

# Using recombinase-mediated cassette exchange to engineer MIN6 insulin-secreting cells based on a newly identified safe harbor locus

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## Keywords

Insulin secretion, Recombination-mediated cassette exchange, Tetracycline-regulated expression

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## ABSTRACT

**Aims/Introduction:** Recent studies have identified genomic and transcript level changes along with alterations in insulin secretion in patients with diabetes and in rodent models of diabetes. It is important to establish an efficient system for testing functional consequences of these changes. We aimed to generate such a system using insulin-secreting MIN6 cells.

**Materials and Methods:** MIN6 cells were first engineered to have a tetracycline-regulated expression system. Then, we used the recombination-mediated cassette exchange strategy to explore the silencing-resistant site in the genome and generated a master cell line based on this site.

**Results:** We identified a site 10.5 kbps upstream from the *Zxdb* gene as a locus that allows homogenous transgene expression from a tetracycline responsible promoter. Placing the Flip/Frt-based platform on this locus using CRISPR/Cas9 technology generated modified MIN6 cells applicable to achieving cassette exchange on the genome. Using this cell line, we generated MIN6 subclones with over- or underexpression of glucokinase. By analyzing a mixed population of these cells, we obtained an initial estimate of effects on insulin secretion within 6 weeks. Furthermore, we generated six MIN6 cell sublines simultaneously harboring genes of inducible overexpression with unknown functions in insulin secretion, and found that *Cited4* and *Arhgef3* overexpressions increased and decreased insulin secretion, respectively.

**Conclusions:** We engineered MIN6 cells, which can serve as a powerful tool for testing genetic alterations associated with diabetes, and studied the molecular mechanisms of insulin secretion.

## INTRODUCTION

It is now widely accepted that pancreatic  $\beta$ -cell impairment, whether in terms of function, mass or both, is of central importance for the development not only of type 1, but also type 2 diabetes mellitus. Recent advances in genome-wide association analysis<sup>1,2</sup> and several studies analyzing transcriptomes or proteomes in insulin secreting cells<sup>3–7</sup> have revealed genes that might be involved in impaired  $\beta$ -cell function and/or mass. These genes are either overexpressed or suppressed in islets or  $\beta$ -cells of diabetes patients. However, research progress beyond identification of these candidate genes has been hampered, and analyses of the functional impacts of these abnormalities have

been limited. Genome-modified murine models and analyses of their pancreatic islets are straightforward approaches to investigating the functions of these candidate genes, but are costly and time-consuming. Although imperfect, utilization of highly differentiated insulinoma cell lines, such as  $\beta$ TC3<sup>8</sup>, MIN6<sup>9,10</sup>, INS1<sup>11–13</sup> or EndoC- $\beta$ H<sup>14</sup>, is an alternative for studying gene functions involved in insulin secretion. However, these cells also present certain difficulties. One of the disadvantages encountered is the low transfection efficiency of nucleotides in these cell lines. When investigating effects of abnormalities in candidate genes, 70–80% of cells, perhaps more, need to be genetically modified, because physiological phenotypes are not anticipated to be particularly large<sup>15</sup>. Using a viral vector is one approach to overcoming this difficulty<sup>16–18</sup>. However, viral

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transduction can affect insulin secretory function if the amounts of virus vectors are not precisely controlled<sup>19</sup>. Therefore, generating stable cell lines would be preferable, offering the advantages of accuracy and reproducibility of experiments, particularly when large numbers of cells are required, such as for metabolome analysis<sup>20,21</sup>. However, generating and using such cell lines can be problematic. One issue is clonal differences, a topic discussed in great detail by Newgard *et al.*<sup>22</sup> Another difficulty is that stable cell line generation is time-consuming, taking a few months, as well as laborious when using highly differentiated insulin secreting cells.

Drug-inducible expression systems can reportedly be applied to overcome clonal differences<sup>23,24</sup>. Functional tests can be carried out 2–7 days after drug treatment and comparisons are then made between genetically identical cells (isogenic cells) differing only in the presence or absence of the drug for short periods. If functional changes are observed, the researcher can confidently attribute these differences to changes in expressions of the genes of interest.

The recombinase-mediated cassette exchange (RMCE) method is being applied to expedite stable cell line generation and eliminate laborious clone selection processes<sup>25,26</sup>. The yeast F1p recombinase with F1p recognition target sites has been widely used, because it has the highest specificity of integration and lowest cross-reactivity of the recombination target sites. The F1p-based RMCE method utilizes a set of hetero-specific F1p recognition target sites to direct a gene of interest toward a unique previously tagged and high-expression locus on the chromosome. The RMCE master cell lines can be selected for a high level of expression, long-term stability and robust cassette exchangeability. Once isolated and stored as master cells, the cell lines can be routinely used to rapidly create multiple generations of stable cell lines. However, there is a problem regarding loci on the genome where the platform should be placed. The mouse *Rosa26* locus is well-known, but transgene expressions, especially from an artificial tetracycline-responsive promoter, seem to be prone to silencing in this locus<sup>27,28</sup>. Therefore, identification of other more appropriate sites for homogeneous transgene expression is desirable.

MIN6 cells were established and characterized more than 30 years ago<sup>9,10</sup>, and have been used worldwide. MIN6 cells grow relatively slowly, however, and it takes more than 2 months to generate stable transformants. In addition, clonal differences are a common problem with other insulin-secreting cells. In the present study, to overcome the challenges of clonal differences, as well as to minimize time-consuming and laborious procedures, we engineered MIN6 cells to have a tetracycline-regulated expression system and the platform for RMCE at a newly identified safe harbor locus.

## METHODS

### Plasmid construction

Detailed construction strategies are described in Supporting Information.

### Genome walking

Genomic deoxyribonucleic acid (DNA) was extracted using the DNeasy kit (Qiagen, Valencia, CA, USA). Integration sites were identified using a Genome Walker kit (Takara, Shiga, Japan).

### MIN6 cell culture and electroporation for RMCE

MIN6 cells were cultured in DMEM, as previously described<sup>9,10</sup>. To examine proliferation rates, 50,000 cells were seeded in wells in 24-multiwell plates, harvested at 1, 3, 5 and 7 days, and then counted.

For RMCE transfection,  $3 \times 10^6$  cells were electroporated with 5  $\mu$ g of exchange vectors and 5  $\mu$ g of pCAG-Flpe<sup>29</sup> using the Nucleofector 2b device (Lonza, Allendale, NJ, USA). Selected antibiotics were added to the cells 4 days later. Ganciclovir treatment, when required, was started 10 days after electroporation and lasted for 6 days. Three weeks after the start of antibiotics, colonies were picked up or combined. For analysis of platform integration, polymerase chain reaction (PCR) was carried out with Q5 DNA polymerase (New England Biolab, Ipswich, MA, USA) using the primers listed in Table S1. Green fluorescent protein (GFP) and red fluorescent protein fluorescence was observed using a fluorescent microscope (BZ-X700; Keyence, Osaka, Japan).

