Serotonin Regulates Adult β-Cell Mass by Stimulating Perinatal β-Cell Proliferation

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A sufficient β-cell mass is crucial for preventing diabetes, and perinatal β-cell proliferation is important in determining the adult β-cell mass. However, it is not yet known how perinatal β-cell proliferation is regulated. Here, we report that serotonin regulates β-cell proliferation through serotonin receptor 2B (HTR2B) in an autocrine/paracrine manner during the perinatal period. In β-cell–specific *Tph1* knockout (*Tph1* βKO) mice, perinatal β-cell proliferation was reduced along with the loss of serotonin production in β-cells. Adult *Tph1* βKO mice exhibited glucose intolerance with decreased β-cell mass. Disruption of *Htr2b* in β-cells also resulted in decreased perinatal β-cell proliferation and glucose intolerance in adulthood. Growth hormone (GH) was found to induce serotonin production in β-cells through activation of STAT5 during the perinatal period. Thus, our results indicate that GH-GH receptor-STAT5-serotonin-HTR2B signaling plays a critical role in determining the β-cell mass by regulating perinatal β-cell proliferation, and defects in this pathway affect metabolic phenotypes in adults.

Diabetes develops when β-cells fail to meet insulin demand; a sufficient β-cell mass is crucial for preventing diabetes, and people with decreased β-cell mass have increased susceptibility to type 2 diabetes (1,2). Despite the importance of the adult β-cell mass, it is not yet clear how a proper amount of β-cell mass is achieved.

During fetal development, insulin-producing β-cells differentiate from endocrine progenitor cells, and the newly developed β-cells actively proliferate during the perinatal period to achieve the adult β-cell mass (3). After the perinatal period, β-cells maintain a low proliferation rate unless sustained metabolic stress increases systemic insulin demand. Therefore, perinatal β-cell proliferation is important in determining the adult β-cell mass and susceptibility to type 2 diabetes. However, we know little

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about how β-cell proliferation is physiologically regulated during the perinatal period.

Serotonin (5-hydroxytryptamine [5-HT]) is a neurotransmitter that is synthesized from tryptophan by the sequential actions of tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase. 5-HT exerts its biological action by binding to 5-HT receptor (HTR) (4,5). Two isoforms of TPH are expressed in a mutually exclusive manner: TPH1 in peripheral nonneuronal tissues and TPH2 in neurons of the central and enteric nervous systems (4,5). In pancreatic β-cells, TPH1 expression and 5-HT production are simultaneously increased during pregnancy. 5-HT released from β-cells acts locally to increase β-cell proliferation and insulin secretion through HTR2B and HTR3, respectively (6,7). Intriguingly, 5-HT production in β-cells also increases robustly during the perinatal period (6,8). These data prompted us to explore the physiological role of 5-HT in perinatal β-cell expansion.

Here, we show that 5-HT regulates the adult β-cell mass by stimulating perinatal β-cell proliferation through HTR2B in an autocrine/paracrine manner. Growth hormone (GH), but not prolactin (PRL), induces 5-HT production in β-cells during the perinatal period. Human β-cells also produce 5-HT during the perinatal period, suggesting that 5-HT may contribute to determining the human β-cell mass and susceptibility to diabetes.

RESEARCH DESIGN AND METHODS

Animal Experiments

All mice used in this study were C57BL/6J background strain. Mice were housed in a specific-pathogen–free facility at the Korea Advanced Institute of Science and Technology, with 12-h light and dark cycles under isothermal and isohumid conditions. Normal chow diet (SCD, D10001; Research Diets) and water were given ad libitum. To generate β-cell–specific knockout (βKO) mice of *Tph1*, *Htr2b*, *Prlr*, and *Stat5*, *Ins2-Cre* (MGI 2387657) mice were mated with mice harboring a *Tph1* floxed allele (MGI 6271993), an *Htr2b* floxed allele (MGI 3837400), a *Prlr* floxed allele, and a *Stat5* floxed allele (MGI 3055318), respectively (9–14). To generate *Ghr* βKO mice, *Ins2-Cre* mice were mated with mice harboring a *Ghr* floxed allele (MGI 5056123) (15,16). All Cre mice used in this study lacked the GH minigene, which has been shown to induce ectopic 5-HT production (17,18). *Htr1b*$^{tm1.1(KOMP)Vlcg}$ (MGI 5463939) and *Htr1d*$^{tm1.1(KOMP)Vlcg}$ (MGI 5529062) mice were purchased from KOMP. For experiments involving adult mice, 9-week-old male mice were used. For high-fat diet (HFD) studies, the diet (60% kcal fat, D12492; Research Diets) was fed to 12-week-old male mice for 8 weeks. For the S-961 (insulin receptor antagonist; Novo Nordisk) study, 100 nmol/kg mouse was given twice a day intraperitoneally for 7 days. Given that glucose levels are comparable in Cre mice and floxed control mice, Cre littermates of the experimental (KO) mice were used as the control, unless otherwise stated.

Human Pancreatic Tissues

Human pancreas autopsy specimens were obtained from the archives of the Department of Pathology and Molecular Medicine, Hirosaki University. All specimens were formalin-fixed and paraffin-embedded for further staining. Conventional hematoxylin and eosin (H&E) staining was performed in all cases. Fetal cases were from a stillborn baby with no apparent malformations on the outer surface. An 8-day-old sample was obtained from a male newborn baby whose cause of death was cardiopulmonary insufficiency secondary to osteogenesis imperfecta. A 3-month-old sample was obtained from a female infant whose cause of death was sudden infant death syndrome. Pancreas samples from a 35-year-old pregnant woman (gestational age 37 weeks; cause of death, pulmonary embolism) and a 72-year-old man (cause of death, myocardial infarction) were used as controls. The use of paraffin blocks and the study design were approved by the ethics committee of the Hirosaki University School of Medicine (approval number #2014-269), and the study conformed to the provisions of the Declaration of Helsinki.

