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Overexpression of Kruppel-Like Factor 7 Regulates Adipocytokine Gene Expressions in Human Adipocytes and Inhibits Glucose-Induced Insulin Secretion in Pancreatic β-Cell Line

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We have identified Kruppel-like factor 7 (*KLF7*) as a new candidate for conferring susceptibility to type 2 diabetes. To ascertain the possible involvement of *KLF7* in the pathogenesis of type 2 diabetes, we examined the functional roles of *KLF7* in various types of cells. In human adipocytes overexpressing *KLF7*, the expression of adiponectin and leptin was decreased compared with that in control cells, whereas expression of IL-6 was increased. In the insulin-secreting cell line (HIT-T15 cells), the expression and glucose-induced secretion of insulin were significantly suppressed in *KLF7*-overexpressed cells compared with control cells, accompanied by the reduction in the expression of glucose transporter 2, sulfonylurea receptor 1, Kir6.2, and pancreatic-duodenal homeobox factor 1. We also found that the overexpression of *KLF7* resulted in the decrease of hexokinase 2 expression in smooth muscle cells, and of glucose transporter 2 expression in the HepG2 cells. These results suggest that *KLF7* may contribute to the pathogenesis of type 2 diabetes through an impairment of insulin biosynthesis and secretion in pancreatic β-cells and a reduction of insulin sensitivity in peripheral tissues. Therefore, we suggest that *KLF7* plays an important role in the pathogenesis of type 2 diabetes, and may be a useful target for new drugs to aid in the prevention and treatment of this disease. (*Molecular Endocrinology* 20: 844–856, 2006)

TYPE 2 DIABETES IS one of the most common diseases, and its prevalence is progressively increasing worldwide, including Japan (1, 2). The pathogenesis of type 2 diabetes appears to be the consequence of insulin resistance in peripheral tissues combined with dysfunction of β-cells in pancreatic islets, although the precise mechanism is still not well understood (3–5). The Kruppel-like transcription factors (KLFs) represent a family of 15 different zinc finger proteins of the Cys2-His2 type and have been reported to regulate cell growth, proliferation, and differentiation (6–8). Some members of the KLF family, *KLF2* and *KLF15*, have been reported to regulate the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and glucose transporter 4 (GLUT4) in adipocytes and have been considered to contribute to the pathogenesis of type 2 diabetes (9, 10). We have recently identified *KLF7*, ubiquitous Kruppel-like factor, on chromosome 2q32 as a new susceptibility gene to type 2 diabetes by a large-scale case-control association study focusing on the genes encoding *KLF* families in Japanese individuals. We also demonstrated that *KLF7* was able to inhibit adipogenesis in 3T3-L1 cells (11). However, the precise mechanism by which *KLF7* contributes to the pathogenesis of type 2 diabetes has not yet been elucidated.

The *KLF7* gene is expressed in adipocytes and various human tissues, including pancreas, liver, and skeletal muscle, which are all considered to be key organs for the pathogenesis of type 2 diabetes. In the study reported here, we examined the effects of *KLF7* on the cellular functions of adipocytes, pancreatic β-cells, skeletal muscle cells, and hepatocytes, and provide evidence that *KLF7* contributes to the pathogenesis of type 2 diabetes by affecting adipogenesis and adipocytokine secretion in adipocytes and by inhibiting insulin expression and secretion in pancreatic β-cells.

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Abbreviations: aP2, Adipocyte protein aP2; C/EBP, CCAAT enhancer binding protein; CMV, cytomegalovirus; GLUT, glucose transporter; HPA, human preadipocyte; IBMX, 3-isobutyl-1-methylxanthine; KLF, Kruppel-like factor; m.o.i., multiplicity of infection; PDX1, pancreatic-duodenal homeobox factor 1; PPAR, peroxisomal proliferator-activated receptor; SUR1, sulfonylurea receptor 1.

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RESULTS

Expression Profile of KLF7 in Human Tissues and in Various Cell Lines

We first examined the expression profile of the *KLF7* gene in human tissues by RT-PCR using cDNA prepared from various human tissues and found that the

expression of *KLF7* was detectable in most human tissues examined, including pancreas, liver, skeletal muscle, and adipose (Fig. 1A). We also observed the expression of *KLF7* in cultured preadipocytes and matured adipocytes [3T3-L1; human preadipocyte (HPA)], insulin-secreting cell lines (HIT-T15, RIN-5F), a skeletal muscle cell line (L6), and HepG2 cells (Fig. 1B). In mouse 3T3-L1 cells, the expression of *KLF7* mRNA

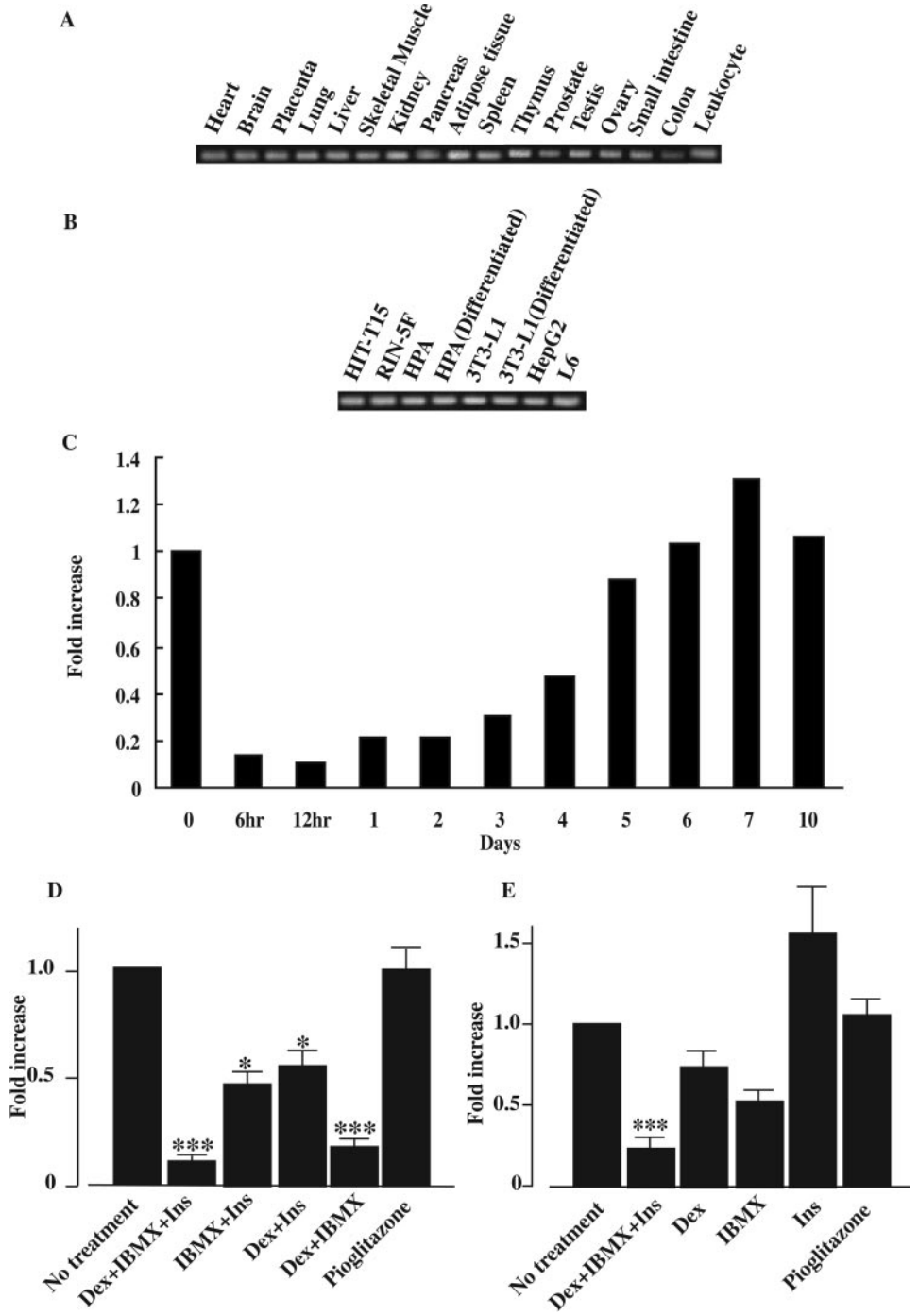

Fig. 1. Expression Profile of *KLF7* mRNA in Human Tissues and in Various Cell Lines

