Glucose metabolism is altered after loss of L cells and α-cells but not influenced by loss of K cells


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Pedersen J, Ugleholdt RK, Jørgensen SM, Windeløv JA, Grunddal KV, Schwartz TW, Füchtbauer EM, Poulsen SS, Holst PJ, Holst JJ. Glucose metabolism is altered after loss of L cells and α-cells but not influenced by loss of K cells. Am J Physiol Endocrinol Metab 304: E60–E73, 2013. First published October 31, 2012; doi:10.1152/ajpendo.00547.2011.—The enteroendocrine K and L cells are responsible for secretion of glucagon-dependent glucinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), whereas pancreatic α-cells are responsible for secretion of glucagon. In rodents and humans, dysregulation of the secretion of GIP, GLP-1, and glucagon is associated with impaired regulation of metabolism. This study evaluates the consequences of acute removal of Gip- or Gcg-expressing cells on glucose metabolism. Generation of the two diphtheria toxin receptor cellular knockout mice, TgN(GIP.DTR) and TgN(GCG.DTR), allowed us to study effects of acute ablation of K and L cells and α-cells. Diphtheria toxin administration reduced the expression of Gip and content of Gip in the proximal jejunum in TgN(GIP.DTR) and expression of Gcg and content of proglucagon-derived peptides in both proximal jejunum and terminal ileum as well as content of glucagon in pancreas in TgN(GCG.DTR) compared with wild-type mice. GIP response to oral glucose was attenuated following K cell loss, but oral and intraperitoneal glucose tolerances were unaffected. Intraperitoneal glucose tolerance was impaired following combined L cell and α-cell loss and normal following α-cell loss. Oral glucose tolerance was improved following L cell and α-cell loss and supernormal following α-cell loss. We present two mouse models that allow studies of the effects of K cell or L cell and α-cell loss as well as isolated α-cell loss. Our findings show that intraperitoneal glucose tolerance is dependent on an intact L cell mass and underscore the diabetogenic effects of α-cell signaling. Furthermore, the results suggest that K cells are less involved in acute regulation of mouse glucose metabolism than L cells and α-cells. L cells; K cells; α-cells; proglucagon; glucose-dependent insulino-tropic polypeptide

PRO-Glucose-dependent Insulino-tropic Polypeptide (proGIP) and proglucagon are classical prohormones encoded by the glucose-dependent insulino-tropic polypeptide (Gip) and glucagon (Gcg) genes. Gip is expressed in the intestinal K cells, although minimal expression has been reported in pancreatic α-cells (14) and in several regions of the cerebral cortex (8). However, the very low extraintestinal expression is probably without physiological relevance. Gcg is expressed in the pancreatic α-cells and intestinal L cells and in neurons of the nucleus of the solitary tract in the brain stem (30). Gip and Gcg are highly conserved, indicating essential physiological roles for the genes (24). Posttranslational processing of proGIP is mediated by prohormone convertase 1/3 and liberates GIP1–42 from proGIP(50). In the body, GIP functions as an incretin hormone. It is released promptly after nutrient ingestion (20), augmenting glucose-stimulated insulin secretion from the pancreatic β-cells (37). GIP is also believed to promote fat deposition (52). Proglucagon is processed differentially in the pancreatic α-cells compared with extrapancreatic tissues and liberates different peptide hormones, several of which play a role in the regulation of the metabolism. In α-cells, proglucagon is cleaved mainly by prohormone convertase 2 (43), resulting in formation of glucagon, glicentin-related pancreatic polypeptide, and major proglucagon fragment (23). Although glucagon is the major counterregulatory hormone of insulin and plays a pivotal role in the regulation of glucose metabolism, the function of glicentin-related pancreatic polypeptide, a major proglucagon fragment, is yet to be determined. In the intestinal L cell and in the central nervous system, proglucagon is processed by prohormone convertase 1/3, resulting in formation of glucin, oxyntomodulin, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) (29, 42). Oxyntomodulin is a dual GLP-1 receptor (GLP-1R) and glucagon receptor (GCGR) agonist that may influence food intake and islet hormone secretion (39). Like GIP, GLP-1 functions as an incretin hormone potentiating glucose-induced insulin secretion but is also involved in the regulation of appetite and body weight and may be cardioprotective and neuroprotective (22). GLP-2 plays a significant role in regulation of intestinal adaptation (27, 45). The functions, if any, of glicentin, except for giving rise to oxyntomodulin, are still undetermined.

During the last decade, the functions of GIP, GLP-1, and glucagon have been studied using GIP receptor (GIPR), GLP-1R, GCGR, and GCG-knockout (KO) mice. Because both the receptors and their ligands are highly conserved during the evolution, it is surprising that the phenotypes of the GIP receptor and GLP-1R-KO mice are so limited. As expected, oral glucose tolerance is impaired in both GIPR and GLP-1R knockout mice (35, 46), and surprisingly, intraperitoneal glucose tolerance is also impaired in the GLP-1R-KO mouse (46). The GIPR-KO mice are reported to be resistant to diet-induced obesity (34), and β-cells were found to be more fragile in the GLP-1R-KO mouse (18). Surprisingly, the glucagon receptor KO mouse is viable, although there is an increased mortality in the neonatal period. Glucose tolerance is supernormal, and also, this mouse is resistant to diet-induced obesity (24). Mice
with a targeted mutation in the glucagon gene also show a modest phenotype; glucose tolerance is supernormal, but unlike the GCGR-KO, it gains more weight on a chow diet (19). Both the GCG-KO and the GCGR-KO show a marked hyperplasia of α-cells(16).

