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BMP4-BMPR1A Signaling in β Cells Is Required for and Augments Glucose-Stimulated Insulin Secretion

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SUMMARY

Impaired glucose-stimulated insulin secretion (GSIS) and perturbed proinsulin processing are hallmarks of β cell dysfunction in type 2 diabetes. Signals that can preserve and/or enhance β cell function are therefore of great therapeutic interest. Here we show that bone morphogenetic protein 4 (*Bmp4*) and its high-affinity receptor, *Bmpr1a*, are expressed in β cells. Mice with attenuated BMPR1A signaling in β cells show decreased expression of key genes involved in insulin gene expression, proinsulin processing, glucose sensing, secretion stimulus coupling, incretin signaling, and insulin exocytosis and develop diabetes due to impaired insulin secretion. We also show that transgenic expression of *Bmp4* in β cells enhances GSIS and glucose clearance and that systemic administration of BMP4 protein to adult mice significantly stimulates GSIS and ameliorates glucose tolerance in a mouse model of glucose intolerance. Thus, BMP4-BMPR1A signaling in β cells plays a key role in GSIS.

INTRODUCTION

Type 2 diabetes develops as a consequence of insulin resistance and impaired β cell function. During the course of the disease, a progressive loss of β cell function and β cell mass triggers the transition from glucose intolerance to overt diabetes (Bergman et al., 2002; Marchetti et al., 2006). β cells of type 2 diabetics exhibit blunted glucose-stimulated insulin secretion (GSIS), with a particular loss or reduction of first-phase insulin release, which in combination with perturbed processing of proinsulin leads to low circulating levels of active insulin (Bergman et al., 2002). Mechanisms of intracellular signaling controlling regulated insulin secretion have been extensively studied,

and transcription factors have been linked to β cell dysfunction in type 2 diabetes and maturity-onset diabetes of the young (MODY) (Edlund, 2002; Marchetti et al., 2006). In contrast, much less is known about secreted signals that maintain and/or enhance GSIS in β cells.

Glucagon-like polypeptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) represent a distinct incretin class of secreted signals that mediate a short-term enhancement of GSIS following a meal (Drucker, 2006). GLP-1 also causes longer-term enhancement of β cell function by stimulating insulin (*Ins*) gene expression, β cell proliferation, and β cell survival (Drucker, 2006). These positive effects of GLP-1 on β cells are mediated at least in part via the transcription factor IPF1/PDX1 (Wang et al., 1999; Stoffers et al., 2000; Perfetti et al., 2000; Drucker, 2006), which has been linked to β cell dysfunction in type 2 diabetes and MODY4 (Stoffers et al., 1997; Ahlgren et al., 1998; Marchetti et al., 2006). Members of the transforming growth factor β (TGF-β)/activin/bone morphogenetic protein (BMP) superfamily of secreted signaling molecules are involved in various cellular processes including proliferation, cell fate specification, and differentiation (Chen et al., 2004). TGF-β/activin signaling has been implicated in pancreatic development and disease (Rane et al., 2006). Pancreatic cancer in particular, but also pancreatitis and diabetes, has been linked to dysregulated TGF-β signaling (Yamaoka et al., 1998; Kim et al., 2000; Smart et al., 2006; Kuang et al., 2006; Rane et al., 2006). Like TGF-β/activin, BMP signaling controls several developmental processes and has been implicated in pancreatic cell proliferation and differentiation (Jiang et al., 2002; Yew et al., 2005; Hua et al., 2006). In contrast, a role for the BMP class of signals and receptors in β cell function, GSIS, and glucose homeostasis has not been established to date.

Here we show that the BMP type 1a receptor (*Bmpr1a*), also known as ALK3, and its high-affinity ligand bone morphogenetic protein 4 (*Bmp4*) are expressed in differentiating and adult β cells. Using multiple genetic approaches, we provide evidence that mice with attenuated BMPR1A signaling in β cells exhibit decreased expression of key

genes involved in insulin gene expression, proinsulin processing, glucose sensing, secretion stimulus coupling, incretin signaling, and insulin exocytosis and consequently develop diabetes due to impaired insulin secretion. We also show that transgenic expression of *Bmp4* in β cells and systemic administration of BMP4 protein to adult mice significantly enhance GSIS. Moreover, systemic administration of BMP4 protein ameliorates glucose tolerance in *Ipf1/Pdx1*^{+/−} mice, a mouse model of glucose intolerance. Thus, BMP4-BMPR1A signaling in β cells is required for regulated insulin secretion, and further stimulation of this pathway can augment GSIS.

RESULTS

Mice with Attenuated BMPR1A Signaling in β Cells Develop Diabetes

Bmpr1a and *Bmp4* expression was first observed in the pancreatic epithelium of embryonic day 13 (E13) embryos; at later fetal stages and the neonatal stage, expression was restricted to the clustering islet cells (Figure 1A). Expression of both *Bmpr1a* and *Bmp4* was maintained in adult mouse islets, and expression of both the receptor and the ligand was also observed in adult human islets (see Figure S1 in the Supplemental Data available with this article online). *Bmp2*, the other high-affinity BMPR1A ligand (Aoki et al., 2001; Miyazono et al., 2005), was not expressed at any stage of pancreatic development (data not shown), and *Bmp7*, which binds only weakly to BMPR1A (Aoki et al., 2001; Miyazono et al., 2005), was highly expressed in the pancreatic epithelium from ∼E9 to ∼E15, but not at later stages of development (data not shown). The overlapping patterns of expression of *Bmpr1a* and *Bmp4* in β cells are suggestive of a role for autocrine BMP4-BMPR1A signaling in pancreatic islet cells. *Bmpr1a* null mutant mice die at ∼E9.5, precluding analyses of a role for pancreatic BMPR1A signaling in these mice (Mishina et al., 1995). Mice with a floxed *Bmpr1a* allele, however, have been generated (Mishina et al., 2002), and we targeted the deletion of *Bmpr1a* in β cells by breeding mice hemizygous for rat insulin promoter 1 (*Rip1*)/*Cre* (Ahlgren et al., 1998) and the *Bmpr1a* null allele (Mishina et al., 1995) with mice homozygous for the floxed *Bmpr1a* allele (Mishina et al., 2002). The resulting mice with *Bmpr1a* deleted in β cells were denoted FIN mice. The deletion of the kinase domain of *Bmpr1a* has been shown to block BMP-induced signal transduction, and forced expression of such dominant-negative forms of *Bmpr1a* is a well-established approach to impairing BMP signaling (Namiki et al., 1997; Katagiri et al., 2002; Brederlau et al., 2004; Kaps et al., 2004). Thus, as a parallel approach to search for a role of BMPR1A signaling in pancreas, we used the *Ipf1/Pdx1* promoter (Apelqvist et al., 1997) that is active in differentiated β cells to drive the expression of a dominant-negative, kinase-deficient form of *Bmpr1a* (*dnBmpr1a*) in transgenic mice, denoted *Ipf1-dnBmpr1a* mice. The *Ipf1/Pdx1* promoter is active also in early (E8–E10) pancreatic epithelial progenitor cells,

but these cells do not express *Bmpr1a* or *Bmp4* (Figure 1A and data not shown).

Both the FIN mice and the *Ipf1-dnBmpr1a* transgenic mice were born alive and initially appeared healthy but showed signs of glucose intolerance from ∼2–3 months of age as assayed by intraperitoneal (i.p.) glucose tolerance tests (Figure 1B) and developed overt diabetes a few months later (data not shown). Overexpression of two other BMP signaling inhibitors, *noggin*, an extracellular BMP antagonist (Chen et al., 2004), and *Smad6*, an intracellular inhibitor that preferentially inhibits BMP signaling (Massague and Gomis, 2006), also caused the resulting transgenic mice to develop glucose intolerance and diabetes (Figure S2). The *Ins* promoter can result in transgene expression also in the arcuate nuclei of the hypothalamus, raising the possibility that *Bmpr1a* could be inactivated in this region of the brain with potential effects on food intake and body weight. However, we did not observe expression of *Bmpr1a* in the arcuate nuclei (data not shown), and the body weight of the FIN mice was not different from that observed for *Ipf1-dnBmpr1a* and control littermates (Figure S3). Taken together, these data provide evidence that BMPR1A signaling in β cells is critical for glucose homeostasis.

