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Multiple Functional Polymorphisms in the *G6PC2* Gene Contribute to the Association with Higher Fasting Plasma Glucose Levels

D. A. Baerenwald[1,*], **A. Bonnefond**[2,3,*], **N. Bouatia-Naji**[2,3,4,*], **B. P. Flemming**[1], **O. C. Umunakwe**[1], **J. K. Oeser**[1], **L. D. Pound**[1], **N. L. Conley**[1], **S. Cauchi**[2,3], **S. Lobbens**[2,3], **E. Eury**[2,3], **B. Balkau**[5,6], **O. Lantieri**[7], **MAGIC Investigators**[8], **P. K. Dadi**[1], **D. A. Jacobson**[1], **P. Froguel**[2,3,9,§], and **R. M. O'Brien**[1,§]

[1]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, 37232 Nashville, Tennessee, USA

[2]CNRS-UMR-8199, Institut Pasteur de Lille, F-59019, Lille, France

[3]University Lille Nord de France, F-59019 Lille, France

[4]INSERM U970, Paris Cardiovascular Research Center PARCC, 56 rue Leblanc, F-75015 Paris, France

[5]INSERM, Centre for research in Epidemiology and Population Health (CESP), U1018, Epidemiology of diabetes, obesity and chronic renal disease over the lifecourse, F-94807, Villejuif, France

[6]Université Paris-Sud 11, UMRS 1018, F-94807 Villejuif, France

[7]Institut inter-régional pour la santé (IRSA), F-37521 La Riche, France

[8]Meta-Analysis of Glucose and Insulin related traits Consortium Investigators (http://www.magicinvestigators.org/)

[9]Department of Genomics of Common Disease, School of Public Health, Imperial College London, W12 0NN London, UK

[§]Correspondence should be addressed to either P.F. at Department of Genomics of Common Disease, School of Public Health, Imperial College London, W12 0NN London, UK (p.froguel@imperial.ac.uk) or R.O'B at Department of Molecular Physiology and Biophysics, 8415 MRB IV, 2213 Garland Ave, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, USA (richard.obrien@vanderbilt.edu).

[*]These authors contributed equally to these studies.

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Author Contributions

D. B. performed gel retardation, fusion gene and minigene analyses and drafted parts of the manuscript.
B. F., O. U., J. O., L. P., and N. C. performed gel retardation, fusion gene and minigene analyses and revised the manuscript.
A.B. and N.B.N. designed and performed genetic analyses and drafted parts of the manuscript.
S. C., S. L. and E. E. contributed to the genetic analyses and revised the manuscript.
B.B. and O.L. managed the D.E.S.I.R. study, designed and performed genetic analyses, and revised the manuscript.
P. D. and D. J. designed and performed isolated islet studies and revised the manuscript.
P. F. was responsible for the conception of the genetic analyses described in this manuscript and revised the manuscript.
R. O'B. was responsible for the conception of the molecular studies and drafted parts of the manuscript.
All authors approved the final version.

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Abstract

Aims—We previously identified the *G6PC2* locus as a strong determinant of fasting plasma glucose (FPG) and showed that a common *G6PC2* intronic single nucleotide polymorphism (SNP) (rs560887) and two common *G6PC2* promoter SNPs (rs573225 and rs13431652) are highly associated with FPG. However, these promoter SNPs have complex effects on *G6PC2* fusion gene expression, and our data suggested that only rs13431652 is a potentially causative SNP. Here we examine the effect of rs560887 on *G6PC2* pre-mRNA splicing and the contribution of an additional common *G6PC2* promoter SNP, rs2232316, to the association signal.

Methods—Mini-gene analyzes characterized the effect of rs560887 on *G6PC2* pre-mRNA splicing. Fusion gene and gel retardation analyses characterized the effect of rs2232316 on *G6PC2* promoter activity and transcription factor binding. The genetic association of rs2232316 with FPG variation was assessed using regression adjusted for age, gender and body mass index in 4,220 Europeans with normal FPG.

Results & Conclusions—The rs560887-G allele was shown to enhance *G6PC2* pre-mRNA splicing while the rs2232316-A allele enhanced *G6PC2* transcription by promoting Foxa2 binding. Genetic analyses provide evidence for association of the rs2232316-A allele with increased FPG (β=0.04 mmol/l; $P=4.3\times10^{-3}$) as part of the same signal as rs560887, rs573225 and rs13431652. As with rs13431652 the *in situ* functional data with rs560887 and rs2232316 are in accord with the putative function of *G6PC2* in pancreatic islets and suggest that all three are potentially causative SNPs that contribute to the association between *G6PC2* and FPG.

Keywords

beta cell; fasting plasma glucose; *G6PC2/IGRP*; pancreatic islet; splicing; transcription

Introduction

The glucose-6-phosphatase catalytic subunit gene family comprises three members, *G6PC*, *G6PC2* and *G6PC3* [1]. All three catalyze the hydrolysis of glucose-6- phosphate to glucose and inorganic phosphate, though with markedly different kinetics [1]. The *G6PC2* gene, also known as *IGRP* [2–4], is principally expressed in the beta cells of pancreatic islets [5]. Recent genome-wide association studies (GWAS) in humans have identified genetic association signals in and near *G6PC2* that are associated with variations in fasting plasma glucose (FPG) and glycated hemoglobin A1C (HbA1c) levels [6, 7]. FPG and HbA1c are important metabolic traits that are correlated with the risk of developing type 2 diabetes [8–10] and cardiovascular-associated mortality [11–13]. Multiple other studies have confirmed that the *G6PC2* locus harbors the strongest common genetic determinant of FPG levels in terms of significance and effect size, particularly with a common single nucleotide polymorphism (SNP), rs560887, that is located in the third intron of *G6PC2*, and that explains ~1% of the total variance in FPG [14–18]. These genetic data are consistent with the ~15% decrease in FPG observed following a global knockout of *G6pc2* in mice [4] and support the hypothesis that genetic variation within the *G6PC2* gene, rather than surrounding genes, directly contribute to variations in FPG in humans. These findings

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suggest that G6PC2 may regulate FPG by opposing the action of glucokinase, thereby modulating beta cell glycolytic flux and glucose-stimulated insulin secretion [19].

A key step in extending the results of these GWAS is establishing a functional link between specific common SNPs in the *G6PC2* locus and variations in FPG. In a previous study, we demonstrated that two common *G6PC2* promoter SNPs, rs13431652 and rs573225, are associated with FPG levels as part of the same association signal as rs560887 [20]. For SNP rs13431652, the A-allele was associated with increased FPG and elevated promoter activity, consistent with the putative function of G6PC2 in pancreatic islets [20]. In contrast, for SNP rs573225, the A-allele was associated with increased FPG but reduced promoter activity, at odds with the putative function of G6PC2 in pancreatic islets [20]. These data suggested that rs13431652 is a potentially causative SNP whereas rs573225 is a functional SNP that opposes the action of causative SNPs [20]. Because the rs573225-A allele negates the effect of the rs13431652-A allele [20], the data also suggested that the *G6PC2* locus must contain additional causative SNPs. The goal of the experiments described here was to identify potentially causative SNPs using a combination of genetics and molecular studies. Specifically we assessed the potential of the intronic SNP rs560887 to alter *G6PC2* RNA splicing. In addition, we examined the contribution of an additional common *G6PC2* promoter SNP, rs2232316, to the association signal as well as its effect on transcription factor binding and *G6PC2* fusion gene expression.

Materials and Methods

Study participants

The Data from the Epidemiological Study on the Insulin Resistance Syndrome (D.E.S.I.R.) cohort is a longitudinal French general population cohort and is fully described elsewhere [6]. We analyzed 4,220 D.E.S.I.R. participants with normal FPG (defined as FPG <6.1mmol/l without hypoglycaemic treatment) who were successfully genotyped. The study protocol was approved by the ethics committee of Bicêtre Hospital (Paris, France).

We also used publicly available data sets from a meta-analysis of European GWAS for FPG levels, which was performed by the MAGIC consortium (Meta-Analysis of Glucose and Insulin related traits Consortium (http://www.magicinvestigators.org), in a total of 46,186 non-diabetic participants [17].

A full list of D.E.S.I.R. and MAGIC investigators is provided in the Electronic Supplementary Material (ESM).

