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Aparna Pal,[1] Thomas P. Potjer,[2] Soren K. Thomsen,[1] Hui Jin Ng,[1] Amy Barrett,[1]
Raphael Scharfmann,[3] Tim J. James,[4] D. Timothy Bishop,[5] Fredrik Karpe,[1,6]
Ian F. Godsland,[7] Hans F.A. Vasen,[8] Julia Newton-Bishop,[5] Hanno Pijl,[9]
Mark I. McCarthy,[1,6,10] and Anna L. Gloyn[1,6]

Loss-of-Function Mutations in the Cell-Cycle Control Gene *CDKN2A* Impact on Glucose Homeostasis in Humans

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At the *CDKN2A/B* locus, three independent signals for type 2 diabetes risk are located in a noncoding region near *CDKN2A*. The disease-associated alleles have been implicated in reduced β-cell function, but the underlying mechanism remains elusive. In mice, β-cell–specific loss of *Cdkn2a* causes hyperplasia, while overexpression leads to diabetes, highlighting *CDKN2A* as a candidate effector transcript. Rare *CDKN2A* loss-of-function mutations are a cause of familial melanoma and offer the opportunity to determine the impact of *CDKN2A* haploinsufficiency on glucose homeostasis in humans. To test the hypothesis that such individuals have improved β-cell function, we performed oral and intravenous glucose tolerance tests on mutation carriers and matched control subjects. Compared with control subjects, carriers displayed increased insulin secretion, impaired insulin sensitivity, and reduced hepatic insulin clearance. These results are consistent with a model whereby *CDKN2A* loss affects a range of different tissues, including pancreatic β-cells and liver. To test for direct effects of *CDKN2A*-loss on β-cell function, we performed knockdown in a human β-cell line, EndoC-bH1. This revealed increased insulin secretion independent of proliferation. Overall, we demonstrated that *CDKN2A* is an important regulator of glucose homeostasis in humans, thus supporting its candidacy as an effector transcript for type 2 diabetes–associated alleles in the region.

Noncoding genetic signals at the *CDKN2A/B* locus have been associated with increased risk of developing type 2 diabetes (1,2). One signal is contained within a long noncoding RNA (*ANRIL*), while two distinct signals map to a region located further upstream of *CDKN2A* and *CDKN2B*. Physiological characterizations of normoglycemic carriers have demonstrated that the risk alleles are associated with reduced β-cell function, yet the underlying "effector" transcript driving these effects has not been established (3,4).

CDKN2A encodes the alternatively spliced proteins p16$^{\mathrm{INK4a}}$ and p14$^{\mathrm{ARF}}$, which are known tumor suppressors acting via distinct signaling pathways (5,6). p16$^{\mathrm{INK4a}}$ is a cyclin-dependent kinase inhibitor involved in the regulation of cell-cycle progression through inhibition of CDK4 and CDK6 (7). p14$^{\mathrm{ARF}}$, in contrast, prevents the degradation of the cell-cycle regulator p53 by forming a stable complex with Mdm2 in the nucleus (8).

Rodent studies have linked *Cdkn2a* to glucose homeostasis, pointing to the gene as a plausible candidate effector transcript at the *CDKN2A/B* locus. In a β-cell–specific knockout mouse, *Cdkn2a* deficiency was found to increase

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[1] Oxford Centre for Diabetes, Endocrinology & Metabolism, University of Oxford, Oxford, U.K.
[2] Department of Clinical Genetics, Leiden University Medical Center, Leiden, the Netherlands
[3] INSERM U1016, Institut Cochin, Université Paris Descartes, Paris, France
[4] Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford, U.K.
[5] Leeds Institute of Cancer and Pathology, University of Leeds, Leeds, U.K.
[6] Oxford National Institute for Health Research Biomedical Research Centre, Churchill Hospital, Oxford, U.K.
[7] Diabetes, Endocrinology and Metabolism, Department of Medicine, Imperial College London, London, U.K.
[8] Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands
[9] Leiden University Medical Centre, Department of Internal Medicine, Leiden, the Netherlands
[10] Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, U.K.

Corresponding author: Anna L. Gloyn, anna.gloyn@drl.ox.ac.uk.

