Original Article

Attenuated Wnt Signaling Perturbs Pancreatic Growth but Not Pancreatic Function

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Mesenchymal-epithelial interactions are pivotal for proper pancreatic growth and development. We have earlier shown that the fibroblast growth factor (FGF) receptor 2 is expressed in pancreatic progenitor cells and that FGF10, the high-affinity ligand of the FGF receptor 2 isoform FGF receptor 2b, promotes expansion of pancreatic progenitors. The Wnt family of ligands, which signal to the Frizzled (Frz) type receptors, have also been shown to mediate mesenchymal-epithelial interactions and cell proliferation in a variety of different systems. Here, we show that Frz3, like FGF receptor 2, is expressed in the pancreatic epithelium during the proliferative phase of the embryonic pancreas in mice and that overexpression of a dominant-negative form of mouse Frz8 in pancreatic progenitors severely perturbs pancreatic growth. Nevertheless, the transgenic mice remain normoglycemic and display normal glucose tolerance and glucose-stimulated insulin secretion when challenged with exogenous glucose. The maintenance of normoglycemia in these mice appears to be the consequence of a relative increase in endocrine cell number per pancreatic area combined with enhanced insulin biosynthesis and insulin secretion. Collectively, our data provide evidence that Wnt signaling is required for pancreatic growth but not adult β-cell function. *Diabetes* 54: 2844–2851, 2005

The embryonic pancreas in mammals forms from a dorsal and ventral protrusion of the primitive gut epithelium (1). These buds grow, branch, and fuse to form the definitive pancreas, a process dependent on mesenchymal-epithelial interactions. Fibroblast growth factor (FGF) signaling has been implicated in the development of many organs that are dependent on mesenchymal-epithelial interactions, including the pancreas (2,3), and several independent studies have demonstrated that FGF signaling is critical for pancreatic growth. FGF ligands have been shown to have a stimulatory effect on pancreatic epithelial cell proliferation in vitro (4), and mice homozygous for a targeted deletion of the *Fgf10* gene present with a hypoplastic pancreas due to impaired proliferation of pancreatic epithelial progenitors (5). In contrast, mice overexpressing *Fgf10* under the *Ipf1/Pdx1* promoter showed enhanced and persistent proliferation of pancreatic progenitors at the expense of pancreatic cell differentiation (6,7).

Other factors that stimulate mesenchymal-epithelial interactions are the Wnt family of secreted glycoproteins. Wnts are expressed in a wide variety of mesenchymal tissues and have been shown to stimulate growth of several organs by signaling to epithelial cells (8). They bind to and activate members of the Frizzled (Frz) family of serpetine receptor proteins and are implicated in a variety of developmental processes such as cell differentiation, cell polarity, cell migration, and cell proliferation (9–12). Several independent studies have demonstrated a key role for Wnt signaling in cell proliferation. During development, Wnt signaling is required in the entire nervous system for expanding the progenitor cell population by simultaneously promoting cell proliferation and blocking apoptosis and differentiation (13). Studies in colorectal cancer cell lines and in transgenic mice in which *Dkk1*, a Wnt antagonist, is ectopically expressed have also demonstrated an essential role of Wnt signaling in the maintenance of intestinal stem cells by promoting proliferation and blocking differentiation (14–16). In addition, Wnt signaling promotes self-renewal of hematopoietic stem cells (HSCs); blocking Wnt signaling by overexpressing *Axin* or by incubating the HSCs in presence of the soluble Frz8 CRD-IgG fusion protein inhibits proliferation in vitro and the ability of HSCs to reconstitute blood cells in vivo (17).

Wnt ligands and Frz receptors have been found to be expressed in the pancreas of mice and humans (18,19). RT-PCR analyses have shown that *Wnt 5a*, *5b*, *2b*, *7b*, and *11* as well as *Frz 2-9* are expressed in the developing pancreas (18). Mice in which Wnt1 and Wnt5a are misexpressed under control of the *Ipf1/Pdx1* promoter show perturbed patterning of the foregut, including the pancreatic domain (18). In *Ipf1/Pdx1-Wnt1* embryos, the foregut region resembled a posterior extension of the stomach associated with complete pancreatic and splenic agenesis. *Ipf1/Pdx1-Wnt5a* embryos displayed decrease in mass of several structures derived from the proximal foregut, including the pancreas, spleen, and stomach, without any apparent shift in the stomach-to-duodenum boundary (18). In addition, a role of Wnt signaling in adult β-cell function has been proposed (20). Mice that lack the gene encoding LDL receptor-related protein 5 (LRP5), a coreceptor involved in β-catenin–dependent Wnt signaling, show impaired glucose tolerance due to perturbed glucose-stimulated insulin secretion (GSIS) (20).

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CPA, carboxypeptidase A; DAPI, 4′,6 diamidino-2-phenylindole; DIG, digoxigenin; FGF, fibroblast growth factor; Frz, Frizzled; GSIS, glucose-stimulated insulin secretion; HSC, hematopoietic stem cell; LRP5, LDL receptor-related protein 5.

Here, we describe the temporal and spatial expression of *Wnt4*, *Wnt7b*, and *Frz3* in the pancreas in mice during the proliferative phase, i.e., embryonic days (e) 10–15. We also show that overexpression of a Frz8 CRD-IgG fusion protein, which functions as a Wnt signaling antagonist by inhibiting the binding of Wnt proteins to the Frz receptors (17,21), in the developing pancreatic epithelium in mice leads to grossly reduced pancreatic mass, suggesting a role for Wnt signaling in pancreatic epithelial cell proliferation. We also provide evidence that perturbation of Wnt signaling, via the expression of the Frz8 CRD domain in adult β-cells, does not affect adult β-cell function.

