



Hepatitis B X-interacting Protein Restrains Hepatic Gluconeogenesis Through Suppressing The Expression of PEPCK*

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Abstract Hepatitis B X-interacting protein (HBXIP) is able to mediate glucose metabolism reprogramming in breast cancer. To investigate the physiological functions of HBXIP in regulation of glucose metabolism, we generated liver-specific HBXIP conditional knockout C57BL/6 mice using Cre/loxP approach. Liver HBXIP^{-/-} mice exhibited a phenotype of glycometabolic dysregulation, such as higher fasting blood glucose level, accumulation of hepatic glycogen, recession of blood glucose profiles, and elevation of gluconeogenesis. The expression levels of PEPCK were remarkably up-regulated in the liver tissues of HBXIP^{-/-} mice. Then, we validated that HBXIP expressions were negatively correlated with those of PEPCK in 30 clinical liver tissues. Mechanistically, luciferase reporter gene assays and ChIP assays showed that HBXIP could inhibit the expression PEPCK at transcription level. Taken together, our findings indicate that HBXIP restrains hepatic gluconeogenesis through suppressing the expression of PEPCK.

Key words HBXIP, PEPCK, gluconeogenesis, glucose metabolism

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1 Introduction

Normal blood glucose levels are tightly maintained within a narrow range by a sophisticated regulatory system to provide a constant fuel supply for the body. The liver displays a unique role in maintaining blood glucose homeostasis^[1]. Gluconeogenesis, the net production of glucose from substrate molecules, is critical for the adaptation to fasting conditions^[2]. Gluconeogenesis takes place mainly in the liver, accounting for up to 80% of total glucose production in healthy individuals during a prolonged fast^[3]. Abnormal activation of hepatic gluconeogenesis accounts for excessive glucose production, contributing to metabolic disorders such as type 2 diabetes^[4]. Thus better understanding of the physiology and physiopathology of hepatic

gluconeogenesis will help us to fight diabetes. The gluconeogenesis pathway includes several key enzymes, such as the first and the last rate-limiting enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P)^[5].

Mammalian hepatitis B X-interacting protein (HBXIP, also named as LAMTOR5) as a conserved

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protein is widely expressed in tissues^[6]. It is originally identified by its interaction with C terminus of the hepatitis B virus X protein^[7]. HBXIP can suppress apoptosis in a survivin-dependent manner^[8]. In addition, HBXIP is required for amino acid sensing by the mTORC1 pathway as a regulator component^[9]. Our group has reported that HBXIP was able to induce glucose metabolism reprogramming through suppressing SCO2 and PDHA1 in breast cancer^[10]. Therefore, we are interested in the role of HBXIP in the regulation of glucose metabolism in normal liver.

In this study, we enrolled liver-specific HBXIP knockout mice to study the role that HBXIP played in liver glucose metabolism. We uncovered that liver HBXIP knockout mice exhibited abnormal glucose metabolism, in which HBXIP suppressed hepatic gluconeogenesis through inhibiting the expression of PEPCK. Our findings provide new insights into glucose metabolism regulation by HBXIP.

2 Materials and methods

2.1 Liver specific HBXIP knockout mice

Liver conditional HBXIP knockout mice were generated using Cre-loxP approach^[11]. LoxP was inserted into the flank regions of the second exon of HBXIP. The liver conditional knockout mice were generated by crossing floxed HBXIP mice with the albumin (Alb) - Cre transgenic mice^[12]. Alb-Cre transgenic mice displayed that Cre recombinase was expressed selectively in liver cells. The genotypes of filial mice were identified by PCR analysis using primers upstream and downstream of the loxP in the intron of HBXIP gene. Analysis of the phenotype was conducted in liver HBXIP^{-/-} mice, and floxed-HBXIP littermates were used as WT controls. The 8-week-old male C57BL/6J mice were used in this study. The mouse housing environment includes a 1 : 1 light-dark cycle within 24 hours, constant room temperature (22–25°C), free access to water and diet. All procedures were performed according to the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The primers used for the loxP and Cre identification were as follows: LoxP forward 5'-TTTTTGTCACCTC-TCGCCTTTG-3' and reverse 5'-GCTGGTATGTAC-TCACCCATT-3'; Cre forward 5'-TCCCACCGT-CAGTACGTGAGATA-3' and reverse 5'-AACGAG-TGATGAGGTTTCGAAGA-3'.

