Dissecting the Role of Glucocorticoids on Pancreas Development

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To determine whether glucocorticoids are involved in pancreas development, glucocorticoid treatment of rat pancreatic buds in vitro was combined with the analysis of transgenic mice lacking the glucocorticoid receptor (GR) in specific pancreatic cells. In vitro treatment of embryonic pancreata with dexamethasone, a glucocorticoid agonist, induced a decrease of insulin-expressing cell numbers and a doubling of acinar cell area, indicating that glucocorticoids favored acinar differentiation; in line with this, expression of Pdx-1, Pax-6, and Nkx6.1 was downregulated, whereas the mRNA levels of Ptf1-p48 and Hes-1 were increased. The selective inactivation of the GR gene in insulin-expressing β-cells in mice (using a RIP-Cre transgene) had no measurable consequences on β- or α-cell mass, whereas the absence of GR in the expression domain of Pdx-1 (Pdx-Cre transgene) led to a twofold increased β-cell mass, with increased islet numbers and size but normal α-cell mass in adults. These results demonstrate that glucocorticoids play an important role in pancreatic β-cell lineage, acting before hormone gene expression onset and possibly also modulating the balance between endocrine and exocrine cell differentiation. ***Diabetes*** **53:2322–2329, 2004**

Increasing evidence from epidemiological studies led to the concept of the early-life origins of adult diseases, suggesting that late-onset disorders such as type 2 diabetes, glucose intolerance, or hypertension may be programmed by nutritional inadequacy in utero (1–5). Excess glucocorticoids also retard fetal growth, and overexposure to these hormones during intrauterine life has been shown to play a role in fetal programming in both humans (6) and rodents (7). The link between glucocorticoid overexposure in utero and the occurrence of metabolic diseases in adulthood has been well documented in rats. Maternal treatment with dexamethasone (DEX), a glucocorticoid agonist, induces in the offspring growth retardation at birth as well as hyperglycemia and increased systolic blood pressure at adult age (8–10). Similarly, inhibition of the placental 11β-hydroxysteroid dehydrogenase type 2, the enzyme that protects the fetus from maternal glucocorticoids, induces intrauterine growth retardation as well as glucose intolerance and hypertension in adults (11).

We have previously shown that maternal general food restriction during late pregnancy decreased the β-cell mass of newborn rats (12). This reduction was irreversible and persisted in adults despite restoration of normal nutrition from weaning (13), ultimately leading to impaired glucose tolerance associated with aging (14) or pregnancy (15). These results sustain the notion that some of the late alterations observed in humans born with intrauterine growth retardation may result from altered β-cell development in utero, as initially suggested by Hales and Barker (2). Additionally, we have recently demonstrated that maternal general food restriction in the rat induced a rise in both maternal and fetal corticosterone levels, which in turn was responsible for the decreased β-cell mass and islet numbers observed in the undernourished fetus (16). These data suggested that glucocorticoids might play a role in pancreas development.

Studies of pancreatic gene expression as well as genetically modified mice have identified a large number of transcription factors that control the development of the endocrine pancreas. Several homeodomain, paired-box, and helix-loop-helix transcription factors such as Nkx6.1, Pax4, Pax6, and Ngn3 are expressed at early stages of development, and their absence leads to alterations in endocrine cell differentiation. Besides, the bHLH transcription factor Ptf1-p48, which is required for exocrine cell differentiation, and the homeodomain protein pancreas duodenum homeobox (Pdx)-1, the earliest marker of pancreatic cells and whose absence results in pancreatic agenesis, are required for the early steps of pancreas development (17–19).

The aims of the present study were *1*) to investigate whether the association between developmental injury of the β-cells and type 2 diabetes may be attributed to a direct effect of glucocorticoids on fetal pancreatic tissue and, if so, *2*) to decipher the cellular and molecular mechanisms by which glucocorticoids could modulate pancreatic development or differentiation. We hypothesized that glucocorticoids act directly on the pancreas and that the transcription factors controlling its normal development and differentiation could be the targets of these hormones. We addressed this hypothesis by combining in vitro and in vivo approaches. First, we used a model of embryonic rat pancreas cultured in vitro in the presence of

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BrdU, bromodeoxyuridine; DEX, dexamethasone; E15.5, embryonic day 15.5; GR, glucocorticoid receptor.

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DEX to study the effect of glucocorticoids on pancreatic differentiation. We then analyzed the consequences of glucocorticoid receptor (GR) inactivation on pancreas development and organization, in mice lacking the GR either in pancreatic precursor cells (Pdx-Cre $GR^{lox/lox}$ mice, denominated $GR^{Pdx\text{-}Cre}$ thereafter) or in cells transcribing the insulin gene, whether immature or fully differentiated β-cells (RIP-Cre $GR^{lox/lox}$ mice, denominated $GR^{RIP\text{-}Cre}$ thereafter).

RESEARCH DESIGN AND METHODS

Mice breeding. $GR^{lox/lox}$ animals (20,21) were maintained on a C57B/L6 and 129Sve/V mixed genetic background. These animals were genotyped by PCR, using primers GR12 (5′-CATGCTGCTAGGCAAATGATCTTAAC) and GR30 (5′-CTTCCACTGCTCTTTTAAA GAAGAC), which amplify a 280-bp fragment for the wild-type allele and a 350-bp fragment for the floxed allele. RIP-Cre and Pdx-Cre lines (22) were originally obtained on a C57B/L6 and CBAJ mixed genetic background. Presence of the Cre transgene was detected by PCR using Cre1 (5′-CCTGTTTTGCACGTTCACCG) and Cre3 (5′-ATGCTTCTGTCCGTTT GCCG) primers (300-bp band). $GR^{lox/lox}$Pdx-Cre and $GR^{lox/lox}$RIP-Cre animals were obtained by mating adequate transgenic lines. To avoid any perturbation in maternal glucose homeostasis induced by gestation, only control females ($GR^{lox/lox}$) were used to obtain experimental animals.

