ISSN 2472-1972

Enhanced Expression of the Key Mitosis Regulator Cyclin B1 Is Mediated by PDZ-Binding Kinase in Islets of Pregnant Mice

Tadayoshi Uesato,[1] Takeshi Ogihara,[2] Akemi Hara,[2] Hitoshi Iida,[2] Takeshi Miyatsuka,[2] Yoshio Fujitani,[2,3,4] Satoru Takeda,[1] and Hirotaka Watada[2,5,6]

[1]Department of Obstetrics and Gynecology, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [2]Department of Metabolism & Endocrinology, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; [3]Laboratory of Developmental Biology and Metabolism, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371-8512, Japan; [4]AMED-JST-CREST Program, Tokyo 100-0004, Japan; [5]Center for Molecular Diabetology, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan; and [6]Center for Therapeutic Innovations in Diabetes, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan

The proliferation of pancreatic β cells is enhanced to enable an increase in β-cell mass and to compensate for insulin resistance during pregnancy. To elucidate the mechanisms involved, we previously investigated islets from pregnant and nonpregnant mice by gene expression profiling and found that the expression of postsynaptic density-95/Discs large/zonula occludens-1 (PDZ)–binding kinase (Pbk), a member of the mitogen-activated protein kinase kinase family, is increased in pregnant mouse islets compared with control mouse islets. Among the pregnancy hormones, treatment with estradiol upregulated *Pbk* expression. Inhibition of *Pbk* expression using a small interfering RNA for *Pbk* reduced bromodeoxyuridine incorporation in mouse insulinoma 6 cells, which was accompanied by a decreased expression of *Ccnb1,* a regulatory gene involved in mitosis. *Ccnb1* expression was augmented in mouse islets during pregnancy. The forced expression of Pbk using an adenovirus system in isolated mouse islets increased *Ccnb1* expression, and the Pbk inhibitor HI-TOPK-032 suppressed *Ccnb1* expression in islets isolated from pregnant mice. Our results suggest that Pbk contributes to the expansion of islets during pregnancy and that *Ccnb1* may assist Pbk in its role in β-cell proliferation.

Freeform/Key Words: cyclin B1, estradiol, islet, p53, Pbk

Pregnancy results in hormonal changes, which in turn induce insulin resistance to shift nutrients from the mother to the fetus. Previous studies using rodents demonstrated that during pregnancy, β-cell mass is increased to enhance insulin secretion and to compensate for the insulin resistance [1, 2]. β cells express receptors that recognize pregnancy hormones, and this likely contributes to the changes in β-cell function and/or survival during pregnancy. Heterozygous prolactin receptor–deficient mice were unable to augment β-cell mass during pregnancy [3, 4]. Prolactin represses the expression of Menin, which is an endocrine tumor suppressor through the upregulation of cyclin-dependent kinase inhibitors, and enhances the

Abbreviations: BrdU, bromodeoxyuridine; Cdk, cyclin-dependent kinase; Cdkn, cyclin-dependent kinase inhibitor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MIN6, mouse insulinoma 6; mRNA, messenger RNA; Pbk, postsynaptic density-95/Discs large/zona occludens-1–binding kinase; PCR, polymerase chain reaction; PDZ, postsynaptic density-95/Discs large/zonula occludens-1; PI3K, phosphoinositide 3-kinase; qRT-PCR, qualitative reverse transcription polymerase chain reaction; RRID, Research Resource Identifier; siRNA, small interfering RNA; Tph1, tryptophan hydroxylase 1; Tph2, tryptophan hydroxylase 2; v/v, volume-to-volume ratio.

Received 15 August 2017
Accepted 25 January 2018
First Published Online 30 January 2018

expression of FoxM1, which plays a crucial role in islet expansion [5, 6]. Furthermore, prolactin elicits serotonin production by upregulating the expression of rate-limiting enzymes of serotonin synthesis, namely tryptophan hydroxylase 1 and 2 (Tph1 and Tph2, respectively) in mouse islets [7, 8], and the increased secretion of serotonin plays a key role in β-cell proliferation. Estrogen is also involved in the hormonal changes that alter β-cell function during pregnancy. The activation of estrogen signaling enhances glucose-stimulated insulin secretion through inhibition of the adenosine triphosphate–sensitive potassium channel and protects oxidative stress- and cytokine-induced β-cell apoptosis [9–11]. These studies suggest that pregnancy hormones play key roles in increasing β-cell mass, enhancing insulin secretion, and preserving β cells. Whereas several mechanisms underlying this phenomenon have been studied, the mechanisms underlying the changes that occur during pregnancy remain unclear.

Postsynaptic density-95/Discs large/zonula occludens-1 (PDZ)–binding kinase (Pbk) is a member of the serine/threonine kinases and was originally identified as a mitogen-activated protein kinase kinase (MAPKK) by two independent groups [12, 13]. Pbk comprises 322 amino acids, of which 89% are conserved between human and mouse. Pbk is expressed in the placenta, testis, heart muscle, and pancreas and is weakly expressed in skeletal muscle, kidney, liver, and lung. Neural progenitor cells also express Pbk during the preneonatal and postneonatal period [14]. It is noteworthy that Pbk is abundantly expressed in malignant neoplasms, including Burkitt lymphoma and leukemia cells, as well as breast, lung, colon, and prostate cancers [12, 15–20]. Previous studies demonstrated that Pbk plays a role in tumor growth and metastasis. Therefore, an increase in Pbk expression could be a predictor of disease progression and poor prognosis [18, 21–23]. Pbk is phosphorylated by the cyclin-dependent kinase (Cdk) 1/cyclin B complex and induces cell proliferation through the activation of p38 mitogen-activated protein kinase (MAPK), Erk, and phosphoinositide 3-kinase (PI3K), or inactivation of p53 [12, 18, 24–26]. Interestingly, the inhibition of Pbk suppressed the growth of transplanted tumors in a mouse model of colon cancer, suggesting that Pbk plays key roles in the proliferation of many cell types [26–28].

In a previous study, we performed comprehensive gene expression analyses using islets isolated from nonpregnant and pregnant mice at gestational day (G)12.5 and found that the expression of Pbk is augmented in mouse islets during pregnancy [7]. Because Pbk is involved in the proliferation of many cell types, we hypothesized that Pbk plays a role in β-cell proliferation during pregnancy. Hence we aimed to elucidate the role of Pbk in β cells during pregnancy.

1. Material and Methods

A. Antibodies and Reagents

The polyclonal rabbit antibody against PDZ-binding kinase (PBK) was purchased from BD Biosciences [Research Resource Identifier (RRID): AB_399542; 12, 13]. Monoclonal mouse antibodies against p53 (RRID: AB_331743), p38 MAPK (RRID: AB_331298), phospho-p38 MAPK (RRID: AB_331296), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (RRID: AB_561053) were purchased from Cell Signaling. The polyclonal goat IgG against cyclin B1 (RRID: AB_1964555) was purchased from R&D Systems. The antibody against p21 was purchased from Proteintech (RRID: AB_11042450). The monoclonal mouse antibody against β-actin was purchased from Sigma-Aldrich (RRID: AB_476692). The Pbk inhibitor HI-TOPK-032 was purchased from Merck Millipore.

