Establishment of a novel hepatic steatosis cell model by Cas9/sgRNA-mediated DGKθ gene knockout

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Abstract. To investigate the role of diacylglycerol kinase θ (DGKθ) in lipid metabolism and insulin resistance, the present study generated an *in vitro* hepatic steatosis cell model by knockout of the DGKθ gene in liver cancer cell line HepG2 using CRISPR/Cas9 technology. The cell line was characterized by Oil Red O staining and shown to exhibit increased intracellular lipid accumulation, compared with that in wild-type liver cancer cell line HepG2. The gene expression levels of signaling proteins in pathways involved in lipid metabolism, insulin resistance and gluconeogenesis were also examined. The DGKθ-knockout HepG2 cells showed increased mRNA and protein expression levels of lipid synthesis-related genes, fatty acid synthase, peroxisome proliferator-activated receptor-γ and sterol regulatory element-binding protein-1c, and decreased expression levels of the lipolysis-related gene, carnitine palmitoyltransferase1A. These changes may account for the increased intracellular lipid content of this cell line. The DGKθ-knockout HepG2 cells also exhibited an increased phosphorylation level of protein kinase Cε and decreased phosphorylation levels of insulin receptor substrate 1, mechanistic target of rapamycin and protein kinase B (also known as Akt). These changes have been reported to mediate insulin resistance. Taken together, an *in vitro* hepatic steatosis cell model was established in the present study, providing a valuable tool for understanding the pathogenesis of nonalcoholic fatty liver disease and associated insulin resistance, and for developing treatment strategies for this disease.

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Key words: diacylglycerol kinase θ, CRISPR/Cas9, lipid accumulation, insulin resistance, nonalcoholic fatty liver disease, type 2 diabetes

Introduction

Diacylglycerol kinases (DGKs) are endogenous lipid regulation enzymes, which are involved in multiple cellular signaling pathways by regulating the levels of intracellular secondary messengers diacylglycerol (DAG) and phosphatidyl acid (PA) (1,2). Currently, 10 DGK isoforms have been documented in mammals, and are grouped into five categories according to their structure and number of specific domains. DGKθ is the sole member of group V (3). Compared with other DGK members, which contain two cysteine-rich domains, DGKθ has three, in addition to an N-terminal proline/glycine-rich domain, a pleckstrin homology domain and a Ras-associating domain (4). DGKθ was initially found to be expressed in mouse brains (4), and was subsequently reported to be the most abundant isoform in hepatocytes (5).

There is evidence that abnormal enzyme activity of DGKθ may be associated with insulin resistance. Hepatic DAG accumulation can activate protein kinase Cε (PKCε) in the liver, which is associated with hepatic insulin resistance (6,7). DGKθ has been identified as the major isoform mediating DAG accumulation (5,8,9). In addition, DGKδ, which has a similar substructure to DGKθ (4), has been shown to be directly linked to insulin resistance in the skeletal muscle of patients with type 2 diabetes (10).

Nonalcoholic fatty liver disease (NAFLD) is an independent risk factor for type 2 diabetes and cardiovascular diseases (11). The prevalence of NAFLD is ~30% in the general population, and up to three times higher in those with type 2 diabetes. Studies have suggested that abnormality of the DAG-PKCε signaling pathway can link NAFLD with hepatic insulin resistance (11). Therefore, it is likely that DGKθ is the key signaling molecule in this pathway and involved in the pathogenesis of NAFLD.

In the present study, CRISPR/Cas9 genome editing technology was used to establish a DGKθ-knockout hepatic cell line. It was found that this cell line had markedly increased intracellular lipid content. The gene expression levels of key proteins in the pathways involved in lipid metabolism were evaluated. These proteins included fatty acid synthase (FAS), peroxisome proliferator-activated receptor-γ (PPARγ), sterol regulatory element-binding protein-1c (SREBP-1c), carnitine palmitoyltransferase1a (CPT1a) and long-chain

Table I. Primer sequences used for sgRNA synthesis and the detection of sgRNA biological activity.

Primer	Sequence (5'-3')
hDGKθ sgRNA1 Forward	ACCGCCCTGCAGGAGGCCGCACTGCGG
hDGKθ sgRNA1 Reverse	AAACCCGCAGTGCGGCCTCCTGCAGGG
hDGKθ sgRNA2 Forward	ACCGGAGGGGGGCGACGGCGCCGACGG
hDGKθ sgRNA2 Reverse	AAACCCGTCGGCGCCGTCGCCCCCCTC
hDGKθ sgRNA3 Forward	ACCGACACAGGCAACTCCGGAGTCCGG
hDGKθ sgRNA3 Reverse	AAACCCGGACTCCGGAGTTGCCTGTGT
hDGKθ sgRNA4 Forward	ACCGAAGCCAGTTCCGCCTCGTCACGG
hDGKθ sgRNA4 Reverse	AAACCCGTGACGAGGCGGAACTGGCTT
hDGKθ sgRNA detection Forward	GCTTCAGCAAGACGCAGAG
hDGKθ sgRNA detection Reverse	CAGGTCCAAACCCAAAAGGT

sgRNA, single-guide RNA; DGKθ, diacylglycerol kinase θ.

