Investigation of the HNF4α Binding Sites in the Promoter of Human Pyruvate Carboxylase Gene

การพิสูจน์หาบริเวณที่จับกับ HNF4α ในโปรโมเตอร์ของยีนเอ็นไซม์ไพรูเวทคาร์บอกซิเลสของมนุษย์

Pakawan Chuayboonya (ภควันต์ ช่วยบุญญะ)* Siriluck Wattanavanitchakorn (ศิริลักษณ์ วัฒนาวาณิชกร)**
Dr.Pinnara Rojvirat (ดร.พินน์นรา โรจน์วิรัตน์)*** Dr.Sarawut Jitrapakdee (ดร.ศราวุฒิ จิตรภักดี)****

ABSTRACT

Pyruvate Carboxylase (PC) is an anaplerotic enzyme which is essential in several biochemical pathways such as gluconeogenesis, lipogenesis and glucose-induced insulin secretion. The present study aims at investigating the regulatory role of HNF4α on the proximal (P1) promoter of human PC gene. To unravel this transcriptional mechanism, reporter gene assay, DNA-protein binding assays, overexpression and knock-down studies were performed. Our results demonstrate that the nucleotides that resemble the direct repeat (DR1), located between -351 to -339 in the P1 promoter of human PC gene is essential for transcriptional activation by the liver-enriched transcription factor, HNF4α. Binding of HNF4α to this binding site was confirmed by an electrophoretic mobility shift assay using recombinant human HNF4α. Functional studies also showed that suppression of HNF4α resulted in marked reduction of endogenous PC expression while overexpression of HNF4α resulted in overexpression of human PC in HepG2 cells.

บทคัดย่อ

ไพรูเวทคาร์บอกซิเลสเป็นเอ็นไซม์สำคัญที่เกี่ยวข้องกับหลายกระบวนการสังเคราะห์สารชีวภาพ โดยเฉพาะอย่างยิ่งกลูโคนีโอเจเนซิสผ่านการคาร์บอกซิเลชันไพรูเวทไปเป็นออกซาโลอะซิเตต ในการศึกษานี้เป็นการพิสูจน์หาบริเวณที่จับกับโปรตีน HNF4α ที่ควบคุมการแสดงออกของยีนเอ็นไซม์ไพรูเวทคาร์บอกซิเลสในเซลล์ตับของมนุษย์ที่บริเวณโปรโมเตอร์ด้านใกล้ ผลการทดลองโดยการใช้วิธีสร้าง gene reporter, DNA-protein binding assays overexpression และ knock-down studies ชี้ให้เห็นว่า ลำดับนิวคลีโอไทด์ที่เป็น DR1 ซึ่งอยู่ระหว่างตำแหน่งที่ -351 ถึง -339 มีความสำคัญต่อการถูกกระตุ้นด้วย HNF4α การวิเคราะห์การจับกันของ DR1 กับ purified HNF4α ด้วยวิธี electrophoretic mobility shift assay ยืนยันปฏิสัมพันธ์ระหว่าง HNF4α กับ DR1 นอกจากนี้ยังพบว่าการยับยั้งการแสดงออกของ HNF4α โดยการใช้ siRNA และการเพิ่มการแสดงออกของ HNF4α ส่งผลให้ระดับการแสดงออกของยีนลดลงและเพิ่มขึ้นตามลำดับแสดงให้เห็นว่า HNF4α มีบทบาทสำคัญในการควบคุมการแสดงออกของยีนไพรูเวทคาร์บอกซิเลส

Keywords: Pyruvate Carboxylase, gluconeogenesis, HNF4α transcription factor
คำสำคัญ: ไพรูเวทคาร์บอกซิเลส กลูโคนีโอเจเนซิส เอชเอ็นเอฟโฟร์แอลฟา

* *Student, Master of Science Program in Biochemistry, Faculty of Science, Mahidol University*
** *Student, Doctor of Philosophy Program in Biochemistry, Faculty of Science, Mahidol University*
*** *Professor, Department of Biochemistry, Faculty of Science, Mahidol University*
**** *Lecturer, Division of Interdisciplinary, Mahidol University (Kanchanaburi Campus)*

