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Hypoxia-inducible factor 1-mediated regulation of PPP1R3C promotes glycogen accumulation in human MCF-7 cells under hypoxia

Guo-Min Shen, Feng-Lin Zhang, Xiao-Ling Liu, Jun-Wu Zhang *

National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China
Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China

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ABSTRACT

Hundreds of genes can be regulated by hypoxia-inducible factor 1 (HIF1) under hypoxia. Here we demonstrated a HIF1-mediated induction of protein phosphatase 1, regulatory subunit 3C gene (*PPP1R3C*) in human MCF7 cells under hypoxia. By mutation analysis we confirmed the presence of a functional hypoxia response element that is located 229 bp upstream from the *PPP1R3C* gene. *PPP1R3C* induction correlates with a significant glycogen accumulation in MCF7 cells under hypoxia. Knockdown of either *HIF1α* or *PPP1R3C* attenuated hypoxia-induced glycogen accumulation significantly. Knockdown of *HIF2α* reduced hypoxia-induced glycogen accumulation slightly (but not significantly). Our results demonstrated that HIF1 promotes glycogen accumulation through regulating *PPP1R3C* expression under hypoxia, which revealed a novel metabolic adaptation of cells to hypoxia.

1. Introduction

The response to hypoxia is certainly the most well-understood aspect of hypoxia-inducible factor (HIF)'s ability to regulate metabolism [1]. HIF is a heterodimeric protein that is composed of a constitutively expressed beta subunit (HIFβ, also called Aryl receptor nuclear translocator, ARNT) and an O_2-regulated alpha subunit (HIFα) [2,3]. There are three HIFα proteins in higher metazoans. HIF1α and HIF2α have been the most extensively studied. They have a similar domain architecture, bind to the same core motif and also are regulated in a similar manner. HIF3α is an inhibitor of HIF1 that may be involved in feedback regulation because its expression is transcriptionally regulated by HIF1 [4]. In well-oxygenated cells, HIFα is hydroxylated at one (or both) of two highly conserved prolyl residues by a family of dioxygenases (EGL nine homologs, EGLNs, also called prolyl hydroxylase domain proteins, PHDs), which uses O_2 and α-ketoglutarate as substrates in a reaction that generates CO_2 and succinate as byproducts [5–8]. Prolylhydroxylated HIFα is bound by the von Hippel–Lindau tumor suppressor protein, which recruits an E3-ubiquitin ligase that targets HIFα for proteasomal degradation. In addition, a specific asparaginyl residue in the transactivation domain is hydroxylated in well-oxygenated cells by factor inhibiting HIF (FIH), which blocks the binding of the coactivators p300 and CCAAT-binding protein (CBP) [9–11]. Under hypoxic conditions, hydroxylation is inhibited and HIFα rapidly accumulates, dimerizes with HIFβ, binds to the core DNA binding sequence 5′-RCGTG-3′ (R is purine A or G) in target genes, recruits co-activators and activates transcription.

The hundreds of genes can be induced by HIF1 under hypoxia. The largest functional group of genes consistently regulated by HIF1 in a number of cell types are associated with glucose metabolism [1]. The glucose metabolism in cells under hypoxia is significantly different from that under normoxia. Glucose uptake significantly increases under hypoxia, primarily in a HIF-dependent manner. HIF1 induces the transcription of solute carrier family 2, member 1 (*SLC2A1*) and *SLC2A3*, which encode the glucose transporter 1 (GLUT1) and GLUT3, respectively [12,13]. HIF1 can also activate the transcription of hexokinase gene 1 (*HK1*) and *HK2* [14]. Intracellular glucose is quickly phosphorylated to glucose-6-phosphate by hexokinase. The charged glucose molecule cannot easily pass back through the plasma membrane and is

Abbreviations: HRE, hypoxia response element; HIF, hypoxia-inducible factor; PPP1R3C, protein phosphatase 1, regulatory subunit 3C; DFOM, deferoxamine mesylate salt; PHD2, prolyl hydroxylase domain protein 2; VHL, von Hippel–Lindau tumor suppressor protein; PTG, protein targeting to glycogen; GYS1, glycogen synthase 1

* Corresponding author at: National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China. Fax: +86 10 65240529.
E-mail address: junwu_zhang@pumc.edu.cn (J.-W. Zhang).

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trapped within the cell. Glucose-6-phosphate has several possible metabolic fates, such as, as a structural component in the synthesis of glycoproteins, metabolized in the pentose shunt to generate ribose, used to synthesize glycogen or, predominantly, broken down to pyruvate in glycolysis [1].