Genotyping of SNP rs2232316

Genotyping of SNP rs2232316 was performed using a custom designed iSELECT array (Metabochip, Illumina; ref#: chr2:169465808 [NCBI36/hg18]), according to the manufacturer's instructions. This array contains ~200,000 SNPs and was developed to support large-scale follow-up of putative associations for metabolic traits and fine-mapping studies of GWAS-identified loci [21]. The genotyping success rate of SNP rs2232316 was 99%. No significant deviation (P>0.05) from Hardy-Weinberg equilibrium was observed. Genotyping of SNPs rs560887 and rs573225 was previously reported [6, 20].

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Minigene construction and splicing analyses

Minigenes were constructed in the RHCglo minigene vector, a generous gift from Dr. T. Cooper (Baylor College of Medicine) [22]. This vector contains the Rous sarcoma virus (RSV) promoter, a 5' exon with a splice junction, an artificial exon surrounded by intron sequences from the human beta-globin gene and a 3' exon with a splice junction (Fig. 1A) [22]. The artificial exon and surrounding human beta-globin gene intron sequences were replaced by *G6PC2* exon 4 and ~300 bp of surrounding intronic sequences. Two minigenes were constructed containing the alternate alleles of rs560887.

These minigenes were expressed in tissue culture cells using transient transfection with RNA isolated 24 hrs post-transfection. cDNA was then generated using reverse transcriptase in conjunction with a primer complementary to the 3' vector exon (Fig. 1A; primer B). Using primer A in combination with primer B (Fig. 1A) splicing between the vector 5' and 3' exons and the inserted *G6PC2* exon was assessed. PCR reactions were performed under conditions where amplification was in the linear range (ESM Fig. 1) and the products (X and Y; Fig. 1A) were then resolved on nondenaturing polyacrylamide gels as described [23]. DNA sequencing confirmed the identity of these products and the presence of the predicted splice junctions. See ESM for a detailed description.

Fusion gene plasmid construction

The originally isolated *G6PC2* promoter contained the rs2232316-G and rs573225-A alleles [3]. The construction of a human *G6PC2*-luciferase fusion gene containing promoter sequence from −324 to +3, has been previously described [20]. A three step PCR strategy [24] was used to introduce the alternate rs2232316-A and/or rs573225-G alleles into the human *G6PC2* promoter within the context of the −324 to +3 promoter fragment. Promoter fragments generated by PCR were completely sequenced to ensure the absence of polymerase errors. All plasmid constructs were purified by centrifugation through cesium chloride gradients.

Cell culture, transient transfection and luciferase assays

Hamster insulinoma tumor (HIT), βTC-3 and Min6 cells were cultured and transfected with various *G6PC2-luciferase* fusion genes in the pGL3 MOD vector (2 μg) and an expression vector encoding *Renilla* luciferase (0.5 μg) using lipofectamine as previously described [3, 25]. Following transfection, cells were incubated for 18–20 hr in serum-containing medium. The cells were then harvested and firefly and *Renilla* luciferase activity were assayed as previously described [25].

Gel retardation assays

The analysis of Foxa2 binding using gel retardation assays was performed exactly as previously described [20]. See ESM for a detailed description.

Statistical analysis

Genetic studies: the effect of SNPs on FPG levels was assessed using linear regression models adjusted for age, sex and body mass index (BMI) or for age, sex, BMI and other

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G6PC2 SNPs (rs573225 and/or rs560887) in conditional regression, under the additive model. All statistical analyses of genetic data were performed with SPSS software (version 14.0). Of note, the MAGIC consortium used linear regressions adjusted for age and sex only [17].

Functional studies: the transfection data were analyzed for differences from the control values, as specified in the Figure Legends. Statistical comparisons were calculated using an unpaired Student's t test. The level of significance was $p < 0.05$ (two-sided test).

Results

SNP rs560887 alters *G6PC2* RNA splicing

Previous studies have shown that multiple *G6PC2* splicing isoforms exist in human islets, with a variant that lacks exon 4 being the most prominent [3, 26]. SNP rs560887 is located 26 nucleotides 5' of *G6PC2* exon 4. A minigene strategy, in which splicing of individual exons can be assessed [27, 28], was used to address the hypothesis that rs560887 affects *G6PC2* RNA splicing (Fig. 1A). Figure 1B shows that the alternate alleles of rs560887 alter the relative abundance of splicing products X and Y (Fig. 1A), with the rs560887-G allele promoting improved splicing, specifically the inclusion of *G6PC2* exon 4. This is consistent with the genetic association between the rs560887-G allele, elevated FPG and the putative function of G6PC2, suggesting that rs560887 is a potentially causative SNP.

The genetic variant rs2232321 also alters *G6PC2* RNA splicing

Another genetic variant, rs2232321, is located immediately downstream of rs560887 and we hypothesized that this variant might also affect splicing. Using the same minigene strategy as described above, Figure 2 shows that the alternate alleles of rs2232321 alter the relative abundance of products X and Y, with the rs2232321-G allele promoting inclusion of *G6PC2* exon 4, though the magnitude of the effect was smaller than seen for rs560887 (compare Fig. 1B and Fig. 2).

The effects of rs560887 and rs2232321 are dependent on the non-consensus exon 4 splice junction

We have previously shown that in both mice and humans the 5' exon 4 splice junction contains the non-consensus sequence tag/A [3, 29] instead of cag/G [30] (intronic sequence in lower case letters). We hypothesized that the presence of this non-consensus splice junction may result in splicing being more susceptible to the influence of rs560887 and rs2232321. This hypothesis was tested using the same minigene approach as discussed above. Figure 3 shows that when the sequence of the non-consensus 5' exon 4 splice junction is converted to tag/G the inclusion of *G6PC2* exon 4 is markedly enhanced, despite the presence of the sub-optimal rs560887-A allele. This indicates that the influence of the alternate alleles of rs560887 and rs2232321 on *G6PC2* RNA splicing is, quantitatively, strongly dependent on the presence of the non-consensus exon 4 splice junction.

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SNP rs2232316 alters *G6PC2* fusion gene expression in βTC-3 cells

We previously demonstrated that the −306 to +3 region of the mouse *G6pc2* promoter confers high fusion gene expression in islet-derived cell lines [24, 29, 31] and is sufficient to initiate transgene expression in mouse islets, predominantly in beta cells, at the expected time in development, around embryonic day 14 [32]. In addition, this region is highly conserved in the human *G6PC2* promoter [3], which likewise confers high fusion gene expression in islet-derived cell lines (ESM Fig. 2), though not in HeLa or hepatoma cells [33]. To identify potentially causative SNPs that affect *G6PC2* transcription we therefore focused on this promoter region. A common SNP in this region, rs2232316, located at −238, relative to the transcription start site [29], lies within a conserved stretches of nucleotides that have the potential to represent an important *cis*-acting element [3]. We therefore investigated whether rs2232316 can affect *G6PC2* fusion gene expression. Figure 4 shows the analysis, by transient transfection of islet-derived βTC-3, HIT and Min6 cells, of fusion genes containing each of the rs2232316 alleles, generated in the context of the −324 to +3 *G6PC2* promoter region. The data show that the rs2232316-A allele was associated with an approximately 50–100% increase in promoter activity in comparison to that observed with the rs2232316-G allele (Fig. 4).

SNP rs2232316 alters Foxa2 binding to the *G6PC2* promoter *in vitro*

We next investigated whether the alternate alleles of rs2232316 affected transcription factor binding. The *G6PC2* promoter region that encompasses rs2232316 was analyzed using MatInspector sequence analysis software [34] with the goal of identifying a *cis*-acting element whose binding of its cognate *trans*-acting factor was likely to be affected by the alternate rs2232316 alleles. This analysis identified a forkhead transcription factor binding motif [35].

While several members of the Fox family are expressed in the pancreas [35], members of the Foxa sub-group have been shown to play important roles in islet development and function [36]. Gel retardation assays were used to investigate whether any of the three Foxa isoforms [36] can bind to this *G6PC2* promoter region *in vitro*. When a labelled double-stranded oligonucleotide, designated rs2232316-A, representing the *G6PC2* promoter sequence from −250 to −226 and containing the rs2232316-A allele (Fig. 5A), was incubated with nuclear extract prepared from βTC-3 cells a single protein-DNA complex was detected (Fig. 5B; lanes 1 & 4). To identify the factor present in this complex a gel retardation assay was performed in which βTC-3 cell nuclear extract was pre-incubated with antisera specific for Foxa2 (lane 2) or, as a control, USF-2 (lane 3). Previous studies have shown that Foxa2 is the predominant Foxa isoform in βTC-3 cells [25]. As can be seen in Figure 5B (arrow), addition of antibodies recognizing Foxa2 resulted in a clear supershift in the migration of the complex whereas addition of antibodies recognizing USF-2 had no effect. This result strongly suggests that the complex represents Foxa2 binding.

