Published in final edited form as:
Diabetes. 2016 February ; 65(2): 527–533. doi:10.2337/db15-0602.

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

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Abstract

At the *CDKN2A/B* locus, three independent signals for type 2 diabetes risk are located in a non-coding 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 whilst 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 controls. Compared with controls, 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 demonstrate that *CDKN2A* is an important regulator of glucose

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Author Contributions

AP, SKT, JNB, MIM, ALG conceived and designed the study. RS, TB provided protocols & clinical data. AP, TPP, SKT, AB, HJN performed the experiments. AP, ST, HJN, IFG analysed the data. AP, TPP, SKT, FK, IFG, HFAV, HP, MIM, ALG interpreted the data. SKT, ALG wrote the first draft of the manuscript. AP, TPP, MIM, HP edited the manuscript. AP, TPP, SKT, AB, HJN, RS, TJJ, IFG, TB, FK, HFAV, JNB, HP, MIM, ALG approved the final manuscript.

The authors declare no conflicts of interest.

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homeostasis in humans, thus supporting its candidacy as an effector transcript for type 2 diabetes-associated alleles in the region.

Introduction

Non-coding 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 non-coding RNA (*ANRIL*), while two distinct signals map to a region located further upstream of *CDKN2A* and *CDKN2B*. Physiological characterisations 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 $p16^{INK4a}$ and $p14^{ARF}$, which are known tumour suppressors acting via distinct signalling pathways (5, 6). $p16^{INK4a}$ is a cyclin-dependent kinase inhibitor (CDKI) involved in the regulation of cell cycle progression through inhibition of CDK4 and CDK6 (7). $p14^{ARF}$, 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 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 (HGP), 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 present study tested the hypothesis that mutation carriers show improved β-cell function compared with non-carriers.

Research design and methods

Study participants

Thirty-one cases diagnosed with FAMMM due to *CDKN2A* mutations were recruited from centres in the UK and the Netherlands. Twenty-eight had been cancer free for at least two years, and the remaining three cases had presented with melanoma between four to twelve months prior to inclusion in the study (supplementary table 1). For a control group of thirty-

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one participants, unaffected first-degree relatives or spouses of carriers were chosen when available and additional controls were recruited from the Oxford Biobank (www.oxfordbiobank.org.uk). Two controls were subsequently excluded based on 2-h OGTT glucose levels diagnosing 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 oral glucose tolerance test (OGTT)

All participants underwent a 75 g OGTT following a 12 hour 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 twelve mutation carriers and twelve controls) also 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 formulae in supplementary table 2.

Intravenous glucose tolerance test (IVGTT)

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

Cellular assays using the EndoC-bH1cell line

The EndoC-bH1 cell line was cultured and passaged as previously described (19). Reverse transfections were performed by adding pre-formed siRNA complexes prepared from ON-TARGETplus siRNA SMARTpools (Dharmacon) at a final concentration of 10 nM siRNA. For gene expression analysis, RNA was extracted and quantitative PCR (qPCR) performed using the TaqMan gene expression kit and assays (Applied Biosystems) on oligo-dT primed cDNA. 72 h after transfection, cells were starved overnight in 2.8 mM glucose followed by 1 h in 0 mM glucose medium. Static insulin secretion assays were then initiated by adding glucose-free growth medium supplemented with the indicated amounts of glucose and IBMX. After 1 h, aliquots of supernatants were removed for later analysis, and ice-cold acid ethanol added to extract insulin content from cells. Sample analysis was performed using the AlphaLISA Human Insulin Immunoassay (Perkin Elmer).

For protein kinase A (PKA) activity assays, cells were harvested following knockdown, as described above, and washed in phosphate-buffered saline. Matching input for number of cells, the samples were then processed according to manufacturer's instructions for the PepTag non-radioactive 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's t-test, except for gender differences where the Chi-squared test was used and for analysis of the IVGTT data where the Mann-Whitney U test was used.

Results

We recruited thirty-one participants carrying inherited *CDKN2A* loss-of-function mutations (supplementary table 1) and thirty-one controls, matched as a group for age (p = 0.99), gender (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 oral glucose tolerance test (OGTT) in all subjects (figure 1a-b). While no difference in glucose levels was detected, insulin levels were significantly increased in carriers throughout the test (p = 0.01 for insulin area under curve [AUC]; 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 non-carriers, both using a dynamic measure of acute insulin response (p = 0.03 for BIGTT-AIR), and in the fasted state (p = 0.05 for HOMA-B, homeostatic model assessment of β-cell function). Corresponding measures of insulin sensitivity, BIGTT-S and HOMA-S (homeostatic model assessment 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 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 controls (p = 0.98). These results were not significantly altered by exclusion of three carriers that had presented with melanoma within two years prior to inclusion in the study (supplementary table 3).

