

## Section 3: $\beta$ -Cell Function and Turnover: Genetic and Metabolic Factors

# Early Development of $\beta$ -Cells Is Impaired in the GK Rat Model of Type 2 Diabetes

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The Goto-Kakisaki (GK) rat is a genetic model of type 2 diabetes obtained by selective inbreeding of mildly glucose-intolerant Wistar rats. Previous studies have shown that at birth, the  $\beta$ -cell mass of the GK rat is severely reduced compared with that of the Wistar rat. Therefore,  $\beta$ -cell deficit could be the primary defect leading to type 2 diabetes in this model. To identify the abnormality at the origin of the  $\beta$ -cell mass deficit, we compared the fetal development of GK and Wistar rats. Our study reveals that during early development (embryonic day 12–14 [E12–14]), GK fetuses present a delayed global growth that progressively recovers: at birth, no size or weight difference persists. However, from E18 onward, the weight and DNA content of the pancreas and liver are reduced by 30% in the GK fetuses. Cell proliferation is reduced in the GK pancreas from E16 to E20. Whereas apoptotic cells are scarce in the Wistar fetal pancreas, a wave of apoptosis from E16 to E18 was detected in the GK pancreas. Analysis of pancreas differentiation revealed that from E12 to E14, there are no significant differences in the number of  $\alpha$ - and  $\beta$ -cells between the GK and Wistar pancreas. However, by E16, the average number of  $\beta$ -cells in the GK pancreas represents only 50% that of the Wistar pancreas, and this difference persists until birth. The number of  $\alpha$ -cells was reduced by 25% from E18 to E21. To determine whether the defect in GK pancreas development depends on intrinsic pancreatic factors or on endocrine extrapancreatic factors, we performed *in vitro* cultures of E12 pancreatic rudiments. The cultures show that *in vitro*, the growth and endocrine differentiation of the GK and Wistar pancreatic rudiments are identical. Thus, impaired development of the GK pancreas probably results from insufficiency of extrapancreatic factor(s) necessary for the growth and survival of fetal pancreatic cells. *Diabetes* 50 (Suppl. 1): S84–S88, 2001

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E, embryonic day; HNF, hepatocyte nuclear factor; PBS, phosphate-buffered saline; PBST, PBS containing 0.5% Tween-20; TUNEL, terminal transferase uridyl nick-end labeling.

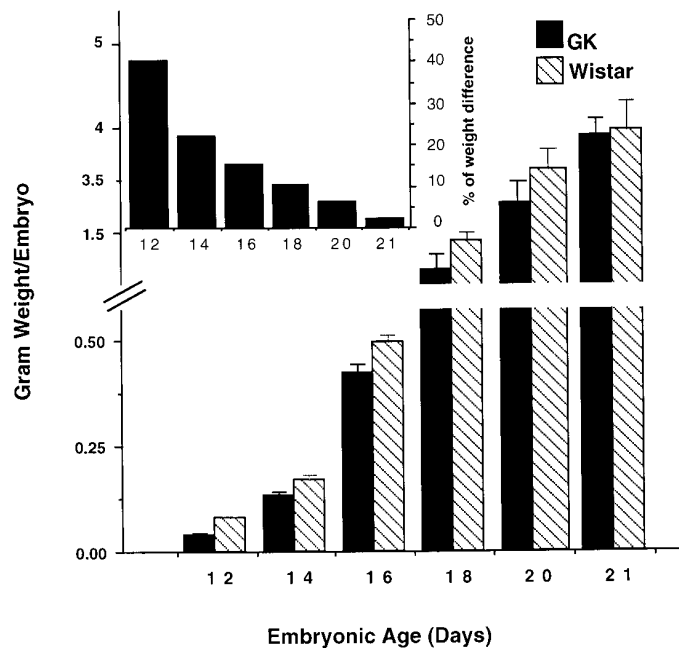
Some studies suggest that total  $\beta$ -cell mass is decreased in type 2 diabetic patients compared with weight-matched control subjects (1–3). This supports the notion that a  $\beta$ -cell mass inadequate to compensate for insulin resistance and/or  $\beta$ -cell secretory defects results in insufficient insulin production and leads to overt diabetes. However, prospective data on the evolution of  $\beta$ -cell mass during the course of type 2 diabetes are not available in humans, and hence it is unknown whether the  $\beta$ -cell mass deficit results from  $\beta$ -cell death and/or inadequate  $\beta$ -cell expansion. This has been addressed in different animal models of type 2 diabetes, although with conflicting conclusions. Thus, in the ZDF rat, the failure of  $\beta$ -cell mass expansion to compensate for insulin resistance is due to increased  $\beta$ -cell apoptosis (4), whereas in the OLETF rat, it seems to be due to impaired  $\beta$ -cell proliferation (5).

