Daniel Oropeza,[1,2] Nathalie Jouvet,[1,3] Lionel Budry,[4] Jonathan E. Campbell,[5] Khalil Bouyakdan,[4,6] Julie Lacombe,[1] Gabrielle Perron,[1,2] Valerie Bergeron,[4,6] Joshua C. Neuman,[7] Harpreet K. Brar,[7] Rachel J. Fenske,[7] Clemence Meunier,[1] Sarah Sczelecki,[3] Michelle E. Kimple,[7] Daniel J. Drucker,[5] Robert A. Screaton,[8] Vincent Poitout,[4,6] Mathieu Ferron,[1,3,4] Thierry Alquier,[4,6] and Jennifer L. Estall[1,2,3,4,6]

Phenotypic Characterization of MIP-CreERT^{1Lphi} Mice With Transgene-Driven Islet Expression of Human Growth Hormone

Diabetes 2015;64:3798–3807 | DOI: 10.2337/db15-0272

ISLET STUDIES

There is growing concern over confounding artifacts associated with β-cell–specific Cre-recombinase transgenic models, raising questions about their general usefulness in research. The inducible β-cell–specific transgenic (MIP-CreERT^{1Lphi}) mouse was designed to circumvent many of these issues, and we investigated whether this tool effectively addressed concerns of ectopic expression and disruption of glucose metabolism. Recombinase activity was absent from the central nervous system using a reporter line and high-resolution microscopy. Despite increased pancreatic insulin content, MIP-CreERT mice on a chow diet exhibited normal ambient glycemia, glucose tolerance and insulin sensitivity, and appropriate insulin secretion in response to glucose in vivo and in vitro. However, MIP-CreERT mice on different genetic backgrounds were protected from high-fat/streptozotocin (STZ)-induced hyperglycemia that was accompanied by increased insulin content and islet density. Ectopic human growth hormone (hGH) was highly expressed in MIP-CreERT islets independent of tamoxifen administration. Circulating insulin levels remained similar to wild-type controls, whereas STZ-associated increases in α-cell number and serum glucagon were significantly blunted in MIP-CreERT^{1Lphi} mice, possibly due to paracrine effects of hGH-induced serotonin expression. These studies reveal important new insight into the strengths and limitations of the MIP-CreERT mouse line for β-cell research.

Cre-lox technology for tissue- and time-specific gene ablation is an invaluable tool in molecular biology, yet it is increasingly apparent that introduction or expression of the Cre-recombinase gene may produce confounding artifacts in different cellular and physiological contexts (1,2). In this regard, β-cells appear particularly sensitive. A few widely used transgenic mouse lines created using the promoters of pancreatic and duodenal homeobox 1 (Pdx1) or the rat insulin promoter (RIP) are reported to have significant transgene-mediated effects on insulin secretion and glucose tolerance (2–4). In addition, ectopic Cre-recombinase expression in the central nervous system (CNS) may cause non–β-cell related physiological effects (5,6).

A major challenge in the field has been to identify a specific promoter expressed exclusively in β-cells of the pancreas. Pdx1-driven gene expression turns on early in pancreatic development, leading to expression of transgenes throughout the pancreas (exocrine and endocrine) under the native promoter. This potential limitation for studies targeted toward particular endocrine cell types appeared largely circumvented

[1]Institut de recherches cliniques de Montréal, Montreal, QC, Canada
[2]Department of Anatomy and Cell Biology, McGill University, Montreal, QC, Canada
[3]Division of Experimental Medicine, McGill University, Montreal, QC, Canada
[4]Montreal Diabetes Research Center, Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, QC, Canada
[5]Department of Medicine, Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada
[6]Département de Médecine, Université de Montréal, Montreal, QC, Canada
[7]Department of Medicine and Interdisciplinary Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, Madison, WI
[8]Department of Cellular and Molecular Medicine, Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, ON, Canada

Corresponding author: Jennifer L. Estall, jennifer.estall@ircm.qc.ca.

Received 26 February 2015 and accepted 24 June 2015.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db15-0272/-/DC1.

D.O. and N.J. contributed equally to this study.

See accompanying article, p. 3648.

by the creation of a tamoxifen-inducible Pdx1-CreERT line, which can be used to limit expression to β-cells in adult mice (7,8). The insulin 2 (Ins2) promoter (commonly referred to as RIP) is expressed predominantly in adult β-cells and has also been used to create both constitutive and inducible lines (9–14). Despite efforts to limit CNS expression using inducible systems, it was recently shown using an in vivo reporter system that significant Cre-mediated recombination in the CNS can still occur in many lines (6), confounding interpretation of results if floxed genes are also expressed at appreciable levels in the CNS.

In an effort to eliminate artifacts due to ectopic Cre-recombinase expression, the inducible mouse insulin promoter (MIP)-CreERT^{1Lphi} mouse line was created using ample upstream regulatory sequence from the mouse insulin 1 promoter (8.5 kb), a gene with limited expression in the CNS compared with the Ins2 gene (15). This promoter drives expression of Cre-recombinase cDNA fused to the hormone-binding domain of a mutant mouse estrogen receptor (Esr1*), preventing nuclear translocation of the recombinase in the absence of tamoxifen (16).

Given the history of confounding issues noted for similar mouse lines and the importance of carefully characterizing each new research model, we closely evaluated the metabolic phenotype and β-cell function of the MIP-CreERT^{1Lphi} mouse (referred to herein as MIP-CreERT) under normal physiological conditions and common in vivo models of diabetes. We also performed gene expression analysis and high-resolution microscopy on multiple regions of the CNS using a definitive reporter system. Our goal was to clearly define the specificity of Cre-recombinase expression and gene ablation to β-cells and determine whether MIP-CreERT mice exhibit abnormalities in whole-body glucose homeostasis, insulin secretion, and β-cell mass.

RESEARCH DESIGN AND METHODS

Experimental Mice

Strains used included mT/mG (17), MIP-CreERT^{1Lphi} (16), RIP-Cre^{Herr} (10), and Pdx1-Cre^{Tuv} (18) (see Supplementary Table 1 for details on genetic background). All mice were hemizygous for the Cre-recombinase allele. Data shown are from MIP-CreERT (C57BL/6J) unless otherwise indicated. All mice were male and gavaged at 6 weeks of age for 10 days with 100 mg/kg tamoxifen (Sigma) suspended in 0.05% methylcellulose/distilled water unless otherwise indicated. A minimum 2-week washout period after tamoxifen was provided. Mice were maintained on a 12-h dark/light cycle and given free access to chow and water. Experiments were performed according to approved protocols from the Institut de recherches cliniques de Montréal.

Histological Analysis of Tissues in mT/mG Reporter Mice

Thirteen-week-old mice were perfused with 10% formalin via cardiac puncture. Brains were fixed overnight in 10% formalin, followed by 10% sucrose/PBS for 24 h prior to freezing in OCT using isopentane and sectioning (7–10 μm). Cells were visualized by fluorescence microscopy at each spectra and images merged.

Static Insulin Secretion and Insulin Content

One-hour static incubations of isolated islets were performed as previously described (19). Briefly, batches of 10 islets were incubated at 37°C 2 × 20 min in Krebs-Ringer bicarbonate HEPES buffer (KRBH) solution containing 0.1% (w/v) BSA and 2.8 mmol/L glucose prior to incubation for 1 h in 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 16.7 mmol/L glucose with 0.5 mmol/L oleate. Oleate was precomplexed with fatty acid–free BSA to a final molar ratio of 1:5 as previously described (20). Control conditions contained identical concentrations of BSA and oleate vehicle (50% [v/v] ethanol). Intracellular insulin content was measured after acid-alcohol extraction. For pancreatic insulin, half of the pancreas (closest to the spleen) was incubated overnight (O/N) (−20°C) in 5 mL acid-ethanol (1.5% HCl in 70% EtOH) prior to homogenization and refreezing. Thawed homogenate was centrifuged prior to neutralization with 1 mol/L Tris pH 7.5. Insulin was measured by immunoassay kit (PerkinElmer or Alpco). Insulin content was normalized to pancreas weight.

Gene Expression Analysis

cDNA was synthesized by reverse transcription (Life Technologies) using RNA isolated from hypothalamic nuclei using Trizol (Invitrogen) or from islets (~120) by RNeasy Mini kit (Qiagen). Gene expression was quantified using SYBR green (Life Technologies) and relative expression calculated by ΔΔct method, normalized to hypoxanthine-guanine phosphoribosyltransferase. Tail genomic DNA or islet cDNA was subject to PCR using primers flanking the start and stop codons of hGH. Cre-recombinase, interleukin-2, and actin primers served as internal controls. Primer sequences are listed in Supplementary Table 2.

hGH Immunoblotting

Islets (~150) or INS-1 cells expressing hGH (pUC plasmid) were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors. Proteins were separated by SDS-PAGE and blotted with mouse anti-hGH (1:500; Abcam) or mouse anti–β-actin (1:10,000; Sigma).

