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SHORT REPORT

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Selective monitoring of insulin secretion after CRISPR interference in intact pancreatic islets despite submaximal infection

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ABSTRACT
Virus-mediated gene knockdown in intact pancreatic islets is technically challenging due to poor infection of the center of the islet. Because the cells that do not have knockdown have normal insulin secretion, measuring changes in insulin secretion after gene knockdown is challenging. We describe a method to monitor insulin secretion from only the beta cells with knockdown of a gene of interest in intact islets using a single lentivirus containing a guide RNA, a luciferase insulin secretion reporter and a dCas9-KRAB cassette. This method allows rapid and inexpensive monitoring of insulin secretion from only those beta cells with knockdown, circumventing the problem of incomplete islet infection.

Introduction

Insulin secretion by the pancreatic islet is critical for glucose homeostasis. Defects in insulin secretion are among the first changes seen in the development of type 2 diabetes[1] and insulin secretion is the target of the majority of diabetes therapeutics. Therefore, a deeper understanding of insulin secretion may lead to new treatment modalities for type 2 diabetes.

While insulin secretion has been studied in transformed beta cell lines, it is known that these cells do not have completely normal insulin secretion. Therefore, the optimal cells in which to study insulin secretion are primary islets. While small molecules can penetrate the three-dimensional structure of the pancreatic islet, gene knockdown has been difficult in islets because viruses are presumably too large to penetrate the outer layer of cells. As such, some protocols call for the dissociation of the islets, infection with adenovirus or lentivirus and then reaggregation of the cells to form pseudoislets which regain insulin secretion.[2] Others involve partial digestion of the islets to allow better penetration of the virus.[3]

While these approaches work, we sought to develop a method to monitor insulin secretion from only the subset of beta cells with knockdown of a gene of interest. Monitoring insulin secretion from subpopulations of beta cells is not new. Co-transfection of human growth hormone (hGH) and measurement of hGH secretion takes advantage of the fact that hGH is co-secreted with insulin.[4] Since it is now appreciated that growth hormone has direct effects on beta cells, this is no longer a viable strategy.[5] Insulin secretion has also been tracked by insertion of GFP into C-peptide.[6,7]

We used the more recently described luciferase-C-peptide fusion strategy[8] to measure insulin secretion only from cells that have knockdown of a gene of interest by creating a single lentiviral vector with a luciferase insulin secretion reporter and a knockdown cassette. Our strategy can measure insulin secretion from <1% of beta cells in an islet, allowing cost and effort effective measurements of insulin secretion following gene knockdown without disruption of the native islet structure.

Results

We developed an insulin secretion reporter vector akin to those previously described[8,9] by placing a copy of the nanoluciferase cDNA into the

C peptide region of the mouse Ins2 cDNA. We included the proximal ~360 bases of the mouse *Ins2* promoter to selectively drive expression of the reporter only in beta cells. We termed this construct mIns2-NL(Figure 1A). We hypothesized that this construct would result in the expression of a pro-insulin -C-peptide nanoluciferase fusion protein that would then be processed into a C-peptide-luciferase fusion protein and mature mouse Ins2 protein (Figure 1B). m*Ins2*-NL was packaged into lentivirus and infected at a low multiplicity of infection (MOI) into MIN6 transformed beta cells. A single cell clone was isolated and a batch glucose stimulated insulin secretion (GSIS) assay was performed at either 2.8 mM glucose or 16.7 mM glucose. As others have found, luciferase counts from the secretion samples closely tracked the insulin measured by ELISA (Figure 1C), demonstrating that our nanoluciferase gives a faithful readout of insulin secretion.

To ensure that the minimal mouse *Ins2* promoter directs exclusive expression of the reporter in beta cells, we cloned a GFP reporter virus (termed mIns2-GFP) where the protein coding sequence of the pre-prosinsulin-nanoluciferase fusion in the mIns2-NL reporter was replaced with GFP (Figure 1D). Dissociated mouse islets were infected with this reporter at an MOI of 10. If all islet cells nonspecifically expressed the mIns2-GFP, we would expect only 70-80% of GFP positive cells to be insulin positive since beta cells make up ~70-80% of all islet cells. We counted 106 GFP positive cells and all 106 were insulin positive by immunofluorescence, suggesting that this reporter is beta cell specific. To confirm this, we counted glucagon positive cells and found that 0 of 36 glucagon positive cells were GFP positive (example shown in Figure 1D) while 90 of 104 insulin positive cells were GFP positive (Fisher's exact test *p* value <.0001). Taken together, these data show that our minimal mouse *Ins2* promoter confers beta cell-specific expression.

