Differential Effects of Prenatal and Postnatal Nutritional Environment on β-Cell Mass Development and Turnover in Male and Female Rats

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Fetal nutrient and growth restriction is associated with development of type 2 diabetes. Although the exact mechanisms responsible for this association remain debated, intrauterine and/or postnatal maldevelopment of β-cell mass has been proposed as a potential mechanism. To address this hypothesis, β-cell mass development and turnover was assessed in rats exposed to either intrauterine and/or postnatal caloric/growth restriction. In total, four groups of male and female Sprague Dawley rats (n = 69) were developed and studied: 1) control rats, *i.e.* control mothers rearing control pups; 2) intrauterine calorically and growth-restricted rats, *i.e.* 50% prenatal calorically restricted pups cross-fostered to control mothers; 3) postnatal calorically and growth-restricted rats, *i.e.* 50% calorically restricted mothers rearing pups born to control mothers; and 4) prenatal and postnatal calorically and growth restricted rats, *i.e.* 50% calorically restricted mothers rearing intrauterine 50% calorically restricted pups. Intrauterine growth restriction resulted in approximately 45% reduction of postnatal β-cell fractional area and mass characterized by reduced rate of β-cell replication and decreased evidence of neogenesis. In contrast, β-cell fractional area and weight-adjusted β-cell mass in postnatal growth restriction was approximately 30% higher than in control rats. Rats exposed to both intrauterine and postnatal caloric and growth restriction demonstrated approximately 80% decrease in β-cell mass, reduction in β-cell replication, and decreased evidence of neogenesis compared with control. Neither intrauterine nor postnatal caloric restriction significantly affected the rate of β-cell apoptosis. These data support the hypothesis that intrauterine maldevelopment of β-cell mass may predict the increased risk of type 2 diabetes in adult life. **(*Endocrinology* 151: 5647–5656, 2010)**

Maternal malnutrition is a major cause of intrauterine growth restriction (IUGR) that afflicts nearly 30 million newborns per year worldwide. It has long been recognized that nutrient availability during fetal and early postnatal life is an important determinant of adult health (1). Specifically, prenatal nutrient insufficiency resulting in low birth weight is associated with increased risk for development of obesity, cardiovascular disease, and type 2 diabetes mellitus (T2DM) (2–4). The association between low birth weight and development of T2DM was first reported in classic studies by Hales *et al.* (4) that demonstrated a several-fold increase in the incidence of glucose intolerance and T2DM in adult males that were born small compared with those who were born at a normal birth weight. These seminal observations have been since consistently reproduced by numerous investigators worldwide (5). Although epidemiological evidence linking low birth weight with increased susceptibility to T2DM is strong (5), the molecular and physiological mechanisms underlying this association are still under investigation (6).

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Abbreviations: BrdU, Bromodeoxyuridine; IPGR, intrauterine and postnatal growth restriction; IUGR, intrauterine growth restriction; PDX-1, pancreatic and duodenal homeobox 1; PNGR, postnatal and growth restriction; T2DM, type 2 diabetes mellitus; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling.

T2DM is a complex polygenic disease that often manifests years before eventual clinical diagnosis (7). T2DM develops as a result of a failure to adequately increase β-cell function and mass to meet the demands of prevailing insulin resistance (8). The contribution of β-cell failure to the pathophysiology of T2DM is supported by islet pathology that reveals a β-cell deficit of approximately 50 and 65% in individuals with impaired fasting glucose and T2DM, respectively (9), suggesting that the loss of β-cell mass is important in the pathophysiology of T2DM. Consistent with these observations, most genes linked to T2DM by genome-wide association scans have been shown to influence some aspects of β-cell biology, such as regulation of β-cell secretory function and development of β-cell mass (10).

It has long been appreciated that low birth weight is associated with adult insulin resistance, which can contribute to the increased risk in development of T2DM (11–13). However, susceptibility to T2DM in low-birth-weight individuals has also been hypothesized to be attributed to inadequate β-cell mass formation (4). Because it is not possible to measure β-cell mass *in vivo*, this hypothesis cannot yet be tested directly in humans. However, evidence suggests that inadequate β-cell formation *in utero* may underlie subsequent susceptibility for T2DM. First, the fetal period is critical for endocrine pancreatic development in rodents and humans (14, 15). Second, animal models of IUGR (due to bilateral uterine artery ligation) develop hyperglycemia as adults characterized by a significant reduction in β-cell mass and secretory function (16). In concert with the animal data, clinical data show that children and adults with low birth weight demonstrate impaired β-cell function compared with their normal-birth-weight counterparts (4, 17).

Our objectives in this study were to first elucidate the effect of maternal nutrient restriction on β-cell mass development immediately after birth and during the suckling postnatal period in the offspring. We intentionally focused on the early postnatal period because it represents the most rapid expansion of β-cell numbers and turnover in rodents and humans (18, 19). Second, we sought to dissect the effect of postnatal *vs.* prenatal nutritional restriction on β-cell development, which was achieved by cross-fostering of newborn control or nutrient-restricted pups to mothers maintained on either control or nutrient-restricted diet. Third, to elucidate the potential mechanisms by which exposure to pre- and/or postnatal nutrient restriction impacts β-cell mass formation, we assessed β-cell turnover (β-cell replication, β-cell apoptosis, and estimated potential β-cell neogenesis). Finally, because almost all pre-/postnatal nutrient restriction animal models focused on male subjects, we investigated potential gender differences in β-cell response to pre-/postnatal nutrient restriction.

