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Abstract

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

Introduction

Pancreatic beta 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 depicted 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 beta cells. Yet, signals to achieve such goals still need to be identified.

The maintenance of the adequate mass of beta cells is mainly performed through 4 mechanisms: apoptosis (2) and dedifferentiation (3) that diminish the number of beta cells, while proliferation of existing beta cells (4) and neogenesis (differentiation of new beta cells from precursors) (5) increase the number of insulin-producing cells. Of particular interest is beta-cell neogenesis since its induction would lead to beta-cell regeneration in situation of beta-cell depletion such as type 1 diabetes. To identify signals that regulate beta-cell neogenesis, previous studies have used animal models. In rats, partial pancreatectomy is a model of beta-cell regeneration through beta-cell replication and neogenesis (6). In mice, pancreatic duct ligation (PDL) induces an increased beta-cell mass due to beta-cell neogenesis from ductal cells through the re-expression of a transcription factor that drives beta-cell differentiation during fetal life, Neurogenin3 (Ngn3) (5). Yet, further studies using a similar model of PDL failed to reproduce these results and concluded that no beta-cell neogenesis was observed in the adult mice, even after pancreatic injury (7). Concerning the nature of pancreatic precursors, it has been proposed that ducts contain undifferentiated cells that can be recruited to differentiate into

beta-cells (5; 8; 9) but recent studies using different lineage tracing experiments demonstrated that duct cells do not contribute to beta-cell neogenesis (10; 11). Thus, whether beta-cell neogenesis from precursors contributes to beta-cell regeneration remains an open question. Alternatively, beta cells can be formed through transdifferentiation from alpha and delta cells (12; 13) after artificial beta-cell depletion using diphtheria toxin in mice. Similarly, alpha cells can transdifferentiate into beta cells in genetically-modified mice when expression of the key transcription factors Pax4 or Arx is altered in alpha cells (14; 15) or in mice treated with GABA (16). Altogether, these studies provided important insight on pancreatic plasticity and beta-cell neogenesis but were mostly obtained after pancreatic injury or genetic manipulation. Moreover, whether beta-cell neogenesis involved precursors located in ducts remains controversial.

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

A pharmacological model that allows a rapid and severe insulin resistance is the administration of glucocorticoids. Glucocorticoids (GC) are hormones that regulate several physiological processes such as behavior, metabolism and immune response. Because of their potent anti-inflammatory properties, they are widely used to treat inflammatory diseases. Unfortunately, they also induce insulin resistance and consequently increase insulin secretion, but a high proportion of GC-treated patients develop diabetes (22). More specifically, GC are deleterious on 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). Concerning insulin secretion, chronic or acute *in vitro* treatment with GC usually inhibits insulin secretion (24 ; 25) through modifications of the alpha-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). Regarding beta-cell mass, chronic GC treatment in rats and mice leads to increased beta-cell mass due to increased beta-cell proliferation (32). In such models, beta-cell neogenesis has not been fully investigated. Thus, the present study was performed to precisely understand pancreatic adaptations and the underlying mechanisms in response to insulin resistance induced by GC.

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

Research design and methods

Animals

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research and Health (INSERM) and were approved by the local Animal Care and Use Committee. C57BL6/J male and female mice were obtained from the French colony of Charles River laboratories (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 Sox9CreERT;ROSA26YFP mice, respectively. ERα-null mice (ERKO) were generated as previously described (36). Mice were allowed to feed *ad libitum* with a chow diet and housed in 12h light/12h darkness cycles.

Chemicals

Corticosterone

Animals were treated with corticosterone (Sigma-Aldrich, St. Louis, MO; CORT) at the concentration of 100 µg/ml or vehicle (1% ethanol, VEH) in drinking water.

Tamoxifen

8-week-old mice Ngn3CreERTM;ROSA26YFP and Sox9CreERTM;ROSA26YFP were submitted to intraperitoneal tamoxifen injections (60 µg/g/day, see protocols in figure 5, tamoxifen solution 10 mg/ml; MP Biomedicals, Illkirch Graffenstaden, France, TMX, dissolved in filtered olive oil).

Canrenoate

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

Streptozotocin

Beta-cell depletion in C57BL6/J mice was obtained by a single intraperitoneal injection of streptozotocin (STZ, 150 mg/kg, Sigma-Aldrich). Mice with blood glucose levels above 300 mg/dl 3 days after STZ injection were selected for CORT treatment.

5-Bromo-2′-deoxyuridine

Beta-cell proliferation was measured in mice that received a single injection of 5-Bromo-2′-deoxyuridine (BrdU, 50 mg/kg, Sigma-Aldrich) 24h before sacrifice.

Glucose and insulin tolerance tests

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

Hormonal assays

Insulin and corticosterone blood levels were measured using mouse immunoassays (Alpco, Salem, NH) and corticosterone (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 GmbH, 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 re-suspended in PBS, 0.5mM EDTA and 2% fetal calf serum and analyzed with an Aria III cell sorter (BD Biosciences, San Jose, CA, USA).

Immunohistochemistry, immunofluorescence and morphometry

Pancreas were fixed in 3.7% formalin solution, embedded in paraffin and cut in 5µm sections. Morphometrical parameters (beta-cell fraction, islet size and density) were evaluated on 8 pancreatic sections per pancreas after immunohistochemistry using the primary antibodies: guinea pig polyclonal anti-insulin (Dako, Agilent, Santa Clara, CA, USA), rabbit polyclonal anti-glucagon (Dako), rabbit polyclonal anti-somatostatin (Dako), chicken polyclonal anti-GFP (Aves lab, Tigard, OR, USA), monoclonal anti-pancytokeratin (Sigma-Aldrich). Secondary antibodies coupled to horseradish peroxidase or to alkaline phosphastase were obtained from Jackson Immunoresearch (Westgrov, PA, USA). Enzyme substrates were DAB+ (Dako) or Fast-red (Sigma-Aldrich). Morphometrical parameters were determined as previously described (39) and are described in details in the supplemental material and on figure S6. For beta-cell proliferation, antigen retrieval was performed in pH6 citrate buffer for 12 minutes at 95°C using a microwave oven. Sections were incubated with an anti-BrdU antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an anti-insulin antibody (Dako). Secondary antibodies coupled to fluorescent molecules (Thermofisher, Waltham, MA) were then incubated. Pictures were taken on a Leica DMRB fluorescent microscope (Leica, Wetzlar, Germany). At least 5000 cells from several sections were analyzed per animal. Beta-cell proliferation was calculated as the percentage of insulin-positive cells that are also positive for BrdU in the whole insulin-positive cell population.

Pancreatic bud culture and analysis

Mouse pancreatic buds at embryonic day 11.5 (E11.5) 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} M 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, Germany) and reverse transcribed into cDNA with superscript transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression was quantified by real-time PCR using SybrGreen supermix (Eurogentec, Seraing, Belgium) in a MyIQ thermocycler (Biorad, Hercules, CA). The value obtained for each specific gene product was normalized for the 18S ribosomal RNA and expressed as a fold change of the value in control condition. Primers sequences are available upon request.

Statistical Analysis

All results are presented as means ± standard deviations (SD). Comparisons were performed using the Mann-Whitney test, one-way or two-way ANOVA tests. The threshold of significance was considered for a p value <0.05.

Results

Corticosterone treatment induces severe insulin resistance

After 8 weeks of corticosterone (CORT) administration, male mice developed a severe insulin resistance (**Figure 1A**). Yet, despite this reduced insulin sensitivity, CORT mice exhibited a better glucose tolerance compared to vehicle (VEH) **(Figure 1B and inset for Areas Under the Curve, AUC)** associated with much higher plasma insulin levels in the basal state and in response to glucose *in vivo* **(Figure 1C)** and *ex vivo* **(Figure 1D)**.

Pancreatic adaptation after CORT-induced insulin resistance

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

Progressive and continuous pancreatic adaptation

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

Beta-cell adaptation after treatment cessation and re-treatment

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

(Figure 3D) but with a further increase of islet density **(Figure 3E)**, emphasizing the role of neogenesis on beta-cell mass increase. Similarly, this reintroduction of CORT treatment during 4 weeks led again to insulin resistance (**Supplementary figure 3E)** and improved glucose tolerance (**Supplementary figure 3F and G)** with again very high blood insulin levels (**Supplementary figure 3H)**.

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 8 weeks while mRNA levels of Sox9, Pdx-1, Pax4 and MafA remained unchanged and levels of Arx were decreased **(Figure 4A)**. mRNA levels of proteins involved in insulin secretion (Slc30A8, Kir6.2, Sur1, Ins2 and Gck) were increased in islets from CORT mice **(Figure 4B)**. Finally, we observed increased mRNA levels of Ki-67 in islets from CORT mice **(Figure 4C)** as well as an increased percentage of insulin-positive cells that are also positive for BrdU **(Figure 4D)** demonstrating beta-cell proliferation. We also observed an increased number of insulin-expressing cells in ducts (stained for pancytokeratin) after one week of treatment **(Figure 4E and 4G)** and an increased number of islets adjacent to ducts after 1 and 8 weeks of treatment **(Figure 4F and 4H)**. Taken together, these observations suggested that beta-cell mass increase results both from beta-cell proliferation and neogenesis in CORT mice**.**

New beta cells do not derive from Sox9-positive cells

Close proximity of islets to ducts and the presence of insulin-positive cells in ducts are only suggestive of neogenesis arising from ducts. To clearly characterize beta-cell neogenesis, we performed lineage tracing. To this end, we first used tamoxifen (TMX)-inducible Sox9-CreERTM;ROSA26YFP mice in which YFP labelling of ductal cells was induced by TMX

injections. At the end of the TMX treatment, we observed the presence of YFP-labelled cells in ducts on pancreatic sections of control mice (**Supplementary figure 4C and D).** Three weeks after TMX treatment, CORT was administrated during 3 weeks **(Figure 5A)**. Mice treated with TMX and CORT exhibited as expected increased beta-cell fraction and mass and islet density when compared to TMX and VEH mice **(Figure 5B-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 (**Figure 5E-G)**. These results indicated that the increase of beta-cell mass does not involve cells deriving from ductal Sox9-positive cells.