To use this luciferase reporter to monitor insulin secretion only in RNA interference (RNAi) knockdown cells, we fused the mIns2-NL to a minimal CMV promoter driving histone H2B fused to GFP (H2B-GFP) with a small hairpin RNA in its 3' untranslated region. From here forward, we refer to this vector as H2B-GFP_mIns2NL (Figure 2A). To determine if the nanoluciferase reporter is properly processed into the C-peptide-nanoluciferase

fusion protein, we performed a western blot on MIN6 cells expressing H2B-GFP_mIns2NL using a nanoluciferase antibody (Figure 2B). We found that the majority of the nanoluciferase reactivity is at the expected size of the processed C-peptide nanoluciferase protein (~20 kDa) while a minority is at the size of the proinsulin nanoluciferase fusion protein (~28 kDa). We speculate that the doublet at 20 kDa may be a degradation or post-translational modification product of the C-peptide nanoluciferase fusion protein.
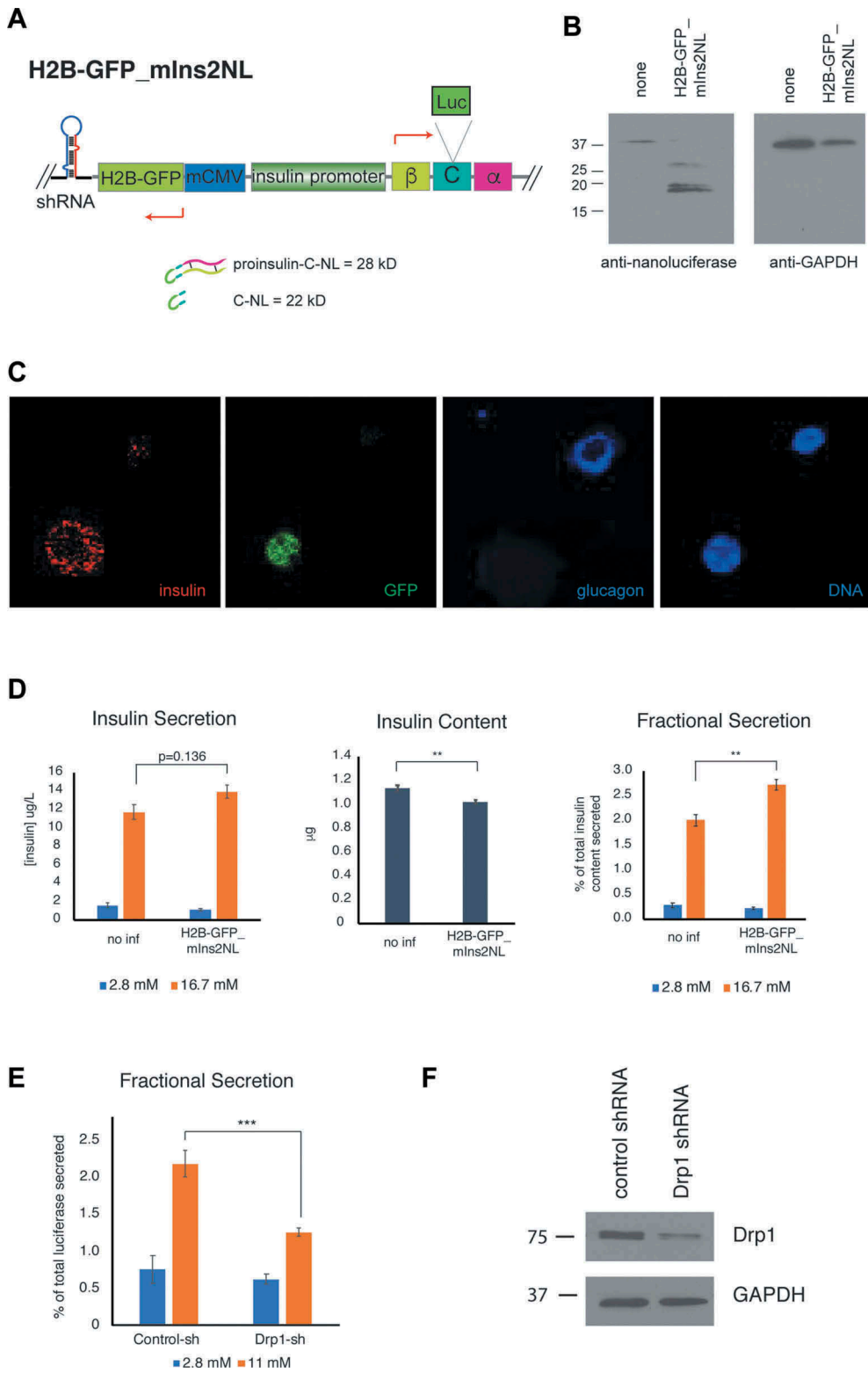
The minimal CMV promoter is reported to maintain the cell-type specificity of its neighboring promoter,[10] potentially allowing us to express the shRNA only in beta cells. To confirm this, we infected dissociated mouse islets with the H2B-GFP_mIns2NL virus containing a control shRNA. Of 99 nuclear GFP positive cells counted, 98 were insulin positive, suggesting that the shRNA and H2B-GFP is predominantly expressed in beta cells. We examined 20 glucagon positive cells and found that none were GFP positive, while 44 of 83 insulin positive cells were GFP positive (Fisher's eact test *p* value < .0001) (example staining in Figure 2C). These data suggest that the anti-sense direction of H2B-GFP_mIns2NL retains beta cell-specific expression.

