

Insights Into the Molecular Mechanism for Type 2 Diabetes Susceptibility at the *KCNQ1* Locus From Temporal Changes in Imprinting Status in Human Islets

Mary E. Travers,¹ Deborah J.G. Mackay,² Marloes Dekker Nitert,³ Andrew P. Morris,⁴ Cecilia M. Lindgren,⁴ Andrew Berry,⁵ Paul R. Johnson,^{1,6,7} Neil Hanley,⁵ Leif C. Groop,³ Mark I. McCarthy,^{1,4,6} and Anna L. Gloyn^{1,6}

The molecular basis of type 2 diabetes predisposition at most established susceptibility loci remains poorly understood. *KCNQ1* maps within the 11p15.5 imprinted domain, a region with an established role in congenital growth phenotypes. Variants intronic to *KCNQ1* influence diabetes susceptibility when maternally inherited. By use of quantitative PCR and pyrosequencing of human adult islet and fetal pancreas samples, we investigated the imprinting status of regional transcripts and aimed to determine whether type 2 diabetes risk alleles influence regional DNA methylation and gene expression. The results demonstrate that gene expression patterns differ by developmental stage. *CDKN1C* showed monoallelic expression in both adult and fetal tissue, whereas *PHLDA2*, *SLC22A18*, and *SLC22A18AS* were biallelically expressed in both tissues. Temporal changes in imprinting were observed for *KCNQ1* and *KCNQ1OT1*, with monoallelic expression in fetal tissues and biallelic expression in adult samples. Genotype at the type 2 diabetes risk variant rs2237895 influenced methylation levels of regulatory sequence in fetal pancreas but without demonstrable effects on gene expression. We demonstrate that *CDKN1C*, *KCNQ1*, and *KCNQ1OT1* are most likely to mediate diabetes susceptibility at the *KCNQ1* locus and identify temporal differences in imprinting status and methylation effects, suggesting that diabetes risk effects may be mediated in early development. *Diabetes* 62:987–992, 2013

The translation of established type 2 diabetes risk variants into an improved understanding of disease pathology is challenging. Progress has been made primarily at the few loci where causal alleles are coding (1–4), but disease mechanisms are unclear for the majority of loci mapping outside coding regions.

The *KCNQ1* locus harbors at least two independent regions of association with type 2 diabetes risk (intron 10 and intron 15), both acting through impaired islet function

(5–11). *KCNQ1* itself encodes the K_v7.1 voltage-gated potassium channel subunit, which is expressed in human β-cells (12) but plays an uncertain role in insulin secretion. Neither patients with cardiac arrhythmia caused by *KCNQ1* mutations nor *Kcnq1*-null mice demonstrates hyperglycemia or glucose intolerance (13,14), whereas *KCNQ1* knockdown in human islets does not alter insulin secretion (15).

In accordance with the location of *KCNQ1* at the imprinted 11p15.5 region, associated alleles at both signals confer disease risk only when maternally inherited (16). It has been demonstrated, primarily through studies of the syntenic region of mouse chromosome 7, that regional gene expression is regulated by differential methylation at the promoter of *KCNQ1* overlapping transcript 1 (*KCNQ1OT1*), a nontranslated antisense RNA that regulates maternal-specific expression of downstream genes (17) (Fig. 1).

Disruption of genomic architecture at the 11p15.5 chromosomal region has a well-established role in Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth syndrome often associated with hypoglycemia (18). Furthermore, unbalanced placental expression of two regional genes, *PHLDA2* and *CDKN1C*, is associated with intrauterine growth retardation (19). We hypothesize that type 2 diabetes risk may be mediated through disruption of methylation and imprinted gene expression within the imprinted cluster. In this study, we perform the first assessment of 11p15.5 regional imprinting in adult human islets and fetal pancreas and investigate the effect of risk genotype status on DNA methylation and imprinted gene expression.

RESEARCH DESIGN AND METHODS

Islet and fetal pancreas isolation and DNA/RNA extraction. Human islets of deceased donors of European descent were obtained (with research consent) from the Oxford Diabetes Research & Wellness Foundation Human Islet Isolation Facility ($n = 30$) and the Human Tissue Laboratory at Lund University Diabetes Centre ($n = 42$). Fetal pancreas samples ($n = 18$) were obtained with informed consent and ethical approval from the North West Regional Ethics Committee. All islet preparations were >80% pure, with RNA integrity numbers >7. Donor and purity details are provided in Supplementary Table 1. DNA and RNA were extracted using TRIzol (Life Technologies).

cDNA synthesis. cDNA was generated through random-primed first-strand synthesis from 1 μg RNA in accordance with standard protocols, including treatment with DNase I.

