Angiotensin I–Converting Enzyme Type 2 (*ACE2*) Gene Therapy Improves Glycemic Control in Diabetic Mice

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OBJECTIVE—Several clinical studies have shown the benefits of renin-angiotensin system (RAS) blockade in the development of diabetes, and a local RAS has been identified in pancreatic islets. Angiotensin I–converting enzyme (ACE)2, a new component of the RAS, has been identified in the pancreas, but its role in β-cell function remains unknown. Using 8- and 16-week-old obese *db/db* mice, we examined the ability of ACE2 to alter pancreatic β-cell function and thereby modulate hyperglycemia.

RESEARCH DESIGN AND METHODS—Both *db/db* and nondiabetic lean control (*db/m*) mice were infected with an adenovirus expressing human ACE2 (Ad-hACE2-eGFP) or the control virus (Ad-eGFP) via injection into the pancreas. Glycemia and β-cell function were assessed 1 week later at the peak of viral expression.

RESULTS—In 8-week-old *db/db* mice, Ad-hACE2-eGFP significantly improved fasting glycemia, enhanced intraperitoneal glucose tolerance, increased islet insulin content and β-cell proliferation, and reduced β-cell apoptosis compared with Ad-eGFP. ACE2 overexpression had no effect on insulin sensitivity in comparison with Ad-eGFP treatment in diabetic mice. Angiotensin-(1–7) receptor blockade by D-Ala^7–Ang-(1-7) prevented the ACE2-mediated improvements in intraperitoneal glucose tolerance, glycemia, and islet function and also impaired insulin sensitivity in both Ad-hACE2-eGFP– and Ad-eGFP–treated *db/db* mice. D-Ala^7–Ang-(1-7) had no effect on *db/m* mice. In 16-week-old diabetic mice, Ad-hACE2-eGFP treatment improved fasting blood glucose but had no effect on any of the other parameters.

CONCLUSIONS—These findings identify ACE2 as a novel target for the prevention of β-cell dysfunction and apoptosis occurring in type 2 diabetes. ***Diabetes*** **59:2540–2548, 2010**

In addition to the systemic renin-angiotensin system (RAS) that regulates blood pressure, the concept of a tissue RAS, modulating local organ function, is now well recognized. Accordingly, most organs express a tissue RAS, capable of locally producing angiotensin (Ang)-II (1). A complete tissue RAS has been identified in the endocrine and exocrine pancreas, and the expression of its various components has been demonstrated in the islets of Langerhans (2–4). While the role of the islet RAS is not completely understood, recent data suggest that it may be important in β-cell homeostasis and function. Indeed, it has been shown to be involved in the regulation of glucose-stimulated insulin secretion, insulin synthesis (3), and islet blood flow (5). Hyperactivity of the ACE/Ang-II/AT1 receptor (AT1R) axis of the RAS leads to a cascade of events implicated in the development of β-cell dysfunction, including the following: increased islet fibrosis (6), oxidative stress (7,8), and inhibition of proinsulin biosynthesis and first-phase and glucose-responsive insulin secretion (3,5,9). Moreover, several studies have demonstrated the effectiveness of RAS blockade at improving islet morphology and function and reducing islet oxidative stress (3,5,8,10) (rev. in 11). Recently, angiotensin 1–converting enzyme (ACE)2, a captopril-insensitive ACE homologue, was identified (12,13) and shown to cleave Ang-II into the biologically active peptide Ang-(1-7) (13). Ang-(1-7) properties are mediated by the G-protein–coupled receptor Mas (14), causing vasodilation, inhibition of fibrosis (15), stimulation of prostaglandin E (PGE)2 (16), and nitric oxide releases (17). The ACE2/Ang-(1-7)/Mas axis is hypothesized to act as a negative regulator for the RAS. Recent data indicate that this alternate pathway may play a compensatory role in the development of type 2 diabetes. ACE2 protein is elevated in the islets of Zucker fatty diabetic rats (10), and ACE2 knockout (ACE2$^{-/y}$) mice exhibit progressive impairments in glucose tolerance and reduced first-phase insulin secretion (18).

Loss of first-phase insulin secretion, an indicator of pancreatic β-cell dysfunction, is considered one of the earliest insults in type 2 diabetes and is evident before the onset of impaired glucose tolerance (19). Defects in insulin sensitivity, glucose tolerance, and glucose uptake exhibited by Mas receptor knockout mice (20) further implicate the loss of Ang-(1-7) signaling in the development of type 2 diabetes and metabolic syndrome. We hypothesized that ACE2 overexpression may ameliorate glucose homeostasis in diabetic mice and prevent the development of pancreatic β-cell dysfunction. Using leptin receptor–deficient obese *db/db* mice, we report that ACE2 overexpression reduced glycemia and increased islet insulin content through Ang-(1-7)–mediated pathways. Our data confirm the pivotal role of this peptide in the pancreas and establish ACE2 as a new target for the treatment of type 2 diabetes.

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RESEARCH DESIGN AND METHODS

Male *db/db* and nondiabetic (*db/m*) mice aged 3, 7, and 15 weeks (BKS.Cg-*m* +/+ *Lepr^{db}*/J; The Jackson Laboratories, Bar Harbor, ME) were infected with an adenovirus coding for human ACE2 (hACE2) upstream of an enhanced green fluorescent protein (eGFP) reporter gene (Ad-hACE2-eGFP) or with the eGFP virus alone (Ad-eGFP) (21) by direct injection (5×10^7 particle forming units [pfu] in a total volume of 100 μl of 0.9% wt/vol saline) into the pancreas ($n = 8$/group). The adenovirus was delivered in five 20-μl injections along the body of the pancreas (Fig. 2*A*). For a subset of animals, D-Ala^7–Ang-(1-7), an Ang-(1-7) receptor antagonist, was infused (600 ng · kg^{-1} · min^{-1} · 7 days^{-1}) using mini-osmotic pumps (Durect, Cuppertino, CA) implanted subcutaneously at the time of virus injection. All procedures were approved by the

TABLE 1
Baseline metabolic parameters in *db/db* and *db/m* mice

	8 weeks old		16 weeks old	
	db/m	*db/db*	*db/m*	*db/db*
Body weight (g)	22.53 ± 0.26	33.65 ± 0.45*	26.08 ± 0.54†	44.06 ± 2.66*‡
Fasting blood glucose (mg/dl)	102.2 ± 5.3	201.0 ± 11.9*	119.8 ± 12.8	398.2 ± 42.8*‡
IPGT AUC (mg · dl^{-1} · min^{-1})	19.32 ± 2.2	48.41 ± 1.1*	20.42 ± 0.8	76.40 ± 4.6*‡

Data represent baseline parameters of *db/m* and *db/db* mice (n = 6 per group) prior to infection with Ad-hACE2-eGFP or Ad-eGFP viruses. Statistical significance: *$P < 0.001$ vs. age-matched *db/m*, †$P < 0.05$ vs. 8-week-old *db/m*, ‡$P < 0.001$ vs. 8-week-old *db/db*. IPGT AUC, intraperitoneal glucose tolerance area under the curve.

