Kr-pok increases gluconeogenesis through upregulation of G6pc and Pck1

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Kr-pok increases gluconeogenesis through upregulation of G6pc and Pck1

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TABLE OF CONTENTS

ABSTRACT……1

I. INTRODUCTION……3

II. MATERIALS AND METHODS

1. Plasmids, antibodies and reagents ……10

2. Site-directed Mutagenesis of the G6pc Promoter ……10

3. Cell culture and transient transfection assay ……11

4. Subcellular fractionation ……12

5. Preparation of recombinant adenovirus overexpressing Kr-pok and shRNA against Kr-pok mRNA ……12

6. Quantitative real-time PCR (qRT-PCR) ……13

7. Western blot analysis……14

8. Co-immunoprecipitation ……14

9. Quantitative chromatin immunoprecipitation (ChIP-qRT-PCR) assays ……15

10. Oligonucleotide pull-down assay……16

11. Glucose and insulin tolerance tests ·····17

12. Primary hepatocyte isolation and culture·····17

13. Glucose production assay·····18

III. RESULTS

1. Levels of blood glucose and expression of gluconeogenic genes, *G6pc* and *Pck1* is decreased in Kr-pok knockout mice·····22

2. Kr-pok expression is increased by fasting and forskolin ·····25

3. Kr-pok activates transcription of *G6pc* and *Pck1* genes ·····29

4. Kr-pok activates transcription of *G6pc* and *Pck1* gene by acting on the promoter region containing IRS·····32

5. Kr-pok increases FoxO1 binding to the IRSs of *G6Pc* and *PCK1* promoters·····38

6. Kr-pok does not change expression and subcellular localization of FoxO1·····41

7. Kr-pok decreases acetylation of FoxO1·····44

8. Forskolin increases protein stability of Kr-pok ·····49

IV. DISCCUSION·····53

V. CONCLUSION·····58

REFERENCES·····60

ABSTRACT (IN KOREAN) ……67

PUBLICATION LIST ……70

LIST OF FIGURES

Figure 1. Levels of blood glucose and expression of gluconeogenic genes, G6pc and Pck1, is decreased in Kr-pok knockout mice·····24

Figure 2. Effects of fasting and forskolin on Kr-pok expression·····27

Figure 3. Kr-pok activates transcription of gluconeogenic genes, *G6pc* and *Pck1*·····30

Figure 4. Kr-pok activates *G6pc* gene by acting on the proximal promoter element containing IRS·····34

Figure 5. Kr-pok activates the transcription of the *PCK1* promoter by acting on the IRS ·····36

Figure 6. Kr-pok increases FoxO1 binding on the IRS of *G6Pc* and *PCK1* promoters ·····39

Figure 7. Kr-pok does not affect expression, subcellular localization and phosphorylation of FoxO1. Kr-pok interacts with FoxO1·····42

Figure 8. Kr-pok recruits HDAC3 to deacetylate FoxO1 ·····46

Figure 9. Kr-pok increases recruitment of FoxO1, Hdac3 and NCoR to the *G6pc* and *Pck1* promoters ·····48

Figure 10. Protein stability of Kr-pok is increased by forskloin ·····50

Figure 11. Hypothetical model of transcriptional regulation of *G6pc* and *Pck1* by Kr-pok·····51

LIST OF TABLES

Table 1. Sequences of oligonucleotide primers used for cloning……19

Table 2. Sequences of oligonucleotide primers used for site-directed mutagenesis……19

Table 3. Sequences of RT-qPCR oligonucleotide primers ……20

Table 4. Sequences of ChIP-qRT-PCR oligonucleotide primers ……20

Table 5. Sequences of oligonucleotide primers used for oligo pull-down……21

ABSTRACT

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Gluconeogenesis is essential for maintenance of blood glucose level during fasting. Gluconeogenesis is tightly regulated by the key enzymes, including glucose-6-phophatase (G6pc) and phosphoenolpyruvate carboxykinase (PEPCK). Transcription of the genes encoding the two enzymes is tightly regulated under fasting condition.

Kr-pok knockout mice showed a decrease in blood glucose level.

Differential gene expression analysis of Kr-pok wild type and knockout mice liver showed that mRNA of *G6pc* and *Pck1* are decreased. I investigated the role of Kr-pok in the regulation of gluconeogenesis. I found that Kr-pok is induced in the liver of fasted mice. Also, forskolin treatment increased Kr-pok expression in mice primary hepatocytes and knockout of Kr-pok decreased transcription of *G6pc* and *Pck1*. These results showed that, under fasting condition, Kr-pok can increase expression of *G6pc* and *Pck1*genes.

I investigated how Kr-pok can increase transcription of *G6pc* and *Pck1* genes. Transient transcription assays of the reporter plasmid showed that Kr-pok increases transcription by acting on the IRSs of the *G6pc* and *Pck1* promoters. ChIP assays revealed that Kr-pok reduces acetylation of FoxO1 and thereby increases FoxO1 binding to the IRS elements of the promoters to activate transcription of *G6pc* and *Pck1*. Kr-pok increased the interaction between FoxO1 and HDAC3 and deacetylated FoxO1. Also, I found that protein stability of Kr-pok is increased by treatment of forskolin. These results suggested that Kr-pok might be a metabolic regulator controlling expression of the two key gluconeogenic enzymes, G6pc and PEPCK.

Key words: Kr-pok, transcription, gluconeogenesis, G6pc, Pck1, FoxO1, HDAC3

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I. INTRODUCTION

The proteins with human BTB/POZ domain are a large and diverse family of regulators including over 200 proteins members. Many other types of protein with BTB/POZ domain have diverse biological functions.[1] In particular, some POK proteins, which have BTB/POZ and C2H2 Krüppel-like zinc-finger domains, are involved in key biological processes such as development, differentiation, hematopoiesis and oncogenesis.[2, 3] POK family proteins were shown to interact with coregulators such as HATs, HDACs, NuRD and BcoR. These interactions are involved in epigenetic changes of gene expression.[4-7]

The POK family proteins such as BCL-6 (B-cell lymphoma 6), FBI-1

(also known as Pokemon and OCZF) and HIC-1 (hypermethylated in cancer-1) function as major regulators of development, malignant transformation and lymphocyte differentiation.[8-12] Recently, it was reported that PLZF (promyelocytic leukemia zinc-finger) overexpression in brown adipocytes leads to the induction of genes involved in fatty acid oxidation, glycolysis, and mitochondrial function.[13] Also, PLZF promotes the expression of gluconeogenic gene and hepatic glucose output, leading to hyperglycemia.[14] However, the biological functions of many other POK family proteins remain uncharacterized. Also, the relationship between POK expression and their roles in differentiation, development, tumor suppression, oncogenesis and metabolism is needed to be investigated.

Kidney cancer–related POK family protein, Kr-pok (ZBTB7c), is a proto-oncoprotein that increases cell proliferation. Kr-pok functions as a negative regulator of CDKN1A expression.[15] Also, Kr-pok increases expression of FASN which is a key enzyme of the fatty acid synthesis.[16] Kr-pok decreases transcription of metabolic regulator gene, SIRT1 in p53-dependent manner. Kr-pok decreases acetylation of Pparγ and Pgc-1α by increasing transcription of Sirt1 (unpublished data). In addition, abdominal fat mass is decreased in Kr-pok knockout mice compared to wild type mice. These findings suggest that Kr-pok may be an important metabolic regulator.

Mammalian cells constantly try to use nutrients efficiently and have mechanisms to adjust excess or deficiency of energy sources. The overall metabolic response to meet demand of energy is accomplished mainly in liver. Glucose which is a main energy source is stored as liver glycogen, adipose tissue triglyceride and muscle protein. In fasting, glycogen is degraded into glucose and glucose is produced from precursors such as amino acids and glycerol derived from triglyceride (gluconeogenesis).[17] In prolonged fasting, ketones synthesized from acetyl-CoA are used as energy sources.[18] An appropriate supply of glucose is particularly important for brain and red blood cells because glucose is the only substrate for these tissues.

Hypoglycemia, a state of low glucose, impairs these tissues, even leading to death.[19] The supply of glucose form gluconeogenesis can protect the tissues from hypoglycemia. Diabetes is characterized by increased hepatic glucose output and high blood glucose levels. The rate of hepatic gluconeogenesis is considerably increased in diabetes, contributing significantly to fasting hyperglycemia.[20] Excessive glucose concentration can cause damage to human body. People with diabetes show complications including endothelial dysfunction and nephrophathy.[21-22] Therefore, maintenance of blood glucose levels is crucial in protecting organisms against hypoglycemia and hyperglycemia.

Gluconeogenesis is required to provide the body with metabolic fuel under physiological conditions. During fasting, mammals show low insulin, high glucagon, glucocorticoids and epinephrine concentrations in plasma. The change of hormones needs to maintain blood glucose levels. The control of gluconeogenesis is mainly achieved by key metabolic enzymes such as PEPCK (phosphoenol-pyruvate carboxykinase), G6P (glucose-6-phosphatase), PC (pyruvate carboxylase) and FBP (fructose biphosphatase). These enzymes are unique to the gluconeogenic pathway, although most of the enzymes involved in glucose metabolism are used in reversible steps of glycolytic and gluconeogenic pathway.[23-24] A major part of the livers' response to fasting is accomplished by transcriptional regulation of gluconeogenic enzymes. During fasting, change of hormones leads to initiate transcriptional program involved in gluconeogenesis.[25]

The two major hormones regulating gluconeogenesis are glucagon and glucocorticoids. Glucocorticoids participate in gluconeogenesis through activating GR (glucocorticoid receptor). GR induces a set of genes encoding fasting-related transcription factors such as C/EBPβ and Krüppel-like factor 15, thereby contributing to gluconeogenesis.[26-27] Glucagon activates adenylate cyclase through binding of membrane receptor, which increases in cellular cAMP levels. This leads to activation of PKA (protein kinase A). Binding of

cAMP to regulatory subunits within PKA heterotetramer stimulates migration of its catalytic subunits to the nucleus. Catalytic subunits of PKA phosphorylates CREB at Ser133. Phosphorylated CREB promotes target gene expression with promoters containing CREs(cAMP-responsive elements). Phosphorylated CREB activates gluconeogenic genes, G6pc (a gene encoding Glucose-6-Phosphatase Catalytic Subunit) and PCK1 (a gene encoding PEPCK) through subsequent recruitment of the coactivators such as CBP (CREB binding protein)/p300, PCAF and PRMT5.[28-29] Also, CREB can induce many transcription factors to activate gluconeogenic genes. For example, CREB induces the expression of the Nr4a gene family which is one of the nuclear receptors and Yin Yang 1 that enhance gluconeogenesis.[30-31] Additionally, CREB activity can be decreased by dephosphorylation through phosphatases such as protein phosphatase 1 (PP1) and PP2A.[32]

FoxO (class O forkhead box) proteins are involved in hepatic glucose production following fasting. FoxO proteins that possess a forkhead box-type DNA binding domain recognize a specific IRS (insulin response sequence) on the promoter of target genes. FoxO1's role in regulation of gluconeogenesis has well characterized. Insulin of gluconeogenesis is largely mediated through the serine/threonine kinase Akt, which phosphorylates and inactivates FoxO family of transcription factors, mainly FoxO1 and FoxO3 in mammalian liver.

Insulin-AKT signaling activates FoxO1 export to cytoplasm. FoxO proteins in cytoplasm are ubiquitinylated and degraded.[33-34] Also, coactivators such as CBP, p300, SRC-1 and PGC-1α were shown to interact with FoxO.[35-37] Acetylation of FoxO1 by coactivator CBP and p300 decreases DNA binding affinity to the promoters of FoxO1 target genes.[38] Glucagon-induced FoxO1 deacetylation by the class IIa HDACs and HDAC3 increases FoxO1 DNA-binding activity to the gluconeogenic gene promoters.[39] Also, deacetylation of FoxO1 by the NAD^+ dependent sirtuin activates transcription of FoxO1 target genes.[40] Loss of insulin signaling in fasting state dephosphorylates the Akt phosphorylation sites in FoxO1 and allows FoxO1 to translocate into the nucleus. Fasting promotes transcriptional activity of FoxO1 by dephosphorylation and deacetylation.

Over the past few decades, studies about molecular mechanisms involved in the regulation of hepatic glucose have been accomplished. The transcriptional regulation of the key gluconeogenic genes such as *G6pc* and *Pck1* has been proved through useful transgenic or knockout mice models. Many studies on the regulation of glucose levels revealed the importance of glucose homeostasis. Also, researchers in this field are trying to find promising targets controlling glucose homeostasis. The increasing studies about gluconeogenesis will provide a solid basis for the maintenance of

glucose homeostasis and development of drug targets.

I investigated regulation of hepatic gluconeogenesis by Kr-pok. Kr-pok is induced in fasting state of mice, and activates transcription of gluconeogenic genes, *G6pc* and *Pck1*. Kr-pok increases deacetylation of FoxO1, which activates G6pc and Pck1 promoter. My study revealed that Kr-pok induced by fasting is an important transcriptional activator of two key gluconeogenic genes, *G6pc* and *Pck1*.