In the present study, rather than examining the consequences of deletions of incretin hormones or their receptors, we decided to develop two different inducible cellular KO mice of proGIP as well as proglucagon-producing cells, allowing a study of the general importance of either K cells or α-cells and L cells on whole body metabolism. Specifically, in the present study, we focused on the glucose homeostasis following ablation of proGIP or proglucagon-producing cells.

MATERIALS AND METHODS

Generation of transgenic mice. The nucleotide sequence encoding a fusion protein between the human diphtheria toxin (DT) receptor (hDTR) and enhanced green fluorescent protein (EGFP) was a kind gift from Dr. Steffen Jung (The Weizmann Institute of Science, Rehovot, Israel). Bacterial artificial chromosome (BAC) from clone RP23–27007 (Bacpac Resource Center, CHORI, Oakland, CA) containing the Gip locus and clone RP23–343C17 containing the Gcg locus were modified by Gene Bridge (Heidelberg, Germany) using the RedEt technology. The clone containing Gip was modified by recombining the sequence of hDTR-EGFP into exon 2 at the site of first ATG from Gip with a STOP codon terminating the hDTR-EGFP sequence in exon 2. Intron 1–6 and exon 1,3–6 were left unmodified. The clone containing the Gcg locus was modified by recombining the sequence of hDTR-EGFP into exon 2 at the site of first ATG from Gcg with a STOP codon terminating the hDTR-EGFP sequence in exon 2, leaving intron 1–6 and exon 1,3–6 unmodified.

The modified BAC clones were purified with the Qiagen large DNA fragments containing the whole modified BAC were used to identify transgenic mice.

Transgenic founder mice were identified by PCR testing for the unique sequence in the modified BAC were used to identify transgenic mice.

All experiments were carried out using only female mice since male mice housed alone or with other male mice are liable to stress. In addition, all experiments were carried out using wild-type littermate controls. Mice were housed in individually ventilated cages under a 12:12-h light-dark cycle with ad libitum access to food and water, and the strains were bred in a specific pathogen-free environment. All experiments were conducted in accordance with the institutional guidelines and the animal experiments inspectorate of the Ministry of Justice in Denmark. The experiments were approved by the animal experiments inspectorate and by the animal welfare committee at the Department of Experimental Medicine at the University of Copenhagen.

DT injections. Mice were injected intraperitoneally with PBS + 0.1% BSA or DT (cat. no. D0564; Sigma-Aldrich, Munich, Germany) dissolved in PBS + 0.1% BSA. The dose was 100 ng DT/g body wt. Oral and intraperitoneal glucose tolerance test. Mice were fasted overnight for 16–18 h with free access to water. The mice received 2 mg glucose/g body wt orally or intraperitoneally, administered as a 50% wt/vol glucose solution through a gavage tube or a 25-gauge needle. Blood samples were drawn into EDTA-coated capillary tubes from the retrobulbar sinus at 0 and 20 min and from tail tip puncture at 0, 20, 40, 60, 90, 120, and 150 min following the glucose load. Plasma glucose concentrations were measured using a hand-held plasma calibrated glucometer (Accu Check Compact Plus; Hoffmann-La Roche, Basel, Switzerland).

GIP response test. Mice were fasted overnight for 16–18 h with free access to water. The mice were dosed orally with 2 mg glucose/g body wt, administered as a 50% wt/vol glucose solution through a gavage tube. Blood samples were drawn into EDTA-coated capillary tubes from the retrobulbar sinus at 0 and 20 min and from tail tip puncture at 0 and 20 min following the oral glucose load. Plasma glucose concentrations were measured using a hand-held, plasma-calibrated glucometer (Accu Check Compact Plus).

Indirect calorimetry, activity, and food and water intake. Indirect calorimetry was performed using an indirect calorimetry system (LabMaster; TSE Systems, Hamburg, Germany). Mice were housed individually in the chambers for 10 days; the first 4 days were considered the acclimation phase, and data were analyzed for only the last 6 days. Sixty hours prior to DT or vehicle injections was considered the baseline. After the injections, the mice were removed from the CaloCages for 36 h and then returned to the CaloCages for another 60 h. Oxygen consumption rate (VO2; ml·h⁻¹·kg⁻¹), respiratory exchange ratio, total activity (beam breaks), and food intake were registered simultaneously for each mouse, using the LabMaster Software (PhenoMaster; TSE Systems).