### Reverse transcription PCR

Total ribonucleic acid (RNA) was extracted using the RNeasy kit (Qiagen). Complementary DNA (cDNA) synthesis was carried out using ReverTra Ace (Toyobo Life Science, Tokyo, Japan). Quantitative reverse transcription PCR analysis was carried out using FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and a LightCycler 96 (Roche). The primers used are listed in Table S2. Transcript levels were normalized with  $\beta$ -actin messenger RNA (mRNA), and the average from three experiments in doxycycline (dox)-untreated cells was defined as 1.0.

### Western blot

Cells were dissolved in sodium dodecyl sulfate sample buffer, and proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Membranes were probed with rabbit anti-GFP antibody (1:1,000; #632592, Takara) or anti-glucokinase antibody (1:1,000; #15629-1-AP; Proteintech, Rosemont, IL, USA) and together with mouse anti- $\beta$ -actin antibody (1:5,000; #60008-1-Ig, Proteintech) overnight at 4°C, and then incubated for 1 h with donkey anti-rabbit immunoglobulin G (1:10,000) and with sheep anti-mouse immunoglobulin G (1:10,000) conjugated with horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA). Detection was accomplished using EZWestLumi plus reagent, and visualized using WSE-6200HLuminoGraphII (ATTO, Tokyo, Japan).

### Southern blot

Southern blot analysis was carried out (by Dr Takahiro Fujino) at the Division of Analytical Bio-Medicine, Ehime University

Advanced Research Support Center (ADRES). Detailed procedures are described in Supporting Information.

### Knockdown by microRNA-embedded short hairpin RNA against glucokinase

A plasmid containing the miRE backbone<sup>30,31</sup> was purchased from Mirimus Inc. (Woodbury, NY, USA). Target sequences of glucokinase were selected by analyzing the mouse glucokinase mRNA sequence on the shERWOOD website<sup>32</sup>. The following target sequences were used: for shGck#1 5'-tggacaagcatcagatgaaca-3', shGck#2 5'-acactcaggtctgctcttca-3' and shGck#1 5'-agctgcgcacacgtggctctt-3'.

### Glucose utilization and static insulin secretion assays

Glucose utilization was measured by following the conversion of [<sup>3</sup>H]glucose into [<sup>3</sup>H]H<sub>2</sub>O, as previously described<sup>10</sup>. Cells were seeded in 24-well plates and subjected to dox treatment. The cells were pre-incubated in HBKRBB<sup>9,10</sup> with 0.1% bovine serum albumin and 5 mmol/L glucose for 0.5 h, and then incubated with HBKRBB with 5, 12.5 or 20 mmol/L [<sup>3</sup>H]glucose. After a 2-h incubation period, a 0.1-mL aliquot of the incubation medium was transferred to a microtube and then placed in plastic scintillation vials containing 0.6 mL of distilled water. The vials were stoppered and kept at 37°C for 36 h to allow the [<sup>3</sup>H]H<sub>2</sub>O in the microtube to equilibrate with the water. Subsequently, the microtube was taken out and 10 mL of scintillation fluid were added.

For insulin secretion, cells were treated as aforementioned and then incubated in HBKRBB supplemented with varying concentrations of glucose, 5 mmol/L glucose (Glc) + 30 mmol/L KCl, 5 mmol/L Glc + 10 mmol/L leucine + 10 mmol/L glutamine or 5 mmol/L Glc + 0.1 μmol/L glimepiride for 1 h. The media were then collected and assayed for immunoreactive insulin by enzyme-linked immunosorbent assay (Merckodia, Uppsala, Sweden). Protein contents were analyzed after extraction with 0.1N NaOH using the Pierce 660 nm protein assay kit (Thermo Fisher Scientific).

### Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical significance was tested using the unpaired Student's *t*-test, unless otherwise described.

## RESULTS

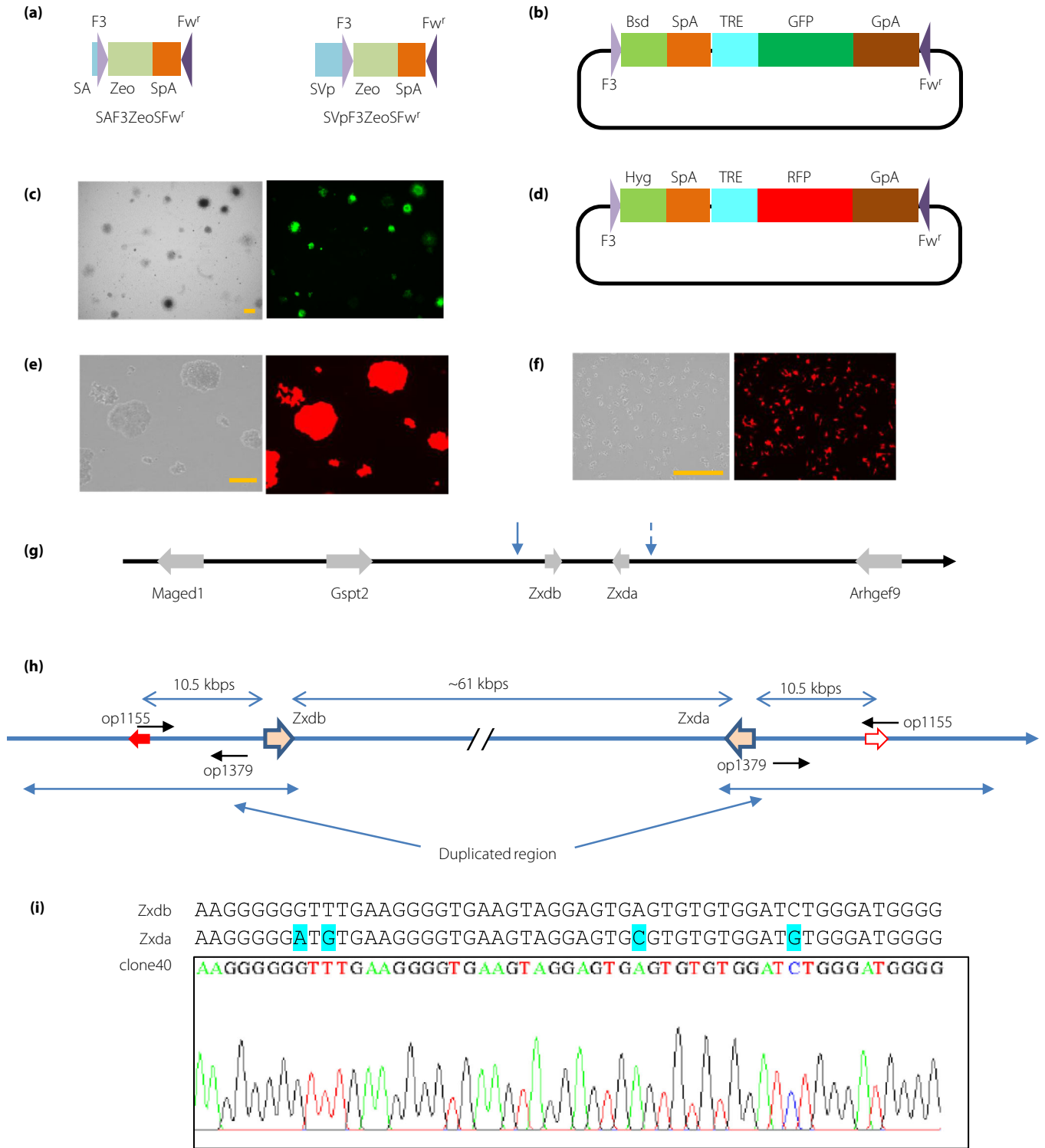
### Identification of a novel genome site appropriate for platform insertion