2.2 RT-PCR and qRT-PCR

Total RNA was extracted from cells or tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized by PrimeScript reverse transcriptase (TaKaRa Bio, Dalian, China) and oligo-(dT) following the manufacturer's instructions. QRT-PCR was performed by a Bio-Rad sequence detection system according to the manufacturer's instructions using double-stranded DNA-specific SYBR GreenPremix Ex Taq™ II Kit (TaKaRa Bio). Experiments were conducted in duplicate in three independent assays. Relative transcriptional folds were calculated as $2^{-\Delta\Delta Ct}$. ACTB was used as an internal control for normalization. The primers used for RT-PCR and qRT-PCR were as follows: HBXIP forward 5'-CGAGGTTTGCGGTGAAGG-3' and reverse 5'-CCACGGCAGCCCAGATTA-3'; PEPCK forward 5'-CATAACGGTCTGGACTTCTCTGC-3' and reverse 5'-GAATGGGATGACATACATGGTG-CG-3'.

2.3 Histological analyses

Mice liver tissues were fixed in formalin, dehydrated and embedded in paraffin for immunohistochemistry (IHC) and Periodicacid-Schiff (PAS) staining. Immunohistochemistry assay was carried out as described previously^[13].

2.4 Cell culture

The Chang liver cells were maintained in Dulbecco's Modified Eagle's medium (Gibco, CA, USA). All cell lines were supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco, CA, USA), 100U/ml penicillin and 100g/L streptomycin and grown at 5% CO₂ and 37°C.

2.5 Western blot analysis and small interference RNA (siRNA)

Whole cell lysates were prepared from liver tissues of mice or cells. Western blot analysis was performed according to the protocols described previously^[14]. Primary antibodies used were rabbit anti-HBXIP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PEPCK antibody (Proteintech Group, Chicago, IL, USA). The siRNA duplexes targeting human HBXIP mRNA were used as previously described^[15].

2.6 Glucose tolerance test (GTT) and pyruvate tolerance test (PTT)

Overnight-fasted mice were injected intraperitoneally with glucose (2g/kg body weight) or

pyruvate (2g/kg body weight). Blood glucose levels were measured from tail vein blood collected at the designated times using Bayer Brand Glucometer (Bayer Health Care, Mishawaka, Indiana, USA). Data were expressed in blood glucose concentration (mg/dL).

2.7 Luciferase reporter gene assays

Adherent Chang liver cells were seeded into 24-well plates, respectively. After 12 h, the cells were transfected with the constructs containing different fragments of PEPCK promoter or pGL3-Basic as a negative control, with the pRL-TK plasmid (Promega) which was used as internal normalization. Cell extracts were harvested after 24 h and lysed using lysis buffer (Promega).

2.8 Chromatin immunoprecipitation (ChIP) assays

The ChIP assays were performed using an EpiQuik™ chromatin immunoprecipitation kit from Epigentek Group Inc. (Brooklyn, NY). Protein/DNA complexes were immunoprecipitated with anti-HBXIP antibody and with mouse IgG as a negative control antibody. Amplification of soluble chromatin prior to immunoprecipitation was used as an input control. DNA from these samples was then subjected to PCR analysis. The primers used for RT-PCR were as follows: PEPCK forward 5'-ATCTGAATACAG-

GAAACATAGCA-3' and reverse 5'-GTCTTAGAG-TTTAGGGAGTGGAG-3'.

2.9 Statistical analysis

Statistical analyses of data were performed by Student's *t*-test. Data were expressed as the mean $\pm$ SEM. $P < 0.05$ were considered statistically significant. The correlation between PEPCK and HBXIP mRNA levels in clinical liver tissues was determined with Pearson's correlation coefficient.