Genotyping. Type 2 diabetes-associated single-nucleotide polymorphisms (SNPs) (rs231362 and rs2237895) were selected as the lead SNPs (strongest evidence for association) in each of the independent signals. Reporter coding SNPs for imprinting analysis were selected to have the highest possible minor allele frequencies, maximizing heterozygous samples capable of differentiating mRNA products from homologous chromosomes. Genotyping was performed

From the ¹Oxford Centre for Diabetes Endocrinology & Metabolism, University of Oxford, Oxford, U.K.; the ²Faculty of Medicine, University of Southampton, Southampton, U.K.; the ³Lund University Diabetes Centre, Malmö, Sweden; the ⁴Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, U.K.; the ⁵Endocrinology and Diabetes, Faculty of Medical & Human Sciences, University of Manchester, Manchester, U.K.; the ⁶Oxford NIHR Biomedical Research Centre, Churchill Hospital, Oxford, U.K.; and the ⁷Nuffield Department of Surgical Sciences, University of Oxford, Oxford, U.K. Corresponding author: Anna L. Gloyn, anna.gloyn@drl.ox.ac.uk.

Received 19 June 2012 and accepted 19 August 2012.

DOI: 10.2337/db12-0819

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0819/-/DC1>.

© 2013 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

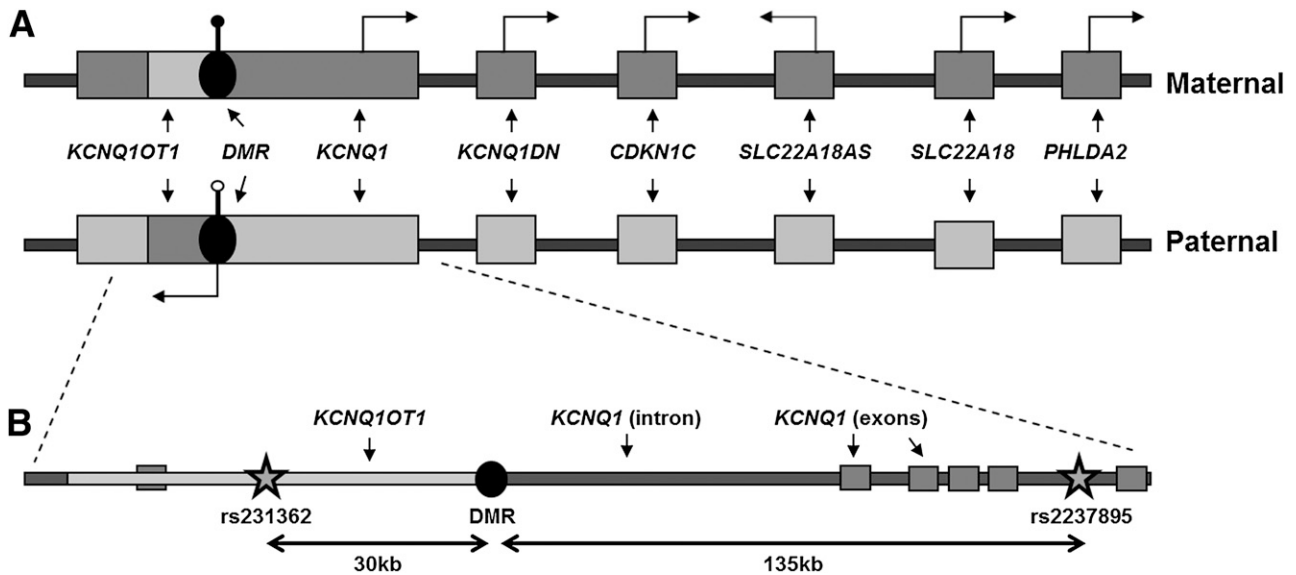


FIG. 1. Schematic representation of imprinting control [as described in (17)] and type 2 diabetes-associated SNPs at 11p15.5. **A:** Chromosome 11:2,450,000–2,960,000. The closed circle at the DMR represents a high level of methylation; the open circle represents a low level of methylation. The transcribed sequence is shown in dark gray, and untranscribed is shown in light gray. Arrows indicate direction of transcription. **B:** Chromosome 11:2,650,000–2,880,000. The smaller region is distinguished by exonic (boxes) and intronic regions of *KCNQ1* (dark gray), a region of *KCNQ1OT1* transcription (light gray), and relative positions of top disease-associated SNPs (rs231362, chr11:2,691,471, and rs2237985, chr11:2,857,194). All genomic coordinates are b37/hg19 (graphics not to scale).

using TaqMan chemistry and SDS2.3 allelic discrimination software (Applied Biosystems). All SNPs reached genotyping pass rates of >95%, were present in accordance with expected (HapMap Centre d'Etude du Polymorphisme Humain) minor allele frequencies, and did not depart from Hardy-Weinberg equilibrium.

