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Original Article

Histone deacetylase 6 promotes insulin resistance via deacetylating phosphatase and tensin homolog (PTEN) in ovarian OVCAR-3 cells

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Abstract: Polycystic ovary syndrome (PCOS) presents a wide range of endocrine and metabolic abnormalities, including various metabolic syndromes (MetS) such as hyperinsulinaemia, hyperglycemia, glucose intolerance. "Insulin resistance (IR)" is considered as the common cause of disorders which affect the long-term health of PCOS patients. In this study, we examined the expression of histone deacetylase 6 (HDAC6) and the acetylation of phosphatase and tensin homolog (PTEN) in PCOS specimens and in ovarian OVCAR-3 cells. And then we investigated the influence of HDAC6 on the glucose uptake and insulin signaling in the OVCAR-3 cells. Results demonstrated that HDAC6 was highly expressed in both mRNA and protein levels, along with a reduced acetylated PTEN in 27 human PCOS tissues. And experiments in human ovarian OVCAR-3 cells confirmed that the HDAC6 knockdown with siRNA inhibited the insulin-induced glucose uptake, the blocked the insulin-promoted AKT phosphorylation, whereas reversed the insulin-downregulated PTEN acetylation in OVCAR-3 cells. In conclusion, the present study recognized the high expression of HDAC6 correlated with the insulin resistance in PCOS via deacetylating PTEN, implying HDAC6 might be a novel target for PCOS treatment, in the context of insulin resistance.

Keywords: Polycystic ovary syndrome (PCOS), histone deacetylase 6 (hdac6), insulin resistance (IR), phosphatase and tensin homolog (PTEN)

Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous gynaecological syndrome, which is often characterized by the manifestation of oligo/anovulation, clinical or biochemical hyperandrogenism and/or polycystic ovaries, and affects 5-10% women of reproductive age [1]. It presents a wide range of endocrine and metabolic abnormalities, including various metabolic syndrome (MetS) such as hyperinsulinaemia, hyperglycaemia, glucose intolerance [2]. "Insulin resistance (IR)" is usually defined as a state which is characterized by reduced glucose-lowering activity of insulin [3]. IR is considered as the common cause of disorders which affect the long-term health of PCOS patients [4], such as defected lipid profile, which is accompanied with 70% PCOS women [5-7]. However, in other investigations, no difference was observed in lipid profile between PCOS women and control participants [8]. PCOS is considered one of the ovarian manifestations of MetS [9], particularly for the PCOS patients with the highest BMIs and insulin levels [10]. And IR seems to contribute mainly to the link between PCOS and MetS [11-13]. However, the molecular pathways underlining the IR in PCOS are not clear.

Histone deacetylases (HDAC) are a group of enzymes that remove acetyl groups from a ε-N-acetyl lysine amino acid on a histone [14], which then regulates the DNA expression. HDACs are classified as class I (HDACs 1, 2, 3, and 8), class IIa (HDACs 4, 5, 7, and 9), class IIb (HDACs 6 and 10), class III HDACs (sirtuins (silent mating type information regulation 2 homolog) 1-7), and class IV (HDAC 11) [15, 16]. And as promising anti-cancer agents, histone deacetylase (HDAC) inhibitors, particularly trichostatin A (TSA), can promote phosphatase and tensin

homolog (PTEN) membrane translocation. Furthermore, the phosphatase activity of PTEN is inhibited by the HDAC6-specific inhibitors [17]. In addition, histone deacetylase HDAC8 promotes insulin resistance in hepatocellular carcinoma [18]. Accordingly, HDAC6 might also contribute to the insulin resistance via deacetylating PTEN.

In this study, we identified histone deacetylase 6 (HDAC6) as a novel IR promoter in ovarian OVCAR-3 cells and further characterized its effects on the proliferation of ovarian OVCAR-3 cells via regulating the acetylation of PTEN. Our findings demonstrate a possible regulation of HDAC6 in PCOS, providing a promising target for therapeutic intervention of PCOS and IR.