New beta cells do not derive from Ngn3-positive cells

As Ngn3 is a transcription factor required for beta-cell formation during fetal life (41) or during adult beta-cell neogenesis (5), and since Ngn3 mRNA level was increased in islets of mice treated with CORT **(Figure 4A)**, we used Ngn3CreERTM;ROSA26YFP mice to define whether the neoformed beta cells in CORT-treated mice derived from Ngn3-positive cells. Mice were injected twice a week with TMX **(Figure 5H)** together with CORT or VEH treatment. At the end of the treatment we observed an increased beta-cell mass and fraction and a strong tendency ($p=0.05$) for an increased islet density in mice treated with TMX and CORT **(Figure 5I-K).** Flow cytometry analysis revealed that the percentage of YFP-positive cells was similar between the two groups **(Figure 5L-N)**, suggesting that new beta cells do not derive from Ngn3-positive cells. To confirm this observation, we used a different strategy. Since beta-cell neogenesis during pancreatic duct ligation (PDL) was shown to rely on Ngn3 re-expression (5) and on the presence of the estrogen receptor alpha (ERα) (42), we tested the implication of ERα in our model. Mice invalidated for ERα (ERKO) and treated with CORT exhibited a severe insulin resistance similarly to WT mice treated with CORT (**Supplementary figure 5A)**

associated with a glucose intolerance (**Supplementary figure 5B and 5C)** and high plasma insulin levels before and after glucose stimulation (**Supplementary figure 5D)**. ERKO mice also presented increased beta-cell fraction, beta-cell mass, mean islet size and islet density in response to CORT **(Figure 6A-D)**, suggesting that beta-cell neogenesis in CORT mice does not require the presence of the ERα.

Beta-cell neogenesis in mice treated with CORT and a mineralocorticoid receptor antagonist

As high doses of GC are able to activate mineralocorticoid receptor (MR) (43), we tested the implication of this receptor. CORT and VEH mice were given canrenoate (CANRE), a MR antagonist in the drinking water. Metabolic tests revealed that in mice treated with CORT and CANRE, insulin resistance, slightly lower than in CORT mice, was observed associated with an improved glucose tolerance **(Supplementary figure 5E-G)** and high plasma insulin levels before and after glucose stimulation (**Supplementary figure 5H).** 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 beta-cell fraction and mass, as well as an augmented mean islet size and islet density **(Figure 6E to 6H)**, demonstrating that MR antagonism did not prevent beta-cell neogenesis in CORT-treated mice.

Partial beta-cell regeneration with CORT treatment after beta-cell depletion

Since CORT was associated with beta-cell neogenesis in normal mice, we then tested whether CORT treatment could regenerate beta cells after their depletion. A single streptozotocin (STZ) injection was performed in wild type mice leading to overt hyperglycemia within 2-3 days **(Figure 7A)**. When hyperglycemia was stable, STZ mice were given VEH or CORT **(arrow on Figure 7A)** during 8 weeks. No change in blood glucose levels was observed

between VEH and STZ-CORT mice **(Figure 7A)**. Pancreatic analysis revealed a 95% depletion of beta-cell fraction and mass in STZ-VEH mice **(Figure 7B-C)**. As compared to STZ-VEH mice, we observed in STZ-CORT mice a slight but significant increase of beta-cell fraction **(Figure 7B)**, beta-cell mass **(Figure 7C)** and islet density **(Figure 7D)** but no change of mean islet size **(Figure 7E)**. These data suggest that beta-cell regeneration occurred, through beta-cell neogenesis in STZ-CORT mice, leading to a trend for increased plasma insulin levels **(Figure 7F)**.

Serum from CORT mice stimulates beta-cell neogenesis *in vitro*

Since we had shown that GC inhibit beta-cell differentiation (44; 45), we postulated that beta-cell neogenesis in CORT mice was not a direct effect of GC. To test this hypothesis, we cultured pancreatic buds of mouse embryos at day 11.5 that are mostly composed of undifferentiated precursors and that differentiate after 7 days of culture into mature exocrine and endocrine cells (40). Treatment of pancreatic buds with 10% of serum from CORT-treated mice for 7 days increased beta-cell fraction **(Figure 8B and 8K)**. Since 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 buds' differentiation. Serum from fed CORT mice contain 4.4 ± 1.4 ng/ml insulin (Figure 1C) and 1.6 ± 0.15 10^{-6} M CORT. We used 10% of these concentrations to mimic insulin and CORT levels present when buds were exposed to 10% of serum from CORT mice. Exposure to CORT alone decreased beta-cell fraction in pancreatic buds **(Figure 8C and 8K)** while insulin alone had no effect **(Figure 8D and 8K)**. Finally, we treated pancreatic buds with both CORT and insulin and observed a decreased beta-cell fraction similar to CORT alone **(Figure 8E and 8K)**. We also observed that serum from CORT-treated mice increased the mRNA levels of Ins1, Ins2, Glucagon, Sox9, Pdx1 and Ngn3 in pancreatic

buds **(Figure 8L)**. Altogether, these results suggest that serum from CORT-treated mice contains factor(s) able to stimulate beta-cell neogenesis *in vitro*.

Discussion

GC are known to modulate glucose homeostasis and to induce tissue remodeling. The pool of pancreatic beta cells is plastic and adapts to changes in insulin demand. In the present study, we showed that chronic GC administration in the adult mouse leads to insulin resistance, increased insulin secretion associated with a massive beta-cell mass increase through beta-cell proliferation and neogenesis, the latter being independent of Sox9 and Ngn3. We also demonstrated that beta-cell neogenesis can partly regenerate beta cells after a chemical depletion. Finally, our data demonstrated that beta-cell neogenesis is not a direct effect of GC but rather an indirect effect through circulating factor(s).

Beta-cell neogenesis refers to the process of differentiation of precursors located in the pancreas into new beta cells. Studies have shown that it does not participate to the normal homeostasis of beta cells in the adult mouse (7) but it may participate to beta-cell expansion in specific murine models such as partial duct ligation (PDL), as shown by the group of Heimberg (5). Yet, other groups have used or generated elegant lineage tracing models in the mouse and reported no beta-cell neogenesis after PDL (7; 10; 46). In the present study, we observed beta-cell neogenesis as revealed by an increased number of islets in the pancreas of CORT-treated mice. Impressively, beta-cell neogenesis together with increased proliferation led to 7-fold increase of beta-cell mass, a result that largely exceeds what is observed in mice fed a high-fat diet feeding for 3 months (47) or in a pharmacological model of insulin resistance in the mouse (18). To our knowledge, our model presents the largest increase of beta-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 beta-cell mass adaptation. Interestingly, our lineage tracing experiments showed that the neoformed beta cells did not originate from Sox9-expressing cells, as observed in the PDL model (10). More surprisingly, lineage tracing also revealed that the neoformed beta cells do not originate from cells having re-expressed Ngn3, a

protein usually described as the key pancreatic pro-endocrine transcription factor, again similarly to what was observed in PDL (48). Further experiments will be required to define the origin of neoformed beta cells in our model. Considering transdifferentiation from other pancreatic endocrine cells, we provide here two arguments against such process: no co-staining 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 alpha and delta cells. These observations are in favor of a global endocrine cell differentiation, rather than a specific beta-cell neoformation.

Here, adult mice exposed to high doses of GC present an increased beta-cell mass in part due to beta-cell neogenesis. Since we have previously shown that GC inhibit beta-cell differentiation *in vivo* and *in vitro* (45; 49), it is very unlikely that GC directly activate beta-cell neogenesis. Alternatively, we propose that factor(s) present in the serum of CORT-treated mice stimulate beta-cell neogenesis. This was demonstrated by our *in vitro* experiments: serum from CORT-treated mice could enhance beta-cell differentiation in pancreatic buds while GC or insulin, at the same levels as measured in the serum of CORT-treated mice, had either a negative or no effect, respectively, on beta-cell differentiation.

Circulating factors able to modulate beta-cell mass have been previously identified. For example, Kulkarni's group showed that in mice deleted for the insulin receptor in the liver, beta-cell proliferation was stimulated by the production of a new hepatokine Serpine B1 (50). In our model of beta-cell neogenesis, we provide evidence that the serum of CORT-treated mice contains one or several factor(s) that can stimulate beta-cell neogenesis *in vitro*. So far, the origin and nature of such factor(s) remain unknown. In an integrated and adaptive point of view, one may think 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 the insulin receptor and for the insulin receptor

substrate present beta-cell hyperplasia that can reach a 30-fold increase (51). Moreover, observations in human have linked insulin resistance with signs for beta-cell neogenesis such as the presence of bi-hormonal cells (52), high frequency of small clusters of insulin-positive cells (53), or co-staining for insulin and the ductal marker cytokeratin 19 (54).

GC are commonly described as diabetogenic hormones. In fact, not all patients treated with GC or not all patients with Cushing syndrome develop diabetes (55). GC 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 subjects were exposed to chronic GC treatment (56). In our model of CORT administration, insulin resistance develops quickly (from the first 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 beta-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. Interestingly, such enhanced insulin production persisted when islets were isolated and stimulated with glucose, suggesting that CORT treatment programmed improved beta-cell function, as previously described in the rat (59). Actually, we observed maximal insulin secretion in islets from CORT mice also at low or high glucose concentration or after KCl exposure, suggesting dysregulated beta-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 beta cells and insulin secretion, as previously described with Gck activators (60) or in congenital hyperinsulinism due to hyperactivation of Gck (61).

One major finding is that treating beta-cell-depleted STZ mice with CORT led to a mildly increased beta-cell mass, resulting from beta-cell neogenesis, as revealed by the

increased islet density. Yet, despite the fact that insulin was detected in the serum of STZ-CORT mice, treatment was not sufficient to recover normoglycemia. This may be due to the fact that CORT treatment also induced insulin resistance in STZ mice, thus reducing the efficiency of the low level of insulin secreted by the regenerated beta cells. Further experiments would be required using other models of beta-cell depletion.