Materials and Methods

Animals

Sprague Dawley rats (7–8 wk old, 200–250 g; Charles River Laboratories, Hollister, CA) were bred and housed individually throughout the study at the University of California, Los Angeles, animal housing facility and subjected to standard 12-h light, 12-h dark cycle and allowed *ad libitum* access to regular chow (6% fat, 20% protein, and 70% carbohydrates; Research Diets Inc., New Brunswick, NJ) and water. The University of California, Los Angeles, Institutional Animal Research Committee approved all experimental procedures.

Maternal nutrient restriction model

To recapitulate intrauterine nutrient restriction resulting in growth restriction, pregnant rats received 50% of their daily food intake beginning on d 11 until d 21 of gestation. This protocol results in fetal caloric restriction during the mid to late gestation period. Control rats received *ad libitum* access to food and water throughout the study.

Postnatal animal maintenance

To maintain constant postnatal nutrition, at birth, the litter size was culled to six. Subsequently, a group of male and female newborn pups born to nutrient-restricted (IUGR; n = 12) or control (n = 12) mothers were euthanized and pancreas was removed for morphological analyses. In addition, the newborn rat pups born to nutrient-restricted mothers were cross-fostered to be fed and reared by either a mother that continued to be nutrient restricted by receiving 50% of daily food intake through lactation (11 g/d) or a control mother with *ad libitum* access to rat chow (~20 g/d). Similarly, newborn pups born to control mothers were cross-fostered to be fed and reared by either a mother that continued to be nutrient restricted by receiving 50% of daily food intake through lactation (11 g/d) or a control mother with *ad libitum* access to rat chow (20 g/d). Thus, in total, four groups of male and female rats were created and studied at 21 d of age as outlined in Fig. 1: 1) control rats (n = 12), *i.e.* control mothers rearing pups born to control mothers; 2) intrauterine caloric and growth-restricted rats (IUGR, n = 12), *i.e.* 50% prenatal calorically restricted pups cross-fostered to control mothers; 3) postnatal caloric and growth-restricted rats [postnatal nutrient and growth restriction (PNGR), n = 12], *i.e.* 50% calorically restricted mothers rearing pups born to control mothers; and 4) prenatal and postnatal caloric and growth-restricted rats [intrauterine and postnatal growth restriction (IPGR), n = 12]. At d 21, the pups were euthanized for pancreas removal and analysis.

Pancreas morphology, immunohistochemistry, and immunofluorescence

Pancreas was rapidly removed from euthanized rats, all nonpancreatic tissue was excised, and pancreas was weighed and immediately fixed in 4% paraformaldehyde overnight at 4 C. The pancreas was embedded in paraffin, and subsequently, complete longitudinal sections (4 μm) of pancreas (head, body, and

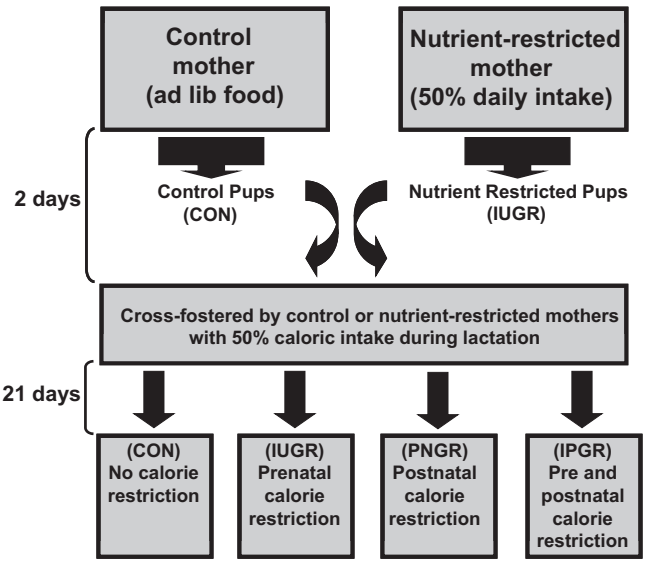

FIG. 1. Schematic representation of the study design. In total, four groups of animals were developed at 21 d: 1) control rats (CON), *i.e.* control mothers rearing control pups; 2) intrauterine calorically restricted rats (IUGR), *i.e.* 50% prenatal calorically restricted pups cross-fostered to control mothers; 3) postnatally calorically restricted rats (PNGR), *i.e.* 50% calorically restricted mothers rearing for control pups; and 4) intrauterine and postnatal calorically restricted rats (IPGR), *i.e.* 50% prenatally and postnatally calorically restricted mothers rearing for intrauterine 50% calorically restricted pups.