Materials and methods

PCOS specimens

There were 27 PCOS patients and 27 women of similar age as controls, who were registered at Department of Obstetrics and Gynecology, Yantai Yuhuangding Hospital of Qingdao University, from April 2013 to Dec 2014. The diagnosis of PCOS patients is according to the 2003 Rotterdam criteria. The presence of two or more of: oligo-ovulation and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries were prerequisite, post the exclusion of such etiologies as congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, 21-hydroxylase-deficient non-classic adrenal hyperplasia, androgenic/anabolic drug use or abuse, thyroid dysfunction, hyperprolactinemia, type 2 diabetes mellitus and cardiovascular disease. There were not any hormonal treatments or insulin-lowering agent before the collection of PCOS specimens via the minimally invasive techniques with Laparoscopy. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital of Qingdao University. And the informed consent was obtained from all participants.

Cell culture and treatment with reagents

The ovarian cancer OVCAR-3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA), which was supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), and with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37°C, with 5% CO_2. Serum-free low-glucose DMEM was used in some experiments. Insulin (as 10 mg/ml solution) was obtained from Sigma-Aldrich (St. Louis, MO, USA). To knockdown HDAC6 in OVCAR-3 cells, HDAC6-specific siRNA (siRNA-HDAC6) or control siRNA (siRNA-Con, scramble siRNA) were purchased from OriGene Technologies (Rockville MD, USA), and were transfected with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA).

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression of HDAC6 or PTEN in PCOS specimens or in OVCAR-3 cells was analyzed by real-time RT-PCR. Total RNA was purified with Trizol™ reagent (Life Technologies, Grand Island, NY, USA), cDNAs of HDAC6 or PTEN were synthesized using the Primescript RT Master Mix Perfect Real-time Kit (Takara, Tokyo, Japan), and the PCR for the mRNA level of each marker was performed on a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using a SYBR™ Premix EX Taq Real-time PCR Master Mix (Takara, Tokyo, Japan). The expression of each marker was normalized to β-actin with $2^{-\Delta\Delta Ct}$ method [19], and was presented as fold change of target mRNA.

Western blotting

PCOS specimens or in OVCAR-3 cells were lyzed with Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA, USA), were supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) and were quantified with Bradford protein assay (Bio-Rad, Hercules, CA, USA). Protein samples were then separated with a 12% polyacrylamide gel in a sodium dodecyl sulfate buffer by electrophoresis. Subsequent to being transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA), the blots were incubated with rabbit polyclone anti-HDAC6 antibody (Abcam, Cambridge, UK), anti-PTEN with or without the acetylation of Lys^{402} (Cell Signaling Technology Inc., Danvers, MA, USA), anti-IRS-1 antibody (Sigma-Aldrich, St. Louis, MO, USA), anti-AKT with or without the phosphorylation of Ser^{473} (Cell Signaling Technology Inc., Beverly, MA, USA).