B. Cell Culture

MIN6 mouse insulinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% [volume-to-volume ratio (v/v)] fetal calf serum, 10 mM HEPES, and 1% (v/v) penicillin and streptomycin (ThermoFisher Scientific) under 5% CO_2 at 37°C. MIN6

cells were treated with or without 100 nM estradiol or 5 μM HI-TOPK-032 for the indicated times.

C. RNA Interference

Stealth small interfering RNAs (siRNAs) against *Pbk* (si-Pbk or si-Pbk2) or nontargeting sequences (si-scramble) were purchased from Life Technologies and transfected into MIN6 cells using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) according to the manufacturer's instructions. Seventy-two hours after transfection, cells were obtained for isolation of total RNA or total protein. Sequences of siRNAs used in this study were as follows: si-Pbk: 5′- GCUUUGGAACUAGAUGGCCAAUGUU -3′; si-Pbk2: 5′- CAGAAGAGACUAACUGAUGAAGCUA -3′; and si-scramble: 5′-UAAAUGUACUGCGCGUGGAGAGGAA-3′.

D. Quantitative Reverse Transcription Polymerase Chain Reaction

MIN6 cells (7×10^5) were seeded in 6-well plates and transfected with si-Pbk, si-Pbk2, or si-scramble. Seventy-two hours after transfection, total RNA was extracted from the cells by using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was synthesized by using SuperScript III Reverse transcription (ThermoFisher Scientific). Reaction mixtures for PCR were prepared by using FAST SYBR Green Master Mix (Life Technologies) according to the manufacturer's instructions, and quantitative PCR was performed by using the 7500 Fast Real-Time PCR System (ThermoFisher Scientific). The condition for PCR was 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Primer sequences for quantitative reverse transcription (qRT)–PCR are shown in the Supplemental Table.

E. Immunoblot Assays

MIN6 cells (1×10^6 cells) were seeded onto 10-cm dishes. After 2 days, the cells were washed twice with phosphate-buffered saline and then lysed by adding cell lysis buffer containing 140 mM of NaCl, 20 mM of Tris, 1 mM of EDTA, 50 mM of NaF, 2 mM of $MgCl_2$, 1 mM of phenylmethylsulfonyl fluoride, 25 U/mL of Benzonase (Merck), and 1 × Halt™ Protease Phosphatase Inhibitor Cocktail (ThermoFisher Scientific). Approximately 20 μg of total protein was separated by using 4% to 15% Mini-PROTEAN TGX Precast Gels (Bio-Rad), transferred to polyvinylidene fluoride membranes and incubated with antibodies against PBK (1:1000), p53 (1:1000), cyclin B1 (1:1000), p38 MAPK (1:1000), phospho-p38 MAPK (1:1000), β-actin (1:5000), p21 (1:500), or GAPDH (1:5,000). Bound primary antibodies were detected with peroxidase-coupled secondary mouse, rabbit, or goat antibodies (RRID: AB_2340069, AB_2313567, AB_2340390), and IRDye®-conjugated secondary mouse or rabbit antibodies (LI-COR Biosciences; RRID: AB_621840, AB_621841). Immunoblots were visualized by using the Odyssey (LI-COR Biosciences) or SuperSignal West DURA system (Pierce) with ECL-Plus™ (Amersham Biosciences).

F. Bromodeoxyuridine Incorporation Assay

Twenty-four hours before transfection, 1×10^4 MIN6 cells were seeded in 96-well plates and then transfected with si-Pbk, si-Pbk2, or si-scramble. Seventy-two hours after transfection or 24 hours after treatment with 5 μM HI-TOPK-032, the proliferation of MIN6 cells was analyzed by using Cell Proliferation ELISA and Bromodeoxyuridine (BrdU; Roche Diagnostics). Briefly, MIN6 cells were incubated with 10 μM BrdU for 2 hours, and the integrated BrdU was detected by using a peroxidase-conjugated anti-BrdU monoclonal antibody. After the addition of tetramethyl-benzidine as a substrate, BrdU incorporation was measured by absorbance at a wavelength of 370 nm by using an xMark™ microplate spectrophotometer (Bio-Rad).

G. Mouse Experiments

C57BL/6J mice were mated at 12 weeks old and euthanized at the indicated gestational ages. Mouse islets were isolated from pregnant mice and nonpregnant littermates according to a previous study [29] and were maintained in RPMI1640 supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin and streptomycin. Isolated mouse islets were treated with 100 nM estradiol, 44.2 mM prolactin, 10 IU/mL human chorionic gonadotropin or 10 μM progesterone, and then total RNA was isolated as previously described. Animal experiments were performed with the approval from the Ethics Review Committee for Animal Experimentation of Juntendo University.

H. Statistical Analysis

All data are presented as the mean ± standard error of the mean. Comparisons involving two groups were analyzed by the Student t test. One-way analysis of variance followed by the Tukey-Kramer test was used for comparisons among three or more groups. A P value < 0.05 was considered to indicate a statistically significant difference between two groups.

2. Results

A. Pbk Expression is Augmented in Mouse Islets During Pregnancy

We previously performed comprehensive gene expression analysis using isolated islets from nonpregnant and pregnant mice on G12.5 and identified that the rate-limiting enzymes for serotonin synthesis, namely Tph1 and Tph2, are upregulated and contribute to β-cell proliferation [7, 8, 30]. To identify other candidate genes regulating maternal β-cell growth and/or function, we focused on Pbk among the genes upregulated during pregnancy, owing to our above results [7]. To confirm the changes in Pbk expression during pregnancy, we isolated islets from nonpregnant and pregnant mice at G14.5 and quantitated Pbk expression by qRT-PCR. As shown in Fig. 1A, expression of *Pbk* and *E2f1*, which is a transcription factor that regulates Pbk expression [31], were increased during pregnancy. In addition, immunoblotting