Gel retardation competition experiments, in which a varying molar excess of unlabelled oligonucleotide was included with the labelled rs2232316-A oligonucleotide, were used to compare the affinity of Foxa2 binding to the rs2232316 variants of this *G6PC2* Foxa2 binding site. Figure 6 (Panels A & B) shows that the rs2232316-A oligonucleotide competed

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effectively for the formation of the Foxa2-DNA complex whereas the rs2232316-G oligonucleotide did not. Consistent with the competition experiment data (Fig. 6A & B), the direct analysis of Foxa2 binding to the rs2232316-A and -G oligonucleotide probes, labelled with the identical specific activity, showed a dramatic difference in Foxa2 binding affinity (Fig. 6C). Interestingly, the original consensus sequence for Foxa2 binding (Fig. 5A; Ref. [37]) indicates that either an 'A' or a 'G' nucleotide at the location of the rs2232316 SNP will support Foxa2 binding, but clearly, for this particular Foxa2 binding site, this is not the case (Fig. 6C). This consensus was established using a sequential selection and amplification of binding sites protocol [37]. In contrast, an analysis of endogenous Foxa2 binding sites based on ChIP-Seq data [38] indicates that an 'A' nucleotide is preferred at the location of the rs2232316 SNP *in vivo*, consistent with the binding data (Fig. 6). The preference for the 'A' nucleotide was evident under multiple distinct assay conditions (Fig. 6C).

SNP rs2232316 does not influence the effect of SNP rs573225 on *G6PC2* fusion gene expression in βTC-3 cells

We recently showed that the alternate alleles of rs573225 also alter Foxa2 binding to the *G6PC2* promoter *in vitro* [20]. The Foxa2 binding sites that encompass rs573225 (−261/−250) and rs2232316 (−246/−235) are located 3 nucleotides apart in the *G6PC2* promoter [3] and this region is highly conserved in the mouse *G6pc2* promoter [3]. Because the Foxa2 binding sites that encompass rs573225 and rs2232316 are so close we hypothesized that Foxa2 binding at one element may modulate the action of Foxa2 at the other. The interaction between these SNPs on *G6PC2* fusion gene expression was therefore investigated (Fig. 7). The results show that the rs573225-G allele and the rs2232316-A allele have an additive and, therefore, apparently independent effect on fusion gene expression (Fig. 7).

SNP rs2232316 is associated with FPG levels but not independently from SNPs rs573225 or rs560887

We next assessed the association of SNP rs2232316 with FPG levels in 4,220 individuals with normal FPG (<6.1 mmol/l) from the French D.E.S.I.R cohort. We found that the minor rs2232316-A allele associates with increased FPG levels (β=0.040 mmol/l, $P=4.3\times10^{-3}$) (Table 1). By using publicly available data sets from the MAGIC consortium, we found that the association between the rs232316-A allele and increased FPG levels was also highly significant in 46,186 non-diabetic participants (β=0.04 mmol/l, $P=7.3\times10^{-10}$) [17].

Conditioned regression model analyses in D.E.S.I.R. to assess the independency from SNPs rs573225 or rs560887 showed a lack of significance of rs2232316 when we took into account the effect of rs573225 or rs560887 (P=0.29 and P=0.35, respectively; Table 1), whereas effects of SNPs rs573225 or rs560887 were unchanged (β=0.076 mmol/l, $P=2.7\times10^{-14}$ and β=0.084 mmol/l, $P=6.8\times10^{-17}$respectively; Table 1). Of note, despite a low correlation between rs2232316 and rs573225 or rs560887 (r2=0.066 and r2=0.046, respectively; in D.E.S.I.R.) due to marked differences in frequency of the effect alleles (Table 1), we observed a very high linkage disequilibrium between rs2232316 and rs573225 or rs560887 (D'>0.95) indicating that the rs2232316 association signal with FPG levels is not independent from association signals for SNPs rs573225 or rs560887. When we added

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SNPs rs2232316, rs573235 and rs560887 in the same linear regression model, the effects of SNPs rs2232316 and rs573235 on FPG levels were not significant (P=0.22 and P=0.95, respectively; Table 1), whereas the effect of the rs560887-G allele on increased FPG levels was unchanged (β=0.084 mmol/l, $P=2.0\times10^{-3}$; Table 1). Therefore, the intronic SNP rs560887 seems to drive the association with FPG levels in the D.E.S.I.R. cohort. However, it will be important to repeat these genetic analyses in other populations.

Discussion

Genome-wide association studies have recently provided important new insights about the genetics of common forms of type 2 diabetes and some of its related quantitative traits such as FPG and HbA1c [6, 7, 14–18, 39–42]. The studies described here represent a key step in extending these discovery phase GWAS data through the analysis of the functional properties of variants that span the *G6PC2* locus (ESM Fig. 3). Using minigenes, in which splicing of individual exons can be assessed [27, 28], we show that a common variant, rs560887, located in the third intron of *G6PC2*, modulates *G6PC2* pre-RNA splicing (Fig. 1), as does a rare variant, rs2232321, located immediately 3' of rs560887 (Fig. 2). Interestingly, the functional effect of these variants is absolutely contingent on the presence of a non-consensus splice junction (Fig. 3). In addition, using fusion genes we show that another common variant, rs2232316, affects *G6PC2* promoter activity (Fig. 4) by modulating Foxa2 binding (Fig. 5 & 6). For both common SNPs, rs560887 and rs2232316, the *in situ* functional data are in accord with the putative function of *G6PC2* in pancreatic islets in that the allele associated with elevated *G6PC2* expression is genetically associated with elevated FPG (Table 1; Ref. [6]), indicating that both variants are potentially causative. Thus based on the analysis of *G6pc2* knockout mice [4] and the resulting hypothesis that G6PC2 opposes the action of glucokinase, elevated *G6PC2* expression is predicted to lead to elevated FPG.

This current study, combined with our previous analysis of the rs573225 and rs13431652 promoter SNPs [20], highlights several limitations in identifying causative SNPs. First, because rs560887, rs2232316 and rs13431652 are all part of the same association signal and because of differences between populations [20], we cannot definitely determine whether one or all of these SNPs are truly causative. Second, we would ideally like to obtain additional support for our conclusions through the analysis of endogenous *G6PC2* expression in pancreatic islets, however, previous experience has shown that the limited sample sizes available from human cadavers are insufficient to detect significant correlations [20]. This appears to be because SNPs in the *G6PC2* gene have relatively subtle effects on *G6PC2* expression (Fig. 4; Ref. [20]). This contrasts with similar studies examining SNPs in the *TCF7L2* gene where significant correlations between genotype and islet expression have been detected because these SNPs markedly alter *TCF7L2* gene expression [43, 44]. Finally, as previously noted [20], confidence in the results of promoter SNP analyses is always tempered by the multiple caveats associated with analyzing *G6PC2* fusion gene transcription in islet-derived cell lines. We attempted to analyze the effect of these SNPs on *G6PC2* fusion gene transcription in primary islet cells but expression was below the limit of detection (data not shown). Similarly, we attempted to repeat the splicing analyses in the islet beta cell-derived βTC-3 and HIT cell lines but minigene expression was below the limit

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of detection (data not shown). Instead HeLa cells were used for the splicing analyses due to their high transfection efficiency. As such further studies will be required to demonstrate that results obtained when analyzing promoter and intronic SNPs using cell lines are relevant to their true effects on endogenous gene expression *in vivo*. We can be more confident that rs560887 is a potentially causative SNP, in comparison with the promoter SNPs, because splicing is largely mediated by ubiquitous factors [45, 46] in contrast to *G6PC2* gene transcription, a process mediated by beta cell-enriched transcription factors whose expression may vary between cell lines and islets [1].

The observation that the rs573225-A allele negates the effect of the rs2232316-A (Fig. 7), just as it negates the effect of the rs13431652-A allele [20], supports the concept that rs573225 is a functional SNP that opposes the action of causative SNPs on *G6PC2* expression [20]. This conclusion contrasts with a previous study by Dos Santos et al. [47] who reported that rs573225 was an epiSNP that was likely to explain the association signal between FPG and *G6PC2*. However, in reaching this conclusion the authors apparently overlooked the fact that their *in situ* functional data with rs573225 [47], like ours, do not correlate with the putative function of *G6PC2* in pancreatic islets. Furthermore, as noted above, a clear limitation of our studies, and theirs [47], remains the high LD between all 4 SNPs, which makes it difficult to conclusively establish a causative link between any/all of these variants in the *G6PC2* locus and variations in FPG.

