Targeted Inactivation of Hepatocyte Growth Factor Receptor c-met in β-Cells Leads to Defective Insulin Secretion and GLUT-2 Downregulation Without Alteration of β-Cell Mass

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Overexpression of hepatocyte growth factor (HGF) in the β-cell of transgenic mice enhances β-cell proliferation, survival, and function. In the current studies, we have used conditional ablation of the c-met gene to uncover the physiological role of HGF in β-cell growth and function. Mice in which c-met is inactivated in the β-cell (MetCKO mice) display normal body weight, blood glucose, and plasma insulin compared with control littermates. In contrast, MetCKO mice displayed significantly diminished glucose tolerance and reduced plasma insulin after a glucose challenge in vivo. This impaired glucose tolerance in MetCKO mice was not caused by insulin resistance because sensitivity to exogenous insulin was similar in both groups. Importantly, in vitro glucose-stimulated insulin secretion in MetCKO islets was decreased by ~50% at high glucose concentrations compared with control islets. Furthermore, whereas insulin and glucokinase expression in MetCKO islets were normal, GLUT-2 expression was decreased by ~50%. These changes in β-cell function in MetCKO mice were not accompanied by changes in total β-cell mass, islet morphology, islet cell composition, and β-cell proliferation. Interestingly, however, MetCKO mice display an increased number of small islets, mainly single and doublet β-cells. We conclude that HGF/c-met signaling in the β-cell is not essential for β-cell growth, but it is essential for normal glucose-dependent insulin secretion. *Diabetes* 54:2090–2102, 2005

Many factors have been identified that are capable of stimulating β-cell hyperplasia in vitro or in vivo: glucose, *reg* (regeneration-associated gene), growth hormone, insulin, IGFs, islet neogenesis-associated peptide, glucagon-like peptide 1 (GLP-1), β-cellulin, transforming growth factor-α (TGF-α), gastrin, prolactin, parathyroid hormone–related protein, placental lactogen, and hepatocyte growth factor (HGF) (1). The fundamental importance of several islet growth factors as physiological regulators of islet growth and function has also been highlighted in studies using generalized knockout mice or transgenic mice overexpressing dominant-negative forms of their receptors in the β-cell (2–5). More recently, conditional disruption strategies using the Cre-LoxP system have allowed the direct deletion of growth factor receptor signaling in the pancreatic β-cell (6–8). β-Cell–specific knockout of the insulin receptor or IGF-1 receptor leads to altered glucose sensing and glucose intolerance in adult mice (7,8). In addition, mice with β-cell–specific knockout of the insulin receptor show an age-dependent decrease in islet size and β-cell mass (7). These studies indicate that insulin and IGF-1 are important for differentiated function, but they do not seem to play a major role in early growth/development of the pancreatic β-cell.

HGF is a mesenchyme-derived protein originally identified as a circulating factor in liver regeneration (9). To accomplish its actions, HGF binds with high affinity to, and induces the dimerization of, a transmembrane receptor encoded by the c-met protooncogene $p190^{MET}$. Dimerization of the c-met receptor is followed by autophosphorylation in *trans* of specific tyrosines in the cytoplasmic portion of the receptor (10,11). The c-met cytoplasmic region includes a kinase domain containing an invariant lysine, K^{1108} (an ATP binding site), that is essential for autophosphorylation of Y^{1234} and Y^{1235}. Phosphorylation of these two tyrosines activates the intrinsic kinase activity of c-met. In addition, the COOH-terminal domain of c-met contains a two-tyrosine docking site (Y^{1349} and Y^{1356}), which, on phosphorylation by the kinase domain, binds multiple SH2-containing transducer proteins that in turn modulate the activity of other adaptors or transcription factors (9).

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BrdU, 5-bromo-2′-deoxyuridine; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; HGF, hepatocyte growth factor; hGH, human growth hormone; KRBB, Krebs-Ringer bicarbonate buffer; RIA, radioimmunoassay; TGF-α, transforming growth factor-α.

In vitro studies have suggested that HGF/c-met has critical roles in epithelia formation, neuroendocrine cell formation, and angiogenesis (12–15). The availability of HGF and c-met knockout mice has unequivocally demonstrated an essential role for HGF/c-met signaling in embryonic development in vivo (16,17). Mice carrying two mutant c-met alleles in which exon 16 was deleted display an embryonic lethal phenotype (17). Histological analysis of these knockout embryos reveals a marked size reduction in the liver; damage of the liver parenchyma; complete absence of myotubes in the limbs, shoulders, and diaphragm; and defects in placental development. These abnormalities result in developmental retardation and embryonic lethality and are identical to the defects observed in HGF knockout mice (16). The early embryonic lethality and the fetal growth retardation make these mice a difficult model for studying the role of HGF/c-met in the development and/or function of other organs, including the pancreas.

HGF has been detected immunohistochemically in rodent pancreatic islet cells (18). In addition, confocal immunofluorescence studies have preferentially colocalized the c-met receptor to insulin-containing cells in the islet (19). Using transgene and ex vivo gene transfer approaches, we have shown that overexpression of HGF in β-cells of transgenic mice increases β-cell proliferation, islet size, and islet mass in vivo, while enhancing islet function and improving islet transplant performance (20–22). These studies have also highlighted the utility of HGF in enhancing β-cell survival because HGF decreases β-cell death rates in islet grafts after transplantation and reduces β-cell death induced by streptozotocin in vitro and in vivo (22).

Taken together, these observations suggest that HGF is a likely candidate for future strategies aimed at the treatment of diabetes. However, the physiological role of HGF/c-met signaling in the β-cell has not been elucidated. Therefore, to directly address the role of c-met in β-cell growth and function, we generated a conditional knockout mouse by using the Cre-LoxP recombination system. Inactivation of c-met in the β-cell was achieved by crossing rat insulin promoter (RIP)-Cre mice with $Met^{lox/lox}$ mice in which exon 16 of the c-met gene is flanked with two LoxP sites (6,23). We report herein that although overexpression of HGF in the pancreatic β-cell has been shown to lead to increased β-cell growth, c-met inactivation in the β-cell does not affect β-cell growth and development. Importantly, loss of c-met function in the β-cell results in decreased insulin secretion and diminished GLUT-2 expression, with resultant impaired glucose tolerance. These effects on β-cell function are exactly the opposite of those induced by HGF overexpression in RIP-HGF mice. These studies demonstrate that HGF/c-met signaling has an essential role in the normal function of the β-cell.

RESEARCH DESIGN AND METHODS

Analysis of c-met mRNA and protein expression in the pancreas of normal mice. Total DNA-free RNA was obtained from islets, ducts, and acini isolated from 8- to 12-week-old CD-1 mice, using a DNA-free RNA isolation kit (Ambion, Austin, TX). Reverse transcription was performed with 1–2 μg total islet RNA, using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA) with oligo-dT and MMLV (Moloney murine leukemia virus) reverse transcriptase. PCR was performed as previously reported (21), using [α-^{32}P]dCTP and primers for the gene of interest and actin gene in the same reaction tube allowing actin RNA to be used as an internal control. Gene-specific primers (400 nmol/l) used in these experiments were mouse c-met (forward primer 5′-GTCCAAGCAGTTCAGCCAG-3′ and reverse primer 5′-GGGATGGCTGAAGTCTTTCATG-3′), mouse HGF (21), mouse insulin (21), mouse carbonic anhydrase (forward primer 5′-GTTCACGGTGTGTGCTCAGAG-3′ and reverse primer 5′-CAGCCTCTGCTCATATCTTATG-3′), and mouse amylase (forward primer 5′-GTTCATAACCCATCAAGACCTTGG-3′ and reverse primer 5′-TTGGATTGAGGTAACTTCCACAGG-3′).

Pancreata from 2-week-old CD-1 mice were removed, fixed in Bouin's solution, embedded in paraffin, and sectioned. After deparaffinization and rehydration, 5-μm sections were then incubated with an anti-insulin antibody (1:50 dilution; Zymed, San Francisco, CA) and an anti–c-met antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Visualization of insulin staining was performed using a tetramethylrhodamine isothiocyanate–conjugated rabbit anti–guinea pig IgG secondary antibody (Sigma, St. Louis, MO). c-met staining was visualized using a fluorescein isothiocyanate–conjugated goat anti-rabbit IgG secondary antibody. All studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh institutional animal care and use committee.

Generation of MetCKO mice and genotyping. We generated MetCKO mice by crossing RIP-Cre transgenic mice with $Met^{lox/lox}$ mice. First, we crossed RIP-Cre mice (CD-1) (6) with $Met^{lox/lox}$ mice (C57BL/6×129/SV), generously provided by Dr. S. Thorgeirsson (National Institutes of Health) (23), generating RIP-Cre/$Met^{lox/+}$ mice. $Met^{lox/lox}$ mice have the exon 16 flanked with two LoxP sites, which allows the excision of exon 16 with Cre recombinase. Exon 16 of the c-met gene contains the invariant K^{1108} essential for the tyrosine kinase activity of the c-met receptor. Second, RIP-Cre/$Met^{lox/+}$ mice were again bred with $Met^{lox/lox}$ mice to generate the final progeny consisting of : *1*) RIP-Cre/$Met^{lox/lox}$ (MetCKO) mice, in which exon 16 of the c-met gene should be deleted in both alleles in Cre-expressing β-cells; *2*) control mice ($Met^{lox/+}$ or $Met^{lox/lox}$) without the RIP-Cre transgene; and *3*) heterozygous RIP-Cre/$Met^{lox/+}$, which should have exon 16 deleted only in one of the c-met alleles. These genotypes follow a normal Mendelian distribution. Only control and MetCKO mice were used for the experiments described in the current study. All mice were maintained on a 12-h light/dark cycle and fed a standard rodent chow diet. Genotyping was performed by PCR amplification of tail DNA using human growth hormone (hGH) primers to detect RIP-Cre transgenic mice (6,24). To detect the presence of the floxed c-met allele, we used LoxP primer 5′-GCAACTGTCTTTTGATCCCTGC-3′, located upstream of the LoxP site in intron 15, and exon 16 primer 5′-TGTCCAGCAAAGTCCCATGATAG-3′, located in exon 16 of the c-met gene. The LoxP/exon 16 primers generate PCR products of 539 bp (wild-type c-met allele) and 600 bp (floxed c-met allele). PCR efficiency was assessed by coamplification of a fragment of the endogenous murine glyceraldehyde-3-phosphate dehydrogenase gene (24).

