Diabetologia (2013) 56:635–643
DOI 10.1007/s00125-012-2807-7

ARTICLE

Adenovirus-mediated overexpression of *Tcfe3* ameliorates hyperglycaemia in a mouse model of diabetes by upregulating glucokinase in the liver

M. Y. Kim · S. H. Jo · J. M. Park · T. H. Kim · S. S. Im · Y. H. Ahn

Received: 4 October 2012 / Accepted: 4 December 2012 / Published online: 27 December 2012

Abstract
Aims/hypothesis Transcription factor E3 (TFE3) has been shown to increase insulin sensitivity by activating insulin-signalling pathways. However, the role of TFE3 in glucose homeostasis is not fully understood. Here, we explored the possible therapeutic potential of TFE3 for the control of hyperglycaemia using a streptozotocin-induced mouse model of diabetes.
Methods We achieved overabundance of TFE3 in streptozotocin mice by administering an adenovirus (Ad) or adeno-associated virus serotype 2 (AAV2). We also performed an oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). To explore molecular mechanisms of blood glucose control by TFE3, transcriptional studies on the regulation of genes involved in hepatic glucose metabolism were performed using quantitative real-time PCR and chromatin immunoprecipitation assay. The binding site of TFE3 in the liver *Gck* gene promoter was identified using deletion and site-specific mutation studies.
Results Overabundance of TFE3 resulted in reduced hyperglycaemia as shown by the OGTT and ITT in streptozotocin-treated mice. We observed that TFE3 can upregulate *Gck* in a state of insulin deficiency. However, glucose-6-phosphatase and cytosolic phosphoenolpyruvate carboxykinase mRNA levels were decreased by Ad-mediated overexpression of *Tcfe3*. Biochemical studies revealed that the anti-hyperglycaemic effect of TFE3 is due to the upregulation of *Gck.* In primary cultured hepatocytes, TFE3 increased expression of *Gck* mRNA. Conversely, small interfering RNA-mediated knockdown of TFE3 resulted in a decrease in *Gck* mRNA.
Conclusions/interpretation This study demonstrates that TFE3 counteracts hyperglycaemia in streptozotocin-treated mice. This effect could be due to the upregulation of *Gck* by binding of TFE3 to its cognitive promoter region.

Keywords Liver-type glucokinase (*Gck*) · TFE3 · Transcription factor E3 · Type 1 diabetes mellitus · Transcriptional regulation · Streptozotocin

Abbreviations

AAV2	Adeno-associated virus serotype 2
Ad	Adenovirus
ChIP	Chromatin immunoprecipitation
GCK	Liver-type glucokinase
GFP	Green fluorescent protein
ITT	Insulin tolerance test
PFU	Plaque-forming units
ph*GCK*	Promoter of human *GCK*
siRNA	Small interfering RNA
SREBP-1c	Sterol regulatory element-binding protein 1c
TFE3	Transcription factor E3

Introduction

The liver is a critical organ for glucose homeostasis because glucose consumption and glucose production occur there

Electronic supplementary material The online version of this article (doi:10.1007/s00125-012-2807-7) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

M. Y. Kim · S. H. Jo · J. M. Park · T. H. Kim · Y. H. Ahn (✉)
Department of Biochemistry and Molecular Biology,
Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu,
Seoul 120-752, Republic of Korea
e-mail: yha111@yuhs.ac

S. H. Jo · J. M. Park · Y. H. Ahn
Brain Korea 21 Project for Medical Sciences,
Yonsei University College of Medicine, Seoul, Republic of Korea

S. S. Im
Department of Physiology, Keimyung University
School of Medicine, Daegu, Republic of Korea

during the feeding and fasting states, respectively. Within the liver, the enzyme liver-type glucokinase (GCK) phosphorylates glucose to generate glucose-6-phosphate [1] and is thought to be a rate-controlling enzyme for the sensing of blood glucose levels [2, 3]. Thus, an understanding of the molecular mechanisms that drive the regulation of the *GCK* gene and the enzymatic activity of GCK is critical for developing glucose-lowering drugs.

Gck expression is reduced in animal models of diabetes with insulin deficiency and insulin resistance [4, 5]. Insulin upregulates, whereas glucagon suppresses *Gck* expression in primary cultured hepatocytes, regardless of glucose concentration [6, 7]. Transcription factors involved in insulin-mediated *Gck* upregulation include sterol regulatory element-binding protein 1c (SREBP-1c), hepatocyte nuclear factor-4 and hypoxia-inducible factor-1α [8, 9].

Transcription factor E3 (TFE3) is a basic helix–loop–helix protein that has been studied in the context of immunology and cancer. TFE3, which is encoded by *Tcfe3* (also known as *Tfe3*) and produced in liver and adipose tissues, activates expression of the immunoglobulin gene [10]. Genes upregulated by TFE3 include *Irs2*, *Akt1*, *Insig1* and *Hk2*, which are involved in insulin-signalling [11]. Specifically, *Irs2* expression is upregulated by the concerted actions of TFE3 and forkhead box protein O1 [11], and downregulated by SREBP-1c [12]. Via this mechanism, TFE3 may help protect against development of the metabolic syndrome and insulin resistance. Despite its role in insulin sensitivity, the precise role of TFE3 in the regulation of blood glucose levels is not fully understood.

In this study, we demonstrate that adenovirus (Ad)-mediated overexpression of *Tcfe3* (Ad-*Tcfe3*) or adeno-associated virus serotype 2-mediated overexpression of *Tcfe3* (AAV-*Tcfe3*) upregulates *Gck* expression in mouse liver and primary cultured hepatocytes. The introduction of Ad-*Tcfe3* or AAV-*Tcfe3* to a mouse model of streptozotocin-induced diabetes led to increased expression of *Gck* in liver, resulting in reduced hyperglycaemia and improved glucose and insulin tolerance. These results indicate that TFE3 could improve glucose disposal by upregulating *Gck* expression under conditions of insulin deficiency. Therefore, TFE3 overabundance may be an alternative therapy for the reduction of hyperglycaemia and improvement of glucose sensitivity without insulin.

Methods

Animal experiments C57BL/6JJmsSlc (C57BL/6J) male mice (9 weeks old) obtained from Shizuoka Laboratory Centre (Hamamatsu, Japan) were fed a regular chow diet and housed under a 12 h light/12 h dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (Seoul, Republic of Korea).