Dissection and culture of rat pancreatic buds at embryonic day 15.5. Pancreatic buds from Wistar rats at embryonic day 15.5 (E15.5) were dissected under the microscope. They were cultured for 3 days in RPMI 1640 (Invitrogen, Cergy Pontoise, France) with 10% FCS in the presence or absence of 100 nmol/l DEX in multiwell culture plates equipped with a 0.4-μm filter insert (Millipore, Bedford, MA). For morphometrical and proliferation rate measurements, the cultured buds were treated with bromodeoxyuridine (BrdU) (10 μmol/l) during the last hour of incubation.

The experiments on mice and rats were carried out according to the Principles of Laboratory Animal Care, National Institutes of Health, and the French laws, authorization number 7612, delivered to B.B. by the French Agricultural Ministry.

RNA extraction and reverse transcription. RNA extraction was performed on batches of eight rat embryonic pancreata cultured as described above using Trizol Reagent (Invitrogen), according to the manufacturer's procedure. After spectrophotometry quantification, 2 μg total RNA was used for reverse transcription in a 20-μl final volume using Superscript II Rnase H^- reverse transcriptase (Invitrogen). For each experiment, a negative control without reverse transcriptase was performed. cDNA was diluted 10 times in sterile water, and PCR was performed on 1.5 μl of this dilution.

Semiquantitative radioactive duplex PCR. PCR was performed in 25 μl final volume containing 1.5 μl cDNA (15 ng RNA equivalent), 1.5 mmol/l $MgCl_2$, 80 μmol/l cold dNTP, 1.3 μCi [α-^{32}P]dCTP, 1× GeneAmp PCR Buffer II, and 1.25 U AmpliTaq Gold "hot start" polymerase (Applied Biosystems, Foster City, CA). The sequences of the primers were as follows: Ngn3 sense, 5′-TGGCGCCTCATCCCTTGGATG; antisense, 5′-CAGTCACCCACTTCTGCT TCG; Hes1 sense, 5′-TCAACACGACACCGGACAAACC; antisense, 5′-GGTAC TTCCCCAACACGCTCG; Ptf1-p48 sense, 5′-ATTAACTTCCTCAGCGAGCT GGT; and antisense, 5′-GTTGAGTTTTCTGGGGTCCTCTG. Primer sequences for Pdx-1, Foxa2, Nkx6.1, Hnf1α, Pax6, cyclophilin, α-tubulin, TBP, and RRPPO were previously described (23). PCR was performed in triplicate by amplifying each transcription factor with an internal control gene. Duplex PCR conditions were set up for each couple of genes and considered satisfactory when similar, and linear amplifications were obtained in simplex and duplex for both genes. PCR products were separated on a 6% acrylamide gel in Tris-borate EDTA buffer. The gel was dried, and the [α-^{32}P]dCTP incorporated in each PCR product was measured on storage phosphor screens by the Packard Cyclone system and quantified by ImageQuant software. The radioactive background was quantified and subtracted from each measurement. The amount of each transcription factor product was normalized to its specific internal control gene, and this average ratio was then expressed as a percent of the mean ratio obtained in the control group tested in the same PCR.

Fixation and tissue processing for immunohistochemistry. Rat pancreatic buds or adult mice pancreata were fixed in 3.7% formalin solution, dehydrated, and embedded in paraffin. Tissues were entirely cut into 5-μm thick sections, which were collected on poly-L-lysin–coated slides. The slides were left at 37°C overnight and stored at 4°C until processed for immunohistochemistry.

Immunohistochemistry. Tissue slides were submitted to a 10-min microwave treatment in a citrate buffer, permeabilized for 20 min with 0.1% Triton X-100 in Tris-buffered saline, and incubated 30 min with a blocking buffer (0.1% Tween20/3% BSA in Tris-buffered saline) before a 4°C overnight incubation with primary antibodies. Secondary antibodies (1:200) were incubated 1–4 h at room temperature. Double immunohistochemistry was performed using fluorescent dye–coupled secondary antibodies visualized under a Leica DMB microscope or, alternatively, using enzyme-linked secondary antibodies revealed by diaminobenzidine (Vector Laboratories, Compiegne, France) or Fast Red (Dako, Carpinteria, CA) substrates. Antibodies used are described below.

Primary antibodies were rabbit anti–Pdx-1 (a gift from Dr O.D. Madsen), mouse anti-insulin (Sigma, St. Louis, MO), mouse anti-BrdU (Amersham Pharmacia Biotech Europe, Saclay, France), rabbit anti-glucagon (Diasorin, Stillwater, MN), rabbit anti-amylase (Sigma), guinea pig anti-insulin (Dako), and rabbit anti-GR (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were fluorescein isothiocyanate anti–guinea pig, fluorescein isothiocyanate anti-rabbit, Texas Red anti-mouse, Texas Red anti-rabbit, peroxidase anti–guinea pig, biotin conjugated anti-rabbit (Jackson Immuno Research Laboratories, West Grove, PA), peroxidase-conjugated anti-rabbit (Promega, Madison, WI), alkaline phosphatase-conjugated streptavidin (Bio-Genex, San Ramon, CA), and peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech Europe).