Immunostaining and β-Cell Area Measurements

Mouse pancreas tissues were harvested and fixed in 10% neutral-buffered formalin solution (Sigma-Aldrich) for 2 h (perinatal) or 4 h (adult) at room temperature and washed with deionized water for 30 min. After washing, the tissues were processed with an automatic tissue processor (TP1020; Leica Biosystems) and embedded in molten paraffin wax. Paraffin-embedded tissues were sliced at 4-μm thickness and mounted on adhesive glass slides (081000; Paul Marienfeld). Sections were manually deparaffinized in xylene and rehydrated in ethanol for further staining. Antigen retrieval was performed by incubating the slides in sodium citrate buffer (10 mmol/L sodium citrate, pH 6.0) with a pressure cooker for 15 min. For immunofluorescent staining, samples were blocked with 2% donkey serum in PBS for 1 h at room temperature. Slides treated with the following primary antibodies were incubated at 4°C overnight: anti-5-HT antibody (rabbit, dilution factor 1:1,000; ImmunoStar), anti-glucagon antibody (1:1,000, mouse; Sigma), anti-insulin antibody (1:1,000, guinea pig; Dako), anti-Nkx6.1 antibody (1:100, mouse; Developmental Studies Hybridoma Bank), anti-Pdx1 antibody (1:100, mouse; Developmental Studies Hybridoma Bank), anti-Ki-67 (1:1,000, rabbit; Abcam), and anti-phospho-Histone H3 antibody (1:1,000, rabbit; Millipore). After incubation and washing, the following secondary antibodies were treated and incubated for 2 h at room temperature: Alexa 594–conjugated donkey anti-mouse IgG (1:1,000; Jackson ImmunoResearch), Alexa 647–conjugated donkey anti-rabbit IgG (1:1,000; Jackson ImmunoResearch), and FITC-conjugated donkey anti-guinea pig IgG (1:1,000; Jackson ImmunoResearch). In

Situ Cell Death Detection Kit, Fluorescein (TUNEL; Sigma-Aldrich) was used to measure apoptosis. Samples were treated with DAPI (1:2,000; Invitrogen) for 5 min and mounted with fluorescence mounting medium (Dako). Pictures were obtained using a confocal microscope (LSM 780; Carl Zeiss) or a fluorescence microscope with a DS-Ri2 camera (Nikon). Immunofluorescence intensity was measured with region-of-interest statistics calculated using Nikon NIS-Elements version 4.3 software (reference range 0–256). 5-HT immunofluorescence intensity was measured in β-cells positive for immunofluorescence staining of insulin. The background intensity was measured in exocrine pancreas to evaluate whether the obtained images were suitable for comparison.

Immunohistochemistry was conducted with a biotin-free polymer detection system for 5-HT (MACH 3; Biocare Medical). After the application of secondary antibody for rabbit IgG, the immunoreaction products were colorized with diaminobenzidine (Thermo Fisher Scientific). Specificity was confirmed by the lack of positive staining after omission of the first antibody or replacement with non-immune serum. The sections were observed and captured by bright-field microscopy (Axio Imager A1 or M1; Carl Zeiss).

For the β-cell mass and proliferation measurements, slides of fresh-frozen paraffin-embedded pancreas were chosen with an 80-μm interval. These slides were immunostained with insulin, and whole slides were scanned with JuLI Stage (NanoEntek). β-Cell mass was measured by insulin immunoreactive area divided by whole-pancreas area (more than eight slides per mouse). β-Cell proliferation was measured by the percentage of insulin and phospho-Histone H3 copositive cells in all β-cells (>2,000 β-cells per mouse).

Glucose Dynamics

For intraperitoneal glucose tolerance test and in vivo glucose-stimulated insulin secretion (GSIS) test, mice were fasted for 16 h, and D-glucose (2 g/kg body weight) was injected intraperitoneally. Blood glucose level was measured from tail vein using a glucometer (GlucoDr.Plus; Allmedicus). For insulin measurement, blood was collected at 0, 15, and 30 min after glucose injection in a heparinized tube, which was immediately centrifuged in 1,500*g* for 10 min at 4°C. Plasma insulin concentration was measured using an ultrasensitive insulin ELISA kit (80-INSMSU-E01; ALPCO). For insulin tolerance test, mice were fasted for 6 h before an intraperitoneal Humulin R (Eli Lilly) injection (0.75 units/kg body weight).

For ex vivo GSIS, mouse islets were isolated using collagenase. The major process of the technique was described previously (19). First, islets were incubated at 37°C in humidified gas of 5% CO_2 and 95% air in RPMI medium (Thermo Fisher Scientific) with 100 units/mL penicillin-streptomycin (Thermo Fisher Scientific) and 10% FBS (Thermo Fisher Scientific). After 4 h of incubation, islets were moved to Krebs-Ringer HEPES (KRH) buffer (pH 7.4, 115 mmol/L NaCl, 25 mmol/L HEPES, 24 mmol/L $NaHCO_3$, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, and 1 mg/mL BSA) containing 2.8 mmol/L glucose for 30 min. Islets were handpicked ($n = 10$ size-matched islets per individual sample) to a 12-well noncoated dish. To measure basal insulin secretion, samples were incubated with 2.8 mmol/L glucose containing KRH buffer for 15 min, and 50 μL of supernatants were obtained for insulin measurements. For high-glucose stimulation, samples were incubated with 16.8 mmol/L glucose containing KRH buffer for 15 min, and supernatants were obtained. Next, the islets were sonicated and incubated in acid-ethanol (1.5% HCl in 100 mL of 70% ethanol) for 18 h at 4°C for intracellular insulin extraction. The same volume of 1 mol/L Tris Cl buffer (pH 8.0) was added for neutralization. All supernatants were immediately snap frozen with liquid nitrogen and kept frozen at −80°C until ELISA experiments. Insulin secretion was calculated by dividing the secreted insulin by the total insulin content extracted from the islets.

GH Studies and 5-HT Measurements

For experiments with GH, islets were isolated from mice at 3 to 4 weeks of age and incubated in RPMI medium. βTC3 and MIN6 cells were maintained in DMEM containing 25 mmol/L glucose and supplemented with 10% or 15% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and, where applicable (i.e., for MIN6 cells), 71.5 μmol/L 2-mercaptoethanol. Cells were incubated at 37°C in a humidified incubator containing 5% CO_2. For GH treatment, cells were incubated in serum-free medium for 12 h, and then recombinant mouse GH (National Hormone and Peptide Program) was added to the medium. RNA was extracted 24 h after the treatment.

