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B-Cell Translocation Gene 2 Regulates Hepatic Glucose Homeostasis via Induction of Orphan Nuclear Receptor Nur77 in Diabetic Mouse Model

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B-cell translocation gene 2 (BTG2) is a member of an emerging gene family that is involved in cellular functions. In this study, we demonstrate that BTG2 regulates glucose homeostasis via upregulation of Nur77 in diabetic mice. Hepatic BTG2 gene expression was elevated by fasting and forskolin. Overexpression of *Btg2* increased the expression of hepatic gluconeogenic genes and blood glucose output and subsequently impaired glucose and insulin tolerance. Upregulation of the transcriptional activity of *Nur77*, gluconeogenic genes, and glucose production by forskolin was observed by *Btg2* transduction, but not in *Btg2* knockdown. BTG2-stimulated glucose production and glucose-6-phosphatase promoter activity were attenuated by dominant-negative *Nur77*. Coimmunoprecipitation and chromatin immunoprecipitation assays showed that BTG2 induced Nur77 occupancy on the glucose-6-phosphatase promoter via a physical interaction. *Btg2* gene expression was increased in streptozotocin-treated and *db*/*db* mice. Finally, impairment of glucose homeostasis, such as the increase of blood glucose, glucose intolerance, and insulin intolerance, was elevated in diabetic mice, whereas this phenomenon was abolished in knockdown of *Btg2*. Together, these data suggest that BTG2 participates in the regulation of hepatic glucose homeostasis, which means that BTG2 might serve as a potential therapeutic target for combating metabolic dysfunction.

The liver plays a crucial role in glucose homeostasis by maintaining a balance between uptake and storage of glucose via glycogenesis and in the release of glucose by both glycogenolysis and gluconeogenesis (1,2). Gluconeogenesis is commonly triggered by key hormones including insulin, glucagon, and glucocorticoid, which contribute to the expression of key metabolic enzymes, such as phosphoenolpyruvate carboxykinase (*Pck1*), fructose biphosphatase (*Fbp*) 1, and glucose-6-phosphatase (*G6pc*) (3). A variety of transcriptional factors and cofactors regulated by the cAMP signaling pathway such as cAMP-responsive element–binding protein (CREB), FoxA 2 (also known as hepatocyte nuclear factor [HNF]-3β), HNF-4α, glucocorticoid receptor (GR), forkhead box protein O1, peroxisome proliferator-activated receptor γ coativator-1α (PGC-1α), and transducer of regulated CREB activity 2 control the expression of hepatic gluconeogenic genes (4–9). Especially CREB acts as a critical transcriptional checkpoint for the upregulation of gluconeogenesis by binding cAMP-response element. Multiple genes involved in the gluconeogenic genes including *Pck1*, *G6pc*, and *Pgc-1α* were

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shown to be increased by recruiting CREB to the promoters of these genes (4,10,11).

The nuclear receptor Nur77/NR4A1 is a member of a family of the NR4A receptor, which has highly conserved DNA- and ligand-binding domains. Nur77 binds to its cognate DNA sequence, called NGFI-B (Nur77)-binding response elements (NBREs) or Nur response elements (NurREs), and induces transcription of target genes involved in the gluconeogenesis and steroidogenesis (12,13). In addition, hepatic expression of NR4A receptors induced by cAMP in response to glucagon and fasting is increased in diabetic mice with elevated gluconeogenesis, and ectopic overexpression of the Nur77 gene induces upregulation of hepatic gluconeogenic genes, glucose production, and increased blood glucose level (12). Consistent with these results, Nur77-null mice on a high-fat diet also show decrease of hepatic glucose production and insulin resistance in the liver (14).

B-cell translocation gene 2 (BTG2) is a member of the BTG/TOB gene family of antiproliferative genes, which are highly conserved among various species (15). BTG2 is reported to be the initial gene induced by growth factors and tumor promoters in several cell types (16,17). Recently, we demonstrated that BTG2 acts as a crucial coactivator of CREB to regulate hepatic gluconeogenesis, and the increase of BTG2 by glucagon–CREB signaling resulted in the elevation of glucose production from hepatocytes (18). However, the interrelationship between BTG2 and Nur77 in terms of the regulation of hepatic gluconeogenesis has not been addressed yet.

In the current study, we identified that transcriptional coregulator BTG2 acts as a novel coactivator of hepatic gluconeogenesis and elucidated a molecular mechanism that links to Nur77 in the regulation of glucose homeostasis in the livers of fasted and diabetic mice.

RESEARCH DESIGN AND METHODS

Reagents

Forskolin and streptozotocin were purchased from Sigma-Aldrich (St. Louis, MO). Cell-culture media were purchased from Gibco-Brl (Grand Island, NY).

Animal Studies

Male C57BL6 mice (8 weeks of age; Samtako, Osan, Republic of Korea) were used in the experiments. For studies in the fasting and feeding conditions, mice were fed and fasted for 12 h. Diabetes was induced in wild-type (WT) and *db/db* mice by intraperitoneal injections of streptozotocin (80 mg/kg body weight). For overexpression of BTG2, WT mice were infected with adenoviral vector expressing *Btg2* (1×10^9 plaque-forming units) by tail vein injection. To disruption BTG2, *db/db* mice were intravenously injected with lentivirus short hairpin *Btg2* (sh*Btg2*; a single dose of 1×10^9 transducing units/mL). Total RNA was prepared from the livers of streptozotocin-treated diabetic mice and adenovirus (Ad)- or lentivirus-infected mice for quantitative reverse transcription-PCR (qPCR) analysis. Before the isolation of the liver, the blood glucose levels of all mice were measured with Glucostix Accu-Check (Roche Diagnostics, Mannheim, Germany). All animal experiments were performed in accordance with the rules and regulations of the Institutional Animal Use and Care Committee at Keimyung University School of Medicine.

Metabolic Parameters

Plasma glucose was measured using a glucometer (Roche Diagnostics) in blood collected from the tail of mice as previously described (19). For the glucose tolerance test, a single dose of 2.5 μg/kg glucose was injected intraperitoneally after a 14-h fast. For the insulin tolerance test, mice fasted for 4 h were administered intraperitoneally with insulin (0.5 units/kg), and glucose concentrations were measured at 0, 30, 60, 90, and 120 min. Blood glucose was analyzed under the same conditions.

Construction of Plasmids and DNA

The reporter plasmids *NBRE*-Luc, *NurRE*-Luc, *Pck1*-Luc, and *G6pc*-Luc were described previously (13,18). Expression vectors of HA-*Nur77*, dominant-negative (DN)-*Nur77*, *Hnf4-α*, *Pgc-1α*, *GR*, and Flag-*Btg2* were described previously (13,18,20–23). The small interfering RNAs for *Btg2* were prepared as described previously (18). The point mutant form of human *G6PC*-Luc or mouse *G6pc*-Luc was constructed using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the following primers: human *G6PC* (h*G6PC*)-Luc forward, 5′-CTGTAACTTTAATTT CATTAAATG-3′ and reverse, 5′-CATTTAATGAAATTAAA GTTACAG-3′ and mouse *G6pc* (m*G6pc*)-Luc forward, 5′-TAACTT-AAAATTTCACTTCCG-3′ and reverse, 5′-CGGAA GTGAAATTTTAAGTTA-3′. All plasmids were confirmed by sequencing analysis.