To explore whether the observed phenotype was driven by underlying effects on $p16^{INK4a}$, $p14^{ARF}$ or both, we re-analysed the data grouping carriers by mutation status (supplementary figure 1). Of the mutations identified, 26 affected both $p16^{INK4a}$ and $p14^{ARF}$, while five were located in regions affecting $p16^{INK4a}$ 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 $AUC_{glucose}$, respectively), suggesting that the observed metabolic phenotype of mutation carriers may be driven either solely by effects on $p16^{INK4a}$, or by effects of similar magnitude on both proteins.

For a subset of participants (twelve carriers and twelve controls) C-peptide measurements were obtained during the OGTT. Despite a tendency towards 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 cases and eight controls available for follow-up studies (supplementary figure 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 $AUC_{insulin}$ 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 around 30 % higher during 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 protein kinase A (PKA) signalling (12). Given the well-characterised effects of PKA on potentiation of insulin secretion, we speculated that such signalling 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 using the proto-oncogene SV40LT, which acts on the Retinoblastoma protein (Rb), thereby masking effects of $p16^{INK4a}$ on cell-cycle control (19).

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 (supplementary figure 3) (21, 22). siRNA-mediated silencing of *CDKN2A* was then performed and efficient knockdown observed both at the mRNA and protein level (figure 2a-b; supplementary figure 4). 96 hours after gene silencing, cells treated with *CDKN2A* or non-targeting 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, $p = 0.04$; figure 2c-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 figure 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 following *CDKN2A* knockdown ($p = 0.02$; supplementary figure 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 oral and intravenous glucose tolerance tests, we found that carriers displayed significantly increased insulin levels compared with matched controls. 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, non-mutually exclusive mechanisms underlying the phenotype of carriers: *(a)* primary β-cell hyperfunction driving progressive insulin resistance; and/or *(b)* primary insulin resistance triggering a compensatory increase in insulin levels (supplementary figure 7). While both explanations are consistent with our data, existing evidence strongly support a role for *CDKN2A* in β-cell function (9). Chronic hyperinsulinemia is known to result in a gradual down-regulation of both insulin receptors and post-receptor signalling 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 UK 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 of *CDKN2A* in a range of tissues (supplementary figure 7) (9, 12).

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 (cis-eQTL) effects for these alleles (24). Our study has shown that both coding *CDKN2A* mutations and the non-coding type 2 diabetes variants are associated with effects on measures of β-cell function. This provides a link between *CDKN2A* and the common GWAS 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 non-coding 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 (supplementary figure 8) (25). This highlights a possible mechanism for the more specific β-cell phenotype caused by common disease-associated

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variants compared with carriers of coding variants. Based on the direction of effect on measures of β-cell function, the non-coding risk alleles would be predicted to increase expression of *CDKN2A* (3, 4). No cis-eQTL 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Linda Whitaker (University of Leeds) and Beryl Barrow (University of Oxford) for their valuable assistance with recruiting FAMMM patients in the UK, Marja Dijk, Bep Ladan and Petra Beckers (Leiden University Medical Centre) for assistance with performing the OGTTs in the Netherlands, and Jonathan Levy (University of Oxford) for advice on designing the study. We thank all volunteers in the UK and the Netherlands for their participation. The Oxford Biobank (www.oxfordbiobank.org.uk), NIHR Oxford Biomedical Research Centre, is part of the NIHR National Bioresource which supported the recalling process of the volunteers.

This study was funded by the Wellcome Trust (095101/Z/10/Z and 098381), the Medical Research Council (G0800467), the National Institute of Health Research Oxford Biomedical Research Centre, Cancer Research UK (C588/A19167) and the ZOLEON foundation (no 12.09). ALG is a Wellcome Trust Senior Fellow in Basic Biomedical Science and MIM is a Wellcome Trust Senior Investigator. ALG is the guarantor of the data in this manuscript.