Sequencing. Primers were designed using web-based software (<http://frodo.wi.mit.edu>). For cDNA sequencing, primers were designed across exons to prevent amplification of residual genomic DNA (gDNA). Samples were amplified using AmpliTaq Gold DNA polymerase enzyme and sequenced on an ABI 3700 genetic analyzer machine using the BigDye Terminator v1.1 Cycle Sequencing Kit (all from Applied Biosystems). Results were analyzed using Mutation Surveyor version 3.4 software (SoftGenetics).

Bisulphite treatment and pyrosequencing. DNA (500 ng) was bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research). Treated DNA was amplified in duplicate using biotinylated primers and Titanium Taq reagents (Clontech) before pyrosequencing on a PSQ 96MA machine (Qiagen). Analysis was performed using PyroMark version 2.0 software, including cytosine bases outside CpG dinucleotides to confirm efficiency of conversion.

Fragment analysis for indel analysis. PCR was performed using HotStar Taq and Q-Solution reagents (Qiagen) with FAM-labeled primers. Products were analyzed on an Applied Biosystems 3130xl machine (Dye set D) and visualized using Peak Scanner version 1.0 software (Applied Biosystems).

Gene expression. Genes were selected for analysis on the basis of inclusion in the GeneImprint database (www.geneimprint.com) and expression regulation by *KCNQ1OT1*'s differentially methylated promoter. Quantitative PCR was performed in triplicate on an AB 7900HT machine, using inventoried (where available) or custom (designed using Primer Express version 3.0 software) TaqMan expression assays and cDNA samples diluted 1:10 in 0.01 mol/L Tris. Total expression reactions multiplexed a FAM-labeled test assay and a VIC-labeled endogenous control assay (one of *RNaseP*, *HPRT*, or *PPIA*). Analysis was performed using the $\Delta\Delta C_t$ method. Allele-specific expression analysis compared the amplification cycle number at which VIC and FAM fluorescence levels crossed a critical threshold, where VIC/FAM probes annealed over the two alleles of a coding SNP.

Statistical analysis. Methylation and expression in islet samples obtained from Lund ($n = 42$) and Oxford ($n = 30$) were not significantly different ($P \geq 0.2$, Kolmogorov-Smirnov independent samples test). All islet samples, therefore, were combined for further analysis. Analysis was conducted by linear regression of expression or methylation on genotype group, with donor age, donor sex, sample purity, and center of origin (for adult islets) or days post-conception (for fetal pancreas) included as covariates. Analysis was also performed using a Kruskal-Wallis analysis of ranks, which retained significance in all cases.

RESULTS

Genes at the 11p15.5 cluster show temporal differences in imprinting status. *KCNQ1*, *KCNQ1OT1*, *CDKN1C*, *PHLDA2*, *SLC22A18*, and *SLC22A18AS* (but not *KCNQ1DN* or *TRPM5*) were quantifiably expressed in both adult islets ($n = 72$) and fetal pancreas ($n = 18$). To determine the imprinting status for each gene, we used common coding SNPs or a coding indel (for *CDKN1C* only) to distinguish between mRNA products from homologous chromosomes (Supplementary Table 2). Imprinted or nonimprinted expression was indicated by mono- or biallelic expression of these SNPs in cDNA from samples that were heterozygous in gDNA.

CDKN1C was monoallelically expressed in both adult and fetal tissues (Fig. 2A), as demonstrated in all samples heterozygous for the coding indel. Imprinted expression, however, was not universal among other genes in the region. *PHLDA2* and *SLC22A18* were expressed biallelically in all adult and all fetal samples (Fig. 2B). For *SLC22A18AS*, where there were relatively few heterozygous samples, cDNA sequencing demonstrated retained heterozygosity at all coding SNPs (Supplementary Fig. 1). *KCNQ1* and *KCNQ1OT1* showed temporal changes in imprinting status. Coding SNPs in both genes showed monoallelic expression in all fetal pancreas samples but were biallelically expressed in all adult islet samples (Fig. 2B). These results were confirmed by sequencing cDNA from all heterozygous samples (Supplementary Fig. 2).