To elucidate whether the impaired glucose tolerance and diabetes displayed by FIN and *Ipf1-dnBmpr1a* mice reflected perturbations in insulin secretion, we next determined serum insulin levels in these mice following i.p. injection of glucose. Wild-type mice showed a distinct biphasic insulin response following glucose injection, whereas both the FIN and *Ipf1-dnBmpr1a* mice displayed a blunted first- and second-phase insulin release (Figure 1C). Serum insulin levels were notably lower in the *Ipf1-dnBmpr1a* mice than in FIN mice, which may be due to different genetic backgrounds and/or a difference in the efficiency of attenuation of BMPR1A signaling by the two transgenic approaches. Consistent with a direct role for BMPR1A signaling in adult β cells, the BMP signaling transducer phospho-Smad1/5/8 was present at high levels in adult β cells of wild-type mice and at reduced levels in β cells of FIN and *Ipf1-dnBmpr1a* mice (Figure 1D). In agreement with a previous report (Brorson et al., 2001) phospho-Smad1/5/8 staining was also observed in α cells. In contrast to the staining in β cells, the level of phospho-Smad1/5/8 staining in α cells was similar in control, FIN, and *Ipf1-dnBmpr1a* mice. Occasional (albeit weaker and more irreproducible) staining was also observed in a subpopulation of exocrine cells (Figure 1D). Collectively, these data provide evidence that attenuation of BMPR1A signaling in adult β cells results in hyperglycemia and diabetes due to impaired GSIS.

FIN and *Ipf1-dnBmpr1a* mice displayed a macroscopically normal pancreas (data not shown) with differentiated endocrine and exocrine cells, as indicated by the expression of insulin and glucagon (Figure 2A) and carboxypeptidase A and amylase (data not shown), respectively. The total number of endocrine cells and the ratio of insulin[+] to glucagon^{+} cells were also normal in the pancreata of these mice (Figures 2B and 2C), but, as observed in several
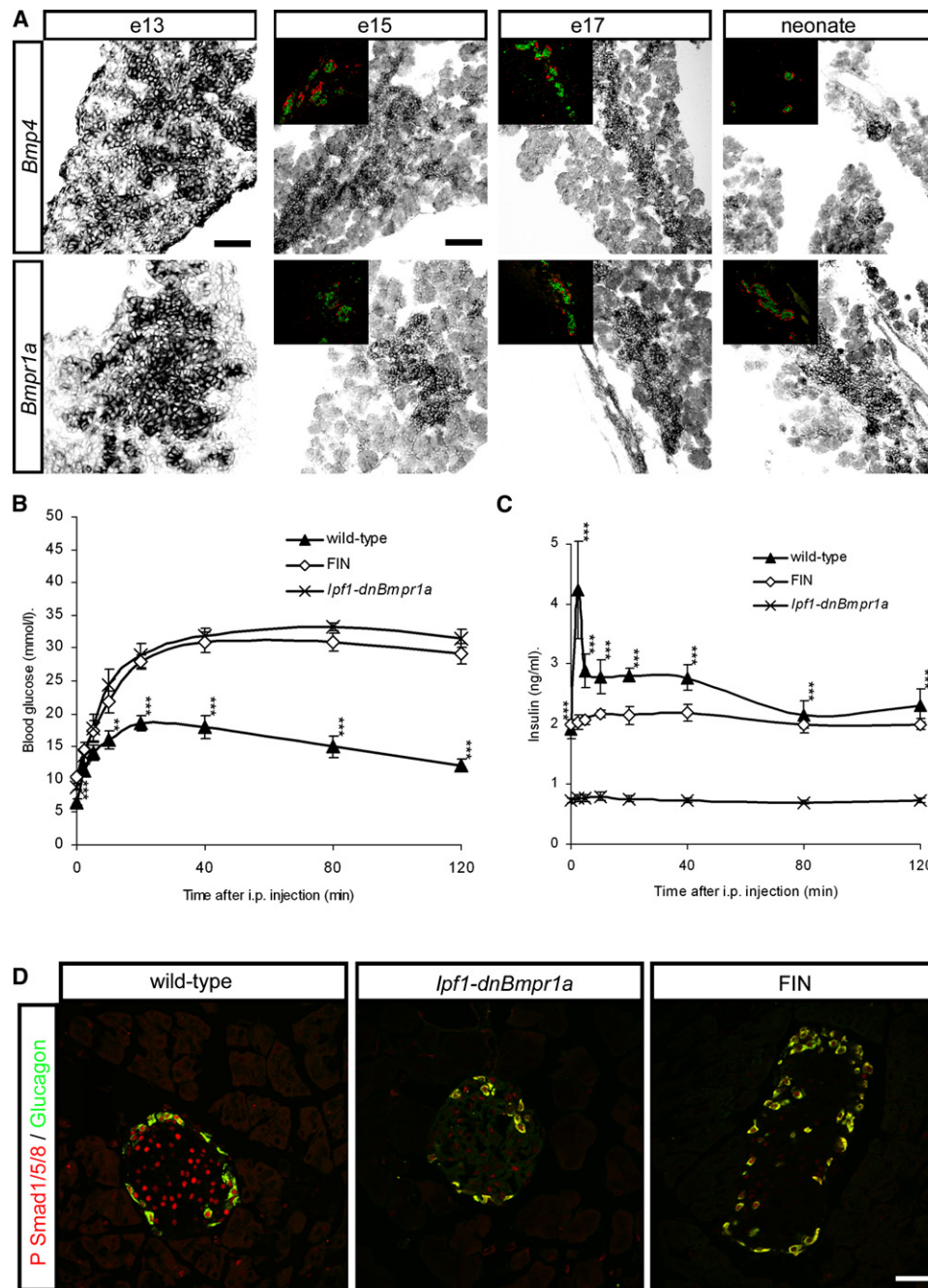
Figure 1. Mice with Attenuated BMPR1A Signaling Are Glucose Intolerant and Exhibit Impaired Glucose-Stimulated Insulin Secretion

(A) In situ hybridization of E13, E15, E17, and neonatal pancreas using DIG-labeled *Bmpr1a* and *Bmp4* probes (dark gray) counterstained with antibodies against insulin (red in inserts) and glucagon (green in inserts). Scale bars = 35 μm and 65 μm for E13 (20× objective) and older (10× objective), respectively.

(B) Blood glucose concentrations at the indicated time points following intraperitoneal (i.p.) injection of glucose in wild-type (n = 9), FIN (n = 6), and *Ipf1-dnBmpr1a* mice (n = 6). In this and all other figures, data represent the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(C) Serum insulin levels after i.p. injection of glucose in wild-type (n = 9), FIN (n = 6), and *Ipf1-dnBmpr1a* mice (n = 6).

