Glucokinase Links Krüppel-Like Factor 6 to the Regulation of Hepatic Insulin Sensitivity in Nonalcoholic Fatty Liver Disease

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The polymorphism, *KLF6-IVS1-27A*, in the Krüppel-like factor 6 (KLF6) transcription factor gene enhances its splicing into antagonistic isoforms and is associated with delayed histological progression of nonalcoholic fatty liver disease (NAFLD). To explore a potential role for KLF6 in the development of insulin resistance, central to NAFLD pathogenesis, we genotyped *KLF6-IVS1-27* in healthy subjects and assayed fasting plasma glucose (FPG) and insulin sensitivities. Furthermore, we quantified messenger RNA (mRNA) expression of *KLF6* and glucokinase (*GCK*), as an important mediator of insulin sensitivity, in human livers and in liver tissues derived from a murine *Klf6* knockdown model (DeltaKlf6). *Klf6* overexpression studies in a mouse hepatocyte line were utilized to mechanistically link KLF6 with *Gck* promoter activity. *KLF6-IVS1-27G*wt (i.e., less KLF6 splicing) was associated with stepwise increases in FPG and insulin and reduced hepatic insulin sensitivity. KLF6 binds to the liver-specific *Gck* promoter and activates a GCK promoter-reporter, identifying GCK as a KLF6 direct transcriptional target. Accordingly, in DeltaKlf6 hepatocytes *Gck* expression was reduced and stable transfection of *Klf6* led to up-regulation of *Gck*. *GCK* and *KLF6* mRNAs correlate directly in human NAFLD tissues and immunohistochemistry studies confirm falling levels of both KLF6 and GCK in fat-laden hepatocytes. In contrast to full-length *KLF6*, splice variant *KLF6-SV1* increases in NAFLD hepatocytes and inversely correlates with glucokinase regulatory protein, which negatively regulates GCK activity. *Conclusion*: KLF6 regulation of GCK contributes to the development of hepatic insulin resistance. The *KLF6-IVS1-27A* polymorphism, which generates more KLF6-SV1, combats this, lowering hepatic insulin resistance and blood glucose. (HEPATOLOGY 2012;55:1083-1093)

The prevalence of nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, is rising dramatically.[1] The factors conferring a risk of progression to cirrhosis include advanced age, increased body mass index (BMI), and an elevated fasting plasma glucose (FPG) associated with insulin resistance (IR).[2] In addition to metabolic and anthropometric factors, genetic factors,

Abbreviations: GCK, glucokinase; GCKR, glucokinase regulatory protein; Hep(IR), hepatic insulin resistance; IR, insulin resistance; KLF6, Krüppel-like factor 6; KLF6-FL, KLF6 full length; KLF6-SV1, KLF6 splice variant 1; NAFLD, nonalcoholic fatty liver disease; NIDDM, noninsulin-dependent diabetes mellitus; SNP, single nucleotide polymorphism.

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such as polymorphisms in adiponutrin (PNPLA3),[3,4] are associated with the progression risk of NAFLD. Among these we have identified the Krüppel-like factor 6 (KLF6) genotype as a predictor of NAFLD histological stage.[5] KLF6 is a ubiquitously expressed transcription factor[6] established as an immediate early gene in activated hepatic stellate cells (HSCs) after liver injury[7,8] and as a tumor suppressor gene in a range of tissues including the liver.[9] A functional intron 1 single nucleotide polymorphism (SNP), *KLF6-IVS1-27G>A* (rs3750861),[10] creates a novel binding site for the splicing factor, SRp40,[11] and promotes alternative splicing of *KLF6* into antagonistic, truncated isoforms.[5] Among these isoforms, KLF6 splice variant 1 (KLF6-SV1) promotes cell growth while reducing differentiation in cancer cells and tumors *in vivo*. The roles of KLF6 and KLF-SV1 in nonmalignant cellular homeostasis, however, are unknown.[12]

To elucidate the mechanisms underlying KLF6's contribution to NAFLD, we explored the association of *KLF6-IVS1-27G>A* with metabolic factors predictive of disease progression. Here we report a significant and independent association of the *KLF6-IVS1-27A* allele with lower FPG and increased hepatic insulin sensitivity. Glucokinase (GCK) is a major determinant of hepatic glucose metabolism and is closely associated with hepatic glucose disposal and production in response to glucose and insulin.[13,14] Further, we suggest that there is a mechanistic link between KLF6 isoforms and the regulation of GCK and the glucokinase regulatory protein (GCKR).

Materials and Methods

Characterization of Human Subjects. A total of 1,276 healthy subjects were recruited from the RISC (Relationship between Insulin Sensitivity and Cardiovascular Disease) cohort, as approved by the local Ethics Committees.[15] Subjects with diabetes, hypertension, dyslipidemia, or a history of liver disease were excluded. Subjects underwent detailed anthropometric assessment and a 75 g oral glucose tolerance test (OGTT).[16] Subjects underwent a hyperinsulinemic-euglycemic clamp, as previously reported and standardized.[15] Insulin sensitivity was assessed as the mean glucose infusion rate over the last 40 minutes of the clamp, corrected for the mean plasma insulin levels achieved during the same period (M/I). Insulin secretion was calculated from the OGTT c-peptide concentrations by deconvolution.[17] An estimate of the endogenous insulin clearance was obtained from the ratio of the areas under the insulin secretion and the insulin concentration curves. Baseline fasting serum measurements included glucose, insulin, triglycerides, and gamma glutamyl transferase. Genotype/phenotype relationships were studied by linear trend analysis corrected for age, sex, and recruitment center. The *KLF6-IVS1-27G>A* SNP was genotyped by Kbiosciences, UK and a complete dataset was available on 1,249 individuals. In a subset of 367 individuals from nine centers, on the clamp day basal endogenous glucose production (EGP) was determined by a primed-constant tracer infusion (6,6-$^2H_2$-glucose). A hepatic insulin resistance index was calculated as (EGP $\times$ fasting insulin).