SNP rs573225 has a paradoxical effect on *G6PC2* fusion gene expression. A block mutation in the *G6PC2* Foxa2 binding site that encompasses rs573225 abolishes Foxa2 binding and also markedly reduces fusion gene expression, indicating that Foxa2 acts an activator when bound to this site in the human *G6PC2* promoter [20], as it does when bound to the equivalent site in the mouse *G6pc2* promoter [25]. Since the loss of Foxa2 binding to this site reduces *G6PC2* promoter activity and because the rs573225-G allele decreases Foxa2 binding [20], we anticipated that the rs573225-G allele would also be associated with reduced *G6PC2* promoter activity. Surprisingly, the rs573225-G allele is associated with increased promoter activity (Fig. 7) [20]. The reason for this unexpected result is unknown but Gao et al. [48] have recently confirmed that Foxa factors also act as activators of endogenous *G6pc2* expression.

In higher eukaryotes, pre-mRNA splicing is mediated by multiple *cis*-elements in the pre-mRNA molecule. These elements comprise the branch point sequence, the polypyrimidine tract, the 5′ and 3′ splice sites and exonic/intronic splicing enhancers/silencers [45, 46]. Visual sequence analyses suggest that SNP rs560887 lies in a branch point sequence, defined as the position where intron lariats form [45, 46]. Importantly, our hypothesis that SNP rs560887 affects FPG by acting in *cis* to directly modulate *G6PC2* expression is at odds with the conclusion of Taneera et al. [49] that SNP rs560887 acts in *trans* to modulate the expression of multiple other genes. Further studies will be required to resolve this discrepancy.

The relationship between *G6PC2* and type 2 diabetes is unclear. In European populations GWAS have linked *G6PC2* to variations in FPG but not risk for the development of type 2 diabetes [17]. This is paradoxical since elevated FPG is considered a risk factor for type 2

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diabetes [8–10]. Indeed variations in *GCK* are linked to both FPG and type 2 diabetes risk [17]. More recently, Taneera et al. [49] reported that *G6PC2* expression was decreased in islets from donors with type 2 diabetes. Based on the GWAS data noted above [17], we would interpret the decrease in *G6PC2* expression reported by Taneera et al. [49] to be a secondary event, specifically a response to the diabetic environment, rather than a causative event that contributes to the development of type 2 diabetes. Thus based on *G6pc2* knockout mouse data [4], the decrease in *G6PC2* expression reported by Taneera et al. [49] would lead to enhanced insulin secretion, which would make sense in terms of a compensatory attempt by unhealthy islets to maintain insulin secretion.

In summary, our study provides genetic and functional evidence supporting an important role for the intronic variant, rs560887 and the promoter variant rs2232316, in addition to the promoter variant rs13431652 [20], as potentially causative SNPs that contribute to the association signal between *G6PC2* and FPG. Future genetic analyses will be required to establish the impact of rs2232321 (Fig. 2) on the variation in FPG levels. However, this variant is very rare: in 4,300 European American participants, the NHLBI Exome Sequencing Project (ESP) database did not show any carrier of the variant and the minor allele frequency was about 0.8% in African Americans according to the same data base. Therefore, only very large sample collections of African origins would be sufficiently powered to detect an association with FPG levels. Future functional studies will focus on the analysis of rare *G6PC2* variants that have the potential to markedly affect enzyme activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMI	body mass index
D.E.S.I.R.	Data from the Epidemiological Study on the Insulin Resistance Syndrome
ESP	Exome Sequencing Project
FPG	fasting plasma glucose
GWAS	genome-wide association studies
HbA1c	hemoglobin A1C
HIT	Hamster insulinoma tumor
MAGIC	Meta-Analysis of Glucose and Insulin related traits Consortium
RSC	Rous sarcoma virus
SNP	single nucleotide polymorphism

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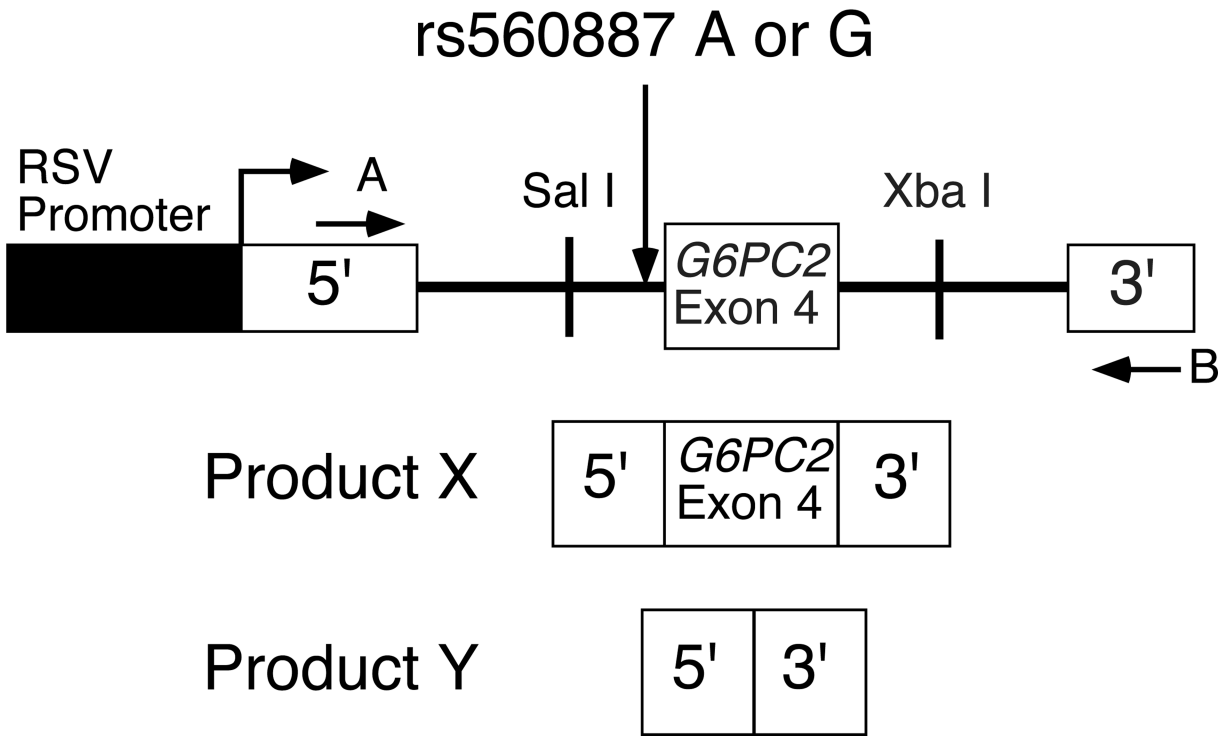

