

# Glucose Intolerance and Reduced Proliferation of Pancreatic $\beta$ -Cells in Transgenic Pigs With Impaired Glucose-Dependent Insulinotropic Polypeptide Function

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**OBJECTIVE**—The insulinotropic action of the incretin glucose-dependent insulinotropic polypeptide (GIP) is impaired in type 2 diabetes, while the effect of glucagon-like peptide-1 (GLP-1) is preserved. To evaluate the role of impaired GIP function in glucose homeostasis and development of the endocrine pancreas in a large animal model, we generated transgenic pigs expressing a dominant-negative GIP receptor (GIPR<sup>dn</sup>) in pancreatic islets.

**RESEARCH DESIGN AND METHODS**—GIPR<sup>dn</sup> transgenic pigs were generated using lentiviral transgenesis. Metabolic tests and quantitative stereological analyses of the different endocrine islet cell populations were performed, and  $\beta$ -cell proliferation and apoptosis were quantified to characterize this novel animal model.

**RESULTS**—Eleven-week-old GIPR<sup>dn</sup> transgenic pigs exhibited significantly reduced oral glucose tolerance due to delayed insulin secretion, whereas intravenous glucose tolerance and pancreatic  $\beta$ -cell mass were not different from controls. The insulinotropic effect of GIP was significantly reduced, whereas insulin secretion in response to the GLP-1 receptor agonist exendin-4 was enhanced in GIPR<sup>dn</sup> transgenic versus control pigs. With increasing age, glucose control deteriorated in GIPR<sup>dn</sup> transgenic pigs, as shown by reduced oral and intravenous glucose tolerance due to impaired insulin secretion. Importantly,  $\beta$ -cell proliferation was reduced by 60% in 11-week-old GIPR<sup>dn</sup> transgenic pigs, leading to a reduction of  $\beta$ -cell mass by 35% and 58% in 5-month-old and 1- to 1.4-year-old transgenic pigs compared with age-matched controls, respectively.

**CONCLUSIONS**—The first large animal model with impaired incretin function demonstrates an essential role of GIP for insulin secretion, proliferation of  $\beta$ -cells, and physiological expansion of  $\beta$ -cell mass. *Diabetes* 59:1228–1238, 2010

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The incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted by enteroendocrine cells in response to nutrients like fat and glucose and enhance glucose-induced release of insulin from pancreatic  $\beta$ -cells (1). The effects of GIP and GLP-1 are mediated through specific receptors, GIPR and GLP-1R, respectively. Both receptors belong to the family of seven transmembrane-domain heterotrimeric G-protein-coupled receptors (2). Activation of the GIPR or GLP-1R leads to enhanced exocytosis of insulin-containing granules (3). Interestingly, variation in the *GIPR* gene influences glucose and insulin responses to an oral glucose challenge in humans (4). Furthermore, findings in insulinoma cells (5–7) and rodent models (8,9) indicate that activation of incretin receptors promotes proliferation and survival of  $\beta$ -cells. Type 2 diabetic patients and ~50% of their first-degree relatives show a reduced incretin effect, mainly due to an impaired insulinotropic action of GIP (10,11). Nearly sustained insulinotropic action of GLP-1 (11) in type 2 diabetic patients revealed its therapeutic potential and initiated the ongoing development of GLP-1R agonists as well as inhibitors of dipeptidyl peptidase-4 (1,12), which rapidly degrades incretin hormones in vivo. The reasons for the reduced response to GIP in type 2 diabetes are unclear (1), but impaired GIP action might be involved in the early pathogenesis of type 2 diabetes (13).

To clarify this point, a mouse model lacking functional GIPR expression was generated by gene targeting (14). *Gipr*<sup>-/-</sup> mice displayed only slightly impaired glucose tolerance and did not develop diabetes. Interestingly, double incretin receptor knockout mice exhibited a similar phenotype (rev. in 15), compensatory regulation of the GLP-1 system or other compensatory mechanisms were discussed. In contrast, transgenic mice overexpressing a dominant-negative GIPR (GIPR<sup>dn</sup>) displayed a severe phenotype (i.e., early-onset diabetes accompanied by a marked fasting hypoinsulinemia and severe reduction of  $\beta$ -cell mass associated with extensive structural alterations of the pancreatic islets) (16).

In light of these discrepant findings in mouse models, we generated a large animal model to address the question whether GIPR signaling plays a role in maintaining pancreatic islet function and structure. Efficient lentiviral vectors (17) were used to generate transgenic pigs expressing a GIPR<sup>dn</sup> under the control of the rat *Ins2*

promoter in the pancreatic islets. This novel animal model, in contrast to GIPR<sup>dn</sup> transgenic mice (16), initially only exhibits a disturbed incretin effect but develops progressive deterioration of glucose control with increasing age, associated with reduced  $\beta$ -cell proliferation and an impairment of physiological age-related expansion of pancreatic  $\beta$ -cell mass.

## RESEARCH DESIGN AND METHODS

**Generation of RIP II-GIPR<sup>dn</sup> transgenic pigs.** The expression cassette consisting of the rat *Ins2* promoter (RIP II) and the cDNA of a human GIPR<sup>dn</sup> (16) was cloned into the lentiviral vector LV-*pGFP* (18) (supplementary Fig. 1 of the online appendix [available at <http://diabetes.diabetesjournals.org/cgi/content/full/db09-0519/DC1>]). Recombinant lentivirus was produced (18) and injected into the perivitelline space of zygotes from superovulated gilts (17). Embryos were transferred into synchronized recipients (19). Offspring were genotyped by PCR and Southern blot analyses using a probe directed toward the RIP II promoter sequence. Expression of GIPR<sup>dn</sup> mRNA in the pancreatic islets was determined by RT-PCR. A total of 400 ng of total RNA were reverse transcribed into cDNA using SuperScriptII reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen) after digestion with DNaseI (Roche). For PCR, the following transgene specific primers were used: sense 5'-TTT TTA TCC GCA TTC TTA CAC GG-3' and antisense 5'-ATC TTC CTC AGC TCC CAG G-3'. All animal experiments were carried out according to the German animal protection law.

**Oral/intravenous glucose tolerance test and GIP/exendin-4 stimulation test.** For the oral glucose tolerance test (OGTT), one central venous catheter (Cavafix Certo; B. Braun) was inserted nonsurgically into the external jugular vein. After an 18-h overnight fast, animals were fed 2 g glucose/kg body weight (BW) (20) mixed with 50/100 g (11-week-old/5-month-old) commercial pig fodder. Blood samples were obtained from the jugular vein catheter at the indicated time points. For the intravenous glucose tolerance test (IVGTT) and GIP/exendin-4 stimulation test, two central venous catheters (Cavafix Certo) were surgically inserted into the external jugular vein under general anesthesia (21). For both tests, a bolus injection of 0.5 g glucose/kg BW (22) was administered through the central venous catheter after an 18-h fasting period. For the GIP/exendin-4 stimulation test, 80 pmol/kg BW of synthetic porcine GIP (Bachem) or 40 pmol/kg BW synthetic exendin-4 (Bachem) were administered intravenously in addition to glucose. Blood samples were collected at the indicated time points. Serum glucose levels were determined using an AU 400 autoanalyzer (Olympus). Serum insulin levels were measured using a porcine insulin radioimmunoassay kit (Millipore).

**Pancreas preparation and islet isolation.** Pancreatic islets were isolated from three 12- to 13-month-old GIPR<sup>dn</sup> transgenic pigs and three littermate control animals (23). After explantation of the pancreas in toto, the left pancreatic lobe was separated from the rest of the organ (supplementary Fig. 2). The left pancreatic lobe was digested using a modification of the half-automated digestion-filtration method as previously described (24). Purification of the isolated islets was performed with the discontinuous OptiPrep density gradient (Progen) in the COBE 2991 cell processor (COBE) (25). Islet numbers were determined using dithizone-stained islet samples (26), which were counted under an Axiovert 25 microscope (Zeiss) with a calibrated grid in the eyepiece. For determination of islet vitality, fluorescein diacetate/propidium iodide (Sigma-Aldrich) staining was performed (27).