Streptozotocin treatment and adenovirus administration Streptozotocin was administered daily for two consecutive days to 9-week-old C57Bl/6J mice (25 g). Administration was done by intraperitoneal injection at a dose of 100 mg/kg body weight in freshly prepared sodium citrate buffer (pH 4.5). An injection of sodium citrate buffer alone was used as a buffer control. Glucose levels were measured every 2 days in blood drawn from the mouse tail vein using a glucose monitor (OneTouch SureStep; Life Scan, Milpitas, CA, USA). At 14 days after streptozotocin treatment, mice with a blood glucose level higher than 25 mmol/l on three consecutive days (n=8) were used as streptozotocin mice and were divided into two subgroups, one receiving Ad-green fluorescent protein (GFP) (n=4) and the other Ad-*Tcfe3* injections (n=4). For control, buffer-treated mice were also divided into two subgroups as in streptozotocin mice (n=4 per subgroup). Ad-*gfp* or Ad-*Tcfe3* (a generous gift from N. Yamada, Department of Internal Medicine, Metabolism and Endocrinology, University of Tsukuba, Tsukuba, Ibaraki, Japan) was injected at a level of 1×10^7 plaque-forming units (PFU) into the tail veins of buffer control or streptozotocin mice, respectively. After an OGTT, mice were housed for an additional 2 days to avoid any stress from OGTT. The incremental AUC of blood glucose was calculated after Ad injection. At 7 days after the Ad injection, during which mice had free access to food, mice were anaesthetised with Zoletil (30 mg/kg, via intramuscular injection) 4 h after an overnight dark cycle and laparotomy was performed. Blood samples were collected from the inferior mesenteric artery, and the whole liver was removed and frozen in liquid nitrogen for subsequent mRNA and protein preparation. The insulin levels in blood from the orbital sinus were measured 1 day before the Ad injection.

AAV2 production and administration Recombinant AAV2-*Tcfe3* was produced as described in the electronic supplementary material (ESM) Methods.

Biodistribution of Ad-Tcfe3 and AAV2-Tcfe3 For details, see ESM Methods.

OGTT and insulin tolerance test At 5 days after the Ad injection, an OGTT was performed on mice that had fasted for 16 h. Glucose (20% wt/vol.) was administered orally using a feeding tube (2 g/kg body weight) and blood glucose levels were monitored at specific time points. For the insulin tolerance test (ITT), mice were fasted for 6 h and insulin (0.75 units/kg Humulin R; Eli Lilly, Indianapolis, IN, USA) was administered intraperitoneally. For the ITT, new sets of

mice were used (n=3 for each subgroup, similarly to OGTT). The measurement of glucose levels and calculation of the AUC were as described above for the OGTT.

Serum metabolite measurements Serum insulin levels were measured by ELISA (Alpco Immunoassays, Salem, NH, USA). Other serum metabolites, such as cholesterol, triacylglycerol and NEFA were measured by an enzymatic method using an autoanalyser (Hitachi 7600: Hitachi Instruments, Tokyo, Japan) as per manufacturer's instructions.

Cell culture HepG2 cells, a human liver hepatocellular carcinoma cell line, were maintained according to a standard protocol. Primary hepatocytes isolated from C57BL/6J mice were prepared as described [13]. After incubation for 4 h, the medium was replaced with serum-free DMEM containing Ad. At 48 h after Ad infection, cells were lysed for RNA and protein preparation.

Small interfering RNAs Details of sequences and transfection of small interfering RNA (siRNA) are described in the ESM Methods.

Total RNA isolation and quantitative PCR Total RNA was isolated from mouse liver or primary cultured hepatocytes using an RNA extraction kit (Easy Spin; iNtRON, Gyeonggi-do, Korea), and cDNA was generated by a reverse transcription system (ImProm-II; Promega, Madison, WI, USA) according to the manufacturer's instructions. Changes in mRNA levels were calculated using the comparative C_t method, with *Rplp0* as the reference gene and buffer-controlled Ad-*gfp* as a calibrator. The sequences for gene-specific PCR primers are provided in ESM Table 1.

Western blot analysis Total protein was extracted from mouse liver following a standard protocol. Anti-GCK (sc-7908 [H-88]; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TFE3 (ab70008; Abcam, Cambridge, MA, USA) or anti-β-actin (A544; Sigma, St Louis, MO, USA) were used as respective specific antibodies. The bands were detected using an imager (LAS-3000 I; Fujifilm, Tokyo, Japan).

Generation of deletion constructs and site-specific mutations Promoter luciferase reporter constructs were generated by amplifying the promoter region of human *GCK* (ph*GCK*) (−1,000 to 158 bp) from HepG2 genomic DNA and inserted into the pGL4.14 vector (Promega). Serial deletion constructs of the *GCK* promoter were prepared by amplifying the indicated regions and subcloned into pGL4.14. Point mutations were introduced into putative TFE3 binding sites in the ph*GCK* (−1,000 to 158 bp) by replacing the sequences with the indicated bases.

Transient transfection and luciferase assays HepG2 cells were plated in 12-well tissue culture dishes at a density of 2×10^5 cells per well in 1 ml medium. Expression plasmids for either wild-type or dominant-negative TFE3, the human *GCK* promoter construct containing the luciferase reporter gene (0.25 μg h*GCK*-Luc, −1,000 to 158 bp) and the *Renilla* luciferase plasmid were transfected using FuGENE 6 (Roche, Mannheim, Germany) at a ratio of 3:1. The total amount of transfected plasmid was adjusted to 0.5 μg by adding empty pcDNA.

Chromatin immunoprecipitation assay Chromatin immunoprecipitation (ChIP) experiments were performed as previously described [13] with HepG2 cells overproducing V5-tagged TFE3 or with mouse liver injected with Ad-*Tcfe3*. The primers used for the PCR of the promoter regions are shown in ESM Table 2.

Statistical analysis In vitro experiments (n=3–5) were each performed in triplicate. For in vivo experiments, three to four animals were used in each subgroup. The data are presented as mean±SEM. All datasets were analysed for statistical significance using a two-tailed unpaired Student's t test. Values of $p<0.05$ were considered significant. Statistical analysis was carried out using SPSS (Ver. 11.5; SPSS, Chicago, IL, USA).