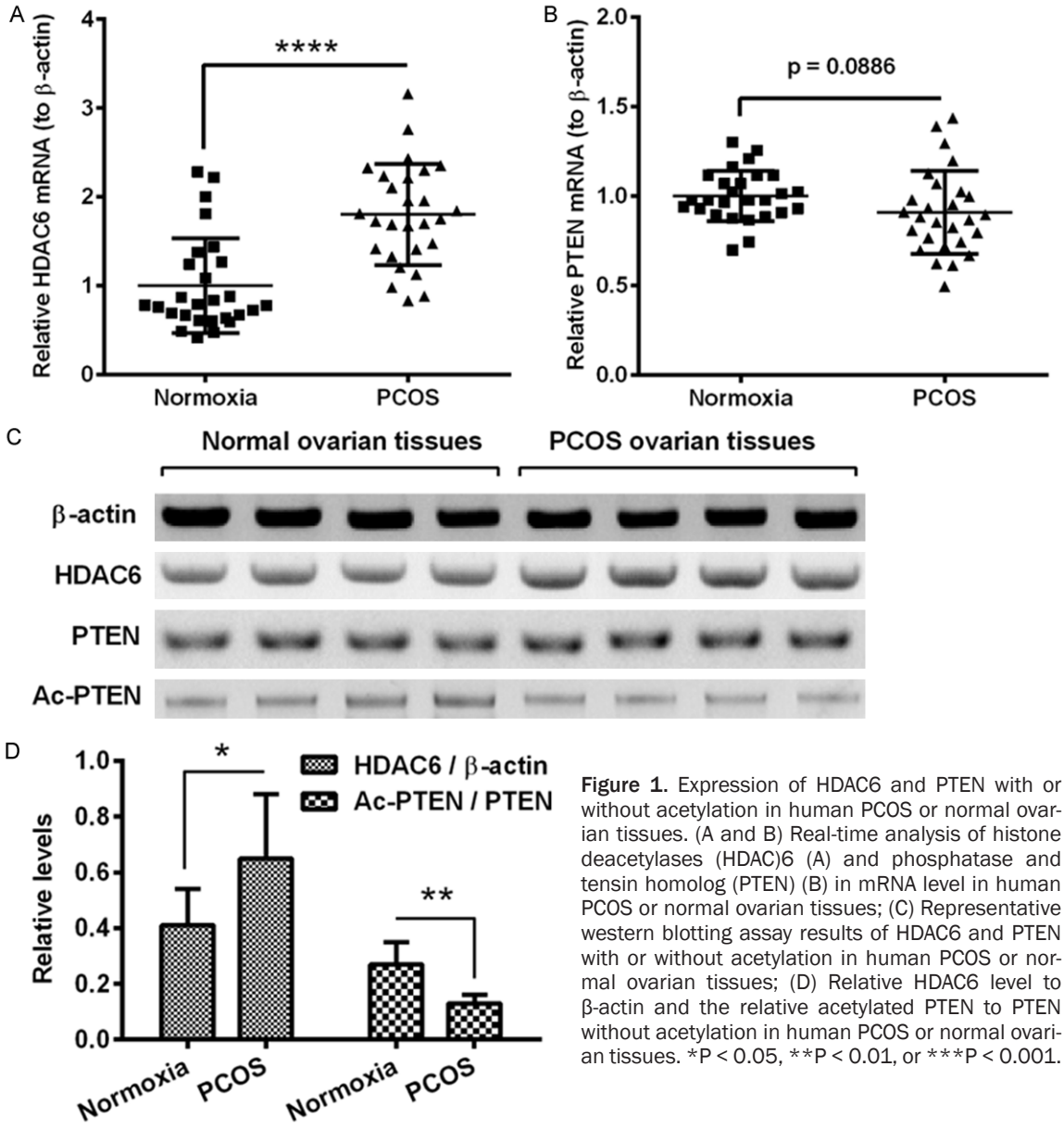

Figure 1. Expression of HDAC6 and PTEN with or without acetylation in human PCOS or normal ovarian tissues. (A and B) Real-time analysis of histone deacetylases (HDAC)6 (A) and phosphatase and tensin homolog (PTEN) (B) in mRNA level in human PCOS or normal ovarian tissues; (C) Representative western blotting assay results of HDAC6 and PTEN with or without acetylation in human PCOS or normal ovarian tissues; (D) Relative HDAC6 level to β-actin and the relative acetylated PTEN to PTEN without acetylation in human PCOS or normal ovarian tissues. *P < 0.05, **P < 0.01, or ***P < 0.001.

And the antigen-antibody binding was revealed by Enhanced chemiluminescence (Thermo Scientific, Rockford, IL, USA), following incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Bio-Rad Laboratories, Hercules, CA, USA). β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the internal control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The difference between two groups was analyzed by Student's t test. A p value < 0.05 or less was considered significance.

Results

HDAC6 is highly expressed, along with the less acetylated PTEN in human PCOS tissues

To investigate the potential role of HDAC6 in the pathogenesis of PCOS, we examined its expression in both mRNA and protein levels by real-time PCR and western blotting methods in normal ovarian tissues and in PCOS tissues. **Figure**