**Immunohistochemistry and quantitative stereological analyses.** After prefixation, the pancreas was cut into 1-cm-thick slices. Slices were tilted to their left side and covered by a 1-cm<sup>2</sup> point-counting grid. Tissue blocks were selected by systematic random sampling, fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. The volume of the pancreas [ $V_{Pan}$ ] before embedding was calculated by the quotient of the pancreas weight and the specific weight of pig pancreas (1.07 g/cm<sup>3</sup>). The specific weight was determined by the submersion method (28). Paraffin sections were routinely prepared, and insulin, glucagon, somatostatin, and pancreatic polypeptide containing cells were stained, using the indirect immunoperoxidase technique (16) and the antibodies described in the online appendix. The volume densities of  $\alpha$ -,  $\beta$ -,  $\delta$ -, and pp-cells in the islets [ $V_{(\alpha\text{-cell}/\text{Islet})}$ ,  $V_{(\beta\text{-cell}/\text{Islet})}$ ,  $V_{(\delta\text{-cell}/\text{Islet})}$ , and  $V_{(\text{pp-cell}/\text{Islet})}$ ], the total volumes of  $\alpha$ -,  $\beta$ -,  $\delta$ -, and pp-cells in the islets [ $V_{(\alpha\text{-cell, Islet})}$ ,  $V_{(\beta\text{-cell, Islet})}$ ,  $V_{(\delta\text{-cell, Islet})}$ , and  $V_{(\text{pp-cell, Islet})}$ ] as well as the total volume of  $\beta$ -cells in the pancreas [referring to  $\beta$ -cells in the islets and isolated  $\beta$ -cells,  $V_{(\beta\text{-cell, Pan})}$ ], and the total volume of isolated  $\beta$ -cells in the pancreas [ $V_{(\text{iso}\beta\text{-cell, Pan})}$ ], a parameter indicative of islet neogenesis (29–31), were determined as described previously (32). Volume densities of the various endocrine cell types in the islets refer to the volume fraction of the particular endocrine cell type in relation to the cumulative

volume of the various endocrine islet cells, thus excluding capillaries and other interstitial tissues in the islets.

Proliferation/apoptosis rates of  $\beta$ -cells were determined by double immunohistochemical staining for insulin and the proliferation marker Ki67 (33) or the apoptosis marker cleaved caspase-3 (34) as detailed in the online appendix. A minimum of 10<sup>4</sup>  $\beta$ -cells per animal was included in the quantification of  $\beta$ -cell proliferation and apoptosis. Cell proliferation/apoptosis index was defined as the number of immunolabeled cell nuclei divided by the total number of cell nuclei counted and expressed as the number of immunolabeled (Ki67+/Casp-3+) cell nuclei per 10<sup>5</sup> nuclei. GIPR and GLP-1R were detected in pancreas sections using the streptavidin-biotin complex technique and the antibodies described in the online appendix.

**Statistics.** All data are presented as means  $\pm$  SE. The results of glucose tolerance tests and incretin stimulation tests were statistically evaluated by ANOVA (linear mixed models; SAS 8.2; PROC MIXED), taking the fixed effects of group (wild type, transgenic), time (relative to glucose or hormone application), and the interaction group  $\times$  time as well as the random effect of animal into account (35). Results of the linear mixed models analysis are shown in supplementary Table 1. The same model was used to compare body weight gain of GIPR<sup>dn</sup> transgenic and control pigs. Pancreas weight and the results of quantitative stereological analyses were evaluated by the general linear models procedure (SAS 8.2) taking the effects of group (wild type, transgenic), age (11 weeks, 5 months, or 1–1.4 years), and the interaction group  $\times$  age into account. Results of the general linear models analysis are shown in Table 1. Calculation of areas under the curve (AUCs) was performed using Graph Pad Prism 4 software. Statistical significance of differences between transgenic and wild-type pigs was tested using the Mann-Whitney *U* test in combination with an exact test procedure (SPSS 16.0, Chicago, IL). *P* values <0.05 were considered significant.

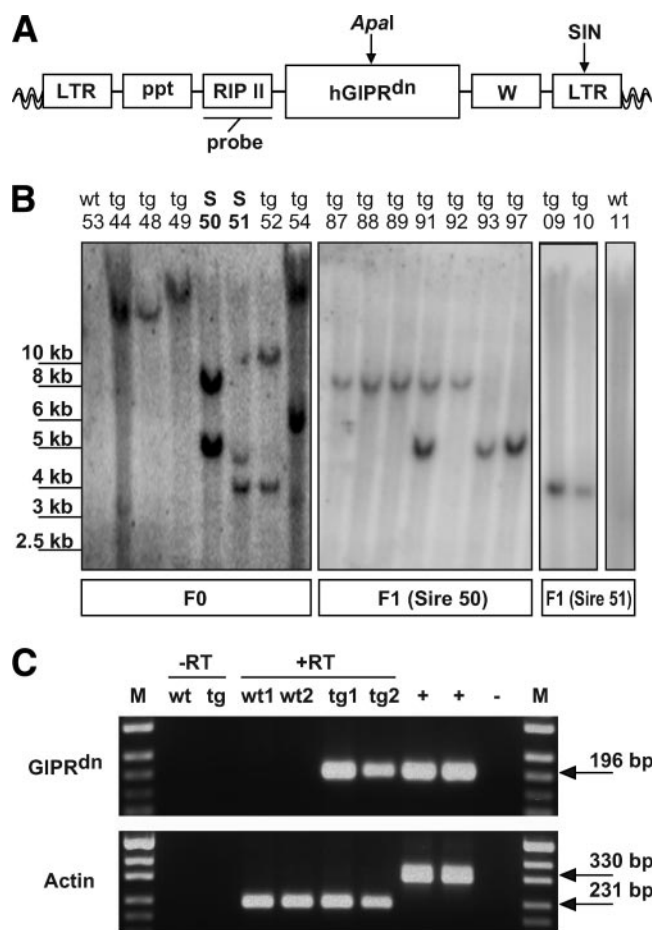
## RESULTS

**Generation of GIPR<sup>dn</sup> transgenic pigs.** A lentiviral vector was cloned that expresses a dominant-negative GIPR (GIPR<sup>dn</sup>) under the control of the rat insulin 2 gene promoter (RIP II) (Fig. 1A). The GIPR<sup>dn</sup> has an eight-amino acid deletion (positions 319–326) and an Ala $\rightarrow$ Glu exchange at amino acid position 340 in the third intracellular loop, which is essential for signal transduction (16). Lentiviral vectors were injected into the perivitelline space of pig zygotes (17). A total of 113 injected zygotes were transferred laparoscopically into the oviducts of three cycle-synchronized recipient gilts. Nineteen piglets (17% of the transferred zygotes) were born. Southern blot analysis identified nine founder animals (47% of the born animals) carrying one or two lentiviral integrants (Fig. 1B), confirming the high efficiency of lentiviral transgenesis in large animals (17).

Two male founder animals (nos. 50 and 51) were mated to nontransgenic females (Fig. 1B). The resulting offspring demonstrated germ line transmission and segregation of the integrants according to Mendelian rules (Fig. 1B). To analyze expression of GIPR<sup>dn</sup> mRNA, pancreatic islets were isolated from transgenic and nontransgenic offspring and analyzed by RT-PCR. Expression of the GIPR<sup>dn</sup> was detected in the islets of all transgenic animals but not in the islets of nontransgenic control animals (Fig. 1C). GIPR<sup>dn</sup> transgenic pigs developed normally and did not show any deviation in body weight gain compared with controls (Fig. 2).