II. MATERIALS AND METHODS

1. Plasmids, antibodies and reagents

The pGL4b-*G6pc*-Luciferase (Luc) plasmid was kindly provided by Dr. Yong-Ho Ahn Young(Yonsei University of Medicine, Seoul, Korea). Also, Dr. Kyung-Sup Kim (Yonsei University of Medicine, Seoul, Korea) kindly provided the HA-HDAC3 plasmid. pcDNA3.0-FLAG-Kr-pok, pcDNA3.1-FoxO1, pGL3-*PCK1*-Luc and pG5-3X(IRS)-Luc were prepared by cloning cDNA. Oligonucleotides used for cloning is listed in Table 1. A series of pGL4b-*G6pc*-Luc plasmids were prepared by cloning mouse *G6pc* promoter DNA fragments (bp, -500 ~ +66; bp, -231 ~ +66; bp, -158 ~ +66; bp, -85 ~ +66) into pGL4b vector (Promega, WI). Various mutant *G6pc*-Luc and *PCK1*-Luc plasmids were prepared using site-directed mutagenesis kit (MGmed, Seoul, Korea). All plasmid constructs were verified by sequencing.

Antibodies against GAPDH, G6pc, Pck1, HA-tag, FLAG-tag, Acetyl-lysine, Hnf4α and FoxO1 were purchased from Cell Signaling Technology (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against Kr-pok was prepared as described previously.

2. Site-directed mutagenesis of the G6pc promoter

Mutations at IRS were introduced into the G6pc and PCK1 promoter sequence using the site-directed mutagenesis kit (MGmed, Seoul, Korea). Sequences of oligonucleotides used to introduce mutations into IRS (Isulin Response Sequence) is listed in Table 2. For site-directed mutagenesis, 18 PCR cycles with denaturation at 94 °C for 30 sec, hybridization at 55 °C for 1 min, and extension at 68 °C for 10 min was used. The amplified mixtures were treated with DpnI (New England Biolabs, MA) at 37 °C for 1 hr, and aliquots were used to transform competent *E. coli*. All constructs were confirmed by DNA sequencing.

3. Cell culture and transient transfection assay

HEK293A, and HepG2 cells were maintained at 37 ºC in a humidified incubator with 5% CO_2 air in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100.0 μg/ml streptomycin and 100 units/ml penicillin. Cell culture medium and supplements were from Invitrogen (Gibco-BRL, MD).

The pGL4b-*G6pc*-Luc, pGL3-*PCK1*-Luc, various pGL4b-*G6pc*-Luc promoter reporter fusion plasmids, pcDNA3.0-FLAG-Kr-pok and HA-HDAC3 in various combinations were transiently transfected into HepG2 cells using Lipofectamine 3000 reagent (Invitrogen, CA). After 24 to 36 hr of

incubation, the cells were harvested and and analyzed for luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold, MD). All the reactions were performed in triplicate. Reporter activity was normalized with protein concentration for transfection efficiency.

4. Subcellular fractionation

The nuclear extract Kit (Active Motif, CA) was used for cytoplasmic and nuclear fraction of cells. Cells were resuspended in 1x hypotonic buffer and incubated for 15 min on ice, followed by adding detergent and vortexing. Cytoplasmic fraction (supernant) collected by centrifugation. The nuclear pellet was resuspended in complete lysis buffer and nuclear fration (supernant) was recovered by centrifugation.

5. Preparation of recombinant adenovirus overexpressing Kr-pok and shRNA against Kr-pok mRNA

The Kr-pok cDNA was cloned into the adenovirus E1 shuttlevector pCA14 (Microbix) to generate pCA14-Kr-pok. To prepare recombinant adenovirus expressing short hairpin RNA (shRNA) against Kr-pok, annealed shRNA DNA sequences [sense: 5'-GATCCCTCCAGTGCATCGTGAATGTTTTTCAAGAGA(loop)-

ACATTCACGATGCACTGGATTTTTTTGGAA(loop)-A-3', antisense: 5'-AGCTTTTCCAAAAA(loop)-AATCCAGTGCATCGTGAATGTTCTCTTGAA(loop)-AAACATTCACGATGCACTGGAGG-3'] were cloned into pSilencer 2.0-U6 (Ambion) and subcloned into the pDE1sp1A vector. The pCA14-Kr-pok shuttle vector or pΔE1sp1A-U6-shKr-pok vector and the adenovirus vector vmdl324Bst were linearized by restriction enzyme digestion. The linearized pCA14-Kr-pok or pΔE1sp1A-U6-shKr-pok was cotransformed into *E. coli* BJ518 with the vmdl324Bst vector for homologous recombination. Homologous recombinant adenoviral plasmid was digested with PacI and transfected into HEK293 cells to generate the adenovirus expressing Kr-pok (dl324-Kr-pok) or shRNA against Kr-pok (dl324-shKr-pok).

6. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from HepG2 cells and mice liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were synthesized using 5.0 μg of total RNA, random hexamer (10 pmol) and Superscript reverse transcriptase II (200 units/μl) in 20 μl using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR was conducted using the SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers.

Reactions were subjected to qRT-PCR quantification using the ABI PRISM 7300 RT-PCR System (Applied Biosystems). All reactions were performed in triplicate. Sequences used for qRT-PCR is listed in Table 3. 18S ribosomal RNA was measured as an invariant control.

7. Western blot analysis

Cells were harvested and lysed in RIPA buffer (50.0 mM Tris-HCl pH 8.0, 1% NP-40, 0.25% sodium deoxycholic acid, 150.0 mM NaCl, 1.0 mM EGTA and complete mini-protease cocktail). Cell extracts (30.0 μg) were separated using 12% SDS-PAGE gel electrophoresis, transferred onto Immun-Blot^{TM} PVDF membranes (Bio-Rad, CA), and blocked with 5% skim milk (BD Biosciences, MD). Blotted membranes were incubated with antibodies against GAPDH, FLAG, HA, Kr-pok, G6pc, PCK1, Hnf4α and FoxO1 and then incubated with anti-mouse, rabbit or goat secondary antibody conjugated with HRP (Vector Laboratory, CA). Protein bands were visualized with ECL solution (Thermo Fisher Scientific, NY).

8. Co-immunoprecipitation

Cells were washed, pelleted, and resuspended in a lysis buffer (20.0 mM Tris-HCl, pH 7.5, 150.0 mM NaCl, 10% glycerol, 1% Triton X-100 and

complete mini-protease cocktail). The cell lysates were pre-cleared, and the supernatant was incubated overnight with anti-FLAG, anti-HA, anti-Kr-pok and anti-FoxO1 antibody on a rotating platform at 4 ºC, followed by incubation with protein A-Sepharose Fast Flow beads. The beads were collected, washed, and resuspended in equal volumes of 5x SDS loading buffer. Immunoprecipitated proteins were separated with 12% SDS-PAGE. Western blot analysis was performed as described above using the appropriate antibodies.

9. Quantitative chromatin immunoprecipitation (ChIP-qRT-PCR) assays

Cells were treated with formaldehyde (final 1%) to cross-link proteins to the DNA promoter sequence. Cells were washed and lysed with SDS lysis buffer (1% SDS, 10.0 mM EDTA, 50.0 mM Tris-HCl, pH 8.0). The lysate was sonicated to shear DNA into fragments ranging from 500 to 1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (1% SDS, 1% Triton X-100, 16.7 mM Tris-HCl, pH 8.1, 167.0 mM NaCl, 1.2 mM EDTA) and incubated with antibody overnight at 4 °C with rotation. To collect DNA-protein-antibody complex, protein A/G-agarose slurry was added to the mixture. The mixture was incubated for 2 hr at 4 °C with rotation and pelleted by brief centrifugation. After extensive washing of the pellet with washing

buffers (low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer) as recommended by the manufacturer, the pellet was dissolved with 500.0 μl elution buffer and spun to remove excess agarose. The supernatant was treated with 20.0 μl 5.0 M NaCl and heated to 65 °C for 2 hr to reverse the protein-DNA cross-link. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover DNA. PCR reactions using the immunoprecipitated DNA were conducted using oligonucleotide primer sets designed to amplify the promoter region. The reactions were performed using the following amplification condition in the ABI PRISM 7300 RT-PCR System (Applied Biosystems, Foster, CA, USA): initial 94 ºC denaturation for 5 min, 50 cycles of amplification reaction 94 ºC, 30 sec; 55 ºC, 30 sec; 72 ºC, 30 sec.

10. Oligonucleotide pull-down assay

Cells were lysed in HKMG buffer (10.0 mM HEPES pH 7.9, 100.0 mM KCl, 5.0 mM $MgCl_2$, 10% glycerol, 1.0 mM DTT, 0.5% NP40 and complete mini-protease cocktail). Cellular extracts were incubated with 1.0 μg biotinylated double stranded oligonucleotides for 16 h. To collect DNA-bound proteins, the mixtures were incubated with NeutrAvidin-agarose beads

(Thermo Fisher Scientific Inc., USA) for 4 hr, washed with HKMG buffer, and precipitated by centrifugation. The precipitate was analyzed by Western blot analysis using the appropriate antibodies. The sequences of the oligonucleotides are listed in table 5. Oligonucleotide probes were annealed by heating at 95 ºC for 5 min and cooling slowly to room temperature.

11. Glucose and insulin tolerance tests

An oral glucose tolerance test (OGTT) was performed on mice that had fasted for 16 hr. Glucose (20% wt/vol.) was administered orally using a feeding tube (2 g/kg body weight) and blood glucose levels were monitored at specific time points. For the insulin tolerance test (ITT), mice were fasted for 6 hr and insulin (0.75 units/kg Humulin R; Eli Lilly, Indianapolis, IN, USA) administered intraperitoneally. Plasma glucose levels were measured using blood drawn from the tail vein at designated time points using an automatic glucose monitor (One Touch, CA).

12. Primary hepatocytes isolation and culture

Hepatocytes were isolated from 8-week-old male mice via collagenase (Sigma, MO) digestion and Percoll gradient purification. The detailed protocol for isolation of hepatocytes was described previously.[41] Cellular

viability of freshly isolated hepatocytes was assessed and only preparations with viability greater than 80% were used.

Hepatocytes were seeded in culture dish. After cell attachment, hepatocytes cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) including 100 nM insulin and 10nM dexamethasone. Cell culture medium and supplements were from Invitrogen (Gibco-BRL, MD).

13. Glucose production assay

Glucose production from primary hepatocytes was measured according to themanufacturer's protocol, using a colorimetric glucose oxidase assay (Invitrogen, CA). Primary hepatocytes were seeded, cultured for 12 hr, the cells were washed three times with phosphate-buffered saline and the medium was then replaced with glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mmol/liter sodium lactate, 1 mmol/liter sodium pyruvate, and 15 mmol/liter HEPES, without phenol red). The glucose assays were performed in triplicate.

Table 1. Sequences of oligonucleotide primers used for cloning

Oligo		Sequence
PCK1 promoter	Sense	5'-GATCCTCGAGCATGGGTTGTTCA-3'
	Antisense	5'-GATCAAGCTTTCTTCCCGCCAGC-3'
FoxO1 CDS	Sense	5'-GATCCTCGAGACCATGGCCGAGGCGCCTCAGGTG-3'
	Antisense	5'-GATCGGATCCGCCTGACACCCAGCTATGTG-3'
3X (IRS)	Sense	5'-CTAGCGCAAAACAAACTTATTT- -TGAAGCAAAACAAACTTATTTTG- -AAGCAAAACAAACTTATTTTGAA-3'
	Antisense	5'-CTAGCTTCAAAATAAGTTT- -GTTTTGCTTCAAAATAAGTTTGTT- -TTGCTTCAAAATAAGTTTGTTTTGCG-3'

Table 2. Sequences of oligonucleotide primers used for site-directed mutagenesis

Oligo		Sequence
G6pc IRS #1	Sense	5'-AATGGCGATCAGGCTCAAAATGTGTGCCTGTTTTG-3'
	Antisense	5'-CAAAACAGGCACACATTTTGAGCCTGATCGCCATT-3'
G6pc IRS #2	Sense	5'-TGTTTTTGTGTGCCTCAAAAGCTATTTTACGTAAA-3'
	Antisense	5'-TTTACGTAAAATAGCTTTTGAGGCACACAAAAACA-3'
G6pc IRS #3	Sense	5'-TGTGCCTGTTTTGCTCAAAAACGTAAATCACCCTG-3'
	Antisense	5'-CAGGGTGATTTACGTTTTTGAGCAAAACAGGCACA-3'
PCK1 IRS	Sense	5'-ATCTTATAGCTGTGGTGAAAACCCAACCAGCAGCTCT-3'
	Antisense	5'-AGAGCTGCTGGTTGGGTTTTCACCACAGCTATAAGAT-3'