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A.P, T.P.P., and S.K.T. are joint first authors.

β-cell proliferation and conferred resistance to chemically induced diabetes (9). Overexpression, in contrast, reduced β-cell proliferation in both young and old mice. This is consistent with the effect of *Cdk4* loss, which has been shown to result in a reduced number of pancreatic β-cells and insulin-deficient diabetes (10). More recent mouse studies have also established a role for *Cdkn2a* and *Cdk4* in hepatic glucose production, demonstrating cell cycle–independent effects on gluconeogenesis under fasted and fed conditions (11,12).

While rodent studies have provided critical clues into the contribution of *Cdkn2a* to diabetes pathogenesis, less is known about the role of *CDKN2A* in glucose homeostasis in humans. The machinery regulating the G1/S transition in adult human β-cells differs from that of mouse β-cells, which do not express CDK6 (13–15). Individuals heterozygous for germline loss-of-function mutations in the *CDKN2A* gene have a high risk of developing (multiple) cutaneous melanoma, a condition known as familial atypical multiple mole melanoma syndrome (FAMMM) (16,17). These subjects provide a unique opportunity to study the effect of *CDKN2A* haploinsufficiency on glucose homeostasis in humans. The current study tested the hypothesis that mutation carriers show improved β-cell function compared with noncarriers.

RESEARCH DESIGN AND METHODS

Study Participants

Thirty-one case subjects diagnosed with FAMMM due to *CDKN2A* mutations were recruited from centers in the U.K. and the Netherlands. Twenty-eight had been cancer free for at least 2 years, and the remaining three case subjects had presented with melanoma between 4 and 12 months prior to inclusion in the study (Supplementary Table 1). For a control group of 31 participants, unaffected first-degree relatives or spouses of carriers were chosen when available and additional control subjects were recruited from the Oxford Biobank (www.oxfordbiobank.org.uk). Two control subjects were subsequently excluded based on 2-h oral glucose tolerance test (OGTT) glucose levels that diagnosed diabetes (serum glucose $>$11 mmol/L). All remaining participants were aged 18–80 years, not suffering from diabetes, and not taking any medication that could interfere with glucose tolerance.

Baseline Clinical Characteristics and OGTT

All participants underwent a 75-g OGTT after a 12-h fast. Blood samples were collected at 0, 15, 30, 60, 90, and 120 min after the oral glucose load to assay plasma glucose, serum insulin, and (for a subset of 12 mutation carriers and 12 control subjects) C-peptide. Insulin and C-peptide were measured using chemiluminescence immunoassays. Measures of insulin sensitivity, β-cell function, and hepatic clearance derived from the OGTT were calculated according to the formulas in Supplementary Table 2.

Intravenous Glucose Tolerance Test

Intravenous glucose tolerance tests (IVGTTs) were performed on a subset of the U.K. subjects (eight) who had attended for OGTT and consented to undergo an IVGTT. Control subjects, matched for age, sex, BMI, and activity, were recruited from the Oxford Biobank. After a 12-h fast, a dose of 50% dextrose (calculated based on weight 0.5 mg/kg) was given over 3 min. Blood samples were then taken at 0, 2, 4, 6, 8, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, and 180 min. These samples were batch analyzed for insulin, glucose, and C-peptide. Data were then analyzed using a minimal model approach, according to an algorithm designed to maximize precision and identification success rate (18).

Cellular Assays Using the EndoC-bH1 Cell Line

The EndoC-bH1 cell line was cultured and passaged as previously described (19). Reverse transfections were performed by adding preformed small interfering (si)RNA complexes prepared from ON-TARGETplus siRNA SMARTpools (Dharmacon) at a final concentration of 10 nmol/L siRNA. For gene expression analysis, RNA was extracted and quantitative PCR performed using the TaqMan gene expression kit and assays (Applied Biosystems) on oligo-dT primed cDNA. Seventy-two hours after transfection, cells were starved overnight in 2.8 mmol/L glucose followed by 1 h in 0 mmol/L glucose medium. Static insulin secretion assays were then initiated by adding glucose-free growth medium supplemented with the indicated amounts of glucose and isobutylmethylxanthine (IBMX). After 1 h, aliquots of supernatants were removed for later analysis and ice-cold acid ethanol was added to extract insulin content from cells. Sample analysis was performed using the AlphaLISA Human Insulin Immunoassay (PerkinElmer).