**Normal fasting glucose and fructosamine levels in GIPR<sup>dn</sup> transgenic pigs.** To evaluate effects of GIPR<sup>dn</sup> expression on glucose homeostasis, fasting blood glucose and serum fructosamine levels were determined in regular intervals from 1 to 7 months of age. No significant differences in blood glucose levels and serum fructosamine levels were detected between GIPR<sup>dn</sup> transgenic and control pigs (supplementary Fig. 3). Fasting blood glucose levels, determined in irregular intervals up to an age of 2

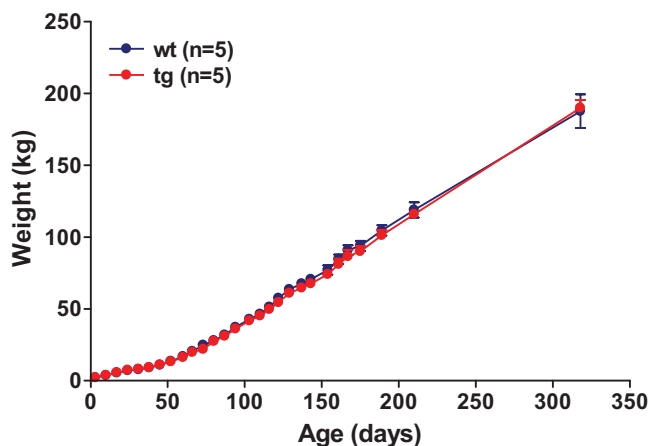




**FIG. 1.** Lentiviral vector, Southern blot analyses, and transgene expression. **A:** The lentiviral vector (LV-*GIPR<sup>dn</sup>*) carrying the cDNA of the dominant-negative *GIPR* (*GIPR<sup>dn</sup>*) under the control of the rat *Ins2* gene promoter (RIP II). *ApaI*, restriction site of *ApaI*; LTR, long terminal repeat; ppt, polypurine tract; SIN, self-inactivating mutation; W, woodchuck hepatitis posttranscriptional regulatory element; probe, probe used for Southern blot analyses; wavy lines, pig genome. **B:** Southern blot analyses of *ApaI*-digested genomic DNA isolated from EDTA blood of piglets generated by subzonal injection of LV-*GIPR<sup>dn</sup>* (transgenic [tg]) and two nontransgenic littermates (wild type [wt]). Pigs of the F0 generation show either one or two single-copy integration sites of the transgene. Sires 50 and 51 (S 50/S 51) were selected to establish two transgenic lines. Note that pigs of the F1 generation show segregation of the integrants according to the Mendelian rules. **C:** Analysis of transgene expression (*GIPR<sup>dn</sup>*) in isolated porcine islets of Langerhans of transgenic (tg) and nontransgenic littermates (wt) by RT-PCR.  $\beta$ -Actin RT-PCR used for confirmation of reverse transcription efficiency. Due to the use of intron-spanning primers to detect  $\beta$ -actin, two different-sized bands are visible differentiating cDNA and genomic DNA. M: pUC Mix Marker; -RT wt: minus RT wild-type pigs; -RT tg: minus RT *GIPR<sup>dn</sup>* transgenic pigs (no signals were obtained from islets of transgenic offspring after omission of the RT step, demonstrating that expressed rather than integrated sequences were detected); +, genomic DNA of *GIPR<sup>dn</sup>* transgenic pig; -, aqua bidest.

years, were unaltered in *GIPR<sup>dn</sup>* transgenic pigs (data not shown).

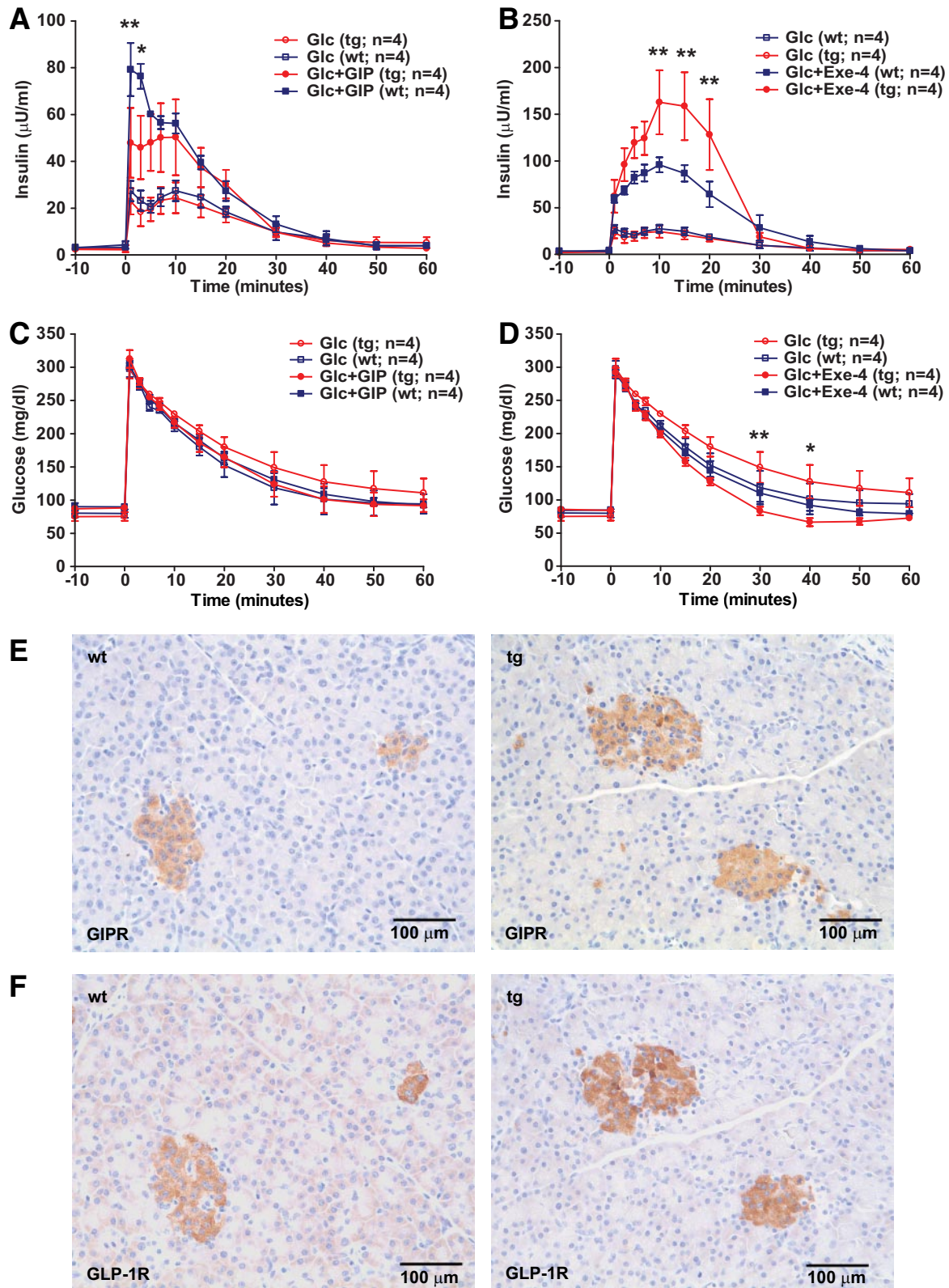
**Reduced insulinotropic effect of GIP but enhanced insulinotropic effect of exendin-4 in *GIPR<sup>dn</sup>* transgenic pigs.** To evaluate whether expression of a *GIPR<sup>dn</sup>* specifically impairs the function of GIP, we performed stimulation tests with GIP and the GLP-1 receptor agonist exendin-4 (1). The insulinotropic effect of GIP, intravenously administered as a bolus, was significantly diminished (Fig. 3A), while insulin secretion in response to exendin-4 was increased in *GIPR<sup>dn</sup>* transgenic versus control pigs (Fig. 3B), leading to a faster decrease of



