Alternative human liver transcripts of TCF7L2 bind to the gluconeogenesis regulator HNF4 α at the protein level

Bernadette Neve · Olivier Le Bacquer · Sandrine Caron · Marlène Huyvaert · Audrey Leloire · Odile Poulain-Godefroy · Cécile Lecoeur · François Pattou · Bart Staels · Philippe Froguel

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Abstract

Aims/hypothesis Gene polymorphisms of *TCF7L2* are associated with increased risk of type 2 diabetes and transcription factor 7-like 2 (TCF7L2) plays a role in hepatic glucose metabolism. We therefore addressed the impact of TCF7L2 isoforms on hepatocyte nuclear factor 4 α (HNF4 α) and the regulation of gluconeogenesis genes.

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B. Neve · O. Le Bacquer · S. Caron · M. Huyvaert · A. Leloire · O. Poulain-Godefroy · C. Lecoeur · F. Pattou · B. Staels · P. Froguel
European Genomic Institute for Diabetes (EGID), Lille, France

B. Neve · O. Le Bacquer · S. Caron · M. Huyvaert · A. Leloire · O. Poulain-Godefroy · C. Lecoeur · F. Pattou · B. Staels · P. Froguel
University Lille 2 of Health and Law, Lille, France

B. Neve · S. Caron · M. Huyvaert · A. Leloire · O. Poulain-Godefroy · C. Lecoeur · B. Staels · P. Froguel
Institut Pasteur de Lille, Lille, France

B. Neve (✉) · M. Huyvaert · A. Leloire · O. Poulain-Godefroy · C. Lecoeur · P. Froguel
CNRS, UMR8199, Génomique et Maladies Métaboliques, Institut de Biologie de Lille & Institut Pasteur de Lille, 1 Rue du Professeur Calmette, CS 50447, 59021 Lille Cedex, France
e-mail: bernadette.neve@good.ibl.fr

O. Le Bacquer · F. Pattou
Inserm, U859, Faculté de Médecine, Lille, France

S. Caron · B. Staels
Inserm, UMR1011, Lille, France

P. Froguel
Department of Genomics of Common Disease, School Of Public Health, Hammersmith Hospital, Imperial College, London, UK

Methods Liver *TCF7L2* transcripts were analysed by quantitative PCR in 33 non-diabetic and 31 type 2 diabetic obese individuals genotyped for *TCF7L2* rs7903146. To analyse transcriptional regulation by TCF7L2, small interfering RNA transfection, luciferase reporter and co-immunoprecipitation assays were performed in human hepatoma HepG2 cells.

Results In livers of diabetic compared with normoglycaemic individuals, five C-terminal *TCF7L2* transcripts showed increased expression. The type 2 diabetes risk allele of rs7903146 positively correlated with *TCF7L2* expression in livers from normoglycaemic individuals only. In HepG2 cells, transcript and TCF7L2 protein levels were increased upon incubation in high glucose and insulin. Of the exon 13 transcripts, six were increased in a glucose dose-responsive manner. TCF7L2 transcriptionally regulated 29 genes related to glucose metabolism, including glucose-6-phosphatase. In cultured HepG2 cells, TCF7L2 did not regulate *HNF4A* and *FOXO1* transcription, but did affect HNF4 α protein expression. The TCF7L2 isoforms T6 and T8 (without exon 13 and with exon 15/14, respectively) specifically interacted with HNF4 α .

Conclusions/interpretation The different levels of expression of alternative C-terminal *TCF7L2* transcripts in HepG2 cells, in livers of normoglycaemic individuals carrying the rs7901346 type 2 diabetes risk allele and in livers of diabetic individuals suggest that these transcripts play a role in the pathophysiology of type 2 diabetes. We also report for the first time a protein interaction in HepG2 cells between HNF4 α and the T6 and T8 isoforms of TCF7L2, which suggests a distinct role for these specific alternative transcripts.

Keywords Gluconeogenesis · HNF4 α · Liver · TCF7L2 isoforms

Abbreviations

ABOS	Biological-Atlas-of-Severe-Obesity
C-clamp	Cysteine clamp
FOXO1	Forkhead box O1
G6PC	Glucose-6-phosphatase
HNF4 α	Hepatocyte nuclear factor 4 α
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
TCF4	T-cell factor 4 alias of TCF7L2
TCF7L2	Transcription factor 7-like 2
TSS	Transcription start site

Introduction

DNA or single-nucleotide polymorphisms (SNPs) in the gene encoding the WNT-signalling transcription factor, transcription factor 7-like 2 (TCF7L2), confer the largest genetic risk known so far for common forms of type 2 diabetes as described in a review by others [1]. Type 2 diabetes-associated *TCF7L2* SNPs have been associated with impaired pancreatic

beta cell function, e.g. reduced insulin exocytosis [2], and a reduction in glucagon-like peptide-1-induced insulin secretion [3–5]. Human carriers of the *TCF7L2* rs7903146 risk allele have increased pancreatic islet size, and altered alpha:beta cell ratios and distribution within pancreatic islets [6]. DNA sequences around the *TCF7L2* rs7903146 SNP contain regulatory elements that are critical in the regulation of *TCF7L2* transcription, suggesting that the type 2 diabetes susceptibility conferred by this locus is related to modified *TCF7L2* expression [7]. At least 20 different TCF7L2 splice variants and isoforms exist (Table 1) [8–12], which may also differ in their transcriptional activation properties [12–14]. We have previously shown that altered TCF7L2 splice variants in the pancreas exert different effects on pancreatic insulin secretion and islet survival [12]. In particular, the medium C-terminal TCF7L2 isoforms T1 and T2 are pro-apoptotic, while the short T3 and medium C-terminal T7 isoforms improve pancreatic beta cell survival and function.