When comparing therapeutical strategies aiming at increasing beta-cell mass, beta-cell neogenesis appears as an interesting choice because in type 1 diabetes, the almost complete depletion of beta cells precludes from using strategies to stimulate beta-cell proliferation and studies have shown that beta-cell proliferation in human is low and hard to stimulate (62). Here, we provide evidence that severe insulin resistance induced by CORT associates with beta-cell neogenesis and that serum from such CORT-treated mice contains factor(s) that can stimulate beta-cell neogenesis. The origin and nature of such factor(s) remain to be fully defined. Once identified, such factor(s) may hold great promises for the treatment of diabetes by generating new beta cells and restoring adequate insulin secretion.

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Author contributions

E.C., A.B. and B.B. wrote the manuscript and researched data. A.L., F.M.A., E.Q. and Ma.Bu. researched data. G.G., T.T.H.D. and Ma.Bu. researched data and reviewed/edited the manuscript. P.G., J.F.G., J.P.R., C.H. and B.F. contributed to discussion and reviewed/edited the manuscript.

B.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of Interest

No potential conflicts of interest relevant to this article were reported.

References

1. Cavaghan MK, Ehrmann DA, Polonsky KS: Interactions between insulin resistance and insulin secretion in the development of glucose intolerance. J Clin Invest 2000;106:329-333

2. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 2003;52:102-110

3. Talchai C, Xuan S, Lin HV, Sussel L, Accili D: Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell 2012;150:1223-1234

4. Bernal-Mizrachi E, Kulkarni RN, Scott DK, Mauvais-Jarvis F, Stewart AF, Garcia-Ocana A: Human beta-cell proliferation and intracellular signaling part 2: still driving in the dark without a road map. Diabetes 2014;63:819-831

5. Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de CM, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharfmann R, Gradwohl G, Heimberg H: Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207

6. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE: A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 1993;42:1715-1720

7. Xiao X, Chen Z, Shiota C, Prasadan K, Guo P, El-Gohary Y, Paredes J, Welsh C, Wiersch J, Gittes GK: No evidence for beta cell neogenesis in murine adult pancreas. J Clin Invest 2013;123:2207-2217

8. Criscimanna A, Speicher JA, Houshmand G, Shiota C, Prasadan K, Ji B, Logsdon CD, Gittes GK, Esni F: Duct cells contribute to regeneration of endocrine and acinar cells following pancreatic damage in adult mice. Gastroenterology 2011;141:1451-1462, 1462 e1451-1456

9. Inada A, Nienaber C, Katsuta H, Fujitani Y, Levine J, Morita R, Sharma A, Bonner-Weir S: Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci U S A 2008;105:19915-19919

10. Kopp JL, Dubois CL, Schaffer AE, Hao E, Shih HP, Seymour PA, Ma J, Sander M: Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development 2011;138:653-665

11. Solar M, Cardalda C, Houbracken I, Martin M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L, Ferrer J: Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. Dev Cell 2009;17:849-860

12. Chera S, Baronnier D, Ghila L, Cigliola V, Jensen JN, Gu G, Furuyama K, Thorel F, Gribble FM, Reimann F, Herrera PL: Diabetes recovery by age-dependent conversion of pancreatic delta-cells into insulin producers. Nature 2014;514:503-507

13. Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL: Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-1154
14. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A: The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 2009;138:449-462
15. Courtney M, Gjernes E, Druelle N, Ravaud C, Vieira A, Ben-Othman N, Pfeifer A, Avolio F, Leuckx G, Lacas-Gervais S, Burel-Vandenbos F, Ambrosetti D, Hecksher-Sorensen J, Ravassard P, Heimberg H, Mansouri A, Collombat P: The inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion into functional beta-like cells. PLoS Genet 2013;9:e1003934
16. Ben-Othman N, Vieira A, Courtney M, Record F, Gjernes E, Avolio F, Hadzic B, Druelle N, Napolitano T, Navarro-Sanz S, Silvano S, Al-Hasani K, Pfeifer A, Lacas-Gervais S, Leuckx G, Marroqui L, Thevenet J, Madsen OD, Eizirik DL, Heimberg H, Kerr-Conte J, Pattou F, Mansouri A, Collombat P: Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis. Cell 2017;168:73-85 e11
17. Golson ML, Misfeldt AA, Kopsombut UG, Petersen CP, Gannon M: High Fat Diet Regulation of beta-Cell Proliferation and beta-Cell Mass. Open Endocrinol J 2010;4
18. Gusarova V, Alexa CA, Na E, Stevis PE, Xin Y, Bonner-Weir S, Cohen JC, Hobbs HH, Murphy AJ, Yancopoulos GD, Gromada J: ANGPTL8/Betatrophin Does Not Control Pancreatic Beta Cell Expansion. Cell 2014;159:691-696
19. Sorenson RL, Brelje TC: Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res 1997;29:301-307
20. Toselli C, Hyslop CM, Hughes M, Natale DR, Santamaria P, Huang CT: Contribution of a non-beta-cell source to beta-cell mass during pregnancy. PLoS One 2014;9:e100398
21. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, Butler PC: Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 2010;53:2167-2176
22. Gulliford MC, Charlton J, Latinovic R: Risk of diabetes associated with prescribed glucocorticoids in a large population. Diabetes Care 2006;29:2728-2729
23. Garabedian MJ, Harris CA, Jeanneteau F: Glucocorticoid receptor action in metabolic and neuronal function. F1000Res 2017;6:1208
24. Lambillotte C, Gilon P, Henquin JC: Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest 1997;99:414-423

25. Rafacho A, Ortsater H, Nadal A, Quesada I: Glucocorticoid treatment and endocrine pancreas function: implications for glucose homeostasis, insulin resistance and diabetes. J Endocrinol 2014;223:R49-62
26. Barseghian G, Levine R: Effect of corticosterone on insulin and glucagon secretion by the isolated perfused rat pancreas. Endocrinology 1980;106:547-552
27. Fransson L, Franzen S, Rosengren V, Wolbert P, Sjoholm A, Ortsater H: beta-Cell adaptation in a mouse model of glucocorticoid-induced metabolic syndrome. J Endocrinol 2013;219:231-241
28. Karlsson S, Ostlund B, Myrsen-Axcrona U, Sundler F, Ahren B: Beta cell adaptation to dexamethasone-induced insulin resistance in rats involves increased glucose responsiveness but not glucose effectiveness. Pancreas 2001;22:148-156
29. Rafacho A, Giozzet VA, Boschero AC, Bosqueiro JR: Functional alterations in endocrine pancreas of rats with different degrees of dexamethasone-induced insulin resistance. Pancreas 2008;36:284-293
30. Rafacho A, Marroqui L, Taboga SR, Abrantes JL, Silveira LR, Boschero AC, Carneiro EM, Bosqueiro JR, Nadal A, Quesada I: Glucocorticoids in vivo induce both insulin hypersecretion and enhanced glucose sensitivity of stimulus-secretion coupling in isolated rat islets. Endocrinology 2010;151:85-95
31. Rafacho A, Quallio S, Ribeiro DL, Taboga SR, Paula FM, Boschero AC, Bosqueiro JR: The adaptive compensations in endocrine pancreas from glucocorticoid-treated rats are reversible after the interruption of treatment. Acta Physiol (Oxf) 2010;200:223-235
32. Protzek AO, Rezende LF, Costa-Junior JM, Ferreira SM, Cappelli AP, de Paula FM, de Souza JC, Kurauti MA, Carneiro EM, Rafacho A, Boschero AC: Hyperinsulinemia caused by dexamethasone treatment is associated with reduced insulin clearance and lower hepatic activity of insulin-degrading enzyme. J Steroid Biochem Mol Biol 2016;155:1-8
33. Gu G, Dubauskaite J, Melton DA: Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447-2457
34. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M: SOX9 is required for maintenance of the pancreatic progenitor cell pool. ProcNatlAcadSciUSA 2007;104:1865-1870
35. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F: Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC developmental biology 2001;1:4

36. Handgraaf S, Riant E, Fabre A, Waget A, Burcelin R, Liere P, Krust A, Chambon P, Arnal JF, Gourdy P: Prevention of obesity and insulin resistance by estrogens requires ERalpha activation function-2 (ERalphaAF-2), whereas ERalphaAF-1 is dispensable. Diabetes 2013;62:4098-4108
37. Valtat B, Riveline JP, Zhang P, Singh-Estivalet A, Armanet M, Venteclef N, Besseiche A, Kelly DP, Tronche F, Ferre P, Gautier JF, Breant B, Blondeau B: Fetal PGC-1alpha overexpression programs adult pancreatic beta-cell dysfunction. Diabetes 2013;62:1206-1216
38. Besseiche A, Riveline JP, Delavallee L, Foufelle F, Gautier JF, Blondeau B: Oxidative and energetic stresses mediate beta-cell dysfunction induced by PGC-1alpha. Diabetes Metab 2018;44:45-54
39. Blondeau B, Sahly I, Massourides E, Singh-Estivalet A, Valtat B, Dorchene D, Jaisser F, Breant B, Tronche F: Novel transgenic mice for inducible gene overexpression in pancreatic cells define glucocorticoid receptor-mediated regulations of Beta cells. PLoS One 2012;7:e30210
40. Filhoulaud G, Guillemain G, Scharfmann R: The hexosamine biosynthesis pathway is essential for pancreatic beta cell development. J Biol Chem 2009;284:24583-24594
41. Gradwohl G, Dierich A, LeMeur M, Guillemot F: neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. ProcNatlAcadSciUSA 2000;97:1607-1611
42. Yuchi Y, Cai Y, Legein B, De Groef S, Leuckx G, Coppens V, Van Overmeire E, Staels W, De Leu N, Martens G, Van Ginderachter JA, Heimberg H, Van de Casteele M: Estrogen Receptor alpha Regulates beta-Cell Formation During Pancreas Development and Following Injury. Diabetes 2015;64:3218-3228
43. Caprio M, Feve B, Claes A, Viengchareun S, Lombes M, Zennaro MC: Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. FASEB J 2007;21:2185-2194
44. Gesina E, Blondeau B, Milet A, Le NI, Duchene B, Czernichow P, Scharfmann R, Tronche F, Breant B: Glucocorticoid signalling affects pancreatic development through both direct and indirect effects. Diabetologia 2006;49:2939-2947
45. Valtat B, Dupuis C, Zenaty D, Singh-Estivalet A, Tronche F, Breant B, Blondeau B: Genetic evidence of the programming of beta cell mass and function by glucocorticoids in mice. Diabetologia 2011;54:350-359
46. Dor Y, Brown J, Martinez OI, Melton DA: Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41-46