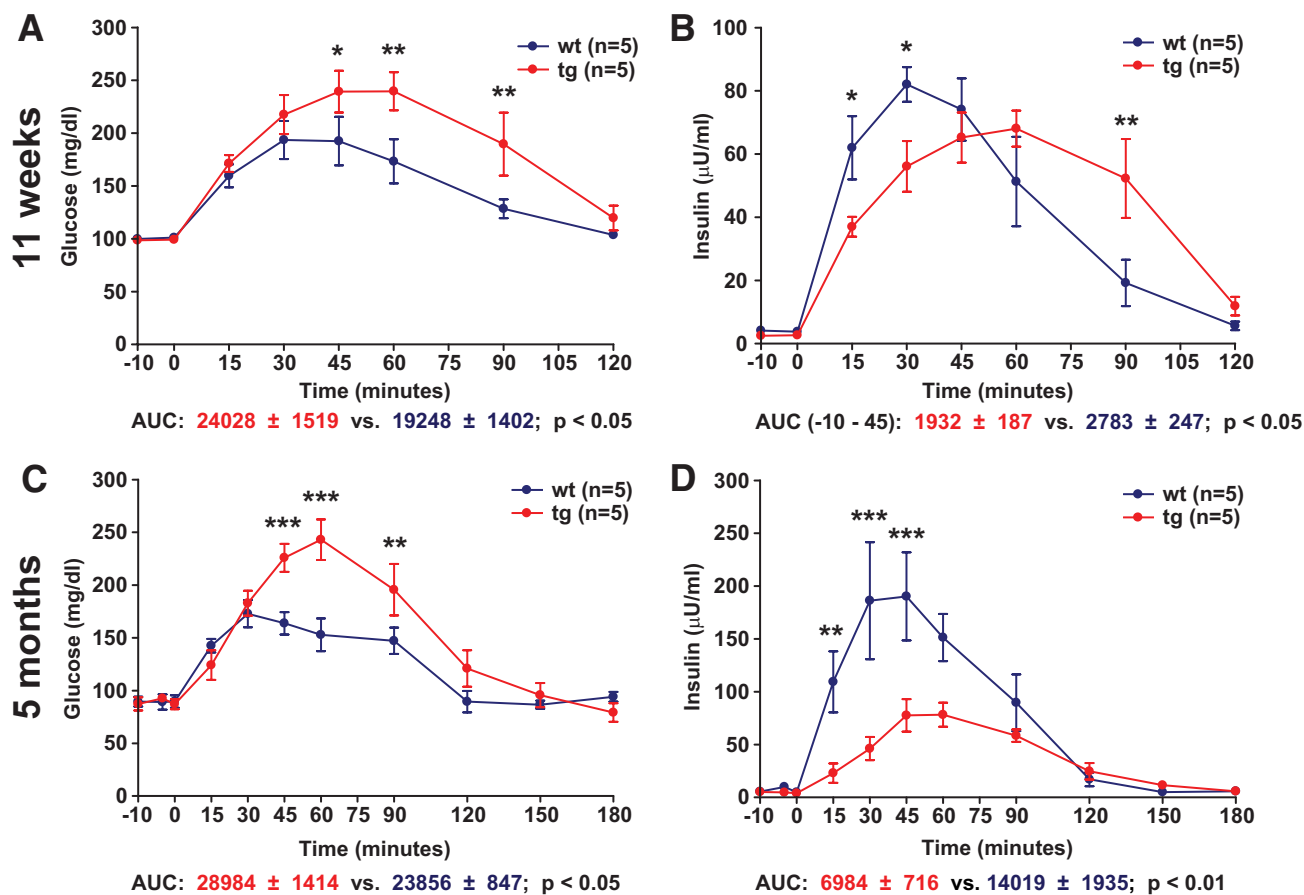
**FIG. 2.** Body weight gain of *GIPR<sup>dn</sup>* transgenic pigs (tg) compared with control pigs (wt). Data are means  $\pm$  SE. (A high-quality digital representation of this figure is available in the online issue.)

serum glucose levels (Fig. 3D). These findings demonstrate that expression of *GIPR<sup>dn</sup>* specifically reduces the insulinotropic action of GIP and does not impair the function of a related G-protein-coupled receptor, namely the GLP-1R. Further, the enhanced insulinotropic effect of exendin-4 in *GIPR<sup>dn</sup>* transgenic versus control pigs indicates a compensatory hyperactivation of the GLP-1/GLP-1R system, which has also been observed in *Gipr<sup>-/-</sup>* mice (rev. in 15). To clarify, whether compensatory mechanisms involve altered expression of incretin receptors, we performed immunohistochemical staining of pancreas sections for *GIPR* (Fig. 3E) and *GLP-1R* (Fig. 3F), which revealed no apparent difference in the abundance and spatial distribution of both receptors comparing *GIPR<sup>dn</sup>* transgenic and control pigs.

**Disturbed incretin function in young *GIPR<sup>dn</sup>* transgenic pigs.** An OGTT (2 g glucose/kg BW) was performed in 11-week-old *GIPR<sup>dn</sup>* transgenic pigs ( $n = 5$ ) and controls ( $n = 5$ ) originating from founder boars nos. 50 and 51. *GIPR<sup>dn</sup>* transgenic pigs exhibited elevated ( $P < 0.05$ ) serum glucose levels (Fig. 4A) as well as a distinct delay in insulin secretion (Fig. 4B) after glucose challenge. The area under the insulin curve (AUC insulin) during the first 45 min following glucose challenge was 31% ( $P < 0.05$ ) smaller in *GIPR<sup>dn</sup>* transgenic pigs than in age-matched controls (Fig. 4B); however, the total amount of insulin secreted during the experimental period (i.e., total AUC insulin until 120 min following glucose load) was not different between the two groups ( $5,155 \pm 763$  vs.  $5,698 \pm 625$ ;  $P = 0.351$ ). These findings indicate that expression of a *GIPR<sup>dn</sup>* in the pancreatic islets of transgenic pigs is sufficient to interfere with the incretin effect but does not initially affect the total AUC insulin. This assumption is supported by the fact that intravenous glucose tolerance was not reduced in *GIPR<sup>dn</sup>* transgenic pigs (Fig. 5A), and the time course and amount of insulin secreted in response to an intravenous glucose load were not different between the two groups (Fig. 5B). Quantitative stereological investigations of the pancreas (32) revealed that the total volume of  $\beta$ -cells in the pancreas was not different between *GIPR<sup>dn</sup>* transgenic pigs and controls (Fig. 6A). Further, the total volume of isolated  $\beta$ -cells (single insulin-positive cells and small clusters of insulin-positive cells not belonging to established islets) was equal in the two groups ( $49 \pm 4$  vs.  $49 \pm 6$  mm<sup>3</sup>;  $P = 0.695$ ). These findings



**FIG. 3.** Functional analysis of GIPR<sup>dn</sup> expression by GIP/exendin-4 stimulation test. Reduced insulinotropic action of GIP but enhanced insulinotropic action of exendin-4 in 11-week-old GIPR<sup>dn</sup> transgenic pigs (tg) compared with nontransgenic control animals (wt). **A:** Serum insulin levels of GIPR<sup>dn</sup> transgenic (tg) and control (wt) pigs after intravenous administration of glucose (Glc)  $\pm$  GIP. **B:** Serum insulin levels of GIPR<sup>dn</sup> transgenic (tg) and control (wt) pigs after intravenous administration of glucose (Glc)  $\pm$  exendin-4 (Exe-4). **C and D:** Corresponding serum glucose levels for the GIP (C) and exendin-4 (D) stimulation test. 0 min = point of Glc/GIP/exendin-4 administration. Data are means  $\pm$  SE. \* $P < 0.05$  vs. control; \*\* $P < 0.01$  vs. control. **E and F:** Immunohistochemical staining of GIPR (E) and GLP-1R (F) in pancreas sections from 11-week-old GIPR<sup>dn</sup> transgenic pigs (tg) and nontransgenic control animals (wt) does not provide evidence for differences in receptor abundance. (A high-quality digital representation of this figure is available in the online issue.)