Immunohistochemistry. Pancreases from wild-type and transgenic mice injected with either vehicle or DT 2 days earlier were fixed by immersion in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Samples were embedded in paraffin and cut into 5-μm sections. The sections were boiled in citric buffer, pH 6, in a microwave oven for 15 min for antigen retrieval. Two consecutive sections were incubated for 5 min in 2% bovine serum albumin, followed by 18 h at 4°C with the primary antibodies, guinea pig anti-insulin (in-house antibody no. 2006) diluted 1:12,800, or rabbit anti-glucagon (in-house side-viewing antibody no. 4304) diluted 1:4,800. Subsequently, the sections were incubated for 1 h with biotinylated goat anti-guinea pig immunoglobulins (cat. no. BA-7000; Vector Laboratories, Burlingame, CA) diluted 1:200 or goat anti-rabbit immunoglobulins (cat. no. BA-1000; Vector Laboratories) diluted 1:200 as the second layer, followed by a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC, cat. no. PK-4000; Vector Laboratories) incubation for 30 min. Finally, the reaction was developed by incubation in 3,3-diaminobenzidine for 15 min, followed by counterstaining with Mayer Hemal.
Small intestines from wild-type and transgenic mice injected with either vehicle or DT 2 days earlier were fixed by perfusion of the lumen with ice-cold 4% paraformaldehyde, and the intestines were subsequently immersed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Samples from the proximal small intestine (1 cm distal to the pyloric sphincter) and distal small intestine (1 cm proximal to the ileocecal valve) were embedded in paraffin and cut into 5-μm sections. The sections were boiled in citric buffer, pH 6, in a microwave oven for 15 min for antigen retrieval. Sections were incubated for 5 min in 2% bovine serum albumin, followed by 18 h at 4°C with the primary antisera, rabbit anti-GIP (in-house antibody no. 80867) diluted 1:40,000, monoclonal rabbit anti-glucagon (cat. no. 2810-1; Epitomics, Burlingame, CA) diluted 1:8,000, or rabbit antichromogranin A (cat. no. ab15160; Abcam, Cambridge, UK) diluted 1:2,000. Subsequently, the sections were incubated for 1 h with goat anti-rabbit immunoglobulins (cat. no. BA-1000; Vector Laboratories) diluted 1:200 as the second layer, followed by a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) (cat. no. PK-4000; Vector Laboratories) incubation for 30 min. Finally, the reaction was developed by incubation in 3,3-diaminobenzidine for 15 min, followed by a counterstaining with Mayer Hemalum.

The size of the islets and their proportion of glucagon- and insulin-immunoreactive cells were estimated using Image Pro Plus 6.0. Images of identical islets stained for glucagon and insulin on neighboring sections were captured by means of a Leica DFC 420 C camera by an investigator not aware of the groups. Three large islets were chosen from each section. The areas of the islets were calculated by tracing around the islets, and the proportion of glucagon- and insulin-positive cells was estimated by means of a color pipette tool.

The numbers of GIP-, proglucagon-, and chromogranin A-positive cells in the small intestine were estimated by counting the positive cells among 5,000 μm of intestine. The counting areas containing well-defined and well-orientated crypt-villus axes were marked on the sections, and the counting was then performed by two independent observers, and the means of the observations were used for further calculations.

Expression analysis. Total RNA was isolated with TRI-Reagent (Molecular Research Center, Cincinnati, OH), using the manufacturer’s protocol. The purity and concentration of the RNA were measured by absorbance at 260 and 280 nm. The integrity of RNA was confirmed by gel electrophoresis.

Table 1. Primer sequences and concentrations

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F, forward; R, reverse. Assay numbers are supplied for the predesigned QuantiTect primer assays. Sequences and concentrations used are primers that were retrieved from Primerbank (http://pga.mgh.harvard.edu/primerbank/).
WAT036810; Waters, Milford, MA), eluted with 70% vol/vol ethanol + 0.1% trifluoroacetic acid, and blown dry under compressed air.

**Peptide extract analysis.** Dried extracts were reconstituted in 80 mM sodium phosphate buffer + 0.1% BSA + 10 mM EDTA, pH 7.5. The content of immunoreactive GIP was determined with RIA employing the R65 antisemur, which recognizes the COOH terminus of GIP, binding to the region 34–42 and therefore also measuring mouse GIP (28), and the content of immunoreactive proglucagon derivatives was determined using the side-viewing 4304 antisemur, which recognizes both proglucagon, glicentin, oxyntomodulin, and glucagon (21).

Insulin was measured with an ELISA (cat. no. 10-1247-01; Mercodia, Uppsala, Sweden).

**Protein isolation and EGFP ELISA.** Snap-frozen tissues from brain, stomach, proximal small intestine, distal small intestine, colon, liver, pancreas, spleen, heart, and skeletal muscle were homogenized in RIPA buffer (50 mM Tris·HCl + 1% Igepal CA630 + 0.25% Na-deoxycholate + 1 mM PMSF) added protease inhibitor (cat. no. P8340; Sigma-Aldrich, Munich, Germany) using a bead mill (TissueLyser; Qiagen Instruments) operated with 5-mm steel beads at 30 Hz for 15 min. One hundred microliters per assay of the supernatants was used and analyzed using a EGFP ELISA kit (cat. no. AKR121R; Cell Biolabs, San Diego, CA).