tail) were obtained through its maximal width. For determination of β-cell area and mass, three complete longitudinal sections spaced by at least 25 μm were stained first for hematoxylin and eosin and insulin (guinea pig antiinsulin IgG, 1:100; Zymed, Carlsbad, CA). The β-cell mass was determined by first quantifying the pancreatic cross-sectional area positive for insulin and multiplying this value by the pancreatic weight. In addition, sections were costained by immunofluorescence for insulin (guinea pig antiinsulin IgG, 1:100; Zymed), terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL method; Roche Diagnostics, Mannheim, Germany) for quantification of β-cell apoptosis and insulin (guinea pig antiinsulin IgG, 1:100; Zymed), and Ki-67 (mouse anti-Ki-67 IgG, 1:50; Dako, Carpinteria, CA) for determination of β-cell replication. Additional sections were costained for the ductal cell marker cytokeratin (mouse anti-pancytokeratin IgG, 1:50; Sigma Chemical Co., St. Louis, MO), pancreatic and duodenal homeobox 1 (PDX-1) (rabbit anti-PDX-1 IgG, 1:1000; Millipore, St. Louis, MO), neurogenin-3 (mouse anti-neurogenin-3 IgG, 1:200; Developmental Hybridoma Studies Bank, University of Iowa, Iowa City, IA), and insulin (guinea pig antiInsulin IgG, 1:100; Zymed) for determination of PDX-1-positive ductal cells. Secondary antibodies labeled with Cy3, Cy5, and fluorescein isothiocyanate were obtained from Jackson Laboratories (West Grove, PA) and used at dilutions of 1:100 for 1-h incubation. All β-cells per pancreatic section (~1200–2500 cells per section) were examined in detail and counted at ×200 magnification (×20 objectives, ×10 ocular) for the total number of TUNEL- and Ki-67-positive β-cells. Expression of Ki-67 protein has been shown to progressively increase during the cell cycle, peaking in the late S/G2 phase (20). Thus, Ki-67 has been extensively validated for the use as a replication marker in pancreatic sections, and unlike the bromodeoxyuridine (BrdU) method (incorporation of thymidine analog BrdU) lacks toxic and mutagenic translational and transcriptional effects on the cell (21, 22). The frequency of TUNEL and Ki-67 are presented as a percent of total β-cells per section. Neurogenin-3 expression was examined and quantified in five separate ×10 microscope visual fields per pancreatic section per animal. Expression was assessed as mean neurogenin-3/cytokeratin-positive cells per visual field per animal. Fluorescent slides were analyzed and imaged using a Leica DM600 microscope (Leica Microsystems, Wetzlar, Germany) and images acquired using OpenLab software (Improvision) for the microscope and analyzed using the ImagePro Plus software.

Statistical analysis

Statistical analysis was performed using the ANOVA with Fisher's *post hoc* when appropriate using Statistica version 6 (Statsoft, Tulsa, OK). Data in graphs and tables are presented as mean ± SEM. Significance was assigned when $P < 0.05$.

Results

At birth, there was no difference in the litter size or male-to-female ratio between control and IUGR pups (data not shown). As expected, the birth weight was significantly reduced in both male and female IUGR pups compared with control (Fig. 2B). Pancreas weight tended to be also decreased in IUGR pups (particularly in males), but this measure failed to reach statistical significance (Fig. 2C). β-Cell fractional area was comparable between control and IUGR newborn pups in both male and female rats ($P = 0.8$ between groups, Fig. 2, A and D). Thus, β-cell mass (a product of β-cell area and pancreatic weight) was also not statistically different between male/female control and IUGR newborns, albeit male newborns demonstrated a tendency for reduced β-cell mass ($P = 0.06$ for male IUGR *vs.* control; Fig. 2E). In concert with these data, the frequency of β-cell replication (6.21 ± 1.25 *vs.* 6.27 ± 0.9% for IUGR *vs.* control; $P = 0.97$, Fig. 3, A, B, and G) and β-cell apoptosis (0.14 ± 0.50 *vs.* 0.14 ± 0.48% for IUGR *vs.* control; $P = 1$; Fig. 3, C, D, and H) as well as islet density (Fig. 3I) and islet size (Fig. 3J) were similar in control *vs.* IUGR male newborns. Furthermore, both control and IUGR newborns demonstrated a comparably high degree of expression of PDX-1-positive ductal cells (Fig. 3, E and F) indicative of β-cell transdifferentiation from putative duct-like precursor cells (23).

At weaning (*i.e.* 21 d), IUGR born male rats demonstrated approximately 15 and 30% decrease in body and pancreatic weight, respectively, compared with control (Fig. 4, A and B). In female IUGR rats, there was no difference in both body and pancreatic weight compared with control (Fig. 4, A and B). On the other hand, postnatal caloric restriction, irrespective of prenatal nutrient availability, in PNGR or IPGR groups resulted in

