Adaptive β-Cell Neogenesis in the Adult Mouse in Response to Glucocorticoid-Induced Insulin Resistance

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Both type 1 and type 2 diabetes are characterized by deficient insulin secretion and decreased β-cell mass. Thus, regenerative strategies to increase β-cell mass need to be developed. To characterize mechanisms of β-cell plasticity, we studied a model of severe insulin resistance in the adult mouse and defined how β-cells adapt. Chronic corticosterone (CORT) treatment was given to adult mice and led to rapid insulin resistance and adaptive increased insulin secretion. Adaptive and massive increase of β-cell mass was observed during treatment up to 8 weeks. β-Cell mass increase was partially reversible upon treatment cessation and reinduced upon subsequent treatment. β-Cell neogenesis was suggested by an increased number of islets, mainly close to ducts, and increased Sox9 and Ngn3 mRNA levels in islets, but lineage-tracing experiments revealed that neoformed β-cells did not derive from Sox9- or Ngn3-expressing cells. CORT treatment after β-cell depletion partially restored β-cells. Finally, β-cell neogenesis was shown to be indirectly stimulated by CORT because serum from CORT-treated mice increased β-cell differentiation in in vitro cultures of pancreatic buds. Altogether, the results present a novel model of β-cell neogenesis in the adult mouse and identify the presence of neogenic factors in the serum of CORT-treated mice.

Pancreatic β-cells secrete insulin that induces nutrient storage, stops energy mobilization, and in fine lowers blood glucose levels. Insulin production is finely tuned and can adapt to increased demand when target tissues, such as liver, muscle, or adipose tissues, become insulin resistant (1). When such adaptation is not sufficient, as observed in type 2 diabetes, or when insulin production is absent, as seen in type 1 diabetes, chronic hyperglycemia occurs and associates with increased morbidity and mortality. Thus, innovative therapies to prevent or treat diabetes rely on strategies to maintain or restore an adequate pool of β-cells. However, signals to achieve such goals still need to be identified.

The maintenance of adequate β-cell mass mainly is performed through four mechanisms: apoptosis (2) and dedifferentiation (3) that diminish the number of β-cells and proliferation of existing β-cells (4) and neogenesis (differentiation of new β-cells from precursors) (5) that increase the number of insulin-producing cells. Of particular interest is β-cell neogenesis because its induction leads to β-cell regeneration in situations of β-cell depletion, such as in type 1 diabetes. To identify signals that regulate β-cell neogenesis, previous studies have used animal models. In rats, partial pancreatectomy is a model

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of β-cell regeneration through β-cell replication and neogenesis (6). In mice, pancreatic duct ligation (PDL) induces an increased β-cell mass as a result of β-cell neogenesis from ductal cells through the reexpression of a transcription factor that drives β-cell differentiation during fetal life, neurogenin3 (Ngn3) (5). However, other studies that used a similar model of PDL failed to reproduce these results and concluded that no β-cell neogenesis was observed in adult mice, even after pancreatic injury (7). With regard to the nature of pancreatic precursors, researchers have proposed that ducts contain undifferentiated cells that can be recruited to differentiate into β-cells (5,8,9), but recent studies using various lineage-tracing experiments have demonstrated that duct cells do not contribute to β-cell neogenesis (10,11). Thus, whether β-cell neogenesis from precursors contributes to β-cell regeneration remains an open question. Alternatively, β-cells can be formed through transdifferentiation from α- and δ-cells (12,13) after artificial β-cell depletion using diphtheria toxin in mice. Similarly, α-cells can transdifferentiate into β-cells in genetically modified mice when expression of the key transcription factors Pax4 or Arx is altered in α-cells (14,15) or in mice treated with GABA (16). Altogether, these studies provide important insight into pancreatic plasticity and β-cell neogenesis but mostly after pancreatic injury or genetic manipulation. Moreover, whether β-cell neogenesis involves precursors located in ducts remains controversial.

In other situations of increased β-cell mass, including high-fat diet (17), drug-induced insulin resistance (18), or the physiological insulin resistance of pregnancy (19), β-cell proliferation, and not neogenesis, has been proposed as the main adaptive mechanism in rodents. However, recent studies have suggested that β-cell neogenesis also may contribute to the β-cell mass increase observed during pregnancy in mice (20) and humans (21).

A pharmacological model that allows a rapid and severe insulin resistance is the administration of glucocorticoids (GCs). GCs are hormones that regulate several physiological processes, such as behavior, metabolism, and immune response. Because of their potent anti-inflammatory properties, GCs are widely used to treat inflammatory diseases. Unfortunately, they also induce insulin resistance and consequently increase insulin secretion, and a high proportion of GC-treated patients develop diabetes (22). More specifically, GCs are deleterious to insulin sensitivity in target tissues, leading to overall decreased glucose uptake, excessive glucose production by the liver, lipolysis by the adipose tissues, and proteolysis in skeletal muscles (23). With regard to insulin secretion, chronic or acute in vitro treatment with GCs usually inhibits insulin secretion (24,25) through modifications of α-adrenergic signaling (26). In contrast, chronic in vivo GC treatment generally leads to increased insulin secretion (25,27) with improved glucose responsiveness (28) and sensitivity (29) as well as modifications of calcium (30) and cholinergic signaling pathways (31). With regard to β-cell mass, chronic GC treatment in rats and mice leads to increased β-cell mass as a result of increased β-cell proliferation (32). In such models, β-cell neogenesis has not been investigated fully. Thus, the current study was performed to understand precisely pancreatic adaptations and underlying mechanisms in response to insulin resistance induced by GCs.

We show that chronic administration of corticosterone (CORT) to mice leads to a massive β-cell mass expansion through both increased β-cell proliferation and β-cell neogenesis, the latter being independent of Sox9 and Ngn3. Moreover, CORT treatment achieves partial β-cell regeneration after β-cell depletion. Finally, we show that serum from CORT-treated mice increases β-cell fraction in an in vitro model of pancreas differentiation, demonstrating the presence of circulating factors that stimulate β-cell neogenesis. Altogether, the results propose a new mode of adaptive β-cell neogenesis.

RESEARCH DESIGN AND METHODS

Animals

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by INSERM and were approved by the local animal care and use committee (Charles Darwin ethic committee, Paris, France). C57BL/6J male and female mice were obtained from the French colony of Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France) at the age of 8 weeks. Mice carrying the transgene Ngn3CreERTM (33) or the transgene Sox9CreERTM (34) were crossed with mice from the ROSA26YFP reporter line (35) to obtain Ngn3CreERTM; ROSA26YFP and Sox9CreERTM;ROSA26YFP mice, respectively. Estrogen receptor α (ERα)–null mice (ERKO) were generated as previously described (36). Mice were chow fed ad libitum and housed in 12-h light/dark cycles.

Chemicals

CORT

Animals were treated with CORT (100 μg/mL) (Sigma-Aldrich, St. Louis, MO) or vehicle (VEH) (1% ethanol) in drinking water.

Tamoxifen

Eight-week-old Ngn3CreERTM;ROSA26YFP and Sox9-CreERTM;ROSA26YFP mice were subjected to intraperitoneal injections of tamoxifen (TMX) dissolved in filtered olive oil (dose 60 μg/g/day [see protocol in Fig. 5], TMX solution 10 mg/mL) (MP Biomedicals, Illkirch Graffenstaden, France).

Canrenoate

Mice were treated with canrenoic acid potassium salt (CANRE) (100 μg/mL) (Sigma-Aldrich) dissolved in the drinking water.