The Goto-Kakisaki (GK) rat is a genetic model of type 2 diabetes without obesity (6). The adult GK rat displays decreased  $\beta$ -cell mass (7) together with mild hyperglycemia, glucose intolerance, impaired glucose-induced insulin secretion (8), hepatic glucose overproduction, and moderate peripheral insulin resistance in muscle and adipose tissue (9). Previous studies in our laboratory have shown that newborn GK rats have normal fasting glucose levels compared with Wistar rats and a drastically reduced  $\beta$ -cell mass (7). Thus, the  $\beta$ -cell mass deficit precedes other manifestations of the disease and could represent the primary defect leading to type 2 diabetes in the adult. This result prompted us to investigate the fetal development of the GK pancreas to clearly define at which stage the  $\beta$ -cell mass deficit appears and try to identify the causes of such a deficit.

### RESEARCH DESIGN AND METHODS

**Animals and pancreatic rudiment dissection.** Pregnant GK and Wistar rats were obtained from our local colony. The morning of the discovery of the vaginal plug was taken as embryonic day 0.5 (E0.5). Pregnant rats were killed by pentobarbital injection, and the fetuses were recovered. Pancreases were obtained by microdissecting the fetuses under a Wild microscope (Wild-Leica, Heerbrugg, Switzerland).

**Immunohistochemistry.** The pancreases were fixed at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h, briefly rinsed with PBS, cryoprotected overnight at 4°C in 30% sucrose, and frozen. Consecutive sections (6  $\mu$ m thick) were cut and collected on gelatinized glass slides. For immunostaining, the sections were first incubated for 30 min in PBS containing 3% bovine serum albumin and subsequently incubated for 2 h at room temperature (or overnight at 4°C) with the primary antibodies. After washing in PBS



**FIG. 1.** Fetal growth. The fetal growth of GK and Wistar rats was compared from E12 to E21. At the first stage of development analyzed (E12), weight of GK fetuses was 40% that of Wistar fetuses. GK fetus weight was lower than that of Wistar at all stages analyzed; however, the difference decreased progressively with age. At birth, there was no significant difference in weight between the GK and Wistar rats. Values represent the means  $\pm$  SE of at least 15 fetuses at each stage of development.

containing 0.5% Tween-20 (PBST), the sections were treated with the appropriate fluorescent secondary antibodies. Finally, the sections were extensively washed in PBST and mounted with a fluorescence protecting medium (Vectashield; Vector Laboratories, Compiègne, France). The sections were examined and photographed with an Olympus BX60 microscope (Hamburg, Germany). The antibodies used in this study were guinea pig anti-porcine insulin (Dako, Trappes, France) at 1:500 dilution, mouse anti-porcine glucagon (Sigma, St. Louis, MO; 1:2000), mouse anti-human insulin (Sigma; 1:2000), mouse monoclonal anti-human mitotic proteins (clone: MPM-2; Dako; 1:30), fluorescein anti-guinea pig antibodies (Dako; 1:500), and Texas-red anti-mouse antibodies (1:200; Jackson Immunoresearch, West Grove, PA).