Human carriers of the *TCF7L2* rs7903146 risk T allele have also been reported to have elevated hepatic glucose production [15–18]. An initial study of alternatively spliced

Table 1 TCF7L2 isoforms

	RefSeq (NCBI)		Alternative exons		
	Mature mRNA transcript	Transcript number	N-terminal	C-terminal	TCF4 nomination [13]
Major pancreatic and liver TCF7L2 isoforms [12]					
T3	N.A.	N.A.	+4 +7b	+13 +14 +15 –16	Short: S2
T5	NM_001198525	7	–4 +7b	+13 +14 +15 –16	Short: S2
T1	NM_0011985311	13	+4 +7b	–13 –14 –15 –16	Medium: M1
T7	NM_001198529	11	–4 +7b	+13 –14 –15 –16	Medium: M2
T2	N.A.	N.A.	+4 –7b	+13 –14 –15 –16	Medium: M2
T6	NM_001198526	8	–4 +7b	–13 –14 +15 –16	Long: E3
T8	NM_001146285	5	–4 +7b	–13 +14 –15 –16	Long: E1
T4	NM_030756	2	–4 +7b	+13 +14 –15 –16	Long: E2
T9	N.A.	NA	–4 –7b	+13 +14 –15 –16	Long: E2
Other TCF7L2 isoforms					
Reported in [13]	N.A.	N.A.	N.A.	–13 +14 +15 –16	Short: S1
N-term. variant T1	NM_001198530	12	–4 –5 +7b	–13 –14 –15 –16	Short: S3
N-term. variant T1	NM_001146283	3	–4 +5b +7b	–13 –14 –15 –16	Medium: M1
N-term. variant T1	NM_001146286	6	–4 +7b	–13 –14 –15 –16	Medium: M1
N-term. variant T2	NM_001146284	4	–4 –7b	+13 –14 –15 –16	Medium: M2
N-term. variant T6	NM_001146274	1	+4 +7b	+13 –14 –15 –16	Long: E3
Pancreas-specific TCF7L2 isoforms [10]					
Reported in [13]	N.A.	N.A.	N.A.	(+16) –13 –14 –15 +16	Short: S3
Reported in [13]	N.A.	N.A.	N.A.	–13 +14 +15 +16	Short: S4
Reported in [13]	N.A.	N.A.	N.A.	–13 +14 +15 +16	Short: S6
T4-like	NM_001198528	10	–4 +7b	+13 +14 –15 +16	Short: S8
T9-like	NM_001198527	9	–4 –7b	+13 +14 –15 +16	Short: S8

N.A., information not available

transcripts in human liver showed a suggestive, but not significant increase of certain *TCF7L2* transcripts in carriers of the rs7903146 risk allele, e.g. isoform T1 [10]. In the liver, the WNT- β -catenin pathway is involved in metabolic zonations, i.e. hepatocytes exhibit different metabolic profiles (glycolysis vs gluconeogenesis) depending on their localisation along the porto–central axis [19]. *TCF7L2* expression is highest in pericentral hepatocytes, where gluconeogenesis is low [20]. As evidence that *TCF7L2* affects liver glucose metabolism [20–22] is accumulating, we sought to further characterise the expression of alternative human *TCF7L2* transcripts in livers of normoglycaemic and type 2 diabetes individuals genotyped for rs7903146. We also analysed expression levels of these transcripts in HepG2 cells incubated with different glucose concentrations in the presence or not of insulin, as well as the mRNA expression of 84 genes related to glucose metabolism, subsequent to downregulation of *TCF7L2* by small interfering RNA (siRNA). Hepatic gluconeogenesis is tightly controlled by rate-limiting enzymes, such as glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase, which are both inhibited by a *TCF7L2*-dependent pathway [20–22]. Hepatocyte nuclear factor 4 α (HNF4 α) and forkhead box O1 (FOXO1) are transcriptional regulators of *G6PC*, which mediates regulation of gluconeogenesis in response to feeding or fasting [23]. FOXO1 may compete for β -catenin binding with *TCF7L2* under low glucose conditions [22, 24]. Therefore, we analysed whether *TCF7L2* isoforms differentially affect the HNF4 α –FOXO1 pathway in the transcriptional regulation of *G6PC*. Our findings, in particular the exclusive interaction of *TCF7L2* isoforms T6 and T8 with HNF4 α protein, suggest a distinct role for alternative *TCF7L2* transcripts.