**Plasma analysis.** Mouse plasma insulin was measured with an ELISA (cat. no. 10-1247-01; Mercodia, Uppsala, Sweden). Mouse plasma GIP was measured with an ultrasensitive ELISA (cat. no. EZRMDP-55K; Millipore, Billerica, MA), recognizing active GIP1–42 and the metabolite GIP3–42 with equal affinity.

**Statistical analysis.** Expression data are presented as log2-transformed means. Medians with 5th and 95th percentiles and back-transformed means and 95% confidence intervals are presented as percentages; all other data are presented as means ± SE unless otherwise stated. For comparison of more than two means, one-way ANOVA was used, and Bonferroni correction was applied for correction of multiple testing. Repeated-measurements ANOVA was used to compare longitudinal data, and Bonferroni was applied for correction of multiple testing. Area under the curve was calculated using the trapezoidal rule. All data were calculated using Graph Pad Prism 5.0 and MS Excel 2007.

**RESULTS**

**Expression of the transgene.** We analyzed the expression of the transgene of both mouse strains. Transgene expression was detected in the brain, pancreas, stomach, jejunum, ileum, and colon of the TgN(GCG.DTR) strain (data not shown). The transgene was expressed in jejurnum, ileum, and colon of the TgN(GIP.DTR) strain (data not shown). We were unable to detect significant levels of expression in either strain in heart, muscle, and liver (data not shown). Next, we quantified the contents of EGFP in tissue homogenates, but we were unable to detect any significant levels of EGFP in homogenates from brain, pancreas, stomach, small and large intestine, spleen, adipose tissue, heart, or muscle tissue (data not shown), indicating that the content of EGFP is very low.

We next analyzed cryosections of pancreas and proximal and distal small intestine using fluorescence microscopy, but we were unable to spot any EGFP-positive cells in the intestinal mucosa or in the pancreatic islets. Because the fusion protein DTR:EGFP is membrane bound, we assume this that the transgene is expressed in rather limited amounts makes the EGFP signal undetectable by fluorescence microscopy, as observed previously by Chen et al. (10).

**Acute reduction of intestinal GIP or intestinal and pancreatic proglucagon-derived peptides.** As shown in Fig. 1A, the relative expression of Gip in the TgN(GIP.DTR) mice that had been injected with DT 2 days earlier was 9.47% (4.64 and 19.34%, P < 0.001 compared with the PBS-injected wild-type mice). The relative expression of peptide YY (Pyy), cholecystokinin (Cck), neurotensin (Nrt), secretin (Sct), and glucagon (Gcg) was equal between the TgN(GIP.DTR) and wild-type mice injected with either DT or PBS (data not shown).

Next, we analyzed tissue extracts from both wild-type and TgN(GIP.DTR) mice that had been injected with DT or vehicle 2 days earlier (Fig. 1B). As expected, we saw a marked reduction in the intestinal content of immunoreactive GIP from 156 ± 10.36 pmol/g in extracts isolated from vehicle-injected wild-type to 15.79 ± 3.46 pmol/g in extracts isolated from DT-injected TgN(GIP.DTR) mice (P < 0.01).

The expression of Gcg, Pyy, Cck, Nrt, Sct, Gip, and Xenin (Copa) in proximal jejunum 2 days after exposure to DT of TgN(GCG.DTR) was reduced to 28% (23 and 33%) for Gcg, 61% (51 and 72%) for Cck, and 46% (33 and 64%) for Nrt compared with the vehicle-injected wild-type mice. The relative expression levels of Pyy, Gip, Copa, and Sct were similar in the proximal jejunum in both the transgenic and the wild-type mice (Fig. 1D). In the distal ileum the relative expression levels were reduced to 8% (4 and 16%) for Gcg, 12% (6 and 25%) for Pyy, 15% (8 and 31%) for Cck, and 4% (2 and 7%) for Nrt in the TgN(GCG.DTR) mice injected with DT compared with the expression levels in the wild-type mice injected with vehicle. The relative expression levels of Gip, Copa, and Sct in the distal ileum were similar in the transgenic and the wild-type mice (Fig. 1E).

Next, we analyzed extracts from distal intestine and pancreas isolated from both transgenic and wild-type mice that had been injected with DT or PBS 2, 7, 14, and 28 days before euthanization. As expected, the contents of proglucagon in distal intestine from DT-injected TgN(GCG.DTR) mice were reduced to 1.9% (4.64 and 15.79%) of the contents from wild-type PBS (Fig. 1F). The contents of proglucagon in pancreatic extracts isolated from DT-injected TgN(GCG.DTR) mice were reduced to 10.2% of the contents from wild-type PBS (Fig. 1F). The intestinal contents of proglucagon were normalized after 7 days, but we observed a slight increase in intestinal proglucagon content in DT-injected wild types 14 days after DT injections (Fig. 1F). The content of pancreatic glucagon was still reduced after a 28-day-long period, and we did not detect any trend toward regeneration of the lost glucagon (Fig. 1F). Because Gcg is expressed in specific nuclei of the brainstem in mice (30), we evaluated the effects on glucagon content in extracts from brainstems isolated from DT- and vehicle-injected wild types and TgN(GCG.DTR), but there was no difference between the DT-injected transgenic and control groups (Fig. 1C).