Introduction

Type 2 diabetes mellitus (T2DM) affects 422 million worldwide and continue to rise in the next decade. A key pathology of T2DM includes insulin resistance in peripheral tissues and impaired insulin secretion, resulting in an overt hyperglycemia. The elevated hyperglycemia is mainly caused by failure of glucose uptake of the peripheral tissues and over-stimulation of hepatic gluconeogenesis (Roden., 2016). Although overexpression of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) is the underlying mechanism of overproduction of hepatic glucose in rodent models (Andrikopoulos & Proietto, 1995; Cool *et al*., 2006; Mendez *et al*., 2013), recent study in humans clearly show this mainly caused by over-expression of pyruvate carboxylase (PC) but not PEPCK or G6Pase (Kumashiro *et al*., 2013). Silencing expression of PC in liver lowered plasma glucose levels, adiposity and improved hepatic insulin sensitivity in high fat diet-induced diabetic rat, underscoring the importance of PC during the development of type 2 diabetes (Weinberg *et al*., 1980; Fang *et al*., 2012; Kumashiro *et al*., 2013). Previous study has shown that human PC gene is regulated by the proximal (P1) and distal (P2) promoters. P1 promoter is active in gluconeogenic tissue while P2 is active in the non-gluconeogenic tissues. Furthermore, overstimulation of P1 promoter activity is linked to overexpression of PC in T2DM patients (Kumashiro *et al*., 2013;). Therefore, understanding transcriptional activation of PC driven by P1 promoter is crucial as this will lead to the identification of key transcription factors that are responsible for overexpression of PC during diabetes and may lead to the development of small molecules that act as transcriptional disruptor that of above key transcription factors. In mouse, hepatocyte nuclear factor 4 (HNF4α) has been shown as the liver-specific transcription factor that directs transcription of PC gene in hepatocytes (Chavalit *et al*., 2013) however, it is unknown whether this is the case in human because the nucleotide sequence of P1 promoter for human PC gene is remarkably different. This raises the question whether the regulatory role of HNF4α is common in humans. The hepatocyte nuclear factor 4 (HNF4α; NR2A1) is one of nuclear receptors (NRs) family, it regulates a variety of metabolic genes mainly in liver. Aberrant expression of HNF4α is associated with several diseases including maturity onset of diabetes of the young 1 (MODY1) (Stoffel and Duncan, 1997). HNF4α is a well-known transcriptional activator that binds to the classical direct repeat-1 (DR1, AGGTCANAGGTCA) nucleotide as a homodimer (Umesono *et al*., 1991) and known as potential drug target for type 2 diabetes (Chen *et al*., 2008; Wang *et al*., 2010; Babeu *et al*., 2014). Therefore, understanding of the regulation of human PC gene expression by HNF4α may provide some useful insights for drug development.

Objectives of the study

To identify HNF4α binding site (s) and investigate the effect of HNF4α transcription factor on P1 promoter of human PC gene in hepatocytes.

Methodology

Generation of hP1 promoter and 5' truncated hP1 promoter-luciferase reporter

According to mouse P1 promoter, the important regions including basal and tissue specific regions located within 0.4 kb upstream of the transcription start site (Rojvirat *et al.*, 2011 and Chavalit *et al.*, 2013). 0.7 kb 5'-upstream sequence of human PC covers all possible enhancer sequences was PCR-amplified. Full-length P1 promoter (-700 to -1) was isolated from human genomic DNA by PCR using a pair of primer designed from the promoter sequence of human PC gene (Table 1). PCR was performed in a 50 µl-total volume containing genomic DNA, 1x PCR buffer [20 mM Tris-HCl pH 8.4, 50 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer and 1 unit of *Taq* DNA polymerase (Invitrogen). The amplification profiles consisted of an initial denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 45 sec and extension at 72ºC for 2 min, followed by a final extension at 72ºC for 10 min in a mini-thermal cycler (BioRad). The PCR product was ligated to pGEM-T easy vector followed by nucleotide sequencing. To precisely identify the importance putative HNF4α binding site, six 5'-truncated P1 promoter fragments, containing 600, 500, 400, 300, 200 and 100 nucleotides upstream of transcription start site of PC gene were generated by using 5'-nested deletion primers (Table 1). The PCR was performed using this thermal profile; initial denaturation at 95°C for 30 s, followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 3 min, extension at 68°C for 5 min prior to a final extension at 68°C for 10 min. The PCR product was digested with *Hind*III and *Xho*I before ligated at the equivalent sites in the pGL4.12-basic reporter plasmid (Promega). All constructs were then transformed into *E. coli* DH5α (Stratagene). The nucleotide sequences of the 5' truncated constructs were verified by nucleotide sequencing.