47. Ahren J, Ahren B, Wierup N: Increased beta-cell volume in mice fed a high-fat diet: a dynamic study over 12 months. Islets 2010;2:353-356
48. Xiao X, Guo P, Shiota C, Prasadan K, El-Gohary Y, Wiersch J, Gaffar I, Gittes GK: Neurogenin3 activation is not sufficient to direct duct-to-beta cell transdifferentiation in the adult pancreas. J Biol Chem 2013;288:25297-25308
49. Gesina E, Tronche F, Herrera P, Duchene B, Tales W, Czernichow P, Breant B: Dissecting the role of glucocorticoids on pancreas development. Diabetes 2004;53:2322-2329
50. El Ouaamari A, Dirice E, Gedeon N, Hu J, Zhou JY, Shirakawa J, Hou L, Goodman J, Karampelias C, Qiang G, Boucher J, Martinez R, Gritsenko MA, De Jesus DF, Kahraman S, Bhatt S, Smith RD, Beer HD, Jungtrakoon P, Gong Y, Goldfine AB, Liew CW, Doria A, Andersson O, Qian WJ, Remold-O'Donnell E, Kulkarni RN: SerpinB1 Promotes Pancreatic beta Cell Proliferation. Cell Metab 2016;23:194-205
51. Bruning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili D, Kahn CR: Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Cell 1997;88:561-572
52. Yoneda S, Uno S, Iwahashi H, Fujita Y, Yoshikawa A, Kozawa J, Okita K, Takiuchi D, Eguchi H, Nagano H, Imagawa A, Shimomura I: Predominance of beta-Cell Neogenesis Rather Than Replication in Humans With an Impaired Glucose Tolerance and Newly Diagnosed Diabetes. J Clin Endocrinol Metab 2013;
53. Hanley SC, Austin E, Assouline-Thomas B, Kapeluto J, Blaichman J, Moosavi M, Petropavlovskaia M, Rosenberg L: {beta}-Cell mass dynamics and islet cell plasticity in human type 2 diabetes. Endocrinology 2010;151:1462-1472
54. Mezza T, Muscogiuri G, Sorice GP, Clemente G, Hu J, Pontecorvi A, Holst JJ, Giaccari A, Kulkarni RN: Insulin resistance alters islet morphology in nondiabetic humans. Diabetes 2014;63:994-1007
55. Hwang JL, Weiss RE: Steroid-induced diabetes: a clinical and molecular approach to understanding and treatment. Diabetes Metab Res Rev 2014;30:96-102
56. Wajngot A, Giacca A, Grill V, Vranic M, Efendic S: The diabetogenic effects of glucocorticoids are more pronounced in low- than in high-insulin responders. Proc Natl Acad Sci U S A 1992;89:6035-6039
57. Boortz KA, Syring KE, Lee RA, Dai C, Oeser JK, McGuinness OP, Wang JC, O'Brien RM: G6PC2 Modulates the Effects of Dexamethasone on Fasting Blood Glucose and Glucose Tolerance. Endocrinology 2016;157:4133-4145

58. Cummings BP, Bremer AA, Kieffer TJ, D'Alessio D, Havel PJ: Investigation of the mechanisms contributing to the compensatory increase in insulin secretion during dexamethasone-induced insulin resistance in rhesus macaques. J Endocrinol 2013;216:207-215
59. Protzek AO, Costa-Junior JM, Rezende LF, Santos GJ, Araujo TG, Vettorazzi JF, Ortis F, Carneiro EM, Rafacho A, Boschero AC: Augmented beta-Cell Function and Mass in Glucocorticoid-Treated Rodents Are Associated with Increased Islet Ir-beta /AKT/mTOR and Decreased AMPK/ACC and AS160 Signaling. Int J Endocrinol 2014;2014:983453
60. Nakamura A, Togashi Y, Orime K, Sato K, Shirakawa J, Ohsugi M, Kubota N, Kadowaki T, Terauchi Y: Control of beta cell function and proliferation in mice stimulated by small-molecule glucokinase activator under various conditions. Diabetologia 2012;55:1745-1754
61. Henquin JC, Sempoux C, Marchandise J, Godecharles S, Guiot Y, Nenquin M, Rahier J: Congenital hyperinsulinism caused by hexokinase I expression or glucokinase-activating mutation in a subset of beta-cells. Diabetes 2013;62:1689-1696
62. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC: Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 2008;57:1584-1594

Legends to figures

Figure 1: Metabolic parameters and pancreatic analysis of mice treated with CORT for 8 weeks

(A) ITT was carried out on mice treated with CORT (black squares, n=8) or VEH (open squares, 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 glycaemia. **(B)** IPGTT was performed after an 18-h fasting. Glucose was injected (2 g/kg) on mice treated with CORT (black squares, n=7) or VEH (open squares, n=10). Areas under the curve are shown in the inset **(C)** Plasma insulin levels during IPGTT (at T0 and T15 following glucose challenge) in mice treated with CORT (black squares, n=7) or VEH (white squares, n=6). **(D)** Insulin secretion was tested in response to 2.8 mM (white squares), 16.7 mM glucose (grey triangles) or 50 mM KCl (black triangles) and measured in isolated pancreatic islets from control (VEH, left, n=4) and CORT-treated mice (CORT, right, n=4). Values are expressed as means $\pm$ SD. # $p<0.05$ VEH 16.7 mM and VEH KCL *versus* VEH 2.8 mM glucose. Representative pictures of insulin immunostaining (brown) on pancreatic sections of mice treated with VEH **(E)** or CORT **(F)** during 8 weeks. Counterstaining was performed using hematoxylin**. (G)** Pancreatic beta-cell fraction, **(H)** beta-cell mass, **(I)** mean islet size, **(J)** islet density, **(K)** islet distribution and **(L)** individual beta-cell area after insulin or glucagon immunostaining on 8 pancreatic slides from each animal, after 8 weeks of treatment with CORT (black squares or bars, n=7) or VEH (white square or bars, n=7). **(M)** Percentage of alpha (white squares) or beta (black squares) cells within islets after insulin and glucagon immunofluorescence on pancreatic slides after 8 weeks of treatment with CORT (right, n=5) or VEH (left, n=5). Values are expressed as the means $\pm$ SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$ CORT *versus* VEH mice.

Figure 2: Pancreatic adaptation throughout corticosterone administration

(A) Pancreatic beta-cell fraction, **(B)** beta-cell mass, **(C)** mean islet size, and **(D)** islet density was quantified after insulin immunostaining on 8 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 weeks (n=7 in VEH and CORT) of VEH (white squares) or CORT (black squares) treatment. Values are expressed as the means $\pm$ SD. * p <0.05; ** p<0.01; *** p<0.001; **** p<0.0001 CORT *versus* VEH mice. ## p<0.01 when comparing CORT-treated mice at different points of treatment.

Figure 3: The beta-cell mass adaptation is reversible and can be re-induced

(A) Scheme of the protocol. During 4 weeks, mice were treated with CORT or VEH (I). Mice were then submitted to a 4-weeks washout (II). Finally, mice were again treated with CORT or VEH for 4 weeks (III). At the end of each stage, pancreata were dissected and analyzed. **(B)** Pancreatic beta-cell fraction, **(C)** beta-cell mass, **(D**) mean islet size, and **(E)** islet density were quantified after insulin immunostaining on 8 pancreatic slides from each animal. Values of mice treated with CORT (black squares, n=5 after the first treatment, n=3 after the washout, n=3 after the retreatment) or VEH (white squares, n=5 after the first treatment, n=3 after the washout, n=3 after the retreatment) are expressed as fold increase relative to the corresponding group of mice treated with VEH. Values are expressed as means $\pm$ SD. * p <0.05; ** p<0.01; *** p<0.001 CORT *versus* VEH mice. $ p <0.05; $$ p<0.01 mice retreated with CORT *versus* mice treated with CORT and submitted to the washout (WO). ## p<0.01 mice retreated with CORT *versus* mice treated with CORT during 4 weeks. WO: Grey background

Figure 4: Chronic corticosterone treatment is associated with signs of beta-cell neogenesis

(A, B and C) mRNA levels in isolated adult pancreatic islets from mice treated with VEH or CORT during 8 weeks; **(A)** mRNA levels of transcription factors involved in pancreatic development and endocrine cell maturation; **(B)** mRNA levels of genes encoding proteins involved in hormone secretion; and **(C)** mRNA levels of Ki67 islets from mice treated with VEH (white squares, n=3) or CORT (black squares, n=3). **(D)** Percentage of cells positive for insulin and BrdU within insulin-positive cells in pancreas from mice treated with VEH (white squares, n=5) or CORT (black squares, n=5). **(E and F)** Pictures of co-immunostaining for insulin (red) and pan-cytokeratin (brown) performed on pancreatic sections from mice treated with CORT during 8 weeks. (d: ducts; arrow points to beta cells in the ductal epithelium). **(G)** Quantification of ducts containing insulin cells and **(H)** pancreatic islets adjacent to ducts in pancreas from mice treated with VEH (white squares, n=3 at 1 week and n=4 at 8 weeks) or CORT (black squares, n=3 at 1 week and n=4 at 8 weeks). Values are expressed as means $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ CORT *versus* VEH mice. # $p < 0.05$ CORT 8 weeks *versus* CORT 1 week.