Cell numbers, area, and morphometrical measurements. On E15.5 rat pancreatic buds treated or not treated with DEX, cells coexpressing Pdx-1 and insulin, and cells expressing only Pdx-1 were counted on every other section throughout the bud; amylase-positive area was morphometrically measured on the other sections. A total of four control and five DEX-treated buds were analyzed. Acinar cell proliferation was studied on 3,000–6,000 amylase-positive cells per bud.

Amylase area on E15.5 rat pancreatic buds was determined by computer-assisted measurements using a DMRB microscope (Leica, Deerfield, IL) equipped with a color video camera coupled with a Q500IW computer (screen magnification, ×24), as previously described (13). Pancreatic tissue area and insulin-positive or glucagon-positive cell area on adult transgenic mice were similarly measured. Briefly, the number of islets (defined as insulin-positive aggregates at least 25 μm in diameter) was scored and used to calculate the islet numerical density (number of islets per square centimeter of tissue). Islets ranging from 25 to 100 μm in diameter were defined as small, those ranging from 101 to 150 μm as medium, and those >150 μm as large. The percent β-cell fraction was measured as the ratio of the insulin-positive cell area to the total tissue area on the entire section. Mean β-cell fraction per pancreas was calculated as the ratio of the sum of insulin-positive area to the sum of pancreatic tissue area. The β-cell mass was obtained by multiplying the β-cell fraction by the weight of the pancreas. α-Cell fraction and mass were similarly measured. Morphometrical analysis was performed on eight sections throughout the pancreas from four $GR^{RIP\text{-}Cre}$ mice or six $GR^{lox/lox}$ and six $GR^{Pdx\text{-}Cre}$ mice.

Statistical analysis. All results are expressed as means ± SE. The statistical significance of variations was evaluated with Statview 4.5 software. Transcription factor mRNA levels were expressed as the percent of their respective control in each experiment and analyzed by a Wilcoxon's nonparametric test. Cell number, amylase cell area, cell proliferation, β-cell and α-cell fraction and mass, islet number, or repartition per size were tested by a Mann-Whitney nonparametric test. P values <0.05 were considered significant.

RESULTS

GR is expressed in pancreatic epithelial cells at E15.5. in the rat. To determine whether GR protein is present in the developing pancreas, we performed immunofluorescence on E15.5 pancreatic paraffin sections. The GR was expressed in a subpopulation of cytokeratin-labeled epithelial cells (Fig. 1*A*). Because GR is already expressed in E15.5 pancreatic buds and because few cells coexpressing insulin and Pdx-1, i.e., differentiated β-cells, can be detected at this stage (Fig. 1*B*), we further used this tissue as a model to study the effects of glucocorticoids on β-cell development and differentiation.

DEX treatment decreases insulin- and-Pdx-1–expressing β-cell numbers but increases the area occupied by acinar cells. Rat pancreatic buds at E15.5 were cultured for 3 days in the presence or absence of DEX (10^{-7} mol/l), a GR agonist. DEX treatment induced a small (25%) but significant decrease of total tissue area ($P < 0.05$). The

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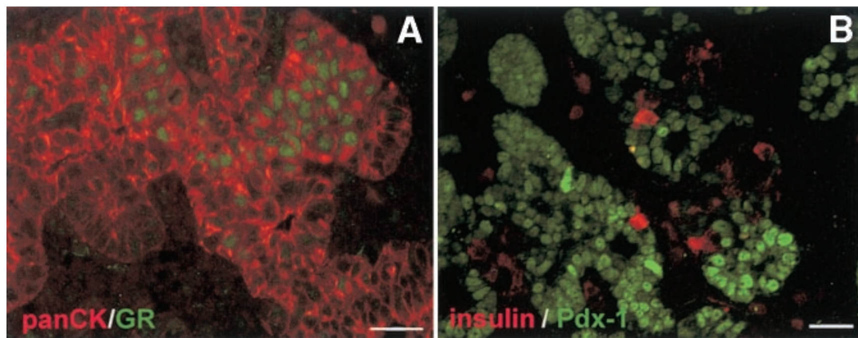

FIG. 1. GR is expressed in E15.5 rat pancreatic bud. ***A*: GR (green) is detected in a subpopulation of epithelial pan-cytokeratin (panCK)-positive cells (red).** ***B*: Few cells coexpressing insulin (red) and Pdx-1 (green) are found in E15.5 rat pancreatic buds. Scale bar = 25 μm.**

consequences of DEX treatment on exocrine and β endocrine cell differentiation were analyzed by immunohistochemistry and RT-PCR. After 3 days of culture in the absence of DEX, all cells expressing insulin coexpressed Pdx-1 and were frequently found organized into small clusters, showing that the tissue had differentiated in culture. In striking contrast, in DEX-treated buds, such β-cell clusters were absent and the number of cells coexpressing insulin and Pdx-1 was decreased, whereas the number of cells expressing only Pdx-1 remained unchanged (Fig. 2*A*). Concomitantly, DEX treatment led to a twofold increase of amylase-containing cells, without altering the histological organization of the acini (Fig. 2*B*). Experiments of BrdU incorporation showed that acinar cell proliferation was decreased after 3 days of treatment with DEX (Fig. 2*C*) but not after 1 day, at which time more amylase-expressing cells were already observed (Fig. 2*D*). Taken together, these observations suggest that the increase in acinar cell area is a direct consequence of glucocorticoid-mediated stimulation of differentiation and not acinar cell proliferation. In both treated and untreated buds, β-cells as well as precursor