Type 2 diabetes risk genotype status influences regional methylation in a developmentally flexible manner. To test the hypothesis that risk SNPs influence gene expression through an effect on DNA methylation, we quantified methylation at five representative regions of sequence. One assay widely used for the clinical diagnosis of BWS (20) maps within the differentially methylated region (DMR), and two were located at CTCF and putative PLAGL1 transcription factor binding sites within the DMR

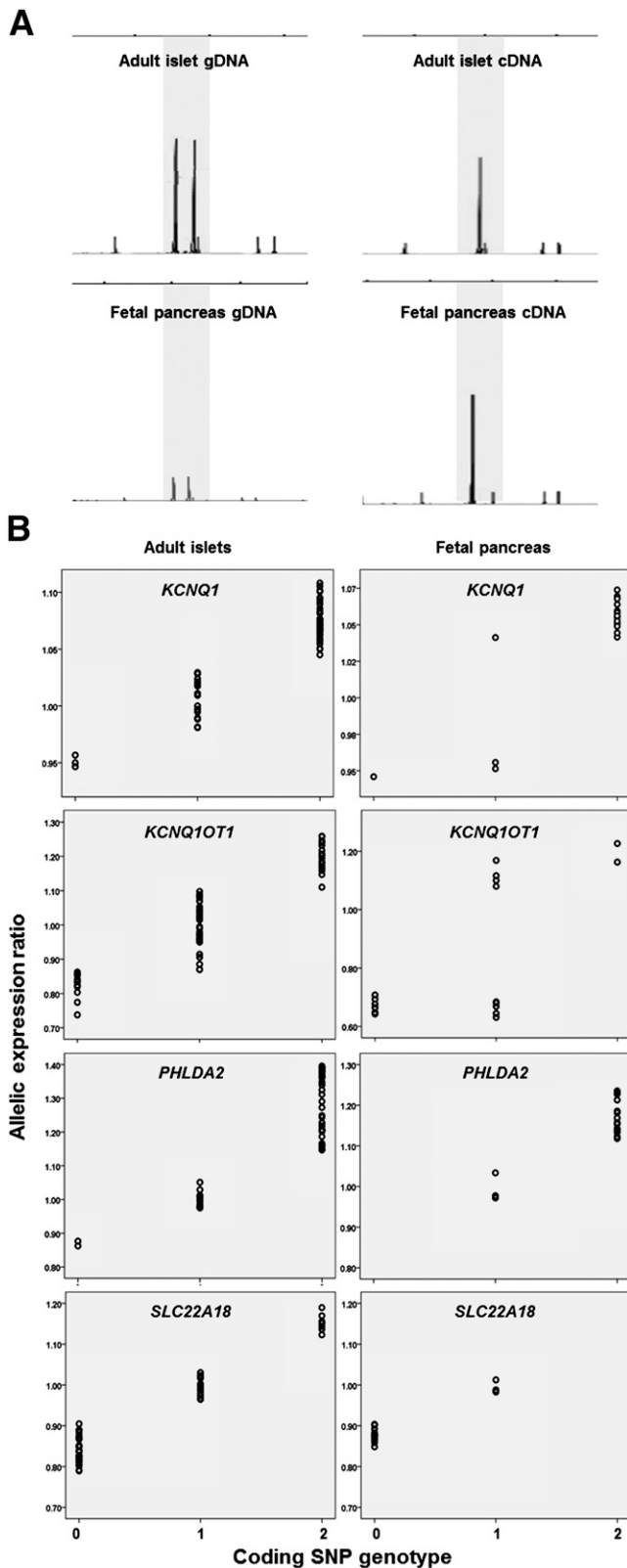


FIG. 2. A: Imprinting status of genes in the 11p15.5 cluster *CDKN1C*. Fragment analysis traces demonstrate monoallelic (imprinted) expression of *CDKN1C* in adult islet (*top*) and fetal pancreas (*bottom*) samples. *Left:* genomic DNA, with the two size peaks characteristic of a heterozygote for del171APVA highlighted. *Right:* cDNA from the same heterozygous samples. In both cases, only one size peak is evident, indicating the presence of mRNA from only one chromosome. Every sample heterozygous for del171APVA at the gDNA level appeared homozygous at the cDNA level, indicating monoallelic expression. **B:** Imprinting status of genes in the 11p15.5 cluster *KCNQ1*, *KCNQ1OT1*,

(21,22). Two other assays were designed at the DMR boundary on the basis of proximity to numerous CpG dinucleotides and disease-associated SNPs (rs231354 and rs2283202, respectively). To ascertain risk genotype status, DNA from each sample was genotyped for SNPs representing the intron 10 (rs231362) and intron 15 (rs2237895) signals.

rs2237895 was nominally associated ($P < 0.1$) with changes in methylation status at three of the sites tested and at differing developmental stages (Fig. 3). At the diagnostic DMR region and CTCF binding site, methylation was higher (7.3 and 5.6%, $P = 0.02$ and 0.08, respectively) in fetal samples homozygous for the risk allele than in fetal nonrisk homozygotes. This effect was not apparent in adult islets, where risk genotype had no effect on methylation levels at these same sites ($P > 0.25$). Conversely, an effect was seen only in adult tissues at the *PLAGL1* binding site. Here, methylation was 1.6% higher in risk genotype homozygotes than in nonrisk homozygotes ($P = 0.006$) in adult islets, but no effect was seen in fetal pancreas ($P = 0.36$). No effects were identified from the top disease-associated SNP within intron 10 of *KCNQ1* (rs231362, $P > 0.1$) (Supplementary Fig. 3) or from either SNP at the two candidate assays ($P > 0.1$) (Supplementary Fig. 4).

