Establishment of liver specific glucokinase gene knockout mice: a new animal model for screening anti-diabetic drugs[1]

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ABSTRACT

AIM: To characterize the liver-specific role of glucokinase in maintaining glucose homeostasis and to create an animal model for diabetes. **METHODS:** We performed hepatocyte-specific gene knockout of glucokinase in mice using Cre-loxP gene targeting strategy. First, two directly repeated loxP sequences were inserted to flank the exon 9 and exon 10 of glucokinase in genomic DNA. To achieve this, linearized targeting vector was electroporated into ES cells. Then G418- and Gancyclovir-double-resistant clones were picked and screened by PCR analysis and the positives identified by PCR were confirmed by Southern blot. A targeted clone was selected for microinjection into C57BL/6J blastocysts and implanted into pseudopregnant FVB recipient. Chimeric mice and their offspring were analyzed by Southern blot. Then by intercrossing the Alb-Cre transgenic mice with mice containing a conditional gk allele, we obtained mice with liver-specific glucokinase gene knockout. **RESULTS:** Among 161 double resistant clones 4 were positive to PCR and Southern blot and only one was used for further experiments. Eventually we generated the liver specific glucokinase knockout mice. These mice showed increased glucose level with age and at the age of 6 weeks fasting blood glucose level was significantly higher than control and they also displayed impaired glucose tolerance. **CONCLUSION:** Our studies indicate that hepatic glucokinase plays an important role in glucose homeostasis and its deficiencies contribute to the development of diabetes. The liver glucokinase knockout mouse is an ideal animal model for MODY2, and it also can be applied for screening anti-diabetic drugs.

INTRODUCTION

Diabetes, characterized by hyperglycemia due to defects in insulin production, insulin action, or both, is a common, serious worldwide health issue. There are two major types of diabetes, type 1 (insulin-dependent diabetes mellitus, IDDM), and type 2 (non-insulin-dependent diabetes mellitus, NIDDM). Type 1 diabetes results from insulin deficiency caused by autoimmune destruction of pancreatic β cells, and generally develops in the young. Type 2 diabetes is a multifactorial disease, depending on a complex interaction between many genetic and environment factors, and generally develops in adults[1]. However, among diabetes, a minority patients result from a single gene mutation that leads to either β-cell dysfunction or, less frequently in-

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sulin resistance. Maturity-onset diabetes of the young (MODY) is a monogenic subtype of diabetes with autosomal dominant inheritance, which makes it an attractive model for studying the genetics of diabetes.

In human, glucokinase (GK) gene mutations cause MODY2 and more than 80 different mutations have been identified to date. Glucokinase, primarily expressed in pancreatic β-cells and hepatocytes, phosphorylates glucose to glucose-6-phosphate which is a rate-limiting step in glucose metabolism, and plays an essential role in maintaining blood glucose homeostasis[2,3-8]. In pancreatic β-cells, glucose metabolism generates signals for insulin secretion and in liver, glucokinase is thought to determine both glucose uptake and glycogen synthesis[9]. Mutant glucokinase protein shows impairment in the enzymatic activity which leads to an increase in the extracellular glucose threshold to trigger insulin secretion, a right shift in the dose-response curve of glucose stimulated insulin release and a decreased accumulation of hepatic glycogen, and then the hyperglycemia of MODY2[10-12].

We believe that the development of animal models is crucial to ultimately find cures for diseases, especially for complex diseases such as type 2 diabetes. A large number of models for type 2 diabetes have been established such as Zucker-Diabetic-Fatty (ZDF) rat[13], Otsuka-Long-Evans-Tokushima -Fatty (OLETF) rat[14], KK mouse[15], db/db mouse[16], ob/ob mouse, New Zealand obese (NZO) mouse[17], Tsumura-Suzuki obese diabetic (TSOD) mouse[18], TallyHo (TH) [19] mouse, and Goto-Kakizaki rat[20]. However, except Goto-Kakizaki rat, all of these animal models are obesity and obesity is essential for the onset of type 2 diabetes in these animals, so they can not explain the pathogenesis of non-obese type 2 diabetes. Even the Goto-Kakizaki rat has been widely used as a good animal model for non-obese type 2 diabetes, pathology of this disease still has not been clearly resolved for it can not be explained by single etiology. Another kind of animal models used for type 2 diabetes is by chemical-induced islets injury and diet-induced obesity[21,22]. These models are either not consistent with the pathogenesis of human disease or not suitable for the non-obese diabetes. Furthermore they can not be controlled well and may result in different levels of pathological changes. As we know, animal model could provide an important insight into the human disease. So it is expected to create a new animal model to enrich model library for type 2 diabetes.