(D) Sections of pancreas from wild-type, FIN, and *Ipf1-Bmpr1a* mice stained for phospho-Smad1/5/8 (red) and glucagon (green). Scale bar = 35 μm.

other diabetic mouse models (Ahlgren et al., 1998; Hart et al., 2000; Yamagata et al., 2002; Steneberg et al., 2005), glucagon cells were scattered throughout the islets

(Figure 1D and Figure 2A). Thus, BMPR1A signaling is not required for pancreatic development per se or for the number of islet cells, which is consistent with the temporal
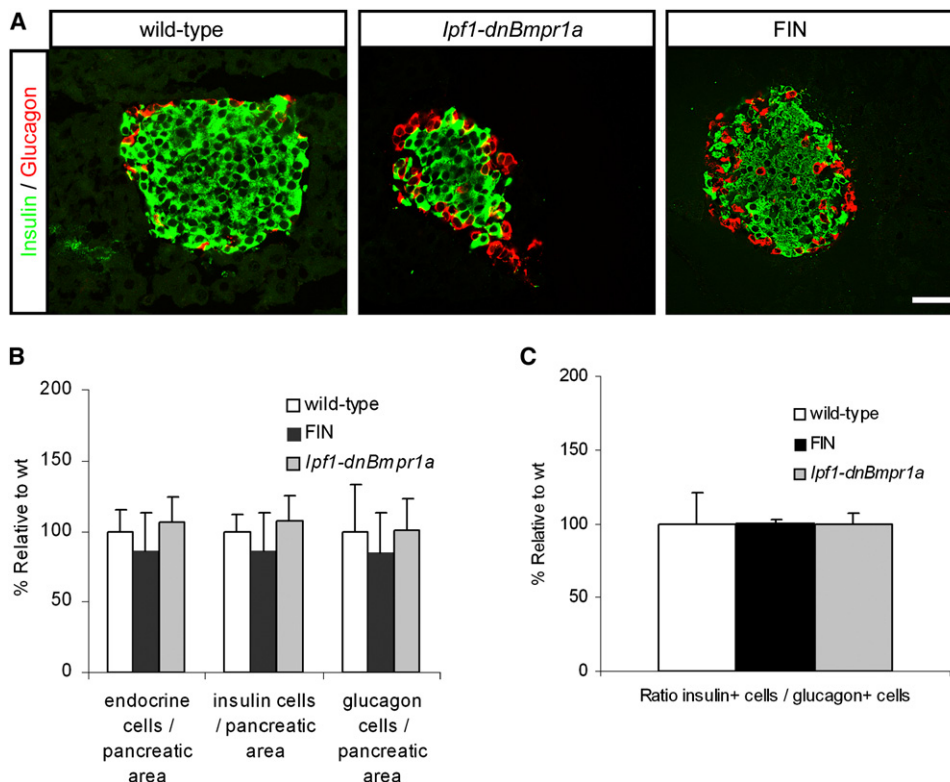
Figure 2. Attenuation of BMPR1A Signaling Does Not Affect Pancreas Development
(A) Immunolocalization analyses of insulin (green) and glucagon (red) in wild-type, FIN, and *Ipf1-dnBmpr1a* mice. Scale bar = 35 μm.
(B and C) Determination of total endocrine, insulin⁺, and glucagon⁺ cells (B) as well as ratio of insulin⁺ to glucagon⁺ cells (C) in pancreatic sections from wild-type (n = 6), FIN (n = 4), and *Ipf1-dnBmpr1a* mice (n = 6).

and spatial patterns of expression of *Bmpr1a* and *Bmp4* in the pancreas.

Autocrine BMPR1A Signaling in β Cells

To begin to unravel the molecular mechanism by which BMPR1A signaling contributes to β cell function and GSIS, we first analyzed the expression of BMP signaling components in FIN and *Ipf1-dnBmpr1a* islets using quantitative real-time PCR. *Id* gene expression is induced in response to BMP stimulation in numerous cell types. Thus, *Id* genes represent one of the most important and general BMP targets. In wild-type islets, *Id1* and *Id2* genes were robustly expressed, whereas *Id3* and *Id4* were expressed at very low, barely detectable levels (Figure 3A and data not shown). In FIN and *Ipf1-dnBmpr1a* islets, *Id2* expression was reduced by ∼80%–85%, whereas *Id1* expression was unchanged (Figure 3A). The reduced islet expression of *Id2* in mice with perturbed BMPR1A signaling is in agreement with the reduced level of phospho-Smad1/5/8 observed in islets of these mice and provides strong evidence for a role for BMPR1A signaling in β cells. Interestingly, endogenous *Bmpr1a* expression was reduced not only in FIN islets, by ∼75%, but also in *Ipf1-dnBmpr1a* islets, by ∼90% (Figure 3A), indicating that the expression of *Bmpr1a* may be positively regulated by BMP signals. In addition, the expression of the BMP

signal transduction components *Smad1* and *Smad4* was decreased by ∼65% and ∼45% in FIN islets and by >90% and >95% in *Ipf1-dnBmpr1a* islets (Figure 3A). In contrast, the expression of the corepressor *Evi-1*, which represses BMP/TGF-β/activin-activated transcription by interacting with the receptor-regulated Smads (i.e., Smad1/5/8 and Smad2/3) (Alliston et al., 2005), was increased 2-fold in FIN islets and 3-fold in *Ipf1-dnBmpr1a* islets (Figure 3A). These results show that the expression of several BMP signaling components in β cells depends on *Bmpr1a* expression/signaling, suggesting that a positive BMPR1A signaling feedback loop operates in β cells.

BMPR1A Signaling Is Required for the Expression of Key Genes Regulating GSIS

To examine at what level BMPR1A signaling is required for maintaining GSIS, we analyzed the expression of genes involved in glucose sensing, glucose metabolism, secretion stimulus coupling, insulin biosynthesis, and exocytosis in β cells. Compared to control islets, the expression of *Ipf1/Pdx1*, which is required for adult β cell function (Stoffers et al., 1997; Ahlgren et al., 1998), was reduced by ∼60% in FIN and ∼65% in *Ipf1-dnBmpr1a* islets (Figure 3B). Consistent with this, the expression of *Ins*, a IPF1/PDX1 target gene, was reduced by ∼90% in FIN islets and ∼95% in *Ipf1-dnBmpr1a* islets, and the expression of
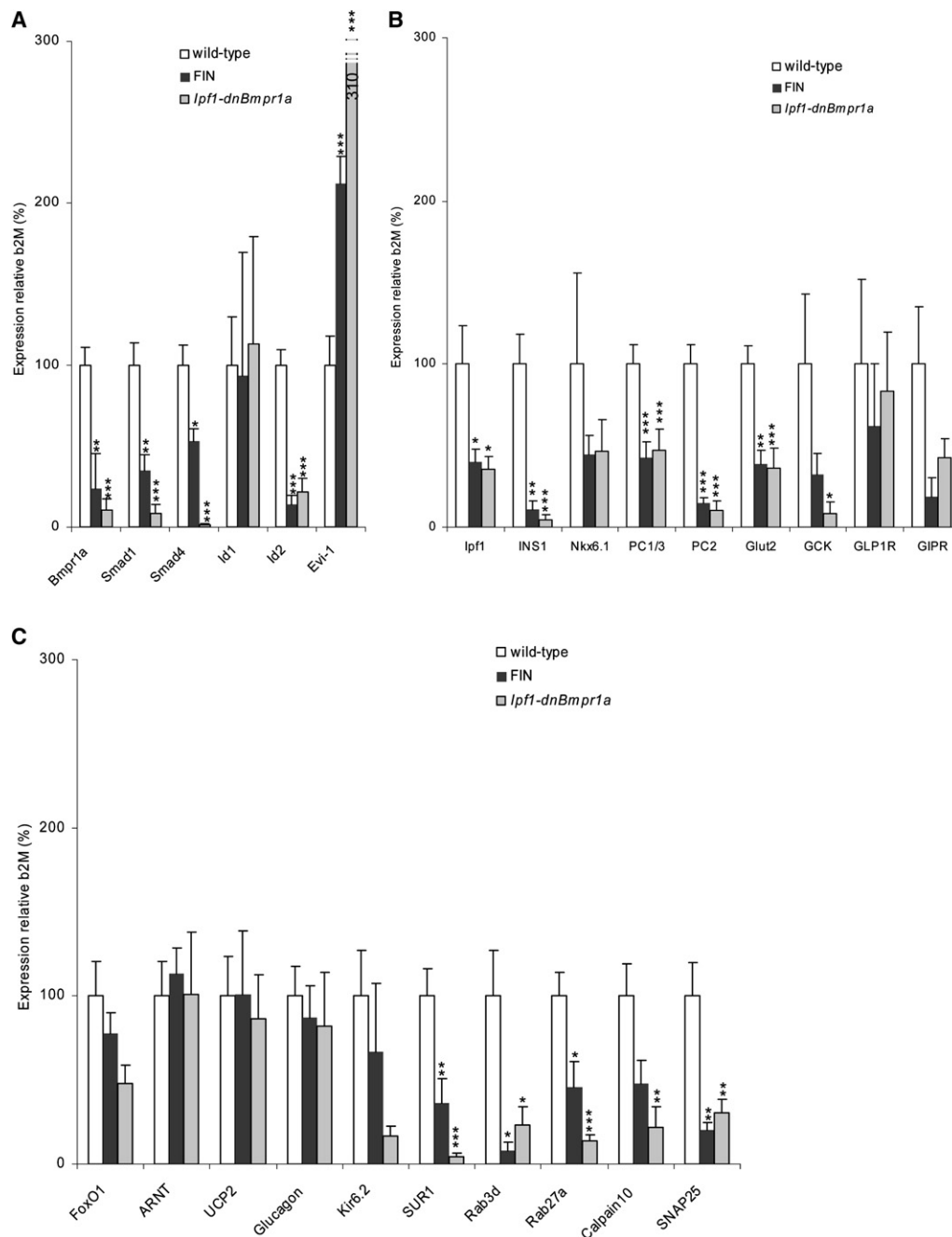
Figure 3. Perturbed Gene Expression Profiles in Mice with Attenuated BMPR1A Signaling