**Quantitative analysis.** The average number of glucagon-, insulin-, MPM-2, or TUNEL-positive cells per section was determined by counting stained cells in one of two sections for pancreases at E12, one of four at E14, one of eight at E16, and one of 15 for E18, E20, and E21. The values shown represent the mean number  $\pm$  SE of positive cells per section from the total number of pancreases analyzed.

**Culture of the dorsal pancreatic rudiments and cytodifferentiation analysis.** Dorsal pancreatic rudiments were grown on top of Millipore filters. The culture medium consisted of M199 supplemented with 10% heat-inactivated

fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Medium was replaced every 48 h. After 7 days of culture, the pancreatic rudiments were processed for immunohistochemistry as described above. To determine the number of endocrine cells per pancreatic rudiment, serial 6- $\mu$ m sections were collected on multiwell glass slides. To avoid counting the same cell twice, one of two consecutive sections (that is, sections separated by 12  $\mu$ m) was analyzed by immunocytochemistry for either glucagon or insulin expression. Only cells with a clearly visible nucleus were counted. Results for each experimental point were obtained by quantifying the absolute number of each endocrine cell type in at least 12 pancreatic rudiments.

**Apoptosis.** Apoptosis detection was performed using the Apotag Plus Peroxidase Kit (Oncor Appligen, Illkirch, France) based on the TUNEL assay (10). **Statistical analysis.** Data are presented as means  $\pm$  SE. Comparison between groups was evaluated using the Student's unpaired *t* test. *P* < 0.05 was considered significant.

## RESULTS

**Fetal growth.** The fetal growth of GK and Wistar rats was compared from E12 to E21 (Fig. 1). At E12, the weight of the GK fetuses was only 40% that of the Wistar fetuses. The weight difference between GK and Wistar fetuses decreased progressively; thus, at E18, it was only 9%, and at birth, no significant weight difference persisted. From E14 to E18, there were no significant differences in the weight or DNA content of different organs between the GK and Wistar embryos. However, by E18 and up to E21 the weight of the pancreas and liver appeared to be reduced by nearly 30% (Table 1). The DNA content corroborated the impaired growth of both organs in GK fetuses (data not shown).

**Pancreatic proliferation.** Cell proliferation in the whole pancreas (that is, epithelial and mesenchymal tissues) was analyzed from E14 to E20 by determining the average number of cells expressing the proliferative antigen MPM-2 (11) per pancreatic section (Fig. 2). At E14, cell proliferation in GK pancreas was similar to that of the Wistar. However, by E16, proliferation in the GK pancreas was reduced by almost 35% (the average number of proliferative cells/pancreas section was  $19 \pm 4$  for GK and  $30 \pm 5$  for Wistar). Reduced pancreatic proliferation was also observed at E18 ( $119 \pm 12$  for GK vs.  $163 \pm 20$  for Wistar) and E20 ( $28 \pm 10$  vs.  $53 \pm 20$ ).

**Apoptosis.** The impaired growth of the pancreas in the GK rat could be the consequence of reduced proliferation but also of increased cell death. We quantified apoptotic cells during pancreatic development by TUNEL (Fig. 3). Very few apoptotic cells were found in the Wistar pancreas from E14 to E20. However, in the GK rat, a wave of apoptosis was detected between E16 and E18. Counterstaining of pancreatic sections with antibodies for insulin and glucagon indicated

**TABLE 1**  
Organ weight

	E21		E18	
	Wistar	GK	Wistar	GK
Liver	351.3 $\pm$ 15.6 (25)	250.1 $\pm$ 10* (30)	144.4 $\pm$ 09.8 (19)	118.4 $\pm$ 10.5 (23)
Lung	79.4 $\pm$ 3.5 (23)	74.5 $\pm$ 4 (30)	55.6 $\pm$ 6.1 (19)	59.2 $\pm$ 8.7 (20)
Kidney	36.2 $\pm$ 1.6 (25)	37.2 $\pm$ 0.9 (25)	ND	ND
Heart	14.8 $\pm$ 0.9 (23)	15.3 $\pm$ 1.2 (25)	10.4 $\pm$ 1.3 (19)	10.7 $\pm$ 0.8 (20)
Spleen	6.2 $\pm$ 0.3 (20)	5.4 $\pm$ 0.5 (25)	ND	ND
Pancreas	30.4 $\pm$ 1.5 (20)	19.2 $\pm$ 2.2† (25)	8 $\pm$ 0.7 (19)	6.0 $\pm$ 0.9 (23)

Data are mean wet weights (mg)  $\pm$  SE (number of animals). \**P* < 0.001; †*P* < 0.01. ND, not determined.