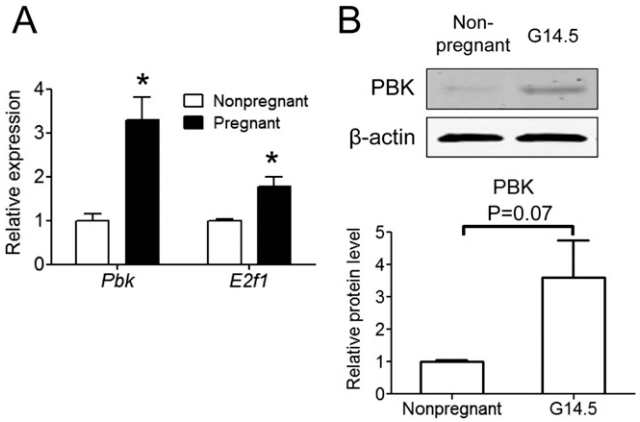

Figure 1. *Pbk* expression is increased in mouse islets during pregnancy. (A) Islets were isolated from nonpregnant and pregnant mice at G14.5 for qRT-PCR analysis. Expression levels of *Pbk* and *E2f1* were normalized to *Tbp* expression levels. Results are shown as the means ± standard error of the mean; n ≥ 3. *Statistically significant difference ($P < 0.05$) compared with the islets isolated from nonpregnant mice. (B) Islets were isolated from nonpregnant and pregnant mice at G14.5 and subjected to immunoblot using PBK and β-actin antibodies. Upper panels show a representative immunoblot. Relative protein levels of Pbk were normalized to β-actin (n = 4).

using an anti-Pbk antibody demonstrated an increasing tendency of Pbk protein expression in the islets from pregnant mice compared with those from nonpregnant mice (Fig. 1B).

B. Estradiol Upregulates Pbk Expression in Mouse Islets

Because pregnancy hormones contribute to the expansion of islets during pregnancy, it is likely that pregnancy hormones regulate Pbk expression. Thus, we incubated islets with various pregnancy hormones at concentrations equivalent to those present in pregnant mice in the second trimester, and we investigated the expression of various genes that are reported to be involved in the expansion of islets during pregnancy. Whereas we observed a robust increase in the expression of *Tph1* by prolactin, as shown previously [7], the expression of *Pbk* was significantly upregulated by estradiol, but not by the other hormones (Fig. 2A). Although mouse islets treated with estradiol showed a tendency of increased E2f1 expression, there was no significant difference from E2f1 levels in islets treated with vehicle. On the other hand, in MIN 6 cells, the expression levels of *Pbk* and *E2f1* were increased by estradiol (Fig. 2B). Thus, during pregnancy, estradiol may enhance Pbk expression in β cells *via* both E2f1-dependent and E2f1-independent mechanisms.

C. Suppression of Pbk Expression Attenuates Cell Proliferation in MIN6 Cells

To investigate the role of Pbk in β-cell growth, we suppressed *Pbk* expression in mouse insulinoma MIN6 cells using the RNA interference method and HI-TOPK-032, a pharmacological inhibitor of Pbk. As shown in Fig. 3A and 3B, treatment of MIN6 cells with an siRNA against *Pbk* (si-Pbk) that we designed effectively decreased *Pbk* messenger RNA (mRNA) levels and protein levels. Under this condition of decreased Pbk expression, we analyzed cell proliferation by BrdU incorporation and found that the proliferation rate of MIN6 cells was decreased by 37% (Fig. 3C). This decrease in proliferation was reproducible in MIN6 cells transfected with a different siRNA against *Pbk* (si-Pbk2) (Fig. 3D and 3E). In

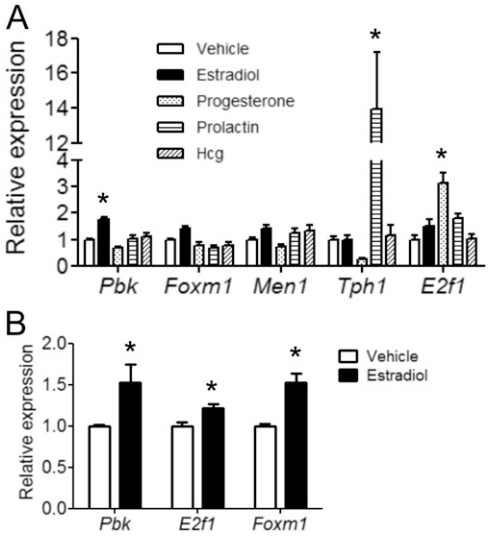

Figure 2. Estradiol upregulates *Pbk* expression in isolated mouse islets and MIN6 cells. (A) Islets were isolated from 12-week-old female mice; treated with 100 nM estradiol, 10 μM progesterone, 44.2 mM prolactin, or 10 IU/mL human chorionic gonadotropin (Hcg) for 24 hours; and then subjected to qRT-PCR ($n \geq 4$). *Statistically significant difference ($P < 0.05$) compared with islets treated with vehicle. (B) MIN6 cells were treated with or without 100 nM estradiol for 24 hours. Total RNA was isolated for qRT-PCR analysis ($n \geq 4$). Expression levels of each gene were normalized to *Tbp* expression. *Statistically significant difference ($P < 0.05$) compared with MIN6 cells treated with vehicle. Results are shown as the means ± standard error of the mean.