Quantitative real-time RT-PCR expression analyses of the indicated genes were performed using islet cDNA prepared from wild-type, FIN, and *Ipf1-dnBmpr1a* mice (n = 4–6 for each mouse line). *p < 0.05; **p < 0.01; ***p < 0.001 for wild-type versus FIN and *Ipf1-dnBmpr1a* mice.

Nkx6.1, another IPF1/PDX1 target gene (Ahlgren et al., 1998; Wang et al., 2005), was also reduced, albeit not significantly, in FIN and *Ipf1-dnBmpr1a* islets (Figure 3B). The expression of the proinsulin-processing enzymes *PC1/3* and *PC2* was reduced by ~55% and ~85% in FIN islets and by ~50% and ~90% in *Ipf1-dnBmpr1a* islets (Figure 3B). Glucose transport and metabolism are key steps in GSIS, and decreased expression of genes controlling

these processes is associated with β cell dysfunction in type 2 diabetes (Marchetti et al., 2006). Expression of the glucose transporter *Glut2* and glucokinase (*GCK*), the rate-limiting enzyme in glucose metabolism, was reduced by ~60% and ~70% in FIN islets and by ~65% and ~90% in *Ipf1-dnBmpr1a* islets (Figure 3B). The incretins GLP-1 and GIP act via their cognate receptors on β cells to stimulate GSIS (Drucker, 2006). GIP receptor (*GIPR*)

expression was reduced, albeit not significantly, in FIN and *Ipf1-dnBmpr1a* islets (Figure 3B), whereas *GLP-1R* expression was virtually unchanged in FIN or *Ipf1-dnBmpr1a* islets (Figure 3B). The expression of several other genes linked to β cell function and/or insulin secretion, including *Foxo1*, *HIF1β/ARNT*, *Ucp2*, and *Glu*, was not significantly affected in FIN or *Ipf1-dnBmpr1a* islets (Figure 3C).

Taken together, these results provide evidence that attenuation of BMP4-BMPR1A signaling in β cells leads to reduced expression of key genes regulating insulin gene expression, proinsulin processing, glucose sensing, and incretin signaling, providing a molecular explanation for the perturbed GSIS observed in mice with impaired BMPR1A signaling.

Ipf1-dnBmpr1a Mice Show a Blunted Response to Secretagogues

The metabolism of glucose ultimately leads to an increase in the ATP:ADP ratio, closure of the K⁺ ATP channel, membrane depolarization, opening of the L-type Ca²⁺ channel, and influx of Ca²⁺, which triggers insulin exocytosis from secretory granules. The expression of K⁺ ATP channel subunits *Kir6.2* and *SUR1* is reduced in mice where *Ipf1/Pdx1* has been inactivated in β cells (Li et al., 2005). Consistent with this observation, expression of *Kir6.2* was reduced by near significant levels in *Ipf1-dnBmpr1a* islets, and there was a tendency toward reduced expression in FIN islets as well (Figure 3C). *SUR1* expression was reduced by ∼60% in FIN islets and ∼95% in *Ipf1-dnBmpr1a* islets (Figure 3C). The expression of the GTPases *Rab3d* and *Rab27a*, genes that are both linked to the exocytosis of insulin from secretory granules (Iezzi et al., 1999; Aizawa and Komatsu, 2005), was decreased by ∼90% and ∼50% in FIN islets and by ∼80% and ∼85% in *Ipf1-dnBmpr1a* islets, whereas the expression of *Rab3a*, *b*, and *c* was not affected (Figure 3C and data not shown). Expression of the type 2 diabetes susceptibility gene *calpain-10*, a member of the family of Ca²⁺-dependent, nonlysosomal cysteine proteases, and its substrate *SNAP-25* (Marshall et al., 2005) was reduced by ∼70 and ∼80% in FIN islets and by ∼80% and ∼70% in *Ipf1-dnBmpr1a* islets (Figure 3C). Thus, attenuation of BMP4-BMPR1A signaling in β cells also leads to reduced expression of key genes regulating secretion stimulus coupling and insulin exocytosis.

The decreased expression of key genes involved in secretion stimulus coupling and insulin exocytosis implies that mice with impaired BMPR1A signaling in β cells might have a perturbed response to secretagogues that uncouple insulin secretion from glucose metabolism. Glibenclamide induces insulin secretion by blocking the SUR1/Kir6.2 K⁺ ATP channel through interactions with SUR1 (Huopio et al., 2002); arginine stimulates insulin secretion by directly depolarizing the β cell membrane (Weinhaus et al., 1997; Thams and Capito, 1999); and carbachol, a cholinergic agonist, is thought to enhance insulin secretion by stimulating the transport of insulin vesicles to the secretory site (Garcia et al., 1988; Guenifi et al., 2001). Given the similar or identical phenotype of the *Ipf1-dnBmpr1a* mice and FIN mice and the less complicated

breeding of the former line, we selected the *Ipf1-dnBmpr1a* mice for studies of response to glibenclamide, arginine, and carbachol. Consistent with the reduced expression of the K⁺ ATP channel component *SUR1*, glibenclamide failed to stimulate insulin secretion in *Ipf1-dnBmpr1a* mice (Figure 4A), which also responded poorly to arginine (Figure 4B) and carbachol (Figure 4C), whereas all three secretagogues effectively stimulated insulin secretion in control mice. Thus, BMPR1A signaling in β cells is required for the ability of secretagogues to stimulate insulin secretion.

Transgenic Expression of *Bmp4* in β Cells Enhances Glucose Tolerance and GSIS

Since attenuation of BMP signaling in β cells impaired β cell function and GSIS, we next examined whether transgenic expression of *Bmp4* in pancreatic β cells could enhance GSIS. For this purpose, we generated mice expressing *Bmp4* under the control of the *Ipf1/Pdx1* promoter, which in the adult pancreas is preferentially expressed at high levels in β cells. The resulting *Ipf1-Bmp4* transgenic mice expressed significant levels of the transgene in adult islets (Figure S6) and were viable and healthy. Both intraperitoneal and oral glucose tolerance tests in *Ipf1-Bmp4* mice revealed a significantly enhanced glucose tolerance (Figure 5A and data not shown) with a parallel increase in GSIS (Figure 5B), which became progressively more prominent with age (data not shown). *Ipf1-Bmp4* transgenic mice displayed a macroscopically normal pancreas with normal organization, number, and size of islets (Figure 5C and data not shown). Thus, expression of *Bmp4* in adult β cells leads to increased glucose tolerance and enhanced GSIS but does not affect pancreas development or pancreatic cell differentiation.