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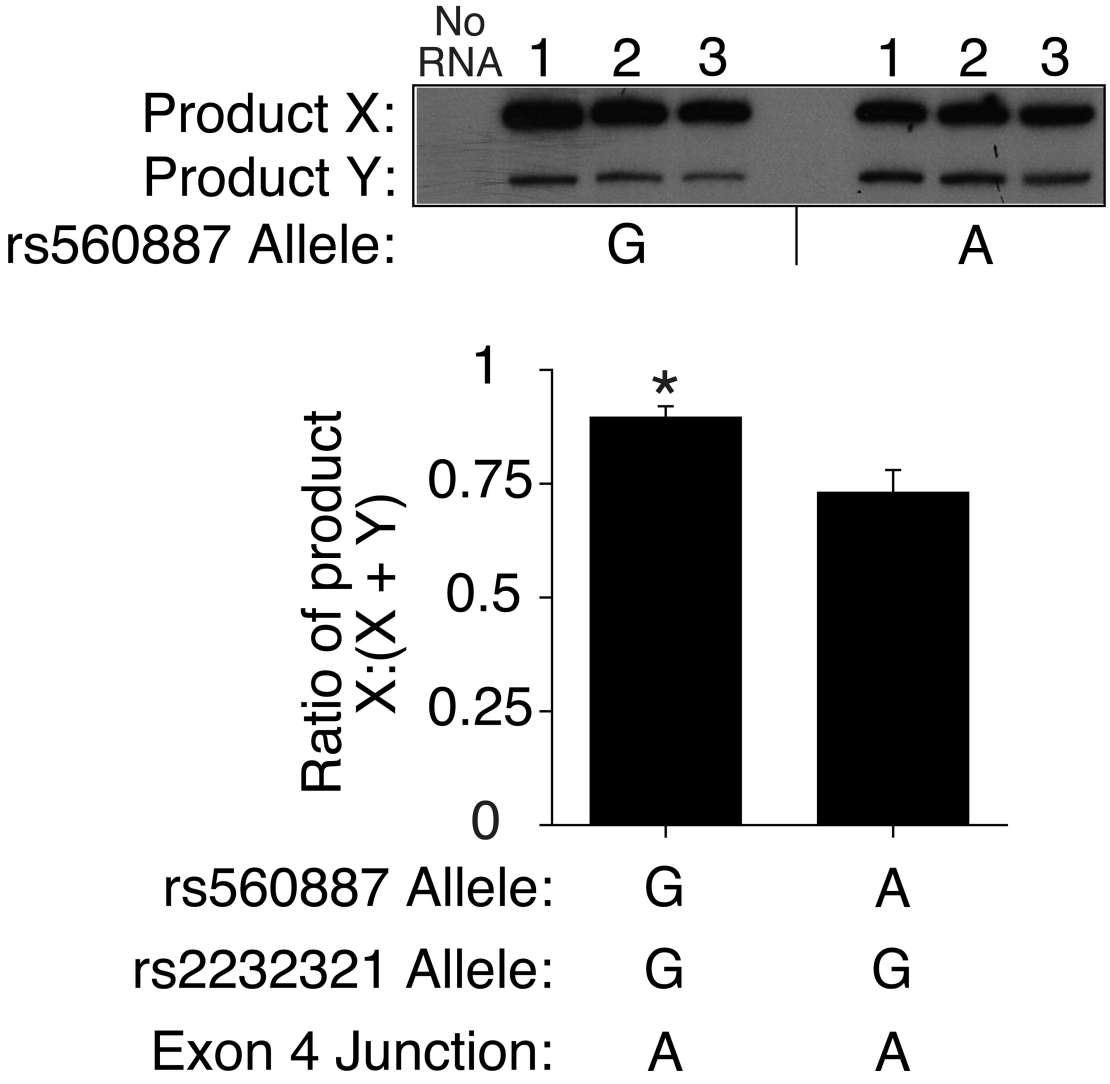

Figure 1. rs560887 alters *G6PC2* exon 4 splicing

Panel A: The RHCglo minigene vector. The vector comprises the RSV promoter, a 5' exon, a 3' exon and *Sal* I plus *Xba* I restriction enzyme sites into which *G6PC2* exon (E) 4 or 5 were ligated along with surrounding 5' and 3' intronic sequences (for exon 4). RT-PCR was used to quantitate the relative abundance of splice products X and Y using primers A and B. **Panel B:** RHCglo minigene vectors containing the alternate alleles of rs560887 were transiently transfected into HeLa cells and RNA expression was quantitated as described in Materials and Methods. Results show the mean data from 3 experiments ± S. E. M. *p<0.05.

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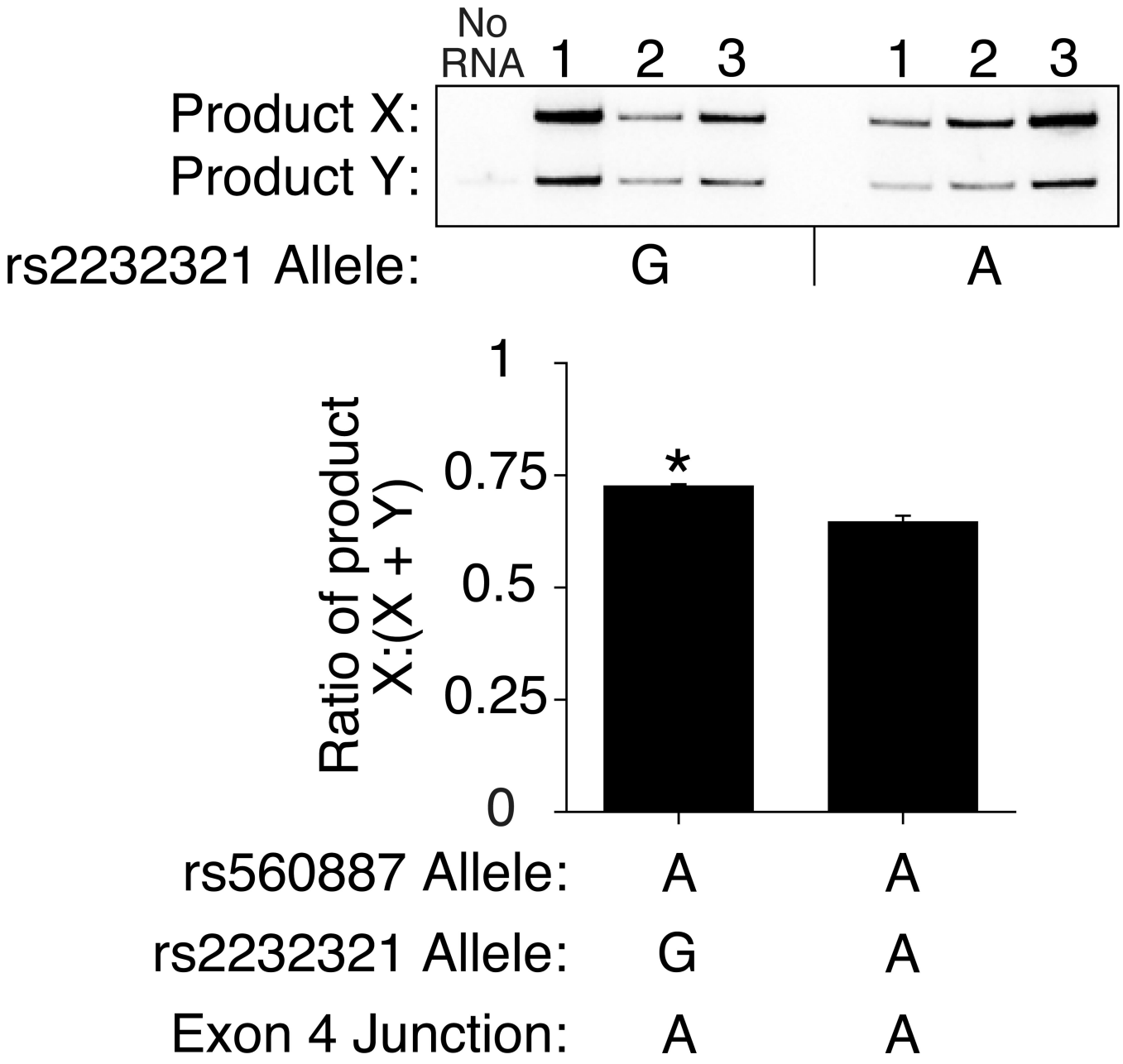

Figure 2. rs2232321 alters *G6PC2* RNA splicing

RHCglo minigene vectors containing the alternate alleles of rs2232321 were transiently transfected into HeLa cells and RNA expression was quantitated as described in Materials and Methods. Results show the mean data from 3 experiments ± S. E. M. *p<0.05.