L-3-hydroxyacyl-coenzyme A dehydrogenase α (HADHα). Key proteins in pathways involved in insulin resistance, including PKCε and insulin receptor substrate 1 (IRS-1), and in gluconeogenesis, including mechanistic target of rapamycin (mTOR) and Akt, were also assessed. This cell line may offer potential for investigating NAFLD and its associated hepatic insulin resistance.

Materials and methods

Cell culture. The human liver cancer cell line HepG2 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C, 5% CO_2 (v/v). A total of ~1x10^5 HepG2 cells were treated with DGKθ inhibitor R59949 at 10 μM, or DGKθ agonist GW4064 (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 1 μM for 24 h at 37˚C.

Plasmid construction. The targeting regions for four pairs of single-guide RNA (sgRNA) located in exon 6, exon 7 or exon 8 of human DGKθ were selected using the CRISPR Design website (http://crispr.mit.edu). The sgRNAs were synthesized at the Beijing Genomics Institute (Beijing, China). The sequences of the oligonucleotides are shown in Table I. According to a previously described method (12), the human U6 promoter and sgRNA backbone were sequentially cloned into pUC19 (Clontech Laboratories, Inc., Mountain view, CA, USA), the obtained plasmid was called the pUC19/U6-BsaI-sgRNA backbone vector. The synthesized oligos were annealed, and ligated into the *Bsa*I sites of the pUC19/U6-BsaI-sgRNA backbone vector under the control of the U6 promoter. The resultant plasmids were referred to as pUC19/U6-DGKθ sgRNA1, pUC19/U6-DGKθ sgRNA2, pUC19/U6-DGKθ sgRNA3 and pUC19/U6-DGKθ sgRNA4.

To construct the donor vector, an up homologous arm, 909 bp in length and located upstream of the targeting sites, was amplified through nest polymerase chain reaction (PCR) using two pairs of primers (Table II) based on a template of human genomic DNA. PCR was conducted in a 50 μl reaction volume, consisting of 5 μl 10X PrimeSTAR buffer, 100 ng genomic DNA template, 0.2 μM each primer, 10 mM dNTPS and 1 unit PrimeSTAR HS DNA Polymerase (all from Clontech Laboratories, Inc.) according to the following conditions: 29 cycles of 94˚C for 30 sec; 98˚C for 10 sec, 58˚C for 15 sec and 72˚C for 1 min and a 10 min extension step at 72˚C. Similarly, a down homologous arm 975 bp in length was obtained using two pairs of primers (Table II). Subsequently, the donor vector pAd5/DGKθ-up/down-arm was constructed by sequentially inserting the up and down homologous arms into the backbone vector according to the previously described method (12). This vector also contained an eGFP-T2A-Neomycin expression cassette between the up and down homologous arms for positive selection, and a PGK-TK-T2A-mCherry expression cassette located at the 3'-terminal of the down homologous arm for negative selection (Fig. 1D).

T7E1 assay. Genomic DNA was extracted using the TIANamp Blood DNA kit (Tiangen Biotech, Inc., Beijing, China). The target site was amplified by nest PCR, the product of which was then purified using an AxyPrep DNA Gel Extraction kit (Axygen Biotechnology, Hangzhou, China). The purified product was then denatured and re-annealed, and digested with T7E1 (New England Biolabs, Ipswich, MA, USA). The digested product was then separated by 1.2% agarose gel electrophoresis. The gel was stained in running buffer containing 0.5 μg/ml ethidium bromide at room temperature for 15-30 min, then the images were captured under FR-98A Gel Imaging System (Shanghai Furi Science & Technology Co., Ltd., Shanghai, China).

Establishment of the DGKθ gene-knockout liver cancer cell line. To construct the human DGKθ gene-knockout Liver cancer cell line, pUC19/CMV-Cas9-U6-sgRNAX (X represents the sgRNA with the highest activity) was generated according to a previously described method (12). A total of ~1.5x10^5 HepG2 cells were then

Table II. Primer sequences used for donor construction and RT-qPCR amplification.