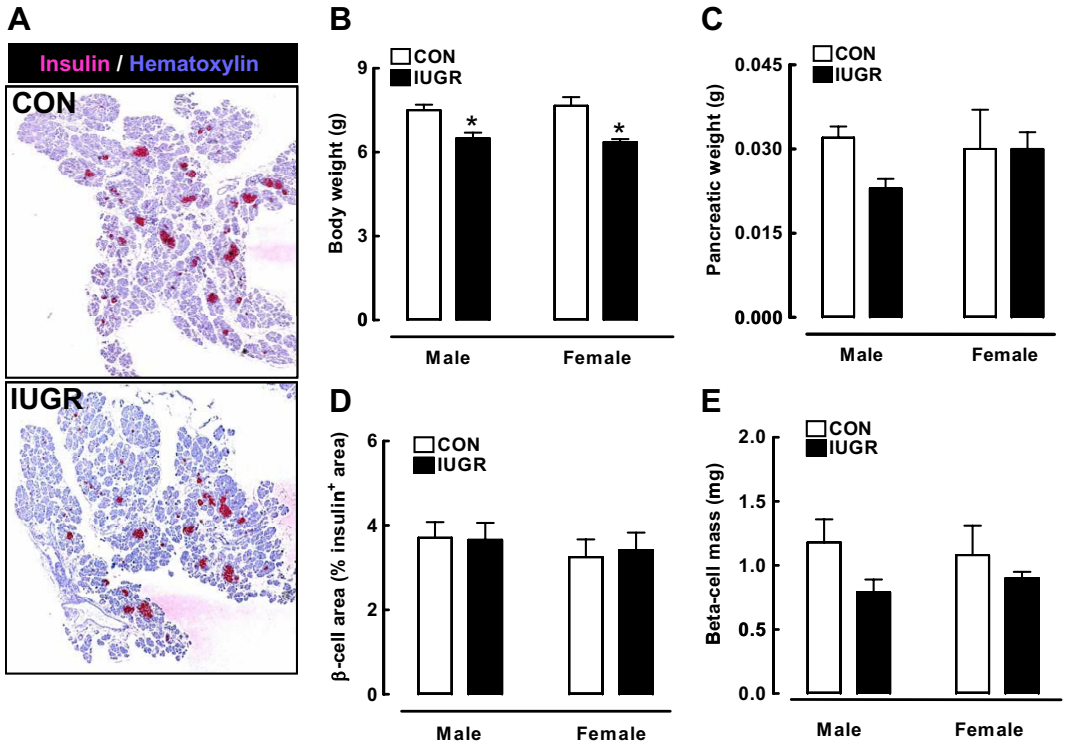

FIG. 2. β-Cell area and mass in 2-d-old control (CON) and IUGR rats. A, Representative pancreatic sections stained for insulin (*pink*) and hematoxylin (*blue*) in control and IUGR rats at 2 d; B–E, body weight (B), pancreatic weight (C), percent β-cell area (D), and β-cell mass (E) in control (n = 10) and *in utero* calorically restricted (IUGR; n = 11) male and female pups at 2 d. Data are expressed as mean ± SE. *, $P < 0.05$ *vs.* control.

a significant reduction in both body (~70%) and pancreatic weight (~60%) compared with control and IUGR groups (Fig. 4, A and B).

Intrauterine caloric and growth restriction in IUGR and IPGR groups resulted in approximately 45% decrease in β-cell fractional area compared with control (0.40 ± 0.02