For protein kinase A (PKA) activity assays, cells were harvested after knockdown, as described above, and washed in PBS. After matching input for number of cells, the samples were then processed according to the manufacturer's instructions for the PepTag nonradioactive PKA assay (Promega) and visualized using the ChemiDoc MP system.

Statistical Analysis

Statistical analysis was performed using R 3.0.2. P values were determined by Welch t test, except for sex differences, where the χ^2 test was used, and for analysis of the IVGTT data, where the Mann-Whitney U test was used.

RESULTS

We recruited 31 participants carrying inherited *CDKN2A* loss-of-function mutations (Supplementary Table 1) and 31 control subjects matched as a group for age (P = 0.99), sex (P = 0.43), and BMI (P = 0.97) (Table 1). To test our hypothesis that *CDKN2A*-loss leads to improved β-cell function, we first performed a 120-min OGTT in all subjects (Fig. 1*A* and *B*). While no difference in glucose levels was detected, insulin levels

Table 1—Baseline characteristics of study participants

	Mutation carriers	Noncarriers	*P*
n	31	31	NA
BMI (kg/m^2)	27.1 (19; 38)	27.1 (19; 36)	0.97
Age (years)	51.8 (21; 71)	51.8 (25; 84)	0.99
Sex (% male)	45	32	0.43

Data are mean and range (minimum; maximum) unless otherwise indicated. *P* values are from Welch *t* test except for sex distribution where the χ^2 test was performed. NA, not applicable.

were significantly increased in carriers throughout the test (P = 0.01 for insulin area under curve [AUC_{ins}]) (Table 2).

Using these data, we derived standard indices of β-cell function and insulin sensitivity (Table 2). This revealed increased β-cell function in carriers compared with noncarriers, both assessed using a dynamic measure of acute insulin response (P = 0.03 for BIGTT-acute insulin response [AIR]) and in the fasted state (P = 0.05 for iHOMA2 of β-cell function). Corresponding measures of insulin sensitivity, BIGTT-S, and iHOMA2 of insulin sensitivity were also both found to be lower in carriers (P = 0.04 and P = 0.05, respectively). Other standard measures, the Belfiore and Matsuda insulin sensitivity indices (ISIs), confirmed the observed reduction in insulin sensitivity of carriers (P = 0.02 and P = 0.02, respectively). As a result, the disposition index, which is an aggregate measure of β-cell function relative to glucose sensitivity, remained unaffected compared with control subjects (P = 0.98). These results were not significantly altered by exclusion of three carriers that had presented with melanoma within 2 years prior to inclusion in the study (Supplementary Table 3).

To explore whether the observed phenotype was driven by underlying effects on p16$^{\text{INK4a}}$, p14$^{\text{ARF}}$, or both, we reanalyzed the data with carriers grouped by mutation status (Supplementary Fig. 1). Of the mutations identified, 26 affected both p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$, while five were located in regions affecting p16$^{\text{INK4a}}$ exclusively. No differences were observed between these two groups in insulin or glucose levels (P = 1.00 for AUC_{ins} and P = 0.49 for $\text{AUC}_{\text{glucose}}$, respectively), suggesting that the observed metabolic phenotype of mutation carriers may be driven either solely by effects on p16$^{\text{INK4a}}$ or by effects of similar magnitude on both proteins.

For a subset of participants (12 carriers and 12 control subjects), C-peptide measurements were obtained during the OGTT. Despite a tendency toward increased C-peptide levels in the fasted state (P = 0.48), the total response was not different for this subset of individuals (P = 1.00 for AUC). Indices of hepatic insulin clearance, derived from the ratio between C-peptide and insulin levels, however, showed significantly decreased hepatic clearance in mutation carriers (P = 0.03) (Table 2) (20).