Primer	Sequence (5'-3')
hDGKθ up arm nest Forward	GGCGAGAGTCAGGAGTGAAG
hDGKθ up arm nest Reverse	GGAGAAGGGCCTGAGCTG
hDGKθ up arm *Sal*I Forward	GTCGACAGAGTTGCGCAGGTGAAGAG
hDGKθ up arm *Cla*I Reverse	AATCGATACCAGGTCCAGGGAAAGACC
hDGKθ down arm nest Forward	CGTACCCTGTGCCTGCTC
hDGKθ down arm nest Reverse	GTGACATCTCACCCCAAAGG
hDGKθ down arm *Sal*I Forward	GTCGACAGGCACGGTGAGTAGACAGC
hDGKθ down arm *BamHI* Reverse	GGATCCCAGAGCCTCTTGGAGGAAGA
hDGKθ knock-in detection nest Forward	TGGTGATTCCACACTGGCTTG
hDGKθ knock-in detection nest Reverse	ATGCCAGATGAAAACAGCGAG
hDGKθ knock-in detection Forward	CACCAGGATCACGTGAGTGTA
hDGKθ knock-in detection Reverse	CAGGTCCAAACCCAAAAGGT
PKCε RT-qPCR Forward	GACGAGTTCGTCACCGATGT
PKCε RT-qPCR Reverse	CTTTAGGGGCTTCACCCGAC
INSR RT-qPCR Forward	GTACCCCGGAGAGGTGTGTC
INSR RT-qPCR Reverse	CCCGGAAGAGCAGCAAGTAA
IRS1 RT-qPCR Forward	CTGGGGGTTTGGAGAATGGT
IRS1 RT-qPCR Reverse	GTCTTCATTCTGCTGTGATGTCC
FAS RT-qPCR Forward	CAGAGCAGCCATGGAGGAG
FAS RT-qPCR Reverse	TTGATGCCTCCGTCCACGAT
PPAR-γ RT-qPCR Forward	ACCCAGAAAGCGATTCCTTCA
PPAR-γ RT-qPCR Reverse	TCCACTTTGATTGCACTTTGGT
SREBP1c RT-qPCR Forward	CTCCGGCCACAAGGTACACA
SREBP1c RT-qPCR Reverse	GAGGCCCTAAGGGTTGACACAG
CPT1a RT-qPCR Forward	GGAATGAAATTCCCACTGTCTGTC
CPT1a RT-qPCR Reverse	CAGTTCAGCCATCGCTGTTGTA
HADHα RT-qPCR Forward	GCCATCAATGGATCCTGCCT
HADHα RT-qPCR Reverse	CAGGCACACCCACCATTTTG
AKT1 RT-qPCR Forward	GGCAAGGTGATCCTGGTGAA
AKT1 RT-qPCR Reverse	ACAGGTGGAAGAACAGCTCG
mTOR RT-qPCR Forward	AAGCCGCGCGAACCTC
mTOR RT-qPCR Reverse	TGGCATCTGAGCTGGAAACC
hDGKθ RT-qPCR Forward	ATCCGGCAGATGTCTGTGC
hDGKθ RT-qPCR Reverse	ATGTGACTCACGGACACCAC

DGKθ, diacylglycerol kinase θ; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PKCε, protein kinase Cε; INSR, insulin receptor; IRS1, insulin receptor substrate 1; FAS, fatty acid synthase, PPAR-γ, peroxisome proliferator-activated receptor-γ; SREBP1c, sterol regulatory element-binding protein-1c; CPT1a, carnitine palmitoyltransferase1A; HADHα, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α.

co-transfected with 4 μg of pUC19/CMV-Cas9-U6-sgRNAX and 8 μg of pAd5/DGKθ-up/down-arm at 37˚C for 48 h at an efficiency of ~20% using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) followed by screening in DMEM containing G418 (1 mg/ml) and GCV (1 mg/ml). A single cell clone was obtained through limited dilution following positive and negative selection, which was then confirmed by PCR that was performed in a 50 μl reaction volume, consisting of 5 μl 10X PrimeSTAR, 100 ng genomic DNA template, 0.2 μM each primer, 10 mM dNTPs, and 1 unit PrimeSTAR HS DNA Polymerase (all from Clontech Laboratories, Inc.) according to the following conditions: 30 cycles of 94˚C for 30 sec; 98˚C for 10 sec, 58˚C for 15 sec and 72˚C for 90 min, followed by a 10 min extension step at 72˚C, then the PCR products were sent to Bejing Genomics Institute Genomics Co., Ltd. (Shenzhen, China) for sequencing.

MTT assay. A total of $1x10^3$ wild-type (WT) HepG2 cells or DGKθ gene-knockout HepG2 cells were cultured in 96-well plates. MTT solution (20 μl; American Type Culture Collection) was added to each well at 37˚C at 24, 48, 72 and 96 h, respectively. Following incubation with MTT for 4 h,

