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Impaired Islet Function in Commonly Used Transgenic Mouse Lines due to Human Growth Hormone Minigene Expression

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SUMMARY

The human growth hormone (*hGH*) minigene is frequently used in the derivation of transgenic mouse lines to enhance transgene expression. Although this minigene is present in the transgenes as a second-cistron, and thus not thought to be expressed, we found that three commonly used lines, *Pdx1-Cre*^{Late}, *RIP-Cre*, and *MIP-GFP*, each expressed significant amounts of hGH in pancreatic islets. Locally secreted hGH binds to prolactin receptors on β cells, activates STAT5 signaling, and induces pregnancy-like changes in gene expression, thereby augmenting pancreatic β cell mass and insulin content. In addition, islets of *Pdx1-Cre*^{Late} mice have lower GLUT2 expression and reduced glucose-induced insulin release and are protected against the β cell toxin

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ACCESSION NUMBERS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.11.004.

streptozotocin. These findings may be important when interpreting results obtained when these and other *hGH* minigene-containing transgenic mice are used.

INTRODUCTION

Conditional inactivation of genes in mice by specific DNA recombination in target tissues was developed in the early 90s (Orban et al., 1992). By then, it was already known that intronic sequences and a polyadenylation signal are essential to achieve efficient expression of the transgene (Brinster et al., 1988). The entire human growth hormone (*hGH*) coding region, including introns and polyadenylation signal (also called *hGH* minigene), was oftentimes inserted downstream of coding regions, such as that of *Cre recombinase*, to generate transgenic mouse models (Orban et al., 1992), some of which are in wide use today.

Prolactin (PRL), placental lactogen (PL), and GH are homologous proteins that display overlapping structure (Goffin et al., 1996) and biological activities (Soares, 2004). The lactogens PRL and PL have a profound effect on the pancreatic islet phenotype of pregnant females (Parsons et al., 1992; Sorenson et al., 1987). During pregnancy, insulin resistance is induced in the mother, which facilitates nutrient flow toward the fetus (Freemark, 2006). To compensate for this increased metabolic demand and to prevent hyperglycemia, maternal pancreatic β cells undergo several structural and functional changes. These changes have been under investigation for several decades and involve multiple β cell parameters, such as increased glucose-stimulated insulin secretion (GSIS) (Green and Taylor, 1972), enhanced β cell proliferation (Van Assche, 1974), accelerated proinsulin biosynthesis (Bone and Taylor, 1976), a higher rate of glucose oxidation and glucose utilization (Green et al., 1978; Weinhaus et al., 1996), and increased gap-junctional coupling of β cells (Sheridan et al., 1988). The importance of lactogens in this phenotypic switch has been demonstrated in mouse models in which β cell-specific overexpression of PL leads to enhanced insulin secretion and increased β cell mass (Fleenor et al., 2000; Vasavada et al., 2000). These signals are mediated by prolactin receptors (PRLR), as was demonstrated in *Prlr*$^{+/-}$ mice (Huang et al., 2009). Growth hormones of primates, but not of other vertebrates, are also able to activate PRLR, a property that is maintained in several heterologous systems (Goffin et al., 1996). Accordingly, it was shown that hGH can mimic the effects of lactogens on mouse and rat β cells (Parsons et al., 1995).

To define the molecular basis of lactogen signaling during pregnancy, we and others recently explored the changes that occur in the gene expression using genome-wide mRNA expression analysis (Kim et al., 2010; Rieck et al., 2009; Schraenen et al., 2010a, 2010b). The largest cluster of upregulated genes during pregnancy regulates β cell mass (Schraenen et al., 2010a). In addition, a strong induction of the serotonin biosynthetic pathway was found in a subset of β cells (Schraenen et al., 2010b). Genes encoding tryptophan hydroxylase-1 (TPH1) and tryptophan hydroxylase-2 (TPH2), catalyzing the rate-limiting step of serotonin biosynthesis, were found to be vastly upregulated (Kim et al., 2010; Schraenen et al., 2010b), resulting in an increase in islet serotonin content greater than 100-fold. The responsible mechanism involved PRLR and activation of its canonical Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signaling pathway

(Schraenen et al., 2010b). An autocrine/paracrine role for serotonin has been suggested in activating β cell proliferation via serotonin receptor 2B (Kim et al., 2010).

Here we describe unexpected functional consequences resulting from the placement of *hGH* minigene in the transgenic constructs. The principal mouse strain used for these studies was the *Tg(Pdx1-cre)1*^{Herr} mouse strain, also known as the *Pdx1-Cre*^{Late} model (Herrera, 2000). This transgenic line contains a 4.5 kb fragment of the *Pdx1* promoter inserted upstream of the *Cre recombinase*-coding region and an *hGH* minigene in order to achieve efficient transgene expression. We studied the expression of *hGH* at the mRNA and protein level and the consequences for islets from *Pdx1-Cre*^{Late} mice, such as changes in β cell mass and insulin secretion. In addition, we examined hGH expression and serotonin biosynthesis in two other lines that are frequently used for pancreatic *25Mgn/J* line (Postic et al., 1999), which is frequently used to generate β cell conditional gene knockouts, and the *B6.Cg-Tg(Ins1-EGFP)1Hara/J* line, which is often used to visualize and/or purify pancreatic β cells (Hara et al., 2003), from now on referred to as *RIP-Cre* and *MIP-GFP*, respectively.

RESULTS

Pancreata from Pdx1-Cre^{Late} Mice Have Increased β Cell Mass and Insulin Content

While working with the *Pdx1-Cre*^{Late} driver line to generate a new β cell-specific knockout strain, we found that circulating blood glucose, in both the random-fed (Figure 1A) and fasted state (Figure 1B), was lower in the *Pdx1-Cre*^{Late} mice than in littermate controls. This difference could not be explained by enhanced insulin sensitivity of *Pdx1-Cre*^{Late} mice (Figure 1C). Instead, we observed an increase in pancreatic β cell mass (Figure 1D), which was accompanied by a nearly doubled pancreatic insulin content (Figure 1E, *Pdx1-Cre*^{Late} 390 ± 19 μg/g versus WT 204 ± 52 μg/g, n = 9–10/genotype, p = 0.006).

Paracrine/Autocrine PRLR Activation by hGH in Islets from Nonpregnant Pdx1-Cre^{Late} Mice