Animal Use and Care Committee at Louisiana State University Health Sciences Center, New Orleans, Louisiana.

Determination of ACE2 expression and activity. To prevent protein degradation, pancreata were first incubated in RNAlater stabilization solution and stored at −80°C. Western blotting for ACE2 expression and ACE2 activity assays were performed as previously described (21).

Measurements of physiological parameters. To assess glucose metabolism in *db/db* mice, we performed an intraperitoneal glucose tolerance test where fasted (12 h) animals were weighed and a bolus intraperitoneal injection of glucose (2 g/kg) was administered to conscious mice. Blood was drawn from the catheterized tail vein and analyzed at 0, 15, 30, 60, and 120 min after glucose administration using a glucometer (Accu-check Aviva; Roche, Mannheim, Germany).

For determinination of first-phase insulin secretion, fasted mice were anesthetized with isoflurane and given a bolus of glucose (1g/kg IP). Blood samples (50 μl) were collected from a catheterized carotid artery at 0, 2, 5, and 10 min following glucose administration. Plasma insulin was then measured using ELISA (Crystal Chem, Downers Grove, IL).

Insulin sensitivity was analyzed following a 1-h fast, and mice were injected subcutaneously with human recombinant insulin (0.3 units/kg; Sigma, St Louis, MO). Blood glucose was measured at 0, 15, 30, 60, and 120 min following injection.

Fasting blood glucose, glucose tolerance and insulin tolerance were measured prior to and 7 days after adenovirus administration. The animals were then killed and the pancreas was removed and rapidly divided, with one-half fixed in 10% formalin in PBS and the other half frozen in liquid nitrogen.

Immunohistochemistry. Pancreas sections (5 μm) were prepared from 10% formalin-fixed, paraffin-embedded tissue. For antigen unmasking, sections were incubated in a citrate buffer solution (100 mmol/l citric acid and 100 mmol/l sodium citrate; Sigma) for 13 min at 100°C. Following washes, sections were incubated for 1 h at room temperature in a blocking solution containing 5% BSA in PBS Tween. Sections were incubated with an anti-insulin primary antibody (1:100; Abcam, Cambridge, MA) for 4 h at 4°C, followed by incubation with biotinylated anti–guinea pig secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were then treated with avidin-biotin complex reagent and developed using alkaline phosphatase red according to the manufacturer's protocol (Vector Laboratories). Pancreatic islet insulin content was calculated as the total insulin staining per islet area. A total of 20 islets per group (n = 6 mice/group) were analyzed.

Additionally, to determine changes in islet proliferation and apoptosis, we assessed proliferating cellular nuclear antigen (PCNA) expression and performed terminal deoxynucleotidyl transferase dUTP nick ended labeling