Perifusion of Primary Islets and Glucagon Secretion

After overnight recovery in RPMI (10% FBS, 1% Pen/Strep), 150 islets were equilibrated in 0.275-mL chambers for 1 h (KRBH, 5 mmol/L glucose) at a flow rate of 100 μL/min (Biorep Perifusion system). Islets were then perifused at 24-min intervals in experimental media (KRBH plus indicated conditions) and glucagon concentrations determined by ELISA (Mercodia) and expressed as percentage of baseline.

Glucose/Insulin Tolerance Tests, Serum Insulin, and Glucagon

Mice were maintained on standard chow (Teklad diet 2018) or provided a high-fat/high-sucrose (HFHS) diet (cat. no. D12451i; Research Diets) starting at 8 weeks old. Physiological tests were performed at 13–17 weeks (chow diet) or 28–30 weeks of age (HFHS diet), as indicated, or within 30 days of streptozotocin (STZ) administration. Oral glucose tolerance

tests (OGTT) were performed after a 16-h fast following oral administration of glucose diluted in water (1.5 g/kg for chow-fed/HFHS and 1 g/kg for HFHS/STZ mice). Insulin tolerance tests (ITT) were performed after injection of human insulin (1 unit/kg i.p. for chow-fed and 1.5 units/kg for HFHS-fed mice; Lilly) in 4-h-fasted mice. Fasted and refed glucose and insulin were measured after 16 h fasting and 2 h chow refeeding. Blood glucose or serum insulin was measured by tail vein using a standard glucometer (FreeStyle Lite, Abbott Diabetes Care) or the mouse ultrasensitive insulin ELISA (Alpco), respectively. Glucagon levels were measured after a 16-h fast in plasma containing aprotinin by glucagon ELISA (Mercodia).

Induction of Hyperglycemia With High-Fat Feeding and/or STZ

A combination of HFHS diet and STZ was administered as previously reported (21), with some modifications. Briefly, 8-week-old mice were provided an HFHS diet for 3 weeks prior to administration of two doses of STZ (Bioshop) (day 0: 50 mg/kg; day 5: 75 mg/kg) in 100 mmol/L sodium citrate, pH 4.5, by intraperitoneal injection after a 4-h fast. Mice were maintained on an HFHS diet until sacrifice. When tamoxifen was not preadministered, a third injection of 100 mg/kg STZ was required to potentiate hyperglycemia in control mice. For single, high-dose STZ administration, chow-fed mice were injected with 150 mg/kg STZ at 10–13 weeks of age after a 4-h fast. Random blood glucose was measured as above at indicated times.

Islet Area/Mass and Immunohistochemistry

For islet area, 10–12 5-μm sections of pancreas (paraffin embedded), separated by at least 200 μm, were stained with hematoxylin-eosin. Islet density was calculated as the number of islets per section divided by section area. Islet mass was calculated as follows: (total islet area per mouse/total section area per mouse) × pancreas weight. Staining of paraffin sections was performed with guinea pig anti-insulin (1:100; Dako), mouse anti-glucagon (1:50; Sigma), mouse anti-serotonin (1:50; Dako), and rabbit Ki67, clone SP6 (1:100; Thermo Scientific) primary antibodies. Ki67 nuclear staining was quantified in each islet and expressed as percentage of total islet cell number. Islets containing <20 cells were excluded.

Statistical Analysis

Data comparing one variable were analyzed by Student *t* tests, corrected for multiple comparisons (Sidak-Bonferroni) ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Normality was determined by Shapiro-Wilk test. Two-way ANOVA followed by post hoc analysis (Bonferroni or Fisher least significant differences test) determined significance of individual points. Analysis was performed using GraphPad Prism. Unless indicated, values are mean ± SEM.

RESULTS

MIP-CreERT Mice Did Not Exhibit Ectopic Recombinase Activity in the Brain

A recent study reports that the insulin 1 (Ins1)/MIP promoter can drive transgene expression in cells isolated from the hypothalamus (22). Although transgenic mice using the Ins1 promoter do not appear to exhibit Cre-recombinase–mediated gene excision using staining/imaging of whole brain sections or quantitative PCR (qPCR) (23–25), these studies lack the single-cell resolution required to conclude whether Cre-recombinase activity is present in distinct neuronal populations. To sensitively detect Cre-mediated recombination in single neurons, we crossed MIP-CreERT mice with the global reporter mT/mG mouse line (17), which constitutively expresses red fluorescent protein (tdTomato) in all cells that are replaced by green fluorescent protein (GFP) after Cre-mediated recombination. Coronal and sagittal sections of brain from mT/mG:MIP-CreERT mice given tamoxifen showed no GFP+ neurons (Fig. 1*A* and *B*). No GFP+ cells were detected in hypothalamic (paraventricular nucleus, arcuate nucleus, ventromedial hypothalamus, dorsomedial hypothalamus) or hindbrain (dorsal motor nucleus of the vagus nerve, nucleus of the solitary tract, parabrachial nucleus) nuclei (Fig. 1*C*–*G*), while pancreata of corresponding mice exhibited a strong GFP signal in islet cells (Fig. 1*H*). These results provide strong evidence that Cre-recombinase does not drive excision of floxed genes in these CNS regions of MIP-CreERT mice in vivo.

Glucose Homeostasis Was Similar in MIP-CreERT Mice Fed Chow or High-Fat Diet

Given concerns that Cre-recombinase expression or activity may lead to artifactual phenotypes (2,4), we next evaluated whole-body glucose homeostasis and in vivo insulin secretion in tamoxifen-treated MIP-CreERT mice on chow and HFHS diets. Compared with wild-type (WT) littermates, chow-fed MIP-CreERT mice showed no significant differences in weight, oral glucose tolerance, insulin tolerance, or fasting or refed glucose (Fig. 2*A*–*D*). There were also no differences in circulating insulin after fasting, refeeding, or oral or intraperitoneal administration of glucose (Fig. 2*E*–*G*). Likewise, HFHS feeding for 20 weeks did not reveal significant differences in weight, glucose tolerance, insulin sensitivity, or insulin secretion after an OGTT (Fig. 2*H*–*K*). However, there was a significant decrease in overnight fasting glucose and 5-h-fasted insulin in HFHS-fed MIP-CreERT mice compared with littermate controls (Fig. 2*L*), which, taken together with ITT data, may suggest a mild increase in insulin sensitivity.

MIP-CreERT Mice on Chow Had Increased Total Pancreatic Insulin Content and Islet Density but Normal In Vitro Glucose-Stimulated Insulin Secretion

On the chow diet, there were no significant differences in general islet architecture (insulin+ and glucagon+ cell distribution) or percentage of glucagon+ cells between WT and MIP-CreERT mice (Fig. 3*A*–*C*). However, there were trends toward increased basal glucose-stimulated insulin secretion (GSIS) and oleate potentiation of insulin secretion in primary islets and increased islet insulin content that did not reach statistical significance (Fig. 3*D* and *E*). Total pancreatic insulin content in MIP-CreERT mice was on average ~27% higher than in WT controls (Fig. 3*F*). When plotted by size, islet numbers were significantly increased in a few size ranges

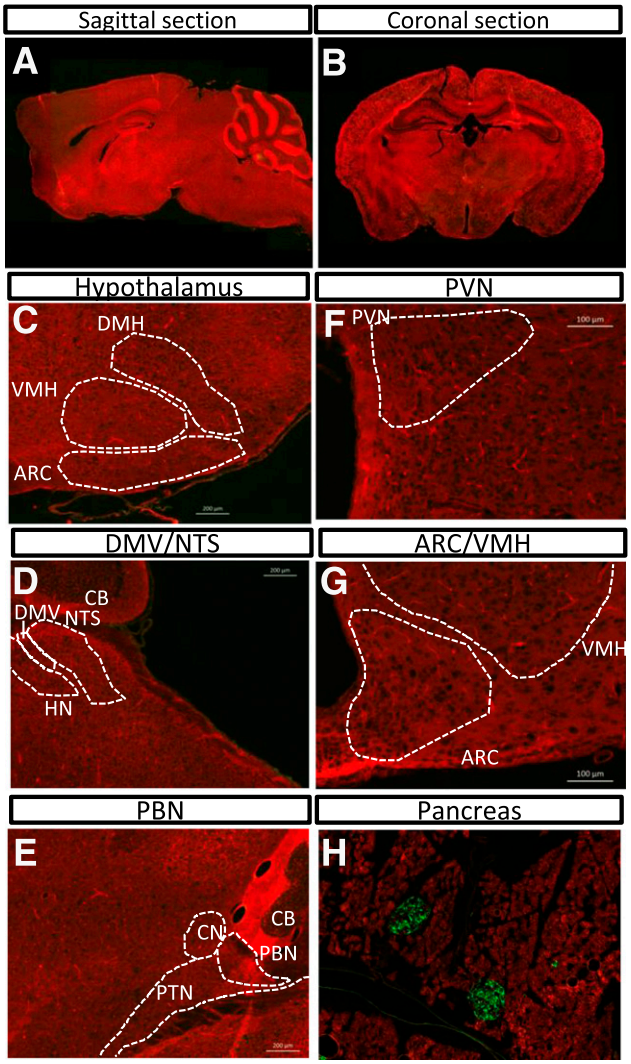

Figure 1—Cre-mediated recombination is not detected in the brain of MIP-CreERT mice. Sagittal (*A*, *C*, *D*, and *E*) and coronal (*B*, *F*, and *G*) brain sections of 13-week-old mT/mG:MIP-CreERT mice gavaged with tamoxifen (representative of $n = 2$). Higher-magnification images of the hypothalamus on coronal sections at the level of the paraventricular nucleus (PVN) and ventrally at the level of the ARC and VMH and dorsomedial hypothalamus (DMH) (representative of $n = 3$). Pancreatic sections (*H*) from same mice. Dorsal motor nucleus of the vagus (DMV), parabrachial nucleus of the pons (PBN), nucleus tractus solitarius (NTS), hypoglossal nucleus (HN), cerebellum (CB), cuneiform nucleus (CN), and pedunculopontine tegmental nucleus (PTN).