Mouse Model. Experiments were approved and conform to regulations of the Institutional Animal Care and Use Committee (IACUC). Mice with a floxed *Klf6* targeting vector (C57BL/6;129Sv, Genentech, San Francisco, CA)[18] were crossed with mice expressing Cre recombinase (Cre) under control of the albumin promoter (B6.Cg-Tg(Alb-cre)21Mgn/J; Jackson Laboratories, Bar Harbor, ME). After backcrossing, male offspring expressing Cre with two floxed KLF6 alleles were used as the experimental group ("DeltaKlf6"). Mice with two floxed alleles and no Cre expression were used as controls (wildtype, WT). Temperature, humidity, and light-dark cycle conditions were controlled, mice were allowed food and water *ad libitum*, and were euthanized at 3 months following an overnight fast. Caval blood was drawn and glucose quantified with a handheld device (LifeScan, Milpitas, CA). Liver tissue was conserved for histology, RNA, and protein isolation. Serum insulin and colorimetric glycogen were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Biovision, Mountain View, CA), whereas GCK was by IF-staining and

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Table 1. KLF6 Genotype NAFLD-Related Associations in Normal Individuals

	KLF6					
	AA	GA	GG	Linear Trend	AA vs. AG+GG	AA+AG vs. GG
1A						
Number	12	155	1082			
Age (years)	42.08±2.95	45.74±0.68	43.57±0.25	na	ns	0.006
Sex (M:F)*	6:6	71:84	481:599	na	ns	ns
BMI	23.72±1.29	25.97±0.33	25.53±0.12	ns	ns	ns
Fat mass (kg/m^2)	15.43±2.37	22.12±0.75	20.94±0.27	ns	0.023	ns
Glucose (mmol/l)	4.79±0.20	5.01±0.05	5.08±0.02	0.003	ns	0.009
FPI (pmol/l)	20.21±3.47	31.36±1.51	32.86±0.68	0.022	ns (0.050)	ns
HOMA-IR	0.95±0.14	1.37±0.07	1.36±0.03	ns	ns	ns
OGIS	486.1±19.7	442.8±5.11	441.5±2.04	0.042	ns	ns
M/I μmol min^{-1} (kg fat-free mass)$^{-1}$ nmol^{-1}	159.8±25.6	143.5±5.48	137.7±2.21	ns	ns	ns
icl_OGTT (l/min/m^2)	2.20±0.29	1.65±0.65	1.62±0.0.56	ns (0.051) KW	0.015	ns
TG (mmol/l)	79.00±7.92	110.05±6.09	107.40±2.23	ns	ns	ns
WC (cm)	82.41±4.76	87.70±1.01	86.58±0.38	ns	ns	Ns
1B						
Number	5	38	327			
Age (years)	36.40±2.65	45.76±1.49	42.75±8.08	na	ns	ns
Sex (M:F)*	5:0	17:21	163:164	na	ns	ns
BMI	25.04±1.67	27.00±0.62	26.01±0.23	na	ns	ns
Fat_mass (kg/m^2)	14.14±3.38	23.26±1.47	21.51±0.51	na	ns	ns
Glucose (mmol/l)	4.72±0.14	4.93±0.08	4.99±0.03	0.027	ns	ns
FPI (pmol/l)	18.54±3.33	26.50±2.52	30.98±1.23	0.005	ns	0.015
EGP	17.06±2.34	16.33±0.79	16.36±0.26	ns	ns	ns
Hepatic insulin resistance	343.46±101.9	422.30±44.3	496.29±19.1	0.022	ns	0.029

Table 1A includes variables classed by genotype for the cohort of 1249 individuals, while Table 1B presents subset data for those in which endogenous glucose production (EGP) was measured. Nonparametric comparisons for quantitative variables was by Mann-Whitney testing. Linear trend analyses were corrected for differences in age, sex, center, and BMI, as were combined group comparisons using the General Linear Model (GLM).

BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; OGIS, oral glucose insulin sensitivity index; M/I, whole-body insulin sensitivity measured by hyperinsulinemic clamp; FPI, Fasting Plasma Insulin; TG, triglycerides; WC, waist circumference; GCR_b, Basal glucose clearance (production/concentration); icl_OGTT, endogenous prehepatic clearance during OGTT. ns: not significant; na: not appropriate; KW, Kruskal Wallis test.

western blot with an anti-GCK antibody (H-88) (Santa Cruz Biotechnology, Santa Cruz, CA).

Supporting Methods. Supporting methods describing stable Klf6 expression in mouse hepatocytes, liver GCK promoter characterization, real-time polymerase chain reaction (PCR) analyses, studies on NAFLD liver tissues, and statistical analyses are detailed as online Supporting information.

Results

KLF6-IVS1-27G>A *Is Associated with Lower Fasting Plasma Glucose and Fasting Plasma Insulin.* We first sought to expand our previous study on the *KLF6-IVS1-27G>A* SNP in NAFLD[5] in an independent cohort. In normal subjects there was a highly significant association between the *KLF6* genotype and fasting blood glucose (FPG) based on linear trend analysis of the genotype groups (AA, AG, and GG) (Table 1A). Additionally, fat mass was lower in the *KLF6-IVS1-27G>A* group by univariate analysis (AA versus AG+GG). Importantly, the association between FPG and KLF6 genotype was significant even when corrected for fat mass or BMI, as well as age, sex, and recruitment center. Trends in fasting plasma insulin (FPI) levels were also significant and paralleled those of FPG.