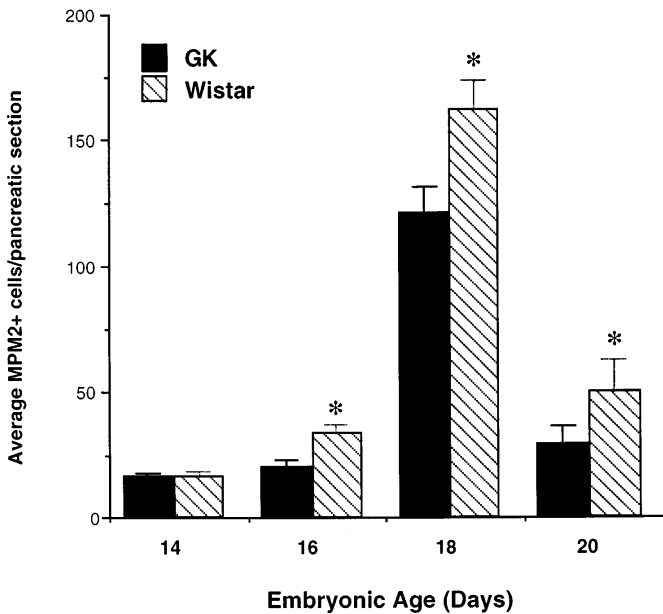


FIG. 2. Analysis of pancreatic proliferation. Cell proliferation throughout pancreas development was quantified by determining the average number of MPM-2<sup>+</sup> cells per pancreatic section. One section (6  $\mu$ m thick) of four was analyzed for pancreases at E14–E16 and one of 15 for E18–E20. Values represent the means  $\pm$  SE of five pancreases for each stage. \* $P < 0.01$ .

that the apoptotic cells in the GK were neither  $\beta$ - nor  $\alpha$ -cells (data not shown).

**Endocrine differentiation.** The differentiation of  $\alpha$ - and  $\beta$ -cells was assessed from E12 to E20 (Fig. 4). During the early

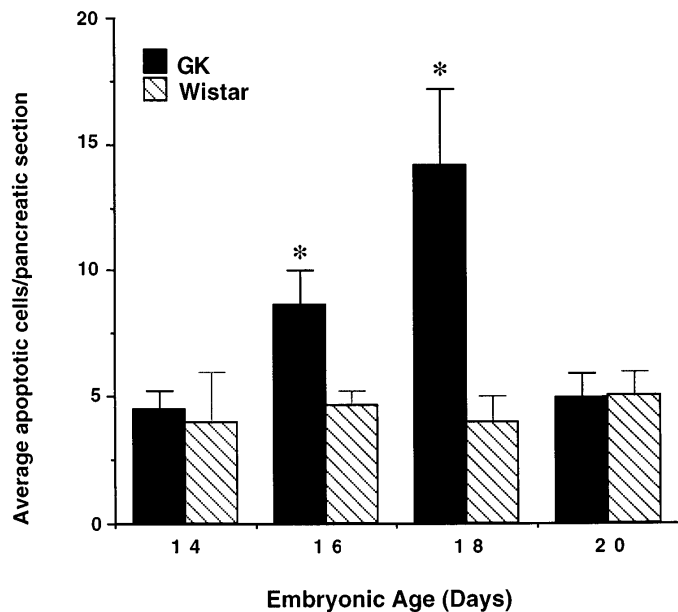


FIG. 3. Pancreatic apoptosis in GK and Wistar fetuses. The average number of apoptotic cells per pancreatic section was analyzed in GK and Wistar pancreases taken at different stages of development. One section of 10 was analyzed between E12 and E16 and one of 20 between E18 and E20. Apoptotic cells were identified by the TUNEL method. Values represent the means  $\pm$  SE of three pancreases for each stage. \* $P < 0.01$ .