Streptozotocin

β-Cell depletion in C57BL/6J mice was obtained by a single-intraperitoneal injection of streptozotocin (STZ) (150 mg/kg)

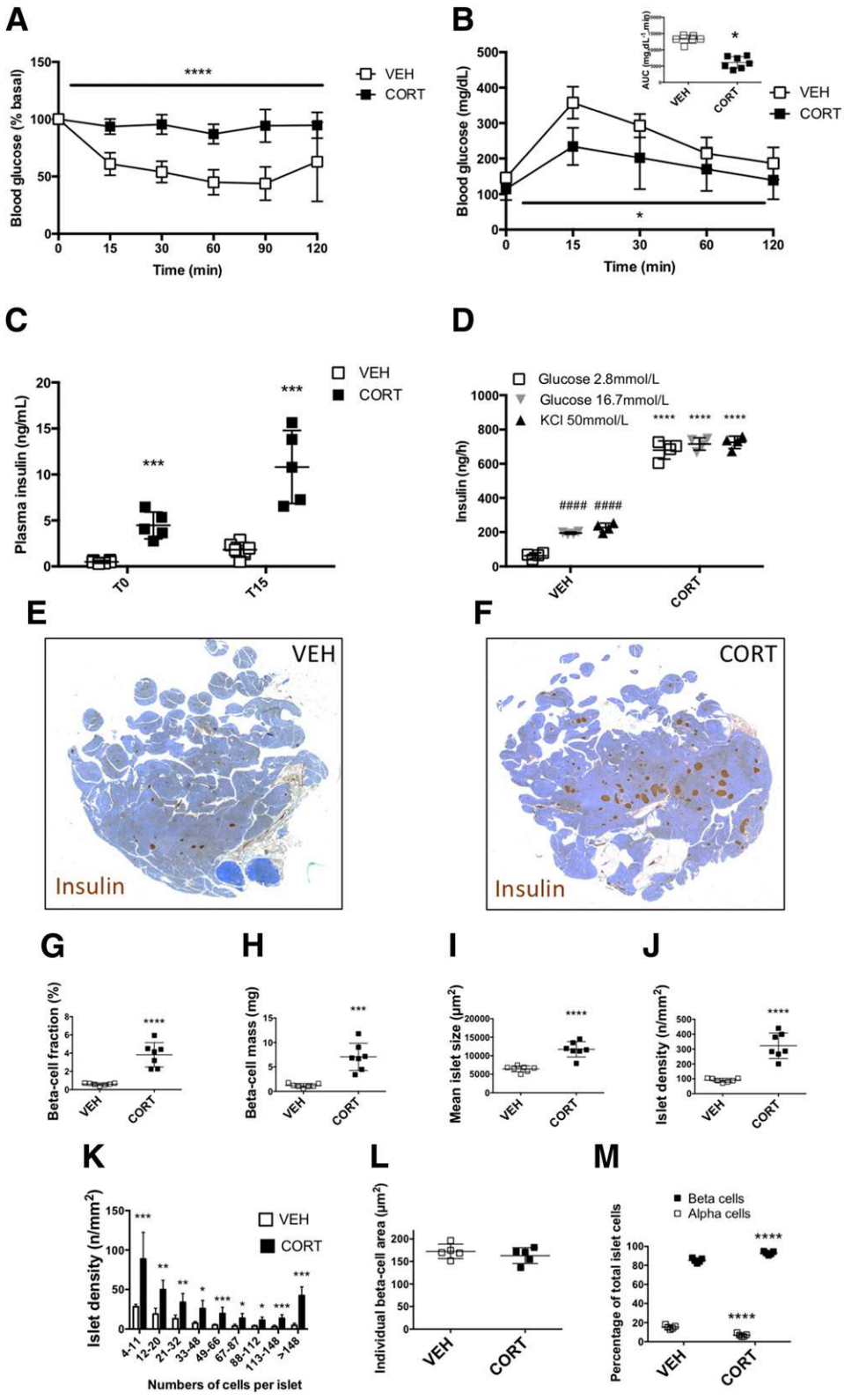

Figure 1—Metabolic parameters and pancreatic analysis of mice treated with CORT for 8 weeks. *A*: Insulin tolerance test was carried out in mice treated with CORT (n = 8) or VEH (n = 7) that were subjected to a 6-h fast and injected with insulin (1 IU/kg). Results are expressed as the percentage of basal glycemia. *B*: IPGTT was performed after an 18-h fast. Glucose was injected (2 g/kg) into mice treated with CORT (n = 7) or VEH (n = 10). AUCs are shown in the inset. *C*: Plasma insulin levels during IPGTT (at time 0 [T0] and T15 after glucose challenge) in mice treated with CORT (n = 7) or VEH (n = 6). *D*: Insulin secretion was tested in response to 2.8 mmol/L glucose, 16.7 mmol/L glucose, or 50 mmol/L KCl and measured in isolated pancreatic islets from VEH-treated (n = 4) and CORT-treated (n = 4) mice. *E* and *F*: Representative images of insulin immunostaining of pancreatic sections of mice treated with VEH or CORT for 8 weeks. Counterstaining was performed using hematoxylin. Magnification is ×2.5. *G–L*: Pancreatic β-cell fraction, β-cell mass, mean islet size, islet density, islet distribution, and individual

(Sigma-Aldrich). Mice with blood glucose levels >300 mg/dL 3 days after STZ injection were selected for CORT treatment.

BrdU

β-Cell proliferation was measured in mice that received a single injection of BrdU (50 mg/kg) (Sigma-Aldrich) 24 h before sacrifice.

Glucose and Insulin Tolerance Tests

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test were performed as previously described (37).

Hormonal Assays

Insulin and CORT blood levels were measured using mouse insulin immunoassay (Alpco, Salem, NH) and CORT immunoassay (Labor Diagnostika Nord, Nordhorn, Germany), respectively.

Islet Isolation and Glucose-Stimulated Insulin Secretion

Mouse islets were isolated after injecting a collagenase solution (1 mg/mL) (Sigma-Aldrich) and handpicked under a binocular microscope (Leica Microsystems, Wetzlar, Germany). Isolated islets were cultured and stimulated as previously described (38).

FACS

Islets from Ngn3CreERTM;ROSA26YFP and Sox9CreERTM; ROSA26YFP mice were isolated and dissociated into single cells by mechanical and enzymatic dispersion using trypsin (0.05 mg/mL) (Eurobio, Courtaboeuf, France). Dissociated cells were resuspended in PBS, 0.5 mmol/L EDTA, and 2% FCS and analyzed with an Aria III cell sorter (BD Biosciences, San Jose, CA).

Immunohistochemistry, Immunofluorescence, and Morphometry

Pancreas were fixed in 3.7% formalin solution, embedded in paraffin, and cut into 5-μm sections. Morphometrical parameters (β-cell fraction, islet size, and density) were evaluated for eight sections per pancreas after immunohistochemistry using the following primary antibodies: guinea pig polyclonal anti-insulin (Dako, Agilent, Santa Clara, CA), rabbit polyclonal antiglucagon (Dako), rabbit polyclonal antisomatostatin (Dako), chicken polyclonal anti-GFP (Aves Labs, Tigard, OR), and monoclonal anti-pancytokeratin (Sigma-Aldrich). Secondary antibodies coupled to horseradish peroxidase or to alkaline phosphastase were obtained from Jackson ImmunoResearch (Westgrove, PA). Enzyme substrates were DAB+ (Dako) or Fast Red (Sigma-Aldrich). Morphometrical parameters were determined as previously described (39) and are described in detail in the Supplementary Data and Supplementary Fig. 6. For β-cell proliferation, antigen retrieval was performed in citrate buffer (pH 6) for 12 min at 95°C using a microwave oven. Sections were incubated with an anti-BrdU antibody (Santa Cruz Biotechnology, Dallas, TX) and an anti-insulin antibody (Dako). Secondary antibodies coupled to fluorescent molecules (Thermo Fisher Scientific, Waltham, MA) were then incubated. Pictures were taken on a Leica DMRB fluorescent microscope. At least 5,000 cells from several sections were analyzed per animal. β-Cell proliferation was calculated as the percentage of insulin-positive cells that were also positive for BrdU in the whole insulin-positive cell population.

Pancreatic Bud Culture and Analysis

Mouse pancreatic buds at embryonic day 11.5 were isolated and cultured as previously described (40) in the presence of 10% of serum from VEH- or CORT-treated mice or with 10^{-7} mol/L CORT, 0.4 ng/mL insulin, or both supplemented in 10% of serum from VEH-treated mice. At the end of the culture period, buds were fixed and sectioned. Immunofluorescence for insulin (Dako) as well as DAPI staining were performed on all sections that were then analyzed as previously described (40).

RNA Analysis

Total RNA was isolated (RNeasy Mini Plus Kit; QIAGEN, Hilden, Germany) and reverse transcribed into cDNA with SuperScript transcriptase (Invitrogen, Carlsbad, CA). Gene expression was quantified by real-time PCR using SYBR Green Supermix (Eurogentec, Seraing, Belgium) in a MyiQ thermal cycler (Bio-Rad, Hercules, CA). The value obtained for each specific gene product was normalized for 18S rRNA and expressed as the fold change of the value in the control condition. Primer sequences are available upon request.