**FIG. 4.** Oral glucose tolerance in 11-week-old and 5-month-old  $GIPR^{dn}$  transgenic pigs (tg) compared with nontransgenic littermates (wt). **A** and **C**: Serum glucose levels; 0 min = point of glucose administration. **B** and **D**: Serum insulin levels. AUC glucose/insulin for transgenic pigs (red) and wild-type pigs (blue). Data are means  $\pm$  SE. \* $P$  < 0.05 vs. control; \*\* $P$  < 0.01 vs. control; \*\*\* $P$  < 0.001 vs. control. Note that in 11-week-old transgenic pigs, there is a delay in insulin secretion leading to a significant reduction of insulin secretion during the first 45 min following oral glucose load, although the total amount of insulin secreted over 120 min is not different from controls. In contrast, 5-month-old transgenic pigs display not only delayed but also reduced insulin secretion. A high-quality digital representation of this figure is available in the online issue.

clearly demonstrate that expression of a  $GIPR^{dn}$  does not exhibit a toxic effect on pancreatic islets and further suggest that pancreatic islet neogenesis is not disturbed. **Glucose control in  $GIPR^{dn}$  transgenic pigs deteriorates with increasing age.** To monitor the long-term consequences of  $GIPR^{dn}$  expression, a second collective of animals was repeatedly investigated. First, an OGTT was performed in 5-month-old ( $20 \pm 1$  weeks)  $GIPR^{dn}$  transgenic pigs ( $n = 5$ ) and littermate controls ( $n = 5$ ) originating from founder boars nos. 50 and 51.  $GIPR^{dn}$  transgenic pigs exhibited elevated glucose levels (Fig. 4C) as well as a distinct reduction of initial insulin secretion after glucose challenge compared with their nontransgenic littermates (Fig. 4D). In addition, peak insulin levels were clearly reduced compared with controls. The AUC glucose was 26% ( $P < 0.05$ ) larger (Fig. 4C), whereas AUC insulin was 49% ( $P < 0.01$ ) smaller in  $GIPR^{dn}$  transgenic pigs (Fig. 4D). The latter finding suggests that, in contrast to 11-week-old animals, the overall insulin secretion following an oral glucose load is reduced in 5-month-old  $GIPR^{dn}$  transgenic pigs and that their islets may undergo progressive functional and/or structural changes. Additionally, an IVGTT was carried out in 5-month-old ( $22.5 \pm 1.5$  weeks)  $GIPR^{dn}$  transgenic and control pigs ( $n = 4$  per group; different collective of animals). Intravenous glucose tolerance (Fig. 5C), as well as insulin secretion (Fig. 5D), in  $GIPR^{dn}$  transgenic pigs was similar to controls. However, a

tendency toward reduced intravenous glucose tolerance and reduced insulin secretion in  $GIPR^{dn}$  transgenic pigs was visible.

Next, we performed an IVGTT in 11-month-old ( $45 \pm 2$  weeks)  $GIPR^{dn}$  transgenic pigs ( $n = 5$ ) and littermate controls ( $n = 4$ ) from the same collective of animals used for OGTT at 5 months of age.  $GIPR^{dn}$  transgenic pigs exhibited a decelerated decline of blood glucose levels (10% larger AUC glucose;  $P < 0.05$ ) (Fig. 5E), going along with significantly reduced insulin release (52% smaller AUC insulin;  $P < 0.05$ ) (Fig. 5F). This observation corroborated the suspicion that impaired GIPR function may cause a general disturbance of insulin secretion and/or alterations in islet structure and/or islet integrity over time.

**Impaired age-related expansion of pancreatic  $\beta$ -cell mass in  $GIPR^{dn}$  transgenic pigs.** To clarify long-term effects of  $GIPR^{dn}$  expression on the islets, we performed quantitative stereological analyses of pancreata from 5-month-old and from 1- to 1.4-year-old  $GIPR^{dn}$  transgenic pigs and controls. Pancreas weight did not differ between  $GIPR^{dn}$  transgenic pigs and control animals in both age-groups (Table 1).

Qualitative histological assessment revealed that pancreatic islet profiles of 5-month-old and 1- to 1.4-year-old  $GIPR^{dn}$  transgenic pigs appeared to be smaller in size and reduced in number (Fig. 6B and C). These findings were



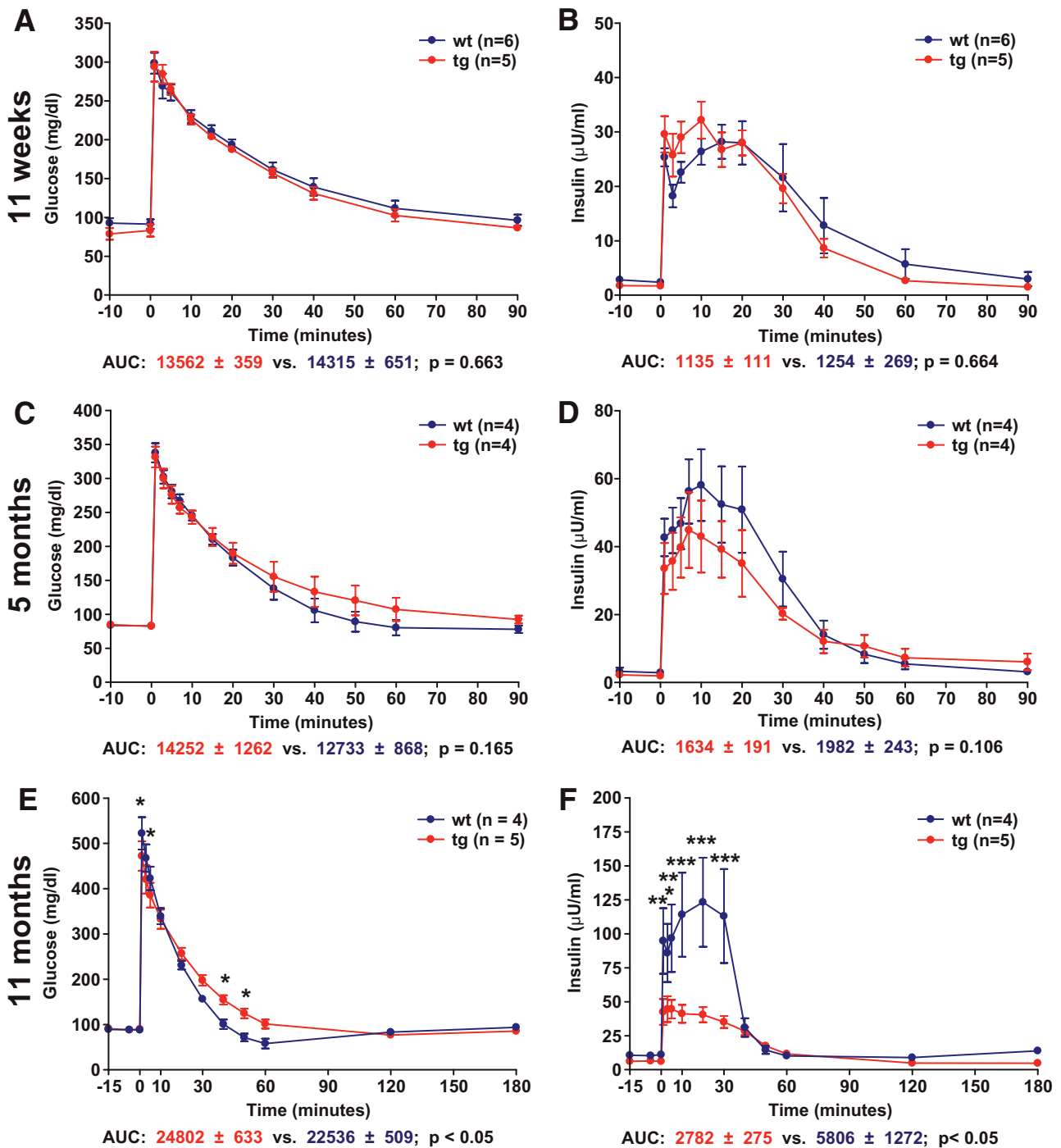


FIG. 5. Intravenous glucose tolerance in GIPR<sup>dn</sup> transgenic pigs (tg) compared with nontransgenic controls (wt). *A*, *C*, and *E*: Serum glucose levels; 0 min = point of glucose administration. *B*, *D*, and *F*: Serum insulin levels. AUC glucose/insulin for transgenic pigs (red) and wild-type pigs (blue). Data are means ± SE. \**P* < 0.05 vs. control; \*\**P* < 0.01 vs. control; \*\*\**P* < 0.001 vs. control. Note that intravenous glucose tolerance (*A*) and insulin secretion (*B*) are not altered in 11-week-old transgenic pigs. In 5-month-old transgenic pigs, a tendency of reduced insulin secretion (*D*) is observed, while 11-month-old transgenic pigs display a significantly reduced intravenous glucose tolerance (*E*) due to a significantly reduced insulin secretion (*F*). (A high-quality digital representation of this figure is available in the online issue.)