Cell Culture and Transient Transfection Assays

AML-12 immortalized mouse hepatocytes were cultured in DMEM/F-12 medium (Gibco-Brl) supplemented with 10% FBS, insulin-transferrin-selenium (Gibco-Brl), dexamethasone (40 ng/mL; Sigma-Aldrich), and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C (24). Transient transfections were carried out as previously described (18,24).

Isolation and Culture of Primary Mouse Hepatocytes

Mouse primary hepatocytes were isolated from the livers of 8-week-old male mice (Samtako). The protocol for isolation of hepatocytes was described previously (8).

Preparation of Recombinant Ad

Ads encoding full-length *Btg2* and green fluorescence protein (GFP) have been described previously (18,24). Recombinant lentiviral delivery of *Btg2*-targeted short hairpin RNA (shRNA) was purchased from Dharmacon (Lafayette, CO). Ad-expressing DN-*Nur77* was gifted from Dr. In-Kyu Lee at Kyungpook National University School of Medicine.

Measurement of mRNA

Total RNA was isolated from liver tissue and used for qPCR as previously described (25,26). The expression of all transcripts using qPCR data was normalized to ribosomal L32 expression.

Glucose Production Assay

Glucose production from mouse primary hepatocytes was measured according to the manufacturer's protocol using a colorimetric glucose oxidase assay (Sigma-Aldrich). Briefly, after designated times, the cells were washed three times with PBS and then incubated for 3 h at 37°C in an atmosphere containing 5% CO_2 in glucose production buffer (glucose-free DMEM [pH 7.4] containing 20 mmol/L sodium lactate, 1 mmol/L sodium pyruvate, and 15 mmol/L HEPES without phenol red). The glucose assays were carried out in triplicate, and the intra-assay coefficient of variation was 5% as described previously (27).

Immunoblotting and Coimmunoprecipitation Assay

Proteins were isolated from mouse primary hepatocytes and liver tissues and analyzed according to the methods described previously (19). The membranes were probed with BTG2 and Nur77 (Santa Cruz Biotechnology, Santa Cruz, CA) and then developed using an ECL Western blot detection kit (Amersham Biosciences, Piscataway, NJ). For immunoprecipitation, total protein extracts from AML-12 cells and mouse liver were probed for BTG2, Nur77, hemagglutinin (HA) (Santa Cruz Biotechnology), and FLAG antibody (Sigma-Aldrich) and then blotted with these antibodies (21). Signals were developed using an ECL Western blot detection kit (Amersham Biosciences).

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed as described previously (18,19). Briefly, 36 h after infection with Ad-*Btg2* in mouse primary hepatocytes, cells were treated with forskolin (10 μmol/L). The cells were subsequently harvested, and the ChIP assay was performed with anti-Nur77. The final DNA extractions were quantified by PCR with two pairs of primers for the proximal (−1,070/−860 and −529/−310) and distal (−2,000/−1,790 and −1,679/−1,460) regions of the h*G6PC* and m*G6pc* promoters. The specific primers used for PCR are as follows: h*G6PC* promoter, proximal, forward, 5′-AGTGAGCCGTGATTATGCCA-3′ and reverse, 5′-TGGGAGATCAACCCCCTTCC-3′; distal, forward, 5′-TGGAATGCTGTC-AACTTTT-3′ and reverse, 5′-CTCAAC CTAGCAAAGCATT-G-3′; m*G6pc* promoter, proximal, forward, 5′-CAGAGGTGGACCCCAGATCC-3′ and reverse, 5′-AGAGTCTGTTT-CAACCATGA-3′; and distal, forward, 5′-GGTGGCTCACAACCATCTGT-3′ and reverse, 5′-TG-AGCTGGTGTCATGTGACT-3′.

Statistical Analysis

Data calculation and statistical analyses were performed using GraphPad Prism 3-5.0 software (GraphPad). The statistical significance of differences between groups were determined using the Student *t* test, and multiple comparisons were analyzed using one-way ANOVA under treatment and experiment as factors. All data are presented as means ± SEM. All P values <0.05 were considered significant.

RESULTS

BTG2 Gene Expression Is Induced by Fasting and Glucagon Signaling in the Liver

BTG2 acts as a coactivator of CREB to positively regulate hepatic gluconeogenesis in vitro (18). To investigate whether BTG2 is regulated by fasting and glucagon stimulation in vivo, we performed qPCR analysis from mouse liver. As shown in Fig. 1*A*, *Btg2* gene expression was induced by fasting along with *Creb*, *Nur77*, *Pck1*, and *G6pc*. Also, protein levels of BTG2 and Nur77 were induced in the fasted state (Fig. 1*B*). *Btg2* mRNA was induced by forskolin, a cAMP agonist, with expression reaching a maximal stimulation at 1 h. Similar results were observed for *Creb*, *Nur77*, *Pck1*, and *G6pc* mRNA levels (Fig. 1*C*), consistent with previous studies (12,18). Interestingly, the increase of BTG2 and Nur77 proteins by 1–6-h treatment of forskolin reached the peak level, and it subsequently declined after 12 h (Fig. 1*D*). These results suggest that BTG2 plays a pivotal role in the upregulation of gluconeogenic genes in vivo.

Overexpression of BTG2 Gene Induces Hepatic Gluconeogenesis

Our findings predict that BTG2 would be involved in the regulation of gluconeogenic genes for hepatic glucose production in liver. To test this hypothesis, we examined the effect of ectopic expression of BTG2 on the transcription of hepatic gluconeogenic genes using an Ad vector to drive expression of *Btg2* (Ad-*Btg2*). Indeed, the expressions of *Nur77*, *Pck1*, and *G6pc* were significantly increased by administration of Ad-*Btg2* in the liver (Fig. 2*A*). However, expression of other transcription factors related to gluconeogenesis like *GR* and *Hnf-4α* was not affected (Fig. 2*A*). Moreover, consistent with the expression of gluconeogenic genes, blood glucose level was significantly increased by Ad-*Btg2* (Fig. 2*B*). To evaluate whether ectopic expression of BTG2 also affects glucose homeostasis and insulin sensitivity, glucose and insulin tolerance tests were performed in the Ad-*Btg2*–infected mice. As predicted, there was significant impairment in glucose tolerance in mice infected with Ad-*Btg2* (Fig. 2*C*). The insulin tolerance test also showed a marked decrease in insulin sensitivity (Fig. 2*D*). Taken together, these results indicate that BTG2 plays a key role in hepatic glucose production and directly influences whole-body glucose metabolism.