Results

Adenovirus-mediated overproduction of TFE3 reduced hyperglycaemia in streptozotocin mice To elucidate the effects of TFE3 on blood glucose control, Ad-*Tcfe3* (1×10^7 PFU) was administered to buffer control and streptozotocin mice whose body weight, and insulin and blood glucose levels were measured (Fig. 1). There were no significant changes in body weight among the test groups, but the weight of streptozotocin mice decreased slightly (data not shown). Serum insulin levels were significantly decreased in streptozotocin mice compared with the non-diabetic control group (113 pmol/l vs <25 pmol/l, $p<0.001$). Streptozotocin mice showed increased blood glucose levels, reaching a maximum of 27.8 mmol/l by 14 days after the streptozotocin injection. In streptozotocin mice, blood glucose levels began to decrease 2 days after introduction of Ad-*Tcfe3*. This trend continued until day6 (Fig. 1b, c). Introduction of Ad-*Tcfe3* resulted in a slight decrease in blood glucose levels in buffer control mice. To study the long-term effect of TFE3 on blood glucose homeostasis, we constructed AAV-*Tcfe3* and observed the long-term effect for 32 days (ESM Fig. 1a, b). Although the blood glucose-lowering effect of AAV-*Tcfe3* was much less efficient than that of Ad-*Tcfe3*, blood glucose levels began to decrease in

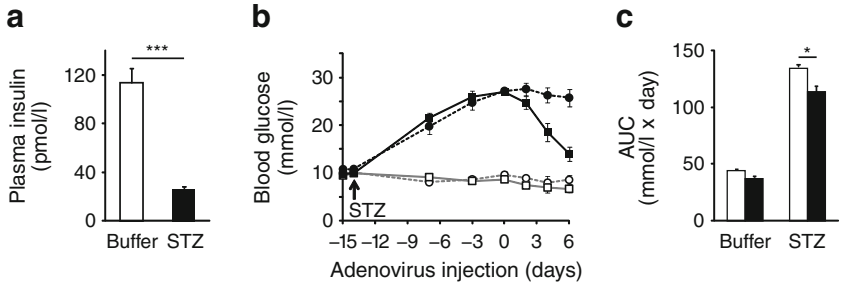


Fig. 1 Effects of TFE3 on blood glucose levels of a streptozotocin-treated mouse model of diabetes. The control mice were treated with sodium citrate buffer (pH4.5), while the streptozotocin group received streptozotocin intraperitoneally. Each group was injected with Ad-*gfp* or Ad-*Tcfe3* (n=4 per subgroup). (**a**) Serum insulin level of buffer-treated control mice and streptozotocin (STZ)-induced diabetic mice. (**b**) Blood glucose levels of buffer-treated (white symbols) and streptozotocin-treated (black symbols) mice before and after Ad-*Tcfe3* (squares) or Ad-*gfp* (circles) administration at the indicated time points. (**c**) AUC for blood glucose levels of the Ad-*gfp* (white bars) and Ad-*Tcfe3* (black bars) groups. All values are expressed as the mean±SEM; *p<0.05 and ***p<0.001

streptozotocin mice after 22 days (from 28.9 mmol/l to 25 mmol/l) (ESM Fig. 1). These data indicate that overabundance of TFE3 can increase the expression of glucose-sensing genes in the liver, in turn contributing to a decrease in blood glucose levels.

TFE3 administration improves glucose tolerance in streptozotocin mice To examine the effects of TFE3 on postprandial glucose disposal, we performed an OGTT. At 15 min after administration of glucose, mice overproducing TFE3 showed improved glucose tolerance compared with GFP controls in the buffer control group (Fig. 2a, b). Similarly, overabundance of TFE3 in streptozotocin mice resulted in significantly improved glucose tolerance (Fig. 2a, b). The OGTT in AAV-*Tcfe3*-treated streptozotocin mice revealed significantly improved glucose tolerance at 60 min after glucose administration (p=0.034) (ESM Fig. 1c, d). An ITT showed that Ad-*Tcfe3* significantly lowered blood glucose levels in streptozotocin mice when compared with Ad-*gfp* (Fig. 2c). The per cent reduction in blood glucose concentration following the insulin injection was approximately 47.6% and 34.9% at 60 min, and 64.7% and 35.1% at 120 min in streptozotocin-treated Ad-*gfp* control and Ad-*Tcfe3* mice, respectively (Fig. 2c). The absolute blood glucose values in the same groups were 7.5 mmol/l and 4.6 mmol/l at 60 min, and 11.7 mmol/l and 4.6 mmol/l at 120 min, respectively (Fig. 2d). These data indicate that TFE3 may improve glucose tolerance and insulin responsiveness by upregulating genes involved in glucose-sensing.

Effects of Ad-Tcfe3 administration on lipid profiles and expression of lipogenic genes To observe the effects of TFE3 on lipid metabolism, we measured the concentrations of serum cholesterol, NEFA and triacylglycerol. As shown in Fig. 3a–c and ESM Fig. 2a–c, levels of these metabolites were not significantly affected by TFE3 levels. Additionally, we monitored mRNA levels of lipogenesis-related genes, including *Srebf1*, *Fasn*, *Acaca*, *Elovl6* and *Scd1*. The expression of a majority of genes involved in lipid metabolism was increased in response to Ad-*Tcfe3* in streptozotocin mice (p<0.05) (Fig. 3d–h). Although lipogenic gene expression showed a modest increase in the liver, the serum lipid profile was not affected. Similarly, AAV mediated *Tcfe3* overexpression did not affect lipogenic gene expression (ESM Fig. 2d–h).

TFE3 upregulates Gck expression in streptozotocin mice Quantitative real-time PCR revealed that *Tcfe3* gene