RESEARCH DESIGN AND METHODS

Generation and genotyping of transgenic mice. The *Frz8CRD-IgG* cDNA (21) (generous gift from Dr. J. Nathans) was cloned after the *Ipf1/Pdxl* promoter and followed by a SV40 polyA site. Transgenic mice were generated by pronuclear injection of the purified fragment (1.8 ng/ml) into F2 hybrid oocytes from B6/CBA parents (M&B A/S) as described previously (22). The genotype was determined by PCR analysis of genomic DNA extracted from tail biopsies using primers (from 5′ to 3′): IPF1, 5′-3(GGGAAGAGGAGATGT AGACTT); FRZ8CRD, 3′(GGTTGAACTGGTTGGGCATGT). Four independent transgenic lines showed pancreatic hypoplasia, and all were used for further analysis.

In situ hybridization and immunohistochemistry. An 800-bp *Hin*dIII-*Eco*RI fragment of the mouse full-length *Frz3* (23) (provided by Dr. J. Nathans) and a 760-bp *Eco*RI fragment representing the entire mouse *Wnt4* (23) cDNA (provided by Dr. A. McMahon) were subcloned into the pBluescript SK (Stratagene) digested with *Eco*RI/*Hin*dIII and *Eco*RI, respectively. A 500-bp *Wnt7b* PCR product was cloned into the pGEMT-Easy Vector (Promega). Digoxigenin (DIG)-labeled antisense probes were synthesized from the T7 promoter on both the pBluescript SK and pGEMT-Easy Vector using the DIG RNA Labeling kit (Roche). DIG-labeled RNA probes for *ngn3* and *Notch1* were used as previously described (24). In situ hybridization using DIG-labeled RNA probes was performed essentially as described previously (24). Immunohistochemical localization of antigens on frozen sections and whole mount was performed as described previously (24). The primary antibodies used were rabbit anti-IPF1 (25), rabbit anti-phospho-Histone H3 (Upstate Biotech), rabbit anti-ISL1 (26), rabbit anti-carboxypeptidase A (anit-CPA) (Anawa), guinea pig anti-insulin (Linco), rabbit anti-glucagon (EuroDiagnostica), rabbit anti-cleaved caspase 3 (Cell Signaling), rabbit anti-ngn3 (27), fluorescein isothiocyanate–conjugated anti-human IgG (Jackson), rat anti–E-cadherin (Zymed), and mouse monoclonal anti-dephosphorylated β-catenin (Upstate Biotech). The secondary antibodies used were fluorescein anti-guinea pig (Jackson), fluorescein anti-rat (Jackson), Cy3 anti-rabbit (Jackson), biotinylated anti-mouse (DAKO), and biotinylated anti-rabbit (DAKO).

Quantification of proliferating cells and endocrine cell types. Pancreas from wild-type and *Ipf1/Frz8CRD* mice at various developmental and neonatal stages were sectioned. Every 10th section was selected for analysis. At least 40 sections, separated by 500 μm, were processed per animal. Proliferating cells were detected using an antibody against phospho-Histone H3, and the different endocrine types were detected using antibodies against insulin and glucagon. 4′,6 Diamidino-2-phenylindole (DAPI) staining was used to label all cells throughout each section, and ISL1 antibodies were used to label all endocrine cells. The ratio between the total area of phospho-histone H3/insulin/glucagon immunoreactivity and the total area of DAPI staining/ISL1 staining was calculated by image software (Image Pro Plus; Media Cybernetics) and used to evaluate the abundance of proliferating cells and the different endocrine cell types.

In vivo glucose and insulin measurements. Overnight-fasted mice were injected with glucose (1 g/kg i.p.), and blood samples were obtained from the tail vein. Blood glucose levels were measured using a Glucometer Elite (Bayer). Serum insulin levels were measured using enzyme-linked immunosorbent assay (Crystal Chem). Total pancreatic insulin was extracted using acid ethanol (75% ethanol and 0.2 mol/l HCl) and measured using Sensitive Rat Insulin RIA kit (Linco). Total pancreatic protein concentration was determined using Bio-Rad protein assay (Bio-Rad).

Quantification of mRNA levels. cDNA was prepared from total RNA prepared from isolated islets essentially as described previously (28). Real-time PCR analysis was performed using the ABI PRISM 7,000 Sequence Detection System and SYBR Green PCR Master Mix (ABI) according to the manufacturer's recommendations. Expression of the 60S Acidic Ribosomal Protein P0, β-2-microglobulin, and glyceraldehyde dehydrogenase was used to normalize expression levels.

Data analysis. Data in the figures are shown as ±SD and means ± SEM. *P* values were calculated using the Student's *t* test, and a value of 0.05 was considered statistically significant. Total insulin secretion for transgenic and wild-type animals was calculated using the trapezium rule for areas bound by a curve and the *x*-axis, with baseline = 0. All calculations and statistical evaluations of results were performed using Excel (2000).

RESULTS

***Wnt7b*, *Wnt4*, and *Frz3* are expressed in the pancreas.** Wnts have been suggested to exert a role during early and late stages of gastrointestinal development as well as in adult islets (18–20). We used a degenerate RT-PCR approach to screen for Wnts and Frz in the mouse embryonic pancreas of stage e12 and e15. The amplicons were subcloned and sequenced to deduce the identity of each amplicon. *Wnt4*, w*nt7b*, and *Frz3* were the most abundantly represented amplicons among the sequenced clones and were therefore selected for further expression analyses.