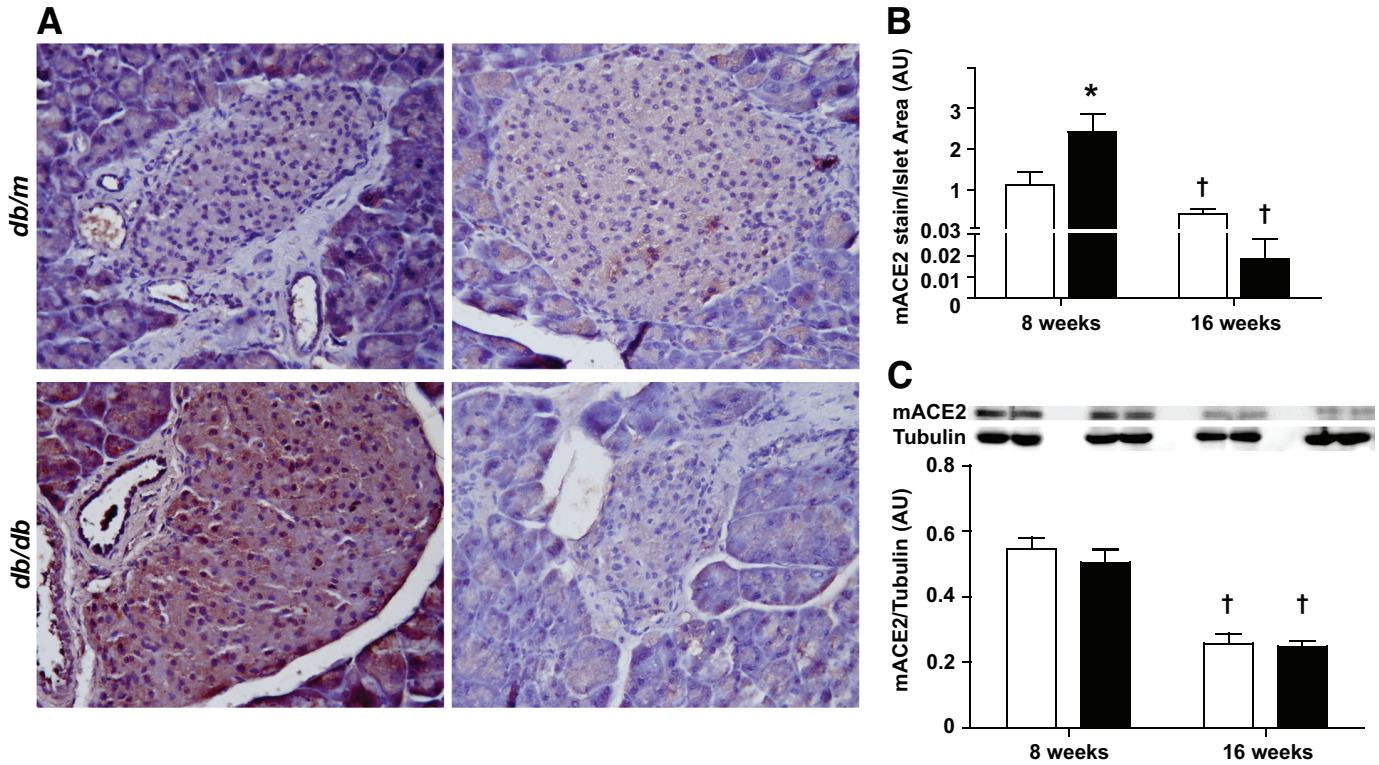

FIG. 1. Mouse ACE2 expression is elevated in the islets of 8-week-old and reduced in 16-week-old *db/db* mice. Immunostaining for mACE2 (*A*) and quantification (*B*) revealed increased mACE2 expression (brown) in the islets of 8-week-old *db/db* mice in comparison with *db/m*. Expression of mACE2 was significantly reduced in the islets of 16-week-old (*A* [*right panel*]) vs. 8-week-old (*A* [*left panel*]) *db/db* mice. Representative Western blot and densitometry (*C*) demonstrating no significant change in pancreatic mACE2 expression in 8-week-old *db/m* □ vs. *db/db* ■ mice and 16-week-old *db/m* vs. *db/db* mice. However, reduced mACE2 expression in the pancreas was observed in 16-week-old mice in comparison with 8-week-old mice. Values are expressed as means ± SEM. Two-way ANOVA statistical significance: †$P < 0.05$ vs. respective 8-week-old mice and *$P < 0.05$ vs. respective *db/m* mice. (A high-quality digital representation of this figure is available in the online issue.)

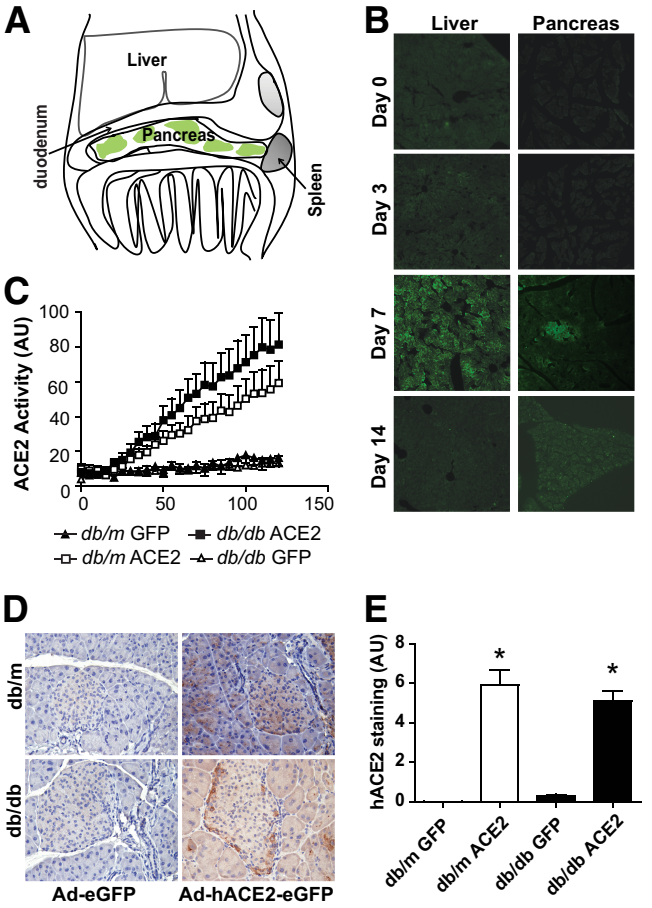

FIG. 2. Adenovirus-mediated expression of hACE2. *A*: Schematic of infection showing Ad-hACE2-eGFP delivery (100 μl) by five microinjections along the pancreas. *B*: Representative immunofluorescence ($n = 3$) showing Ad-hACE2-eGFP transgene expression in the pancreas and liver of *db/m* mice at 0, 3, 7, and 14 days following adenovirus delivery. Expression of hACE2 peaked at 7 days, as also evidenced by activity (*C*) and ACE2 (*D* and *E*) immunostaining in the pancreas of both *db/m* and *db/db* mice infected with Ad-hACE2-eGFP. Immunostaining for hACE2 revealed high expression in both endocrine and exocrine tissue (*D*). *E*: hACE2 immunostaining in pancreatic islets was quantified. Values are expressed as means ± SEM. Two-way ANOVA statistical significance: *$P < 0.05$ vs. Ad-eGFP–treated mice. (A high-quality digital representation of this figure is available in the online issue.)