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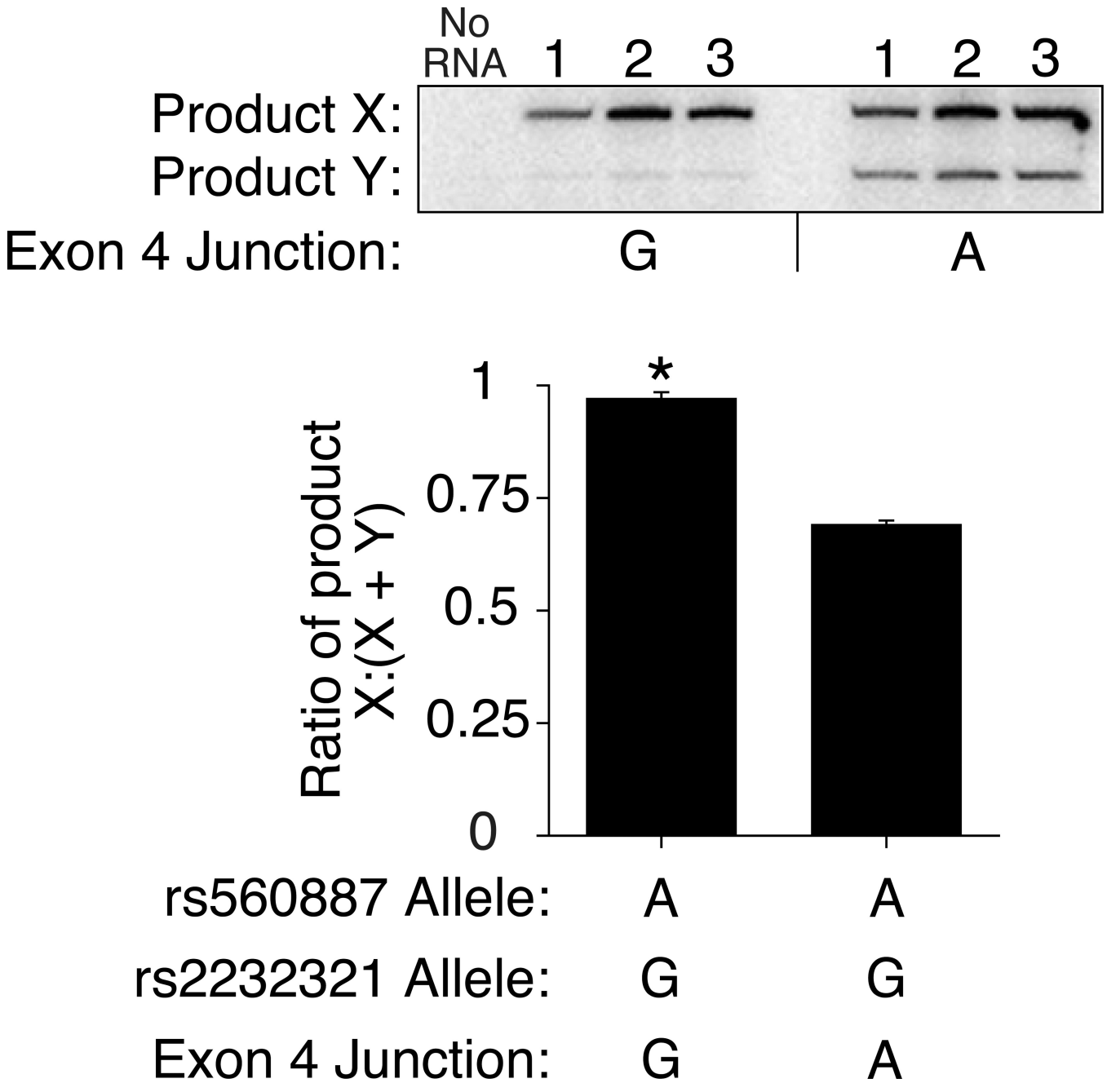

Figure 3. The effects of rs560887 and rs2232321 are dependent on the non-consensus exon 4 splice junction

RHCglo minigene vectors containing the native (A) or consensus (G) exon 4 5' splice junction were transiently transfected into HeLa cells and RNA expression was quantitated as described in Materials and Methods. Results show the mean data from 3 experiments ± S. E. M. *p<0.05.

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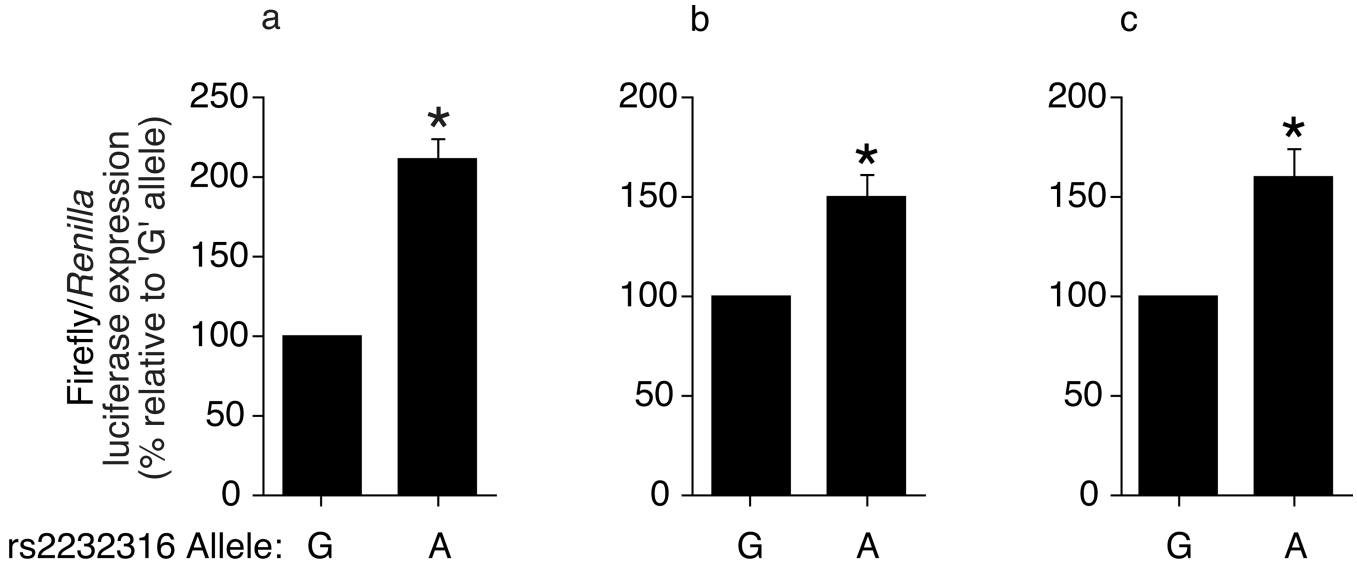

Figure 4. The human *G6PC2* rs2232316-A allele is associated with increased *G6PC2* promoter activity relative to the rs2232316-G allele

βTC-3 (**Panel A**), HIT (**Panel B**) and Min6 (**Panel C**) cells were transiently co-transfected with various *G6PC2-luciferase* fusion genes and an expression vector encoding *Renilla* luciferase as described in Materials and Methods. The *G6PC2*-luciferase fusion genes represented the rs2232316-A or G alleles present in the context of the human *G6PC2* promoter sequence located between −324 and +3. Results are presented as the ratio of firefly:*Renilla* luciferase activity, expressed as a percentage relative to the value obtained with the rs2232316-G allele, and represent the mean of 3 experiments ± S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *, $p < 0.05$ *versus* rs2232316-G allele.

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a

```
rs2232316-A  -250 ATCCAACTGTTCaCACAAAAGGGAG -226
rs2232316-G  -250 ATCCAACTGTTCgCACAAAAGGGAG -226

Foxa Consensus     AAATGTTGGCTT
                   C T A  TAT C
                   G
```

b

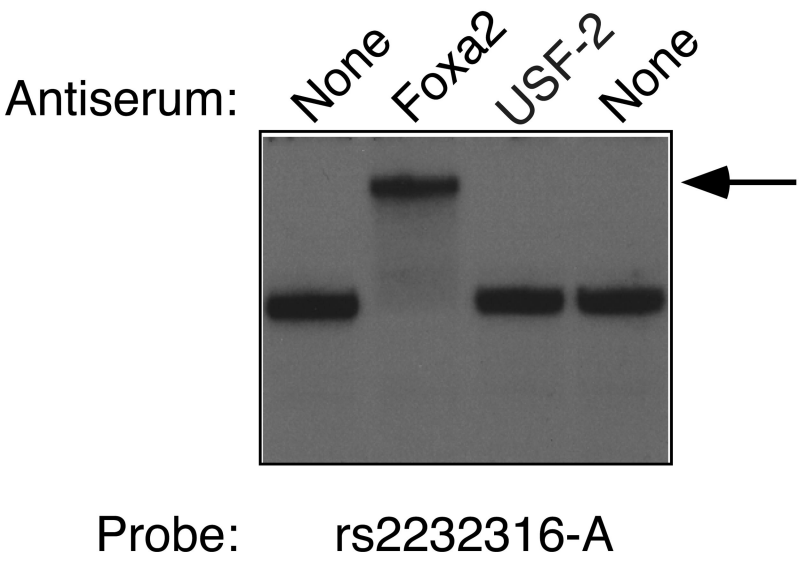

Figure 5. Foxa2 binds the human *G6PC2* −250/−226 promoter region *in vitro*

Panel A: The sense strand sequences of the oligonucleotides used in gel retardation assays are shown. SNP base pairs are shown in bold lower case letters. The consensus Foxa binding motif, (A/G)A(G/A)(C/T)(C/A)AA(C/T)A(T/A)T(T/G/C), is taken from Ref. [37].