Statistical Analysis

All results are presented as mean ± SD. Comparisons were performed using the Mann-Whitney *U* and one-way or two-way ANOVA tests. $P < 0.05$ was considered significant.

RESULTS

CORT Treatment Induces Severe Insulin Resistance

After 8 weeks of CORT administration, male mice developed severe insulin resistance (Fig. 1*A*), but despite this reduced insulin sensitivity, CORT mice exhibited a better glucose tolerance than VEH mice (Fig. 1*B* and inset showing the areas under the curve [AUCs]) that was associated with much higher plasma insulin levels in the basal state and in response to glucose in vivo (Fig. 1*C*) and ex vivo (Fig. 1*D*).

β-cell area after insulin or glucagon immunostaining of eight pancreatic slides from each animal after 8 weeks of treatment with CORT ($n = 7$) or VEH ($n = 7$). *M*: Percentage of α- or β-cells within islets after insulin and glucagon immunofluorescence on pancreatic slides after 8 weeks of treatment with CORT ($n = 5$) or VEH ($n = 5$). Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ CORT vs. VEH mice; $^{\#\#\#\#}P < 0.001$ VEH 16.7 mmol/L glucose or VEH 50 mmol/L KCl vs. VEH 2.8 mmol/L glucose.

Pancreatic Adaptation After CORT-Induced Insulin Resistance

After 8 weeks of treatment, pancreatic sections of male CORT mice presented more and larger islets stained for insulin (Fig. 1*E* and *F*). Morphometric quantification revealed a massive increase of β-cell fraction and β-cell mass (Fig. 1*G* and *H*) in CORT mice associated with a higher mean islet size, an indirect marker of islet cell proliferation (Fig. 1*I*), and an increased islet density, an indirect marker of islet neogenesis (Fig. 1*J*), in islets of all sizes (Fig. 1*K*). Individual β-cell area remained unchanged (Fig. 1*L*), but islet composition changed with more β-cells (93 ± 1.8% vs. 85.2 ± 2.4%; $P < 0.0001$) and fewer α-cells (6.8 ± 1.7% vs. 15.1 ± 2.1%; $P < 0.0001$) in islets from CORT mice (Fig. 1*M*) compared with islets from VEH mice. In females, CORT treatment led to insulin resistance (Supplementary Fig. 1*A*), normal glucose tolerance (Supplementary Fig. 1*B* and *C*), and similar pancreatic adaptation compared with males (Supplementary Fig. 1*D–H*), demonstrating that β-cell mass adaptation in response to CORT treatment is sex independent. In males, α-cell fraction tended to be increased, whereas α-cell mass was significantly increased in CORT mice (Supplementary Fig. 2*A* and *B*). A trend for an increased δ-cell fraction and mass also was observed in CORT mice (Supplementary Fig. 2*C* and *D*).

Progressive and Continuous Pancreatic Adaptation

Analysis of pancreatic adaptation at several time points during CORT treatment revealed no change after 1 week, but a massive increase of β-cell fraction and mass was found after 4, 6, and 8 weeks of treatment compared with VEH (Fig. 2*A* and *B*). Mean islet size reached a plateau after 4 weeks of treatment (Fig. 2*C*), whereas islet density still increased after 6 and 8 weeks of treatment (Fig. 2*D*). Thus, these observations suggest that the massive β-cell mass adaptation in CORT-treated mice may involve β-cell proliferation (reflected by mean islet size that plateaued at 4 weeks of treatment) and a continuous increase of β-cell neogenesis (reflected by islet density that increases throughout the whole 8 weeks of CORT treatment).

β-Cell Adaptation After Treatment Cessation and Retreatment

Four to 8 weeks of CORT treatment led to a massive increased β-cell mass without any change in pancreas weight (data not shown). To test whether such an increase is reversible, we treated male mice with CORT for 4 weeks (Fig. 3*A*, stage I), stopped the treatment for 4 weeks (Fig. 3*A*, stage II [washout]), and treated again with CORT (Fig. 3*A*, stage III). Effects of CORT on β-cell mass and fraction were found to be partly reversible after the 4-week washout period (Fig. 3*B* and *C*). This reduction was associated with a decrease of mean islet size (Fig. 3*D*), whereas islet density remained unchanged after washout (Fig. 3*E*). Of note, after 4 weeks of washout, mice previously treated with CORT exhibited a normal insulin sensitivity (Supplementary Fig. 3*A*) and glucose tolerance (Supplementary

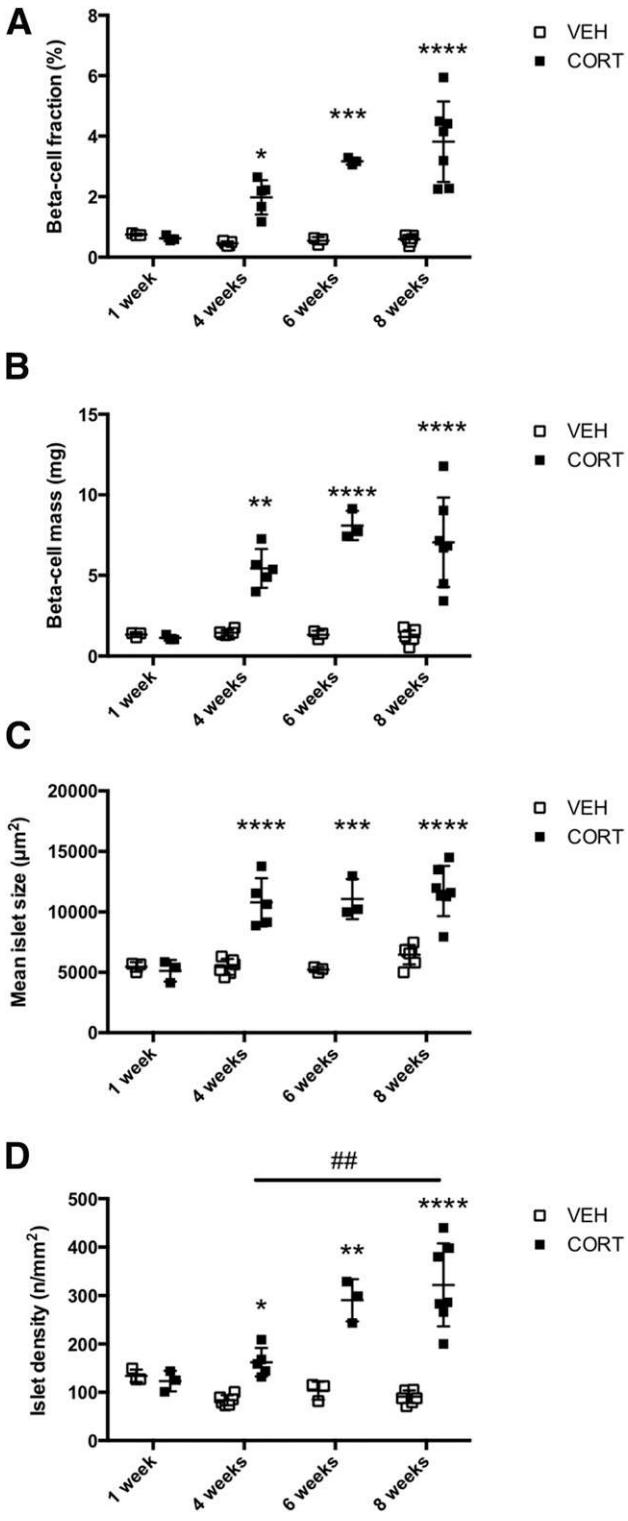

Figure 2—Pancreatic adaptation throughout CORT administration. *A–D*: Pancreatic β-cell fraction, β-cell mass, mean islet size, and islet density was quantified after insulin immunostaining of eight pancreatic slides from each animal after 1 ($n = 3$ in VEH and CORT), 4 ($n = 6$ in VEH and $n = 5$ in CORT), 6 ($n = 3$ in VEH and CORT), or 8 ($n = 7$ in VEH and CORT) weeks of treatment. Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ CORT vs. VEH; $^{\#\#}P < 0.01$ comparing CORT-treated mice at various points of treatment.