Table 1 Oligonucleotides used for generating hP1 (Full length) and 5'-deletion mutagenesis

Construct name	Primer name	Oligonucleotides	Sequence (5'⟶3')
pGL4-700hP1	700hP1F	-700/-1	CTCGAGTTACTGGGGTGCCCTTTAGGAT
pGL4-600hP1	600hP1F	-600/-1	CTCGAGTACTGGGTCACCAGCTCACACT
pGL4-500hP1	500hP1F	-500/-1	CTCGAGTTCCCAGGTGGACAACCCAAGG
pGL4-400hP1	400hP1F	-400/-1	CTCGAGTTCCTTAGGCCAGATGCCCTGT
pGL4-300hP1	300hP1F	-300/-1	CTCGAGCTTTCCACCAGAACTTGTGAGC
pGL4-200hP1	200hP1F	-200/-1	CTCGAGGGCCCACCCCTAAGCCAGAGAT
pGL4-100hP1	100hP1F	-100/-1	CTCGAGCTAGAACATCAATTATAAACAG
-	hP1R	-	AAGCTTCACGGCAGTTGGTCTGCAGAGT

Underline indicates *Hind*III or *Xho*I restriction sites.

Cell culture and transactivation assay

Hepatocellular carcinoma cell line, HepG2 (ATCC: HB-8065) was grown as monolayer in DMEM-high glucose medium (Gibco) supplied with 10% fetal bovine serum (Gibco) and antibiotic-antimycotic (100 units/ml penicillin and 100 µg/ml streptomycin) (Gibco) in an incubator at 37°C with 5% CO_2. For transactivation assays,

$1x10^5$ cells were seeded in 24-well that containing DMEM medium supplemented with 10% FBS without antibiotics before transfection. Two cocktails were prepared as follows the first cocktail contained 2 µl of LipofectAMINE™ 2000 reagent (Invitrogen) diluted in 50 µl of Opti-MEM® I, reduced serum medium (Invitrogen) while the second cocktail contained 0.07 pmol promoter-luciferase reporter construct, 0.07 pmol pcDNA-HNF4α (the plasmid overexpressing of HNF4α) and 250 ng pRSV-β -gal plasmid diluted in 50 µl of OptiMEM®I. Both cocktails were combined and incubated at room temperature for 5 min. The transactivated cells were incubated at 37°C with 5% CO_2 for 48 hours. The transactivation of each construct was done duplicate in each experiment. The transfected cells were then harvested and lysed in 100 µl of 1x cell culture lysis reagent (Promega). Luciferase activity and β -Galactosidase activity were measured as previously described (Boonsaen *et al.*, 2007). The relative luciferase activity was normalized with β -Galactosidase activity and shown as relative luciferase activity.

Statistical analysis

The results were shown as means of relative luciferase activity ± standard deviation. To verify the significant difference between groups, statistical analysis was determined by One Way ANOVA using SigmaStat 3.5 (Systat Software, Inc.).

Electrophoretic mobility shift assay (EMSA)

Purified HNF4α were obtained from Dr. Tanit Chavalit (Chavalit *et al*., 2013). Non-radioactive EMSA was performed using the LightShift chemiluminescent EMSA kit (Pierce). The 3'-end biotin-labeled oligonucleotides were synthesized which contain putative DR-1 site (5'- AGCCCTGGCCCTGGCCCTCCCTT -3') of PC gene. The 6 pmol of each oligonucleotide was annealed in 1x annealing buffer (1 mM EDTA, 10 mM Tris pH 7.4, 100 mM NaCl) producing double-strand oligonucleotide probes. The DNA-protein binding reaction was performed at 4°C for 30 min in a 20 µl reaction mixture containing 1x binding buffer (1 mM DTT, 10 mM HEPES pH 7.8, 50 mM NaCl), 1% (v/v) NP-40, 10% (v/v) glycerol, 2 µg of poly(dI·dC), 250 ng purified HNF4α and 120 fmol of DNA probe. The EMSA was used 5% native polyacrylamide gel in 0.5x TBE (10 mM EDTA, 0.45 M boric acid, 0.45 M Tris pH 8.0) at 4°C, 100 V for 120 min to separate DNA-protein complexes. The specificity of DNA-protein complex was examined by 50-fold excess unlabeled oligonucleotide probes and non-specific oligonucleotide probes. To specify the binding protein, supershift assay was performed using 0.5 µg of HNF4α antibody (Santa Cruz Biotechology, Inc.) in the binding reaction. The DNA-protein complexes on the gel were transferred to Biodyne® B nylon membrane (Pierce) at 150 mA for 90 min using Semi Phore™ Semi-Dry Transfer Units (Hoeffer). The DNA-protein complexes were detected using the Non-radioactive Nucleic Acid Detection Kit (Pierce).