MIN6 cell subclone 4, MIN6c4, retained glucose-stimulated insulin secretion after long-term culture<sup>3</sup>. MIN6c4 cells were transfected with pCMV-Tet3G (Takara Bio.) and selected against G418. Then, transiently transfecting pTRE3g-Luc expressing luciferase under the control of the tetracycline-responsive promoter and luciferase activity analysis yielded clones with high inducible luciferase expression on dox treatment. We selected one clone, named MIN6Tet3G9, which

showed the highest luciferase activity, as well as robust glucose-responsiveness of insulin secretion and cellular insulin content similar to those of parental MIN6c4 cells.

Transgene expressions, especially those from a tetracycline-responsive promoter, reportedly tends to be heterogeneous when expression units are placed at the *Rosa26* locus<sup>27,28</sup>. Therefore, we explored novel loci that allow homogeneous expression from a tetracycline-responsive promoter. We used a construct, SAF3ZeoSFw<sup>r</sup>, having a splice acceptor (Figure 1a) to trap an intrinsic transcript. We also created a construct with the SV40 promoter instead of a splice acceptor, SVpF3ZeoSFw<sup>r</sup> (Figure 1a). The platform, either SAF3ZeoSFw<sup>r</sup> or SVpF3ZeoSFw<sup>r</sup>, was introduced into MIN6Tet3G9 cells, and zeocin-resistant colonies were harvested and combined 3 weeks later. These pooled zeocin-resistant cells were then subjected to RMCE with an exchange plasmid; that is, pF3BsdSTreGFPGFw<sup>r</sup> (Figure 1b). The rationale for this strategy aimed at RMCE-mediated insertion of GFP after chromosomal integration of the platform, but not direct insertion of GFP embedding in the platform, is as follows: when the platform fragment was introduced by electroporation, the fragment was integrated randomly into chromosomal sites in the cells. Although the recipient platform should be integrated as a single copy for reproducible cassette exchange, random integration could result in not only single copy integration at single genome sites, but also multiple tandem integrations at a single site, or multiple integrations at multiple genome sites. Tandem copies can reportedly be changed to a single copy after RMCE<sup>33</sup>. In addition, simultaneous cassette exchange at multiple genome sites on single RMCE reportedly did not consistently take place<sup>33</sup> or was even rare<sup>34</sup>. Therefore, our assumption was that clones with multiple tandem integrations at a single site and those with integration at multiple genome sites could be eliminated by appropriate phenotype selection after RMCE. These considerations led us to a strategy that involved searching for clones with a single recipient platform by initially integrating the smaller recipient platform randomly and then examining antibiotic phenotypes after RMCE with pF3BsdSTreGFPGFw<sup>r</sup> (Figure 1b).

Colonies growing against blasticidin after RMCE with a pF3BsdSTreGFPGFw<sup>r</sup>, showed variable GFP intensity in response to dox treatment (Figure 1c). GFP expressing colonies were picked up and examined for zeocin-sensitivity. Among 52 clones sensitive to zeocin and resistant to blasticidin, we selected one clone from among the cells transfected with SAF3ZeoSFw<sup>r</sup>, and three clones from those transfected with SVpF3ZeoSFw<sup>r</sup> based on their robust and uniform GFP expression after dox induction. We then tested whether these clones produced, after another RMCE with pF3HygSTreRFPGFw<sup>r</sup>, colonies expressing red fluorescent protein homogeneously (Figure 1e,f). In the clone identified from cells transfected with SAF3ZeoSFw<sup>r</sup>, the genomic walking method identified that the platform was inserted into intron 2–3 of the islet amyloid polypeptide gene. In two clones from cells transfected with SVpF3ZeoSFw<sup>r</sup>, the insertion



**Figure 1** | Strategy for identifying novel genetic sites for homogenous expression from tetracycline-dependent promoter. (a) The recipient platforms for recombinease-mediated cassette exchange containing a mutated FRT (F3) and a wild-type FRT in reverse orientation (Fw<sup>r</sup>). SA, splice acceptor of rabbit β-globin; SpA, SV40 polyA region; SVp, SV40 promoter; Zeo, zeocin-resistant gene. (b) The exchange vector making cells blasticidin (Bsd)-resistant and expressing green fluorescent protein (GFP) under the tetracycline-regulated promoter (TRE). (c) GFP expression in growing colonies of MIN6Tet3G cells transfected with the platform and then subjected to recombinease-mediated cassette exchange with the construct shown in (b) and treated with 1 μg/mL dox. Bar, 100 μm. (d) The exchange vector making cells hygromycin (Hyg)-resistant and

expressing red fluorescent protein (RFP) under the tetracycline-regulated promoter. (e) RFP expression in colonies of MIN6 cassette exchange clone 40 cells subjected to recombinase-mediated cassette exchange with the construct shown in (d) and treated with 1  $\mu\text{g}/\text{mL}$  doxycycline. Bar, 100  $\mu\text{m}$ . (f) RFP expression in dispersed cells from a single colony shown in (e). Bar, 100  $\mu\text{m}$ . (g) Gene organization on the X chromosome around the *Zxda* and *Zxdb* loci. Blue vertical solid and broken arrows indicate the region where a Basic Local Alignment Search Tool research hit using the sequence obtained from clone 40. (h) Polymerase chain reaction strategy for determining the integration site. The platforms at the candidate integration sites are shown by a bold red arrow and a white arrow framed with red. Oligo primers, op1155 and op1379, were used to amplify duplicated regions containing different nucleotides. (i) Amplified sequence of the duplicated region from MIN6 cassette exchange clone 40 matched to the sequence of *Zxdb*.

occurred at intron 2–3 of the *Gng12* (guanine nucleotide binding protein, gamma 12) gene and at intron 3–4 of the *Nup93* (nucleoporin 93) gene. As integrations at sites within genes might affect gene expression, we considered these sites to not be appropriate for platform insertion.