To elucidate the molecular mechanism underlying the enhanced GSIS, we analyzed the expression of the same set of genes in *Ipf1-Bmp4* islets that we previously examined in FIN and *Ipf1-dnBmpr1a* islets. Expression of the BMP target gene *Id2* was increased ∼2-fold in *Ipf1-Bmp4* islets, whereas *Id1* was unchanged (Figure 5D). *Bmpr1a* expression was enhanced ∼65-fold in *Ipf1-Bmp4* islets (Figure 5D), and expression of *Smad1* and *Smad4* was increased ∼10-fold and ∼8-fold (Figure 5D). In contrast, *Evi-1* expression was reduced by ∼85% in *Ipf1-Bmp4* islets (Figure 5D). In agreement with a stimulation of BMP signaling, western blot analyses revealed an increased level of phospho-Smad1/5/8 and Id2 expression in *Ipf1-Bmp4* islets compared to control islets (Figure S5). Thus, genes involved in BMPR1A signaling were affected in an opposite manner in islets with perturbed BMPR1A signaling and *Ipf1-Bmp4* islets, supporting the idea of a positive BMPR1A signaling feedback loop in β cells.

Moreover, all genes involved in various aspects of insulin secretion that showed a reduced expression in *Ipf1-dnBmpr1a* islets showed an increased expression in *Ipf1-Bmp4* islets. Expression of *Ipf1/Pdx1*, *Nkx6.1*, and *Ins* was increased ∼2-fold, ∼65-fold, and ∼4-fold (Figure 5E). *PC1/3* and *PC2* expression was increased ∼2-fold and ∼7-fold, and *Glut2* and *GCK* expression
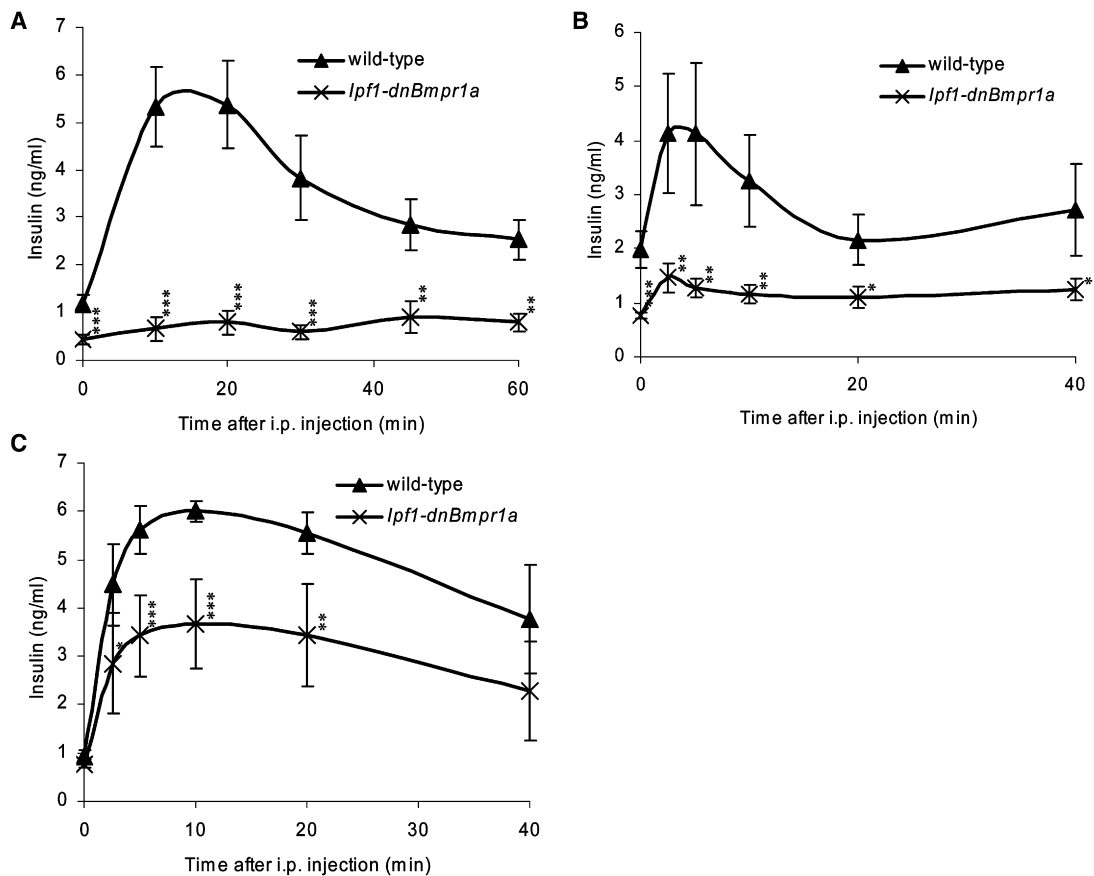
Figure 4. *Ipf1-dnBmpr1a* Mice Show an Impaired Response to Secretagogues
Serum insulin levels were determined after i.p. injection of glibenclamide (A) (wild-type n = 8; *Ipf1-dnBmpr1a* n = 8), arginine (B) (wild-type n = 4; *Ipf1-dnBmpr1a* n = 8), and carbachol (C) (wild-type n = 5; *Ipf1-dnBmpr1a* n = 4). *p < 0.05; **p < 0.01; ***p < 0.001 for wild-type versus *Ipf1-dnBmpr1a* mice.

was increased ∼4-fold and ∼2-fold (Figure 5E). Expression of *GLP-1R* and *GIPR* was increased ∼90-fold and ∼50-fold, *Kir6.2* expression was increased ∼12-fold, and *SUR1* expression was increased by nearly ∼50% (Figure 5E); an ∼2-fold increase in Kir6.2 protein expression was also observed (Figure S5). Immunohistochemical analyses of GIPR expression indicated that, like Glut2, the expression was upregulated in β cells, although an increased expression of GIPR in α cells cannot be excluded (Figure S5). The expression of *Rab3d*, *Rab27a*, and *calpain-10* was increased ∼15-fold, ∼40-fold, and ∼8-fold, and *SNAP-25* expression was increased by ∼75% (Figure 5E). The increased expression of key β cell genes in *Ipf1-Bmp4* mice and the complementary decreased expression of these genes in mice with attenuated BMPR1A signaling provide strong evidence that these genes are bona fide BMP4-BMPR1A signaling target genes in β cells. Thus, BMP4 enhances β cell function by stimulating the expression of key genes involved at various levels of GSIS.

Systemic Administration of BMP4 to Mice Enhances GSIS

To assess whether repeated administration of BMP4 protein to mice could enhance GSIS, we monitored insulin secretion following glucose injection (1.5 g/kg body weight) in 4 hr fasted CBA mice that had received two daily i.p. injections of 10 or 20 μg BMP4/kg body weight for 3 days and a single injection at day 4. Under these conditions, 20 μg, but not 10 μg, BMP4/kg body weight resulted in a significant stimulation of insulin secretion with a concomitant improvement in glucose tolerance (Figures 6A and 6B). Treatment of mice with BMP4 injections did not provoke hypoglycemia, suggesting that the stimulatory effects of BMP4 on insulin secretion are glucose dependent. Administration of 20 μg BMP4/kg body weight also ameliorated the impaired glucose tolerance of *Ipf1/Pdx1* null heterozygous mice (Ahlgren et al., 1998) to near normal levels (Figures 6C and 6D). Taken together, these data demonstrate that administration of BMP4 protein to mice enhances GSIS and is capable of improving glucose clearance in a glucose-intolerant mouse model.