For measurement of 5-HT secretion, neonatal pancreas was harvested at postnatal day 0 (P0) and washed with PBS. The P0 pancreas was incubated in 1 mL of KRH buffer supplemented with protease inhibitor cocktail for 10 min in a CO_2 incubator. Supernatants were obtained, and the secreted 5-HT concentration was measured using ELISA (LDN, Nordhorn, Germany). This value was normalized to the protein content, which was measured using the bicinchoninic acid technique (Thermo Fisher Scientific).

Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's protocol. One microgram of total RNA was used to generate cDNA High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed with Fast SYBR Green Master Mix (Applied Biosystems) using a Viia 7 Real-Time PCR System (Applied Biosystems). Gene expression was calculated by relative gene expression analysis using the ΔCt (threshold cycle) method. *Actb* was used as an internal control. Primer sequences are listed in Supplementary Table 1.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical significance was obtained by Student *t* test (two-tailed) or

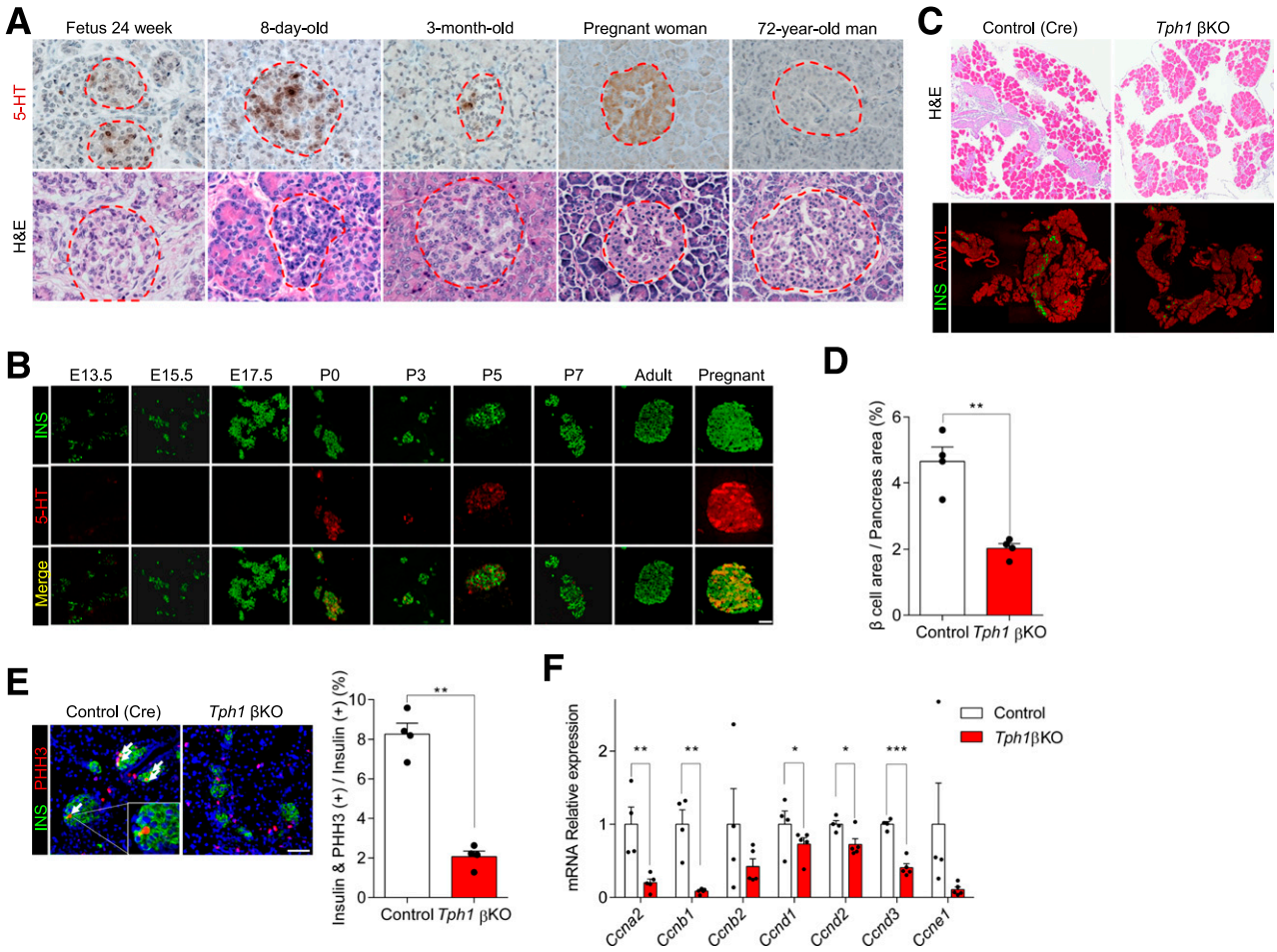

Figure 1—5-HT regulates β-cell proliferation during the perinatal period. *A*: Immunohistochemical staining (5-HT in brown) and H&E staining in perinatal human pancreata. Fetal (gestational age 24 weeks), postnatal 8-day-old, and 3-month-old pancreata were stained. Pancreas samples from a 35-year-old pregnant woman (gestational age 37 weeks) and a 72-year-old man were used as a positive and negative control, respectively. *B*: Immunofluorescent (IF) staining of perinatal pancreas from C57BL6/J mice labeled insulin (green) and 5-HT (red). *C–F*: Pancreata of control (Cre) and *Tph1* βKO mice were assessed at P0. *C*: Representative IF staining labeled insulin (green) and amylase (AMYL) (red) at P0. Images of a whole-slide section were obtained using a slide scanner. *D*: β-Cell mass was quantified as the percentage of insulin-positive area over whole pancreas area at P0 (n = 4 mice/group). *E*: IF staining labeled insulin (green) and phospho-Histone H3 (PHH3) (red). Arrows indicate insulin and PHH3 copositive cells. β-Cell proliferation was quantified by counting the percentage of insulin-positive cells labeled with PHH3 at P0 (n = 4 mice/group). *F*: mRNA levels of cyclin families were measured by qRT-PCR in RNAs extracted from pancreata at P0 (n = 4–5 mice/group). Scale bars = 50 μm. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. INS, insulin.