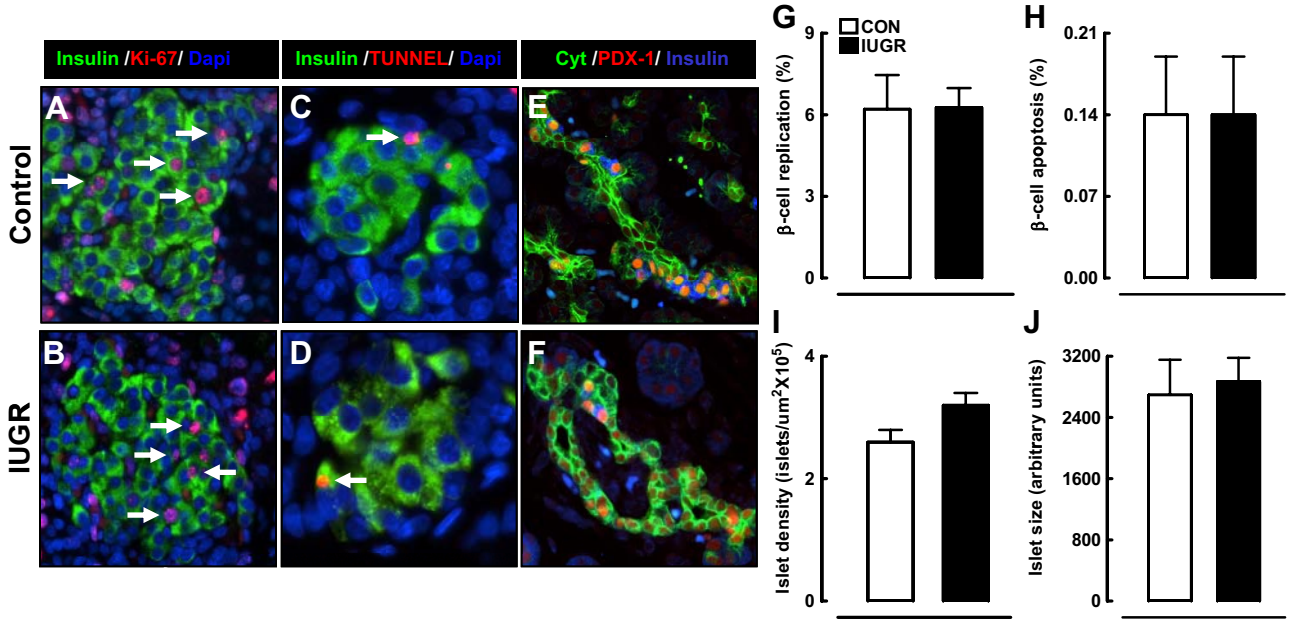

FIG. 3. β-Cell turnover and neogenesis in male control and IUGR rats at 2 d. A and B, Examples of representative islets stained for replication marker Ki-67 (*red*), insulin (*green*), and nuclear marker 4′,6-diamidino-2-phenylindole (Dapi, *blue*) imaged at ×20 magnification in control and IUGR male rats at 2 d; C and D, examples of representative islets stained for apoptosis marker TUNEL (*red*), insulin (*green*), and nuclear marker Dapi (*blue*) and imaged at ×20 magnification in control and IUGR male rats at 2 d; E and F, examples of representative islets stained for ductal cell marker (cytokeratin, *green*), endocrine precursor marker PDX-1 (*red*), and insulin (*blue*) and imaged at ×20 magnification. No discernable differences between control and IUGR groups at 2 d of age were observed. G–J, Quantification of β-cell replication (G), β-cell apoptosis (H), mean islet density (I), and size (J) in control (CON; n = 5) and *in utero* calorically restricted (IUGR; n = 6) male pups at 2 d. Data are expressed as mean ± SE.

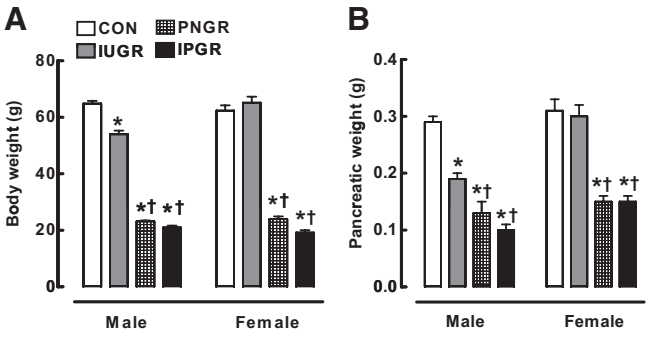

FIG. 4. Body and pancreatic weight in control (CON), IUGR, PNGR, and IPGR male and female rats at 21 d. A and B, Body weight (A) and pancreatic weight (B) in control (n = 12), IUGR (n = 12), PNGR (n = 12), and IPGR (n = 12) male and female rats at 21 d. Data are expressed as mean ± SE. *, $P < 0.05$ *vs.* control; †, $P < 0.05$ *vs.* IUGR.

and 0.40 ± 0.03% *vs.* 0.69 ± 0.12 for female IUGR and IPGR *vs.* control; $P < 0.05$, Fig. 5, A and B) in both male and female rats. In contrast, postnatal caloric and growth restriction alone in both female and male rats failed to negatively affect β-cell fractional area compared with control rats (0.61 ± 0.1 *vs.* 0.69 ± 0.12% for female PNGR *vs.* control; $P = 0.46$, Fig. 5, A and B). Also, in male and female rats, both intrauterine (IUGR) or postnatal (PNGR) caloric restriction resulted in approximately 50% reduction in absolute β-cell mass compared with control rats at 21 d (1.26 ± 0.13 and 1.43 ± 0.24 mg *vs.* 2.79 ± 0.16 mg for male IUGR and PNGR *vs.* control; $P < 0.05$, Fig. 5C). Interestingly, when we expressed β-cell mass as percent of body weight (taking in consideration the reduced body size of pups exposed to postnatal caloric restriction), weight-adjusted β-cell mass in PNGR animals was significantly higher compared with all the other groups ($P < 0.05$ *vs.* all groups) in males and comparable to that of control in female rats (Fig. 5D). However, β-cell mass expressed as percentage of body weight was still decreased by approximately 50% in the IUGR group compared with control in both male and female rats (Fig. 5D). In rats exposed to both intrauterine and postnatal caloric and growth restriction (IPGR), absolute β-cell mass was decreased by approximately 80% (Fig. 5C), and β-cell mass expressed as percentage of body weight was decreased by approximately 50% (Fig. 5D).