Real-Time PCR (qPCR)

To determine the effect of HNF4α on the expression of PC mRNA in human HepG2 hepatocytes, real-time PCR was performed. The HNF4α knockdown and HNF4α overexpressed cDNAs were obtained from Ms. Siriluck Wattanavanitchakorn. The qPCR was using for detected PC and HNF4α expressions. The 12 µl reaction mixture consists of 2x SYBR® PCR Master Mix, 0.2 µM forward primer and reverse primer and 2 µl of cDNA. The primers used to detect PC mRNAs in HepG2 are 5' -ACCAACTGCCGTGATGCTGA-3' (forward primer) and 5' -ACACACGGATGGCAATCTCACC -3' (reverse primer). PCR was carried out in a BioRad MyIQ Real-Time

Detection System with SYBR Premix Ex Taq (RR041Q) (Takara) with two-steps SYBR thermal profile; initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, extension at 55°C for 30 s, and final extension at 95 °C for 30 sec. The PC and HNF4α expressions were normalized to the expression of 18s rRNA and presented as relative gene expression.

Results

Identification of functional HNF4α binding site by deletion analysis and transactivation assay.

To identify the HNF4α binding site, bioinformatics analysis of 700 bp human P1 promoter sequence using JASPAR2018 (Khan *et al*., 2017) was performed. The result revealed that it contained four putative HNF4α binding sites at -351/-339, -287/-275, -251/-239 and -73/-61 as shown in Figure 1.

-700 TTACTGGGGTGCCCTTTAGGATAACACTTTGTTAAATGAACTGAGCTGGGTGGTTTCATAGTATGTTCAAGCATGTGAACATCTGCTTGCTGATAAATCG -601

-600 TACTGGGTCACCAGCTCACACTAATCACCAGGTAACATGTGCCCTGGTGAGGGATCCAGTCTTCTGGCCCTGTGGGGAGACAGGCCCATGATGTCACCAG -501

-500 TTCCCAGGTGGACAACCCAAGGCATAGTGAGGTCTCGGCAGGAAGGAGGAAGACATTAGACTCCTTCTTTTTTCTTCTCCCCATTGAATCTTCTGCTCCT -401

-400 TTCCTTAGGCCAGATGCCCTGTCTCTCTCCTGGCTGGCTTTCCCAGCCC**TGGCCCTGGCCCT**CCCTTCTTTGTGAAACTCGAACACCACTGCCCTTTCAT -301

-300 CTTTCCACCAGAACTTGTGAGCAG**TGGCTTTTGACCG**CCGCTCTTCCAGC**TCACCCCTGACCT**AGTGTTTGTTTTTTTATCCTTCCCTCAGTCTACTCAA -201

-200 GGCCCACCCCTAAGCCAGAGATTGGCTCCCAAGCAGCCTCTCTCTCCTGGGAGCGGTAAAGGAGGGGACAGTTCTGGAAAGTCATTGACAGGGGATTAAC -101

-100 CTAGAACATCAATTATAAACAGGCAGC**AGGCCTCAGGCCC**AGACTTGGGTGTTACAGGAACAGTGTGGCCTCTCTGGAACTCTGCAGACCAACTGCCGTG -1

Figure 1 Nucleotide sequence of 0.7 kb of P1 promoter of human PC gene. Underlines are the putative HNF4α binding sites.