Although mechanism of regulating glycolysis under hypoxia has been well defined, the effect of hypoxia on other glucose metabolic fates is yet to be addressed. Now, some reports have described the glycogen accumulation in cells under hypoxia conditions [15–18]. Pescador et al. demonstrated that hypoxia promotes glycogen accumulation through HIF1-mediated induction of glycogen synthase 1 [18]. They also showed that hypoxia upregulates the expression of UTP: glucose-1-phosphate urydylyltransferase (UGP2) and 1,4-α glucan branching enzyme (GBE1), in a cell-type specific manner [18]. Does hypoxia induce other genes that promote glycogen accumulation? Some reports showed that protein phosphatase 1, regulatory subunit 3C (*PPP1R3C*) could be induced by hypoxia [19,20], and Ortiz-Barahona et al. predicted that *PPP1R3C* is a new HIF-targeted gene [21]. *PPP1R3C* was also called protein targeting to glycogen (PTG) and regulated glycogen metabolism [22]. Here we demonstrated that hypoxia leads to glycogen accumulation in human MCF7 cells through HIF1-PPP1R3C pathway, a new HIF-dependent pathway of glycogen accumulation in cells under hypoxia conditions.

2. Materials and methods

2.1. Cell culture and hypoxic exposures

MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS and 100 units/ml penicillin, 100 μg/ml streptomycin. Normoxic cells were maintained at 37 °C in a 5% CO_2 and 95% air incubator. The cells were exposed to hypoxia in three gas incubator (YCP-50S) in 5% CO_2, 94% N_2 and 1% O_2 at 37 °C.

2.2. Plasmid constructs

The nucleotide sequences encoding HIF1α-CA5 [23], HIF1α-DN [23] and HIF2α-DPA [24] were amplified by PCR and cloned into FLAG-taged pcDNA6-V5-HisB vector (Invitrogen), respectively. Promoter region of *PPP1R3C* were amplified from human genomic DNA by PCR and cloned into the luciferase reporter vector pGL3-promoter (Promega). As a positive control, the nucleotide sequence containing the identified hypoxia response element (HRE) of EPO gene [25] was also cloned into pGL3-promote.

2.3. Western blotting

Cell lysate was subjected to SDS–PAGE and transferred to a PVDF membrane. Primary antibodies against the following proteins were used: HIF1α (Santa Cruz), HIF2α (Novus Biologicals), β-actin (Proteintech), and PPP1R3C (Santa Cruz). HRP-conjugated secondary antibodies were used. Signal was detected using ECL kit (Millipore).

2.4. Transient transfection and luciferase assay

MCF7 cells were plated into 24-well plates to reach approximately 40–50% confluence on the next day. The cells were cotransfected with pGL3-promoter-based construct and pRL-TK plasmid DNAs using Lipofectamine Plus (Invitrogen). The transfection medium was replaced with complete medium after 6 h. The cells were incubated in normoxia and hypoxia for an additional 24 h. Then cells were lysed with PLB and reporter gene expression was assessed using the Dual-Luciferase Reporter Assay System (Promega).

2.5. RNA isolation and PCR analysis

Cells were dissolved in TRIZOL reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. Total RNA was converted into cDNA using the M-MLV reverse-transcriptional system (Invitrogen) in the presence of oligo$(dT)_{18}$. The cDNA was used for conventional PCR or quantitative real-time PCR (qrt-PCR) with specific gene primers: PPP1R3C forward: 5′-GCGTTGTGTTTGCTGACTCC, reverse: 5′-CGGTTGAAGGCTGAGGGAAAT-3′; β-actin forward: 5′-CTGGCACCACACCTTCTACA-3′, reverse: 5′-AGCACAGCCTGGATAGCAAC-3′; HIF1α forward: 5′-AGGTGGATAT GTCTGGGTTG-3′, reverse: 5′-AAGGACACATTCTGTTT GTTG-3′; HIF2α forward: 5′-GTCTCTCCACCCCATGTCTC-3′, reverse: 5′-GGTTCTTCAT CCGTTTCCAC-3′. SYBR green PCR mix (Transgen) was used for the real-time PCR. The relative abundance of PPP1R3C transcript was quantified using the comparative Ct method with β-actin as an internal control.

2.6. Chromatin immunoprecipitation (ChIP)

MCF7 cells were plated into 15 cm plate and grown to about 70% confluence. Then the cells were exposed to hypoxia for 24 h and ChIP was performed as previous described [18]. The precipitated DNA was amplified by PCR using the following primers: P1: 5′-ACCCGCAGTGACCCACAAAG-3′, P2: 5′-CGCGGGGAGGTTCTGGGACT-3′, P3: 5′-TTGAATCTGGCAACCGAGAGG-3′, P4: 5′-CGAGTCTT TGGGACATTTGTGC-3′. For a positive control, the PDK1 promoter region that contains a known HIF1-binding site was amplified using the forward primer, 5′-CGCCCTGTCCTTGAGCC-3′ and the reverse primer, 5′-CGGTATGGAGCGTCCCCT-3′ [26].