(Fig. 3*G*) and there was a strong trend toward increased overall islet mass and density (Fig. 3*H* and *I*). Taken together, these results imply that MIP-CreERT mice had more pancreatic islets containing more insulin, leading to significantly higher pancreatic insulin content. However, this was not accompanied by a corresponding increase in insulin released, in agreement with in vivo data.

MIP-CreERT Mice Were Protected Against STZ-Induced Hyperglycemia Independent of Cre-Recombinase Activity

MIP-CreERT mice were also challenged with a combination of HFHS diet and STZ, a model of type 2 diabetes (21). Compared with controls, MIP-CreERT mice were significantly protected against resulting hyperglycemia (Fig. 4*A*) while maintaining similar weights (Fig. 4*B*). Of note, tamoxifen administration was not required for their resistance to HFHS/STZ (Fig. 4*C*) and a similar phenotype was observed in MIP-CreERT mice on an FVB background (Fig. 4*D*). In contrast, the Pdx1-Cre^{Tuv} mouse line, which also expresses Cre-recombinase in β-cells, remained sensitive to HFHS/STZ-induced hyperglycemia compared with littermates (Fig. 4*E*). MIP-CreERT mice were also significantly resistant to hyperglycemia induced by a single high dose of STZ (Fig. 4*F*), which was not the case for RIP-Cre^{Herr} mice (Fig. 4*G*). Taken together, these data suggest that expression and/or activity of Cre-recombinase in β-cells alone cannot explain the significant resistance to STZ-induced hyperglycemia in MIP-CreERT mice. HFHS/STZ-treated MIP-CreERT mice also had significantly improved glucose tolerance, yet unexpectedly, no differences in fasted or refed serum insulin levels (Fig. 4*H* and *I*), suggesting a non–insulin-dependent mechanism underlying normoglycemia.

hGH Protein Was Expressed From the MIP-CreERT Transgene

It was recently demonstrated that hGH can be translated from the hGH mini-gene commonly used as a polyadenylation sequence in transgene constructs and suggested that this ectopic hGH impairs islet function (3). For determination of whether the MIP-CreERT transgene contained the entire hGH mini-gene, PCR amplicons from genomic DNA were produced using primers flanking the coding sequence of hGH (Fig. 5*A*) or the 3′ region of the Ins1 promoter to the 3′-UTR of the hGH mini-gene. The sequence contained the entire hGH gene from start to stop codon (Supplementary Fig. 1) (GenBank accession no. KR632635). In silico analysis of the sequenced MIP-CreERT transgene did not identify any previously characterized internal ribosomal entry sites or cryptic promoters (Supplementary Fig. 1). Various spliced variants of the hGH gene were detected in MIP-CreERT islet cDNA, with the major band corresponding to a fully spliced hGH transcript (Fig. 5*B*). As expected, the hGH cassette was amplified from CD11b-Cre mice (26) but not from Pdx1-Cre^{Tuv} (18) genomic DNA (Fig. 5*A* and *B*) or cDNA of Pdx1-Cre^{Tuv} or RIP-Cre^{Herr} (10) mice (not shown). MIP-CreERT primary islets expressed high levels of hGH protein (Fig. 5*C*) and mRNA (Fig. 5*D*) that were undetectable in controls. mRNA expression of tryptophan hydroxylase 1 (*Tph1*) and *Tph2*, enzymes that produce serotonin and are surrogate markers of hGH signaling via the prolactin receptor (3,27), were also increased in MIP-CreERT (Fig. 5*E*) but not RIP-Cre^{Herr} islets (not shown). This was consistent with a high level of serotonin immunoreactivity in MIP-CreERT islets (Fig. 5*F*), although the intensity of the signal varied greatly throughout the β-cell population.

Low-level Ins1 promoter activity is detected in the brain (15,22), suggesting that hGH may also be expressed in regions of MIP-CreERT CNS. Although we detected no Cre-mediated gene recombination in the CNS, transgene

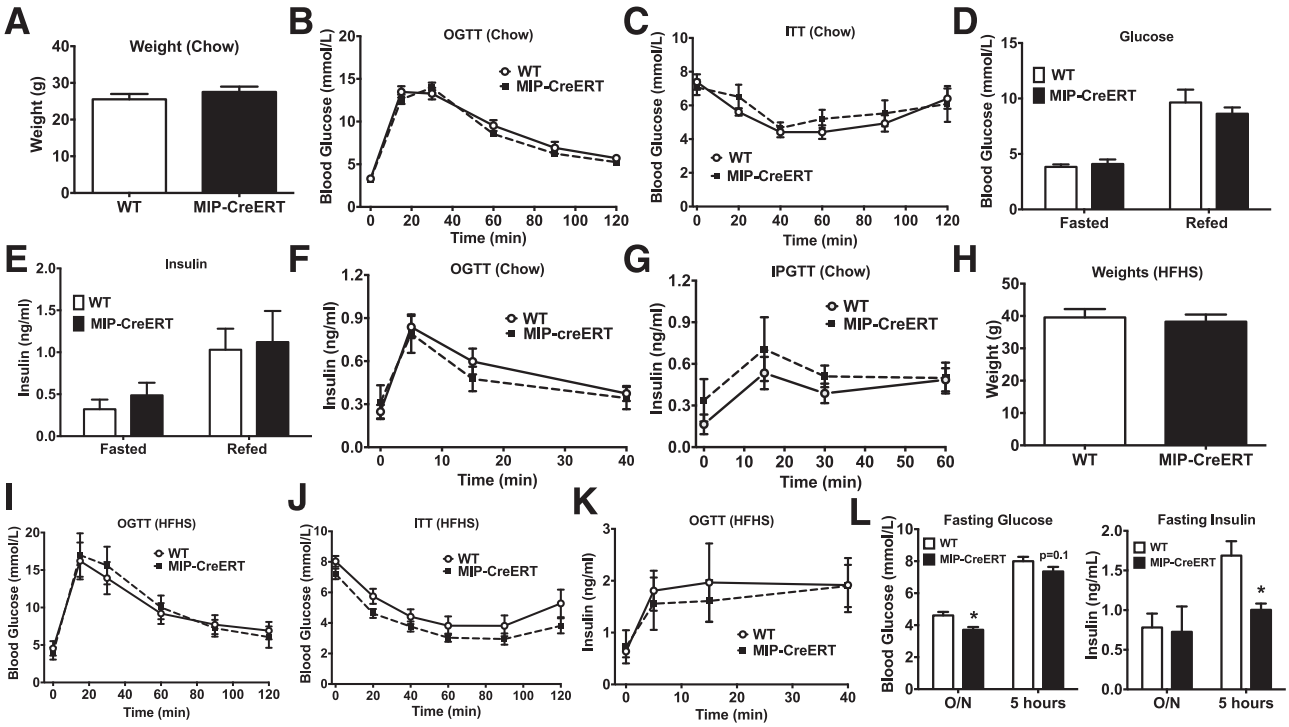

Figure 2—MIP-CreERT mice on a chow or HFHS diet have normal glucose and insulin tolerance and GSIS. *A*: Weights of 13-week-old mice on chow diet (n = 9–10). *B*: OGTT in 16-h-fasted mice (n = 9–10). *C*: ITT of 4-h-fasted mice (n = 8). The 16-h-fasted and 2-h-refed blood glucose (*D*) and insulin levels (*E*) in chow-fed mice (n = 7). Serum insulin levels after OGTT (*F*) and intraperitoneal glucose tolerance test (IPGTT) (*G*) in 16-h-fasted mice (n = 7–10). For mice on chow, all tests were performed at 14–17 weeks of age. *H*: Weights of mice on HFHS-diet for 20 weeks (n = 9). *I*: Blood glucose after an OGTT in 16-h-fasted mice. *J*: ITT in 4-h-fasted mice. *K*: Serum insulin after an OGTT in 16-h-fasted mice. *L*: Fasting blood glucose and serum insulin levels in mice fasted for 16 h (O/N) or for 5 h in HFHS-fed mice 28–30 weeks of age. $^*P < 0.05$.

expression in this inducible system is not controlled by tamoxifen and hGH activity does not depend on drug-induced nuclear localization, as is the case for the Cre-recombinase. mRNA encoding Cre-recombinase and hGH were detected in both primary islets and microdissected arcuate nucleus (ARC) and ventromedial hypothalamus (VMH); however, levels in islets were 100- to 200-fold higher (Fig. 5*G* and *H*). Unlike in islets, there were no significant increases in *Tph1* or *Tph2* mRNA in ARC or VMH of MIP-CreERT mice compared with controls (Fig. 5*I*), suggesting that levels of hGH in hypothalamic nuclei may not be high enough to elicit physiological effects.