KLF6-IVS1-27G>A *Is Associated with Enhanced Insulin Sensitivity and Insulin Clearance.* We next sought to elucidate the mechanisms underlying the protective effect of *KLF6-IVS1-27G>A* on glucose homeostasis. Insulin sensitivity assessed by the hyperinsulinemic clamp (M/I), a measure primarily of extrahepatic glucose disposal in euglycemic conditions, was not significantly different in the three genotypes. Because hepatic glucose disposal is markedly dependent on the route of glucose delivery and its concentration in the portal vein,[19] the OGTT, and insulin sensitivity based on the OGTT data (OGIS),[16] provides greater insight into hepatic insulin sensitivity. The glucose profile during the OGTT was identical in all three KLF6 genotype groups (Fig. 1A). Remarkably, however, the initial insulin plasma level was significantly lower in the *KLF6-IVS1-27AA* group (30-min; $P = 0.006$ relative to GG; Fig. 1B) and there was a trend toward lower insulin levels at later timepoints

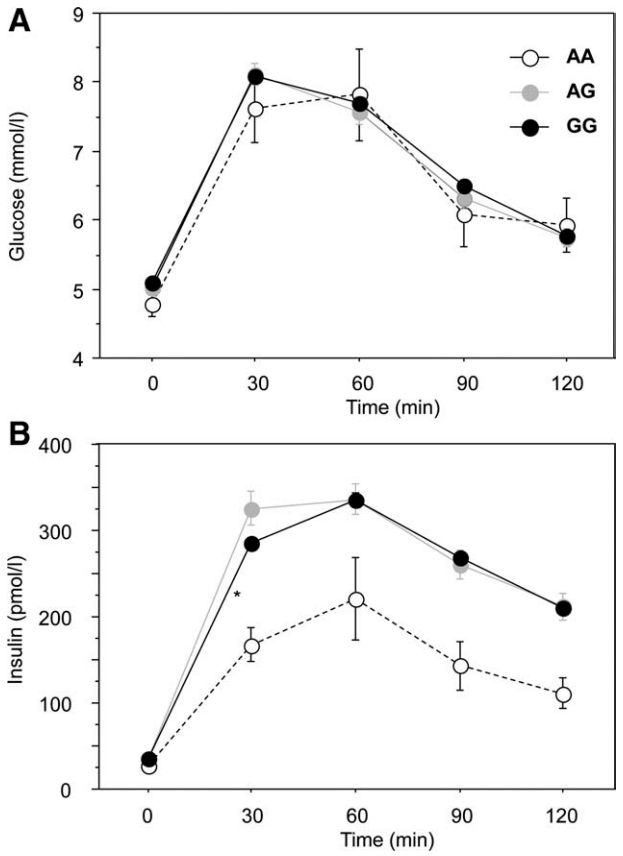

Fig. 1. *KLF6_IVS1-27AA* individuals have a normal glucose profile during the OGTT, despite lower plasma insulin levels. The profile of plasma glucose levels during the OGTT was unchanged between the three KLF6_IVS1-27G>A genotype groups, depicted as homozygous variant (AA), heterozygous variant (AG), and wildtype (GG) (A). In contrast, the plasma insulin levels were lower, significantly so at 30 minutes, in the homozygous variant AA individuals (*$P < 0.05$) (B).

(n = 12; area under curve $P = 0.07$). Insulin secretion was the same across the genotype groups (data not shown), indicating that lower plasma insulin levels (Fig. 1B) for the *KLF6-IVS1-27AA* genotype were not due to insulin secretion. This suggests a greater hepatic clearance of insulin, resulting in a lower peripheral level. Endogenous "hepatic" clearance of insulin (icl-OGTT) has been estimated from insulin secretion and concentration during the OGTT; this parameter was significantly higher in *KLF6-IVS1-27AA* individuals (Table 1A).

Despite the reduced insulin concentration attributed to increased insulin clearance in *KLF6-IVS1-27AA* individuals, the glucose concentrations during the OGTT were not increased, suggesting increased insulin sensitivity. Although the hyperinsulinemic clamp data reflecting extrahepatic glucose disposal showed no significant differences in peripheral insulin sensitivity, insulin sensitivity estimated by OGIS during the OGTT was significantly associated with KLF6 genotype (Table 1A). The association of *KLF6* genotype with OGIS but not M/I is again suggestive of a difference in the hepatic insulin clearance and sensitivity, rather than peripheral reaction to insulin.

KLF6-IVS1-27G>A *Is Associated with Increased Hepatic Insulin Sensitivity.* To explore an association with hepatic insulin sensitivity, EGP was measured after an overnight fast by infusion of 6,6-$^2H_2$-glucose in a subgroup of 367 patients from nine centers. EGP was identical in the KLF6 genotype groups (Table 1B). However, when EGP was corrected for the insulin concentration (EGP × fasting insulin), which is a measure of hepatic insulin resistance, there was a stepwise change indicating increased insulin sensitivity associated with the *KLF6 IVS1-27<A* allele (*KLF6_AA* 343 ± 102; *KLF6_GA* 422 ± 44; *KLF6_GG* 494 ± 19; $P = 0.023$). Thus, *KLF6-IVS1-27AA* individuals have both increased hepatic insulin sensitivity and insulin clearance. The hepatic insulin resistance index was correlated with the insulin clearance estimate (Spearman rho −0.44, $P < 0.0001$).