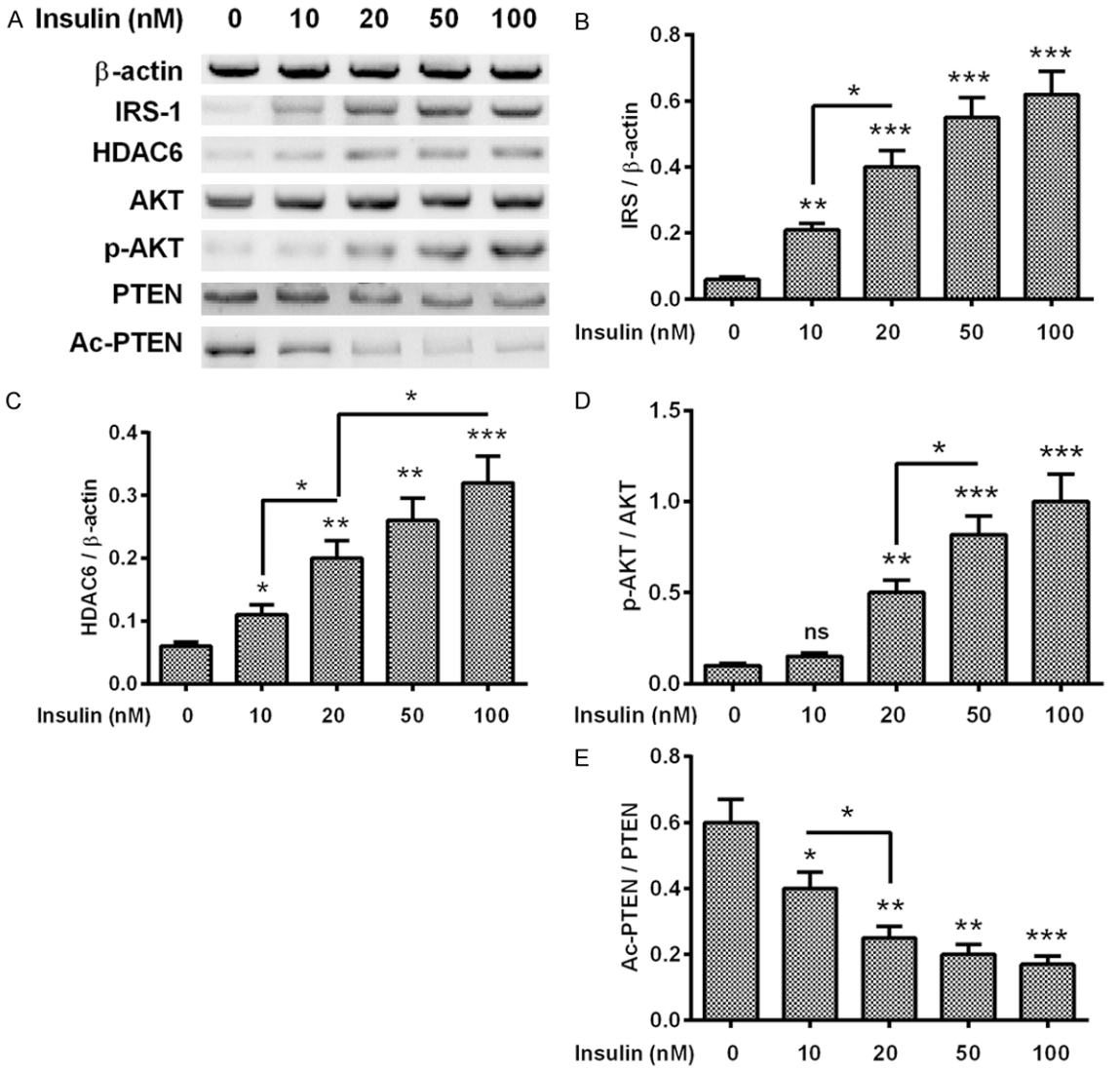

Figure 2. Insulin signaling, HDAC6 and PTEN acetylation in insulin-treated OVCAR-3 cells. (A) Western blot analysis of insulin signaling markers, such as Insulin receptor substrate 1 (IRS-1) and AKT with or without phosphorylation, HDAC6 and PTEN with or without acetylation in the OVCAR-3 cells, which were treated with 0, 10, 20, 50 or 100 nM insulin for 24 hours; (B and C) Relative level of IRS-1 (B) and HDAC6 (C) to β-actin in the insulin-treated OVCAR-3 cells; (D) Relative level of AKT with phosphorylation to AKT without phosphorylation in the insulin-treated OVCAR-3 cells; (E) Relative level of PTEN with acetylation to PTEN without acetylation in the insulin-treated OVCAR-3 cells; Quantitative data was averaged for triple independent results. *P < 0.05, **P < 0.01, ***P < 0.001, or ns: no significance.