A, Expression of *KLF7* mRNA in various human tissues. B, Expression of *KLF7* mRNA in several cell lines. C, Expression of the *KLF7* gene during differentiation into adipocytes in 3T3-L1 cells. Quantitative real-time PCR was used to evaluate the amount of *KLF7* mRNA. Total RNA was extracted from 3T3-L1 at 0, 6 h, and 12 h, and 1, 2, 3, 4, 5, 6, 7, and 10 d after induction of differentiation. D, Effects of elimination of each component from differentiation cocktail on the expression of the *KLF7* gene. E, Effects of individual component of differentiation cocktail on the expression of the *KLF7* gene. Total RNA was extracted 6 h after the addition of each component. Dex, Dexamethasone; Ins, insulin.

was abundant in the cells before differentiation was induced, and markedly decreased at 6 h after induction of differentiation. After this period, the expression of *KLF7* continued to increase according to the degree of differentiation (Fig. 1C). The elimination of dexamethasone or 3-isobutyl-1-methylxanthine (IBMX) from the differentiation media could significantly reduce the inhibitory effects of differentiation cocktail on the reduction of *KLF7* expression, whereas the absence of insulin seemed not to affect the expression of the *KLF7* (Fig. 1D). As shown in Fig. 1E, dexamethasone and IBMX seemed to have an independent effect on the suppression of the *KLF7* expression, although the decrease of the *KLF7* in these cells was not statistically significant. Pioglitazone also did not have any effect on the expression of *KLF7* (Fig. 1E).

Effect of KLF7 Overexpression on Adipogenesis

To ascertain the possible role of *KLF7* in adipogenesis, the effect of *KLF7* overexpression on adipocyte differentiation was examined in human preadipocytes. The human preadipocytes were infected with adenovirus vectors encoding *KLF7* or LacZ (control) and introduced to differentiate into adipocytes as described in *Materials and Methods*. As shown in Fig. 2A, differentiation of the human adipocytes was remarkably inhibited in the cells overexpressing *KLF7* compared with control cells at 14 d after induction of differentiation. Quantification of the accumulated lipid droplets revealed that the amount of lipid droplets in the cells overexpressing *KLF7* was less than 0.5% of that in the control cells (Fig. 2B). The expression of PPAR-γ, CCAAT/enhancer binding protein (C/EBP)α, adipocyte protein aP2 (aP2), and adipsin was significantly reduced in *KLF7*-overexpressing cells compared with control cells, whereas those of C/EBPβ and C/EBPδ were not affected by the *KLF7* overexpression (Fig. 2C).

Expression of Adipocytokines in Differentiated Human Adipocytes

We next examined the effects of *KLF7* on the expression of several adipocytokine genes in differentiated human adipocytes. Five days after infection of differentiated adipocytes with adenovirus vectors encoding *KLF7* or LacZ, total RNA was extracted, and the mRNA expression of the genes encoding several adipocytokines in these cells was analyzed by quantitative real-time PCR. The mRNA expression of the IL-6 gene in *KLF7*-overexpressing human adipocytes was increased compared with that in control cells (Fig. 3A; 1.217 ± 0.241 and 14.206 ± 5.219 for LacZ and *KLF7*, respectively; $P < 0.05$), whereas the expression of adiponectin and leptin was significantly reduced in *KLF7*-overexpressing cells (adiponectin: 1.072 ± 0.112 and 0.407 ± 0.059 for LacZ and *KLF7*, respectively; $P < 0.01$; leptin: 1.158 ± 0.125 and 0.241 ± 0.012 for LacZ and *KLF7*, respectively; $P < 0.001$; Fig. 3A). The change in expression of resistin and type 1 plasminogen activator inhibitor was not statistically significant between *KLF7*-overexpressing cells and the control cells (Fig. 3A). The expression of PPAR-γ and aP2 was not different between *KLF7*-overexpressing and control adipocytes (Fig. 3A).

As shown in Fig. 3B, the excretion of IL-6 protein into culture media was significantly increased in the *KLF7*-overexpressing cells compared with the control cells (2.9-fold; $P < 0.01$ *vs.* LacZ; Fig. 3B), whereas that for adiponectin or leptin protein was decreased in *KLF7*-overexpressing cells (adiponectin, 43.6%; leptin, 21.2%; $P < 0.01$ *vs.* LacZ; Fig. 3B).

Effects of KLF7 on Insulin Expression and Secretion

To ascertain the possible role of *KLF7* in pancreatic β-cells, we next examined the role of *KLF7* in an insulin-secreting cell line (HIT-T15). HIT-T15 cells were infected with *KLF7*- or LacZ-encoding adenovirus vector for 1 h, and total RNA was prepared for quantitative real-time PCR 2 d after infection. As shown in Fig. 4A, the expression of the insulin gene was significantly reduced in the cells overexpressing *KLF7* in a dose-dependent manner [53.5 ± 4.2, 41.5 ± 5.8, 32.3 ± 6.3, 25.5 ± 4.1: percent of control in the cells infected with *KLF7* adenovirus at 1, 3, 10, and 30 multiplicity of infection (m.o.i.), respectively; $P < 0.001$]. The content of insulin protein was also significantly decreased in *KLF7*-overexpressing HIT-T15 cells (LacZ, 26.38 ng/mg protein; *KLF7*, 9.34 ng/mg protein; $P = 0.0005$; Fig. 4B).

To examine the capability of *KLF7* to directly inhibit the transcription of the insulin gene, the effect of *KLF7* on the transcriptional activity of the insulin gene promoter was examined. As shown in Fig. 4C, the transcriptional activity for the insulin gene promoter (-692 to $+25$) was remarkably suppressed by the overexpression of *KLF7* ($32.1 \pm 2.3\%$ of control; $P < 0.0001$). Subsequent analyses using various kinds of deletion mutant revealed that *KLF7* could inhibit the promoter activity of all constructs, whereas *KLF7* did not affect the activity of the cytomegalovirus (CMV) promoter.