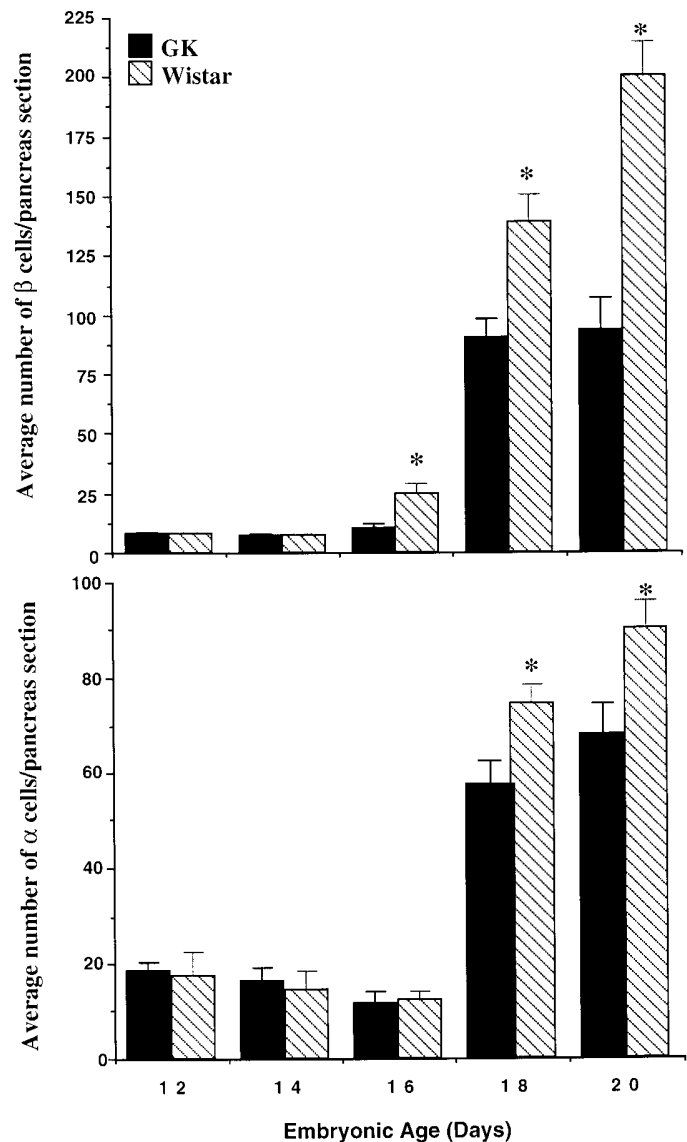
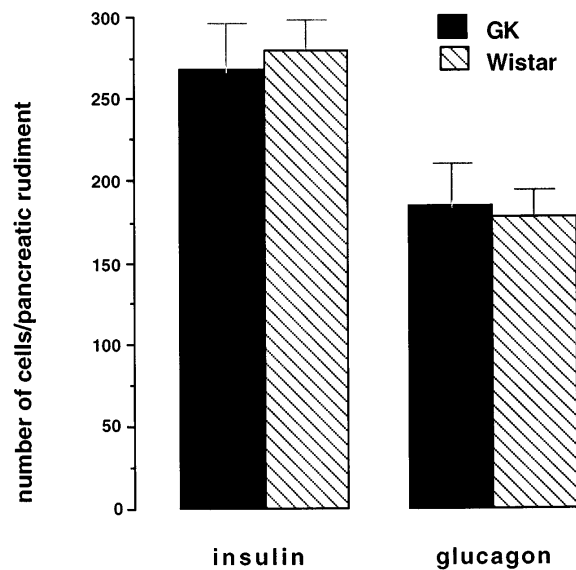