3 Results

3.1 Generation of liver-specific HBXIP^{-/-} mice

HBXIP, which is a conserved ubiquitously expressed protein, is able to induce glucose metabolism reprogramming through suppressing SCO2 and PDHA1 in breast cancer^[10,16]. To investigate the role of HBXIP in liver, we developed an HBXIP knockout allele using Cre-loxP approach. According to the model (Figure 1a), the loxP was inserted into the flank regions of the HBXIP exon2 and a floxed allele of HBXIP (HBXIP^{fllox}) was generated by homologous recombination in embryonic stem (ES) cells. The expression of Cre recombinase deleted the exon2 of HBXIP and generated a deletion allele (HBXIP^{-/-}), disrupting the transcription of HBXIP. Our data showed that

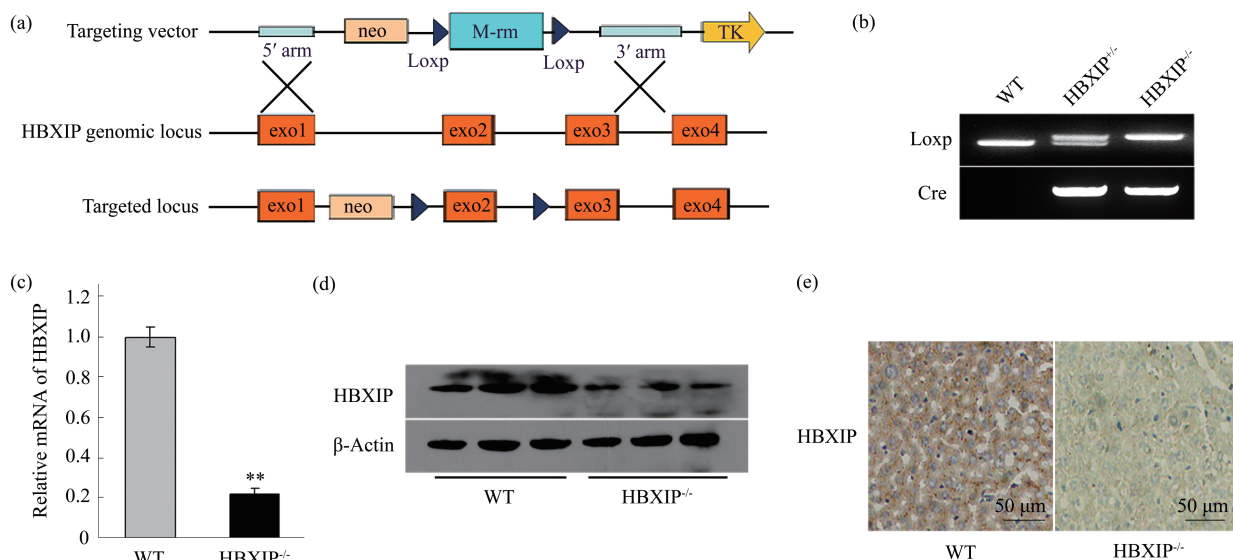


Fig. 1 Generation of liver-specific HBXIP^{-/-} mice

(a) The liver specific HBXIP knockout (KO) mice construction model was shown. (b) Genotyping of WT, HBXIP^{+/+} and HBXIP^{-/-} mice was performed. (c) The relative mRNA levels of HBXIP in liver tissues were examined in WT and liver HBXIP^{-/-} mice ($n=4$ /genotype). All data are presented as means $\pm$ SEM. ** $P < 0.01$, versus WT control. (d) The expression levels of HBXIP was tested in the liver tissues of WT and liver HBXIP^{-/-} mice by Western blotting. (e) The expression levels of HBXIP were examined in the liver tissues of WT and liver HBXIP^{-/-} mice by IHC assay.

wild-type (WT) allele resulted in a -500 bp PCR product and floxed allele shift to a -540 bp PCR product (Figure 1b). The expression levels of HBXIP in liver were remarkably decreased at the mRNA and protein levels in liver HBXIP^{-/-} mice relative to those of WT mice (Figure 1c, d). Moreover, the minute expression of HBXIP was validated by immunohistochemistry (IHC) staining in the liver of HBXIP^{-/-} mice (Figure 1e), suggesting that liver-specific HBXIP knockout mice were successfully established.