We further evaluated the effects of *KLF7* on glucose-stimulated insulin secretion using the HIT-T15 cells infected with adenovirus vectors encoding *KLF7* or LacZ. Two days after infection, the level of glucose-stimulated insulin secreted into the culture media was determined as described in *Materials and Methods*. As shown in Fig. 5A, the insulin secretion from the cells overexpressing *KLF7* was significantly reduced compared with that of the control cells (LacZ: 7.910 ± 1.060, 10.095 ± 0.906, 11.946 ± 0.556, 14.990 ± 1.221; *KLF7*: 5.141 ± 1.330, 5.057 ± 0.643, 5.439 ± 1.313, 5.131 ± 0.609; nanograms/h·mg protein at 0, 2.7, 10, 16.7 mM, respectively). The response to glucose concentrations was also suppressed in *KLF7*

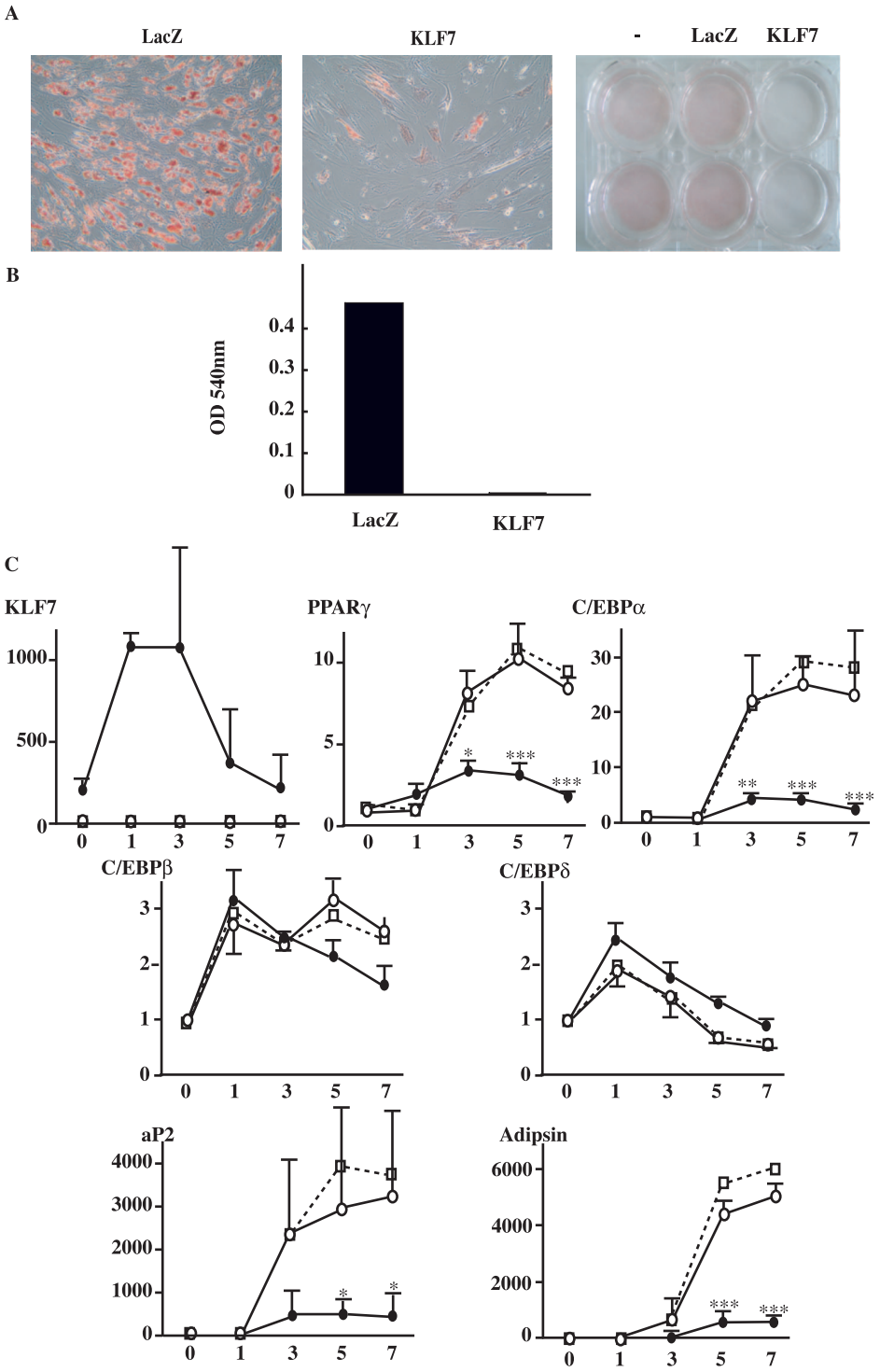

Fig. 2. Effects of *KLF7* on Adipogenesis

A, Effects of *KLF7* overexpression on adipogenesis in HPAs. Cells were infected with adeno-*KLF7* or -LacZ 48 h before the induction of differentiation, and Oil red O staining was performed on d 14 of differentiation. −, Cells without infection; LacZ, cells infected with adenovirus vectors encoding LacZ (control); *KLF7*, cells infected with adenovirus vectors encoding *KLF7*. B, Quantification of oil red O staining. Absorbance was measured at 540 nm. C, The expression of adipogenic markers in *KLF7*-overexpressing cells. Confluent 3T3-L1 cells were infected with adenovirus vectors 2 d before induction of differentiation. At the indicated days after induction of differentiation, total RNA was extracted from no infecting (□), adeno-*KLF7*-infecting (●), and adeno-LacZ-infecting (○) cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$, *vs.* control cells.