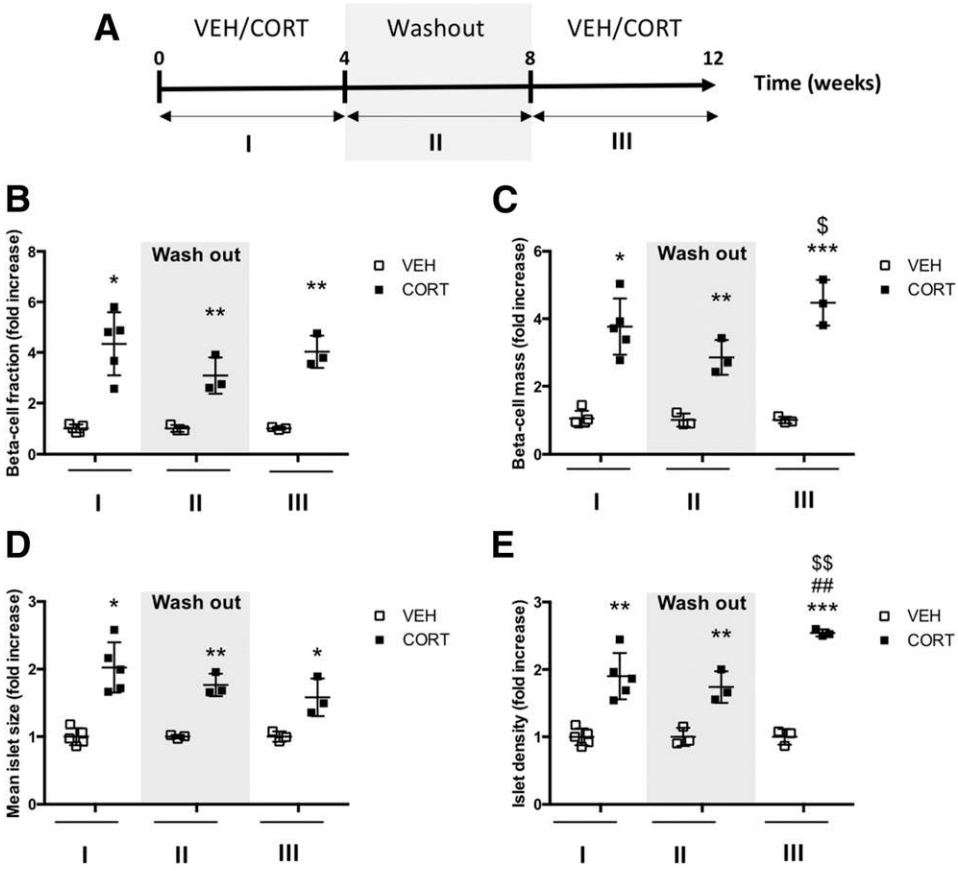

Figure 3—The β-cell mass adaptation is reversible and can be reinduced. *A*: Scheme of the protocol. During 4 weeks, mice were treated with CORT or VEH (stage I). Mice were then subjected to a 4-week washout period (stage II). Finally, mice were again treated with CORT or VEH for 4 weeks (stage III). At the end of each stage, pancreata were dissected and analyzed. *B–E*: Pancreatic β-cell fraction, β-cell mass, mean islet size, and islet density were quantified after insulin immunostaining of eight pancreatic slides from each animal. Number of mice treated with CORT were five after the first treatment, three after the washout, and three after the retreatment and of those treated with VEH, five after the first treatment, three after the washout, and three after the retreatment; values are fold increase relative to the corresponding group of mice treated with VEH. Data are mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ CORT vs. VEH; $\$P < 0.05$, $\$\$P < 0.01$ CORT vs. CORT and then subjected to washout; $\#\#P < 0.01$ CORT retreatment vs. CORT during 4 weeks.

Fig. 3*B* and *C*) but high blood insulin levels (Supplementary Fig. 3*D*). After the washout period, an additional 4-week CORT treatment led to increased β-cell fraction and mass (Fig. 3*B* and *C*) without any change in mean islet size (Fig. 3*D*) but with a further increase in islet density (Fig. 3*E*), emphasizing the role of neogenesis on β-cell mass increase. Similarly, this reintroduction of CORT treatment for 4 weeks again led to insulin resistance (Supplementary Fig. 3*E*) and improved glucose tolerance (Supplementary Fig. 3*F* and *G*) with very high blood insulin levels (Supplementary Fig. 3*H*).

Proliferation and Neogenesis in Pancreata of CORT-Treated Mice

mRNA levels of transcription factors Ngn3, Nkx2.2, Nkx6.1, Insm1, and Rfx6 were increased in islets from male CORT-treated mice during the 8 weeks of CORT treatment, whereas mRNA levels of Sox9, Pdx-1, Pax4, and MafA remained unchanged and that of Arx decreased (Fig. 4*A*). mRNA levels of proteins involved in insulin secretion (Slc30A8, Kir6.2, Sur1, Ins2, and Gck) were increased in islets from CORT mice (Fig. 4*B*). Finally, we observed increased mRNA levels of Ki67 in islets from CORT mice (Fig. 4*C*) as well as an increased percentage of insulin-positive cells also positive for BrdU (Fig. 4*D*), demonstrating β-cell proliferation. We also observed an increased number of insulin-expressing cells in ducts (stained for pancytokeratin) after 1 week of treatment (Fig. 4*E* and *G*) and an increased number of islets adjacent to ducts after 1 week and 8 weeks of treatment (Fig. 4*F* and *H*). Taken together, these observations suggest that β-cell mass increase results from both β-cell proliferation and β-cell neogenesis in CORT mice.

New β-Cells Do Not Derive From Sox9-Positive Cells

The proximity of islets to ducts and the presence of insulin-positive cells in ducts are suggestive only of neogenesis arising from ducts. To characterize β-cell neogenesis clearly, we performed lineage tracing. To this end, we first used TMX-inducible Sox9-CreERTM;