In the original description of the *Pdx1-Cre*^{Late} mouse model (Her-rera, 2000), the complete coding *hGH* gene sequence, including exons, introns, and its polyadenylation signal, is present downstream of the *Pdx1* promoter and *Cre recombinase* (Figure 2A). Results from Figures 2B–2F demonstrate that the *hGH* minigene is specifically and highly expressed in mouse β cells of the *Pdx1-Cre*^{Late} strain. We quantified *hGH* expression at both the mRNA level (Figure 2B) and protein level (Figures 2C–2E). We found significant hGH expression only in pancreatic islets, but not in the other tissues (Figure 2B), including the exocrine part of the pancreas (Figure 2D). Western blots (Figure 2C) indicated that the mature hormone of the expected molecular weight (22 kDa) was produced in *Pdx1-Cre*^{Late} islets. Quantification of hGH in extracts from isolated islets and in conditioned medium (Figure 2F) shows that approximately 4% of cellular hGH content is released per hour from 20 mM glucose-stimulated *Pdx1-Cre*^{Late} islets. Figure 2G supports the idea that locally secreted hGH has functional effects on pancreatic islet in vivo. Indeed, a significant increase (3.7-fold, n = 3, p = 0.005) in STAT5 phosphorylation was detected in freshly isolated *Pdx1-Cre*^{Late} islets compared to control islets (Figure 2G). Because hGH can bind to GH receptors (GHR) and to PRLR (Goffin et al., 1996), and since both receptors are strongly expressed in rodent β cells (Brelje et al., 2002; Møldrup et al., 1993), we next investigated the mechanism by which hGH causes phenotypic changes in β cells. We first used MIN6 cells as a surrogate model for mouse β cells (Miyazaki et al., 1990) and quantified *Tph1* as a marker for lactogen-mediated PRLR activation, taking ovine PL (oPL) as a positive control (Schraenen et al., 2010b). As is shown in Figure 2H, both hGH and oPL were very potent ligands in this assay, as a significant induction of *Tph1* mRNA was already observed with 25 ng/ml (~1 nM). In comparison, the minimal effective concentration of mouse (m)GH, which only binds to GHR, was 500 ng/ml, indicating that synthetic hGH was at least 20-fold more potent. Similar results were obtained with primary islets: a significant induction of *Tph1* mRNA was found in islet monolayers treated with 500 ng/ml oPL or hGH, but not after treatment with mGH (Figure 2I). To confirm that hGH stimulation of *Tph1* expression is mediated by PRLR signaling, MIN6 cells stimulated or not with 25 ng/ml hGH were cotreated with increasing concentrations of the synthetic PRLR antagonist Δ1-9-G129R-hPRL (Bernichtein et al., 2003). A concentration-dependent antagonism was observed (Figure 2I). Together, our data indicate that lactogen-like increment of pancreatic β cell mass and insulin content in *Pdx1-Cre*^{Late} mice is initiated by local hGH release and mediated by activation of PRLR.

Activation of a Pregnancy-Related Phenotypic Switch in Islets from Pdx1-Cre^{Late} Mice

The autocrine/paracrine PRLR stimulation by local release of hGH in pancreatic islets not only enhanced the β cell mass but also upregulated more than 100 genes that are also upregulated in islets from *C57BL/6J* pregnant mice (Figure 3A). This was measured by comparison of the global islet gene expression profile in 12-week-old *Pdx1-Cre*^{Late} mice and control nonpregnant littermates as well as islet from *C57BL/6J* pregnant mice not carrying the *Pdx1-Cre*^{Late} insertion. More than 50% (121/218) of the upregulated genes in nonpregnant *Pdx1-Cre*^{Late} islets were also upregulated during pregnancy (Figure 3A). Overlap was complete for a pregnancy gene expression signature, consisting of 12 known genes described previously to be highly up-regulated during pregnancy (fold increase ≥ 3 and $p < 0.001$ at pregnancy day 12.5) (Figure S1, available online). For this strongly induced pregnancy gene expression signature, we confirmed by quantitative RT-PCR (qRT-PCR) analysis that the mRNA signal in *Pdx1-Cre*^{Late} islets was significantly higher than in control islets (Figure 3B). The two transcripts that encode the nonallelic paralogs of tryptophan hydroxylase, *Tph1* and *Tph2*, were highly induced in *Pdx1-Cre*^{Late} islets: 346.3 ± 69.4-fold ($p = 0.004$) and 14.3 ± 3.2-fold ($p = 0.011$), respectively. To assess the importance of the PRLR in the pregnancy switch in vivo, expression of the 12 genes in the gene expression signature was quantified in islets from Pdx1-Cre^{Late};PRLR$^{-/-}$ mice versus Pdx1-Cre^{Late} littermates by qRT-PCR. A significant reduction in gene expression was observed for most of the 12 genes in the Pdx1-Cre^{Late};PRLR$^{-/-}$ mouse model as compared to Pdx1-Cre^{Late} controls (Figure 3C), again stressing the crucial role of the PRLR in the pregnancy switch.

Because DOPA decarboxylase, the second enzyme needed for serotonin biosynthesis from tryptophan, is constitutively and highly expressed in mouse pancreatic islets, the strong up-regulation of the tryptophan hydroxylase step indicates that islets of *Pdx1-Cre*^{Late} mice are competent to synthesize serotonin under nonpregnant conditions. This is in contrast to control mice, which only produce serotonin during pregnancy. Therefore, we quantified islet serotonin content of control and *Pdx1-Cre*^{Late} mice under nonpregnant conditions and observed a striking difference (Figure 3D). This difference between mouse genotypes contrasted with islet gamma-amino butyric acid (GABA) (Figure 3E), a neurotransmitter that is constitutively produced by decarboxylation of glutamate in rodent and human β cells (Sorenson et al., 1991). Consequently, the islet serotonin/GABA molar ratio increased by at least one order of magnitude in islet extracts from the nonpregnant *Pdx1-Cre*^{Late} strain (Figure 3F). To analyze the serotonin production at the cellular level in nonpregnant *Pdx1-Cre*^{Late} mice, we performed immunostaining on pancreatic islet sections. A heterogeneous pattern of serotonin immunoreactivity was found in islet β cells of nonpregnant *Pdx1-Cre*^{Late} mice (Figure 3G), similar to the production observed in wild-type *C57BL/6J* mice during pregnancy (Schraenen et al., 2010b). No serotonin immunoreactivity was detected in nonpregnant control mice. Together, these data show that the normal serotonin biosynthetic pathway, observed in a subpopulation of β cells in the pancreas of pregnant mice, is induced independently of pregnancy in islets from *Pdx1-Cre*^{Late} mice.

Impaired Glucose Tolerance and Reduced Islet GLUT2 Expression in Pdx1-Cre^{Late} Mice

Overlap between genes that are downregulated in islets from pregnant wild-type *C57BL/6J* mice versus nonpregnant *Pdx1-Cre*^{Late} mice was much weaker (Figure 3A). One example was the mRNA encoding the glucose transporter GLUT2, which was repressed in *Pdx1-Cre*^{Late} islets (Figure 4A), but not in islets from pregnant mice. GLUT2 acts as a glucose sensor protein in rodent pancreatic β cells and is essential for normal glucose homeostasis in mice (Guillam et al., 1997; Thorens et al., 1988). This reduction in *Glut2* mRNA expression correlated with a strong reduction in immunoreactive protein on islet β cell membranes (Figure 4B) but contrasted with that of other genes involved in GSIS, which remained at the control level in islets isolated from *Pdx1-Cre*^{Late} mice (Figure S2A). The reduction of GLUT2 expression also coincided with a partial loss of GSIS with either 20 mM glucose or 20 mM glucose plus the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) as well as higher basal release (Figure 4C). This abnormality was associated with a slightly decreased glucose tolerance (Figure 4D) and lower circulating insulin levels (Figure 4E) after 2.5 mg/g body weight (BW) intraperitoneal (i.p.) glucose injection. Glucose intolerance was even more pronounced in older *Pdx1-Cre*^{Late} mice (20 weeks old) (Figure S2B). However, glycemia turned back to starting levels at the same time point as that for control mice. Together, these data indicate that in addition to some pregnancy-related phenotypic changes, local release of hGH induces other changes in pancreatic islets, as exemplified by reduced GLUT2 and partial loss of GSIS in *Pdx1-Cre*^{Late} islets.

