The Tetracycline-Controlled Transactivator (Tet-On/Off) System in β-Cells Reduces Insulin Expression and Secretion in Mice

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Controllable genetic manipulation is an indispensable tool in research, greatly advancing our understanding of cell biology and physiology. However in β-cells, transgene silencing, low inducibility, ectopic expression, and off-targets effects are persistent challenges. In this study, we investigated whether an inducible Tetracycline (Tet)-Off system with β-cell–specific mouse insulin promoter (MIP)-itTA–driven expression of tetracycline operon (TetO)-Cre$^{Jaw/J}$ could circumvent previous issues of specificity and efficacy. Following assessment of tissue-specific gene recombination, β-cell architecture, in vitro and in vivo glucose-stimulated insulin secretion, and whole-body glucose homeostasis, we discovered that expression of any tetracycline-controlled transactivator (e.g., improved itTA, reverse rtTA, or tTA) in β-cells significantly reduced *Insulin* gene expression and decreased insulin content. This translated into lower pancreatic insulin levels and reduced insulin secretion in mice carrying any tTA transgene, independent of Cre recombinase expression or doxycycline exposure. Our study echoes ongoing challenges faced by fundamental researchers working with β-cells and highlights the need for consistent and comprehensive controls when using the tetracycline-controlled transactivator systems (Tet-On or Tet-Off) for genome editing.

Mice are invaluable research tools, leading to translatable discoveries and greatly expanding our understanding of physiology and disease. Cell-specific and drug-controlled transgene expression systems, such as the estrogen receptor tamoxifen-dependent ligand domain (ERT) and the tetracycline (Tet) operon (TetO)/repressor bitransgenic systems, are widely used. However, there are numerous challenges associated with targeting transgene expression to pancreatic β-cells (1).

Multiple promoters achieve high levels of cell-type specificity; yet, even limited ectopic expression can cause off-target effects (2,3). The rat insulin (*RIP* or *Ins2*) and pancreatic and duodenal homeobox 1 (*Pdx-1*) promoters fragments used in the construction of some transgenes also drive expression in the central nervous system (2,4) and/or in the digestive track (3). Regardless of the promoter, transgene expression level can be highly variable (5,6), while exogenous drugs and transgenic element expression (e.g., human growth hormone minigene) can alter β-cell function and islet mass (7–10).

In light of ongoing challenges with current mouse models, including cell-specific targeting (2–4) or poor transgene expression (5,6), we sought to design an indu-cible transgenic model with enhanced efficiency and optimal β-cell specificity. Following doxycycline removal, the Tet-Off transgenic system allows continuous transgene expression in the absence of drug. This would be advantageous for long-term studies where maintained transgene expression is desired and could enhance recombination efficiency in inducible LoxP/Cre systems. In contrast to the *RIP*/*Ins2* promoter, the mouse insulin promoter (*MIP*

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or *Ins1*) does not appear to be expressed in brain or other neuroendocrine cell types (2,6,7,11). Thus, using a combination of the Tet-Off system and the MIP promoter (MIP-itTA [12]:TetO-Cre$^{Jaw/J}$ [13]), we evaluated tissue expression patterns and effects of this system on β-cell function and glucose homeostasis. Our data show that different components of this targeting system have significant effects on insulin expression and secretion, highlighting important limitations of the tetracycline-controlled transactivator models (Tet-On/Off) for cell-specific targeting, particularly in β-cells.

RESEARCH DESIGN AND METHODS

Mouse Models

Unless specified, hemizygote littermate male and female MIP-itTA (12) or TetO-Cre$^{Jaw/J}$ (13) mice on a mixed C57Bl/6J:C57Bl/6N:129Sv background, or male RIP-rtTA and wild-type controls on a C57Bl/6J background, were used (14). Mice were maintained on a 12-h dark/light cycle and had free access to water (containing doxycycline where indicated, amber bottles changed weekly) and standard chow (Teklad Rodent Diet 2018 or Purina Lab Diet 5LJ5). Experiments were approved by the Institut de recherches cliniques de Montréal and the Vanderbilt University Institutional Animal Care Committees.

Histological Analysis and Immunohistochemistry

Tissues were collected at 18–20 weeks of age as previously described (7). Antibodies for immunofluorescence are listed in Supplementary Table 1. Images were ×20 magnification for islets and confocal images of intestine and ×10 for other tissues.

Glucose/Insulin Tolerance Tests

Glucose tolerance tests (GTTs) and insulin tolerance tests were performed at 6–8 (RIP-rtTA) or 13–18 weeks (MIP-itTA) of age, as previously described (7,15). Areas under the curve were calculated using baseline fasting glucose.

Gene Expression Analysis

RNA from INS-1 cells or islets (∼120) was reverse transcribed (Life Technologies) and mRNA was quantified by quantitative PCR using SYBR Green (Bioline). Relative expression was calculated by ΔΔcycle threshold method and normalized to TATA-box binding protein (*Tbp*). Primers are listed in Supplementary Table 2.

Cell Culture

INS-1 cells (16) were transduced (multiplicity of infection 0.1) with pLJM1-EGFP vector (Addgene #19319), EGFP was replaced by itTA (gift from Rolf Sprengel [17]), rtTA (Addgene #25434), or tTA (Addgene #14901) or ligated without EGFP (empty vector). INS-1 stable clones were cultured in complete RPMI 1640 with puromycin (Bio Basic Inc., 0.5 μg/mL).

Insulin Content

INS-1 clones were washed and incubated at −20°C with acid–ethanol (1.5% HCl in 70% ethanol) for 24 h. Pancreas was harvested as described (7). Insulin was measured by STELLUX Chemiluminescent Rodent Insulin ELISA (Alpco), and values were normalized to tissue weight, protein (pancreas), or DNA content (cells).