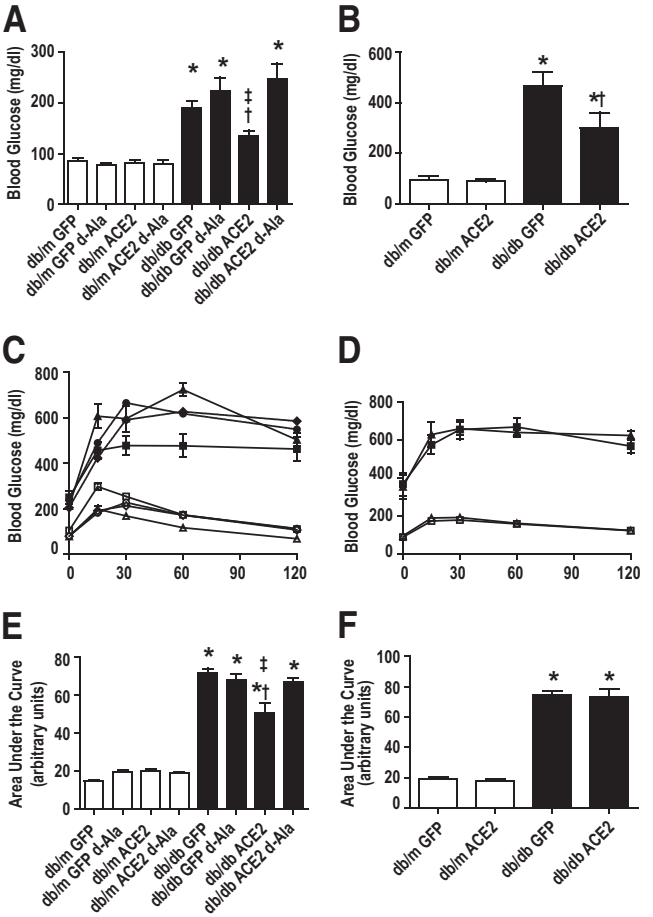

FIG. 3. ACE2 overexpression ameliorates glycemic homeostasis. *A*: Ad-hACE2-eGFP had no effect on the fasting blood glucose of 8-week-old *db/m* mice (open bars; $n = 12$). Similarly, the Mas antagonist D-Ala^7–Ang-(1-7) (600 ng · kg^{-1} · min^{-1} subcutaneously) had no effect on the fasting blood glucose of *db/m* mice ($n = 6$). Diabetic mice (solid bars) treated with Ad-eGFP had elevated fasting blood glucose levels in comparison with *db/m* mice. Mas blockade did not produce significant changes in the fasting blood glucose of Ad-eGFP–treated *db/db* mice. Ad-hACE2 treatment, however, significantly reduced fasting blood glucose levels in *db/db* mice ($n = 12$) to levels not significantly different from *db/m* mice. Mas blockade with D-Ala^7–Ang-(1-7) prevented ACE2-mediated improvements in fasting blood glucose of *db/db* mice. *B*: Ad-hACE2-eGFP had no effect on the fasting blood glucose of 16-week-old *db/m* mice in comparison with Ad-eGFP ($n = 6$). Ad-hACE2-eGFP infection reduced fasting blood glucose in *db/db* mice vs. that in Ad-eGFP–treated mice ($n = 6$). *C* and *E*: Glucose tolerance was determined as the area under the curve (mg · dl^{-1} · min^{-1}) of blood glucose levels following a bolus of glucose (2g/kg) over a 2-h period. Ad-hACE2-eGFP delivery had no effect on the glucose tolerance of *db/m* mice (open squares) in comparison with Ad-eGFP–treated *db/m* mice (open triangles). D-Ala^7–Ang-(1-7) administration had no effect on Ad-eGFP (open diamonds) or Ad-hACE2-eGFP–treated (open circles) *db/m* mice. Ad-eGFP–treated *db/db* (solid triangles) mice had significantly impaired glucose tolerance that was not affected by Mas blockade (solid diamonds). Ad-hACE2-eGFP delivery improved glucose tolerance in diabetic mice (solid squares) in comparison with Ad-eGFP–treated *db/db* mice. D-Ala^7–Ang-(1-7) administration attenuated ACE2-mediated improvements in glucose tolerance in *db/db* mice (solid circles). *D* and *F*: Ad-ACE2-eGFP delivery had no effect on the glucose tolerance of 16-week-old *db/m* mice (open squares) vs. Ad-eGFP–treated *db/m* (open triangles) mice. Ad-hACE2-eGFP delivery failed to improve glucose tolerance in 16-week-old *db/db* mice (solid squares) vs. Ad-eGFP—treated *db/db* mice (solid triangles). Values are expressed as means ± SEM. Two-way ANOVA statistical significance: *$P < 0.05$ vs. respective *db/m*; †$P < 0.05$ vs. *db/db* GFP; ‡$P < 0.05$ vs. *db/db* ACE2 d-Ala.

(TUNEL) staining. These antibodies were incubated simultaneously with anti-insulin primary antibodies for 36 h at 4°C. Biotinylated anti-goat and anti-rabbit secondary antibodies (1:200; Vector Laboratories) were incubated at room temperature for 1 h. The sections were then treated with avidin-biotin complex reagent and developed using 3,3′-diaminobenzidine (Vector labs). TUNEL staining was performed according to the manufacturer's instructions (Roche, Indianapolis, IN), and the staining was developed using 3,3′-diamiobenzidine. Image capture was performed using a Nikon eclipse E600 light microscope. All images were quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). For a semiquantitative assessment of islet insulin, mouse ACE2 (mACE2), and hACE2, staining per islet (n = 16–20/group) area was used to quantify protein content from immunohistochemistry (10). For PCNA and TUNEL staining, positive cells per islet were counted. To assess pancreatic β-cell mass, the mean density of islet insulin staining was multiplied by the mean islet area per pancreatic section, adjusted for wet organ weight per animal (six sections per organ) (10).

Statistical analysis. Data are expressed as means ± SEM. Data were analyzed by Student's t test or two-way ANOVA (Bonferroni post hoc tests to compare replicate means) when appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Animal model. To address the involvement of ACE2 in β-cell function and the development of type 2 diabetes, we

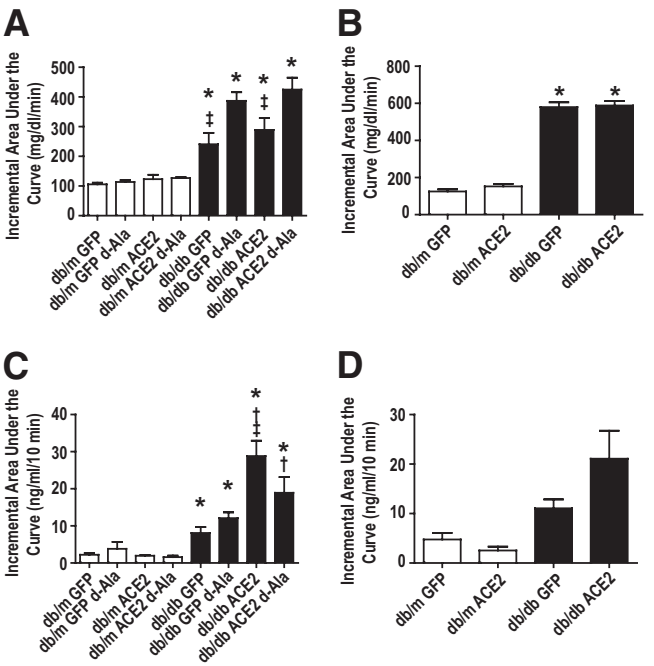

FIG. 4. ACE2 overexpression improves insulin secretion but not insulin sensitivity in diabetic mice. Insulin tolerance was expressed by the area under the curve ($mg \cdot dl^{-1} \cdot min^{-1}$), measuring blood glucose over a 2-h period after administration of an insulin bolus (0.3 IU/kg subcutaneously). *A*: 8-week-old diabetic mice (solid bars) had significantly impaired insulin sensitivity in comparison with *db/m* mice (open bars). Ad-hACE2-eGFP delivery did not improve insulin sensitivity. D-Ala^7–Ang-(1-7) (600 $ng \cdot kg^{-1} \cdot min^{-1}$ subcutaneously) significantly worsened insulin tolerance in both Ad-eGFP– and Ad-hACE2-eGFP–treated *db/db* mice. *B*: 16-week-old diabetic mice (solid bars) had significantly impaired glucose tolerance in comparison with *db/m* mice (open bars). Ad-hACE2-eGFP delivery had no effect on insulin sensitivity in *db/m* or *db/db* mice vs. respective Ad-eGFP–treated mice. First-phase insulin secretion was measured as area under the curve ($ng \cdot ml^{-1} \cdot min^{-1}$) of insulin concentration in response to a glucose bolus (1g/kg) over a 10-min period. *C*: Ad-hACE2-eGFP delivery had no effect on first-phase insulin secretion of 8-week-old *db/m* mice in comparison with Ad-GFP–treated *db/m* mice. ACE2 overexpression significantly enhanced insulin secretion in diabetic mice in comparison with Ad-eGFP–treated *db/db* mice. Administration of D-Ala^7–Ang-(1-7) attenuated the ACE2-mediated improvements on first-phase insulin secretion. *D:* ACE2 overexpression did not significantly enhance insulin secretion in 16-week-old *db/m* mice (open bars) vs. Ad-eGFP treatment. While Ad-ACE2-eGFP treatment did not significantly increase first-phase insulin secretion in *db/db* mice (solid bars) vs. Ad-eGFP–treated mice, there was a trend toward enhanced first-phase insulin secretion. Values are expressed as means ± SEM. Two-way ANOVA statistical significance: *$P < 0.05$ vs. respective *db/m*, †$P < 0.05$ vs. *db/db* GFP, and ‡$P < 0.05$ vs. respective *db/db* mice treated with D-Ala^7–Ang-(1-7).