A Basic Local Alignment Search Tool search using a sequence of another clone, designated MIN6 cassette exchange (MIN6CE) clone 40, obtained by genome walking, hit two sites in the X chromosome. These sites are approximately 10.5 kbps upstream from the initial codons of *Zxda* and *Zxdb* (zinc finger, X-linked, duplicated A and B) on the proximal short arm of the X chromosome<sup>35</sup>. These regions containing *Zxda* and *Zxdb* are duplicated, and *Zxda* and *Zxdb* are located 61 kbps apart from each other and in opposite directions (Figure 1g,h). As MIN6 cells were derived from male mice, the insertion sites could be one of those upstream from *Zxdb* or *Zxda*. PCR was carried out for a primer within the platform and one in the common region of the *Zxdb* and *Zxda* genes, and the resulting product was sequenced. The sequence showed several bases of clone 40 to be identical to those of *Zxdb*, but not *Zxda*, identifying that the integration site as being upstream from *Zxdb* (Figure 1i).

#### CRISPR/Cas9-mediated integration of the platform into the *Zxdb* site

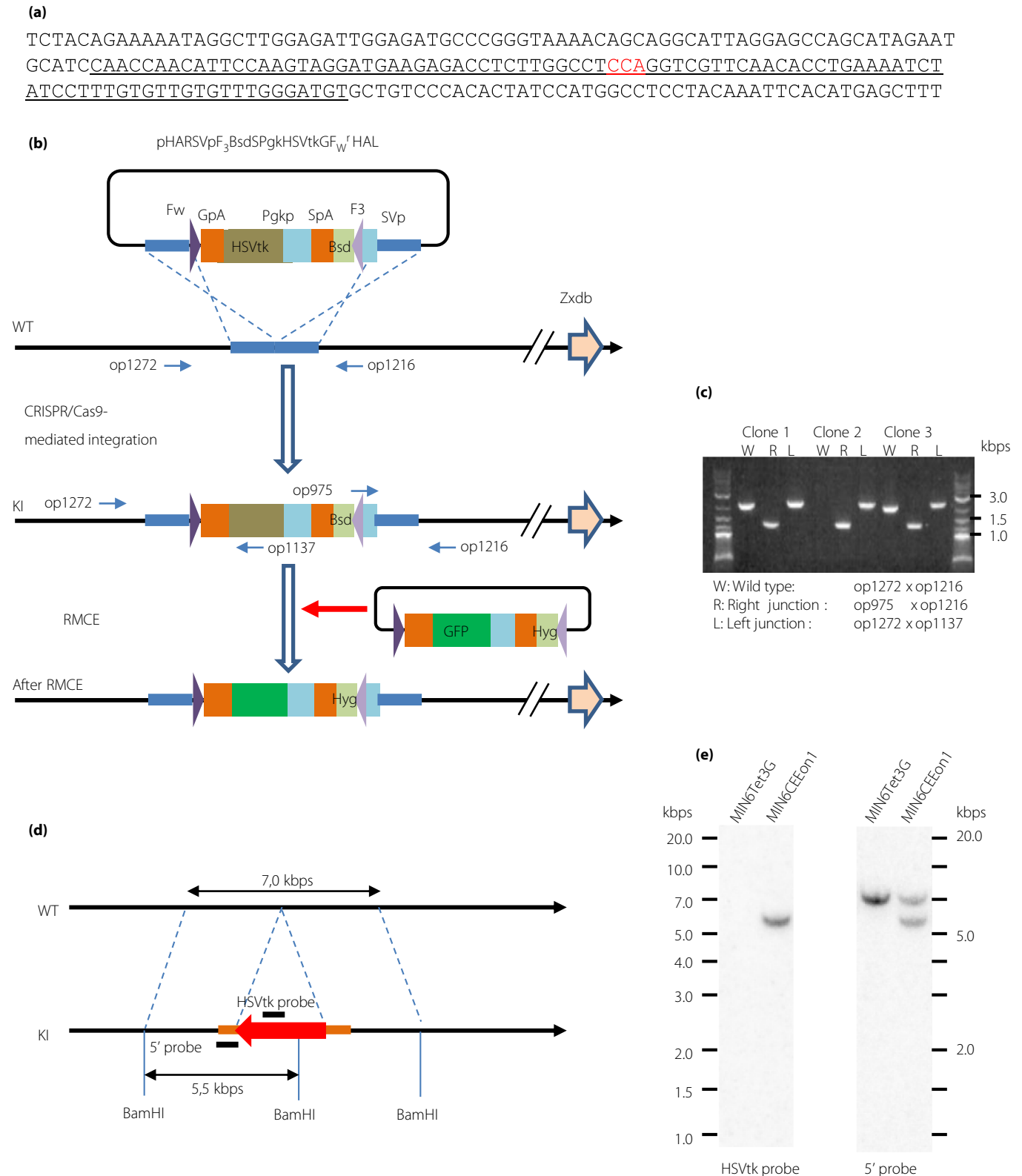
To make the *Zxdb* site a general application target site in any of the mouse cell lines, we generated a set of plasmids that allow platform insertion using the CRISPR/Cas9 system at the locus. Sequencing the genome of the MIN6CE clone 40 cells showed that the platform had been inserted with deletion of 92 bp (Figure 2a, underlined). We selected a protospacer adjacent motif sequence in this deleted region (Figure 2a), after screening of candidate sites by a program presented on the Integrated DNA Technologies website. We also improved the platform by including the Herpes simplex virus thymidine kinase (*HSVtk*) gene in the new platform for negative selection during RMCE<sup>36</sup> and changed the antibiotic marker from zeocin to blasticidin (Figure 2b), because the latter kills MIN6 cells more efficiently. A plasmid containing the platform flanked with ~800 bps homology arms (Figure 2b) and those expressing guide RNA and Cas9 were electroporated and selected against blasticidin. We screened more than 10 clones by PCR (just three clones are shown) for proper platform integration in the *Zxdb* locus by PCR and sequencing (Figure 2b,c). One of the selected clones (clone 1 in Figure 2c) was designated MIN6CEon1. To confirm correct insertion of

the platform, Southern blot analysis was carried out (Figure 2d,e). An inner HSVtk1 probe yielded a single band, indicating that off target or random integrations did not take place. The expected shift of the BamHI fragment of one allele shown by the 5' probe, together with results of the PCR analysis (Figure 2b,c), showed the platform to have been correctly integrated.