Wnt4 expression was not observed in the early pancreatic progenitors (Fig. 1*A*). *Wnt4* expression was, however, distinct in the later-appearing ngn3$^+$ pro-endocrine cells and differentiated endocrine cells as well as the intestinal and stomach epithelia (Fig. 1*B* and data not shown). At later stages of development, *Wnt4* expression became restricted to the islets with preferential high expression in non–β-cells (Fig. 1*C* and data not shown). In agreement with previous findings, *Wnt7b* expression was apparent in the lung buds (29) and spinal cord (30) but not in the pancreas (18) of e10 embryos (Fig. 1*D* and *E*). By e13, *Wnt7b* expression was, however, expressed in the pancreatic epithelium (Fig. 1*F*).

In contrast to a report by Heller et al. (18), we found *Frz3* to be expressed in the early e10 pancreatic buds (Fig. 1*G*). At this stage, the majority of the *Frz3*-expressing cells in the pancreatic bud coexpressed IPF1/PDX1 (Fig. 1*G*). A lower level of *Frz3* expression was also observed in the adjacent IPF1/PDX1$^-$ mesenchyme (Fig. 1*G*). *Frz3* expression was maintained in the developing pancreatic epithelium until e15 (Fig. 1*H* and data not shown) but appeared reduced and barely detectable at later stages (Fig. 1*I*). The temporal expression of *Frz3* in the pancreatic epithelium between e10 and e15 overlaps with the major phase of pancreatic growth, implying a potential role for Wnt signaling in pancreatic epithelial cell proliferation.

The *Ipf1/Frz8CRD* mice show reduced pancreatic mass. To elucidate a potential role for Wnt signaling in pancreatic growth and/or differentiation, we next generated transgenic mice expressing the mouse Frz8 CRD IgG fusion protein (17,21) in the developing and adult pancreas using the *Ipf1/Pdx1* promoter. Wnts bind to Frz receptors via the CRD domain, which is highly conserved between different Frz members, and thus the CRD domain alone functions as an antagonist of Wnt signaling by inhibiting binding of Wnt ligands to Frz receptors (17,21,31). Expression of the transgene was confirmed in early pancreatic progenitors and adult islets by monitoring the expression of human IgG, which is fused to the CRD domain thus enabling secretion of the transgene product (Fig. 2*A*). *Ipf1/Frz8CRD* neonates were born alive and appeared indistinguishable from their littermates but presented with a drastically reduced pancreas (Fig. 2*B*). The net wet weight of the transgenic pancreas of 10-week-old *Ipf1/Frz8CRD* mice was merely 25% of that of the wild type (~8 mg, $n = 5$ for the transgenic; ~30 mg, $n = 4$ for the wild

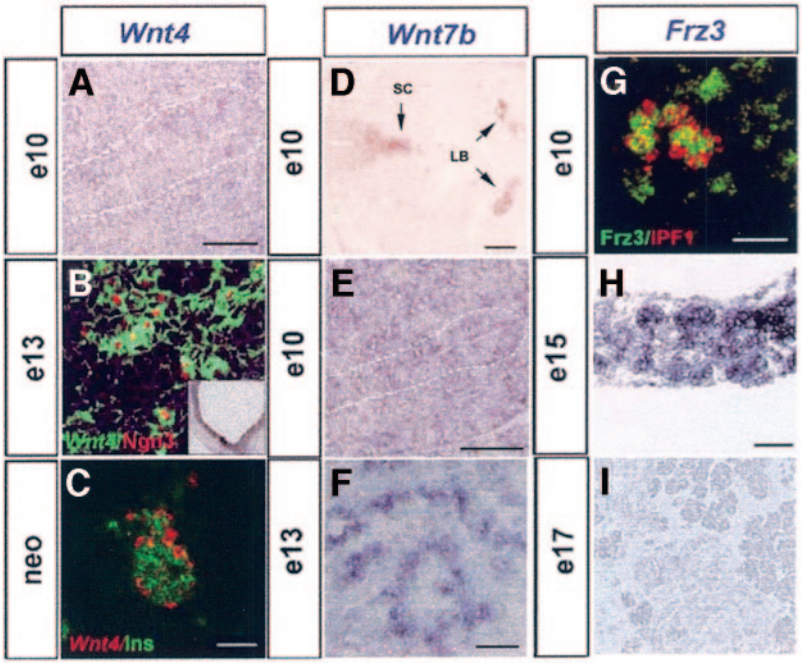

FIG. 1. *Wnt4*, *Wnt7b*, and *Frz3* expression in the developing and neonatal pancreas. *A–C*: In situ hybridization of e10, e13, and neonatal pancreas and e13 stomach (*inset* in *B*) using a DIG-labeled *Wnt4* probe (dark blue in *A* and inset in *B*, green pseudo-color in *B*, and red pseudo-color in *C*) counterstained with antibodies against ngn3 (red in *B*) and insulin (green in *C*). *D–F*: In situ hybridization of e10 spinal cord (SC) and lung buds (LB) (*D*), e10 pancreas (*E*), and e13 pancreas (*F*) using a DIG-labeled *Wnt7b* probe (dark blue in *E* and *F*). *G–I*: In situ hybridization of e10, e15, and e17 pancreas using a DIG-labeled *Frz3* probe (green pseudo-color in *G* and dark blue in *H*) counterstained with antibodies against IPF1/PDX1 (red) in *D*. The dotted lines indicate the pancreatic region. Scale bars in *A* and *B*, 0.06 mm; *C*, 0.04 mm; *D*, 0.03 mm; *E*, 0.07 mm; *F*, 0.04 mm; *G*, 0.07 mm; *H* and *I*, 0.04 mm.

type; Fig. 2*C*). Together, these data indicate that Wnt signaling is critical for pancreatic growth.

The reduction in pancreatic size observed in the *Ipf1/Frz8CRD* mice is due to attenuated pancreatic epithelial cell proliferation. To investigate whether the reduction in pancreatic mass displayed by the *Ipf1/Frz8CRD* mice reflected perturbations in initial pancreatic specification, proliferation, apoptosis, or premature differentiation, we set out to explore each of these possibilities separately.

