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

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The molecular basis of type 2 diabetes predisposition at most established susceptibility loci remains poorly understood. \textit{KCNQ1} maps within the 11p15.5 imprinted domain, a region with an established role in congenital growth phenotypes. Variants intrinsic to \textit{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. \textit{CDKN1C} showed monoallelic expression in both adult and fetal tissue, whereas \textit{PHLDA2}, \textit{SLC22A18}, and \textit{SLC22A18AS} were biallelically expressed in both tissues. Temporal changes in imprinting were observed for \textit{KCNQ1} and \textit{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 \textit{CDKN1C}, \textit{KCNQ1}, and \textit{KCNQ1OT1} are most likely to mediate diabetes susceptibility at the \textit{KCNQ1} locus and identify temporal differences in imprinting status and methylation effects, suggesting that diabetes risk effects may be mediated in early development.

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 \textit{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). \textit{KCNQ1} itself encodes the K\textsubscript{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 \textit{KCNQ1} mutations nor \textit{Kcnq1-null} mice demonstrates hyperglycemia or glucose intolerance (13,14), whereas \textit{KCNQ1} knockdown in human islets does not alter insulin secretion (15).

In accordance with the location of \textit{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 \textit{KCNQ1} overlapping transcript 1 (\textit{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, \textit{PHLDA2} and \textit{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**

\textit{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

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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 heterogeneous samples capable of differentiating mRNA products from homologous chromosomes. Genotyping was performed 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 database (www.hapmap.org)) and expression regulation using Peak Scanner version 1.0 software (Applied Biosystems). Analysis was performed using PyroMark version 2.0 software, including cytosine 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 ≥ 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 (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 non-risk 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 non-risk 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 alleledspecific gene expression.** We identified no relationship between rs2237895 risk allele number and total gene expression of KCNQ1, KCNQ1OT1, CDKN1C, PHLDA2, SLC22A18, or SLC22A1AS 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 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. 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,
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 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 p57KIP2), imprinted at both developmental time points, emerges as a particularly strong regional candidate. *CDKN1C* is expressed by 30–40% of $\beta$-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 $\beta$-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.

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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.
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M.T., D.J.G.M., A.P.M., C.L.M., 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.T., M.D.N., and A.B. performed the research. M.T., M.D.N., and A.B. analyzed the data. M.T., M.D.N., and A.B. 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.

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