et al (2008) SMRT repression of nuclear receptors controls the adipogenic set point and metabolic homeostasis. *Proc Natl Acad Sci USA* 105:20021–20026
44. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet Supplement* 33:245–254
45. Tremblay J, Hamet P (2008) Impact of genetic and epigenetic factors from early life to later disease. *Metabolism* 57:S27–S31
46. Jirtle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 8:253–262
47. Park JH, Stoffers DA, Nicholls RD, Simmons RA (2008) Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of *Pdx1*. *J Clin Invest* 118:2316–2324
48. Dabelea D, Mayer-Davis E, Lamichhane A et al (2008) Association of intrauterine exposure to maternal diabetes and obesity with type 2 diabetes in youth: the SEARCH Case-Control Study. *Diabetes Care* 31:1422–1426
49. Gill-Randall R, Adams D, Ollerton R, Lewis M, Alcolado J (2004) Type 2 diabetes mellitus—genes or intrauterine environment? An embryo transfer paradigm in rats. *Diabetologia* 47:1354–1359
50. Dabelea D, Pettitt D (2001) Intrauterine diabetic environment confers risks for type 2 diabetes mellitus and obesity in the offspring, in addition to genetic susceptibility. *J Pediatr Endocrinol Metab* 14:1085–1091
51. Diakoumopoulou E, Tentolouris N, Kirlaki E et al (2005) Plasma homocysteine levels in patients with type 2 diabetes in a Mediterranean population: relation with nutritional and other factors. *Nutr Metab Cardiovasc Dis* 15:109–117
52. Poulsen P, Kyvik KO, Vaag A, Beck-Nielsen H (1999) Heritability of type II (non insulin-dependent) diabetes mellitus and abnormal glucose tolerance—a population-based twin study. *Diabetologia* 42:139–145