Methods

Patients We analysed liver samples from white morbidly obese French patients who underwent abdominal surgery and were included in the Biological Atlas of Severe Obesity (ABOS) cohort realised at the Centre Hospitalier Régional Universitaire de Lille, France (ClinicalGov NCT01129297) (Table 2). Informed consent was obtained from all individuals and the experimental design was approved by the Hospital's Ethics Committee. Type 2 diabetic patients were defined by fasting plasma glucose levels ≥ 7.0 mmol/l and/or current treatment for diabetes; normoglycaemic individuals were defined by fasting glucose levels of < 5.6 mmol/l. All patients underwent preoperative evaluation (medical history and physical examination) and were devoid of any condition that could have increased abdominal surgery risks. The majority of patients suffered from obesity-related complications, like arthritis and hypertension.

Liver biopsies were immediately frozen in liquid nitrogen and stored at -85°C . A total of 250 individuals were genotyped for variant rs7903146 using Taqman SNP-assay C_29347861_10 on a 7900 HT Sequencer (Applied Biosystems/Life Technologies, Saint Aubin, France), following the manufacturer's instructions. The genotyping error rate was 0% as previously confirmed by automated sequencing using 768 duplicates [25]. Samples from all homozygous risk allele carriers (TT) were selected and equivalent numbers of individuals were selected from the other rs7903146 genotype classes (CC and CT) to have balanced groups. To minimise potential confounding factors, these individuals were selected within similar age and BMI ranges and sex

Table 2 Characteristics of ABOS study participants

rs7903146 allele	Normoglycaemic			Type 2 diabetic		
	CC	CT	TT	CC	CT	TT
<i>n</i>	14	10	9	11	11	9
Sex ^a						
Men (<i>n</i>)	4	3	2	1	2	2
Women (<i>n</i>)	10	7	7	10	9	7
Age ^{b, c} (years)	38.7 $\pm$ 6.8	46.1 $\pm$ 10.3	43.7 $\pm$ 14.5	45.0 $\pm$ 8.8	49.4 $\pm$ 6.7	51.2 $\pm$ 8.2
BMI ^b (kg/m ²)	47.4 $\pm$ 6.7	48.7 $\pm$ 7.3	49.7 $\pm$ 7.9	47.7 $\pm$ 5.7	48.2 $\pm$ 5.9	46.6 $\pm$ 5.3
Fasting glucose (mmol/l)	5.1 $\pm$ 0.5	5.1 $\pm$ 0.7	4.9 $\pm$ 0.6	8.0 $\pm$ 1.9	9.8 $\pm$ 4.8	9.3 $\pm$ 3.8
Fasting insulin (pmol/l)	94.3 $\pm$ 65.3	91.3 $\pm$ 58.5	100 $\pm$ 57.9	189 $\pm$ 162	194 $\pm$ 42.4	140 $\pm$ 255

Unless otherwise stated, values are mean $\pm$ SD

^a No significant differences were observed between genotype groups (CC, CT, TT) in the two categories (normoglycaemic or type 2 diabetes) of obese patients using Fisher's exact test for sex ratio

^b No significant differences were observed between genotype groups (CC, CT, TT) in the two categories (normoglycaemic or type 2 diabetes) of obese patients using the Kruskal–Wallis test. The Levene test indicated homogeneity of variances for all groups

^c The age of the individuals in the normoglycaemic and type 2 diabetes groups was significantly different (median 41 [range 21–65] years vs 50 [33–62] years, respectively; $p=0.02$ by Mann–Whitney test)

distribution per normoglycaemic or type 2 diabetes groups (Table 2).

RNA expression Total RNA was extracted using a kit (RNeasy Lipid Tissue Kit; Qiagen, Courtaboeuf, France) and transcribed using a high-capacity cDNA reverse transcription kit (Invitrogen/Life Technologies). Gene expression was analysed by quantitative real-time PCR standardised to *RPLP0* expression ($2^{-\Delta C_t}$). We studied *TCF7L2* exon expression using 16 assays covering exon junctions (electronic supplementary material [ESM] Fig. 1a, b); these assays were based on Taqman Gene Signature Arrays (Applied Biosystems/Life Technologies) or Stratagene Sybr green technology (Invitrogen/Life Technologies), and primers have been described by Prokunina-Olsson [9]. Other Taqman assays used were for: *G6PC* Hs006619178_m1; *PCK1* (*PEPCK*) Hs00159918_m1; *ALDOB* Hs00163626_m1; *HK3* Hs00240827_m1; *HNF4A* Hs00604435_m1; and *RPLP0* (*36B4*) Hs99999902_m1.

HepG2 cell culture and transfection HepG2 cells were cultured on 1% wt/vol. gelatine-coated plates in DMEM (31966; Gibco/Life Technologies) supplemented with 10% vol./vol. heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and non-essential amino acids; culture was at 37°C in humidified air containing 5% vol./vol. CO₂. For experiments, cells were incubated for 16 h in DMEM without glucose (11966; Gibco/Life Technologies) and supplemented with 1 mmol/l glucose and 0.1% wt/vol. fatty acid-free BSA (Sigma-Aldrich, Lyon, France); cells were then incubated with the indicated glucose and insulin concentrations for 24 h.