HFHS/STZ Revealed Potential MIP-CreERT Transgene-Dependent Paracrine Effects on α-Cell Number and Function

As MIP-CreERT mice did not develop severe hyperglycemia in response to STZ, we first hypothesized that they were resistant to the cytotoxic effects of STZ. In contrast to findings reported in Pdx1-Cre^{Late} mice (3), we saw no significant difference in *Glut2* expression in MIP-CreERT islets, suggesting sufficient entry of the toxin into β-cells (Fig. 6*A*). In addition, islet mass and density were reduced to comparable levels in both genotypes (mass −78.4% ± 2.5% for WT and −72.7% ± 5.7% in MIP-CreERT; density −58.8% ± 4.8% for WT and −49.7% ± 7.4% in MIP-CreERT) after HFHS/STZ, consistent with the action of STZ to induce β-cell death (Fig. 6*B* and *C*). There was an overall increase in the total number of islets in the MIP-CreERT pancreata, with some fractions reaching statistical significance (Fig. 6*D*). However, when expressed as a percentage of total islet number (not shown), the proportion of islets in each size was identical, suggesting the increase in islet mass resulted from having more, not larger, islets. There were no differences in the incidence of Ki67+ islet cells 30 days after HFHS/STZ (Fig. 6*E*), and the proportion of these costaining positive for insulin was also similar (not shown), indicating that rates of islet/β-cell proliferation were equivalent.

Similar to mice on chow, there was a significant increase in total pancreatic insulin content in MIP-CreERT mice on HFHS/STZ (Fig. 6*F*). Furthermore, MIP-CreERT mice subject to HFHS/STZ had fewer α-cells and lower levels of serum glucagon after fasting (Fig. 6*G* and *H*), offering a plausible explanation for the significantly reduced glycemia despite similar circulating insulin (Fig. 4*I*). Consistent with this possibility, glucagon secretion from WT islets in response to low glucose (1 mmol/L) was modestly reduced after addition of serotonin (5-hydroxytryptamine [5-HT]) and rebounded upon serotonin washout, while 5-HT had

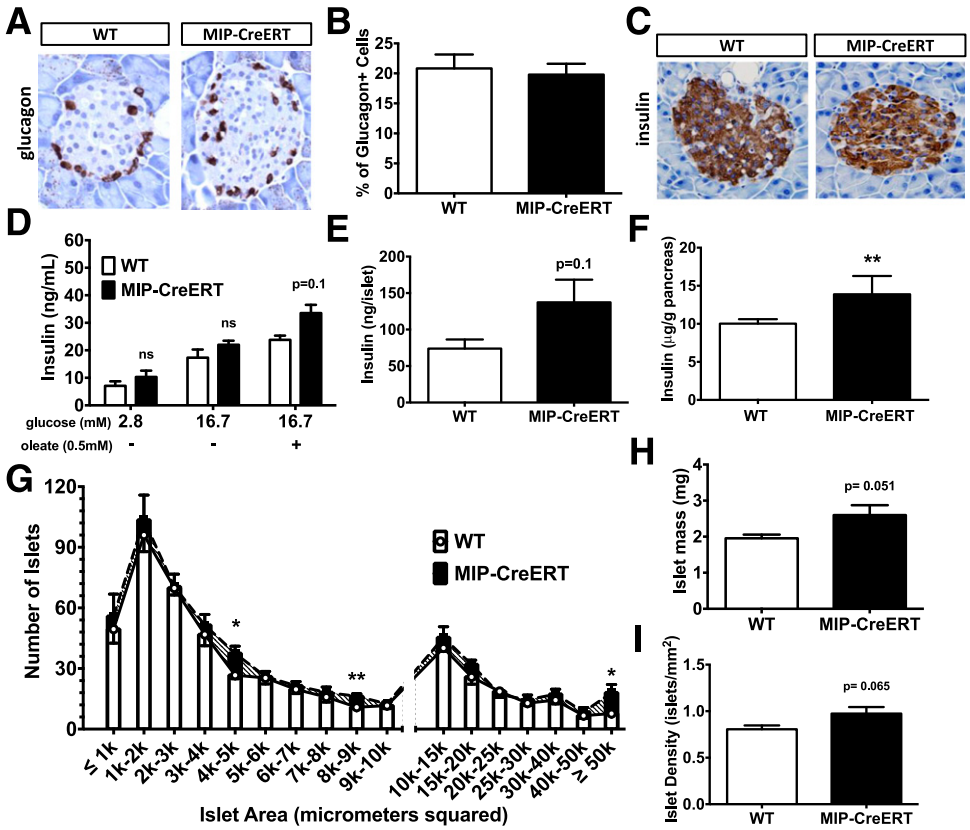

Figure 3—MIP-CreERT mice have normal islet architecture and nutrient-stimulated insulin secretion in vitro, yet increased total pancreatic and islet insulin content. *A*: Representative images of glucagon staining (n = 7). *B*: Quantification of glucagon-stained positive cells (n = 7). *C*: Representative images of insulin staining (n = 6). Static GSIS (*D*) and islet insulin content (*E*) (n = 4) in primary islets of mice on a C57BL/6N background. *F*: Total pancreatic insulin content (n = 8–9). *G*: Distribution of islet area expressed as number of islets (n = 6). *H*: Islet mass (n = 6). *I*: Islet density (number of islets per mm^2) (n = 6). *P < 0.05, **P < 0.01.

no inhibitory effect on secretion in response to arginine (Fig. 6*I*). These data support our hypothesis that high levels of local islet serotonin may have a paracrine effect on α-cell function.

DISCUSSION

Our study demonstrates that under many physiological circumstances, the MIP-CreERT transgenic mouse line can be a useful tool for in vivo and in vitro studies investigating biological effects of β-cell–specific gene ablation. We report high specificity of tamoxifen-inducible Cre-recombinase activity to islets, with no detectable recombinase activity in multiple regions of the CNS. The MIP-CreERT transgene did not have significant effects on glucose homeostasis, insulin secretion, or insulin sensitivity in chow-fed mice, despite increased pancreatic insulin content and transgene-driven expression of hGH. However, use of these mice in certain contexts should be pursued with caution, as the mice exhibit abnormalities in fasting glycemia and insulin upon high-fat feeding and significant resistance to HFHS/STZ-induced hyperglycemia.

Increased insulin content and islet mass and resistance to STZ-induced hyperglycemia in MIP-CreERT mice resemble aspects of a phenotype recently ascribed to Pdx1-Cre^{Late} mice (3), and these similarities are likely attributable to ectopic expression of hGH from the transgene cassette—not Cre-recombinase. This is consistent with STZ resistance in Pdx1-Cre^{Late} (3) and MIP-CreERT mice but not Rip-Cre^{Herr} or Pdx1-Cre^{Tuv} mice, whose transgenes do not contain the hGH mini-gene. hGH can bind and activate the mouse growth hormone receptor, which can have significant physiological consequences including somatotrophic and lactotrophic effects (28). Consistent with our observations in MIP-CreERT mice, a two- to threefold increase in circulating growth hormone in mice has been shown to increase islet insulin content (29), and transgenic mice expressing hGH under the metallothionein promoter have increased islet size and number (30). Furthermore, culture of β-cells with recombinant hGH increases β-cell replication, insulin secretion, and insulin biosynthesis (31).

In β-cells, hGH can signal via the prolactin receptor (3). Prolactin increases β-cell proliferation and neogenesis during pregnancy and improves β-cell function (GSIS) at physiological doses; however, pharmacological levels can impair insulin secretion (32). Consistent with the former scenario, we observed increased insulin content and a modest