Panel B: βTC-3 nuclear extract was incubated in the absence (−) or presence of the indicated anti-serum for 10 minutes on ice. A labelled oligonucleotides representing the −250/−226 *G6PC2* promoter region and containing the rs2232316-A allele (rs2232316-A; **Panel A**) was then added and incubation continued for 20 min at room temperature. Protein binding was then analyzed using the gel retardation assay as described in Materials and Methods. In the representative audioradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. A single major complex was detected that represents Foxa2 binding. The arrow indicates a supershifted complex.

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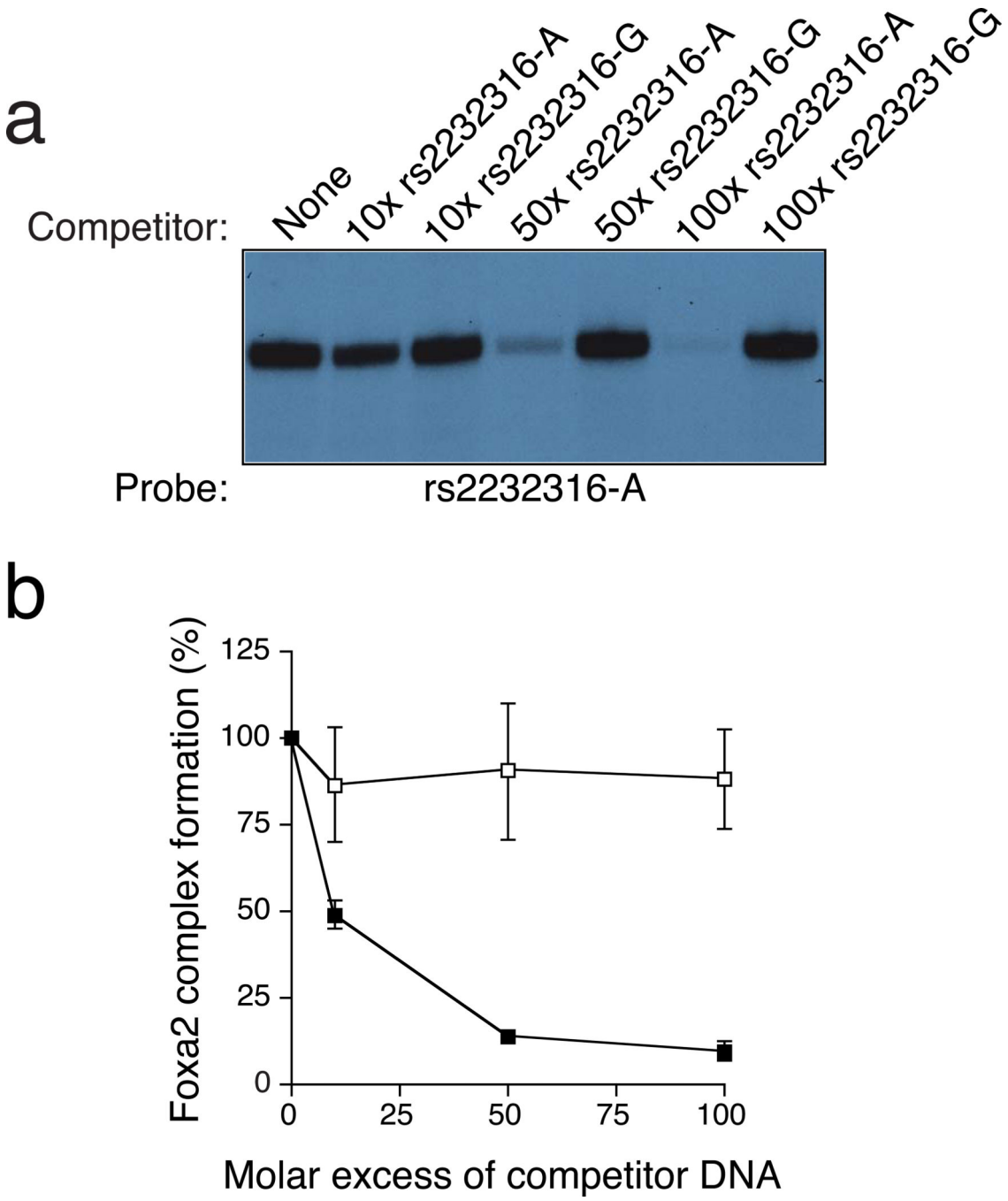

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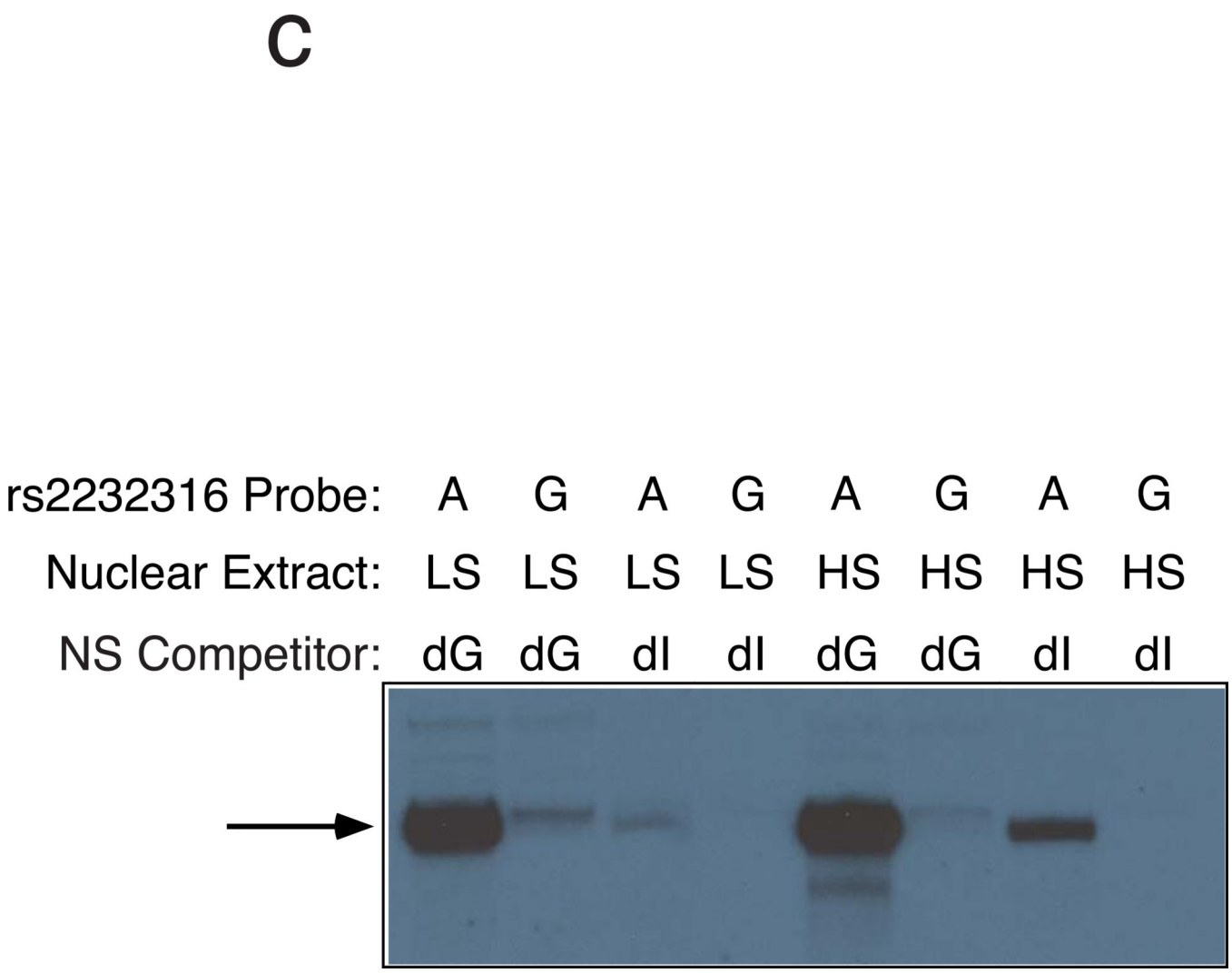

Figure 6. Comparison of the effect of the rs2232316-A and -G alleles on Foxa2 binding affinity *in vitro*

Panel A: The labelled rs2232316-A oligonucleotide (Fig. 2A) was incubated in the absence or presence of the indicated molar excess of the unlabelled human rs2232316-A or -G oligonucleotide competitors (Fig. 2A) prior to the addition of βTC-3 cell nuclear extract. Protein binding was then analyzed using the gel retardation assay as described in Materials and Methods. In the representative autoradiograph shown only the retarded complex is visible and not the free probe, which was present in excess.