Figure 5: New beta-cells did not derive from Sox9- or Ngn3-positive cells after CORT treatment

(A) Scheme of Sox9CreERTM;ROSA26YFP mice generation (left panel) and the lineage tracing experiment (right panel). Tamoxifen (TMX) was administrated by 4 daily injections during the first week. After 3 weeks, CORT or VEH was administrated during 3 weeks. **(B)** Pancreatic beta-cell fraction, **(C)** beta-cell mass and **(D)** islet density were calculated by morphometrical analysis in VEH-treated mice (white squares, n=5) and in CORT-treated mice (black squares, n=8). Representative panels of flow cytometry based on (FITC-A; y-axis) and cellular granulation (FSC-A; x-axis) of trypsinized cells from islets isolated from a mouse treated with VEH **(E)** or with CORT **(F)**. **(G)** Quantification of YFP-positive cells in VEH

(VEH TMX, white squares, n=3) and CORT (CORT TMX, black squares, n=4). **(H)** Scheme of Ngn3CreERTM; ROSA26YFP mice generation (left panel) and the lineage tracing experiment (right panel). Tamoxifen (TMX) was administrated 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)** Pancreatic beta-cell fraction, **(J)** beta-cell mass and **(K)** islet density were calculated by morphometrical analysis in VEH-treated mice (white squares, n=3) and in CORT-treated mice (black squares, n=3). Representative panels of flow cytometry based on (FITC-A; y-axis) and cellular granulation (FSC-A; x-axis) of trypsinized cells from islets isolated from a mouse treated with VEH **(L)** or with CORT **(M)**. **(N)** Quantification of YFP-positive cells in VEH (VEH TMX, white squares, n=3) and CORT (CORT TMX, black squares, n=4). Values are expressed as means ± SD. * $p < 0.05$; ** $p < 0.01$ CORT *versus* VEH mice.

Figure 6: Beta-cell neogenesis did not involve the estrogen receptor α (ERα) or the mineralocorticoid receptor (MR)

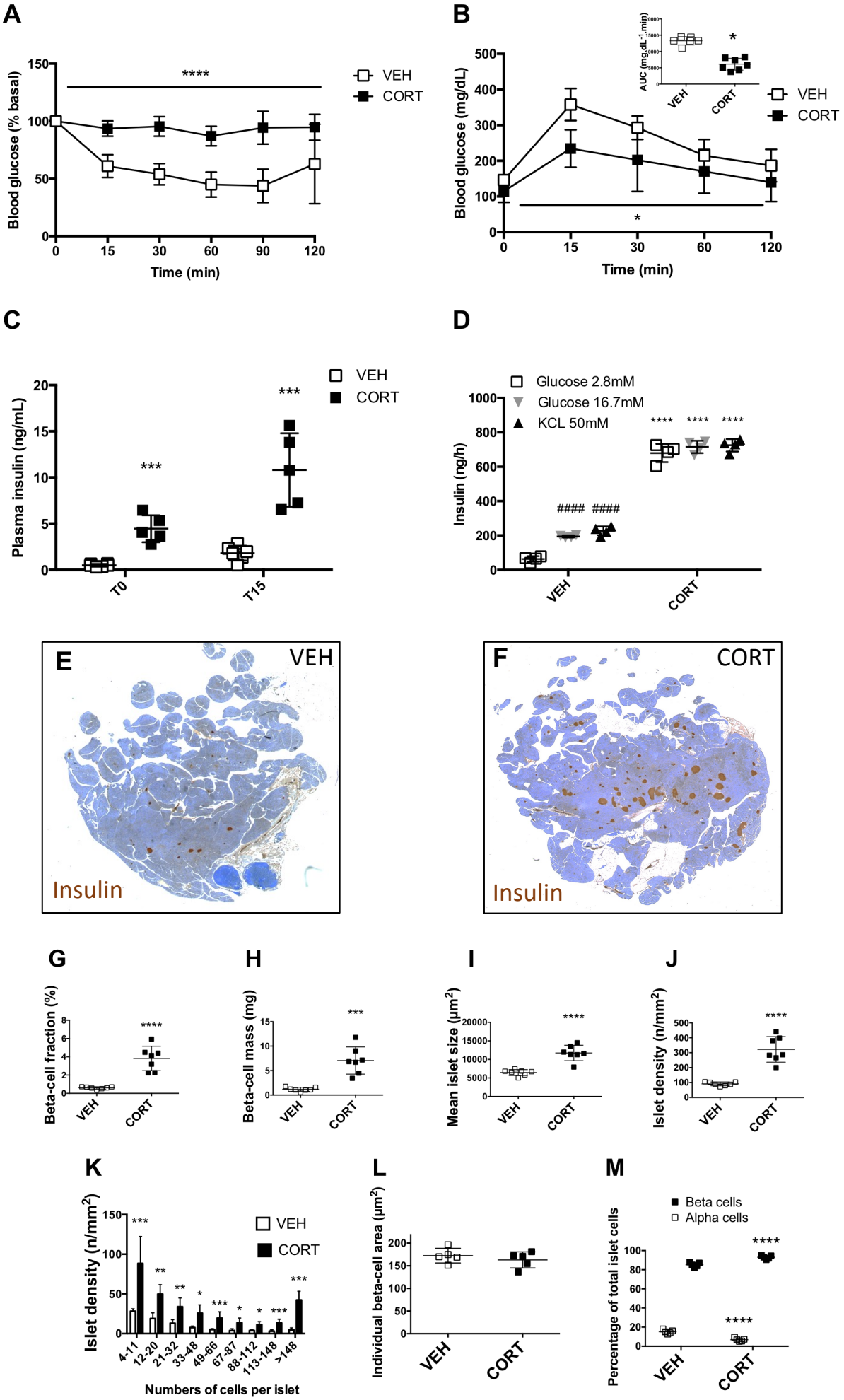
(A) Pancreatic beta-cell fraction, **(B)** beta-cell mass, **(C)** mean islet size and **(D)** islet density were calculated by morphometrical analysis after insulin immunostaining on pancreatic sections from ERKO mice treated with VEH (white squares, n=3) or with CORT (black squares, n=3). **(E)** Pancreatic beta-cell fraction, **(F)** beta-cell mass, **(G)** mean islet size and **(H)** islet density were calculated by morphometrical analysis after insulin immunostaining on pancreatic sections from mice treated with VEH and CANRE (white squares, n=3) or mice treated with CORT and CANRE (black squares, n=3). Values are expressed as means ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ CORT *versus* VEH mice.

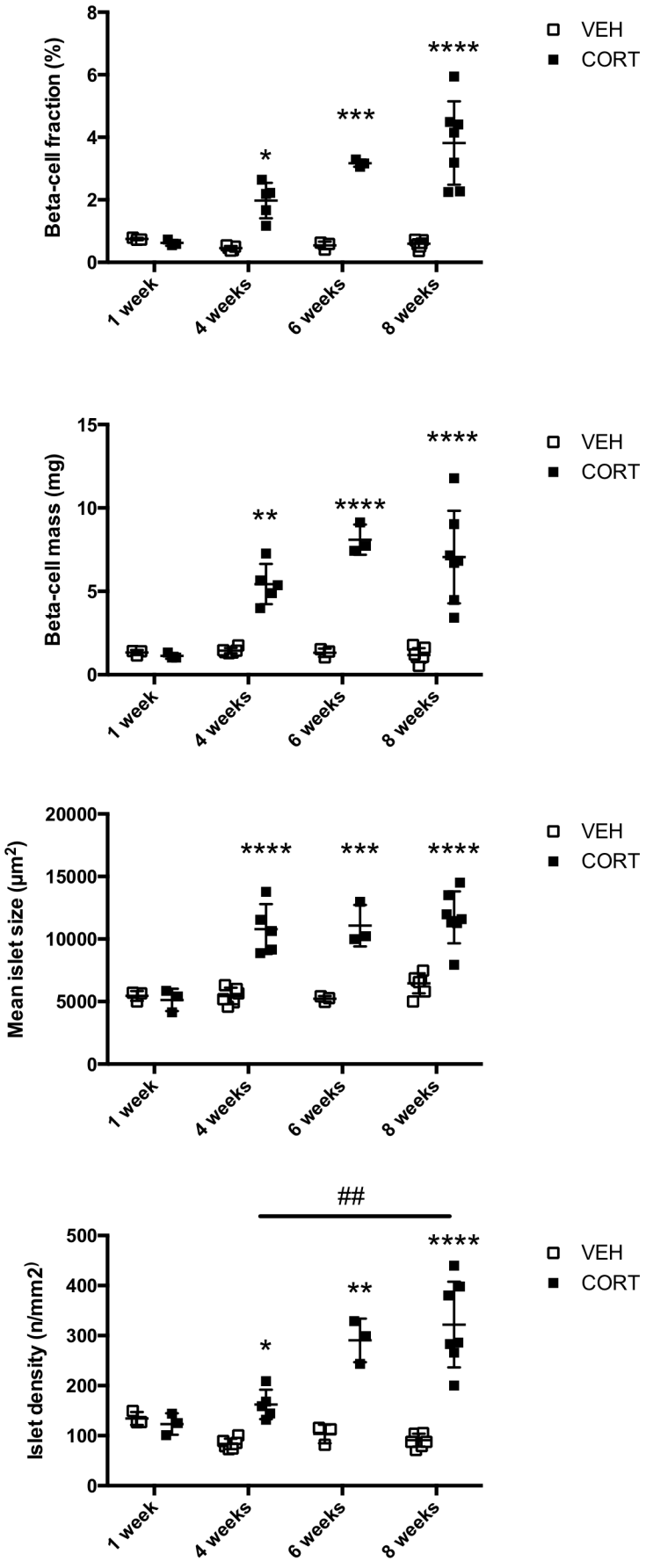
Figure 7: Corticosterone treatment led to partial beta-cell regeneration after chemical depletion