used leptin receptor–deficient (*db/db*) and control (*db/m*) mice. As shown in Table 1, *db/db* mice weight and fasted glycemia were significantly increased, at both 8 and 16 weeks of age, compared with those of lean *db/m* mice, while the ability of *db/db* mice to metabolize glucose was dramatically reduced, confirming both obese and diabetic phenotypes in these animals. In addition, there were no significant differences in pancreatic mACE2 expression among 8- or 16-week-old *db/db* and *db/m* mice. Sixteen-week-old *db/m* and *db/db* mice, however, had lower pancreatic mACE2 expression in comparison with respective 8-week old mice (Fig. 1*C*). Immunohistochemistry (Fig. 1*A* and *B*) revealed increased mACE2 expression in the islets of Langerhans in 8-week-old *db/db* compared with *db/m* mice. In 16-week-old mice, however, mACE2 expression was decreased in the islets of *db/db* compared with those of 8-week-old *db/db* mice (Fig. 1*A* and *B*). Although not significant, there was a trend toward decreased islet mACE2 expression in 16-week-old *db/db* mice in comparison with *db/m*.

ACE2 viral expression. Following injection of the adenovirus (Fig. 2*A*), hACE2 expression and activity were assessed at various time points. Immunofluorescence reveals that hACE2 expression in the pancreas peaks between 7 and 14 days after infection (Fig. 2*B*), as previously observed in the brain (21). A stronger signal was also observed in the liver, suggesting that a significant amount of virus is carried out of the pancreas. However, hACE2 expression in the liver disappeared more rapidly, probably resulting from increased protein turnover in this tissue. ACE2 activity was significantly elevated (Fig. 2*C*) in the pancreas of both *db/db* (slope 69.7 ± 5.9 vs. 8.6 ± 1.8; $P < 0.05$) and *db/m* (45.9 ± 4.3 vs. 5.7 ± 1.4; $P < 0.05$) mice infected with Ad-hACE2-eGFP compared with Ad-eGFP–infected mice. Morphological analysis of hACE2 immunoreactivity showed that the enzyme was expressed in both exocrine and endocrine pancreas of the *db/db* and control mice (Fig. 2*D* and *E*) without significant difference between genotypes. Ad-hACE2-eGFP and Ad-eGFP treatments, as well as D-Ala^7–Ang-(1-7) infusion, did not significantly change body weight in either *db/db* or *db/m* mice at either 8 or 16 weeks of age. Adenoviral delivery did not cause significant pancreatic inflammation or $CD3^+$ lymphocyte infiltration (supplemental Fig. 1, available in an online appendix [http://diabetes.diabetesjournals.org/cgi/content/full/db09-1297/DC1]).

Glycemic control. Ad-eGFP treatment had no effect on *db/db* or *db/m* fasting blood glucose levels (Fig. 3 *A* and *B*) or glucose tolerance (Fig. 3*C* and *D*) at 4 (supplemental Table 1), 8 (Fig. 3*A* and *C*), or 16 weeks (Fig. 3*B* and *D*). On the other hand, ACE2 overexpression significantly decreased fasting blood glucose in diabetic mice at both 8 ($P < 0.05$) (Fig. 3*A*) and 16 weeks of age ($P < 0.05$) (Fig. 3*B*). Ad-hACE2-eGFP improved intraperitoneal glucose tolerance in 8-week-old ($P < 0.05$) (Fig. 3*C* and *E)* but not 16-week-old (Fig. 3*D* and *F*) mice. These beneficial effects of ACE2 were significantly prevented following blockade of the Ang-(1-7) receptor in 8-week-old mice (Fig. 3*A* and *C*). At both 8 and 16 weeks, Ad-hACE2-eGFP had no effect on insulin sensitivity in comparison with Ad-eGFP–treated mice (Fig. 4*A* and *B*). Ad-hACE2-eGFP significantly increased first-phase insulin secretion in 8-week-old mice (Fig. 4*C*), and this effect was blocked by the Ang-(1-7) receptor antagonist. ACE2 overexpression also tended to increase insulin secretion, albeit statistically non-significant, in 16-week-old *db/db* mice (Fig 4*D*). While glucose tolerance was impaired in both Ad-eGFP– and Ad-hACE2–treated 4-week-old mice, Ad-hACE2-eGFP expression had no effect on glucose tolerance in 4-week-old *db/m* or *db/db* mice. There was not a significant difference in insulin sensitivity of 4-week-old *db/m* and *db/db* mice treated with Ad-eGFP or Ad-hACE2-eGFP. Fasting blood glucose was significantly elevated in Ad-hACE2-eGFP–treated *db/db* mice in comparison with Ad-GFP–treated control (supplemental Table 1).

Islet insulin content and β-cell mass. At 8 and 16 weeks of age, there were no differences in insulin immunoreactivity between *db/m* mice treated with the Ad-hACE2-eGFP or Ad-eGFP. However, Ad-eGFP–treated *db/db* mice exhibited reduced insulin staining (Fig. 5*A* and *C*). In both 8- (Fig. 5*A* and *C*) and 16-week-old (supplemental Fig. 2*A* and *C*) mice, Ad-hACE2-eGFP treatment significantly increased the islet insulin content in *db/db* mice in comparison with that in Ad-eGFP–treated *db/db* mice. In 8-week-