To identify the functional HNF4α binding site in the P1 promoter, 5'-truncated constructs were generated using primers designed to delete every 100 nucleotides interval. These constructs were co-transfected with an empty vector (pcDNA) or with plasmid overexpressing HNF4α (pcDNA-HNF4α) into HepG2 cells. Positive activation by HNF4α would be indicated by an increased luciferase activity compared with those transfected with empty vector. As shown in Figure 2, HNF4α can increase the activity of luciferase activity of 700 hP1 by 5-fold. Truncation of the first 100 nucleotides (600hP1) only slightly reduced transactivation of promoter activity however, further deletion (500hP1) markedly reduced HNF4α transactivation activity by 50%. Further deletion to -400 (400hP1) brought the HNF4α -transactivation back to the similar level as the wild type. Further deletion to -300 (300hP1) caused the rapid decrease of HNF4α -transactivation more than 50%, suggesting the presence of an important *cis*-acting element that mediates HNF4α -transactivation ability. Further deletion to -200 (200hP1) or -100 (100hP1) totally abolished HNF4α transactivation ability, suggesting that the primary determinant for HNF4α activation is located between nucleotides -400 to -300.

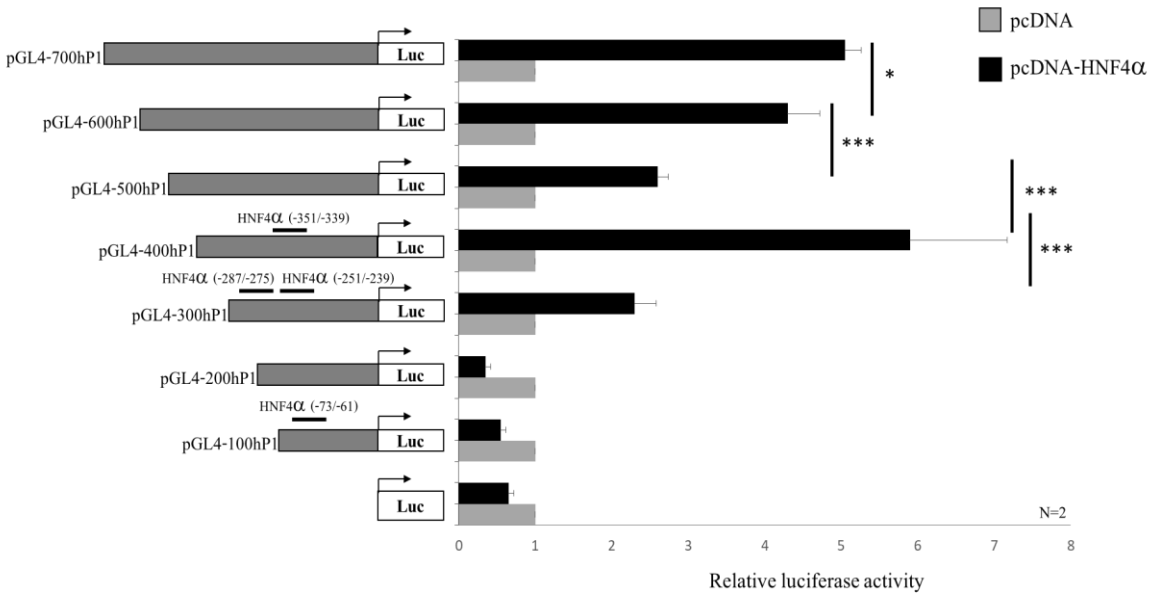

Figure 2 Transactivation of the full length and 5'-truncated promoter-luciferase reporter constructs by HNF4α. Seven constructs were transiently co-transfected with empty vector (pcDNA, grey bar) or plasmid expressing human HNF4α (pcDNA-HNF4α, black bar) into HepG2 cells. The luciferase activity of each construct was normalized by β-galactosidase activity and expressed as relative luciferase activity. The black bars represent the transactivation of pcDNA-HNF4α plasmid comparison with the gray bars represent the transactivation of pcDNA using as the control group, which were set to 1. (*p<0.05, ***p<0.001)

Examination of binding ability of HNF4α to -351/-339 of P1 promoter of human PC gene