Table 3. Sequences of RT-qPCR oligonucleotide primers

Oligo		Sequence
Kr-pok	Sense	5'-CCCATCTGCCACAAAGTCATC-3'
	Antisense	5'-TGGTGCACATGTATGGCTTCTC-3'
Human G6Pc	Sense	5'-GGCTCAACCTCGTCTTTAAGTG-3'
	Antisense	5'-CTCCCTGGTCCAGTCTCACA-3'
Mouse G6pc	Sense	5'-TCGGAGACTGGTTCAACCTC-3'
	Antisense	5'-TCACAGGTGACAGGGAACTG-3'
Human PCK1	Sense	5'-ACGGATTCACCCTACGTGG-3'
	Antisense	5'-CCCCACAGAATGGAGGCATTT-3'
Mouse Pck1	Sense	5'-CTGGCACCTCAGTGAAGACA-3'
	Antisense	5'-TCGATGCCTTCCCAGTAAAC-3'
Human FoxO1	Sense	5'-GGCTGGAAGAATTCAATTCGTC-3'
	Antisense	5'-ACCCTCTGGATTGAGCATCCAC-3'
Mouse FoxO1	Sense	5'-TTCAATTCGCCACAATCTGTCC-3'
	Antisense	5'-GGGTGATTTTCCGCTCTTGC-3'

Table 4. Sequences of ChIP-qRT-PCR oligonucleotide primers

Oligo		Sequence
Human G6Pc	Sense	5'-CTTGCACTGCCAAGAAGCAT-3'
	Antisense	5'-ATTGATCAAAGGTGCATCAC-3'
Mouse G6pc	Sense	5'-TGGCTTCAAGGACCAGGAAG-3'
	Antisense	5'-TGCAAACATGTTCAGGGTGA-3'
Human PCK1	Sense	5'-GGGTGCATCCTTCCCATGAA-3'
	Antisense	5'-GACTTCGAGCCCTCAACCAA-3'
Mouse Pck1	Sense	5'-TGGCTCAGAGCTGAATTTCC-3'
	Antisense	5'-CCTGTTGCTGATGCAAACTG-3'

Table 5. Sequences of oligonucleotide primers used for oligo pull-down

Oligo		Sequence
G6pc IRS #1	Sense	5'-GATCAGGCTGTTTTTGTGTGC-3'
	Antisense	5'-GCACACAAAAACAGCCTGATC-3'
G6pc IRS #2,3	Sense	5'-GCCTGTTTTGCTATTTTACGTA-3'
	Antisense	5'-TACGTAAAATAGCAAAACAGGC-3'
PCK1 IRS	Sense	5'-AGCTGTGGTGTTTTGCCAACCAG-3'
	Antisense	5'-CTGGTTGGCAAAACACCACAGCT-3'

III. RESULTS

1. Levels of blood glucose and the expression of gluconeogenic genes, *G6pc* and *Pck1*, is decreased in Kr-pok knockout mice

Kr-pok increases expression of FASN (fatty acid synthase), a key enzyme in fatty acid synthesis, showing that Kr-pok plays important roles in fatty acid metabolism. I investigated metabolic change of Kr-pok knockout mice. To examine the effects of Kr-pok expression on levels of blood glucose, glucose and insulin tolerance tests were performed in Kr-pok wild type and knockout mice. These studies showed that blood glucose level of Kr-pok knockout mice was lower than that of wild type mice, but Kr-pok wild type and knockout mice showed similar rates of recovery in blood glucose (Fig. 1A, B). Because Kr-pok regulates blood glucose level, I investigated whether Kr-pok can affect expression of gluconeogenesis related genes. Western blot analysis of livers in Kr-pok wild type and knockout mice showed that gluconeogenic genes, *G6pc* and *Pck1* were decreased in Kr-pok knockout mice. However, expression of other transcription factors related to gluconeogenesis like Hnf-4a and FoxO1 was not changed (Fig. 1C). I also tested whether Kr-pok regulates glucose production in primary hepatocytes. Primary hepatocytes of Kr-pok knockout mice showed decreased hepatic glucose production compared with those of

wild type mice (Fig. 1D). Taken together, these results indicate that Kr-pok might play a key role in hepatic glucose production by increasing expression of gluconeogenic genes, *G6pc* and *Pck1*.

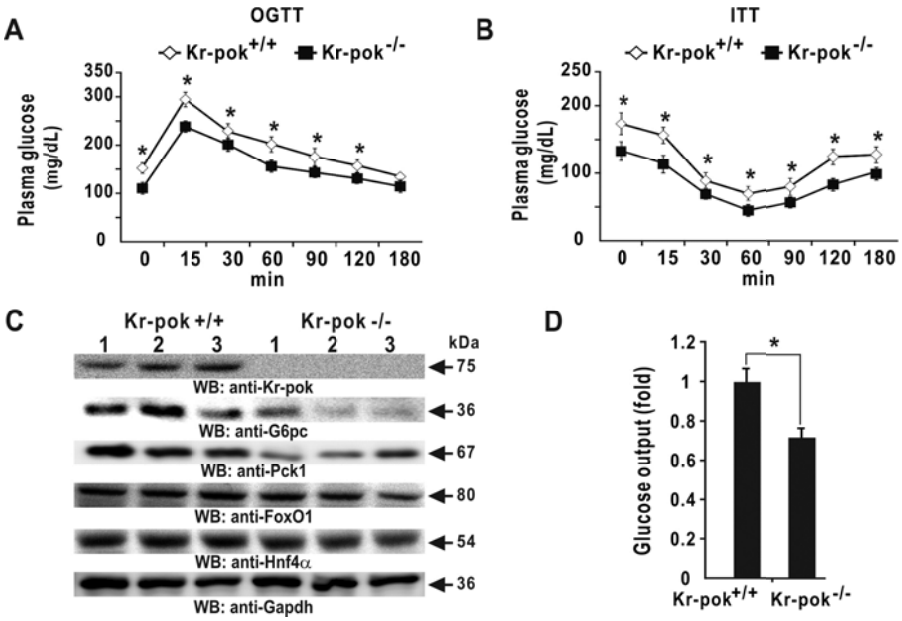

Figure 1. Levels of blood glucose and expression of gluconeogenic genes, G6pc and Pck1, is decreased in Kr-pok knockout mice. (A, B) OGTT (Oral Glucose Tolerance Test) was performed in mice fasted for 16 hr, with glucose injected orally. For the ITT (Insulin Tolerance Test), mice were fasted for 6 hr and insulin was injected intraperitoneally (n=3 per group). Error bars represent standard deviations. (C) Total proteins were prepared from the liver tissues of Kr-pok wild and knockout mice (n=3 per group). The proteins were analyzed by western blot. (D) A glucose production assay in primary hepatocytes.

2. Kr-pok expression is increased by fasting and forskolin

Gluconeogenic genes, *G6pc* and *Pck1* were decreased in livers of Kr-pok knockout mice. To test whether fasting can affect expression of Kr-pok, I investigated a change of Kr-pok expression in mouse liver under feeding and fasting conditions. The hepatic expression of Kr-pok mRNA was not changed in mice subjected to feeding and fasting. But, protein expression of Kr-pok in fasting state for 12 hr was more increased than in feeding state. Also, mRNA and protein levels of G6pc and Pck1 were induced in the fasted state (Fig. 2A). To investigate the expression of Kr-pok during fasting, primary hepatocytes were treated with 10 μM of forskolin, an adenylate cyclase activator which increases intracellular levels of cAMP and create a situation similar to fasting state. Primary hepatocytes treated with forskolin showed increase in endogenous Kr-pok expression in a time dependent manner. Also, Kr-pok mRNA expression was not affected by forskolin treatment in primary hepatocytes (Fig. 2B). These results suggested that Kr-pok might increase gluconeogenesis during fasting.

Being intrigued by the observations, I investigated expression of Kr-pok in diabetes mouse model induced by streptozotocin, which shows consistent increase in gluconeogenesis. Streptozotocin injection strongly induced expression of Kr-pok protein, suggesting that Kr-pok might play a role in

gluconeogenesis in diabetes. But, increase of Kr-pok mRNA expression was not observed in streptozotocin-treated mice (Fig. 2C). These results suggest that Kr-pok may be involved in gluconeogenesis.

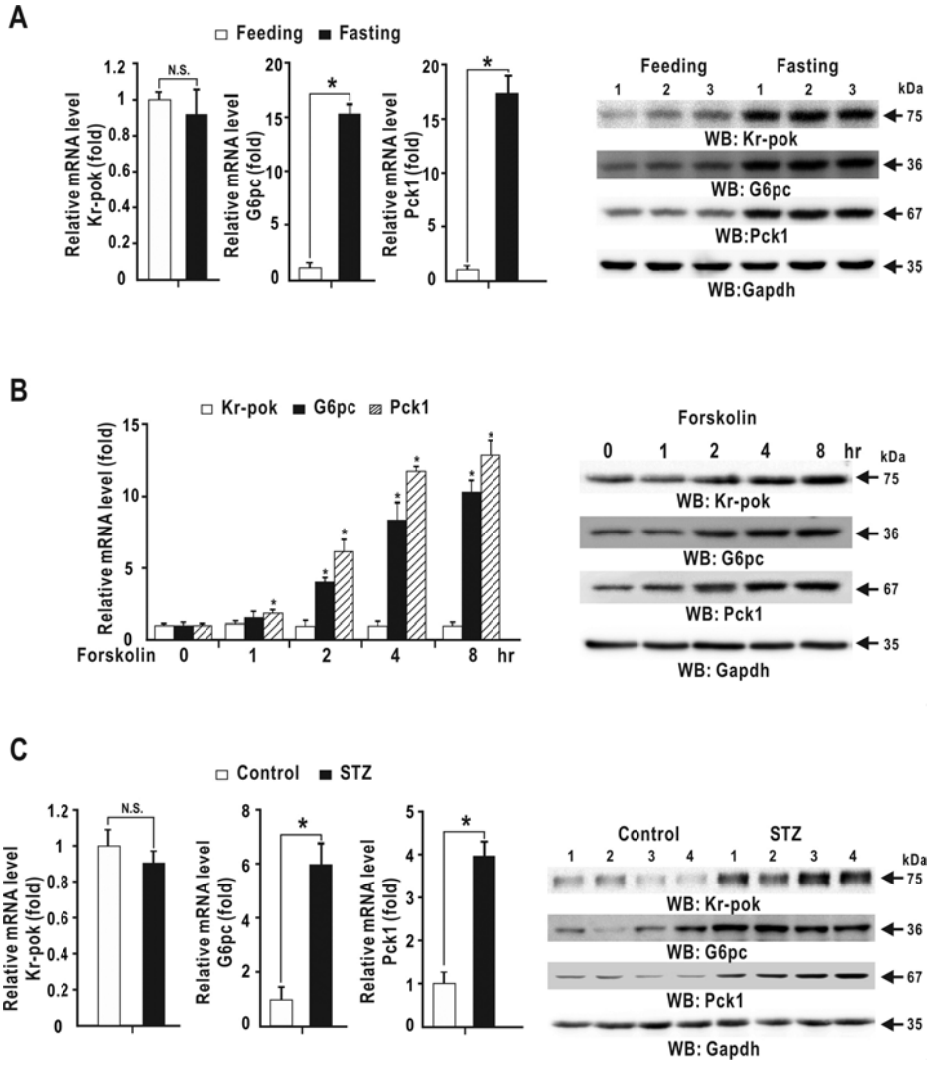

Figure 2. Effects of fasting and forskolin on Kr-pok expression. (A) C57BL6 mice (n=3 per group) were fed and fasted for 12 hr. Total RNAs were prepared from the livers of the mice. The expression of Kr-pok, *G6pc* and *Pck1* were measured by qRT-PCR and analyzed by western blot. Error bars

represent standard deviations. (B) Primary hepatocytes were treated with 10 μM forskolin for the indicated time periods. The lysates of primary hepatocytes were used for analysis of qRT-PCR and western blot. Error bars represent standard deviations. (C) qRT-PCR analysis and western blot of Kr-pok expression from livers of streptozotocin (STZ)-induced diabetic mice (n=4 per group). Error bars represent standard deviations. *, $p<0.05$; N.S., Not Significant; GAPDH, control.

3. Kr-pok activates transcription of *G6pc* and *Pck1* genes

G6pc and Pck1, two key enzymes involved in gluconeogenesis are controlled at transcriptional levels. Based on the facts that the expression of *G6pc* and *Pck1* is decreased in livers of Kr-pok knockout mice, I investigated whether Kr-pok regulates transcription of *G6pc* and *Pck1* genes, using promoter-Luc fusion plasmids. Transient transfection assay showed that Kr-pok activate transcription of both *G6pc* and *PCK1* genes in HepG2 cells by 4-7 fold (Fig. 3A and B). Also, Knockdown of Kr-pok mRNA by adenovirus sh-Kr-pok decreased transcription of *G6pc* and *PCK1* genes in forskolin treated HepG2 cells (Fig. 3A and B). Forskolin increased mRNA expression of *G6pc* and *Pck1* in wild type primary hepatocytes. But, mRNA expression of *G6pc* and *Pck1* in primary hepatocytes of Kr-pok knockout mice was less increased by forskolin (Fig. 3C). Taken together, these data suggested that Kr-pok activates transcription of *G6pc* and *Pck1* genes involved in gluconeogenesis.