Defect pancreatic specification would reflect a role for Wnt signaling in controlling and/or mediating the molecular cues that define and/or induce the pancreatic domain along the primitive gut endoderm. If Wnt signaling is involved in the early specification of the pancreatic program, the phenotype would be evident already at e9–e10. To address this question, we performed whole-mount immunohistochemistry on transgenic ($n = 5$) and wild-type ($n = 4$) e10 embryos and compared the size of the pancreas at this stage. No obvious difference was apparent in the size of the dorsal and ventral buds of e10 transgenic versus wild-type embryos (Fig. 3*A*), providing evidence that the reduced pancreatic size of the *Ipf1/Frz8CRD* mice is not due to perturbed pancreatic specification.

Activated, intracellular Notch blocks pancreatic cell differentiation by repressing the expression of the pro-endocrine gene *ngn3* (24,32). In the absence of activated Notch, pancreatic progenitor cells differentiate prematurely into the endocrine lineage at the expense of pancreatic progenitor cell expansion (24). To exclude that the reduced pancreatic size observed in *Ipf1/Frz8CRD* mice was the consequence of premature differentiation, we examined the expression of differentiation markers in e13 pancreases. No increase in the expression of the pro-endocrine marker *ngn3* (Fig. 3*A*) or the endocrine marker ISL1 (data not shown) was observed, and the expression of *Notch1* was indistinguishable from that observed in wild-type embryos of the same stage (Fig. 3*A*). Together, these data provide evidence that the reduced pancreatic

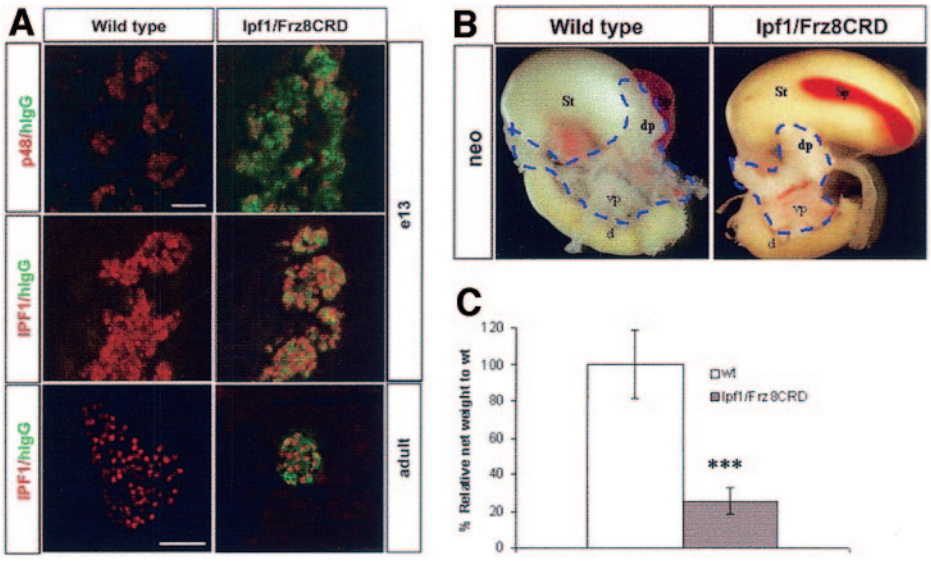

FIG. 2. Pancreatic hypoplasia in *Ipf1/Frz8CRD* mice. *A*: Expression of the *Ipf1/Frz8CRD* transgene was detected using antibodies against human IgG (hIgG) (green) and counterstained with antibodies against Ptf1a/p48 (red) and IPF1/PDX1 (red) on e13 and adult pancreatic sections from wild-type and transgenic mice. *B*: Gross morphological analyses of wild-type and *Ipf1/Frz8CRD* transgenic mice. The area of the pancreas is indicated by the dotted line. *C*: Determination of pancreatic weight in 10-week-old wild-type (wt) and *Ipf1/Frz8CRD* mice. St, stomach; Sp, spleen; Dp, dorsal pancreas; Vp, ventral pancreas; d, duodenum. Scale bars in *A*, 0.04 mm (*top row*) and 0.05 mm (*last row*). Data are means ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ vs. control animals.

