The KLF14 transcription factor regulates hepatic gluconeogenesis in mice

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Krüppel-like factor 14 (KLF14) is a member of the Cys_2/His_2 zinc-finger DNA-binding proteins. Despite strong evidence showing that a polymorphism in the *Klf14* gene is closely linked to the development of type 2 diabetes, the physiological and metabolic functions of KLF14 still remain unclear. In the present study, we investigated the role of KLF14 in the regulation of hepatic gluconeogenesis. Adenoviruses expressing KLF14 (Ad-*Klf14*) or KLF14-specific shRNAs (Ad-sh*Klf14*) were injected into normal C57BL/6J, *db/db* diabetic, or high-fat diet–induced obese (DIO) mice. Gene expression, hepatic glucose production, glucose tolerance, and insulin resistance were tested in C57BL/6J, *db/db*, and DIO mice and primary hepatocytes. Our results demonstrate that KLF14 expression is induced in the livers of normal C57BL/6J mice upon fasting and significantly up-regulated in the livers of *db/db* mice, suggesting a physiological link between KLF14 and gluconeogenesis. Adenovirus-mediated overexpression of KLF14 in primary hepatocytes increased both the mRNA and protein levels of peroxisome proliferator-activated receptor-γ coactivator 1α (*Pgc-1α*, also known as Ppargc1a), thereby stimulating cellular glucose production. Conversely, knockdown of KLF14 expression led to a reduction in PGC-1α, subsequently inhibiting glucose output in primary hepatocytes. Finally, forced expression of KLF14 in the livers of normal mice increased the plasma glucose levels and impaired glucose tolerance; in contrast, KLF14 knockdown in diabetic mouse livers improved glucose tolerance. Taken together, our data strongly indicate that KLF14 modulates hepatic gluconeogenesis.

Type 2 diabetes (T2D)[5] is a pandemic metabolic disease that affects millions of people worldwide. Systemic dysregulation of micro- and macronutrients is closely associated with the development of chronic metabolic diseases, such as T2D, obesity, and cardiovascular disease (1–3). It is well known that hepatic glucose production is highly elevated after overnight fasting, and its abnormal increase is positively associated with fasting hyperglycemia (4–6). Hepatic glucose production is central to metabolic adaptation during fasting, and its abnormal elevation is a major determinant of fasting hyperglycemia in diabetes (6, 7). During short-term fasting, the liver produces glucose by glycogenolysis and gluconeogenesis, whereas upon prolonged fasting, glucose is synthesized almost exclusively from gluconeogenesis (8).

Krüppel-like factors (KLFs) belong to a subclass of Cys_2/His_2 zinc-finger DNA-binding proteins and play an essential role in health and diseases (9). For example, *Klf15* knockout mice display severe hypoglycemia after prolonged fasting (10). Our recent studies have demonstrated that KLF11 is involved in the regulation of hepatic glucose and lipid metabolism *in vivo* (11, 12). Seventeen similar but distinct KLF family members have been identified, and they are tightly linked to disease phenotypes in different organisms ranging from flies to humans by regulating the target cellular network (13). Several studies have also suggested that KLF14 is an important regulator of metabolic diseases, such as diabetes and obesity (14, 15). Human genetic studies have shown that DNA variants near the *Klf14* gene are strongly associated with HDL cholesterol levels, trig-

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This article contains Tables S1–S4 and Figs. S1–S6.

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[5] The abbreviations used are: T2D, type 2 diabetes; Ad-*gfp*, adenovirus containing green fluorescent protein; Ad-*Klf14*, adenovirus expressing KLF14; Ad-shCtrl, control adenovirus expressing short-hairpin RNA against luciferase; Ad-sh*Klf14*, adenovirus expressing short hairpin RNA against KLF14; DEX, dexamethasone; DIO, diet-induced obese; FSK, forskolin; GTT, glucose tolerance test; ITT, insulin tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PTT, pyruvate tolerance test; TG, triglyceride; FFA, free fatty acid; HFD, high-fat diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; qPCR, quantitative PCR.

lyceride (TG) levels, risk of T2D mellitus, and risk of coronary artery disease (16). Due to the strong link between KLF14 and metabolic diseases, KLF14 has been referred to as a "conductor of the metabolic syndrome orchestra" (14). KLF14 was recently identified as an activator protein and novel regulator of lipid signaling by binding to GC-rich regions in the sphingosine kinase 1 (*Sk1*) promoter and regulating fibroblast growth factor 2 (FGF2)-stimulated *Sk1* promoter activity (17). A previous study has proven that KLF14 increased plasma HDL-C levels and cholesterol efflux capacity by increasing ApoA-I expression (18). An *in vitro* study showed that KLF14 could improve insulin sensitivity and increase glucose uptake in Hepa1-6 cells (19). Although the role of KLF14 in regulating HDL-C levels has now been clearly established, its role in glucose metabolism and insulin actions remains largely unknown.

Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a transcription coactivator that interacts with a broad range of transcription factors, regulating a subset of genes involved in cellular metabolisms, such as mitochondrial biogenesis, glucose metabolism, fatty acid metabolism, adaptive thermogenesis, etc. (20). In the liver, PGC-1α expression is tightly regulated in response to low systemic glucose levels. Under normal or fed conditions, PGC-1α is expressed at a barely low level. In contrast, during fasting, PGC-1α is robustly increased to promote hepatic glucose production by stimulating hepatic gluconeogenesis and fatty acid oxidation (21). The induction of PGC-1α is activated by glucagon and catecholamines through the cAMP pathway and cAMP-responsive element–binding protein transcription factor. PGC-1α transactivates gluconeogenic gene expression by coactivating a variety of transcription factors, including hepatic nuclear factor-4α (HNF4α), glucocorticoid receptor, forkhead box O1 (FOXO1), and cAMP-responsive element–binding protein (22). These transcription factors bind to the promoter regions of genes that encode key gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (23).

In the present study, we examined the physiological and molecular functions of KLF14 in liver gluconeogenesis. Our results indicated that adenovirus-mediated overexpression of KLF14 in mouse primary hepatocytes increased the master regulator of hepatic gluconeogenesis, PGC-1α, as well as PGC-1α target gene expression. In contrast, knockdown of KLF14 in primary hepatocytes led to the down-regulation of corresponding target gene expression. Our results also indicated that Ad-*Klf14* infection increased glucose production in mouse primary hepatocytes. We also demonstrate that Ad-*Klf14*-mediated overexpression of KLF14 in the liver significantly increased fasting blood glucose and impaired glucose tolerance and insulin sensitivity in mice. The present study demonstrated that the KLF14 is a critical physiological regulator of hepatic gluconeogenesis.

Results

Hepatic Klf14 gene expression is regulated by nutritional status and dysregulated in diabetic mice

Genetic evidence from various studies has shown that *Klf14* is closely related to metabolic diseases, including neonatal/juvenile diabetes and metabolic syndrome with obesity-associated insulin resistance, leading to adult T2D (15, 16, 24). Recent studies have demonstrated that KLF14 could regulate lipid metabolism and is involved in glucose uptake and insulin actions *in vitro* (18, 19). However, whether KLF14 regulates glucose homeostasis *in vivo* remains unknown. To better understand the role of KLF14 in hepatic glucose metabolism, *in vivo* KLF14 expression was investigated under different nutritional statuses. The results demonstrated that overnight fasting led to a significant induction in both *Klf14* mRNA and protein levels in the liver and that the induction was restored upon refeeding (Fig. 1*A*). Next, KLF14 expression levels were further investigated in a mouse model of diabetes and obesity utilizing *db/db* and high fat diet (HFD)–induced obese (DIO) mice. Compared with the KLF14 expression levels in the control group, the mRNA and protein expression levels unexpectedly increased in both the *db*/*db* and DIO mouse groups (Fig. 1, *B* and *C*). A similar induction of KLF14 expression was observed in the genetic obesity model, the *ob/ob* mice (Fig. S1*A*). Previous studies have shown that FSK could activate the cAMP signaling pathway, which mimics the fasting action of glucagon (25). Here, our results demonstrated that the treatment of mouse primary hepatocytes with FSK significantly induced *Klf14* mRNA and protein levels, whereas the presence of insulin decreased KLF14 expression levels (Fig. 1, *D* and *E*); these results were similar to those in HepG2 cells (Fig. S1, *B* and *C*). However, KLF14 expression was only modestly induced by DEX (data not shown), which mimics the functions of glucocorticoids and activates the gluconeogenic pathway (26). Collectively, these results demonstrated that KLF14 may be involved in hepatic glucose metabolism and T2D pathogenesis.