confirmed by quantitative stereological investigations. In 5-month-old GIPR<sup>dn</sup> transgenic pigs (*n* = 4), the total volume of β-cells [ $V_{(\beta\text{-cell}, P_{\text{pan}})}$ ] was diminished by 35% (*P* < 0.05) versus controls (*n* = 4) (Fig. 6*B*). In 1- to 1.4-year-old GIPR<sup>dn</sup> transgenic pigs (*n* = 5), the reduction of total β-cell volume compared with controls (*n* = 5) was even more pronounced (58%; *P* < 0.01) (Fig. 6*C*). Reduced β-cell mass of young adult GIPR<sup>dn</sup> transgenic pigs compared with controls was confirmed by islet isolation

experiments. The number of islet equivalents recovered from pancreas samples of GIPR<sup>dn</sup> transgenic pigs (*n* = 3) was reduced by 93% (*P* < 0.05) as compared with littermate controls (*n* = 3) (supplementary Table 2).

In contrast, volume density (data not shown) as well as the total volume of isolated β-cells were not different between GIPR<sup>dn</sup> transgenic and control pigs, neither at 5 months of age ( $121 \pm 18$  vs.  $127 \pm 15$  mm<sup>3</sup>; *P* = 0.883) nor at 1–1.4 years of age ( $77 \pm 8$  vs.  $71 \pm 5$  mm<sup>3</sup>; *P* = 0.844).

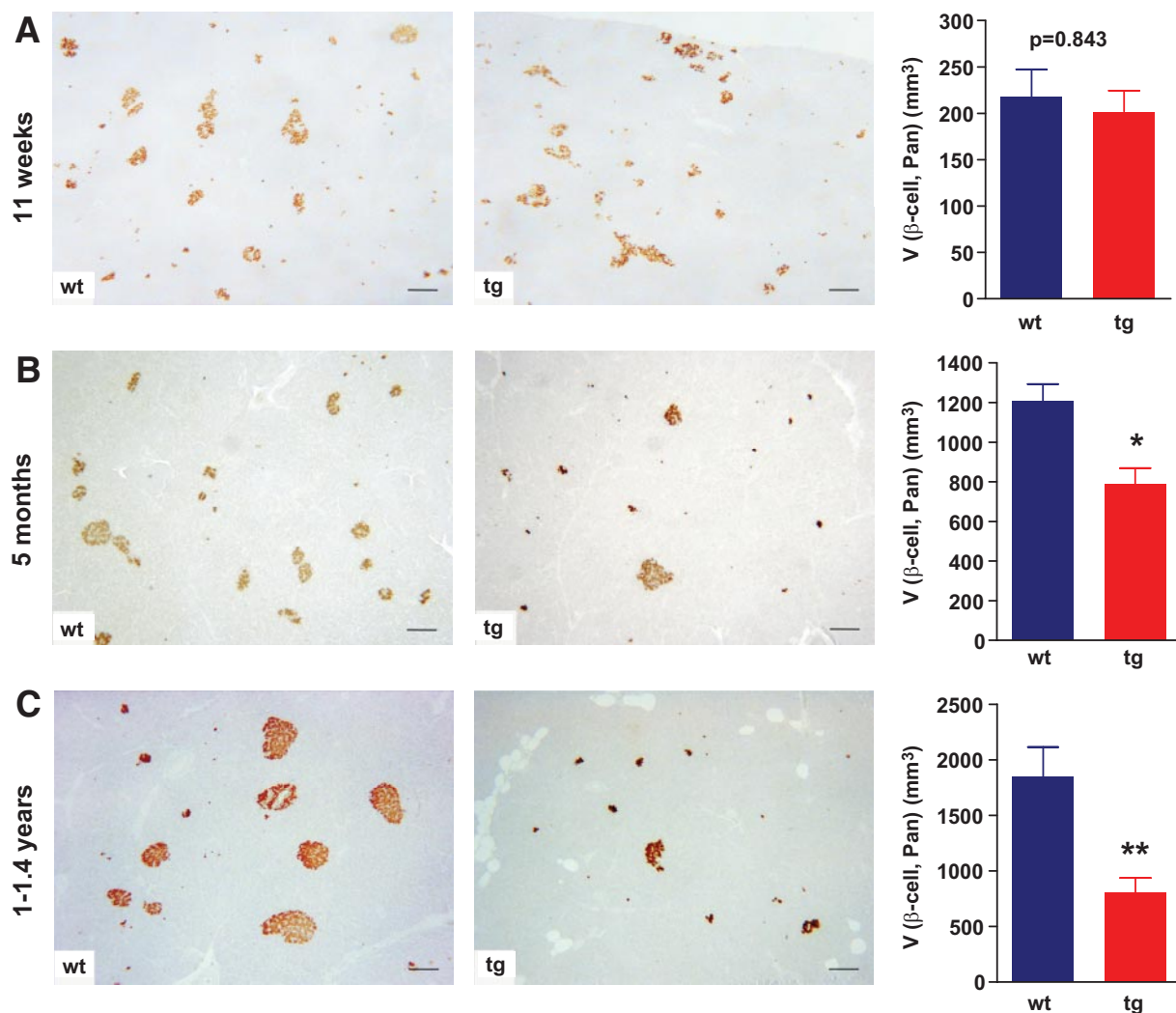


FIG. 6. Immunohistochemistry for insulin and total  $\beta$ -cell volume in the pancreas [ $V_{(\beta\text{-cell, Pan})}$ ] determined with quantitative stereological methods. A–C: Representative histological sections of pancreatic tissue from a control (wt) and a  $\text{GIPR}^{\text{dn}}$  transgenic pig (tg); scale bar = 200  $\mu\text{m}$ . Unaltered total  $\beta$ -cell volume in 11-week-old  $\text{GIPR}^{\text{dn}}$  transgenic pigs ( $n = 5$  per group) (A) but reduction of the total  $\beta$ -cell volume in 5-month-old ( $n = 4$  per group) (B) and young adult (1–1.4 years old) ( $n = 5$  per group)  $\text{GIPR}^{\text{dn}}$  transgenic pigs (C) compared with controls. Data are means  $\pm$  SE. \* $P < 0.05$  vs. control; \*\* $P < 0.01$  vs. control. A high-quality digital representation of this figure is available in the online issue.

These data clearly demonstrate an age-related reduction of pancreatic  $\beta$ -cell mass expansion in  $\text{GIPR}^{\text{dn}}$  transgenic pigs, which is in line with previous evidence for a trophic action of GIP on  $\beta$ -cells in vitro (5–7).

**Altered cellular composition of islets in  $\text{GIPR}^{\text{dn}}$  transgenic pigs.** To evaluate effects of  $\text{GIPR}^{\text{dn}}$  expression on the volume densities of the various endocrine islet cells and their total volumes, we performed detailed stereological analyses of the pancreatic islets in all three age classes investigated. In control animals, total volumes of  $\alpha$ -,  $\beta$ -,  $\delta$ -, and pp-cells in established islets increased significantly with age (Table 1). In  $\text{GIPR}^{\text{dn}}$  transgenic pigs, a similar age-dependent increase was seen for the total volumes of  $\alpha$ -,  $\delta$ -, and pp-cells. However, in comparison with controls, the increase of total  $\beta$ -cell volume of  $\text{GIPR}^{\text{dn}}$  transgenic pigs was less pronounced from 11 weeks to 5 months of age. Importantly, there was no further augmentation of total  $\beta$ -cell volume in 1- to 1.4-year-old  $\text{GIPR}^{\text{dn}}$  transgenic pigs, demonstrating that impaired GIPR function interferes with the physiological expansion of pancreatic  $\beta$ -cells. In addition, the fractional volume of  $\beta$ -cells in the islets was decreased, while that of  $\alpha$ - and  $\delta$ -cells was

increased in 1- to 1.4-year-old  $\text{GIPR}^{\text{dn}}$  transgenic pigs. However, the total volumes of these non- $\beta$ -cell populations were not different from those of age-matched control pigs (Table 1).