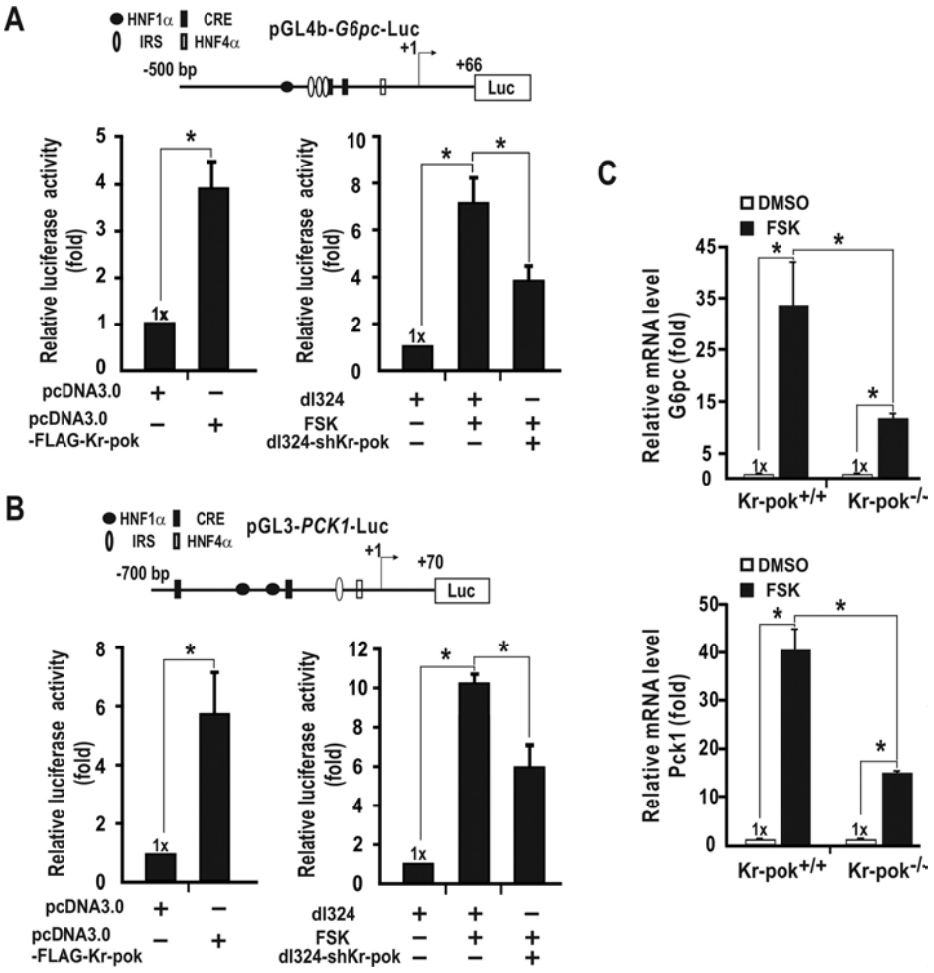

Figure 3. Kr-pok activates transcription of gluconeogenic genes, *G6pc* and *Pck1*. (A, B) The transcriptional activity of Kr-pok on the promoter of *G6pc* and *PCK1* in HepG2 cells. Kr-pok expression plasmids and reporter plasmid of *G6pc* or *PCK1* promoter fused with the luciferase gene were transiently co-transfected and luciferase activity was measured. HepG2 cells were infected with adenovirus dl324 or dl324-shKr-pok and transfected with

reporter plasmid of *G6pc* or *PCK1* promoter. After transfection for 24 hr, cells were treated with FSK for 6 hr prior to harvest and then luciferase activity was measured. Error bars represent standard deviations. (C) Primary hepatocytes of Kr-pok wild and knockout were treated with forskolin for 6 hr and analyzed for *G6pc* and *Pck1* mRNA expression by qRT-PCR. Error bars represent standard deviations. 18S RNA was measured as a control. FSK, Forskolin; *, $p<0.05$; N.S., Not Significant.

4. Kr-pok activates transcription of *G6pc* and *PCK1* gene by acting at the promoter region containing IRS

Since Kr-pok activates transcription of the *G6pc* gene, I tried to map the regulatory elements of the *G6pc* gene promoter responsible for transcriptional activation by Kr-pok. Four *G6pc*-reporter gene fusion reporter plasmid constructs differing in the length of the 5' upstream sequence were prepared. Transient transfection and transcription assays in HepG2 cells showed that ectopic Kr-pok increases transcription of #1 and #2 promoter constructs, but showed little effect on transcription in the two of the constructs with deletion of -500 ~ - 231 region (Fig. 4A). Transcription of *G6pc* by Kr-pok was not changed when regions containing IRSs (Insulin Response Sequences; bp, -202 ~ - 196; bp, -188 ~ -182; bp, -180 ~ -175) were deleted. To examine the effects of IRS in transcriptional activation by Kr-pok, reporter assays were executed with the IRS mutated *G6pc* promoter constructs. While Kr-pok increased reporter activity of the promoter with intact IRS binding sites, it could not increase transcription of the promoter with the mutation at second and third IRS. The mutation at first IRS showed little decrease in transcriptional activation by Kr-pok (Fig. 4B). Also, oligonucleotide pull-down assays showed that Kr-pok binds to the IRS#1 and IRS#2/3 region on *G6pc* promoter. Kr-pok preferentially binds to the IRS#2/3 probe over the

IRS#1 probes tested (Fig. 4C). Based on these results, I investigated whether Kr-pok is targeted to the promoter region containing IRSs by ChIP assays. ChIP assay of the HepG2 cells with ectopic Kr-pok expression showed that Kr-pok binds to the region containing IRSs in *G6pc* promoter (Fig. 4D). Also, ChIP assays showed decrease in Kr-pok binding to the promoter region containing IRSs by Kr-pok Knockdown (Fig. 4D).

Also, the *PCK1* promoter contains one IRS, I examined the functions of IRS (bp, -363 ~ -358) in the transcription activation by Kr-pok. Kr-pok could not activate transcription of *PCK1* promoter with mutated IRS (Fig. 5A). Kr-pok was shown to bind to the IRS region of the *PCK1* promoter by oligonucleotide pull-down assays (Fig. 5B). HepG2 cells were transfected with a FLAG-Kr-pok expression plasmid and ChIP assays showed that Kr-pok binds to the region containing IRS. Also, ChIP assays showed that Kr-pok knockdown decreases Kr-pok binding to the region (Fig. 5C). These data suggested that the promoter regions containing IRSs are important for transcriptional activation of the *G6pc* and *PCK1* gene by Kr-pok.

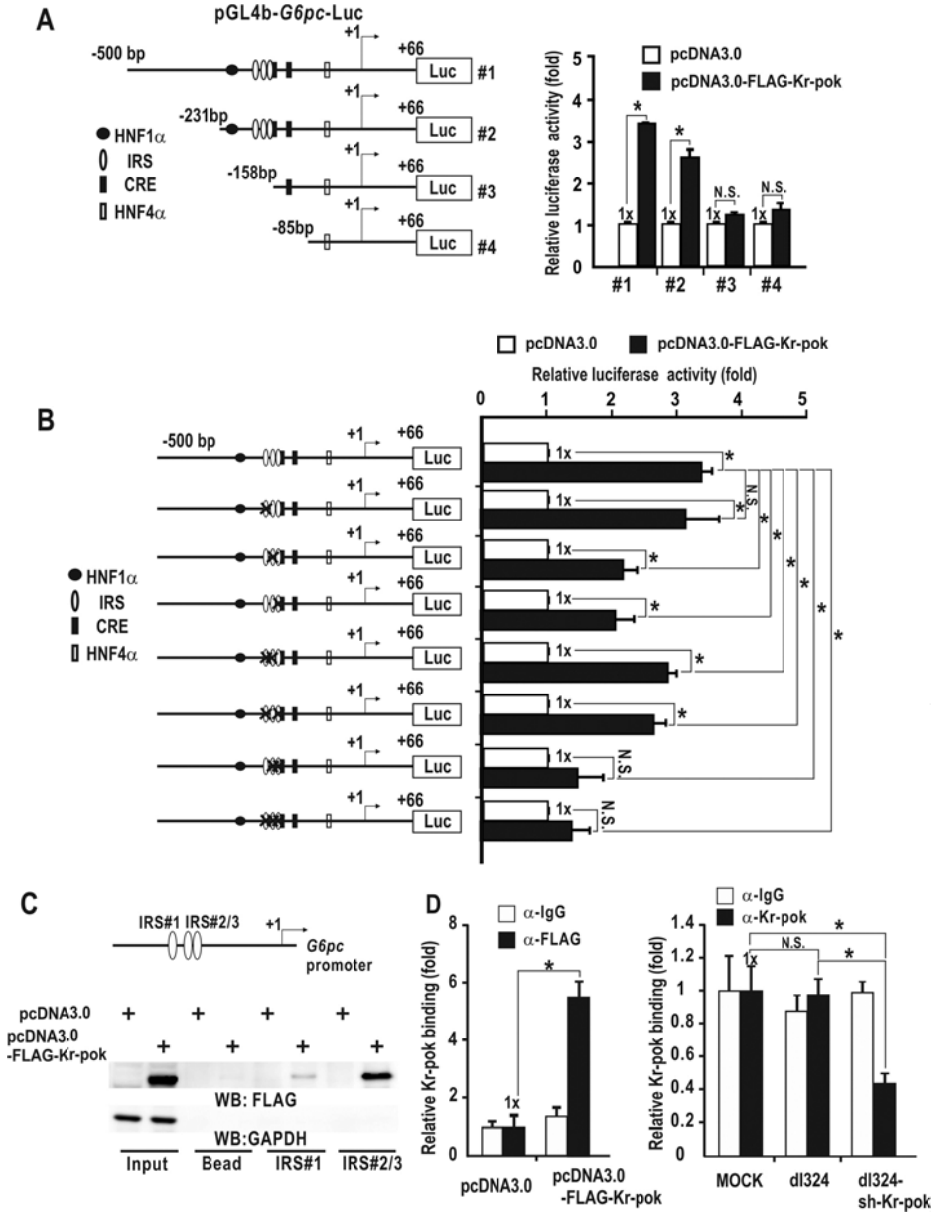

Figure 4. Kr-pok activates *G6pc* gene by acting on the proximal promoter element containing IRS. (A) Transient transcription assays of *G6pc* gene. Structures of various *G6pc* gene promoter constructs fused with luciferase gene, varying in 5' upstream regulatory sequence length. HepG2 cells were transiently co-transfected with Kr-pok expression vector and various pGL4b-*G6pc*-Luc reporter plasmids and analyzed for luciferase activity. All assays were performed in triplicate. Filled circle, HNF1α binding sites; open circle, IRS (Insulin Response Sequence); filled square, CRE(cAMP Response Element); open square, HNF4α binding sites. (B) Transient transcription assays and luciferase gene fusion constructs of *G6pc* promoter designed to test function of IRS in transcriptional regulation by Kr-pok. (C) Oligonucleotide pull-down assays of Kr-pok binding to IRSs of G6pc promoter. These assays were analyzed by western blot using antibody against FLAG. (D) qChIP assays of Kr-pok binding on promoter region (ChIP region: bp, -250 ~ -100) of endogenous *G6pc* gene in HepG2 cells. The ChIP antibodies used were anti-FLAG for FLAG-Kr-pok, anti-Kr-pok or control IgG. Error bars represent standard deviations. *, $p<0.05$; N.S., Not Significant.