Expression of an additional transgenic copy of the insulin gene could interfere with endogenous insulin secretion. We infected MIN6 cells at an MOI of 10 with the H2B-GFP_mIns2NL virus with a control shRNA and saw 85% of cells expressing the reporter by GFP fluorescence (not shown). We then performed batch GSIS and measured insulin secretion by ELISA for mouse insulin. We found that absolute insulin secretion was unchanged by expression of the reporter (Figure 2D, left). While we expected increased total insulin in the reporter expressing cells, we found a 10% reduction in total insulin content (Figure 2D, middle). This reduced total insulin caused an increase in fractional insulin secretion (absolute insulin secretion normalized to total cellular insulin content) (Figure 2D, right). These data show that while absolute insulin secretion is unaffected by expression of the H2B-GFP_mIns2NL reporter, total insulin is slightly decreased.

We then infected MIN6 cells with the H2B-GFP_mIns2NL reporter containing an shRNA
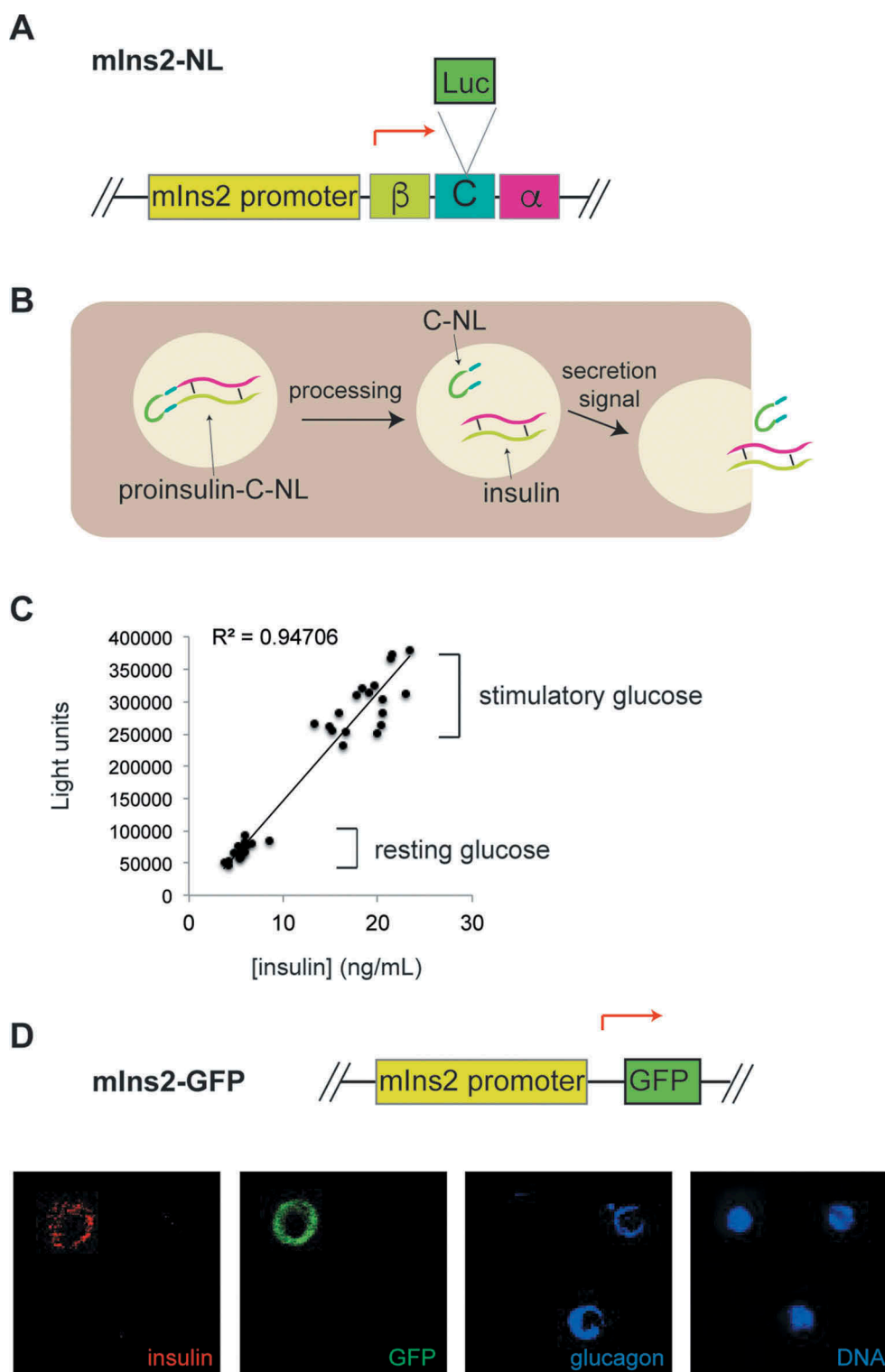
Figure 1. Luciferase insulin secretion reporter accurately reflects insulin secretion and is specifically expressed in beta cells. (A) Schematic of the base reporter, m*Ins2*-NL. mIns2 promoter is the proximal 360 bases of the mouse *Ins2* gene. β, C, and α indicate the coding regions that will give rise to the indicated chains of the insulin protein. (B) Diagram of processing of the insulin luciferase reporter. (C) Correlation between mouse insulin and luciferase counts at resting glucose (2.8 mM) or stimulatory glucose (16.7 mM) from MIN6 cell after batch stimulation. (D) Beta cell specific expression driven by the mIns2 promoter sequence. GFP was cloned downstream of the mIns2 minimal promoter. Lentivirus containing this reporter was used to infect dissociated primary mouse islets. Two days after infection, the cells were stained for insulin, GFP, glucagon and DNA. An example field is shown.