Islet Isolation and Insulin Secretion

Primary mouse islets were isolated as described (16). Handpicked islets were cultured 48 h and insulin secretion and content assessed following 1-h static incubations (7) or by perifusion (18) in indicated media.

Statistical Analysis

Data comparing two or more groups on one or two variables were analyzed by one- or two-way ANOVA, respectively, corrected for multiple comparisons (Dunnett). Analyses were performed using GraphPad Prism software. Unless indicated, values are mean ± SEM.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article (and its online supplementary files).

RESULTS

Inducible Cre Recombinase Is Efficiently Targeted to Islets of MIP-itTA:TetO-Cre$^{Jaw/J}$ Mice

To design a Tet-Off–based mouse model expressing Cre recombinase only in β-cells, we paired MIP-itTA (12) and TetO-Cre$^{Jaw/J}$ (13) mice. In this system, Cre recombinase is expressed under the 7× tetracycline response element (TRE or TetO) promoter when doxycycline is removed (Fig. 1*A* and *B*). We confirmed that the human growth hormone minigene was not present in MIP-itTA or TetO-Cre$^{Jaw/J}$ genomes (Fig. 1*C*).

To measure efficiency of recombination in β-cells, we crossed MIP-itTA:TetO-Cre$^{Jaw/J}$ mice with the mTmG reporter line (19) (Fig. 1*B*), where red fluorescent protein is ubiquitously expressed until Cre recombinase mediates its replacement with green fluorescent protein (GFP). Mice received doxycycline from initiation of mating until 8 weeks of age, and tissues were collected or a group continued without the drug for 4 more weeks before tissue harvest (Fig. 1*D*). As expected, mice lacking one transgene had no GFP$^+$ cells in islets (i.e., MIP-itTA:mTmG or TetO-Cre$^{Jaw/J}$:mTmG mice) (Fig. 1*E* and *F*). MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice receiving doxycycline had a few green islet cells (∼2–5% cells of 60 islets analyzed), representing a low level of "leaky" itTA activity (Fig. 1*G*, white arrows). In contrast, removal of doxycycline for 4 weeks led to ∼75% Cre$^+$ cells within the islet (Fig. 1*H* and *I*), while mice that never received doxycycline (NEVER DOX, Fig. 1*J*) showed close to 100% recombination efficiency. Interestingly, while the vast majority of GFP$^+$ cells expressed insulin, we noted