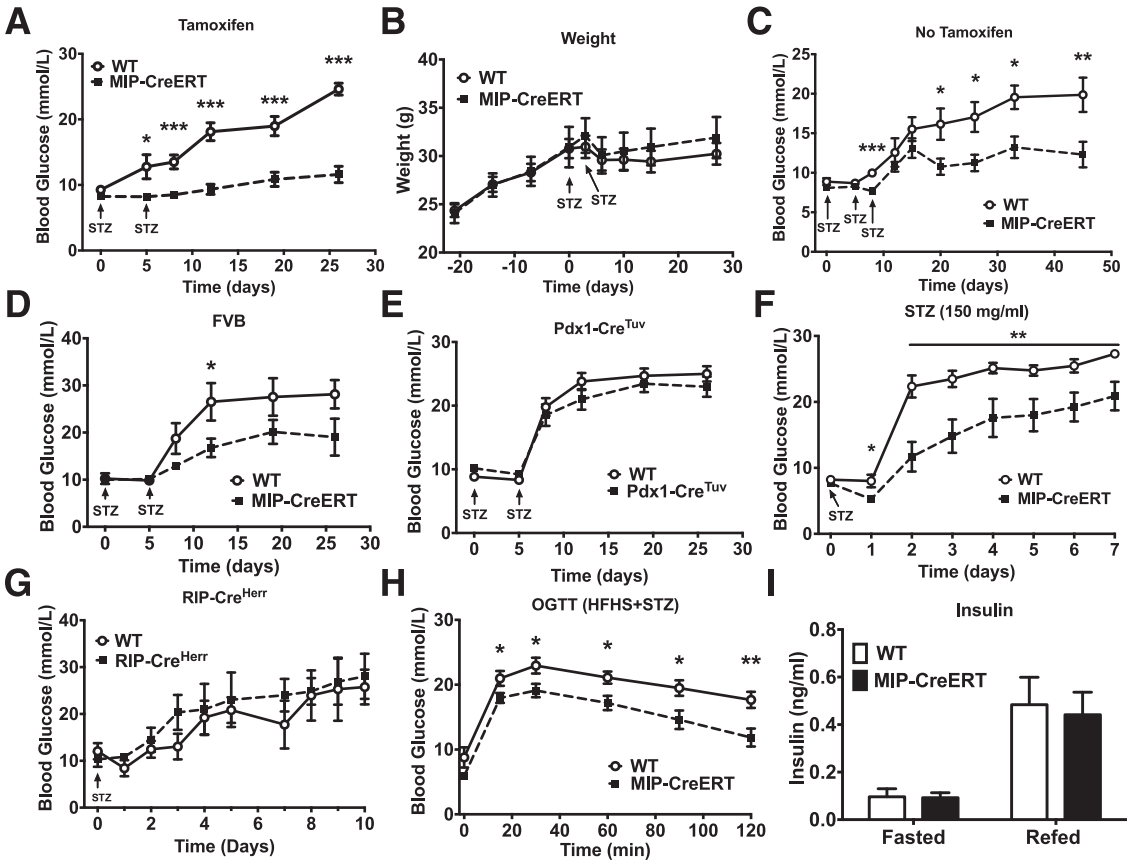

Figure 4—MIP-CreERT mice are protected against STZ-induced hyperglycemia. *A*: Random fed blood glucose levels of MIP-CreERT mice (C57BL/6J) fed HFHS diet and administered two injections of STZ (n = 8). *B*: Weight gain from mice in *A*. *C*: Random blood glucose levels of MIP-CreERT mice (C57BL/6J) not gavaged with tamoxifen, fed HFHS diet, and administered three injections of STZ (n = 7). *D*: Random blood glucose levels of MIP-CreERT mice (FVB/n) fed HFHS diet and administered two injections of STZ (n = 7). *E*: Random blood glucose levels of Pdx1-Cre^{Tuv} mice (C57BL/6J/N) fed HFHS diet and administered two injections of STZ (n = 12–14). *F*: Random blood glucose levels of MIP-CreERT mice (C57BL/6J) fed chow diet and after administration of high-dose STZ by single injection (n = 8–11). *G*: Random blood glucose levels of RIP-Cre^{Herr} (C57BL/6J) fed a chow diet and administered a single high dose of STZ (n = 4). *H*: OGTT of tamoxifen-treated MIP-CreERT mice 1 week after second STZ injection. *I*: Fasted and 2-h refed insulin levels in 16-h-fasted MIP-CreERT mice from (*C*) 28 days following the third STZ injection (n = 7). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

trend toward enhanced GSIS in MIP-CreERT islets. In light of described effects of growth and prolactin hormones on β-cells, increased islet neogenesis in MIP-CreERT mice is probable and consistent with the higher islet numbers and total mass. Increased islet numbers were more pronounced 30 days post-HFHS/STZ and not accompanied by higher rates of islet cell proliferation; however, our data cannot rule out potential contributing effects of hGH on β-cell proliferation that may occur at other times during treatment and/or development. Given the role of the prolactin receptor in Pdx1-Cre^{Late} STZ resistance (3) and our data demonstrating that mice expressing Cre-recombinase without hGH remain sensitive to STZ, we predict that the effects on glucagon secretion, α-cell number, islet number, and insulin content in MIP-CreERT mice are most likely the result of the ectopic hGH expression. However, it remains possible that they occur independently due to other factors specific for this mouse, such as positional effects of transgene insertion.

Interestingly, we did not observe impairment in glucose tolerance or GSIS, as was reported for Pdx1-Cre^{Late} mice (3), supporting previous studies using MIP-CreERT mice on various genetic backgrounds (16,33–35). This difference may be due to transgene-specific differences in the level of hGH or secretion or other factors independent of hGH and serotonin action. Thus, it remains unclear whether ectopic hGH in β-cells is the underlying cause of cellular and metabolic abnormalities (including impaired β-cell function) reported for other Cre-recombinase transgenic lines (2–4).

Our high-resolution imaging using a definitive reporter (red/green) provides strong evidence that MIP-CreERT mice have no Cre-mediated recombination in the CNS in vivo, extending previous observations (6,23,25,36) and supporting the argument that inducible, Ins1/MIP promoter-driven Cre-recombinase expression is currently the best approach for β-cell–specific gene ablation (5). Nonetheless, we detected, albeit at very low levels compared with islets, transcripts containing both Cre-recombinase and hGH mRNA in the VMH and ARC. Cre-recombinase activity, in this system, depends on sufficient levels of recombinase

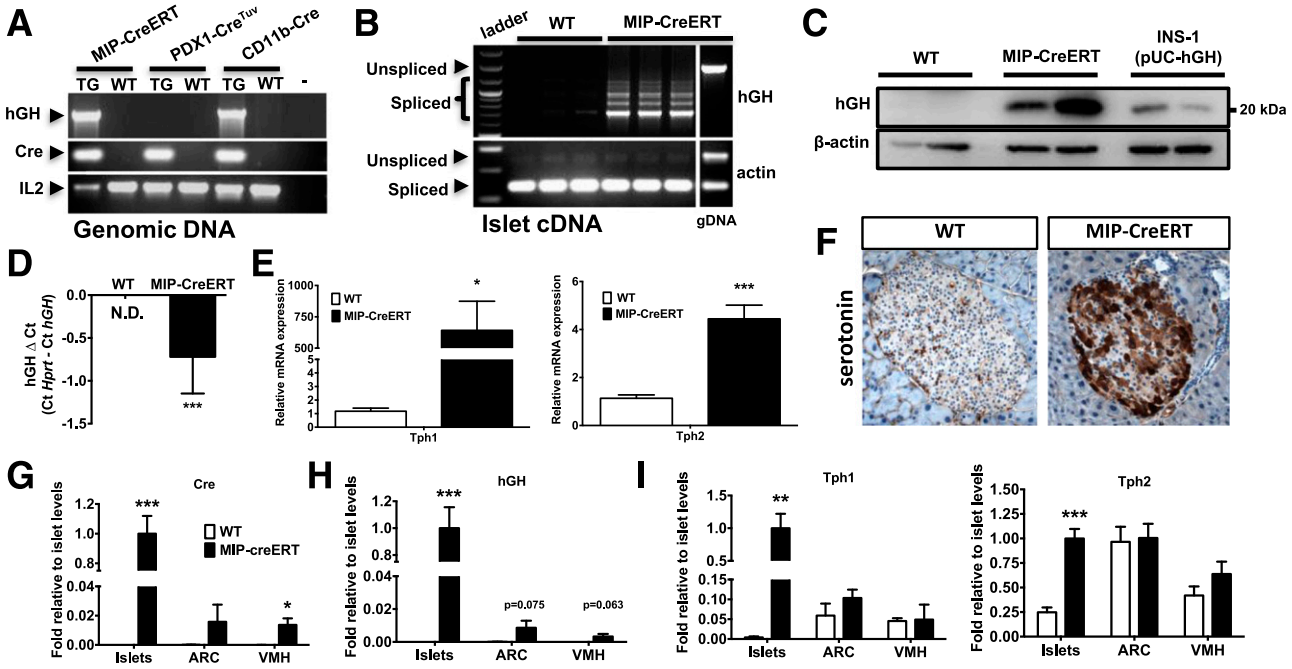

Figure 5— MIP-CreERT mice express high levels of hGH hormone in islets and low levels in brain. *A*: Full hGH coding region amplified from genomic tail DNA. Interleukin-2 primers were used as an internal control. *B*: Spliced cDNA transcripts detected in islet RNA. *C*: Western blot for hGH from isolated islets and from INS-1 cells transfected with a plasmid encoding hGH cDNA (pUC-hGH) as a positive control. *D* and *E*: qPCR of primary islets (n = 9–10 mice). *F*: Serotonin immunostaining in islets from chow-fed mice (n = 4). *G–I*: qPCR in islets compared with dissected nuclei of the ARC and VMH (n = 6 mice). N.D., cycle threshold value was not determined. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression and local tamoxifen to drive nuclear translocation and efficient excision of accessible LoxP loci. Thus, our data suggest that while not sufficient to mediate gene ablation, the Ins1/MIP promoter can still have low or leaky activity in the CNS, which may be of concern if used to drive expression of other biologically active proteins. However, unlike islets, we did not see significant induction of *Tph1/2* gene expression, suggesting (at least using this surrogate marker of hGH activity) that transcripts generated in the brain may not be expressed at meaningful levels to have biologically relevant consequences. However, since Pdx1 and RIP promoters can drive significant transgene expression in the CNS (6), it is possible that the disruption of whole-body glucose homeostasis noted for Pdx1-Cre^{Late} (3) and certain RIP-Cre mouse lines (2) may, at least in part, be due to hGH expression in the brain. Newly generated knock-in mice (25) and transgenic (23) mice with constitutive or conditional Cre-recombinase expression driven by the Ins1 promoter are a promising alternative to the MIP-CreERT mouse to avoid potentially confounding effects of hGH expression on endocrine cell mass. However, efficiency of gene ablation in the β-cell population in these new conditional lines appears lower than achieved with the MIP-CreERT line in our hands. This may be due to differences in drug administration and dosing that will be revealed after further molecular and physiological characterization.