To confirm these findings, we performed IVGTTs on eight case and eight control subjects available for follow-up studies (Supplementary Fig. 2). None of the measures derived from this test reached statistical significance, but directions of effect were confirmed for both insulin secretion (P = 0.14 for AUC_{ins} 10–180 min) and hepatic insulin clearance (P = 0.21) (Supplementary Table 4). The insulin response was found to be 66% and 110% higher for carriers during the first and second phase of secretion, respectively. In contrast, the C-peptide response (which is unaffected by hepatic clearance) was ~30% higher during

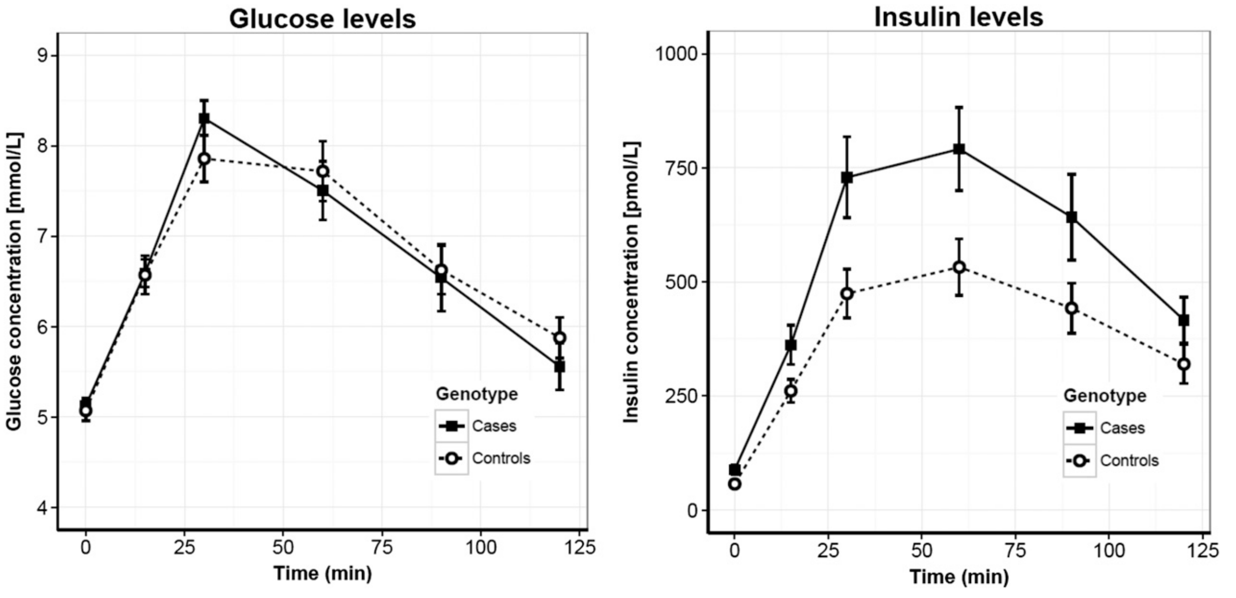

Figure 1—Serum glucose and insulin levels during a 120-min OGTT in 31 carriers and 31 control subjects. Data shown as mean ± SEM.

both phases of secretion, indicating a direct contribution of improved β-cell function to the elevated circulating insulin levels of carriers.

Finally, we sought to establish the extent to which cell cycle–independent effects of *CDKN2A* on the regulation of insulin secretion could contribute to the phenotype of mutation carriers. Recent work in rodent hepatocyte models has suggested a role of *Cdkn2a* in the regulation of PKA signaling (12). Given the well-characterized effects of PKA on potentiation of insulin secretion, we speculated that such signaling events could have a direct effect on β-cell function. To test this hypothesis, we performed knockdown and secretion studies in the human pancreatic β-cell line, EndoC-bH1. This cell line was transformed by Ravassard et al. (19) using the protooncogene SV40LT, which acts on the Retinoblastoma (Rb) protein, thereby masking effects of $p16^{INK4a}$ on cell-cycle control.