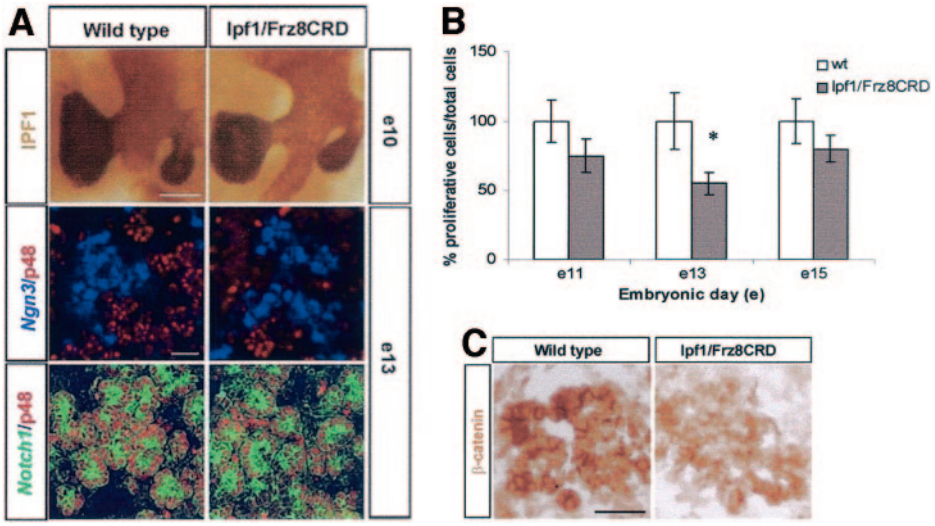

FIG. 3. ***A*** **and** ***B*****: The reduced pancreas of** ***Ipf1/Frz8CRD*** **mice is the consequence of decreased epithelial cell proliferation.** ***A*****: Whole-mount immunostaining using anti-IPF1/PDX1 antibodies (brown) on e10 wild-type and** ***Ipf1/Frz8CRD*** **embryos. In situ hybridization of e13 wild-type and** ***Ipf1/Frz8CRD*** **pancreatic sections using DIG-labeled** ***ngn3*** **(blue pseudo-color;** ***middle panel*****) and** ***Notch 1*** **(green pseudo-color;** ***bottom panel*****), counterstained with antibodies against Ptf1a/p48 (red).** ***B*****: Determination of proliferating cells over total pancreatic cells in e11, e13, and e15, wild-type (wt) and** ***Ipf1/Frz8CRD*** **pancreatic sections.** ***C*****: Immunohistochemical analyses of e13 wild-type (***left panel***) and** ***Ipf1/Frz8CRD*** **(***right panel***) pancreatic sections using a monoclonal antibody against dephosphorylated (i.e., activated) β-catenin. Scale bars in** ***A*****, 0.05 mm (***top row***) and 0.04 mm (***second row***); in** ***C*****, 0.07 mm (***left* **and** ***right panels***). Data are means ± SD. ******P* **< 0.001; *****P* **< 0.01; ****P* **< 0.05 vs. control animals.**

size in *Ipf1/Frz8CRD* mice is not due to premature endocrine differentiation.

To investigate whether an increase in apoptosis could explain the reduced pancreatic mass observed in the *Ipf1/Frz8CRD* mice, transgenic and wild-type pancreases at stages e13–17 were stained for the apoptotic marker Caspase 3. We did not find evidence of increased apoptosis in the embryonic pancreas of transgenic mice, suggesting that enhanced apoptosis did not cause the reduction in pancreatic size in the transgenic mice (data not shown).

Finally, we performed immunohistochemistry using the mitotic marker phospho-Histone H3 and calculated the ratio of phospho-Histone $H3^+$ cells over DAPI, i.e., total cell number (for all stages; transgenic, $n = 4$; wild type, $n = 4$). We detected a 25% reduction in the proliferating cells in the pancreas of e11 transgenic embryos and an even greater reduction, by 50%, at e13 (Fig. 3*B*). At e15, when pancreatic progenitor cell proliferation is declining also in wild-type mice, the difference in the proliferation rate was merely 20% (Fig. 3*B*). Taken together, these data provide evidence that the reduced size in *Ipf1/Frz8CRD* mice is the consequence of decreased pancreatic cell proliferation rather than perturbed specification, premature differentiation, or increased apoptosis.

The *Ipf1/Frz8CRD* mice display reduced levels of nonphosphorylated β-catenin in the pancreatic epithelium. Canonical Wnt signaling results in accumulation of nonphosphorylated β-catenin in the cytoplasm that then can be translocated to the nucleus and activate downstream target genes by interacting with the LEF/TCF transcription factors. Thus, accumulation of nonphosphorylated β-catenin is indicative of active, canonical Wnt signaling (33). Immunohistochemical analyses of e13 wild-type and transgenic pancreases using a monoclonal antibody specific for the nonphosphorylated form of β-catenin demonstrated a strong immunoreactivity in the pancreatic epithelium of wild-type mice (Fig. 3*C*). In support of an attenuated Wnt signaling, provoked by the expression of the Frz8CRD protein, β-catenin immunoreactivity was distinctly weaker in the pancreatic epithelium of the *Ipf1/Frz8CRD* mice (Fig. 3*C*). The weak β-catenin staining observed in the transgenic pancreas also implies that canonical, rather than noncanonical, Wnt signaling is active in the pancreatic epithelial cells at this stage.

The *Ipf1/Frz8CRD* mice show normal endocrine and exocrine function. To analyze in detail the differentiated state of the pancreas in *Ipf1/Frz8CRD* neonates, we examined the expression of transcription factors, hormones, and enzymes. Expression of the transcription factors IPF1/PDX1, ISL1, the hormones insulin and glucagon, and the exocrine enzyme CPA was apparently normal in the pancreas of the *Ipf1/Frz8CRD* mice (Fig. 4*A–H*). The islets of the *Ipf1/Frz8CRD* mice appeared somewhat smaller in size but showed normal organization with the β-cells residing in the core of the islet and the α-cells in the periphery (Fig. 4*A* and *B*). The transgenic mice also presented with fully developed acinar structures, and CPA expression in transgenic pancreas appeared indistinguishable from that of the controls (Fig. 4*G* and *H*). Collectively, these data show that although attenuated Wnt signaling perturbs pancreatic epithelial cell proliferation, it does not affect differentiation of pancreatic endocrine and exocrine cells.

***Ipf1/Frz8CRD* mice are normoglycemic.** Despite the drastic reduction in size, the transgenic mice did not display signs of either hyperglycemia or diabetes and showed a normal glucose tolerance when challenged with exogenous glucose (Fig. 5*A*). To test whether the normal glucose homeostasis in *Ipf1/Frz8CRD* mice reflected enhanced insulin secretion and/or glucose sensing, we monitored insulin levels in 10- to 12-week-old transgenic mice at fasted conditions and serum insulin levels in response to glucose challenge. Serum insulin levels were normal in fasted *Ipf1/Frz8CRD* mice, and the mice showed a normal first and second phase insulin release upon glucose challenge (Fig. 5*B*).