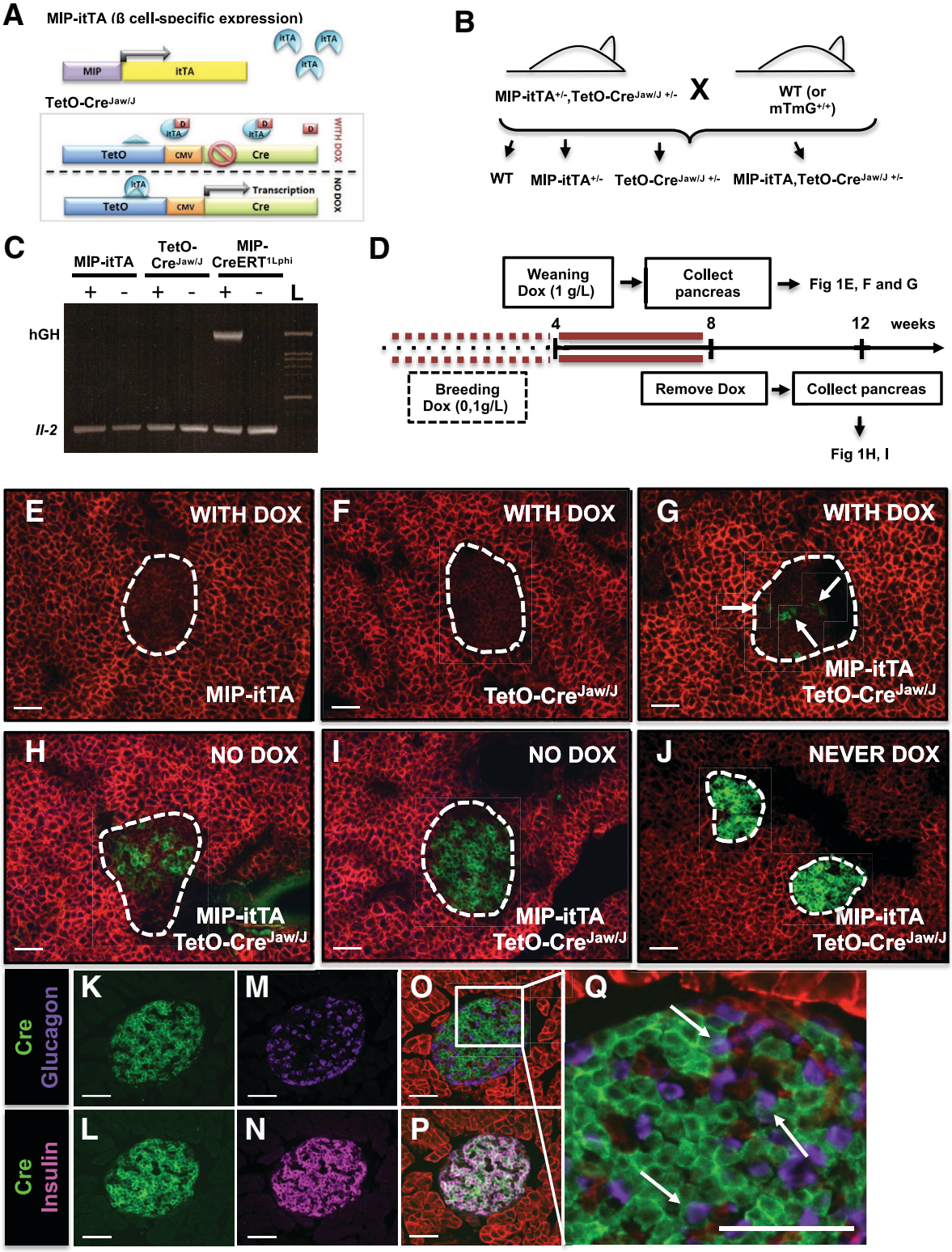

Figure 1—Islet-specific Cre recombination in MIP-itTA:TetO-Cre$^{Jaw/J}$ mice. *A*: β-Cell–specific Tet-Off system schematic. CMV, cytomegalovirus minimal promoter; D, doxycycline; Cre, Cre-recombinase. *B*: Hemizygous double transgenic MIP-itTA:TetO-Cre$^{Jaw/J}$, males or females) were crossed with WT or homozygous mTmG mice to generate all the littermate, age- and sex-matched experimental mice. Mendelian ratios and expected litter sizes were observed, regardless of parental genotype. *C*: PCR analysis to detect the human growth hormone (hGH) minigene in genomic DNA from MIP-itTA or TetO-Cre$^{Jaw/J}$ mice. Tail DNA from MIP-CreERT^{Lphi} and from WT littermate mice were used as positive and negative controls, respectively. *IL-2* is used as a positive control. *D*: Schematic of the experimental design and time frame. Pancreatic sections from MIP-itTA:mTmG (*E*), TetO-Cre$^{Jaw/J}$:mTmG (*F*), or MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice (*G*) kept on doxycycline treatment (in utero, 0.1 g/L dox) and 8 weeks after birth (1 g/L dox) (WITH DOX), or 12-week-old MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice

occasional glucagon/GFP$^+$ cell in islets from MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice (Fig. 1*K–Q*) following hormone labeling.

To determine cell-type specificity, we assessed Cre recombinase activity in other tissues. Ectopic Cre expression was found outside of β-cells; however, this was not due to the MIP-itTA transgene (Supplementary Fig. 1) but instead caused by innappropriate expression of the TetO-Cre transgene alone (Fig. 2). Recombination of the reporter was absent in the paraventricular nucleus or nuclei of the brain stem (Fig. 2*A–D*); however, female mice carrying a TetO-Cre$^{Jaw/J}$ transgene had GFP$^+$ cells in the arcuate nucleus (Fig. 2*E,F*), and both sexes had green cells in the choroid plexus (Fig. 2*G* and *H*). We did not detect GFP$^+$ cells in the muscle, liver, or heart (Fig. 2*I–K*). TetO-Cre$^{Jaw/J}$:mTmG mice also had many green cells in the stomach (Fig. 2*L*) and intestine (Fig. 2*M–P*), localized to the submucosa of the duodenum and ileum (Fig. 2*Q–T*) by confocal microscopy. We had similar results in the complete MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG model (Supplementary Fig. 1) and no spontaneous reporter activity in mTmG control mice (Supplementary Fig. 2). These data indicate that expression of the TetO-Cre$^{Jaw/J}$ transgene can occur, in the absence of a transactivator, in areas of the brain and digestive tract.

MIP-itTA Mice Are Glucose Intolerant Due to Insufficient Insulin Secretion

There is increasing evidence that transgenes believed to be inert (e.g., Cre recombinase, GFP, bacterial transactivators) can have significant effects on cell function and viability (20,21). Based on these concerns, we investigated the impact of Tet-Off system components on glucose metabolism. Glucose tolerance, insulin sensitivity, and glucose-stimulated insulin secretion (GSIS) were assessed following a 5-week doxycycline wash out (Fig. 3*A*). We observed significant glucose intolerance in mice of both sexes expressing the MIP-itTA transgene following oral glucose challenge (Fig. 3*B–E*), regardless of Cre recombinase expression. There were no significant differences in mouse weights or fasting glucose (4 or 18 h) for any genotype within each sex (Supplementary Fig. 3*A–F*), although there was a trend toward higher glycemia in MIP-itTA:TetO-Cre$^{Jaw/J}$ female mice (Supplementary Fig. 3*D*).

To determine whether glucose intolerance was due to defects in insulin secretion versus insulin action, we measured plasma insulin during the oral glucose tolerance test and noted that MIP-itTA$^+$ male and female mice had deficiencies in GSIS (Fig. 3*F* and *G*). In line with a primary β-cell defect and not insulin resistance, all mice had similar responses to exogenous insulin (Fig. 3*H* and *I*). Drug exposure, route of glucose administration, and mouse genetic background can influence glucose metabolism (22) and cause off-target effects (23). To test whether phenotypes were affected by the mixed genetic background of the mouse or doxycycline exposure, mice were bred onto a pure C57Bl/6N background. In the absence of doxycycline, we again observed glucose intolerance correlating with presence of the MIP-itTA transgene (Supplementary Fig. 3*G* and *H*) in inbred male mice. Similar impairments were seen following i.p. administration of glucose (Supplementary Fig. 4), suggesting that decreased GSIS in MIP-itTA$^+$ mice was not the result of a defective incretin response. In short, we report that the MIP-itTA transgene causes glucose intolerance correlating with low glucose-stimulated circulating insulin. This phenotype was independent of the TetO-Cre$^{Jaw/J}$ transgene, which, in our hands, had no significant impact on glucose tolerance or GSIS in vivo.