**Reduced proliferation rate of  $\beta$ -cells in  $\text{GIPR}^{\text{dn}}$  transgenic pigs.** To clarify the mechanism of impaired  $\beta$ -cell expansion in  $\text{GIPR}^{\text{dn}}$  transgenic pigs, we determined  $\beta$ -cell proliferation by double immunohistochemical staining for insulin and the proliferation marker Ki67 in all three age-groups. Indeed,  $\beta$ -cell proliferation was significantly reduced by 60% ( $P < 0.05$ ) in 11-week-old  $\text{GIPR}^{\text{dn}}$  transgenic pigs (Fig. 7A and B). In addition, we performed double immunohistochemical staining for insulin and the apoptosis marker cleaved caspase-3 to evaluate a potential impact of  $\text{GIPR}^{\text{dn}}$  expression on cell death in the  $\beta$ -cell compartment. Overall, the proportion of cleaved caspase-3 positive cells was very low, with no significant difference between  $\text{GIPR}^{\text{dn}}$  transgenic pigs and controls of all age classes. However, there was a trend ( $P = 0.075$ ) of more cleaved caspase-3 positive  $\beta$ -cells in 1- to 1.4-year-old  $\text{GIPR}^{\text{dn}}$  transgenic pigs as compared with age-matched controls (Fig. 7C and D).

TABLE 1  
Quantitative stereological analyses of the endocrine pancreas of GIPR<sup>dn</sup> transgenic pigs (tg) and wild-type control pigs (wt)

Parameter	11 weeks	5 months	1–1.4 years	ANOVA		
	( <i>n</i> = 5 wt, 5 tg) Means ± SE	( <i>n</i> = 4 wt, 4 tg) Means ± SE	( <i>n</i> = 5 wt, 5 tg) Means ± SE	Group	Age	Group × age
Pancreas weight (g)						
Wild type	34.5 ± 4.2	115.3 ± 5.6	183.4 ± 13.8	NS	<0.0001	NS
Transgenic	32.7 ± 3.1	125.7 ± 6.1	206.1 ± 4.9			
<i>V</i> <sub>V(β-cell/islet)</sub> (%)				0.0066	<0.0001	0.0024
Wild type	69.8 ± 2.2	89.0 ± 1.5	90.2 ± 1.2			
Transgenic	70.8 ± 1.3	87.0 ± 1.2	76.4 ± 3.2*			
<i>V</i> <sub>V(α-cell/islet)</sub> (%)				0.0122	<0.0001	0.0008
Wild type	14.1 ± 1.2	5.0 ± 0.8	5.0 ± 0.7			
Transgenic	12.2 ± 0.7	6.5 ± 0.7	13.8 ± 2.3*			
<i>V</i> <sub>V(δ-cell/islet)</sub> (%)				NS	<0.0001	NS
Wild type	13.5 ± 2.8	4.3 ± 0.8	2.2 ± 0.6			
Transgenic	13.0 ± 1.0	5.5 ± 0.9	5.8 ± 0.9†			
<i>V</i> <sub>V(pp-cell/islet)</sub> (%)				NS	0.0355	NS
Wild type	2.7 ± 0.7	1.8 ± 0.3	2.8 ± 0.8			
Transgenic	3.9 ± 0.5	1.3 ± 0.3	3.8 ± 1.1			
<i>V</i> <sub>V(β-cell/islet)</sub> (mm <sup>3</sup> )				0.0002	<0.0001	0.0024
Wild type	168.7 ± 29.5	1,088.2 ± 82.0	1,694.6 ± 251.7			
Transgenic	152.1 ± 17.1	664.1 ± 74.5†	663.7 ± 130.5*			
<i>V</i> <sub>V(α-cell/islet)</sub> (mm <sup>3</sup> )				NS	<0.0001	NS
Wild type	32.6 ± 8.7	58.4 ± 6.3	95.7 ± 17.4			
Transgenic	24.5 ± 1.7	47.7 ± 4.5	112.8 ± 14.5			
<i>V</i> <sub>V(δ-cell/islet)</sub> (mm <sup>3</sup> )				NS	0.0014	NS
Wild type	26.6 ± 3.9	49.5 ± 6.1	36.8 ± 6.3			
Transgenic	25.9 ± 1.8	39.6 ± 3.5	47.5 ± 5.2			
<i>V</i> <sub>V(pp-cell/islet)</sub> (mm <sup>3</sup> )				NS	<0.0001	NS
Wild type	6.0 ± 1.8	20.7 ± 2.9	52.4 ± 12.6			
Transgenic	8.2 ± 1.7	9.3 ± 2.1	30.3 ± 7.9			

Data were analyzed by the general linear models procedure (SAS Institute) taking the effects of group (wild type, transgenic), age (11 weeks, 5 months, 1–1.4 years), and the interaction group × age into account. For significant effects of these factors *P* values are indicated in the last three columns. In addition, significant differences between groups within age classes are marked by: \**P* < 0.01; †*P* < 0.05; NS, not significant.

## DISCUSSION

This study established the first transgenic large animal model of impaired incretin function. The cDNA of the human *GIPR* was mutated at the third intracellular loop, where a deletion of eight amino acids (positions 319–326) and a point mutation at position 340 was introduced. In stably transfected Chinese hamster lymphoblast (CHL) cells, GIPR<sup>dn</sup> bound GIP with normal affinity but failed to increase intracellular cAMP levels. Thus, the GIPR<sup>dn</sup> expressed in transgenic pigs is capable of ligand binding but not of signal transduction (16) and competes with the endogenous GIPR for GIP. Consequently, the insulinotropic effect of GIP is highly reduced but not completely eliminated, mirroring the situation in human type 2 diabetes.