Transactivation of the Pgc-1α promoter by KLF14

To demonstrate whether KLF14 regulates glucose and lipid metabolism in the liver, primary hepatocytes were isolated and infected with a KLF14 expression vector or the empty vector, followed by RNA isolation and RNA-seq to assess the global gene expression profile. From the RNA-seq results, glucose and lipid metabolism–related genes, such as *Pgc-1α*, *G6pc*, and *Ucp1*, were dysregulated in the hepatocytes upon KLF14 overexpression (Fig. 2*A*). KLF14 expression is correlated with *Pgc-1α* and *G6pc* expression, and these genes were significantly induced in the fasted state and decreased in the refed state (Fig. S2). To further understand whether KLF14 directly regulates these glucose and lipid metabolism-related genes, the promoter sequences from these genes were extracted from the database and further analyzed. We speculated that KLF14 may regulate *Pgc-1α* gene expression. The characteristic feature of KLFs is the presence of three Krüppel-like zinc fingers that bind to CACCC elements or GC-rich sequences in the promoter region to mediate transcriptional activation and/or repression (27). A previous study identified that KLF14 is able to bind to the CACCC sequence in the *Apoa1* promoter and drive promoter activity (18). Thus, promoter analysis was performed to explore the mechanistic and physiological roles of KLF14 in the up-regulation of *Pgc-1α*. Promoter sequence analysis indicated the presence of two potential putative KLF14-binding sites

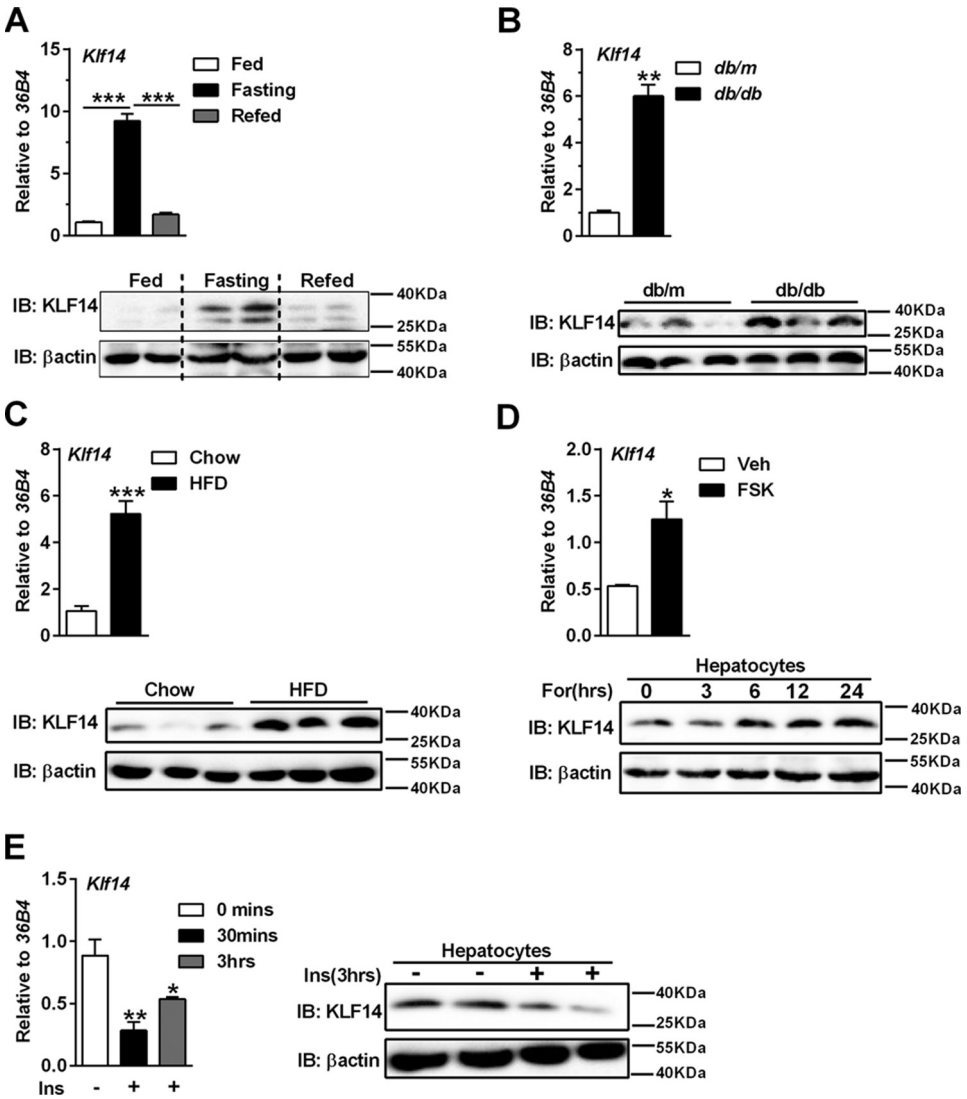

Figure 1. Klf14 expression is regulated by nutritional status and is induced in *db/db* and DIO mouse livers. *A*, real-time qPCR analysis of hepatic *Klf14* mRNA (*top*) and protein (*bottom*) levels in C57BL/6J mice under *ad libitum* feeding, 24-h fasting, and 24-h fasted/12-h refed conditions (n = 4/group). *B*, the real-time qPCR and Western blot analysis for *Klf14* mRNA (*top*) and protein (*bottom*) levels in livers from *db/m* or *db/db* mice (n = 4/group). *C*, the real-time qPCR and Western blot analysis for *Klf14* mRNA (*top*) and protein (*bottom*) levels in livers from C57BL/6J fed normal chow or an HFD for 16 weeks (DIO mice) (n = 4/group). *D*, real-time qPCR (*top*) and Western blot (*bottom*) analyses for *Klf14* expression in mouse primary hepatocytes treated with 10 μM FSK for various lengths of time. *E*, the real-time qPCR (*left*) and Western blot (*IB*; *right*) analyses for *Klf14* expression in mouse primary hepatocytes exposed to 100 nM insulin (*Ins*) for different lengths of time. Values represent the means ± S.E. (*error bars*) of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(CACCC elements) located from −1306 to −1301 and from −1048 to −1043 bp in the 2015-bp *Pgc-1α* promoter; these sequences were subsequently cloned into the pGL3-luciferase vector. The reporter assay was performed in HepG2 cells by co-transfecting the *Pgc-1α* promoter construct and KLF14 expression vector. An ~3.5-fold increase in *Pgc-1α* promoter activity was induced with KLF14 overexpression, suggesting that *Pgc-1α* may be a target of KLF14 (Fig. 2*B*). To search for the potential region in the *Pgc-1α* promoter necessary for the KLF14-mediated induction, deletion analysis of the *Pgc-1α* promoter was performed to generate shorter *Pgc-1α* promoter fragments by deletion of multiple CACCC elements. Three shorter promoter constructs were generated and co-transfected with the KLF14 expression vector into HepG2 cells. The deletion of the upstream 600 bp (p-*Pgc-1α*-1402) did not affect KLF14-mediated induction compared with that of the longest promoter construct. However, further deletion (p-*Pgc-1α*-907 and -253) led to the ablation of KLF14-mediated induction of promoter activity (Fig. 2*B*), suggesting that this promoter region may serve as a putative binding sequence necessary for the KLF14-dependent activation of the *Pgc-1α* promoter. As expected, mutation of the −1048/−1043 nucleotide site greatly reduced promoter activity (Fig. 2*C*); however, the −1306/−1301 nucleotide mutation did not affect the KLF14-mediated induction (Fig. S3). We also performed a ChIP assay to determine whether exogenous KLF14 protein could be recruited to the *Pgc-1α* promoter. First, mouse primary hepatocytes were isolated and infected with adenoviruses expressing FLAG-tagged KLF14. As a result, the *Pgc-1α* promoter fragment containing the CACCC elements could be amplified from DNA complexes precipitated with the anti-FLAG antibody in primary hepatocytes (Fig. 2*D*) but not when using normal mouse