A

H2B-GFP_mIns2NL

shRNA

proinsulin-C-NL = 28 kD

C-NL = 22 kD

B

anti-nanoluciferase   anti-GAPDH

C

insulin   GFP   glucagon   DNA

D

Insulin Secretion

p=0.136

[insulin] ug/L

no inf   H2B-GFP_mIns2NL

■ 2.8 mM ■ 16.7 mM

Insulin Content

**

μg

no inf   H2B-GFP_mIns2NL

Fractional Secretion

**

% of total insulin content secreted

no inf   H2B-GFP_mIns2NL

■ 2.8 mM ■ 16.7 mM

E

Fractional Secretion

***

% of total luciferase secreted

Control-sh   Drp1-sh

■ 2.8 mM ■ 11 mM

F

Drp1

GAPDH

targeting *Dnml1* (or Drp1), a gene required for mitochondrial fission and optimal GSIS,[11,12] or a H2B-GFP_mIns2NL reporter containing control shRNA to achieve ~30% infection as measured by GFP fluorescence (not shown). We performed batch GSIS on the unsorted cells and measured luciferase to monitor secretion from cells with knockdown of Drp1. We found a 40% reduction in fractional luciferase secretion in the Drp1 shRNA expressing cells as compared to the control shRNA expressing cells (Figure 2E). We sorted GFP+ cells by flow cytometry and measured Drp1 by western blot (Figure 2F) and found modest knockdown of Drp1 protein.

Since the degree of knockdown was not optimal using this Drp1 shRNA and shRNAs are often less efficient than more modern CRISPR approaches, we adapted our system for CRISPR interference-(CRISPRi), where a catalytically inactive Cas9 protein fused to the KRAB transcriptional repressor silences transcription directed by a small guide RNA (sgRNA).[13] In this case, we placed a U6 pol III promoter to drive the expression of a sgRNA 5' to our luciferase reporter and added a dCas9-KRAB using a T2A ribosomal skip sequence (Figure 3A). While the U6 promoter is active in all cells, the mouse *Ins2* promoter ensures that dCas9-KRAB expression, and therefore target silencing, only occurs in beta cells. We were unable to incorporate a fluorescent marker in this construct as the virus is already near or above the lentivirus packaging limit. Instead, we monitored infection rates using droplet PCR quantitation of the lentiviral DNA from genomic DNA (see Methods).

To test this construct, we cloned a sgRNA targeting the glucokinase gene (*Gck*). This sgRNA gives >99% knockdown in MIN6 cells that stably express dCas9-KRAB (Figure 3B). We infected MIN6 cells with either a *Gck*-targeting sgRNA insulin secretion reporter virus or a non-targeting insulin secretion reporter virus, achieving 0.3% infection for the control sgRNA and 0.6% infection for the *Gck* targeting sgRNA. We deliberately chose to infect the cells at a very low MOI since this would allow us to simulate the conditions in suboptimal islet infection. We found that insulin secretion from these cells as measured by luciferase was reduced by 40% as compared to the non-targeting sgRNA (Figure 3C). Since the vast majority of cells are not infected and should have normal insulin secretion, we predicted that a measurement of mouse insulin secretion should not be different between the sgRNAs. Indeed, there was no difference in fractional insulin secretion as measured by ELISA from the same secretion samples used in Figure 3C (Figure 3D).