Cells, at 60–80% confluence, were transfected with siRNA (negative control 46-2000 and *TCF7L2*_overall [exon 17] HSS110547; Invitrogen/Life Technologies) using Lipofectamine RNAimax (Invitrogen/Life Technologies) and/or with plasmid constructs [12] (FuGENE HD; Roche Diagnostics, Mannheim, Germany). RNA analyses were performed 48 h after transfection. For gene reporter assays, cells were lysed with passive lysis buffer (Promega, Charbonnières, France) and luciferase activities were measured by a dual luciferase reporter assay system (Promega) with a luminometer (Centro LB 960; Berthold, Thoiry, France). Firefly luciferase activity was normalised to *Renilla* luciferase activity and results expressed relative to control.

Co-immunoprecipitation and western blot analysis HepG2 cells were transfected with the previously described ([12]) *TCF7L2* splice variants (Fig. 1a) using a transfection agent according to the manufacturer's instructions (FuGENE HD; Roche Diagnostics). Alternative liver *TCF7L2* transcripts are listed in Table 1. The T3 and T5 constructs both have a short C-terminal end that includes exon 14,

which encodes a CRARF cysteine clamp (C-clamp) domain, but they differ in exon 4, which encodes 23 amino acids. The T1, T2 and T7 constructs have a medium C-terminal end and no C-clamp domain, but T1 and T2 contain exon 4; T1 also contains exon 13, which encodes 17 amino acids, and T2 and T7 contain the alternative exon 7b, which encodes the LVPQ motif [26, 27]. All T6, T8, T4 and T9 constructs have a long C-terminal end and no exon 4, but do have either exon 14, which encodes a CRARF (T8, T4, T9), or exon 15, which encodes a CRALF (T6) C-clamp domain. Constructs T4 and T9 also have exon 13, and T4 has the longer exon 7b.

The co-immunoprecipitation protocol was performed as previously described [28]. Briefly, cells were lysed for 30 min on ice with a 50 mmol/l Tris HCl (pH 8.0) buffer containing 150 mmol/l NaCl, 5 mmol/l EGTA, 50 mmol/l NaF, 10% vol./vol. glycerol, 1.5 mmol/l MgCl₂, 1% vol./vol. Triton X-100 and freshly added 1× protease inhibitor cocktail (Complete; Roche Diagnostics). Protein lysates (2 mg) were clarified by 5 min centrifugation at 12,000 g and 4°C, and incubated overnight at 4°C with 5 µg of the following antibodies: normal rabbit immunoglobulin G (2729; Cell Signaling Technology, Danvers, MA, USA); and rabbit anti-HNF4α (H-171, sc-8987; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, 50 µl protein magnetic beads (A/G Dynal; Invitrogen/Life Technologies), previously washed with 2% wt/vol. BSA in PBS, were added to the incubation medium for 1 h. Co-immunoprecipitates were washed four times with 50 mmol/l Tris HCl (pH 8.0) buffer containing 150 mmol/l NaCl, 5 mmol/l EGTA and 1% vol./vol. NP-40, followed by boiling for 10 min in SDS-sample buffer. Proteins were applied to 6% SDS-PAGE and western blot immunolabelling performed with mouse monoclonal anti-HNF4α (PP-K9218-00; R&D Systems, Lille, France) or goat anti-TCF7L2 (TCF-4[N20], sc-8631; Santa Cruz). Other antibodies used for western blot immunolabelling were mouse anti-β-actin (sc-1616; Santa Cruz) and rabbit anti-β-tubulin (2148; Cell Signaling). Proteins were visualised with a western blot detection system (Amersham ECL; GE Healthcare, Freiburg, Germany).

Statistical analyses For mRNA expression analysis in human samples, we applied a non-parametric Mann–Whitney test (comparison of normoglycaemic vs type 2 diabetes) or a Kruskal–Wallis test (comparison of three genotype groups). Linear regression analysis of mRNA expression was performed using SPSS 14.0 (IBM Corporation, Armonk, NY, USA) with rs7903146 genotype, age, BMI and sex as covariates. Results of HepG2 experiments are expressed as means ± SEM, with statistical significance determined by Student's *t* test using Prism 5

Fig. 1 Expression of *TCF7L2* transcripts in liver samples of patients genotyped for rs7903146. **(a)** Schematic representation of the nine *TCF7L2* transcripts previously cloned from human liver RNA [12]. **(b–p)** Expression of *TCF7L2* mRNA transcripts (arbitrary units relative to the expression of housekeeping gene *RPLP0*) in liver samples of 33 normoglycaemic (NGT) and 31 type 2 diabetic (T2D) obese patients according to rs7903146 allele-group (T=risk allele) for **(b, e, h, k, n)** normoglycaemic and **(c, f, i, l, o)** type 2 diabetic participants, or **(d, g, j, m, p)** according to study group as indicated. Additional *TCF7L2* transcript expressions are presented in ESM Fig. 1. Values are mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$

software (GraphPad Software, La Jolla, CA, USA). Significance is indicated by asterisks as follows: * $p<0.05$,

** $p<0.01$ or *** $p<0.001$. We declared statistical significance as given at $p<0.05$.

Results

Expression of several TCF7L2 alternative transcripts is increased in livers of normoglycaemic carriers of the rs7903146 risk allele and in those of type 2 diabetes individuals We identified nine *TCF7L2* homozygous risk allele carriers (TT) in the normoglycaemic group and nine among type 2 diabetes individuals from ABOS; they were compared with individuals of wild-type (CC) and heterozygous (CT) genotypes (Table 2). As previously shown [12], nine major *TCF7L2* transcripts are expressed in the human liver and can be divided into three classes according to the length of their protein C-terminal end (short, medium and long) (Fig. 1a). We examined transcript expression in 64 liver samples using quantitative real-time PCR assays specific to the presence of exon junctions. Several assays measured a combination of *TCF7L2* transcripts with the same exon junction (ESM Fig. 1b).