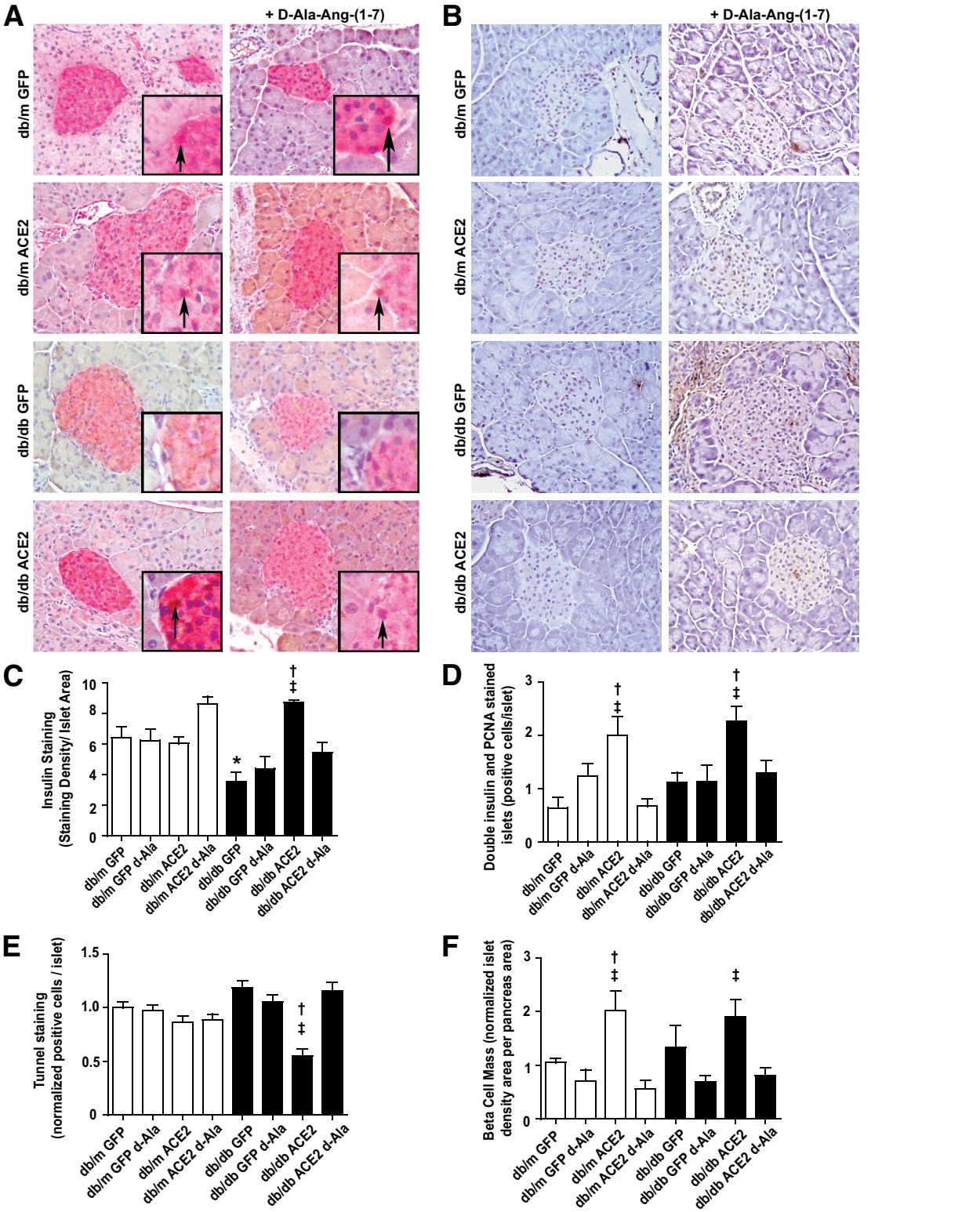

FIG. 5. ACE2 overexpression enhances islet insulin content and pancreatic β-cell proliferation and reduces apoptosis. *A:* Representative immunostaining for PCNA (3,3′-diaminobenzidine) and insulin (alkaline phosphatase red) (n = 15). *B*: Representative TUNEL staining (n = 18). *C*: Ad-hACE2-eGFP had no effect on islet insulin content (insulin staining/islet area) in 8-week-old *db/m* mice (open bars) vs. Ad-eGFP treatment. D-Ala^7–Ang-(1-7) (600 ng · kg^{-1} · min^{-1} subcutaneously) administration had no effect on islet insulin content in *db/m* mice. Ad-eGFP–treated *db/db* mice (solid bars) had significantly reduced islets insulin content vs. *db/m* mice, which was unaffected by Mas blockade. Ad-hACE2-eGFP increased islet insulin content in *db/db* mice vs. Ad-eGFP–treated mice. Islet insulin content in Ad-hACE2-eGFP–treated *db/db* mice was not significantly different from that in *db/m* mice. D-Ala^7–Ang-(1-7) administration significantly reduced ACE2-mediated increases in islet insulin content. However, islet insulin content of D-Ala^7–Ang-(1-7)– and Ad-hACE2–treated mice was not significantly different from that of *db/m* mice. *D*: proliferating pancreatic β-cells were determined as cells staining positive for both insulin and PCNA. Ad-hACE2-eGFP significantly increased pancreatic β-cell proliferation in both *db/m* and *db/db* mice in comparison with their respective Ad-eGFP–treated controls. Administration of D-Ala^7–Ang-(1-7) significantly attenuated ACE2-mediated stimulation of pancreatic β-cell proliferation. *E*: Neither Ad-hACE2-eGFP nor Mas blockade treatment modified apoptosis in *db/m* mice. Ad-eGFP–treated *db/db* mice had no significant increase in apoptosis in comparision with

old *db/db* mice, D-Ala^7–Ang-(1-7) treatment prevented ACE2-mediated increases in islet insulin content (Fig. 5*A* and *C*). Ad-hACE2-eGFP expression resulted in increased pancreatic β-cell mass in *db/m* mice. D-Ala–Ang-(1-7) treatment resulted in significantly less pancreatic β-cell mass in Ad-ACE2-eGFP–treated mice. While there was a trend toward increased pancreatic β-cell mass in 8- and 16-week-old *db/db* mice, this finding was not statistically significant. Similarly, while there was a trend toward decreased pancreatic β-cell mass in D-Ala–Ang-(1-7)–treated mice, this difference was not statistically significant in Ad-eGFP–expressing mice (Fig. 5*E*).

Islet cell proliferation and apoptosis. In both *db/m* and *db/db* mice, Ad-hACE2-eGFP expression increased proliferation of pancreatic β-cells (Fig. 5*D*), as evidenced by the increase in double-stained cells for insulin and PCNA (Fig. 5*A*). D-Ala^7–Ang-(1-7) treatment prevented ACE2-mediated increase in β-cell proliferation (Fig. 5*A* and *D*). At 8 weeks of age, there was no significant change in apoptosis in Ad-eGFP–treated *db/db* mice compared with *db/m* mice. ACE2 overexpression and D-Ala^7–Ang-(1-7) treatment had no effect on apoptosis in *db/m* mice in comparison with their Ad-eGFP–treated counterparts (Fig. 5*B* and *E*). ACE2 overexpression did, however, significantly reduce apoptosis in *db/db* mice in comparison with the Ad-eGFP–treated group (1.18 ± 0.07 vs. 0.55 ± 0.06, normalized ratio positive nuclei to total nuclei per islet, $P < 0.05$; $n = 12$), and this improvement was prevented by Ang-(1-7) receptor blockade (1.16 ± 0.08, $P < 0.05$; $n = 12$) in Ad-hACE2-eGFP–treated *db/db* mice. In 16-week-old mice, Ad-hACE2-eGFP expression in *db/db* mice did not significantly improve pancreatic β-cell proliferation or apoptosis rates in comparison with Ad-GFP–treated *db/db* mice (supplemental Fig. 2).