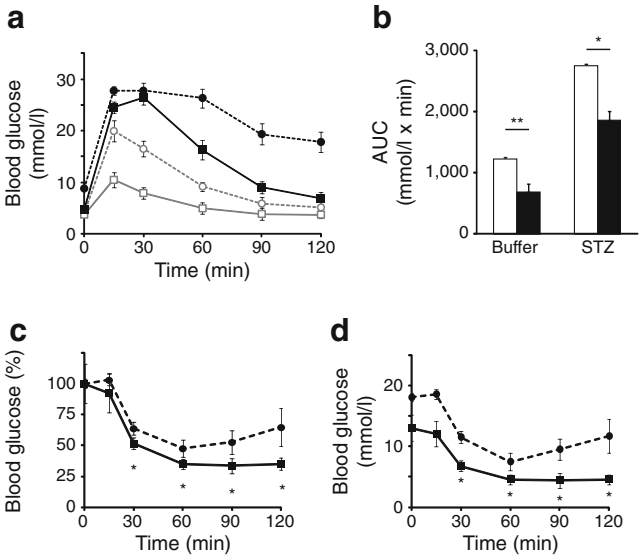


Fig. 2 TFE3 improves glucose and insulin tolerance in a streptozotocin-treated mouse model of diabetes. (**a**) OGTT results for glucose. White circles, buffer control injected with Ad-*gfp*; white squares, buffer control with Ad-*Tcfe3*; black circles, streptozotocin mice with Ad-*gfp*; black squares, streptozotocin mice with Ad-*Tcfe3*; n=4 per subgroup. (**b**) AUC values for glucose from the OGTT in the Ad-*gfp* (white bars) and Ad-*Tcfe3* (black bars) groups. (**c**) Results of the ITT as percentage of blood glucose (time 0, 100%) and (**d**) as absolute values of blood glucose concentrations. Streptozotocin-treated Ad-*gfp* (black circles); streptozotocin-treated Ad-*Tcfe3* (black squares) (n=3 per subgroup). All values are expressed as the mean±SEM; *p<0.05 and **p<0.01

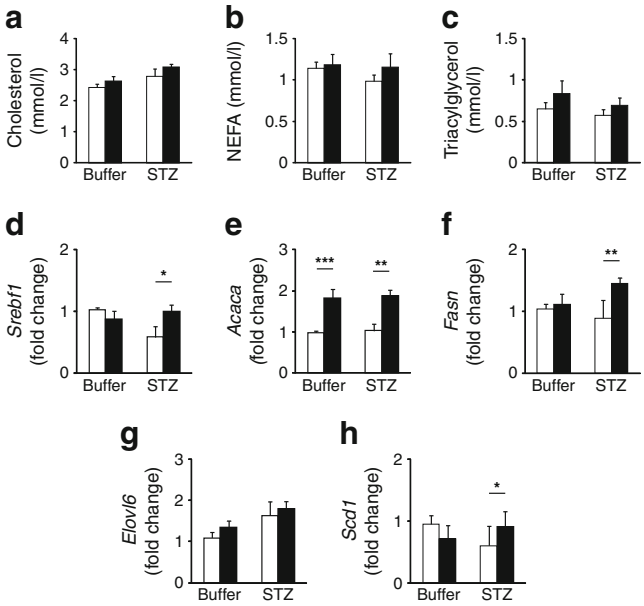


Fig. 3 Effects of TFE3 on serum metabolites and lipogenic gene expression in the liver. (**a**) Concentrations of serum cholesterol, (**b**) NEFA and (**c**) triacylglycerol in the Ad-*gfp* (white bars) and Ad-*Tcfe3* (black bars) groups (n=5–6 per group). (**d–h**) mRNA levels of lipogenic genes as indicated in the livers of Ad-*gfp* and Ad-*Tcfe3* groups. All values are expressed as the mean±SEM; *p<0.05, **p<0.01 and ***p<0.001

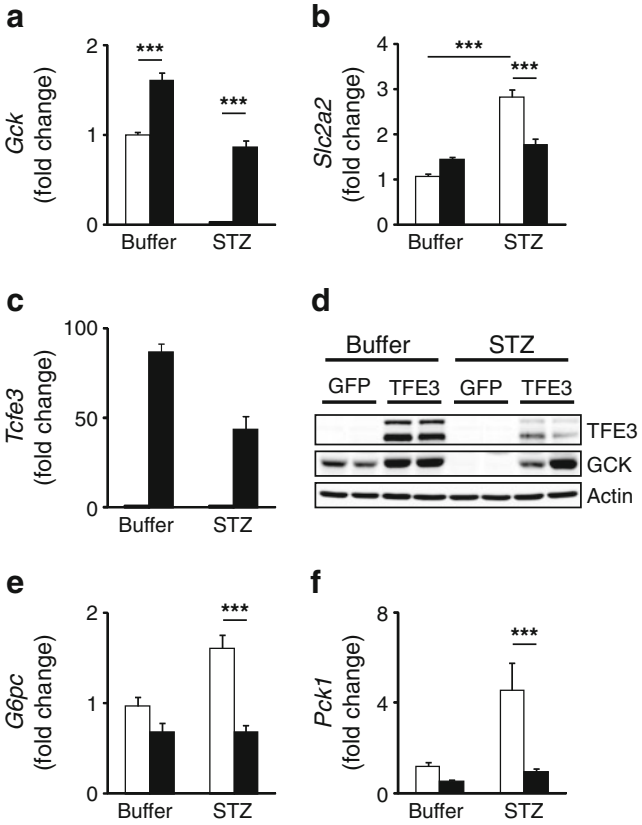


Fig. 4 Effects of Ad-*Tcfe3* on the expression of genes involved in hepatic glucose metabolism. **(a)** mRNA fold changes of *Gck*, (**b**) *Slc2a2*, (**c**) *Tcfe3*, (**e**) *G6pc* and (**f**) *Pck1* from liver extracts of buffer control or streptozotocin-treated mice that were injected with Ad-*gfp* (white bars) or Ad-*Tcfe3* (black bars). (**d**) Western blot of GCK from liver extracts of mice as above (**a–c**, **e**, **f**). β-Actin was used as an internal control. Quantitative real-time PCR and western blot were performed 7 days after Ad-*Tcfe3* administration. Data are expressed as the mean±SEM; n=7–8 per group; ***p<0.001

expression was maintained 6 days after the Ad-*Tcfe3* injection (Fig. 4c). Gene delivery of *Tcfe3* to the liver may have been restricted because *Tcfe3* mRNA levels were very low in skeletal muscle (ESM Fig. 3c) and adipose tissue (data not shown). In the liver of control mice, *Gck* mRNA was increased in response to TFE3 overabundance. Although *Gck* mRNA expression was significantly reduced in streptozotocin mice, the introduction of Ad-*Tcfe3* restored it to a level comparable to that of the control Ad-*gfp* group (Fig. 4a). *Slc2a2* (which encodes GLUT2) mRNA expression was not changed in the control group by Ad-*Tcfe3* administration (Fig. 4b). However, *Slc2a2* expression in the Ad-*gfp* control group was increased in streptozotocin mice and decreased by TFE3 overabundance (Fig. 4b). AAV-mediated *Tcfe3* gene transfer resulted in the expression of *Tcfe3* in liver, in turn resulting in increased *Gck* expression (ESM Fig. 3a, b). Western blot revealed that GCK levels were well correlated with those of TFE3 (Fig. 4d, ESM Fig. 3d). In contrast, mRNA expression of *G6pc* and *Pck1* were significantly decreased by Ad-*Tcfe3* (p<0.05) (Fig. 4e, f).