First, we compared *TCF7L2* expression in type 2 diabetic vs normoglycaemic individuals irrespective of their genotype and differences of age (Mann–Whitney tests) (Fig. 1d, g, j, m, p). Total *TCF7L2* expression was similar in type 2 diabetic vs normoglycaemic individuals (exons 7 to 8; including transcription start site [TSS] 1 to 3 transcripts). *TCF7L2* transcripts with TSS1 showed a significant increase in type 2 diabetic individuals compared with normoglycaemic individuals, as did transcripts containing exons 13–14, 13–17, 12–14 and 14–17 (Fig. 1m, p, j, ESM Fig. 1y); this represents all transcripts, except T1 and T6. Alternative C-terminal transcripts containing exons 12–15 (T6), 12–17 (T1) and 14–15 (T3, T5) may have had slightly decreased expression, but were not significantly different expressed in type 2 diabetes vs normoglycaemic individuals (ESM Fig. 1t, w, z). Linear regression analysis of *TCF7L2* expression in type 2 diabetes vs normoglycaemic individuals, with age, BMI and sex as covariates, showed that the observed differences were only significant ($p < 0.05$) when excluding samples from homozygous risk allele carriers (TT), in whom expression was similar in both groups. The combination of the observed exon junction expression values suggests that in type 2 diabetes individuals expression is increased in five of nine *TCF7L2* transcripts (T2, T7, T8, T4 and T9).

Next, we compared *TCF7L2* expression in relation to the presence of the rs7903146 risk allele in the normoglycaemic and type 2 diabetes groups (Fig. 1, ESM Fig. 1). In normoglycaemic individuals carrying two rs7903146 risk alleles, the overall *TCF7L2* (exon 7–8) assay, as well as eight alternative transcript assays, showed significantly increased expression (Fig. 1e, h, k, n, ESM Fig. 1l, r, u, x, α). Analysis of *TCF7L2* expression in a linear regression model, with rs7903146 genotype, age, BMI and sex as covariates, generated similar results ($p < 0.05$). In livers of type 2 diabetes individuals, where expression of *TCF7L2* was increased on average compared with normoglycaemic individuals, the

expression of splice variants was similar in all genotypes (Fig. 1c, f, i, l, o, ESM Fig. 1j, m, p, s, v, y, β). These results suggest that having type 2 diabetes or carrying the rs7903146 risk allele is associated with increased liver *TCF7L2* transcript expression, but that individuals carrying two risk alleles have higher expression levels, regardless of whether they suffer from diabetes or not.

Glucose and insulin affect TCF7L2 splice variant expression in HepG2 cells

To analyse whether the liver *TCF7L2* transcripts correlating with *TCF7L2* rs7903146 and type 2 diabetes also play a role in liver glucose metabolism, their expression was examined in human liver HepG2 cells. First, we established that this cell line is heterozygous for rs7903146 (ESM Fig. 2a). The induction of liver pyruvate kinase mRNA expression by glucose showed that genes related to glucose metabolism can indeed be induced in glucose-depleted HepG2 cells (data not shown). When incubated in the presence of 100 nmol/l insulin and either 1, 5 or 11 mmol/l glucose for 24 h, the full-length *TCF7L2* transcript showed significantly increased expression compared with incubation in 1 mmol/l glucose alone (TSS1, exon 7–8) (Fig. 2a, b), (exon 11–12) (ESM Fig. 2b). A similar induced expression pattern was observed for alternative *TCF7L2* transcripts with exon junctions 4–5 (representing T1, T2, T3) (ESM Fig. 2c), 12–14 (T8) (Fig. 2c), 12–17 (T1) (Fig. 2d), 12–15 (T6) (ESM Fig. 2d) and 14–17 (T4, T8, T9) (ESM Fig. 2e). In addition, the expression of *TCF7L2* transcripts with exon junctions 14–15 (T3, T5) (Fig. 2f), 13–17 (T2, T7) (ESM Fig. 2e) and 13–14 (T3, T5, T4, T9) (ESM Fig. 2f) was increased in a glucose dose-responsive manner (all major transcripts with exon 13). Since changes in mRNA expression do not always correlate with protein expression, we also performed western blot analysis (Fig. 2g, h). Levels of *TCF7L2* isoforms with a molecular mass of 58 and 78 kDa were increased by the presence of 100 nmol/l insulin upon incubation of HepG2 cells with 1 or 5 mmol/l glucose. At 11 mmol/l glucose incubation, protein expression was increased in the 58 and 78 kDa isoforms, compared with incubation at 1 and 5 mmol/l glucose (Fig. 2g), and we also observed weak induction of additional ~65 kDa *TCF7L2* proteins (Fig. 2g, h). Our results show that *TCF7L2* mRNA and *TCF7L2* protein expression is increased in HepG2 cells by incubation with high glucose and insulin.