We first confirmed expression of $p16^{INK4a}$ by immunofluorescence and found that, consistent with previous reports, the protein localized to both the nucleus and cytoplasm (21,22) (Supplementary Fig. 3). siRNA-mediated silencing of *CDKN2A* was then performed and efficient knockdown observed both at the mRNA and protein levels (Fig. 2*A* and *B* and Supplementary Fig. 4). Ninety-six hours after gene silencing, cells treated with *CDKN2A* or nontargeting siRNAs were incubated under different conditions to assess the glucose responsiveness of the cells. In addition to basal and high-glucose conditions, the effect of the phosphodiesterase inhibitor (IBMX) on insulin secretion was tested. For all three conditions, *CDKN2A* knockdown was found to increase insulin secretion as a fraction of total content (basal, $P = 0.02$; high, $P = 0.01$; high glucose with IBMX, $P = 0.04$) (Fig. 2*C* and *D*), and as expected, no effect on proliferation was detected. We also observed a small but significant reduction in the total insulin content per cell ($P < 0.01$) (Supplementary Fig. 5). Finally, we performed PKA activity assays to directly assess the effect of *CDKN2A* silencing on the potentiating pathway of insulin secretion. Consistent with an increase in insulin secretion, this revealed a corresponding 23% increase in the activity of PKA after *CDKN2A* knockdown ($P = 0.02$) (Supplementary Fig. 6).

DISCUSSION

Individuals carrying heterozygous loss-of-function mutations in the *CDKN2A* gene provide a unique opportunity to study the role of $p16^{INK4a}$ and $p14^{ARF}$ in glucose homeostasis in humans. Through OGTT and IVGTT, we found that carriers displayed significantly increased insulin levels compared with matched control subjects. In a subset of individuals, measurements of C-peptide levels established a contribution of both decreased hepatic insulin clearance and increased β-cell function to the elevated circulating insulin. Further, grouping carriers by mutation status showed the effects to be driven either by $p16^{INK4a}$ exclusively or through similar effects on both $p16^{INK4a}$ and $p14^{ARF}$.

Overall, these results are consistent with a combination of two nonmutually exclusive mechanisms underlying the

Table 2—OGTT-derived measures of β-cell function, insulin sensitivity, and hepatic clearance

	Mutation carriers	Noncarriers	*P*
Fasting glucose (mmol/L)	5.2 (4.3; 6.3)	5.1 (3.2; 6.4)	0.65
Fasting insulin (pmol/L)	87 (15; 337)	55 (22; 150)	0.01
Fasting C-peptide (nmol/L)	0.44 (0.24; 0.81)	0.39 (0.20; 0.60)	0.48
iHOMA-B	124 (38.1; 452.4)	96 (45; 236.7)	0.05
iHOMA-S	91 (18; 328.7)	120 (35; 235.6)	0.05
BIGTT-AIR ($*10^3$)	6.4 (0.9; 28)	3.0 (1.2; 12)	0.03
BIGTT-S	5.8 (0.4; 12.8)	7.8 (1.1; 17.8)	0.04
Belfiore ISI	0.78 (0.17; 1.35)	0.97 (0.35; 1.77)	0.02
Matsuda ISI	4.3 (0.8; 11.1)	6.3 (1.5; 20.9)	0.02
$AUC_{glucose}$	839 (563; 1,449)	829 (502; 1,086)	0.79
AUC_{ins} ($*10^4$)	7.3 (2.4; 25)	4.7 (1.1; 15)	0.01
$AUC_{C\text{-}peptide}$	212 (106; 333)	212 (115; 260)	1.00
Insulinogenic index	203 (39; 561)	152 (53; 360)	0.08
C-peptidogenic index	0.45 (0.15; 1.12)	0.45 (0.17; 1.78)	1.00
Disposition index	2.3 (1.1; 3.8)	2.3 (1.0; 3.7)	0.98
Fasting insulin clearance	0.66 (0.12; 1.27)	0.88 (0.65; 1.29)	0.07
Insulin clearance	0.35 (0.16; 0.51)	0.56 (0.32; 1.05)	0.03

Data are mean and range (minimum; maximum). All indices based on C-peptide measurements are based on data from a subset of individuals only (*n* = 12 carriers and *n* = 12 control subjects; all U.K.). Details on definitions of physiological measures are listed in Supplementary Table 2. iHOMA-B, interactive HOMA of β-cell function; iHOMA-S, interactive HOMA of insulin sensitivity.