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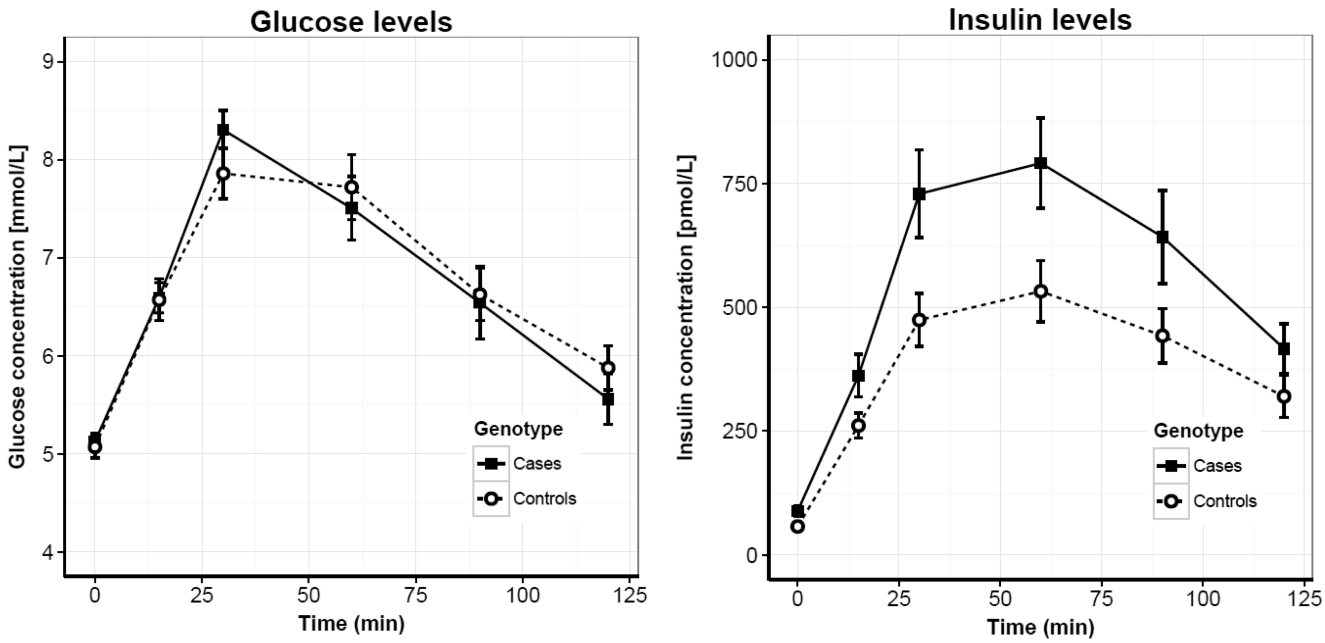

Figure 1.

Serum glucose (*left panel*) and insulin (*right panel*) levels during a 120-min OGTT in thirty-one carriers (*black squares, solid line*) and thirty-one controls (*white circles, dashed line*). Data shown as mean +/− SEM.