Glucokinase Expression Is Reduced in DeltaKlf6 Mouse Livers. GCK is the rate-limiting enzyme in glycolysis and its expression in the liver is closely associated with hepatic glucose metabolism in response to glucose and insulin.[20,21] An hepatic GCK promoter polymorphism is associated with altered hepatic insulin resistance and underscores the potential role of GCK as a KLF6-regulated target.[22] Moreover, GCK activity is closely linked to the expression of GCKR, which determines GCK activity, subcellular location, and GCK protein stability.[23]

To explore the role of KLF6 in the regulation of GCK, we generated mice with reduced expression of *Klf6* (DeltaKlf6) in hepatocytes by crossing *Klf6* floxed mice[18] with animals expressing albumin-Cre. Cre expression was gradually increased during the first 6 weeks and tissues were harvested at 11-12 weeks of age. Immunohistochemistry confirmed a clear reduction in hepatocyte expression of Klf6 (Fig. 2). There were no clear phenotypic differences (hematoxylin and eosin, Fig. 2), although there was a trend toward higher FBG, HOMA-IR, and triglyceride levels (Supporting Table 3). In whole liver lysates, *Klf6* messenger RNA (mRNA) was not in fact reduced in the DeltaKlf6 mice (Fig. 3A) and this was attributed to the reduction in hepatocytes being masked by a relative increase in KLF6 protein in hepatic perisinusoidal cells, as evident from immunohistochemistry of liver sections (Fig. 2) and as reported for other genes targeted in the albumin-Cre model.[24,25] In contrast, in hepatocytes isolated from DeltaKlf6 mice, Klf6 was

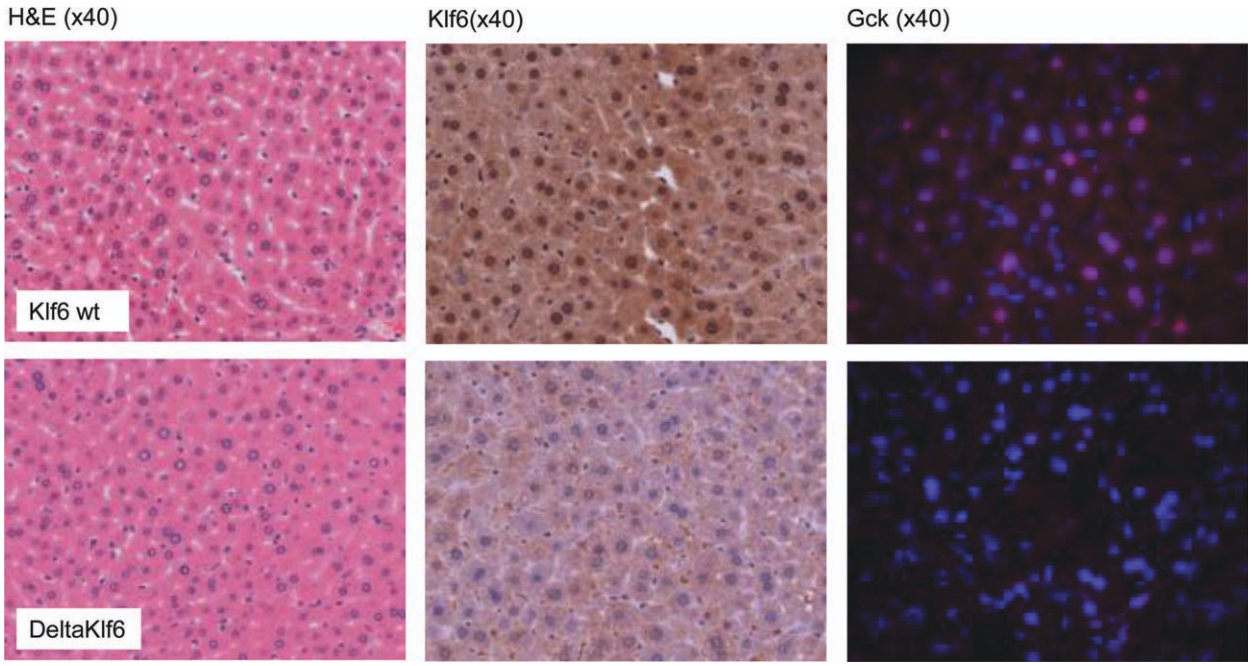

Fig. 2. Reduced Klf6 and glucokinase expression in vivo DeltaKlf6 mice. Hematoxylin and eosin (H&E) stains for WT and DeltaKlf6 mice were similar with no apparent phenotypic differences at 3 months of age. KLF6 IHC revealed a decreased expression of KLF6 within hepatocytes (4.1-fold fewer positive nuclei by manual counting; $P = 0.002$) rather than nonparenchymal cells. GCK immunofluorescent staining (1,300 ms exposure time, DAPI blue, GCK red) confirmed a relative abundance (1.92-fold by integrated density quantification, $P = 0.038$) of hepatocyte Gck expression in WT versus DeltaKlf6 mice.

markedly reduced at mRNA and protein levels (Fig. 3D,F). Both *in vivo* and in hepatocytes *in vitro*, *Gck* mRNA and protein were markedly down-regulated in the DeltaKlf6 mice relative to WT littermates (Figs. 2, 3D,F). Notably, in whole tissues there were no significant changes in the expression of other transcription factors that regulate *Gck*, including *Hnf4a, Hnf6*, or *Usf1*, or in hexokinase 2, which is expressed at low levels in hepatocytes[26] (Fig. 3B). In isolated hepatocytes, the negative regulator *Gckr* was reduced