To determine whether other tetracycline transactivators cause similar defects, we performed a preliminary study in mice expressing rtTA in β-cells (14). RIP-rtTA mice showed trends toward reduced insulin secretion in response to i.p. glucose and decreased total pancreatic insulin content (Supplementary Fig. 5*A–E*); however, these effects were milder than itTA and did not reach statistical significance (possibly due to low number). Regardless, these preliminary data suggest that effects on insulin expression and secretion might be shared by other tTA proteins to varying degrees.

Tetracycline-Controlled Transactivators Reduce Insulin Expression in β-Cells

Reduced GSIS can result from low levels of insulin, defects in glucose sensing, or reduced secretion capacity. Islet architecture by hematoxylin-eosin (H&E) staining in pancreatic sections showed no visible differences between genotypes (Fig. 4*A–D*). However, hormone-labeling irregularities (e.g., increased α-cells in the islet core or cells that were neither insulin- nor glucagon-positive) were seen in >30% of islets with the MIP-itTA transgene (Fig. 4*E–I*). Total insulin content of the pancreas (Fig. 4*J*) and primary islets (Fig. 4*K*) was also reduced in MIP-itTA$^+$ mice compared with wild-type (WT) littermates.

To determine whether transactivators directly impact insulin expression, we first measured mRNA in primary islets from MIP-itTA mice. Correlating with reduced pancreatic and islet insulin content, MIP-itTA$^+$ islets had low expression of both the insulin 1 (*Ins1*) and insulin 2 (*Ins2*) genes compared with controls (Fig. 5*A*). We also

without doxycycline for the last 4 weeks (NO DOX) (*H* and *I*). *J*: Pancreatic sections from MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice never exposed to doxycycline (NEVER DOX) (representative images of $n = 4$ mice, male or female). Islet from MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG mice where Cre$^+$ cells are green (*K–L*) and colabeled with glucagon (purple) (*M*) or insulin (magenta) (*N*) and merged images (*O* and *P*). *Q*: Magnification showing double-positive (Cre$^+$ glucagon$^+$) cells (arrows) (representative image of $n = 2$ mice). Scale bars, 58 μm.