Islet isolation. Islets from control and MetCKO mice were isolated as previously described (20). Briefly, pancreata injected through the pancreatic duct with Hank's buffered saline solution containing collagenase P were collected and incubated at 37°C, and the digest was separated by density gradient in Histopaque (Sigma). After several washes with Hank's buffered saline solution, islets were handpicked under a microscope.

Assessment of Cre-mediated recombination by PCR and immunohistochemical analysis. To analyze at a gene level whether recombination occurs in islets from MetCKO mice, PCR analysis of genomic DNA isolated from control ($Met^{lox/lox}$) and MetCKO mouse littermate islets was performed. Isolation of genomic DNA and PCR analysis were performed with a Redextract-N-Amp tissue PCR kit (Sigma), following the manufacturer's procedure and using the LoxP/exon 16 and glyceraldehyde-3-phosphate dehydrogenase primers mentioned above. If recombination occurs in the β-cells of MetCKO mice, a profound decrease in intensity of the 600 bp band (floxed c-met alleles) is expected.

To asses whether deletion of exon 16 of the c-met gene in MetCKO islets correlated with a decrease in the expression of phosphorylated c-met protein, staining with antibodies against insulin and phospho-$Tyr^{1234/1235}$ c-met was performed. Pancreata were obtained from 1-day-old MetCKO and control mice, fixed in Bouin's solution, embedded in paraffin, and sectioned. After deparaffinization and rehydration, 5-μm sections were then incubated with the anti-insulin antibody mentioned above and an anti–phospho-$Tyr^{1234/1235}$ c-met antibody (Santa Cruz) at 1:25 dilution. Visualization of insulin and phospho-$Tyr^{1234/1235}$ c-met staining was performed using the tetramethylrhodamine isothiocyanate–conjugated rabbit anti–guinea pig IgG secondary antibody and the fluorescein isothiocyanate–conjugated goat anti-rabbit IgG secondary antibody mentioned above, respectively. Staining was not observed when primary antibodies were omitted.

Blood glucose and plasma insulin analysis. Blood was obtained from control and MetCKO mice in fasting (16 h) or in the random-fed state by retro-orbital bleeding, as described previously (25). Briefly, blood glucose was

determined using a Precision QID glucometer (Medisense, Bedford, MA). Plasma insulin levels were measured by radioimmunoassay (RIA; Linco, St. Louis, MO).

Intraperitoneal glucose tolerance test. Glucose tolerance was analyzed in 16-h–fasted 8- to 12-week-old mice by intraperitoneal injection of 2 g glucose/kg body wt, as previously reported (21). Blood glucose was measured from the snipped tail at 0, 15, 30, 60, and 120 min after glucose injection by a portable glucometer. In two different sets of mice, blood was obtained from the snipped tail at 0 and 30 min after glucose (2 g/kg body wt) or L-arginine (0.3 g/kg body wt) intraperitoneal injections, and plasma insulin concentrations were measured by RIA (Linco).

Intraperitoneal insulin sensitivity test. Insulin sensitivity tests were performed on 8- to 12-week-old mice in the random-fed state, as previously reported with some modifications (8). Mice were injected with 0.75 units/kg body wt i.p. bovine insulin. Blood glucose was measured from the snipped tail at 0, 15, 30, and 60 min after insulin injection by a portable glucometer.

Glucose-stimulated insulin secretion. Insulin release from control and MetCKO isolated islets was measured as previously described (21), with some modifications. Briefly, Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 10 mmol/l HEPES was prepared and continuously bubbled with a mixture of O_2/CO_2 (95/5%) to a final pH of 7.4. Isolated islets were then preincubated in KRBB with 1% BSA and 2.5 mmol/l glucose for 1 h at 37°C in a 5% CO_2 incubator. After washing the islets once in KRBB plus 1% BSA, groups of 10 islets of similar size from control and MetCKO mice were incubated in 1 ml of fresh KRBB plus 1% BSA and different glucose concentrations (0, 2.5, 5, 11, and 22 mmol/l) for 30 min at 37°C in the 5% CO_2 incubator. In a different set of experiments, islets were incubated with KRBB plus 1% BSA and 8 mmol/l glucose plus 100 nmol/l GLP-1 (6-36) amide (Bachem Biosciences, King of Prussia, PA) or 20 mmol/l L-arginine. All the experiments were performed in triplicate at each glucose concentration tested. After incubation, buffer was removed and frozen at −20°C until insulin determination by RIA (Linco). Islets were then washed three times with PBS and digested overnight in 1 ml of 0.1 N NaOH at 37°C. After neutralization with 0.1 N HCl, protein was measured by the Bradford method. Results are expressed as a percentage of insulin secreted by control islets incubated in the absence of glucose.

Quantitation of pancreas and islet insulin content. Insulin was extracted from pancreata and isolated islets from 8- to 12-week-old control and MetCKO mice, using the acid/ethanol method as previously reported (20,25). Briefly, pancreata or 50 islet equivalents (IE; 1 IE = 125 μm diameter) were homogenized in acid/ethanol (0.18 mol HCl in 70% ethanol) and extracted at −20°C for 24 h. Tubes were then centrifuged at 2,500 rpm for 10 min at 4°C and the supernatant stored at −20°C until insulin determination by RIA (Linco). Protein concentrations in the pancreas extracts were determined using the Bradford method.

Western blot analysis of glucokinase and GLUT-2 expression. Western blot analysis of GLUT-2 and glucokinase in isolated control and MetCKO islets was performed as previously described (26). Briefly, whole-islet extracts were made in freshly prepared lysis buffer (5% SDS, 80 mmol/l Tris-HCl, pH 6.8, 5 mmol/l EDTA, and 0.5 mmol/l phenylmethylsulfonyl fluoride). Islets were sonicated, the supernatant containing the cell lysate was separated by centrifugation, and protein concentrations were measured using a MicroBCA assay (Pierce). Next, 40 μg of protein from each sample was added to loading buffer and analyzed using 10% SDS-polyacrylamide gels. Proteins were transferred from the gels to Immobilon-P membrane (Millipore, Bedford, MA) using standard techniques. Blots were incubated with primary antibodies against GLUT-2 (1:200 dilution; Santa Cruz), glucokinase (1:200 dilution; Santa Cruz), and actin (1:400 dilution; Sigma). Chemiluminescence was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemical analysis of GLUT-2, insulin, glucagon, and somatostatin. Pancreata removed from MetCKO and control mice were fixed in Bouin's solution, embedded in paraffin, sectioned, and immunostained after deparaffinization and rehydration. Immunostaining for GLUT-2 was performed using an affinity-purified goat anti-human GLUT-2 antibody (1:500 dilution; Santa Cruz), as previously described (21). Immunostaining for insulin, glucagon, and somatostatin was performed as previously reported (25).

Quantitative islet histomorphometry. Histomorphometric analysis on insulin-stained sections from control and MetCKO littermates was performed as previously reported, with some modifications (20,21). Briefly, quantitation of islet number and islet volume as a function of pancreatic volume was performed in insulin-stained sections, using the MetaMorph Imaging System Analysis software package (Universal Imaging, Downingtown, PA). Islet size distribution analysis was performed in two different insulin-stained sections separated by 50 μm.

Quantitation of β-cell proliferation rates. β-Cell replication was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation as described previously (20), with minor modifications. Briefly, MetCKO and control littermates were injected intraperitoneally with BrdU (Cell Proliferation Kit, Amersham Pharmacia Biotech) and killed after 6 h. Pancreata were removed and immediately fixed in Bouin's solution for 6 h, and then they were embedded, sectioned, deparaffinized, and stained with an anti-BrdU antibody and with an antibody against insulin (see above). After counterstaining with hematoxylin, the sections were counted in a blinded fashion. Islet cells that stained for BrdU and insulin were considered BrdU-positive β-cells. At least 1,000 islet nuclei were counted per pancreas. The results are expressed as the percentage of BrdU-positive β-cell nuclei per total number of β-cell nuclei.

Statistical analysis. Data are expressed as the means ± SE. Unpaired two-tailed Student's t tests were used to determine statistical significance. Differences were considered significant at $P < 0.05$.