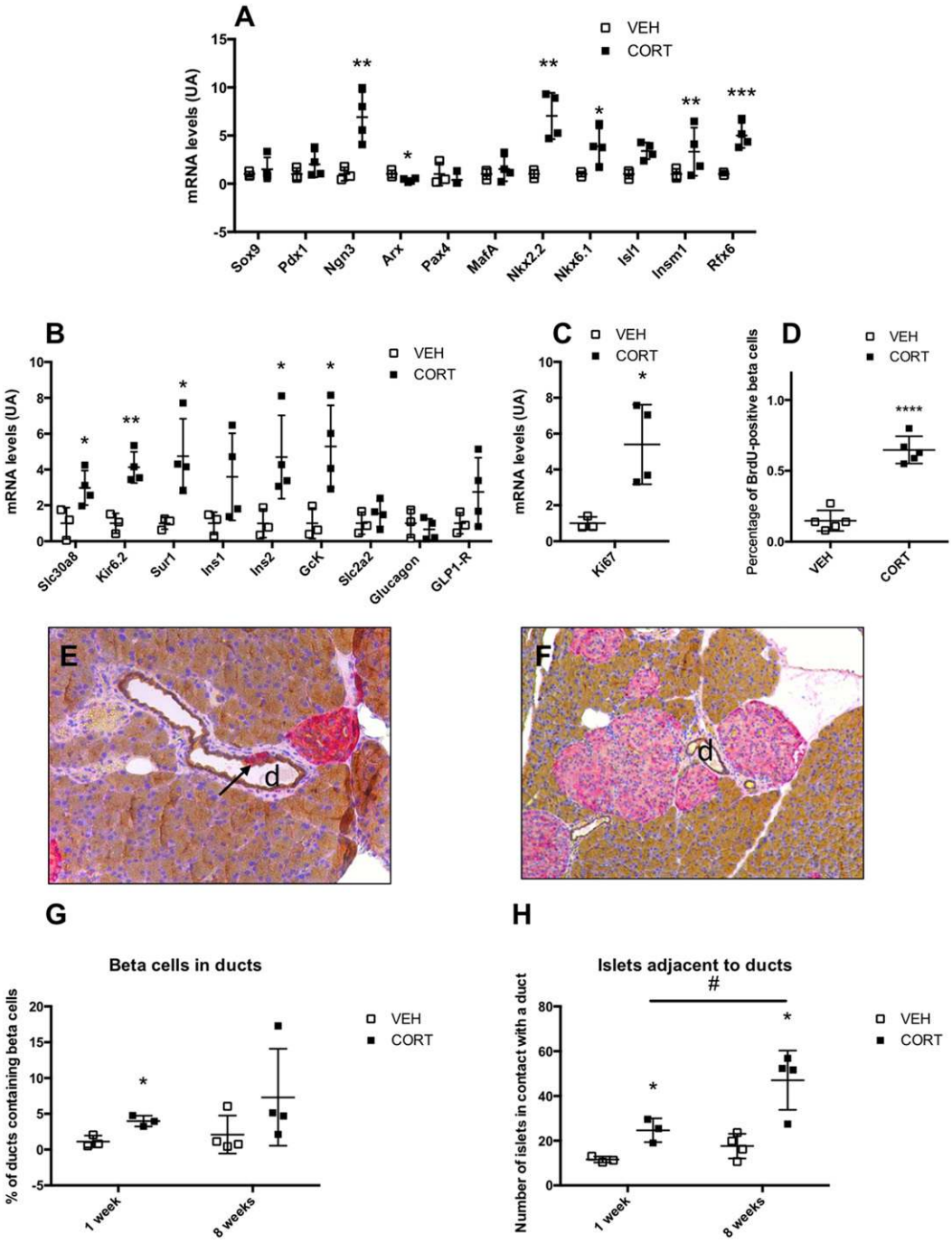

Figure 4—Chronic CORT treatment is associated with signs of β-cell neogenesis. *A–C*: mRNA levels in isolated adult pancreatic islets from mice treated with VEH or CORT for 8 weeks, including transcription factors involved in pancreatic development and endocrine cell maturation (*A*), genes encoding proteins involved in hormone secretion (*B*), and Ki67 islets (*C*) from mice treated with VEH ($n = 3$) or CORT ($n = 3$). *D*: Percentage of cells positive for insulin and BrdU within insulin-positive cells in pancreas from mice treated with VEH ($n = 5$) or CORT ($n = 5$). *E* and *F*: Images of coimmunostaining for insulin (red) and pancytokeratin (brown) performed on pancreatic sections from mice treated with CORT for 8 weeks (arrow points to β-cells in the ductal epithelium). Magnification is ×20. *G* and *H*: Quantification of ducts containing insulin cells and pancreatic islets adjacent to ducts in pancreata from mice treated with VEH ($n = 3$ at 1 week and $n = 4$ at 8 weeks) or CORT ($n = 3$ at 1 week and $n = 4$ at 8 weeks). Data are mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ CORT vs. VEH mice; #$P < 0.05$ CORT at 8 weeks vs. CORT at 1 week. d, duct; UA, arbitrary unit.

ROSA26YFP mice in which yellow fluorescent protein (YFP) labeling of ductal cells was induced by TMX injections. At the end of the TMX treatment, we observed the presence of YFP-labeled cells in ducts on pancreatic sections of control mice (Supplementary Fig. 4*C* and *D*). Three weeks after TMX treatment, CORT was administrated for 3 weeks (Fig. 5*A*). Mice treated with TMX and CORT exhibited as expected increased β-cell fraction and

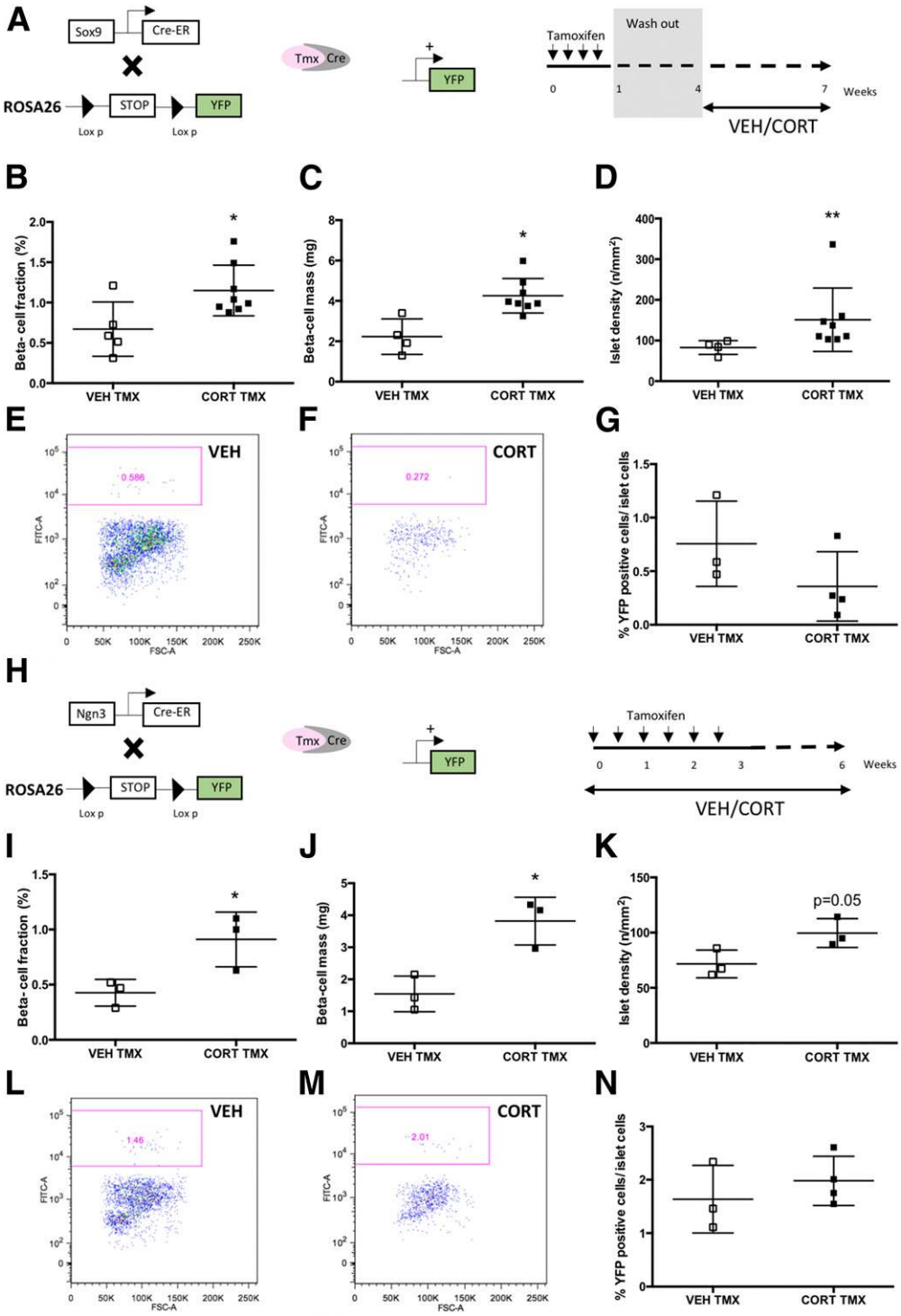

Figure 5—New β-cells did not derive from Sox9- or Ngn3-positive cells after CORT treatment. *A*: Schematic of Sox9CreERTM;ROSA26YFP mice generation (left) and the lineage-tracing experiment (right). TMX was administered by four daily injections during the 1st week. After 3 weeks, CORT or VEH was administrated during 3 weeks. *B–D*: Pancreatic β-cell fraction, β-cell mass, and islet density were calculated by morphometrical analysis in VEH-treated ($n = 5$) and CORT-treated ($n = 8$) mice. *E* and *F*: Representative graphs of flow cytometry on the basis of FITC-A and cellular granulation (forward-scattered light [FSC]-A) of trypsinized cells from islets isolated from a mouse treated with VEH or CORT. *G*: Quantification of YFP-positive cells in VEH TMX ($n = 3$) and CORT TMX ($n = 4$) mice. *H*: Schematic of Ngn3CreERTM;ROSA26YFP mice generation (left) and the lineage-tracing experiment (right). TMX was administered by injection twice a week during 3 weeks simultaneously with CORT or VEH treatment. Treatment was continued during 3 weeks after stopping TMX injections. *I–K*: Pancreatic β-cell fraction, β-cell mass, and islet density were calculated by morphometrical analysis in VEH-treated ($n = 3$) and CORT-treated ($n = 3$) mice. *L* and *M*: Representative graphs of flow cytometry on the basis of FITC-A and cellular granulation (FSC-A) of trypsinized cells from islets isolated from a mouse treated with VEH or CORT. *N*: Quantification of YFP-positive cells in VEH TMX ($n = 3$) and CORT TMX ($n = 4$) mice. Data are mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$ CORT vs. VEH mice.

mass and islet density compared with TMX and VEH mice (Fig. 5*B–D*). Flow cytometry analysis of YFP-positive cells in isolated and dissociated islets revealed no difference between mice treated with TMX and CORT and mice treated with TMX and VEH (Fig. 5*E–G*). These results indicate that the increase of β-cell mass does not involve cells that derive from ductal Sox9-positive cells.