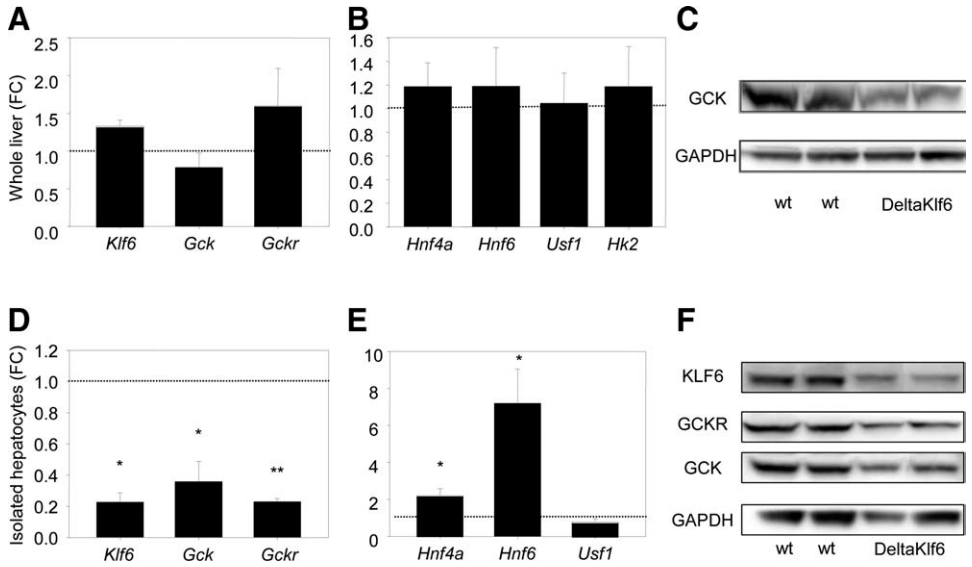

Fig. 3. Reduced *Klf6* and glucokinase expression in DeltaKlf6 mice. Although expression of *Klf6, Gck*, and *Gckr* mRNA was not significantly suppressed in whole liver tissues in DeltaKlf6 mice compared to WT littermates (n = 7) (A), levels were markedly suppressed in primary hepatocytes isolated from DeltaKlf6 mice (n = 3) (D). Data are presented as fold change (FC) relative to WT littermates. Known transcriptional activators of *Gck* were not reduced in the presence of reduced *Gck* in deltaKlf6 mice (B,E). Western blot of both whole tissues and isolated hepatocytes confirmed decreased Gck protein in association with reduced Klf6 (*$P < 0.05$; **$P < 0.005$).

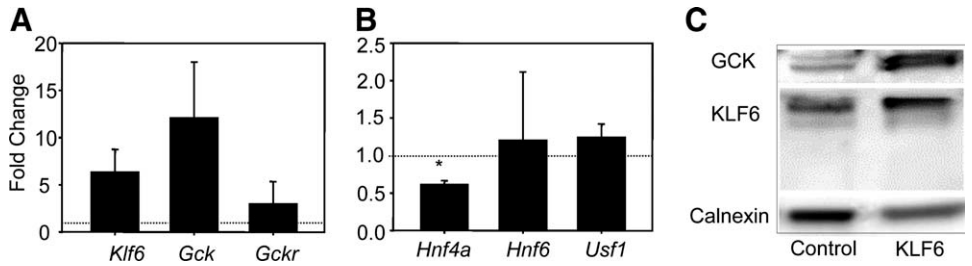

Fig. 4. Increased *Klf6* and glucokinase expression in vitro in *Klf6* transfected AML12 cells. Increased *Klf6* and *Gck* expression were present in the murine hepatoma cell line, AML12, after transfection with a plasmid vector expressing *Klf6* (A,C). Known transcriptional activators of *Gck* remained unchanged. Hnf4a mRNA was down-regulated indicating counterregulatory mechanisms (B) ($*P < 0.05$).

(Fig. 3D,F), whereas significant increases in *Hnf4α* and *Hnf6* mRNAs were observed (Fig. 3E). It is unlikely, therefore, that these factors contributed to the Gck decrease in the DeltaKlf6 mice.

Klf6 Expression in Mouse Hepatocytes Increases Gck. To further link Klf6 directly to Gck expression in hepatocytes, we examined the impact of stable Klf6 complementary DNA (cDNA) expression on *Gck* expression in the AML12 mouse hepatocyte cell line, which retains major hepatocyte characteristics.[27] In cells stably expressing full-length Klf6 cDNA, an increase in *Gck* mRNA and protein was evident (Fig. 4A,C). Consistent with the observations in DeltaKlf6 mice, *Hnf4α* expression was significantly decreased in these cells, most likely as part of a counterregulatory mechanism (Fig. 4B). Immunofluorescence confirmed hepatocyte accumulation of GCK protein (Supporting Fig. 1).

Gck Promoter Is a Direct Transcriptional Target of Klf6. To test for direct transcriptional activation of the GCK promoter by Klf6, AML12 cells were cotransfected with the Klf6 cDNA and a Gck-luc promoter reporter containing candidate KLF6 binding sites.[28] Cotransfection with Klf6 increased *Gck* promoter activity (Supporting Fig. 2A). Interaction of KLF6 with the endogenous *Gck* promoter at two defined sites containing putative KLF6 binding sites was confirmed (Supporting Fig. 2B) by chromosomal immunoprecipitation (ChIP).

KLF6 and GCK Expression Are Directly Correlated in NAFLD Livers. We analyzed *KLF6* and *GCK* expression in 28 precirrhotic NAFLD biopsy samples classed as having mild (grade 1) or severe (grades 2 and 3) hepatocyte steatosis. Although we have previously shown increased *KLF6* expression in NAFLD livers in association with inflammation and fibrosis,[5] the data presented here demonstrate a significant reduction of the full-length isoform in association with increased steatosis (Pearson correlation KLF6-FL and steatosis: -0.445; $P = 0.018$; 1.34 ± 0.126 versus 0.91 ± 0.122; t test $= 0.018$, Fig. 5C). This apparent discrepancy is clarified by immunohistochemistry (IHC) data which reveal differential KLF6 expression in distinct populations of liver cells. IHC studies in normal liver using KLF6 monoclonal 1A9 antibody (detecting all KLF6 isoforms) demonstrate predominantly nuclear KLF6 expression in hepatocytes, portal tract cells, and occasional sinusoidal cells (Fig. 6Ai). In more advanced disease (Fig. 6Ci), there is a dramatic increase in KLF6 expression, but this is notably in a mixed inflammatory cell population within portal tracts and in sinusoidal cells, rather than hepatocytes. Parallel IHC with a KLF6-SV1 isoform specific monoclonal antibody (2A2) shows the opposite, with no parallel increase in inflammatory cells, but with much more prominent expression in hepatocytes (Fig. 6Ci+ii). These data parallel isoform specific real-time PCR data in which, unlike KLF6-FL,