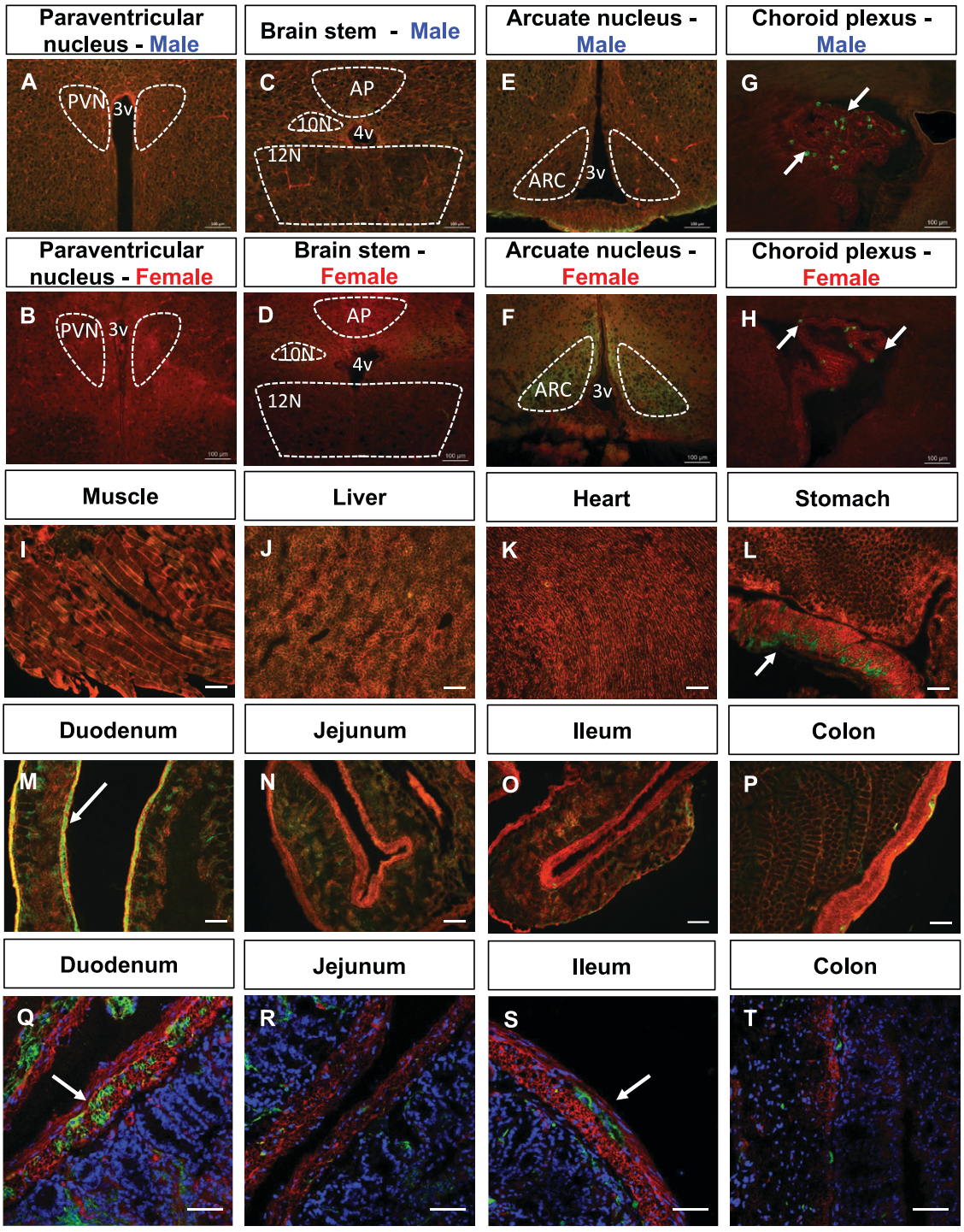

Figure 2—Cre-mediated recombination detected in the arcuate nucleus of female TetO-Cre$^{Jaw/J}$:mTmG mice and in the choroid plexus and duodenum of all TetO-Cre$^{Jaw/J}$:mTmG mice. Coronal sections of brain from 18- to 20-week-old TetO-Cre$^{Jaw/J}$:mTmG mice 4 weeks after doxycycline removal. *A* and *B*: Paraventricular nucleus (PVN). *C* and *D*: Brain stem. *E* and *F*: Arcuate nucleus (ARC). *G* and *H*: Choroid plexus. 3v, third ventricle; 4v, fourth ventricle; AP, area postrema; 10N, dorsal motor nucleus of the vagus; 12N, hypoglossal nucleus from male or female (n = 3 mice, per sex). Scale bars, 100 μm. Sections of the muscle (*I*), liver (*J*), heart (*K*), stomach (*L*), duodenum (*M*), jejunum (*N*), ileum (*O*), and the colon (*P*) from 18- to 20-week-old TetO-Cre$^{Jaw/J}$:mTmG mice 4 weeks after doxycycline removal. Scale bars, 120 μm. Higher magnification, confocal images of duodenum (*Q*), jejunum (*R*), ileum (*S*), and colon (*T*) (representative images of n = 4 male or female mice). Scale bars, 50 μm.

found the solute carrier family 2 member 2 (*Slc2a2* or *Glut2*) gene, encoding a transporter important for glucose entry and sensing, was significantly lower (Fig. 5*A*). In contrast, genes for pancreatic and duodenal homeobox 1 (*Pdx-1*) and potassium inwardly rectifying channel subfamily J member 11 (*Kcnj11*) were unchanged (Fig. 5*A*).