3.2 Liver HBXIP^{-/-} mice display glucose metabolism dysregulation involving gluconeogenesis

To better understand the physiological function of HBXIP in regulation of glucose metabolism in liver, we firstly monitored the change of blood glucose levels in mice. Interestingly, liver HBXIP^{-/-} mice, as opposed to wild-type (WT) mice, exhibited significantly higher blood glucose levels (Figure 2a) and increased hepatic glycogen (Figure 2b) in fasting conditions. In response to glucose tolerance test (GTT), liver HBXIP^{-/-} mice displayed attenuated blood glucose profiles (Figure 2c), suggesting that the

liver HBXIP deletion results in glucose metabolism dysregulation in mice. It has been reported that abnormal activation of hepatic gluconeogenesis contributes to hyperglycemia^[4]. Accordingly, we concerned whether gluconeogenesis was involved in the event. Pyruvate tolerance test (PTT) showed that liver HBXIP^{-/-} mice exhibited higher blood glucose levels compared with WT mice (Figure 2d), suggesting that HBXIP depletion leads to glucose metabolism dysregulation through promoting gluconeogenesis in liver. Thus, we concluded that liver HBXIP^{-/-} mice displayed abnormal glucose metabolism with suppressed hepatic gluconeogenesis.

3.3 HBXIP is able to suppress PEPCK expression in liver

PEPCK is a key enzyme in the gluconeogenesis pathway^[17-18]. Accordingly, we evaluated the mRNA levels of PEPCK in liver HBXIP^{-/-} mice. Our data showed that the mRNA levels of PEPCK were remarkably increased in liver HBXIP^{-/-} mice relative to those of WT mice (Figure 3a). Moreover, we showed that the protein levels of PEPCK was up-regulated in the liver of HBXIP^{-/-} mice (Figure 3b, c), consistent with elevated gluconeogenesis in the liver

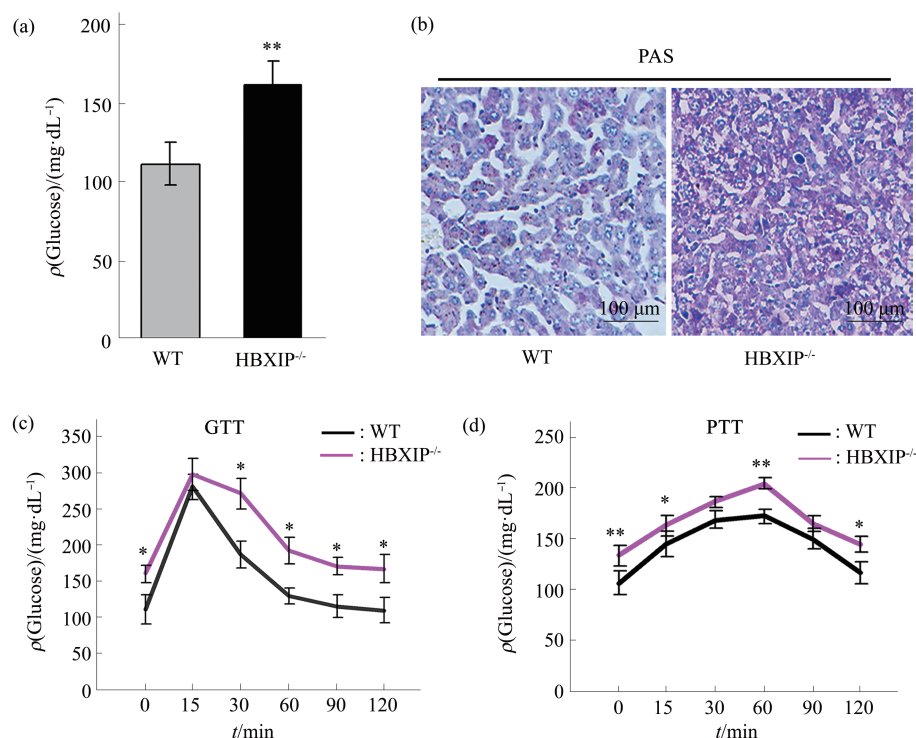


Fig. 2 Liver HBXIP^{-/-} mice display glucose metabolism dysregulation involving gluconeogenesis