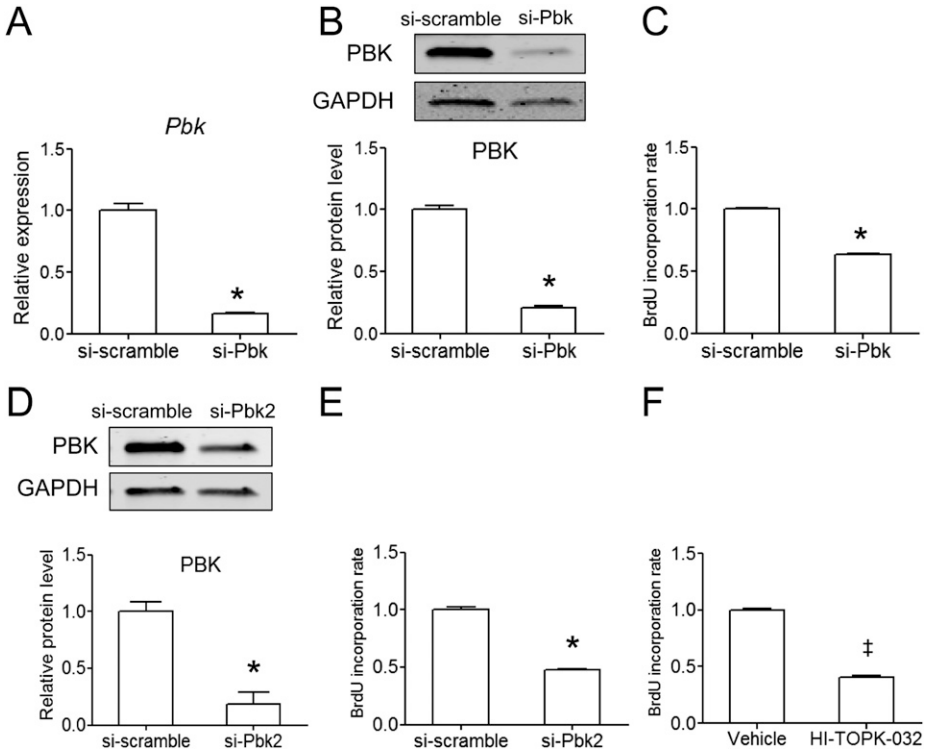

Figure 3. Pbk knockdown reduces cell proliferation in mouse insulinoma MIN6 cells. (A) Seventy-two hours after transfection with si-Pbk or si-scramble, total RNA was isolated from MIN6 cells for analysis of *Pbk* expression. Expression levels of *Pbk* were normalized to *Tbp* expression ($n \geq 3$). (B) After knockdown using si-Pbk or si-scramble, total protein was isolated from MIN6 cells and subjected to immunoblot for PBK and GAPDH. Upper panels show a representative immunoblot. Relative protein levels of Pbk were normalized to Gapdh ($n \geq 3$). (C) Seventy-two hours after the transfection of MIN6 cells with si-Pbk or si-scramble, the BrdU incorporation assay was performed. (D) Seventy-two hours after transfection with si-Pbk2 or si-scramble, total protein was isolated from MIN6 cells and subjected to immunoblot for PBK and GAPDH. (E) After knockdown using si-Pbk2 or si-scramble, MIN6 cells were subjected to BrdU incorporation analysis. (F) Twenty-four hours after treatment with HI-TOPK-032 (5 μM) or vehicle, BrdU incorporation analysis was performed. Results are shown as the mean ± standard error of the mean. *Statistically significant difference ($P < 0.05$) compared with MIN6 cells treated with si-scramble. ‡Statistically significant difference ($P < 0.05$) compared with MIN6 cells treated with vehicle.

addition, treatment with HI-TOPK-032 also attenuated BrdU incorporation in MIN6 cells (Fig. 3F).

D. Inhibition of Pbk Does Not Alter the Expression of p53-Target Genes in MIN6 Cells

Pbk is a member of the MAPKK family, which phosphorylates p38 MAPK and contributes to cell growth in breast cancer Michigan Cancer Foundation 7 cells [32]. Although we performed immunoblotting using an antiphospho-p38 MAPK antibody, the phosphorylated form of p38 MAPK could not be detected in MIN6 cells at the steady state, as previously reported (data not shown) [33]. In the HT-1080 fibrosarcoma cell line, Pbk interacts with p53 and the ectopic expression of Pbk induces the destabilization of p53 [34]. In colon cancer cells, pharmacological inhibition of Pbk increased p53 protein levels and inhibited cell proliferation [26]. In contrast, knockdown of Pbk using short hairpin RNA increased p53 activity without affecting p53 protein levels, and upregulated the p53-target gene *Cdkn1a*, which encodes p21, in colorectal carcinoma HCT-166 cells [27]. To investigate the effect of Pbk on p53 expression, we performed immunoblotting for p53 in MIN6 cells treated with si-Pbk or HI-TOPK-032 and found that the expression of p53 was augmented by both methods of Pbk suppression (Fig. 4A

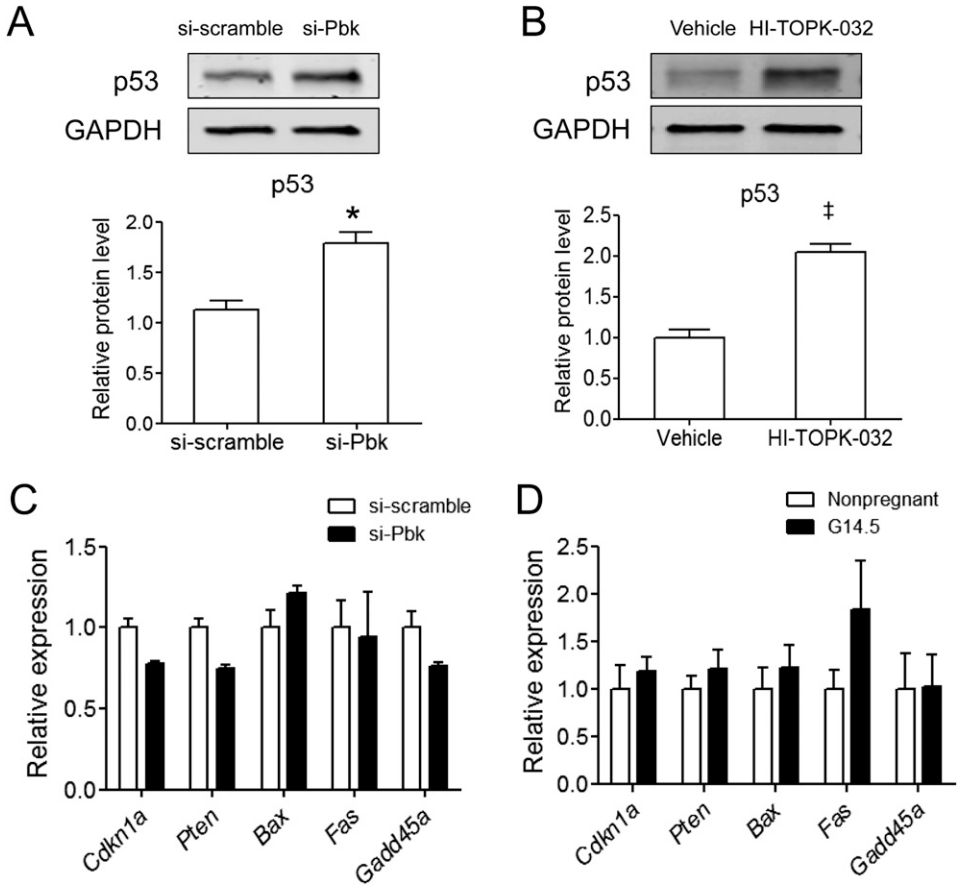

Figure 4. Expression levels of p53-target genes are not altered by Pbk knockdown in MIN6 cells. (A) Seventy-two hours after transfection of MIN6 cells with si-Pbk or si-scramble, total protein was isolated for immunoblot analysis. Upper panels show representative immunoblots of p53 and GAPDH. Relative protein levels of p53 were normalized to those of Gapdh (n ≥ 4). *Statistically significant difference ($P < 0.05$) compared with MIN6 cells transfected with si-scramble. (B) Six hours after treatment with HI-TOPK-032 (5 μM) or vehicle, total protein was isolated for immunoblot analysis using anit-p53 and GAPDH antibodies. ‡Statistically significant difference ($P < 0.05$) compared with MIN6 cells treated with vehicle. (C) After knockdown using si-Pbk or si-scramble, total RNA was isolated from MIN6 cells to analyze the expression levels of p53-target genes, including *Cdkn1a*, *Pten*, *Bax*, *Fas*, and *Gadd45a*. Expression levels of each gene were normalized to those of *Tbp*. Results are shown as the means ± standard error of the mean; n ≥ 3. (D) Total RNA was isolated from islets of pregnant or nonpregnant mice to analyze the expression levels of p53-target genes (n ≥ 4).

and 4B). p53 contributes to cell cycle arrest and apoptosis through the transactivation of *Cdkn1a, Pten*, *Bax*, *Fas*, and *Gadd45a* [35–38]. Thus, we analyzed the expression of p53 target genes by qRT-PCR. However, Pbk suppression did not alter the expression of p53 target genes, such as *Cdkn1a, Pten*, *Bax*, *Fas*, and *Gadd45a*, nor p21 protein levels (Fig. 4C and data not shown). In addition, pregnancy did not change the expression of these genes in mouse islets (Fig. 4D). These results suggest that the role of Pbk on β-cell proliferation may be independent of the p53 target genes mentioned above.