In contrast to the study of Brouwers et al. (3), MIP-CreERT mice did not have decreased *Glut2* gene expression, implying that resistance to STZ-induced hyperglycemia was not mediated by inability of STZ to enter β-cells. Instead, we report differences in α-cell mass and glucagon secretion that may explain lower levels of glycemia in MIP-CreERT mice after HFHS/STZ. Ablation of glucagon receptor signaling in mice prevents STZ-induced hyperglycemia, despite equivalent reductions in β-cell mass and circulating insulin (37). We propose that the high levels of serotonin, which is secreted along with insulin (38), inhibit glucagon secretion from neighboring α-cells (39), preventing development of hyperglucagonemia and possibly affecting α-cell number. This hypothesis is supported by our perfusion data in islets, as well as previous demonstration that serotonin antagonists increase circulating glucagon concentrations in men (40).

It still remains a mystery how the hGH protein is translated from these transgenic constructs, highlighting that we have yet to fully grasp or predict all potential molecular and physiological changes caused by manipulation of the genome. Our findings highlight the importance of using appropriate controls, including mice expressing only the Cre-transgene. In conclusion, MIP-CreERT mice and other similar β-cell–specific lines, like most research tools, possess both strengths and caveats, yet remain an invaluable resource to dissect many aspects of β-cell biology when potentially confounding variables

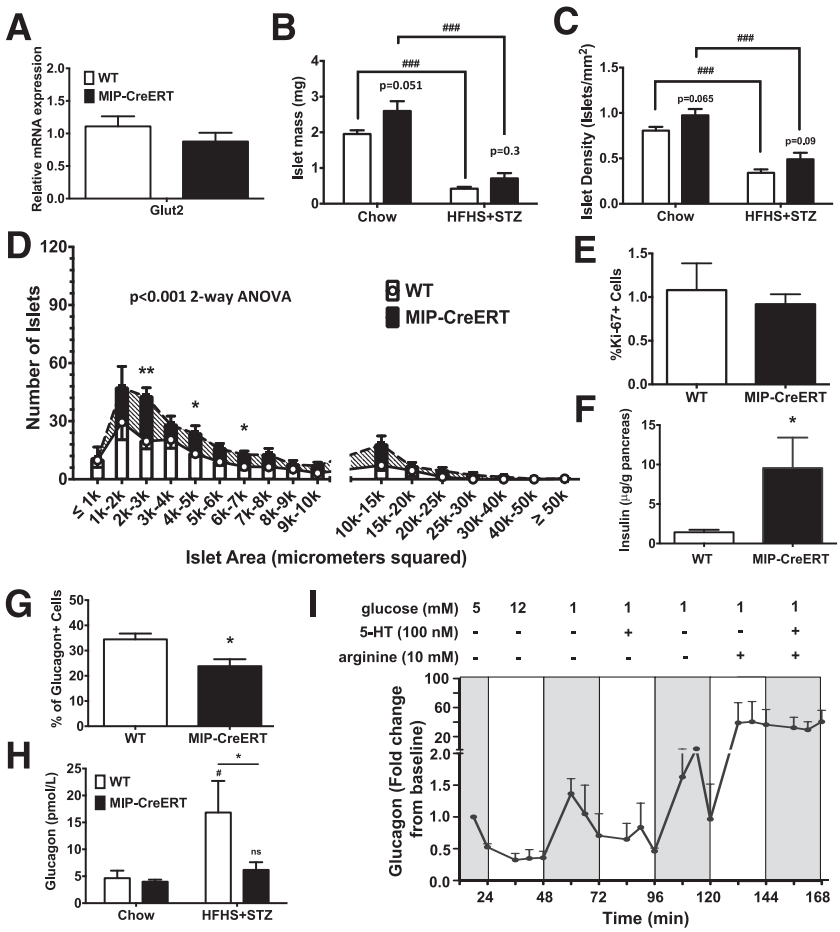

Figure 6—MIP-CreERT mice had similarly decreased islet mass, yet had decreased α-cell number and function after HFHS/STZ. *A*: qPCR analysis of *Glut2* from islets of mice on a chow diet (n = 10). Islet mass (*B*) or density (*C*) in chow-fed mice or 30 days after HFHS/STZ (n = 6). *D*: Distribution of islet size by number in HFHS/STZ mice (n = 6). *E*: Ki67+ cells in islets of mice 30 days after HFHS/STZ. Total pancreatic insulin content (*F*) and percentage of glucagon-positive cells per islet (*G*) in HFHS/STZ mice (n = 5–7). *H*: Fasted plasma glucagon from MIP-CreERT on chow or HFHS/STZ (n = 5–7). $^*P < 0.05$ comparing genotype on similar diet. $\#P < 0.05$ comparing diet effect in similar genotype. *I*: Glucagon secretory responses to glucose, serotonin (5-HT) (100 nmol/L), and arginine (10 mmol/L) during perifusion of WT mouse islets (n = 3). ns, not significant. $^*P < 0.05$ and $^{**}P < 0.01$, for comparisons between genotype. $\#\#\#P < 0.001$, comparisons of diet effect.

are considered within the context of appropriate control experiments.

Acknowledgments. The authors thank Dr. Louis H. Philipson for the MIP-CreERT^{1Lphi} mice. The pUC:hGH plasmid and the parental INS-1 cell line were generous gifts from Drs. Jacques Drouin (Institut de recherches cliniques de Montreal) and Chris Newgard. The authors give special thanks to Marie-Claude Lavallée, Courtney Reeks, Alexandra Abboud, Noura Kabbara, Naveen Khan, and Edwige Marcinkiewicz for technical assistance and Dawn B. Davis for helpful discussion and comments.

Funding. This study was supported by operating funds to M.E.K. from the National Institutes of Health (NIH) (R01-DK-102598) and American Diabetes Association (114BS115); to D.J.D. from the Canadian Institutes of Health Research (CIHR) (MOP 136942); to R.A.S. from CIHR (MOP111186); to V.P. from CIHR (MOP86454); to M.F. from CIHR (MOP133652), the J.A. DeSève Foundation, and Diabète Québec; to T.A. from CIHR (MOP115042); and to J.L.E. from the Canadian Diabetes Association (CDA) (OG3123843JE), Fonds de recherche du Québec - Santé (FRQS) (26721), Canadian Foundation for Innovation, and the J.A. DeSève Foundation. K.B. was supported by a Diabète Québec studentship. J.L. was supported by a CDA fellowship. J.C.N. was supported by a training grant (University of Wisconsin-Madison Institute on Aging: NIH T32 AG000213). D.J.D. holds a Canada Research Chair in Regulatory Peptides and the Banting and Best Diabetes Centre Novo Nordisk Chair in Incretin Biology. V.P. holds the Canada Research Chair in Diabetes and Pancreatic Beta-Cell Function. M.F. holds the Canada Research Chair in Bone and Energy Metabolism. CIHR and FRQS provided salary awards to T.A. and J.L.E.

Duality of Interest. No potential conflicts of interest relevant to this study were reported.

Author Contributions. D.O., N.J., and J.L.E. conceived and designed the study and wrote the manuscript. D.O., N.J., L.B., J.E.C., K.B., J.L., G.P., V.B., J.C.N., H.K.B., R.J.F, C.M., S.S., M.E.K., D.J.D., R.A.S., V.P., M.F., T.A., and J.L.E. performed experiments and analyzed data. D.O., N.J., M.E.K., D.J.D., R.A.S., V.P., M.F., T.A., and J.L.E. reviewed and revised the manuscript. J.L.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 75th Scientific Sessions of the American Diabetes Association, Boston, MA, 5–9 June 2015.