Pdx1-Cre^{Late} Mice Are Protected against the Diabetogenic Toxin Streptozotocin

The GLUT2 transporter on the β cell surface is also responsible for the uptake of the β cell toxin streptozotocin (STZ) (Schnedl et al., 1994). Consequently, in control mice, which have very high levels of GLUT2 expression on β cells, STZ can induce an acute β cell necrosis after single injection of a high dose or chronically after multiple low-dose injections (Lenzen, 2008). As the *Glut2* gene expression is reduced in *Pdx1-Cre*^{Late} islets, the effect of STZ might be diminished. Moreover, PRL-induced activation of the JAK2/STAT5 pathway was reported to protect mice against multiple low doses of STZ (Holstad and Sandler, 1999; Jackerott et al., 2006). The effect of these two protective changes in islets of *Pdx1-Cre*^{Late} mice on blood glucose levels was tested in the multiple low-dose STZ model (50 mg/kg BW for 5 consecutive days and 1 month follow up of blood glucose levels; Figure 4F) as well as in the single high-dose STZ model (150 mg/kg BW and 5 days of follow up; Figure 4G). *Pdx1-Cre*^{Late} mice remained normoglycemic during the whole follow-up period in both models, whereas all control animals developed diabetes. However, the protection of *Pdx1-Cre*^{Late} mice against a single high-dose STZ injection was completely lost when *Pdx1-Cre*^{Late} mice were crossed to *Prlr*$^{-/-}$, further emphasizing the involvement of the PRLR in the hGH-induced phenotypic changes observed in *Pdx1-Cre*^{Late} mice (Figure 4H).

hGH and Serotonin Production Also Occurs in Islets from RIP-Cre and MIP-GFP Mice

Since many transgenic mouse models, in addition to the *Pdx1-Cre*^{Late} animals, incorporated a *hGH* minigene in their design (see Table 1), we also examined whether hGH protein is produced in *MIP-GFP* and *RIP-Cre* islets, two transgenic lines that are commonly used in studies of β cell biology and cellular mechanisms of diabetes. While expression of the *hGH* mRNA was expected given the placement of the gene sequences in the transgene construct (Figure 5A), hGH protein immunoreactivity was also observed (Figures 5B and S3). Furthermore, we found evidence for a pregnancy-related phenotypic switch as illustrated by a strong induction of *Tph1* mRNA (Figure 5A) and immunoreactive serotonin in islets from *MIP-GFP* and *RIP-Cre* islets (Figure 5C and quantified in Figure S4). Therefore, the presence and functional activity of hGH protein is not limited to *Pdx1-Cre*^{Late} mice, but also occurs in these two other lines. Similar to what was observed in the Pdx1-Cre^{Late} mice, 9-week-old *MIP-GFP* mice had lower blood glucose levels after an overnight fast (Figure S5A) and lower plasma insulin levels 30 min after the start of the glucose tolerance test (Figures S5B and S5C), whereas insulin sensitivity was normal (Figure S5D).

DISCUSSION

The Cre/LoxP system is a powerful tool to conditionally inactivate or overexpress genes in transgenic animals with a number of specific advantages over whole-body knockouts. However, there have been many reports describing specific limitations of some Cre driver strains. These include variegated Cre expression in target organs (Gannon et al., 2000; Ryding et al., 2001), ectopic expression in undesired tissues (Delacour et al., 2004; Song et al., 2010; Wicksteed et al., 2010), and unwanted effects related to the integration site (Cartwright and Wang, 2009). For central nervous system and pancreas-specific Cre driver lines, these limitations and their likely causes have been reviewed recently (Harno et al., 2013; Magnuson and Osipovich, 2013). In particular for the *RIP-Cre* mouse model (Postic et al., 1999), a multicenter study showed glucose intolerance in these mice in the absence of genes targeted by loxP sites (Lee et al., 2006). Moreover, younger *RIP-Cre* mice exhibited β cell hypoplasia, whereas older mice showed β cell hyperplasia (Pomplun et al., 2007).

In the present study, we describe a mechanism whereby inclusion of an *hGH* minigene as a component of the transgene construct can impair β cell function. Since the *hGH* minigene is the second cistron in the transgene-encoded mRNA, it was believed for a long time that it was not expressed. Our studies provide compelling data otherwise. Expression of hGH may have a profound influence on the interpretation of certain types of experiments, especially those pertaining to the control of pancreatic β cell mass and the regulation of insulin secretion, both of which are very active fields of diabetes research.

In islets from *Pdx1-Cre*^{Late}, the *MIP-GFP*, and the *RIP-Cre* mouse models, we observed expression of hGH from the *hGH* minigene sequences placed downstream of the *Cre* or *GFP* coding regions (Brinster et al., 1988). As a result, hGH of the expected molecular weight (22 kDa) is synthesized and secreted from islets of these mice, causing Cre- or GFP-independent effects by autocrine/paracrine stimulation of β cells. An insertional effect has been postulated before (Lee et al., 2006; Pomplun et al., 2007) but is unlikely since we observe *Tph1* expression and serotonin immunoreactivity in two other frequently used mouse lines (*RIP-Cre* and *MIP-GFP*). Figure 6 proposes a model in which locally secreted hGH acts as a lactogen by activating abundantly expressed PRLR on mouse β cells and initiating the JAK2/STAT5 signaling pathway. A first group of effects is therefore pregnancy like and includes induction of serotonin biosynthesis and a doubling of β cell mass and pancreatic insulin content. The most important of pregnancy-unrelated effects is a partial loss of GSIS, decreasing glucose tolerance. This loss of function could be partially caused by downregulation of GLUT2, a transporter responsible for the rapid equilibration between extra- and intracellular glucose concentration and thus the first step of glucose sensing in rodent islets (Guillam et al., 1997). This is in contrast with the pregnancy state, as β cell *Glut2* levels have been reported to increase as part of the adaptive maternal response (Weinhaus et al., 1996). GLUT2 is also needed for rapid uptake of the β cell toxins STZ (Schnedl et al., 1994) and alloxan (De Vos et al., 1995), so it seems reasonable to attribute the protection of *Pdx1-Cre*^{Late} islets against STZ-induced diabetes to reduced toxin uptake rates caused by fewer GLUT2 channels (Figure 5). In addition, enhanced β cell mass and greater potential to regenerate new β cells may also be involved.

As the *hGH* minigene is frequently used to enhance expression of transgenes, we did a literature search for mouse lines generated using the same genetic strategy (Table 1). At the time of preparation of this manuscript, we listed a total of 22 mouse models. Due to the lack of information available for several other β cell-specific mouse strains, our table may be an underestima tion of the total amount of models involved. In some of the models listed in Table 1, expression of the *hGH* minigene was mentioned, at the level of either mRNA (Miyazaki et al., 2010; Postic et al., 1999; Sanvito et al., 1995) or immunoreactive protein (Klee et al., 2011). In none of them, however, was the potential influence on pancreatic islet morphology, β cell function, or glucose homeostasis considered.

The investigation of cellular and physiological mechanisms that regulate β cell mass and β cell function is currently a very active area of diabetes research that makes extensive use of genetically altered mice. Moreover, there is already a large body of experimental work that has made use of mice containing *hGH* minigene. Our current observations therefore seem to have potential implications on the interpretation of a large body of published data, in particular with respect to changes in functional β cell mass, insulin secretion, and protection against the diabetogenic effect of STZ.

EXPERIMENTAL PROCEDURES

Mice

Pdx1-Cre^{Late} transgenic mice (Herrera, 2000) (Dr. Herrera, University of Geneva, Switzerland) were crossed with *C57BL/6J* (Janvier) for at least eight generations. PRLR knockout mice (designated *Prlr*$^{-/-}$) on a 129Sv background were described previously (Freemark et al., 2002). *Pdx1-Cre*^{late} females were bred with *Prlr*$^{+/-}$males to generate *Pdx1-Cre*^{Late}; *Prlr*$^{+/-}$mice, which were subsequently crossed with *Prlr*$^{+/-}$ to obtain *Pdx1-Cre*^{Late}; *Prlr*$^{-/-}$ mice. Institutional guidelines for animal welfare and experimental conduct were followed. All experiments with laboratory animals were approved by the committee for animal welfare at the KU Leuven. *RIP-Cre* mice (Postic et al., 1999) and *MIP-GFP* mice (Hara et al., 2003) were maintained on C57Bl and CD1 backgrounds, respectively. All animal procedures and husbandry were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Cell Cultures

MIN6 cells were incubated in Dulbecco's modified Eagle's medium (DMEM; 25 mmol/l glucose, 2% fetal calf serum [FCS], 4 mmol/l glutamax) (Invitrogen, Gibco) with hGH (Calbiochem), mGH, or oPL (Prospec). For the experiments with the PRLR antagonist Δ1-9-G129R-hPRL MIN6 cells were preincubated for 30 min with the antagonist. Thereafter, recombinant hGH (from Vincent Goffin) was added to the medium. RNA was extracted 24 hr later.