TFE3 activates Gck gene expression in primary hepatocytes The administration of Ad-*Tcfe3* to primary cultured hepatocytes resulted in an increase in *Gck* mRNA in a dose-dependent manner in serum-free media (Fig. 5a). However, *Slc2a2* mRNA expression was not affected (Fig. 5b). Levels of GCK were increased proportionately to the amount of Ad-*Tcfe3* (Fig. 5d). One extra immunoreactive band of TFE3 was observed, which could be a variant form of TFE3 [11]. Administration of *Tcfe3* siRNA to hepatocytes resulted in a corresponding decrease in *Gck* and *Tcfe3* mRNA levels; however, *Slc2a2* expression was not affected (Fig. 5e–g). These data suggest that TFE3 may directly activate *Gck* expression in primary cultured hepatocytes.

Gck promoter activity is directly upregulated by TFE3 To evaluate the role of TFE3 in *GCK* expression, the promoter reporter construct, ph*GCK* 1000, was co-transfected with the TFE3 expression vector into HepG2 cells. As shown in Fig. 6a, wild-type TFE3 activated ph*GCK* 1000. However, this effect was suppressed, in a dose-dependent manner, by a dominant-negative form of TFE3, which lacks a transactivation domain [11]. A computer search (using Transcription Element Search Software; www.cbil.upenn.edu/cgi-bin/tess/tess, 16 April 2009) suggested the presence of three

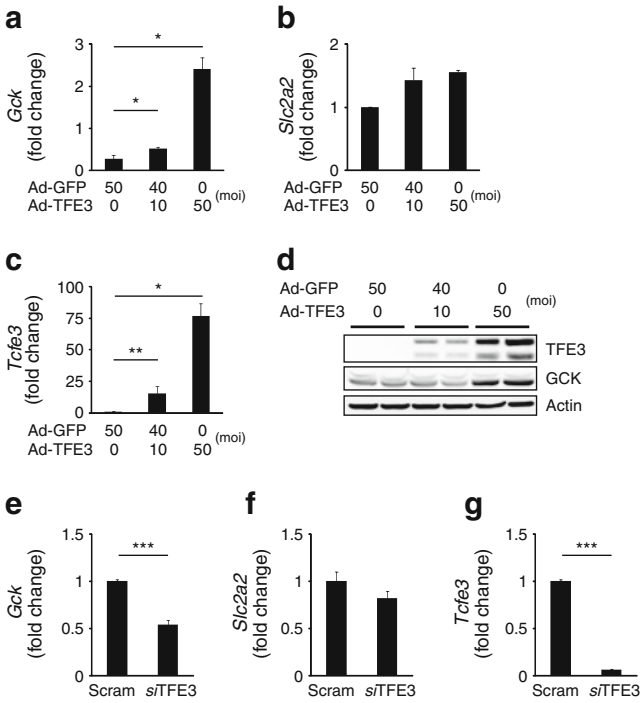


Fig. 5 Overabundance of TFE3 increases *Gck* mRNA levels in mouse primary cultured hepatocytes. (**a**) Quantitative real-time PCR of *Gck*, (**b**) *Slc2a2* and (**c**) *Tcfe3*. Each value represents the amount of mRNA relative to that of the Ad-*gfp*-treated sample in the same experiment, which was arbitrarily defined as 1. moi, multiplicity of infection. (**d**) Western blot of TFE3, GCK and β-actin. (**e**) Effect of siRNA-mediated knockdown of TFE3 on mRNA levels of *Gck*, (**f**) *Slc2a2* and (**g**) *Tcfe3*. Scram, scramble. Values are mean±SEM for three plates per group, experiments performed in triplicate; *$p<0.05$, **$p<0.01$ and ***$p<0.001$

potential TFE3-binding sites within the −1,000 bp region of the *GCK* promoter: −808 to −803 bp, −613 to −608 bp and −83 to −78 bp (Fig. 6b). Deletion of the promoter from −1,000 bp down to −800 bp or −50 bp resulted in a significant loss of TFE3-dependent luciferase activities, suggesting the presence of TFE3-response elements in the regions between −1,000 to −800 bp and −200 to −50 bp (Fig. 6c). Site-specific mutations in the TFE3-binding sites of the *GCK* promoter revealed that the −808 to −803 bp and the −83 to −78 bp sites are responsible for the TFE3-dependent activation of the *GCK* promoter (Fig. 6d). Direct binding of TFE3 to these regions was confirmed by ChIP assay in HepG2 cells overproducing human TFE3 and in Ad-*Tcfe3*-transduced murine liver (Fig. 6e, f).