As shown above, truncation analysis suggests the presence of the main HNF4α binding sites located between nucleotides -400 and -300. Investigation of nucleotide sequences between these regions identified the putative binding site of HNF4α located between nucleotides –400 and –301 as shown in Figure 1. This sequence resembles the classical DR1 at nucleotide position, -351/-339 on the reverse strand (5'-**AGGGCC**A**GGGCCA**-3). To examine whether HNF4α interacts with this DR-1 site located between -351 and -339 of human P1 promoter, electrophoretic mobility shift assay (EMSA) was performed using biotinylated probe containing (-351/-339) DR-1 with purified HNF4α. We designed the 3'-end biotin-labeled oligonucleotides probe to cover this putative DR1 site. The nucleotide sequences of oligonucleotide probe/competitor are shown in Figure 3. As shown in Figure 3, incubating DR1 probe with purified HNF4α formed DNA-protein complex, designated as C (lane 1). The binding specificity of HNF4α and DR-1 probe was determined by the competition assay in which excess amounts of unlabeled competitor (50x WT) and unlabeled oligonucleotides non-specific sequence competitor (50x non-specific) were included in the binding reaction. As shown in Figure 3 (lanes 2), the DNA-protein complex was disappeared when 50x excess amounts of unlabeled DR-1 probe were included in the binding reaction. On the other hand, the formation of DNA-protein complex was not inhibited when excess amounts of unlabeled oligonucleotides non-specific sequence competitor were present in the binding reaction (lane 4). To confirm whether DNA-protein complex was associated with HNF4α, a supershift assay using anti-

HNF4α polyclonal antibodies was performed. After pre-incubation of purified HNF4α with anti-HNF4α antibody added in the binding reaction, the DNA-protein complex was disappeared concomitant with the formation of the supershift band. These results suggested that DR1 located at -351/-339 acts as binding site for HNF4α.

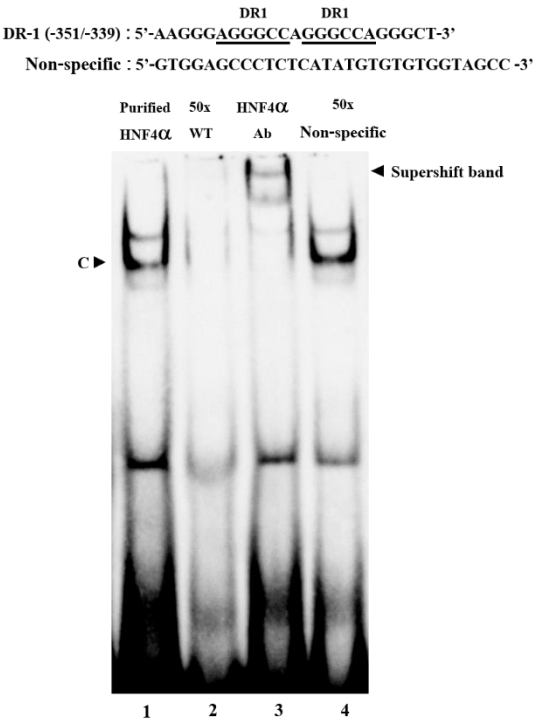

Figure 3 Electrophoretic mobility shift assay of HNF4α -351/-339 of the human P1 with purified HNF4α. The 3'-end biotin-labeled oligonucleotides probe was incubated in the 250 ng of purified HNF4α nuclear extract (lane 1), the presence of excess amount of unlabeled oligonucleotides competitor (50x) (lane 2), presence of anti-HNF4α antibody (lane 3) and presence of excess amount of non-specific sequence competitor (50x) (lane4). The nuclear protein/DNA complex (C) and supershift band are indicated by triangle sign.

Investigation of the effect of HNF4α transcription factor on human PC gene expression

To examine the functional significance of the HNF4α transcription factor on human PC gene expression, the overexpression and knock-down experiments were performed in which the plasmid overexpressing human HNF4α or siRNA targeted HNF4α was transiently transfected into HepG2 cells. The expression of HNF4α and PC were measured by quantitative real time reverse transcription PCR (qRT-PCR). As expected, overexpression of HNF4α caused 2.5-fold increase of PC mRNA expression (Figure 4 B and C) while suppression of HNF4a by siRNA resulted in a 35% reduction of human PC mRNA. These results suggested that HNF4α moderately regulates expression of PC gene.