Finally, we infected the same *Gck* targeting or non-targeting lentiviruses into intact primary mouse primary islets, achieving 2% infection for control sgRNA and 0.5% for Gck sgRNA. We found that fractional luciferase secretion from mouse islets infected with Gck knockdown virus was markedly reduced as compared to the secretion from cells infected with a non-targeting virus (Figure 4A). In contrast, measurement of mouse insulin by ELISA demonstrated no change in insulin secretion (Figure 4B). It is particularly important to measure fractional luciferase secretion to compare secretion between two different sgRNAs

Figure 2. Bidirectional shRNA-coupled insulin luciferase reporter. (A) Schematic of the mIns2NL reporter coupled to a nuclear GFP-shRNA cassette. (B) Anti-nanoluciferase and anti-GAPDH western blots of MIN6 cells expressing the H2B-mIns2NL reporter with a control shRNA or uninfected MIN6 cells. Numbers to the left are molecular weight markers in kDa. (C) As in Figure 1D except dissociated mouse islets were infected with a H2B-GFP_mIns2NL virus. (D) Batch GSIS from MIN6 cells without and with infection with the H2B-GFP_mIns2NL reporter virus. Mouse insulin was measured by ELISA. (Left) Absolute insulin secretion. (Middle) Total insulin per 40,000 cells. (Right) Fractional insulin secretion. For absolute secretion: Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 16)} = 5.919$, $p = .027$; glucose $F_{(1, 16)} = 442.99$, $p < .0001$; reporter expression $F_{(1, 16)} = 2.597$, $p = .1266$. $p = .136$ between high glucose samples and $p = .34$ between low glucose samples after Bonferroni corrected post-hoc testing. n = 5 per condition. Standard error is plotted. For total insulin, n = 10, **p < .01 by Student's t-test. For fractional insulin secretion, two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 16)} = 21.36$, $p = .000283$; glucose $F_{(1, 16)} = 658.06$, $p = 2e-14$; reporter expression $F_{(1, 16)} = 14.79$, $p = .00146$. $p = .004$ between high glucose samples and $p = .556$ between low glucose samples after Bonferroni corrected post-hoc testing. n = 5 per condition. (E) Batch GSIS from MIN6 cells infected with the indicated viruses. Data presented as the fraction of total cellular luciferase secreted. n = 8 for each virus. Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 20)} = 8.658$, $p = .0081$; glucose $F_{(1, 20)} = 58.301$, $p < .0001$; gene knockdown $F_{(1, 20)} = 15.663$, $p = .0008$. ***p < .0001 after Bonferroni corrected post-hoc testing. (F) Western blot for Drp1 from sorted GFP+ cells from MIN6 cells infected with the indicated H2B-GFP_mIns2NL virus. Numbers to the left are molecular weight markers in kDa.
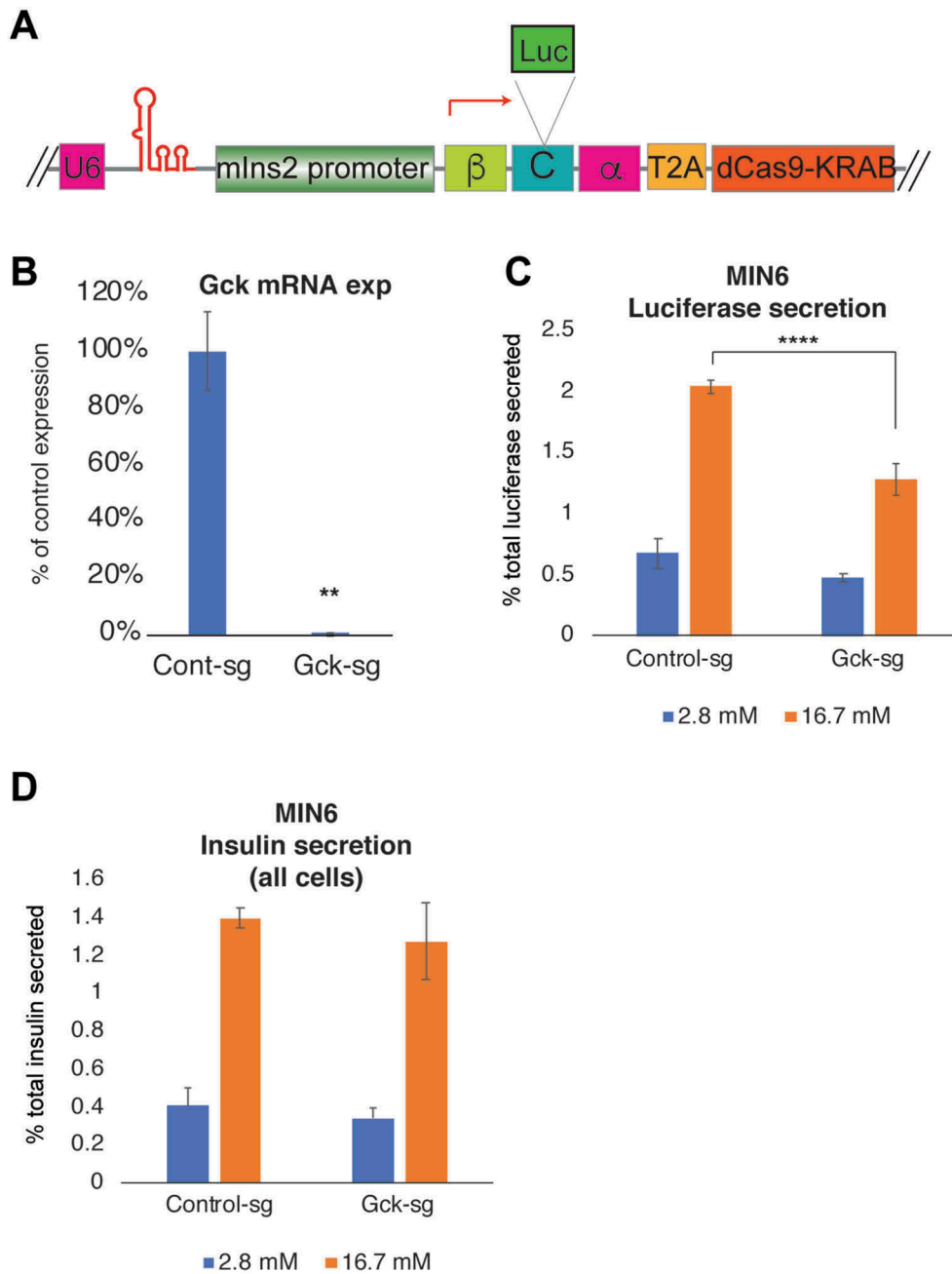
Figure 3. CRISPRi-coupled insulin nanoluciferase reporter. (A) Schematic of the reporter coupled to a sgRNA expression cassette and a dCas9-KRAB cDNA. (B) Validation of a potent *Gck* sgRNA. MIN6 expressing dCas9KRAB were infected with a virus expressing a *Gck* sgRNA or a control sgRNA selected with hygromycin and RT-qPCR was performed for *Gck*. n = 3. As determined by Student's t-test, **p < .01 (C) Batch GSIS from MIN6 cells infected with the indicated viruses. Data presented as a fractional luciferase. n = 8 for each virus. Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 24)}$ = 10.097, p = .0041; glucose $F_{(1, 24)}$ = 150.887, p < .0001; gene knockdown $F_{(1, 24)}$ = 30.625, p < .0001. ****p < .00001 after Bonferroni correction during post-hoc testing. (D) As in C, but mouse insulin was measured by ELISA. Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 20)}$ = 0.062, p = .8055; glucose $F_{(1, 20)}$ = 65.487, p < .0001; gene knockdown $F_{(1, 20)}$ = 0.652, p = .4288. NS, not significant.