ANOVA to compare differences among three or more groups. Tukey honestly significant difference method was used for post hoc analysis. Statistical analyses were conducted using SPSS version 22 software (IBM Corporation). The levels of statistical significance were $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Data and Resource Availability

All data that support the findings of this study are available from the authors upon reasonable request.

RESULTS

5-HT Production in β-Cells During the Perinatal Period

We detected the presence of 5-HT in human pancreatic islets during the perinatal period, ranging from a gestational age of 24 weeks to a postnatal age of 19 months (Fig. 1*A* and Supplementary Fig. 1*A* and *B*). 5-HT–positive cells were more frequently observed during the perinatal period, and 5-HT staining was confined to endocrine cells. Similarly, 5-HT staining was observed in mouse β-cells at P0–P5, and the expression pattern of the *Tph1* mRNA in pancreas paralleled the change in 5-HT immunostaining (Fig. 1*B* and Supplementary Fig. 1*C–E*). In humans, the mRNA expression of *TPH1* was higher than that of *TPH2* in β-cells, and *TPH1* mRNA expression was higher in fetal β-cells than in adult β-cells (20) (Supplementary Table 2). These data suggest that 5-HT may have a common function in human and rodent β-cells during the perinatal period and that 5-HT production mainly depends on TPH1 than on TPH2.

To elucidate the physiological role of 5-HT in β-cells, we generated *Tph1* βKO mice (12). *Tph1* βKO was confirmed

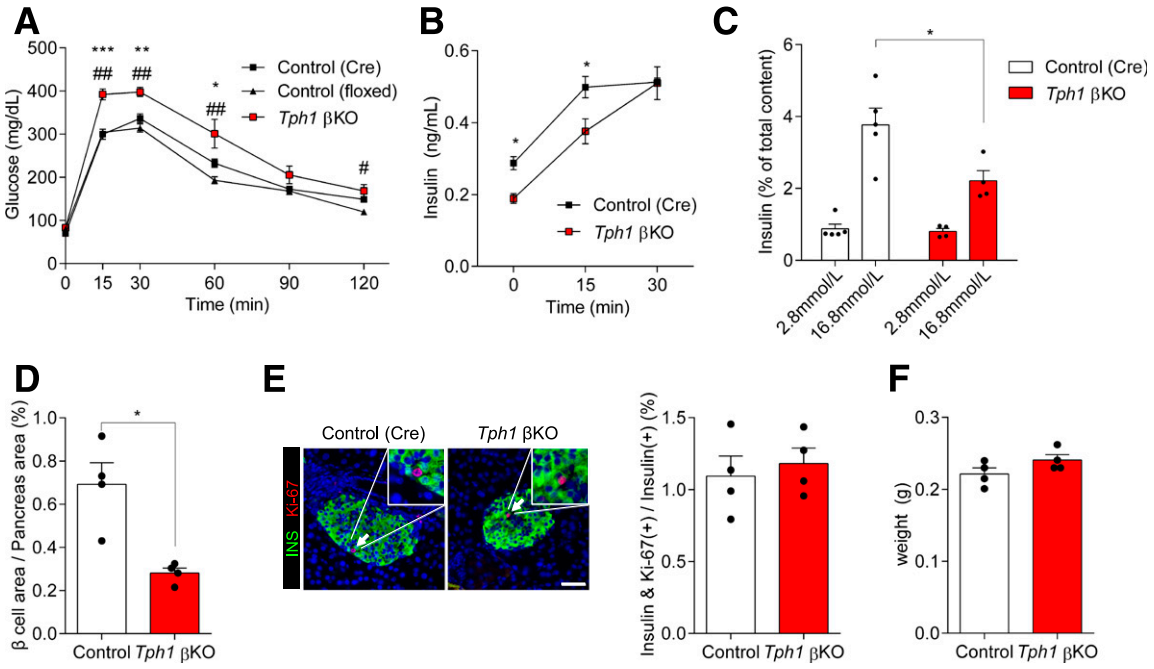

Figure 2—5-HT regulates adult β-cell mass and is necessary to maintain glucose homeostasis. Metabolic phenotypes were evaluated in 9-week-old male control and *Tph1* βKO mice. *A* and *B*: Blood glucose and plasma insulin levels were measured after intraperitoneal glucose injection (2 g/kg) after a 16-h fast. Both Cre-only and floxed-only mice were used as controls for the glucose tolerance test. Because glucose levels were comparable between the two groups, Cre-only mice were used as the control in further experiments (n = 4–8 mice/group). *C*: Secreted insulin was measured with ELISA after a 15-min incubation of islets isolated from 9-week-old control (Cre) and *Tph1* βKO mice in 2.8 or 16.8 mmol/L glucose. Insulin secretion was normalized to the total insulin content extracted from the islets (n = 5/group). *D*: β-Cell mass was quantified as the percentage of insulin-positive area over whole-pancreas area of 9-week-old mice (n = 4 mice/group). *E*: Immunofluorescent staining labeled insulin (green) and Ki-67 (red), with arrows indicating insulin and Ki-67 copositive cells. β-Cell proliferation was quantified by counting the percentage of insulin-positive cells labeled with Ki-67 in 9-week-old mice (n = 4 mice/group). *F*: Pancreas weight was measured after harvest in 9-week-old mice (n = 4 mice/group). Scale bar = 50 μm. Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, control (Cre) vs. KO; $^{\#\#}P < 0.01$, control (floxed) vs. KO. INS, insulin.

by decreased *Tph1* mRNA expression in the pancreas at P0 and the reduction of 5-HT production to a level undetectable by immunofluorescence staining in β-cells during the perinatal period and pregnancy (Supplementary Fig. 2*A* and *B*). Next, we evaluated 5-HT secretion from β-cells in the neonatal P0 pancreas (Supplementary Fig. 2*C*). Glucose did not stimulate 5-HT secretion in the P0 pancreas because β-cells were not mature enough to sense the increase in glucose levels. Tolbutamide treatment increased 5-HT secretion in the P0 pancreas, but this response was perturbed in the *Tph1* βKO pancreas. These results suggest that 5-HT is produced and secreted in the β-cell during the perinatal period and can activate HTR in an autocrine/paracrine manner.