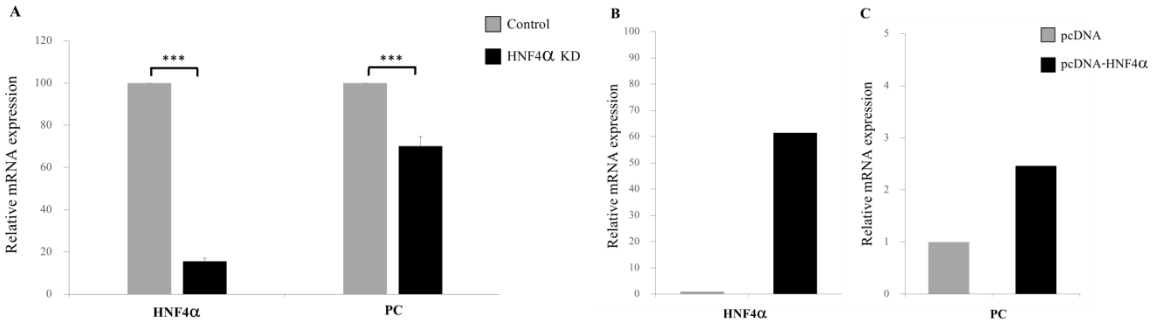

Figure 4 Suppression or overexpression of HNF4α in HepG2 cells. **A**, qRT-PCR of HNF4α and PC expression of HepG2 cells. The grey bars represent the control group (cells transfected with scrambled control siRNA) and the black bars represent the cells transfected with HNF4α siRNA. The relative mRNA expressions were normalized with 18sRNA, which are set to 100. **B**, **C**, qRT-PCR of HNF4a or PC mRNA expression in HepG2 cells transfected with empty vector (pcDNA3.1) or HNF4α expression plasmid (pcDNA-HNF4a) The relative mRNA expressions were normalized with 18sRNA, which was arbitrarily set to 1. Statistical analysis was performed by Paired t-test using SigmaStat 3.5. (***$p < 0.001$).

Discussion and Conclusions

In human, PC gene uses two alternative promoters, the proximal (P1) and the distal (P2) as in the mouse gene to regulate PC gene expression. The P1 promoter is responsible for generating mRNA transcript that is expressed in gluconeogenic tissue while mRNA transcript generated from P2 promoter is expressed in pancreatic β-cells. The regulation of human PC gene expression by P2 promoter has been studied (Thonpho *et al*., 2013) however, there was no information available regarding the regulation of the P1 promoter. Although HNF4a has been shown to be a major transcription factor that directs transcription of PC gene in mouse liver, its sequence is remarkably different from that of the human PC gene. This raises the possibility whether the regulatory role of HNF4α is restricted to mouse only. HNF4α plays an important role in the central metabolism by regulating glucose homeostasis. Mutation of this transcription factor causes the development of a monogenic form of diabetes known as the Maturity Onset of the Young 1 (MODY-1) (Yamagata *et al*., 1996). Despite established knowledge that mouse P1 promoter of PC gene is regulated by HNF4α, various differences between mouse and human promoter sequence (data not shown) make it important to also carry out these investigations in the human P1 promoter. The results will give multiple beneficial insights into the effectiveness of human P1 promoter regulation in the PC gene as a therapeutic target in diabetes. Therefore, firstly, JASPAR was used to identify four HNF4α binding sites located within the 400 nucleotides proximal to transcription start site of PC gene. All of these putative binding site are classical DR1, consisting of two direct repeats of the hexameric half sites separated by one nucleotide (AGGTCAxAGGTCA). It is not surprised to see the presence of several putative binding sites of HNF4α in human P1 promoter because mouse P1 promoter also contain three functional HNF4α binding sites. Next, to test functional significance of all putative HNF4α binding sites in transcriptional regulation under HNF4α -transactivation

conditions, transient transfection of seven 5'-truncated promoter constructs with pcDNA-HNF4α was performed. The result revealed that the functional putative HNF4α located at -351/-339 DR1. EMSA has confirmed that this DR1 bind HNF4α. Finally, the functional importance of HNF4α in the regulation of endogenous PC expression in hepatocytes has been determined by siRNA and overexpress HNF4α were performed. Suppression or overexpression of HNF4α had significant effect on PC gene expression. Overall, although the P1 promoter sequences of mouse and PC genes are remarkably different, they are both regulated by HNF4α via different binding sites. In mouse, HNF4α regulated PC gene via the HNF4α -specific motif (Chavalit *et al*., 2013) while it regulates human gene via the DR1.