Glucagon Signal–Dependent Hepatic Gluconeogenic Gene Expression Is Mediated by BTG2

To determine whether *Btg2* was directly regulated by the fasting hormone glucagon, as shown in Fig. 3*A*, *Btg2* gene expression was overexpressed well by Ad-*Btg2*, and

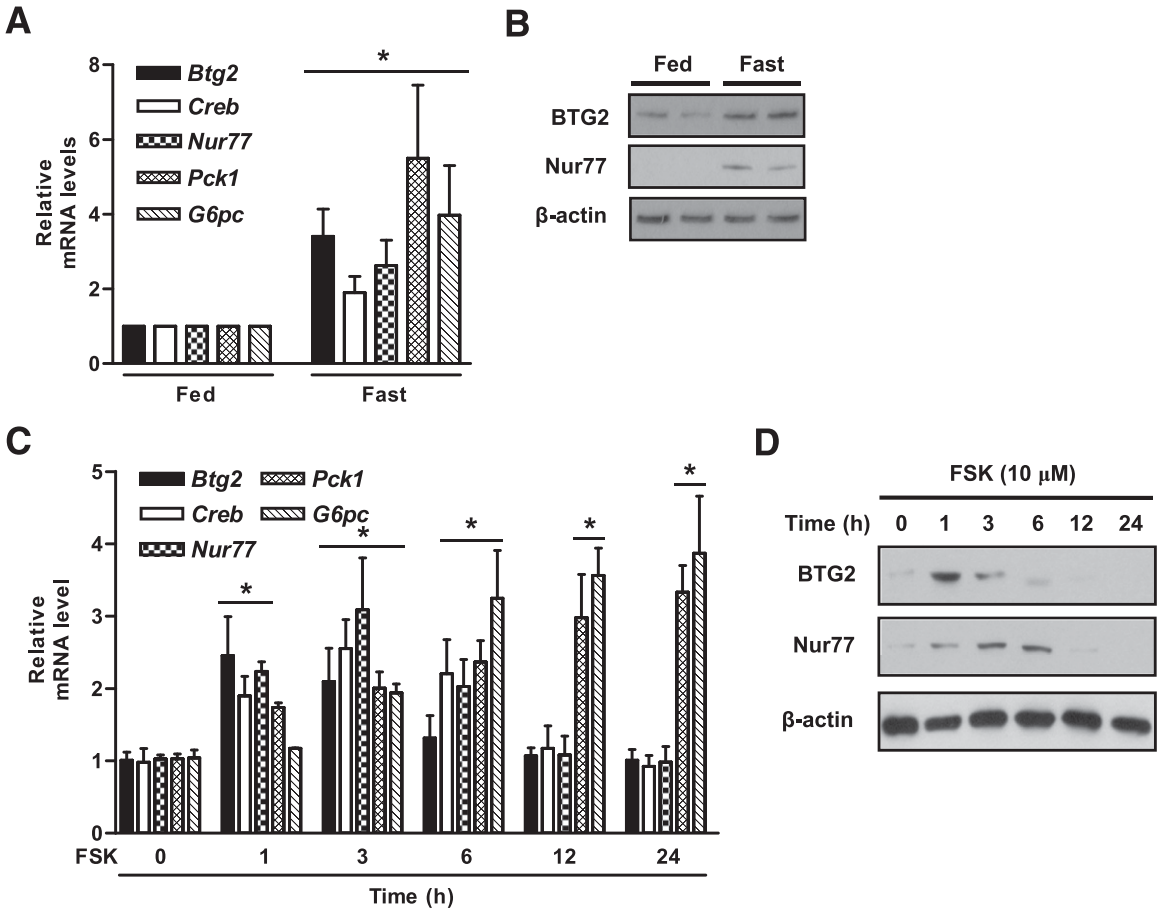

Figure 1—Effect of fasting and forskolin in the expression of BTG2 in the liver. *A*: C57BL6 mice (n = 5) were fed ad libitum and fasted for 12 h. Total RNAs were prepared from the livers of the mice. *Btg2* and gluconeogenic gene expression were measured by qPCR. *B*: Whole-cell extracts were prepared from mouse livers and then Western blot analysis performed. *C* and *D:* qPCR and Western blot of BTG2 and gluconeogenic pathway genes in mouse primary hepatocytes treated with forskolin (FSK) for the indicated time periods. *P < 0.05 vs. fed mice or untreated control.

glucagon signaling–induced *Btg2* gene expression was successfully decreased by sh*Btg2*. In addition, forskolin-stimulated gluconeogenic gene expression was significantly reduced by knockdown of endogenous *Btg2* in primary hepatocytes (Fig. 3*B–D*). We also tested whether BTG2 regulates glucose production in primary hepatocytes. Interestingly, like forskolin treatment, Ad-*Btg2* significantly increased hepatic glucose production compared with untreated control and Ad-GFP–infected cells, whereas this effect was attenuated by silencing of *Btg2* (Fig. 3*E*).

Because Nur77 is one transcription factor involved in gluconeogenesis, we hypothesized that BTG2 might function as a coactivator along with Nur77. To test this prediction, luciferase assays were performed with the Nur77 reporter plasmids *NBRE*-Luc and *NurRE*-Luc. *Btg2* significantly increased reporter activity of both *NBRE*- and *NurRE*-mediated transcription in a dose-dependent manner (Fig. 3*F*). Moreover, forskolin did not activate these reporters when *Btg2* was suppressed by small interfering *Btg2* (si*Btg2*) (Fig. 3*F*). Ectopic expression of *Btg2* significantly increased promoter activities of the *Pck1* and *G6pc* (Fig. 3*G*). Interestingly, forskolin-stimulated promoter activities *of G6pc* and *Pck1* were significantly decreased when si*Btg2* was transfected, and overexpression of *Btg2* rescued the promoter activities (Fig. 3*G*). These results indicate that BTG2 regulates glucagon-mediated gluconeogenic gene expression. This phenomenon raises an interesting possibility that BTG2 may serve as a major player in regulating Nur77-mediated transcription of gluconeogenic genes in liver.

BTG2 Physically Interacts With Nur77 and Mediates Nur77 Occupancy on the G6pc Promoter

Based on our results of the role of BTG2 regulating *NBRE*- and *NurRE*-mediated transcription, we observed a physical interaction between BTG2 and Nur77 using a coimmunoprecipitation assay. Flag-*Btg2* was coprecipitated with HA-*Nur77* (Fig. 4*A*). In the coimmunoprecipitation assay, endogenous Nur77 was coprecipitated with endogenous BTG2 (Fig. 4*B*). Collectively, these results demonstrate that BTG2 physically interacts with Nur77 both in vitro and in vivo.