E. Pbk Regulates Ccnb1 Expression in Islets of Pregnant Mice

Because the suppression of Pbk reduced cell proliferation in MIN6 cells and p53-targeted genes showed no change in Pbk suppression in MIN6 cells, we analyzed the expression of genes regulating the cell cycle to identify Pbk-target gene candidates. As shown in Fig. 5A,

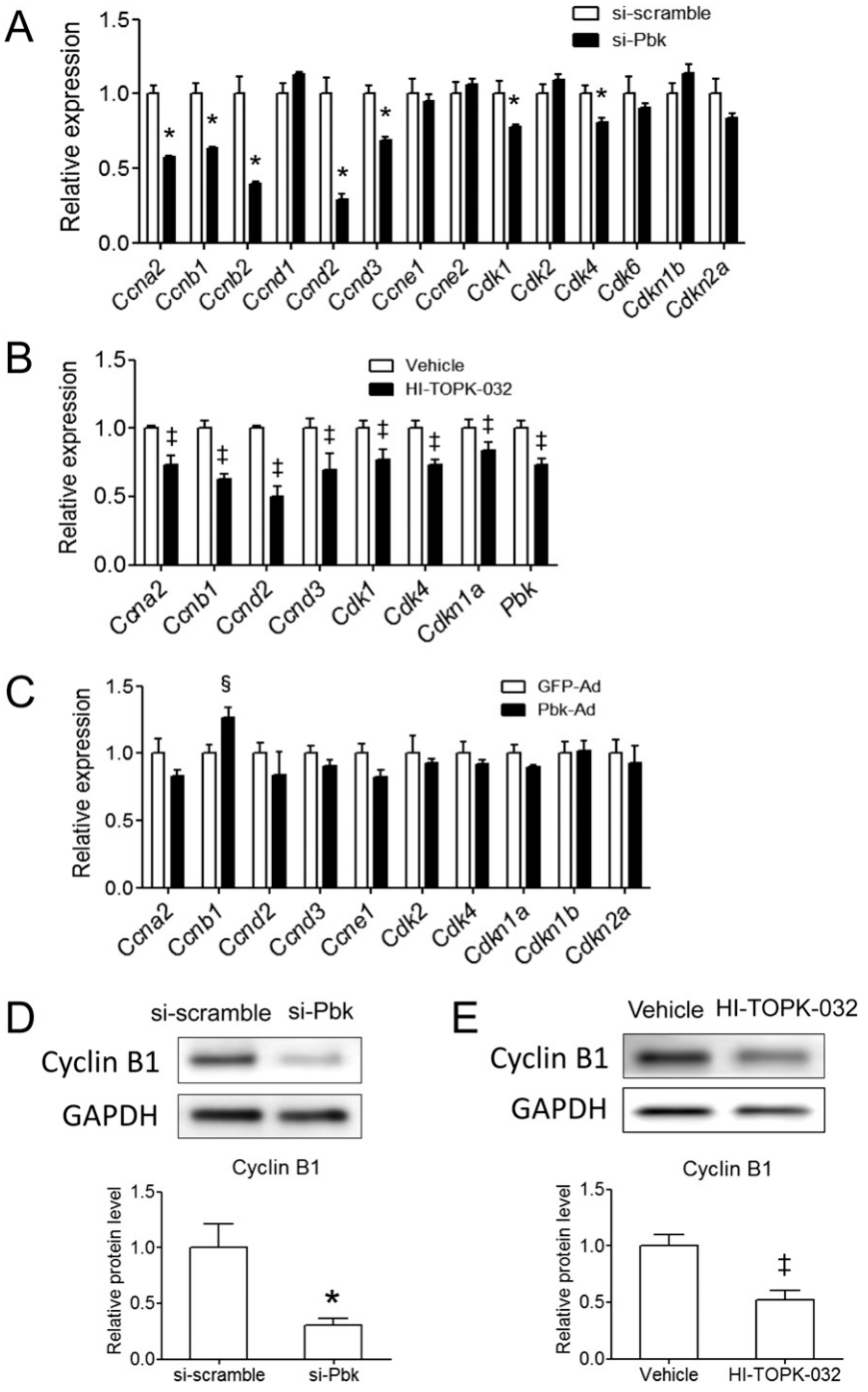

Figure 5. Pbk knockdown attenuates *Ccnb1* expression in MIN6 cells. (A) Expression levels of genes regulating the cell cycle were analyzed by qRT-PCR in MIN6 cells transfected with si-Pbk or si-scramble. *Statistically significant difference ($P < 0.05$) compared with MIN6 cells transfected with si-scramble. (B) Six hours after treatment with HI-TOPK-032 (5 μM) or vehicle, total RNA was isolated from MIN6 cells for qRT-PCR analysis (n ≥ 3). ‡Statistically significant difference ($P < 0.05$) compared with MIN6 cells treated with vehicle. (C) Isolated mouse islets were incubated with adenoviruses expressing Pbk or GFP and then subjected to qRT-PCR analysis (n = 3). Expression levels of each gene were normalized to those of *Tbp*. Results are shown as the mean ± standard error of the mean. §Statistically significant difference ($P < 0.05$) compared with islets treated with green fluorescent protein–expressing adenoviruses. (D) MIN6 cells were transfected with si-Pbk or si-scramble, followed by immunoblotting for PBK and GAPDH.

Upper panels show representative immunoblots. The lower panel shows relative protein levels of Pbk, which were normalized to those of Gapdh (n ≥ 3). *Statistically significant difference ($P < 0.05$) compared with MIN6 cells transfected with si-scramble. (E) Six hours after treatment with HI-TOPK-032 or vehicle, total protein was isolated from MIN6 cells for immunoblot analysis. ‡Statistically significant difference ($P < 0.05$) compared with vehicle-treated cells. Results are shown as the mean ± standard error of the mean; n ≥ 3.