In summary, our results have shown that regulation of transcription of human P1 promoter of PC gene was mediated through HNF4α binding to (-351/-339) DR1 of P1 promoter.

Acknowledgements

Molecular metabolism and gene regulation lab, Department of Biochemistry, Faculty of Science, Mahidol University.

References

Andrikopoulos S., & Proietto J. (1995). The biochemical basis of increased hepatic glucose production in a mouse model of type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia, 38*(12), 1389-1396.

Babeu J. P., & Boudreau F. (2014). Hepatocyte nuclear factor 4-alpha involvement in liver and intestinal inflammatory networks. *World journal of gastroenterology: WJG*, *20*(1), 22.

Boonsaen T., Rojvirat P., Surinya K. H., Wallace J. C., & Jitrapakdee S. (2007). Transcriptional regulation of the distal promoter of the rat pyruvate carboxylase gene by hepatocyte nuclear factor 3β /Foxa2 and upstream stimulatory factors in insulinoma cells. *Biochemical Journal*, *405*(2), 359-367.

Chavalit T., Rojvirat P., Muangsawat S., & Jitrapakdee S. (2013). Hepatocyte nuclear factor 4α regulates the expression of the murine pyruvate carboxylase gene through the HNF4-specific binding motif in its proximal promoter. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1829*(10), 987-999.

Chen T. (2008). Nuclear receptor drug discovery. *Current opinion in chemical biology*, *12*(4), 418-426.

Cool B., Zinker B., Chiou W., Kifle L., Cao N., Perham,M., … & Zhao G. (2006). Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell metabolism*, *3*(6), 403-416.

Fang B., Mane-Padros D., Bolotin E., Jiang T., & Sladek F. M. (2012). Identification of a binding motif specific to HNF4 by comparative analysis of multiple nuclear receptors. *Nucleic acids research*, *40*(12), 5343-5356.Han, J., & Liu, Y.Q. (2010). Reduction of islet pyruvate carboxylase activity might be related to the development of type 2 diabetes mellitus in Agouti-K mice. *J Endocrinol, 204*(2), 143-152.

Khan A., Fornes O., Stigliani A., Gheorghe M., Castro-Mondragon J. A., van der Lee R., ... & Baranasic D. (2017). JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic acids research*.

Kumashiro N., Beddow S.A., Vatner D.F., Majumdar S.K., Cantley J.L., Guebre-Egziabher F., *et al*. (2013). Targeting pyruvate carboxylase reduces gluconeogenesis and adiposity and improves insulin resistance. *Diabetes, 62*(7), 2183-2194.

Mendez-Lucas A., Duarte J.A., Sunny N.E., Satapati S., He T., Fu X., *et al*. (2013). PEPCK-M expression in mouse liver potentiates, not replaces, PEPCK-C mediated gluconeogenesis. *J Hepatol, 59*(1), 105-113.

Roden M. (2016). [Diabetes mellitus: definition, classification and diagnosis]. *Wien Klin Wochenschr, 128 Suppl 2*, S37-40.

Rojvirat P., Chavalit T., Muangsawat S., Thonpho A., & Jitrapakdee S. (2011). Functional characterization of the proximal promoter of the murine pyruvate carboxylase gene in hepatocytes: role of multiple GC boxes. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1809*(10), 541-548.

Stoffel M., & Duncan S. A. (1997). The maturity-onset diabetes of the young (MODY1) transcription factor HNF4α regulates expression of genes required for glucose transport and metabolism. *Proceedings of the National Academy of Sciences*, *94*(24), 13209-13214.

Thonpho A., Rojvirat P., Jitrapakdee S., & MacDonald M. J. (2013). Characterization of the distal promoter of the human pyruvate carboxylase gene in pancreatic beta cells. *PloS one*, *8*(1), e55139.

Umesono K., Murakami K. K., Thompson C. C., & Evans, R. M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell*, *65*(7), 1255-1266.

Weinberg M.B., & Utter M.F. (1980). Effect of streptozotocin-induced diabetes mellitus on the turnover of rat liver pyruvate carboxylase and pyruvate dehydrogenase. *Biochem J, 188*(3), 601-608.

Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, et al. (1996), Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature*, *384(6608),* 458-60.