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Targeted demethylation at the \textit{CDKN1C}/p57 locus induces human β cell replication

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The loss of insulin-secreting β cells is characteristic among type I and type II diabetes. Stimulating proliferation to expand sources of β cells for transplantation remains a challenge because adult β cells do not proliferate readily. The cell cycle inhibitor p57 has been shown to control cell division in human β cells. Expression of p57 is regulated by the DNA methylation status of the imprinting control region 2 (ICR2), which is commonly hypomethylated in Beckwith-Wiedemann syndrome patients who exhibit massive β cell proliferation. We hypothesized that targeted demethylation of the ICR2 using a transcription activator–like effector protein fused to the catalytic domain of TET1 (ICR2-TET1) would repress p57 expression and promote cell proliferation. We report here that overexpression of ICR2-TET1 in human fibroblasts reduces p57 expression levels and increases proliferation. Furthermore, human islets overexpressing ICR2-TET1 exhibit repression of p57 with concomitant upregulation of Ki-67 while maintaining glucose-sensing functionality. When transplanted into diabetic, immunodeficient mice, the epigenetically edited islets show increased β cell replication compared with control islets. These findings demonstrate that epigenetic editing is a promising tool for inducing β cell proliferation, which may one day alleviate the scarcity of transplantable β cells for the treatment of diabetes.

Introduction

In 2014, 422 million people worldwide lived with diabetes (1). While patients with type I or severe forms of type II diabetes can manage extreme glucose excursions by administering insulin shortly before a meal, only β cell replacement therapy through transplantation of healthy pancreatic islets obtained from carefully screened deceased organ donors provides long-term diabetes reversal without the need for insulin injections (2, 3). However, the use of β cell replacement therapy is limited by the availability of transplantable donor islets. As a result, there is a concerted effort to produce functionally mature β cells from embryonic stem cells and induced pluripotent stem cells, or to identify strategies to enhance β cell proliferation (4, 5).

Although inducing β cell proliferation would appear to be a straightforward approach, it is severely limited by the fact that the proliferative capacity of human β cells declines dramatically after early childhood (6, 7). This correlates with an age-related accumulation of the senescence effector p16 that induces cell cycle arrest in β cells (8). Strikingly, the overgrowth disorder Beckwith-Wiedemann syndrome (BWS) is characterized by an expansion of β cell mass — in addition to other tissue types such as the tongue, kidneys, liver, and other abdominal organs — that is associated with decreased protein levels of the cell cycle inhibitor p57 (9), a recognized negative regulator of β cell replication (10–12).

The cell cycle inhibitor p57 is the protein product of the \textit{CDKN1C} gene, which is imprinted and regulated by the DNA methylation status of the nearby imprinting control region 2 (ICR2). The ICR2 is a CpG-dense region located on chromosome 11p15.5 that is hypomethylated on the paternal allele, and hypermethylated on the maternal allele (13). This asymmetrical methylation signature is linked to preferential expression of \textit{CDKN1C} from the maternal allele through molecular mechanisms that are still not well understood. In the majority of patients with BWS, the ICR2 is hypomethylated on both alleles (12), correlating with deactivation of \textit{CDKN1C} and an increase in proliferation of β cells.

By mimicking the molecular alterations observed in BWS via transcription activator–like effector (TALE) epigenome editing (Figure 1A), we were able to target and demethylate the ICR2 in β cells of human islets. We show in this proof-of-principle study that targeted epigenetic editing can be harnessed to induce β cell proliferation and model critical aspects of human imprinting disorders.

Results and Discussion

A TALE-TET1 effector causes specific demethylation of the ICR2 at the \textit{CDKN1C} locus. TALE proteins are commonly utilized for epigenome editing owing to their customizable yet highly specific DNA-recognition domain and compatibility with numerous chromatin modifiers (14, 15). Indeed, a prior study demonstrated the high specificity and limited off-target effects of TALE proteins fused to the catalytic domain of the methylcytosine dioxygenase
demethylation at its binding site in the ICR2 (Figure 1B), demonstrating local specificity of the epimutation achieved. Methylation at regions 2 and 3 of the ICR2, including the KCNQ1OT1 promoter, was not changed by the ICR2-TET1 protein. Furthermore, the untethered TET1-cd had no effect on DNA methylation at the targeted locus. These results establish that the ICR2-targeting TALE-TET1 protein is functional and a suitable tool for investigating the relationship between the ICR2 methylation status, p57 expression, and proliferative capacity of epigenetically edited cells. It is important to note that the demethylation effect of the TALE-TET1 TET1 (16), which facilitates the passive and active demethylation of methylated CpGs. We designed a TALE-TET1 fusion protein targeting the ICR2 (ICR2-TET1) at chr11:2,720,607–2,720,625 (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI99170DS1). As controls, we engineered a fusion protein with an identical TALE DNA-binding domain ligated to an enzymatically dead TET1 mutant protein (ICR2-TET1\textsuperscript{dead}), and an untethered TET1 catalytic domain (TET1-cd). By performing targeted bisulfite sequencing in sorted HEK293T cells (17), we found that the ICR2-TET1 protein induced