Male IUGR and IPGR animals demonstrated a significantly reduced rate of β-cell replication, which was, respectively, 30 and 50% lower compared with control rats (1.12 ± 0.21 and 0.76 ± 0.20% *vs.* 1.66 ± 0.15 for male IUGR and IPGR *vs.* control; $P < 0.05$, Fig. 6, A and B). The rate of β-cell replication was also significantly reduced in postnatal calorically restricted rats (PNGR) compared with control ($P < 0.05$, Fig. 6B). In contrast, neither intrauterine nor postnatal caloric restriction alone or in combination augmented the rate of β-cell apoptosis ($P = 0.96$ between groups; Fig. 6, A and C). To assess potential

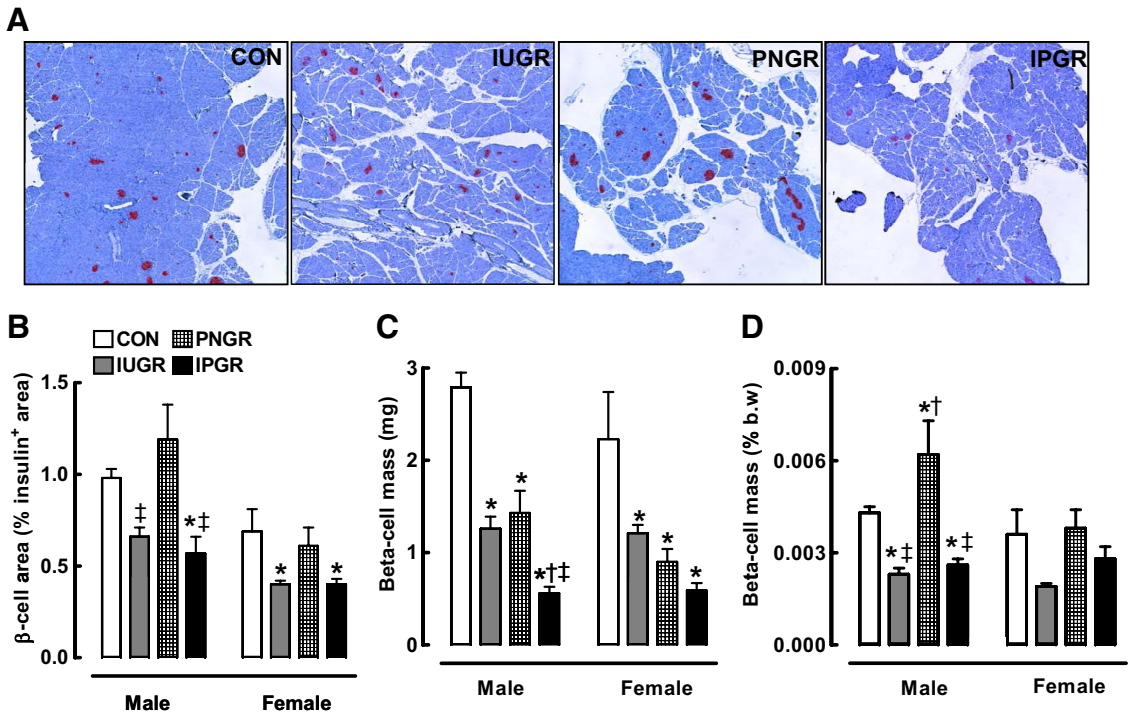

FIG. 5. β-Cell area and mass in control (CON), IUGR, PNGR, and IPGR male and female rats at 21 d. A, Representative pancreatic sections imaged at ×4 from control, intrauterine calorically restricted (IUGR), postnatally calorically restricted (PNGR), and intrauterine and postnatal calorically restricted (IPGR) 21-d-old rats stained for insulin (*pink*) and hematoxylin (*blue*); B–D, β-cell fractional area (B), β-cell mass in milligrams (C) and β-cell mass expressed as a percentage of body weight (D) in control (n = 12), IUGR (n = 12), PNGR (n = 12), and IPGR (n = 12) male and female rats at 21 d. Data are expressed as mean ± SE. *, $P < 0.05$ *vs.* control; †, $P < 0.05$ *vs.* IUGR; ‡, $P < 0.05$ *vs.* PNGR.