DISCUSSION

All the classic components of the RAS (renin, angiotensinogen, ACE, and Ang-II type 1 and 2 receptors) have been identified in the pancreas, where they are thought to modulate β-cell function (3,4). Several studies implicate RAS overactivity in the development of islet dysfunction (8,10). Notably, in vitro (22.2 mmol/l glucose) and genetic (Zucker diabetic fatty rat) models of type 2 diabetes show increased expression of ACE and AT1R in islets, supporting the idea of a feed-forward mechanism ultimately resulting in β-cell dysfunction (7,10). While ACE2 has been shown to be elevated in renal tubules and cortex of *db/db* mice, prior to the onset of diabetic nephropathy (22), its relationship with β-cell function has not been studied. Our study shows the following: *1*) islet ACE2 expression is upregulated at 8 weeks and tends to be reduced at 16 weeks of age in *db/db* mice islets compared with *db/m* controls; *2*) Ad-hACE2-eGFP significantly increased ACE2 expression and activity in the mouse pancreas; *3*) ACE2 overexpression was associated with reduced hyperglycemia, improved glucose tolerance, increased insulin secretion and β-cell proliferation, and reduced apoptosis in 8-week-old *db/db* mice; and *4*) the beneficial effects of ACE2 overexpression were prevented by Ang-(1-7) receptor blockade, suggesting that the favorable effects of ACE2 on β-cell function are mediated by the Ang-(1-7) peptide.

db/db mice, a classic model of type 2 diabetes, have previously been reported to have early increase (22) and late decrease (23) in renal ACE2 expression during diabetic nephropathy. These observations are consistent with our data showing that ACE2 levels in the islets are increased in 8-week-old but decreased in 16-week-old *db/db* mice, in comparison with age-matched *db/m* mice, and support our hypothesis that ACE2 may be part of a compensatory mechanism during β-cell dysfunction (11). In addition, our observations supply a rationale for ACE2 gene therapy in the pancreas. Our experiments were performed in the C57BLKS/J background of *db/db* mice. These mice are obese at 4 weeks of age and develop persistant hyperglycemia and diabetes between 4 and 8 weeks of age. It is well known that islet compensation peaks in these animals between 8 and 12 weeks of age and that β-cell failure occurs between 5 and 8 months of age (24). We therefore studied 4-, 8- and 16-week-old *db/db* mice to determine the effects of ACE2 on islets prior to and during compensation and in decompensated islets. Adenoviral vectors are a useful tool for gene delivery in endocrine cells because of their ability to transfer genes with high efficiency to both dividing and nondividing cells (25). While these tools may be desirable in treatment of diabetes as a result of their ability to preferentially infect pancreatic β-cells over α-cells (26), the method has been limited by evidence of significant inflammation, tissue damage, and short duration of viral expression (27,28). Adenoviral delivery directly into the pancreas and infusion through the common bile duct at the entrance of the duodenum have been shown to be effective methods, although both induce acute pancreatitis, the severity of which correlates to viral load (27). Alternatively, systemic adenoviral delivery in mice, with clamped hepatic circulation, does not induce inflammation while providing high levels of viral infection. However, because isolation of the bile duct and hepatic vasculature resulted in significantly increased mortality in *db/db* mice, we opted for direct injection underneath the pancreas capsule. In our hands, adenovirus administration did not result in the development of inflammation and there was only minor CD3$^+$ cell infiltration (supplemental Fig. 1), consistent with previous observations that this adenoviral backbone induced mild infiltration of CD-3 F4/80 into the brain without causing tissue or cellular damage (29). Therefore, this particular viral vector may be less immunogenic than those used by other groups (27,28).

We hypothesized that ACE2 overexpression would ameliorate the impaired glucose homeostasis in diabetic mice. The current study demonstrates that ACE2 reduces fasting blood glucose and improves glucose tolerance in this model. Glucose tolerance is determined by both insulin secretion and peripheral insulin sensitivity. Insulin secretion has been hypothesized to be a more important factor than insulin sensitivity in determining glucose tolerance

_db/m_ controls. ACE2 overexpression, however, reduced apoptosis in _db/db_ mice vs. Ad-eGFP–treated mice. While D-Ala^7–Ang-(1-7) had no effect on apoptosis in Ad-eGFP-treated _db/db_ mice, it prevented ACE2-mediated reduction of apoptosis. _F_: Ad-hACE2-eGFP expression resulted in significant increases in pancreatic β-cell mass in _db/m_ mice and trended toward an increase in pancreatic β-cell mass in _db/db_ mice. While D-Ala^7–Ang-(1-7) treatment did not significantly reduce pancreatic β-cell mass in Ad-eGFP–expressing mice, it prevented ACE2-mediated enhancement of pancreatic β-cell mass. Values are expressed as means ± SEM. Two-way ANOVA statistical significance: *$P < 0.05$ vs. respective _db/m_, †$P < 0.05$ vs. respective mice treated with Ad-eGFP, ‡$P < 0.05$ vs. respective mice treated with D-Ala^7–Ang-(1-7). (A high-quality digital representation of this figure is available in the online issue.)

(30). First-phase insulin secretion is considered a reliable measure of pancreatic β-cell function. Moreover, impairment in first-phase insulin secretion is a sensitive marker for reduced pancreatic β-cell function and is evident before the onset of type 2 diabetes (19). Interestingly, there is significant evidence to implicate the ACE2/Ang-(1-7)/Mas axis in the prevention of insulin resistance. Mas-deficient mice develop a metabolic syndrome–like state that includes hyperinsulinemia and impaired glucose tolerance (20). Moreover, a recent study demonstrated that Ang-(1-7) prevents fructose-induced insulin resistance by stimulating phosphorylation of the insulin receptor, the insulin receptor substrate-1, and activation of Akt and phosphatidylinositol 3-kinase (31). In our study, ACE2 overexpression had no effect on insulin tolerance but increased first phase-insulin secretion, suggesting an improvement of β-cell function rather than insulin sensitivity. These findings are supported by another study showing that loss of ACE2 had no effect on insulin sensitivity but impaired first-phase insulin secretion (18). These data demonstrate that the primary effect of ACE2 overexpression in the pancreas and liver was mediated by changes in islet function and not hepatic insulin sensitivity. Of particular interest, Ang-(1-7) receptor inhibition worsened insulin sensitivity in all *db/db* mice. An explanation for this effect is that while our adenovirus was not expressed in the skeletal muscle or adipose tissue, D-Ala^7–Ang-(1-7) was administered systemically and therefore would be expected to reduce insulin signaling in all tissues, thus worsening insulin sensitivity.