1A demonstrated that the relative HDAC6 mRNA level was 1.803 ± 0.3096 (N = 27) in the human PCOS ovarian tissues, significantly higher than 1.000 ± 0.1027 (N = 27) in the normal ovarian tissues (P < 0.0001). However, there was no significant difference in the relative PTEN mRNA level between the PCOS ovarian tissues and the normal ovarian tissues (**Figure 1B**). We then examined the both markers with western blotting assay. It was demonstrated in **Figure 1C** and **1D** that HDAC6 was also overexpressed in protein level in the PCOS tissues than in the normal ovarian tissues (P < 0.05). And the PTEN in protein level was also not markedly different between the PCOS and normal ovarian tissues. However, the PTEN with acetylation was markedly reduced in the PCOS tissues than in the normal ovarian tissues (P <

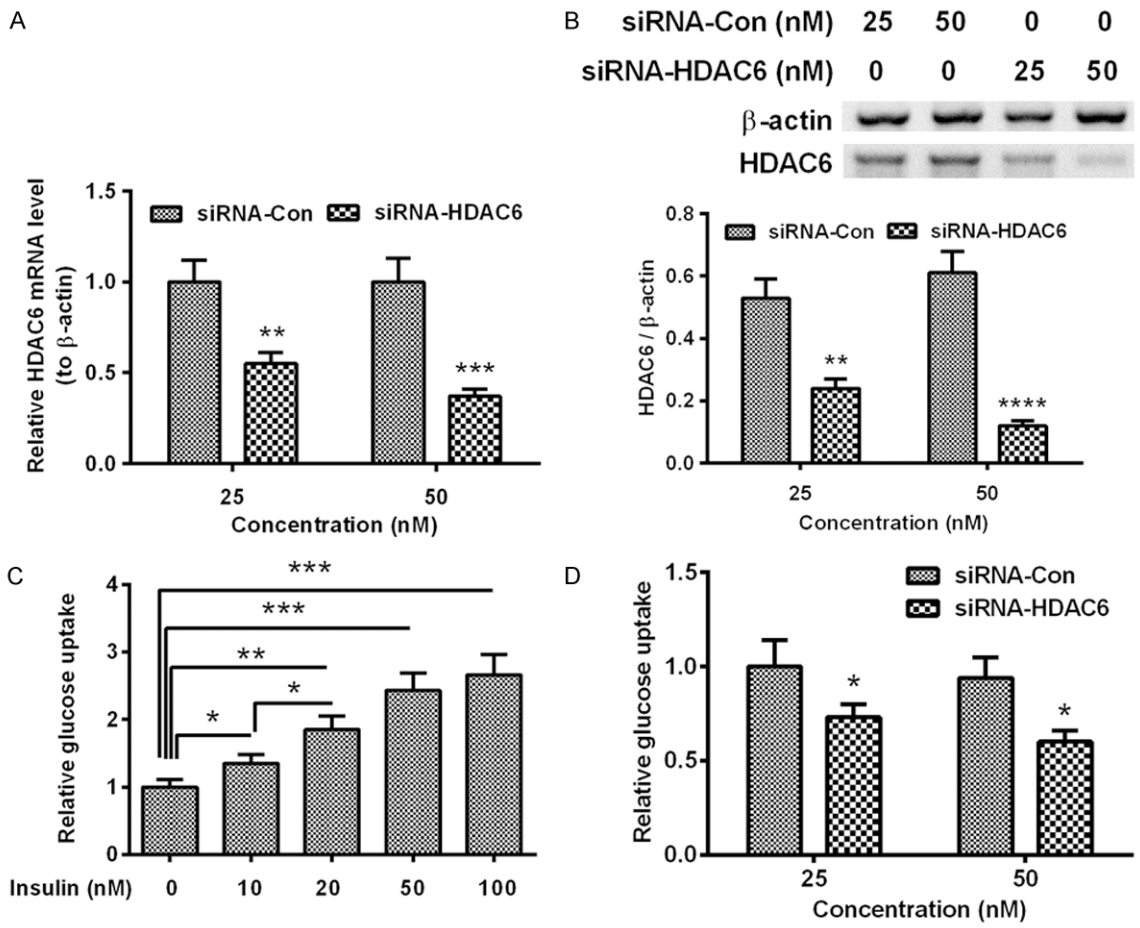

Figure 3. HDAC6 knockdown inhibits the glucose uptake in human ovarian OVCAR-3 cells. A: Relative mRNA level in the OVCAR-3 cells which were transfected with 25 or 50 nM siRNA-HDAC6 or siRNA-Con for 12 hours; B: Western blot analysis and the protein level of HDAC6 in the siRNA-HDAC6- or siRNA-Con-transfected OVCAR-3 cells for 24 hours; C: Relative glucose uptake in the OVCAR-3 cells which were treated with 0, 10, 20, 50 or 100 nM insulin for 24 hours; D: Relative glucose uptake in the insulin-treated (50 nM) OVCAR-3 cells, which were transfected with 25 or 50 nM siRNA-HDAC6 or siRNA-Con for 24 hours. Each experiment was repeated independently in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 or ns: no significance.