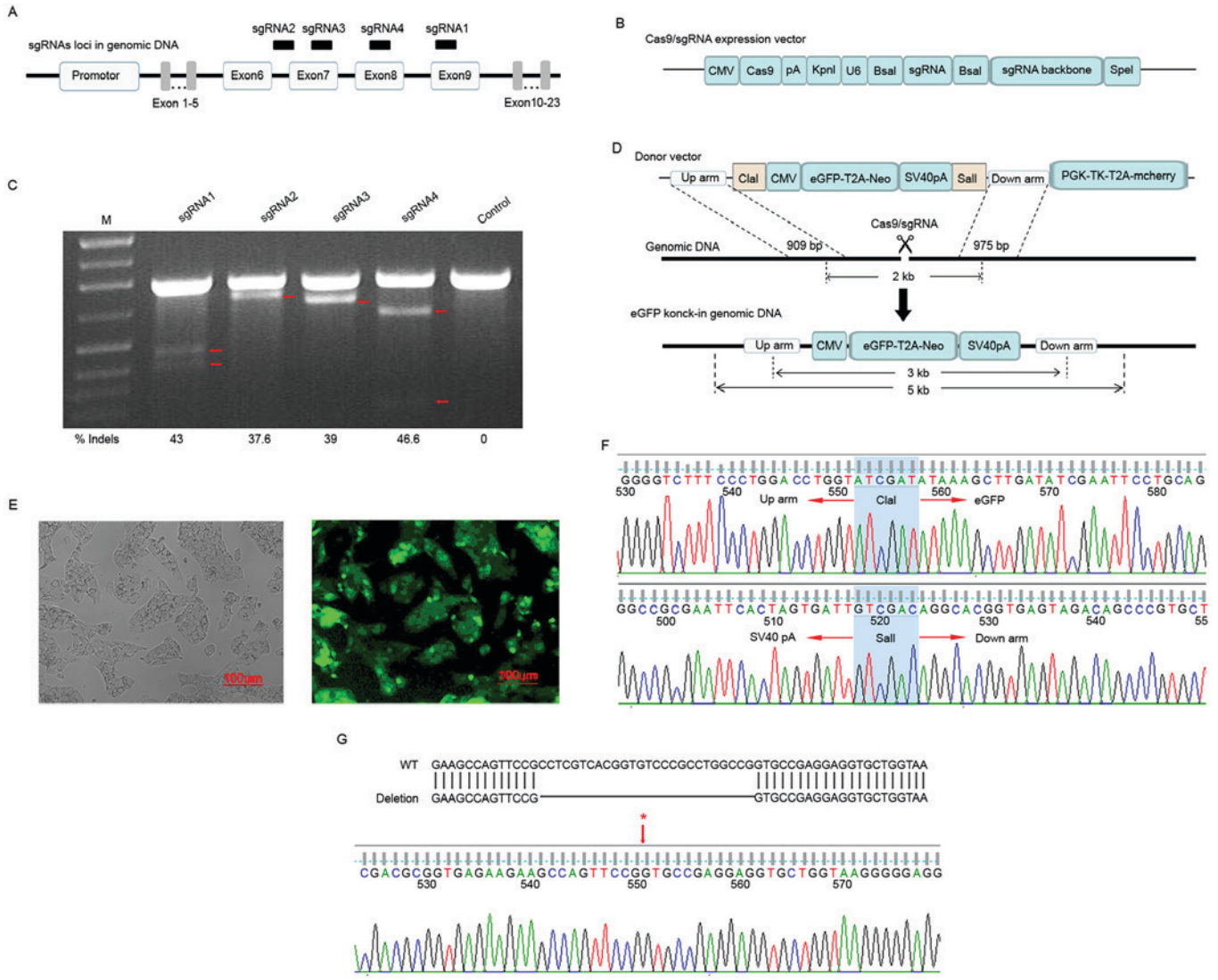

Figure 1. Establishment of the DGKθ-knockout liver cancer cell line HepG2 using the Cas9/sgRNA technique. (A) Schematic diagram of the target sites for the four pairs of sgRNAs in DGKθ loci. (B) Illustration of target vector carrying Cas9 and sgRNA expression cassettes. (C) Efficacy of sgRNAs was determined using a T7E1 assay. Indel frequencies of sgRNAs 1-4 are shown below the gel. Red arrows indicate expected positions of DNA bands cleaved by T7E1. (D) Diagram of donor vector and integration of exogenous fragments into the genome by homologous recombination. (E) Dark field (left) and fluorescence images of liver cancer cells with proper homologous recombination stably expressing the eGFP reporter gene. (F) Sequencing results of clone 8 showing one allele containing the exogenous fragment (or 'fragments') integrated into the genome by homologous recombination, (G) and the other allele with a 26-bp deletion in the DGKθ loci. The red arrow indicates the position of the deletion. DGKθ, diacylglycerol kinase θ; sgRNA, single-guide RNA; WT, wild-type.

200 μl DMSO was added to each well for 35 min at 37˚C. The absorbance in each well was then measured at 570 nm on a microplate reader (Thermo Fisher Scientific, Inc.). Each group contained six replicates and the experiment was repeated three times.

Oil Red O staining and determination of optical density (OD) values. The WT HepG2 cells and DGKθ-knockout HepG2 cells grown in 24-well plates were harvested. The cells were stained with Oil Red O (Sigma-Aldrich; Merck KGaA), and quantification of Oil Red O-based steatosis was performed, as previously described (13). The cell nuclei were stained with hematoxylin for 15 sec and washed with saturated Li_2CO_3 solution. Images were captured using a Leica DFC 420 C microscope (Leica Microsystems GmbH). The experiments were performed in triplicate.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA (1 μg), purified with an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) was used to synthesize cDNA, followed by amplification of the target gene that was carried out in a 25 μl reaction volume, consisting of 150 ng cDNA, 0.2 μM each primer, 12.5 μl 2X SYBR buffer (Takara Biotechnology Co., Ltd., Dalian, China) containing 10 mM dNTPs and 1 unit DNA Taq polymerase according to the following conditions: 39 cycles of 95˚C for 30 sec; 95˚C for 5 sec and 60˚C for 30 sec. The sequences of the primers used are listed in Table II. All tests were performed in triplicate and the data were normalized to GAPDH and quantified using the $2^{-\Delta\Delta Cq}$ method (14). ΔCq was calculated by subtracting the Cq value of GAPDH from the Cq value of the target gene. The fold change was generated using the formula $2^{-\Delta\Delta Cq}$.