Type 2 diabetes risk genotype and total and allele-specific gene expression. We identified no relationship between rs2237895 risk allele number and total gene expression of *KCNQ1*, *KCNQ1OT1*, *CDKN1C*, *PHLDA2*, *SLC22A18*, or *SLC22A18AS* in the samples available for study ($P > 0.1$ for all genes) (Fig. 4). There was also no detectable relationship between rs231362 risk allele number and total gene expression (Supplementary Fig. 5) or between methylation at any of the five tested sites and total gene expression ($P > 0.1$ in all cases). Power for this analysis was reduced by undetermined risk allele parent of origin. We also examined the effect of risk genotype on allele-specific expression levels but identified no impact on the balance of expression between chromosomes for any tested gene ($P > 0.2$).

DISCUSSION

There is compelling evidence that diabetes risk at the *KCNQ1* locus is mediated through a gene with imprinted expression (16). By demonstrating that *PHLDA2*, *SLC22A18*, and *SLC22A18AS* are biallelically expressed in both adult and fetal pancreas and islets, we show that they are unlikely to be involved in a proximal molecular mechanism for diabetes risk. Likewise, any diabetes susceptibility

PHLDA2, and *SLC22A18*. Plots demonstrate flexibility of imprinting at the 11p15.5 cluster. The x-axis represents genotypes of reporter coding SNPs within the relevant gene (numbering arbitrary). The left- and right-hand clusters contain the two homozygote groups, whereas the center cluster contains heterozygous samples. The y-axis represents the ratio of mRNA expression level of a fluorescent probe specific to one allele of the coding SNP against the expression level of a differently labeled probe specific to the alternative allele. The two homozygous groups are therefore expected to have relatively high and low ratios, representing substantially more amplification of the probe specific for one allele. Under biallelic expression (all adult islet samples and *PHLDA2* and *SLC22A18* fetal pancreas samples), heterozygous samples form a central cluster with a ratio of ~1, indicating equal expression from homologous chromosomes. Under monoallelic (imprinted) expression (*KCNQ1* and *KCNQ1OT1* fetal pancreas samples), heterozygote samples separate into two distinct groups, corresponding to the two homozygous clusters and indicating expression from only one chromosome.

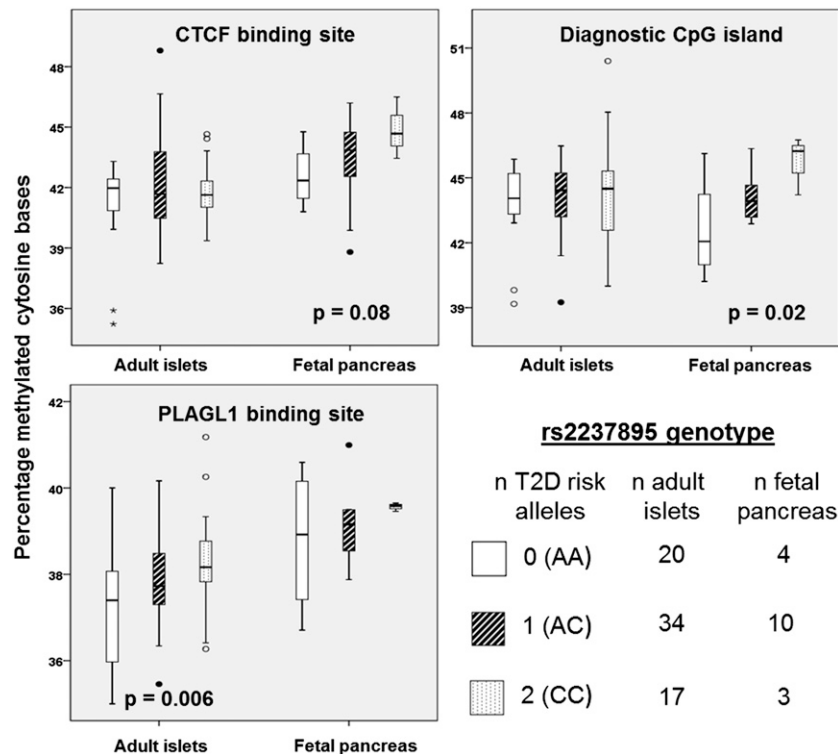


FIG. 3. Methylation according to rs2237895 type 2 diabetes risk genotype. Box plots show the effect of risk allele number (x-axis) on methylation level (y-axis) separated by tissue type. Boxes represent quartiles; whiskers encompass values within 1.5 times the interquartile range. Risk genotype is significantly associated with increased methylation levels at the CTCF binding site and Beckwith-Wiedemann diagnostic CpG island in fetal samples only and at the PLAGL1 binding site in adult islets only. T2D, type 2 diabetes.