0.01). Taken together, HDAC6 is highly expressed, along with the less acetylated PTEN in human PCOS specimens.

Insulin promotes HDAC6 expression and PTEN deacetylation in human ovarian OVCAR-3 cells

We then determined the insulin signaling and the regulation on HDAC6 expression and PTEN acetylation by insulin in human ovarian OVCAR-3 cells. Insulin receptor substrate 1 (IRS-1) [20] and AKT (also known as Protein kinase B, PKB) [21, 22] have been confirmed to be two key markers in insulin signaling. And the western blot analysis in **Figure 2A** indicated that both IRS-1 and the phosphorylated AKT were significantly promoted by the insulin treatment with 10, 20, 50 or 100 nM ($P < 0.01$ or $P < 0.001$, **Figure 2B** and **2C**), dose-dependently ($P < 0.05$ for column 2 vs column 3 in **Figure 2B** or for column 3 vs column 4 in **Figure 2C**). Moreover, it was indicated in **Figure 2A** and **2D** that the HDAC6 expression was markedly promoted by the insulin treatment with more than 10 nM, with dose dependence ($P < 0.05$ for column 2 vs column 3 or for column 3 vs column 5 in **Figure 2D**). However, the acetylated PTEN was markedly downregulated by the insulin treatment ($P < 0.05$, $P < 0.01$ or $P < 0.001$, **Figure 2E**). Thus, we confirmed that insulin promoted HDAC6 expression and PTEN deacetylation in OVCAR-3 cells.