2.7. Cell transfection with small interference RNA (siRNA)

One day before transfection, MCF7 cells were plated into 6-well plates. The cells were grown to 50% confluence and then transfected with 25 nM (final concentration) of siGENOME non-targeting siRNA2, human HIF1A siGENOME SMART pool, or human HIF2A siGENOME SMART pool, or human PPP1R3C siGENOME SMART pool (Dharmacon) using Dharma FECT1 transfection reagent according to the manufacturer's instructions (Dharmacon). After 24 h incubation at normoxia, the transfection medium was replaced with complete medium and then the cells were incubated at hypoxia for another 24 h. Total RNA and cell lysates were collected for real-time PCR and Western blot, respectively.

2.8. PAS staining and glycogen determination

Coverslips were placed into 6-well plate, then MCF7 cells were plated. After incubation and treatment, medium was removed and the cells were fixed with Carnoy's fixative for 1 h. The cells were rinsed with absolute alcohol and 66% alcohol once for 2 min, followed by rinsing with deionized water for 90 s (3 × 30 s). The cells were treated with periodic acid solution for 10 min and then rinsed with deionized water for 90 s (3 × 30 s). Then the cells were treated with Shiff reagent for 15–20 min followed by 5 min with running tap water. For visualising nuclei, the cells were counterstained using Mayer's hematoxylin for 1 min followed by running tap water for 5 min. After air-drying, the stained cells were covered with a glass slides using pure glycerol as mounting medium. For glycogen determination, glycogen was extracted as previous described [27] and the amount of glycogen was assessed by glycogen assay kit (Biovision).

2.9. Statistics

Student's *t*-test (two-tailed) was performed to analyze Data. *P*-values <0.05 were considered significant.

3. Results

3.1. Hypoxia induces PPP1R3C expression in an HIF-dependent manner

To examine whether *PPP1R3C* gene expression is O_2-regulated in human cells, MCF7 cells were incubated at normoxia (21% O_2), hypoxia (1% O_2) or medium containing 100 μM deferoxamine mesylate salt (DFOM) for 24 h. Both conventional RT-PCR (Fig. 1A) and quantitative real-time PCR (qrtPCR; Fig. 1B) assay revealed an increased PPP1R3C mRNA level in the cells under hypoxic or DFOM treatment conditions. PPP1R3C protein level in the MCF7 cells was also be determined by immunoblot assay (Fig. 1C). An increased PPP1R3C protein level in company with increased HIF1α and HIF2α levels was observed in the cells under hypoxic or DFOM treatment conditions. These results indicated that *PPP1R3C* was a hypoxia-inducible gene. In agreement, a significantly decreased PPP1R3C mRNA (Fig. 1D) and protein levels were (Fig. 1E) detected in the MCF7 cells transfected with siRNAs targeting *HIF1α* or *HIF2α*, which suggested that the inhibition of the HIF genes (especially *HIF1α*) reduced hypoxia induction of *PPP1R3C*. An increased PPP1R3C mRNA level was also detected in HepG2 cells under hypoxic or DFOM treatment conditions (Supplementary Fig. 1). Taken together these results demonstrated that PPP1R3C gene could be induced in response of cells to hypoxia and this response is regulated by HIF.

3.2. Identification and validation of hypoxia-response element in the human PPP1R3C gene

We searched the promoter region of human PPP1R3C gene for matches to a consensus HRE sequence as previous described [28]. Four putative HREs (Fig. 2A) were identified. To determine which one is a functional HRE, we amplified the promoter region of PPP1R3C gene and inserted into luciferase reporter plasmid pGL3-promoter. As shown in Fig. 2C, the region of −317 to −44 could dramatically increase while the region of −212 to −44 could not increase luciferase activity in MCF7 cells under hypoxia condition. In addition, the region of −49 to +249 was also failed to increase luciferase activity in MCF7 cells under hypoxia condition (Supplementary Fig. 2). Mutation of the putative HRE1 motif completely impaired the induction of luciferase activity by hypoxia (Fig. 2C), but mutation of the HRE2 or(and) HRE3 did not impaire the luciferase activity in MCF7 cells under hypoxia condition (Supplementary Fig. 2), which suggested that HRE1 is a functional HRE.