(luciferase secretion normalized to the total luciferase content of the islets tested) since a lower infection rate in one sgRNA will result in a lower absolute luciferase secretion, giving the false impression that this sgRNA decreases secretion.

Furthermore, if knockdown of the gene reduces activity of the m*Ins2* promoter in the reporter construct, it will also appear to reduce secretion. Both effects can be controlled for by normalization to the cellular luciferase expression.
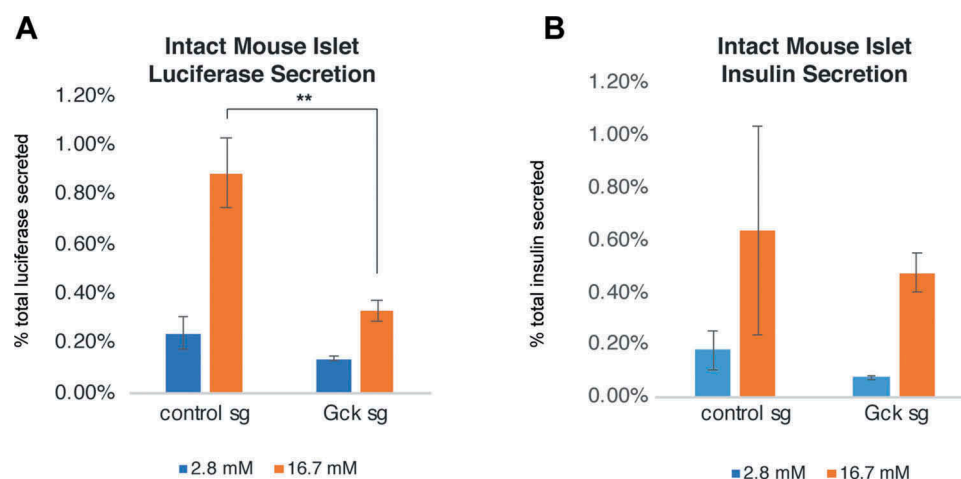
Figure 4. CRISPRi-coupled insulin nanoluciferase reporter in intact mouse islets. (A) Mouse islets were infected with the indicated viruses and cultured for 7 days. Batch GSIS was performed and fractional luciferase secretion is plotted for the indicated glucose concentrations. n = 4. Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 12)} = 8.057$, $p = .0149$; glucose $F_{(1, 12)} = 27.762$, $p = .0002$; gene knockdown $F_{(1, 12)} = 17.021$, $p = .0014$. **$p < .001$ after Bonferroni correction during post-hoc testing. (B) As in A, but mouse insulin was measured by ELISA. Two-way ANOVA with repeated measures data are as follows: interaction $F_{(1, 12)} = 0.034$, $p = .8562$; glucose $F_{(1, 12)} = 2.932$, $p = .1125$; gene knockdown $F_{(1, 12)} = 0.932$, $p = .3535$.