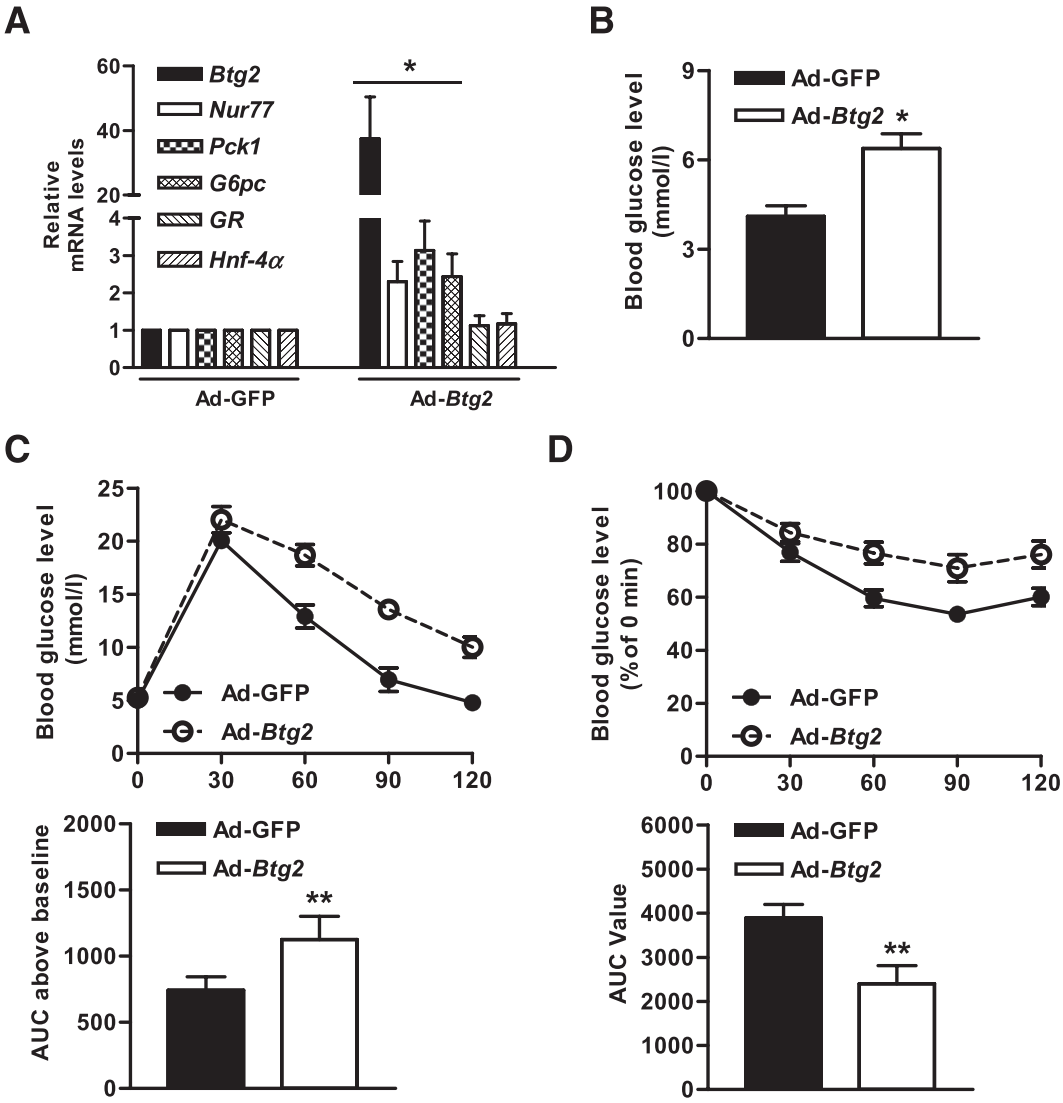

Figure 2—Overexpression of BTG2 resulted in an increase in hepatic gluconeogenesis. *A:* qPCR of gluconeogenic gene expression. *B:* Blood glucose levels from Ad-GFP– and Ad-*Btg2*–infected mice for 7 days (n = 4–6). *C* and *D:* Glucose and insulin tolerance tests from mice infected with Ad-*Btg2* at day 4 intravenously. *P < 0.05; **P < 0.01 vs. Ad-GFP. AUC, area under the curve.

Next, we investigated whether *Btg2* modulates the promoter activity of mouse *G6pc* gene as well as human *G6PC* gene through *Nur77*. Like forskolin, overexpression of Nur77 significantly increased the promoter activity of *G6pc* in a dose-dependent manner and luciferase activity by *Btg2* was synergistically increased by cotransfection of the *Nur77*. Introduction of mutation at the NBRE resulted in a decrease of promoter activity (Fig. 4*C* and *D*). To further confirm whether interaction of BTG2 with Nur77 affects the direct DNA binding of Nur77 protein on the promoters of these target genes, a ChIP assay was performed. The endogenous binding activity of Nur77 to its proximal region by forskolin treatment was significantly increased by Ad-*Btg2* compared with that of control (Fig. 4*E* and *F*). However, the nonspecific distal region of the h*G6PC* and m*G6pc* promoter was unable to recruit Nur77 under all conditions (Fig. 4*E* and *F*). These results indicate that BTG2 could contribute to the Nur77-dependent regulation of *G6pc* gene expression via a physical interaction.

BTG2-Mediated Induction of Hepatic Gluconeogenesis Is Mediated by Nur77

To determine whether the BTG2-induced regulation of hepatic gluconeogenesis is mediated by Nur77, we evaluated the effect of Nur77 on BTG2-mediated hepatic glucose production and gluconeogenic gene expression in primary hepatocytes. Increased glucose production and gene expression of *Creb* as well as *G6pc* by Ad-*Btg2* were attenuated by DN-*Nur77*, but not *Pck1* gene expression (Fig. 5*A* and *B*). Next, we confirmed whether Nur77 also regulates transcriptional activity of gluconeogenic genes.