It has been reported that HIF1α-CA5 and HIF2α-DPA, which encode constitutively active form of HIF1α and HIF2α, respectively, can activate target gene expression under non-hypoxic conditions [23,24]. As shown in Fig. 2D, cotransfection of the expression vector encoding HIF1α-CA5 or HIF2α-DPA increased the luciferase activity of the construct containing HRE1 at normoxia. Mutation of HRE1 resulted in a complete loss of HIF1α-CA5 or HIF2α-DPA-mediated

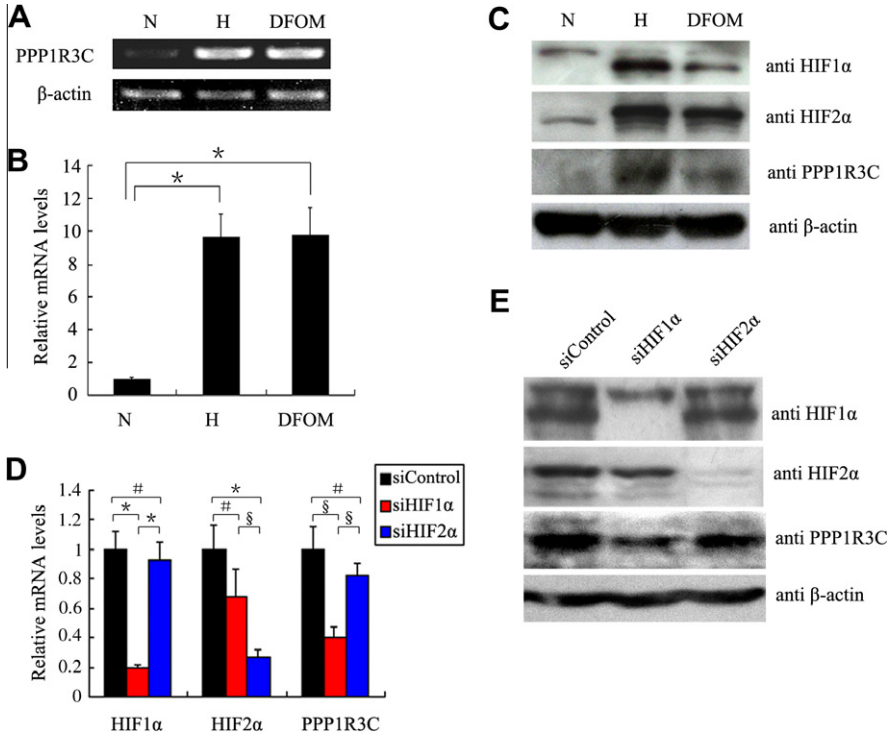

Fig. 1. *PPP1R3C* is induced by hypoxia in an HIF-dependent manner. (A) PPP1R3C mRNA level was analyzed by RT-PCR in MCF7 cells that were cultured under normoxia (N), hypoxia (H) or medium containing 100 μM deferoxamine mesylate salt (DFOM) for 24 h. (B) PPP1R3C mRNA level was analyzed by quantitative real-time PCR (qrt PCR) in the MCF7. The PPP1R3C mRNA level was normalized to β-actin mRNA level. The relative PPP1R3C mRNA expression was shown as the fold values of hypoxic over normoxic mRNA levels. (C) HIF1α, HIF2α, PPP1R3C and β-actin protein levels in the MCF7 cells were determined by immunoblot assays. Anti-β-actin antibody was used as a control for an indication of equal protein loading. (D) HIF1α, HIF2α and PPP1R3C mRNA levels were determined by Qrt-PCR in the MCF7 cells transfected with different siRNAs. The cells were transfected with siRNA of control, HIF1α or HIF2α. After 24 h, the cells were incubated at hypoxia for an additional 24 h. The expression of each mRNA in each transfection experiment was normalized to β-actin mRNA expression. The relative expression of each mRNA was shown as the fold values of mRNA levels in cells transfected with siHIF1α or siHIF2α over that in the cells transfected with sicontrol. (E) HIF1α, HIF2α and PPP1R3C protein levels were determined by immunoblot assays in the MCF7 cells described in (D). Data shown (B and D) are their mean of three independent experiments. Error bars represent one standard deviation. The experiments (A, C, and E) were repeated twice with similar results. #$P > 0.05$, §$P < 0.05$, *$P < 0.01$.