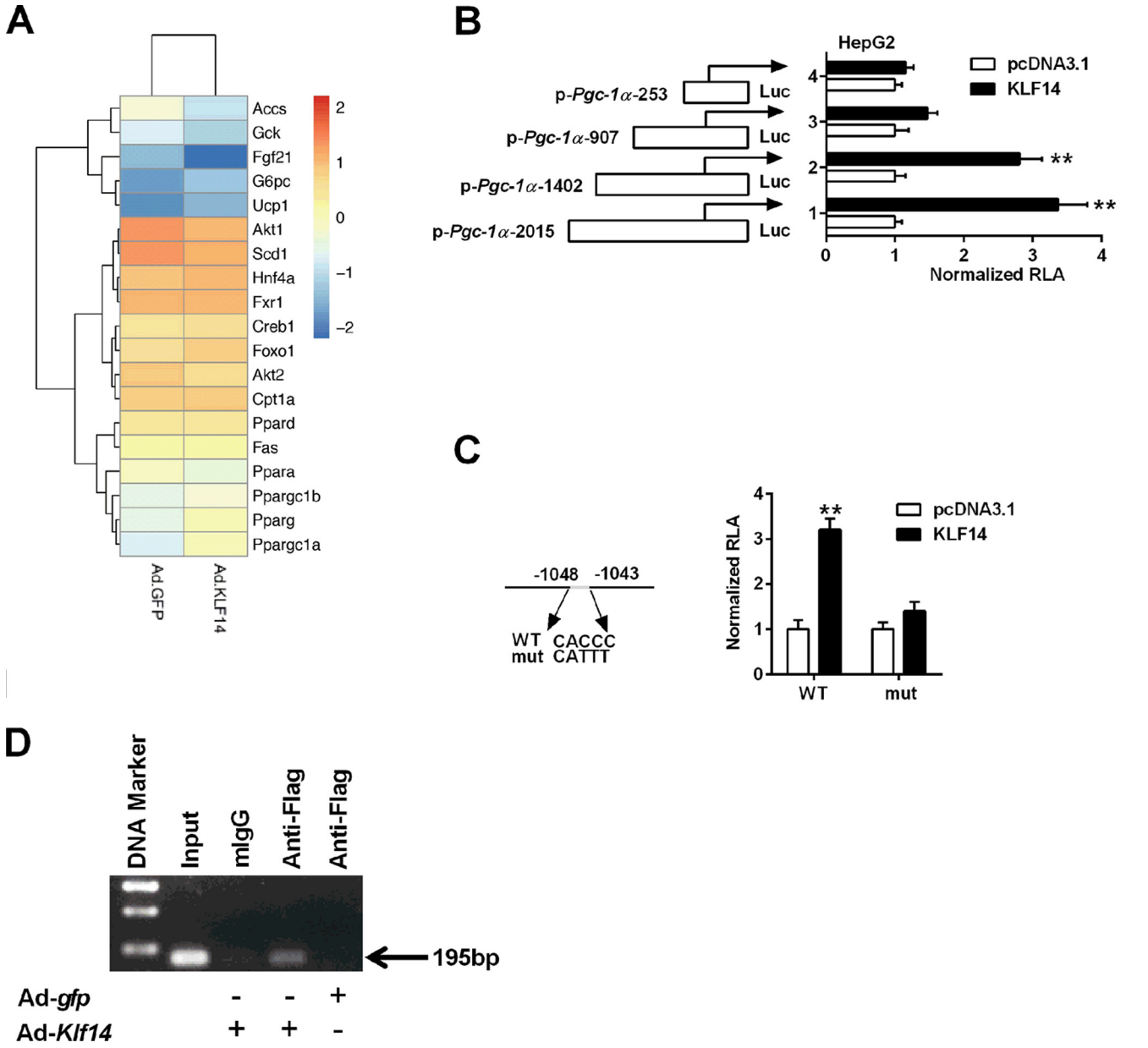

Figure 2. KLF14 regulates *Pgc-1α* gene promoter activity in HepG2 cells. *A*, heat map of the expression of dysregulated mRNAs involved in glucose and lipid metabolism in mouse primary hepatocytes infected with either the Ad-*gfp* or Ad-*Klf14* adenovirus. *B*, the 2015-bp *Pgc-1α* promoter was PCR-amplified and cloned into the pGL3 firefly luciferase reporter vector. The subsequent deletion constructs were generated (p-1402, -907, and -253-*Pgc-1α*). The luciferase constructs were co-transfected with the KLF14 expression vector or pcDNA3.1 as control vector into the HepG2 cells. After 48 h, the luciferase activity was measured. *C*, the pGL3-*Pgc-1α*-2015 promoter construct and mutant construct (pGL3-*Pgc-1α*-mut) were transfected into HepG2 cells together with the pcDNA3.1-KLF14 expression plasmids or pcDNA3.1 (control). After 48 h, the relative luciferase activity (*RLA*) was measured. *D*, mouse primary hepatocytes were infected with adenoviruses expressing KLF14-FLAG fusion protein (Ad-*Klf14*). ChIP assays were performed as described under "Experimental procedures." Protein-DNA complexes were immunoprecipitated with control mouse IgG (control) or anti-FLAG antibody. The resultant DNA was analyzed by PCR with primers to amplify the promoter region flanking the *Pgc-1α* CACCC elements. Amplified DNA fragments are indicated by *arrows*. Values represent the means ± S.E. (*error bars*) of three independent experiments. **, $p < 0.01$.

IgG. Indeed, ChIP results showed significant KLF14 binding to the −1189 to −993 region in the *Pgc-1α* promoter. These data suggest that exogenous KLF14 proteins could bind to the *Pgc-1α* gene promoter.

KLF14 overexpression promotes gluconeogenic and hepatic glucose production in mouse primary hepatocytes

To determine the regulatory effects of KLF14 on endogenous *Pgc-1α*, a gain- and loss-of-function assay was performed to determine the physiological and functional importance of KLF14 on gluconeogenic programming in cultured primary hepatocytes. Mouse primary hepatocytes were isolated and infected with Ad-*Klf14* expressing FLAG-tagged Ad-*Klf14* or Ad-*gfp*. Western blot analysis indicated that this adenovirus effectively increased KLF14 protein levels (Fig. 3*B*). Consistent with the results from the luciferase reporter gene assay, KLF14 was also able to activate expression of endogenous gluconeogenic genes, including *Pgc-1α*, *Pck1*, and *G6pc* (Fig. 3, *A* and *B*). Accordingly, KLF14 overexpression markedly enhanced glucose production in primary hepatocytes (Fig. 3*C*). Additionally,