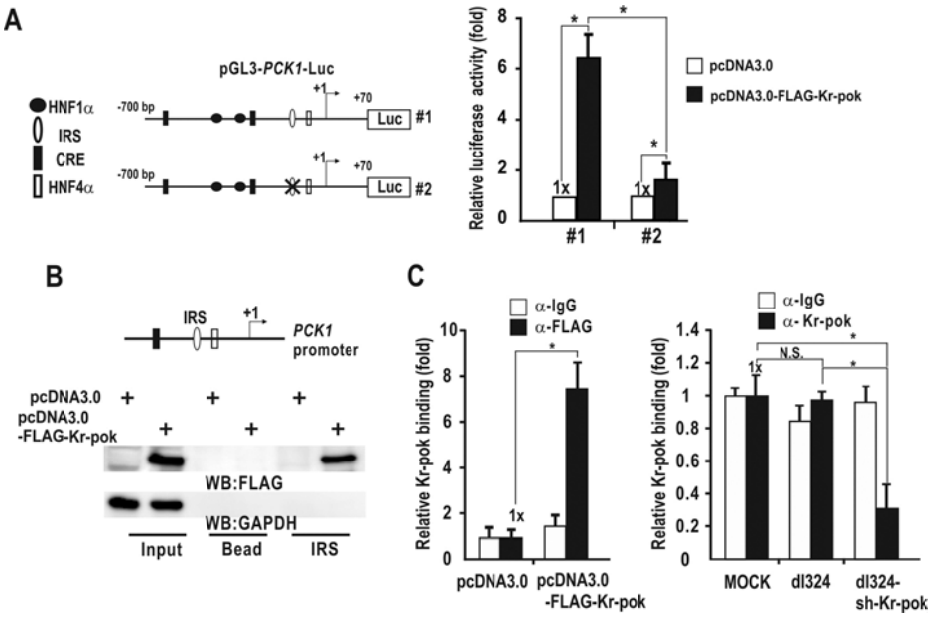

Figure 5. Kr-pok activates the transcription of the *PCK1* promoter by acting on the IRS. (A) Structures of luciferase gene fusion constructs of PCK1 promoter tested and transient transcription assays. HepG2 cells are co-transfected with pcDNA3.0, pcDNA3.0-FLAG-Kr-pok, pGL3-PCK1-Luc (wild type, WT) or pGL3-PCK1-Luc mutant plasmids and luciferase activity was measured. Error bars represent standard deviations. (B) Oligonucleotide pull-down assay of Kr-pok binding to the PCK1 promoter IRS region. (C) qChIP assay of Kr-pok binding on the promoter region containing IRS of endogenous PCK1 gene in HepG2 cells. HepG2 cells were transfected with FLAG-Kr-pok or infected adenovirus dl324-sh-Kr-pok and

immunoprecipitated using the antibodies indicated. Error bars represent standard deviations. *, $p<0.05$; N.S., Not Significant.

5. Kr-pok increases FoxO1 binding to the IRSs of *G6Pc* and *PCK1* promoters

It was reported that transcription factor FoxO1 activates the transcription of *G6pc* and *Pck1* by directly binding to IRSs. In the above, I have shown that Kr-pok could not activate transcription of *G6pc* and *PCK1* when the IRSs were mutated. So, I tested effects of Kr-pok on the 3X IRS FoxO1 response element-luciferase reporter construct in HepG2 cells. Kr-pok alone activated the construct weakly, but Kr-pok more increased transcriptional activity of FoxO1 on the 3X IRS construct (Fig 6A). I investigated whether Kr-pok increases binding of FoxO1 to the region spanning IRSs of *G6pc* and *Pck1* promoters. HepG2 cells were transfected with a Kr-pok expression plasmid. ChIP assays showed that the binding of FoxO1 to the IRS regions of *G6Pc* and *PCK1* promoter is increased in the presence of Kr-pok (Fig. 6B and C).

Fasting increases FoxO1 binding to the IRSs of *G6pc* and *Pck1* promoter. I also examined the effects of Kr-pok of FoxO1 binding to IRSs in forskolin treated cells, HepG2 cells were infected with adenovirus dl324-Kr-pok or dl324- sh-Kr-pok for knockdown, followed by treatment with forskolin for 6 hr. Overexpressed Kr-pok with forskolin treatment increased FoxO1 binding more compared to with forskolin only. However, Kr-pok knockdown by sh-Kr-pok attenuated the increase in FoxO1 binding by forskolin treatment (Fig. 6D and E).

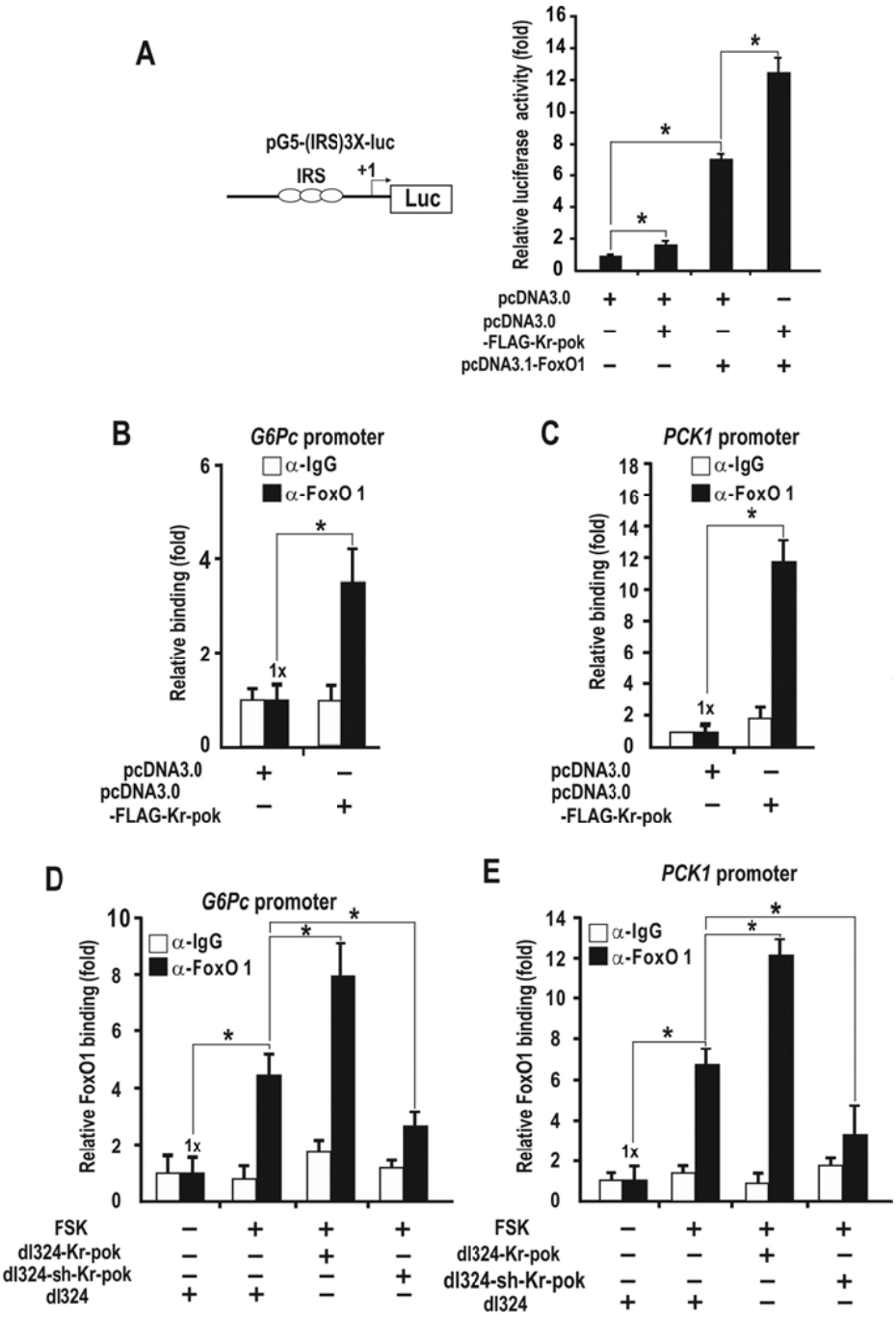

Figure 6. Kr-pok increases FoxO1 binding on the IRS of *G6Pc* and *PCK1* promoters. (A) The transcriptional activity of Kr-pok and/or FoxO1 on 3X IRS reporter constructs in HepG2 cells. Kr-pok and/or FoxO1 expression plasmids and reporter plasmid of 3X IRS fused with the luciferase gene were transiently co-transfected and luciferase activity was measured. (B, C) qChIP assay of endogenous FoxO1 binding to the IRSs of the G6Pc and PCK1 promoter in HepG2 cells. The cells were transfected with Kr-pok expression vector, fixed, and the chromatins were subjected to immunoprecipitation using the indicated antibodies: anti-FLAG for Kr-pok, anti-FoxO1 and control IgG. Error bars represent standard deviations. (D, E) ChIP assay for the recruitment of FoxO1 on the G6Pc and PCK1 gene promoter. HepG2 cells were infected with adenovirus dl324-Kr-pok or dl324-sh-Kr-pok. After 36 hr, the cells were treated with FSK for 6 hr. Cell lysates were precipitated with anti-FoxO1 and control IgG. Error bars represent standard deviations. FSK, Forskolin; *, $P<0.05$.

6. Kr-pok does not change expression and subcellular localization of FoxO1

Since Kr-pok increased FoxO1 binding to *G6Pc* and *PCK1* promoters, I investigated how Kr-pok increases FoxO1 binding to the *G6Pc* and *PCK1* promoters. I tested whether Kr-pok regulates expression of *FoxO1* gene. qRT-PCR showed that overexpression and Knockout of Kr-pok could not increase expression of FoxO1 (Fig. 7A).

Subcellular localization of FoxO1 by signals such as insulin and cAMP is changed. I investigated whether Kr-pok can change subcellular localization of FoxO1. Fractionation in Kr-pok overexpressed HepG2 cell showed that protein level of FoxO1 in cytoplasm and nucleus is not affected by Kr-pok (Fig. 7B). It is reported that insulin is involved in phosphorylation and nucleus exclusion of Foxo1, leading to degradation of FoxO1. Also, I tested whether Kr-pok affect phosphorylation of FoxO1 by insulin. Overexpression and knock down of Kr-pok did not affect phosphorylation of FoxO1 in insulin-treated primary hepatocytes (Fig. 7C). But, co-immunoprecipitation of Kr-pok wild and knockout livers showed that the Kr-pok and FoxO1 proteins interact with each other (Fig. 7D).

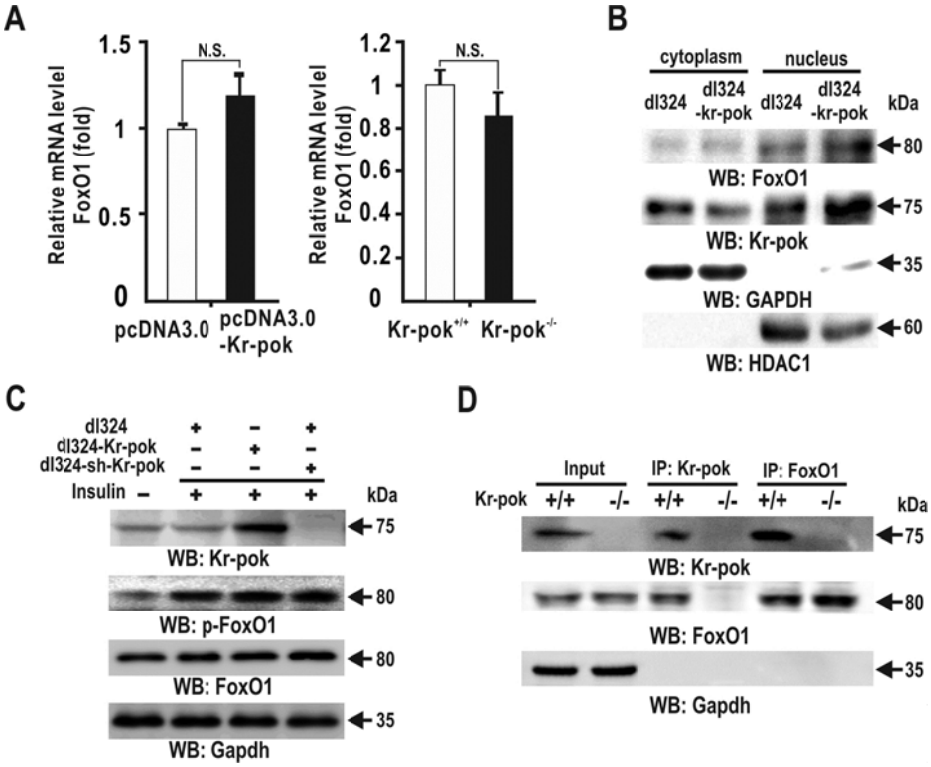

Figure 7. Kr-pok does not affect expression, subcellular localization and phosphorylation of FoxO1. Kr-pok interacts with FoxO1. (A) qRT-PCR of endogenous FoxO1 expression. cDNA for qRT-PCR were prepared from the cell lysates transfected with a Kr-pok expression plasmid. And cDNA from wild and Kr-pok knockout mice livers was used for qRT-PCR. Error bars represent standard deviations. (B) Subcellular localization of FoxO1 by expression of Kr-pok. HepG2 cells infected with control dl324 or dl324-Kr-pok adenovirus were fractionated followed by western blot analysis of

cytoplasm and nucleus fractions. GAPDH, Cytoplasm marker protein; HDAC1, Nucleus marker protein. (C) Change of phosphorylation of FoxO1 by Kr-pok. Primary hepatocytes were infected with adenovirus dl324-Kr-pok or dl324-sh-Kr-pok. After 36 hr, the hepatocytes were treated with 10 μM insulin for 6 hr. The lysates of primary hepatocytes were used for analysis of western blot. (D) Co-immunoprecipitation of Kr-pok and FoxO1. Lysates of Kr-pok wild and knockout livers were immunoprecipitated using anti-Kr-pok antibody, IgG and analyzed by western blot using anti-FoxO1 and anti-Kr-pok. GAPDH, control; IP, immunoprecipitation. *P<0.05; N.S., Not Significant.