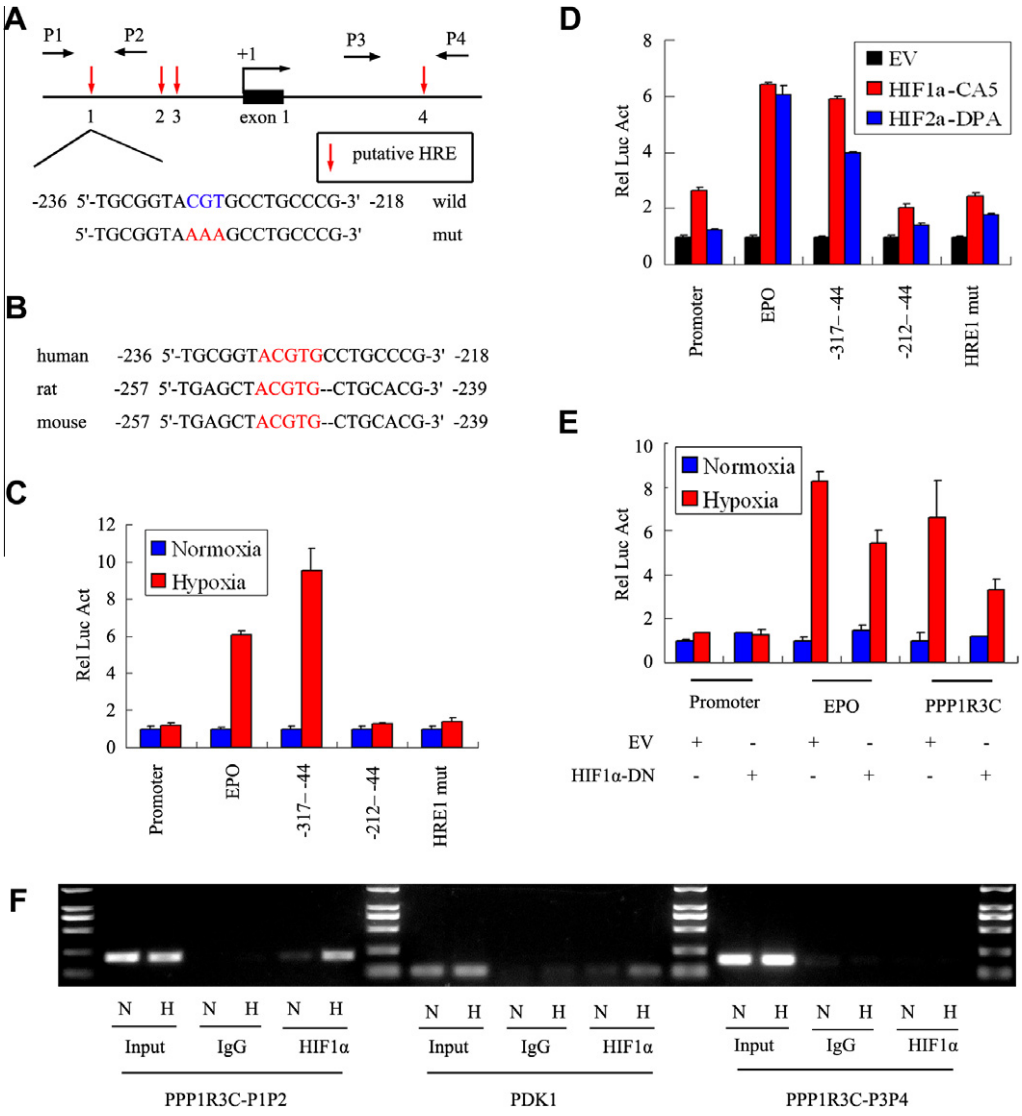

Fig. 2. Identification and validation of HREs in the *PPP1R3C* gene. (A) Skech map of *PPP1R3C* promoter region. The putative HREs are pointed out by the arrows. The nucleotides of HRE1 and flanking sequences were shown and HRE1 was mutated as indicated (red). The nucleotide sequences are numbered in relation to the transcription initiation site, which is designated "+1". P1, P2, P3 and P4 indicated primers that are used for PCR amplification of the immunoprecipitated chromatin fragments in Fig. 2F. (B) Comparison of the hypoxia response element (red) and flanking nucleotides identified in the human, rat and mouse PPP1R3C gene promoter regions. (C) Luciferase reporter assays were performed in MCF7 cells transfected with the constructs containing the indicated sequences from the human *PPP1R3C* gene promoter region. Each transfection experiment was performed triplicate. The relative mean luciferase activity in the cells under hypoxia is shown as fold over the mean activity in the cells under normoxia. Error bars represent one standard deviation. "HRE1 mut" contains the promoter region of PPP1R3C from −317 to −44 with the HRE1 mutation as indicated in Fig. 2A. (D) Reporter plasmids were co-transfected into MCF7 cells along with pcDNA6V5HisB empty vector plasmid (EV), pcDNA6V5 HisB-HIF1α-CA5 or HIF2α-DPA expression plasmid. Mean luciferase activity of three independent experiments is shown relative to activity in the cells transfected with empty vector and incubated at normoxia. Error bars represent one standard deviation. E. Reporter plasmids were co-transfected into MCF7 cells along with a pcDNA6V5HisB empty plasmid (EV) or HIF1α-DN expression plasmid that encodes a dominant-negative form of HIF1α, and were incubated at normoxia or hypoxia for 24 h. Mean luciferase activity of three independent experiments is shown as fold relative to the activity in the cells transfected with empty vector pcDNA6V5HisB and incubated at normoxia. Error bars represent one standard deviation. (F) ChIP-PCR assays were performed using the indicated primers and antibodies to demonstrate binding of HIF1α to HRE1 of *PPP1R3C* in the MCF7 cells under normoxia and hypoxia. H and N represent hypoxia and normaxia, respectively.