Pancreatic β-cell decompensation and death occur during the progression of type 2 diabetes. While traditionally glucotoxicity- and lipotoxicity-mediated oxidative stress have been hypothesized to be the cause of β-cell death in type 2 diabetes (32), ACE inhibitors and AT1R blockers enhance islet insulin content in both Zucker diabetic fatty rats and *db/db* mice and prevent pancreatic β-cell loss, independently of changes in plasma glucose levels, by reducing intraislet apoptosis and enhancing pancreatic β-cell proliferation (10,33) Here, ACE2 overexpression increased islet insulin content in *db/db* mice above the level observed in *db/m* mice. In addition, we found that the enhanced insulin content, in *db/db* mice overexpressing ACE2, was due to enhanced pancreatic β-cell proliferation and reduced apoptosis. We found an increase in pancreatic β-cell mass in *db/m* and a trend toward increased pancreatic β-cell mass in *db/db* mice, supporting the hypothesis that ACE2 enhances pancreatic β-cell proliferation. While increases in β-cell mass were not found in *db/db* mice, we hypothesize that a long-term ACE2 expression model would demonstrate maintained or enhanced pancreatic β-cell mass. The main function of ACE2 is to transform Ang-II into Ang-(1-7), whose antiproliferative effects have been demonstrated in tumor growth (34), cardiac remodeling, and vascular injury (35). Consequently, ACE2 might be expected to have antiproliferative effects on pancreatic β-cells. Although our data showing that, in both lean and diabetic mice, ACE2 stimulates β-cell proliferation may seem paradoxical at first, a very recent study described the ability of Ang-(1-7) to activate growth-stimulatory pathways in human mesangial cells (36). Moreover, Ang-(1-7) has been implicated in the beneficial effects of both AT1R blockers (37) and ACE inhibitors (38). RAS blockade has also been reported to stimulate pancreatic β-cell proliferation (10). Accordingly, these data suggest that enhanced Ang-(1-7) and reduction of Ang-II signaling may be a putative mechanism for the increase in pancreatic β-cell proliferation associated with ACE2 overexpression. Alternatively, while the direct effects of ACE2 on downstream cell signaling are unknown, the enzyme shares 47.8% sequence homology with collectrin (39) which enhances insulin exocytosis (40), stimulates pancreatic β-cell proliferation, and increases islet insulin content (41). ACE2 may therefore act similarly to collectrin in stimulating pancreatic β-cell proliferation, although the mechanism remains unknown.

Very little is known about the role of ACE2 and Ang-(1-7) in the regulation of apoptosis. Mas knockout mice have increased cardiac apoptosis in comparison with controls (42). Moreover, left ventricular device–mediated enhancement of ACE2 activity, in end-stage heart failure, has been associated with reduced myocyte apoptosis in vivo, and Ang-(1-7)/Mas activation has been shown to reduce cardiomyocyte apoptosis in vitro (43). Moreover, oxidative stress directly induces pancreatic β-cell death. A recent study by Chu and Leung demonstrated that ACE inhibition causes a reduction in intraislet apoptosis and enhances pancreatic β-cell proliferation as a result of reduced uncoupling protein-2–driven oxidative stress (44). ACE2, therefore, may preserve pancreatic β-cell mass by reducing oxidative stress. Consistent with these observations, we show that ACE2 overexpression reduced apoptosis in 8-week-old diabetic mice. Moreover, we demonstrated that Ang-(1-7) receptor inhibition prevented the ACE2-mediated reduction in apoptosis, indicating that the antiapoptotic effects of ACE2 on pancreatic β-cells are mediated by Ang-(1-7). Although not the focus of this study, ACE2 could potentially regulate several pathways involved in apoptosis, including uncoupling protein 2 (44), bradykinin (45), extracellular signal–regulated kinase 1/2 and p38 signaling (46), and Akt phosphorylation (47). While a decrease in fasting blood glucose was observed in 16-week-old *db/db* mice, there were no significant changes regarding glucose tolerance, insulin secretion, or insulin sensitivity. Moreover, despite increased islet insulin content after ACE2 overexpression, there was not a significant increase in pancreatic β-cell mass, proliferation, or apoptosis. Taken together, these data indicate that ACE2 overexpression is not able to rescue β-cell function in late type 2 diabetes. During the pre-diabetic state, pancreatic β-cells undergo a compensatory phase during which β-cell mass and insulin output increase (48); then, at the onset of hyperglycemia, up to 50–75% of β-cell secretory function is lost (49). Finally, in late type 2 diabetes, pancreatic β-cells undergo decompensation, which results in loss of up to 60% of β-cell mass and failure (48). Given that significant loss of β-cell function and mass, added to deleterious changes in islet morphology, is evident in 15-week-old *db/db* mice, it is conceivable that ACE2 overexpression might be too late to reverse these changes. Indeed, a hypothetical window during which β-cell function and islet morphology can be modified has been proposed to exist before the onset of hyperglycemia (50). ACE2 overexpression for 7 days did, however, increase islet insulin content and reduce fasting blood glucose. While we did not see a significant effect of ACE2 overexpression in 4-week-old mice, these animals have only mild hyperglycemia and impaired glucose tolerance. We hypothesize that longer ACE2 overexpression may have resulted in enhanced β-cell function. In light of the increased β-cell proliferation and reduced apoptosis following administration of the adenovirus during the peak of

maximal β-cell compensation, long-term ACE2 gene therapy either before the onset of hyperglycemia or in the early stages of type 2 diabetes may potentially result in improved islet function and glucose homeostasis at 16 weeks of age through maintenance of islet compensation.

In summary, islet ACE2 expression increased early and decreased late in type 2 diabetes. This is consistent with observations in the diabetic kidney, where ACE2 is thought to act as a compensatory mechanism for hyperglycemia-induced RAS activation. In the *db/db* mouse model, ACE2 overexpression significantly improved glucose tolerance, enhanced islet function, increased β-cell proliferation and insulin content, and prevented β-cell apoptosis in 8-week-old *db/db* mice. These findings suggest that *ACE2* gene therapy could be a novel therapeutic approach for prevention of β-cell dysfunction and loss in type 2 diabetes.

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