7. Kr-pok decreases acetylation of FoxO1

Because Kr-pok could not change the expression, subcellular localization and phosphorylation of FoxO1, I investigated how Kr-pok can increase FoxO1 binding to *G6Pc* and *PCK1* promoters. It was reported that deacetylation of FoxO1 by gluconeogenic signals increases its transcriptional activity. Co-immunoprecipitation and western blots in mice livers showed that Kr-pok and FoxO1 proteins interact with each other (Fig. 7D). I investigated whether Kr-pok affects acetylation of FoxO1. Acetylation of FoxO1 was increased in Kr-pok knockout mice livers (Fig. 8A). Co-immunoprecipitation revealed that FoxO1 acetylation was decreased in Kr-pok overexpressed HepG2 cells (Fig. 8B). Also, forskolin treatment decreased FoxO1 acetylation, while Kr-pok knockdown with forskolin treatment did not decrease FoxO1 acetylation (Fig. 8C).

Based on previous studies that Kr-pok represses transcription of *CDKN1A* gene through interaction with HDAC3, I investigated that Kr-pok interacts with FoxO1 and HDAC3. Co-immunoprecipitation and western blot assays of the HEK293A cells transiently transfected with HA-HDAC3 expression plasmid and/or FLAG-Kr-pok expression plasmid showed that Kr-pok interacts with FoxO1 and HDAC3 (Fig. 8D). Kr-pok knockdown by sh-Kr-pok reduced the interaction between FoxO1 and HDAC3 (Fig. 8E). As

previous studies showed that FoxO1 deacetylation through interaction with HDAC3 increases its ability to bind DNA, I examined the association of HDAC3 with *G6Pc* and *PCK1* promoters by Kr-pok. Forskolin treatment resulted in increased binding of HDAC3 to *G6pc* and *PCK1* promoters, which is consistent with the increased deacetylation and binding of FoxO1 to the promoters. On the contrary, ChIP assays showed that binding of HDAC3 to the *G6Pc* and *PCK1* promoters were decreased by Kr-pok knock down upon treatment with forskolin, leading to reduced deacetylation and binding of FoxO1 to the promoters (Fig. 8F and G). ChIP assays further showed that Kr-pok, FoxO1, Hdac3 and NCoR bind to *G6pc* and *Pck1* promoters in Kr-pok wild and knockout mice liver tissues. Fasting increased binding of Kr-pok, FoxO1, Hdac3 and NCoR to the promoters in Kr-pok wild livers. However, the binding of FoxO1, Hdac3 and NCoR was less increased in Kr-pok knock out livers compared to in Kr-pok wild type livers (Fig. 9A and B). Liver tissues used in ChIP assays showed that knockout of Kr-pok decrease protein levels of G6pc and Pck1 (Fig. 9C).

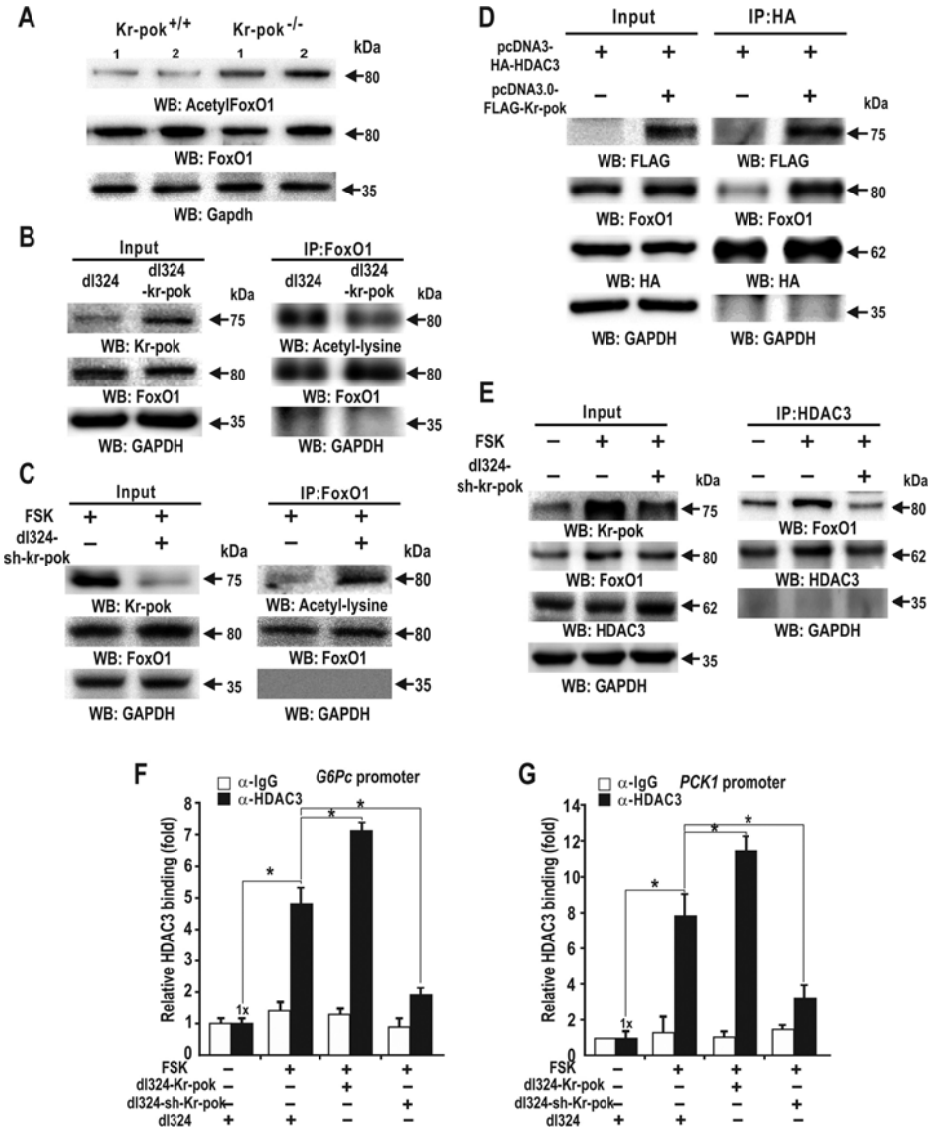

Figure 8. Kr-pok recruits HDAC3 to deacetylate FoxO1. (A) Western blot analysis of acetylation FoxO1 in Kr-pok wild and knockout mice livers. (B) Cell lysates prepared from HepG2 cells transfected with FLAG-Kr-pok

expression plasmids were immunoprecipitated using anti-FoxO1 antibody and analyzed by western blot using the antibodies indicated. Anti-acetyl-lysine was used to detect acetylation of FoxO1. (C) HepG2 cells were infected with adenovirus dl324-sh-Kr-pok for 36 hr and then treated with FSK for 6 hr. The cell lystes were immunoprecipitated and analyzed by western blot as depicted in (B). (D) Co-immunoprecipitation and western blot assays of protein interaction between HDAC3 and FoxO1. HEK293A cell extracts with ectopic HA-HDAC3 and/or FLAG-Kr-pok expression were immunoprecipitated with ant-HA antibody. The precipitates were analyzed by western blotting with anti-HA, anti-FLAG, anti-FoxO1 and anti-GAPDH antibody. (E) HepG2 cells infected with adenovirus dl324-sh-Kr-pok and treated with FSK for 6 hr were immunoprecipitated using anti-HDAC3 antibody and analyzed by western blot using the antibodies indicated. (F, G) qChIP assay of endogenous HDAC3 binding to the IRSs of the G6Pc and PCK1 promoter in HepG2 cells. The cells were infected with adenovirus dl324-sh-kr-pok for 36 hr and treated with FSK for 6 hr. The cells were fixed, and the chromatins were subjected to immunoprecipitation using the antibodies indicated: anti-HDAC3 for HDAC3 and control IgG. Error bars represent standard deviations. FSK, Forskolin; *$P<0.05$.

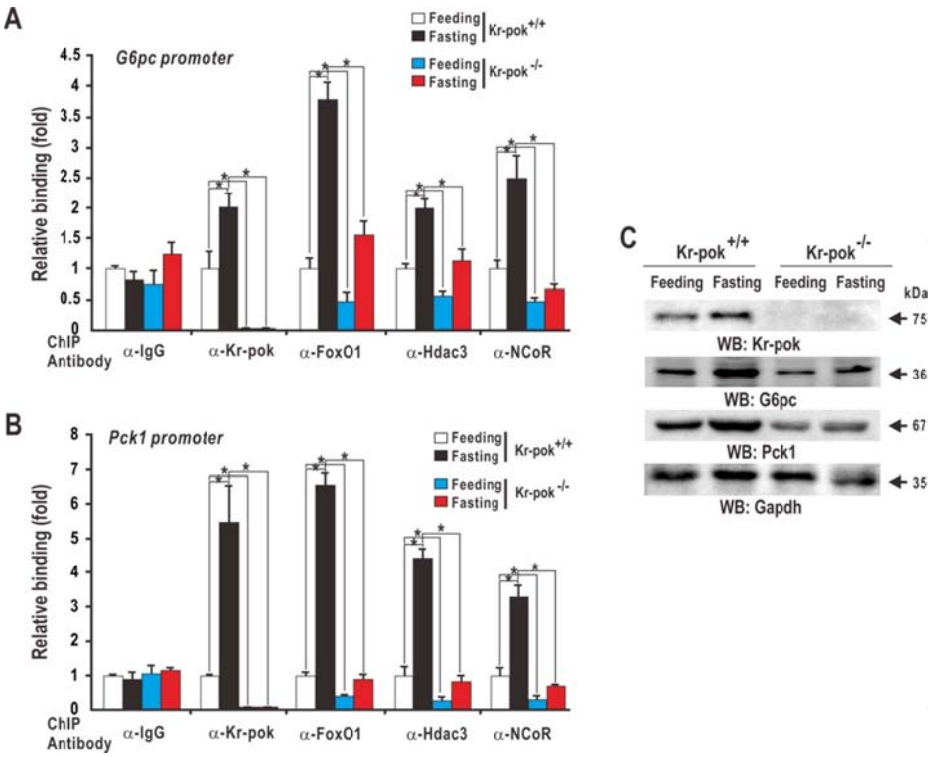

Figure 9. Kr-pok increases recruitment of FoxO1, Hdac3 and NCoR to the *G6pc* and *Pck1* promoters. (A, B) qChIP assay of endogenous Kr-pok, FoxO1, Hdac3 and NCoR binding to the IRSs of the *G6pc* and *Pck1* promoter in wild and Kr-pok knockout livers. The livers were washed and fixed by perfusion. The lysates were sonicated and subjected to immunoprecipitation using the indicated antibodies: anti-, Kr-pok, FoxO1,Hdac3, NCoR and control IgG (C) Western blot analysis of the liver tissue used for qChIP assay. GAPDH, control; *P<0.05.

8. Forskolin increases protein stability of Kr-pok

Above results showed that forskolin treatment of HepG2 cells and fasting of mice increase Kr-pok protein expression. But, mRNA expression of Kr-pok was not increased by treatment of forskolin and fasting. I investigated whether protein stability of Kr-pok is modulated by forskolin. Exogenous Kr-pok transfected with FLAG-Kr-pok expression plasmid in HEK 293A cells was increased in protein levels by forskolin treatment, while mRNA expression of transfected Kr-pok was not changed (Fig. 10A). I also measured the protein stability of Kr-pok by a cycloheximide chase assay of HepG2 cells treated with forskolin. Endogenous Kr-pok of the forskolin treated cells was degraded more slowly than that of the control cells (Fig. 10B). These results suggested that Kr-pok expression may be regulated at the post-transcriptional level.

