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.

Key words: diacylglycerol kinase 0, 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 1 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 isofrom 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 (PKCe) 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, DGKθ, 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-PKCe 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
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 and 100 µg/ml streptomycin at 37˚C, 5% CO₂.

Plasmid construction. The targeting regions for four pairs of single-guide RNA (sgRNA) are shown in Table I. According to a previously described method (12), the human U6 promoter 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.

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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 ~1.5x10⁵ HepG2 cells were then

| Primer | Sequence (5’-3’) |
|--------|------------------|
| hDGKθ sgRNA1 Forward | ACCGCCCTGCAGGAGGCCGACTGCGG |
| hDGKθ sgRNA1 Reverse | CCCGAGCGAGCCGACGCGAG |
| hDGKθ sgRNA2 Forward | ACCGGAGGGGGGGCGAAGGGCAGCCG |
| hDGKθ sgRNA2 Reverse | A AACCCGTGGCCGGTGGCCCTGGG |
| hDGKθ sgRNA3 Forward | ACCGACAGCCAACTCCGAGTCCGG |
| hDGKθ sgRNA3 Reverse | AAAAAGGGCTTGTGCTTGGT |
| hDGKθ sgRNA4 Forward | ACCGAAGCCATTCGGCTGTCAGG |
| hDGKθ sgRNA4 Reverse | AAAACGGGTAGGCGGAACTTGGG |
| hDGKθ sgRNA detection Forward | GCCCTACGACAGGAGAGAG |
| hDGKθ sgRNA detection Reverse | CAGGTCACAAAAACCCAAAGGT |

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

Table I. Primer sequences used for sgRNA synthesis and the detection of sgRNA biological activity.
co-transfected with 4 µg of pUC19/CMV-Cas9-U6-sgRNA 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 Beijing 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,
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 Li2CO3 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^ΔΔCq 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^(-ΔΔCq).
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...
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...
and SREBP-1c) and genes associated with lipolysis (CPT1a and HADHα) in the DGK0-knockout HepG2 cells at the mRNA and protein levels. The results indicated that DGK0 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 DGK0 inhibitor, R59949. However, the DGK0 agonist, GW4064, had an opposite effect at the mRNA level for FAS only, compared with the knockout of DGK0 and treatment with DGK0 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 DGK0-knockout affected neither the mRNA nor the protein levels of mTOR and Akt (Fig. 3D-F). The effect of DGK0-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 DGK0-knockout group (Fig. 3G-I). In addition, the DGK0 inhibitor R59949 decreased the level of p-IRS-1, and the DGK0 agonist GW4064 significantly decreased the level of p-PKCr.

Discussion

In the present study, a DGK0 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 DGK0 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 DGK0 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 DGK0-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 DGK0 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 DGKθ-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 transfection 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-PKCe, possibly due to increased intracellular DAG, and a decreased level of p-IRS-1. These changes have been reported to be mediated 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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