References

1. Harno E, Cottrell EC, White A. Metabolic pitfalls of CNS Cre-based technology. Cell Metab 2013;18:21–28
2. Lee JY, Ristow M, Lin X, White MF, Magnuson MA, Hennighausen L. RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. J Biol Chem 2006;281:2649–2653
3. Brouwers B, de Faudeur G, Osipovich AB, et al. Impaired islet function in commonly used transgenic mouse lines due to human growth hormone minigene expression. Cell Metab 2014;20:979–990
4. Teitelman G, Kedees M. Mouse insulin cells expressing an inducible RIPCre transgene are functionally impaired. J Biol Chem 2015;290:3647–3653
5. Magnuson MA, Osipovich AB. Pancreas-specific Cre driver lines and considerations for their prudent use. Cell Metab 2013;18:9–20
6. Wicksteed B, Brissova M, Yan W, et al. Conditional gene targeting in mouse pancreatic ß-Cells: analysis of ectopic Cre transgene expression in the brain. Diabetes 2010;59:3090–3098
7. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447–2457
8. Zhang H, Fujitani Y, Wright CV, Gannon M. Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. Genesis 2005;42:210–217
9. Ray MK, Fagan SP, Moldovan S, DeMayo FJ, Brunicardi FC. Development of a transgenic mouse model using rat insulin promoter to drive the expression of CRE recombinase in a tissue-specific manner. Int J Pancreatol 1999;25:157–163
10. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 2000;127:2317–2322
11. Postic C, Shiota M, Niswender KD, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J Biol Chem 1999;274:305–315
12. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41–46
13. Crabtree JS, Scacheri PC, Ward JM, et al. Of mice and MEN1: Insulinomas in a conditional mouse knockout. Mol Cell Biol 2003;23:6075–6085
14. Leiter EH, Reifsnyder P, Driver J, et al. Unexpected functional consequences of xenogeneic transgene expression in beta-cells of NOD mice. Diabetes Obes Metab 2007;9(Suppl 2):14–22
15. Mehran AE, Templeman NM, Brigidi GS, et al. Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metab 2012;16:723–737
16. Tamarina NA, Roe MW, Philipson L. Characterization of mice expressing Ins1 gene promoter driven CreERT recombinase for conditional gene deletion in pancreatic β-cells. Islets 2014;6:e27685
17. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis 2007;45:593–605
18. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4:437–450
19. Ferdaoussi M, Bergeron V, Zarrouki B, et al. G protein-coupled receptor (GPR)40-dependent potentiation of insulin secretion in mouse islets is mediated by protein kinase D1. Diabetologia 2012;55:2682–2692
20. Briaud I, Harmon JS, Kelpe CL, Segu VB, Poitout V. Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids. Diabetes 2001;50:315–321
21. Mu J, Woods J, Zhou YP, et al. Chronic inhibition of dipeptidyl peptidase-4 with a sitagliptin analog preserves pancreatic beta-cell mass and function in a rodent model of type 2 diabetes. Diabetes 2006;55:1695–1704
22. Wang ZC, Wheeler MB, Belsham DD. Isolation and immortalization of MIP-GFP neurons from the hypothalamus. Endocrinology 2014;155:2314–2319
23. Cheng Y, Su Y, Shan A, et al. Generation and Characterization of Transgenic Mice Expressing Mouse Ins1 Promoter for Pancreatic β-Cell-Specific Gene Overexpression and Knockout. Endocrinology 2015;156:2724–2731
24. Hasegawa Y, Daitoku Y, Mizuno S, et al. Generation and characterization of Ins1-cre-driver C57BL/6N for exclusive pancreatic beta cell-specific Cre-loxP recombination. Exp Anim 2014;63:183–191
25. Thorens B, Tarussio D, Maestro MA, Rovira M, Heikkilä E, Ferrer J. Ins1(Cre) knock-in mice for beta cell-specific gene recombination. Diabetologia 2015;58:558–565
26. Ferron M, Vacher J. Targeted expression of Cre recombinase in macrophages and osteoclasts in transgenic mice. Genesis 2005;41:138–145
27. Matsuda M, Imaoka T, Vomachka AJ, et al. Serotonin regulates mammary gland development via an autocrine-paracrine loop. Dev Cell 2004;6:193–203
28. Kopchick JJ, Bellush LL, Coschigano KT. Transgenic models of growth hormone action. Annu Rev Nutr 1999;19:437–461
29. Cordoba-Chacon J, Majumdar N, Pokala NK, Gahete MD, Kineman RD. Islet insulin content and release are increased in male mice with elevated endogenous GH and IGF-I, without evidence of systemic insulin resistance or alterations in β-cell mass. Growth Horm IGF Res. 7 April 2015 [Epub ahead of print]. DOI: 10.1016/j.ghir.2015.04.002
30. Parsons JA, Bartke A, Sorenson RL. Number and size of islets of Langerhans in pregnant, human growth hormone-expressing transgenic, and pituitary dwarf mice: effect of lactogenic hormones. Endocrinology 1995;136:2013–2021
31. Nielsen JH, Linde S, Welinder BS, Billestrup N, Madsen OD. Growth hormone is a growth factor for the differentiated pancreatic beta-cell. Mol Endocrinol 1989;3:165–173
32. Park S, Kim S, Daily JW, Kim SH. Serum prolactin concentrations determine whether they improve or impair β-cell function and insulin sensitivity in diabetic rats. Diabetes Metab Res Rev 2011;27:564–574
33. Zhao S, Mugabo Y, Iglesias J, et al. α/β-Hydrolase domain-6-accessible monoacylglycerol controls glucose-stimulated insulin secretion. Cell Metab 2014;19:993–1007
34. Kaihara KA, Dickson LM, Jacobson DA, et al. β-Cell-specific protein kinase A activation enhances the efficiency of glucose control by increasing acute-phase insulin secretion. Diabetes 2013;62:1527–1536
35. Smith EP, An Z, Wagner C, et al. The role of β cell glucagon-like peptide-1 signaling in glucose regulation and response to diabetes drugs. Cell Metab 2014;19:1050–1057
36. Smith PA, Proks P, Ashcroft FM. Quantal analysis of 5-hydroxytryptamine release from mouse pancreatic beta-cells. J Physiol 1999;521:651–664
37. Lee Y, Berglund ED, Wang MY, et al. Metabolic manifestations of insulin deficiency do not occur without glucagon action. Proc Natl Acad Sci U S A 2012;109:14972–14976
38. Gylfe E. Association between 5-hydroxytryptamine release and insulin secretion. J Endocrinol 1978;78:239–248
39. Adeghate E, Ponery AS, Pallot D, Parvez SH, Singh J. Distribution of serotonin and its effect on insulin and glucagon secretion in normal and diabetic pancreatic tissues in rat. Neuroendocrinol Lett 1999;20:315–322
40. Marco J, Hedo JA, Martinell J, Calle C, Villanueva ML. Potentiation of glucagon secretion by serotonin antagonists in man. J Clin Endocrinol Metab 1976;42:215–221

SUPPLEMENTARY DATA

Supplementary Table 1. Mouse strains, MGI name and genetic background.

	MGI name	C56Bl/6J	C56Bl/6N	129	CD1	FVB/n
mT/mG:MIP-CreERT	Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo	56.25%		31.25%	12.5%	
MIP-CreERT (6J)	Tg(Ins1-cre/ERT)1Lph	98.50%	0.75%	0.75%		
MIP-CreERT (FVB)	Tg(Ins1-cre/ERT)1Lph	6.2%				93.8%
MIP-CreERT (6N)	Tg(Ins1-cre/ERT)1Lph		100%			
Pdx1-Cre^{Tuv}	Tg(Ipf1-cre)1Tuv	50%	50%			
RIP-Cre^{Herr}	Tg(Ins2-cre)23Herr	100%				

SUPPLEMENTARY DATA

Supplementary Table 2. Primers used for qPCR, PCR and transgene sequencing designed in the 5' to 3' direction.

Gene	Forward Primer	Reverse Primer
qPCR		
Cre	GCGGTCTGGCAGTAAAAACTATC	GTGAAACAGCATTGCTGTCACTT
Glut2	AGGTCCAATCCCTTGGTTCATGGT	AATGTACTGGAAGCAGAGGGCGAT
hGH	CCAGGAGTTTGAAGAAGCC	GGAGGTCATAGACGTTGCTGT
Tph1	ACTGCGAAGGAAGACGTTATG	CGTCAAGTTCGGATCCATACAA
Tph2	CGTATGGAGCAGGGTTACTTTC	CGTCCTGAAAGGTGGTGATTAG
HPRT	TGAAAGACTTGCTCGAGATGTCA	CACACAGAGGGCCACAATGT
PCR		
hGH	CCTAGCTGCAATGGCTACAG	GCACTGGAGTGGCAACTTCC
Cre	GCGGTCTGGCAGTAAAAACTATC	GTGAAACAGCATTGCTGTCACTT
IL-2	CTAGGCCACAGAATTGAAAGATCT	GTAGGTGGAAATTCTAGCATCATCC
β-actin	GACCTCTATGCCAACACAGT	AGTACTTGCGCTCAGGAGGA
Transgene Sequencing		
A	TAAGGGCCCAGCTATCAATGGGAA	GTGAAACAGCATTGCTGTCACTT
B	AAGTGACAGCAATGCTGTTTCAC	GATACTCACATTCAGAAGCC
C	TACGGCGCTAAGGATGACTC	AGGCCAAAAGCCAGGAGCAG
D	CCTAGCTGCAATGGCTACAG	GCACTGGAGTGGCAACTTCC

SUPPLEMENTARY DATA

Supplementary Figure 1. Sequenced MIP-CreERT transgene locus

Partial genomic sequence of the MIP-CreERT locus obtained from mouse tail DNA. PCR products amplified using primers flanking consecutive regions (Primers included in Supplementary Table 2) of the locus were sequenced and assembled. Sequence listed in 5'-3' orientation. Promoter and cDNA regions are indicated as follows: Grey: 3' region of *Ins1* (MIP) promoter and first exon of *Ins1* gene (underlined); White: intergenic sequences; Green: Cre-recombinase cDNA (START codon in red); Blue: partial mESR1 cDNA (mouse estrogen receptor, STOP codon in red): Yellow: hGH (human growth hormone) genomic sequence (START and STOP codons in red, exons underlined).