MIN6CEon1 cells showed robust insulin secretion in response to high glucose (Figure 3a). Insulin secretory responses of these cells to non-glucose nutrients and a sulfonylurea were observed to be very similar to those in MIN6c4 cells (Figure 3b). Their growth rate was identical to that of MIN6c4 cells (Figure 3c). When MIN6CEon1 cells were used for RMCE with pF3Hyg-STreGFPGFw<sup>r</sup> (Figure 2b), and selected against hygromycin (200  $\mu\text{g}/\text{mL}$ ) and ganciclovir (10  $\mu\text{g}/\text{mL}$ ), a mixed cell population consisting of essentially 100% GFP-positive cells was obtained (Figure 3d). Induction of GFP expression in these cells was regulated in a dox concentration-dependent manner (Figure 3e,f).

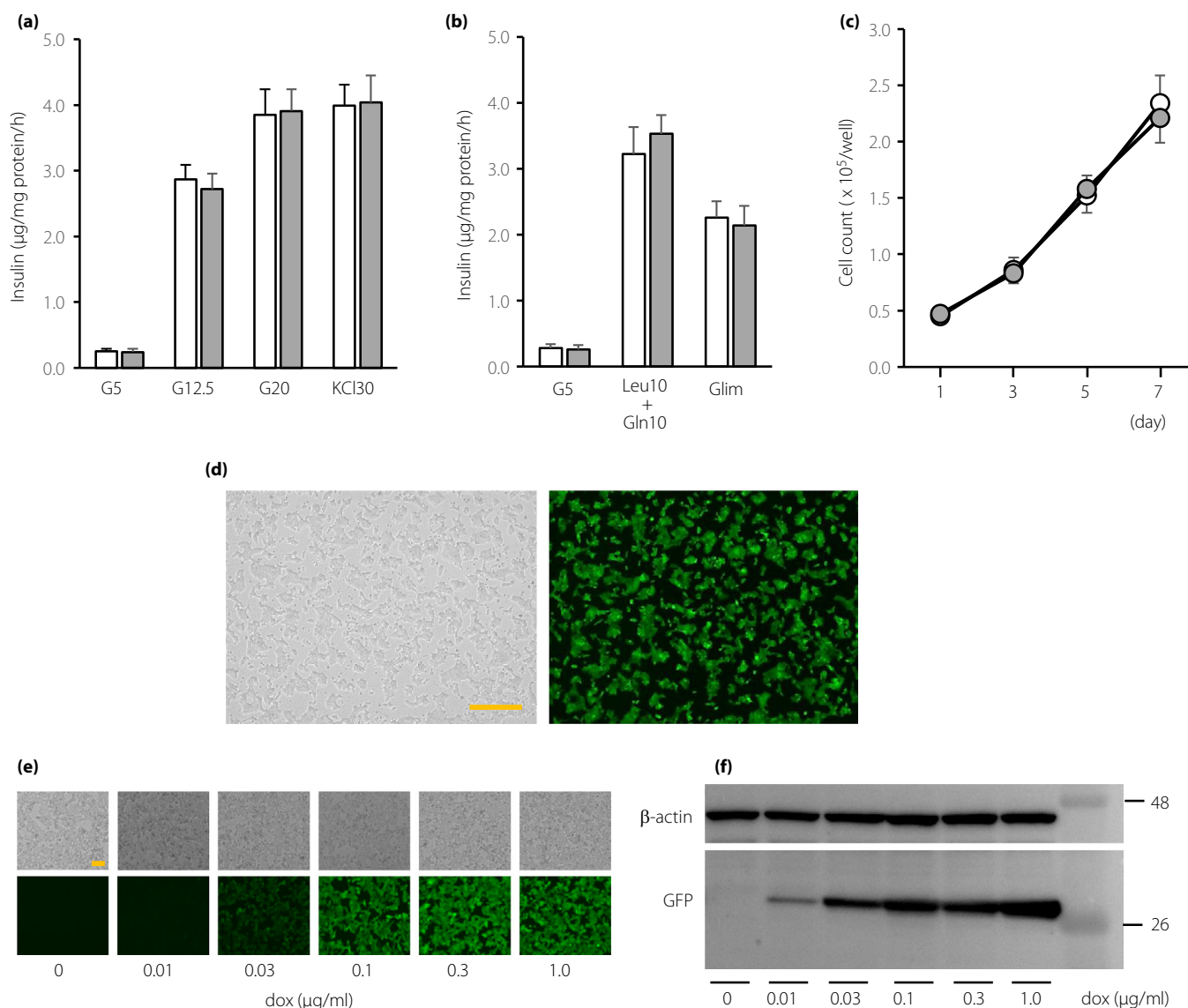
#### Overexpression of glucokinase in MIN6CEon1 cells

To validate the system, we generated MIN6 cells overexpressing  $\beta$ -cell type glucokinase. Complementary DNA of glucokinase with  $\beta$ -cell specific exon 1 was subcloned into the pF3Hyg-STreGFPGFw<sup>r</sup> after deleting GFP cDNA. MIN6CEon1 cells were subjected to RMCE with pF3HygSTrebGckGFw<sup>r</sup> (Figure 4a), and treated with hygromycin and ganciclovir. Because nearly 100% of cells with proper cassette exchange survive, the combined cell population should be sufficient to obtain an initial estimate of the effects of overexpressing genes of interest. Therefore, 3 weeks after transfection, we picked up six colonies, followed by combination and expansion of other colonies. These cells were named MIN6CEon1oeGck. Treatment with 0.3  $\mu\text{mol}/\text{L}$  dox for 2 days increased glucokinase mRNA (Figure 4b) and protein (Figure 4c) levels by threefold and 1.5-fold, respectively. We observed stronger induction with 1.0  $\mu\text{mol}/\text{L}$  dox (Figure 4c) to cause cell death (data not shown). When combined cells were subjected to [<sup>3</sup>H]glucose utilization assay, glucose metabolism more than doubled at all three glucose concentrations tested in MIN6CEon1oeGck cells treated with 0.3  $\mu\text{mol}/\text{L}$  dox (Figure 4c). We also observed that insulin secretion was increased at an intermediate glucose concentration (12.5 mmol/L), but not at either basal (5 mmol/L) or high (20 mmol/L) glucose concentrations (Figure 4d). These cell viability and insulin secretion results were consistent with regulated glucokinase expression in INS-1 cells<sup>23</sup>. We obtained