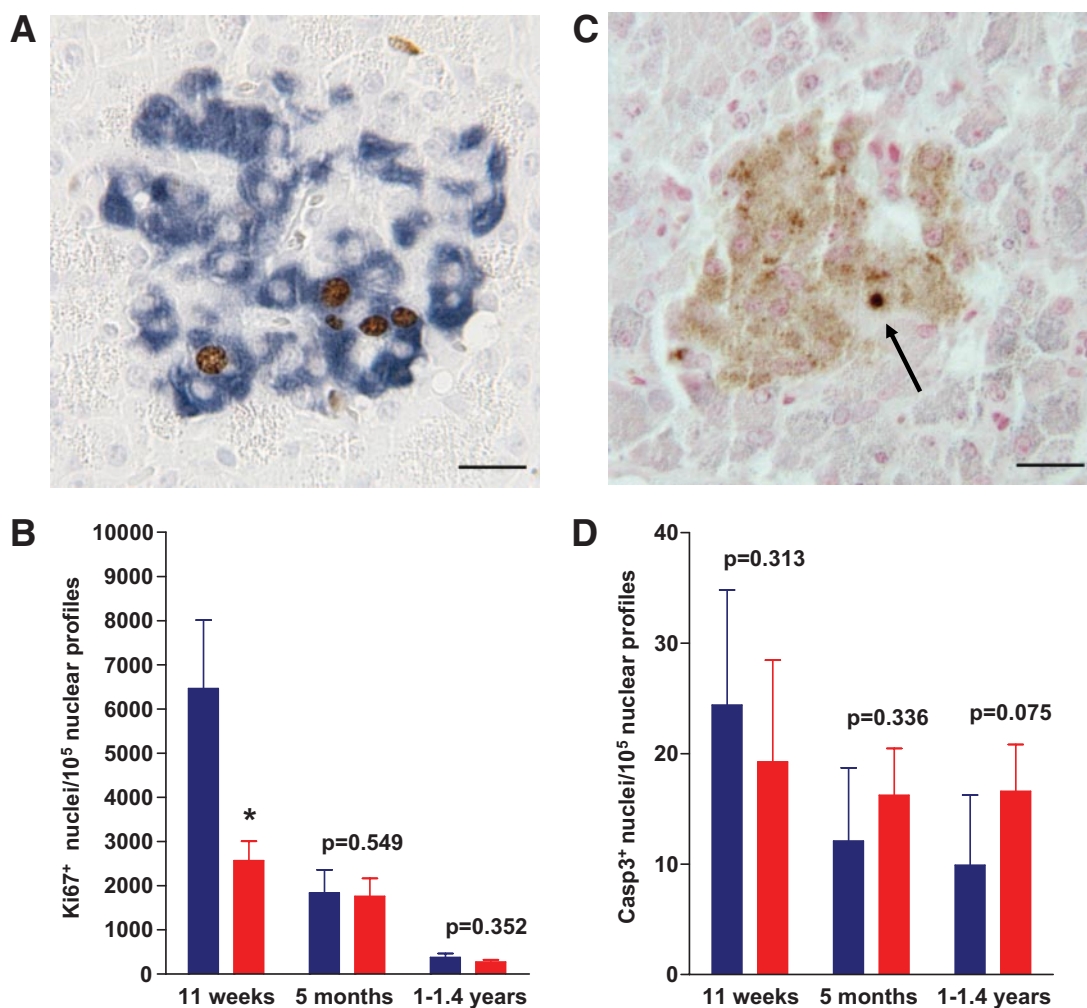
In view of the severe, early-onset diabetes of GIPR<sup>dn</sup> transgenic mice (16), we tested the oral and intravenous glucose tolerance of young (11-week-old) GIPR<sup>dn</sup> transgenic and control pigs and determined their pancreatic β-cell mass by quantitative stereological analyses. The finding of reduced oral glucose tolerance associated with delayed insulin secretion is in line with a disturbance of the incretin effect. Reduced oral glucose tolerance in GIPR<sup>dn</sup> transgenic pigs is also consistent with previous observations in *Gipr*<sup>-/-</sup> mice (14). Importantly, normal intravenous glucose tolerance, insulin secretion, and unchanged β-cell mass in 11-week-old GIPR<sup>dn</sup> transgenic pigs strongly argue against a toxic effect of GIPR<sup>dn</sup> expression. The reasons for different outcomes in the GIPR<sup>dn</sup> trans-

genic pig and the GIPR<sup>dn</sup> transgenic mouse model remain unclear but may be related to different methods of transgenesis (lentiviral transgenesis versus pronuclear DNA microinjection) or different copy numbers and/or integration sites leading to different expression levels of the transgene.

To evaluate long-term effects of GIPR<sup>dn</sup> expression in the pancreatic islets, we performed a longitudinal study of a collective of animals, involving OGTTs and IVGTTs. These revealed a progressive deterioration of glucose control in GIPR<sup>dn</sup> transgenic pigs, although none of our transgenic pigs has developed fasting hyperglycemia up to an age of 2 years. Quantitative stereological investigations of pancreata from 5-month-old and from 1- to 1.4-year-old GIPR<sup>dn</sup> transgenic pigs showed a reduced pancreatic β-cell mass, which was confirmed by quantitative islet isolation experiments.

Quantitative stereological analyses of the pancreatic islets demonstrated a marked increase of total β-cell volume from 11 weeks to 5 months (6.4-fold) and from 5 months to 1–1.4 years (1.6-fold) of age in control pigs. In contrast, the expansion of total β-cell volume in GIPR<sup>dn</sup> transgenic pigs was less pronounced between 11 weeks and 5 months of age (4.3-fold), with no further increase in 1- to 1.4-year-old animals. These findings are explained by a markedly reduced proliferation rate of β-cells in 11-week-old GIPR<sup>dn</sup> transgenic pigs, a developmental stage characterized by massive expansion of β-cells in pigs (36). Staining for cleaved caspase-3 did not show a significantly





**FIG. 7.**  $\beta$ -Cell proliferation and apoptosis. *A* and *C*: Representative histological sections doublestained for insulin (blue) and Ki67 (brown) (*A*) or for insulin (light brown) plus cleaved caspase-3 (dark blue; see arrow) (*C*). *B* and *D*: Determination of the number of Ki67- (*B*) and cleaved caspase-3-positive  $\beta$ -cells (*D*). Wild type: blue bars, transgenic: red bars. Wild type:  $n = 5$ , transgenic:  $n = 5$  for 11-week-old and 1- to 1.4-year-old pigs; wild type:  $n = 4$ , transgenic:  $n = 4$  for 5-month-old pigs. Data are means  $\pm$  SE. \* $P < 0.05$  vs. control; scale bar = 20  $\mu$ m. Note the significantly ( $P < 0.05$ ) reduced  $\beta$ -cell proliferation rate in 11-week-old GIPR<sup>dn</sup> transgenic pigs. (A high-quality digital representation of this figure is available in the online issue.)

increased rate of cell death in the  $\beta$ -cell compartment of GIPR<sup>dn</sup> transgenic pigs versus controls, although a trend of higher numbers of cleaved caspase-3 positive  $\beta$ -cells was visible in 1- to 1.4-year-old GIPR<sup>dn</sup> transgenic pigs. This may suggest a contribution of apoptosis to the reduction of total  $\beta$ -cell volume in mature GIPR<sup>dn</sup> transgenic pigs.

Due to the reduced volume fraction of  $\beta$ -cells in the islets of 1- to 1.4-year-old GIPR<sup>dn</sup> transgenic pigs, the relative volumes of  $\alpha$ - and  $\delta$ -cells in the islets were increased. However, since the islet volume was concomitantly reduced, the absolute volumes of  $\alpha$ - and  $\delta$ -cells were not different between GIPR<sup>dn</sup> transgenic and control pigs.

The numbers of animals investigated in our study are, in part, smaller than in some rodent studies. However, due to the large size of the pig, several blood-based parameters could be measured repeatedly with short time intervals in the same animals, providing an important advantage for statistical analysis. Further, the stereological data of all animals ( $n = 28$ ) were evaluated together by ANOVA, demonstrating significant group effects (Table 1) with  $P$  values  $< 0.01$  for many data/differences supporting our core statements.

Interestingly, *Gipr*<sup>-/-</sup> mice provided no evidence that GIPR action is required for the maintenance of islet and  $\beta$ -cell integrity in vivo (15,37). These mice exhibited an increase in relative  $\beta$ -cell area referring to pancreas area (37), leading to the conclusion that in vivo the function of GIP is primarily restricted to that of an incretin (15). However, the relatively mild phenotype of *Gipr*<sup>-/-</sup> mice may result from compensatory mechanisms (15). Although mice lacking both GIPR and GLP-1R exhibited more severe glucose intolerance than the individual mutants (38,39) these double mutant animals did not develop diabetes, raising the suspicion of the existence of compensatory mechanisms other than the GIP/GLP-1 system (38). The findings in GIPR<sup>dn</sup> transgenic pigs suggest that, in addition to its role as an incretin hormone, GIP is necessary for the expansion of  $\beta$ -cell mass and that its partial loss of function cannot be fully compensated by hyperactivation of the GLP-1/GLP-1R system.

In conclusion, GIPR<sup>dn</sup> transgenic pigs resemble characteristic features of human type 2 diabetic patients very closely in the following ways: disturbed GIP function, glucose intolerance, and reduced pancreatic  $\beta$ -cell mass. Moreover, GIPR<sup>dn</sup> transgenic pigs may be an attractive

animal model for the development and preclinical evaluation of incretin-based therapeutic strategies (40). Another potential application of GIPR<sup>dn</sup> transgenic pigs is the development of novel techniques for dynamic in vivo monitoring of pancreatic islet mass (41). Due to their size and close physiological and anatomical similarities to humans (42), pigs represent attractive animal models for translating novel therapeutic and diagnostic principles into clinical practice.

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