RESULTS

Expression of c-met mRNA and protein in normal mouse pancreas. To analyze the expression and localization of c-met mRNA and protein in the mouse pancreas, we performed RT-PCR analysis for c-met mRNA in total RNA extracted from isolated ducts, acini, and islets, and we performed immunohistochemical staining of c-met in mouse pancreatic sections (Fig. 1). As shown in Fig. 1*A*, c-met mRNA expression was detected in isolated ducts and islets from murine pancreas but was absent from acini (Fig. 1*A*). Interestingly, HGF mRNA expression was detected mainly in ducts and also, though at a lower level, in mouse islets (Fig. 1*A*). Immunofluorescent staining of mouse pancreatic sections confirmed the colocalization of insulin and c-met in mouse islets (Fig. 1*B*), previously observed in human and rat islets (18,19).

Generation of RIP-Cre/Met$^{lox/lox}$ mice. RIP-Cre and Met$^{lox/lox}$ mice were crossed to generate β-cell conditional c-met knockout mice (MetCKO; RIP-Cre/Met$^{lox/lox}$) and control mice (Met$^{lox/lox}$ or Met$^{lox/+}$ without the RIP-Cre transgene). A schematic representation of the floxed exon 16 of the c-met allele in Met$^{lox/lox}$ mice (23) and the resultant product after its excision with Cre recombinase is shown in Fig. 2*A*. The genotypes of the MetCKO mice and control littermates were identified using tail DNA PCR. LoxP/exon 16 primers were used to distinguish between the floxed and wild-type c-met alleles, which result in distinct bands of 600 and 539 bp, respectively (Fig. 2*B*). The presence of the RIP-Cre transgene was determined using hGH primers (Fig. 2*B*) because the RIP-Cre transgene contains untranslated sequences of the hGH gene (6). As shown in Fig. 2*B*, MetCKO mice are positive for RIP-Cre transgene and have both c-met alleles floxed (*lane 3*), whereas control mice are negative for RIP-Cre transgene and could have both c-met alleles floxed (*lane 1*) or one floxed and one wild-type c-met allele (*lane 2*). Mice were born with normal Mendelian frequency, indicating that there is no embryonic lethality in MetCKO animals. Body weights (g) were similar in male and female MetCKO and control mice when analyzed at 8 weeks of age (29.1 ± 0.5, n = 13, and 23.7 ± 0.3, n = 16, for wild-type males and females, respectively, vs. 28.6 ± 0.4, n = 13, and 22.8 ± 0.6, n = 8, for MetCKO males and females).

To assess the efficiency of Cre-mediated exon 16 deletion from pancreatic β-cells in MetCKO mice, PCR analysis of islet genomic DNA from MetCKO (RIP-Cre/Met$^{lox/lox}$) and control (Met$^{lox/lox}$) mice was performed (Fig. 2*C*). Importantly, the intensity of the 600-bp band (floxed c-met

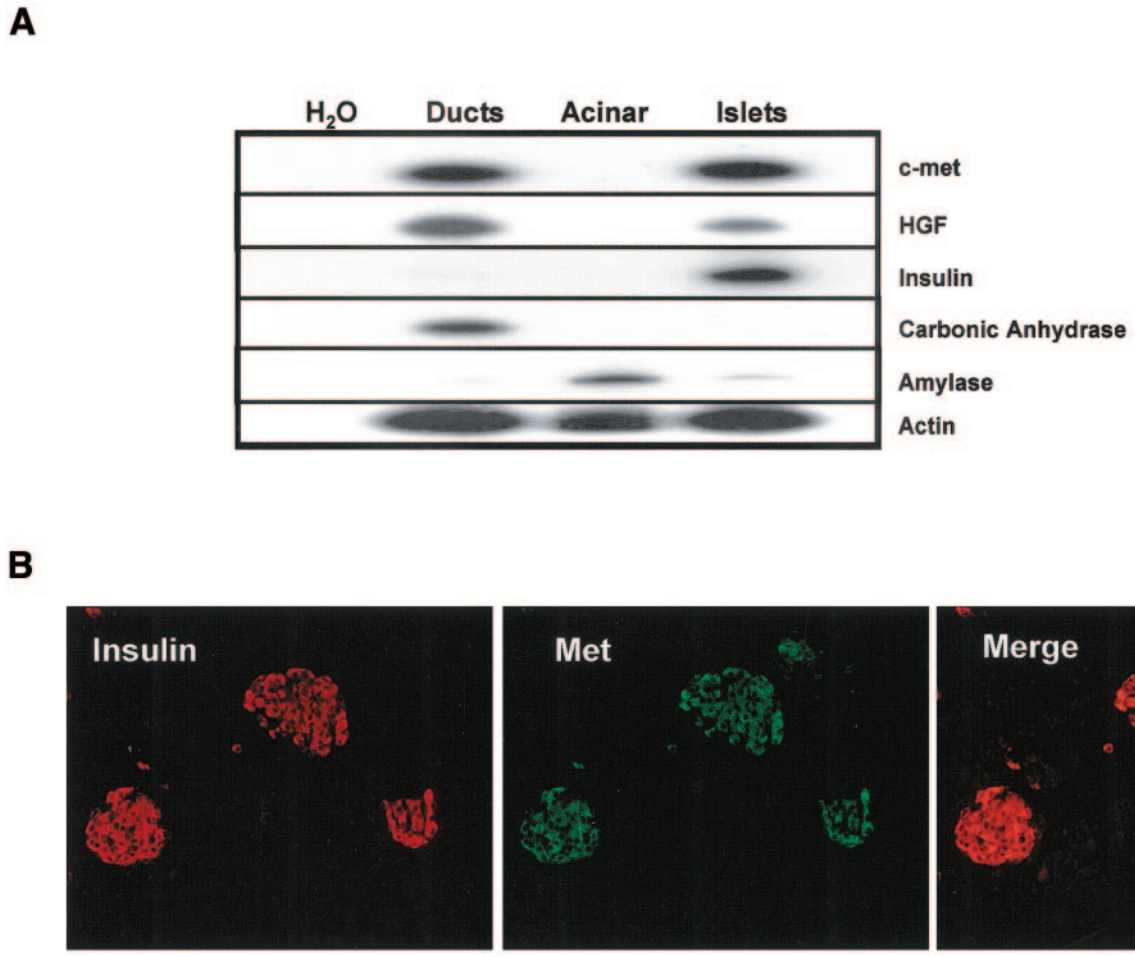

FIG. 1. Detection of HGF mRNA and c-met mRNA and protein in murine pancreas. *A*: RT-PCR analysis of c-met and HGF mRNA expression in isolated ducts, acini, and islets obtained after collagenase digestion of pancreata from 8- to 12-week-old CD-1 mice. PCR was performed for 35 cycles with primers for c-met, HGF, carbonic anhydrase (ductal marker), amylase (acinar marker), and actin as housekeeping gene and for 20 cycles with primers for insulin (islet marker). Notice that ducts and islets but not acini express c-met mRNA. HGF mRNA is expressed in ducts and at a lower level in islets but not in the acinar tissue of these mice. *B*: Representative immunofluorescent staining for insulin and c-met in pancreatic sections of 2-week-old CD-1 mice. Visualization of insulin (red) and c-met (green) staining was performed with a tetramethylrhodamine isothiocyanate–conjugated and a fluorescein isothiocyanate–conjugated secondary antibody, respectively. Merge of both images reveals colocalization of c-met and insulin in the islet.

alleles) in MetCKO mice (*lanes 3* and *4*) was markedly decreased (80–85%) compared with the intensity observed in control samples (*lanes 1* and *2*), indicating that efficient deletion of the exon 16 in the c-met alleles had occurred. To confirm that this effective deletion had also occurred at the protein level, immunohistochemical staining of pancreatic sections from MetCKO and control mice was performed with anti–phospho-Tyr$^{1234/1235}$ c-met and anti-insulin antibodies (Fig. 2*D*). As shown in the representative microphotographs in Fig. 2*D*, a clear decrease in phospho-Tyr$^{1234/1235}$ c-met immunofluorescent staining was observed in the islets from MetCKO mice compared with those in control mouse pancreas. Taken together, these results indicate that Cre-mediated deletion of exon 16 in the c-met gene has efficiently occurred, resulting in markedly decreased levels of phospho–c-met protein in the islets of MetCKO mice.

MetCKO mice display normal blood glucose and plasma insulin levels but impaired glucose tolerance. As can be seen in Fig. 3*A* and *B*, blood glucose and plasma insulin concentrations were similar in MetCKO mice and control littermates in both nonfasting and 16-h fasting conditions. To assess the impact of inactivation of c-met on glucose-regulated insulin secretion in vivo, we examined the response of 8- to 12-week-old MetCKO and control mice to a glucose challenge by performing an intraperitoneal glucose tolerance test after a 16-h overnight fast. As shown in Fig. 3*C*, blood glucose levels were similar before administration of glucose (0 min) and 15 min after the glucose load. However, compared with control littermates, blood glucose levels were significantly increased in MetCKO mice for the remaining time points. Moreover, calculation of the area under the curve of the intraperitoneal glucose tolerance tests performed in both types of mice confirmed that MetCKO mice display significantly increased area under the curve (mmol/l $\times$ min $\times$ 10^3) compared with control mice (1.7 ± 0.06, $n = 18$, vs. 1.3 ± 0.03, $n = 18$; $P < 0.01$) when subjected to a glucose challenge.

These results indicate that MetCKO mice have delayed glucose disposal that could be the result of decreased insulin secretion and/or decreased insulin sensitivity in vivo. To determine whether insulin sensitivity is affected in MetCKO mice, insulin sensitivity tests were performed.