Determination of the relative insulin content in total pancreatic extracts (nanograms insulin per milligram pancreatic protein) from 12-week-old transgenic ($n = 4$) and wild-type mice ($n = 4$) revealed that there was a ~50% increase in relative insulin content in the transgenic mice compared with the controls (Fig. 5*C*). These results suggest that insulin biosynthesis and/or the relative number of the insulin-positive cells is increased in the transgenic mice. Therefore, we quantified numbers of differentiated endocrine cell types against total endocrine cell count and against total pancreatic area in neonatal pancreas (Fig. 5*D*). There was an ~50% reduction in the absolute numbers of endocrine cells (i.e., ISL1-positive cells), however, there were ~30% more endocrine cells per pancreatic

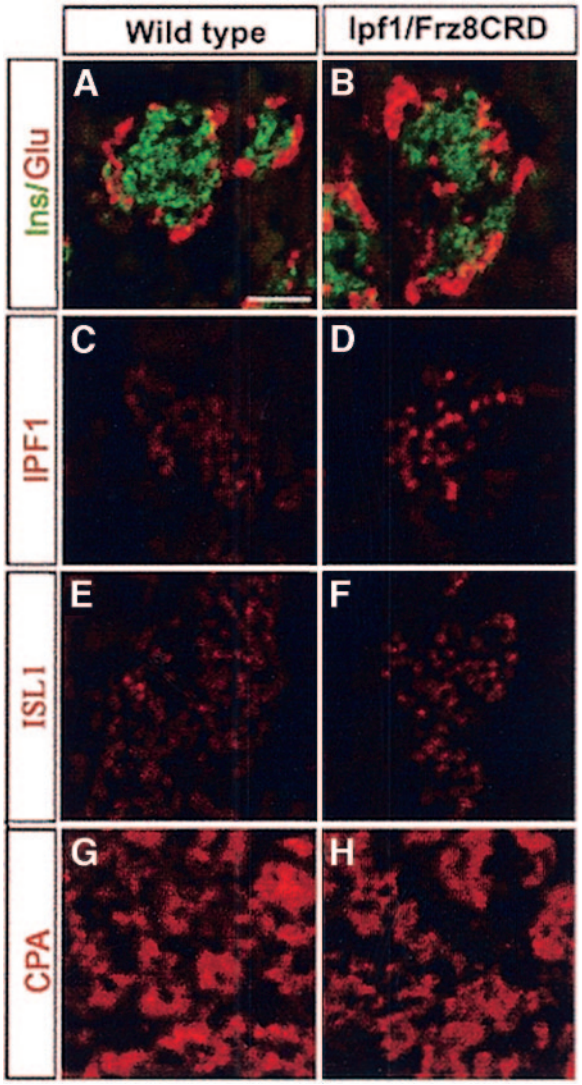

FIG. 4. Pancreatic cell differentiation is normal in *Ipf1/Frz8CRD* mice. *A* and *B*: Immunohistochemical analyses of differentiated pancreatic markers in wild-type (*A*, *C*, *E*, and *G*) and *Ipf1/Frz8CRD* (*B*, *D*, *F*, and *H*) transgenic pancreases using antibodies against insulin (green) and glucagon (red) (*A* and *B*); IPF1/PDX1 (*C* and *D*); ISL1 (*E* and *F*); and CPA (*G* and *H*). Scale bars in *A–H*, 0.04 mm.

area, and no difference was observed in the proportion of β- versus α-cells over total endocrine cells (Fig. 5*D*).

The relative increase in β-cells observed in transgenic compared with wild-type mice might explain the normal serum insulin levels observed in the transgenic mice and the 50% increase observed in the relative total insulin content in transgenic pancreases. However, improved insulin biosynthesis and enhanced insulin secretion could also contribute to the increase in relative total insulin content and normal insulin levels in the transgenic mice. To explore this, we calculated the total relative insulin content and total insulin secretion per unit of β-cell mass, i.e., per individual β-cell, and observed a fourfold increase in insulin content and a twofold increase in insulin secretion per unit of β-cell mass for the transgenic mice compared with control mice (Fig. 5*E*). Together, these data provide evidence that the reduced pancreatic mass of *Ipf1/Frz8CRD* mice is compensated by a parallel increase in relative β-cell mass, insulin biosynthesis, and insulin secretion.

In agreement with an enhanced insulin biosynthesis, the expression of *prohormone convertase 1/3* (*PC1/3*), which promotes processing of proinsulin to mature insulin, was increased threefold in islets of 10-week-old *Ipf1/Frz8CRD* mice compared with wild-type littermates (Fig. 5*F*). *PC1/3* expression has been shown to be dependent on *Ipf1/Pdx1* and FGF receptor 1c signaling, and mice that express a dominant-negative form of *FRFR1c* or lack *Ipf1/Pdx1* in β-cells display reduced PC1/3 expression (25,34). Consequently, analysis of *Ipf1/Pdx1* expression by quantitative RT-PCR showed that its expression was increased nearly 2.5-fold in transgenic islets compared with that of controls (Fig. 5*F*). The increased mRNA expression of *Ipf1/Pdx1* in transgenic islets is also consistent with the strong IPF1/PDX1 immunoreactivity observed in the islets of transgenic mice (Fig. 4*C* and *D*), although quantitative information cannot be deduced from immunohistochemical data. The expression of *glucose transporter type 2* and *glucokinase* mRNA, two key mediators of the β-cell response to glucose, and *insulin* mRNA were only moderately increased in transgenic islets (Fig. 5*F*).

These data suggest that the improved β-cell function in *Ipf1/Frz8CRD* mice is mediated, at least in part, by the increased expression of *Ipf1/Pdx1* and *PC1/3*. In summary, our data showed that attenuation of Wnt signaling in adult β-cells does not affect glucose sensing and GSIS and that normoglycemia in the *Ipf1/Frz8CRD* mice was likely achieved by combination of increased relative endocrine cell number, enhanced insulin biosynthesis, and insulin secretion per unit of β-cell mass.