(a) Blood glucose levels were examined in WT and liver HBXIP^{-/-} mice under fasting conditions ($n=6/\text{genotype}$, 16 h fasting). (b) PAS staining was conducted in the liver tissues of WT and HBXIP^{-/-} mice. (c, d) Glucose tolerance test and Pyruvate tolerance test were performed in mice ($n=8/\text{genotype}$, 16 h fasting). All data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, versus WT control.

of HBXIP^{-/-} mice. Then, we validated that the expression levels of HBXIP were negatively correlated with those of PEPCK in 30 clinical liver tissues (Figure 3d). Next, we observed that the overexpression of HBXIP markedly down-regulated PEPCK at mRNA and protein levels in a dose-

dependent manner in Chang liver cells (Figure 3e, f), suggesting that HBXIP inhibits gluconeogenesis through down-regulating PEPCK in liver. Therefore, we concluded that HBXIP was able to down-regulate the expression of PEPCK in liver.

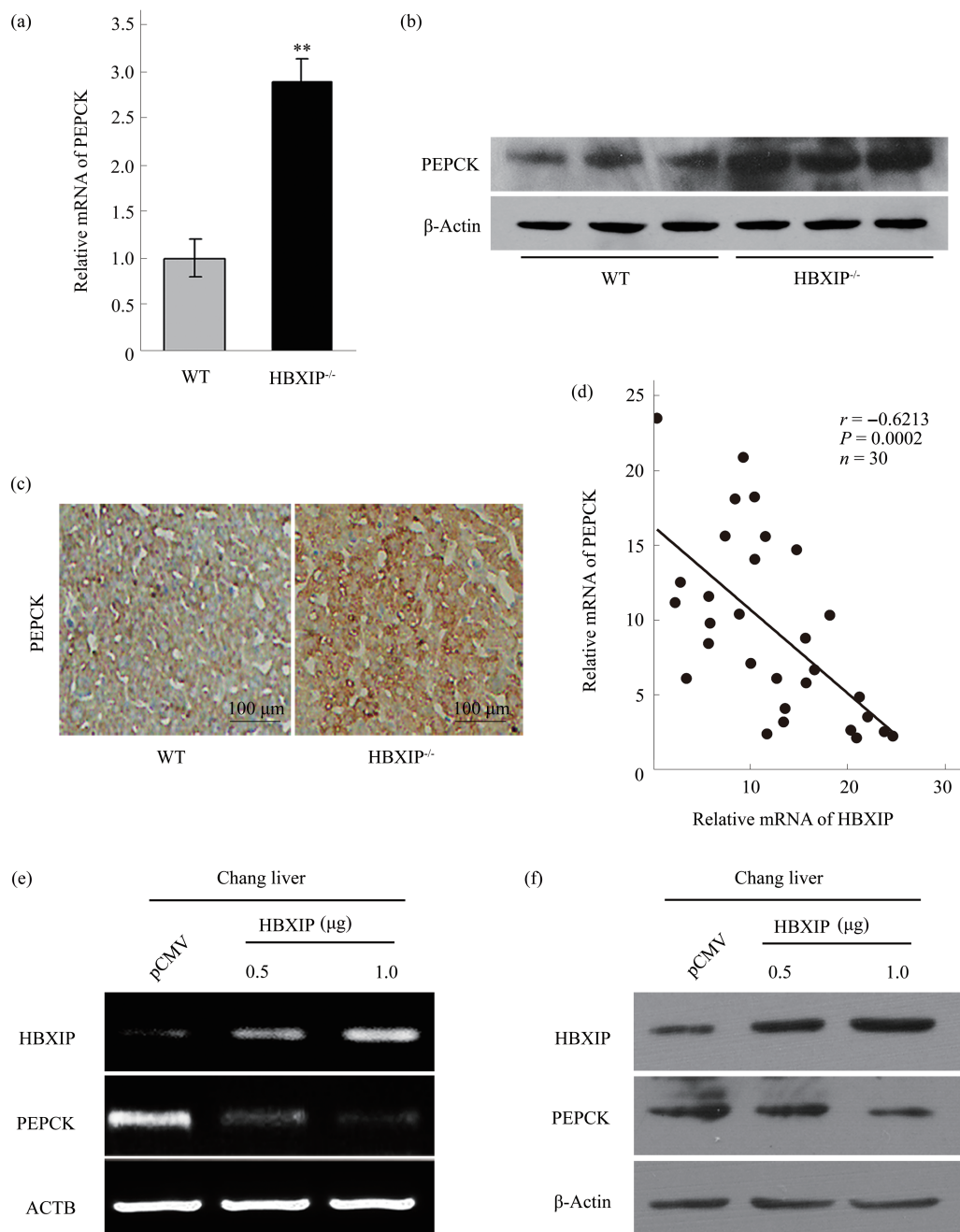


Fig. 3 HBXIP is able to down-regulate PEPCK in liver

(a) The relative mRNA levels of PEPCK were detected in the liver tissues of WT and HBXIP^{-/-} mice. ($n=4/\text{genotype}$). All data are presented as means $\pm$ SEM. ** $P<0.01$, versus WT control. (b, c) The expression levels of PEPCK were detected in the liver tissues of WT and HBXIP^{-/-} mice by (b) Western blot and (c) IHC. (d) The correlation