**K and L cell numbers.** Jejunum and ileum from both transgenic mouse strains were analyzed by immunohistochemistry (Fig. 2, E–H). The DT-injected TgN(GIP.DTR) mice had reduced numbers of GIP-positive cells in jejunum: 15 ± 1 cells/5,000 μm intestine compared with 59 ± 6 cells/5,000 μm intestine (P < 0.001; Fig. 3A). As expected, the number of GIP-positive cells in the distal ileum was very limited (7 cells/5,000 μm intestine in the PBS-injected wild types), and we were unable to detect a significant reduction in the number
Fig. 1. Acute reduction of K cell or L cell and α-cell markers. A: relative expression of glucose-dependent insulinotropic polypeptide (GIP) in the proximal intestine in TgN(GIP.DTR) or wild-type mice after injection with diphtheria toxin (DT; 100 ng/g) or PBS (vehicle) 2 days before. Relative expressions are presented on a logarithmic scale; n = 12–16. B: content of immunoreactive GIP in tissue extracts from the proximal intestine from wild-type or TgN(GIP.DTR) mice injected with DT or PBS 2 days before; n = 5–8. C: content of proglucagon in brainstem 2 days after injection with DT or PBS; n = 6–8. D and E: relative expression of peptide YY (Pyy), cholecystokinin (Cck), neurotensin (Nrt), secretin (Sct), xenin (Copa), glucagon (Gcg), and Gip in proximal (n = 5–11; D) or distal (n = 7; E) small intestine from TgN(GCG.DTR) or wild-type mice 2 days after injection with DT or PBS presented as Log2-transformed values. *P < 0.05 and **P < 0.01 vs. wild-type PBS. F: %proglucagon content in terminal ileum (n = 6–8) and pancreas (n = 5–17) 2–28 days after injection with DT or PBS. *P < 0.05 and ***P < 0.001 vs. ileum from TgN(GCG.DTR) DT. •P < 0.05, ••P < 0.01, and •••P < 0.001 vs. pancreas from TgN(GCG.DTR) DT.
of GIP-positive cells in the DT-injected TgN(GIP.DTR) mice in the distal ileum (Fig. 3B). The number of proglucagon-positive cells in the proximal jejunum was reduced from 33 ± 6 cells/5,000 μm intestine in PBS-injected wild types to 15 ± 2 cells/5,000 μm in DT-injected TgN(GCG.DTR) (P < 0.05; Fig. 3C). In the distal ileum, the number of proglucagon-positive cells was reduced from 86 ± 18 cells/5,000 μm intestine in PBS-injected wild types to 14 ± 4 cells/5,000 μm in the DT-injected TgN(GCG.DTR) mice (P < 0.01; Fig. 3D). The number of proglucagon-positive cells in jejunum and ileum was normalized 1 wk after DT injections (Fig. 3, C and D). Furthermore, we did not observe any impact on the number of GIP- or proglucagon-positive cells in the DT-injected wild types.

**α-Cell and β-cell area.** Pancreases from TgN(GCG.DTR) were evaluated immunohistochemically (Fig. 2, A–D). Pancreases from both DT- and vehicle-injected mice appeared to be normal, without any lymphocyte infiltration, necrosis, or apoptosis. We stained the sections for glucagon to visualize loss of α-cells and observed a marked but not complete reduction in
the number of α-cells in pancreases from DT-injected TgN(GCG.DTR) mice. To quantify the loss of α-cells, we measured the α-cell, β-cell, and total islet areas; however, α-cells in rodents are located almost exclusively in the mantel zone of the islet, and the counting of all islets would introduce a section bias from islets cut through the periphery. Hence, we counted three large diameter islets from each section of the pancreas. Unexpectedly, we observed that islets from the transgenic mice were generally larger compared with the wild types (13,948 ± 1,817 vs. 8,157 ± 746 μm², *P < 0.01; Fig. 4A).

Hence, we decided to calculate the ratio of α-cell and β-cell areas to the total islet area. The normalized α-cell areas of the largest islets were significantly smaller in transgenic mice injected with DT 2 days earlier compared with the other groups (Fig. 4B). Furthermore, the α-cell/islet area in islets from transgenic mice injected with DT 7 days earlier (0.9%; 0.2 and 3.4%) was even more reduced compared with the α-cell/islet area in islets from transgenic mice injected with DT 2 days earlier (3.7%; 2.6 and 5.2%) (P < 0.02). We did not observe any effect of DT injection in either TgN(GCG.DTR) or wild-type mice on the normalized β-cell area, indicating that the DT injection targeted only the α-cells in the TgN(GCG.DTR) (Fig. 4C). Next, we quantified the content of insulin in extracts from pancreas, and although the islets were generally larger, the content of insulin was not different between the groups (Fig. 4D).

Indirect calorimetry, activity, and food and water intake. Injection of wild-type or transgenic mice with DT did not influence oxygen consumption, respiratory exchange ratio, or ambulatory activity, nor did it influence food and water intake (data not shown).