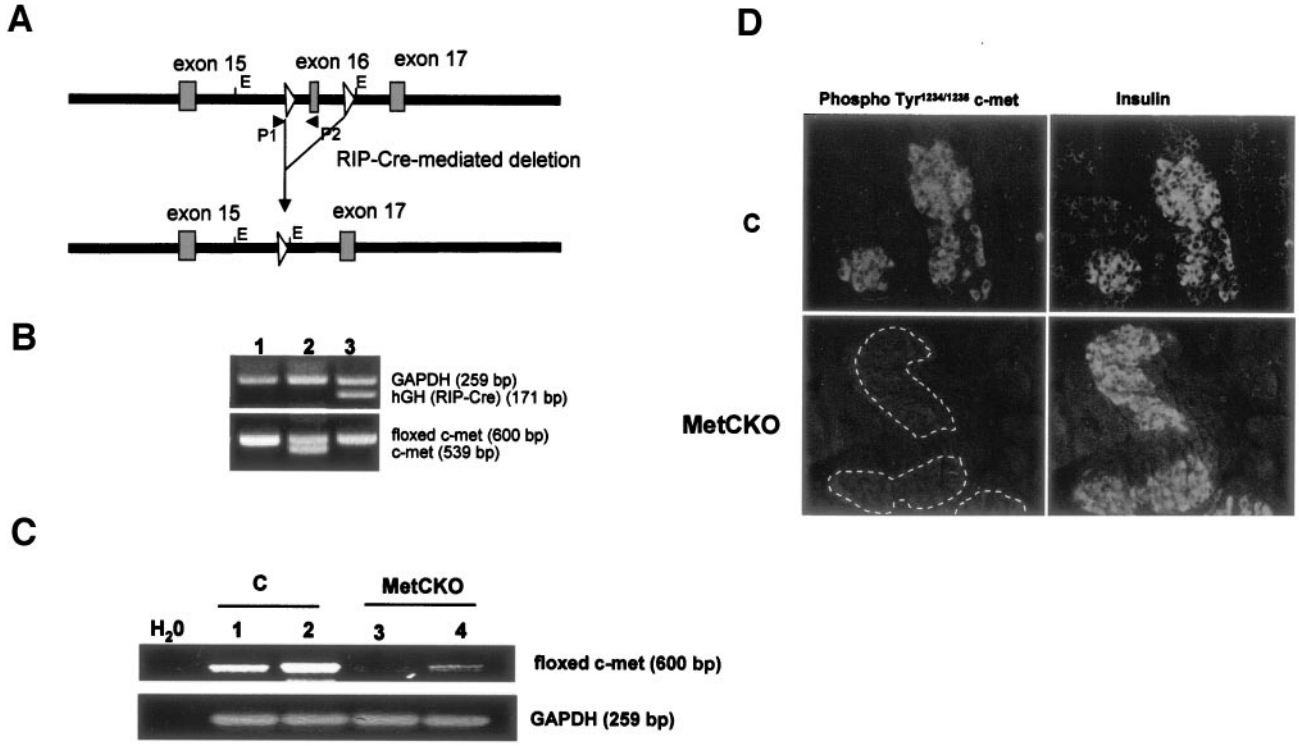

FIG. 2. Assessment of exon 16 deletion and c-met inactivation in pancreatic islets of MetCKO mice. *A*: Schematic illustration of Cre-mediated deletion of exon 16 from the c-met allele. Cre action removes exon 16 and intron sequences between the two LoxP sites (white arrowheads). E, *Eco*RI restriction sites; P1, LoxP primer used for genotyping and assessment of c-met deletion from islet genomic DNA; P2, exon 16 primer used for genotyping and assessment of c-met deletion from islet genomic DNA. *B*: PCR analysis of tail DNA from control mice (*lane 1*, $Met^{lox/lox}$; *lane 2*, $Met^{lox/+}$) and from MetCKO mice (*lane 3*, RIP-Cre/$Met^{lox/lox}$). PCR fragments corresponding to the housekeeping gene GADPH (259 bp) and hGH (171 bp; RIP-Cre transgene) are shown (*upper panel*). PCR fragments corresponding to wild-type (539 bp) and floxed (600 bp) c-met alleles are shown (*lower panel*). *C*: PCR analysis of genomic DNA from islets isolated from control mice (*lanes 1* and *2*, $Met^{lox/lox}$) and MetCKO mice (*lanes 3* and *4*, RIP-Cre/$Met^{lox/lox}$). PCR was performed for 35 cycles with LoxP/exon 16 primers. Notice the highly reduced band intensity of the floxed c-met PCR fragment in both MetCKO islet DNA samples compared with control samples. Densitometric analysis of bands corresponding to floxed c-met fragments revealed an 80–85% reduction in intensity. *D*: Fluorescent immunohistochemical analysis for phospho-$Tyr^{1234/1235}$ c-met and insulin on pancreatic sections from 1-day-old MetCKO mice and control littermates. A profound decrease in immunofluorescent staining for phospho-$Tyr^{1234/1235}$ c-met was observed in islets of MetCKO mouse pancreata.

Identical decreases in blood glucose levels were observed in MetCKO mice and control littermates after intraperitoneal administration of insulin (Fig. 3*D*), indicating similar insulin sensitivity in both types of mice. This result rules out the possibility of increased insulin resistance as the cause of the glucose intolerance observed in MetCKO mice. To determine whether insulin secretion is affected in vivo in MetCKO mice, plasma insulin levels were measured before and 30 min after administration of glucose. As shown in Fig. 3*E*, glucose administration induced a quantitatively large and significant increase in plasma insulin levels in control mice. In contrast, MetCKO mice displayed a significantly attenuated response to the same dose of glucose (Fig. 3*E*). On the other hand, no significant differences in the insulin secretory response to L-arginine were observed between the two types of mice (Fig. 3*F*), indicating a glucose-specific secretory defect in MetCKO mice. Collectively, these findings reveal that glucose-induced insulin secretion in vivo in MetCKO mice is defective.

β-Cell mass, islet morphology, and islet cell composition are not altered in MetCKO mice. The decrease in glucose tolerance and insulin secretion in MetCKO mice described above could result from a decrease in β-cell mass. We therefore performed immunohistological examinations of insulin-stained pancreatic sections from MetCKO mice and control littermates. As shown in Fig. 4*A*, no striking differences were observed between these two types of mice. Furthermore, pancreatic insulin content in acid-ethanol extracts was identical in the two groups (Fig. 4*B*). Detailed histomorphometric analysis corroborated these observations because β-cell area per pancreatic area was similar in 8- to 12-week-old MetCKO mice and control littermates (Fig. 4*C*). These studies indicate that, in contrast to the increased β-cell mass observed in RIP-HGF transgenic mice (20), inactivation of the HGF/c-met signaling pathway in the β-cell does not result in changes in total pancreatic β-cell mass. To determine whether alterations in islet function in vivo in MetCKO mice were associated with any changes in morphology or islet cell composition, we performed immunohistochemical staining on pancreatic sections of these mice. The pattern of distribution, the intensity of staining, and the ratio of β- to non–β-cells at 8–12 weeks of age were not different in MetCKO mice and control littermates (Fig. 4*D*).

MetCKO mouse islets display defective glucose-stimulated insulin secretion and decreased GLUT-2 levels. MetCKO mice display normal β-cell mass, normal insulin sensitivity, impaired glucose tolerance and decreased plasma insulin levels after a glucose challenge. Taken together, these observations suggest that glucose-stimulated insulin secretion (GSIS) could be defective in