Recently the overexpression of genes as transgenic and the disruption of genes as knockout approaches have provided a powerful tool to study individual gene contributions to the pathogenesis of diseases *in vivo*. So gene targeting into mammalian genomes by means of homologous recombination is a useful tool for characterizing gene function and generating animal models. Except conventional loss-of-function gene disruption, the use of Cre-loxP system allows production of tissue- or time-specific gene knockout animal[23]. In these applications, a Cre transgenic mouse in a tissue-specific or inducible expression manner using cell type-restricted or inducible promoters is created[24]. Through crossbred, specific deletion of DNA flanked by loxP occurs only in those tissues that expressed Cre recombinase or in expected phase when added inducing substance[25,26].

In this study we used Cre-loxP system to create an animal model which was a heterozygous knockout of glucokinase in liver. This knockout mouse had some characteristic features of type 2 diabetes, such as spontaneous hyperglycemia, glucose intolerance, but not obesity. Therefore this mouse not only was an ideal model for MODY2 but also a potential model for type 2 diabetes without obesity. And it could be used to further evaluate the function of glucokinase in hepatocytes.

MATERIALS AND METHODS

Materials Targeting Vector, pGK/BOB was kindly provided by Dr Magnuson (Vanderbilt University School of Medicine, USA). ES cell line was from 129/Sv strain. Restriction endonucleases, DMEM and fetal calf serum (ES cell grade) *etc* were obtained from Invitrogen. DIG-High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche. LIF and G418 were from Sigma. Gancyclovir was provided by Syntex. *Taq* DNA polymerase, PCR primer synthesis, DNA sequencing were served by Shanghai Bioasia (China). All other chemicals were of analytical grade.

Gene targeting, blastocyst injections and production of GK floxed mice ES cells were cultured on mouse embryo fibroblasts feeder layers in DMEM supplemented with *L*-glutamine 2 mmol/L, non-essential amino acid 0.1 mmol/L, β-mercaptoethanol 0.1 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, 15 % heat-inactivated FCS and LIF 10^6 U/L under a humidified condition of 5 % CO_2/95 % air at 37 ℃. The targeting vector (50 μg), linearized with *Not*I and then purified using phenol/$CHCl_3$ method, was

electroporated into 2×10^7 ES cells. After 7 d, clones that are resistant to both G418 and gancyclovir were picked and screened by PCR. Positive clones identified by PCR were further confirmed by Southern blot analysis. From them, a single targeted ES cell clone was selected for microinjection into C57BL/6J blastocysts and implanted into pseudopregnant FVB female recipient. Chimeric mice were mated with C57BL/6J and their offspring were analyzed for the lox-type allele (gk^{lox}), which underwent the expected homologous recombination, by Southern blot of tail DNA. After first generation gk^{lox} mice were maintained as inbred lines[27].

PCR analysis ES cells genomic DNA was prepared by the conventional method. Two different gk alleles (gk^w, gk^{lox}) were routinely characterized by PCR analysis. Primers (pNeo 5′-CCTACCGGTGGATGTGGAATGTGTG and $pGK_{2\alpha}$ 5′-ATCTGTTCCAATGTGGTGAGTGTGCC) located in positive-selection neomycin resistance gene (neo^r) and short homologous arm (Fig 1). PCR was carried out in 25 μL reactions containing 1×Buffer [KCl 10 mmol/L, $(NH_4)_2SO_4$ 8 mmol/L, Tris-HCl (pH 9.0) 10 mmol/L, 1 % NP-40], $MgCl_2$ 1.5 mmol/L, dNTPs 0.2 mmol/L, the primers 0.5 μmol/L, 2.5 units of *Taq* DNA polymerase and 1 μg of genomic DNA. PCR consisted of 10 min denaturation at 95 ºC followed by 35 cycles of 1 min denaturation at 94 ºC, 45 s annealing at 55 ºC and 30 s extension at 72 ºC. After a final extension at 72 ºC for 10 min, the final reaction (10 μL) was mixed with 1 μL of gel loading buffer, separated on a 2.0 % agarose gel and visualized by ethidium bromide staining. A 264 bp fragment was amplified from the gk^{lox} allele, and no products from the wild-type allele (gk^w).