phenotype of carriers: *1*) primary β-cell hyperfunction driving progressive insulin resistance and/or *2*) primary insulin resistance triggering a compensatory increase in insulin levels (Supplementary Fig. 7). While both explanations are consistent with our data, existing evidence strongly supports a role for *CDKN2A* in β-cell function (9). Chronic hyperinsulinemia is known to result in a gradual downregulation of both insulin receptors and postreceptor signaling efficiency, thereby causing general insulin resistance and reduced insulin clearance (23). Our data are therefore in agreement with the expected physiological adaption to chronic hyperinsulinemia. However, due to limitations on the design of our clinical study, we cannot conclusively address the cause and effect between hyperinsulinemia and insulin resistance in mutation carriers. The IVGTT is well validated against clamp-based techniques, but power calculations based on our results suggest that an impractically high number of 50–60 individuals would be required to establish significant differences. Given the rarity of the disease, this exceeds the number of carriers available in the U.K. and Dutch cohorts recruited for our study.

To test for a cell cycle–independent role of *CDKN2A* in the regulation of insulin secretion, we performed knockdown studies in the human β-cell line, EndoC-bH1. This identified cell cycle–independent increases in insulin secretion under three conditions. These changes were found to be accompanied by increased PKA activity, in agreement with previous studies establishing such an effect of *CDKN2A* knockdown in liver (11,12). This suggests a possible contribution of the PKA-dependent potentiating pathway to the secretory effects observed in the EndoC-bH1 cell line. Taken in combination with existing data, our clinical and cellular studies indicate that the phenotype of carriers may arise out of a complex interplay between both cell cycle–independent and –dependent roles