5-HT Stimulates Perinatal β-Cell Proliferation

We further explored the physiological role of 5-HT in *Tph1* βKO mice. The loss of 5-HT production did not affect β-cell differentiation, as evidenced by normal expression of PDX1 and NKX6.1 (Supplementary Fig. 2*D*). Intriguingly, the β-cell mass was remarkably reduced in *Tph1* βKO mice, whereas the size of the pancreas was unchanged (Fig. 1*C* and *D* and Supplementary Fig. 2*E*). Immunofluorescence staining of proliferation markers (phospho-Histone H3 and Ki-67) and TUNEL assays revealed that β-cell proliferation was reduced by ~75% in *Tph1* βKO mice without obvious induction of β-cell death (Fig. 1*E* and Supplementary Fig. 2*F* and *G*). The expression levels of cyclin family members (i.e., D1, D2, D3, A2, B1) were reduced in the pancreas of *Tph1* βKO mice at P0 (Fig. 1*F*). Collectively, these data suggest that 5-HT increases the β-cell mass by promoting perinatal β-cell proliferation.

To examine the long-term impacts of the reduced perinatal β-cell proliferation seen in *Tph1* βKO mice, we evaluated the metabolic phenotypes of *Tph1* βKO mice at 9 weeks of age. *Tph1* βKO mice showed normal growth and insulin sensitivity (Supplementary Fig. 3*A* and *B*); however, their glucose tolerance was impaired (Fig. 2*A*). Plasma insulin levels at fasting and 15 min after glucose administration were lower, and the insulin secretory capacity was lower in *Tph1* βKO islets than in control islets (Fig. 2*B* and *C*). In adult *Tph1* βKO mice, the β-cell proliferation and pancreas size were comparable to those of control mice, but the β-cell mass was reduced by more than half (to ~40%) compared with that of control mice (Fig. 2*D*–*F*). To investigate the mechanism underlying the reduction in the β-cell mass of adult *Tph1* βKO mice, we evaluated