New β-Cells Do Not Derive From Ngn3-Positive Cells

Because Ngn3 is a transcription factor required for β-cell formation during fetal life (41) or during adult β-cell neogenesis (5) and Ngn3 mRNA level was increased in islets of mice treated with CORT (Fig. 4*A*), we used Ngn3CreERTM;ROSA26YFP mice to define whether the neoformed β-cells in CORT-treated mice derived from Ngn3-positive cells. Mice were injected twice a week with TMX (Fig. 5*H*) together with CORT or VEH treatment. At the end of the treatment, we observed an increased β-cell mass and fraction and a strong tendency ($P = 0.05$) for an increased islet density in mice treated with TMX and CORT (Fig. 5*I–K*). Flow cytometry analysis revealed that the percentage of YFP-positive cells was similar between the two groups (Fig. 5*L–N*), suggesting that new β-cells do not derive from Ngn3-positive cells. To confirm this observation, we used a different strategy. Because β-cell neogenesis during PDL has been shown to rely on Ngn3 re-expression (5) and on the presence of ERα (42), we tested the implication of ERα in our model. ERKO mice treated with CORT exhibited a severe insulin resistance similarly to wild-type mice treated with CORT (Supplementary Fig. 5*A*) that was associated with glucose intolerance (Supplementary Fig. 5*B* and *C*) and high plasma insulin levels before and after glucose stimulation (Supplementary Fig. 5*D*). ERKO mice also presented increased β-cell fraction, β-cell mass, mean islet size, and islet density in response to CORT (Fig. 6*A–D*), suggesting that β-cell neogenesis in CORT mice does not require the presence of the ERα.

β-Cell Neogenesis in Mice Treated With CORT and a Mineralocorticoid Receptor Antagonist

Because high doses of GCs are able to activate mineralocorticoid receptor (MR) (43), we tested the implication of this receptor. CORT and VEH mice were given CANRE, an MR antagonist, in drinking water. Metabolic tests revealed that in mice treated with CORT and CANRE, slightly lower insulin resistance was observed in CANRE mice than in CORT mice, which was associated with an improved glucose tolerance (Supplementary Fig. 5*E–G*) and high plasma insulin levels before and after glucose stimulation (Supplementary Fig. 5*H*). The reason why glucose tolerance and insulin levels are similar while insulin resistance is different remains unknown. Mice treated with CORT and CANRE presented an increased β-cell fraction and mass as well as an augmented mean islet size and islet density (Fig. 6*E–H*), demonstrating that MR antagonism did not prevent β-cell neogenesis in CORT-treated mice.

Partial β-Cell Regeneration With CORT Treatment After β-Cell Depletion

Because CORT was associated with β-cell neogenesis in normal mice, we then tested whether CORT treatment could regenerate β-cells after their depletion. A single STZ injection was performed in wild-type mice, leading to overt hyperglycemia within 2–3 days (Fig. 7*A*). When hyperglycemia was stable, STZ mice were given VEH or CORT (arrow on Fig. 7*A*) for 8 weeks. No change in blood glucose levels was observed between VEH and STZ-CORT mice (Fig. 7*A*). Pancreatic analysis revealed a 95% depletion of β-cell fraction and mass in STZ-VEH mice (Fig. 7*B* and *C*). Compared with STZ-VEH mice, STZ-CORT mice showed a slight, but significant increase of β-cell fraction (Fig. 7*B*), β-cell mass (Fig. 7*C*), and islet density (Fig. 7*D*) but no change of mean islet size (Fig. 7*E*). These data suggest that β-cell regeneration occurred through β-cell neogenesis in STZ-CORT mice, leading to a trend for increased plasma insulin levels (Fig. 7*F*).

Serum From CORT Mice Stimulates β-Cell Neogenesis In Vitro

Because we had shown that GCs inhibit β-cell differentiation (44,45), we postulated that β-cell neogenesis in CORT mice is not a direct effect of GCs. To test this hypothesis, we cultured pancreatic buds of mouse embryos at day 11.5, which mostly comprise undifferentiated precursors and which differentiate after 7 days of culture into mature exocrine and endocrine cells (40) (Fig. 8). Treatment of pancreatic buds with 10% of serum from CORT-treated mice for 7 days increased β-cell fraction (Fig. 8*B* and *K*). Because the serum of CORT mice contains high levels of CORT and insulin, we tested in vitro the effect of exact same levels of CORT or insulin on pancreatic bud differentiation. Serum from fed CORT mice contained 4.4 ± 1.4 ng/mL insulin (Fig. 1*C*) and $1.6 \pm 0.15 \times 10^{-6}$ mol/L CORT. We used 10% of these concentrations to mimic insulin and CORT levels present when buds are exposed to 10% of serum from CORT mice. Exposure to CORT alone decreased β-cell fraction in pancreatic buds (Fig. 8*C* and *K*), whereas insulin alone had no effect (Fig. 8*D* and *K*). Finally, we treated pancreatic buds with both CORT and insulin and observed a decreased β-cell fraction similar to CORT alone (Fig. 8*E* and *K*). We also observed that serum from CORT-treated mice increased the mRNA levels of Ins1, Ins2, glucagon, Sox9, Pdx1, and Ngn3 in pancreatic buds (Fig. 8*L*). Altogether, these results suggest that serum from CORT-treated mice contains factors able to stimulate β-cell neogenesis in vitro.

DISCUSSION

GCs are known to modulate glucose homeostasis and to induce tissue remodeling. The pool of pancreatic β-cells is plastic and adapts to changes in insulin demand. In the current study, we show that chronic GC administration in the adult mouse leads to insulin resistance and increased insulin secretion associated with a massive β-cell mass