between HBXIP and PEPCK was detected by qRT-PCR in 30 cases of clinical human liver tissues ($r=-0.6213$, $P=0.0002$, Pearson's correlation). (e, f) The expression levels of PEPCK were detected in Chang liver cells transiently transfected with HBXIP expression constructs by (e) qRT-PCR or (f) Western blotting.

3.4 HBXIP inhibits PEPCK at transcriptional level in liver

Our laboratory reported that HBXIP could regulate gene transcription^[19-20]. To gain insight into the mechanism by which HBXIP down-regulated PEPCK, we constructed the promoter of PEPCK. Luciferase reporter gene assays showed that overexpression of HBXIP dose-dependently reduced promoter activities of PEPCK in Chang liver cells

(Figure 4a). Conversely, depletion of HBXIP elevated promoter activities of PEPCK in a dose-dependent manner (Figure 4b). ChIP assays indicated that HBXIP could occupy the promoter of PEPCK, suggesting that HBXIP might be a transcriptional suppressor of PEPCK (Figure 4c). Thus, we concluded that HBXIP inhibited PEPCK at transcription level.

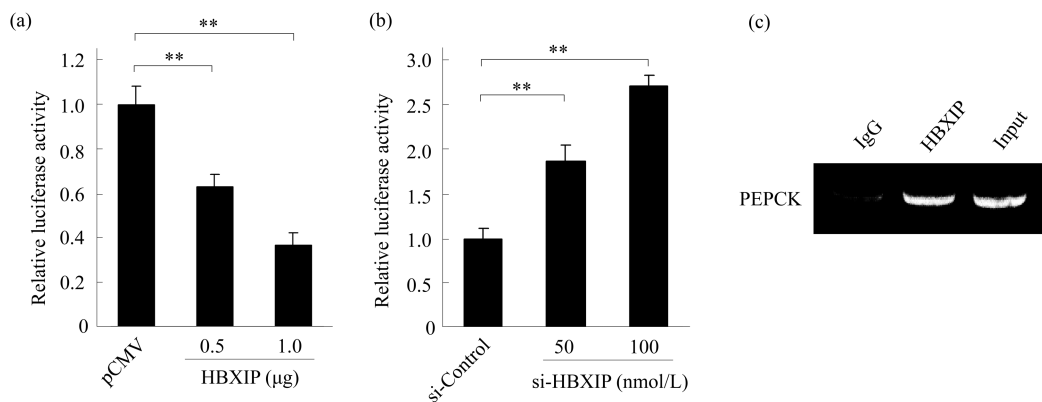


Fig. 4 HBXIP inhibits PEPCK at transcriptional level in liver

(a, b) The promoter activities of PEPCK in Chang liver cells transiently transfected with (a) HBXIP expression constructs or (b) HBXIP siRNA. (c) The enrichment of PEPCK promoter region after ChIP with HBXIP antibody or relative control IgG.