Islet Isolation

Pancreatic islets were isolated after infusion and digestion of the pancreata by collagenase P (Roche) as described previously (Lemaire et al., 2009).

Islet Monolayers

Islet monolayers were performed as described previously (Schraenen et al., 2010b). Isolated islets were cultured for 7 days in RPMI medium (10% [v/v] de-complemented FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mmol/l glutamax, 10 mM HEPES [pH 7.4]) to form monolayers. On day 7, they were stimulated with 0 or 500 ng/ml oPL, mGH, or hGH.

Microarray Expression Analysis

Microarray analysis was performed on RNA of isolated islets using MoGene_1.0_ST arrays (Affymetrix). For RNA extraction, see Supplemental Experimental Procedures. Total islet RNA (100 ng) was used to hybridize the arrays according to manufacturer's manual 701880Rev4 as described previously (Lemaire et al., 2009). Samples were analyzed pairwise, using $p < 0.001$ and fold change ≥ 1.5 as selection criteria. A list of up- and downregulated genes in islets isolated from *Pdx1-Cre*^{Late} transgenic mice versus control mice is provided as an excel table (see Table S1), and genes related to pregnancy are marked.

Quantitative RT-PCR

Following cDNA synthesis using a reverse transcriptase kit (RevertAid H Minus; Fermentas), qRT-PCR (Absolute QPCR mix; Abgene, Thermo Fisher Scientific) was performed on a Rotorgene (Corbett Research). For primers and probes, see Supplemental Experimental Procedures. For *Tph2*, a Taqman gene expression assay (Mm00557717_m1;

Applied Biosystems) was used. When a probe was used, data were analyzed according to the Pfaffl method; without a probe, delta Ct was used.

Histology

Pancreata were fixed overnight in 4% formaldehyde and embedded in paraffin. Sections were rehydrated and heated for 20 min in Target Retrieval Solution (pH 6.1, Dako). After blocking with 20% normal goat serum (Dako) in PBS, slides were incubated with 1/1,000 anti-hGH monoclonal antibody (ab15317, Abcam) in Antibody Diluent (Dako). For double immunofluorescent labeling, 1/50,000 rabbit anti-serotonin (Immunostar, #20080) or 1/2,000 polyclonal anti-GLUT2 (07-1402, Millipore) was combined with 1/10,000 diluted guinea pig anti-insulin antibody (a gift of Dr. Van Schravendijk, VUB, Brussels) and detected with anti-rabbit Cy3 and anti-guinea pig FITC, respectively (both from Jackson ImmunoResearch Laboratories). For *MIP-GFP* and *RIP-Cre* mice, frozen sections were stained with 1/10,000 rabbit anti-serotonin or 1/200 mouse anti-hGH (blocking with Mouse on Mouse [M.O.M.] Basic Kit from Vector Laboratories) and costained with 1/2,000 guinea pig anti-insulin in sections from *RIP-Cre* mice. For *MIP-GFP*, direct fluorescence for GFP was used to detect β cells.

Glucose and Insulin Tolerance Tests

Overnight (glucose tolerance test [GTT]) or 6 hr fasted (insulin tolerance test [ITT]) mice were injected i.p. with 2.5 mg/g BW D-glucose or 0.75 mU/g BW human insulin, respectively, and glycemia was measured by tail-blood analysis using a Contour glucose meter (Bayer). For GTT, tail blood was collected at the indicated time points, and plasma was analyzed.

Islet Serotonin and GABA Content

A total of 80 islets were homogenized by 3 min sonication in a buffer containing 0.01 mol/l HCl, 1 mmol/l EDTA, and 4 mmol/l sodium metabisulfite. After centrifugation (20,000 × *g*) for 15 min at 4°C and addition of 0.1% (w/v) ascorbic acid (Acros), lysates were stored at −80°C. Serotonin and GABA concentrations were determined via high-performance liquid chromatography (HPLC) (see Supplemental Experimental Procedures).

Islet hGH Content and Release

Freshly isolated islets were incubated in batches of 100 at 37°C. Incubation was in HEPES Krebs buffer (20 mmol/l HEPES [pH 7.4], 119 mmol/l NaCl, 4.75 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 5 mmol/l $NaHCO_3$, 0.5% [w/v] BSA) containing 20 mmol/l glucose. After 1 hr, half of the medium was removed for measurement of hGH release. For the content, Triton X-100 (final concentration: 0.5%) was added to the other half of the medium with the islets. Islets were sonicated for 3 min, and lysates were stored at −20°C. To measure concentrations, an hGH ELISA was used (Invitrogen).

Islet Insulin Content and Release

For insulin secretion measurements, size-matched islets (n = 5 per tube) were placed in glass tubes containing HEPES Krebs solution containing 0.5% BSA supplemented with glucose 5 mM (G5), 20 mM (G20), or G20 with 250 μM IBMX. Supernatant was collected after 1 hr incubation at 37°C. The islets were sonicated for 3 min after adding acid ethanol (final concentration: 75% EtOH, 0.1 N HCl, 1% Triton). Samples were stored at −20°C, and the ELISA kit used for insulin determination was from Crystal Chem.

Total Pancreas Insulin Content

Pancreata were dissected, and acid-ethanol extracts were diluted 1/1,000 in PBS and analyzed for insulin using an insulin high-range ELISA (Mercodia). Absolute insulin content per pancreas as well as relative content (corrected for pancreas weight) were quantified.

β Cell Mass Quantification

Total pancreas from 24-week-old *Pdx1-Cre*^{late} and littermate control mice (four males per group) was processed, and six sections separated by 200 μm were stained for insulin as described above. The total surface area of insulin-positive cells (in pixels) was quantified with Zeiss Axiovision software (Micro Imaging). The relative insulin surface area per section (total insulin area [pixels]/total pancreas area [pixels]) was multiplied by the pancreas weight (mg) to obtain the β cell mass (mg).

Western Blot

Islets were isolated as described above and homogenized in lysis buffer (Cell Signaling Technology) by sonication. Protein extracts were separated by SDS-PAGE (10% [v/v] Bis/Tris gel; Life Technologies), blotted on a nitrocellulose membrane, blocked in 4% (w/v) milk, and incubated with primary antibody (anti-hGH, ab15317, 1/1,000; anti-GAPDH, 1/15,000; clone 6C5, both from Abcam). The blot was subsequently incubated with peroxidase-conjugated secondary antibody (Dako), and proteins were detected using the Western Lightning ECL System (PerkinElmer). For hGH staining on islets from *MIP-GFP* and *RIP-Cre* mice, a similar protocol was used with adjustments: 10 μg of islet protein sample in 1× Laemmli sample buffer was resolved on 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore).