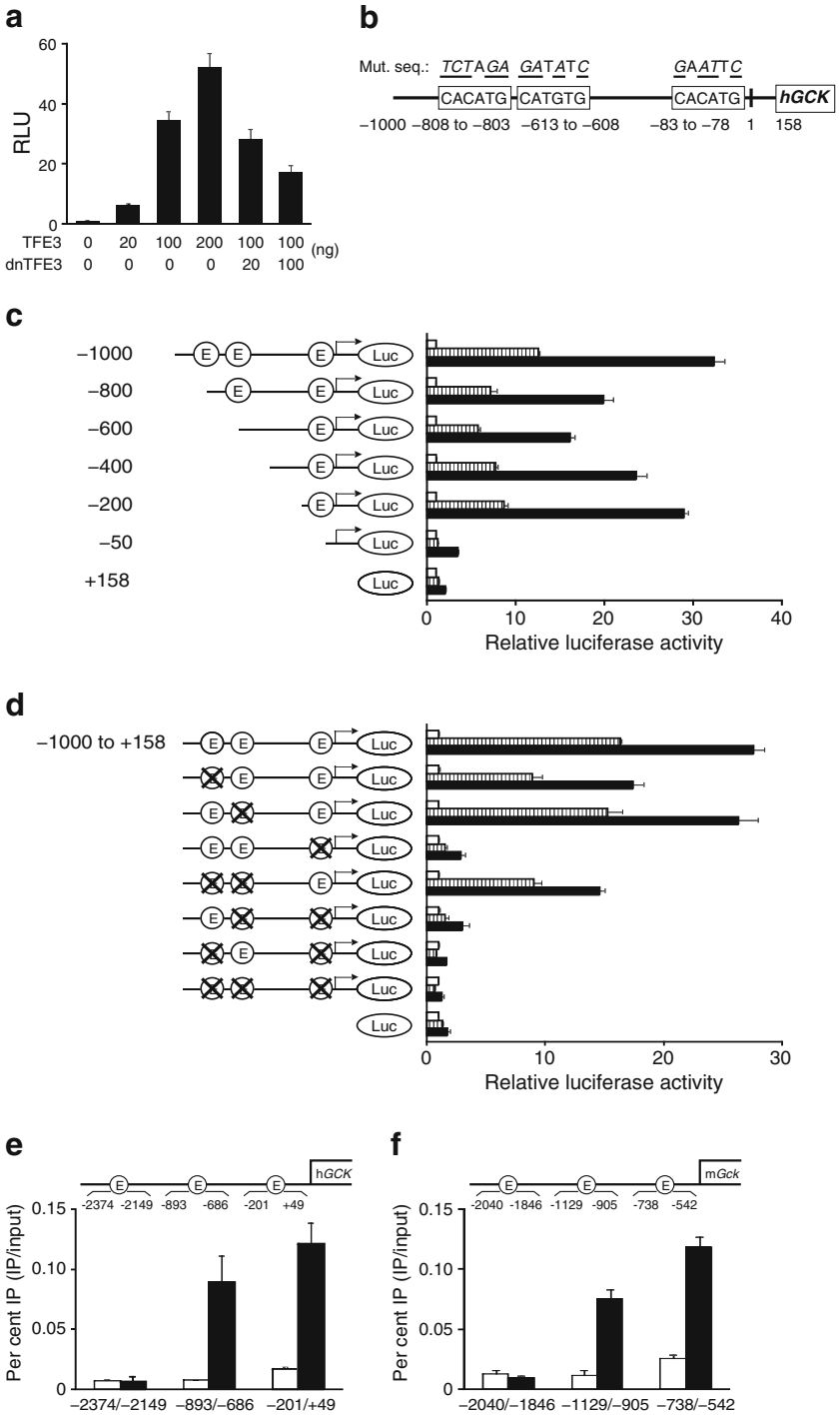
Discussion

In the murine model, Ad-*Tcfe3* was introduced to buffer control or streptozotocin-treated mice. In response to overabundance of TFE3, control mice showed a 1.5-fold increase in *Gck* mRNA (Fig. 4a) and protein (Fig. 4d). This

Fig. 6 Effects of TFE3 on promoter activities of *GCK* in HepG2 cells. (**a**) Effects of TFE3 on the promoter activities of human (h) *GCK* promoter constructs. HepG2 cells were transfected with firefly luciferase fusion gene containing the human *GCK* (h*GCK*) promoter sequence from −1,000 to 158 bp and expression vectors encoding *Renilla* luciferase (Luc) and either wild-type *TCFE3* or a dominant-negative (dn) form of *TCFE3* (dn*TCFE3*). Results are the mean±SEM of five experiments, with each sample assayed in triplicate. (**b**) Consensus search of putative TFE3-binding sites in the h*GCK* gene promoter. The number indicates the distance of nucleotides from the transcription start site (1) of *GCK*. The mutated sequences (mut. seq.; underlined, italic) of each TFE3-binding site are indicated above the consensus TFE3-binding sequence. (**c**) Localisation of TFE3-binding sites in the promoter regions of h*GCK*, and relative luciferase activity of serial deletion constructs or (**d**) site-specific mutations. To exclude length effects of individual promoters, the luciferase activity of each promoter construct was arbitrarily defined as 1. Normalised luciferase activity is shown as the mean±SEM of three independent experiments performed in triplicate and expressed as fold increase relative to basal activity. White bars, TFE3 0 ng; striped bars, TFE3 50 ng; black bars, TFE3 200 ng. (**e**) ChIP assays performed in V5-tagged HepG2 cells that overproduced hTFE3 and (**f**) in mouse liver administered with Ad-*Tcfe3*. Normal IgG was used as a negative control for immunoprecipitation (IP). The regions amplified were −893 to −686 bp and −201 to 49 bp for h*GCK*, and −1,129 to −905 bp and −738 to −542 bp for m*Gck*. The regions −2,374 to −2,149 bp for h*GCK*, and −2,040 to −1,846 bp for m*Gck*, which do not contain any TFE3 binding sites, were amplified as negative controls. The quantity of immunoprecipitated DNA was normalised to total input DNA (1% of the chromosomal DNA used for immunoprecipitation). White bars, precipitation with IgG antibody; black bars, precipitation with V5 antibody (**e**) or TFE3 antibody (**f**)

modest increase in *Gck* expression caused a slight decrease in blood glucose levels (Fig. 1b) and a significant improvement of glucose tolerance (Fig. 2a). These results are supported by previous studies showing that a mild increase of hepatic *Gck* expression in control mice led to a corresponding decrease in blood glucose levels [14] without altering the basal metabolic profile. A mild increase in *Gck* expression also resulted in glucose tolerance dominated by hepatic mechanisms [15]. Previously, the injection of Ad-*Tcfe3* did not induce *Gck* expression in control mice [11]. However, as shown by our OGTT and ITT (Fig. 2), the administration of Ad-*Tcfe3* significantly improved glucose disposal in control mice, an observation supported by the finding that small changes in hepatic *Gck* expression have a considerable impact on blood glucose levels in transgenic mice [2, 3, 16].

The TFE3-dependent increase in blood glucose disposal was more prominent in streptozotocin-treated mice than in control mice. In this model, severely depleted *Gck* mRNA expression was restored by overabundance of TFE3. In turn, symptoms of hyperglycaemia were relieved (Fig. 1c) due to improved glucose and insulin tolerance (Fig. 2). In the streptozotocin mice, which have nearly fully depleted insulin levels (<25 pmol/l), the upregulation of *Gck* can be explained by the direct binding of TFE3 to the *Gck* promoter (Fig. 4, Fig. 6). Furthermore, administration of Ad-*Tcfe3* to primary cultured hepatocytes maintained in serum-free



media resulted in increased *Gck* mRNA levels (Fig. 5). These data suggest that the anti-hyperglycaemic effect of TFE3 might be, in part, due to direct upregulation of *Gck*.