Figure 1. Targeted demethylation of the ICR2 at the CDKN1C/p57 locus causes increased proliferation of human fibroblasts. (A) Schematic of the imprinted chr11p15.5 locus. The ICR2 is methylated (depicted by black circles) at the promoter of the long noncoding RNA KCNQ1OT1 on the maternal allele, and correlates with maternal allele-specific expression of CDKN1C. A TALE-TET1 fusion protein was designed to target the ICR2 and remove the methylated CpGs at the ICR2 in order to deactivate CDKN1C and increase cell proliferation. (B) Three regions within the ICR2 were amplified for methylation analysis by targeted bisulfite sequencing. Percentage CpG methylation at 3 regions of the ICR2 are shown (n = 3 for each condition). (C) p57 mRNA and protein levels in fibroblasts overexpressing ICR2-TET1\textsuperscript{dead} or ICR2-TET1 (n = 3 for each condition). VCL, vinculin. (D) EdU incorporation in fibroblasts 72 hours after transduction with the ICR2-TET1\textsuperscript{dead} or ICR2-TET1 lentivirus (n = 5 for each condition). Scale bar: 100 μm. *P < 0.05; **P < 0.01 by 1-way ANOVA (B), 1-tailed t test (C), or 2-tailed t test (D). NS, not significant.
The mechanism by which the methylation status of the ICR2 regulates CDKN1C expression is unclear. The long noncoding RNA KCNQ1OT1, which is transcriptionally active when the ICR2 is hypomethylated, has been suggested to silence CDKN1C in cis (18, 19). Although we observed a slight increase in expression levels of KCNQ1OT1 in both transfected HEK293T cells and transduced fibroblasts (Figure 1C and Supplemental Figure 2), we did not find any significant increase in bi-allelic expression of KCNQ1OT1 in response to ICR2 demethylation by DNA-RNA FISH (Supplemental Figure 3), indicating that deactivation of CDKN1C is mediated by a mechanism independent of the in cis function of KCNQ1OT1 (20, 21).

Demethylation of the ICR2 increases proliferation in human fibroblasts. Having established that targeted demethylation of the ICR2 causes inactivation of p57 and increased expression of Ki-67 in human fibroblasts, we wanted to evaluate the phenotypic outcome of TALE-TET1-induced demethylation of the ICR2. We performed

Figure 2. ICR2-TET1 deactivates p57 in human β cells. (A) CDKNIC mRNA expression in islets transduced for 72 hours with the ICR2-TET1dead or ICR2-TET1 adenovirus. Islets were obtained from 7 non-diabetic, non-obese, deceased organ donors. (B) CDKNIC mRNA expression in islets grouped by adenoviral treatment. Each data point represents the relative expression of CDKNIC from one of the donors from A (n = 7 for each condition). *P < 0.02 by Wilcoxon’s matched-pairs signed-rank test. (C) Immunocytochemical staining of transduced β cells. White arrowheads indicate transduced GFP+ β cells, while purple arrowheads indicate untransduced controls. Unlike the Ad.ICR2-TET1dead-transduced β cells, Ad.ICR2-TET1-transduced β cells stained weakly for p57. Scale bar: 50 μm.
expression resulting from ICR2-TET1 overexpression primarily occurred in β cells. Donor-to-donor variability in CDKNIC deactivation can be attributed to variability in transduction efficiency, where on average only 20%-40% of β cells were successfully transduced. Despite variation between the individual donors assayed, there was a significant decrease in CDKNIC transcript levels in the ICR2-TET1-treated islets when all donors were analyzed collectively (P < 0.05) (Figure 2B). Decreased p57 protein levels were further verified through immunocytochemical staining of transduced compared with nontransduced β cells (Figure 2C).

Epigenetically edited β cells retain glucose-sensing functionality. Next, we wanted to determine whether p57 deactivation impacted β cell function. We first performed whole islet perfusion to determine differences in glucose-stimulated insulin secretion (GSIS) between Ad.ICR2-TET1dead– and Ad.ICR2-TET1–transduced islets. We found that GSIS was not impaired in Ad.ICR2-TET1–transduced islets compared with Ad.ICR2-TET1dead controls (Figure 3A). However, only a subset of β cells were transduced in the whole islet, necessitating the need for an additional technique to assess functionality of the transduced β cells specifically. We measured single-cell intracellular calcium concentrations as a surrogate for insulin secretion in transduced β cells by using Fura-2 as a calcium indicator. Basal [Ca2+]i, as well as calcium response to high-glucose stimulation were similar between GFP+, Ad.ICR2-TET1–transduced β cells and GFP+, Ad.ICR2-TET1dead control cells (Figure 3B). Together, these results demonstrate that targeted demethylation of the ICR2 does not negatively impact β cell function.