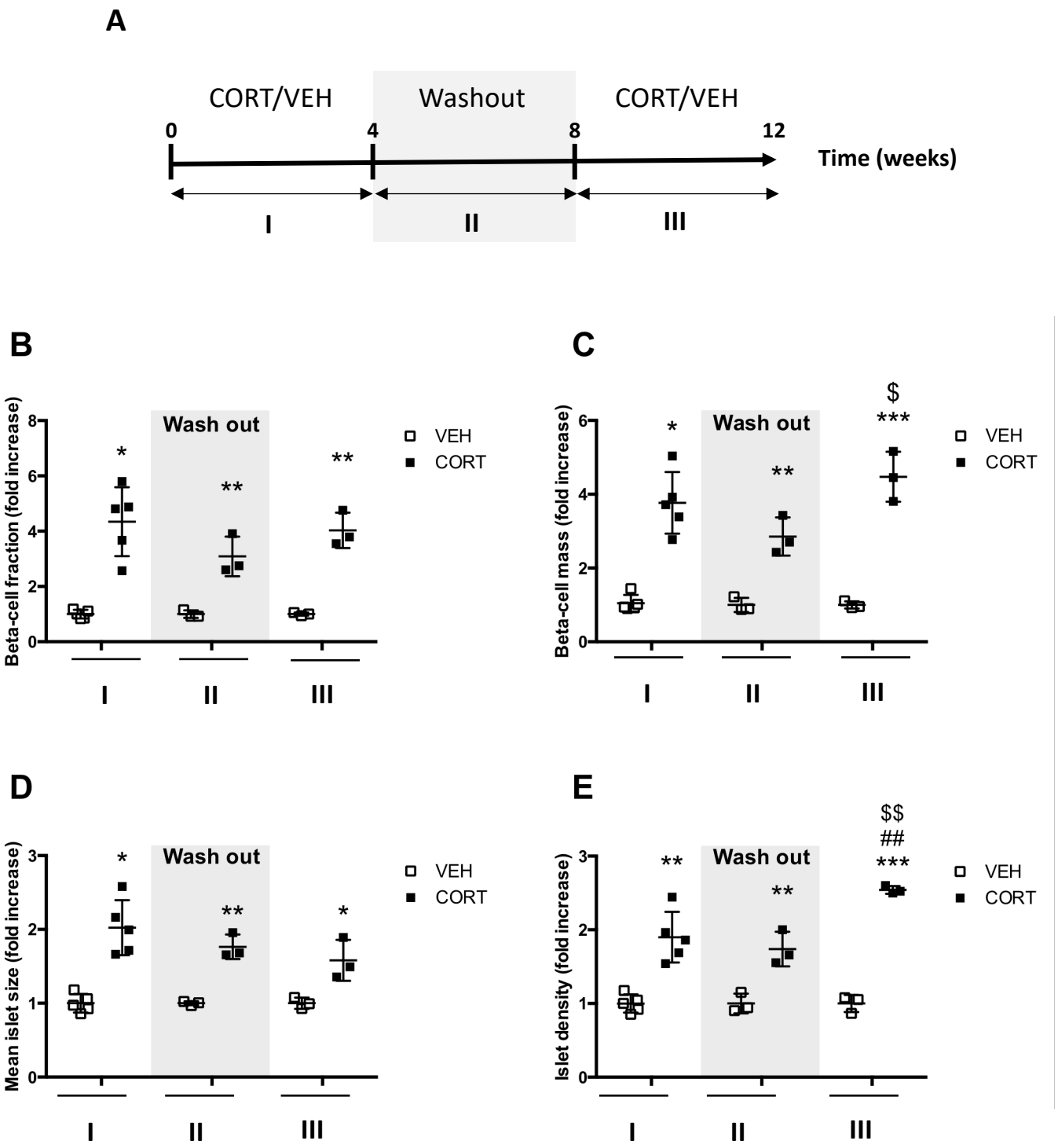
(A) Blood glucose levels in mice after STZ injection (day 0) and treatment with VEH (white circles, n=10) or with CORT (black squares, n=10). The arrow indicates the start of CORT or VEH treatment **(B)** Pancreatic beta-cell fraction, **(C)** beta-cell mass, **(D)** islet density and **(E)** mean islet size were calculated by morphometrical analysis after insulin immunostaining on pancreatic sections from mice injected with STZ and treated with VEH (white squares, n=3) or mice injected with STZ and treated with CORT (black squares, n=4). **(F)** Blood insulin levels at the end of the treatment in mice injected with STZ and treated with VEH (white squares, n=10) or mice injected with STZ and treated with CORT (black squares, n=11). Dotted lines represent mean value for mice without STZ injection. n = 3 to 10 per condition. Values are expressed as means $\pm$ SD.** $p<0.01$; *** $p<0.001$ CORT *versus* VEH mice.

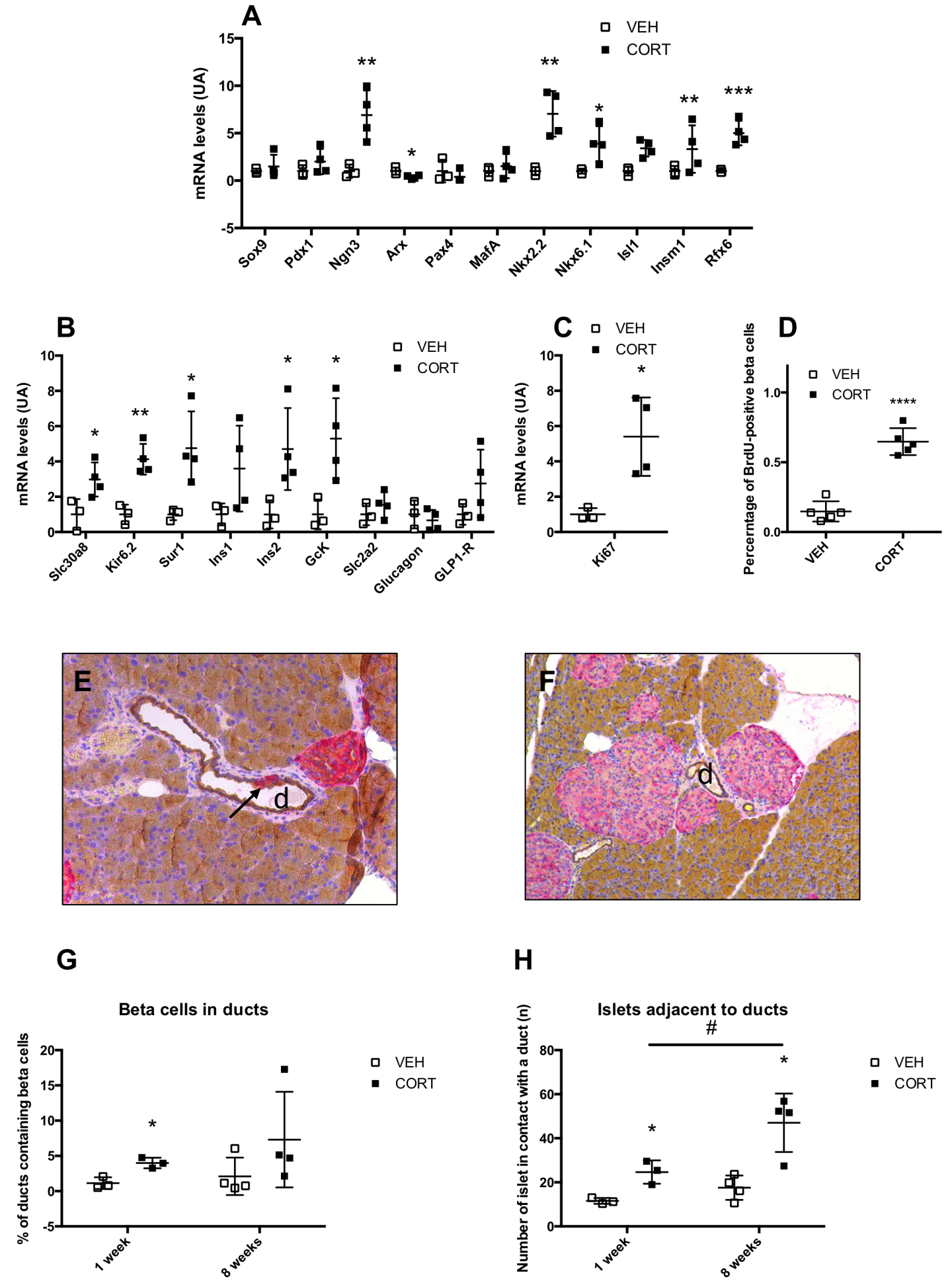
Figure 8: Serum from CORT treated mice stimulates beta-cell neogenesis in embryonic pancreatic buds. Immunofluorescence for insulin (green) and DAPI nuclear staining on representative sections from pancreatic buds cultured 7 days with 10% VEH serum **(A, F)**, 10% CORT serum **(B, G)**, 10^{-7} M CORT alone **(C, H)**, 0.4 ng/ml insulin **(D, I)** or 10^{-7} CORT and 0.4 ng/ml insulin **(E, J)** supplemented in the serum of VEH mice**. (K)** Beta-cell fraction in embryonic pancreatic buds after 7 days of culture in the same conditions with 10% VEH serum (white squares, n=6), 10% CORT serum (black squares, n=4), 10^{-7} M CORT alone (black circles, n=3), 0.4 ng/ml insulin (black triangles, n=3) or 10^{-7} CORT and 0.4 ng/ml insulin (black diamonds, 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% VEH serum (white squares, n=3) or CORT serum (black squares, n=3). Values are expressed as means $\pm$ SD. * $p<0.05$; when comparing CORT serum-treated buds *versus* VEH