Statistical Methods

When not differently stated in the legend or text, data are presented as mean ± SEM, and significance is shown on graphs as *$p < 0.05$, **$p < 0.01$, or $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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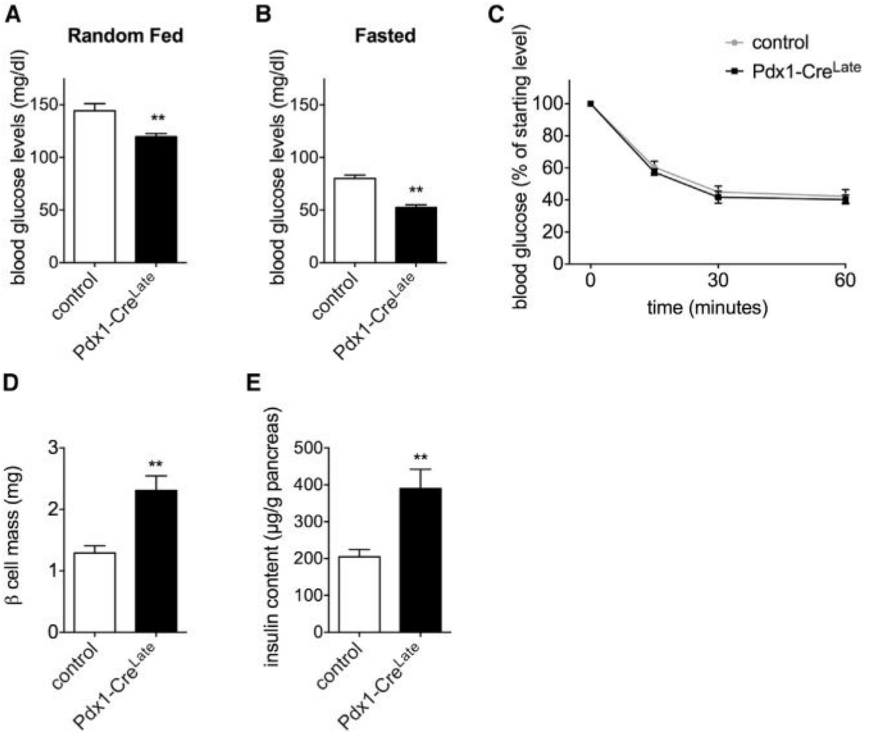

Figure 1. Increased β Cell Mass and Insulin Content in Nonpregnant *Pdx1-Cre*^{Late} Mice

(A and B) Blood glucose levels in random-fed (A) and fasted (B) *Pdx1-Cre*^{Late} versus control mice. Data are represented as mean ± SEM, n = 7 mice per genotype, **p < 0.01.

(C) Insulin tolerance test (ITT) on 10-week-old *Pdx1-Cre*^{Late} versus control mice. Values are presented as a percentage compared to the starting glucose level. Data are represented as mean ± SEM, n = 7–8 mice per genotype.

(D) Quantification of β cell mass in 24-week-old *Pdx1-Cre*^{Late} versus control mice, performed as described in the Experimental Procedures. Data are represented as mean ± SEM, n = 4 mice per genotype, **p < 0.01.

(E) Pancreatic insulin content in 24-week-old *Pdx1-Cre*^{Late} versus control mice. Data are represented as mean ± SEM, n = 9–10 mice per genotype, **p < 0.01.

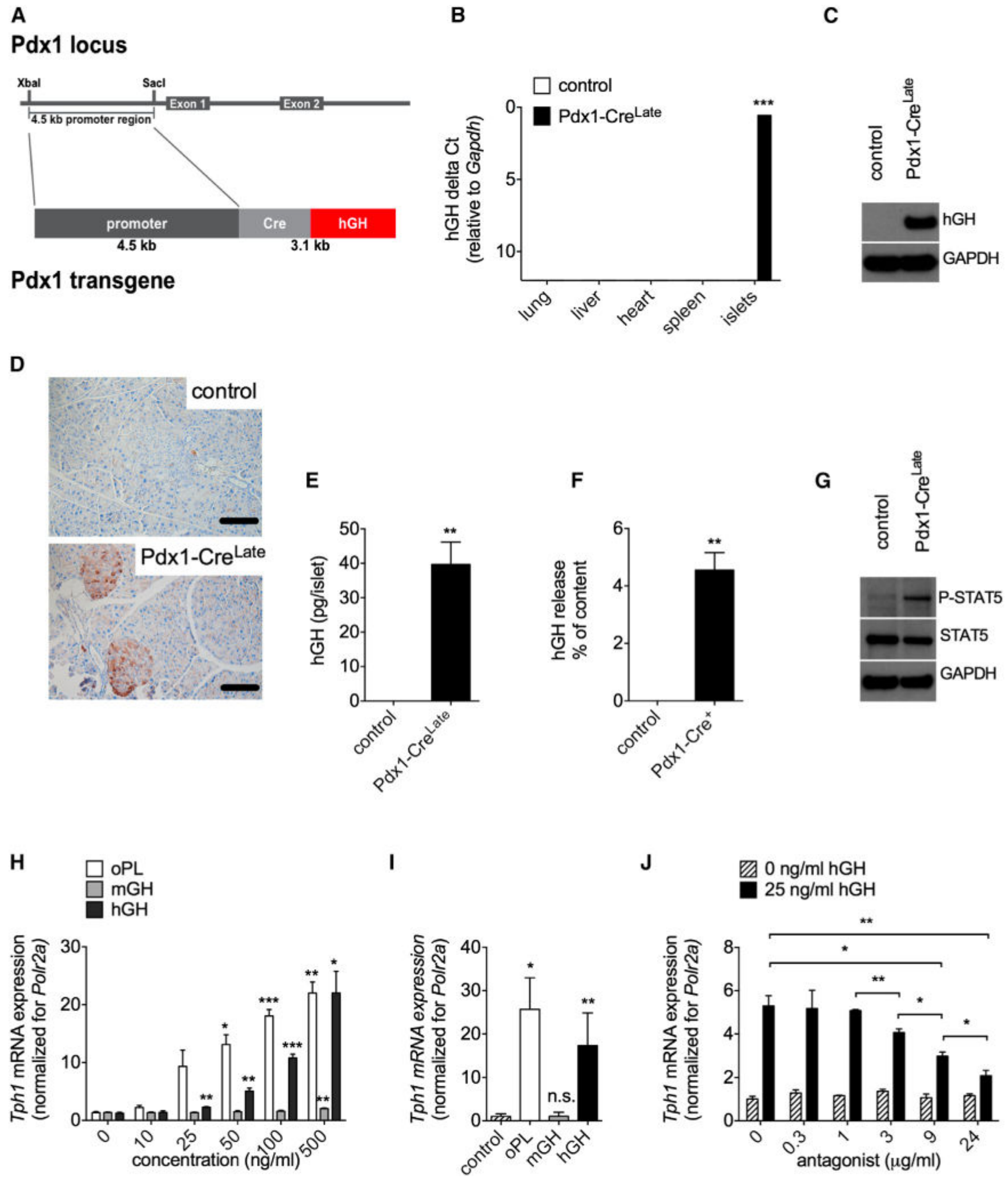

Figure 2. Pregnancy-Related Phenotypic Changes Are Caused by Local Production and Secretion of hGH in Islets from Nonpregnant *Pdx1-Cre*^{Late} Mice

(A) Schematic representation of the *Pdx1-Cre* construct used to generate the *Pdx1-Cre*^{Late} mouse model. A 4.5 kb *Pdx1* promoter fragment was inserted upstream of the *Cre* transgene. The *hGH* minigene, containing introns, exons, and polyadenylation signal, is located directly downstream of the *Cre* fragment and indicated in red. This figure was based on the materials and methods from Herrera (2000).

(B) qRT-PCR analysis of *hGH* mRNA in different tissues, including isolated islets from control and nonpregnant *Pdx1-Cre*^{Late} mice. Data were quantified as delta Ct values (relative to *Gapdh*). Data are represented as mean ± SEM, n = 3–6 per condition, $***p < 0.001$.

(C) Western blot analysis of hGH expression in islets of control and nonpregnant *Pdx1-Cre*^{Late} mice. GAPDH was used as a loading control.