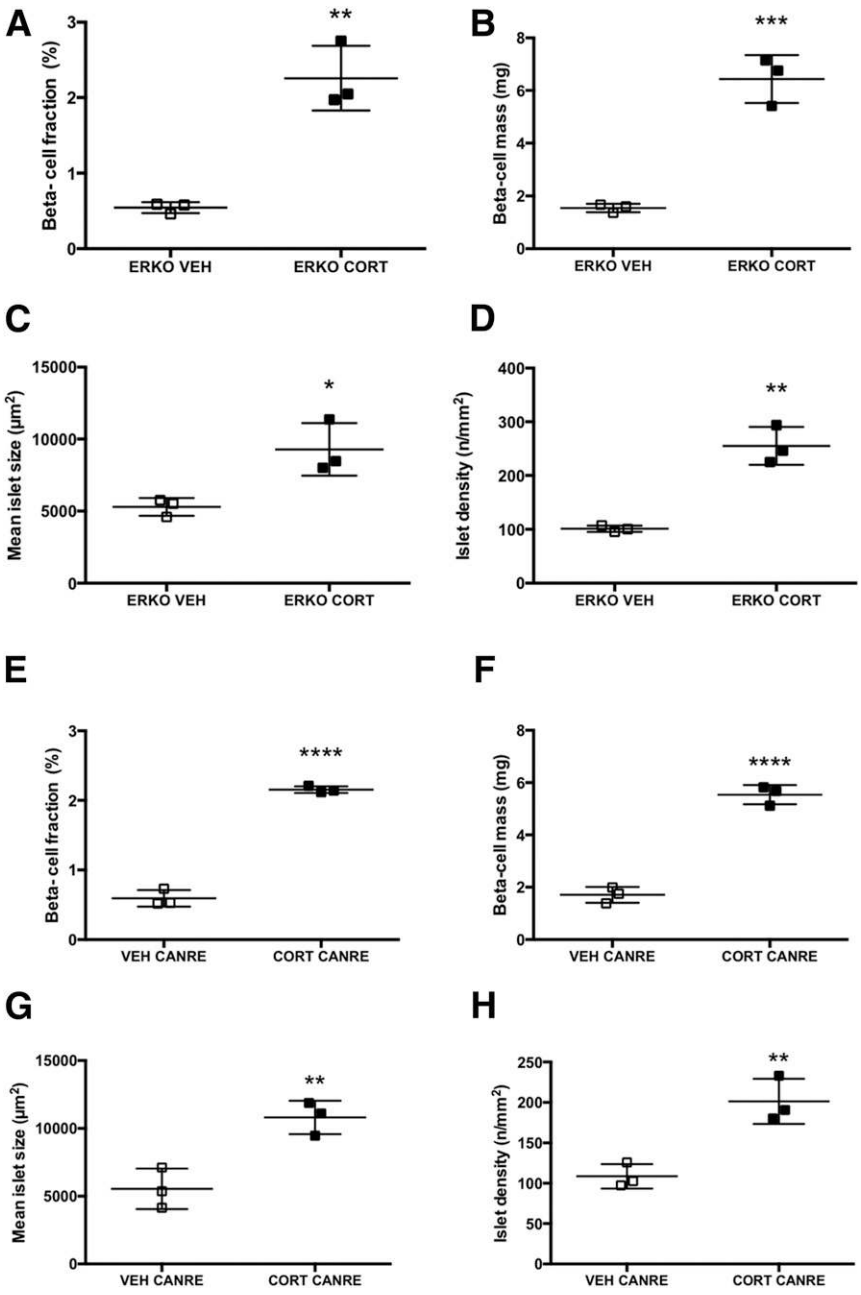

Figure 6— β-Cell neogenesis did not involve the ERα or MR. *A–D*: Pancreatic β-cell fraction, β-cell mass, mean islet size, and islet density were calculated by morphometrical analysis after insulin immunostaining of pancreatic sections from ERKO mice treated with VEH ($n = 3$) or CORT ($n = 3$). *E–H*: Pancreatic β-cell fraction, β-cell mass, mean islet size, and islet density were calculated by morphometrical analysis after insulin immunostaining of pancreatic sections from mice treated with VEH and CANRE ($n = 3$) or CORT and CANRE ($n = 3$). Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ CORT vs. VEH mice.

increase through β-cell proliferation and neogenesis, the latter being independent of Sox9 and Ngn3. We also demonstrate that β-cell neogenesis can partly regenerate β-cells after a chemical depletion. Finally, the data demonstrate that β-cell neogenesis is not a direct effect of GCs but rather, an indirect effect through circulating factors.

β-Cell neogenesis refers to the process of differentiation of precursors located in the pancreas into new β-cells. Studies have shown that it does not participate in the normal homeostasis of β-cells in the adult mouse (7) but may participate in β-cell expansion in specific murine models such as PDL, as shown by Xu et al. (5). Other groups, however, have used or generated elegant lineage-tracing models in the mouse and reported no β-cell neogenesis after PDL (7,10,46). In the current study, we observed β-cell neogenesis as revealed by an increased number of islets in the pancreas of CORT-treated mice. Of note, β-cell neogenesis together with increased proliferation led to a sevenfold increase of β-cell mass, a result that largely exceeds what is observed in mice fed a high-fat diet for

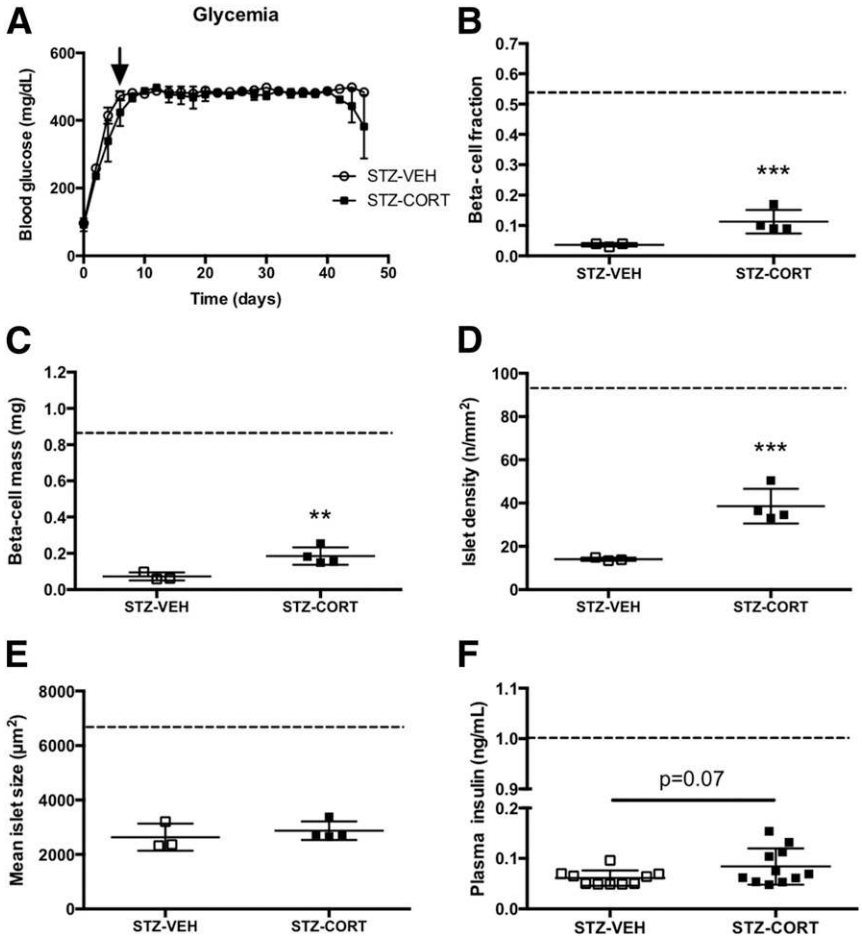

Figure 7—CORT treatment led to partial β-cell regeneration after chemical depletion. *A*: Blood glucose levels in mice after STZ injection (day 0) and treatment with VEH ($n = 10$) or CORT ($n = 10$). The arrow indicates the start of CORT or VEH treatment. *B–E*: Pancreatic β-cell fraction, β-cell mass, islet density, and mean islet size were calculated by morphometrical analysis after insulin immunostaining of pancreatic sections from mice injected with STZ and treated with VEH ($n = 3$) or CORT ($n = 4$). *F*: Blood insulin levels at the end of the treatment in mice injected with STZ and treated with VEH ($n = 10$) or CORT ($n = 11$). Dashed lines represent mean value for mice without STZ injection ($n = 3$–10 per condition). Data are mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ CORT vs. VEH mice.

3 months (47) or in a pharmacological model of insulin resistance in mouse (18). To our knowledge, our model presents the largest increase of β-cell mass in an adult mouse model without genetic modification or pancreatic injury; thus, it represents a unique model to identify mechanisms and signals of β-cell mass adaptation. Of note, our lineage-tracing experiments showed that the neoformed β-cells do not originate from Sox9-expressing cells, as observed in the PDL model (10). Moreover, lineage tracing revealed that neoformed β-cells do not originate from cells having reexpressed Ngn3, a protein usually described as the key pancreatic proendocrine transcription factor, again similarly to what was observed in PDL (48). Further experiments will be required to define the origin of neoformed β-cells in our model. With consideration of transdifferentiation from other pancreatic endocrine cells, we provide here two arguments against such a process: no costaining with other pancreatic cell markers (glucagon or amylase [data not shown]) and, in contrast to other studies (13,14), no depletion, but rather an increase, of α- and δ-cells. These observations favor a global endocrine cell differentiation rather than a specific β-cell neoformation.

In the current study, adult mice exposed to high doses of GCs present an increased β-cell mass in part as a result of β-cell neogenesis. Because we have shown previously that GCs inhibit β-cell differentiation in vivo and in vitro (45,49), it is unlikely that GCs directly activate β-cell neogenesis. Alternatively, we propose that factors present in the serum of CORT-treated mice stimulate β-cell neogenesis. This was demonstrated by our in vitro experiments wherein serum from CORT-treated mice could enhance β-cell differentiation in pancreatic buds, whereas GCs or insulin at the same levels as measured in the serum of CORT-treated mice had either a negative or no effect, respectively, on β-cell differentiation.