No influence on glucose metabolism following acute K cell loss. We evaluated the impact of an acute partial depletion of the K cells on oral (Fig. 5A) and intraperitoneal glucose tolerance (Fig. 5C), and we also evaluated the effects on oral (Fig. 5B) and intraperitoneal glucose tolerance (Fig. 5D) of DT in wild-type mice. Surprisingly, there were no differences in plasma glucose between any of the mice, and insulin responses were also comparable between all of the groups (data not shown), indicating that acute K cell loss and DT injection have no influence on glucose metabolism. The GIP response to the standard glucose load (2 mg/kg) was reduced by 70% in the DT-injected transgenic mice compared with the PBS-injected transgenic mice (Fig. 6A). Fasting concentrations were also reduced by 80% in the DT-injected transgenic mice compared with the PBS-injected transgenic mice. Furthermore, there were no differences between PBS-injected wild types and DT-injected wild types (Fig. 6B).
Glucose metabolism is altered following L cell and α-cell loss. The physiological impact of the acute reduction of L cells and α-cells was addressed by applying a glucose tolerance test 2 days after injection with either DT or PBS in both TgN(GCG.DTR) (Fig. 7, A and C) and wild-type mice (Fig. 7, B and D). There were no differences in fasting plasma glucose concentrations between the TgN(GCG.DTR) and wild-type mice. During the oral glucose tolerance test, the DT-injected TgN(GCG.DTR) mice had a lower concentration of plasma glucose 20 min after glucose delivery compared with the PBS-injected TgN(GCG.DTR) mice; however, the overall glucose clearance was not affected, as indicated by the comparable AUC\textsubscript{glucose} (0–150 min) values (Fig. 7A, inset). We were unable to detect any effect on oral glucose tolerance of DT injections in wild-type mice (Fig. 7B). We detected an impairment of the intraperitoneal glucose tolerance in the TgN(GCG.DTR) mice injected with DT 2 days prior to the test compared with TgN(GCG.DTR) injected with vehicle, which was indicated by higher glucose concentrations 40 and 60 min after glucose delivery mice (Fig. 7C); however, the AUC\textsubscript{glucose} (0–150 min) values were not different (P < 0.14; Fig. 7C, inset). Injection of wild types with DT induced a minor improvement in the intraperitoneal glucose tolerance, as indicated by the slightly lower peak plasma glucose concentration of 26.2 ± 1.0 mM compared with 28.9 ± 0.7 mM in the vehicle-injected wild types (P < 0.05; Fig. 7D). The AUC\textsubscript{glucose} (0–150 min) values were not different between the vehicle- and DT-injected wild types (P < 0.08; Fig. 7D, inset). Although we were able to detect alterations in the excursions of the time-glucose curves, there were no differences in the insulin responses following oral or intraperitoneal glucose delivery in any of the groups (data not shown). Because the L cells in the intestine were fully regenerated 7 days after DT and the pancreatic α-cells showed no tendency toward regeneration in this time period, we performed an oral glucose tolerance test in TgN(GCG.DTR) mice with a reduced α-cell mass 7 days after DT injection. Oral glucose tolerance showed a marked improvement in mice with reduced α-cell mass, as indicated by the lower concentration of plasma glucose observed 20 and 40 min after glucose delivery in the DT-injected TgN(GCG.DTR) mice (Fig. 8A). Injection with DT 7 days prior to the test had no effect on plasma glucose concentration during an oral glucose tolerance test in wild-type mice (Fig. 8B). Next, we analyzed the intraperitoneal glucose tolerance 7 days after injections with DT, and surprisingly, intraperitoneal glucose tolerance was normalized in mice with reduced α-cell mass and regenerated L cells (Fig. 8C). Intraperitoneal glucose tolerance was unaffected in wild-type mice that received DT injection 7 days prior to the test.

**DISCUSSION**

We created two, genetically modified mouse models for studying the role of *Gip*- and *Gcg*-expressing cells in lean mice. Employing these models, we show that reduction of K
cells does not alter the glucose metabolism in lean female mice. Furthermore, we show that reduction of L cells and α-cells slightly improves oral and impairs intraperitoneal glucose tolerance in lean female mice. In addition, we are able to show that reduction of pancreatic α-cells markedly improves oral glucose tolerance in lean female mice.

Our mouse models differ from other mouse models used previously to dissect the function of GIP and the proglucagon-derived peptide in the whole organism by focusing on 1) the cells producing the ligands rather than the physiological functions of a specific receptor or ligand and 2) the inducible ablation of the target cells. Hence, we were able to study...
modulation of K or L cell and α-cell signaling without interference from the biological compensation that invariably follows permanent gain- or loss-of-function mouse models.