(D) Immunoreactive hGH in pancreatic sections from control and nonpregnant *Pdx1-Cre*^{Late} mice. The hGH signal is most intense in pancreatic islets and not uniformly distributed over their different islet cells. Scale bar, 100 μm.

(E and F) Quantification of hGH content (E) and release (F) (measured as the percentage of cellular content per hour) in islets isolated from *Pdx1-Cre*^{Late} mice. For the release experiments, isolated islets were incubated with 20 mM D-glucose for 1 hr. Data are represented as mean ± SEM, n = 3 per condition, $**p < 0.01$.

(G) Representative immunoblot of islet phospho-STAT5 (P-STAT5) and total STAT5 protein in islets from control and nonpregnant *Pdx1-Cre*^{Late} mice. GAPDH was used as a loading control. Mean density ratios of P-STAT5/total STAT5 were increased 3.72 ± 0.38-fold in *Pdx1-Cre*^{Late} islets compared to controls. Data are represented as mean ± SEM, n = 3 per condition, $p = 0.005$.

(H and I) Induction of *Tph1* expression in MIN6 cells (H) and primary islet monolayers (I) by hGH and oPL. Cells were treated with the indicated concentrations of oPL, mGH, or hGH, and islet monolayers with vehicle (control) or 500 ng/ml oPL, mGH, or hGH. Expression of *Tph1* was quantified by qRT-PCR. *Polr2a* was used as a reference gene. Data were calculated via the Pfaffl method, and the ratio of the control sample was normalized to 1. Data are represented as mean ± SEM, n = 3–5 independent measurements per condition; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

(J) Inhibition of hGH-induced *Tph1* expression in MIN6 cells by a specific PRLR inhibitor. MIN6 cells preincubated (30 min) with different concentrations of PRLR-antagonist Δ1-9-G129R-hPRL were stimulated with 0 or 25 ng/ml hGH; *Tph1* mRNA expression was used as readout for the pregnancy signature, and *Polr2a* was used as a reference gene. Data were calculated via the Pfaffl method, and the average ratio of 0 ng/ml hGH was set to 1. Data are represented as mean ± SEM, n = 3 per condition; $*p < 0.05$, $**p < 0.01$.

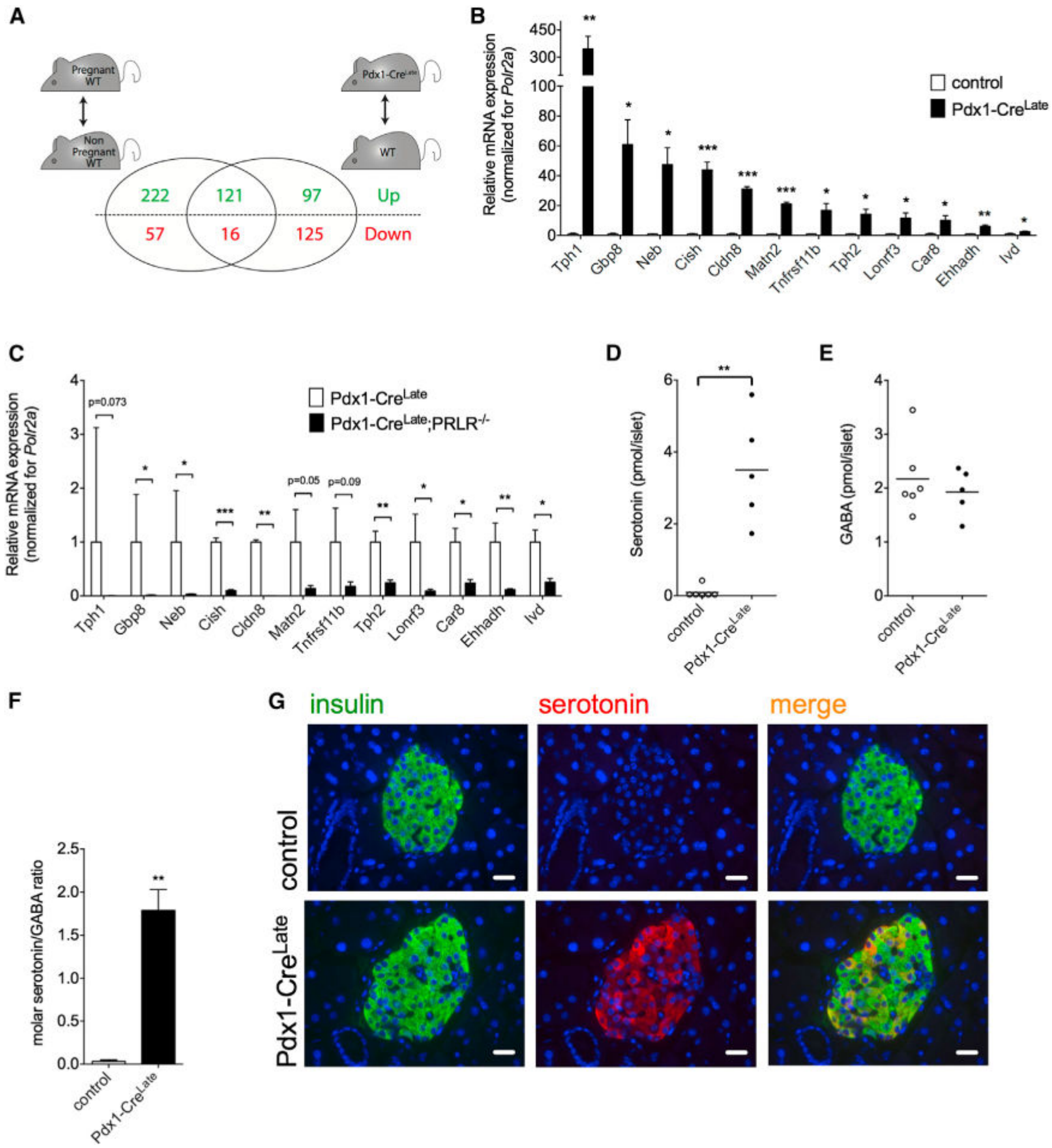

Figure 3. Pregnancy-Related Phenotypic Switch in Islets Isolated from Nonpregnant *Pdx1-Cre^{Late}* Mice

(A) Microarray analysis of islet mRNA expression. A substantial overlap of >100 differentially expressed genes ($p < 0.001$ and fold change of ≥1.5) was found when comparing pregnant versus nonpregnant mice on the one hand (left) and nonpregnant *Pdx1-Cre^{Late}* mice versus nonpregnant control mice on the other hand (right). This overlap contains almost 10-fold more upregulated than downregulated genes.

(B) qRT-PCR analysis of the pregnancy gene signature in nonpregnant *Pdx1-Cre^{Late}* versus control mice. For each of the top 12 upregulated genes during pregnancy at P12.5, we observed significant upregulation in islets from nonpregnant *Pdx1-Cre^{Late}* mice. *Polr2a* was used as a reference gene. Data were calculated via the Pfaffl method, and the average ratio of each gene was set to 1 for the control mice. Two strongly induced genes in islets from *Pdx1-*

Cre^{Late} mice encode the two isoforms of tryptophan hydroxylase (TPH1 and TPH2). Data are represented as mean ± SEM, n = 4–6 per group; *p < 0.05, **p < 0.01, ***p < 0.001.

(C) qRT-PCR analysis on all 12 genes from the pregnancy gene expression signature, in islets from Pdx1-Cre^{Late};PRLR$^{-/-}$mice versus Pdx1-Cre^{Late} littermates, n = 3 mice per genotype. Data are represented as mean ± SEM, n = 3 per group, *p < 0.05, **p < 0.01, ***p < 0.001.