Southern blot analysis PCR-positive clones were further confirmed by Southern blot. Genomic DNA of PCR-positive ES cells or mice tail was prepared by the conventional method. The probe was cloned with a PCR-amplified DNA fragment including mouse GK partial intron 8, exon 9, intron 9, and partial exon 10 (Fig 1) and then labeled with digoxigenin using DIG-High Prime DNA Labeling and Detection Starter Kit I. Genomic DNA was digested with *Bgl*II and *Xmn*I respectively overnight, subjected to electrophoresis on a 0.8 % agarose gel, and then transferred to a Hybond nylon membrane. Membranes were hybridized and visualized by a NBT/BCIP reagent detection system.

Use of Alb-Cre transgenic mice to generate liver GK knockout mice The heterozygous liver-specific knockout mice ($gk^{w/del}$) were generated from cross-breeding $gk^{w/lox}$ mice with Alb-Cre transgenic mice. In Alb-Cre transgenic mice, the Cre transgene was controlled by the enhancer/promoter of the rat albumin gene so that Cre recombinase was specially expressed in hepatocytes and shown to be efficient for performing liver-specific gene knockout using the Cre-loxP system.

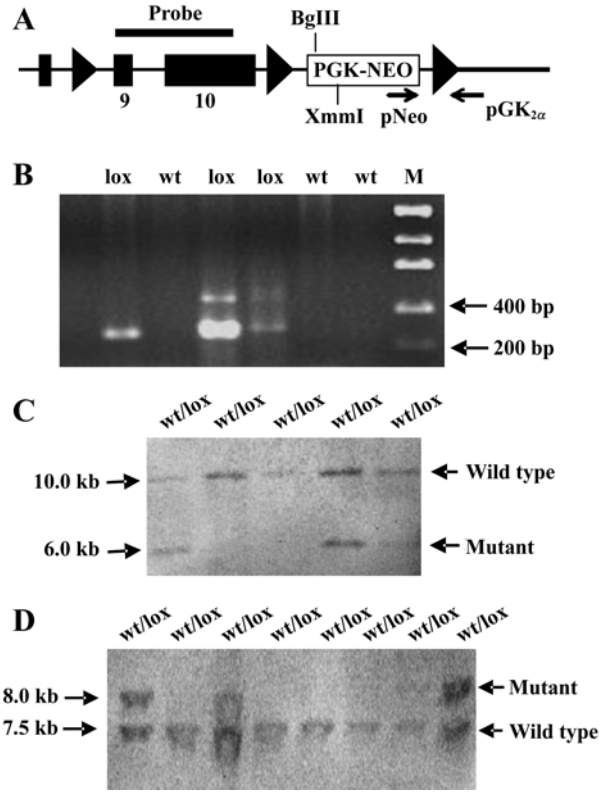

Fig 1. Results of PCR and Southern blot identification A) Gene targeting and the strategy for molecular identification. B) PCR analysis, A 264-bp DNA fragments corresponding to gk^{lox} allele was amplified using specific primers as shown in A. C, D) Southern blot analysis, genomic DNA were digested with *Bgl*II (C) or *Xmn*I (D) and then hybridization (probe location was shown in A).