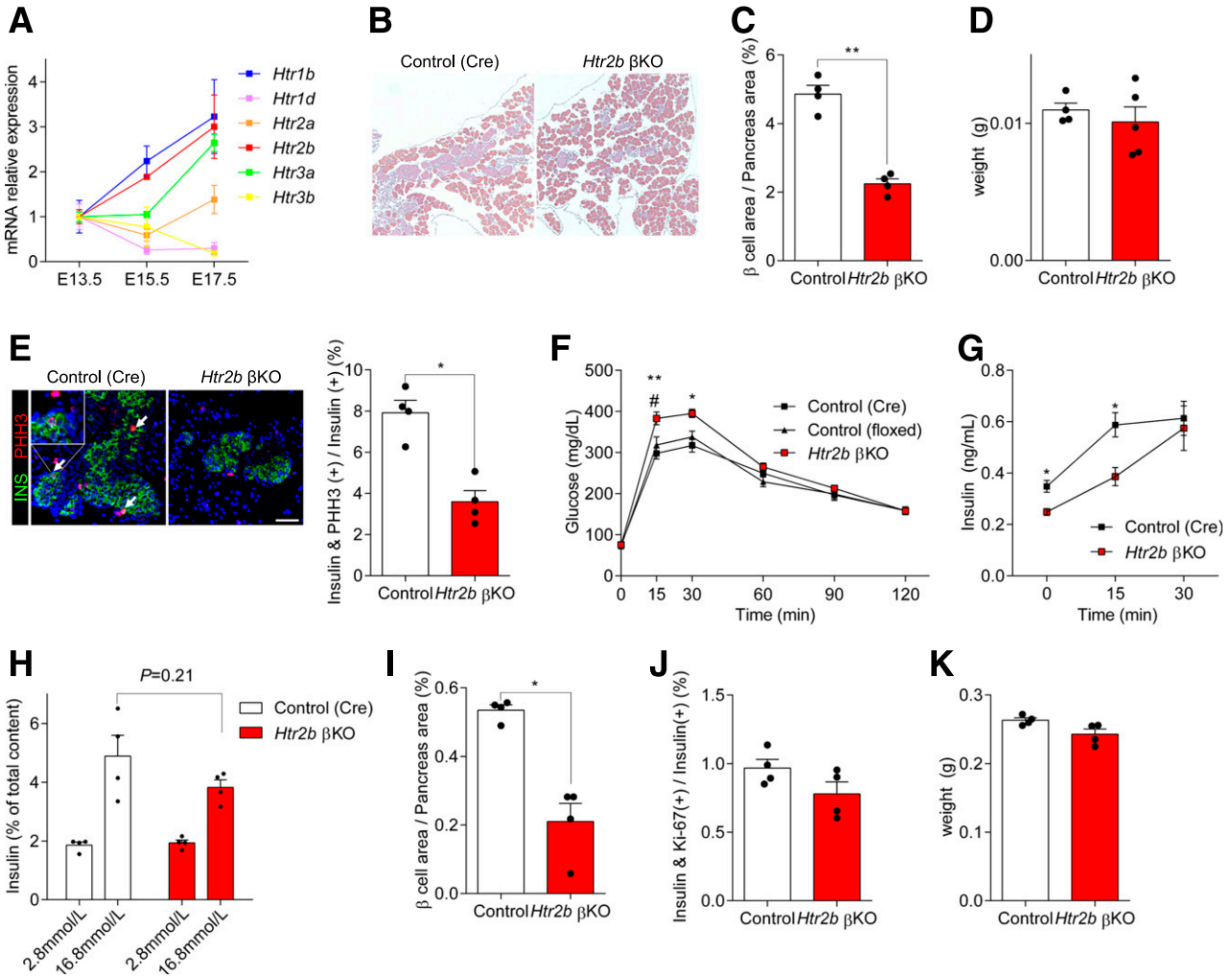

Figure 3— HTR2B is the downstream target of 5-HT in perinatal β-cells. *A*: mRNA expression levels of indicated HTRs were assessed by qRT-PCR in RNAs extracted from pancreata during embryonic development ($n = 4$ mice/group). *B–E*: Pancreata of control (Cre) and *Htr2b* βKO at P0 were evaluated. *B*: H&E staining was performed in control (Cre) and *Htr2b* βKO pancreas at P0. *C*: β-Cell mass was quantified as the percentage of insulin-positive area over whole-pancreas area at P0 ($n = 4$ mice/group). *D*: Pancreas weight was measured after harvest at P0 ($n = 4–5$ mice/group). *E*: Immunofluorescent staining labeled insulin (green) and phospho-Histone H3 (PHH3) (red), with arrows indicating insulin and PHH3 copositive cells. β-Cell proliferation was quantified by counting the percentage of insulin-positive cells labeled with PHH3 at P0 ($n = 4$ mice/group). *F–J*: Metabolic phenotypes were evaluated in 9-week-old male control and *Htr2b* βKO mice. *F* and *G*: Blood glucose and plasma insulin levels were measured after intraperitoneal glucose injection (2 g/kg) after a 16-h fast. Both Cre-only and floxed-only mice were used as controls for glucose tolerance tests. Because glucose levels were comparable between the two, Cre-only mice were used as the control in further experiments ($n = 4$ mice/group). *H*: Secreted insulin was measured with ELISA after a 15-min incubation of islets isolated from 9-week-old control (Cre) and *Htr2b* βKO mice in 2.8 or 16.8 mmol/L glucose. Insulin secretion was normalized to the total insulin content extracted from the islets ($n = 3$/group). *I*: β-Cell mass was quantified as the percentage of insulin-positive area over whole-pancreas area of 9-week-old mice ($n = 4$ mice/group). *J*: β-Cell proliferation was quantified by counting the percentage of insulin-positive cells labeled with Ki-67 in 9-week-old mice ($n = 4$ mice/group). *K*: Pancreas weight was measured after harvest in 9-week-old mice ($n = 4$ mice/group). Scale bar = 50 μm. Data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ control (Cre) vs. KO; $\#P < 0.05$, control (floxed) vs. KO. INS, insulin.

apoptosis and endoplasmic reticulum stress. However, we did not observe any significant difference in the TUNEL-positive cell numbers or endoplasmic reticulum stress gene expression levels in islets of *Tph1* βKO mice, and mature β-cell markers were appropriately expressed (Supplementary Fig. 3*C–F*). These data indicate that the reduced β-cell mass seen in adult *Tph1* βKO mice can be attributed to the reduction in perinatal β-cell proliferation. Thus, 5-HT stimulation of perinatal β-cell proliferation is necessary to achieve the adult β-cell mass needed to accomplish normoglycemia.

5-HT Signaling Through HTR2B Regulates β-Cell Proliferation

Among the 14 HTRs in rodents, 6 are expressed in pancreatic islets (6). To determine the downstream HTRs involved in perinatal β-cell proliferation, we examined the expression levels of these 6 (HTR1B, 1D, 2A, 2B, 3A, and

3B) in embryonic pancreas. The expression levels of *Htr1b*, *Htr2b*, and *Htr3a* mRNAs showed increasing trends during pancreatic development (Fig. 3*A*). Among them, HTR3 is known to be involved in insulin secretion but not in β-cell proliferation (7), and the loss of HTR1B or HTR1D did not decrease β-cell proliferation at P0 (Supplementary Fig. 4). In contrast, the β-cell mass was reduced by ~55% in *Htr2b* βKO mice at P0, while the weight of the pancreas remained unchanged (Fig. 3*B–D*). The β-cell proliferation was reduced in *Htr2b* βKO mice without any obvious increase in β-cell death (Fig. 3*E* and Supplementary Fig. 2*G*). Thus, reduced perinatal β-cell proliferation was responsible for the reduction of β-cell mass in *Htr2b* βKO mice during the perinatal period.

We further analyzed the adult phenotypes of *Htr2b* βKO mice. *Htr2b* βKO mice became glucose intolerant in adulthood while exhibiting normal body weight and insulin sensitivity (Fig. 3*F* and Supplementary Fig. 5*A* and *B*). Plasma insulin levels at fasting and 15 min after glucose administration were lower in *Htr2b* βKO mice (Fig. 3*G*). However, GSIS from isolated *Htr2b* βKO islets was not significantly impaired (Fig. 3*H*). Instead, the β-cell mass was persistently reduced in adult *Htr2b* βKO mice to ~40% of control mice (Fig. 3*I*). The β-cell proliferation and pancreas size were similar between control and *Htr2b* βKO mice, and β-cell markers were appropriately expressed in *Htr2b* βKO mice (Fig. 3*J* and *K* and Supplementary Fig. 5*C*). Thus, *Htr2b* βKO mice phenocopied *Tph1* βKO mice except for the ex vivo GSIS. These data support the conclusion that 5-HT stimulates β-cell proliferation through HTR2B during the perinatal period. Furthermore, defects in 5-HT–dependent perinatal β-cell proliferation are associated with decreases in the adult β-cell mass, indicating that 5-HT plays an important role in determining the adult β-cell mass.

5-HT Is Not Involved in HFD-Induced β-Cell Proliferation

HFD is known to induce β-cell proliferation to compensate for the increased insulin demand. To further assess whether 5-HT is necessary for the ability of β-cells to endure metabolic stress, we fed mice an HFD for 8 weeks beginning at 12 weeks of age. Neither *Tph1* expression nor 5-HT production was induced in β-cells by HFD (21) (Supplementary Fig. 6*A*). *Tph1* βKO mice showed a similar body weight gain and insulin sensitivity compared with control mice (Supplementary Fig. 6*B* and *C*). However, glucose intolerance was more rapidly aggravated upon HFD in *Tph1* βKO mice compared with control mice, whereas β-cell proliferation was comparable (Fig. 4*A* and *B*). These data suggest that 5-HT is not involved in the induction of compensatory β-cell proliferation in response to metabolic stress. This conclusion was further supported by measuring β-cell proliferation in mice with insulin resistance induced by the insulin receptor antagonist S-961. Induction of β-cell proliferation was comparable between control and *Tph1* βKO mice upon S-961 administration (Fig. 4*C* and Supplementary Fig. 6*D*). Thus, 5-HT stimulates β-cell proliferation not in response to metabolic stress but, rather, in response to physiological stimuli during the perinatal period. This notion prompted us to explore the upstream signals that regulate 5-HT production in β-cells during the perinatal period.