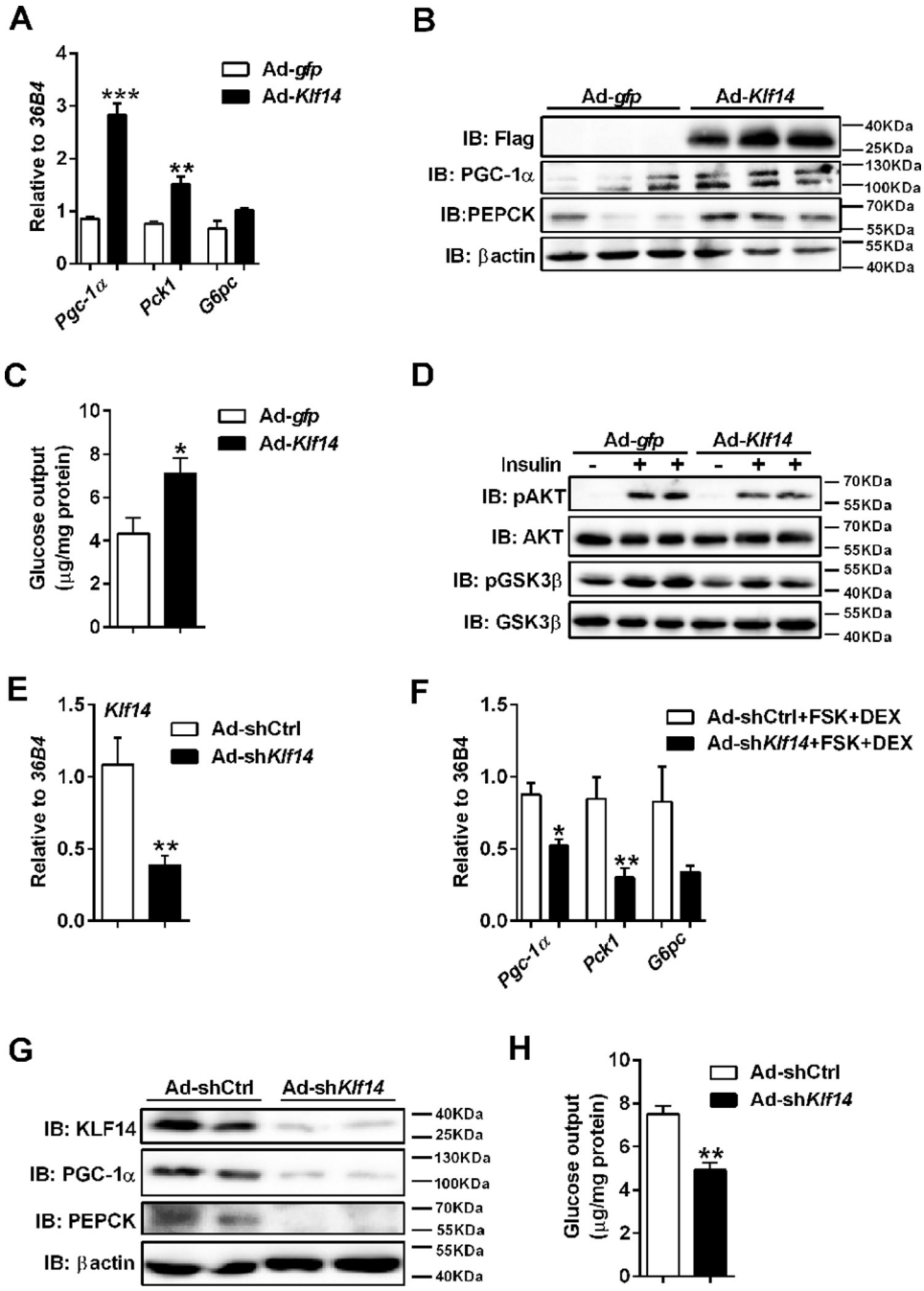

Figure 3. KLF14 regulates the expression of gluconeogenic genes in mouse primary hepatocytes. *A*, real-time qPCR analysis for *Klf14* and gluconeogenic gene mRNA expression levels in mouse primary hepatocytes infected with either the Ad-*gfp* or Ad-*Klf14* adenovirus. *B*, Western blot analysis (*IB*) of KLF14, PGC-1α, and PEPCK protein levels in mouse primary hepatocytes infected with either the Ad-*gfp* or Ad-*Klf14* adenovirus. *C*, measurement of cellular glucose production in the primary hepatocytes described in *A*. *D*, forced expression of *Klf14* in primary hepatocytes significantly decreased insulin-stimulated phosphorylation of AKT and GSK-3β. *E*, real-time qPCR analysis of KLF14 mRNA expression levels in primary hepatocytes infected with either the Ad-shCtrl or Ad-sh*Klf14* adenovirus. *F*, real-time qPCR analysis for gluconeogenic gene mRNA expression levels in primary hepatocytes infected with either the Ad-shCtrl or Ad-sh*Klf14* adenovirus. At 36 h after infection, hepatocytes were switched to starvation medium for 6 h, followed by treatment with 10 μM FSK and 1 μM DEX for 3 h. *G*, Western blot analysis of PGC-1α and PEPCK protein levels in mouse primary hepatocytes as described in *F*. *H*, measurement of cellular glucose production in primary hepatocytes as described in *F*. Values represent the means ± S.E. (*error bars*) of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

we also demonstrated that forced expression of KLF14 in primary hepatocytes significantly decreased the insulin-stimulated phosphorylation of AKT and GSK3β (Fig. 3*D*). Next, we used loss-of-function studies to further determine whether KLF14 is required for the increase in gluconeogenic gene expression. We generated an adenovirus expressing KLF14-specific shRNAs (Ad-sh*Klf14*). Treatment of primary hepatocytes with Ad-sh*Klf14* effectively decreased *Klf14* mRNA and protein expression (Fig. 3, *E–G*). Conversely, the Ad-sh*Klf14*-mediated reduction in KLF14 expression in primary hepatocytes significantly reduced *Pgc-1α* and *Pck1* expression levels in the presence of FSK and DEX; these drugs mimic the fasting

actions of glucagons and glucocorticoids, respectively (Fig. 3, *F* and *G*). The mRNA levels of *G6pc* were also decreased in Ad-sh*Klf14*-infected primary hepatocytes, although the difference was not statistically significant (Fig. 3*F*). Accordingly, KLF14 knockdown markedly decreased glucose production in mouse primary hepatocytes (Fig. 3*H*). These data clearly indicated that endogenous *Pgc-1α* is a KLF14 target in primary hepatocytes and that KLF14 increases cellular glucose production by up-regulating gluconeogenic gene expression.

KLF14 overexpression in the C57BL/6J mouse liver impairs glucose homeostasis and insulin tolerance

We have shown that manipulation of KLF14 levels in primary cultured hepatocytes dysregulates glucose homeostasis-related genes. However, the detailed physiological function of KLF14 *in vivo* is still unclear. Thus, adenovirus carrying a KLF14 expression cassette was injected into the tail veins of C57BL/6J mice; this effectively augmented *Klf14* mRNA and protein levels in the liver (Fig. 4, *A* and *C*) but did not affect KLF14 expression in the other tissues examined, including adipose and muscle tissues (Fig. S4*A*). Subsequently, the overexpression of KLF14 in mice led to a induction of the expression of gluconeogenic genes, including *Pgc-1α*, *Pck1*, and *G6pc* (Fig. 4, *B* and *C*). Meanwhile, the induction of liver KLF14 expression also led to a significant increase in fasting plasma glucose and insulin levels (Fig. 4, *D* and *E*). In addition, forced expression of the hepatic *Klf14* gene resulted in an increase in serum TG levels, hepatic cholesterol levels, and circulating non-esterified fatty acid (free fatty acid; FFA) while decreasing the liver weight/body weight ratio (Table S2). However, Ad-*Klf14*-infected mice did not display obvious differences in serum cholesterol levels, hepatic TG levels, body weight, or markers of liver damage (ALT or AST levels in the serum) compared with the Ad-GFP–treated (control) mice (Table S2). The glucose tolerance test (GTT) and insulin tolerance test (ITT) experiments indicated that hepatic overexpression of KLF14 significantly impaired glucose tolerance and insulin tolerance compared with the expression in control mice (Fig. 4, *F* and *G*). We also utilized a pyruvate tolerance test (PTT) in these mice. The PTT confirmed that hepatic gluconeogenesis was increased in the Ad-*Klf14*–infected mice (Fig. 4*H*), indicating that an increase in hepatic gluconeogenesis contributes to higher glucose levels. The AUC significantly increased in the GTT, ITT, and PTT in the mice with hepatic overexpression of KLF14 (Fig. S4, *B–D*). We next examined whether the up-regulation of KLF14 expression levels in the mouse liver affected the insulin signaling pathway and led to impaired glucose homeostasis. Indeed, we found that up-regulation of KLF14 impaired the phosphorylation levels of insulin-stimulated AKT and its downstream target GSK3β but did not change the total AKT and GSK3β levels (Fig. 4*I*). A recent study reported that perhexiline, a *Klf14* activator (18), could increase KLF14 expression levels. Thus, we tested the effects of perhexiline-mediated activation of the KLF14 pathway on glucose metabolism in normal C57BL/6J mice. The mice were treated five times with either perhexiline (10 mg/kg) or DMSO for 7 days via gavage administration. Western blot showed that perhexiline could increase KLF14 and PGC-1α protein levels (Fig. S5*A*). Compared with the controls, the perhexiline-treated mice had significantly increased fasting blood glucose levels (Fig. S5*B*). Together, our data reveal that forced expression of KLF14 in the liver promoted hepatic gluconeogenesis in C57BL/6J mice, resulting in higher blood glucose levels and impaired glucose tolerance.