FIG. 4. Analysis of endocrine cell differentiation. The average number of insulin- or glucagon-positive cells per section was determined by counting antibody-labeled cells in one section of two for pancreas at E12, one of eight at E16, and one of 15 at E18–E20. Values represent the means  $\pm$  SE of hormone-positive cells per section. At least seven pancreases were analyzed for each stage of development. In the GK fetuses, differentiation of  $\beta$ -cells was decreased by almost 50% as early as E16 onward. From E18, a significant difference was also observed in the number of  $\alpha$ -cells. \* $P < 0.01$ .

stages of pancreatic development,  $\alpha$ - and  $\beta$ -cell differentiation was similar in the GK and Wistar pancreases. However, by E16, the average number of  $\beta$ -cells per pancreatic section in the GK rat was reduced by  $>50\%$  compared with the Wistar rat ( $10 \pm 3$  vs.  $25 \pm 4$ ); this difference persisted until birth. The average number of  $\alpha$ -cells in the GK pancreas was also significantly reduced at E18 ( $57 \pm 10$  vs.  $75 \pm 8$ ) and E20 ( $69 \pm 6$  vs.  $93 \pm 12$ ). Although this quantitative analysis clearly revealed an important impairment in the differentiation of endocrine cells in the GK pancreas, the morphogenesis of the islets of Langerhans was essentially normal, with the first organized islets appearing at E20. However, these islets were smaller in the GK pancreases.



**FIG. 5.** In vitro development of GK and Wistar pancreatic rudiments. GK and Wistar pancreatic rudiments (E12) were cultured for 7 days in M199 medium supplemented with 10% fetal calf serum. The endocrine differentiation of the rudiments was analyzed by immunohistochemistry. Values represent the means  $\pm$  SE of 12 pancreatic rudiments in each graph.

**Pancreatic development in vitro.** To determine whether the defect in the GK rat leading to reduced growth of the pancreas and endocrine differentiation is dependent on intrinsic pancreatic factors or endocrine extrapancreatic factors, we compared the in vitro development of E12 pancreatic rudiments of GK and Wistar fetuses (Fig. 5). This study revealed that after 7 days of culture, the DNA content and endocrine cytodifferentiation of the GK and Wistar pancreatic rudiments were identical.

## DISCUSSION

At birth, the GK rat presents severely reduced  $\beta$ -cell mass compared with the Wistar rat. The aim of this work was to investigate the fetal development of the GK rat to understand the reason of this impairment. Comparison of the growth of GK and Wistar fetuses has shown that during the early stages of development, GK fetuses display smaller size and weight. Indeed, at E12, the weight of the GK fetuses represents only 40% of their Wistar counterpart. However, this delayed growth is progressively recovered: at E18, the difference of weight represents only 9% and disappears at birth. This striking observation suggests that GK rats lack factor(s) of importance for the somatic growth during early stages of development and that later, this insufficiency can be compensated for by other factor(s). The nature of such factor(s) is not elucidated in the present study. However, previous studies in our laboratory have shown a link between the type 2 diabetes phenotype of the GK rat and a genomic locus containing the gene for IGF-II (12). Moreover, recent studies in our laboratory suggest that at E21, the serum level of IGF-II in the GK rat is reduced by more than threefold compared with the Wistar rat (P. Serradas, personal communication). Thus, low circulating levels of IGF-II during early embryonic life could explain the delay of the global growth of GK fetuses at E12. Study of IGF-I-deficient mice has

demonstrated that IGF-I also plays a significant role in fetal life (13). Thus, a compensatory effect of IGF-I could account for the recovery later during gestation. In this sense, it is significant that circulating levels of IGF-I rise by more than eightfold from E11 to E14 (14).