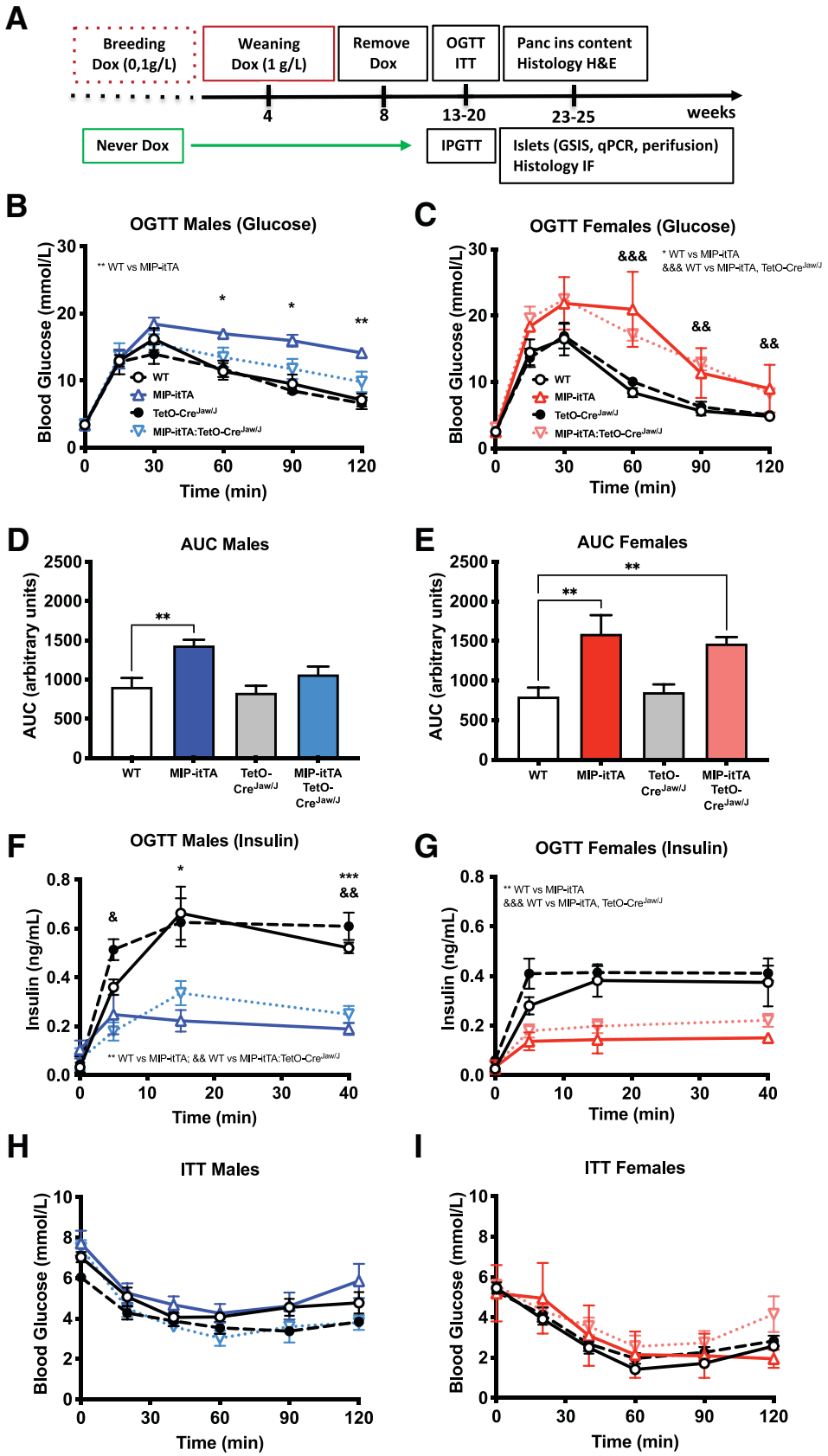

Figure 3—Mice carrying the MIP-itTA transgene are glucose intolerant and exhibit defective insulin secretion. *A*: Schematic of the experimental design and time frame. dox, doxycycline; IF, immunofluorescence; ins, insulin; IPGTT, intraperitoneal glucose tolerance test; panc, pancreas. Blood glucose following oral glucose tolerance test (OGTT; 1.5 g/kg) in 13- to 15-week old males (n = 4–6) (*B*) and females

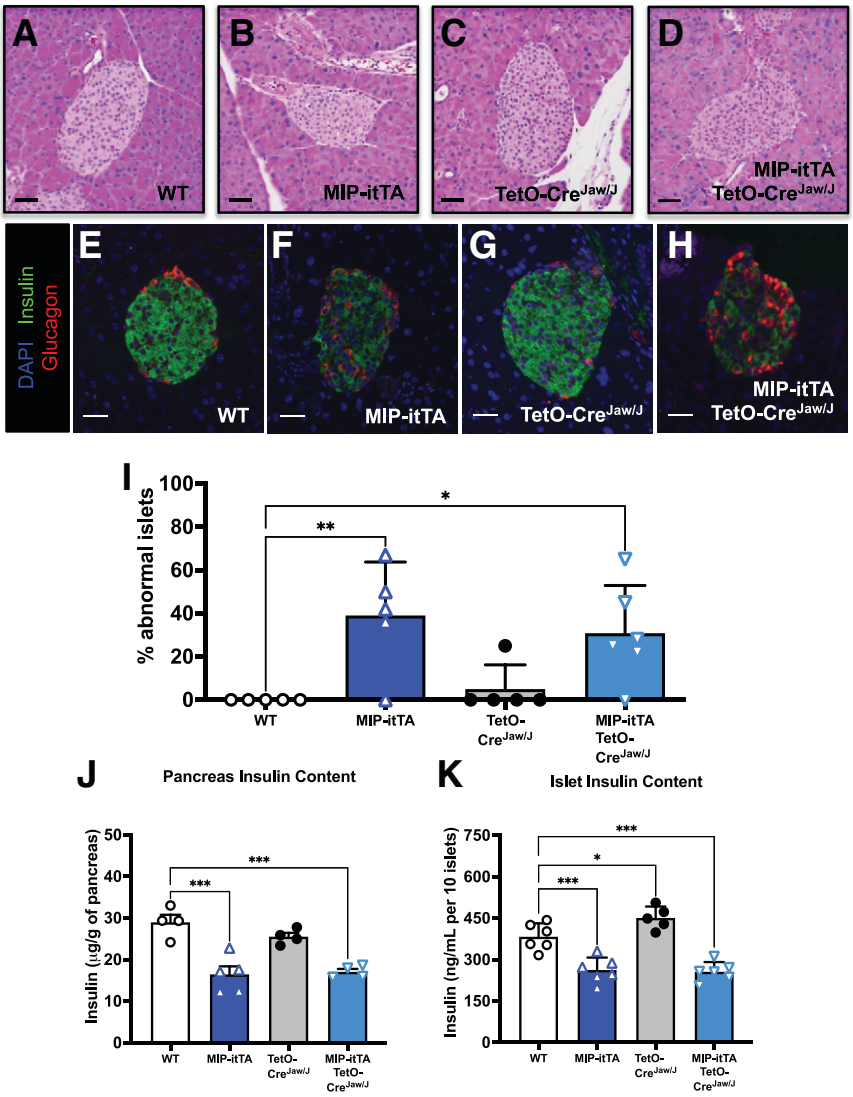

Figure 4—Islets from mice with the MIP-itTA transgene have abnormal hormone immunolabeling and decreased insulin content. Representative images of pancreatic islets of mice from each genotype following H&E, scale bars, 50 μm (*A–D*); or immunofluorescence labeling with antibodies against insulin (green), glucagon (red), or DAPI (blue) (*E–H*); scale bars, 30 μm. *I*: Scoring of islets analyzed after immunofluorescence (n = 5–6 C57Bl/6N males never on doxycycline, 60 islets per genotype). Abnormal islets are defined as having significant irregularities, such as >5% α-cells in the islet core and/or >10% unlabeled cells. *J*: Total pancreatic insulin content from males (n = 4–5). *K*: Insulin content of isolated islets from C57Bl/6N male mice from each genotype never on doxycycline (pooled islets from n = 3 mice, values are means ± SD). Following ANOVA, post hoc statistical comparisons are performed vs. WT. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Considered with in vivo data, our results suggest that tTA proteins may have detrimental effects on insulin expression or glucose-regulated gene expression.