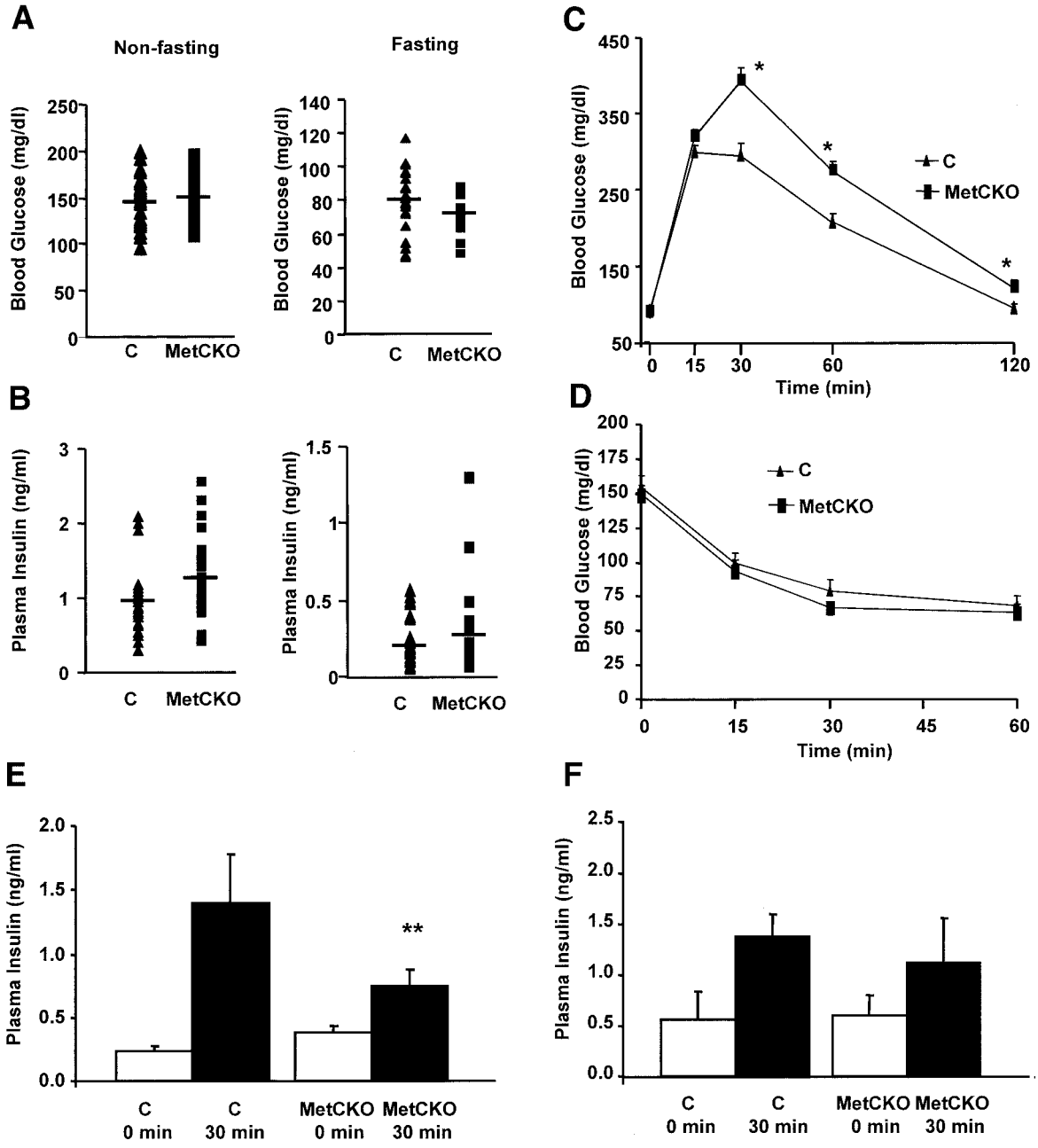

FIG. 3. Glucose homeostasis in 8- to 12-week-old MetCKO mice. *A* and *B*: Scatter plot showing blood glucose concentrations and plasma insulin levels in nonfasting and 16-h–fasted MetCKO mice (n = 21) and control (C) littermates (n = 29). Averages are indicated by horizontal lines. Under both conditions no significant differences were observed in these parameters. *C*: Intraperitoneal glucose tolerance test in MetCKO mice (n = 18) and control littermates (n = 18). After fasting overnight, mice were injected with glucose (2 g/kg body wt i.p.) and blood glucose levels were measured at the time points indicated in the figure. MetCKO mice exhibited reduced glucose tolerance, as revealed by significantly increased blood glucose levels at the time points indicated (*P < 0.01). *D*: Insulin sensitivity tests were performed on random-fed MetCKO (n = 10) and control littermates (n = 10). No significant differences were found in blood glucose levels before and after insulin administration in both types of mice. *E*: Plasma insulin concentrations in MetCKO mice (n = 7) and control littermates (n = 7) injected with glucose (2 g/kg body wt i.p.). Blood was obtained before (0 min) and 30 min after glucose administration. At 30 min after glucose administration, a significant decrease in plasma insulin levels was observed in MetCKO mice. *F*: Plasma insulin concentrations in MetCKO mice (n = 5) and control littermates (n = 5) injected with L-arginine (0.3 g/kg body wt i.p.). Blood was obtained before (0 min) and 30 min after arginine administration. No significant differences were observed between both types of mice before or after L-arginine injection. **P < 0.05.

MetCKO islets compared with control littermate islets. We therefore studied GSIS in islets isolated from MetCKO mice and control littermates (Fig. 5*A*). As expected, MetCKO islets secreted significantly less insulin than control islets at the highest glucose concentrations tested, 11 and 22 mmol/l. No significant differences in insulin secretion were observed in the absence of glucose, at a low glucose concentration (2.5 mmol/l), or at a physiological glucose concentration (5 mmol/l) (Fig. 5*A*). To determine whether the insulin secretory response was altered in the presence of other secretagogues, we tested the effect of 20 mmol/l L-arginine and 100 nmol/l GLP-1 in islets incubated at 8 mmol/l glucose. MetCKO and control islets displayed similar secretory response to these insulin secretagogues (Fig. 5*B*).

Overexpression of HGF in the pancreatic β-cell of

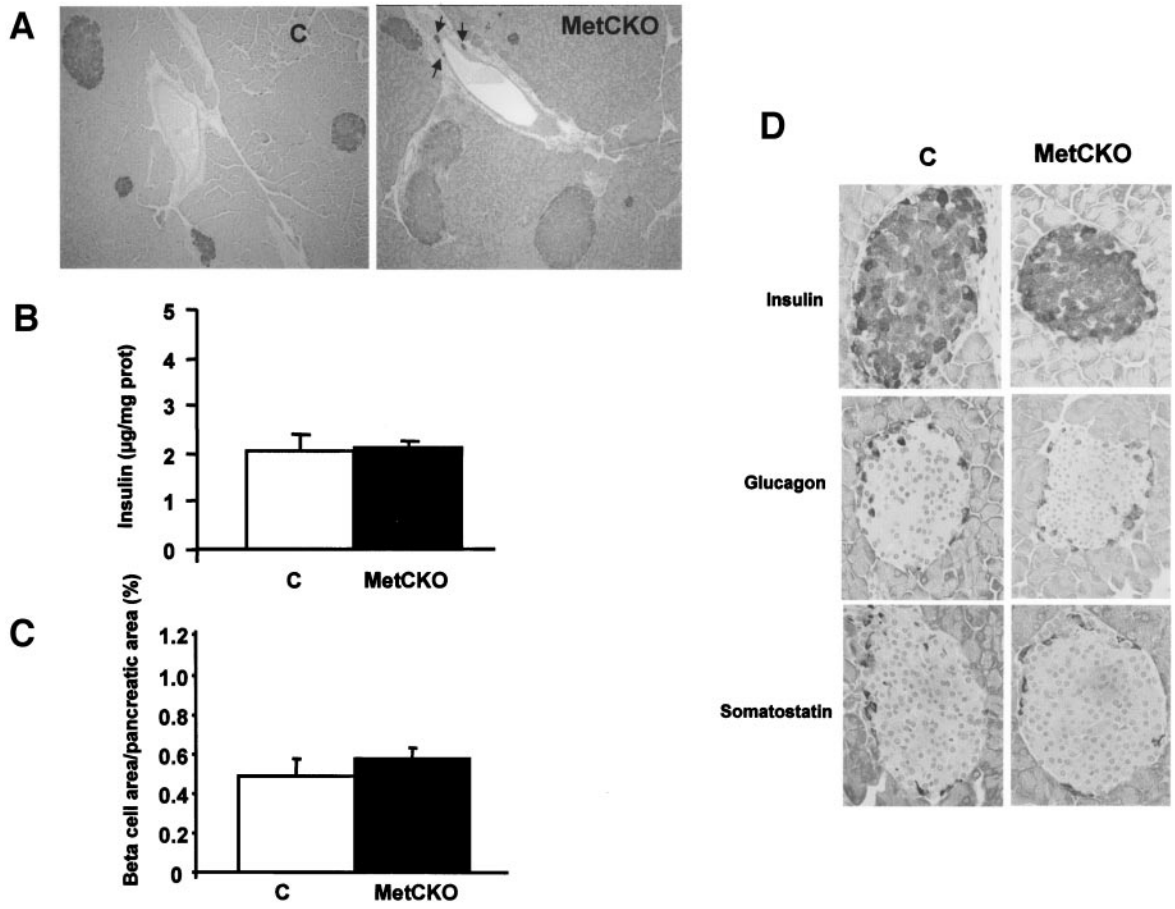

FIG. 4. MetCKO mice (8–12 weeks of age) show normal pancreas histology, pancreas insulin content, β-cell mass (β-cell area/pancreatic area), islet morphology, and islet cell composition. *A*: Representative microphotographs of MetCKO and control (C) pancreata stained for insulin and counterstained with hematoxylin. No obvious differences in islet size and mass were found between both types of mice, except for an increased number of small islets and insulin-positive cells in the ducts (arrows) in MetCKO mice (see Fig. 7). *B*: Insulin content in pancreata from MetCKO ($n = 4$) and control ($n = 4$) mice extracted with acid/ethanol solution at −20° for 24 h. Almost identical values were obtained in both types of mice. *C*: Histomorphometric analysis of MetCKO ($n = 6$) and control ($n = 5$) mouse pancreata. The percentage of β-cell area is expressed as a function of total pancreas area in pancreatic sections of MetCKO and control mice stained for insulin and counterstained with hematoxylin. No significant differences were found between both types of mice. *D*: Representative microphotographs showing immunohistochemical staining for insulin, glucagon, and somatostatin in pancreatic islets from control and MetCKO mice. The pattern and intensity of staining of these three hormones in MetCKO islets is indistinguishable from the staining in islets from control mice.

transgenic mice results in increased expression of insulin, GLUT-2, and glucokinase, three key proteins implicated in insulin secretion (20). Alterations in the levels of these three proteins in MetCKO islets could account for the observed reduced GSIS observed in MetCKO islets. Insulin mRNA expression (Fig. 5*C*) and insulin protein content (Fig. 5*D*) were similar in islets isolated from MetCKO mice and control littermates. Similar glucokinase levels were also observed in these two types of mouse islets, as demonstrated by Western blot analysis (Fig. 6*A*). On the other hand, Western blot analysis revealed a 50% decrease in the GLUT-2 expression levels in these same islet protein extracts from MetCKO mice compared with those obtained from control littermates (Figs. 6*A* and *B*). This decrease in GLUT-2 expression levels in MetCKO islets was confirmed by immunohistochemical staining of pancreatic sections from MetCKO mice and control littermates performed under identical conditions (Fig. 6*C*). Whereas GLUT-2 staining is mainly membrane-associated in normal islets, GLUT-2 staining in islets from MetCKO mice was less intense and less clearly associated with the β-cell membrane (Fig. 6*C*). These results, together with our previous results demonstrating an HGF-mediated upregulation of GLUT-2 in transgenic mouse islets (21), indicate that the HGF/c-met system is required for normal GLUT-2 expression and β-cell function.