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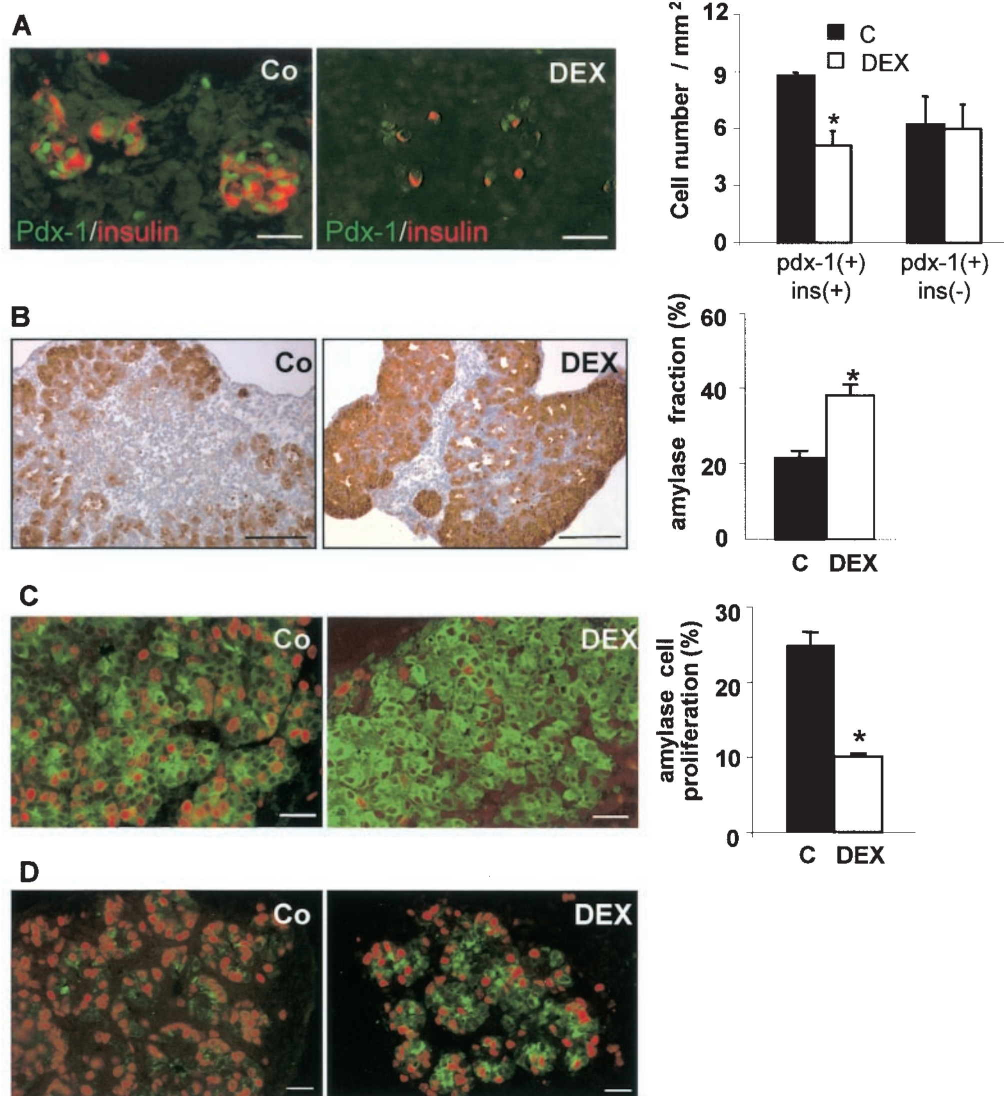

FIG. 2. DEX favors in vitro pancreatic differentiation into exocrine tissue and represses β-cell differentiation. The effect of a 3-day treatment of E15.5 rat pancreatic buds with 10^{-7} mol/l DEX on differentiation into β-cells or acinar cells is shown. ***A*: Clusters of cells coexpressing insulin (red) and Pdx-1 (green) are abundant in control buds (Co, C), but not in DEX-treated buds (DEX). Scale bar = 25 μm. The number of differentiated β-cells coexpressing Pdx-1 and insulin was decreased upon DEX treatment, whereas the number of precursor cells expressing only Pdx-1 remained unchanged.** ***B*: Amylase cell area (brown) was increased twofold in DEX-treated buds. Scale bar = 50 μm.** ***C*: BrdU$^+$ nuclei (red) were counted in amylase immunoreactive cells (green): acinar cell proliferation was decreased in DEX-treated E15.5 embryonic pancreas after 3 days.** ***D*: After 1 day of DEX treatment, acinar cell proliferation was similar to that in untreated buds (Co), but amylase-expressing cell numbers were already increased. Scale bar = 50 μm. Results are expressed as means ± SE; *P < 0.05 compared with the untreated group.**