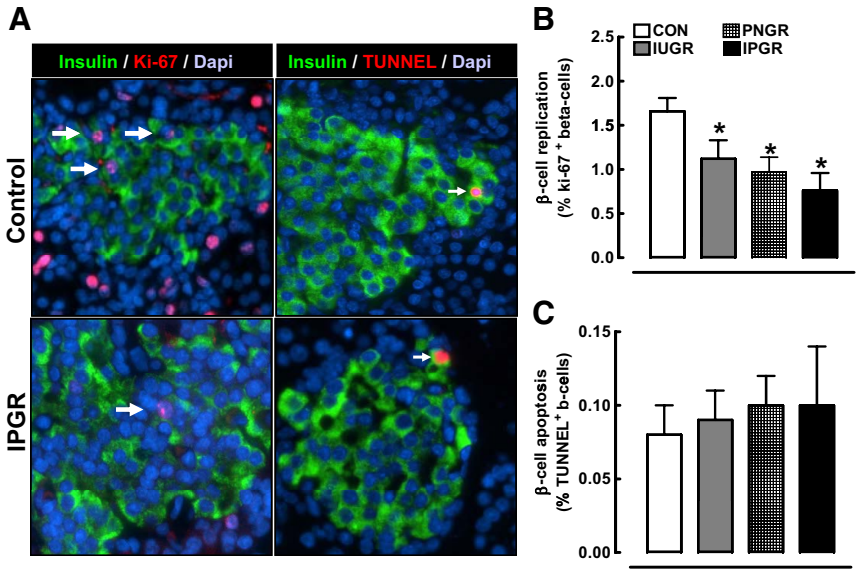

FIG. 6. β-Cell turnover in control, IUGR, PNGR, and IPGR rats at 21 d. A, Examples of islets stained for replication marker Ki-67 (*red*), insulin (*green*), and nuclear marker 4′,6-diamidino-2-phenylindole (Dapi, *blue*) as well as apoptosis marker TUNEL (*red*), insulin (*green*), and Dapi (*blue*) imaged at ×20 magnification in control (CON) and *in utero* and postnatally calorically restricted (IPGR) male rat at 21 d. B and C, Quantification of β-cell replication (B) and β-cell apoptosis (C) in control (CON; n = 6), IUGR (n = 6), PNGR (n = 6), and IPGR (n = 6) male rats at 21 d. Data are expressed as mean ± SE. *Arrows* mark examples of Ki-67 or TUNEL-positive/insulin-positive cells. *, $P < 0.05$ *vs.* control.

contribution of β-cell neogenesis/differentiation to postnatal β-cell mass, we examined PDX-1 and neurogenin-3 ductal cell immunoreactivity. Intrauterine caloric and growth restriction (IUGR) was associated with the greatest decline in PDX-1 ductal immunoreactivity (~80% compared with control; $P < 0.05$, Fig. 7, A–E). Additionally, ductal cell immunoreactivity for PDX-1 was also significantly reduced after postnatal caloric restriction in either PNGR or IPGR groups (~50%; $P < 0.05$ *vs.* control; Fig. 7, A–E). Furthermore, ductal cell immunoreactivity for neurogenin-3, a critical islet development transcription factor, was also significantly reduced (51 ± 6, 54 ± 6, and 65 ± 4 *vs.* 100 ± 10 neurogenin-3/cytokeratin cells per field, $P < 0.05$ *vs.* control; Fig. 7, F–K) in IUGR, PNGR, and IPGR groups, respectively, compared with control.

Discussion

In the present studies, we sought to elucidate the role of prenatal and/or postnatal nutritional environment on β-cell mass development and turnover at birth and during the early postnatal period in rats. We purposefully focused on the early postnatal period of β-cell expansion because this period has been shown to represent the greatest expansion of β-cell numbers and turnover in rodents and humans (18, 19). We report that fetal caloric restriction during mid to late gestation period leads to a significant reduction in postnatal β-cell mass formation, despite exposure to normal postnatal nutrition. This decrease in β-cell mass formation is largely due to a reduction in β-cell fractional area and characterized by a reduced rate of β-cell replication and a potential decrease in β-cell neogenesis.

On the other hand, selective caloric restriction during the postnatal period results in diminished absolute β-cell mass (expressed in mg) attributed solely to the decline in pancreatic weight, which was proportional to the decline in body weight with no change in β-cell fractional area. Consequently, when corrected for decreased body size, animals exposed to selective postnatal caloric restriction exhibit β-cell mass significantly higher than control animals. Animals that were exposed to both pre- and postnatal nutritional restriction exhibited diminished absolute and weight-corrected β-cell mass and significantly reduced β-cell replication and β-cell neogenesis. The effect of intrauterine and/or postnatal caloric restriction on β-cell fractional area and mass was consistent between male and female rats.

In human infants, IUGR is seen in Western countries mainly due to aberrant placental health (24). However, worldwide, the most common cause of IUGR (which afflicts as many as 30 million newborns worldwide) is maternal malnutrition related to diminished calories in her diet similar to our rat IUGR group (1). Separate from IUGR, continued malnutrition of the mother during lactation predisposes the breastfeeding infant to malnutrition postnatally, similar to our IPGR group. Thus, IPGR human infants, who express a slow growth rate from 0–2 yr that is superimposed on a low birth weight, have a higher incidence of glucose intolerance and T2DM (25). Although the pure PNGR group in our study may be difficult to find in relation to maternal malnutrition in the Western world, this condition is still prevalent in developing countries exhibiting substantial seasonal variations in food availability (26, 27). In Western countries, PNGR is rampant in neonatal intensive care units where babies who are critically ill cannot feed and are parenterally nourished (28). Most of these infants suffer from postnatal caloric restriction resulting in postnatal growth restriction mimicking our PNGR group. Although pre- and postnatal growth-restricted human infants are all lumped together as children, it is important to separate these groups when considering interventions.