KLF14 knockdown in db/db and DIO mouse livers improves glucose tolerance

KLF14 expression is highly induced in the livers of *db/db* and DIO mice. Therefore, we sought to investigate whether the down-regulation of KLF14 in the liver could improve systemic glucose metabolism. Thus, Ad-sh*Klf14* was injected into *db/db* mice; this led to a reduction in KLF14 expression in the liver, followed by decreased expression of gluconeogenic genes, including *Pgc-1α*, *Pck1*, and *G6pc* (Fig. 5, *A* and *B*), and lower fasting blood glucose levels compared with the control group (Fig. 5*C*). These data suggested that the KLF14-mediated reduction in the blood glucose levels was associated with fasting hepatic glucose production. Moreover, a reduction in fasting insulin levels was also observed in the Ad-sh*Klf14*–injected group (Fig. 5*D*). GTT and PTT experiments indicated that KLF14 knockdown in *db/db* mouse livers significantly ameliorated glucose tolerance and decreased hepatic gluconeogenesis levels; in contrast, ITT experiments barely affected insulin sensitivity, as the baseline glucose levels in these mice were lower than those in the control mice (Fig. 5, *E–G*). The AUC significantly decreased in the GTT and PTT experiments and decreased slightly but did not reach significant difference in the ITT experiment in mice with hepatic knockdown of KLF14 (Fig. S6, *A* and *B*). Meanwhile, KLF14 knockdown in the *db/db* mouse livers was also accompanied by an increase in AKT and GSK3β phosphorylation in the liver in response to insulin (Fig. 5*H*). Biochemical analysis indicated that the Ad-sh*Klf14* adenovirus treatment increased the liver weight/body weight ratio while decreasing hepatic TG and cholesterol levels. The serum TG, cholesterol, and total FFA contents were not affected (Table S3).

Similar to the results in the DIO mice (Fig. 6), the knockdown of KLF14 in the liver of DIO mice inhibited the expression of gluconeogenic genes (Fig. 6, *A* and *B*) and decreased plasma glucose levels (Fig. 6*C*). However, Ad-sh*Klf14* infection in the DIO mice, compared with that in the control mice, did not significantly alter fasting insulin levels (Fig. 6*D*). The GTT and PTT assays have identified that KLF14 knockdown improves glucose tolerance (Fig. 6, *E* and *G*), whereas ITT experiments demonstrated that down-regulation of KLF14 ameliorated insulin sensitivity (Fig. 6*F*). The AUC was reduced in the GTT, ITT, and PTT experiments in mice with hepatic KLF14 knockdown (Fig. S6, *C–E*). We once again measured AKT and GSK3β phosphorylation levels in the livers of Ad-sh*Klf14*- and Ad-shCtrl-infected DIO mice. Indeed, knockdown of KLF14 in DIO mouse livers enhanced insulin-induced AKT and GSK3β phosphorylation levels but without changing the total AKT or GSK3β levels (Fig. 6*G*). Biochemical analysis also confirmed that the adenovirus-mediated knockdown of KLF14 significantly increased serum cholesterol levels and decreased hepatic cholesterol contents (Table S4). The concentrations of serum

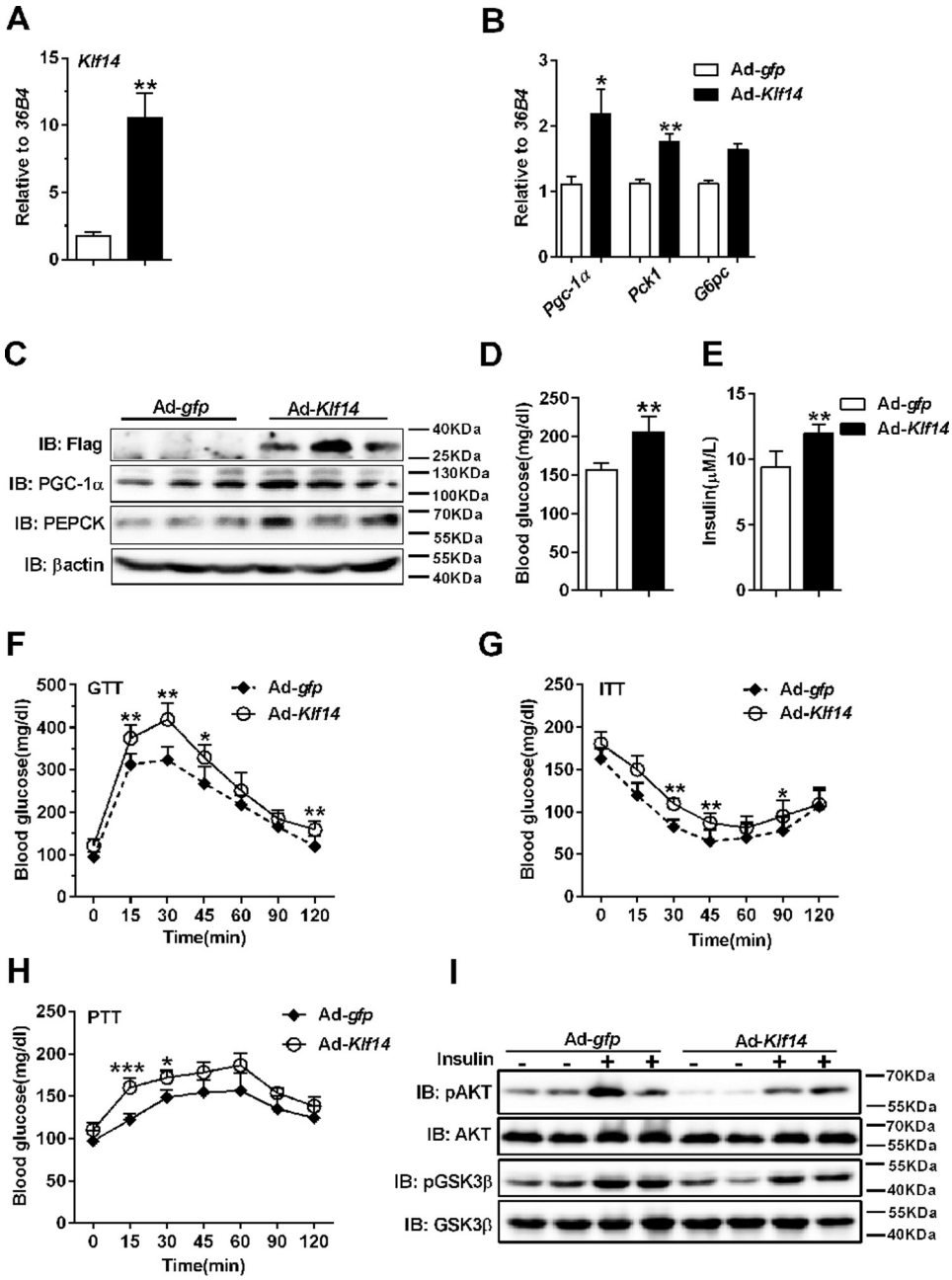

Figure 4. Overexpression of KLF14 in *C57BL/6J* mice livers impairs glucose homeostasis. *A*, real-time qPCR analysis showing *Klf14* mRNA and protein levels in livers from Ad-*gfp*– or Ad-*Klf14*–injected C57BL/6J mice 7 days after injection ($n = 7$/group). *B* and *C*, real-time qPCR (*B*) and Western blot (*IB*) (*C*) analyses showing the mRNA and protein levels, respectively, of the gluconeogenic genes in livers from the same mice as in *A* ($n = 7$/group). *D*, blood glucose levels in the control Ad-*gfp*– or Ad-*Klf14*–injected C57BL/6J mice 7 days after injection under fasting conditions ($n = 7$/group). *E*, serum insulin contents in the control Ad-*gfp*– or Ad-*Klf14*–injected C57BL/6J mice 7 days after injection under fasting conditions ($n = 7$/group). *F–H*, GTT (*F*), ITT (*G*), and PTT (*H*) in the control Ad-*gfp*– or Ad-*Klf14*–injected C57BL/6J mice 7 days after injection ($n = 7$/group). *I*, C57BL/6J mice were treated as described in *A*. Seven days after adenovirus infection (Ad-*Klf14* or Ad-*gfp*), mice were fasted overnight and anesthetized with tribromoethanol followed by injection of 5 units of insulin or saline (as a control) via the inferior vena cava. Five minutes later, the animals were sacrificed, and the liver protein lysates were subjected to Western blot analysis. The data shown are the means ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

and hepatic TG levels, FFA, or markers of liver damage (ALT or AST levels in the serum) were not affected by KLF14 knockdown (Table S4).

Taken together, these data demonstrate that reducing KLF14 expression levels in the liver suppresses hepatic gluconeogenesis in diabetic mice, eventually leading to decreases in fasting blood glucose levels and improved glucose tolerance. Based on our data, we propose a model for the mechanism of KLF14 action on hepatic gluconeogenesis (Fig. 7).