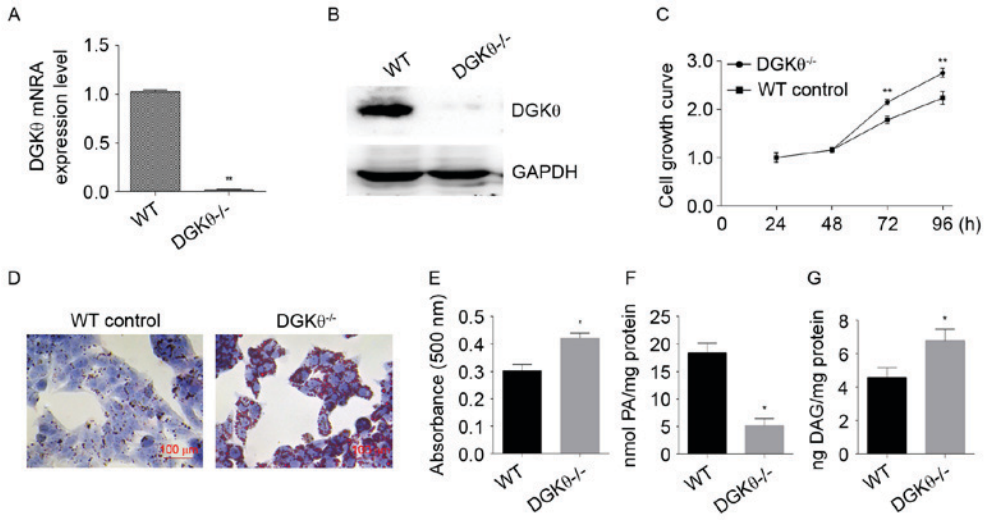

Figure 2. Characterization of the DGKθ-knockout liver cancer cell line. Knockout of the DGKθ gene in liver cancer cell line HepG2 was confirmed using (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analyses. (C) Growth rates of WT and DGKθ knockout liver cancer cell line HepG2 were measured using an MTT assay. (D) Cells were stained with Oil Red O and DGKθ-knockout cells exhibited increased intracellular lipids. (E) For quantification of Oil Red O-based steatosis, intracellular lipids stained with Oil Red O were released and optical density values at 500 nm were measured. Intracellular (F) PA and (G) DAG were detected using the corresponding kits. *P<0.05 and **P<0.01, vs. WT. All results were derived from three independent experiments. DGKθ, diacylglycerol kinase θ; sgRNA, single-guide RNA; WT, wild type control; DGKθ-/-, DGKθ gene-knockout; PA, phosphatidyl acid; DAG, diacylglycerol.

Western blot analysis. The proteins were extracted from the cells using extraction buffer as previously described (13) and quantified using Pierce bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.), which were then applied (80 μg/lane) to a gel for 10% SDS-PAGE and subsequently electrotransferred onto methanol-pretreated polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with PBS buffer containing 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and 0.5% v/v Tween-20 for 1 h at room temperature. The membranes were then incubated with primary antibodies targeting FAS (1:500; cat no. ab82419), CPT1a (1:300; cat no. ab128568), PPARγ (1:500; cat no. ab66343), mTOR (1:500; cat no. ab25880), phosphorylated (p-)mTOR (1:300; cat no. ab109268), PKCε (1:500; cat no. ab63638), p-PKCε (S729; 1:500; cat no. ab63387), IRS1 (1:500; cat no. ab52167) and p-IRS1 (Y632; 1:300; cat no. ab109543) from Abcam (Cambridge, UK), and primary antibodies targeting DGKθ (1:500; cat no. 17885-1-AP), SREBP-1c (1:500; cat no. 14088-1-AP), HADHα (1:500; cat no. 10758-1-AP), AKT (1:500; cat no. 10176-2-AP) and p-AKT (1:300; cat no. 66,444-1-Ig) from ProteinTech Group, Inc. (Chicago, IL, USA) at 37˚C for 1 h. Finally, the membranes were incubated with secondary antibodies, horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) polyclonal antibody (1:10,000; cat no. ZB-2301; Beijing Zhongshan Jinqiao Biological Technology Ltd., Beijing, China) or HRP-conjugated goat anti-mouse IgG polyclonal antibody (1:10,000; cat no. ZB-2305; Beijing Zhongshan Jinqiao Biological Technology Ltd., Beijing, China) at 37˚C for 1 h and visualized on a Tanon 5500 Chemiluminescence Imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China), and the protein levels were visualized using a Supersignal West Pico chemiluminescent detection system (Tanon Science and Technology Co., Ltd.), according to the manufacturer's protocol. Protein levels were determined using ImageCal software (version 4.0; Tanon Science and Technology Co., Ltd.).

PA and DAG assay. The WT HepG2 cells and DGKθ-knockout HepG2 cells were grown on 60 mm plates for 48 h and lysed with RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and 1 mM phenylmethanesulfonyl fluoride. The total lipids in the lysates were harvested by centrifugation at 10,000 x g for 10 min at 4˚C. The PA content was quantified using a Total PA kit (HZbscience, Shanghai, China) according to the manufacturer's protocol. The quantity of DAG in each sample was determined using a Human DAG ELISA kit (Cusabio Biotech Co., Ltd., Barksdale, DE, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences between the means of each group were analyzed using one-way analysis of variance with Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. The statistical analyses were performed using Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS Statistics 20 software (IBM SPSS, Armonk, NY, USA).