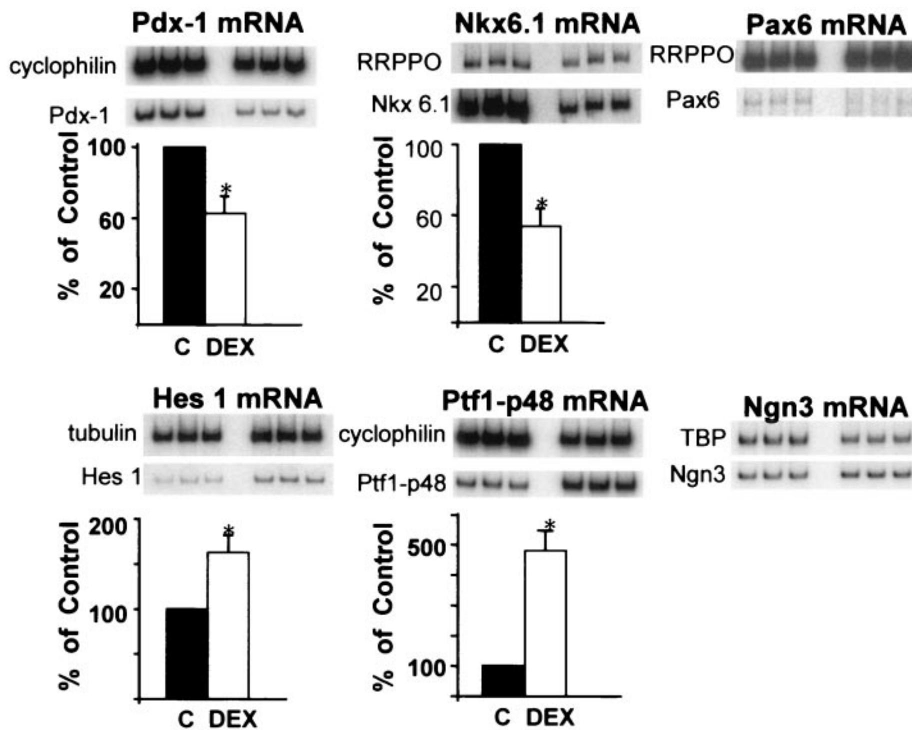

FIG. 3. DEX treatment modifies the expression levels of transcription factors involved in pancreas development. E15.5 rat pancreatic buds were similarly treated with 10^{-7} mol/l DEX. For each transcription factor, a representative gel of radioactive duplex RT-PCR is shown, on which the first three lanes are triplicates for the control group and the three others are triplicates of the DEX-treated group in the same experiment. The ratio of the transcription factor to the internal control (tubulin, cyclophilin, RRPPO, and TBP) mRNA levels was calculated for each triplicate. The ratio for the DEX-treated group (□) was expressed as a percentage of the same ratio in the untreated group (■). Results of five independent experiments are shown and expressed as means ± SE; *$P < 0.05$ compared with control group. C, control.

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cells were too few to allow any reliable quantification of their proliferation rate.

DEX affects the expression levels of transcription factors involved in pancreas development. To further characterize the changes induced by glucocorticoids on E15.5 rat pancreata, we determined the mRNA levels of some of the transcription factors involved in pancreatic development and differentiation using semiquantitative duplex RT-PCR analysis (Fig. 3). DEX treatment clearly decreased the mRNA levels of Pdx-1 (60% of the control, $P < 0.05$) and Nkx6.1 (50% of the control, $P < 0.05$). Pax6 mRNA was almost undetectable in the presence of DEX. Upon DEX treatment, no changes could be detected in either Ngn3 mRNA levels (Fig. 3) or Foxa2 and Hnf1α (data not shown). In striking contrast, the mRNA levels of Ptf1-p48 and Hes1, encoding transcription factors involved in exocrine cell differentiation, were increased 5-fold ($P < 0.05$) and 1.7-fold ($P < 0.05$), respectively, in the presence of DEX (Fig. 3).

These data suggest that glucocorticoids modulate the balance of pancreatic differentiation into endocrine or exocrine cells in vitro. We next investigated whether pancreas development would be disturbed in vivo, by studying the consequences of the selective inactivation of GR in pancreatic cells.

Disruption of GR in pancreatic precursor cells increases β-cell mass. Mice carrying the GR^{lox} allele (21) were crossed with mice expressing the Cre recombinase either in pancreatic precursor cells (Pdx1-Cre) or specifically in cells expressing the insulin gene (rat insulin promoter, RIP-Cre). The efficiency and specificity of GR gene recombination (i.e., inactivation) were assessed by immunohistochemistry with anti-GR antibodies on pancreatic sections (Fig. 4). In $GR^{Pdx\text{-}Cre}$ mice, GR staining was

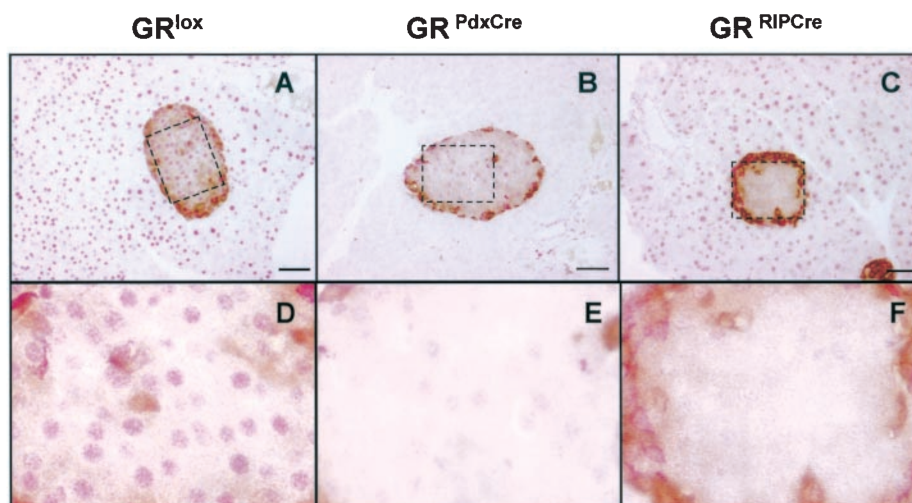

FIG. 4. Assessment of the GR deletion in control $GR^{lox/lox}$, $GR^{Pdx\text{-}Cre}$, and $GR^{RIP\text{-}Cre}$ adult mice. Double immunohistochemistry was performed on pancreatic sections for GR (pink) and glucagon (brown). In control mice, the GR was normally expressed in exocrine and endocrine cells (*A*). In $GR^{Pdx\text{-}Cre}$ mice, the GR was deleted in all exocrine cells and most of the β-cells (*B*). In $GR^{RIP\text{-}Cre}$ mice, the GR was specifically deleted in all the differentiated β-cells (*C*) but present in other pancreatic cells. *D*, *E*, and *F* are higher magnification of the boxed area shown in *A*, *B*, and *C*, respectively. Scale bar = 50 μm.