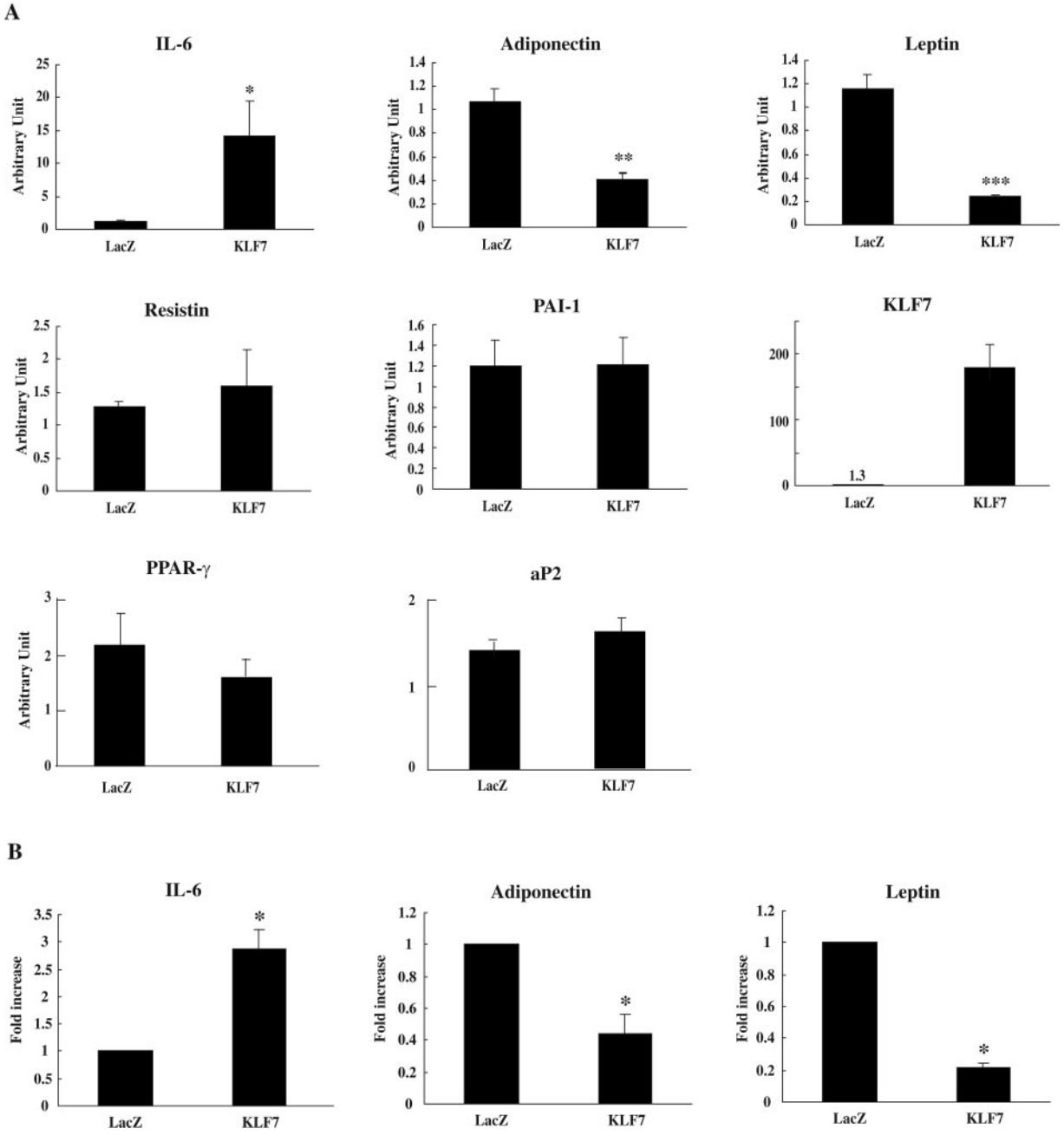

Fig. 3. Effects of *KLF7* on Adipocytokine Expressions

A, Effects of *KLF7* overexpression on adipocytokine gene expression in differentiated human adipocytes. Human adipocytes were infected with adenovirus vectors 14 d after induction of differentiation, and samples were obtained 5 d after adenovirus infection. Relative amounts of each gene are indicated as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ *vs.* control cells. B, Effects of *KLF7* overexpression on the protein secretion of IL-6, adiponectin, and leptin in human adipocytes. *, $P < 0.01$ *vs.* control cells (LacZ). PAI-1, Type 1 plasminogen activator inhibitor.

-overexpressing cells (Fig. 5B; LacZ: 1.384 ± 0.079, 1.510 ± 0.150 and 1.895 ± 0.170-fold increase for 2.7, 10, and 16.7 mM, respectively; *KLF7*: 0.984 ± 0.119, 1.058 ± 0.017, and 0.998 ± 0.147-fold increase for 2.7, 10, and 16.7 mM, respectively). In contrast, KCl-induced insulin secretion was not affected by the *KLF7* overexpression (Fig. 5, C and D).

Effects of KLF7 on the Expression of Genes Related to Glucose Metabolism in HIT-T15 Cells

To elucidate the possible mechanism for the suppression of insulin secretion by *KLF7*, we examined the effects of *KLF7* on the expression of genes related to glucose metabolism in HIT-T15. As shown in Fig. 6, the

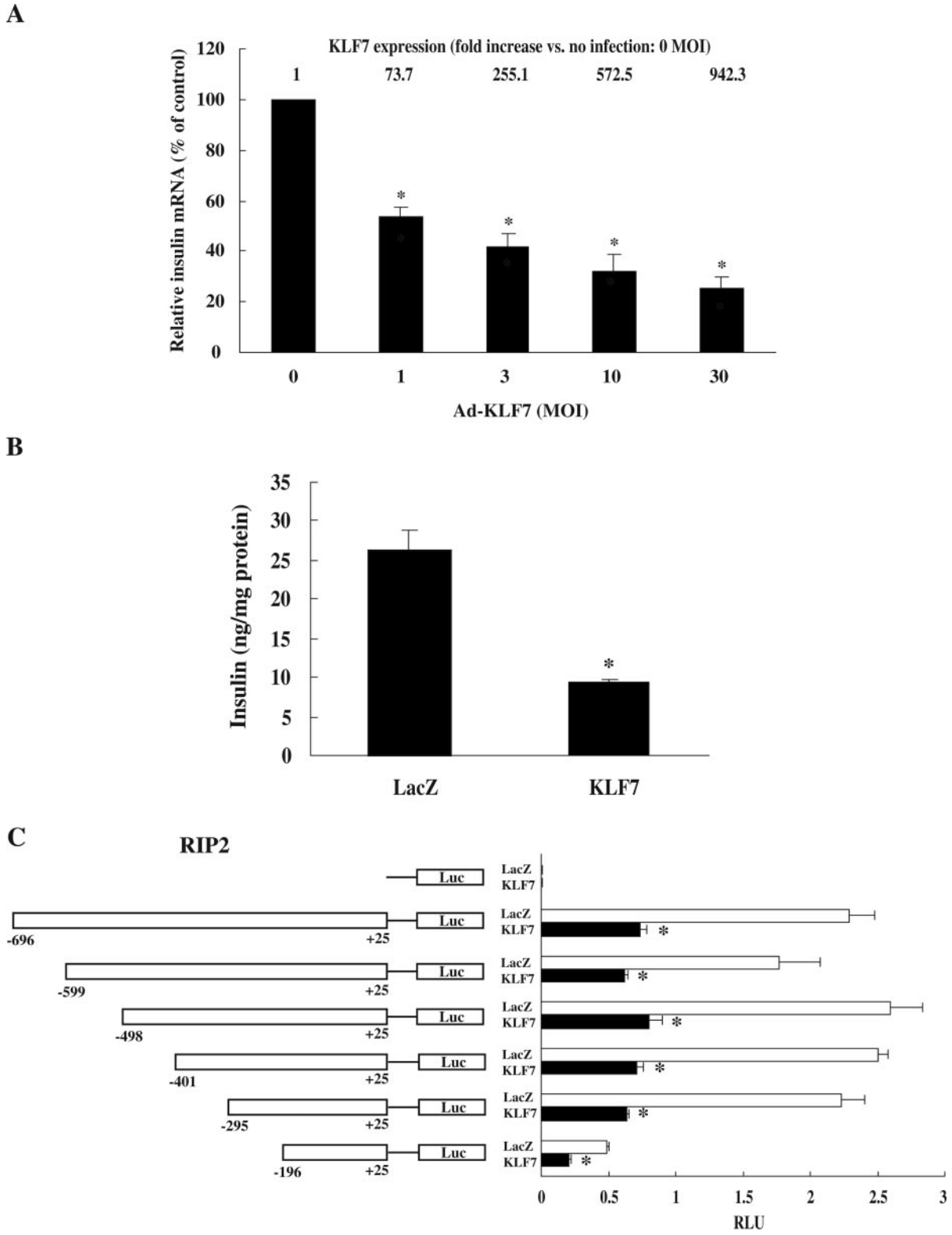

Fig. 4. Effects of *KLF7* Overexpression on Insulin Expression in Pancreatic β-Cell HIT-T15

A, After infection with 0, 1, 3, 10, or 30 m.o.i. of adenovirus vectors encoding *KLF7*, cells were cultured for 2 d, and total RNA was isolated from each cell for quantitative real-time PCR. Relative amounts of the insulin gene are indicated as mean ± SEM. *, $P < 0.001$ *vs.* uninfected cells. B, The contents of intracellular insulin in *KLF7*-overexpressing HIT-T15 cells. *, $P < 0.001$ *vs.* control cells. C, Luciferase activity in various constructs of the insulin gene promoter in *KLF7*-overexpressing HIT-T15 cells. Promoter activity was shown as a ratio between firefly and *Renilla* luciferase activity. *, $P < 0.0005$ *vs.* control cells. Ad-, Adenovirus; RIP2, rat insulin 2 promoter; RLU, relative light units.