Discussion

Our approach allows rapid and inexpensive interrogation of insulin secretion after gene knockdown. The development time of the luciferase assay is <5 minutes as compared to the 150 minutes for a standard mouse insulin ELISA. Furthermore, there is no special manipulation of the islets necessary to ensure optimal infection. Each assay can cost as little as 5 cents as opposed to ~3-4 dollars per insulin ELISA. The luciferase assay also has a much greater linear range than ELISA, meaning fewer dilutions and fewer pilot assays to ensure accurate measurements. Finally, we show that as few as 0.5% of cells can be infected in the intact mouse islet with interpretable secretion results. We do not suggest attempting lower infection rates because at this level of infection, we observe a luciferase signal 5-fold above background at 2.8 mM glucose. Reducing to less than an infection rate of <0.5% is likely to reduce the signal to noise ratio to unacceptable levels.

While this reporter accurately reports on insulin secretion, several potential caveats exist. First, while expression of this secretion reporter does not change absolute insulin secretion, it does slightly decrease total insulin protein per cell. We speculate that the expression of this additional copy of the mouse *Ins2* promoter might compete for transcriptional activators of the endogenous insulin promoters, thereby reducing transcription of the endogenous insulin mRNA. In addition, we suspect that the insulin nanoluciferase mRNA is less efficiently translated than the endogenous insulin mRNA since is lacks the 3'UTR sequences that stabilize the endogenous insulin mRNA.[14] In combination, the net effect would be reduced total insulin content per cell. Normalization of secretion of luciferase to the total luciferase and comparison of fractional luciferase secretion to that of a control sgRNA should minimize artifacts, but we cannot rule out rare false positives or negatives due to the reporter's effect on total insulin. Second, since the nanoluciferase must be properly processed in order to be secreted, false positives or negatives may arise from the knockdown of any gene that disrupts folding or processing of the nanoluciferase. Third, many authors have demonstrated that there is beta cell heterogeneity[15-18] and our method could theoretically be misleading if a particular subtype of beta cell is more susceptible to lentiviral infection and this subtype turns out to be unimportant for insulin secretion of the islet as a whole. Although we are not aware of this possibility currently, it should be considered as more is discovered about beta cell heterogeneity. A fourth potential caveat is that the electrical connectivity of the beta cells within an islet may mask defects

from gene knockdown. That is, an uninfected beta cell with normal electrical oscillations (since it does not have knockdown of any gene) could trigger a neighboring beta cell that expresses the luciferase reporter (via gap junctions) even when it itself might not be able to do so due to knockdown of a particular gene. This does not appear to be the case in the example used here. Glucokinase knockdown should generate a defect upstream of membrane depolarization[19] and there does not appear to be rescue of insulin secretion by neighboring cells in our experiments. For the potential caveats described above, we do not believe this technique supplants transgenic mouse models or other methods that are capable of 100% efficient gene silencing in the islet, but it does offer a lower cost and effort strategy that allows screening larger numbers of genes prior to committing to the time and expense of a transgenic model.

In the future, our system could be easily adapted to the growing number of Cas9 based interventions that have been described. Alternatively, a cDNA can be cloned in place of the dCas9-KRAB cDNA to allow for direct over-expression. Finally, though our experiments were conducted in batch GSIS, islets with the luciferase reporter could be used in a perifusion system where the frequency of sampling is high and costly.

In conclusion, our CRISPRi luciferase insulin secretion reporter virus allows rapid and inexpensive monitoring of insulin secretion from intact mouse islets and will facilitate our understanding of insulin secretion.

Methods

Molecular biology

The insulin luciferase vector was created by insertion of the nanoluciferase cDNA (Promega) in-frame, into the SmaI and BstEII sites of the mouse *Ins2* cDNA. We extended the cDNA to the minus 360 base of the genomic DNA sequence to capture the minimal mouse *Ins2* promoter. For the mIns2-GFP reporter, we replaced the ATG to stop codon of the preproinsulin-nanoluciferase fusion with GFP using HiFi cloning (New England Biolabs). For the minCMV H2B-GFP shRNA vector, the minCMV GFP polyA signal was cloned from the bidirectional vector minCMV-PGK, a gift from L. Naldini.[10] The mini-mir30 based shRNA cassette inserted into the 3' UTR of the GFP was cloned from pSicoR-mCh-mir30, a gift from Michael McManus.[20] The U6 sgRNA cassette and dCas9-KRAB cDNA were a gift from Michael McManus.[21]

GSIS

40,000 MIN6 cells were plated into 96 well Corning Cellbind plates for 48 hours. Cells were rested in Krebs-Ringer Bicarbonate HEPES buffer (KRBH; 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 25 mM NaHCO$_3$, 20 mM HEPES, pH 7.4, 0.2% BSA) with 2 mM glucose for 2 hours, washed 3 times with KRBH and incubated in KRBH with the indicated glucose concentration for 1 hour. Total cell extracts were made with addition of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitors (cOmplete, Roche). For mouse islets, 20–30 islets were hand-picked into 1.5 mL tubes with 500 microliters of KRBH. These were rested at 2.8 mM glucose for 1 hour and then changed to new KRBH with 2.8 mM glucose for one hour. This sample was collected and the islets were changed to KRBH with 16.7 mM glucose for one hour. This sample was collected and the islets were then lysed in the lysis buffer listed above for measurements of insulin by ELISA and/or luciferase (see below). Insulin was measured by ELISA (Mercodia, 10-1247-01). This insulin ELISA assay cross reacts with proinsulin I (43%) and proinsulin II (60%) per the manufacturer.