TABLE 1
Comparative analysis of body weight, pancreas weight, and fasted glycemia in $GR^{lox/lox}$, $GR^{Pdx\text{-}Cre}$, and $GR^{RIP\text{-}Cre}$ adult mice

	$GR^{lox/lox}$	$GR^{Pdx\text{-}Cre}$	$GR^{RIP\text{-}Cre}$
n	5	6	4
Body weight (g)	19.2 ± 1.4	21.1 ± 0.4 (0.14)	19.0 ± 0.4 (0.90)
Pancreas weight (mg)	225 ± 21	277 ± 10 (0.05)	217 ± 10 (0.80)
Pancreas weight (mg/g body wt)	11.7 ± 0.4	13.1 ± 0.5 (0.04)	11.4 ± 0.7 (0.80)
Fasted glycemia (mg/dl)	79 ± 3	76 ± 3 (0.31)	68 ± 4 (0.06)

Data are means ± SE or means ± SE (*P*). Statistical differences between each mutant group and the control mice were assessed using the Mann-Whitney nonparametric test.

almost totally absent in all pancreatic cell types, although a faint labeling was sometimes detected in islets (Fig. 4*B* and *E*). In $GR^{RIP\text{-}Cre}$ mice, the GR was specifically deleted in all differentiated β-cells but remained well expressed in all other pancreatic cell types, as expected (Fig. 4*C* and *F*). Female mice were analyzed at adult age (3–4 months) and compared with age-matched control females (Table 1). GR deletion in β-cells did not alter body or pancreatic weight, but a tendency to decreased fasted glycemia was observed. GR deletion in Pdx-1–expressing cells did not alter the glycemia or the body weight but slightly increased pancreatic weight (Table 1).

Four to six females at 3–4 months of age were used for morphometric analysis on immunostained paraffin sections. The β-cell fraction increased nearly twofold in $GR^{Pdx\text{-}Cre}$ mice (1.06 ± 0.14 vs. 0.65 ± 0.13% in controls, $P < 0.01$), in line with an increase in β-cell mass (2.82 ± 0.36 vs. 1.50 ± 0.52 mg in controls, $P < 0.01$) (Fig. 5). In contrast, α-cell fraction and mass from $GR^{Pdx\text{-}Cre}$ animals were similar to those of controls (Fig. 5*C*). Further characterization showed that the increased β-cell mass arose from increased islet numbers, mainly small and large islets (Fig. 6), and increased area of the large islets (giant islets >300 μm equivalent diameter were often observed) (Fig. 5). Individual β-cell area was unchanged in $GR^{Pdx\text{-}Cre}$ mice (188 ± 4 vs. 177 ± 9 μm^2 in controls, $P = 0.27$), indicating that the β-cells were not hypertrophied. The increased β-cell fraction in $GR^{Pdx\text{-}Cre}$ mice was already present in neonates at 2.5 days of age (3.73 ± 0.19 vs. 3.08 ± 0.05% in controls, $n = 4$ in each group, $P < 0.05$). A mutation restricted to β-cells did not have any major consequences on pancreas morphology, and $GR^{RIP\text{-}Cre}$ mice were similar to the control group for all parameters analyzed (Figs. 5 and 6), suggesting that glucocorticoids do not play a major role in differentiated β-cells.

DISCUSSION

In a previous study, we had shown that decreased β-cell mass was observed under conditions of fetal overexposure