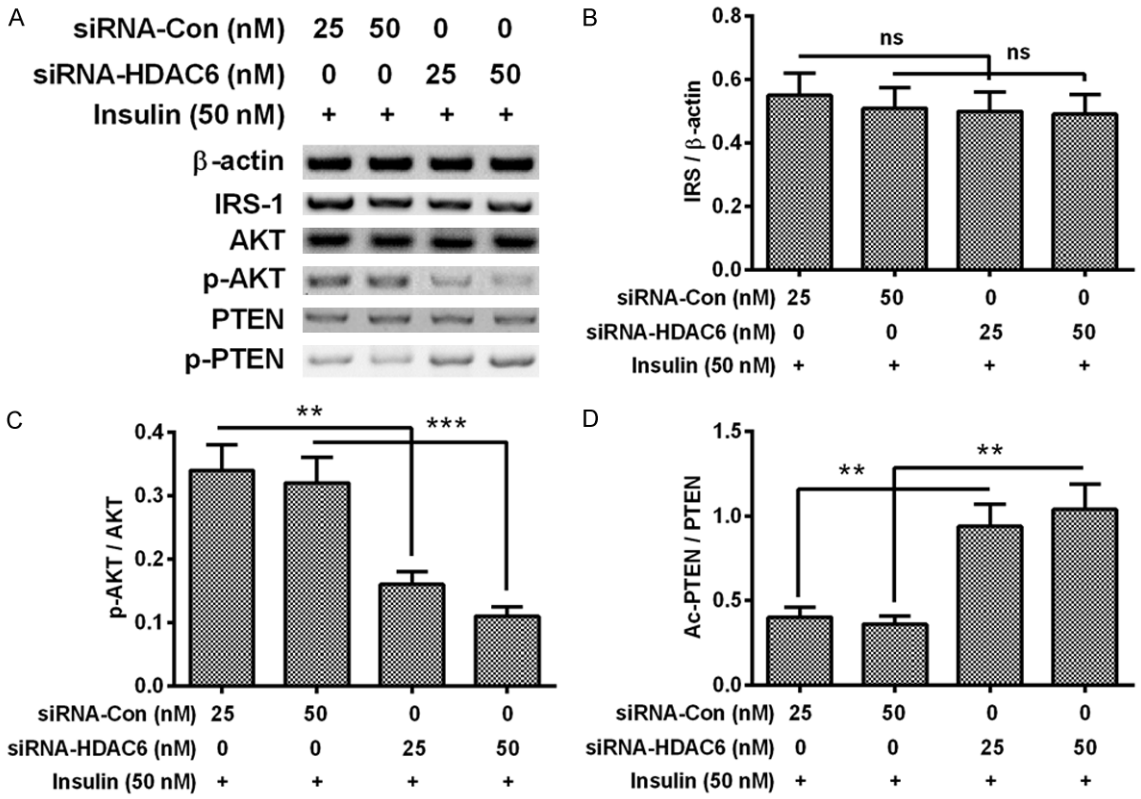

Figure 4. HDAC6 knockdown reverses the promoted AKT phosphorylation and the reduced PTEN acetylation in the insulin-treated OVCAR-3 cells. A: Western blot analysis of insulin signaling markers, such as Insulin receptor substrate 1 (IRS-1) and AKT with or without phosphorylation, HDAC6 and PTEN with or without acetylation in the OVCAR-3 cells, which were treated with 50 nM insulin for 24 hours, post a transfection with 25 or 50 nM siRNA-HDAC6 or siRNA-Con; B: Relative level of IRS-1 to β-actin in the insulin-treated (50 nM) OVCAR-3 cells, post the HDAC6 knockdown; C: Relative level of AKT with phosphorylation to AKT without phosphorylation in the insulin-treated OVCAR-3 cells, post the HDAC6 knockdown; D: Relative level of PTEN with acetylation to PTEN without acetylation in the insulin-treated OVCAR-3 cells, post the HDAC6 knockdown; Each experiment was performed independently in triplicate. **P < 0.01, ***P < 0.001, or ns: no significance.

HDAC6 knockdown inhibits the insulin-induced glucose uptake in OVCAR-3 cells

To investigate the role of the promoted HDAC6 in the insulin resistance in PCOS, we knocked down HDAC6 with HDAC6-specific siRNA in OVCAR-3 cells, and then evaluated the influence of HDAC6 knockdown on the glucose uptake in the insulin-treated OVCAR-3 cells. **Figure 3A** indicated that the transfection with 25 or 50 nM siRNA-HDAC6 significantly reduced the HDAC6 expression in mRNA level (P < 0.01 or P < 0.001). And the western blot analysis reconfirmed the knockdown of HDAC6 in protein level (P < 0.01 or P < 0.0001, **Figure 3B**). **Figure 3C** indicated that the insulin treatment with more than 10 nM markedly upregulated the glucose uptake in OVCAR-3 cells (P < 0.05, P < 0.01 or P < 0.001), with a dose-dependence (P < 0.05, for column 2 vs column 3 in **Figure 3C**). However, **Figure 3D** demonstrated that the promoted glucose by insulin treatment was significantly inhibited by the HDAC6 knockdown with siRNA-HDAC6 transfection. Therefore, HDAC6 knockdown inhibits the insulin-induced glucose uptake in OVCAR-3 cells.