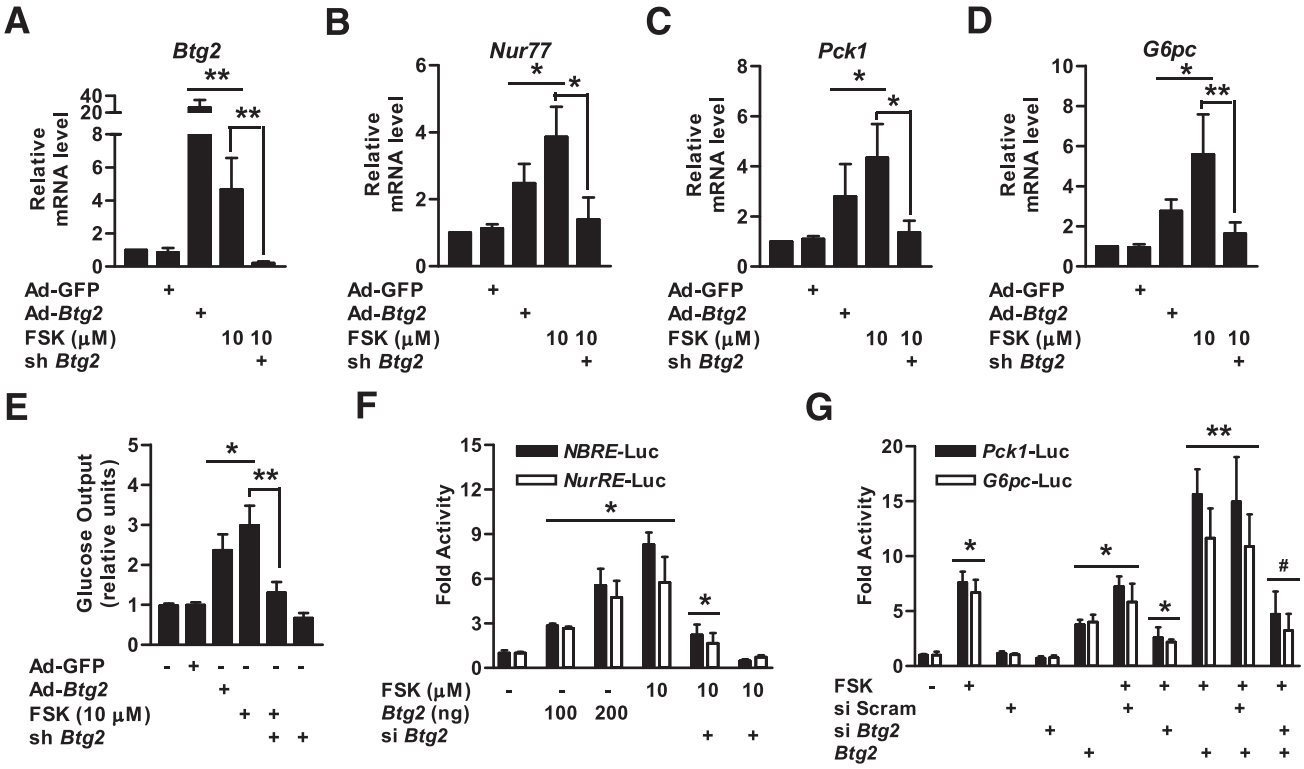

Figure 3—Induction of hepatic gluconeogenesis by glucagon is BTG2-dependent. *A–D:* Relative mRNA levels of *Btg2* and gluconeogenic pathway genes in mouse primary hepatocytes infected with Ad-GFP, Ad-*Btg2*, and lentivirus-sh*Btg2* (sh *Btg2*) at a multiplicity of infection of 60 and then exposed for 3–6 h in the presence or absence of forskolin (FSK). Total RNAs extracted from hepatocytes were subjected to qPCR analysis. *E:* Measurement of hepatic glucose production. A glucose output assay in primary hepatocytes was performed using glucose-free media supplemented with sodium lactate (20 mmol/L) and sodium pyruvate (1 mmol/L). *F* and *G:* AML-12 cells were transfected with si*Btg2* and siScram. After transfection for 36 h, cells were cotransfected with the indicated reporter genes and *Btg2* and then treated with FSK for 6 h. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. untreated control and/or FSK-treated cells; #$P < 0.05$ vs. FSK- and si*Btg2*-exposed cells.

G6pc and *Pck1* promoter activities were synergistically increased by *Btg2*, *Nur77*, and *Creb*. It is notable that *G6pc* promoter activity was decreased by DN-*Nur77* (Fig. 5*C*). Although *Pck1* gene promoter was significantly upregulated by *Btg2*, its promoter activities were not affected by DN-*Nur77* (Fig. 5*C*). Interestingly, while overexpression of *Hnf-4α*, *GR*, and *Pgc-1α* genes significantly increases the promoter activities of *Pck1* and *G6pc*, which is consistent with previous reports (4,5,7,9). Remarkably, this stimulatory effect of *Btg2* was not synergistically increased by cotransfection of the *Hnf-4α*, *GR,* and *Pgc-1α* (Fig. 5*D*). Thus, it is speculated that BTG2 acts as a coactivator upregulating the gluconeogenesis network with Nur77 and CREB (18), whereas it does not act as a crucial coactivator of *Hnf-4α*, *GR*, and *Pgc-1α*. Taken together, these results indicate that BTG2 functions together with Nur77 in the upregulation of gluconeogenesis in the primary hepatocytes.

Expression of Gluconeogenic Genes in Diabetic Models Are Increased by BTG2

Excessive hepatic gluconeogenesis contributes to the fasting hyperglycemia in diabetes (28). To access the functional contribution of BTG2 to the increase of hepatic glucose production in diabetes, streptozotocin-treated and *db/db* mice were used. qPCR analysis showed that streptozotocin injection strongly induced expression of the *Btg2* as well as the *Creb*, *Nur77*, *Pck1*, and *G6pc* genes in liver (Fig. 6*A*). Moreover, mRNA levels of *Btg2*, *Creb*, *Nur77*, *Pck1*, and *G6pc* are increased in the liver of *db/db* mice when compared with those of WT mice (Fig. 6*B*). These data indicate that BTG2 contributes to elevated gluconeogenesis in diabetes. When *db/db* mice were infected with sh*Btg2*, *Btg2* was significantly reduced in the liver. mRNA levels of *Btg2*, *Creb*, *Nur77*, *Pck1*, and *G6pc* were significantly elevated in liver of *db/db* mice, and these mRNAs were attenuated by sh*Btg2* (Fig. 6*B*). These findings suggest that BTG2 plays a pivotal role in transcriptional regulation of hepatic gluconeogenic genes in diabetic mouse models.

Hepatic Gluconeogenesis in *db/db* Mice Is Altered by BTG2

Finally, we investigated the crucial role of BTG2 on glucose homeostasis in fasted *db/db* mice. Administration of sh*Btg2* to *db/db* mice resulted in the amelioration of glucose tolerance (Fig. 7*A*). An insulin tolerance test revealed that *db/db* mice were significantly improved by