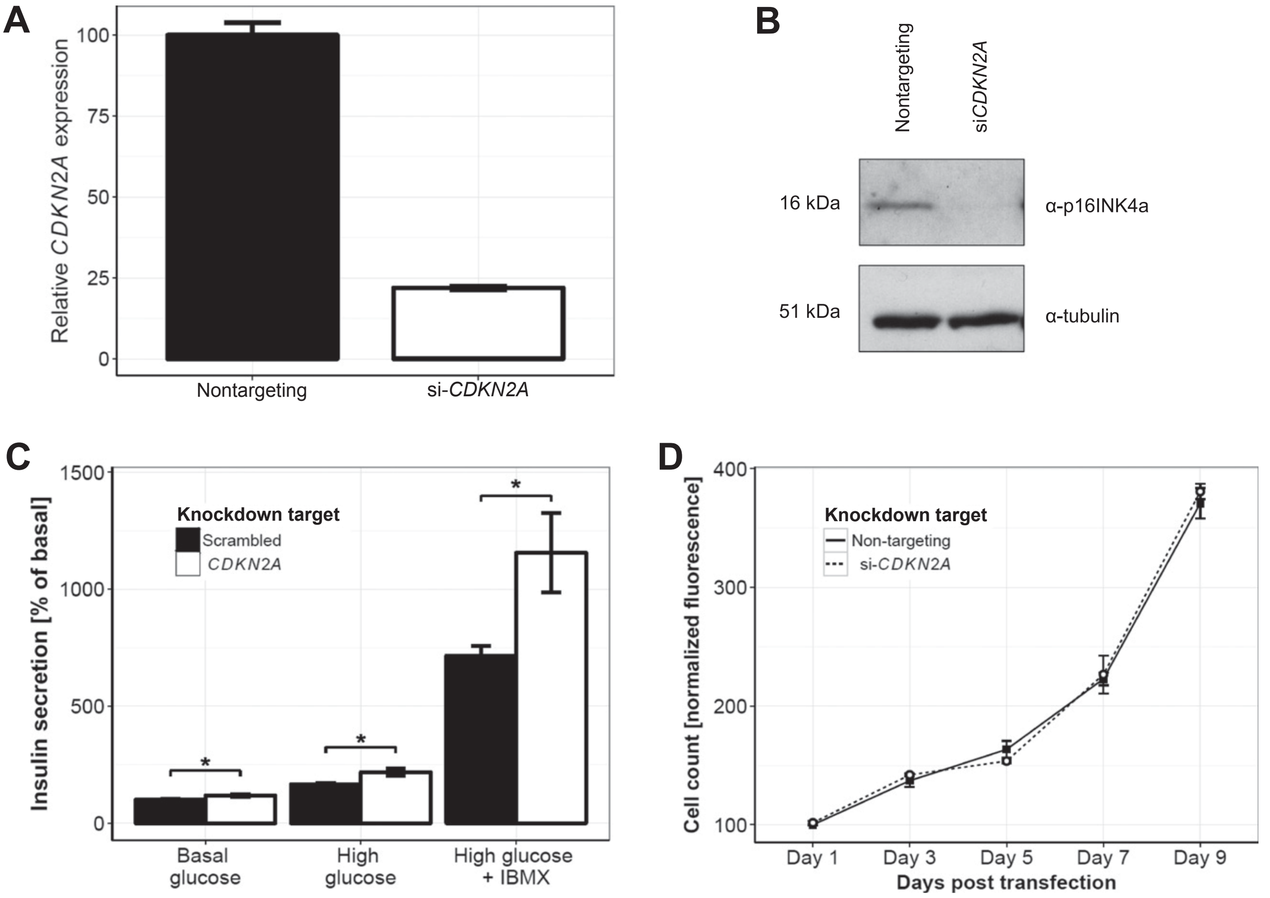

Figure 2—*CDKN2A* knockdown in the human β-cell line, EndoC-bH1. Silencing of *CDKN2A* and nontargeting sequence was performed using pools of siRNA (10 nmol/L) and knockdown confirmed both at the mRNA level (*A*) and at the protein level (*B*) after 72 h. Static insulin secretion assays were performed under culturing conditions of basal glucose (2.8 mmol/L glucose), high glucose (20 mmol/L), and high glucose with IBMX (100 μmol/L) (*C*). All secretion results were normalized to total insulin content per well. Cellular proliferation (*D*) after treatment with si-*CDKN2A* and nontargeting siRNA was measured using the CyQUANT Direct Cell Proliferation assay and normalized to the respective counts for day 1. Bars represent means for $n = 11$ (si-*CDKN2A*) and 18 (nontargeting) generated in three independent experiments. Data points for cellular proliferation are means for $n = 3$, and error bars are SEM. $^*P < 0.05$.

of *CDKN2A* in a range of tissues (9,12) (Supplementary Fig. 7).

Upstream of the *CDKN2A* and *CDKN2B* genes, several independent association signals for type 2 diabetes risk have been identified. The underlying effector transcript and disease mechanism has remained elusive, and prior studies have not reported any *cis*-expression quantitative trait loci effects for these alleles (24). Our study has shown that both coding *CDKN2A* mutations and the noncoding type 2 diabetes variants are associated with effects on measures of β-cell function. This provides a link between *CDKN2A* and the common genome-wide association study alleles and thus points to the gene as a likely effector transcript at this locus.

Interestingly, type 2 diabetes–associated variants at the *CDKN2A/B* locus have consistently been linked to a more "classic" β-cell phenotype than that observed for carriers of coding mutations in our study, with no evidence for an impact on measures of insulin resistance (3,4). We speculated that any *cis*-regulatory effect exerted on *CDKN2A* could achieve a more restricted β-cell phenotype through tissue-specific regulation of gene expression. To address this hypothesis, we interrogated existing genome annotations and found that the noncoding disease-associated variants map to a cluster of islet enhancer activity and open chromatin. Specifically, the association signals overlap a strong enrichment for islet- and melanocyte-specific FOXA-2 binding (25) (Supplementary Fig. 8). This highlights a possible mechanism for the more specific β-cell phenotype caused by common disease-associated variants compared with carriers of coding variants. Based on the direction of effect on measures of β-cell function, the noncoding risk alleles would be predicted to increase expression of *CDKN2A* (3,4). No *cis*-expression quantitative trait loci effects have previously been reported in islets for this region, but larger studies currently underway may be able to shed further light on this hypothesis (26).

Taken together, our data establish *CDKN2A* as an important regulator of glucose homeostasis in humans. We have shown that our data are consistent with loss-of-function mutations in *CDKN2A* affecting a range of tissues, including both pancreatic β-cells and liver. Our study thus supports the candidacy of *CDKN2A* as the effector transcript of the type 2 diabetes–associated alleles in the region, and we have proposed a mechanism to account for the apparent tissue specificity of the β-cell dysfunction caused by diabetes risk alleles.

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