Luciferase assay

Ten microliters of sample was added to 10 microliters of nanoluciferase substrate diluted per the protocol (Promega). Luminescence was read 10 minutes after addition with a Victor II (Perkin Elmer).

Lentivirus

Lentivirus was produced using 3rd generation packaging vectors in 293T cells.[22] The titer of GFP expressing viruses were measured on MIN6 cells using flow cytometry to determine infection.

For the non-fluorescent viruses, titer was determined by droplet PCR from genomic DNA as described below post-hoc. We note that the titers of viruses from dCas9-KRAB expressing viruses are low due to their size.

Islet isolation

Islets were isolated from C57B6J mice by intraductal collagenase injection and density centrifugation as previously described.[23] Islets were infected 24 hours after isolation and cultured in RPMI with 10% fetal bovine serum, HEPES, and penicillin/streptomycin.

Cell culture

MIN6 cells were a gift from Osaka University. They were maintained in 15% fetal bovine serum in DMEM high glucose with sodium pyruvate (Gibco) and 50 micromolar beta-mercaptoethanol (Sigma), penicillin/streptomycin (Gibco).

qRT-PCR

A MIN6 line stably expressing dCas9KRAB was infected with a virus containing a control or Gck sgRNA and hygromycin resistance. Cells were selected with hygromycin before RNA isolation and qPCR using Taqman Unviersal Master Mix II (ThermoFisher) and GUS and Gck probes as previously described.[11]

Droplet PCR

Genomic DNA was isolated from MIN6 cells or islets using a genomic DNA isolation kit (Nucleospin Tissue, Machery Nagel). Thirty nanograms of genomic DNA was digested with HindIII (NEB) and droplet PCR was performed to determine lentiviral copy number using the following probes to recognize the lentiviral provirus. RRE-F = GGCAAAGAGAAGAGTGGTGC; RRE-R = GACGGTACAGGCCAGACAAT; RRE-probe = CCATAGTGCTTCCTGCTGCTCCC. Droplets were read on a BioRad QX100. Lentiviral copy number was normalized to a probe recognizing a gDNA locus (AP3B1, Biorad) and divided by 2. We note that this is likely an overestimate of the percent of beta cells infected as non-beta cells will also be detected with this assay.

Western blotting

MIN6 cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitors (cOmplete, Roche) for 15 minutes at 4 degrees, clarified by centrifugation at 21,000xg for 10 minutes. The supernatant was mixed with LDS sample buffer (Life Technologies) to 1x final with a final concentration of 100 mM DTT. After heating at 70 degrees Celsius for 10 minutes, the sample was run on a 15% SDS-PAGE gel and blotted with mouse anti-nanoluciferase (R and D systems) at 1:250 or anti-GAPDH-horseradish peroxidase (Sigma) at 1:10,000.

Immunofluorescence

Mouse islets were rested overnight and then incubated with 0.05% trypsin with EDTA (ThermoFisher) for 15 minutes at 37 degrees Celsius, then triturated with a P1000 pipette 10 times to achieve >80% single cells. Trypsin was inactivated with RPMI 1640 + 10% fetal bovine serum, and then cells were plated on glass coverslips. Two days after plating, the cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at room temperature. The cells were then blocked with 10% donkey serum in PBS for 1 hour at room temperature and incubated with primary antibody overnight in 1% donkey serum in PBS at 4 degree Celsius. Secondary antibodies were used at 1:500 in 1% donkey serum in PBS for one hour at room temperature and coverslips were mounted with Vectashield with DAPI and imaged on a Leica SP5 confocal with a single plane of imaging where maximal cytosolic staining was observed. Primary antibodies were: guinea pig anti-insulin (1:250, Dako), rabbit anti-glucagon (1:250, Dako), chicken anti-GFP (1:1000, Aves). Secondary antibodies were: anti-chicken Alexa 488 (ThermoFisher), anti-guinea pig Alexa 555 (ThermoFisher), and anti-rabbit Alexa 647 (ThermoFisher).

Statistical analysis

For two-way ANOVA analyses with replication, the results of Bonferroni corrected unpaired Student's t-tests were used to make comparisons between gene knockdown groups (Control-sh and Drp1-sh, Control-sg and Gck-sg) at high and low glucose conditions, as displayed in the figures.

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