GH Induces 5-HT Production in β-Cells During the Perinatal Period

During pregnancy, placental lactogen stimulates *Tph1* expression through the PRL receptor (PRLR)-STAT5 pathway (6,22), and plasma PRL levels remain high during the perinatal period (23). To determine whether PRL induces *Tph1* expression in perinatal β-cells through the PRLR-STAT5 pathway, we investigated β-cell–specific *Prlr* KO (*Prlr* βKO) and *Stat5* KO (*Stat5* βKO) mice. Similar to observations made during pregnancy, 5-HT was not detectable in the β-cells of *Stat5* βKO mice, but in contrast to pregnancy, the β-cells in *Prlr* βKO mice still produced 5-HT at P0 (Fig. 5*A*). These findings suggest that different upstream signals induce *Tph1* expression during the perinatal period.

Similar to PRL signaling through PRLR, GH has been shown to act through the GH receptor (GHR) on β-cells to stimulate STAT5 phosphorylation and β-cell proliferation

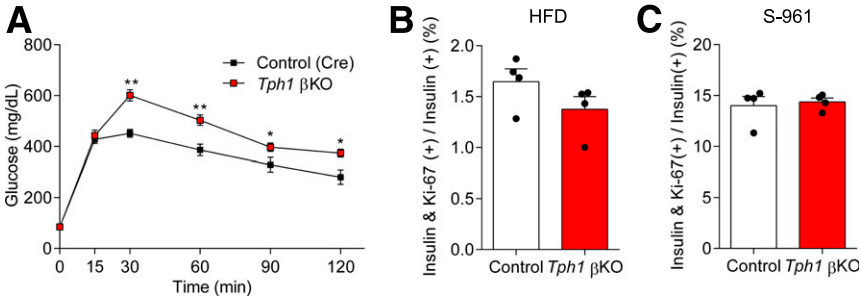

Figure 4—5-HT is not involved in β-cell proliferation induced by metabolic stress. HFD was fed to 12-week-old mice for 8 weeks. *A*: Blood glucose levels were measured after intraperitoneal glucose injection (2 g/kg) after a 16-h fast (n = 4–5 mice/group). *B*: β-Cell proliferation was quantified by counting the percentage of insulin-positive cells labeled with Ki-67 (n = 4 mice/group). *C*: β-Cell proliferation upon S-961 administration (100 nmol/kg mouse twice a day) for 7 days was quantified by counting the percentage of insulin-positive cells labeled with Ki-67 (n = 4 mice/group). Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$.

(16,24). Disruption of *Ghr* in β-cells results in a decrease in the β-cell mass and insulin secretory function upon HFD (16). *GHR* expression is increased in human fetal β-cells, whereas *PRLR* expression is not (Supplementary Table 2). Therefore, we generated β-cell–specific *Ghr* KO (*Ghr* βKO) mice and found that they failed to produce 5-HT in β-cells at P0 (Fig. 5*A*). Consistently, *Tph1* mRNA expression was decreased in pancreas at P0 in both *Stat5* βKO mice and *Ghr* βKO mice but not in *Prlr* βKO mice (Supplementary Fig. 6*E–G*). The loss of 5-HT production was associated with a reduction in β-cell proliferation in both *Ghr* βKO mice and *Stat5* βKO mice but not in *Prlr* βKO mice (Fig. 5*B–E*). Consistent with these reductions in β-cell proliferation, the β-cell mass was reduced in *Ghr* βKO mice and *Stat5* βKO mice but not in *Prlr* βKO mice (Fig. 5*F–H*).

Next, we examined whether GH can directly induce 5-HT production in β-cells during the perinatal period. Plasma GH levels peaked at embryonic day 17.5 (E17.5) and gradually declined after birth (Supplementary Fig. 6*H*). GH treatment directly increased the expression of *Tph1* and *Mki67* in wild-type islets, and this response was perturbed in *Tph1* βKO islets (Fig. 5*I* and *J*). GH also