Pbk knockdown attenuated the expression of *Ccna2*, *Ccnb1*, *Ccnb2*, *Ccnd2*, *Ccnd3*, *Cdk1*, and *Cdk4*. To confirm these findings, we used HI-TOPK-032 and found that treatment with this inhibitor also attenuated the expression of these genes in MIN6 cells (Fig. 5B). In addition, we isolated mouse islets and enhanced their expression of mouse *Pbk* using an adenovirus vector system. Interestingly, among these genes, *Ccnb1* was the only gene to be upregulated in response to the forced expression of Pbk (Fig. 5C). Similar to the changes in *Ccnb1 mRNA,* immunoblotting analysis showed that cyclin B1 was reduced in MIN6 cells by Pbk knockdown as well as treatment with HI-TOPK-032 (Fig. 5D and 5E). Previous data showed that p53 interrupts the cell cycle through the suppression of *Ccnb1* [39]. To investigate whether a similar system is involved in the regulation of β-cell proliferation, we suppressed Pbk expression and then treated MIN6 cells with the p53 inhibitor Pifithrin-α. However, inhibition of p53 did not reverse the reduction in *Ccnb1* expression (data not shown).

Finally, we investigated the expression of these genes in islets isolated from pregnant mice. As shown in Fig. 6, *Ccna2* and *Ccnb1* levels were increased in the islets of pregnant mice, and treatment with HI-TOPK-032 attenuated *Ccnb1* expression.

3. Discussion

Pbk is expressed in proliferative fetal tissues, and its expression is observed in limited types of adult tissues [15]. Interestingly, we found that the expression of *Pbk* is augmented in islets during pregnancy. As Pbk contributes to tumor cell growth, we hypothesized that Pbk is involved in the expansion of β-cell mass in pregnancy. In this study, we found that estradiol enhanced the expression of Pbk. In addition, the forced expression of Pbk enhanced the expression of *Ccnb1* in islets, and the augmented expression of *Ccnb1* in islets from pregnant mice was suppressed by the inhibition of Pbk. Although we could not directly investigate the effect of Pbk on the cell proliferation of pregnant islets, the suppression of Pbk attenuated BrdU incorporation in MIN6 cells, indicating that Pbk is involved in the regulation of β-cell proliferation. Furthermore, Pbk knockdown reduced the mRNA expression levels of several cell-cycle genes, including *Ccnb1*.

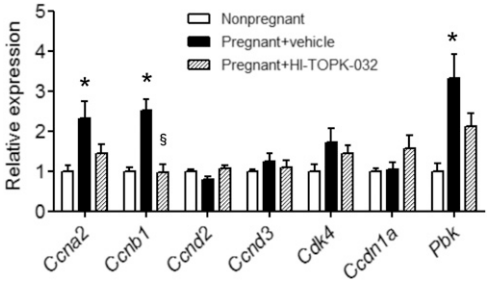

Figure 6. Attenuation of *Ccnb1* expression by a Pbk inhibitor in isolated islets of pregnant mice. Isolated mouse islets were incubated with 5 μM HI-TOPK-032 or vehicle for 24 hours and then subjected to qRT-PCR analysis. Expression levels of each gene were normalized to *Tbp* expression (n ≥ 4). *Statistically significant difference ($P < 0.05$) compared with islets of nonpregnant mice. §Statistically significant difference ($P < 0.05$) compared with islets of pregnant mice treated with vehicle.

To identify the upstream signal of Pbk expression, we treated isolated mouse islets with several pregnancy hormones and identified that estradiol upregulates *Pbk* expression. The role of the estrogen signal on β cells is controversial. Whereas Le May *et al.* [40] showed that treatment with estradiol inhibited β-cell apoptosis without affecting β-cell proliferation in a streptozotocin-induced diabetic mouse model, replacement therapy for estradiol was reported to contribute to β-cell proliferation in a mouse model of ovariectomy with subtotal pancreatectomy [41]. In addition, treatment with a selective estrogen receptor β agonist increased the number of BrdU-positive cells in mouse islets [42]. Given that in pregnancy, estrogen induces β-cell proliferation by cooperating with other humoral factors, our results suggest that Pbk is involved in the estrogen signaling pathway.

Although the necessity of *Ccnb1* for β-cell proliferation has not yet been elucidated, previous studies showed that the expression of cyclin B1 is associated with β-cell proliferation. Ackermann *et al.* [43] demonstrated that the expression of *Ccnb1* was increased during the expansion of mouse islets in a mouse partial pancreatectomy model. In addition, the enhanced expression of cyclin B1 is also observed with the overexpression of the oncoprotein MafB in cultured βTC3 cells [44]. Thus, the enhanced expression of *Ccnb1* by Pbk during pregnancy may play a role in the expansion of β-cells mass. Further analyses to investigate the role of *Ccnb1* on β-cell proliferation are required in the future.

As previously shown, we found that *Ccna2* expression is increased in mouse islets during pregnancy [7]. *Ccna2* overexpression was reported to enhance β-cell proliferation [45], and thus *Ccna2* is a possible candidate downstream target of Pbk that mediates β-cell proliferation [45]. Pbk knockdown attenuated the expression of *Ccna2* in MIN6 cells and the pharmacological inhibition of Pbk also demonstrated a decreasing tendency of *Ccna2* expression in isolated islets from pregnant mice. However, the forced expression of Pbk was unable to upregulate *Ccna2* in mouse islets. This phenomenon can be explained if Pbk is able to enhance *Ccna2* expression, by cooperating with other essential factors that are upregulated in pregnancy. Thus, it is possible that *Ccna2* is the main mediator of Pbk function in pregnant islets.

p53 is a tumor suppressor that plays important roles in cell cycle arrest and apoptosis, and its fundamental functions are well established in cancer research [46, 47]. Pbk directly binds p53 and inhibits the functions of p53. Previous studies demonstrated that the inhibition of p53 increased the expression of *Cdkn1a*, the gene encoding p21, which inhibits the activity of cyclin/Cdk complexes. p53 also contributes to mitochondrial function and proliferation in β cells [48, 49]. Whereas knockdown of Pbk increased p53 protein levels in MIN6 cells, *Cdkn1a* expression levels were not altered. The expression of *Cdkn1a* may be regulated independently of p53 in β cells [50]. We evaluated the expression of other p53-target genes regulating cell proliferation or apoptosis. However, the suppression of Pbk did not alter the expression of those genes. Because p53 plays an important role in tumor growth and controls the transcription of many genes, it is possible that p53 might contribute to the effects of Pbk other than on the cell cycle in β cells.

4. Conclusion

Here, we propose a role of Pbk in the expansion of islets in pregnant mice. During pregnancy, an increased estrogen level upregulates the expression of Pbk. This enhanced expression of Pbk increases *Ccnb1* and possibly *Ccna2*. The enhanced expression of *Ccnb1* and *Ccna2* may induce β-cell proliferation. Although direct evidence that Pbk and *Ccnb1* are involved in the expansion of β-cell mass in pregnancy is lacking, here we showed evidence to support such a role.

Acknowledgments

We acknowledge Ms. Naoko Daimaru, Ms. Hiroko Hibino, and Ms. Eriko Magoshi for assistance with the experiments.