Western blot analysis of liver glucokinase About 100 mg liver tissues were homogenized in 1 mL lysis buffer containing Tris-HCl (pH 8.0) 50 mmol/L, NaCl 150 mmol/L, 0.02 % NaN_3, 0.1 % SDS, EDTA 1 mmol/L, PMSF 100 mg/L, leupeptin 1 mg/L and 1 % NP-40. The mixture was incubated at 4 ºC for 30 min, and then removed the supernatant by centrifuging at 10 000×*g* for 10 min. Protein was quantified using Bradford method. Equal amount of protein with equal volume of 2×SDS loading buffer containing Tris-HCl (pH 6.8) 100 mmol/L, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol and DTT 200 mmol/L mixed and

boiled for 10 min. The supernatant was transferred after centrifuged at 10 000×*g* for 10 min. Proteins were separated by 10 % SDS-PAGE and electrotransferred to nitrocellulose membranes in a cooled chamber. After that the membranes were blocked with 5 % non-fat milk and then incubated overnight at 4 ºC with primary polyclonal antibodies (sc-7908, Santa Cruz Biotechnology, Inc CA), washed three times with TBST for 10 min each, incubated for 2 h with the alkaline phosphatase-conjugated secondary antibodies, and finally washed thoroughly with TBST. Protein bands were visualized by a NBT/BCIP reagent detection system. Band intensities were quantified by Gel Doc 2000 densitometer (Bio-RAD).

Assessment of plasma parameters, blood glucose, insulin, TG, TC, ALT, AST, and glucose tolerance test Plasma glucose, insulin, and some metabolic parameters were measured. From the age of two weeks, fasting blood glucose concentrations and glucose tolerance tests were measured from mice tail with a Roche blood glucose monitor (Glucotrend 2). Serum insulin levels were determined by radioimmunoassay (RIA) using a human insulin RIA kit. To determine the effects of diminished hepatocyte glucokinase activity on the function of liver, plasma triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were experimentally detected.

Morphological analysis of the pancreas, liver and heart The pancreas, liver and heart were obtained from mice at the age of 6 weeks, fixed by formaldehyde, embedded in paraffin, and cut into sections (about 5 μm thick). Sections were stained with hematoxylin-eosin (HE) and visualized using a microscope. At least 10 fields were randomly selected and analyzed.

Statistical analysis Values were shown as mean± SD and tested by one-way ANOVA. $P<0.05$ was considered to be statistical significance.

RESULTS

Generation of conditional GK mice We picked 161 ES cell clones that were G418- and gancyclovir-double-resistance and then successfully isolated 4 ES cell clones with the expected homologous recombination identified by PCR and Southern blot (Fig 2). This conditional allele (gk^{lox}) contained two loxP flanking exons 9 and 10, and the third loxP flanking neo gene. For PCR analysis, the gk^{lox} was amplified for a 264 bp

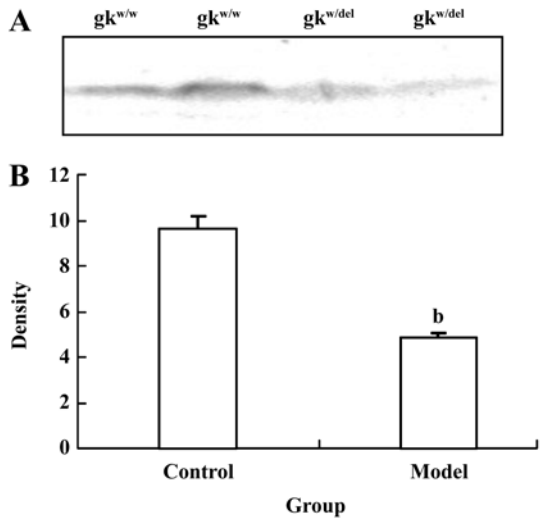

Fig 2. Comparison of Protein Levels of Liver Glucokinase Using Western Blot Analysis. A) Representative Western blot of glucokinase. B) Quantification of immunoblots of glucokinase in liver ($^bP<0.05$ *vs* control, $n=4$). Control: $gk^{w/w}$; model: $gk^{w/del}$.

fragment. In Southern blot analysis, we detected the 10.0 kb *Bgl*II fragment from the gk^w and 6.0 kb from gk^{lox}, and for *Xmn*I 7.5 kb fragment from gk^w and 8.0 kb from gk^{lox}. Then a targeted ES cells whose genotype was $gk^{w/lox}$ was used to create mice. Chimeric and germline-transmitted heterozygous mice were characterized by Southern blot of tail genomic DNA as described above. The offspring were normal at birth and no abnormalities were observed through adulthood.