(D–F) Quantification of serotonin and GABA in islets isolated from nonpregnant control and nonpregnant *Pdx1-Cre*^{Late} mice. (D) Basal islet serotonin levels are near the detection limit of the assay in control mice and dramatically upregulated in nonpregnant *Pdx1-Cre*^{Late} mice. (E) In contrast, GABA is as abundant in islets of control as in islets of nonpregnant *Pdx1-Cre*^{Late} mice. (F) Consequently, the molar serotonin/GABA ratio is about 100-fold higher in islets from *Pdx1-Cre*^{Late} mice compared to control islets. Data are represented as mean ± SEM, n = 5–6 per group; *p < 0.05, **p < 0.01, ***p < 0.001.

(G) Heterogeneous serotonin immunoreactivity in islets from nonpregnant *Pdx1-Cre*^{Late} mice. No serotonin immunoreactivity is detected in control mice, while in nonpregnant *Pdx1-Cre*^{Late} mice serotonin is only present in islet β cells with marked differences between neighboring β cells. Data are representative sections from pancreata analyzed from five nonpregnant control mice and four nonpregnant *Pdx1-Cre*^{Late} mice (scale bar, 20 μm). See also Figure S1 and Table S1.

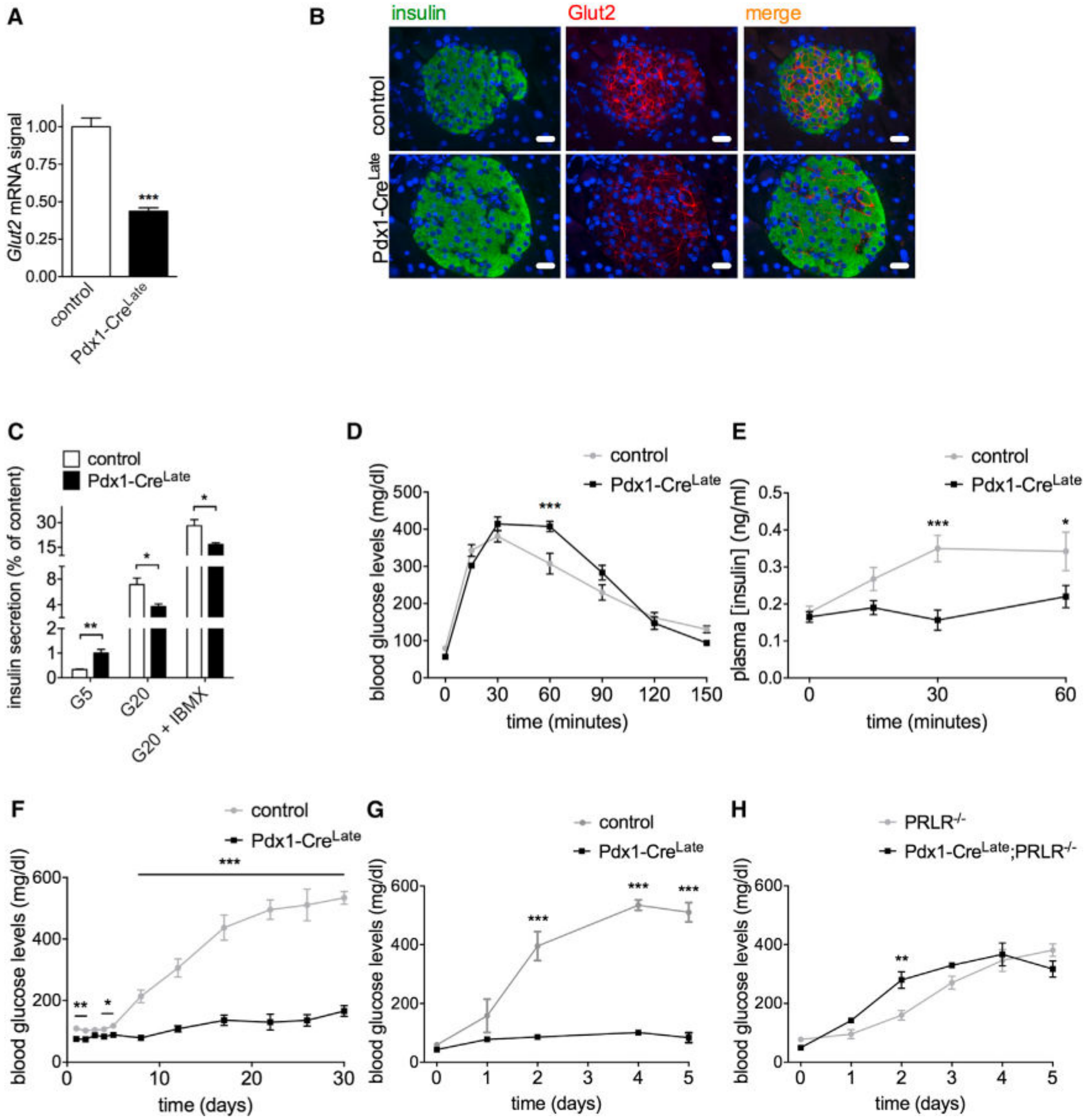

Figure 4. Reduced Islet GLUT2 Expression, Loss of GSIS, and Protection against STZ in *Pdx1-Cre^{Late}* Mice

(A) *Glut2* mRNA signal in control versus *Pdx1-Cre^{Late}* islets. *Gapdh* was used as a reference gene. Data are represented as mean ± SEM, n = 3–4 mice per genotype, $^{***}p < 0.001$.

(B) Representative immunofluorescence staining for insulin (green signal), GLUT2 (red signal), and merge on pancreatic sections from control versus *Pdx1-Cre^{Late}* mice. Overall, the GLUT2 signal is much weaker in *Pdx1-Cre^{Late}* islets compared to controls. Scale bar, 20 µm.

(C) Ex vivo insulin secretion assay. Islets from *Pdx1-Cre^{Late}* and control mice were isolated and incubated for 1 hr with 5 mM (G5), 20 mM (G20), or 20 mM D-glucose with the phosphodiesterase inhibitor IBMX (G20 + IBMX) Insulin release was quantified as the percentage of insulin secreted compared to islet insulin content. Data are represented as mean ± SEM, n = 6 mice per genotype; $^{*}p < 0.05$, $^{**}p < 0.01$.

(D) Intraperitoneal glucose tolerance test (IPGTT) on 10-week-old *Pdx1-Cre*^{Late} versus control mice, n = 5 per genotype. Overnight fasted mice were injected i.p. with 2.5 mg/g BW D-glucose and blood glucose levels were measured at the indicated time points. Data are represented as mean ± SEM, n = 5 mice per genotype; ***$p < 0.001$, repeated measures ANOVA.

(E) Circulating plasma insulin levels in blood samples obtained at the indicated time points from IPGTT. Data are represented as mean ± SEM, n = 5 mice per genotype; *$p < 0.05$, ***$p < 0.001$, repeated measures ANOVA.

(F) Multiple low-dose (MLD) treatment of the diabetogenic agent streptozotocin (STZ) in *Pdx1-Cre*^{Late} versus control mice. Mice aged 12 weeks were injected i.p. with 50 mg STZ/kg BW for 5 consecutive days, and random-fed blood glucose levels were measured at the indicated time points. Data are represented as mean ± SEM, n = 5 mice per genotype; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, repeated measures ANOVA.

(G) Single high dose of STZ in *Pdx1-Cre*^{Late} versus control mice. Mice were injected i.p. with 150 mg STZ/kg BW, and fed blood glucose levels were measured. Data are represented as mean ± SEM, n = 5–6 mice per genotype; ***$p < 0.001$, repeated measures ANOVA.

(H) Single high dose (150 mg/kg BW) of STZ in *Prlr*$^{-/-}$ versus *Pdx1-Cre*^{Late}; *Prlr*$^{-/-}$ mice. Data are represented as mean ± SEM, n = 3–4 mice per genotype; **$p < 0.01$, repeated measures ANOVA. See also Figure S2.