Panel B: Protein binding, specifically 32P associated with the retarded complex, was quantified as described in Materials and Methods. The data represents the mean + S.E.M. of three experiments. Closed squares: rs2232316-A oligonucleotide; open squares: rs2232316-G oligonucleotide.

Panel C. The labelled rs2232316-A and -G oligonucleotides (Fig. 2A) were incubated with either high salt (HS) or low salt (LS) βTC-3 cell nuclear extract and in the presence of either the poly(dI-dC)·poly(dI-dC) (dI) or poly(dG-dC)·poly(dG-dC) (dG) non-specific competitors, which influence the strength of Foxa2 binding [50]. Protein binding was then

analyzed using the gel retardation assay as described in Materials and Methods. The arrow points to the specific Foxa2-DNA complex that preferentially forms with the rs2232316-A oligonucleotide.

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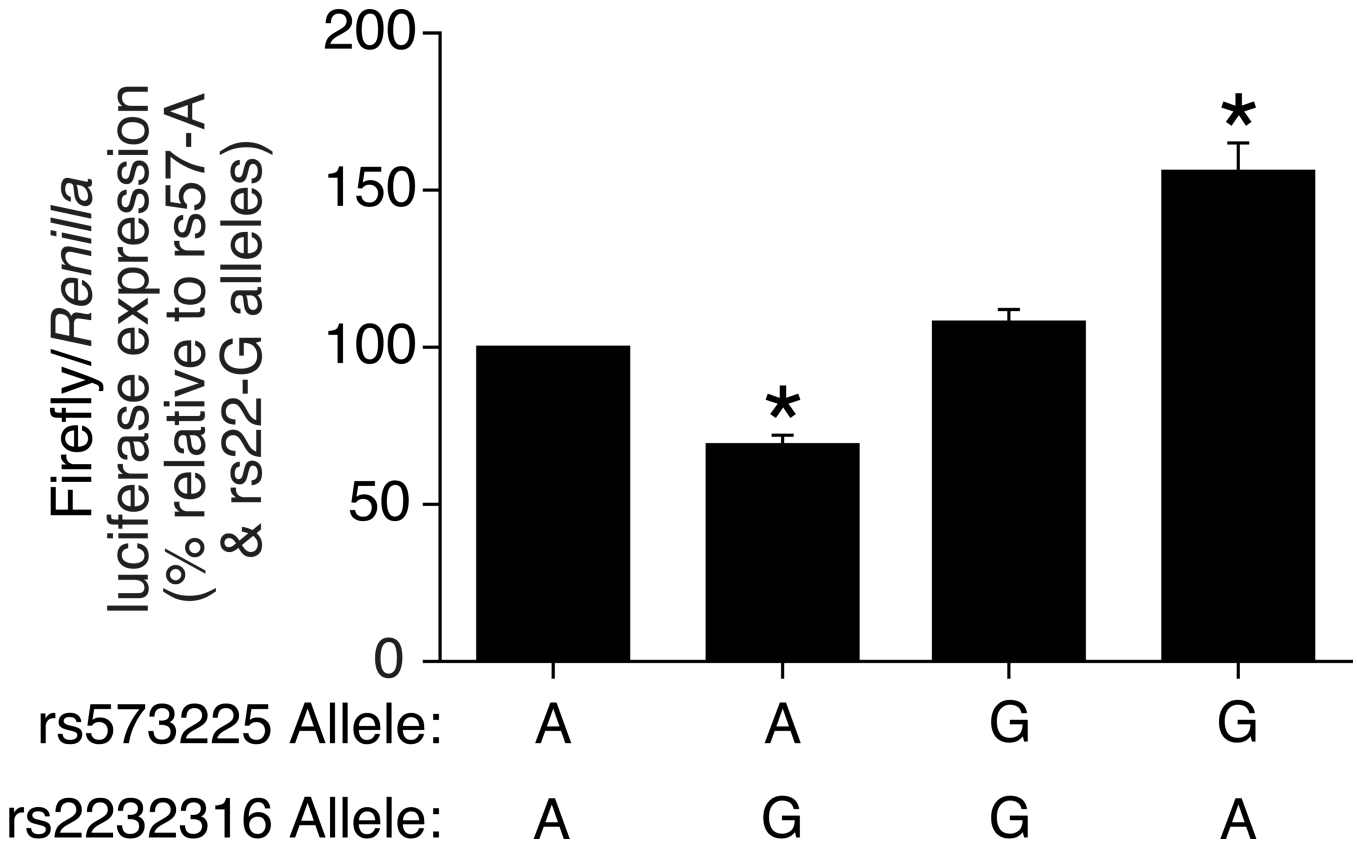

Figure 7. The rs2232316 and rs573225 SNPs have independent effects on *G6PC2* promoter activity in βTC-3 cells

βTC-3 cells were transiently co-transfected with various *G6PC2*-luciferase fusion genes and an expression vector encoding *Renilla* luciferase as described in Materials and Methods. The *G6PC2*-luciferase fusion genes represented the rs2232316-A or -G alleles and rs573225-A or -G alleles present in the context of the human *G6PC2* promoter sequence located between −324 and +3. Results are presented as the ratio of firefly:*Renilla* luciferase activity, expressed as a percentage relative to the value obtained with the rs2232316-G and rs573225-A allele, and represent the mean of 3 experiments ± S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *, $p < 0.05$ *versus* rs2232316-G and rs573225-A allele. **, $p < 0.05$ *versus* (rs2232316-G and rs573225-G) and (rs2232316-A and rs573225-A) alleles.

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Table 1

Association between *G6PC2* SNPs and fasting plasma glucose levels in 4,220 normoglycemic French participants from D.E.S.I.R.

SNP	Adjustments	Frequency of the allele increasing FPG (Y)	Mean FPG level by genotype (mmo/l) XX	Mean FPG level by genotype (mmo/l) XY	Mean FPG level by genotype (mmo/l) YY	β (SE)[a]	P-value
rs2232316 X-allele=G Y-allele=A	Age, sex, BMI	11.4%	5.19±0.45	5.25±0.43	5.22±0.46	0.040 0.1)	4.3×10^{-3}
rs573225 X-allele=G Y-allele=A	Age,sex, BMI	67.0%	5.11±0.45	5.17±0.46	5.25±0.44	0.079 (0.01)	2.8×10^{-16}
rs560887 X-allele=A Y-allele=G	Age, sex, BMI	69.5%	5.09±0.45	5.16±0.46	5.25±0.44	0.086 (0.01)	1.8×10^{-18}
rs2232316 X-allele=G Y-allele=A	Age, sex, BMI, rs573225	11.4%	NA	NA	NA	0.016 (0.01)	0.29
rs573225 X-allele=G Y-allele=A	Age, sex, BMI, rs2232316	67.0%	NA	NA	NA	0.076 (0.01)	4.0×10^{-14}
rs2232316 X-allele=G Y-allele=A	Age, sex, BMI, rs560887	11.4%	NA	NA	NA	0.014 (0.01)	0.35
rs560887 X-allele=A Y-allele=G	Age, sex, BMI, rs2232316	69.5%	NA	NA	NA	0.083 (0.01)	2.9×10^{-16}
rs2232316 X-allele=G Y-allele=A	Age, sex, BMI, rs573225, rs560887	11.4%	NA	NA	NA	0.018 (0.01)	0.22
rs573225 X-allele=G Y-allele=A	Age, sex, BMI, rs2232316, rs560887	67.0%	NA	NA	NA	0.000 (0.03)	0.99
rs560887 X-allele=A Y-allele=G	Age, sex, BMI, rs573225, rs2232316	69.5%	NA	NA	NA	0.082 (0.03)	2.5×10^{-3}

[a]Per Y-allele effect size: coefficient β from additive linear regression models adjusted for age, sex and BMI, or for age, sex, BMI and other *G6PC2* SNPs in conditional regressions. Data are presented as mean ± standard deviation. *SE*, standard error; *NA*, not applicable; *BMI*, body mass index; *FPG*, fasting plasma glucose.