Generation of liver-specific GK knockout mice To determine the role of hepatic glucokinase in the blood glucose homeostasis, $GK^{w/lox}$ mice were crossbred with Alb-Cre transgenic mice to give rise heterozygous liver-specific GK knockout mice ($GK^{w/del}$). These heterozygous mutant pups were normal in size, appearance and body weight at birth, and showed an increase with age.

Western blot analysis To further assess the phenotype of hepatocytes, Western blot analysis of protein from liver was performed. The results showed that the expression of glucokinase in liver was indeed decreased and the protein levels were twice as low as the control (Fig 2). So the mice we got were heterozygous and glucokinase alleles in liver were partially deleted.

Metabolic parameters from $GK^{w/del}$ From the age of 2 weeks, fasting blood glucose levels were measured. $GK^{w/del}$ showed an increased glucose level in an age-dependent manner (3.8±0.8 mmol/L in 2 weeks, 7.5±0.4 mmol/L in 4 weeks and 8.9±0.9 mmol/L in 6 weeks, Fig 3) and at the age of 6 weeks glucose

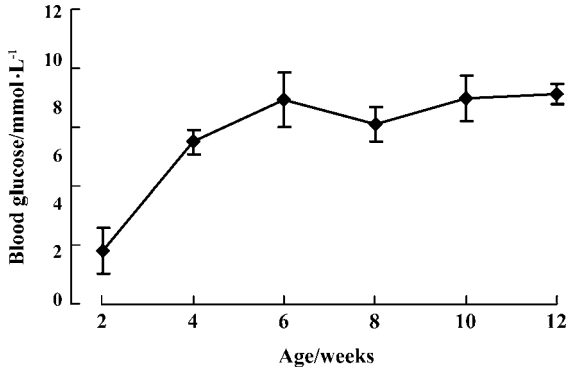

Fig 3. Blood glucose levels of model mice at the age of different weeks. *n*=6. Mean±SD.

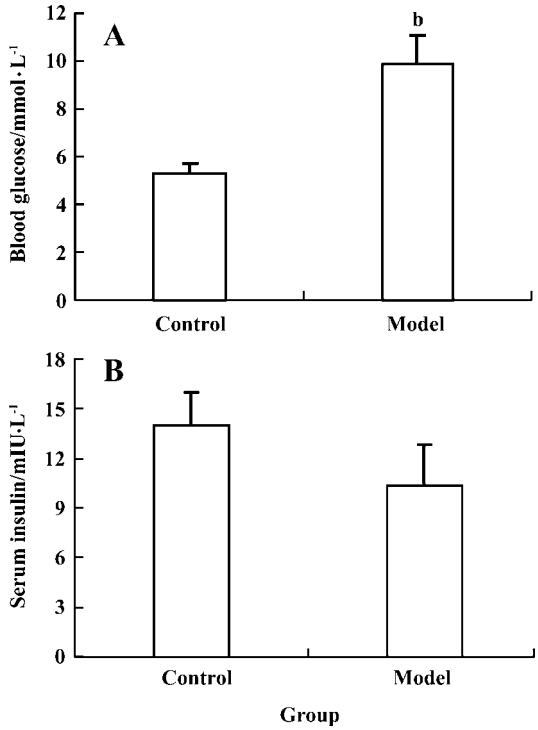

Fig 4. Fasting blood glucose concentrations (A) and serum insulin concentrations (B) of mice at 6 weeks of age. *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control.

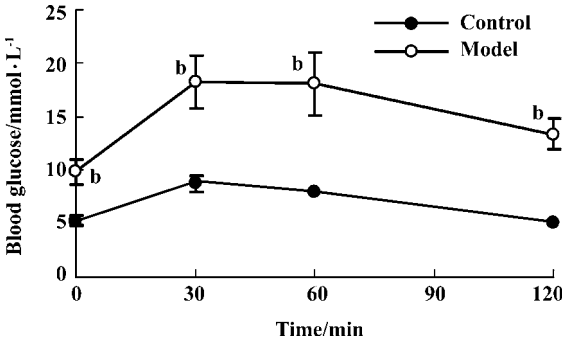

Fig 5. Glucose tolerance tests in control and model mice at 6 weeks of age. *n*=6. Mean±SD. $^{b}P<0.05$ *vs* control.

concentrations were significantly higher compared with controls (5.3±0.4 mmol/L, Fig 4) and they also displayed glucose intolerance (Fig 5). The mean serum insulin concentrations seemed to incline to decrease, but did not display significance (Fig 4) which indicated that insulin secretion of pancreas was normal at the age of 6 weeks. Plasma TG, TC, ALT, and AST showed no difference with controls, and the function of liver in these mutant mice did not alter (Tab 1). Furthermore, there was no obvious difference in the HE-sections of pancreas, liver and heart between the model and control groups (data not shown).