The delay in global growth of the GK fetus seems to have no major consequence for the development of most organs. Indeed, at birth, the size and weight of the spleen, lung, kidney, and heart were identical in GK and Wistar neonates. However, two organs were considerably affected: the liver and the pancreas. In the GK neonates, the weight of the liver and pancreas are reduced by >30%. It is remarkable that of all organs, only these two are so profoundly affected. This result suggests that GK fetuses lack factor(s) playing an important role in the development of both organs. This is a plausible hypothesis because the liver and pancreas originate from very close regions of the foregut endoderm, and many transcription factors essential for the development of the liver, such as hepatocyte nuclear factor HNF-3 $\beta$ , HNF-4 $\alpha$ , and CCAAT enhancer binding protein  $\beta$  (C/EBP $\beta$ ), are also implicated in the development of the pancreas (15–19).

Comparison of the ontogeny of the pancreas in GK and Wistar rats indicates that the differentiation of the early endocrine cells that appear between E12 and 14 (20) is preserved in GK rats, with the number of insulin- or glucagon-expressing cells being similar to that of the Wistar pancreases. Furthermore, analysis of cell proliferation and apoptosis revealed no differences between Wistar and GK pancreases at this stage. Throughout E12–E16, the pancreatic epithelial buds spread into the surrounding mesenchyme, branching and forming an extensive ductal network. A second population of endocrine cells, to become the main one, develops from the ductal network between E16 and E20. Our study indicates that as early as E16, the number of  $\beta$ -cells differentiating from the ductal network is reduced by >50% in the GK rat. This deficit persists throughout the fetal life. Moreover, during this period in the GK pancreas, cell proliferation appeared decreased, whereas a significant number of apoptotic cells were detected. Decreased proliferation mainly affected the exocrine compartment (both ductal and acinar cells). Proliferating  $\beta$ -cells were not detected during the E12–E20 period, in either the GK or Wistar pancreas. This is in accordance with other studies and with the notion that during fetal life,  $\beta$ -cells originate essentially by differentiation from precursor cells (21,22). Our study also indicates that the apoptotic cells in the E16–E18 GK pancreases are not endocrine cells, because we did not detect TUNEL<sup>+</sup> cells expressing insulin or glucagon; morphological analysis of the sections suggests that these are ductal cells. Thus, the  $\beta$ -cell deficit starts as early as E16. Decreased proliferation and increased apoptosis in the ductal compartment of the pancreas, in which putative endocrine precursor cells reside (20,23), suggests that impaired  $\beta$ -cell development in GK could result from the failure of the proliferative and survival capacities of these endocrine precursor cells. In newborn rats,  $\beta$ -cell destruction by streptozotocin is followed by spontaneous regeneration of the cell mass through both differentiation of precursor cells (neogenesis) and proliferation of surviving  $\beta$ -cells (24–27). Previous studies in our laboratory have shown that  $\beta$ -cell mass regeneration is impaired in streptozotocin-treated newborn GK rats;  $\beta$ -cell proliferation in streptozotocin-treated GK and Wistar neonates was iden-

tical, indicating that the defect in the GK pancreas resulted from defective neogenesis (27). This is consistent with the results of the present study. Poor proliferation and/or survival of endocrine precursor cells during fetal life leads to defective development of  $\beta$ -cell mass and to a decrease in the pool of endocrine precursors needed to maintain a normal capacity of regeneration by neogenesis in later life.

Another important issue addressed in the present study was whether the anomaly leading to impaired differentiation of the endocrine cells in the GK pancreas is dependent on intrinsic factors (produced by either the pancreatic epithelium or mesenchyme) or on extrapancreatic endocrine factors. To address this question, we performed a comparative study of the development of GK and Wistar pancreatic rudiments *in vitro*, expecting that intrinsic pancreatic factors would also express themselves *in vitro* in the GK pancreatic rudiments. Our results show that the *in vitro* growth and endocrine differentiation of GK and Wistar pancreatic rudiments are identical. Thus, the anomaly of the GK rat leading to deficient endocrine cell differentiation seems to result from the deficiency of an extrapancreatic factor. Because endocrine pancreatic precursors localize in the developing ducts, we suspect that this putative factor is necessary for the growth and survival of the endocrine precursor cells.

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