mechanism working through *KCNQ1* or *KCNQ1OT1* is likely to be early in islet development because these transcripts are imprinted in fetal pancreas but not in adult islets.

The cyclin-dependent kinase inhibitor *CDKN1C* (encoding p57^{KIP2}), imprinted at both developmental time points, emerges as a particularly strong regional candidate. *CDKN1C* is expressed by 30–40% of β -cells in healthy individuals but never concurrently with the Ki67 marker of cell proliferation (23). *CDKN1C* expression is abolished in the hyperproliferative pancreatic lesions of focal hyperinsulinemia, suggesting a key role in regulating pancreatic β -cell proliferation (23). Loss-of-function mutations in *CDKN1C* cause BWS and hypoglycemia, whereas gain-of-function mutations in the PCNA-binding domain have recently been shown to cause congenital undergrowth (18,24). A higher level of DNA methylation in individuals carrying more risk alleles is consistent with a disease model of reduced *KCNQ1OT1* transcription, diminished repressive histone modifications (25), and increased *CDKN1C* expression, leading to impaired islet proliferation or development.

We demonstrate that risk genotype status is related to DNA methylation in a developmentally variable manner. Although the relationship between genotype and methylation is statistically stronger in adult than in fetal samples (probably attributable to improved power from a larger sample size), the magnitude of effect appears larger in fetal samples. It is noteworthy that the true allele-specific effect size is likely to be underestimated because data were obtained by pyrosequencing PCR products amplified from both chromosomes. Further work in larger numbers of

human islet samples, when they become available, will be required to explore in more detail the relationship between DNA methylation and expression.

By use of the largest cohort of human islets currently available, we have performed the first assessment of imprinting status at 11p15.5 in human adult islets and fetal pancreas. The data provide insights into the complexity of imprinting at 11p15.5, highlighting the necessity of performing functional studies in relevant tissues and at appropriate developmental stages. The data have significant implications for the molecular mechanism by which associated variants in this region exert their effect on diabetes risk.

ACKNOWLEDGMENTS

The work in Oxford was supported by the Medical Research Council (G0700222/81696 to A.L.G.), the Wellcome Trust (095101/Z/10/Z to A.L.G.), and the European Foundation for the Study of Diabetes (Albert Reynold Travel Fellowship to M.E.T.). The work in Oxford and Manchester was funded by the National Institute for Health Research Biomedical Research Centres in Oxford and Manchester, respectively. The work in Southampton was funded by Diabetes UK (BDA:RD08/0003611 to D.J.G.M.). The work at Lund University Diabetes Centre was funded by the Swedish Research Council (Dnr 521-2007-4037, Dnr 2009-1039, and Dnr 349-2008-6589 to L.C.G.). M.E.T. was funded by a Medical Research Council studentship. M.D.N. was funded by the Swedish Foundation for Medical Research. N.H. is a Wellcome Trust Senior Fellow in Clinical Science. A.L.G. is a Wellcome Trust Senior Fellow in Basic and Biomedical Research.