HDAC6 knockdown reverses the insulin-promoted AKT phosphorylation and the -reduced PTEN acetylation

Then we determined the influence of the HDAC6 knockdown on the insulin signaling and PTEN acetylation. Western blotting (**Figure 4A**) demonstrated that the transfection with 25 or 50 nM siRNA-HDAC6 did not markedly influence the IRS-1 level in the OVCAR-3 cells, which were treated with 50 nM insulin (**Figure 4B**). However,

the promoted AKT phosphorylation was significantly downregulated by the transfection with 25 or 50 nM siRNA-HDAC6, compared to the transfection with 25 or 50 nM siRNA-Con ($P < 0.01$ or $P < 0.001$, **Figure 4C**), though the level of AKT without phosphorylation was not markedly influenced by the siRNA-HDAC6 transfection. And the reduced PTEN acetylation was also markedly reversed by such knockdown of HDAC6, compared to the PTEN without acetylation ($P < 0.01$ respectively, **Figure 4D**). Therefore, the HDAC6 knockdown reverses the promoted AKT phosphorylation and the reduced PTEN acetylation.

Discussion

Insulin resistance induces hyperglycemia and decreases the activation of AKT signaling *in vitro* [23, 24] or *in vivo* [25, 26]. The downstream signaling of insulin signaling is mediated by the serine/threonine kinases Akt/PKB [27, 28] and PKC [29, 30], both of which are activated by PI3-kinase via phosphoinositide-dependent protein kinase-1 (PDK1). And both Akt and PKC are negatively regulated by the phosphatase and tensin homolog (PTEN) [31, 32]. And the insulin stimulation of Akt and PKC is impaired along with increased expression of PTEN in the alcohol-stimulated skeletal muscle [33, 34]. And peroxisome proliferator-activated receptor-coactivator-1 (PGC-1), which regulates gluconeogenic enzymes, promotes insulin resistance in liver via induction of TRB3 [35]. PTEN has been reported to enhance gluconeogenesis [36], whereas acetylated PTEN has been shown to reduce its enzymatic activity [37]. It suggests that the deacetylation of PTEN might increase gluconeogenesis. Moreover, the hepatic insulin resistance induced by prenatal alcohol exposure is associated with reduced PTEN acetylation in adult rat offspring [38]. Therefore, the acetylation and deacetylation of PTEN regulate insulin resistance.

In the present study, we investigated the potential role of HDAC6 and PTEN in the pathogenesis of PCOS. Our results demonstrated that HDAC6 was highly expressed in both mRNA and protein levels, along with a reduced acetylated PTEN in 27 human PCOS tissues. And experiments in human ovarian OVCAR-3 cells confirmed that the HDAC6 knockdown with siRNA inhibited the insulin-induced glucose uptake, the blocked the insulin-promoted AKT phosphorylation, whereas reversed the insulin-downregulated PTEN acetylation in OVCAR-3 cells. Interestingly, the HDAC6 knockdown did not regulate the insulin-induced IRS-1, though both IRS-1 [20] and AKT [21, 22] have been confirmed to be two key markers in insulin signaling. Our results firstly demonstrated that the HDAC6 was significantly upregulated, and the acetylated PTEN was markedly reduced in the PCOS tissues.

HDAC6 has been indicated to be associated with many cell functions including tubulin stabilization, cell motility, and regulation of the binding between Hsp90 and its cochaperone [39]. It also regulates the interaction between Ku70 and Bax in neuroblastoma cells [40], or deacetylates survivin to promote its nuclear exit in estrogen receptor-positive breast cancer cells [41]. Moreover, a high level of HDAC6 has also been associated with ovarian cancer [42]. And the oncogenic role of HDAC6 has been indicated to be associated with the deacetylation of PTEN [17]. Recently, a decreased PTEN with acetylation status, in association with increased HDAC activity, was recognized in hepatic insulin resistance in adult rat offspring [43]. Together with the finding in the present study, we speculated that the high expression of HDAC6 and low level of deacetylated PTEN correlated with the insulin resistance in PCOS.

In conclusion, the present study recognized the high expression of HDAC6 correlated with the insulin resistance in PCOS via deacetylating PTEN, implying HDAC6 might be a novel target for PCOS treatment, in the context of insulin resistance.

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Disclosure of conflict of interest

None.

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