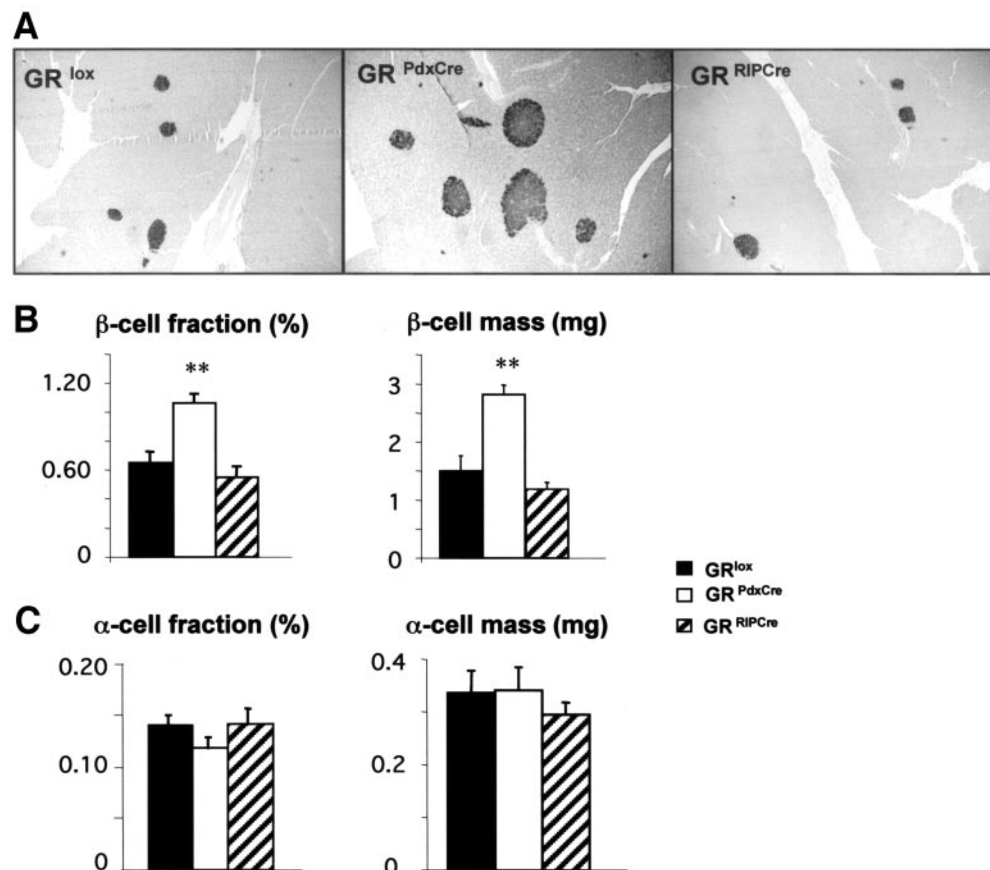

FIG. 5. Increased β-cell mass in $GR^{Pdx\text{-}Cre}$ mice. *A*: $GR^{Pdx\text{-}Cre}$ mice have giant islets. *B*: β-Cell fraction and β-cell mass are increased in $GR^{Pdx\text{-}Cre}$ adult female mice, whereas $GR^{RIP\text{-}Cre}$ mice are undistinguishable from control mice. *C*: α-Cell fraction and mass are unaffected in both mutants compared with the controls. Values are means ± SE; *$P < 0.05$, **$P < 0.01$ compared with the control group.

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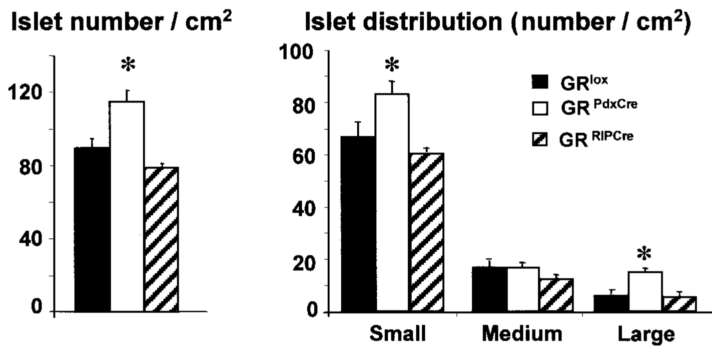

FIG. 6. Increased β-cell mass in $GR^{Pdx\text{-}Cre}$ mice arises from increased numbers of small and large islets. Total islet numbers per centimeter squared as well as numbers of large and small islets per centimeter squared are increased in $GR^{Pdx\text{-}Cre}$ mice. $GR^{RIP\text{-}Cre}$ mice are undistinguishable from control mice. Data are means ± SE; *$P <$ 0.05 compared with the control group.

to glucocorticoids, such as that observed during fetal undernutrition, whereas large β-cell numbers were associated with low corticosterone levels (16). The aim of this work was to investigate whether glucocorticoids were implicated directly in pancreatic development and to determine the cellular and molecular targets of these hormones. We hypothesized that the hormonal steroid imbalance generated by undernutrition would affect the developmental programming by modifying the balanced level of transcription factors modulating pancreas development. This hypothesis was investigated by in vitro studies of treatment with glucocorticoids and by studying mouse models lacking the GR in specific pancreatic cell populations.

In the present work, we show that in vitro treatment of the embryonic rat pancreas with DEX did not affect the number of precursor cells but decreased the number of differentiated β-cells and increased the differentiated acinar cell area. These results suggest that glucocorticoids decreased the differentiation of the embryonic pancreas into β-cells while favoring its differentiation into acinar cells. This conclusion was further sustained by the finding of decreased proliferation of amylase-expressing cells upon DEX treatment, a result suggesting that glucocorticoids could also control the proliferation of already differentiated acinar cells and thereby prevent their overgrowth. Taken together, our in vitro data suggest that the differentiation process from precursor to differentiated endocrine or exocrine cell is altered, suggesting that the precursor cells but not the differentiated β-cells are potential targets for glucocorticoids. Whether this in vitro situation also applies in vivo remains to be fully investigated. In line with this idea, rats undernourished during their perinatal life and thereby exposed to increased corticosterone levels in utero show increased pancreatic weight at adult age (14,15).

The finding that β-cell differentiation is impaired in glucocorticoid excess situations is reinforced in the mirror situation found in conditional mutant mice where the GR signaling is absent, such that the deletion of the GR in Pdx-1–expressing precursor cells ($GR^{Pdx\text{-}Cre}$) led to a twofold increase of β-cell mass, with increased islet numbers. This increased β-cell mass in $GR^{Pdx\text{-}Cre}$ animals was already observed in neonates, although to a lesser extent than in adults. Surprisingly, the decreased exocrine cell differentiation, which would have been expected from the in vitro data, was not observed in the $GR^{Pdx\text{-}Cre}$ mutants, since their pancreatic weight was on the contrary slightly increased. Other factors, coming either from maternal environment or adjacent tissue interactions might modulate this effect in vivo. Interestingly, while the deletion of the GR in pancreatic precursor cells led to increased β-cell mass, α-cell mass remained unaffected, indicating that glucocorticoid action was restricted to the β-cell lineage. On the other hand, the specific GR deletion in differentiated β-cells in $GR^{RIP\text{-}Cre}$ mice had no measurable consequences on β-cell or α-cell mass. Taken together, these findings support the idea that glucocorticoids act on undifferentiated endocrine pancreatic cells having expressed the proendocrine marker Ngn3, but before insulin gene expression onset.