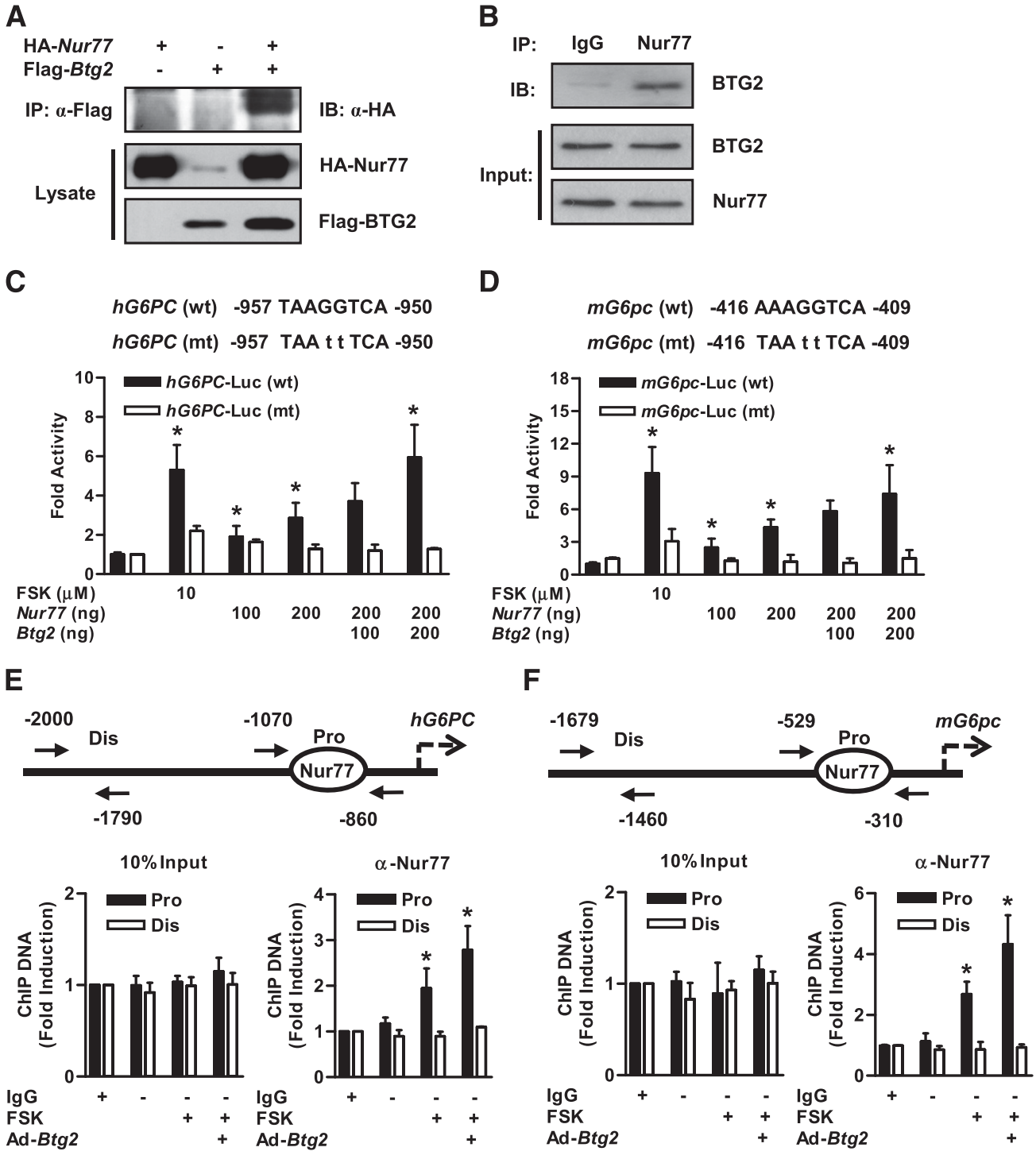

Figure 4—Identification of interaction between BTG2 and Nur77. *A:* Coimmunoprecipitation assays. Flag-*Btg2* and HA-*Nur77* were transfected to AML-12 cells. Cell lysates were precipitated (immunoprecipitation [IP]) with anti-Flag antibody and immunoblotted (IB) with HA antibody. *B:* Interaction between BTG2 and Nur77 in the liver of mice fasted for 12 h. Liver extracts were precipitated with Nur77 and immunoblotted with anti-BTG2 antibody. *C* and *D:* Effect of NurRE mutation on the Nur77-mediated activation of h*G6PC* and m*G6pc* promoters. Site-directed mutation was introduced into the h*G6PC* and m*G6pc*-NurRE sequence located at −957/−950 bp and −416/−409 bp, respectively. AML-12 cells were cotransfected with wild-type (wt) and mutant (mt) forms of the h*G6PC* and m*G6pc* gene promoter and Nur77. Forskolin (FSK) was used as a positive control. ChIP assay for the recruitment of Nur77 on the h*G6PC* (*E*) and m*G6pc* (*F*) gene promoter. Primary hepatocytes were infected with Ad-*Btg2* for 36 h and then treated with FSK for 6 h. Cell lysates were precipitated with anti-Nur77 antibody, and DNA was amplified using PCR primers binding to the specific proximal (Pro) and nonspecific distal (Dis) regions on these gene promoters. The quantity of immunoprecipitated DNA was normalized to input chromatin (10% of the chromosomal DNA used for immunoprecipitation). *$P < 0.05$ vs. untreated control or forskolin- and Nur77-treated cells. IgG, immunoglobulin G.