Discussion

The physiological and metabolic roles of the KLF family in glucose and lipid metabolism have been extensively investigated since the discovery of these genes (9). We have previously demonstrated that the hepatic *Klf11* gene is an important regulator of hepatic lipid and glucose metabolism (11, 12). Despite the strong evidence linking KLF14 with T2D and insulin resistance (15, 24, 28), the precise physiological function of KLF14,

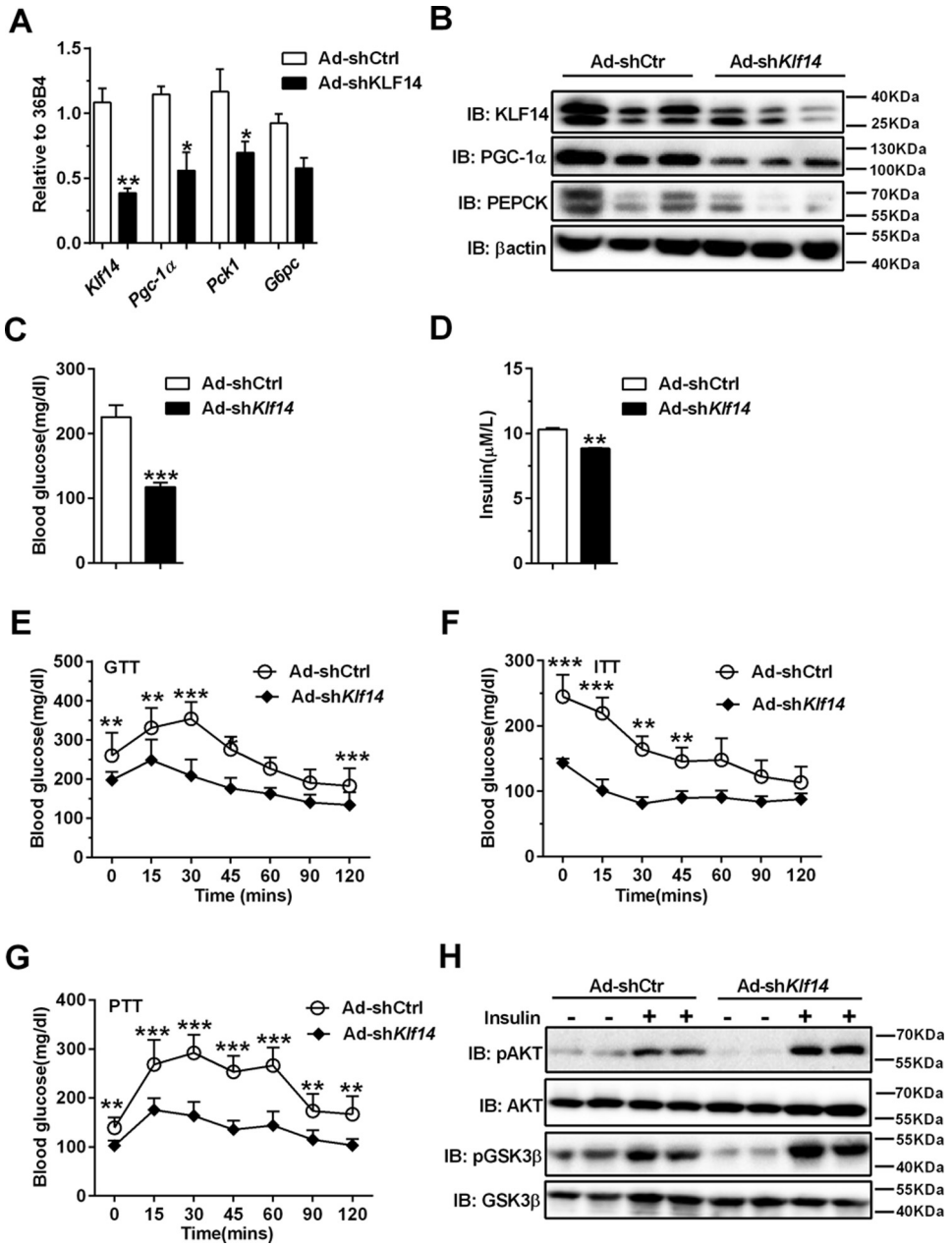

Figure 5. Knockdown of KLF14 in *db/db* mouse livers decreases blood glucose levels and improves glucose tolerance. *A* and *B*, real-time qPCR analysis of mRNA levels (*A*) and Western blot analysis (*IB*) of protein levels (*B*) of *Klf14* and gluconeogenic genes in livers from *db/db* mice 9 days after infection with either the Ad-shCtrl or Ad-sh*Klf14* adenovirus ($n = 7$/group). *C*, plasma glucose levels in the 6-h-fasted *db/db* mice described in *A*. *D*, serum insulin concentrations in the 6-h-fasted *db/db* mice as described in *A*. *E–G*, GTT (*E*), ITT (*F*), and PTT (*G*) in the control Ad-shCtrl– or Ad-sh*Klf14*–injected *db/db* mice 9 days after injection ($n = 7$/group). *H*, *db/db* mice were treated as described in *A*. Nine days after adenovirus infection (Ad-sh*Klf14* or Ad-shCtrl), the mice were fasted overnight and anesthetized with tribromoethanol, followed by the injection of 5 units of insulin or saline (as a control) via the inferior vena cava. Five minutes later, the animals were sacrificed, and the liver protein lysates were subjected to Western blot analysis. Values represent the means ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

especially in glucose homeostasis, remains largely unknown *in vivo*. KLF14 differs from other KLFs that are imprinted and expressed only from the maternal allele, indicating a possible role in growth and development (14). KLF14 is highly expressed in neurons, and its function may be related to metabolic control in the brain and adipose tissue (29). de Assuncao *et al.* (17) reported that KLF14 is an activator protein involved in the regulation of lipid signaling. In this study, we explored the function of KLF14 in regulating hepatic glucose metabolism *in vitro* and *in vivo*. To our knowledge, this is the first report showing that KLF14 regulates glucose metabolism *in vivo*.

It is well known that abnormal blood glucose levels are closely associated with the development of T2D. In the present work, our results indicated that hepatic KLF14 expression is regulated by nutritional status; fasting increases its expression, whereas refeeding after fasting restores *Klf14* mRNA and protein levels. In the fasted state, insulin levels decrease, whereas the secretion of glucocorticoids and glucagon increases, resulting in increased hepatic gluconeogenesis (26, 30). Our data demonstrate that glucagon or insulin could regulate the expression of KLF14; however, glucocorticoids only have a minor effect on KLF14 expression. These findings demonstrate that