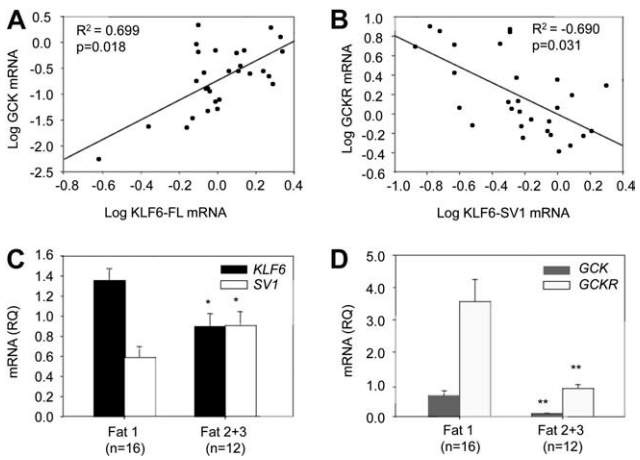

Fig. 5. *KLF6-FL* and *GCK* are reduced with advanced steatosis, whereas elevated *KLF6-SV1* is associated with suppressed *GCKR*. Expression of the KLF6 full-length isoform (*KLF6-FL*) correlated positively with *GCK* expression (A). In contrast, expression of *KLF6-SV1* was associated with a dramatic reduction in expression of *GCKR* (B). *KLF6-FL* expression was significantly suppressed with more advanced steatosis (grade 2+3), whereas *KLF6-SV1* was significantly up-regulated (C). Both *GCK* and *GCKR* were significantly down-regulated in patients with advanced steatosis (grade 2+3) (D) ($*P < 0.05$; $**P < 0.01$).