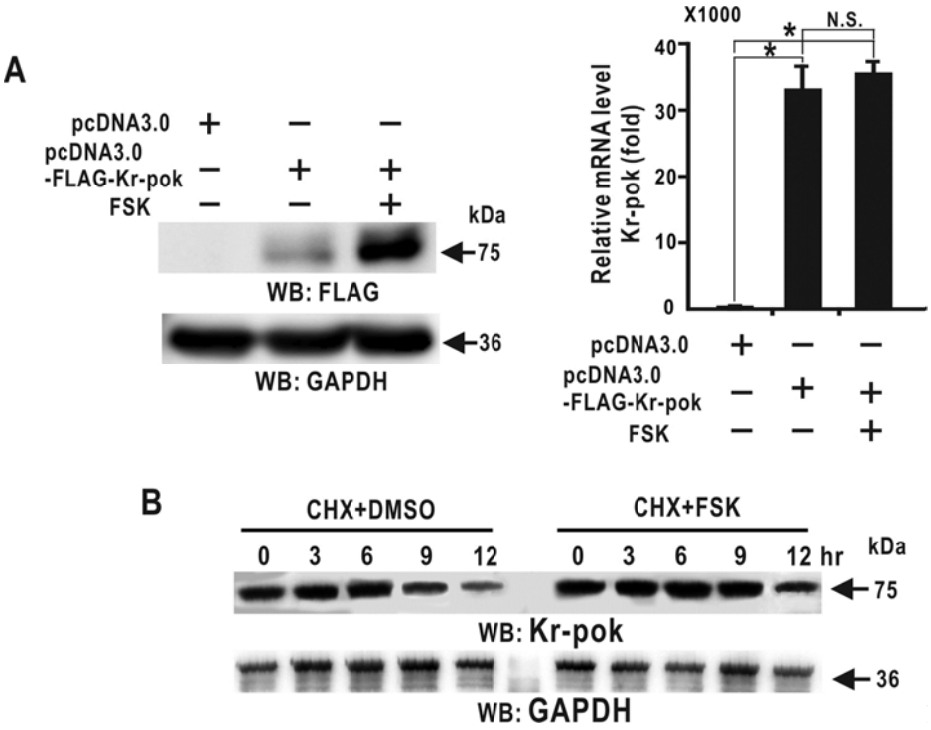

Figure 10. Protein stability of Kr-pok is increased by forskloin. (A) HepG2 cells were transfected with FLAG-Kr-pok expression plasmid, incubated for 24 hr and then treated with FSK for 6 hr. Exogenous Kr-pok expression was analyzed by western blot using anti-FLAG Ab and qRT-PCR. Error bars represent standard deviations. (B) The HepG2 cells were treated with vehicle or 10 μM FSK. Then, the cells were treated with 10 μM cycloheximide. The cells were lysed at the indicated times for western blot. Endogenous Kr-pok expression was analyzed by western blot using Kr-pok antibody. FSK, Forskolin; GAPDH, control.

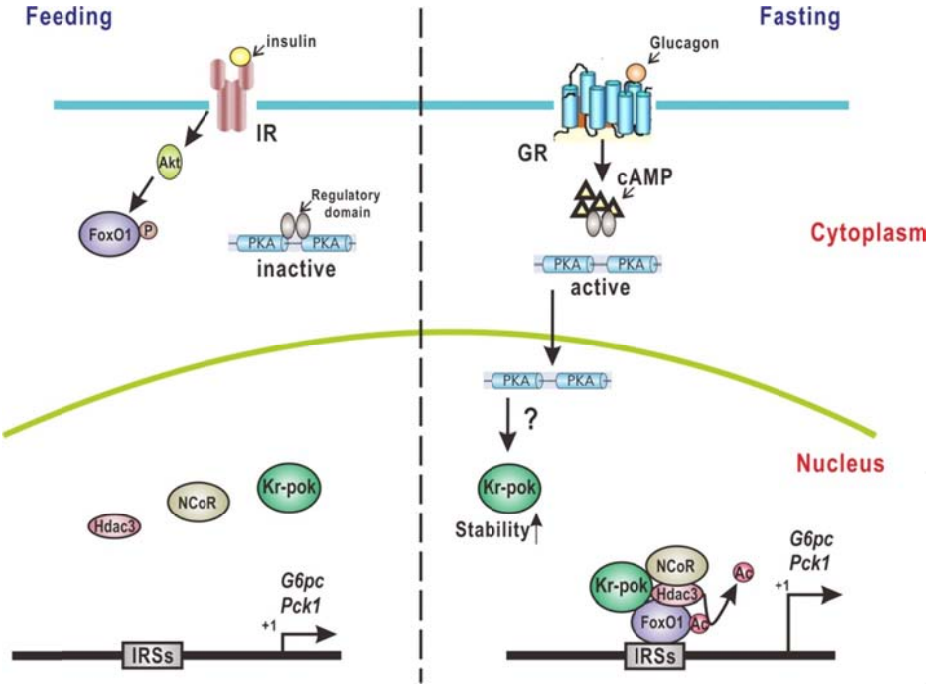

Figure 11. Hypothetical model of transcriptional regulation of *G6pc* and *Pck1* by Kr-pok. Feeding leads to enhanced secretion of pancreatic insulin and insulin binds to insulin receptor (IR) to activate of insulin signaling pathways in liver. Activated Akt also directly phosphorylates FoxO1, inhibiting translocation into nucleus. In fasting, binding of glucagon to glucagon receptor (GR) triggers the activation of adenylyl cyclases, which catalyse the production of cAMP from ATP. Binding of cAMP to protein kinase A (PKA) migrates PKA to the nucleus. This hormone signal increases

protein stability of Kr-pok. Kr-pok increases recruitment of FoxO1, Hdac3 and NCoR to *G6pc* and *Pck1* promoters. Deacetylation of Foxo1 by Hdac3 promotes transcription of *G6pc* and *Pck1*.

IV. DISCUSSION

It is reported that the POK family proteins regulate many biological processes, including embryonic development, cell differentiation, inflammation, apoptosis, and oncogenesis. Kr-pok was first identified as one of the POK family proteins with growth inhibitory activity in a cervical carcinoma cell line.[42] Recent studies have revealed the biological functions of Kr-pok. Kr-pok activates transcription of FASN (fatty acid synthase) involved in synthesis of fatty acids.[16] Also, Kr-pok function as a proto-oncoprotein repressing transcription of *CDKN1A*.[15] Kr-pok mRNA is expressed in various tissues of the mouse including the brain, liver, heart, kidney, WAT, BAT, muscle and very little in spleen. Normal physiology function of Kr-pok has not yet been reported. The size of the gonadal white adipocytes of the old Kr-pok knock-out mice is significantly decreased compared to that of Kr-pok wild type mice (unpublished data) and blood glucose level of Kr-pok knockout mice was lower than that of that of the wild type mice. The study of Kr-pok knockout mice provides clues about functions of Kr-pok involved in metabolism.

Intrigued by these initial observations, I tried to analyze the metabolic changes of Kr-pok knockout mice. Glucose, insulin tolerance tests and

glucose production assays of primary hepatocytes showed decreases of blood glucose level in Kr-pok knockout mice. Glycogenolysis (breakdown of glycogen to glucose) and gluconeogenesis mainly contribute to production of glucose.[23] Also, Glucose transporter 2 (Glut 2) in livers is involved in regulation of blood glucose level.[43] Microarray differential gene expression analysis showed that expression of Glut 2 and the genes involved in glycogenolysis and gluconeogenesis such as Pc, Fbp, Pyg 1 and Pgm 5 is not changed in Kr-pok knockout livers. I found that loss of Kr-pok in mice livers decreases the expression of gluconeogenic enzymes such as G6pc and Pck1, and *G6pc* and *Pck1* genes are the transcriptional activation target of Kr-pok.

Because expression of *G6pc* and *Pck1* is activated by cAMP/PKA signaling during fasting,[44-45] I investigated whether fasting affects Kr-pok expression in cultured primary hepatocytes. Forskolin which increases cAMP/PKA signaling in mice primary hepatocytes increased expression Kr-pok expression. Also, Kr-pok expression was increased in mice livers by fasting. Interestingly, mRNA expression of Kr-pok was not changed in forskolin-treated primary hepatocytes and also in livers isolated form fasted mice. Kr-pok expression was also increased in hyperglycaemic streptozotocin-induced mice diabetes models, suggesting Kr-pok may be involved in glucose production.

I also studied how Kr-pok regulates transcription of *G6pc* and *Pck1* gene. Kr-pok could activate *G6pc* and *Pck1* gene transcription by acting on the IRSs. Glucocorticoid receptor, HNF4α, Nur77 and FOXO1 are transcription factors activating transcription of *G6pc* and *Pck1*. In particular, FoxO1 is known to increase gluconeogenesis by acting on IRSs.[46-47] Co-transfection with FoxO1, Kr-pok and pG5-(IRS)3X-Luc reporter construct increased promoter activity significantly. Kr-pok increased FoxO1 binding to IRSs on the promoters of *G6pc* and *Pck1*. I investigated whether Kr-pok affects the expression and subcellular localization of FoxO1. Expression of FoxO1 was not changed in Kr-pok knockout mice livers. Also, Kr-pok could not affect the subcellular localization of FoxO1 in HepG2 cells. Insulin treatment phosphorylates and inactivates FoxO1 through serine/threonine kinase Akt, in mammalian liver.[33-34] Kr-pok could not increase or decrease phosphorylation of FOXO1 by insulin treatment in hepatocytes.

Once FoxO1 enter into nucleous, it binds to the promoters of gluconeogenic target genes and activates transcription, at least in part through deacetylation and activation of FoxO1. Dephosphorylation of HDAC3 results in deacetylation of nuclear FOXO1, enhancing FOXO1 DNA-binding activity to the gluconeogenic gene promoters.[39] It was reported that Kr-pok interacts with HDAC3 and NuRD complex. I found that Kr-pok increases interaction

between FoxO1 and HADC3, promoting deacetylation of FoxO1, which activates transcription of *G6pc* and *Pck1*. Recruitment of FoxO1, Hdac3 and NCoR to *G6pc* and *Pck1* promoters was decreased in Kr-pok knockout mice livers subjected to fasting compared to that of wild type mice.

Proteins are often subjected to posttranslational modifications in response to signals, including acetylation, methylation, phosphorylation, and SUMOylation. These modifications can regulate stability and localization of proteins. Stability of Kr-pok was increased by forskolin treatment, suggesting posttranslational modifications of Kr-pok. Also, my studies demonstrated a role for Kr-pok in deacetylation of FoxO1 by interaction with HDAC3 during fasting in mice liver. FoxO1 was previously shown as a target of SIRT1 in a number of cell types, such as in muscle.[48] Also, Kr-pok represses transcription of SIRT1(unpublished data). It will be interesting to investigate relative contributions of SIRT1 versus HDAC3-Kr-pok complex in the control of FoxO1 acetylation in liver.

The expression of gluconeogenesis enzymes such as PEPCK and G6Pase could be activated by glucagon via cAMP/PKA and CREB because of CREB-response elements on their promoters. However, a variety of other transcription factors were also shown to play key roles in the regulation of PEPCK or G6Pase, such as CREBH, HNF4α, FOXO1, and KLF15.

Deficiency of one of these genes resulted in fasting hypoglycemia and reduced expression of gluconeogenesis enzymes, suggesting that these transcription factors could not compensate each other and that all of them are required for the liver to fully activate expression of gluconeogenic genes. Also, G6pc and Pck1 are mainly expressed in livers and their expression is increased during fasting. Accumulating evidences showed the importance of gluconeogenesis regulation for glucose homeostasis. Most of glycolytic enzymes are used for both glycolysis and gluconeogenesis. G6Pc and PCK1 are enzymes only for gluconeogenesis. Therefore, transcriptional regulation of *G6pc* and *Pck1* is important for glucose homeostasis. The significance of the results presented here is that Kr-pok is a new transcription factor increasing gluconeogenesis through deacetylation of FoxO1. Kr-pok activated transcription of *G6pc* and *Pck1*, increasing deacetylation of FoxO1 through promoting the interaction of FoxO1 and HDAC3. Kr-pok increases glucose production through transcriptional regulation of *G6pc* and *Pck1*. Identification of Kr-pok's role in hepatic gluconeogenesis will expand our knowledge on the molecular mechanism of the regulatory factors involved in blood glucose homeostasis.

V. CONCLUSION

Analysis of Kr-pok knockout mice showed that Kr-pok increases blood glucose levels and glucose production in cultured primary hepatocytes. Also, I found that expression of Kr-pok was induced in fasted mice and forskolin-treated cultured primary hepatocytes. I investigated whether and how Kr-pok can regulate expression of the genes involved in gluconeogenesis and found that Kr-pok is involved in glucose homeostasis through transcriptional activation of gluconeogenic genes, G6pc and Pck1. Kr-pok binds to IRSs which is FoxO1 binding sites to activate transcription of G6pc and Pck1. Kr-pok decreases FoxO1 acetylation through interaction with HDAC3, which increases transcriptional activity of FoxO1. ChIP assays of Kr-pok wild and knockout mice liver tissues showed that Kr-pok increases recruitment of FoxO1, HDAC3 and NCoR to the *G6pc* and *PCK1* promoters, which results in the transcriptional activation of these genes via deacetylation of FoxO1 (Fig. 11). I investigated how fasting increases expression of Kr-pok. I found that forskolin treatment which mimics fasting states increases protein stability of Kr-pok.

My results suggest that Kr-pok may be one of the glucose metabolism regulators and Kr-pok increases gluconeogenic genes, G6pc and PCK1 by

decreasing FoxO1 acetylation.