However, because insulin levels in streptozotocin mice are not absolutely zero, it is possible that residual insulin in these mice still affects *Gck* expression. Indeed, it has been demonstrated that insulin-dependent activation of Akt in primary cultured hepatocytes was sufficient to induce *Gck* expression [17]. In addition, Akt, when overabundant due to TFE3 administration, needs insulin for its phosphorylation [11], which is a key modification step in transmitting signals for downstream gene regulation. In streptozotocin-treated mice, the almost fully depleted levels of insulin could phosphorylate Akt through IRS-2. However, because insulin may not be the only Akt phosphorylation signalling pathway [18], the precise mechanism of Akt overabundance and its accompanying phosphorylation when induced by TFE3 needs to be explored.

It is speculated that the increased expression of *Srebf1* induced by TFE3 in streptozotocin mice (Fig. 3d) may increase *Gck* mRNA together with TFE3 levels. Overexpression of *Gck* resulted in resistance to streptozotocin [3] and to high-fat diet-induced diabetes in mice [19, 20]. Furthermore, the restoration of *Gck* expression in Zucker diabetic fatty rats [21] and in mice deficient in liver-specific 3-phosphoinositide-dependent kinase-1, which have defects in the metabolic actions of insulin [22], normalised plasma glucose levels.

GCK plays a role in maintaining normal glucose levels in liver in the postprandial state. In this study, blood glucose levels of randomly fed, streptozotocin-treated mice were significantly decreased by Ad-*Tcfe3* (Fig. 1b). The OGTT and ITT (Fig. 2) showed that Ad-*Tcfe3* decreased blood glucose levels in streptozotocin mice, an observation explained by the fact that Ad-*Tcfe3* increased *Gck* expression. These results indicate that if hepatic levels of GCK are maintained, it is possible to dispose of glucose in the absence of insulin. Furthermore, Ad-*Tcfe3*-mediated overabundance of GCK did not cause hypoglycaemia (Fig. 1b).

Expression of *Slc2a2*, a partner of GCK in the transport of glucose into hepatocytes, was significantly decreased by Ad-*Tcfe3* injection into streptozotocin mice. Because *Slc2a2* expression is increased in the state of hyperglycaemia [23], it can be speculated that TFE3 downregulates *Slc2a2* expression (Fig. 4b), causing a decrease in hepatocyte glucose output. Additionally, the introduction of Ad-*Tcfe3* and the subsequent increase in *Gck* expression may also have led to increased glucose trapping in hepatocytes, as inferred from the decreased blood glucose levels in streptozotocin mice.

Generally, streptozotocin mice show a disrupted hormone ratio, in which insulin levels are severely decreased and glucagon levels are higher, resulting in increased gluconeogenesis [24]. Even under high glucagon levels (data not shown), the lower blood glucose levels observed in fasting streptozotocin mice injected with Ad-*Tcfe3* (Fig. 2a, d) could have been due to decreased expression of *G6pc* and *Pck1* (Fig. 4e, f).

The ITT showed that insulin decreased blood glucose levels in mice injected with Ad-*gfp* or Ad-*Tcfe3* (Fig. 2c). In streptozotocin mice, increased blood glucose levels are caused by a depletion of insulin, rather than by insulin resistance. With regard to the ability of insulin to reduce blood glucose levels, mice injected with Ad-*Tcfe3* exhibited a stronger response than those injected with Ad-*gfp* alone. In vivo, insulin stimulates the secretion of counterregulatory hormones, such as glucocorticoids, resulting in an increase in blood glucose levels. Therefore, because the injected insulin has a short half-life and is rapidly degraded, the inhibitory effects on gluconeogenesis in the liver may disappear. However, in streptozotocin mice injected with Ad-*Tcfe3*, blood glucose levels were maintained at lower levels until 120 min because TFE3 suppresses the expression of *G6pc* and *Pck1* (Fig. 4e, f).

Ad-*Tcfe3* administration to streptozotocin mice led to a slight increase in hepatic lipogenic gene expression (Fig. 3d–h). Because of the activation of *Gck* expression, increased glycolytic flux is likely to increase lipogenesis and esterification of NEFA [14]. However, the administration of Ad-*Tcfe3* did not alter concentrations of cholesterol, NEFA and triacylglycerol in serum (Fig. 3a–c).

To observe the long-term effect of TFE3 on glucose homeostasis, we used AAV-mediated *Tcfe3* gene transfer to streptozotocin mice. To this end, we adopted AAV serotype 2 as a gene delivery vehicle because of its excellent safety and the greater duration of its transgene expression capability [25]. AAV2 has been described as an efficient in vivo delivery vector for hepatic expression for the treatment of metabolic disease [26, 27]. In AAV2-*Tcfe3*-treated streptozotocin mice, plasma glucose levels were significantly decreased compared with the saline-treated control group. In addition, the OGTT results were improved by AAV-*Tcfe3* administration to streptozotocin mice (ESM Fig. 1). The patterns of the various genes involved in lipid metabolism in the AAV-*Tcfe3* treatment group were similar to those in animals given Ad-*Tcfe3*. Furthermore, lipid profiles in blood were not significantly changed (ESM Fig. 2). A possible explanation of these phenomena is that TFE3 might regulate the expression of various genes involved in lipid metabolism, and also of genes related to mitochondrial functions or fatty acid oxidation [28].

However, the AAV2-mediated *Tcfe3* gene transfer did not induce a greater reduction of hyperglycaemia in streptozotocin mice. The superior effect of Ad treatment over that of AAV treatment could be due to a higher transduction of the liver and higher *Tcfe3* expression, as compared by quantitative real-time PCR (data not shown). The reduced effect of AAV-mediated gene transfer could also be due to the use of AAV2, rather than AAV8 or 9, which are better known vectors for hepatic gene expression [29]. Another reason for reduced response could be the use of the cytomegalovirus promoter instead of liver-specific promoter(s) for long-term expression of *Tcfe3*. Thus, the use of AAV8 or AAV9 with liver-specific promoter and the use of higher doses of the vector might have resulted in better glucose control.

GCK expression is decreased in diabetic patients and animals with streptozotocin-induced diabetes [30]. Therefore, an understanding of the molecular mechanisms that regulate *GCK* expression is critical for the development of treatments for diabetes and other conditions causing hyperglycaemia. As shown in this study, overabundance of TFE3 efficiently decreases blood glucose levels in vivo by upregulating *Gck* expression in the liver during beta cell destruction. Taken together, overproduction of TFE3 could be a good therapeutic approach to reducing high blood glucose levels and improving glucose tolerance in diabetes patients, without affecting their serum lipid profile.