PPP1R3C promoter activity. These results suggested that HIF could contribute to increased PPP1R3C promoter activity in hypoxic cells. To further test this, MCF7 cells were cotransfected with pcDNA6V5-HisB-HIF1α-DN, which encodes a dominant-negative form of HIF1α that competes with endogenous HIF1α and HIF2α for dimerization with HIF1β and thus abolishes trans-activation effect of the heterodimers [23]. As expected HIF1α-DN inhibited hypoxia-induced PPP1R3C promoter activity in MCF7 cells (Fig. 2E) and HeLa cells (Supplementary Fig. 3).

To demonstrate that HIF1α binds to the HRE1 within living cells, we performed chromatin immunoprecipitation (ChIP) assays using hypoxia-treated MCF7 cells and HIF1α antibodies (using IgG as a negative control). The amplicon containing the HRE1 of the PPP1R3C gene was detected from chromatin fragments immunoprecipitated with HIF1α (Fig. 2F). For a positive control, we demonstrated binding of HIF1 to a known HIF1 target gene PDK1 [26]. For a negative control, amplicon with HRE4 of the PPP1R3C gene was detected.

Taken together, these results showed that PPP1R3C is induced by HIF1′ combining to the HRE1 in MCF7 cells under hypoxia.

3.3. Hypoxia induces glycogen accumulation in MCF7 cells

PPP1R3C is also known as PTG. By overexpression studies and transgenic animal models PPP1R3C has been shown to potently regulate glycogen levels [29–37]. In order to test the functional

relevance of increased glycogen levels, MCF7 cells were exposed to hypoxia or normoxia and their glycogen levels were determined at different time points. Both PAS staining (Fig. 3A) and glycogen quantity determination (Fig. 3B) explored a significant increase of cellular glycogen concentration under hypoxia conditions. These results confirmed that the hypoxic induction of PPP1R3C correlates with the increase in glycogen reserves in MCF7 cells.

3.4. Hypoxia-induced glycogen accumulation is HIF-dependent in MCF7 cells

To test the role of HIF in hypoxia-induced glycogen accumulation, MCF7 cells were transfected with small interference RNAs (siRNAs). The cell transfection with siRNA that specifically target *HIF1α* (siHIF1α) significantly attenuated the hypoxia-induced glycogen accumulation (Fig. 4A and B). The cell *transfection with siRNA that specifically target *HIF2α* (siHIF2α) attenuated the hypoxia-induced glycogen accumulation slightly (Fig. 4A and B). These results demonstrated that HIF1α is necessary for the hypoxia-induced glycogen accumulation.

3.5. PPP1R3C mediates the hypoxic accumulation of glycogen

To further test the role of PPP1R3C in hypoxia-induced glycogen accumulation, we transfected MCF7 cells with siRNAs. In comparison with the cells transfected with non-targeting siRNA (sicontrol), a significant reduction of glycogen accumulation (Fig. 5A and B), accompanying a reduction of both PPP1R3C mRNA and protein levels (Fig. 5C and D), was observed in the cells transfected with the siRNA that specifically targets to *PPP1R3C* (siPPP1R3C). These results suggested a significant effect of PPP1R3C on the hypoxia-induced glycogen accumulation.