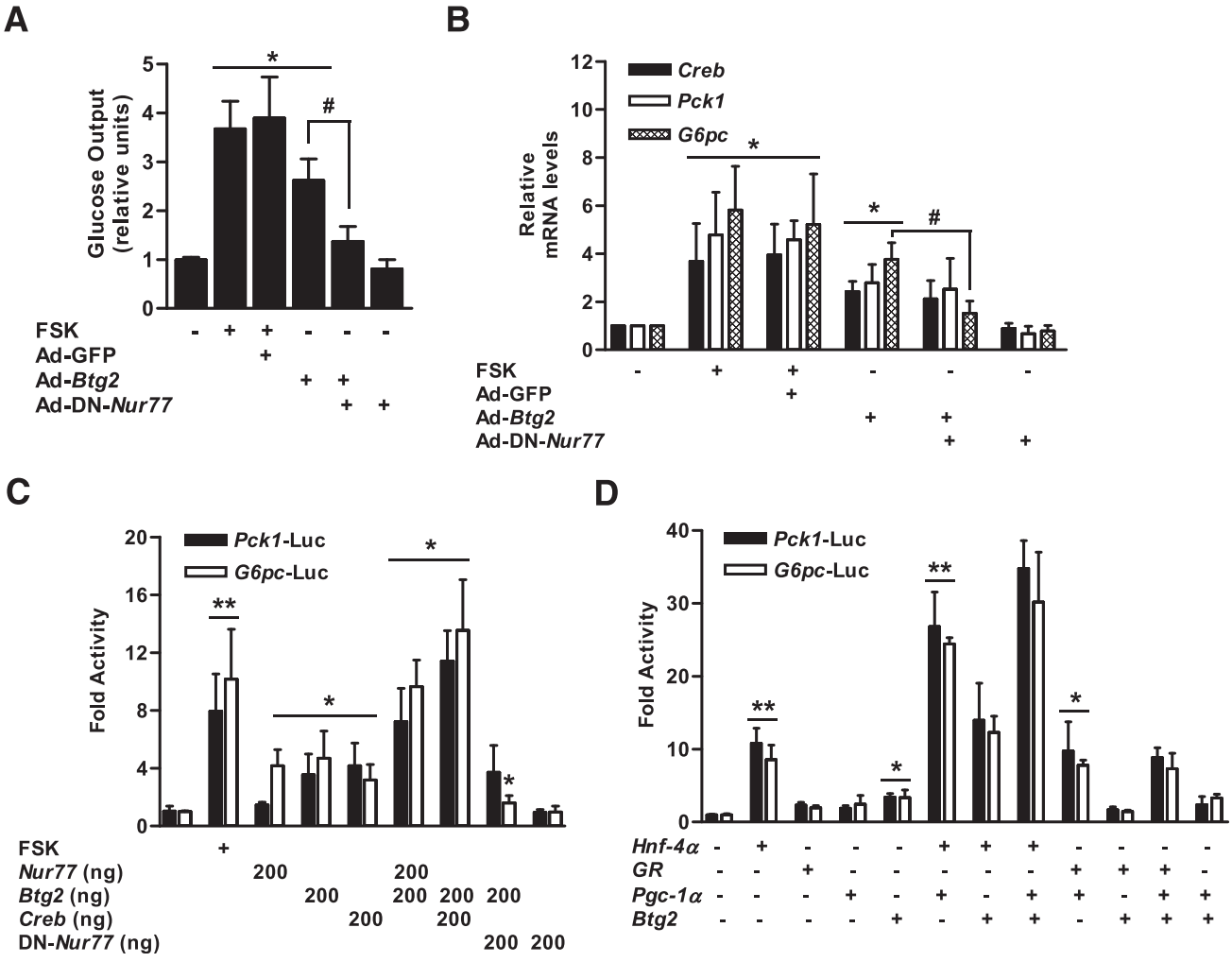

Figure 5—BTG2-mediated hepatic gluconeogenesis is mediated by Nur77. *A:* Mouse primary hepatocytes were infected with Ad-GFP, Ad-*Btg2*, and Ad-DN-*Nur77* at a multiplicity of infection of 60, and then hepatic glucose output was measured using glucose-free media supplemented with the sodium lactate (20 mmol/L) and sodium pyruvate (1 mmol/L). *B:* qPCR analysis for expression of *Creb* and hepatic gluconeogenic genes. Total RNAs were prepared from primary hepatocytes under the same conditions. *C* and *D:* AML-12 cells were transiently transfected with *Btg2*, *Nur77*, *Creb*, DN-*Nur77*, *Hnf-4α*, *GR*, *Pgc-1α*, and the indicated reporter genes. **P* < 0.05, ***P* < 0.01 vs. untreated control or individual transfected cells; #*P* < 0.05 vs. Ad-*Btg2*. FSK, forskolin.

sh*Btg2* (Fig. 7*B*). In addition, the increase of blood glucose levels in *db/db* mice was markedly reduced by silencing of *Btg2* (Fig. 7*C*). These results indicate that systemic glucose homeostasis in both physiology and diabetes is mediated by BTG2.

DISCUSSION

Our recent study revealed another physiological function of BTG2, namely hepatic gluconeogenesis in primary hepatocytes (18). BTG2 gene expression was increased during fasting, resulting in elevated transactivity of hepatic gluconeogenic genes. Moreover, the function of BTG2 has not been addressed in metabolic diseases like diabetes. In this study, Nur77 was identified as a new target of BTG2 involved in hepatic gluconeogenesis. The current study demonstrates that BTG2 increases hepatic glucose production through the upregulation of *G6pc* gene expression by physical interaction with Nur77 during both fasted and diabetic conditions. Overexpression of *Btg2* induced impaired glucose and insulin tolerances during glucose tolerance testing and insulin tolerance testing in mice. Administration of sh*Btg2* significantly improved glucose disposal in *db/db* mice.

Time-sequential expression of the *Btg2* gene increased soon after forskolin treatment, with mRNA expression of *Creb* and *Nur77* genes induced concurrently. Finally, both *Pck1* and *G6pc* genes were expressed in primary hepatocytes (Fig. 1*C*). Based on these data, we speculate that BTG2 may play a key role in modulation of gluconeogenesis at the upstream level of glucagon signaling.

A recent study suggested that BTG2 increases *Pck1* gene transcription through the induction of CREB in primary hepatocytes (18). CREB is a pivotal regulator of hepatic glucose production during fasting in the liver

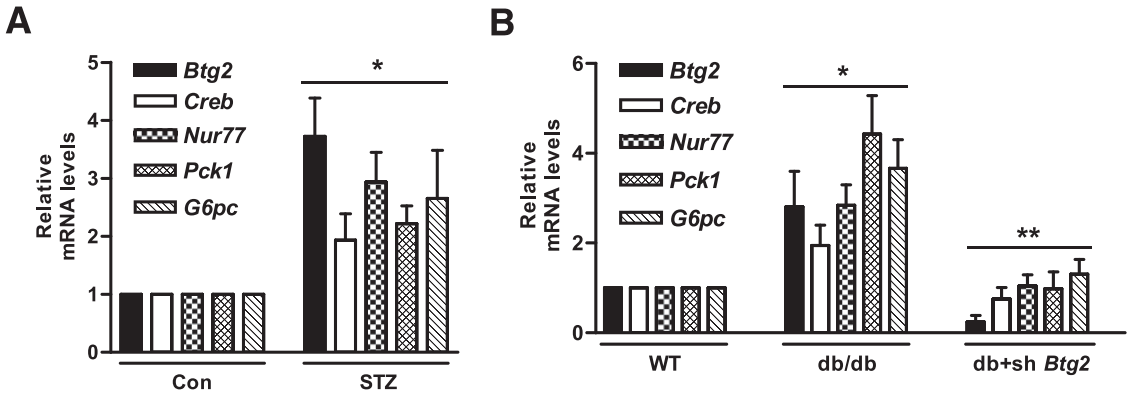

Figure 6—BTG2 regulates gluconeogenic gene expression in diabetic mouse models. qPCR analysis of *Btg2* and gluconeogenic genes from streptozotocin (STZ)-induced (80 mg/kg) diabetic mice (n = 4–6) (*A*) and *db/db* mice (*B*). At 7 days after shRNA administration, total RNA was extracted from 12-h fasted livers. *P < 0.05 vs. control or WT mice; **P < 0.01 vs. *db/db* mice. Con, control.

through binding to the *cis*-element of CREB on the target gene promoter. Gluconeogenesis is activated by regulation of *Pck1* through the interaction of BTG2 and CREB as well as glucagon signaling with cAMP and dexamethasone (4,12,18). However, even though *G6pc* gene expression was presently increased by overexpression of *Btg2* in primary hepatocytes, the regulatory mechanism by BTG2 has not been clear to explain an increase of *G6pc* gene expression both in vitro and in vivo. The current study expands upon these findings to show that transcription of *G6pc* gene is activated by BTG2 through regulation of Nur77 gene expression, which is a novel copartner of BTG2 in the liver during fasted and diabetic conditions.

Nur77 is induced by multiple extracellular signals in a tissue-specific manner. Nur77 is also involved in 3T3-L1 adipogenesis response to cAMP signaling without peroxisome proliferator–activated receptor γ agonist (29). In the liver, Nur77 modulates hepatic gene expression (10). Nur77 is also a mediator of cAMP action in the regulation of hepatic glucose metabolism (12). The expression of Nur77 in hepatocytes is increased by cAMP in vitro and by glucagon during fasting in vivo (12). In this study, *Nur77* mRNA level was induced by *Btg2* overexpression and abolished by its knockdown using shRNA (Fig. 3*B*). Moreover, *Nur77* gene expression was accelerated by *Creb* in fasted liver, consistent with a previous report (30). These results suggest that Nur77 gene expression is stimulated by the BTG2–CREB axis during fasting and diabetes. Therefore, Nur77 is not only increased in mRNA expression, but also induces protein interaction with BTG2 to elevate hepatic glucose production.