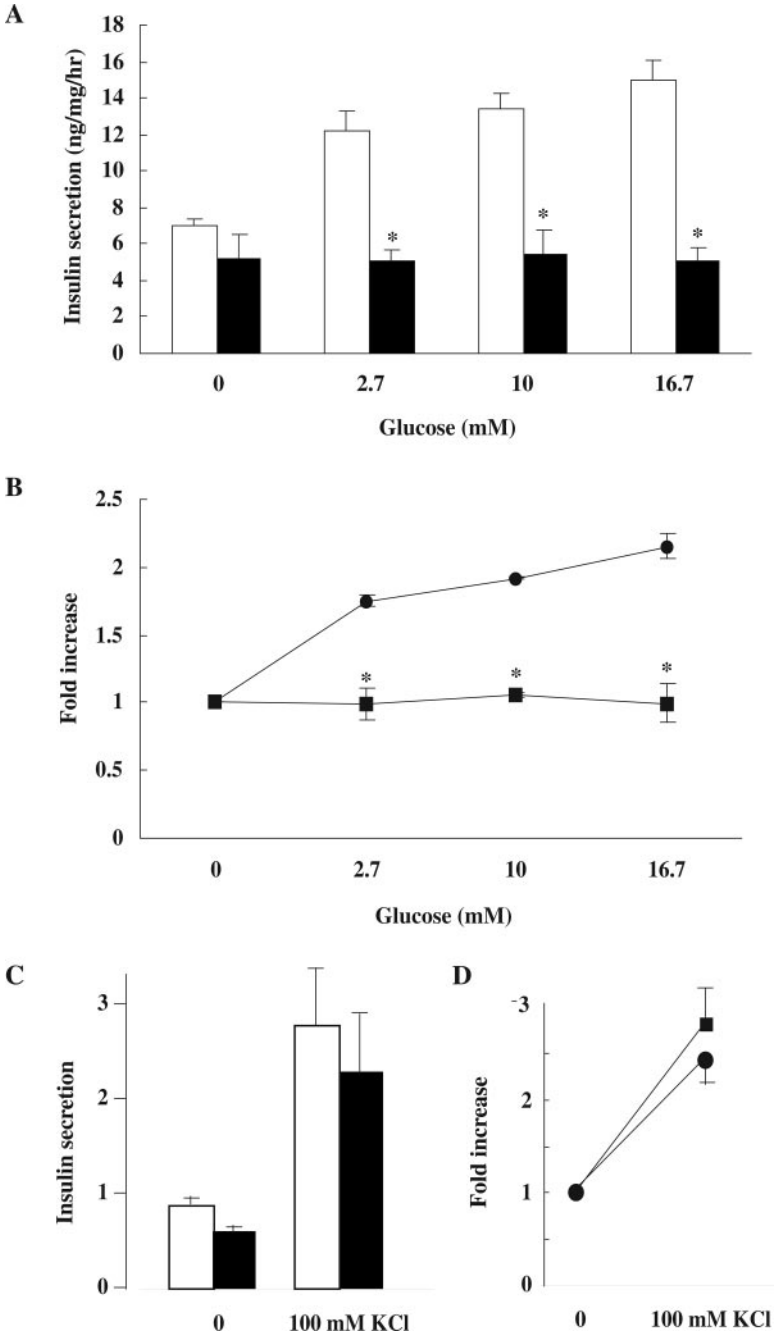

Fig. 5. Glucose-Induced Insulin Secretion in HIT-T15 Cells

A, LacZ (□), KLF7 (■)-overexpressing HIT-T15 cells were incubated in the media containing various concentrations of glucose (0, 2.7, 10, and 16.7 mM) for 60 min. Data were presented as mean ± SEM. *, $P < 0.01$ *vs.* control cells. B, Glucose-induced increase of insulin secretion in LacZ (●)- or *KLF7*- (■)-overexpressing HIT-T15 cells. *, $P < 0.01$ *vs.* control cells. C, KCl-induced insulin secretion in HIT-T15 cells overexpressing LacZ (□) or *KLF7* (■). D, KCl-induced increase of insulin secretion in LacZ (●)- or KLF7 (■)-overexpressing HIT-T15 cells.

mRNA expression of sulfonylurea receptor 1 (SUR1), Kir6.2, pancreatic duodenal homeobox factor 1 (PDX-1), and GLUT2 genes in *KLF7*-overexpressing HIT-T15 cells was significantly reduced compared with that in the control cells (SUR1: 0.993 ± 0.076 and 0.675 ± 0.032 for LacZ and *KLF7*, respectively, $P < 0.05$; Kir6.2: 1.023 ± 0.066 and 0.589 ± 0.106 for LacZ and *KLF7*, respectively, $P < 0.05$; PDX-1: 1.030 ± 0.042 and 0.472 ± 0.059 for LacZ and *KLF7*, respectively, $P < 0.01$; GLUT2: 1.067 ± 0.073 and 0.167 ± 0.079 for LacZ and *KLF7*, respectively; $P < 0.01$; Fig. 6). The change in expression of the glucokinase and NeuroD1 genes was not statistically significant between the LacZ- and *KLF7*-overexpressing cells (Fig. 6).

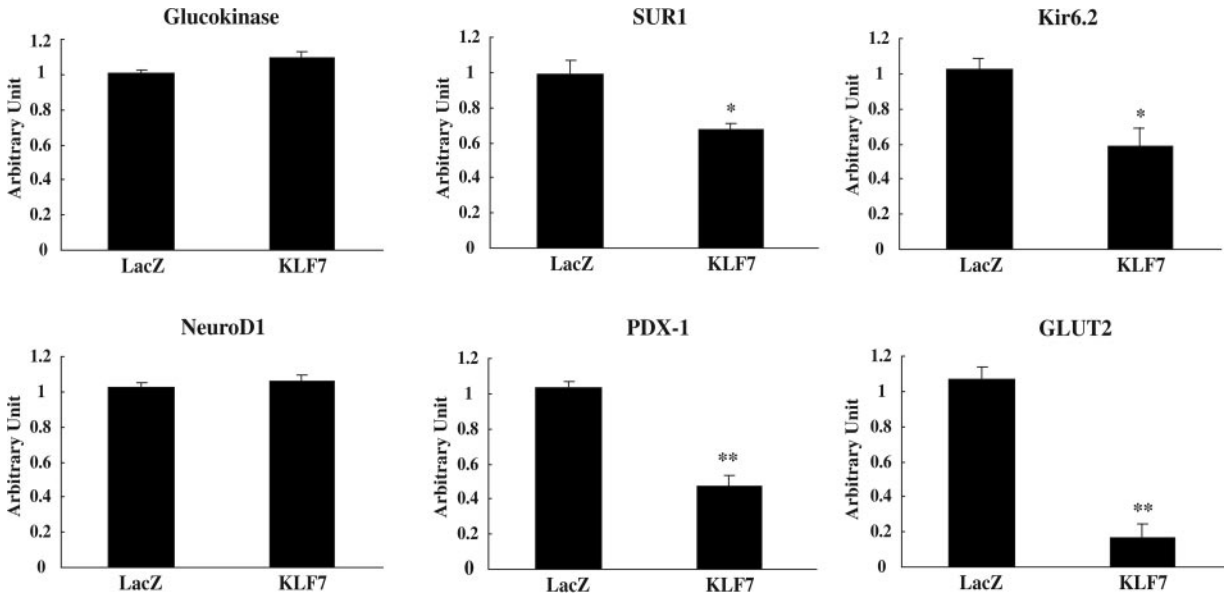

Fig. 6. Effects of *KLF7* Overexpression on the Expression of Glucokinase, SUR1, Kir6.2, NeuroD1, PDX-1, and GLUT2 in HIT-T15 Cells

Data are indicated as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ *vs.* control cells.