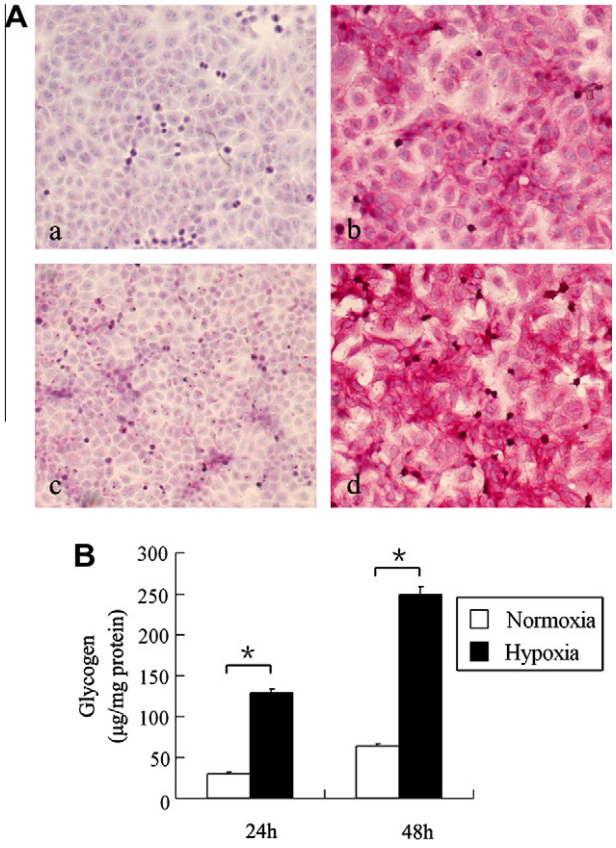

Fig. 3. Hypoxia induces glycogen accumulation in MCF7 cells. (A) PAS staining of the cells that were exposed to normoxia or hypoxia for different times. (a) at normoxia for 24 h; (b) at hypoxia for 24 h; (c) at normoxia for 48 h; (d) at hypoxia for 48 h. The experiment was repeated twice with similar results. (B) Glycogen content determination in the cells that were exposed to normoxia or hypoxia for the point times. Data shown are their mean of three independent experiments and error bars represent one standard deviation. *$P < 0.01$.

4. Discussion

In this study we demonstrated that *PPP1R3C* was regulated by HIF under hypoxia and hypoxia promotes glycogen accumulation through HIF-mediated induction of *PPP1R3C* in MCF7 cells. HIF1α should play a main role in this process. Although dual-luciferase reporter assays showed that PPP1R3C promoter is induced by HIF2α (Fig. 2D), the siRNA that especially targets HIF2α mRNA (siHIF2α) did not reduced PPP1R3C mRNA level (Fig. 1D) significantly. In addition, the siRNA that especially targets HIF1α mRNA (siHIF1α) rather than siHIF2α can reduce glycogen accumulation significantly (Fig. 4B). So the PPP1R3C's induction by hypoxia is mainly attributed to HIF1α, and *PPP1R3C* may be a selective target gene of HIF1α. Pescador's results indicated that hypoxia induced a coordinated response that affects glycogen metabolism at multiple levels. Our results supported these.

Ortiz-Barahona and colleagues used a computational strategy based on the combination of phylogenetic footprinting and transcription profiling meta-analysis for a genome-wide identification of HIF binding sites and target genes, and identified a potential HRE that is from −14 to −10 in the PPP1R3C promoter [21]. In this study we confirmed a functional HRE that is from −230 to −226 in the PPP1R3C promoter using report gene assay and ChIP-PCR analysis. The discrepancy on the HRE identity may be caused by the difference in defining the conditions of HIF-binding sites (HBSs).

It was reported that PTG regulates glycogen metabolism through directly binding to protein phosphatase 1 (PP1). Theoretically, PTG-PP1 complex could act via regulation of glycogen synthase, glycogen phosphorylase, or both enzymes [37]. Cynthia et al. reported PTG-PP1 complex resulted in cellular glycogen accumulation through regulating glycogen phosphorylase but not glycogen synthase [37]. Other reports also supported this [30,32,38]. Pescador et al. demonstrated that under hypoxia, the increased GS activity is a consequence of the incremented amount of enzyme, rather than of a shift in its activation state. They also found reduction of glycogen phosphorylase (GP) activity in cells exposed to hypoxia [18]. Reduction of GP activity was also observed during prolonged hypoxia [16]. So we hypothesize that under hypoxia PPP1R3C interacts with PP1 dephosphorylates GP and reduces GP activity, thus repressing glycogen breakdown.

Glycogen accumulation maybe involves in longterm adaptation to hypoxia. Exposure of cells to hypoxia, prior to the anoxic treatment in the absence of glucose, resulted in increased survival [18]. O_2 concentrations are significantly reduced in many human tumors compared with the surrounding normal tissue. Reduced O_2 mediates adaptive responses of tumor cell to changes in tissue oxygenation [39]. Glycogen accumulation in some cancers had been reported [40–49]. Glycogen accumulation in cancer maybe involves in adaptation to tumor microenvironment Exposure of cells to hypoxia, prior to the anoxic treatment in the absence of glucose, resulted in increased survival [18]. Further work is required to test whether glycogen accumulation exists in tumors widely and PPP1R3C plays a role in the process.

In summary, our results demonstrated that HIF1 promotes glycogen accumulation through regulating *PPP1R3C* expression under hypoxia, which revealed a novel metabolic adaptation of cells to hypoxia.