Targeted demethylation of the ICR2 leads to increased replication of β cells. After verifying that epigenetically edited β cells displayed normal glucose-sensing behavior, we next assessed the effect of targeted ICR2 demethylation on β cell replication. In addition to decreased CDKNIC transcript levels, ICR2-TET1–treated human islets exhibited increased expression of the proliferation marker MKI67, which was significantly anticorrelated with CDKNIC transcript levels (Figure 4A). We were also able to detect Ki-67 protein in transduced β cells, as well as quantify an increase in expression of several cell cycle genes controlling the G2/M transition (Supplemental Figure S).

In order to study the long-term effect of TALE-TET1–induced epimutation on β cell proliferation, we utilized the immunodeficient NOD-scid IL2rγnull (NSG) mouse model rendered diabetic with treatment of streptozotocin (Figure 4B). To control for the variability of mitogenic stimuli provided by the host, each diabetic NSG mouse was transplanted simultaneously with 100 Ad.ICR2-TET1– and Ad.ICR2-TET1dead–transduced islets (placed in opposite subcutaneous pockets). After transplantation, mice were administered BrdU in the drinking water for 3 weeks in order to label every cell that entered S-phase during this time frame. The transplanted islets were able to correct the hyperglycemic glucose levels of the NSG mice, indicating that they remain functional (Supplemental Figure 6). After 3 weeks, the islet xenografts were harvested, processed for paraffin sectioning, and stained for C-peptide and BrdU to identify β cells and cells that replicated their DNA, respectively. Strikingly, we observed an increase in the percentage of BrdU-positive β cells in the islet fraction that had received the ICR2-TET1 compared with the ICR2-TET1dead control in 4 out of the 5 donors that underwent the transplant study (Figure 4, C and D). Furthermore, we detect-
late expression of disease-related genes. Already, several studies have demonstrated the promise of epigenetic editing in targeting genes involved in cancer and neurological disorders (25–27). In the context of stimulating β cell replication, targeting p57 through epigenetic editing is an attractive strategy for several reasons. First, p57 is difficult to inhibit pharmacologically due to its nuclear localization, necessitating intervention at the gene regulation level. Secondly, the DNA methylation signature of the epigenetically edited parent cell is inherited by the daughter cells after cell division, which suggests that \( CDKN1C \) will remain silenced after multiple rounds of cell division. However, additional studies are needed to investigate how long the induced epimutation and p57 deactivation can be maintained, and to evaluate the risk for hyperinsulinism from continuous β cell expansion. Our data suggest that epigenetically edited β cells have functional K\(_{ATP} \) channels, and thus should be responsive to diazoxide to attenuate insulin secretion if needed. Altogether, our results for using epigenetic editing to promote β cell replication is an encouraging approach.

We demonstrate that epigenetic editing can be applied to promote replication in human pancreatic β cells. We successfully demonstrate that targeted demethylation of the ICR2 directly results in the downregulation of p57, aligning with the underlying epigenetic cause of BWS. In β cells, this epimutation causes increased Ki-67 expression as well as increased cell cycle entry as detected by BrdU incorporation in the xenografts of transplanted NSG mice.

Epigenetic editing holds great therapeutic potential due to its effectiveness at reprogramming stable epigenetic marks to modulate expression of disease-related genes. Already, several studies have demonstrated the promise of epigenetic editing in targeting genes involved in cancer and neurological disorders (25–27). In the context of stimulating β cell replication, targeting p57 through epigenetic editing is an attractive strategy for several reasons. First, p57 is difficult to inhibit pharmacologically due to its nuclear localization, necessitating intervention at the gene regulation level. Secondly, the DNA methylation signature of the epigenetically edited parent cell is inherited by the daughter cells after cell division, which suggests that \( CDKN1C \) will remain silenced after multiple rounds of cell division. However, additional studies are needed to investigate how long the induced epimutation and p57 deactivation can be maintained, and to evaluate the risk for hyperinsulinism from continuous β cell expansion. Our data suggest that epigenetically edited β cells have functional K\(_{ATP} \) channels, and thus should be responsive to diazoxide to attenuate insulin secretion if needed. Altogether, our results for using epigenetic editing to promote β cell replication is an encouraging approach.
starting point towards increasing the supply of transplantable β cells for diabetic patients.

Methods

For complete methods, see supplemental material.

Statistics. Student’s t test using a 2-tailed distribution, unless otherwise stated in the text or figure legends, was used to determine statistical significance. For all tests, P < 0.05 was considered statistically significant. Data represent mean ± SEM. All analyses were conducted using GraphPad Prism 6.

Study approval. The experiments using islets obtained from cadaveric organ donors were declared exempt by the IRB of the University of Pennsylvania. The transplantation studies were approved by the IACUC of the University of Pennsylvania.

Author contributions

KHK, BG, and DA conceived the study. KO, MY, KHK, BG, and DA designed the experiments. KO, MY, CJ, YJW, and NGM acquired the data. KO and YJW analyzed and interpreted the data. KO wrote the manuscript and performed the statistical analyses. AWW, SCN, EFJ, VK, EF, and AN provided material support. KHK, BG, YJW, and EFJ reviewed and edited the manuscript. KHK supervised the study.

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