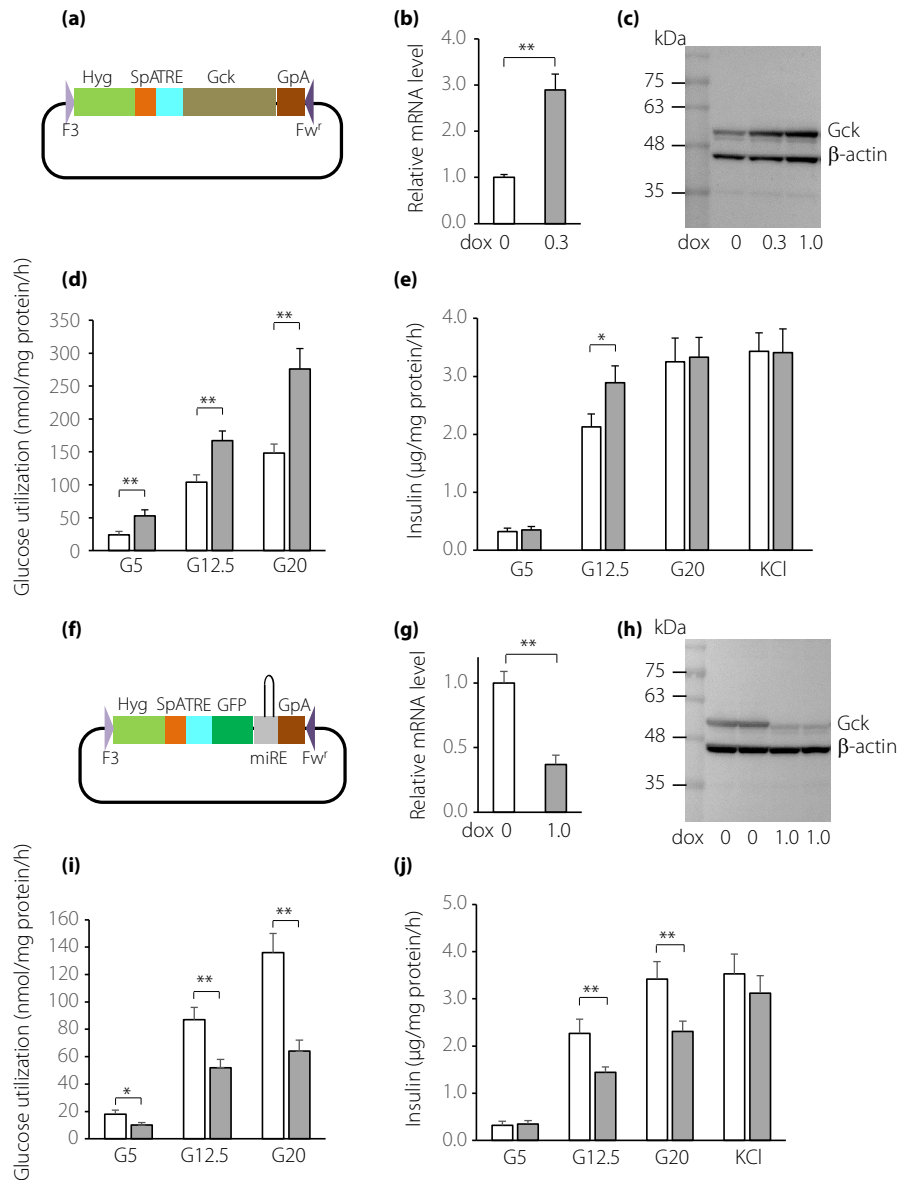


**Figure 2** | CRISPR/Cas9-mediated integration of the recombinase-mediated cassette exchange (RMCE) platform at the site upstream from *Zxdb*. (a) The nucleotide sequence around the platform integration site in MIN6 cassette exchange clone 40 cells. The underlined portion was deleted in clone 40 cells. The protospacer adjacent motif sequence used for targeted integration is shown in red. (b) Schema of targeted integration into the *Zxdb* upstream site. The platform donor vector pHARSVP<sub>F3</sub>BsdSPgkHSVtkGF<sub>W</sub><sup>r</sup>HAR (4 μg) was electroporated together with a guide ribonucleic acid

plasmid (2  $\mu\text{g}$ ) and a Cas9 expressing plasmid (4  $\mu\text{g}$ ). The oligo primers shown are used for analyzing proper integrations. Note that the platform is integrated in the antisense direction relative to the genome direction. (c) Polymerase chain reaction genotyping of clones transfected with the platform using the oligo primers shown in (b). W: the wild type (intact) region was amplified using op1216 and op1272, generating a 2,018 bps band. Left junction: amplified by op1216 and op975, 1,296 bps. Right junction: amplified by op1137 and op1272, 2,271 bps. (d) Wild-type (WT) and knock-in (KI) alleles showing BamHI sites, and a 5' probe and a HSVtk probe used in Southern blot. (e) Southern blot analysis of genomic deoxyribonucleic acid from parental MIN6Tet3G and MIN6CEon1 cells probed with HSVtk (left panel) and 5' probe (right panel).



**Figure 3** | Characteristics of MIN6CEon1 cells. (a,b) Insulin secretion stimulated with varying concentrations of glucose or (a) 5 mmol/L glc + 30 mmol/L KCl, and (b) 5 mmol/L Glc + 10 mmol/L leucine (Leu) + 10 mM glutamine (Gln) or 0.1  $\mu\text{mol/L}$  glimepiride (Glim) in MIN6c4 (white columns) and MIN6CEon1 cells (grey columns). Data represent the mean  $\pm$  standard error of the mean,  $n = 3$  experiments. (c) Proliferation of MIN6c4 (white circles) and MIN6CEon1 cells (grey circles). Data represent the means  $\pm$  standard error of the mean,  $n = 3$  experiments. (d) Doxycycline (dox; 1  $\mu\text{g/mL}$ )-induced green fluorescent protein (GFP) fluorescence of MIN6CEon1 cells after recombinase-mediated cassette exchange with pF3HygTreRFPGFw<sup>r</sup> (as shown in Fig. 2b). Dox treatment was applied for 2 days. Bar, 100  $\mu\text{m}$ . (e,f) Dox concentration-dependent expression of GFP shown by (e) fluorescent microscope and (f) western blots. Bar, 100  $\mu\text{m}$ .