Results

Generation of a DGKθ-knockout liver cancer cell line using the Cas9/sgRNA technique. In the present study, the DGKθ gene-knockout liver cancer cell line HepG2 was established using the Cas9/sgRNA technique, as follows. Firstly, the pUC19/CMV-Cas9-U6-sgRNA4 vector was constructed according to the previously described method (12). The vector carried sgRNA4, which had the highest cleavage activity among the four pairs of human

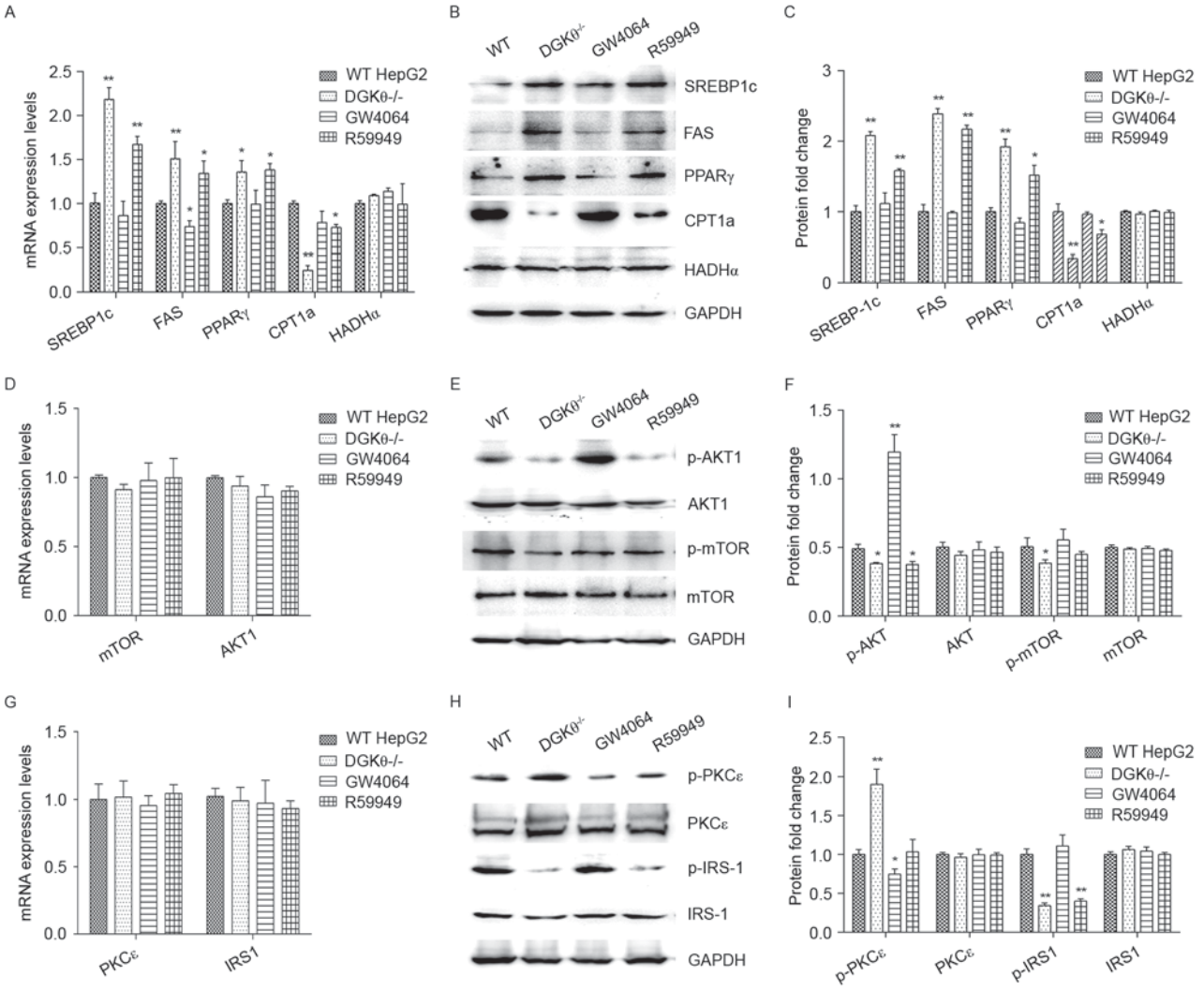

Figure 3. Effects of the knockout of DGKθ on the expression levels of signaling proteins involved in lipid metabolism, insulin resistance and gluconeogenesis pathways. (A) Expression levels of proteins involved in lipid metabolism pathway signaling, SREBP-1c, FAS, PPARγ, CPT1a and HADHα, were analyzed using RT-qPCR and (B) western blot analyses with (C) semi-quantification. (D) Expression levels of the proteins involved in the gluconeogenesis pathway signaling, mTOR and AKT, were detected by RT-qPCR and (E) western blot analyses with (F) semi-quantification. (G) The expression levels of the insulin resistance pathway signaling proteins PKCε and IRS-1 were measured by RT-qPCR and (H) western blot analyses with (I) semi-quantification. For western blot analysis, 80 μg of proteins were loaded in each lane. Protein expression values were normalized to GAPDH and data are presented as the mean ± standard error of the mean of three independent experiments, each performed in triplicate. *P<0.05 and **P<0.01, vs. control. DGKθ, diacylglycerol kinase θ; WT, wild-type control; DGKθ-/-, DGKθ gene-knockout; GW4064, WT Liver cancer cells treated with 1 μM GW4064; R59949, WT Liver cancer cells treated with 10 μM R59949; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SREBP1c, sterol regulatory element-binding protein-1c; FAS, fatty acid synthase, PPAR-γ, peroxisome proliferator-activated receptor-γ; CPT1a, carnitine palmitoyltransferase1A; HADHα, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α; mTOR, mechanistic target of rapamycin; PKCε, protein kinase Cε; IRS1, insulin receptor substrate 1; p-, phosphorylated.