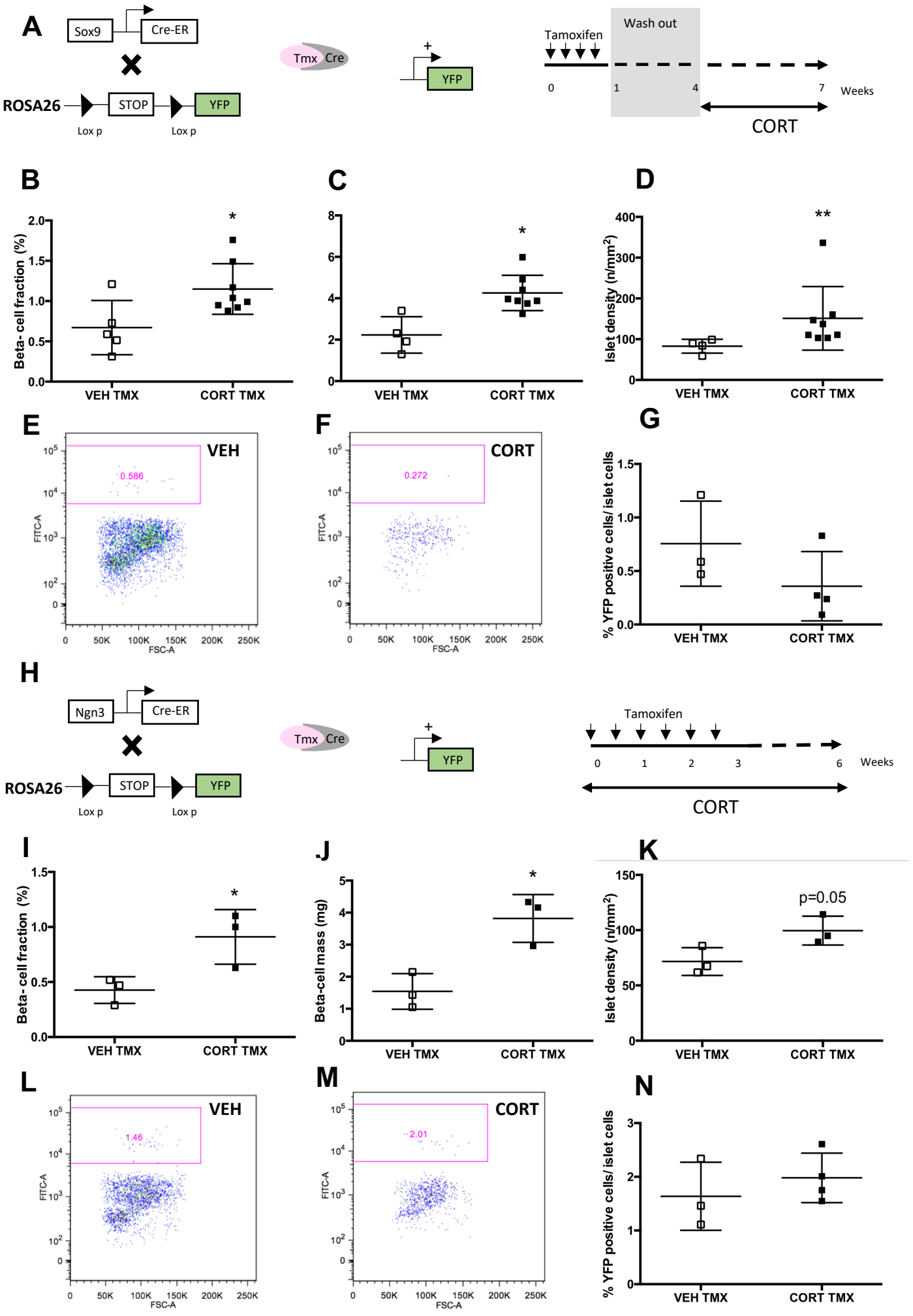
serum-treated buds. # $p<0.05$ when comparing CORT-treated buds *versus* VEH serum-treated buds. $ $p<0.05$ when comparing CORT+Insulin-treated buds *versus* VEH serum-treated buds.

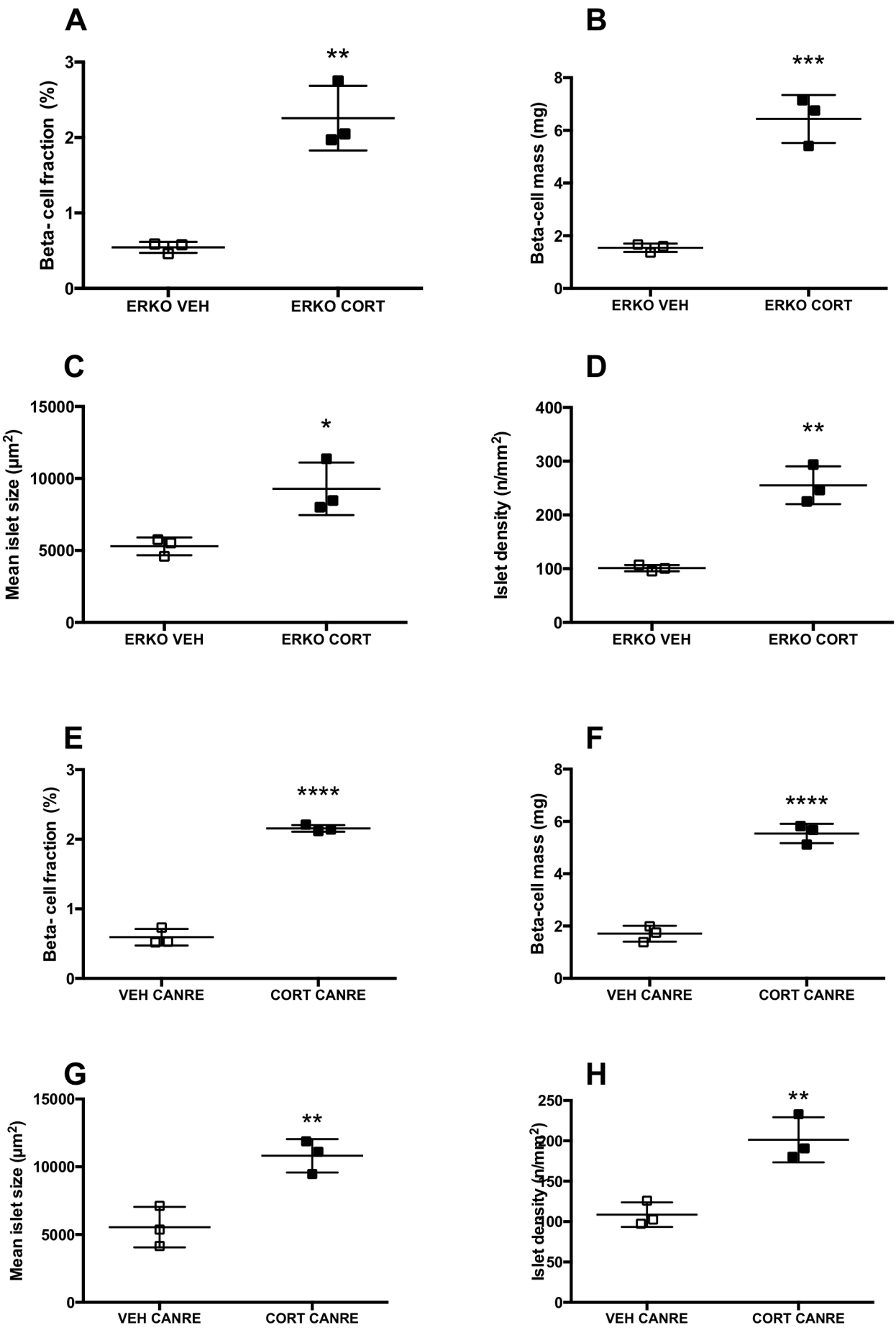

A
Blood glucose (% basal)
Time (min)
VEH
CORT
****
B
Blood glucose (mg/dL)
Time (min)
AUC (mg.dL-1.min)
VEH
CORT
*
C
Plasma insulin (ng/mL)
T0
T15
***
D
Glucose 2.8mM
Glucose 16.7mM
KCL 50mM
Insulin (ng/h)
####
****
E
VEH
Insulin
F
CORT
Insulin
G
Beta-cell fraction (%)
H
Beta-cell mass (mg)
I
Mean islet size (µm2)
J
Islet density (n/mm2)
K
Islet density (n/mm2)
4-11
12-20
21-32
33-48
49-66
67-87
88-112
113-148
>148
Numbers of cells per islet
L
Individual beta-cell area (µm2)
M
Beta cells
Alpha cells
Percentage of total islet cells