The development of the intestinal endocrine cells is a complex process that involves expression of various hormones before the cells are completely mature (41); therefore, execution of cellular knockout in the gastrointestinal epithelium is associated with a risk of also eliminating enteroendocrine progenitor cells. To circumvent this risk, and inspired by the work of Chen et al. (10), we designed our constructs to target only cells with a strong Gip or Gcg promoter activity. By omitting a poly(A) site next to the STOP codon introduced in exon 2 of the modified Gip or Gcg gene, we generated a powerful posttranscriptional downregulation of the transcript. Therefore, only cells with a strong activity of the Gip or Gcg promoter were targeted by DT injections rather than every Gip- or Gcg-expressing cell in the organism. Massive overexpression of the human DT receptor is not as seamless as originally thought, as several observations from different groups have reported unexpected effects of cell-specific overexpression of the human DT receptor, which appears to function as a growth factor in the different organisms. The very limited expression of the hDTR could reduce the effects that invariably follow overexpression. The strategy, however, has a particular disadvantage because the expression of the transgene is too low to generate a detectable EGFP signal. However, considering the modest expression of the transgene, this is expected. It is commonly accepted that EGFP requires a relatively high expression and relatively high intracellular concentration before it is detectable, as reviewed recently by Boubabe (6).

The use of bacterial toxin receptor-mediated cellular knockout models always gives rise to concerns about the off-target effects of the bacterial toxin. DT-induced cell death in genetically modified mice has been employed for almost a decade in studies of the immune system (7, 12, 25, 26, 51), and until recently, all studies reported DT used in comparable doses to be inert in wild-type mice (9, 44). However, Meyer Zu Hörste et al. (33) recently reported a 3-day-delayed DT-induced weight loss in wild-type C57Bl/6 mouse. In agreement with the findings by Meyer Zu Hörste et al. (33), we also found small but significant off-target effects of DT in mice. In contrast to the finding in the transgenic mice, we found that DT injection in wild-type mice improves the intraperitoneal glucose tolerance, and we also found that DT injection in wild-type mice augments GIP response to a high dose of oral glucose. Because the findings in the wild types are opposite of the findings in the

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**Fig. 7. Oral and intraperitoneal glucose tolerance after combined L cell and α-cell reduction.**

A and B: PG concentration following oral glucose load (2 mg/g) in TgN(GCG.DTR) (n = 8; A) or wild-type mice (n = 7–8; B) injected with DT (100 ng/g) or PBS (vehicle) 2 days prior to the test. Blood samples were drawn from the tail vein at the indicated time points for measurement of glucose. *P < 0.05 vs. TgN(GCG.DTR) PBS. C and D: PG concentration following intraperitoneal glucose load (2 mg/g). Blood samples were drawn from the tail vein at the indicated time points for measurement of glucose. C: TgN(GCG.DTR) (n = 14–15) injected with DT or PBS 2 days prior to the test. *P < 0.05 vs. TgN(GCG.DTR) PBS. D: wild-type mice (n = 12–15) injected with DT or PBS 2 days prior to the test. *P < 0.05 vs. wild-type PBS. A–D, insets: area under glucose concentration curve.
transgenic mice, we consider the DT off-target effects irrelevant for the conclusions we have drawn in this study.

Our TgN(GCG.DTR) mouse differs from the mouse in the study conducted recently by Thorel et al. (48) by the different design of the transgene: 1) the mouse published by Thorel et al. (48) uses a minimal rat glucagon promoter directing the expression of the transgene to α-cells only, 2) the expression of the transgene is not dampened by posttranslational downregulation of the transcript, resulting in a more extensive removal of the α-cells, and 3) the mouse in the study by Thorel et al. (48) uses the glucagon promoter to drive the expression of heparin-binding EGF-like growth factor (HB-EGF). Therefore, it is reasonable to assume that the HB-EGF of this particular mouse expresses massive amounts of the growth factor HB-EGF, which, although not addressed by Thorel et al. (48), is known to induce changes (5, 32, 40). We designed our models to include posttranslational downregulation of HB-EGF to circumvent the risk from HB-EGF, and we also included littermate wild types into all of our studies. Although we did not detect any differences between wild-type and transgenic mice regarding glucose metabolism, we observed that the islets in the TgN(GCG.DTR) were slightly larger compared with islets from wild-type mice. To prevent potentially erroneous conclusions based on comparisons between wild-type and transgenic mice, we limited our data interpretation regarding glucose metabolism to comparisons between transgenic mice with reduced K cell, α-cell, or L cell and α-cell mass and transgenic mice with intact K cell, α-cell, or L cell and α-cell mass.

Reduction of K cells in TgN(GIP.DTR) by DT injections did reduce the relative expression of Gip but did not influence the expression of various other peptide hormones. Injection with DT in TgN(GCG.DTR) resulted in reduction of Gcg mRNA in proximal as well as distal small intestine, but the reduction of Gcg expression in the proximal intestine was not as powerful as in the distal intestine. In the proximal intestine, reduced Gcg expression also led to a reduction of Nrt and Cck, but not to the same extent as Gcg, whereas in the distal intestine reduction of Gcg expression induced a comparable reduction of Pyy, Cck, and Nrt expression. These findings illustrate the complex nature of the enteroendocrine cells and show that L cells in the proximal intestine are different from L cells in the distal intestine, consistent with recent findings by Egerod et al. (13).