MetCKO mouse pancreas displays increased number of small islets. Although we did not observe significant differences in total β-cell mass in 8- to 12-week-old MetCKO mice, quantitation of the number of islets per pancreatic area revealed that the islet number was significantly increased in MetCKO mice compared with control littermates (Fig. 7*A*). An increase in islet number without a concomitant increase in islet mass suggested an increase in the number of very small islets, whose contribution to islet mass would be negligible. To analyze this hypothesis, we performed detailed histomorphometric evaluation of islet size and number in insulin-stained pancreatic sections from 8- to 12-week-old MetCKO mice and control littermates. Microscopic examination of MetCKO pancreata at high magnification confirmed an increased number of small islets (mainly single and doublet β-cells), as shown in the representative microphotographs in Fig. 7*B*. Furthermore, numerous insulin-positive cells budding from

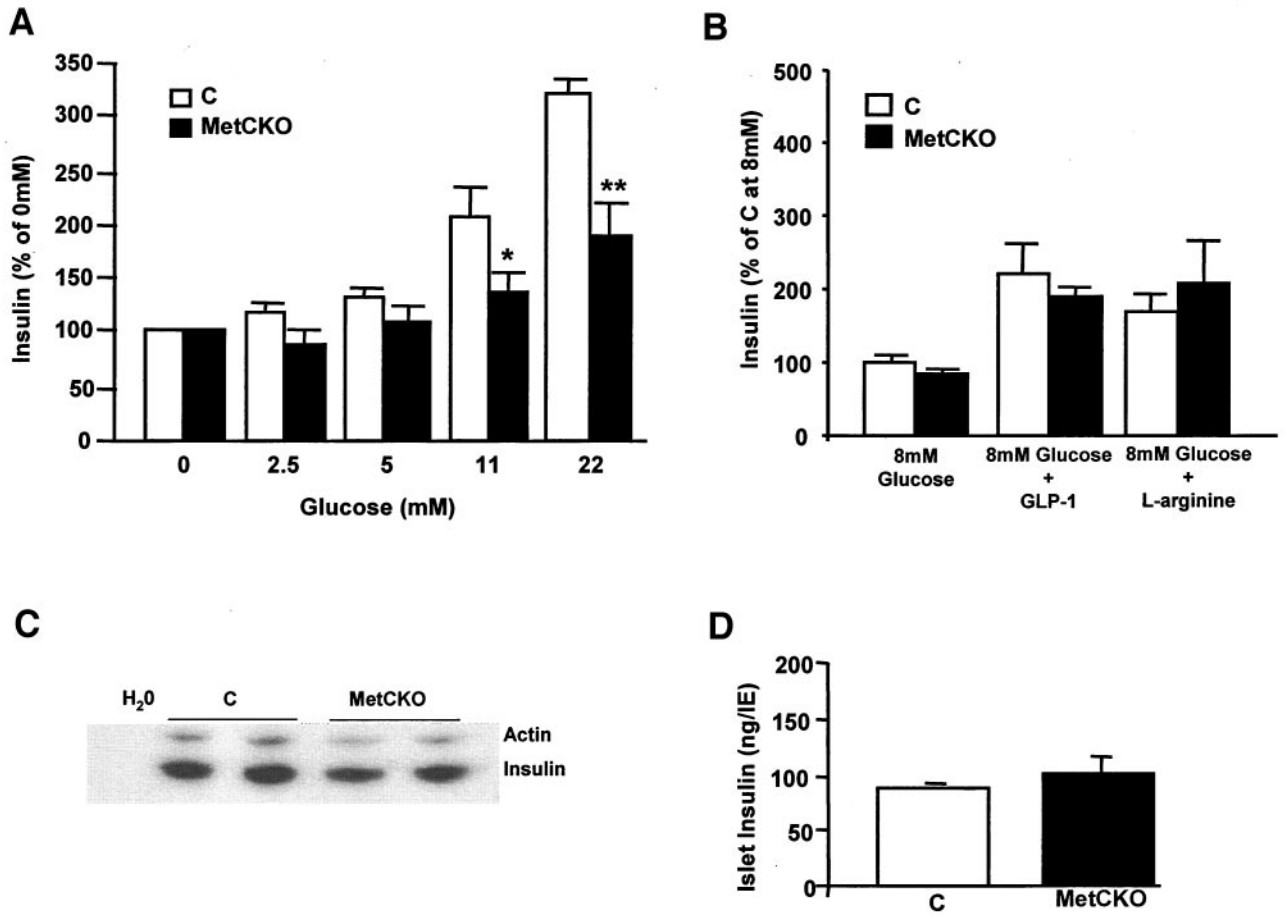

FIG. 5. GSIS and insulin content in islets from MetCKO mice and control (C) littermates. *A*: GSIS performed in groups of 10 islets of similar sizes obtained from control ($n = 5$) and MetCKO ($n = 5$) mice and incubated for 30 min with different glucose concentrations as indicated in the figure. Experiments were performed in triplicate, and insulin was measured by RIA. Significant decreases in GSIS were observed in MetCKO islets incubated at 11 and 22 mmol/l glucose compared with control islets at the same glucose concentrations. *B*: GSIS performed in groups of 10 islets of similar sizes obtained from control ($n = 4$) and MetCKO ($n = 6$) mice and incubated for 30 min with 8 mmol/l glucose and 20 mmol/l L-arginine or 100 nmol/l GLP-1. No significant differences were observed in the insulin secretory response of these two types of islets. *C*: Expression of insulin mRNA in control and MetCKO mouse islets. Relative semiquantitative RT-PCR was performed with 1–2 μg DNA-free islet RNA from both types of mice. Mouse actin was used as an internal control for loading. *D*: Islet insulin content in aliquots of 50 islet equivalents (IE; 1 islet equivalent = 125 μm diameter) isolated from MetCKO ($n = 6$) and control ($n = 6$) mice. Insulin content was quantified as described under RESEARCH DESIGN AND METHODS. No significant differences were observed in islet insulin content in both types of mice. *$P < 0.05$, **$P < 0.02$.

ducts were observed in MetCKO compared with control mice (Fig. 4*A* and 7*B*). Quantitative histomorphometric analysis of these pancreatic sections indicated that the number of islets <500 μm^2 and between 500 and 7,500 μm^2 were significantly increased in MetCKO mice compared with control littermates (Fig. 7*C*). Interestingly, no significant changes were observed in the number of islets with areas $>7,500$ μm^2 (Fig. 7*C*).

β-Cell proliferation rates are not affected in MetCKO mice. It has previously been shown that overexpression of HGF in the pancreatic β-cell of RIP-HGF transgenic mice increases β-cell proliferation (20). We hypothesized that a potential decrease in β-cell proliferation could occur in MetCKO mice, and therefore we analyzed β-cell proliferation rates in pancreatic sections obtained from 8- to 12-week-old mice. As shown in Fig. 8*A*, β-cell proliferation rates were not significantly different in MetCKO mice compared with the rates observed in control littermates. Because β-cell proliferation rates are low in adult mice, detection of a further decrease in β-cell proliferation rates in MetCKO mice may be difficult. We therefore measured β-cell proliferation in 1-day-old mice, when proliferation rates are higher (27,28). As seen in Fig. 8*B*, β-cell proliferation in 1-day-old control mice is 10- to 12-fold higher than in normal adult mice (Fig. 8*A*). However, even at this age, β-cell proliferation in MetCKO mice, though slightly decreased, was not significantly different from their control littermates (Fig. 8*B*). Taken together, these results suggest that although HGF is able to stimulate β-cell proliferation in vitro and in vivo, the loss of HGF signaling is not essential for maintaining normal β-cell mass or proliferation rates.