DISCUSSION

The data presented here suggest that Wnt signaling plays a role in pancreatic epithelial cell proliferation. We show that the Wnt signaling components *Wnt4*, *Wnt7b*, and *Frz3* are expressed in the developing pancreas of mice and that overexpression of a Wnt signaling antagonist in the embryonic pancreas perturbs pancreatic epithelial cell proliferation. In agreement with Heller et al. (18), we do not observe expression of *Wnt7b* in the early pancreatic buds. However, at later stages of development, *Wnt7b* expression could be observed in the pancreatic epithelium; and in contrast to Heller et al. (18), we find *Frz3* to be expressed in the pancreatic epithelium during the phase of extensive growth. Consistent with previous reports, we also observed expression of these genes in the lung buds (*Wnt7b*) and spinal cord (*Wnt7b* and *Frz3*) of e10 embryos (29,30). The presence of *Frz3* in the early pancreatic progenitor cells is in agreement with a potential role for this receptor in mediating Wnt signals that promote subsequent growth and morphogenesis of the early embryonic pancreas. Because several different *Wnts* and *Frzs* genes are expressed in the developing pancreas (18), the expression of the dominant-negative form of mouse Frz8 (i.e., the Frz8 CRD-IgG fusion protein), which appears to function as a general antagonist of Wnt signaling (17,21,31), is advantageous compared with gene inactivation approaches because it is less sensitive to potential redundant effects.

Unlike mice overexpressing *FGF10* under control of the *Ipf1/Pdx1* promoter (6,7), mice overexpressing *Wnt1* or *Wnt5a* under the control of the same promoter do not show signs of pancreatic hyperplasia (18). Instead, the mice overexpressing these Wnt ligands show signs of perturbed foregut specification and/or patterning (18), suggesting that enhanced Wnt signaling perturbs early

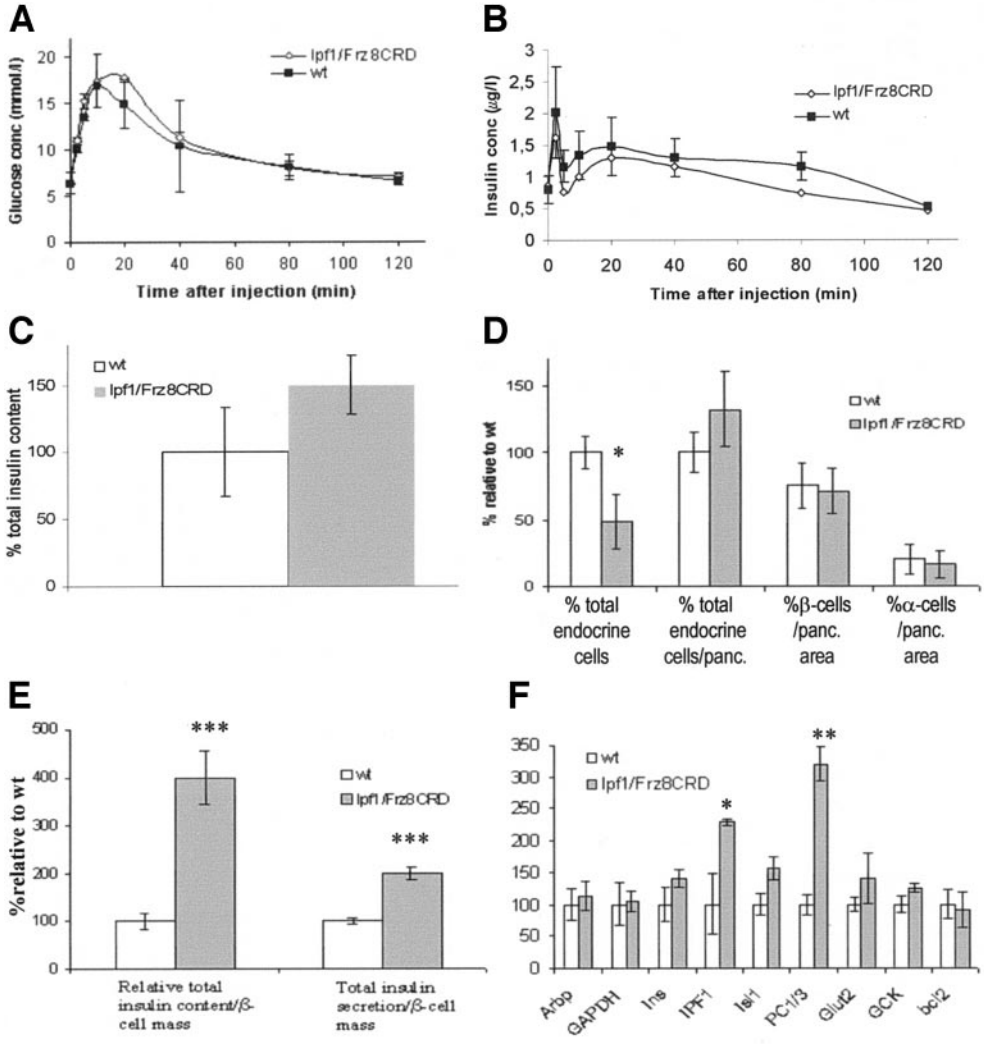

FIG. 5. The *Ipf1/Frz8CRD* mice are normoglycemic and show improved insulin content and secretion. *A*: Blood glucose concentrations were determined after intraperitoneal injection of glucose in fasted wild-type (wt) (n = 8) and *Ipf1/Frz8CRD* (n = 10) mice. *B*: Serum insulin levels were determined after intraperitoneal injection of glucose in fasted wild-type (n = 10) and *Ipf1/Frz8CRD* (n = 10) mice. *C*: Determination of total insulin content (ng insulin/mg pancreatic protein) in isolated pancreas from wild-type (n = 4) and *Ipf1/Frz8CRD* (n = 4) mice. *D*: Determination of total and relative endocrine cells in pancreatic sections from wild-type and *Ipf1/Frz8CRD* mice as well as ratios of β- and α-cells over total endocrine cells. *E*: Determination of total relative insulin content and total insulin secretion over β-cell mass. *F*: Quantitative RT-PCR analyses of the indicated genes were performed using islet cDNA prepared from *Ipf1/Frz8CRD* (n = 4) and wild-type (n = 4) mice. Data are shown as means ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ vs. control animals.