Considering the results from Mortensen et al. (36) showing that GLP-1 was colocalized with either GIP or PYY, it was surprising that expression of Gip and Pyy was not reduced together with Gcg in the proximal intestine of the DT-injected TgN(GCG.DTR) mice. Conversely, in our TgN(GIP.DTR) mice, Gcg expression was unaffected by a 10-fold downregulation of Gip in the proximal intestine. The discrepancy between the study by Mortensen et al. (36) and our study

Fig. 8. Oral and intraperitoneal glucose tolerance after α-cell reduction. Plasma glucose concentration curves following oral glucose tolerance test (2 mg/g, n = 6–10; A and B) and intraperitoneal glucose tolerance test (2 mg/g, n = 7–8; C and D). TgN(GCG.DTR) (A and C) or wild-type mice (B and D) injected with DT (100 ng/g) or PBS (vehicle) 7 days prior to the test. Blood samples were drawn from the tail vein at indicated time points for measurement of glucose. **P < 0.01 and ***P < 0.001 vs. TgN(GCG.DTR) DT. A–D, insets: area under glucose concentration curves.
probably reflects the different expression levels of Gip and Gcg in the “pure” L and K cells and in the so-called K/L cell, since our mouse models were designed to target only cells with a high expression of Gip or Gcg.

The repopulation of the enteroendocrine cells 1 wk after ablation is in good agreement with the kinetics of mucosal renewal described by Bjerknes and Cheng (4) and indicates that stem cells and endocrine progenitor cells of the intestine are not targeted by the DT injection. In contrast to the intestinal regeneration of K and L cells and in agreement with the findings by Thorel et al. (49), we did not observe any tendency for repopulation of the pancreatic α-cell following DT injection after 28 days.

Our finding that a 10-fold reduction of GIP content in the intestine associated with a 70% reduction of the GIP response to orally ingested glucose had no detectable impact on the glucose tolerance is in conflict with previous findings in other different mouse models with introduced defects in GIP signaling. A GIP receptor knockout mouse was first described in 1999 by Miyawaki et al. (35), and the disrupted GIP signaling resulted in impaired oral glucose tolerance in male mice. In line with our results, Hansotia et al. (17) found only a modest impairment in oral glucose tolerance in the GIPR-KO mice. Also, it was shown by Fulurija et al. (15) that disrupted GIP signaling due to vaccination against GIP protected against diet induced obesity but did not affect glucose metabolism. Our findings, however, are in complete contrast to the findings in the GIP-DT mouse (2); the GIP-DT mice are born without Gip-expressing cells due to unconditional expression of the A-subunit of DT in Gip-expressing cells (2), and these mice showed a marked impairment in oral glucose tolerance. A possible reason for the discrepancy between the two mouse models, which in theory have comparable phenotypes, should probably be found in the lifelong elimination of K cells in the GIP-DT mouse in contrast to the inducible and partial loss in our mouse.

Injection of TgN(GCG.DTR) with DT ablates both L cells and α-cells; hence, the impact of a DT injection on glucose metabolism is complex, comprising opposing hormonal actions, which probably explain the modest effects on glucose metabolism in our study. The changes in glucose tolerance after L cell and α-cell reduction are minor compared with the changes described in the GLP-1R-knockout mouse (46) and GCGR-knockout mouse. Because the GCGR-knockout mouse shows improved intraperitoneal (16) and oral glucose tolerance (11) and the GLP-1R knockout mouse shows impaired tolerance in both tests, our results are in good agreement with the expected outcome of an acute combined disruption of GLP-1 and glucagon signaling. However, it was shown recently that the GLP-1R and GCGR double-knockout mouse has a normal intraperitoneal and a supernormal oral glucose tolerance (1), but it was also shown that the β-cells of the double knockouts reacted differently compared with β-cell from wild types, reflecting the marked compensation due to the inborn defects. Importantly, our results show that intraperitoneal glucose tolerance, a surrogate marker of β-cell function, is dependent on an intact L cell mass, as we show that intraperitoneal glucose tolerance was impaired following combined L cell and α-cell loss, whereas intraperitoneal glucose tolerance was normal following isolated α-cell loss. The latter is in agreement with the recently published study showing that fasting blood glucose and intraperitoneal glucose tolerance are not affected by extensive α-cell loss (48). Considering the known phenotype of the GCGR-knockout mouse, the observed supernormal oral glucose tolerance observed following α-cell reduction was expected and underscores the diabetogenic effects of α-cell signaling.

In conclusion, we introduce two new genetically modified mouse models, one allowing the study of K cell reduction and the other allowing combined L cell and α-cell reduction as well as an isolated α-cell reduction. For the first time, we were able to study the effects following acute reduction of K cells, and surprisingly, this does not alter glucose metabolism. In contrast, combined acute L cell and α-cell reduction resulted in significant changes in the glucose metabolism following combined L cell and α-cell loss. Additionally, we showed that isolated α-cell loss resulted in supernormal oral glucose tolerance and normal intraperitoneal glucose tolerance. These observations suggest that K cells are less involved in acute regulation of mouse glucose metabolism than L cells and α-cells and underscore the diabetogenic effects of α-cell signaling.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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