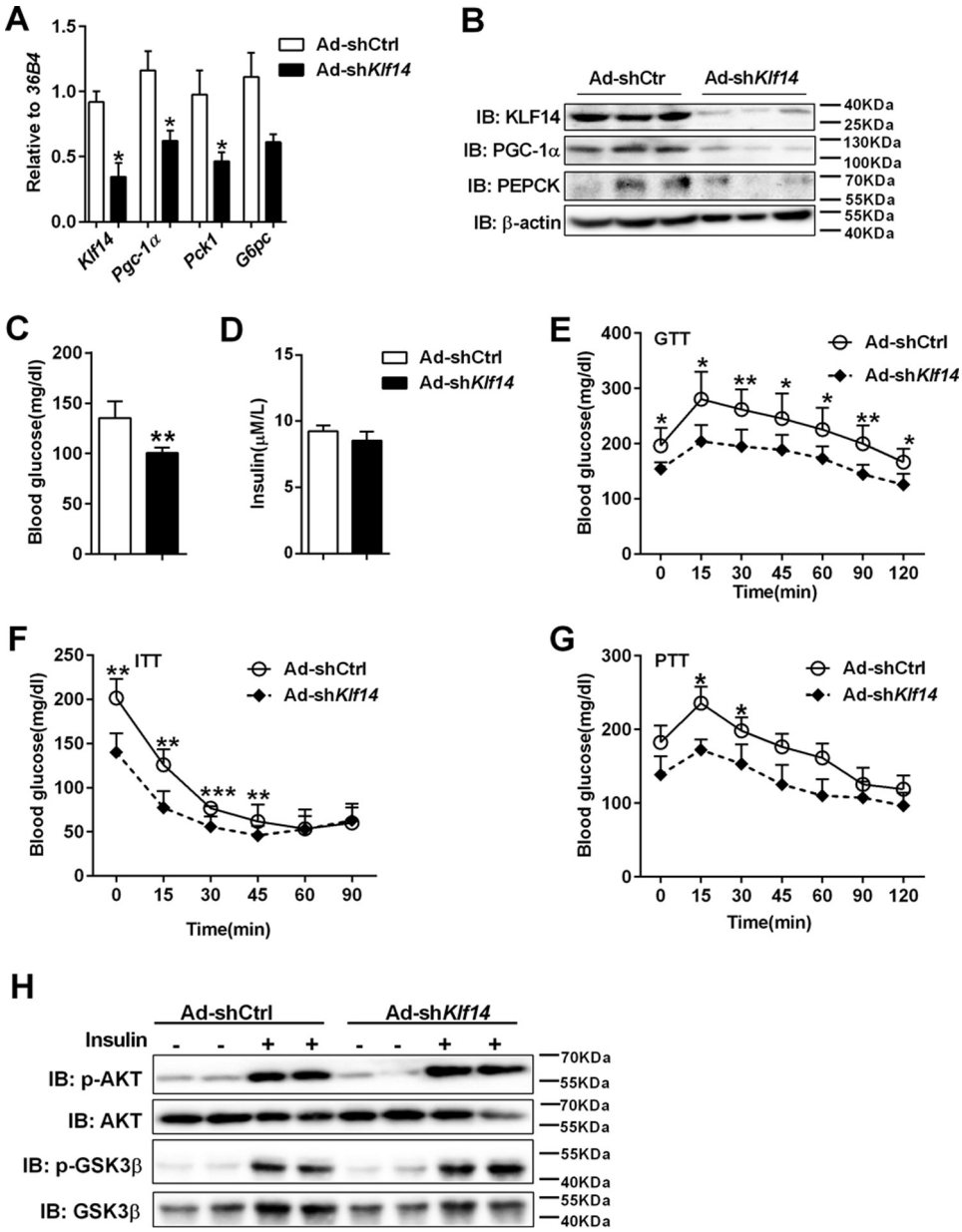

Figure 6. Hepatic silencing of KLF14 in the liver of DIO mice suppresses hepatic gluconeogenesis and alleviates glucose intolerance. *A*, real-time qPCR analysis of *Klf14*, *Pgc-1α*, *Pck1*, and *G6pc* mRNA levels in livers from Ad-shCtrl– or Ad-sh*Klf14*–injected DIO mice ($n = 7$/group). *B*, Western blotting analysis (*IB*) for KLF14, PGC-1α, and PEPCK protein levels in livers from Ad-shCtrl– or Ad-sh*Klf14*–injected DIO mice ($n = 7$/group). *C*, fasting (6 h) blood glucose levels in DIO mice on day 9 after injection with the indicated adenovirus ($n = 7$/group). *D*, serum insulin concentrations in DIO mice as described in *C*. *E–G*, GTT (*E*), ITT (*F*), and PTT (*G*) in the control Ad-shCtrl– or Ad-sh*Klf14*–injected DIO mice 9 days after injection ($n = 7$/group). *H*, DIO mice were treated as described in *A*. Nine days after adenovirus infection (Ad-sh*Klf14* or Ad-shCtrl), mice were fasted overnight and anesthetized with tribromoethanol, followed by injection with 5 units of insulin or saline (as a control) via the inferior vena cava. Five minutes later, the animals were sacrificed, and the liver protein lysates were subjected to Western blot analysis. Values represent the means ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

KLF14 may be involved in the regulation of glucose metabolism, especially in health and disease contexts. RNA-seq was performed to determine the KLF14-mediated molecular mechanism. The RNA-seq results indicated that KLF14 overexpression in hepatocytes dysregulated a group of glucose and lipid metabolism-related genes. Among them, gluconeogenesis-related genes are dysregulated in mouse primary hepatocytes (Fig. 2*A*). KLF14 can function as either an activator or a repressor, depending on the cellular context, the promoters it binds to, and the cofactor it recruits (17, 18). Here, we demonstrate that KLF14 proteins can up-regulate the expression of PGC-1α by directly binding to CACCC elements mapped in the *Pgc-1α* gene promoter, allowing them to serve as an important positive regulator of hepatic gluconeogenesis. Indeed, PGC-1α is proposed to be a critical master regulator of hepatic gluconeogenesis by activating the entire pathway of key gluconeogenic enzymes, including PEPCK and glucose-6-phosphatase, through the co-activators HNF4α and FOXO1 (26, 31). Thus, the increased PGC-1α activity contributes to the induction of hepatic glucose output and the development of hyperglycemia. Our results clearly demonstrate that the overexpression of mouse KLF14 promoted PGC-1α and its target genes' mRNA

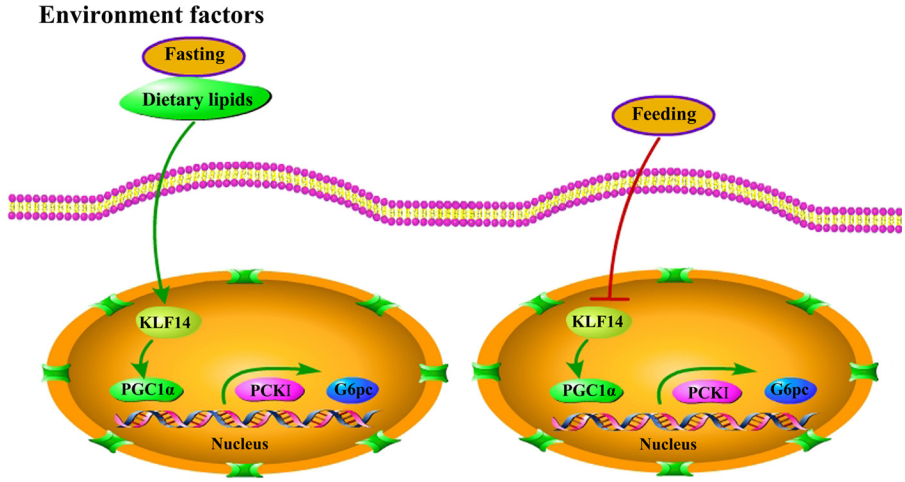

Figure 7. Proposed model for the role of fasting-dependent activation of KLF14 in hepatic gluconeogenesis. Under fasting conditions (*left*), KLF14 expression and PGC-1α protein levels increased, thereby increasing the expression of hepatic gluconeogenic genes. However, under feeding conditions (*right*), inhibited KLF14 expression decreased PGC-1α protein abundance, which subsequently inhibits the expression of hepatic gluconeogenic genes and contributes to reduced hepatic glucose production.

and protein levels in primary hepatocytes, which is consistent with a previous report (19). Accordingly, KLF14 overexpression markedly promoted glucose production in mouse primary hepatocytes. Furthermore, the up-regulation of KLF14 expression significantly decreased the insulin-stimulated phosphorylation levels of AKT and GSK3β in primary hepatocytes. However, a recent study has shown that overexpression of KLF14 in Hepa1-6 cells improved insulin sensitivity through activation of the PI3K/AKT signaling pathway (19). Hepa1-6 cells are mouse-derived hepatoma cells, whereas the primary hepatocytes used in our study are normal liver cells. Thus, different energy metabolism mechanisms in the two different cell types could explain the different observations upon KLF14 function.

Our results clearly demonstrate that forced expression of mouse KLF14 in C57BL/6J mice livers markedly increases fasting plasma glucose levels and impairs glucose tolerance and insulin tolerance. Our data (from RNA-seq, luciferase reporter gene assay, and ChIP) indicate that KLF14 exerts its promoting effects on the expression of gluconeogenic genes by regulating PGC-1α. However, Guo *et al.* (18) reported that up-regulation of human KLF14 in DIO mice has no effect on blood glucose levels. A possible explanation might be the differences in the mouse models and source of *Klf14*. The Ad-*Klf14* adenovirus from our study comes from mice. The serum TG and hepatic cholesterol levels in the Ad-*Klf14*-treated mice were increased. However, hepatic TG and serum cholesterol displayed no differences. Further studies will be required to determine the molecular mechanisms underlying the KLF14-mediated change in serum TG levels. Conversely, knockdown of KLF14 in *db/db* and DIO mice significantly decreased fasting blood glucose levels and improved glucose tolerance but did not markedly affect insulin tolerance; this is not consistent with the results in the C57BL/6J mice but may reflect genetic differences in the mouse models. Thus, the specific mechanisms for the effects on insulin actions and sensitivity should be further investigated. Accordingly, down-regulation of KLF14 markedly inhibited the expression of gluconeogenic genes. However, we should note that in the present study, we employed the adenovirus system to effectively force exogenous *Klf14* gene expression in mouse livers, which only allowed us to observe the short-term effects of hepatic KLF14 activation on glucose metabolism. To address the precise mechanisms of KLF14 in regulating glucose metabolism, both *Kfl14* knockout and transgenic mice should be used.