Tab 1. Several plasma metabolic parameters from model and control mice at 6 weeks of age. *n*=3. Mean±SD.

Group	TG/mmol·L^{-1}	TC/mmol·L^{-1}	ALT/U·L^{-1}	AST/U·L^{-1}
Control	0.79±0.15	3.00±0.21	29±8	84±13
Model	0.84±0.19	3.3±0.4	35±3	87±13

DISCUSSION

Glucokinase (also called hexokinase IV), encoded by a single gene in rodents, which catalyses glucose phosphorylation in hepatocytes and pancreatic β cells, is a key component of the glucose sensing machinery in mammals and plays a fundamental role in blood glucose homeostasis. According to experimental data, GK gene mutants are associated with MODY2 and mutations in β cell isoform cause impairment in insulin secretion. Furthermore liver is another major organ to maintain glucose homeostasis, so our concern is what effects would have when mutations appeared in hepatic GK isoform and the contributions of it in the pathogenesis of type 2 diabetes.

To investigate the liver-specific roles of GK in glucose homeostasis we used Cre-loxP gene targeting strategy to generate a new animal model for MODY2, in which glucokinase was selectively knockout in liver without affecting expression in pancreatic β cells[28,29].

Type 2 diabetes models mentioned above provided novel insights into the pathophysiology of obese-related diabetes, and these animals suggested an idea that combination of defects in insulin secretion and insulin action can lead to diabetes. However these polygenic models can not provide a single factor in the develop-

ment of diabetes, so knockout models with monogenic impairment or with tissue-specific alterations in glucose metabolism can dissect the individual contributor in the pathogenesis of diseases. As we know, high levels of blood glucose stimulate glucose uptake and glycogen synthesis through enhancing glucose phosphorylated by glucokinase in hepatocytes. Therefore decreased glucokinase activity can cause impaired glucose usage, especially after meal. From analysis we indicated that hepatic glucokinase, through either direct or indirect, played an important role in glucose homeostasis.

Taken together, liver-specific GK gene knockout mice we got through crossbreeding $GK^{w/lox}$ with Alb-Cre transgenic mice was an ideal mouse model for MODY2 and a potential model for non-obese type 2 diabetes. They showed normal at birth, and exhibited no sign of obesity throughout their lives. To characterize these mice, glucose levels was first measured. We found that the fasting blood glucose levels increased with age and the mice spontaneously developed hyperglycemia after 6 weeks of age. Meanwhile we performed intraperitoneal glucose tolerance test (IPGTT) to examine glucose tolerance of mice and they showed an impaired glucose tolerance. Moreover no evidence of autoimmunity of pancreatic islets exhibited in these knockout mice. Measurement of plasma insulin, triglyceride, cholesterol, ALT, and AST showed no difference compared with control at the age of 6 weeks.

Lastly, these studies allowed us to investigate directly the role of liver glucokinase in glucose homeostasis. Our animal model in which one allele of glucokinase gene in hepatocytes was disrupted clearly indicated that variations in the amount of hepatic glucokinase can significantly increase blood glucose concentration and cause hyperglycemia. In addition, abnormal glucose metabolism in liver also contributed to some forms of type 2 diabetes. So, this diabetic animal model can not only be an ideal animal model for MODY2 but also be applied as a non-obese type 2 diabetes for screening whether an anti-diabetic drug can restore normal glucose usage in liver.

In general, transgenic and knockout animals have become an irreplaceable tool for the study of individual genes in the complex pathways[30]. And the genotype of these genetically modified mice can be inherited and their phenotype is stable. So they can serve as models allowing of testing pharmaceutical and gene therapeutic approaches to human disease and will certainly become a major direction in the future.

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