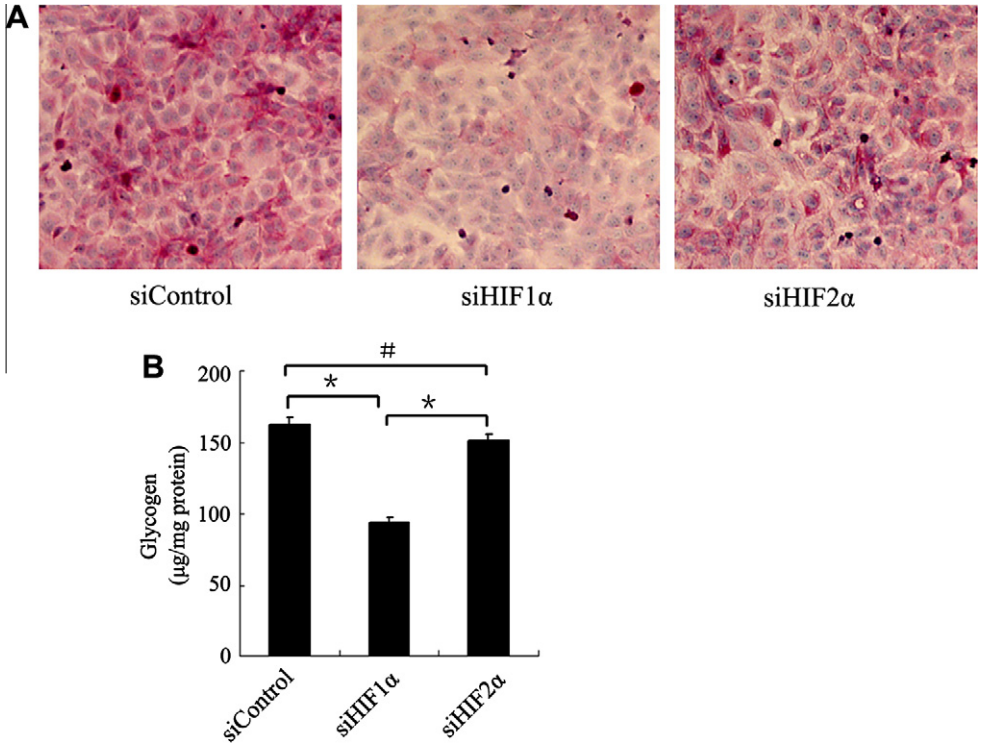

Fig. 4. Glycogen accumulation is attenuated by knockdown of HIF1α or HIF2α. MCF7 cells were transfected with siRNA of control, HIF1α or HIF2α for 24 h, then incubated at hypoxia for an additional 24 h. (A) PAS staining of cells. The experiment was repeated twice with similar results. (B) Glycogen content determination of cells. Data shown are their mean of three independent experiments and error bars represent one standard deviation. #$P > 0.05$, *$P < 0.01$.

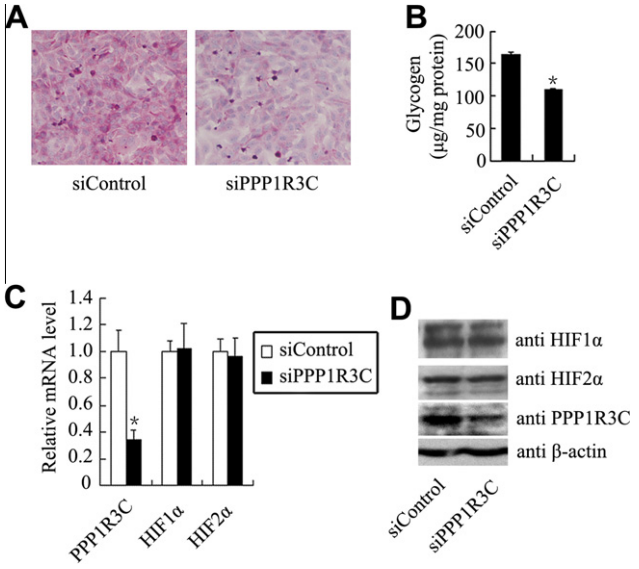

Fig. 5. Glycogen accumulation is attenuated by knockdown of *PPP1R3C*. MCF7 cells were transfected with siRNA of control and PPP1R3C for 24 h, then incubated at hypoxia for an additional 24 h. (A) PAS staining of cells. The experiment was repeated twice with similar results. (B) Glycogen content determination of cells. Data shown are their mean of three independent experiments and error bars represent one standard deviation. (C) PPP1R3C, HIF1α and HIF2α mRNA levels were determined by qrtPCR. The PPP1R3C, HIF1α and HIF2α mRNA levels were normalized to β-actin mRNA levels. The relative expression of each mRNA was shown as the fold values of mRNA levels in cells transfected with siPPP1R3C over that in the cells transfected with sicontrol. Data shown are their mean of three independent experiments and error bars represent one standard deviation. *$P < 0.01$. (D) PPP1R3C, HIF1α, HIF2α and β-actin protein levels were determined by immunoblot assays. Anti-β-actin antibody was used as a control for an indication of equal protein loading. The experiment was repeated twice with similar results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.040.

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