In summary, the present study demonstrated that KLF14 plays a critical role in regulating hepatic gluconeogenesis *in vitro* and *in vivo*. Taken together, our findings demonstrate KLF14 as a new therapeutic target for the treatment of T2D and metabolic syndromes.

Experimental procedures

Animals and experimental design

Male *db/db* and C57BL/6J mice at the age of 8–9 weeks, purchased from the Animal Research Center of Nanjing University (Nanjing, China), were housed and maintained in a 12-h light/12-h dark cycle clean animal facility at Anhui Medical University. C57BL/6J mice were fed either a normal chow diet (9% fat; Lab Diet) or HFD (45% fat; Research Diets) *ad libitum*, with free access to water. Mice were tail vein–injected with adenovirus ($0.5–1.0 \times 10^9$ active viral particles in 200 μl of PBS). 7–9 days after infection, mice were fasted for 6 h, and the liver was collected for additional biochemical analysis. All animal experiments were carried out strictly following the guidelines of the Animal Center of Anhui Medical University, and all animal experimental procedures were approved by the Experimental Animal Ethical Committee of Anhui Medical University.

Preparation of expression plasmids and recombinant adenoviruses

The full-length mouse *Klf14* gene was amplified by PCR from the C57BL/6J mouse liver cDNA library. Then the FLAG-tagged *Klf14* was subcloned into pcDNA3.1 using the following PCR primer pairs: 5′-ATGTCGGCCGCCGTGGCTT-3′ (forward) and 5′-CTACAGGCAAGCAGTGAAG-3′ (reverse) for

Klf14. Recombinant adenoviruses expressing KLF14 were generated as described before (32).

RNA interference

shRNAs were synthesized by Invitrogen (Carlsbad, CA) and subcloned into the adenovirus plasmids. The adenoviruses were prepared according to the supplier's instructions as described before (11). The sequence of siRNA against luciferase (siControl) was 5′-CTTACGCTGAGTACTTCGA-3′; siRNA against mouse *Klf14* (siKLF14) was 5′-ATAGACACCAG-GCACTCGG-3′.

In vivo glucose, insulin, and pyruvate tolerance tests

For *in vivo* infections, adenoviruses (Ad-*gfp*, Ad-*Klf14*, Ad-shControl (Ad-shCtrl), or Ad-sh*Klf14*) were delivered by tail vein injection into *db/db*, DIO or control mice, both from C57BL/6J mice. Seven days after injection, mice were injected with D-glucose (1–2 g/kg body weight) or pyruvate sodium (1–2 g/kg body weight) via i.p. injection after 16 h of fasting, and blood glucose levels were determined using a glucose monitor (OneTouch; LifeScan, Inc.). For the ITT, mice were injected with insulin (0.5–0.75 units/kg) via i.p. injection after 6 h of fasting.

Analytical procedures and chemicals

The hepatic TG and cholesterol content was measured using a colorimetric diagnostic kit (Applygen Technologies, Inc., Beijing, China). Serum insulin concentrations were determined by ELISA (Mlbio Corp., Shanghai, China). Serum concentrations of TG, cholesterol, FFAs, ALT, and AST were determined using an automated Monarch device (First Affiliated Hospital of Anhui Medical University, Hefei, China).

Cell culture

Mouse primary hepatocytes were isolated from the livers of male C57BL/6J mice (8 weeks old) and cultured as described previously (11). Mouse primary hepatocytes were infected with the indicated adenoviruses. 24 h (Ad-*Klf14* and Ad-*gfp* treatment) or 48 h (Ad-sh*Klf14* and Ad-shCtrl treatment) after infection, cells were switched to the serum-free medium and treated with dexamethasone (DEX, 1 μmol/liter; Sigma-Aldrich) and forskolin (FSK, 10 μmol/liter; Sigma-Aldrich) for 6 h before harvest for further analysis.

Luciferase reporter gene assay

Mouse *Pgc-1α* promoter constructs (PGL3-*Pgc-1α*) were cotransfected into HepG2 cells, together with KLF14 expression plasmid or empty vectors (pcDNA3.1) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. A *Renilla* luciferase expression vector was used as an internal control to adjust for transfection efficiency. After 48 h, cells were harvested to assess luciferase activity using the Dual-Luciferase reporter assay system (Promega), following the manufacturer's instructions.

ChIP assay

Briefly, mouse primary hepatocytes were infected with adenoviruses expressing KLF14-FLAG fusion protein (Ad-*Klf14*). After 36 h, the cells were treated using the SimpleChIP™ enzymatic chromatin IP kit (Cell Signaling Technology), following the manufacturer's instructions. The promoter region of *Pgc-1α* was amplified by PCR using the following primer pair: 5′-TGGTTATGTCCTCTGTCTGTAATG-3′ as a forward primer and 5′-GCCTTGGGCTGTACAGTT-3′ as a reverse primer.

Glucose production assay

For glucose output assays, primary hepatocytes were prepared from livers of male C57BL/6J mice as described previously (11). Cells were cultured in RPMI1640 with 10% FBS and then infected with adenovirus for 24 or 48 h. Cells were washed five times with PBS and then stimulated with or without FSK/DEX for 3 h in phenol red-free, glucose-free DMEM containing 20 mM sodium pyruvate. Glucose concentrations in the medium were measured with a glucose assay kit (Applygen Technologies Inc., Beijing, China).

In vivo insulin signaling

After an overnight fasting, mice were anesthetized with 2,2,2-tribromoethanol in PBS and injected with 5 units of regular human insulin (Sigma-Aldrich) via the inferior vena cava injection. Five or 10 min after the insulin bolus, livers were removed and frozen in liquid nitrogen. Immunoblot analysis of insulin signaling molecules was performed using tissue homogenates prepared in a tissue homogenization buffer supplemented with the Complete protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor.

Real-time quantitative PCR (qPCR)

Total RNA was isolated from cells or pulverized liver using TRIzol (Invitrogen). Primers are listed in Table S1.

RNA-seq and bioinformatics analysis

Total RNA was isolated from hepatocytes, which were infected with Ad-*gfp* or Ad-*Klf14* 48 h before the harvest. Oligo(dT) beads were used to enrich the poly(A)-tailed mRNA, which was further used for sequencing library construction by utilizing the TruSeq® RNA LT Sample Prep kit version 2 (AmpureBeads, Beckman) according to the manufacturer's instructions. High-throughput sequencing was performed on an Illumina Hiseq 2000 instrument. The raw sequencing file (fastq) was further processed for quality control with Fastqc, followed by removing the low-quality reads by fastx-toolkit. The high-quality reads were then aligned to the mouse genome (mm10) by Tophat, followed by differential analysis with Cuffdiff (33). A heat map was generated by R, utilizing the differentially expressed gene from the Cuffdiff analysis.

Western blot analysis

Liver tissues or cultured hepatocytes were lysed in radioimmune precipitation assay lysis buffer, and 30–50 μg of proteins were resolved by SDS-PAGE and eletrotransferred to PVDF membranes. Western blot assays were performed using antibodies specific for KLF14, FLAG (Sigma), PGC-1α (Millipore), PEPCK, AKT, phospho-AKT (Ser-473), glycogen synthase kinase 3β (GSK3β), phospho-GSK3β β-actin (Cell Signaling

Technology), and GAPDH (Santa Cruz Biotechnology). Protein on membrane was visualized by enhanced chemiluminescence reagent (Thermo Fisher) and autoradiography.

Statistical analysis

Data are presented as means ± S.E. of more than three independent duplicates. Statistical analysis was performed with Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Author contributions—H. Z. designed the experiments, performed the experiments and the data analysis, and wrote the manuscript. L. W. performed the experiments and the data analysis. X. T. and L. Z. performed the experiments. L. X., F. G., W. C., and X. C. performed the experiments and the data analysis and critically revised the manuscript. Y. C. provided funding support and critically revised the manuscript.

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