TCF7L2 represses a large set of genes that regulate gluconeogenesis

To further analyse *TCF7L2*'s role in liver glucose metabolism, we decreased *TCF7L2* expression in HepG2 cells by siRNA and analysed the mRNA expression of 84 genes related to glucose metabolism (SABioscience QPCR pathway-focused array PAHS006, Qiagen Courtaboeuf, France). HepG2 cells were cultured either with 1 mmol/l glucose without insulin (with siRNA negative control or *TCF7L2*) or with 11 mmol/l glucose with 100 nmol/l insulin

Fig. 2 Expression of *TCF7L2* transcripts in HepG2 cells cultured with different concentrations of glucose with or without insulin. **(a–f)** *TCF7L2* transcript expression of HepG2 cells cultured for 48 h in DMEM supplemented with 0.1% BSA in the presence of 1, 5 or 11 mmol/l glucose, with (black bars) or without (white bars) 100 nmol/l insulin. mRNA expression of *TCF7L2* transcripts containing the indicated exon junctions is given as arbitrary units relative to the housekeeping gene *RPLP0* and to the relevant transcript expression in HepG2 cells cultured with 1 mmol/l glucose. The expression of additional *TCF7L2* transcripts is presented in ESM Fig. 2b–f. Values are mean $\pm$ SEM * p <0.05, ** p <0.01 and *** p <0.001. **(g, h)** Representative western blots of total cell lysate from HepG2 cells cultured as above **(a–f)**

(with siRNA negative control or *TCF7L2*). As expected, in HepG2 cells cultured with insulin the expression of gluconeogenesis-stimulating genes (e.g. *G6PC*, *PCK1*) was lower and that of genes linked to glycolysis (e.g. *PFKL*, *PKLR*) higher than when incubated with glucose alone (ESM Fig. 3a). In HepG2 cells incubated with 1 mmol/l glucose, 29 genes related to glucose metabolism showed a ± 1.5 -fold change in expression when *TCF7L2* was downregulated by siRNA (ESM Fig. 3b, c). We also analysed the expression of *G6PC* and *PCK1* in *TCF7L2* siRNA-transfected HepG2 cells incubated with 1, 5 or 11 mmol/l glucose, with or without 100 nmol/l insulin (Fig. 3). As previously described for *G6PC* [29], we observed a dose-dependent increase of mRNA expression by glucose, which was decreased by insulin (Fig. 3a, c). siRNA-mediated downregulation of *TCF7L2* expression significantly increased expression of *G6PC* and *PCK1*, independently of insulin (Fig. 3b, d).

Glucose-6-phosphatase gene expression is repressed by TCF7L2 independently of FOXO1 Expression of *G6PC* is

regulated by FOXO1 and HNF4α [30], and the β-catenin–FOXO1 complex stimulates *G6PC* only in the absence of insulin [24]. We observed that in HepG2 cells with reduced *TCF7L2* expression, *G6PC* mRNA expression was increased independently of the presence or absence of insulin (Fig. 3b, d), suggesting that the regulation of *G6PC* by *TCF7L2* may be independent of FOXO1. This prompted us to analyse *FOXO1* and *HNF4A* expression in our experimental conditions. *FOXO1* mRNA expression was unchanged when *TCF7L2* was downregulated by siRNA transfection (Fig. 4a). Moreover, although *TCF7L2* may bind to distant enhancer elements of *HNF4A* to regulate its expression [31], *HNF4A* mRNA expression was not altered by *TCF7L2* siRNA in our study (Fig. 4a). To further characterise the effect of *TCF7L2* on *G6PC* expression, we measured *G6PC* promoter activity by using luciferase reporter constructs previously used to analyse the synergistic activation of HNF4α and FOXO1 [30]. In HepG2 cells treated with *TCF7L2* siRNA, both the wild-type *G6PC* promoter and the *G6PC* promoter construct lacking the FOXO1 binding region (*G6PC*Δ123) showed a

Fig. 3 mRNA expression of gluconeogenesis genes *G6PC* and *PCK1* in HepG2 cells after downregulation of *TCF7L2* transcripts. HepG2 cells were transfected with negative control (N.C.) or *TCF7L2* siRNA and incubated in medium containing 1, 5 or 11 mmol/l glucose alone (white bars), or 100 nmol/l insulin (black bars). After 48 h the expression of *TCF7L2*, *G6PC* and *PCK1* was measured by quantitative real-time PCR Taqman assays. Values are mean expression $\pm$ SEM of (a) *G6PC* and (c) *PCK1* given as arbitrary units relative to the housekeeping gene *RPLP0* and to their own expression in HepG2 cells cultured with 1 mmol/l glucose (fold change). (b) Expression of *G6PC* and (d) *PCK1* in HepG2 cells transfected with siRNA *TCF7L2*. Values are mean expression $\pm$ SEM given as arbitrary units relative to the housekeeping gene *RPLP0* and to the expression of *G6PC* and *PCK1*, respectively, in cells transfected with negative control siRNA cultured in the same medium. The fold change in *TCF7L2* expression was between 0.20 and 0.27 (data not shown). * $p < 0.05$, ** $p < 0.01$