Pei et al. (12) demonstrated that various genes involved in the gluconeogenesis including *G6pc*, *Fbp1*, and *Fbp2* were upregulated by *Nur77*, but not *Pck1*. Moreover, promoter activity of these genes was increased by direct binding of Nur77 (12). In the current study, the Nur77 binding region was confirmed on the *G6pc* promoter, and deletion of the Nur77 response element site abolished BTG2-induced *G6pc* transcription. Interestingly, although a Nur77 response *cis*-element has been not identified on the *Pck1* gene promoter, endogenous *Pck1* gene expression as well as promoter activity was induced by overexpression of *Btg2* (Figs. 2*A* and 3*G*). Thus, it is speculated that Nur77 directly upregulates the expression of multiple genes involved in glucose metabolism, whereas *Pck1* may be regulated by Nur77 in an indirect manner both in vitro and in vivo. In addition, a previous study addressed not only BTG2-induced physical interaction with CREB in fasting, but also discovered the binding region of the CREB protein on the *Pck1* promoter (18). The data indicate that *Pck1* gene expression might be induced by BTG2 through CREB, not Nur77, in liver during fasting and in diabetes. Administration of sh*Btg2* into *db/db* mice ameliorated blood glucose levels by inhibiting *Pck1* and *G6pc* gene expression via decrease of Nur77 and CREB. In addition, gene silencing of *Btg2* by lenti-shRNA significantly improved both glucose and insulin tolerance in *db/db* mice. These findings in vivo are consistent with our previous study that reported the function of BTG2 in vitro (18). Although the physiological role of BTG2 has been verified in diabetic mouse model, the role of BTG2 still needs to validate among human samples to develop as a new therapeutic agent in diabetes.

In conclusion, the current study demonstrates that Nur77 is a novel target of BTG2 and BTG2 regulates hepatic glucose production through Nur77-mediated *G6pc* gene expression. We characterized the role of BTG2 as a novel transcriptional coactivator of hepatic gluconeogenesis in vivo and elucidated the molecular mechanism of regulation of glucose homeostasis mediated by a link of BTG2 and Nur77 as a key regulator of gluconeogenesis. Moreover, disruption of BTG2 ameliorates glucose disposal and insulin resistance in diabetic animal model. Thus, as we depict in the novel schematic model shown in Fig. 7*D*, regulation of BTG2 may offer an effective

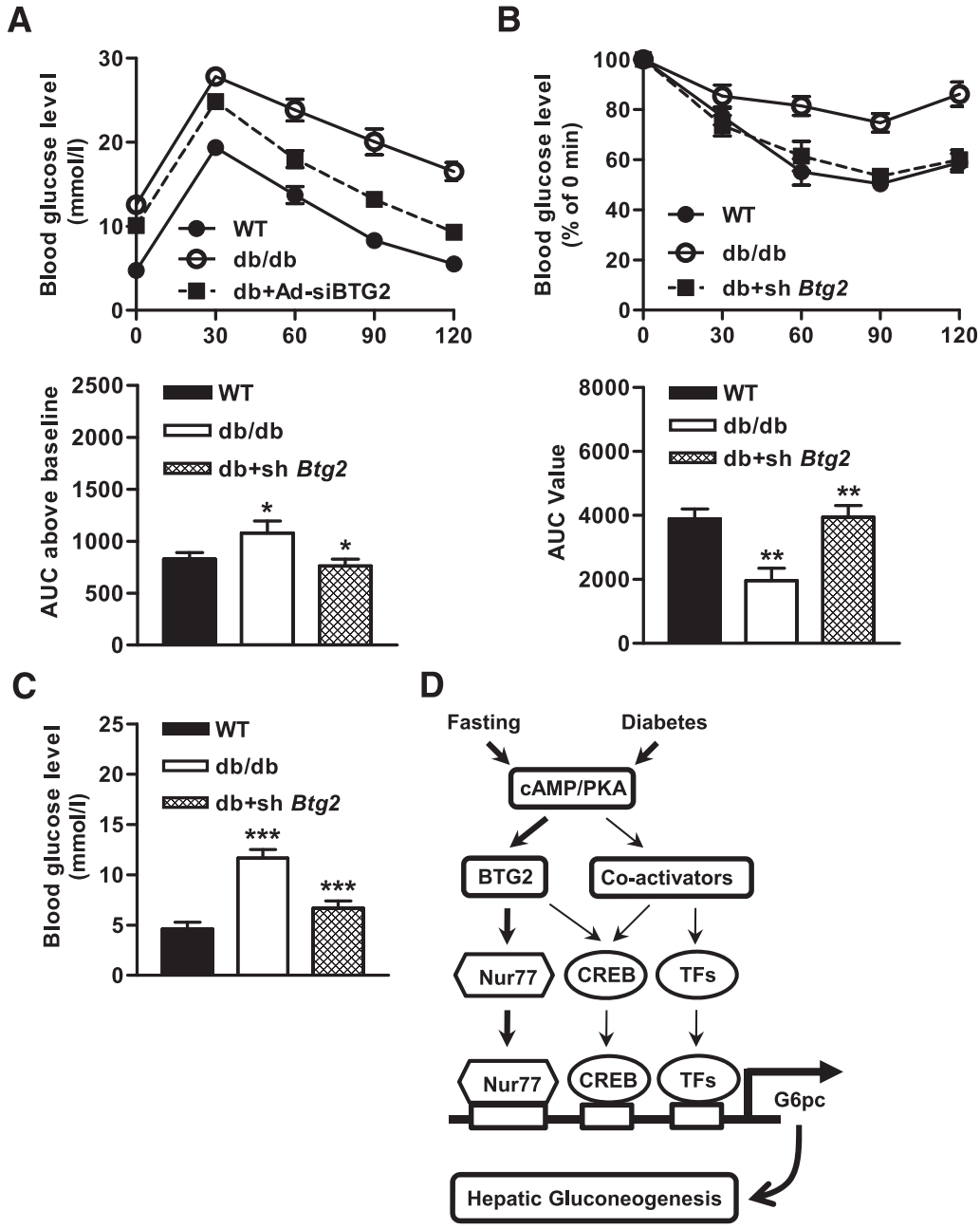

Figure 7—Disruption of BTG2 improves glucose homeostasis in diabetic mice. *A* and *B:* Blood glucose profiles of glucose and insulin tolerance tests at day 4 after sh*Btg2* infection. The respective area under the curve (AUC) values for glucose and insulin tolerance tests were represented. *C:* Blood glucose levels measured 7 days after sh*Btg2* infection. Blood was withdrawn from fasted mice (n = 5–10). *D:* Scheme of the role of BTG2 in the regulation of glucose homeostasis. In the fasting and diabetic states, *G6pc* gene expression is induced by BTG2-Nur77 as well as BTG2 transcription factors (TFs) and a coactivator signaling system for which the mechanism involves either upregulation of Nur77 or TF occupancy on its promoter. *P < 0.05; **P < 0.01; ***P < 0.001 vs. WT or *db/db* mice. PKA, protein kinase A.

means to control hepatic glucose homeostasis in diabetes and may guide the development of novel therapeutic target for metabolic diseases like diabetes.

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