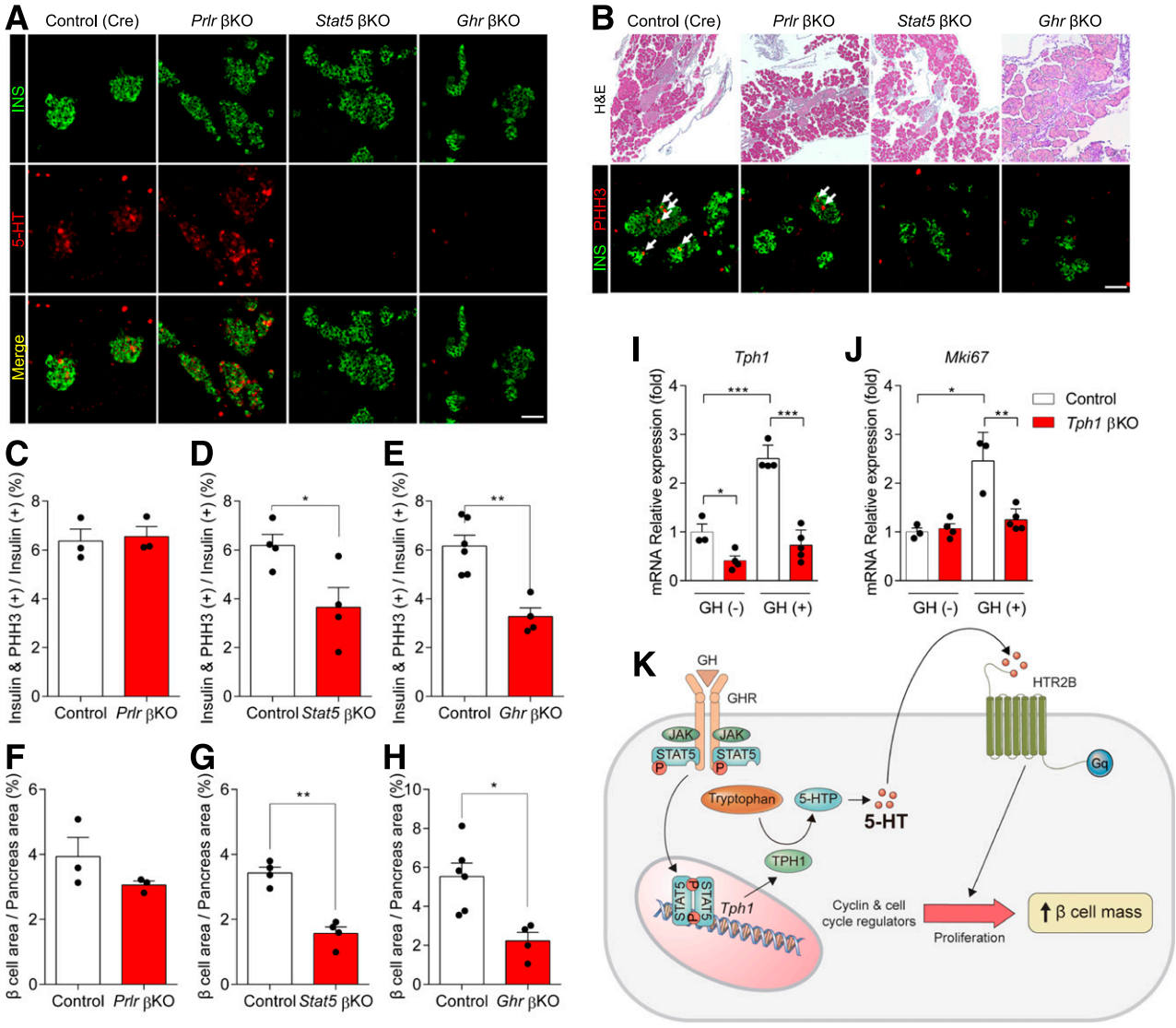

Figure 5—GH regulates 5-HT production in β-cells during the perinatal period. Pancreas of control (Cre), *Prlr* βKO, *Stat5* βKO, and *Ghr* βKO mice at P0 were examined. *A*: 5-HT production in β-cells at P0 were assessed by immunofluorescent (IF) staining, which labeled 5-HT (red) and insulin (green). *B*: Representative H&E staining and IF staining, labeled insulin (green) and phospho-Histone H3 (PHH3) (red) at P0. Arrows indicate insulin and PHH3 copositive cells. *C–E*: β-Cell proliferation of *Prlr* βKO, *Stat5* βKO, and *Ghr* βKO mice at P0 was quantified by counting the percentage of insulin-positive cells labeled with PHH3 (n = 4–6 mice/group). *F–H*: β-Cell mass was quantified as the percentage of insulin-positive area over whole-pancreas area in *Prlr* βKO, *Stat5* βKO, and *Ghr* βKO mice at P0 (n = 4–6 mice/group). *I* and *J*: Induction of *Tph1* or *Mki67* in islets isolated from 3-week-old control (Cre) and *Tph1* βKO mice was measured by qRT-PCR. Islets were incubated with recombinant mouse GH (1,000 ng/mL) for 24 h (n = 3–5/group). *K*: Schematic describing the GHR-STAT5-TPH1-HTR2B axis for β-cell proliferation during the perinatal period. Scale bars = 50 μm. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. INS, insulin.

increased *Tph1* expression in βTC3 and MIN6 cells (Supplementary Fig. 6*I*). However, no significant difference in HTR expression was observed upon GH treatment (Supplementary Fig. 6*J*). These results suggest that GH signals through GHR-STAT5 to stimulate TPH1 expression during the perinatal period, which in turn induces 5-HT production in β-cells.

Taken together, we identified that 5-HT plays a key role in determining the adult β-cell mass by regulating β-cell proliferation during the perinatal period (Fig. 5*K*). GH binds to GHR and activates STAT5 to stimulate 5-HT production by increasing TPH1 expression in perinatal β-cells. In turn, 5-HT stimulates β-cell proliferation through HTR2B in an autocrine/paracrine manner. Remarkably, defects in this pathway lead to a reduction in adult β-cell mass and glucose intolerance, which is exacerbated by metabolic stress.

DISCUSSION

Pancreatic β-cells undergo physiological mass expansion during the perinatal period and pregnancy. Intriguingly, 5-HT production increases robustly in β-cells during both periods, and 5-HT/HTR2B works as a common mechanism for β-cell proliferation in response to different external upstream signals (6,8). 5-HT production in human β-cells has also been reported by multiple groups (6,25–27), although this has been argued, as the expression of *TPH1* is low and STAT5 functions differently in human β-cells (28,29). Recent advances in β-cell transcriptomics support our hypotheses that 5-HT is produced from perinatal β-cells to stimulate its proliferation through HTR2B (Supplementary Table 2). Blodgett et al. (20) performed RNA sequencing analysis of sorted human β-cells, which showed that *TPH1* expression was enriched in fetal β-cells compared with adult β-cells, and *GHR* expression was 10-fold higher in fetal β-cells. *HTR2B* was among the most abundant HTR transcripts in human fetal β-cells, although potential contributions by other HTRs should be further investigated. Qiu et al. (30) performed transcriptomic analysis of mouse β-cells sorted from different perinatal periods and found that *Tph1* expression peaks at P0, which is in accordance with our findings. We also directly demonstrated the presence of 5-HT in fetal and perinatal human β-cells.

GH production increases during fetal development and surges at birth (10–50 ng/mL) (31,32). After birth, the GH level falls within 1 month to <10 ng/mL (32,33). Transient and robust 5-HT production in β-cells during the perinatal period parallels these dynamic changes in GH levels in both humans and mice. Our results showing defects in β-cell proliferation in each βKO model of the GHR-STAT5-TPH1-HTR2B axis are in accordance with previous reports showing that β-cell proliferation and cyclin D2 expression are altered in models of reduced GH signaling (34–37). In humans, children requiring GH replacement have a six- to eightfold higher incidence of diabetes relative to age-matched untreated children (38,39). Inadequate GH stimulation at an earlier age in these children may increase the risk of diabetes by reducing the perinatal expansion of the β-cell mass. In addition, the yield of islet clusters increases when human fetal pancreas are cocultured with GH (40). These data support the potential implication of GH in the expansion of the β-cell mass.

In summary, we show that 5-HT plays a critical role in GH signaling to determine the β-cell mass by regulating β-cell proliferation through HTR2B during the perinatal period. Future studies of this pathway may provide new insights into the genetics and pathophysiology of diabetes and could suggest new methods for regenerating β-cells for people with diabetes.

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