Financial Support: AstraZeneca Schweiz (T.O.), Eli Lilly and Company (T.O.), Mitsubishi Tanabe Pharma (T.O.), and Sanofi-Aventi (T.O.).

Author Contributions: T.O. and H.W. designed the research; T.U., A.H., and H.I. conducted the research; T.O., H.W., T.M., Y.F., and S.T. analyzed the data; T.O., T.U., and H.W. wrote the manuscript; H.W. holds primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

Correspondence: Takeshi Ogihara, MD, PhD, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail: take@juntendo.ac.jp.

Disclosure Summary: T.O. has received research funding from AstraZeneca, Eli Lilly and Company, Sanofi-Aventis, and Mitsubishi Tanabe Pharma. The remaining authors have nothing to disclose.

References and Notes

1. Buchanan TA, Xiang AH. Gestational diabetes mellitus. *J Clin Invest*. 2005;**115**(3):485–491.
2. Rieck S, Kaestner KH. Expansion of beta-cell mass in response to pregnancy. *Trends Endocrinol Metab*. 2010;**21**(3):151–158.
3. Huang C, Snider F, Cross JC. Prolactin receptor is required for normal glucose homeostasis and modulation of beta-cell mass during pregnancy. *Endocrinology*. 2009;**150**(4):1618–1626.
4. Hughes E, Huang C. Participation of Akt, menin, and p21 in pregnancy-induced beta-cell proliferation. *Endocrinology*. 2011;**152**(3):847–855.
5. Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, Fontaine M, Yen MH, Kim SK. Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. *Science*. 2007;**318**(5851):806–809.
6. Banerjee RR, Cyphert HA, Walker EM, Chakravarthy H, Peiris H, Gu X, Liu Y, Conrad E, Goodrich L, Stein RW, Kim SK. Gestational diabetes mellitus from inactivation of prolactin receptor and MafB in islet β-cells. *Diabetes*. 2016;**65**(8):2331–2341.
7. Kim H, Toyofuku Y, Lynn FC, Chak E, Uchida T, Mizukami H, Fujitani Y, Kawamori R, Miyatsuka T, Kosaka Y, Yang K, Honig G, van der Hart M, Kishimoto N, Wang J, Yagihashi S, Tecott LH, Watada H, German MS. Serotonin regulates pancreatic beta cell mass during pregnancy. *Nat Med*. 2010;**16**(7):804–808.
8. Iida H, Ogihara T, Min MK, Hara A, Kim YG, Fujimaki K, Tamaki M, Fujitani Y, Kim H, Watada H. Expression mechanism of tryptophan hydroxylase 1 in mouse islets during pregnancy. *J Mol Endocrinol*. 2015;**55**(1):41–53.
9. Soriano S, Ropero AB, Alonso-Magdalena P, Ripoll C, Quesada I, Gassner B, Kuhn M, Gustafsson JA, Nadal A. Rapid regulation of K(ATP) channel activity by 17beta-estradiol in pancreatic beta-cells involves the estrogen receptor beta and the atrial natriuretic peptide receptor. *Mol Endocrinol*. 2009;**23**(12):1973–1982.
10. Liu S, Le May C, Wong WP, Ward RD, Clegg DJ, Marcelli M, Korach KS, Mauvais-Jarvis F. Importance of extranuclear estrogen receptor-alpha and membrane G protein-coupled estrogen receptor in pancreatic islet survival. *Diabetes*. 2009;**58**(10):2292–2302.
11. Kumar R, Balhuizen A, Amisten S, Lundquist I, Salehi A. Insulinotropic and antidiabetic effects of 17β-estradiol and the GPR30 agonist G-1 on human pancreatic islets. *Endocrinology*. 2011;**152**(7):2568–2579.
12. Abe Y, Matsumoto S, Kito K, Ueda N. Cloning and expression of a novel MAPKK-like protein kinase, lymphokine-activated killer T-cell-originated protein kinase, specifically expressed in the testis and activated lymphoid cells. *J Biol Chem*. 2000;**275**(28):21525–21531.
13. Gaudet S, Branton D, Lue RA. Characterization of PDZ-binding kinase, a mitotic kinase. *Proc Natl Acad Sci USA*. 2000;**97**(10):5167–5172.
14. Dougherty JD, Garcia AD, Nakano I, Livingstone M, Norris B, Polakiewicz R, Wexler EM, Sofroniew MV, Kornblum HI, Geschwind DH. PBK/TOPK, a proliferating neural progenitor-specific mitogen-activated protein kinase kinase. *J Neurosci*. 2005;**25**(46):10773–10785.
15. Simons-Evelyn M, Bailey-Dell K, Toretsky JA, Ross DD, Fenton R, Kalvakolanu D, Rapoport AP. PBK/TOPK is a novel mitotic kinase which is upregulated in Burkitt's lymphoma and other highly proliferative malignant cells. *Blood Cells Mol Dis*. 2001;**27**(5):825–829.
16. Nandi A, Tidwell M, Karp J, Rapoport AP. Protein expression of PDZ-binding kinase is up-regulated in hematologic malignancies and strongly down-regulated during terminal differentiation of HL-60 leukemic cells. *Blood Cells Mol Dis*. 2004;**32**(1):240–245.