**Figure 4** | Generation of glucokinase-overexpressing and -knock down cells by recombinase-mediated cassette exchange. (a) A recombinase-mediated cassette exchange vector for glucokinase overexpression under the TRE3g promoter. (b) Glucokinase messenger ribonucleic acid (mRNA) expressions in MIN6CEon1oeGck cells treated with (grey column) and without (white column) doxycycline (dox; 0.3 µg/mL for 2 days). Data are the mean ± standard error of the mean,  $n = 3$  experiments. \*\* $P < 0.01$ . (c) Dox-induced overexpression of glucokinase protein shown by a western blot. Antibodies against glucokinase and β-actin were added simultaneously. (d) Glucose utilization in MIN6CEon1oeGck cells treated with (grey columns) and without (white columns) dox (0.3 µg/mL for 2 days). Data are the mean ± standard error of the mean,  $n = 3$  experiments. \*\* $P < 0.05$ . \* $P < 0.05$ . (e) Glucose-stimulated insulin secretion in MIN6CEon1Gck cells treated with (grey columns) and without (white columns) dox. Data are the mean ± standard error of the mean,  $n = 3$  experiments. \* $P < 0.05$ . (f) A recombinase-mediated cassette exchange vector for expression of miRE embedded short hairpin ribonucleic acid against glucokinase. (g) Glucokinase mRNA expressions in MIN6CEon1shGck cells treated with (grey column) and without (white column) dox (1.0 µg/mL for 6 days). Data are the mean ± standard error of the mean,  $n = 3$  experiments. \*\* $P < 0.01$ . (h) Dox-induced suppression of glucokinase protein shown by a western blot. (i) Glucose utilization in MIN6CEon1shGck cells treated with (white columns) and without (grey columns) dox (1.0 µg/mL for 6 days). Data are the mean ± standard error of the mean,  $n = 3$  experiments. \* $P < 0.05$ . \*\* $P < 0.01$ . (j) Glucose-stimulated insulin secretion in MIN6CEon1shGck cells treated with (grey columns) and without (white columns) dox. Data are mean ± standard error of the mean,  $n = 3$  experiments. \*\* $P < 0.01$ .



similar results using individual clones from the same electroporation procedure (data not shown).

### Knockdown of glucokinase in MIN6CEon1 cells

We then applied this system to a loss-of-function study by utilizing shRNA-mediated knockdown. For this purpose, we took advantage of a modified system of miR30-based primicroRNA expression by RNA polymerase II, as a tetracycline-regulated system can be applied to achieve regulated knockdown<sup>30</sup>. As shown in Figure 4e,f, the exchanging plasmid contains a shRNA-encoding sequence embedded in miRE<sup>30</sup>. We targeted glucokinase again and looked for three target sequences by using the shERWOOD program<sup>32</sup>. Dox-induced expression of glucokinase-targeting shRNA shGck#2 for 6 days caused a 70% reduction in both mRNA and protein levels (Figure 4g,h), whereas shGck#1 and #3 caused just 20–30% reductions in a mixed cell population. The former cells were named MIN6CEon1shGck. [5-<sup>3</sup>H]glucose utilization showed a more than twofold reduction in glucokinase-knockdown cells at all three glucose concentrations tested (Figure 4i). Glucose-stimulated insulin secretion was significantly reduced in MIN6CEon1shGck cells treated with dox (Figure 4h,j), similar to results obtained using islets from mice with heterozygous knockout of the  $\beta$ -cell glucokinase gene<sup>37</sup>.

### Simultaneous generation of six stable sublines overexpressing genes with unknown roles in insulin secretion

To examine whether this system is applicable to screening for novel genes with the potential to be important for glucose-stimulated insulin secretion, we endeavored to generate six stable cell lines simultaneously. A preliminary microarray comparison of transcripts in highly glucose-responsive MIN6c4 cells and those of long passaged poorly-responsive MIN6 cells, identified more than 1,000 genes differentially expressed between the two cell populations. We confirmed differential expression in several genes by semiquantitative reverse transcription PCR. Transcript levels of *Cited4* (Cbp/P300-interacting transactivator 4), *Lyve1* (lymphatic vessel endothelial hyaluronan receptor 1) and *Rspo4* (R-spondin 4) were significantly higher in cells showing high responsiveness (Figure 5a), and those of *Arhgef3* (Rho guanine nucleotide exchange factor 3), *Folr1* (folate receptor 1) and *Plin5* (perilipin 5) were significantly higher in cells showing low responsiveness (Figure 5b). cDNAs were amplified from MIN6c4 mRNA with appropriate primers and cloned into the exchange plasmid. MIN6CEon1 cells were electroporated with these exchange vectors. Three weeks after electroporation, two-thirds of the colonies were combined. Dox-induced expressions of these genes were verified with primers used for cloning cDNAs (Figure 5c). Then, combined cells were subjected to testing for glucose-stimulated insulin secretion. Static insulin secretion analysis showed that *Cited4* overexpression resulted in increased glucose-stimulated insulin secretion, whereas overexpression of *Arhgef3* decreased insulin

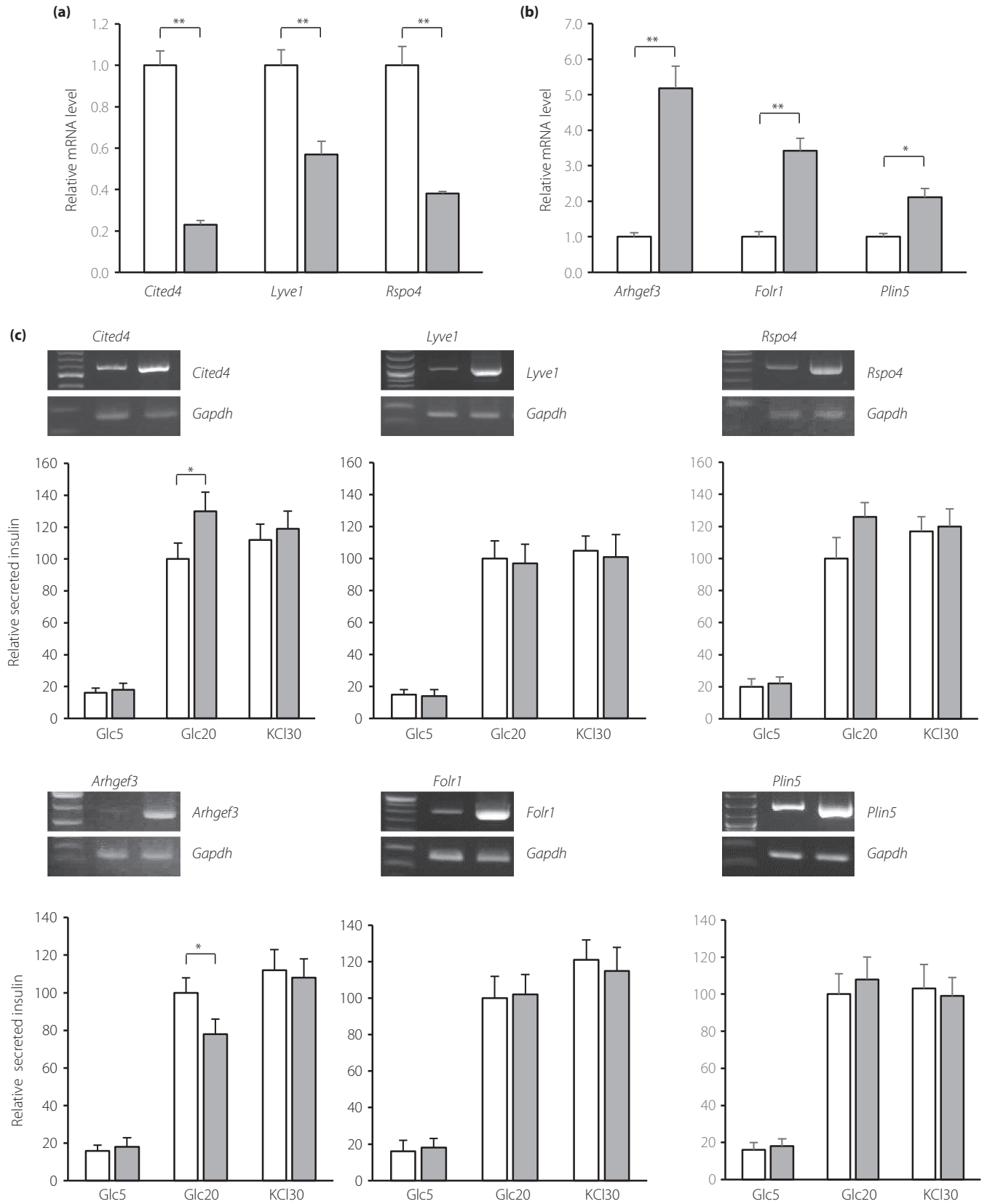
secretion. *Lyve1*, *Rspo4*, *Folr1* and *Plin5* had no significant impacts on insulin secretion (Figure 5c).