4 Discussion

In glucose metabolism, liver is primarily responsible for the maintenance of blood glucose levels by its ability to produce glucose from gluconeogenesis or glycogen breakdown and to store glucose as glycogen^[21-22]. Recently, our group reported that HBXIP induces glucose metabolism reprogramming through down-regulating SCO2 and PDHA1 in breast cancer cells^[10]. In this study, we uncovered that HBXIP was involved in the regulation of glucose metabolism in physiological condition through suppressing PEPCK.

To address the role of HBXIP in glucose metabolism, we developed liver-specific HBXIP knockout mice. Interestingly, we observed that hepatic HBXIP depletion resulted in higher blood glucose level, increased hepatic glycogen, attenuated glucose tolerance and elevated gluconeogenesis in mice under fasting condition. PEPCK and G6P are two key enzymes in the gluconeogenesis pathway^[23]. We

tested the effect of HBXIP on PEPCK in the liver of HBXIP^{-/-} mice. Our data showed that the expression of PEPCK was dramatically increased in liver HBXIP^{-/-} mice. Then, we validated that the expression levels of HBXIP were negatively correlated with those of PEPCK in clinical liver tissues. Next, we demonstrated that HBXIP was able to down-regulate PEPCK in liver cells. We have reported that HBXIP, as a transcriptional modulator, is able to regulate gene transcription^[24-25]. Intriguingly, we identified that HBXIP suppressed the promoter activities of PEPCK in liver cells. Therefore, we concluded that HBXIP could regulate PEPCK expression at transcription level.

Taken together, HBXIP could physiologically inhibit gluconeogenesis to regulate glycometabolism in liver. Our data showed that HBXIP was able to down-regulate PEPCK in liver cells. Mechanistically, HBXIP was capable of inhibiting activation of PEPCK promoter in liver cells. Thus, our finding provides new insights into the mechanism of glucose metabolism regulation mediated by HBXIP in liver.

Moreover, our results highlight the potential of HBXIP as therapeutic targets for the treatment of hyperglycemia in type 2 diabetes.

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乙肝病毒X蛋白结合蛋白通过下调PEPCK的表达抑制肝脏糖异生*

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摘要 乙肝病毒X蛋白结合蛋白 (hepatitis B X-interacting protein, HBXIP) 可以调节乳腺癌中糖代谢重编程. 为了研究HBXIP在生理条件下对糖代谢的调节作用及机制, 本研究利用Cre/loxP重组酶系统成功构建了肝脏组织中HBXIP特异敲除小鼠. 当小鼠接受刺激后, 与正常组小鼠相比, 肝脏HBXIP敲除小鼠表现基础糖代谢功能异常, 如葡萄糖、丙酮酸; 相对于对照小鼠, 肝脏HBXIP敲除小鼠对糖异生和胰岛素耐受性减弱. RT-PCR、Western blot实验和免疫组化实验结果表明, HBXIP敲除小鼠肝脏组织中糖异生关键酶磷酸烯醇式丙酮酸羧化酶 (phosphoenolpyruvate carboxykinase, PEPCK) 表达显著增加. QRT-PCR分析30例临床肝组织中HBXIP mRNA和PEPCK mRNA表达水平发现, HBXIP与PEPCK表达水平呈负相关. 荧光素酶报告基因实验和ChIP实验结果表明HBXIP可以在基因转录水平调节PEPCK表达. 以上结果表明, HBXIP通过调节糖异生关键酶PEPCK的表达参与调控小鼠肝脏糖异生.

关键词 乙肝病毒X蛋白结合蛋白, 磷酸烯醇式丙酮酸羧化酶, 糖异生, 葡萄糖代谢

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