DISCUSSION

The mature β cell responds to elevated glucose levels by secreting insulin in a tightly controlled manner. This physiological response of the β cell to elevated blood glucose levels is critical for maintenance of normoglycemia, and
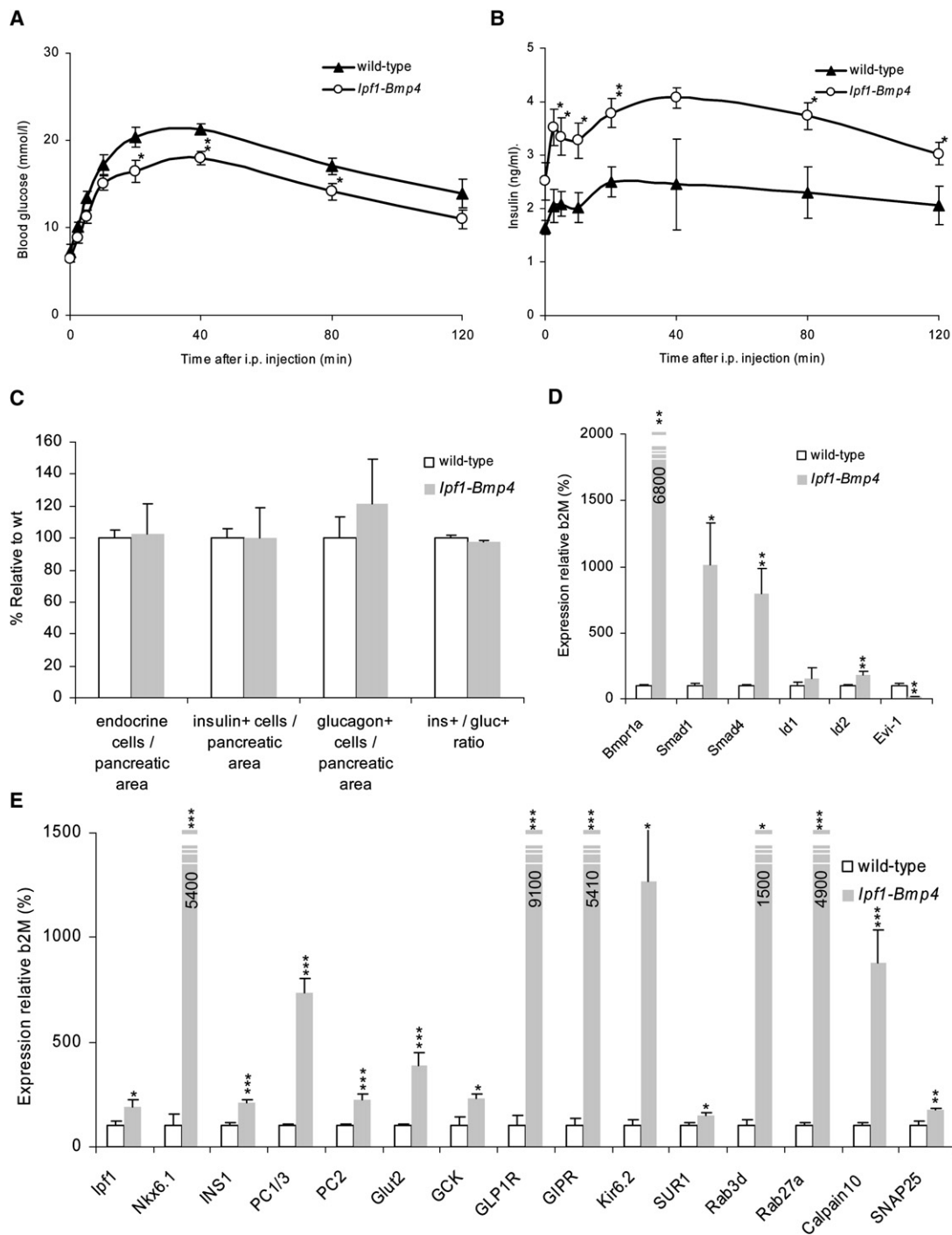
Figure 5. *Ipf1-Bmp4* Mice Show Improved Glucose Tolerance and Enhanced Glucose-Stimulated Insulin Secretion

(A) Blood glucose concentrations at the indicated time points following i.p. injection of glucose in wild-type (n = 6) and *Ipf1-Bmp4* animals (n = 6).

(B) Serum insulin levels during glucose tolerance test in wild-type (n = 6) and *Ipf1-Bmp4* mice (n = 6).

(C) Determination of total endocrine, insulin[+], and glucagon^{+} cells as well as ratios of insulin[+] to glucagon^{+} cells from wild-type (n = 5) and *Ipf1-Bmp4* mice (n = 5).

(D and E) Quantitative real-time RT-PCR expression analyses of the indicated genes were performed using islet cDNA prepared from wild-type and *Ipf1-Bmp4* mice (n = 4–6 for each mouse line). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ for *Ipf1-Bmp4* versus wild-type mice.
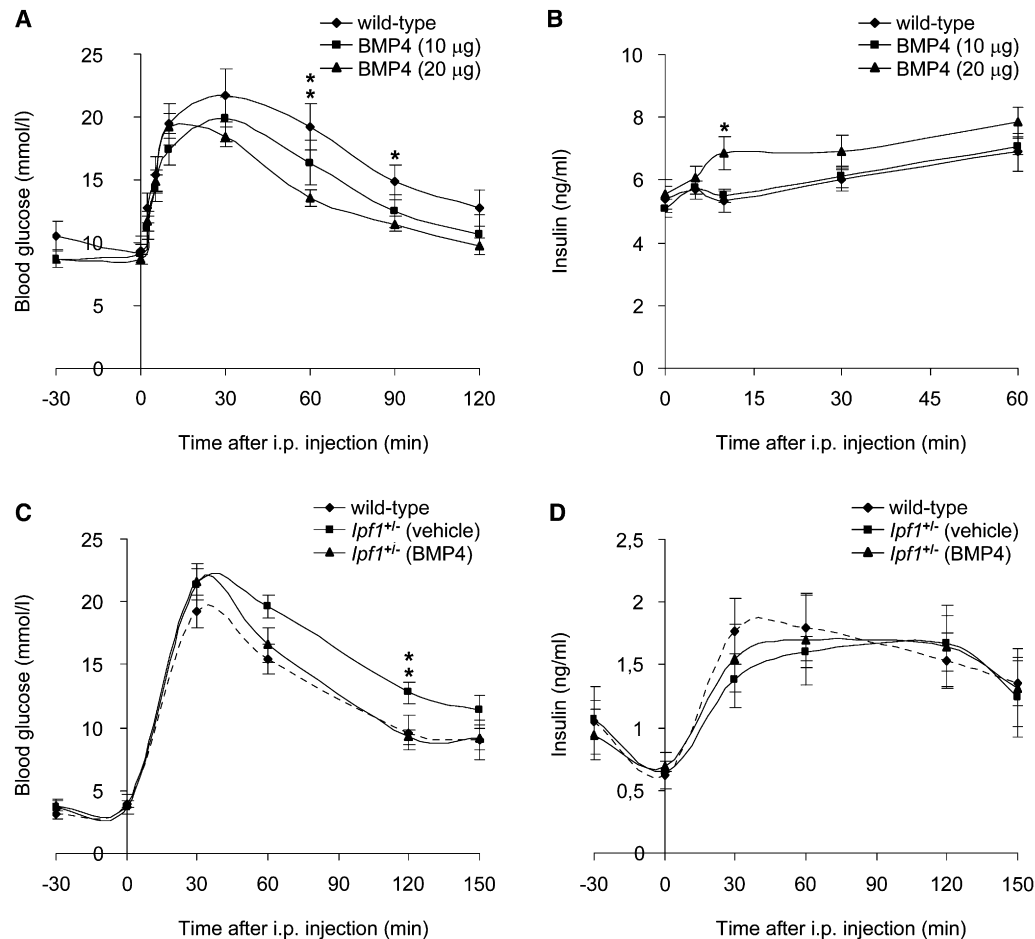
Figure 6. BMP4 Administration Improves Glucose Tolerance and Stimulates Glucose-Stimulated Insulin Secretion
(A) Blood glucose concentrations at the indicated time points following i.p. injection of glucose 30 min after the final i.p. injection of 10 and 20 μg/kg body weight of BMP4 (n = 10 for each concentration) or vehicle (n = 10) in CBA mice.
(B) Serum insulin levels during glucose tolerance tests in the BMP4- and vehicle-treated CBA mice in (A).
(C) Blood glucose concentrations were measured at the indicated time points following i.p. injection of glucose 30 min after the final i.p. injection of 20 μg/kg body weight of BMP4 (n = 6) or vehicle (n = 5) in $Ipf1/Pdx1^{+/-}$ animals. As a reference, a glucose tolerance curve for wild-type littermates (n = 7) is included (broken line).
(D) Serum insulin levels during glucose tolerance tests in the BMP4- and vehicle-treated $Ipf1/Pdx1^{+/-}$ animals in (C). As a reference, serum insulin levels in wild-type littermates (n = 7) during glucose tolerance tests are included (broken line). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ for BMP4-treated versus vehicle-treated mice.

impaired GSIS is a prominent feature of overt type 2 diabetes. Thus, the identification of signals and pathways that ensure and stimulate GSIS in β cells is of great clinical interest. Here we show that BMPR1A and its high-affinity ligand BMP4 are expressed in fetal and adult islets. We also provide evidence that BMPR1A signaling in adult β cells is required for GSIS and that both transgenic expression of *Bmp4* in β cells and systemic administration of BMP4 protein to mice enhance GSIS. Thus, BMP4-BMPR1A signaling in β cells positively regulates the genetic machinery that ensures GSIS.