REFERENCES

1. Aravind L, Koonin EV. Fold prediction and evolutionary analysis of the POZ domain: structural and evolutionary relationship with the potassium channel tetramerization domain. J Mol Biol. 1999;285:1353–61
2. Costoya JA. Functional analysis of the role of POK transcriptional repressors. Brief Funct Genomic Proteomic. 2007;6:8–18
3. Chevrier S1, Corcoran LM1. BTB-ZF transcription factors, a growing family of regulators of early and late B-cell development. Immunol Cell Biol. 2014;92:481-8
4. Kelly KF, Daniel JM. POZ for effect-POZ-ZF transcription factors in cancer and development. Trends Cell Biol. 2006;16:578–87
5. Heinzel T, Lavinsky RM, Mullen TM, Söderstrom M, Laherty CD, Torchia J, et al. A complex containing NCoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature. 1997;387:43–8
6. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH, Evans RM, et al. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature. 1998;391:811–4
7. Zullo JM, Demarco IA, Pique-Regi R, Gaffney DJ, Epstein CB, Spooner CJ, et al. DNA sequence-dependent compartmentalization and silencing of

chromatin at the nuclear lamina. Cell. 2012;149: 1474–87

8. Chen Z, Brand NJ, Chen A, Chen SJ, Tong JH, Wang ZY, et al. Fusion between a novel Krüppe-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. EMBO J. 1993;12:1161–7
9. Chen W, Cooper TK, Zahnow CA, Overholtzer M, Zhao Z, Ladanyi M, et al. Epigenetic and genetic loss of Hic1 function accentuates the role of p53 in tumorigenesis. Cancer Cell. 2004;6:387–98
10. Issa JP, Zehnbauer BA, Kaufmann SH, Biel MA, Baylin SB. HIC1 hypermethylation is a late event in hematopoietic neoplasms. Cancer Res. 1997;57:1678–81
11. Maeda T, Hobbs RM, Merghoub T, Guernah I, Zelent A, Cordon-Cardo C, et al. Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. Nature. 2005;433:278–85
12. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature. 2004;432:635–9
13. Plaisier CL, Bennett BJ, He A, Guan B, Luis AJ, Reue K, et al. Zbtb16 has a role in brown adipocyte bioenergetics. Nutr Diabetes. 2012;2:e46
14. Control of Hepatic Gluconeogenesis by the Promyelocytic Leukemia Zinc Finger Protein. Mol Endocrinol. 2014;28:1987-98

15. Jeon BN, Kim MK, Choi WI, Koh DI, Hong SY, Kim KS, et al. KR-POK Interacts with p53 and Represses Its Ability to Activate Transcription of p21WAF1/CDKN1A. Cancer Res. 2012;72:1137-48

16. Jeon BN, Kim YS, Choi WI, Koh DI, Kim MK, Yoon JH, et al. Kr-pok increases FASN expression by modulating the DNA binding of SREBP-1c and Sp1 at the proximal promoter. J Lipid Res. 2012;53:755-66

17. Pilkis SJ, el-Maghrabi MR, Claus TH. Hormonal regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Biochem. 1988;57:755-83

18. Cahill, GF Jr. Fuel metabolism in starvation. Annu Rev Nutr. 2006;26:1-22

19. John CW, Greg JB. Gluconeogenesis. eLS. 2016;1-10

20. Giugliano D, Ceriello A, Esposito K. Glucose metabolism and hyperglycemia. Am J Clin Nutr. 2008;87:217-22

21. Giacco F, Brownlee M. Oxidative Stress and Diabetic Complications. Circ Res. 2010;107:1058-70

22. Brownlee M. The Pathobiology of Diabetic Complications A Unifying Mechanism. Diabetes. 2005;54:1615-25

23. Nordlie RC, Foster JD, Lange AJ. (1999) Regulation of glucose production by the liver. Annu. Rev. Nutr. 1999;19:379-406

24. Pilkis SJ, Claus TH. Hepatic gluconeogenesis/ glycolysis: regulation and

structure/function relationships of substrate cycle enzymes. Annu Rev Nutr. 1991;11:465-515

25. Goldstein I, Hager GL. Transcriptional and Chromatin Regulation during Fasting – The Genomic Era. Trends Endocrinol Metab. 2015;26:699-710
26. Roesler WJ. The role of C/EBP in nutrient and hormonal regulation of gene expression. Annu Rev Nutr. 2001;21:141-65
27. Takashima M, Ogawa W, Hayashi K, Inoue H, Kinoshita S, Okamoto Y, et al. Role of KLF15 in regulation of hepatic gluconeogenesis and metformin action. Diabetes. 2010;59:1608–15
28. Oh KJ, Han HS, Kim MJ, Koo SH. CREB and FoxO1: two transcription factors for the regulation of hepatic gluconeogenesis. BMB Rep. 2013;46:567-74
29. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol. 2011;12:141-51
30. Pei L, Waki H, Vaitheesvaran B, Wilpitz DC, Kurland IJ, Tontonoz P. NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. Nat Med. 2006;12:1048–55
31. Lu Y, Xiong X, Wang X, Zhang Z, Li J, Shi G, et al. Yin Yang 1 promotes hepatic gluconeogenesis through upregulation of glucocorticoid receptor.

Diabetes. 2013;62:1064–73

32. Alberts AS, Arias J, Hagiwara M, Montminy MR, Feramisco, JR. Recombinant cyclic AMP response element binding protein (CREB) phosphorylated on Ser-133 is transcriptionally active upon its introduction into fibroblast nuclei. J Biol Chem. 1994;269:7623-30
33. Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell. 2004;117:421-6
34. Barthel A, Schmoll D, Unterman TG. (2005) FoxO proteins in insulin action and metabolism. Trends Endocrinol Metab. 2005;16:183-9
35. Mahmud DL, G-Amlak M, Deb DK, Platanias LC, Uddin S, Wickrema A. Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. Oncogene. 2002;21:1556–62
36. Nasrin N, Ogg S, Cahill CM, Biggs W, Nui S, Dore J, et al. DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc Natl Acad Sci USA. 2000;97:10412–7
37. Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, et al. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature. 2003;423:550–5

38. Matsuzaki H, Daitoku H, Hatta M, Aoyama H, Yoshimochi K, Fukamizu A. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. Proc Natl Acad Sci USA. 2005;102:11278-83

39. Mihaylova MM, Vasquez DS, Ravnskjaer K, Denechaud PD, Yu RT, Alvarez JG, et al. Class IIa Histone Deacetylases Are Hormone-Activated Regulators of FOXO and Mammalian Glucose Homeostasis. Cell. 2011;145:607-21

40. Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, et al. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc. Natl Acad Sci USA. 2004;101:10042-7

41. Zhang L, Rubins NE, Ahima RS, Greenbaum LE, Kaestner KH. Foxa2 integrates the transcriptional response of the hepatocyte to fasting. Cell Metab. 2005;2:141-8

42. Reuter S1, Bartelmann M, Vogt M, Geisen C, Napierski I, Kahn T, et al. APM-1, a novel human gene, identified by aberrant co-transcription with papillomavirus oncogenes in a cervical carcinoma cell line, encodes a BTB/POZ-zinc finger protein with growth inhibitory activity. EMBO J. 1998;17215-22

43. Zhao FQ, Aileen FK. Functional Properties and Genomics of Glucose

Transporters. Curr Genomics. 2007;8:113–28

44. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, et al. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature. 2005;437:1109–11
45. Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol. 2011;12:141–51
46. Nakae J, Biggs WH, Kitamura T, Cavenee WK, Wright CV, Arden KC, et al. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor FoxO1. Nat Genet. 2002;32:245–53
47. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, et al. Inhibition of FoxO1 function is associated with improved fasting glycemia in diabetic mice. Am J Physiol Endocrinol. Metab. 2003;285:718–28
48. Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature. 2009;458:1056

ABSTRACT (IN KOREAN)

Kr-pok의 *G6pc*, *Pck1* 유전자 전사활성화를 통한 포도당 신생합성 조절

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윤재현

포도당 신생합성은 금식 시 혈중 포도당 유지에 중요하다. 포도당 신생합성은 주로 glucose-6-phophatase(G6pc)와 phosphoenolpyruvate carboxykinase(PEPCK) 효소의 유전자 발현 변화를 통해 조절된다.

Kr-pok knockout 생쥐의 혈중 포도당 농도는 대조군 생쥐의 비하여 낮다. 또한 Kr-pok knockout 생쥐와 정상 대조군 생쥐 간에서의 유전자 발현 분석을 통해 Kr-pok knockout 생쥐에서 포도당 신행합성 유전자 *G6pc*와 *Pck1* 발현 감소를 관찰하였다. 본 연구자는

포도당 신생합성 조절에서의 Kr-pok의 역할을 연구하였다.

Kr-pok 발현은 금식 시 생쥐 간에서 증가하였다. 그리고 생쥐 간 세포에서의 forskolin 처리는 Kr-pok 발현을 증가시켰다. 이와 같은 결과는 Kr-pok가 식이 제한 시에 발현이 증가되는 *G6pc*와 *Pck1*의 전사 활성인자로서 작용할 수 있음을 의미한다.

나아가 본 연구자는 Kr-pok가 어떻게 *G6pc*와 *Pck1*의 전사를 증가시키는지 연구하였다. 전사 조절 기전 연구를 통해 Kr-pok는 *G6pc*와 *Pck1* promoter의 IRS (Isulin Response Sequence)에 작용함을 알 수 있었다. IRS는 식이 제한 시 FoxO1이 작용하여 유전자의 전사 활성화에 관여하는 promoter 조절 부위로 Kr-pok는 FoxO1의 IRS에 대한 결합을 증가시켰다.

또한 Kr-pok에 의하여 FoxO1의 결합과 전사활성화가 어떻게 증가되는지 연구하였다. Kr-pok는 FoxO1과 FoxO1의 탈아세틸화에 관여하는 HDAC3의 상호작용을 증가시키어, Kr-pok는 *G6pc*와 *Pck1* promoter에 FoxO1, HDAC3, NCoR의 작용을 증가시켰다. Kr-pok는 FoxO1의 *G6pc*와 *Pck1*에 대한 전사조절 능력을 활성화 시키도록 FoxO1의 아세틸레이션 감소에 기여함을 알 수 있다. 이와 함께 Forskolin 처리 시에 Kr-pok 발현 증가는 단백질 안정성 증가에

의하여 이루어졌다.

본 연구를 통하여 Kr-pok가 금식 시 발현이 증가하여 포도당 신생합성의 주요 효소인 *G6pc*와 *Pck1* 유전자들의 전사를 활성화시킴으로써 포도당 합성을 유도하여 포도당 대사 항상성 유지에 관여하는 대사 조절자로 작용함을 발견하였다.

핵심되는 말: Kr-pok, 전사, 포도당신생합성, G6pc, Pck1, FoxO1, HDAC3

PUBLICATION LIST

1. Lee KM, Choi WI, Koh DI, Kim YJ, Jeon BN, Yoon JH, et al. The proto-oncoprotein KR-POK represses transcriptional activation of CDKN1A by MIZ-1 through competitive binding. Oncogene. 2012. Mar 15;31(11):1442-58.
2. Jeon BN, Kim YS, Choi WI, Koh DI, Kim MK, Yoon JH, et al. Kr-pok Increases FASN Expression by Modulating the DNA Binding of SREBP-1c and Sp1 at the Proximal Promoter. J Lipid Res. 2012. Apr;53(4):755-66.
3. Choi WI, Jeon BN, Yoon JH, Koh DI, Kim MH, Yu MY, et al. The proto-oncoprotein FBI-1 interacts with MBD3 to recruit the Mi-2/NuRD-HDAC complex and BCoR and to silence p21WAF/CDKN1A by DNA methylation. Nucleic Acids Res. 2013 Jul;41(13):6403-20.
4. Kim HY, Yang DH, Shin SW, Kim MY, Yoon JH, Kim S, et al. CXXC5 is a transcriptional activator of Flk-1 and mediates bone morphogenic protein-induced endothelial cell differentiation and vessel formation. FASEB J. 2014 Feb;28(2):615-26.
5. Koh DI, Yoon JH, Kim MK, An H, Kim MY, Hur MW. Kaiso is a key regulator of spleen germinal center formation by repressing Bcl6 expression in splenocytes. Biochem Biophys Res Commun. 2013 Dec

13;442(3-4):177-82.

6. Yoon JH, Choi WI, Jeon BN, Koh DI, Kim MK, Kim MH, et al. Human Kruppel-related 3 (HKR3) is a novel transcription activator of alternate reading frame (ARF) gene. J Biol Chem. 2014 Feb 14;289(7):4018-31.
7. Choi WI, Yoon JH, Kim MY, Koh DI, Licht JD, Kim K, et al. Promyelocytic leukemia zinc finger-retinoic acid receptor α (PLZF-RARα), an oncogenic transcriptional repressor of cyclin-dependent kinase inhibitor 1A (p21WAF/CDKN1A) and tumor protein p53 (TP53) genes. J Biol Chem. 2014 Jul 4;289(27):18641-56.
8. Jeon BN, Kim MK, Yoon JH, Kim MY, An H, Noh HJ, et al. Two ZNF509 (ZBTB49) isoforms induce cell-cycle arrest by activating transcription of p21/CDKN1A and RB upon exposure to genotoxic stress. Nucleic Acids Res. 2014 Oct;42(18):11447-61.