DISCUSSION

Type 2 diabetes is characterized by two principal components: insulin resistance coupled with β-cell dysfunction. Several growth factors are involved in the physiological regulation of β-cell mass and function, and HGF appears to be potentially important among them (1,4,5,7,8,19–22). To gain insight into the role of HGF in normal β-cell growth and function, the current study analyzed the impact of conditional inactivation of its receptor, c-met, in the pancreatic β-cell. Disruption of HGF/c-met signaling in

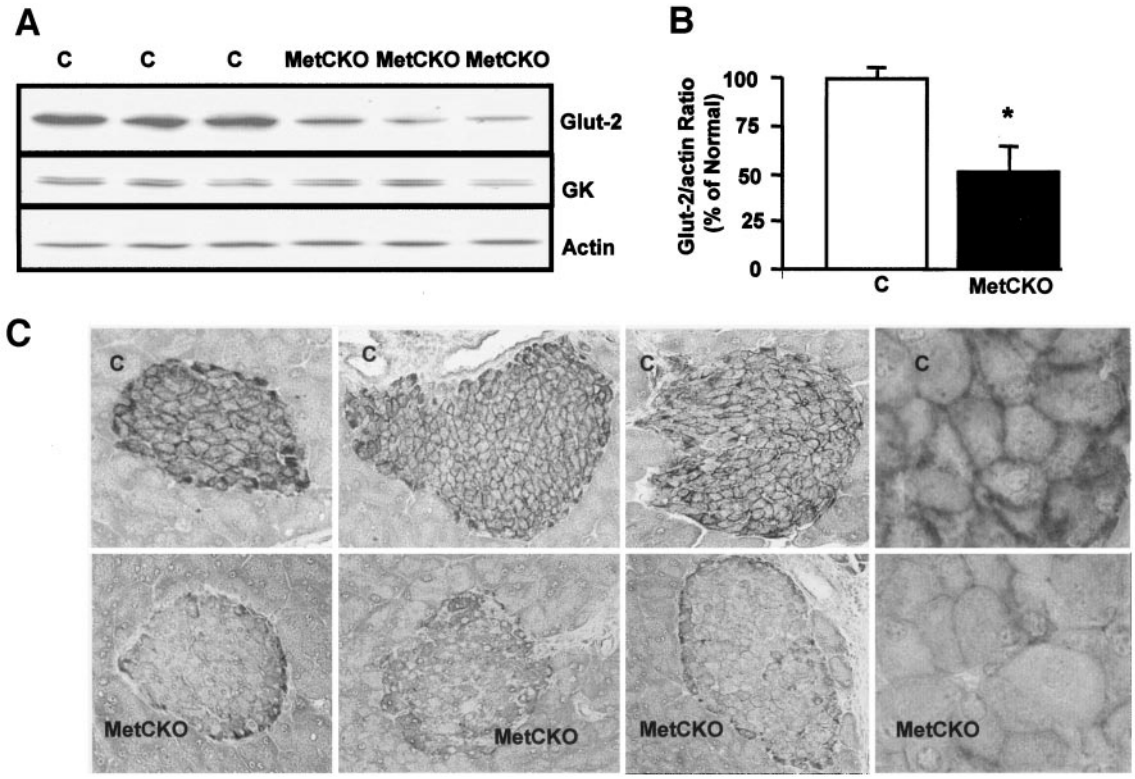

FIG. 6. Analysis of glucokinase and GLUT-2 expression in MetCKO and control (C) islets. *A*: Representative Western blot performed with islet extracts from three control and three MetCKO mice using GLUT-2 and glucokinase antibodies together with actin antibody as an internal control. Decreased band intensities were observed with GLUT-2 but not with glucokinase in MetCKO islet extracts compared with control islets. *B*: Densitometric scanning and quantitation of GLUT-2–to–actin ratios in MetCKO ($n = 5$) and control ($n = 5$) mouse samples. Results are the means ± SE. *C*: Representative microphotographs showing the immunohistochemical detection of GLUT-2 in pancreatic islets from control and MetCKO littermates at 200× (*left three panels*) and 1,000× (*right panel*) magnifications. Diffused and decreased intensity of staining for GLUT-2 in MetCKO mice contrasts with the stronger and membrane-localized staining observed in control islets under identical staining conditions. *$P < 0.05$.

the mouse β-cell decreased glucose tolerance and glucose-mediated insulin secretion in vivo, diminished GSIS in vitro, and downregulated GLUT-2 expression in β-cells. On the other hand, no changes in β-cell mass, islet morphology, or islet cell composition were observed in MetCKO mice in this study. Taken together, these findings indicate that HGF can be added to the short list of growth factors, including IGF-1, insulin, fibroblast growth factors, and TGF-β (4,5,7,8), that are required for complete physiological expression of the β-cell phenotype.

RIP-HGF transgenic mice overexpressing HGF in the pancreatic β-cell display mild insulin-mediated decreases in blood glucose levels, markedly improved glucose tolerance, and enhanced GSIS (20,21). In contrast, whereas MetCKO mice display normal blood glucose levels under fasting and nonfasting conditions, these mice display obviously impaired glucose tolerance and markedly decreased GSIS. These results indicate that in some respects, MetCKO mice and RIP-HGF mice are mirror images of one another.

How does inactivation of c-met in the β-cell impair GSIS? HGF has been shown to increase β-cell expression of insulin mRNA and protein in RIP-HGF mice (20,21). Therefore, one possibility is that HGF signaling deficiency in the β-cell reduces insulin gene transcription and insulin content, resulting in diminished insulin secretion. This is unlikely because, as observed in Figs. 4 and 5, pancreatic insulin content, islet insulin mRNA expression, and islet insulin content were normal in MetCKO mice. Thus, whereas excess HGF can have an impact on insulin expression, its absence is not essential for the normal regulation of its expression. This is perhaps not surprising because reduction in the expression of the key insulin transcription factor PDX-1 (duodenal homeobox factor 1) has no adverse effect on the level of insulin expression in PDX-1 heterozygote knockout mice or MIN-6 cells (29,30), suggesting the presence of redundant systems to maintain normal expression levels of such an essential hormone. HGF increases glucokinase expression and glucose metabolism in RIP-HGF mouse β-cells (21). Thus, an alternate potential explanation for the decreased GSIS in MetCKO mice is that disruption of HGF/c-met signaling reduces glucokinase expression in MetCKO mouse β-cells. However, this does not seem to be the case because glucokinase levels were normal in MetCKO mice.

HGF overexpression in pancreatic β-cells of RIP-HGF mice results in a striking increase in GLUT-2 mRNA expression, as well as increased glucose transport in the β-cell (21). Others have shown that HGF is also capable of inducing GLUT-2 expression in pancreatic acinar cells in vitro (31) and upregulating the Na^+/glucose cotransporter SGLT1 and the facilitative glucose transporter GLUT-5 in the small intestine in vivo (32). Interestingly, a 50% decrease in the expression level of GLUT-2 was observed in MetCKO islets by Western blot analysis. Moreover, less intense GLUT-2 staining was observed in MetCKO islets

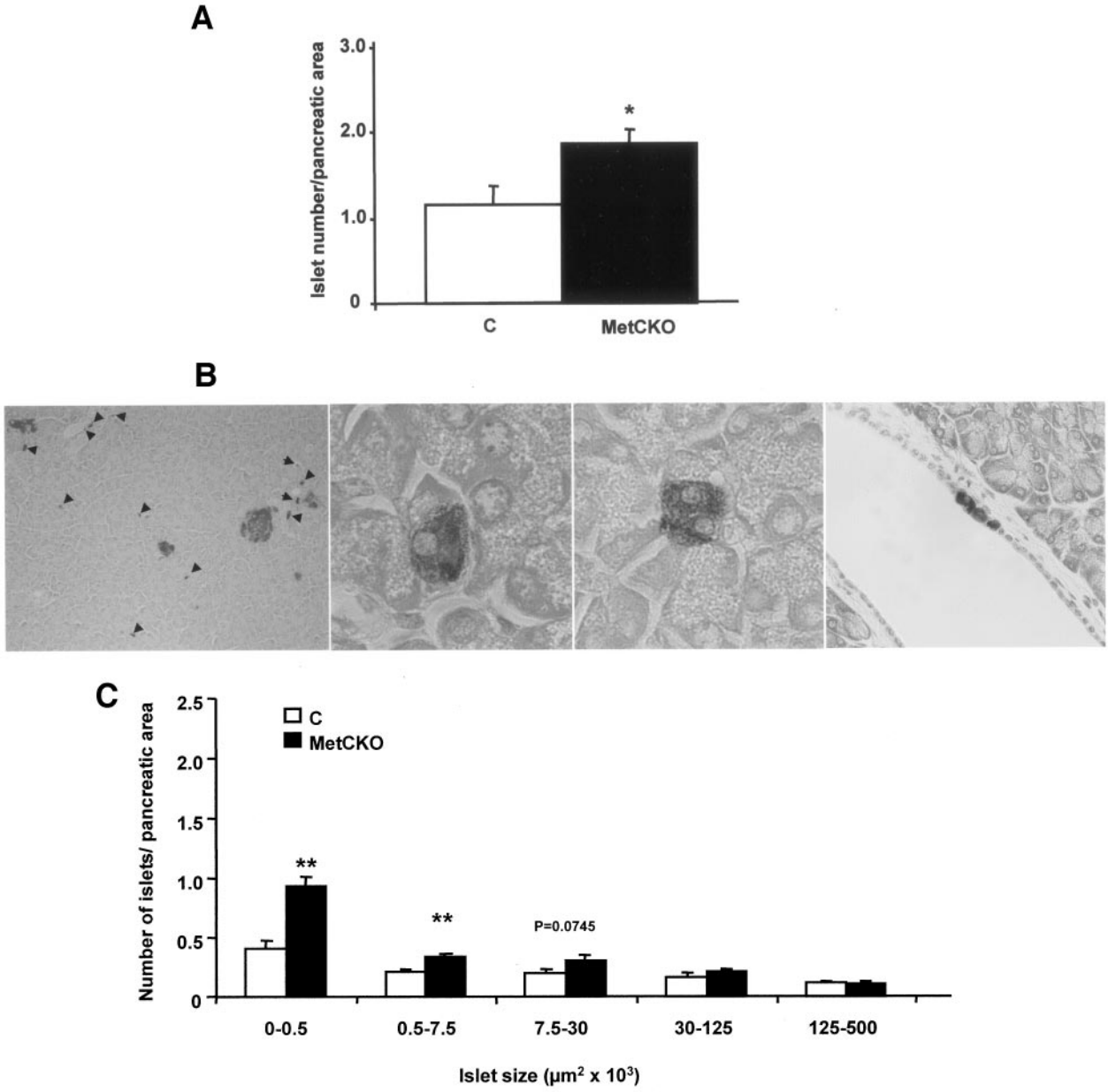

FIG. 7. Histomorphometric analysis of islet numbers in MetCKO mice ($n = 6$) and control (C) ($n = 5$) littermates. *A*: Number of islets per square millimeter of total pancreatic area. Results are the means ± SE. The analysis of these results reveals that the number of islets is significantly increased in MetCKO mouse pancreas. *B*: Representative microphotographs of a pancreatic section from a MetCKO mouse stained for insulin and showing the presence of multiple single and doublet β-cells (*left panel*, arrows). Representative microphotographs of two of these doublets (*two middle panels*) and several insulin-positive cells in a pancreatic duct (*right panel* and arrows in Fig. 4*A*) in an insulin-stained pancreatic section from a MetCKO mouse. *C*: Histomorphometric analysis of the size and number of islets in these pancreatic samples from MetCKO and control mice. Notice the significant increase observed in the number of small islets (<7.5 μm² ×10³) in MetCKO pancreatic sections. *$P < 0.02$, **$P < 0.01$.