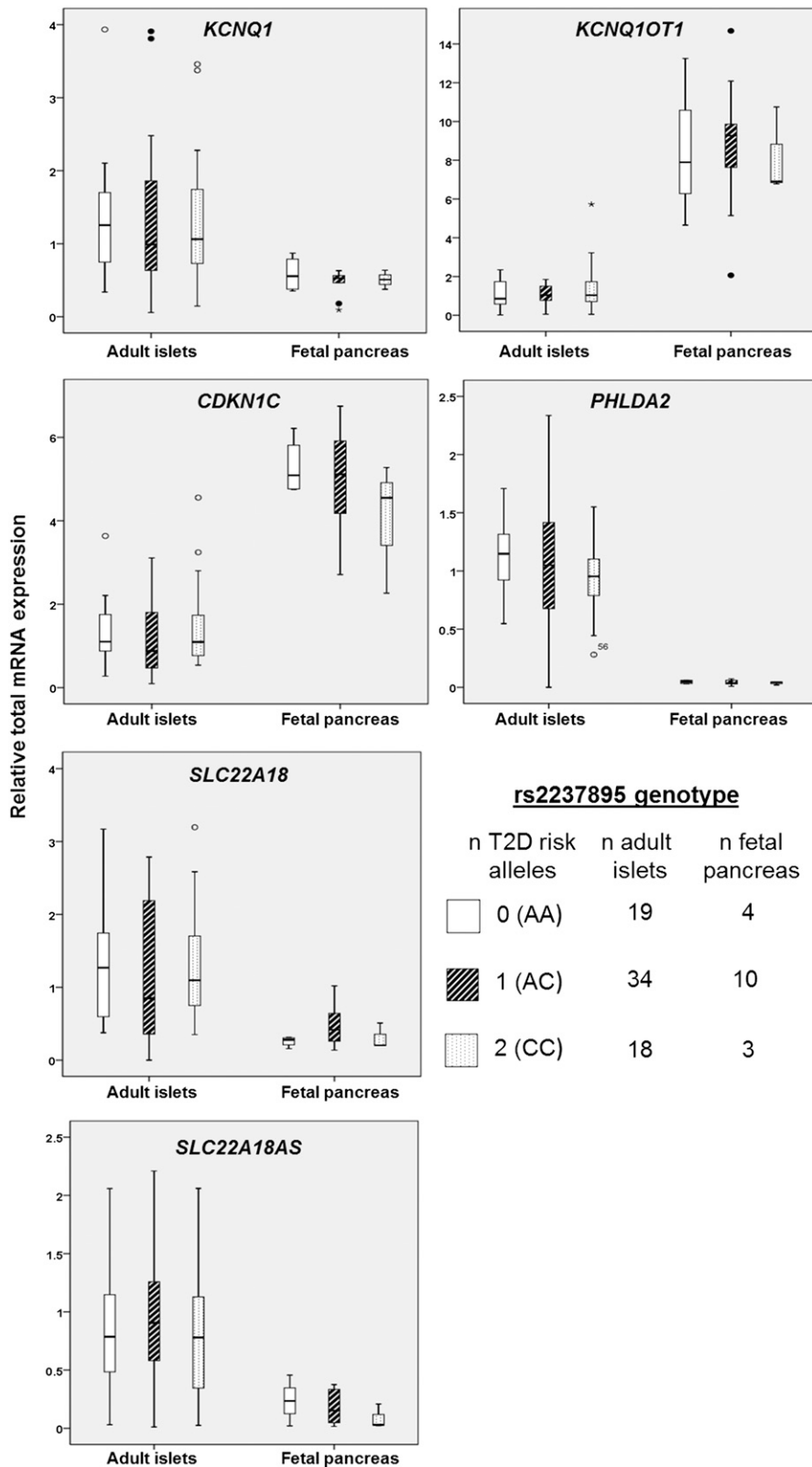


FIG. 4. Total expression according to rs2237895 type 2 diabetes risk genotype. Box plots show the effect of risk allele number (x-axis) on total mRNA expression level (y-axis), separated by tissue type. Boxes represent quartiles; whiskers encompass values within 1.5 times the interquartile range. There was no evidence for an effect of risk allele number on expression levels of any of the tested genes in either tissue type ($P > 0.05$ in all cases). T2D, type 2 diabetes.

The funder played no role in the conduct of the study, collection of data, management of the study, analysis of data, interpretation of data, or preparation of the manuscript.

No potential conflicts of interest relevant to this article were reported.

M.E.T., D.J.G.M., A.P.M., C.M.L., M.I.M., and A.L.G. designed the project. P.R.J. and L.C.G. provided human islet samples. N.H. provided fetal pancreas samples. M.E.T., M.D.N., and A.B. performed the research. M.E.T., M.I.M., and A.L.G. analyzed the data. M.E.T., M.I.M., and A.L.G. wrote the manuscript. D.J.G.M., M.D.N., A.P.M., C.M.L., A.B., N.H., and L.C.G. contributed to the manuscript. A.L.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented at the 46th Annual Meeting of the European Association for the Study of Diabetes (EASD), Stockholm, Sweden, 20–24 September 2010.

The authors thank the Nordic Network for Clinical Islet Transplantation (JDRF award 31-2008-413) and the tissue isolation teams and human tissue laboratory within EXO-DIAB/Lund University Diabetes Centre. The authors also thank the Islet Isolation team in Oxford, particularly Stephen Hughes and Sarah Cross, for providing human islets for this work.