A search for IRES elements or cryptic promoters using the following resources yielded no known/predicted motifs:

http://iresite.org/
http://www.cbs.dtu.dk/services/Promoter/

ATCAATGGGAACTGTGAAACAGTCCAAGGGACATCAATATTAGGTCCCTAACAACTGCAGT
TTCCTGGGGAATGATGTGGAAAATGCTCAGCCAAAGATGAAGAAGGTCTCACCTTCTGGGA
CAATGTCCCCTGCTGGGAACTGGTTCATCAGGCCATCTGGTCCCTTATTAAGACTATAATAA
CCCTAAGACTAAGTAGATGTGTTGATGTCCAATGAGTGCTTTCTGCAGACCTAGCACCAGG
CAAGTGTTTGGAAACTGCAGCTTCAGCCCCTCTGGCCATCTGCCTACCCACCCCACCTGGA
GACCTTAATGGGCCAAACAGCAAAGTCCAGGGGGCAGAGAGGAGGTACTTTGGACTATAA
AGCTGGTGGGCATCCAGTAACCCCCAGCCCTTAGTGACTAGAGGGCCCGGATCCCTCGACG
GTATCGATAAGCTTGATATCGAATTCGGGCCCCCCCTCGAGGGGCAGAGCCGATCCTGTAC
ACTTTACTTAAAACCATTATCTGAGTGTGAAATGTCCAATTTACTGACCGTACACCAAAATT
TGCCTGCATTACCGGTCGATGCAACGAGTGATGAGGTTCGCAAGAACCTGATGGACATGTT
CAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTGCCGGTCGT
GGGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTCG
CGATTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAAACTATCCAGCAACATTTGG
GCCAGCTAAACATGCTTCATCGTCGGTCCGGGCTGCCACGACCAAGTGACAGCAATGCTGT
TTCACTGGTTATGCGGCGGATCCGAAAAGAAAACGTTGATGCCGGTGAACGTGCAAAACA
GGCTCTAGCGTTCGAACGCACTGATTTCGACCAGGTTCGTTCACTCATGGAAAATAGCGAT
CGCTGCCAGGATATACGTAATCTGGCATTTCTGGGGATTGCTTATAACACCCTGTTACGTAT
AGCCGAAATTGCCAGGATCAGGGTTAAAGATATCTCACGTACTGACGGTGGGAGAATGTTA
ATCCATATTGGCAGAACGAAAACGCTGGTTAGCACCGCAGGTGTAGAGAAGGCACTTAGC
CTGGGGGTAACTAAACTGGTCGAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGA
ATAACTACCTGTTTTGCCGGGTCAGAAAAAATGGTGTTGCCGCGCCATCTGCCACCAGCCA
GCTATCAACTCGCGCCCTGGAAGGGATTTTTGAAGCAACTCATCGATTGATTTACGGCGCT
AAGGATGACTCTGGTCAGAGATACCTGGCCTGGTCTGGACACAGTGCCCGTGTCGGAGCCG
CGCGAGATATGGCCCGCGCTGGAGATTCAATACCGGAGATCATGCAAGCTGGTGGCTGGAC
CAATGTAAATATTGTCATGAACTATATCCGTAACCTGGATAGTGAAACAGGGGCAATGGTG
CGCCTGCTGGAAGATGGGGATCAGGCTGGTGCCATGGGCGATCCACGAAATGAAATGGGT
GCTTCAGGAGACATGAGGGCTGCCAACCTTTGGCCAAGCCCTCTTGTGATTAAGCACACTA
AGAAGAATAGCCCTGCCTTGTCCTTGACAGCTGACCAGATGGTCAGTGCCTTGTTGGATGC
TGAACCGCCCATGATCTATTCTGAATATGATCCTTCTAGACCCTTCAGTGAAGCCTCAATGA
TGGGCTTATTGACCAACCTAGCAGATAGGGAGCTGGTTCATATGATCAACTGGGCAAAGAG
AGTGCCAGGGCTTTGGGGACTTATGATCAGGTCCACCTTTCTCGAGTGTGCCTGGCTGGAG
ATTCTGATGATTGGTCTCGTCTGGCGCTCCATGGAACACCCGGGGAAGCTCCTGTTTGCTCC

SUPPLEMENTARY DATA

TAACTTGCTCCTGGACAGGAATCAAGGTAAATGTGTGGAAGGCATGGTGGAGATCTTTGAC
ATGTTGCTTGCTACGTCAAGTCGGTTCCGCATGATGAACCTGCAGGGTGAAGAGTTTGTGT
GCCTCAAATCCATCATTTTGCTTAATTCCGGAGTGTACACGTTTCTGTCCAGCACCTTGAAG
TCTCTGGAAGAGAAGGACCACATCCACCGTGTCCTGGACAAGATCACAGACACTTTGATCC
ACCTGATGGCCAAAGCTGGCCTGACTCTGCAGCAGCAGCATCGCCGCCTAGCTCAGCTCCT
TCTCATTCTTTCCCATATCCGGCATATGAGTAACAAACGCATGGAGCATCTCTACAACATGA
AATGCAAGAACGTGGTACCCCTCTATGACCTGCTCCTGGAGATGTTGGATGCCCACCGCCT
TCATGCCCCAGCCAGTCGCATGGGAGTGCCCCCAGAGGAGCCCAGCCAGACCCAGCTGGC
CACCACCAGCTCCACTTCAGCACATTCCTTACAAACCTACTACATACCCCCGGAAGCAGAG
GGCTTCCCCAACACGATCTGAGAGCTCCTACTAGTGAATTCCTGCAGCCCGGGGGATCCAC
TAGTTCTAGAGCGGCCCCGGTCGAGGTCGACCACTCAGGGTCCTGTGGTCAGCTCACCTAG
CTGCAATGGCTACAGGTAAGCGCCCCTAAAATCCCTTTGGGCACAATGTGTCCCGAGGGGA
GAGGCAGCGACCTGTAGATGGGACGGGGGCACTAACCCTCAGGTTTGGGGCTTCTGAATGT
GAGTATCGCCATGTAAGCCCAGTATTTGGCCAATCTCAGAAAGCTCCTGGTCCCTGGAGGG
ATGGAGAGAGAAAAACAAACAGCTCCTGGAGCAGGGAGAGTGCTGGCCTCTTGCTCTCCG
GCTCCCTCTGTTGCCCTCTGGTTTCTCCCCAGGCTCCCGGACGTCCCTGCTCCTGGCTTTTGG
CCTGCTCTGCCTGCCCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATTCCCTTATCCAGGC
TTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTGGCCTTTGACACCTACCAG
GAGTTTGTAAGCTCTTGGGGAATGGGTGCGCATCAGGGGTGGCAGGAAGGGGTGACTTTCC
CCCGCTGGGAAATAAGAGGAGGAGACTAAGGAGCTCAGGGTTTTTCCCGAAGCGAAAATG
CAGGCAGATGAGCACACGCTGAGTGAGGTTCCCAGAAAAGTAACAATGGGAGCTGGTCTC
CAGCGTAGACCTTGGTGGGCGGTCTTTCTCCTAGGAAGAAGCCTATATCCCAAAGGAACAG
AAGTATTCATTCCTGCAGAACCCCCAGACCTCCCTCTGTTTCTCAGAGTCTATTCCGACACC
CTCCAACAGGGAGGAAACACAACAGAAATCCGTGAGTGGATGCCTTCTCCCCAGGCGGGG
ATGGGGGAGACCTGTAGTCAGAGCCCCCGGGCAGCACAGCCAATGCCCGTCCTTCCCCTGC
AGAACCTAGAGCTGCTCCGCATCTCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCA
GTTCCTCAGGAGTGTCTTCGCCAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTAT
GACCTCCTAAAGGACCTAGAGGAAGGCATCCAAACGCTGATGGGGGTGAGGGTGGCGCCA
GGGGTCCCCAATCCTGGAGCCCCACTGACTTTGAGAGCTGTGTTAGAGAAACACTGCTGCC
CTCTTTTTAGCAGTCAGGCCCTGACCCAAGAGAACTCACCTTATTCTTCATTTCCCCTCGTG
AATCCTCCAGGCCTTTCTCTACACCCTGAAGGGGAGGGAGGAAAATGAATGAATGAGAAA
GGGAGGGAACAGTACCCAAGCGCTTGGCCTCTCCTTCTCTTCCTTCACTTTGCAGAGGCTGG
AAGATGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAA
ACTCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGA
CATGGACAAGGTCGAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGCTGT
GGCTTCTAGCTGCCCGGGTGGCATCCCTTGTGACCACTCCCCAGTGCCTCTCCTGGCCCTGG
AAGT