DGKθ-targeting sgRNAs (Fig. 1A-C). Secondly, the donor vector pAd5/DGKθ-up/down-arm, which contained up- and down-homologous arms for homologous recombination, a neomycin-T2A-eGFP expression cassette for positive selection and a TK expression cassette for negative selection (Fig. 1D), was generated according to the previously described method (12). The Liver cancer cell line was then transfected with pUC19/CMV-Cas9-U6-sgRNA4 and pAd5/DGKθ-up/down-arm donor vector followed by screening with G418. As the donor vector contained an eGFP expression cassette, the cells with homologous recombination exhibited green fluorescence (Fig. 1E). Finally, the DGKθ gene-knockout liver cancer cell line HepG2 carrying a targeted integration in one allele (Fig. 1F) and a 26 bp deletion in the other allele was confirmed by sequencing (Fig. 1G).

Characterization of the DGKθ-knockout liver cancer cell line. RT-qPCR and western blot analyses were performed to confirm the knockout of the DGKθ gene in the liver cancer cell line HepG2 (Fig. 2A and B). The effect of knockout of the DGKθ gene on the growth of liver cancer cells was then investigated using an MTT assay. The knockout of the DGKθ gene promoted the growth of the liver cancer cells (Fig. 2C). Oil Red O staining showed that the DGKθ gene-knockout HepG2 cells had 32% higher intracellular lipid content (Fig. 2D), compared with the WT HepG2 cells (Fig. 2E). As expected, the content of intracellular PA was significantly decreased (Fig. 2F), whereas the content of DAG was increased (Fig. 2G) in the DGKθ gene-knockout HepG2 cells.

Subsequently, the present study examined the expression levels of genes associated with lipid synthesis (FAS, PPARγ

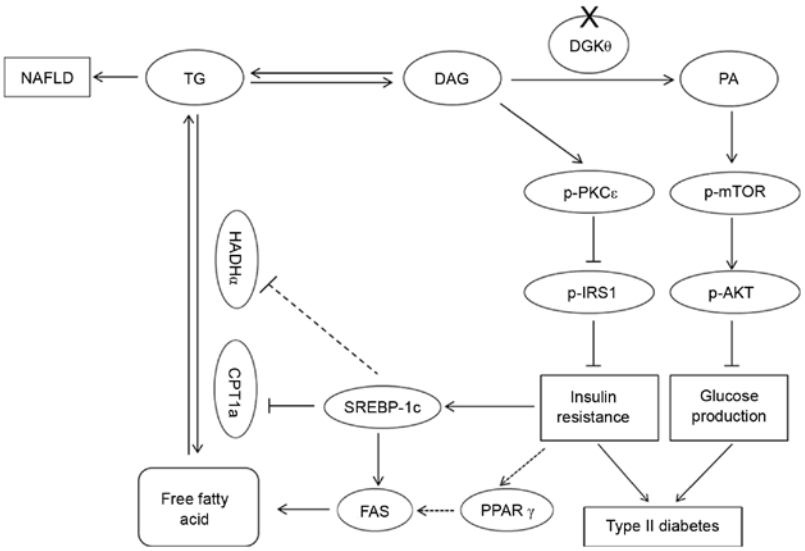

Figure 4. Illustration demonstrating the roles of DGKθ in lipid accumulation, insulin resistance and glucose production. DGKθ gene knockout leads to a decrease in the level of PA, which causes type 2 diabetes by increasing the levels of p-mTOR and AKT, and an increase in the level of DAG, which causes insulin resistance, type 2 diabetes and NAFLD. Solid lines indicate confirmed regulatory associations, while dotted lines indicate undetermined hypotheses. DGKθ, diacylglycerol kinase; p-, phosphorylated; NAFLD, nonalcoholic fatty liver disease; PA, phosphatidyl acid; DAG, diacylglycerol; mTOR, mechanistic target of rapamycin; PKCε, protein kinase Cε; IRS1, insulin receptor substrate 1; PPAR-γ, peroxisome proliferator-activated receptor-γ; SREBP1c, sterol regulatory element-binding protein-1c; FAS, fatty acid synthase; CPT1a, carnitine palmitoyltransferase1A; HADHα, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α; TG, triglyceride.