REFERENCES

1. Hamming KS, Soliman D, Maternisz LC, et al. Coexpression of the type 2 diabetes susceptibility gene variants *KCNJ11* E23K and *ABCC8* S1369A alter the ATP and sulfonylurea sensitivities of the ATP-sensitive K(+) channel. *Diabetes* 2009;58:2419–2424
2. Nicolson TJ, Bellomo EA, Wijesekara N, et al. Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes* 2009;58:2070–2083
3. Rees MG, Wincovitch S, Schultz J, et al. Cellular characterisation of the *GCKR* P446L variant associated with type 2 diabetes risk. *Diabetologia* 2012;55:114–122
4. Beer NL, Tribble ND, McCulloch LJ, et al. The P446L variant in *GCKR* associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver. *Hum Mol Genet* 2009;18:4081–4088
5. Tsai FJ, Yang CF, Chen CC, et al. A genome-wide association study identifies susceptibility variants for type 2 diabetes in Han Chinese. *PLoS Genet* 2010;6:e1000847
6. Unoki H, Takahashi A, Kawaguchi T, et al. SNPs in *KCNQ1* are associated with susceptibility to type 2 diabetes in East Asian and European populations. *Nat Genet* 2008;40:1098–1102

7. Voight BF, Scott LJ, Steinthorsdottir V, et al.; MAGIC investigators; GIANT Consortium. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* 2010;42:579–589
8. Yasuda K, Miyake K, Horikawa Y, et al. Variants in *KCNQ1* are associated with susceptibility to type 2 diabetes mellitus. *Nat Genet* 2008;40:1092–1097
9. Holmkvist J, Banasik K, Andersen G, et al. The type 2 diabetes associated minor allele of rs2237895 *KCNQ1* associates with reduced insulin release following an oral glucose load. *PLoS ONE* 2009;4:e5872
10. Jonsson A, Isomaa B, Tuomi T, et al. A variant in the *KCNQ1* gene predicts future type 2 diabetes and mediates impaired insulin secretion. *Diabetes* 2009;58:2409–2413
11. Tan JT, Nurbaya S, Gardner D, Ye S, Tai ES, Ng DP. Genetic variation in *KCNQ1* associates with fasting glucose and β -cell function: a study of 3,734 subjects comprising three ethnicities living in Singapore. *Diabetes* 2009;58:1445–1449
12. MacDonald PE, Wheeler MB. Voltage-dependent K(+) channels in pancreatic beta cells: role, regulation and potential as therapeutic targets. *Diabetologia* 2003;46:1046–1062
13. Jespersen T, Grunnet M, Olesen SP. The *KCNQ1* potassium channel: from gene to physiological function. *Physiology (Bethesda)* 2005;20:408–416
14. Pan Q, Ma J, Zhou Q, et al. *KCNQ1* loss-of-function mutation impairs gastric acid secretion in mice. *Mol Biol Rep* 2010;37:1329–1333
15. Rosengren AH, Braun M, Mahdi T, et al. Reduced insulin exocytosis in human pancreatic β -cells with gene variants linked to type 2 diabetes. *Diabetes* 2012;61:1726–1733
16. Kong A, Steinthorsdottir V, Masson G, et al.; DIAGRAM Consortium. Parental origin of sequence variants associated with complex diseases. *Nature* 2009;462:868–874
17. Thakur N, Tiwari VK, Thomassin H, et al. An antisense RNA regulates the bidirectional silencing property of the *Kcnq1* imprinting control region. *Mol Cell Biol* 2004;24:7855–7862
18. Weksberg R, Shuman C, Beckwith JB. Beckwith-Wiedemann syndrome. *Eur J Hum Genet* 2010;18:8–14
19. McMinn J, Wei M, Schupf N, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* 2006;27:540–549
20. Mackay DJ, Callaway JL, Marks SM, et al. Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in *ZFP57*. *Nat Genet* 2008;40:949–951
21. Arima T, Kamikihara T, Hayashida T, et al. *ZAC*, *LIT1* (*KCNQ1OT1*) and *p57^{KIP2}* (*CDKN1C*) are in an imprinted gene network that may play a role in Beckwith-Wiedemann syndrome. *Nucleic Acids Res* 2005;33:2650–2660
22. Fitzpatrick GV, Pugacheva EM, Shin JY, et al. Allele-specific binding of CTCF to the multipartite imprinting control region KvDMR1. *Mol Cell Biol* 2007;27:2636–2647
23. Kassem SA, Ariel I, Thornton PS, et al. *p57^{KIP2}* expression in normal islet cells and in hyperinsulinism of infancy. *Diabetes* 2001;50:2763–2769
24. Arboleda VA, Lee H, Parnaik R, et al. Mutations in the PCNA-binding domain of *CDKN1C* cause IMAGE syndrome. *Nat Genet* 2012;44:788–792
25. Redrup L, Branco MR, Perdeaux ER, et al. The long noncoding RNA *Kcnq1ot1* organises a lineage-specific nuclear domain for epigenetic gene silencing. *Development* 2009;136:525–530