similar increase in luciferase activity (Fig. 4b), confirming that FOXO1 is not involved in TCF7L2-mediated repression of *G6PC* expression. Analysis of HNF4 α protein levels in these cell lysates revealed an increase of HNF4 α protein upon downregulation of *TCF7L2* by siRNA transfection (data not shown). In additional experiments, we confirmed that *TCF7L2* downregulation increases the accumulation of nuclear HNF4 α protein in HepG2 cells cultured with 1, 5 and 11 mmol/l glucose (with or without insulin) (Fig. 4c). Since in our HepG2 culture conditions, the transcription of *HNF4A* was not altered by TCF7L2, these data suggest that TCF7L2 may interfere directly with HNF4 α protein expression.

Two specific TCF7L2 isoforms interact with HNF4 α protein No direct TCF7L2 binding on the *G6PC* promoter region was reported in ChIP binding studies [21, 31–33]. Therefore, we hypothesised that TCF7L2 could regulate *G6PC* transcription by interacting with HNF4 α protein. We

immunoprecipitated HNF4 α protein from whole-cell lysates to analyse a potential interaction with TCF7L2. After immunolabelling western blots with TCF7L2 antibodies, we observed a weak signal that suggested interaction of endogenous TCF7L2 proteins with HNF4 α (data not shown). We then transfected HepG2 cells with the nine different TCF7L2 isoforms in the presence of 5 mmol/l glucose and 100 nmol/l insulin, and immunoprecipitated HNF4 α from whole-cell lysates (ESM Fig. 4). The co-immunoprecipitation assay shows that solely the long C-terminal TCF7L2 isoforms T6 and T8 co-immunoprecipitated with HNF4 α . Next, we analysed T6-transfected HepG2 cells incubated with 1, 5 or 11 mmol/l glucose, and with or without insulin, conditions where endogenous T6 mRNA (represented by exons 12–15 assays) was also increased (Fig. 2a). The western blot analyses of the co-immunoprecipitation assays show that the interaction of TCF7L2 with HNF4 α was more pronounced in the presence of insulin and at higher glucose concentrations (Fig. 5b), suggesting this represents a relevant control mechanism in liver glucose metabolism.

Discussion

We report a significant correlation between increased expression of alternative *TCF7L2* liver transcripts and the type 2 diabetes-associated rs7901346 risk allele in obese normoglycaemic individuals. Such a correlation has also been found in other tissues important for glucose homeostasis (e.g. pancreas and adipose tissue [8, 9, 25]), but a previous analysis in human liver samples [10] included fewer homozygous rs7901346 risk allele carriers and thus may have been underpowered to observe this correlation. Statistically, our study has a small sample size. Thus a larger size with a more stringent threshold for statistical significance may be needed to guarantee solid evidence against the null hypotheses. The observed association between the rs7901346 risk allele and increased TCF7L2 liver expression in obese normoglycaemic individuals was not observed in obese diabetic individuals. Our study suggests that having type 2 diabetes is associated with increased liver *TCF7L2* transcript expression, although the functional causes of this association could not be assessed in our cross-sectional study. Another weakness is that we matched individuals within the normoglycaemic and diabetic groups, and were limited by the number, phenotypic characteristics of homozygous rs7901346 risk allele carriers, and individual diversity of the volunteers. We were unable to determine whether the regulation of TCF7L2 expression in livers of diabetic patients involved different mechanisms related to hyperglycaemia or insulin resistance, or whether the higher TCF7L2 expression levels were a consequence of other confounding factors such as differences in medication of type 2 diabetic individuals.

Fig. 4 Effects of *TCF7L2* downregulation on *G6PC* regulation. **(a)** HepG2 cells were transfected with negative control (N.C.) or *TCF7L2* siRNA, and incubated with 1, 5 or 11 mmol/l glucose, without insulin (white bars) or with 100 nmol/l insulin (black bars). After 48 h RNA was isolated, and the fold change in expression of the *TCF7L2* and the *G6PC* transcription regulators, *HNF4A* and *FOXO1*, measured in each experiment by quantitative real-time PCR Taqman assays. Values are mean expression $\pm$ SEM represented as arbitrary units relative to *RPLP0* and to expression of the relative genes in cells transfected with negative control siRNA cultured in the same medium. **(b)** HepG2 cells cultured in DMEM supplemented with 0.1% BSA and with 1 mmol/l glucose

(without insulin) were transfected with negative control (white bars) or *TCF7L2* siRNA (black bars), and either the luciferase reporter construct pGVB2-empty, pGVB2-G6Pase-Wt (G6PC) or pGVB2-G6Pase-ΔIRS123. Mean relative luciferase activity (RLU) $\pm$ SEM measured after 48 h was calculated as per cent of negative control siRNA ($n=3$). * $p<0.05$. **(c)** Representative western blots of nuclear extract proteins from HepG2 cells cultured for 48 h in DMEM supplemented with 0.1% BSA in the presence of 1, 5 or 11 mmol/l glucose with (+) or without (–) 100 nmol/l insulin, and immunolabelled with goat anti-*TCF7L2*, mouse anti-HNF4α or rabbit anti-tubulin