foregut development. The pancreatic hypoplasia observed in the *Ipf1/Frz8CRD* mice provide evidence that Wnt signaling also stimulates pancreatic cell proliferation; and the observation that nonphopshorylated β-catenin immunoreactivity is weaker in e13 *Ipf1/Frz8CRD* mice than that observed in stage-matched wild-type mice indicates that canonical rather than noncanonical Wnt signaling mediates pancreatic epithelial cell proliferation.

FGFs, Wnts, and epidermal growth factors are known to act in synergy to promote proliferation in a variety of systems (35–37). Gross morphological analysis of *Ipf1/Frz8CRD* neonates revealed pancreatic hypoplasia, as early as e14. *Fgf10*$^{-/-}$ mice also display severe pancreatic hypoplasia, indicating a role for both signaling pathways in pancreatic growth. However, there is a distinct difference between the *Ipf1/Frz8CRD* and *Fgf10*$^{-/-}$ mouse models. The *Fgf10*$^{-/-}$ mice show complete pancreatic agenesis and severely reduced expression of IPF1/PDX1 in early pancreatic progenitors (5). The pancreatic growth arrest observed in the *Fgf10*$^{-/-}$ mice in fact resembles that observed in *Ipf1/Pdx1*$^{-/-}$ (38,39) mice. In contrast, IPF1/PDX1 expression is not perturbed in the *Ipf1/Frz8CRD* mice, and pancreatic epithelial cell proliferation still occurs in these mice albeit at a reduced rate. Nevertheless, the fact that FGF and Wnt signaling appear to stimulate proliferation of pancreatic progenitor cells leaves open the possibility that these pathways act together, possibly in synergy, to ensure optimal growth of the embryonic pancreas.

Despite the 75% drastic reduction in overall pancreatic mass and 50% reduction in absolute β-cell numbers, adult *Ipf1/Frz8CRD* mice were normoglycemic with an apparently normal endocrine and exocrine pancreatic function. The normoglycemia displayed by *Ipf1/Frz8CRD* mice suggests that Wnt signaling is not critical for normal glucose metabolism and insulin secretion. Analyses of mice lacking a functional copy of *LRP5*, a coreceptor involved in Wnt signaling, have suggested that Wnt/LRP5 signaling contributes to GSIS (20). *LRP5*$^{-/-}$ mice showed an impaired glucose tolerance and GSIS when glucose challenged, and pretreatment of isolated islets with Wnt-conditioned media resulted in enhanced GSIS from wild-type but not *LRP5*$^{-/-}$ islets (20). The difference in glucose tolerance and insulin secretion in *LRP5*$^{-/-}$ mice was, however, age dependent and developed first after 6 months, leaving open the possibility that the β-cell defects in *LRP5*$^{-/-}$ mice are secondary rather than primary.

Although attenuation of Wnt signaling in the pancreas of *Ipf1/Frz8CRD* mice resulted in a reduced total endocrine cell count, the relative number of endocrine cells (i.e., endocrine cells per pancreatic area) was increased, and the ratio between β- and α-cells was normal. Transgenic mice challenged with exogenous glucose responded with releasing insulin at amounts similar to those of wild-type

littermates. The latter suggests that the β-cells of transgenic mice somehow compensate for the reduced absolute numbers of β-cells to ensure glucose homeostasis. In agreement with such a scenario, there was a ~50% increase in the relative pancreatic insulin content and a threefold increase in *PC1/3* expression in transgenic islets compared with controls. Similarly, the expression of *Ipf1/Pdx1*, which is known to transactivate the *insulin* gene promoter and control *Glut2* and *PC1/3* expression (25,40), was increased. The β-cells of transgenic mice produce four times more and secrete twice as much insulin as that of wild-type littermates, which strongly suggests the presence of compensatory mechanisms adopted by the β-cells of the transgenic mice to achieve and maintain normoglycemia.

There have been numerous reports on compensatory adaptations by adult β-cells in response to variations in insulin demands (41). In rodents, a 40–60% reduction in β-cell mass does not result in abnormal glucose homeostasis; 85–95% pancreatectomy is required to induce hyperglycemia in these rodents (42,43). Mice with a targeted disruption of the insulin receptor substrate 1 develop insulin resistance but maintain normoglycemia via a compensatory increase in β-cell mass (44,45). Rats infused with glucose or lipids were shown to achieve maintenance of normoglycemia by dramatically increasing their insulin secretory response paralleled by enhanced β-cell proliferation 1–2 days after infusion, leading to subsequent increase in β-cell mass (46). Finally, chemical reduction of β-cell mass in minipigs, which provokes glucose intolerance, resulted in an increased insulin response from the residual β-cell population during a mixed-meal tolerance test (47). Our data suggest that the *Ipf1/Frz8CRD* mice also display compensatory β-cell adaptations, and thus these mice could provide a useful tool for unraveling the cellular and molecular mechanisms underlying such β-cell compensatory adaptations.

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