17. Park JH, Lin ML, Nishidate T, Nakamura Y, Katagiri T. PDZ-binding kinase/T-LAK cell-originated protein kinase, a putative cancer/testis antigen with an oncogenic activity in breast cancer. *Cancer Res*. 2006;**66**(18):9186–9195.
18. Shih MC, Chen JY, Wu YC, Jan YH, Yang BM, Lu PJ, Cheng HC, Huang MS, Yang CJ, Hsiao M, Lai JM. TOPK/PBK promotes cell migration via modulation of the PI3K/PTEN/AKT pathway and is associated with poor prognosis in lung cancer. *Oncogene*. 2012;**31**(19):2389–2400.
19. Zhu F, Zykova TA, Kang BS, Wang Z, Ebeling MC, Abe Y, Ma WY, Bode AM, Dong Z. Bidirectional signals transduced by TOPK-ERK interaction increase tumorigenesis of HCT116 colorectal cancer cells. *Gastroenterology*. 2007;**133**(1):219–231.
20. Sun H, Zhang L, Shi C, Hu P, Yan W, Wang Z, Duan Q, Lu F, Qin L, Lu T, Xiao J, Wang Y, Zhu F, Shao C. TOPK is highly expressed in circulating tumor cells, enabling metastasis of prostate cancer. *Oncotarget*. 2015;**6**(14):12392–12404.
21. Brown-Clay JD, Shenoy DN, Timofeeva O, Kallakury BV, Nandi AK, Banerjee PP. PBK/TOPK enhances aggressive phenotype in prostate cancer via β-catenin-TCF/LEF-mediated matrix metalloproteinases production and invasion. *Oncotarget*. 2015;**6**(17):15594–15609.
22. Kwon CH, Park HJ, Choi YR, Kim A, Kim HW, Choi JH, Hwang CS, Lee SJ, Choi CI, Jeon TY, Kim DH, Kim GH, Park Y. PSMB8 and PBK as potential gastric cancer subtype-specific biomarkers associated with prognosis. *Oncotarget*. 2016;**7**(16):21454–21468.
23. Wang MY, Lin ZR, Cao Y, Zheng LS, Peng LX, Sun R, Meng DF, Xie P, Yang JP, Cao L, Xu L, Huang BJ, Qian CN. PDZ binding kinase (PBK) is a theranostic target for nasopharyngeal carcinoma: driving tumor growth via ROS signaling and correlating with patient survival. *Oncotarget*. 2016;**7**(18): 26604–26616.
24. Matsumoto S, Abe Y, Fujibuchi T, Takeuchi T, Kito K, Ueda N, Shigemoto K, Gyo K. Characterization of a MAPKK-like protein kinase TOPK. *Biochem Biophys Res Commun*. 2004;**325**(3):997–1004.
25. Fujibuchi T, Abe Y, Takeuchi T, Ueda N, Shigemoto K, Yamamoto H, Kito K. Expression and phosphorylation of TOPK during spermatogenesis. *Dev Growth Differ*. 2005;**47**(9):637–644.
26. Kim DJ, Li Y, Reddy K, Lee MH, Kim MO, Cho YY, Lee SY, Kim JE, Bode AM, Dong Z. Novel TOPK inhibitor HI-TOPK-032 effectively suppresses colon cancer growth. *Cancer Res*. 2012;**72**(12): 3060–3068.
27. Hu F, Gartenhaus RB, Eichberg D, Liu Z, Fang HB, Rapoport AP. PBK/TOPK interacts with the DBD domain of tumor suppressor p53 and modulates expression of transcriptional targets including p21. *Oncogene*. 2010;**29**(40):5464–5474.
28. Joel M, Mughal AA, Grieg Z, Murrell W, Palmero S, Mikkelsen B, Fjerdingstad HB, Sandberg CJ, Behnan J, Glover JC, Langmoen IA, Stangeland B. Targeting PBK/TOPK decreases growth and survival of glioma initiating cells in vitro and attenuates tumor growth in vivo. *Mol Cancer*. 2015;**14**(1): 121.
29. Iwashita N, Uchida T, Choi JB, Azuma K, Ogihara T, Ferrara N, Gerber H, Kawamori R, Inoue M, Watada H. Impaired insulin secretion in vivo but enhanced insulin secretion from isolated islets in pancreatic beta cell-specific vascular endothelial growth factor-A knock-out mice. *Diabetologia*. 2007; **50**(2):380–389.
30. Schraenen A, Lemaire K, de Faudeur G, Hendrickx N, Granvik M, Van Lommel L, Mallet J, Vodjdani G, Gilon P, Binart N, in't Veld P, Schuit F. Placental lactogens induce serotonin biosynthesis in a subset of mouse beta cells during pregnancy. *Diabetologia*. 2010;**53**(12):2589–2599.
31. Hu F, Gartenhaus RB, Zhao XF, Fang HB, Minkove S, Poss DE, Rapoport AP. c-Myc and E2F1 drive PBK/TOPK expression in high-grade malignant lymphomas. *Leuk Res*. 2013;**37**(4):447–454.
32. Ayllón V, O'connor R. PBK/TOPK promotes tumour cell proliferation through p38 MAPK activity and regulation of the DNA damage response. *Oncogene*. 2007;**26**(24):3451–3461.
33. Lee JS, Kim YR, Song IG, Ha SJ, Kim YE, Baek NI, Hong EK. Cyanidin-3-glucoside isolated from mulberry fruit protects pancreatic β-cells against oxidative stress-induced apoptosis. *Int J Mol Med*. 2015;**35**(2):405–412.
34. Nandi AK, Ford T, Fleksher D, Neuman B, Rapoport AP. Attenuation of DNA damage checkpoint by PBK, a novel mitotic kinase, involves protein-protein interaction with tumor suppressor p53. *Biochem Biophys Res Commun*. 2007;**358**(1):181–188.
35. Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S, Mak TW. Regulation of PTEN transcription by p53. *Mol Cell*. 2001;**8**(2):317–325.
36. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*. 1995;**80**(2):293–299.

37. Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E, et al. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol*. 1995;**15**(6):3032–3040.
38. Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ, Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*. 1992;**71**(4):587–597.
39. Innocente SA, Abrahamson JL, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. *Proc Natl Acad Sci USA*. 1999;**96**(5):2147–2152.
40. Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, Mauvais-Jarvis F. Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proc Natl Acad Sci USA*. 2006;**103**(24):9232–9237.
41. Choi SB, Jang JS, Park S. Estrogen and exercise may enhance beta-cell function and mass via insulin receptor substrate 2 induction in ovariectomized diabetic rats. *Endocrinology*. 2005;**146**(11): 4786–4794.
42. Alonso-Magdalena P, Ropero AB, García-Arévalo M, Soriano S, Quesada I, Muhammed SJ, Salehi A, Gustafsson JA, Nadal A. Antidiabetic actions of an estrogen receptor β selective agonist. *Diabetes*. 2013; **62**(6):2015–2025.
43. Ackermann Misfeldt A, Costa RH, Gannon M. Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of FoxM1. *Diabetes*. 2008;**57**(11):3069–3077.
44. Lu J, Hamze Z, Bonnavion R, Herath N, Pouponnot C, Assade F, Fontanière S, Bertolino P, Cordier-Bussat M, Zhang CX. Reexpression of oncoprotein MafB in proliferative β-cells and Men1 insulinomas in mouse. *Oncogene*. 2012;**31**(31):3647–3654.
45. Song WJ, Schreiber WE, Zhong E, Liu FF, Kornfeld BD, Wondisford FE, Hussain MA. Exendin-4 stimulation of cyclin A2 in beta-cell proliferation. *Diabetes*. 2008;**57**(9):2371–2381.
46. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer*. 2002;**2**(8):594–604.
47. Vousden KH, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol*. 2007;**8**(4):275–283.
48. Hoshino A, Ariyoshi M, Okawa Y, Kaimoto S, Uchihashi M, Fukai K, Iwai-Kanai E, Ikeda K, Ueyama T, Ogata T, Matoba S. Inhibition of p53 preserves Parkin-mediated mitophagy and pancreatic β-cell function in diabetes. *Proc Natl Acad Sci USA*. 2014;**111**(8):3116–3121.
49. Hobson A, Draney C, Stratford A, Becker TC, Lu D, Arlotto M, Tessem JS. Aurora Kinase A is critical for the Nkx6.1 mediated β-cell proliferation pathway. *Islets*. 2015;**7**(1):e1027854.
50. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer*. 2009;**9**(6): 400–414.