Recent reports have shown that mouse *TCF7L2* expression is insulin-responsive [20, 22] and that in insulin-resistant mice expression of the short and medium C-terminal transcripts (e.g. including T3 and T5) was reduced [21]. Our data on human liver transcript expression show that the expression of five liver *TCF7L2* transcripts (T2, T4, T7, T8, T9) was increased in type 2 diabetic compared with normoglycaemic individuals. Furthermore, both short and long C-terminal *TCF7L2* transcripts containing exon 13 (T2, T3, T4, T5, T7 and T9) were increased in a glucose dose-responsive manner in human HepG2 cells. Owing to the similar molecular masses of *TCF7L2* protein isoforms, it is not clear which specific isoform is mainly induced by incubation of HepG2 cells with insulin. Indeed, our data may indicate that in type 2 diabetic individuals mRNA expression of the short C-terminal transcripts T3 and T5 in the liver is dysregulated, as expression of these transcripts was induced by high glucose in HepG2 cells, but not upregulated by high glucose levels in type 2 diabetic individuals.

Results from our siRNA experiments in HepG2 cells show that *TCF7L2* inhibits a substantial number of genes involved in the human liver gluconeogenesis pathway, including the gene encoding the rate-limiting *G6PC* enzyme. Transcription of some genes may be directly regulated by *TCF7L2* binding to their promoter and/or enhancer sequences, as has been reported for *PCK1* and *GYS2*, while others (*G6PC*) have not been reported to have *TCF7L2* recognition sites [21, 31–33]. Therefore, we analysed the role of *TCF7L2* in the HNF4α–FOXO1-mediated transcriptional regulation of *G6PC*. Under our culture conditions, *TCF7L2* affected HNF4α protein levels independently of any transcriptional regulation, suggesting a complex interaction with this major hepatic transcriptional regulator. Moreover, we report for the first time that solely *TCF7L2* protein isoforms T6 and T8 interacted directly with HNF4α protein in human HepG2 cells cultured in 5 mmol/l glucose with 100 nmol/l insulin. The *TCF7L2* isoform T6 contains the unique C-clamp sequence CRALF, while other isoforms (T3, T5, T8, T4 and T9) contain the

Fig. 5 Co-immunoprecipitation of TCF7L2 with HNF4α in HepG2 cells. **(a)** Schematic representation of the alternative TCF7L2 isoforms used, with the regions encoded by exons 12, 13, 14, 15 and 17. The different amino acids of the CRA/R/LF domain are highlighted in white. **(b)** HepG2 cells were cultured in the presence of 1, 5 or 11 mmol/l

glucose, either with (w) or without (w/o) 100 nmol/l insulin, and transfected with TCF7L2 construct T6. After 48 h, proteins were immunoprecipitated (IP) from the total cell lysate with either rabbit control IgG or rabbit anti-HNF4α IgG. Western blots of protein samples were immunolabelled with goat anti-TCF7L2 or mouse anti-HNF4α

CRARF C-clamp domain (Fig. 5a). This C-clamp sequence does not alter the DNA-binding capacity of TCF7L2, but may affect its transcriptional activity [13, 34]. From the nine major human liver transcripts analysed by us [12], the long isoforms T6 and T8 are the only C-clamp-containing proteins that lack exon 13, suggesting that this combination may permit HNF4α interaction. Protein sequence analysis predicted no domains/motifs encoded by exon 13. Three other short isoforms containing the C-clamp domain without exon 13 have been reported (T-cell factor 4 alias of TCF7L2 [TCF4]S1, TCF4S4 and TCF4S6 [13]), although they were not expressed in mouse liver tissue. These long and short transcripts were assessed by exon-junction 12–15 and 12–14 expression assays. The corresponding mRNA transcript expression levels slightly increased in HepG2 cells cultured in the presence of a high insulin concentration; however, this did not occur in a glucose-responsive manner as seen in exon 13-containing transcripts. This reveals a

new and distinct role of particular C-terminal TCF7L2 isoforms in the pathogenic mechanisms leading to type 2 diabetes.

In conclusion, human alternative C-terminal *TCF7L2* transcripts are induced in HepG2 cells by high glucose and insulin, and in liver of normoglycaemic individuals carrying the rs7901346 type 2 diabetes risk allele, as well as in diabetic individuals compared with normoglycaemic individuals (independently of their genotype). This suggests that these liver transcripts may play a role in the pathophysiology of type 2 diabetes. Two of the induced alternative mRNA transcripts encode TCF7L2 isoforms that bind to HNF4α and therefore may interfere with the transcriptional regulation of gluconeogenesis genes by HNF4α. These results underscore the importance of studying the regulation and function of alternative *TCF7L2* transcripts with a view to characterising the role of TCF7L2 in the pathogenic mechanisms leading to type 2 diabetes.

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