### DISCUSSION

For stable integration of an artificial expression unit, it is important to select a proper genome site. It has been reported that integration of an artificial expression unit with the tetracycline-regulated system at mouse *Rosa26*<sup>27,28</sup>, human *AAVS1*<sup>36</sup> locus or another unknown mouse locus<sup>38</sup> reportedly resulted in unsatisfactory mosaic expression or silencing of tetracycline-controlled genes. Thus, we randomly integrated the recipient platform, and searched for clones expressing GFP uniformly and at a high level on dox treatment. We found that the platform located at the site 10.5 kbps upstream of *Zxdb* escapes silencing. Special characteristics of the *Zxda* and *Zxdb* gene regions (being duplicated and reciprocally connected) could be the reason for this. Further studies are required to clarify the underlying mechanisms. Regarding safety, there are no reports of genes located at this site. In addition, so far as we were able to ascertain, the insulin secretion, cell morphology and growth of MIN6CEon1 cells are indistinguishable from those of parental MIN6c4 cells. Therefore, the site upstream from *Zxdb* might be a safe harbor site for foreign gene integration. We reached this site by chance after random integration of the platform. However, owing to progress in the genome editing technology, the RMCE platform can now be integrated at this site in any mouse cell genome, allowing the site to be easily modulated by RMCE. Use of this site might be applicable to other species including humans, although genome organization might differ among species.

MIN6CEon1, a MIN6 master cell line, has been generated by integrated integration of the platform by means of genome engineering. These cells are equipped with a tetracycline-regulated gene expression system. This allowed us to examine the effects of modifying gene expressions on insulin secretion, based on control (non-induced) and modified (induced) cells differing only in the single gene expression being examined; that is, with the other components being the same. Thus, clonal differences can be overcome.

This system is especially useful for gain-of-function studies. As essentially 100% of cells are isogenic, a mixed cell population allows an initial estimate of the effects of a modified gene expression to be examined within 6 weeks, which is shorter by approximately 1 month than the time frame for conventional methods. The initial estimates need to be confirmed using clones expanded from the appropriate single colonies. One can obtain multiple expresser clones by analyzing three to six clones. Given this ease-of-use, one researcher can generate six or even more cell lines simultaneously. In our earlier attempts to establish and refine this method, we found that *Cited4* could be positive regulators and *Arhgef3* a negative regulator of glucose-stimulated insulin secretion. Details of the mechanisms by which these genes modulate insulin secretion merit future analysis. For loss-of-function studies, we took advantage of the microRNA-embedded shRNA expression system. As prediction of the effective target sites for



**Figure 5** | Generation of stable cell lines overexpressing six genes with unknown effects on insulin secretion. (a) Relative messenger ribonucleic acid (mRNA) expressions of *Cited4*, *Lyve1* and *Rspo4* in highly glucose-responsive MIN6c4 cells (white bars) and long passaged poorly glucose-responsive MIN6 cells (grey bars). The average mRNA levels normalized with  $\beta$ -actin mRNA in MIN6c4 cells is defined as 1.0. Data are the mean  $\pm$  standard error of the mean,  $n = 3$  experiments.  $**P < 0.01$ . (b) Relative RNA expressions of *Arhgef3*, *Folr1* and *Plin5* as in (a). Data represent means  $\pm$  standard error of the mean,  $n = 3$  experiments.  $*P < 0.05$ ,  $**P < 0.01$ . (c) Effects of the overexpressions of six genes differentially expressed between highly and poorly glucose-responsive MIN6 cells. Overexpressions were induced by treatment with doxycycline for 2 days (grey columns). The secreted insulin amount at 20 mmol/L glucose from doxycycline-untreated cells (white columns) is defined as 100. Expressions of transgenes were confirmed by reverse transcription polymerase chain reaction. Data are the mean  $\pm$  standard error of the mean,  $n = 3$ –4 experiments.  $*P < 0.05$ , paired Student's *t*-test. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RNA interference remains imperfect, greater efforts are necessary than gain-of-function analysis. We have knocked down several genes (data not shown) and found, for practical application, that it appears to be most appropriate to select at least three target regions in a single gene.

In conclusion, we generated MIN6CEon1 cells for RMCE with an inducible expression system. These cells are anticipated to be useful for studying functional impacts of genome mutations revealed by genetic studies and to thereby contribute to progress in diabetes research.

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## DISCLOSURE

Approval of the research protocol: This research was approved by the Nihon University Recombinant DNA Advisory Committee (approval number 2018Med5, 5 September 2018). Approval by Nihon University ethics committee was not required, as the study involves neither patients nor normal control subjects.

Informed consent: N/A.

Approval date of registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

Conflict of interest: HI has served on the advisory board of Astellas Pharma, has received lecture fees from Astellas Pharma, MSD, Mitsubishi Tanabe Pharma, Nippon Boehringer Ingelheim and Novartis Pharma, and has received grants from Ono Pharmaceutical, Nippon Boehringer Ingelheim, Sanofi, Mitsubishi Tanabe Pharma, Eli Lilly, Daiichi-Sankyo, Novo Nordisk Pharma and MSD. The other authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** | Oligonucleotide primers for analysis of platform integration.

**Table S2** | Primers for reverse transcription polymerase chain reaction.