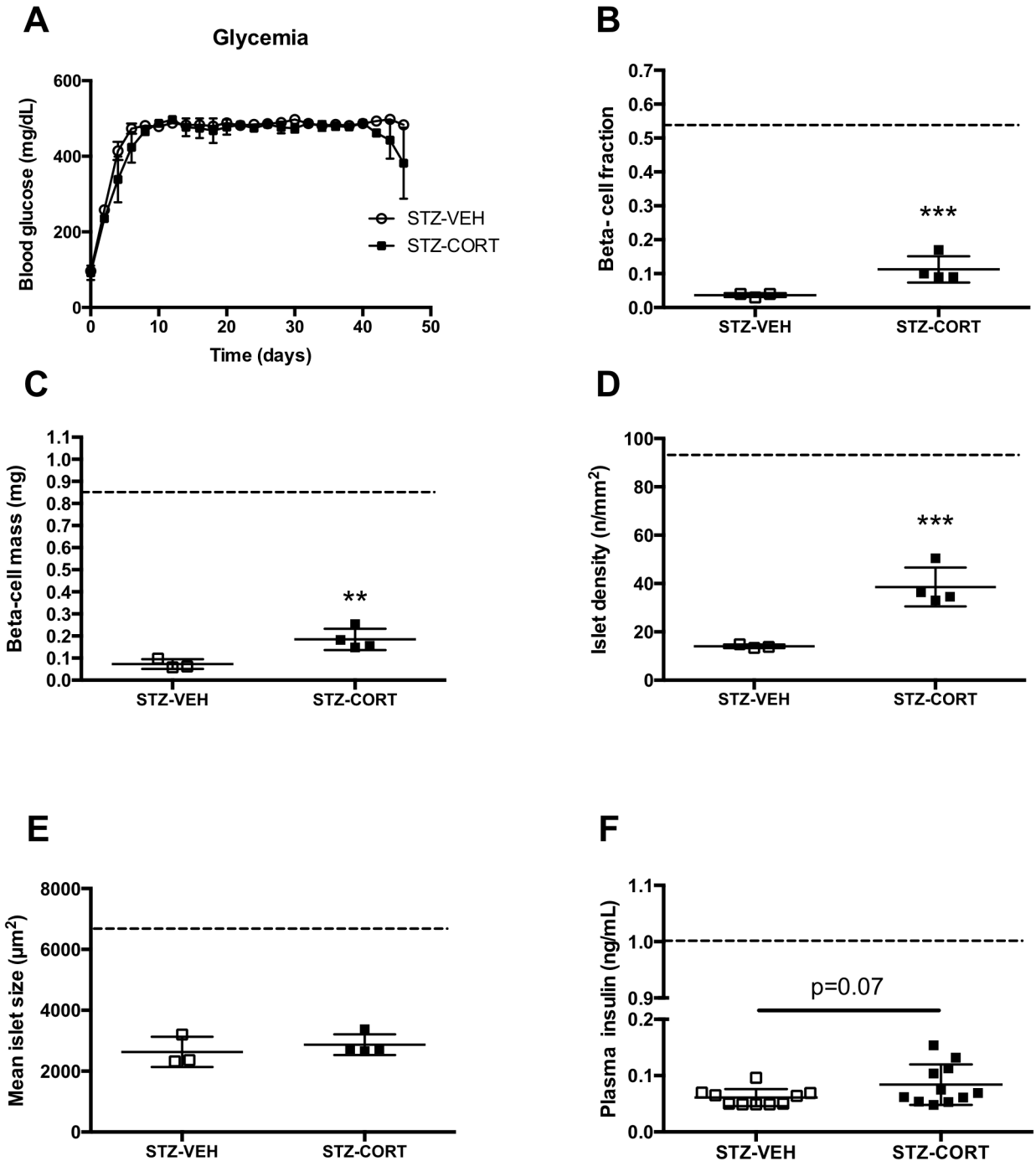

Beta-cell fraction (%)
8
6
4
2
0
*
***
****
1 week
4 weeks
6 weeks
8 weeks
VEH
CORT
Beta-cell mass (mg)
15
10
5
0
**
****
****
1 week
4 weeks
6 weeks
8 weeks
VEH
CORT
Mean islet size (µm2)
20000
15000
10000
5000
0
****
***
****
1 week
4 weeks
6 weeks
8 weeks
VEH
CORT
##
Islet density (n/mm2)
500
400
300
200
100
0
*
**
****
1 week
4 weeks
6 weeks
8 weeks
VEH
CORT

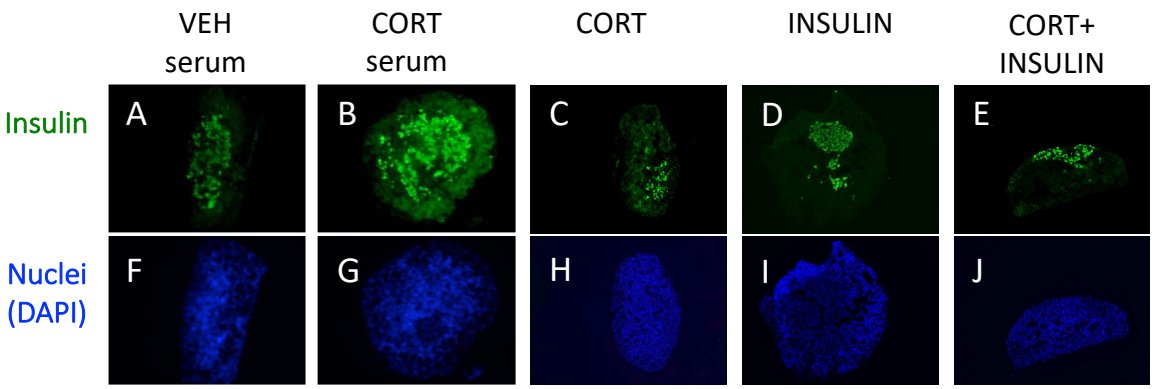

A
CORT/VEH
Washout
CORT/VEH
0
4
8
12
Time (weeks)
I
II
III
B
Beta-cell fraction (fold increase)
Wash out
VEH
CORT
C
Beta-cell mass (fold increase)
Wash out
VEH
CORT
D
Mean islet size (fold increase)
Wash out
VEH
CORT
E
Islet density (fold increase)
Wash out
VEH
CORT

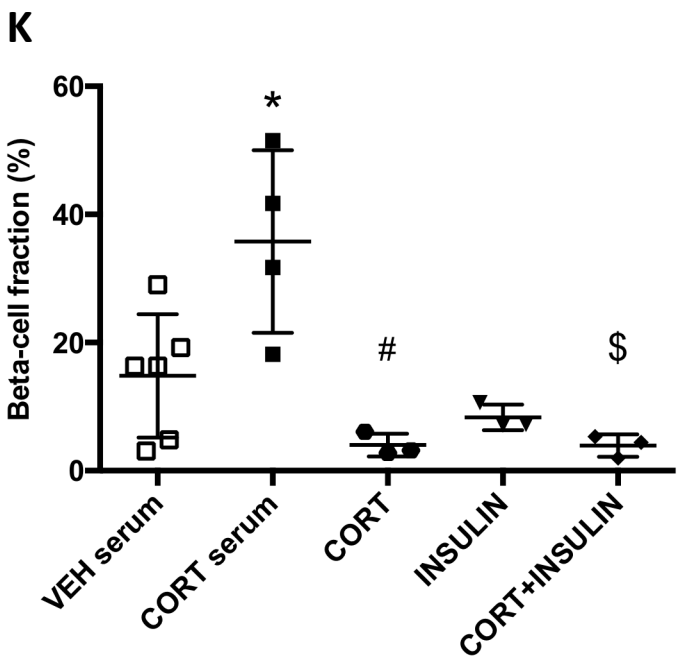

A
VEH
CORT
mRNA levels (UA)
Sox9
Pdx1
Ngn3
Arx
Pax4
MafA
Nkx2.2
Nkx6.1
Isl1
Insm1
Rfx6
B
Slc30a8
Kir6.2
Sur1
Ins1
Ins2
GcK
Slc2a2
Glucagon
GLP1-R
C
Ki67
D
Percerntage of BrdU-positive beta cells
****
E
d
F
d
G
Beta cells in ducts
% of ducts containing beta cells
1 week
8 weeks
H
Islets adjacent to ducts
Number of islet in contact with a duct (n)
#

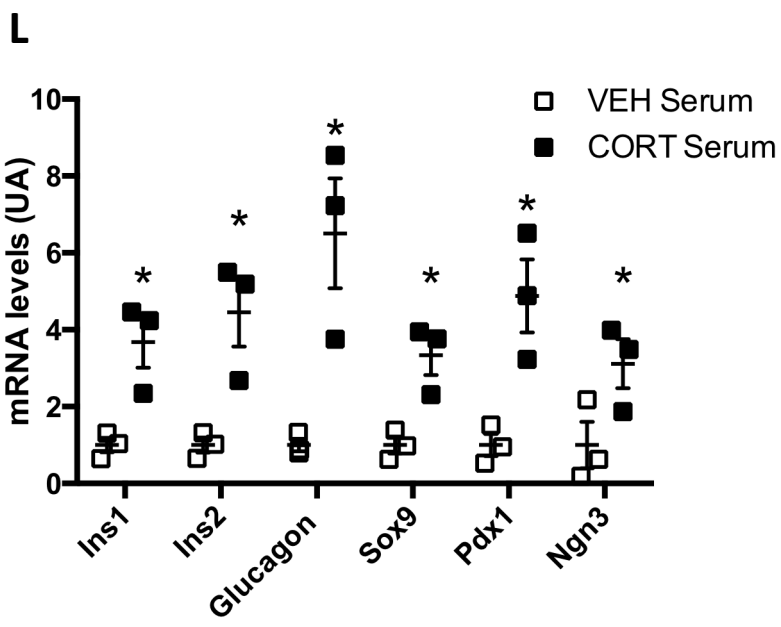

A
Sox9
Cre-ER
ROSA26
STOP
YFP
Lox p
Lox p
Tmx
Cre
YFP
Tamoxifen
Wash out
0
1
4
7
Weeks
CORT
B
Beta- cell fraction (%)
VEH TMX
CORT TMX
C
VEH TMX
CORT TMX
D
Islet density (n/mm²)
VEH TMX
CORT TMX
E
VEH
0.586
FITC-A
FSC-A
F
CORT
0.272
FITC-A
FSC-A
G
% YFP positive cells/ islet cells
VEH TMX
CORT TMX
H
Ngn3
Cre-ER
ROSA26
STOP
YFP
Lox p
Lox p
Tmx
Cre
YFP
Tamoxifen
0
1
2
3
6
Weeks
CORT
I
Beta- cell fraction (%)
VEH TMX
CORT TMX
J
Beta-cell mass (mg)
VEH TMX
CORT TMX
K
Islet density (n/mm²)
p=0.05
VEH TMX
CORT TMX
L
VEH
1.46
FITC-A
FSC-A
M
CORT
2.01
FITC-A
FSC-A
N
% YFP positive cells/ islet cells
VEH TMX
CORT TMX


A
Beta- cell fraction (%)
3
2
1
0
**
ERKO VEH
ERKO CORT
B
Beta-cell mass (mg)
8
6
4
2
0
***
ERKO VEH
ERKO CORT
C
Mean islet size (µm2)
15000
10000
5000
0
*
ERKO VEH
ERKO CORT
D
Islet density (n/mm2)
400
300
200
100
0
**
ERKO VEH
ERKO CORT
E
Beta- cell fraction (%)
3
2
1
0
****
VEH CANRE
CORT CANRE
F
Beta-cell mass (mg)
8
6
4
2
0
****
VEH CANRE
CORT CANRE
G
Mean islet size (µm2)
15000
10000
5000
0
**
VEH CANRE
CORT CANRE
H
Islet density (n/mm2)
250
200
150
100
50
0
**
VEH CANRE
CORT CANRE


A
Glycemia
Blood glucose (mg/dL)
Time (days)
STZ-VEH
STZ-CORT
B
Beta- cell fraction
***
STZ-VEH
STZ-CORT
C
Beta-cell mass (mg)
**
STZ-VEH
STZ-CORT
D
Islet density (n/mm2)
***
STZ-VEH
STZ-CORT
E
Mean islet size (µm2)
STZ-VEH
STZ-CORT
F
Plasma insulin (ng/mL)
p=0.07
STZ-VEH
STZ-CORT


VEH serum
CORT serum
CORT
INSULIN
CORT+ INSULIN
Insulin
Nuclei (DAPI)
A
B
C
D
E
F
G
H
I
J


K
Beta-cell fraction (%)
60
40
20
0
*
#
$
VEH serum
CORT serum
CORT
INSULIN
CORT+INSULIN


L
mRNA levels (UA)
10
8
6
4
2
0
VEH Serum
CORT Serum
*
Ins1
Ins2
Glucagon
Sox9
Pdx1
Ngn3