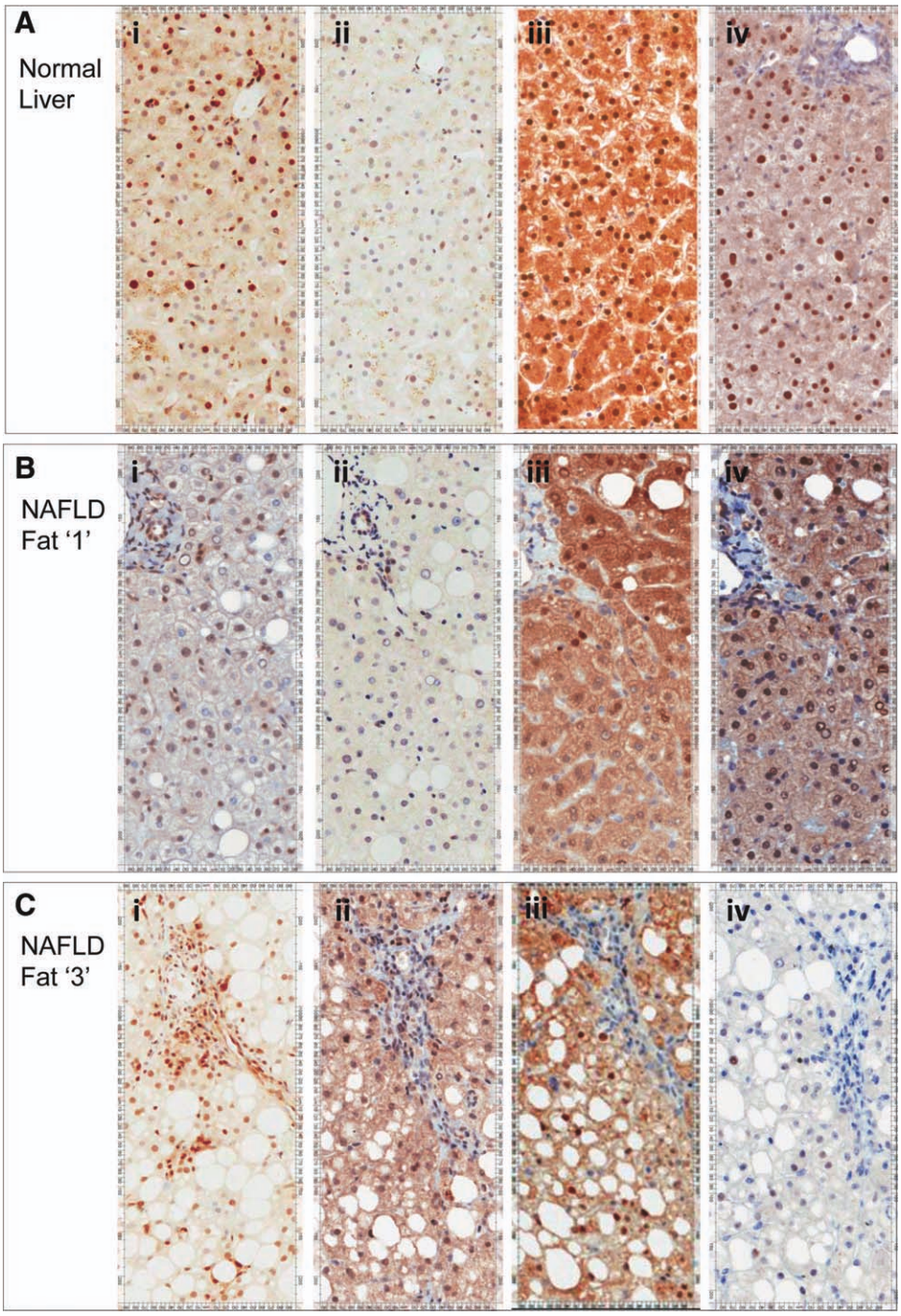

Fig. 6. *KLF6-FL* falls in hepatocytes with advanced steatosis, but increases in inflammatory and sinusoidal cells. KLF6 expression detected by the 1A9 monoclonal (all isoforms) is evident in hepatocytes and nonparenchymal cells in normal liver (Ai). A fall in hepatocytes in the presence of steatosis is contrary to an increase in inflammatory and sinusoidal cells (Bi+Ci). In contrast, KLF6-SV1 increases preferentially in hepatocytes in the presence of more advanced steatosis (Aii-Cii). These changes in representative sections parallel mRNA data in Fig. 5, as do changes in GCK and GCKR. Both GCK and GCKR are present in normal liver (Aiii+iv), with notable nuclear localization in this fasted state. The pattern is similar but less dramatic in the presence of fat grade 1 simple steatosis (Biii+iv). In the presence of more severe steatosis, expression of both is notably reduced (Ciii+iv). Quantification of nuclear expressions was performed using imaging software and these additional data are included as Supporting figures.

the KLF6-SV1 isoform is significantly increased with more advanced steatosis (Fig. 5C).

We explored the relationship between KLF6 and its target gene, GCK, in these NAFLD biopsies. Bivariate analysis of real-time PCR analyses of mRNA extracted from whole human liver confirmed a highly significant association between *KLF6* and *GCK* (Pearson correlation 0.687; $P < 0.0001$). By linear regression, corrected for other significantly associated variables (Supporting Table 4: center, fat and *GCKR*) the association between *GCK* and *KLF6* remained independently significant (Fig. 5A). IHC studies in normal liver confirm GCK expression in hepatocytes, which is notably sequestered in the nucleus in association with nuclear GCKR. Individuals with severe steatosis had decreased expression of KLF6 and GCK in hepatocytes (Fig. 6Aiii-Ciii). Although the role of KLF6-SV1 in NAFLD is presently unknown, our SNP association studies suggest a protective role in both chronic liver disease progression[5] and enhanced hepatic sensitivity

to insulin. It is interesting, therefore, that KLF6-SV1 is increasingly expressed in hepatocytes in the presence of steatosis, a marker not just of the severity of the insult, but possibly also of the livers ability to tolerate it. Further, there is a highly significant negative correlation between *KLF6-SV1* and *GCKR* at mRNA levels (Fig. 5B). This is apparent also at the protein level, as when KLF6-SV1 expression is evident in the presence of increased steatosis, GCKR is not detectable (Fig. 6Cii+iv).

Discussion

Although NAFLD prevalence is increasing in Western societies, established predictors of disease progression to advanced stages are scarce.[2] We previously demonstrated that the functional *KLF6-IVS1-27G>A* polymorphism correlates inversely with disease progression in NAFLD. Individuals with *KLF6-IVS1-27G>A* were less likely to have significant fibrosis when compared to those with the wildtype allele.[5] In the work presented here, we have functionally linked KLF6 to components of the hepatic insulin response, which is central to the progression of NAFLD.

The association between the KLF6 genotype groups and FPG is novel and striking. Although the *KLF6-IVS1-27A* allele was also associated with a lower FPI, the euglycemic clamp data discount peripheral insulin resistance as the cause of the variation with genotype. Together, the OGIS and OGTT-derived data and absence of a difference in pancreatic beta cell insulin secretion support increased hepatic clearance of insulin and increased hepatic insulin sensitivity in *KLF6-IVS1-27A* individuals. This was subsequently confirmed by assessing endogenous glucose production relative to fasting insulin levels.

IR is an established key feature of the metabolic syndrome and increased resistance associated with fasting hyperglycemia is believed to contribute to hepatic steatosis in obese individuals.[29] There is evidence, however, that insulin resistance is not essential for the development of steatosis. In hypobetalipoproteinemia, for example, fatty liver develops because of defective very low-density lipoprotein (VLDL) export in the absence of IR.[30] Furthermore, whereas a fatty liver genome-wide association study (GWAS) identified the rs738409 C>G SNP in PNPLA3, which leads to a missense mutation (I148M), and is associated with increased liver fat, it is not associated with IR.[3,31,32] Additional GWAS data in patients with fatty liver disease has further highlighted our incomplete understanding of the relationship between fatty liver disease and features of the metabolic syndrome. Although some of the steatosis-associated variations of candidate genes are associated with high triglycerides and plasma LDL-cholesterol, for example, the opposite is true of other variants.[33,34] Furthermore, the PP1R3B and GCKR polymorphisms are associated with a lower rather than a higher fasting glucose, in keeping with a relatively enhanced, rather than reduced, sensitivity to insulin. Therefore, whereas IR indisputably contributes to the progression of NAFLD to fibrosis and cirrhosis, its role in the development of steatosis is not as clear. Rather than being indicative of disease severity, in some individuals steatosis may instead be a biomarker of an enhanced ability to convert glucose to hepatic fat, conferring protection from an elevated blood glucose and reflecting enhanced insulin sensitivity.