Effects of KLF7 on the Expression of Genes Related to Glucose Metabolism in L6 and HepG2 Cells

The mRNA expression of the hexokinase 2 gene in *KLF7*-overexpressing L6 cells was significantly reduced compared with that in the control cells (1.009 ± 0.011 and 0.657 ± 0.032 for LacZ and *KLF7*, respectively; $P < 0.001$; Table 1), whereas the change in expression of GLUT1, GLUT4, and glycogen synthase was not statistically significant between the LacZ- and *KLF7*-overexpressing cells. In HepG2 cells, the mRNA expression of the GLUT2 was significantly reduced in KLF7-overexpressing cells compared with that in control cells (1.148 ± 0.014 and 0.283 ± 0.021 for LacZ and *KLF7*, respectively; $P = 0.0001$; Table 1), whereas the expression of GLUT1, phosphoenol pyruvate carboxykinase, and PPAR coactivator 1 was not different between the LacZ- and *KLF7*-overexpressing cells.

Table 1. The Expression of Genes Related to Glucose Metabolism in *KLF7* Overexpressing L6 and HepG2 Cells

Gene	Arbitrary Unit		*P* Value
	LacZ	KLF7	
L6 cells			
GLUT1	0.982 ± 0.048	0.992 ± 0.092	
GLUT4	1.025 ± 0.068	0.821 ± 0.129	
Hexokinase	1.009 ± 0.011	0.657 ± 0.032	0.0005
Glycogen Synthase	0.963 ± 0.033	0.991 ± 0.062	
KLF7	1.028 ± 0.049	33.9 ± 5.6	<0.0001
HepG2 cells			
GLUT1	1.204 ± 0.245	1.226 ± 0.135	
GLUT2	1.148 ± 0.014	0.283 ± 0.021	0.0001
PEPCK	1.451 ± 0.045	1.087 ± 0.190	
PGC-1	1.041 ± 0.055	1.179 ± 0.149	
KLF7	1.255 ± 0.111	172.9 ± 54.2	<0.0001

PEPCK, Phosphoenolpyruvate carboxykinase; PGC-1, PPAR-γ coactivator 1.

DISCUSSION

In the present study, we examined the effects of the *KLF7* gene on the functions of various types of cells related to glucose metabolism, and found that *KLF7* could affect the expression and excretion of adipocytokines in cultured human adipocytes, and inhibit the expression and excretion of insulin in an insulin-secreting cell line.

We recently reported that *KLF7,* a member of the KLF family, is a novel candidate for conferring susceptibility to type 2 diabetes (11). We also reported that the overexpression of *KLF7* resulted in a remarkable inhibition of adipogenesis in mouse 3T3-L1 cells, suggesting *KLF7* may play a role in the regulation of adipocyte function. However, until now, no evidence has emerged clarifying the role of *KLF7* in the pathogenesis of type 2 diabetes. In the present study, we examined the expression profile of this gene in various human tissues and several types of cultured cell lines and obtained a similar result with that reported previously (12). In 3T3-L1 cells, however, we additionally identified that the expression of *KLF7* was abundant in the cells before inducing differentiation, and rapidly decreased after inducing differentiation, followed by the gradual increase according to the degree of dif-

ferentiation into adipocytes. This result suggested that *KLF7* might play a role in the regulation of cell functions not only in preadipocytes but also in mature adipocytes. The transient decrease of *KLF7* gene expression after the induction of differentiation might be due to the direct effect of the component of the differentiation medium. To evaluate this possibility, we examined the effect of each component on the expression of the *KLF7* gene. The result indicated that dexamethasone and IBMX might have some independent effects, but this result also seemed to reflect the degree of differentiation. Pioglitazone did not have any effect on adipocyte differentiation and *KLF7* expression by itself in our experiment, but rats treated with pioglitazone had reduced adipocyte expression of *KLF7* (our unpublished observation). Therefore, we thought *KLF7* might be a physiological inhibitor of adipocyte differentiation, although the possibility for the direct effect of differentiation cocktail could not be excluded completely.

We then examined the effect of overexpression of *KLF7* in human preadipocytes, and identified that adipogenesis was remarkably suppressed in *KLF7*-overexpressing cells, indicating that the *KLF7* gene is a strong regulator of adipogenesis. We further examined the role of the *KLF7* gene in matured human differentiated adipocytes. Because differentiated adipocytes have been known to be capable of secreting several cytokines, known as adipocytokines (13), we examined the expression of adipocytokines in *KLF7* overexpressing human adipocytes. Our results clearly indicated that *KLF7* could also regulate the expression of several adipocytokine genes in differentiated human adipocytes. Because the expression of PPAR-γ and aP2 was not different between *KLF7*-overexpressing and control human adipocytes, it is likely that the alteration of adipocytokine gene expression in *KLF7*-overexpressing human adipocytes was due to the direct or indirect effect of *KLF7* on the regulation of these gene expressions rather than the consequence of the inhibition of further adipocyte differentiation. Because *KLF7* overexpression resulted in the increase of IL-6 expression, and in the reduction of adiponectin and leptin, we suggest that the excess of *KLF7* may contribute to the development of systemic insulin resistance.

In an insulin-secreting cell line (HIT-T15), we also demonstrated that the expression and glucose-induced excretion of insulin were remarkably suppressed in HIT-T15 cells overexpressing *KLF7*. The effect of *KLF7* on insulin gene expression was likely to be due to the inhibition of transcriptional activation of the gene. The expression of PDX-1 was also decreased in *KLF7* overexpressing cells, but the results using various deletion constructs, including the fragment deleting the PDX-1-binding site, suggested *KLF7* could affect basal promoter activity for insulin gene transcription. It would be worthwhile to investigate the mechanism by which *KLF7* is able to inhibit the glucose-induced insulin secretion from the cells. Although the content of insulin in the *KLF7*-overexpressing cells was remarkably decreased, and this might lead to a reduction in insulin reserves, a sufficient amount of insulin was still considered to exist because KCl-induced insulin secretion was not inhibited in the KLF7-overexpressing cells. Therefore, we suggest that *KLF7* may directly affect the secretory process induced by extracellular glucose. Next, we examined the expression of genes related to glucose transport, glucose metabolism, and insulin secretion in a *KLF7*-overexpressing β-cell line with quantitative real-time PCR. As a result, we found that the expression of GLUT2, SUR1, and Kir6.2 was reduced in *KLF7*-overexpressing cells. It has been reported that the expression of GLUT2 was remarkably reduced in glucose-unresponsive islets from different animal models of diabetes (14–19), and mice lacking GLUT2 reportedly exhibited impaired glucose-induced insulin secretion (20). It has also been reported that the mutation of SUR1 or Kir6.2 in humans causes hyperinsulinemic hypoglycemia in infancy, and SUR1 or Kir6.2 null mice could not secrete insulin in response to glucose (21, 22). Mice introducing a loss of function mutation of Kir6.2 (G132S) were also reported to show hyperglycemia due to the disability for the secretion of insulin in response to glucose (23). These reports also demonstrated that the heterozygous subjects and heterozygous mice did not exhibit an abnormal glucose homeostasis, which suggests that complete disruption of each gene might be required for exhibition of the reported phenotype. Because the overexpression of *KLF7* could not completely suppress the expression of these genes in our study, the reduction in the expression of these genes may cooperate with each other to mediate the inhibitory effect of *KLF7* on glucose-induced insulin secretion in pancreatic β-cells.

In a rat skeletal muscle cell line, we observed a significant decrease in the expression of hexokinase 2 in *KLF7*-overexpressing cells. It has been reported that expression and activity of hexokinase in the skeletal muscle were reduced in patients with type 2 diabetes compared with control subjects (24). Therefore, the *KLF7*-induced reduction of the hexokinase gene expression in skeletal muscle cells may also contribute to the impaired response to insulin.