To address this hypothesis, we stably expressed tTA, improved tTA (itTA), or reverse tTA (rtTA) in the rat insulinoma (INS-1) β-cell line (Supplementary Fig. 6*A* and *B*). Consistent with data in pancreas and primary islets, insulin content in INS-1 cell lines expressing any of the tetracycline-controlled transactivators was decreased compared with levels in cells expressing empty vector (Fig. 5*B*). To test whether overexpression of any protein could decrease insulin, we also overexpressed EGFP, which conversely increased

(n = 2–5) (*C*) and corresponding area under the curve (AUC) for males (*D*) and females (*E*) or serum insulin levels for males (*F*) and females (*G*). *H* and *I*: Insulin tolerance test (ITT) (0.8 units/kg) performed on mice from *B* and *C*. Two-way ANOVA results and post hoc comparisons are shown for WT vs. MIP-itTA ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) and WT vs. MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG ($\&P < 0.05$, $\&\&P < 0.01$, $\&\&\&P < 0.001$). WT, white circle/solid line; MIP-itTA, open, red/dark blue triangle/solid line; TetO-Cre$^{Jaw/J}$, black circle/dashed line; MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG, open, inverted pink/light blue triangle/dashed line.

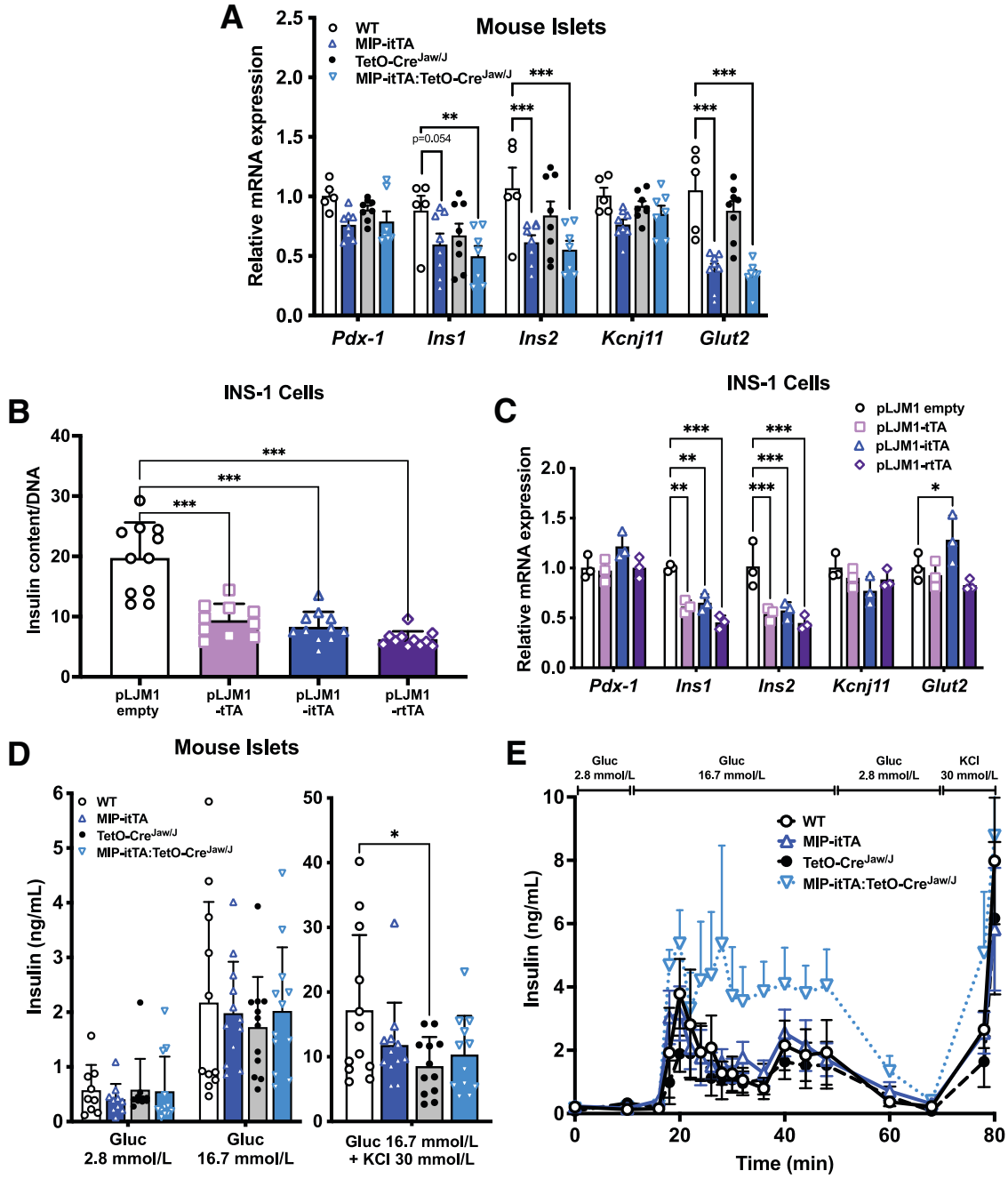

Figure 5—Expression of tetracycline-controlled transactivators in cultured β-cells reduces insulin mRNA and protein levels and affects insulin secretion. *A*: Gene expression of primary islets of C57Bl/6N male mice never on doxycycline expressed relative to *Tbp* (n = 5–8 mice per genotype), shown as means ± SEM. Comparison are vs. WT following post hoc analysis. Insulin content (*B*) and gene-expression analysis (*C*) of INS-1 cells stably overexpressing empty vector, tTA, itTA, or rtTA. Values are means ± SD normalized to *Tbp* and representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represent comparisons to pLJM1 empty, as indicated. *D*: Static glucose-stimulated insulin secretion from primary islets of C57Bl/6N male mice never on doxycycline. Values are means ± SD of replicates of 10 islets from a pool of n = 3 mice per genotype. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. WT. *E*: Insulin secretion from primary islets of C57Bl/6N male mice never on doxycycline in perifusion experiments. Values are means ± SEM of replicates (n = 4) of 50 islets from a pool of n = 5–6 mice per genotype. The assays were performed in Krebs-Ringer bicarbonate HEPES buffer supplemented with 2.8 mmol/L or 16.7 mmol/L of D-glucose (Gluc) or 30 mmol/L KCl.