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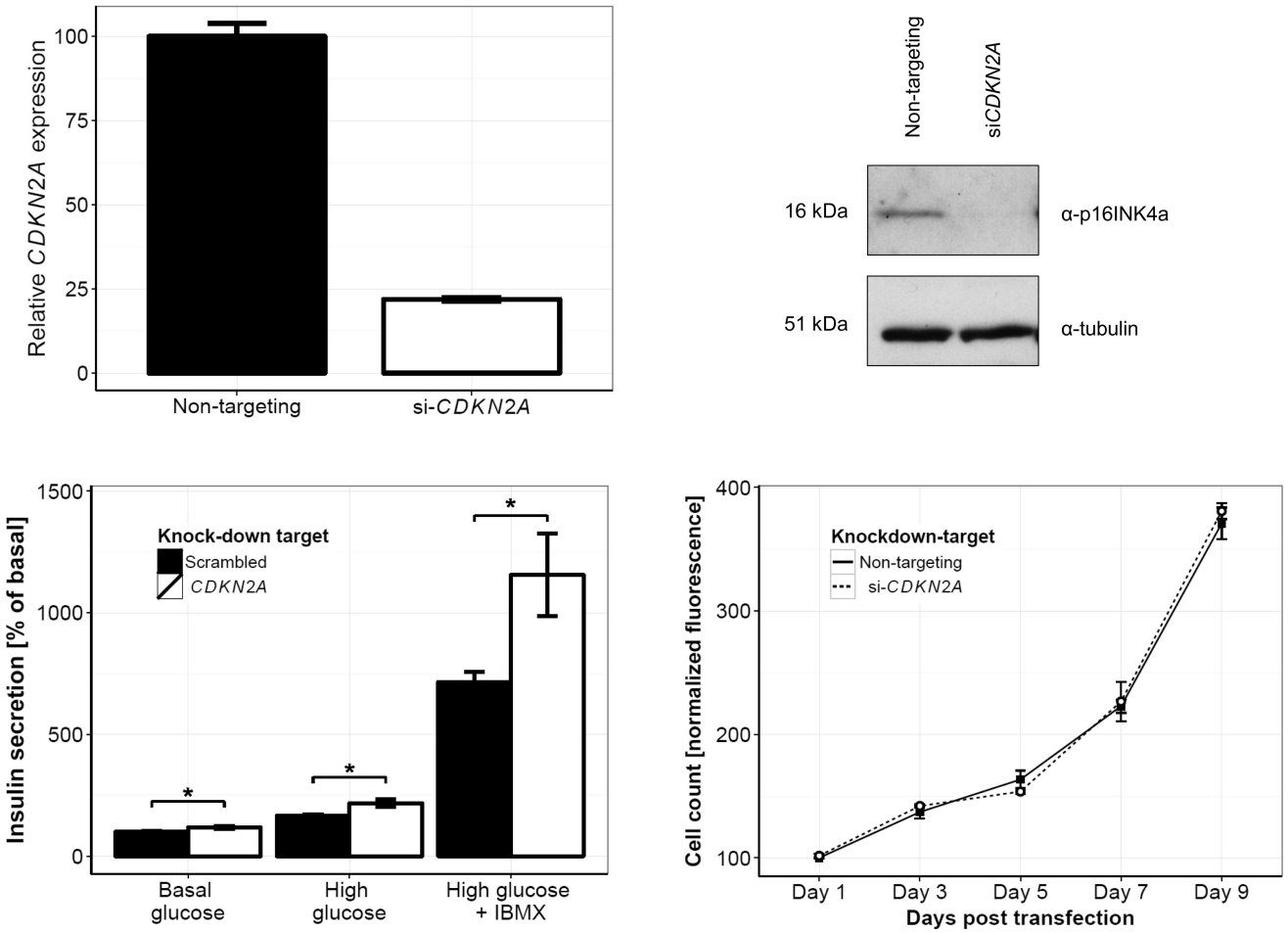

Figure 2.

CDKN2A knockdown in the human β-cell line, EndoC-bH1. Silencing of *CDKN2A* (*white bars*) and non-targeting sequence (*black bars*) was performed using pools of siRNA (10 nM), and knockdown confirmed both at the mRNA level (*upper left panel*) and at the protein level (*upper right panel*) after 72 h. Static insulin secretion assays were performed under culturing conditions of basal glucose (2.8 mM glucose), high glucose (20 mM) and high glucose with IBMX (100 μM) (*lower left panel*). All secretion results were normalized to total insulin content per well. Cellular proliferation (*lower right panel*) following treatment with si-*CDKN2A* (*white circles, dashed line*) and non-targeting siRNA (*black squares, solid line*) was measured using the CyQUANT Direct Cell Proliferation assay, and normalized to the respective counts on day 1. Bars represent means for n = 11 (si-*CDKN2A*) and 18 (non-targeting) generated in three independent experiments. Data points for cellular proliferation are means for n = 3, and error bars are SEM.

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Table 1

Baseline characteristics of study participants. Data are given as mean and range [min; max]. P-values are from Welch's t-test except for gender distribution where the Chi-squared test was performed.

	Mutation carriers	Non-carriers	P-value
n	31	31	*NA*
BMI / [cm/kg^2]	27.1 [19; 38]	27.1 [19; 36]	0.97
Age / [yrs]	51.8 [21; 71]	51.8 [25; 84]	0.99
Gender / [% male]	45	32	0.43

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Table 2

OGTT-derived measures of β-cell function, insulin sensitivity and hepatic clearance. Data are given as mean and range [min; max]. All indices based on C-peptide measurements are based on data from a subset of individuals only (n = 12 carriers and n = 12 controls; all UK). Details on definitions of physiological measures are listed in supplementary table 2.

	Mutation carriers	Non-carriers	P-value
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; 1449]	829 [502; 1086]	0.79
$AUC_{insulin}$ [$*10^4$]	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