Our own study focused on GCK, whose expression in the liver is closely associated with hepatic insulin sensitivity.[20,21] In conjunction with GCKR, it is a major determinant of insulin responsive hepatic glucose metabolism, catalyzing the production of glucose-6-phosphate.[23] Although the hepatic expression of GCK protein is reduced in cirrhosis attributed to both alcohol excess and primary biliary cirrhosis,[35,36] its contribution to NAFLD has not previously been characterized. GCK's vital role in determining blood glucose is underscored by the discoveries of over 600 hereditary mutations of GCK associated with glycemic disease.[37,38] Subjects with a single inactivating mutant allele have a mild form of diabetes, associated with elevated FPG.[39,40] Furthermore, recent data suggest a role for KLF6 in response to glucose stimulation.[41] GCK activity is closely linked to the abundance of its regulator, GCKR, and small changes in the molar ratio of GCK/GCKR protein markedly impact hepatic glucose metabolism.[42] In the fasting state GCK is sequestered in an inactive state in the nucleus, bound to GCKR. However, after a meal glucose and insulin act synergistically in causing rapid dissociation of GCK from GCKR and translocation to the cytoplasm.[43]

Our *in vivo* and *in vitro* murine data demonstrate that down-regulation of *Klf6* in hepatocytes is associated with reduced *Gck*, whereas *Klf6* overexpression increases *Gck*. ChIP and reporter studies confirm direct transactivation of the *Gck* promoter by *Klf6*, leading us to propose *Klf6* as an additional mediator of glucose homeostasis. The normal phenotype of DeltaKlf6 mice is consistent with published data from liver-specific *Gck* knockout mice as well as *Gckr* knockout mice, in which no differences in fasting glucose levels were detected.[44-46] Biopsy studies from patients with histologically scored NAFLD confirm a significant

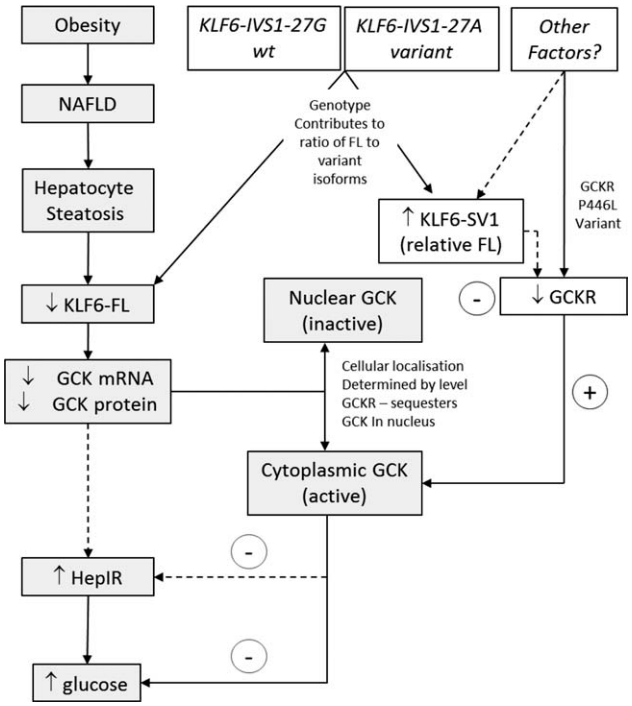

Fig. 7. Proposed mechanisms for *KLF6* regulation of hepatic glucose control. KLF6 is a ubiquitously expressed immediate early gene expressed in response to stressful stimuli. It is regulated by alternative splicing to dominant negative splice forms. The *KLF6-IVS1-27A* polymorphism favors alternative splicing. In the obesity-related metabolic syndrome, fat accumulates in hepatocytes. Although KLF6 expression increases in inflammatory and nonparenchymal cells in NASH, it falls in fat-laden hepatocytes. Consequently, expression of its transcriptional target GCK falls, leading to reduced GCK protein in hepatocytes, an increase in hepatic insulin resistance, and an elevated plasma glucose. Importantly, however, the activity of the GCK that is expressed is determined by its localization, being active in the cytoplasm, but inactive in the nucleus. GCKR is its negative regulator, responsible for its nuclear sequestration. A relative increase in KLF6-SV1 expression in steatotic hepatocytes is associated with a fall in GCKR, which we propose facilitates GCK activity, antagonizing the KLF6-FL (or WT genotype) association with elevated serum glucose and HepIR.

association between *KLF6-FL* and *GCK* mRNA expression in human tissues. Although the significant reductions of KLF6-FL and GCK in individuals with more advanced versus mild steatosis may simply be representative of more advanced disease, they may also represent effectors of the development of resistance of hepatocytes to insulin.

In human tissues, several alternative splice forms of *KLF6* have been identified, and the presence of the *KLF6-IVS1-27G>A* allele promotes their generation.[5] To date, *Klf6* splicing in the mouse has not been described, precluding our ability to study the effects of murine dominant negative splice variants on *Gck* or *Gckr* in an *in vivo* model. The mechanism underlying the enhanced hepatic insulin sensitivity in human individuals with the KLF6-SV1-promoting *KLF6-IVS1-27G>A* SNP is presently unknown. However, our expression data identifying a highly significant negative correlation between KLF6-SV1 and GCKR suggests that antagonism of GCKR, the negative regulator of GCK, is one potential mechanism. Here we are able to draw a link to the recently published data on SNPs in GCKR. The GCKR rs780094 GWAS identified SNP[34,47] was previously associated with higher triacylglycerol, reduced insulin levels, and a reduced risk of type 2 diabetes.[48,49] GCKR rs780094 is commonly inherited with a GCKR coding SNP, rs1260326 (Pro446Leu), which codes for a mutant and inactive form of GCKR.[50] The consequences include enhanced GCK activity and reduced FPG, as well as increased *de novo* lipogenesis attributed to enhanced production of malonyl coA, the substrate for fatty acid synthesis.[51] Similar to this phenotypically altered GCKR variant, we hypothesize that KLF6-SV1 contributes to the lowering of FPG as a result of increased GCK activity brought about by the antagonism of GCKR, as summarized in Fig. 7.

In conclusion, we propose KLF6 as an additional regulator of fasting plasma glucose and hepatic insulin sensitivity. Understanding the interactions between KLF6 and KLF6-SV1, as well as their roles in regulation of expression of GCK and GCKR, may help us to clarify the role(s) of NAFLD in the metabolic syndrome, as well as its progression to more advanced disease.

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