insulin content (Supplementary Fig. 6*C*), suggesting that reductions linked to transactivator expression were not an artifact of the system. Similar to islet data, we saw a significant decrease in *Ins1* and *Ins2* gene expression following expression of the transactivators in INS-1 cells, and *Pdx-1* and *Kcnj11* were unaltered (Fig. 5*C*). In contrast to data in primary islets, *Slc2a2* (*Glut2*) expression was not reduced in this system (Fig. 5*C*). These data suggest that any

tetracycline-controlled transactivator may cause reductions in insulin gene expression leading to lower islet insulin content.

Alterations in Insulin Secretion Linked to Tetracycline-Controlled Transactivator Expression Were Limited Ex Vivo

Finally, we tested whether defects in GSIS in vivo were preserved ex vivo. Surprisingly, primary islets isolated from mice of each genotype had comparable insulin secretion in response to low (2.8 mmol/L) or high (16.7 mmol/L) glucose or in response to the general insulin secretagogue KCl (except a mild decrease of the TetO-Cre$^{Jaw/J}$ islets compared with WT) (Fig. 5*D*), despite lower insulin content (Fig. 4*K*). This suggests that primary islets contained sufficient stocks of insulin to respond to an ex vivo glucose challenge or that additional factors in vivo contributed to altered insulin secretion. Similar results were obtained following glucose challenge by perifusion, yet this assay also revealed a potential for insulin hypersecretion from double transgenic MIP-itTA:TetO-Cre$^{Jaw/J}$:mTmG islets in response to glucose (Fig. 5*E*). These data highlight unpredictable differences in transgene effect across assays and suggest that insulin reserve levels, compensatory islet changes, and/or other factors in vivo contribute to defective GSIS linked to tetracycline-controlled transactivator expression in β-cells.

DISCUSSION

Our work illustrates that a TetO-Cre/MIP-itTA approach effectively targets β-cells and allows efficient, drug-controllable expression of transgenes, in line with previous reports (12,24,25). However, we find that the TetO-Cre$^{Jaw/J}$ transgene causes unregulated expression of Cre recombinase in multiple regions of the brain and gastrointestinal tract that can vary between sexes. Moreover, we show that expression of tetracycline-controlled transactivators in β-cells can significantly reduce insulin expression, potentially manifesting as defective GSIS, glucose intolerance, and lower pancreatic insulin content. Reduction in insulin content correlates with decreases in *Ins1/2* expression in transgenic islets that is replicated in cultured β-cells expressing the transactivators alone.

The MIP-itTA/TetO-Cre system was specific and well-controlled by doxycycline in β-cells. Despite multiple positive aspects of this targeting system, the MIP-itTA transgene alone reduced insulin levels and glucose tolerance in both sexes, similar to Pdx1-tTA mice (26) but in contrast to MIP-driven rtTA^{+} mice (25). In our small cohort, RIP-rtTA mice also had normal glucose tolerance but mildly decreased insulin content and GSIS in vivo. Differences between models could be due to control groups, mouse strain, age of the mice, or sex- or transgene-specific effects (e.g., level of transactivator expression or position of transgene insertion). However, we could reproduce reductions in β-cell insulin content and expression by overexpression of any tetracycline-controlled transactivator in vitro. Being transcriptional coactivators by design, it is possible that these foreign tTA proteins interact with and/or regulate transcription of genes in unpredictable ways. To our knowledge, chromatin binding/activity studies to determine whether the original or improved transactivators can have TRE-independent activity across various cell-types or genomes are not widely available or reported.

Despite significant effects of transactivator expression on insulin secretion in vivo, we were surprised to see little effect on GSIS ex vivo. A single glucose challenge may not be sufficient to reveal secretion deficiencies linked to low insulin content ex vivo, and repeated stimulations may be necessary (27). Depletion of insulin content might become more pertinent if these genetic tools are used in murine models of diabetes, where insulin resistance and β-cell stress lead to insulin hypersecretion and/or deficiency. Unpredictably, islets expressing both MIP-itTA and TetO-Cre exhibited enhanced insulin secretion during perifusion experiments only, further emphasizing the importance of controlling for transgene expression during all assays and conditions.

Mounting evidence illustrates that there is no perfect system, but measures can be taken to control for expected and unexpected off-targets effects. Novel tools are continuously being developed, but with new technology inevitably comes new caveats that require further characterization and unique controls. While the Tet-On/Off system remains an extremely useful tool, our evidence illustrates that the required transgenes, even without doxycycline, can impact β-cell function and insulin content, reinforcing the need to carefully control this system in all assays.

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Author Contributions. N.J. contributed to the study design and conducted all experiments. N.J. and J.L.E. designed the research, interpreted data, and co-wrote and edited the manuscript. K.B. and T.A. processed the brains and analyzed corresponding immunofluorescence images. S.A.C. performed the islet perifusion experiments. C.B. created expression constructs and lentiviral vectors and helped perform metabolic tests. S.E.T. and M.A.G. performed the

experiments on RIP-rtTA mice and analyzed the results. M.A.G., V.P., and T.A. edited the manuscript. All authors gave final approval of the version to be published. N.J. and J.L.E. are the guarantors of this work and, as such, had full access to all data in the study and take responsibility for the integrity of the data and accuracy of data analysis.

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