Finally, we observed a significant decrease in the expression of GLUT2 in *KLF7*-overexpressing hepatocytes compared with control cells. Because it has been reported that glucose uptake was almost completely suppressed in hepatocytes of GLUT2-null mice (25), *KLF7* may also participate in the impaired glucose uptake in hepatocytes through the reduction of GLUT2 expression.

From these results, it is suggested that *KLF7*, which has been shown to be a new candidate gene for type 2 diabetes, plays an important role in the onset and the progression of type 2 diabetes through multiple mechanisms, including an impairment of insulin biosynthesis and secretion in pancreatic β-cells, a dysregulation of adipocytokine gene expression in adipose, a sup-

pression of hexokinase 2 gene expression in skeletal muscle, and a suppression of GLUT2 gene expression in hepatocyte. Therefore, the inhibition of *KLF7* expression and/or activity may be a target for a new therapeutic and/or preventative approach for treating type 2 diabetes, although the elucidation of the precise mechanism requires further study.

In summary, we have demonstrated that *KLF7* can regulate the function of adipocytes, pancreatic β-cells, and skeletal muscle cells and is involved in the pathogenesis of type 2 diabetes. Our present results suggest that *KLF7* is a new target for drug discovery in type 2 diabetes.

MATERIALS AND METHODS

Materials

HIT-T15, RIN-5F, and L6 cells were obtained from Dainippon Pharmaceutical (Osaka, Japan). Mouse 3T3-L1 and HepG2 cells were obtained from The Health Science Research Resources Bank (Suita, Japan). HPA was purchased from ZenBio, Inc. (Research Triangle Park, NC) and insulin, indomethacin, cesium chloride, glycerol, formaldehyde, and oil red O were purchased from Wako (Osaka, Japan). Fetal bovine serum was purchased from Gemini Bio-Products (St. Woodland, CA). Dexamethasone and 3-isobutyl-1-methylxanthine were obtained from Sigma Chemical Co. (St. Louis, MO). Pioglitazone was provided by Shionogi & Co. Ltd. (Toyonaka, Japan).

3T3-L1 Cell Culture

Mouse 3T3-L1 preadipocytes were cultured at 37 C in DMEM containing 10% fetal bovine serum, 50 IU/ml penicillin G, and 50 μg/ml streptomycin in an atmosphere of 5% CO_2. To induce differentiation, confluent preadipocytes were cultured in differentiation medium (DMEM; 10% fetal bovine serum, 1 μM insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine) for 2 d. At the indicated number of days after inducing differentiation, total RNA was extracted and used for quantitative real-time PCR.

Construction of Adenovirus Vector Encoding Human KLF7

cDNA for human *KLF7* (837 bp: GenBank accession no. NM_003709) was generated by amplification from Human Adipocyte Marathon-Ready cDNA (CLONTECH Laboratories, Inc., Palo Alto, CA) using the following primers: sense, 5′-TGA GCC AGA CAG ACT GAC AA-3′; antisense, 5′-CCT TTA GAC ACT AGC CGA TG-3′. Amplified products were separated on a 0.8% agarose gel and the desired band was purified with the MinElute Gel Extraction Kit (QIAGEN, Valencia, CA). The purified human *KLF7* cDNA was subcloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and verified by direct sequencing to confirm that the obtained fragment corresponded to human *KLF7*.

Adenovirus vector encoding human *KLF7* was prepared using the Adenovirus Expression Vector Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Briefly, human *KLF7* cDNA was inserted into the pAxCAwt plasmid, which contains a CAG promoter (CMV enhancer, chicken β-actin promoter, and part of an untranslated region of rabbit β-globin), and the plasmid was introduced into human embryonic kidney 293 cells using the FuGENE6 transfection procedure. The adenovirus from a single plaque was expanded and purified twice by cesium chloride gradient ultracentrifugation, and dialyzed in a solution of 10% glycerol in PBS. After determination of the viral titer by a plaque assay, the viral solution was stored at −80 C until use. Control adenovirus, which carries β-galactosidase cDNA (LacZ), was prepared using the same procedure as described above.

Preparation of Human Adipocytes and Infection

Human preadipocytes were cultured at 37 C in DMEM:HAM-F10 (1:1, vol/vol) containing 10% fetal bovine serum, 50 IU/ml penicillin G, and 50 μg/ml streptomycin in an atmosphere of 5% CO_2. Cells were introduced to differentiate into adipocytes in 1:1 DMEM:HAM-F10 containing 10% fetal bovine serum, 2 μM insulin, 0.2 mM indomethacin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. For differentiation study, cells were transduced with adenovirus vectors encoding either *KLF7* or LacZ at a m.o.i. of 100 plaque-forming units/cell 48 h before the induction of differentiation, and samples were obtained at the indicated days after inducing differentiation. To determine the effect of *KLF7* on adipocytokine gene expressions, human adipocytes were infected with adenovirus vectors at the indicated m.o.i. 14 d after inducing differentiation.

Oil Red O Staining

At the indicated number of days after inducing differentiation, human adipocytes were washed three times with PBS and then fixed for 1 h with 3.7% formaldehyde. Fixed cells were incubated with oil red O for 15 min at room temperature. After washing the cells four times with water, the stained lipid droplets in the cells were visualized by light microscopy. For quantification, the dye was extracted with isopropyl alcohol, and the absorbance was measured at 540 nm.

Measurement of Adipocytokines Secretion by ELISA

HPA cells were seeded in six-well plates, and confluent preadipocytes were stimulated with differentiation medium. Fourteen days after induction of differentiation, human adipocytes were infected with LacZ or *KLF7* adenovirus at 100 m.o.i. overnight and cultured for 4 d. Culture medium was changed to a 500 μl volume of new medium, and the medium was collected after 24 h. The protein content of adipocytokines in the supernatants was measured by ELISA using commercially available kits according to the manufacturer's specifications. Total protein was determined by a modified Lowry method using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA).

Insulin-Secreting Cell Culture and Infection

The insulin-secreting cell line, HIT-T15, was maintained at 37 C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin G, and 50 μg/ml streptomycin in an atmosphere of 5% CO_2. Cells plated into 24-well tissue culture dishes (1×10^5 cells per well) were infected with adenovirus vectors encoding *KLF7* or LacZ at 10 m.o.i. for 1 h.

Measurements of Insulin Secretion

Forty eight hours after infection, the cells were washed three times with HEPES-Krebs buffer containing 0.2% BSA and incubated for 2 h in HEPES-Krebs buffer containing 0.2% BSA. Subsequently, cells were incubated in HEPES-Krebs buffer containing 0.2% BSA with 0, 2.7, 10, or 16.7 mM glucose, or 100 mM KCl for 1 h at 37 C, and the incubation

media were collected to measure insulin secreted from the cells. Next, the cells were treated with radioimmune precipitation assay buffer to measure intracellular insulin or total protein contents. The concentration of insulin was determined by ELISAs using commercially available kits according to the manufacturer's specifications (MORINAGA, Yokohama, Japan). Total protein was determined by a modified Lowry method using the DC Protein Assay (Bio-Rad).

Construction of Rat Insulin Promoter and Transfection Experiments

The various fragments identical to the rat insulin 2 promoter were amplified by PCR using rat genomic DNA as a template. The primers used for the amplification are shown in Table 2. The amplified products were purified and inserted into pCR2.1-TOPO vector (Invitrogen), and the identity of the fragments was checked by direct sequencing. DNA fragments digested with *Mlu*I and *Xho*I were subcloned into a luciferase reporter vector (pGL3-Basic; Promega Corp., Madison, WI) at its multiple cloning site upstream of the luciferase reporter gene.