During the last decade, cell lineage studies in the pancreas have shown the requirement of a group of transcription factors for normal pancreatic development (19,24,25) even though their chronology of action is not fully understood yet. Pdx-1 is acknowledged as the earliest, because Pdx-1–expressing cells give rise to all types of adult pancreatic cells (26,27) before being restricted to mature β-cells also expressing Nkx6.1, Pax6, and other markers. Ngn3 is the common endocrine precursor cell marker (27,28), whereas Ptf1-p48, despite its early expression in pancreatic precursor cells (29), is an absolute prerequisite to drive exocrine cell differentiation (30). Moreover, Hes1 can also be considered a proexocrine transcription factor because it inhibits Ngn3 in the delta/notch pathway (25,31,32).

To further characterize the mechanisms by which glucocorticoids act on pancreas development, and hypothesizing that the hormonal steroid imbalance affects the developmental programming by modifying the level of the genes modulating pancreas development, we studied the expression of these transcription factors after in vitro treatment with DEX. Interestingly, the transcription factors implicated in β-cell differentiation, such as Pdx-1, Pax6, and Nkx6.1, were downregulated, whereas the exocrine-specific transcription factors Ptf1-p48 and Hes1 were upregulated upon glucocorticoid treatment. These results suggest that glucocorticoids impair β-cell development by favoring exocrine differentiation and that transcription factors could be their molecular targets.

The modulation of exocrine/endocrine differentiation balance had already been suggested in older in vitro studies of rat pancreatic explants treated with corticosterone, showing a decreased insulin secretion and islet mass while exocrine enzyme contents and acinar mass were enhanced (33,34). Additionally, the AR42J cell line, which shares some characteristics of multipotency with precursor cells, has been shown to differentiate into acinar cells when exposed to DEX (35). The decreased Pdx-1 mRNA levels we observed are also in good agreement with similar

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findings obtained after treatment of mouse pancreatic buds with DEX (36). However, the latter work argues in favor of a transdifferentiation of β-cells into hepatocytes without any changes in exocrine tissue. The processes involved in the two studies appear quite different. In the experiments of Shen et al. (36), the treatment begins earlier, when more undifferentiated cells are likely to maintain a multipotency, rendering them more susceptible to de-differentiate into another tissue cell fate, whereas cells at a later stage, such as those used in our model, are more likely committed to a pancreatic cell fate.

The mechanisms by which glucocorticoids modulate the levels of the transcription factors remain to be determined. In HIT-T15 cells, it has been shown that glucocorticoids decreased the expression of Pdx-1 by inhibiting Hnf3β (37). In our cultured pancreatic buds, as well as in adult rat islets (E.G., unpublished data), DEX treatment decreased Pdx-1 without inducing any changes in Hnf3β mRNA levels, suggesting that the mechanisms regulating *Pdx-1* gene transcription could be slightly different between mature islets and β-cell lines. Alternatively, the transcription factor or transactivator environment could differ between precursor cells and mature β-cells, thereby allowing a different transcriptional control of the *Pdx-1* gene. Surprisingly, the mRNA levels of the proendocrine marker Ngn3 were unaffected by in vitro DEX treatment, despite increased Hes1 mRNA levels. It is possible that the 1.6-fold increase of Hes-1 was insufficient to inhibit Ngn3; alternatively, other still unknown transcription factors controlling Ngn3 transcription could also operate, thereby interfering with the Hes-1 inhibitory effect. Further studies in the conditional $GR^{Pdx\text{-}Cre}$ and $GR^{RIP\text{-}Cre}$ mutants would help us understand how glucocorticoids affect β-cell lineage at the molecular level.

The present study shows that glucocorticoids are important modulators of lineage commitment in the pancreas, acting during the differentiation process rather than on mature β-cells. The increased islet numbers and size observed in $GR^{Pdx\text{-}Cre}$ mice also shows that glucocorticoids repress signals that normally control β-cell numbers or islet size. Despite normal β-cell mass in $GR^{RIP\text{-}Cre}$ mice, glucocorticoids could also play a role on differentiated β-cells or in postnatal life. Many reports have shown the importance of glucocorticoids on β-cell function: GLUT2 protein has been shown to be decreased (38), glucose-stimulated insulin release is also altered in adult islets treated with DEX (38–41), and a negative glucocorticoid response element was identified on the insulin promoter (42).

Taken together, our data show that glucocorticoids have profound effects on β-cell development and differentiation in vivo. Even though the molecular mechanisms by which glucocorticoids mediate their effects are only partly elucidated at this time, these results demonstrate that glucocorticoids play an important role on pancreatic β-cell lineage during specific developmental windows, acting before hormone gene expression onset and possibly also modulating the balance between endocrine and exocrine cell differentiation. Glucocorticoid hormones should therefore be considered as major hormones involved in normal pancreatic development. These results, together with the previously demonstrated associations of altered β-cell development with impaired glucose tolerance at adult age, strongly support the concept that impaired glucose homeostasis in adulthood can be programmed by glucocorticoid-induced alterations in pancreas differentiation.

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