and SREBP-1c) and genes associated with lipolysis (CPT1a and HADHα) in the DGKθ-knockout HepG2 cells at the mRNA and protein levels. The results indicated that DGKθ gene-knockout increased the expression levels of FAS, PPARγ and SREBP-1c, and suppressed the expression of CPT1a (Fig. 3A-C), compared, with the levels in the WT liver cancer cell line HepG2. These changes were observed at the mRNA and protein levels. Similar results were found in WT HepG2 cells treated with the DGKθ inhibitor, R59949. However, the DGKθ agonist, GW4064, had an opposite effect at the mRNA level for FAS only, compared with the knockout of DGKθ and treatment with DGKθ inhibitor. No effects on HADHα were observed in any of the treatment groups (Fig. 3A-C).

The expression levels of the signaling proteins involved in the glucose metabolism pathway, mTOR and Akt, were also analyzed. The results showed that DGKθ-knockout affected neither the mRNA nor the protein levels of mTOR and Akt (Fig. 3D-F). The effect of DGKθ-knockout on the levels of protein phosphorylation were then determined. No significant changes were observed in the total protein levels of mTOR and Akt, however, the phosphorylation levels of these proteins were significantly decreased in the DGKθ-knockout group (Fig. 3E and F). Treatment with the DGKθ inhibitor R59949 decreased the level of p-Akt, whereas the DGKθ agonist GW4064 significantly increased the level of p-Akt. Neither R59949 nor GW4064 treatment affected the level of p-mTOR.

Finally, the present study examined whether the DGKθ-knockout affected the expression levels of insulin resistance mediators, PKCε and IRS-1. The results showed no significant change in the expression levels of PKCε and IRS-1 by DGKθ knockout at the mRNA or protein levels. However, the level of p-PKCε (serine 729) was significantly increased, and the level of p-IRS-1 at tyrosine 632 (a stimulatory site for insulin signaling) was significantly decreased in the DGKθ-knockout group (Fig. 3G-I). In addition, the DGKθ inhibitor R59949 decreased the level of p-IRS-1, and the DGKθ agonist GW4064 significantly decreased the level of p-PKCε.

Discussion

In the present study, a DGKθ gene-knockout liver cancer cell line HepG2 was produced using CRISPR/Cas9 technology, which exhibited a marked increase in the accumulation of intracellular lipids. This cell line was then evaluated for the expression of genes associated with lipid and glucose metabolism, confirming that the established cell line offers potential for investigating NAFLD and its associated hepatic insulin resistance.

CRISPR/Cas9 is a next-generation targeted genome editing technology. Compared with ZFN technology or TALEN technology, it is easier to manipulate (15-17). In the present study, an efficient CRISPR/Cas9 system designed. Four pairs of sgRNA targeting the human DGKθ gene were first obtained with an indel frequency up to 46.6%. The donor vector, which carried the positive and negative selection markers, improved the selection efficiency. The DGKθ gene-knockout Liver cancer cell line was successfully generated by integrating the exogenous fragment into one allele, and deleting a 26-bp base on the other allele.

The results of the present study showed that the DGKθ-knockout liver cancer cell line HepG2 exhibited increased expression of all three of the lipid synthesis-related genes examined (FAS, PPARγ and SREBP-1c) and decreased the expression of the lipolysis-related gene, CPT1a. This may be the cause of the increased intracellular lipid content of this cell line. Of note, Cai *et al* reported that DGKθ gene-knockdown using short hairpin RNA led to a decrease in the expression of SREBP-1c; however, this was performed in human adrenocortical cells (18), which may have a lipid metabolism pathway

differing from that of human hepatocytes. The overexpression of FAS has been shown to promote not only lipogenesis but also the growth of breast cancer cells (19). This may explain why the DGθ-knockout Liver cancer cells exhibited an increased growth rate. The increased FAS and decreased CPT1a of the DGKθ-knockout liver cancer cells may also be caused by the increased activity of SREBP-1c in this cell line. SREBP-1c has been reported to activate the transcription of FAS (19) and downregulate lipolytic enzyme genes (20).

In a previous study, DGKθ was shown to modulate cellular DAG and PA, which further modulated DAG-sensitive proteins associated with hepatic insulin resistance, including PKCε (6), and PA-sensitive proteins, including mTOR and Akt, which are associated with glucose production (21-24). As expected, the results of the present study showed that the DGKθ-knockout HepG2 cells expressed an increased level of p-PKCε, possibly due to increased intracellular DAG, and a decreased level of p-IRS-1. These changes have been reported to be mediate insulin resistance (6,25). Consistent with a previous study on DGKθ silencing (24,26), the DGKθ-knockout HepG2 cells in the present study expressed lower levels of p-mTOR and p-AKT, which may have been caused by decreased PA. Based on the results from the present study, the roles of DGKθ in lipid accumulation, insulin resistance and glucose production are summarized in Fig. 4.

In conclusion, the present study successfully generated a DGKθ-knockout Liver cancer cell line using the CRISPR/Cas9 technique. This cell line provides a valuable tool for investigating the pathogenesis of, and developing treatments for, NAFLD and type 2 diabetes.

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