HIT-T15 cells were plated in a 24-well cell culture plate at a density of 1×10^5 cells per well and cultured for 2 d. The cells were infected with adenovirus encoding *KLF7* or LacZ at 10 m.o.i. for 1 h, followed by further incubation in the RPMI 1640 media for 24 h. Next, each construct was introduced into the cells together with a *Renilla* luciferase control vector, phRL-CMV (Promega), using the liposome transfection procedure (FuGENE6; Roche, Basel, Switzerland). The cells were harvested 48 h after transfection, and luciferase activities were determined by the Dual Luciferase Reporter Assay System (Promega). The luminescence of firefly luciferase was corrected by that of *Renilla* luciferase, which reflected transfection efficiency.

L6 and HepG2 Cell Culture and Infection

L6 cells and HepG2 were maintained at 37 C in DMEM supplemented with 10% fetal bovine serum (vol/vol), 50 IU/ml penicillin G, and 50 μg/ml streptomycin in an atmosphere of 5% CO_2. L6 cells were differentiated in DMEM supplemented with 2% fetal bovine serum in myotubes within 7 d after seeding. HepG2 cells and differentiated L6 cells were infected with adenovirus vectors encoding *KLF7* or LacZ, and 2 d after infection, total RNA was extracted for quantitative real-time PCR.

Table 2. Primers Used for PCR or Quantitative Real-Time PCR

	Forward (5′–3′)	Reverse (5′–3′)
For cloning		
RIP2(−696 to +25)	TAACGCGTGGATCCCCCAACCACTCCAA	TACTCGAGCGACTGTAGCTGGTCACTTA
RIP2(−599 to +25)	TAACGCGTCAAGCACCTCTTATGGAGAG	TACTCGAGCGACTGTAGCTGGTCACTTA
RIP2(−401 to +25)	TAACGCGTGAGGACACAGCTATCAGTG	TACTCGAGCGACTGTAGCTGGTCACTTA
RIP2(−295 to +25)	TAACGCGTCAGCCAAGGACAAAGAAGGC	TACTCGAGCGACTGTAGCTGGTCACTTA
RIP2(−196 to +25)	TAACGCGTACCCTAAGGCTAAGTAGAGG	TACTCGAGCGACTGTAGCTGGTCACTTA
For real-time PCR		
Human KLF7	GGTGAGCCAGACAGACTGACAA	GAAGTAGCCGGTGTCGTGGA
Human adiponectin	CTTTGGAGTGTTGGTAGGTGTCTG	AAAGGCATGAGAAGGGACATAGG
Human leptin	TGTGAGCAGTGAGTTACAGCGAG	CCTGATTAGGTGGTTGTGAGGAT
Human IL-6	AATAACCACCCCTGACCCAACC	TGACCAGAAGAAGGAATGCCC
Human resistin	GAAGAAGCCATCAATGAGAGGATC	GCAGTGACATGTGGTCTCGG
Human PAI-1	ATGCAGATGTCTCCAGCCCTCAC	TGAATCTGGCTCTCTCCACCTC
Human PPARγ	GCAAGAAGCCCGGGATCTA	AAAGACCAACCAGATGCAGGA
Human aP2	TTGCTACCAGGCAGGTGGCC	CCAGTGTGGTCTCTTGCCCG
Human PEPCK	CTTTTTCGGTGTCGCTCCTG	GACACCTGAAGCTAGCGGCT
Human GLUT1	GCAGCCTGTGTATGCCACC	CGCGATGGTCATGAGTATGG
Human PGC-1	TGATCACAGGACTAGCAGTG	ATTGACCGCTGCTTGGCTG
Human β-actin	CACGGCATCGTCACCAACT	TGATCTGGGTCATCTTCTCGC
Rat GLUT1	TATAAAAAGGCAGCTCCGCG	GCTGTGAAACGGAGAATGGAC
Rat GLUT4	GGAGTCATCAACGCCCCACAG	TGCCACCCACAGAGAAGATGG
Rat Hexokinase	TGGTTTCAAAGCGGTCGAAC	TCTGCACTTGGTTTTGGTTGAG
Rat Glycogen synthase	TTGGTCTGTGTCTGTGCCGA	CTCTCGCCTGCTTCCTTGTC
Rat GAPDH	TGGGCTACACTGAGGACCAGG	GGCAACTGAGGGCCTCTCTC
Hamster Insulin	AGAAGCCATCAGCAAGCAGG	AGAGTGCCTCCACAAGGTGG
Hamster GLUT2	TCTCTCCTTCCAGTACATCG	TCTCTTCTTCCGGAATTCTG
Hamster Glucokinase	AGGTAGAGCAGATCCTGGCA	ACTTCTGAGCCTTCTGGGGT
Hamster SUR1	CCACGTCAGCTTCTTCAAAG	ATCTCTGCACTGGACAGGAA
Hamster Kir6.2	CTGCACCACCACCAGGACCT	CCAAATTTGGAGTAGTCCACA
Hamster NeuroD1	GCTCAGCATCAATGGCAACT	TGTCTATGGGGATCTCGCAG
Hamster PDX-1	AAAGCTCACGCGTGGAAAGG	TCATGCGACGGTTTTGGAAC
Hamster 36B4	CAATGGCAGCATTTACAACCC	CCCATTGATAATGGAGTGTGG
Mouse PPARγ	TTTGAAAGAAGCGGTGAACCAC	ACCATTGGGTCAGCTCTTGTG
Mouse C/EBPα	AAAGCCAAGAAGTCGGTGGAC	GGTCATTGTCACTGGTCAACTCC
Mouse C/EBPβ	TCACCTTAAAGATGTTCCTGCG	CAAATATACATACGCCTCTTTTCTCATAG
Mouse C/EBPδ	CGAATCGCTAGTTTCTTTGGGAC	GTCTCTTCCTCTTATCTACAAAAGTCTGTC
Mouse aP2	AACACCGAGATTTCCTTCAA	TCACGCCTTTCATAACACAT
Mouse adipsin	GCAGTCGAAGGTGTGGTTACG	TTTTCGATCCACATCCGGTAG

Quantitative Real-Time PCR

Total RNA was extracted from the cells using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions, and 5 μg of total RNA was reverse transcribed with the SuperScript first-strand synthesis system (Invitrogen). Real-time PCR was carried out using the Mx3000P Multiplex Quantitative PCR system (Stratagene, La Jolla, CA). PCRs were performed in a 22-μl reaction mixture containing 1× Ex *Taq* buffer, 200 nM deoxynucleotide triphosphate, 800 nM primer, 0.05 U/μl Ex Taq HS polymerase, 1/20000 SYBR green (CAMBREX, Rockland, ME). The thermal profile consisted of 2 min at 50 C, 10 min at 95 C, followed by 40 cycles of 30 sec at 95 C, 30 sec at 68 C, and 30 sec at 72 C for human *KLF7*, phosphoenolpyruvate carboxykinase, rat glycogen synthase, and hamster insulin; 40 cycles of 30 sec at 95 C, 30 sec at 60 C, 30 sec at 72 C for human adiponectin, leptin, resistin, type 1 plasminogen activator inhibitor, IL-6, PPAR-γ, aP2, GLUT1, GLUT2, PPAR-γ coactivator 1, rat GLUT1, GLUT4, and hexokinase 2; 40 cycles of 30 sec at 95 C, 30 sec at 65 C, 30 sec at 72 C for hamster glucokinase, SUR1, Kir6.2, NeuroD1, and PDX-1; or 50 cycles of 30 sec at 95 C, 30 sec at 60 C for hamster GLUT2. The primers used for the amplification are shown in Table 2.

Statistical Analysis

Statistical analysis of the difference between the two groups was performed using Student's unpaired *t* test, where $P < 0.05$ was considered significant.

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