Our results demonstrate that BMP4-BMPR1A signaling in β cells plays a key role in GSIS in vivo by positively regulating key genes involved in glucose sensing, glucose-metabolism-coupled secretion, incretin signaling, proin-

sulin processing, and insulin exocytosis. The complementary gene expression profiles displayed by mice with attenuated BMPR1A signaling as compared to mice overexpressing *Bmp4* provide evidence that the expression of these genes is directly affected by BMPR1A signaling rather than changes in blood glucose levels. Many of these genes have been previously linked to diabetes in animal models and humans (Edlund 2002; Marchetti et al., 2006). In particular, *Ipf1/Pdx1* null heterozygosity leads to glucose intolerance in mice (Ahlgren et al., 1998), and heterozygosity for a nonsense mutation in the human *IPF1* gene has been linked to MODY4 in humans (Stoffers et al., 1997). Our results establish that BMP4-BMPR1A signaling promotes *Ipf1/Pdx1* expression in β cells, which in turn has been shown to stimulate the expression of *Ins*,

Glut2, *Nkx6.1*, *GLP-1R*, *PC1/3*, and the K⁺ ATP channel subunits *Kir6.2* and *SUR1*. In contrast, the wide expression of *PC2*, *calpain-10*, *Rab3d*, *Rab27a*, and *SNAP-25* suggests that these genes are not direct targets of IPF1/PDX1. Collectively, these results indicate that BMP4-BMPR1A signaling positively regulates genes that mediate GSIS, in part by stimulating *Ipf1/Pdx1* expression. Fetal β cells respond poorly to glucose stimulation but progressively attain adequate glucose responsiveness after birth (Norlin et al., 2005). The onset of expression of both *Bmpr1a* and *Bmp4* in fetal β cells leaves open the possibility that BMP4-BMPR1A signaling is involved in the acquisition and/or maintenance of GSIS. However, the enhanced GSIS observed in mice overexpressing *Bmp4* in β cells—and in particular, in adult mice injected with BMP4 protein—provides evidence that the BMP4-BMPR1A signaling pathway can enhance GSIS in adult β cells.

The coexpression of *Bmp4* and *Bmpr1a* in β cells indicates that autocrine BMP4-BMPR1A signaling operates in β cells. The finding that the expression of *Bmpr1a* and other BMP signaling components in β cells is positively regulated by BMP4-BMPR1A signaling implies the existence of a positive feedback loop. Autocrine BMP4-BMPR1A signaling has also been suggested to occur in the developing distal lung epithelium (Eblaghie et al., 2006), and the existence of a positive BMPR1A signaling feedback loop has been previously proposed based on the observation that both BMP4 and BMP2 stimulate *Bmpr1a*, *Bmpr2*, and *Smad4* expression in cultured primary muscle cells (Nakamura et al., 2005). Formal evidence for a positive BMP4-BMPR1A signaling feedback loop in β cells, however, will have to await the generation and analyses of mice in which *Bmp4* has been specifically inactivated in β cells. BMP4 can also stimulate signaling via BMPR1B. Whereas our data on the selective inactivation of *Bmpr1a* in β cells demonstrate an important role for *Bmpr1a* signaling in β cell function and regulated insulin secretion and argue against a potential functional redundancy between *Bmpr1a* and *b* in β cells, they do not exclude a potential role for *Bmpr1b* in pancreas development or β cell function. The elucidation of a role, if any, for *Bmpr1b* in β cells, however, will have to await analyses of mice in which *Bmpr1b* has been specifically inactivated in β cells.

BMP4 can stimulate the proliferation of a pancreatic exocrine cancer cell line, and injection of anti-BMP4 antibodies into a mouse model of pancreatic hyperplasia appears to reduce the proliferation of pancreatic ductal cells (Hua et al., 2006). We did not observe a decrease in pancreatic mass in mice with attenuated BMPR1A signaling, nor did we observe an increase in pancreatic mass in the *Ipf1-Bmp4* mice, arguing against a prominent role for BMP4 in pancreatic ductal cell proliferation in normal pancreas. Moreover, BMP2—which, like BMP4, signals via BMPR1A—has been shown to stimulate proliferation of pancreatic ductal cell lines only in the absence of normal Smad4 activity, and *Smad4* is deleted in a vast majority of pancreatic cancer cell lines (Rane et al., 2006). Hence, the mitogenic effect of BMP2/4 on pancreatic ductal cells

appears to be preferentially observed under pathological conditions in which *Smad4* is mutated or absent.

Both *Bmp6* and *Bmp7* have been reported to be expressed in the developing pancreas (Dichmann et al., 2003), but, unlike BMP4, these BMPs bind preferentially to ActR1A, also known as ALK2 (Aoki et al., 2001; Miyazono et al., 2005). Overexpression of *Bmp6* under the control of the *Ipf1/Pdx1* promoter results in pancreatic hypoplasia (Dichmann et al., 2003). In contrast, *Bmp6*-deficient mice are viable and fertile and show no overt abnormalities (Solloway et al., 1998), arguing against a prominent role for *Bmp6* in the pancreas. *Bmp7* is expressed in the developing pancreatic epithelium between ~E9 and E15, but *Bmp7* mutant mice, which die shortly after birth, show no signs of pancreatic defects (Edlund 1998), arguing against a role for *Bmp7* in pancreatic development or β cell function. Thus, further studies are needed to elucidate a potential role for ALK2 signaling during pancreas development.

In summary, our results in mice provide evidence that an autocrine, positive BMP4-BMPR1A signaling feedback loop in β cells plays a key role in maintaining and enhancing GSIS by regulating genes involved in glucose sensing, glucose-metabolism-coupled secretion, incretin signaling, proinsulin processing, and insulin exocytosis. We also demonstrate that exogenous BMP4 administration enhances GSIS in normal mice and improves glucose tolerance in mice with impaired glucose tolerance. *BMPR1A* and *BMP4* are also expressed in human islets, suggesting that BMP4 or signals that stimulate BMPR1A signaling may represent important novel therapeutic approaches for the prevention and/or restoration of GSIS in type 2 diabetics.