compared with the strong and primarily membrane-localized staining observed in control mouse islets. Similar alterations in the pattern of GLUT-2 expression and distribution have been reported in several animal models of hyperglycemia and diabetes (33–35). Furthermore, GLUT-2 null mice develop early diabetes and defective GSIS (36). However, partial re-expression (20–50%) of GLUT-2 in islets of GLUT-2 null mice restores normal GSIS (37,38). In addition, human islets contain 100-fold lower GLUT-2 and exhibit 10-fold lower glucose transport than rat islets (39). Nevertheless, glucose transport capacity has been demonstrated to exceed glucose phosphorylation in both rodent and human pancreatic β-cells (39,40). Based on this evidence, it seems unlikely that a 50% decrease in GLUT-2 alone could explain the diminished GSIS observed in MetCKO islets, suggesting that other events required for GSIS might be altered in these mice. Collectively, these results suggest that three key β-cell genes implicated in insulin secretion—insulin, glucokinase, and GLUT-2—display differential sensitivity to the level of HGF action, with GLUT-2 gene expression being altered by both HGF reduction and overexpression, and expression of insulin and glucokinase being influenced only by HGF overexpression.

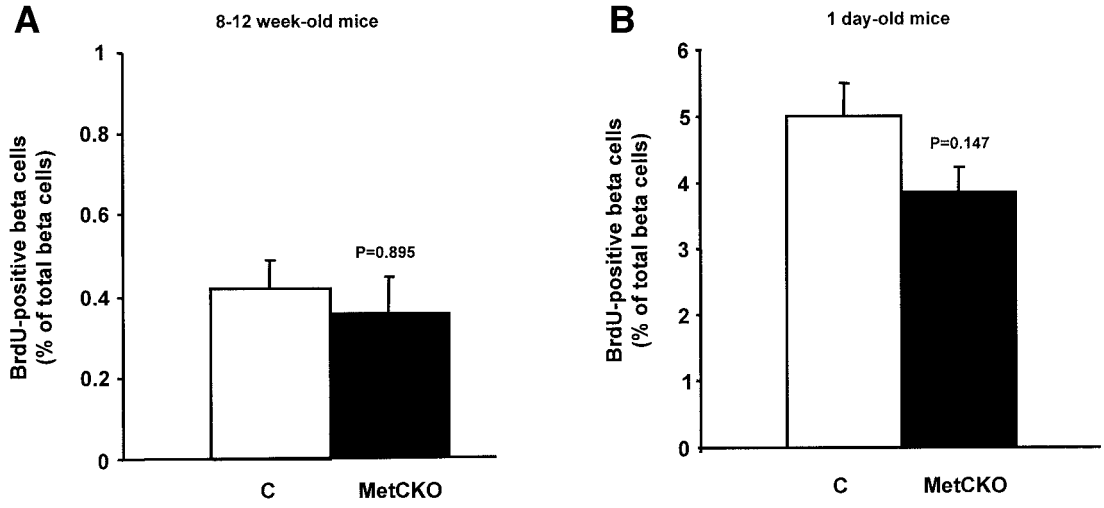

FIG. 8. β-Cell proliferation rates in 8- to 12-week-old MetCKO mice (n = 6) and control (C) (n = 4) littermates (A) and in 1-day-old MetCKO (n = 3) and control (n = 3) mice (B). Control and MetCKO pancreatic sections were stained for BrdU and insulin. After counterstaining, 500–1,000 β-cell nuclei were counted per pancreas. The data are presented as percentage of replicating β-cells. P values are indicated in the figure.

HGF has been repeatedly demonstrated to be a potent mitogen for the β-cell in vitro (19,41,42). Furthermore, we have recently demonstrated that overexpression of HGF in the β-cell of RIP-HGF mice induces an impressive augmentation in β-cell mass and an increase in β-cell proliferation rates in vivo (20). Based on these results, and because HGF is expressed in the pancreas (Fig. 1) (18) and c-met is localized in the β-cell (Fig. 1) (19), we hypothesized that HGF might play a paracrine and/or autocrine physiological role in the growth and development of the pancreatic islet. To our surprise, the studies presented herein demonstrate that inactivation of c-met in β-cells does not alter β-cell mass or proliferation under basal conditions, indicating that HGF is not crucial for β-cell growth in the mouse. In addition, inactivation of c-met does not alter the morphology or cell composition of the islet, as previously observed in RIP-HGF mice (20), suggesting that HGF does not affect the normal differentiation of pancreatic islet cells in mice. Using the same Cre-LoxP system, deletion of insulin and IGF-1 receptors in the β-cell have been performed (7,8). These studies have revealed that insulin and IGF-1 are important for differentiated β-cell function but do not seem to play a major role in early growth of the pancreatic β-cells, as we have observed in MetCKO mice. Collectively, these studies suggest a number of scenarios regarding β-cell growth. First, it could be that HGF is not important for normal β-cell growth, whereas other growth factors (e.g., fibroblast growth factors and TGF-β) are essential for this task (4,5). Second, it is possible that redundancy exists among the growth factors required for maintaining normal β-cell growth. Third, because the Cre-LoxP system using RIP-Cre mice would disable ligand/receptor interactions only after the cell becomes differentiated and insulin-producing, this would preclude elucidating any role HGF may have in the differentiation/development of the β-cell during embryogenesis (13). Finally, these studies were performed under basal physiological conditions and do not address the impact that deletion of HGF signaling in the β-cell might have in response to diabetes, insulin resistance, obesity, or partial pancreatectomy.

Interestingly, we found a striking increase in the number of single and doublet β-cells throughout the exocrine pancreas and the presence of multiple insulin-positive cells in the ducts of MetCKO mice that do not translate into significant increases in β-cell mass. These characteristics are consistent with an increase in the rate of β-cell neogenesis, a process difficult to define and more difficult to quantitate (43,44). However, islet neogenesis has been potentially associated with an increased number of β-cells budding from the ducts or scattered as single and doublet cells throughout the exocrine pancreas (28,44). Several scenarios may explain these findings. First, because HGF is a mitogen for β-cells (20,41,42), it may be that the absence of HGF action in β-cells in MetCKO mice could impede the proliferation of newly formed β-cells. Against this interpretation is the observation that β-cell proliferation rates were normal in the islet of METCKO mice (Fig. 8). Second, because HGF is an antiapoptotic factor for the β-cell (22), it might be possible that the life span of β-cells is shortened in MetCKO mice. However, β-cell death assessed by insulin and TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) staining in 1-day- and 3-month-old mice under basal conditions is not different in MetCKO and control littermates (data not shown). Third, an alternative explanation is that HGF acts as a physiological suppressor of β-cell differentiation, and its absence permits increased generation of new β-cells. This seems unlikely because: *1*) HGF action would be deleted only in differentiated β-cells because we used the RIP promoter for these studies, and it should not be deleted from β-cell precursors before they express insulin (45); *2*) HGF overexpression in transgenic mice results in a small but significant increase in islet number (20); and *3*) HGF has been shown to induce in vitro the differentiation of pancreatic acinar and islet-derived epithelial cells into insulin-expressing cells (32,46). Finally, it is possible that the decreased β-cell function and diminished glucose tolerance in MetCKO mice induce compensatory β-cell neogenesis. Along these lines, similarly increased β-cell neogenesis has been observed in pancreatic samples obtained postmortem from type 2 diabetic

patients compared with those from nondiabetic subjects (47,48). Unfortunately, it is impossible to know whether this increased β-cell neogenesis is a compensatory response that occurs in the pre-diabetic or in the diabetic state of these patients.

In summary, removal of HGF/c-met signaling from the β-cell in conditional knockout mice results in obvious β-cell dysfunction but no evidence of disordered β-cell proliferation. MetCKO mice display an acute decrease in insulin secretion and diminished glucose tolerance with decreased GLUT-2 expression in β-cells. These results provide evidence for an essential role for HGF/c-met in the maintenance of normal β-cell function. The MetCKO phenotype is similar to early phases of β-cell failure in type 2 diabetes. Genetic alterations in HGF, c-met, and its downstream signaling pathways combined with environmental factors may be implicated in the predisposition to develop type 2 diabetes. Conversely, the HGF–c-met system may represent an attractive pharmacological target for enhancing β-cell function in type 2 diabetes.

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