Acknowledgements We are grateful to N. Yamada (Department of Internal Medicine, University of Tsukuba, Tsukuba, Japan) for providing Ad-*Tcfe3*. We also thank to K.S. Kim, (Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul, Republic of Korea) and J.H. Jang (Department of Chemical and Biomolecular Engineering, Yonsei University, Seoul, Republic of Korea) for their generous gifts of pAAV-RC and AAV 293 cells, respectively.

Funding This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) and the Ministry of Education, Science and Technology (MEST), Republic of Korea (2009-0080655 and 2011-0030706 to Y.H. Ahn).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MYK, substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; SHJ, analysis and interpretation of data, drafting the article, final approval of the version to be published; JMP, acquisition of data, revising it critically for important intellectual content, final approval of the version to be published; THK, analysis and interpretation of data, revising it critically for important intellectual content, final approval of the version to be published; SSI, substantial contributions to conception and design, revising it critically for important intellectual content, final approval of the version to be published; YHA, contributions to conception, drafting the article, interpretation of data, revising it critically for important intellectual content, final approval of the version to be published.

References

1. Walker DG, Rao S (1964) The role of glucokinase in the phosphorylation of glucose by rat liver. Biochem J 90:360–368
2. Niswender KD, Shiota M, Postic C, Cherrington AD, Magnuson MA (1997) Effects of increased glucokinase gene copy number on glucose homeostasis and hepatic glucose metabolism. J Biol Chem 272:22570–22575
3. Ferre T, Riu E, Bosch F, Valera A (1996) Evidence from transgenic mice that glucokinase is rate limiting for glucose utilization in the liver. FASEB J 10:1213–1218
4. Iynedjian PB, Gjinovci A, Renold AE (1988) Stimulation by insulin of glucokinase gene transcription in liver of diabetic rats. J Biol Chem 263:740–744
5. Michael MD, Kulkarni RN, Postic C et al (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol Cell 6:87–97
6. Iynedjian PB, Jotterand D, Nouspikel T, Asfari M, Pilot PR (1989) Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. J Biol Chem 264:21824–21829
7. Magnuson MA, Andreone TL, Printz RL, Koch S, Granner DK (1989) Rat glucokinase gene: structure and regulation by insulin. Proc Natl Acad Sci U S A 86:4838–4842
8. Kim TH, Kim H, Park JM et al (2009) Interrelationship between liver X receptor alpha, sterol regulatory element-binding protein-1c, peroxisome proliferator-activated receptor gamma, and small heterodimer partner in the transcriptional regulation of glucokinase gene expression in liver. J Biol Chem 284:15071–15083
9. Roth U, Curth K, Unterman TG, Kietzmann T (2004) The transcription factors HIF-1 and HNF-4 and the coactivator p300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem 279:2623–2631
10. Beckmann H, Su LK, Kadesch T (1990) TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer muE3 motif. Genes Dev 4:167–179
11. Nakagawa Y, Shimano H, Yoshikawa T et al (2006) TFE3 transcriptionally activates hepatic IRS-2, participates in insulin signaling and ameliorates diabetes. Nat Med 12:107–113
12. Ide T, Shimano H, Yahagi N et al (2004) SREBPs suppress IRS-2-mediated insulin signalling in the liver. Nat Cell Biol 6:351–357
13. Im SS, Kim MY, Kwon SK et al (2011) Peroxisome proliferator-activated receptor α is responsible for the up-regulation of hepatic glucose-6-phosphatase gene expression in fasting and db/db Mice. J Biol Chem 286:1157–1164
14. O'Doherty RM, Lehman DL, Telemaque-Potts S, Newgard CB (1999) Metabolic impact of glucokinase overexpression in liver: lowering of blood glucose in fed rats is accompanied by hyperlipidemia. Diabetes 48:2022–2027
15. Magnuson MA, She P, Shiota M (2003) Gene-altered mice and metabolic flux control. J Biol Chem 278:32485–32488
16. Hariharan N, Farrelly D, Hagan D et al (1997) Expression of human hepatic glucokinase in transgenic mice liver results in decreased glucose levels and reduced body weight. Diabetes 46:11–16
17. Ribaux PG, Iynedjian PB (2003) Analysis of the role of protein kinase B (cAKT) in insulin-dependent induction of glucokinase and sterol regulatory element-binding protein 1 (SREBP1) mRNAs in hepatocytes. Biochem J 376:697–705
18. Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7:85–96
19. Shiota M, Postic C, Fujimoto Y et al (2001) Glucokinase gene locus transgenic mice are resistant to the development of obesity-induced type 2 diabetes. Diabetes 50:622–629
20. Desai UJ, Slosberg ED, Boettcher BR et al (2001) Phenotypic correction of diabetic mice by adenovirus-mediated glucokinase expression. Diabetes 50:2287–2295
21. Torres TP, Catlin RL, Chan R et al (2009) Restoration of hepatic glucokinase expression corrects hepatic glucose flux and normalizes plasma glucose in zucker diabetic fatty rats. Diabetes 58:78–86
22. Okamoto Y, Ogawa W, Nishizawa A et al (2007) Restoration of glucokinase expression in the liver normalizes postprandial glucose disposal in mice with hepatic deficiency of PDK1. Diabetes 56:1000–1009
23. Im SS, Kang SY, Kim SY et al (2005) Glucose-stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. Diabetes 54:1684–1691
24. Hanson RW, Reshef L (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66:581–611
25. Mingozzi F, High KA (2011) Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nat Rev Genet 12:341–355
26. Xiao W, Berta SC, Lu MM, Moscioni AD, Tazelaar J, Wilson JM (1998) Adeno-associated virus as a vector for liver-directed gene therapy. J Virol 72:10222–10226
27. Grimm D, Zhou S, Nakai H et al (2003) Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. Blood 102:2412–2419
28. Meikle PJ, Christopher MJ (2011) Lipidomics is providing new insight into the metabolic syndrome and its sequelae. Curr Opin Lipidol 22:210–215
29. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 99:11854–11859
30. Jourdan T, Djaouti L, Demizieux L, Gresti J, Verges B, Degrace P (2009) Liver carbohydrate and lipid metabolism of insulin-deficient mice is altered by trans-10, cis-12 conjugated linoleic acid. J Nutr 139:1901–1907