EXPERIMENTAL PROCEDURES

Mice

All animal studies were approved by the Institutional Animal Care and Use Committee of Umeå University and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals. The dominant-negative deletion mutant of *Bmpr1a* that encodes 186 amino acids (aa) of BMPR1A plus 9 aa for an influenza virus hemagglutinin (HA) epitope at the C terminus was constructed by RT-PCR using mouse full-length *Bmpr1a* cDNA as a template. The resulting truncated *dnBmpr1a* and full-length mouse *noggin* (kindly provided by R. Harland) and *Smad6* cDNAs (a gift from P. Sideras) were individually cloned behind the 4.5 kb *Ipf1/Pdx1* promoter (Apelqvist et al., 1997). For BMP4 expression, a mouse *Bmp2/4* cDNA fragment combining the *Bmp2* precursor segment with the mature *Bmp4* sequence (Ericson et al., 1998) was cloned behind the *Ipf1/Pdx1* gene promoter. Transgenic mice were generated by pronuclear injections into oocytes derived from a CBA×C57BL/6 cross as described elsewhere (Hogan et al., 1994). Genotyping of all offspring was performed by PCR on genomic DNA isolated from tail biopsies of 3-week-old mice. The primers used for genotyping were 5′-GGGAAGAGGGAGATGTAGACTT-3′ (*Ipf1/Pdx1* primer), 5′-CTATTGTCCTGCGTAGCTGG-3′ (*dnBmpr1a* primer), 5′-AGAAGAGACAGCAGTCA-3′ (*Smad6* primer), 5′-GATGTGTAGAT AGTGCTGGC-3′ (*noggin* primer), and 5′-CCTCAACTCAAATTCGC GT-3′ (*Bmp4* primer). Two or more independent founders with a definite phenotype (diabetes for the *Ipf1-dnBmpr1a*, *Ipf1-Smad6*, and *Ipf1-noggin* founders; improved glucose tolerance for *Ipf1-Bmp4* founders) were obtained for each transgenic construct. Genotyping of *Ins-Cre*, *Bmpr1a* flox, and *Bmpr1a* null mice has been described elsewhere (Ahlgren et al., 1998; Mishina et al., 1995, 2002). The *Ins-Cre*,

Ipf1/Pdx1$^{+/-}$, *Bmpr1a* flox, and *Bmpr1a* null mice have been previously described (Ahlgren et al., 1998; Mishina et al., 1995, 2002). All analyses were performed on male mice.

In Situ Hybridization and Immunohistochemistry

Immunofluorescence localization of antigens, double-label immuno-fluorescence, and confocal microscopy were carried out essentially as previously described (Apelqvist et al., 1997). The primary antibodies used were guinea pig anti-insulin (Linco), rabbit anti-glucagon (Euro-Diagnostica), rabbit anti-carboxypeptidase A (Anawa), rabbit anti-amylase (Sigma), rabbit anti-phospho-Smad1/5/8 (Cell Signaling), rabbit anti-Smad1/5/8/9 (Imgenex), rabbit anti-Id2 (Santa Cruz), rabbit anti-Kir6.2 (gift of S. Seino and K. Takata), rabbit anti-Glut2 (raised against peptides 512–523 of mouse GLUT2 as described in Thorens et al. [1992] by AgriSera AB), and rabbit anti-GIPR (kindly provided by T. Kieffer). The secondary antibodies used were Alexa 488 anti-guinea pig (Molecular Probes) and Cy3 anti-rabbit (Jackson). In situ hybridization using DIG-labeled RNA probes for *Bmpr1a* and *Bmp4* was performed essentially as previously described (Apelqvist et al., 1997).

Western blot analyses

Protein was prepared from isolated islets (75 to 80 islets from each mouse) essentially as described elsewhere (Boucher et al., 2006). Protein concentrations were measured using bicinchoninic acid (BCA) reagent from Nordic Biolabs AB with bovine serum albumin as standard. Equal amounts of proteins were separated by SDS-PAGE, and proteins were detected immunologically after electrotransfer onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% nonfat dry milk and 0.05% Tween 20 for 1 hr at 25°C. Membranes were then incubated with appropriate primary antibodies in blocking solution followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing in PBS-0.05% Tween, blots were visualized with chemiluminescence reagent.

Glucose and Insulin Measurements

For glucose tolerance tests, blood samples were obtained from the tail vein of overnight-fasted and anesthetized mice to measure glucose levels using a Glucometer Elite (Bayer Inc.) immediately before and 2.5, 5, 10, 20, 40, and 120 min after intraperitoneal (i.p.) injection of (dose/body weight) glucose (2 g/kg), glibenclamide (5 mg/kg, Sigma G-0639), carbachol (0.16 μmol/kg, Sigma C-4382), or L-arginine (1 g/kg, Sigma A-5131). Effects of BMP4 administration were analyzed following two daily i.p. injections of either vehicle or BMP4 (10 or 20 μg/kg body weight) for 3 consecutive days, 4 hr fast, and a final i.p. injection of BMP4 or vehicle 30 min before i.p. injection of glucose (1.5 g/kg body weight). Blood samples were obtained from the tail vein, and glucose levels were measured immediately before injection of BMP4 and vehicle control prior to (=0 min) glucose injection and at the indicated time intervals following glucose injection. *Ipf1/Pdx1*$^{+/-}$ mice received two daily i.p. injections of either vehicle or BMP4 (20 μg/kg body weight) for 3 consecutive days, were fasted overnight, and received a final i.p. injection of BMP4 or vehicle 30 min before i.p. injection of glucose (2 g/kg body weight). For analysis of insulin, blood samples were collected in parallel with glucose measurements, and serum insulin levels were determined using ELISA (Crystal Chem Inc.). Total pancreatic insulin was extracted using acid ethanol (75% EtOH, 0.2 M HCl) and measured by Sensitive Rat Insulin RIA Kit (Linco). Pancreatic protein concentration was determined by Bio-Rad Protein Assay Kit (Bio-Rad).

Confocal Microscopy

Longitudinal sections of dorsal pancreata were immunostained as describe above. Images were collected first on a Leica TCS SP confocal microscope fitted with a spectrophotometer for emission band wavelength selection and a dual detector with both argon/krypton (Ar/Kr) and neon (Gre/Ne) lasers for simultaneous scanning of two different fluorochromes, then on a Nikon Eclipse 800 with the same lasers but using Nikon EZ-C1 1.6 software to characterize the different emissions.

Cell Counting

Images from longitudinal sections of dorsal pancreata were immuno-stained as described above. Pictures were taken with a 5× objective with a system composed of a Zeiss AxioPlan 2 imaging microscope coupled with an AxioCam linked to AxioVision 3.0.6 (Carl Zeiss) and analyzed with Image-Pro Plus 4.1 (Media Cybernetics) or a Zeiss Axiovert 200 M with a 40× objective coupled with PALMRobo software. Five to six pancreata from 2- to 3-month-old *Ipf1-dnBmpr1a*, FIN, and wild-type littermates (average weight 0.1005 ± 0.0085 g [*Ipf1-dnBmpr1a* and FIN] and 0.1058 ± 0.0114 g [wild-type]) and from 4- to 5-month-old *Ipf1-Bmp4* and wild-type littermates (average weight 0.1375 ± 0.096 g [*Ipf1-Bmp4*] and 0.1322 ± 0.0191 g [wild-type]) were processed. At least six sections, separated by 240 μm, were analyzed for each pancreas.

Quantitative Real-Time PCR Analysis

cDNA was prepared from total RNA derived from isolated islets as described elsewhere (Steneberg et al., 2005) using a NucleoSpin RNA II kit (635990, Macherey-Nagel) and SuperSmart PCR (635000, Clontech). Real-time PCR analysis was performed using the ABI PRISM 7000 Sequence Detection System and SYBR Green PCR Master Mix (ABI) according to the manufacturer's recommendations. Oligonucleotide primers were design using Primer Express software (Applied Biosystems). Expression levels were normalized to expression of β2 microglobulin (b2M). Primer sequences are described in Supplemental Data.

Reverse Transcriptase-PCR Analysis

cDNA was prepared from human islets, kindly provided by Dr. A. Pileggi (Diabetes Research Institute, Miller School of Medicine at University of Miami), and mouse islets were prepared essentially as described elsewhere (Steneberg et al., 2005). RT-PCR was performed using the sequence primers (forward and reverse) 5′-CACTGGTCCCTGGGATGTTC-3′ and 5′-GATCCACAGCACTGGTCTTGACTA-3′ (*hBmp4*), 5′-GGTCCAGGAAGAAGAATAA-3′ and 5′-GGTACAACATGGAAATGG-3′ (*mBmp4*), 5′-ACCTGGGCCTGCTGTTAAAT-3′ and 5′-GTGCCCACCCTGGTATTCAA- 3′ (*hBmpr1a*), and 5′-GAAGTTGCTGTATTGCTGA-3′ and 5′-GTAATACAACGACGAGCC-3′ (*mBmpr1a*).

Statistical Analysis

Statistical significance was calculated using Student's t test for pairwise comparison or one-way ANOVA for comparison of groups ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/3/207/DC1/.

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