Circulating factors able to modulate β-cell mass have been identified previously. For example, El Ouaamari et al. (50) showed that in mice deleted for insulin receptor in the liver, β-cell proliferation is stimulated by the production of a new hepatokine, serpineB1. In our model of β-cell

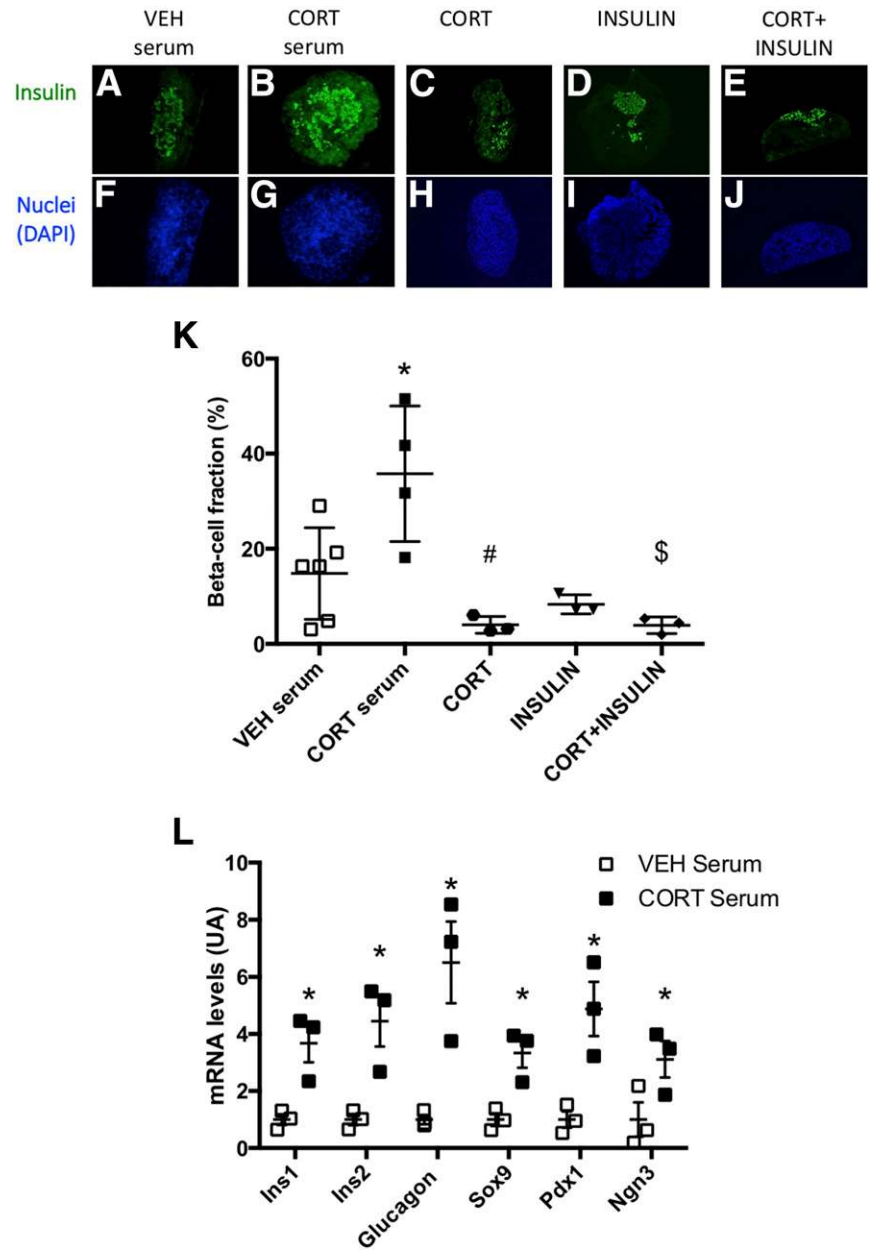

Figure 8— Serum from CORT-treated mice stimulates β-cell neogenesis in embryonic pancreatic buds. *A–J*: Immunofluorescence for insulin and DAPI nuclear staining on representative sections from pancreatic buds cultured 7 days with 10% of VEH serum (*A* and *F*), 10% of CORT serum (*B* and *G*), 10^{-7} mol/L CORT alone (*C* and *H*), 0.4 ng/mL insulin (*D* and *I*), or 10^{-7} mol/L CORT and 0.4 ng/mL insulin (*E* and *J*) supplemented in the serum of VEH mice. Magnification is ×10. *K*: β-Cell fraction in embryonic pancreatic buds after 7 days of culture in the same conditions with 10% of VEH serum ($n = 6$), 10% of CORT serum ($n = 4$), 10^{-7} mol/L CORT alone ($n = 3$), 0.4 ng/mL insulin ($n = 3$), or 10^{-7} mol/L CORT and 0.4 ng/mL insulin ($n = 3$) supplemented in the serum of VEH mice. *L*: mRNA levels coding proteins of endocrine cell differentiation and maturation in pancreatic buds cultured 7 days in the presence of 10% of VEH serum ($n = 3$) or CORT serum ($n = 3$). Data are mean ± SD. *$P < 0.05$ CORT serum–treated buds vs. VEH serum–treated buds; #$P < 0.05$ CORT-treated buds vs. VEH serum–treated buds; \$$P < 0.05$ CORT + insulin–treated buds vs. VEH serum–treated buds. UA, arbitrary unit.

neogenesis, we provide evidence that the serum of CORT-treated mice contains one or several factors that can stimulate β-cell neogenesis in vitro. So far, the origin and nature of such factors remain unknown. In an integrated and adaptive point of view, one may believe that tissues that become insulin resistant upon CORT treatment produce signals to increase insulin production to compensate for the resistance. In fact, a previous study showed that mice with a combined deficiency for insulin receptor and insulin receptor substrate present β-cell hyperplasia that can reach a 30-fold increase (51). Moreover, observations in human have linked insulin resistance with signs for β-cell neogenesis, such as the presence of bihormonal cells (52), high frequency of small clusters of insulin-positive cells (53), or costaining for insulin and the ductal marker cytokeratin 19 (54).

GCs commonly are described as diabetogenic hormones. In fact, not all patients treated with GCs or not all patients with Cushing syndrome develop diabetes (55). GCs induce significant insulin resistance, but if the endocrine pancreas adapts to the increased demand for insulin, normal glucose control is preserved. In humans, low insulin secretion was predictive of diabetes when patients were exposed to chronic GC treatment (56). In our model of CORT administration, insulin resistance develops quickly (from the 1st week of treatment [data not shown]) and associates at 8 weeks with a strong pancreatic adaptation with simultaneous high insulin secretion and a dramatic increase of β-cell mass. In agreement with previously reported studies in mice (57) and macaques (58), CORT-treated mice do not develop hyperglycemia and present improved glucose tolerance. Therefore, CORT treatment can lead to insulin resistance and pancreatic adaptation with high insulin production. Of note, such enhanced insulin production persists when islets are isolated and stimulated with glucose, suggesting that CORT treatment programs improved β-cell function, as previously described in rats (59). We also observed maximal insulin secretion in islets from CORT mice at low- or high-glucose concentration or after KCl exposure, suggesting dysregulated β-cell function. Such abnormal insulin secretion may originate from the augmented Gck expression measured in islets from CORT mice, a change that may trigger enhanced glycolysis flow in β-cells and insulin secretion, as previously described with Gck activators (60) or in congenital hyperinsulinism as a result of hyperactivation of Gck (61).

One major finding is that treating β-cell–depleted STZ mice with CORT leads to a mildly increased β-cell mass resulting from β-cell neogenesis, as revealed by the increased islet density. Despite that insulin was detected in the serum of STZ-CORT mice, however, treatment was not sufficient to recover normoglycemia. This may be due to CORT treatment also inducing insulin resistance in STZ mice, thus reducing the efficiency of the low level of insulin secreted by the regenerated β-cells. Further experiments would be required using other models of β-cell depletion.

When comparing therapeutic strategies aimed at increasing β-cell mass, β-cell neogenesis appears as a choice because in type 1 diabetes, the almost complete depletion of β-cells precludes the use of strategies to stimulate β-cell proliferation, and studies have shown that β-cell proliferation in human is low and difficult to stimulate (62). We provide evidence that severe insulin resistance induced by CORT is associated with β-cell neogenesis and that serum from CORT-treated mice contains factors that can stimulate β-cell neogenesis. The origin and nature of such factors remain to be fully defined. Once identified, these factors may hold great promise for the treatment of diabetes by generating new β-cells and restoring adequate insulin secretion.

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