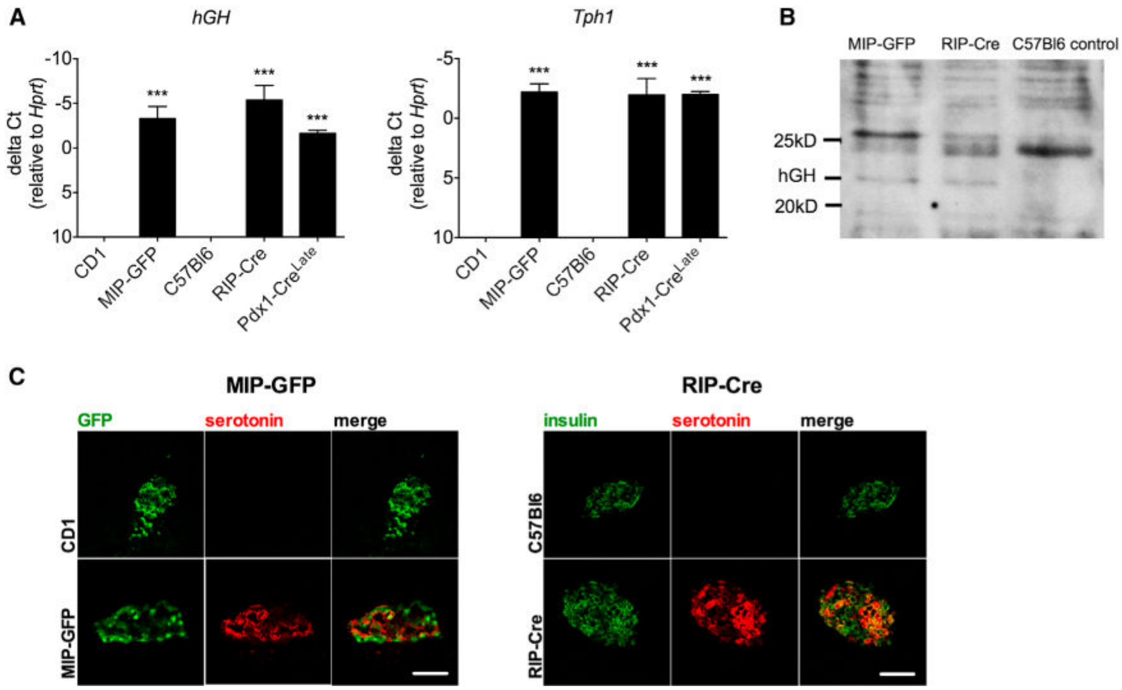

Figure 5. Growth Hormone and *Tph1* Expression and Islet Serotonin Immunoreactivity in Pancreatic Islets from Two Other Commonly Used Mouse Driver Strains, *MIP-GFP* and *RIP-Cre*

(A) *hGH* and *Tph1* mRNA signal in isolated islets from *MIP-GFP* and *RIP-Cre* mice, quantified as delta Ct values relative to a housekeeping mRNA signal (*Hprt*). Data are represented as mean ± SEM, n = 3–6 mice per genotype, $^{***}p < 0.001$.

(B) Western blot analysis of hGH expression in islets from *MIP-GFP*, *RIP-Cre*, and *C57Bl6* control mice. Predicted hGH weight = 22 kDa. GAPDH was used as a loading control. See also Figure S3.

(C) Representative immunofluorescence micrographs show serotonin immunoreactivity in islets from *MIP-GFP* and *RIP-Cre* mice, whereas no signal was observed in respective littermate controls. Scale bar, 50 µm. See also Figures S4 and S5.

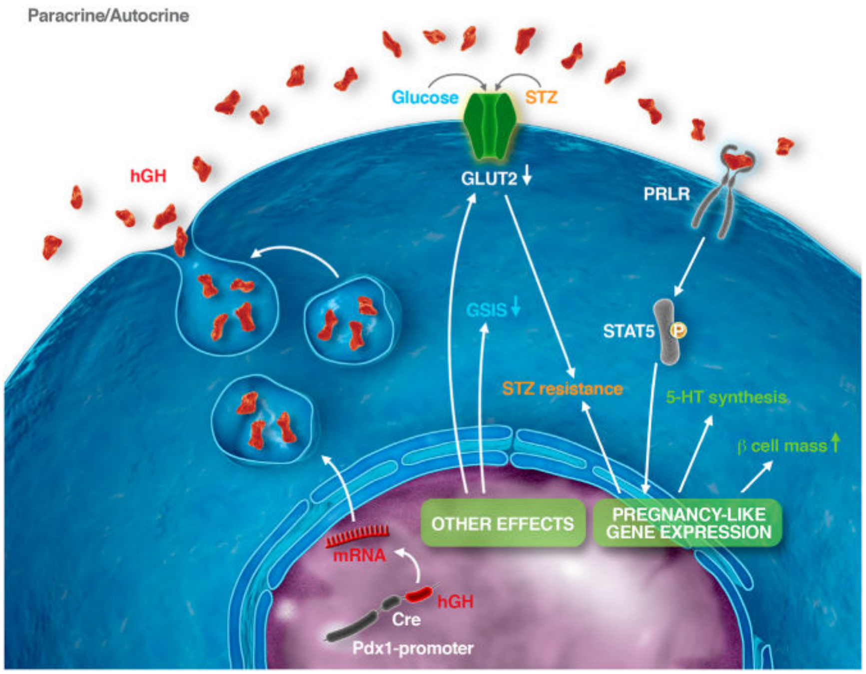

Figure 6. Model of hGH-Induced Phenotypic Changes in *Pdx1-Cre*^{Late} β Cells

Pdx1-promoter-driven expression of the *hGH* minigene causes biosynthesis and secretion of hGH, which exerts autocrine or paracrine effects after binding to PRLR on β cells. This causes STAT5 phosphorylation and pregnancy-like phenotypic changes, such as enhanced β cell mass and serotonin (5-HT) production. In addition, expression of the *hGH* minigene causes pregnancy-unrelated changes, such as reduction of GLUT2 expression and partial loss of glucose-induced insulin release. Lower GLUT2-mediated uptake and higher β cell mass protect against the diabetogenic effect of the β cell toxin STZ.

Table 1

β Cell-Specific Transgenic Mice Generated Based on a Similar Strategy, Namely Using the hGH Minigene as Transgene Enhancer

Common Strain Name	**Reference**
Ins-rtTA/TetO-RXRβΔC2	Miyazaki et al., 2010
MIP-CFP	Hara et al., 2006
MIP-Cy3.3er	Hara et al., 2004
MIP-GFP	Hara et al., 2003
MIP-HIMP1	Zhang et al., 2012
MIP-hProCpepGFP	Hodish et al., 2010
MIP-Luc	Park et al., 2005
MIP-Phogrin-pHluorin-mCherryhip-rxr	Lu et al., 2009
MIP-RFP	Hara et al., 2006
MIP-sr39tk	McGirr et al., 2011
Pdx1-Cre^{Late}	Herrera, 2000
Pdx1-eGFP	Sylvestersen et al., 2011
RIP-Cx32	Charollais et al., 2000
RIP-Cx43	Klee et al., 2011
RIP-HGF	Garcia-Ocaña et al., 2000
RIP-PL	Vasavada et al., 2000
RIP-PTHrP	Vasavada et al., 1996
RIP-TGFb1	Sanvito et al., 1995
RIP-VegfA165	Gannon et al., 2002
RIP1-Podo	Wicki et al., 2006
RIP1-VEGFD	Kopfstein et al., 2007
RIP2-Cre	Postic et al., 1999

This table includes the names of the strains that were identified and the references of the first report.