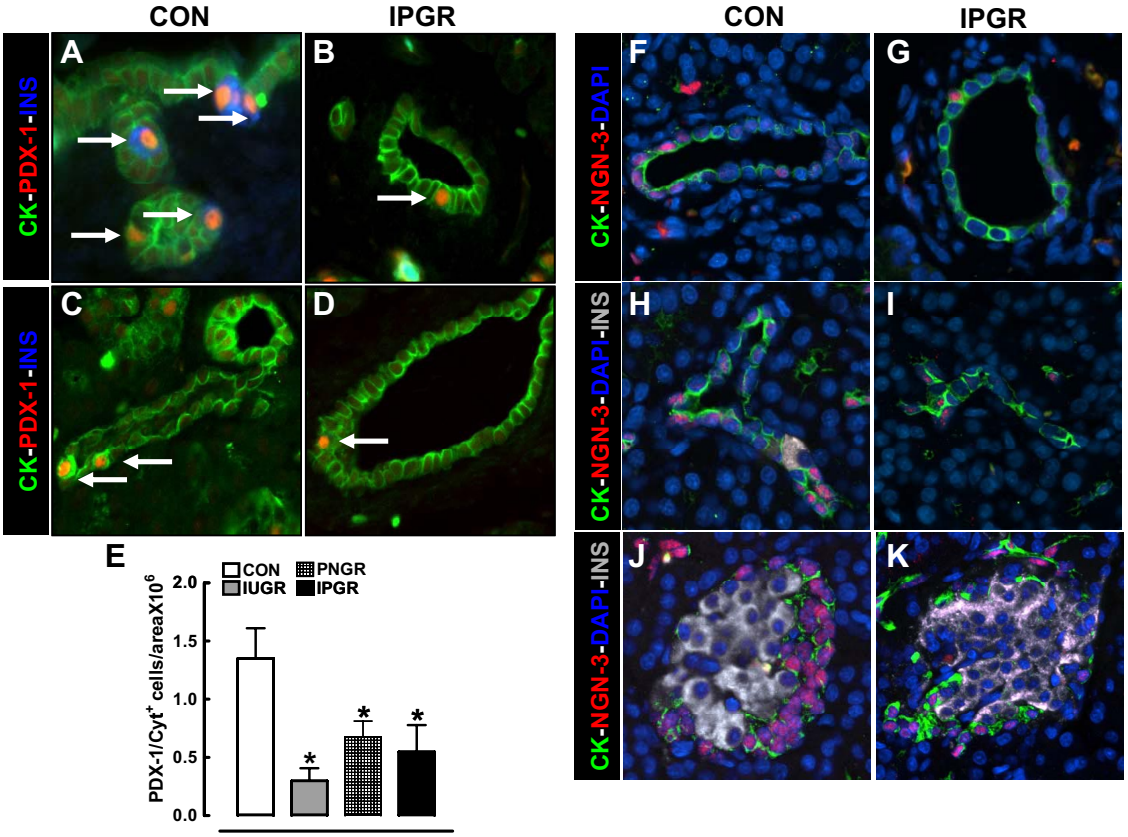

FIG. 7. Estimation of β-cell neogenesis in control (CON), IUGR, PNGR, and IPGR rats at 21 d. A–D, Examples of pancreatic exocrine ducts stained at ×20 for cytokeratin (*green*), insulin (*blue*), and PDX-1 (*red*) in control (A and C) and *in utero* and postnatally calorically restricted (IPGR, B and D) rat at 21 d. *Arrows* mark expression of cytokeratin-positive/PDX-1-positive cells. E, Quantification of the frequency of cytokeratin-positive/PDX-1-positive cells within the exocrine pancreas in control (n = 6), IUGR (n = 6), PNGR (n = 6), and IPGR (n = 6) rats at 21 d. F–K, Examples of exocrine ductal tissue stained at ×20 for cytokeratin (*green*), neurogenin-3 (*red*), and nuclear stain 4′,6-diamidino-2-phenylindole (Dapi, *blue*) and insulin (*gray*) in control (F–J) and *in utero* and postnatally calorically restricted (IPGR, G–K) rat at 21 d. Data are expressed as mean ± SE. *, $P < 0.05$ *vs.* control.

Maternal malnutrition and consequent IUGR is a strong predictor of future development of T2DM (5). The mechanisms by which fetal undernutrition increases the risk for T2DM are still largely unknown, but it has been proposed that inadequate prenatal nutritional environment can influence development of β-cell mass and thus predispose individuals to developing T2DM later in life (4). Our data support this hypothesis. Specifically, we report that intrauterine caloric restriction in rats during the mid to late gestational period leads to a significant (~45%) decrease in β-cell fractional area and β-cell mass irrespective of postnatal nutritional environment. Furthermore, we demonstrate that the mechanisms for impaired postnatal β-cell formation after IUGR is a reduction in β-cell replication and a potential decrease in β-cell neogenesis from putative ductal precursors, with no detected change in β-cell apoptosis. It is important to note that the contributions of β-cell replication *vs.* β-cell neogenesis/differentiation (from putative ductal cell precursors) to maintenance and expansion of postnatal β-cell mass in rodents and humans still remains debated (29, 30). Our observations of reduced PDX-1 and neurogenin-3 expression in pancreatic ducts of IUGR-IPGR groups are consistent with a previously proposed postulate that pre- and postnatal malnutrition impairs β-cell neogenesis/differentiation (31, 32). However, without genetic lineage tracing studies, it is not possible to directly demonstrate contribution of ductal cell precursors to new β-cell formation, and thus, additional studies are needed to address the role of pre-/postnatal nutritional environment on β-cell neogenesis/differentiation.

Previous reports on the effects of pre- and postnatal caloric restriction on rat β-cell mass development and turnover at birth and after weaning exist (31–35). Most (31, 32, 34, 35) but not all (33) reports in rats demonstrate approximately 30–50% decrease in β-cell mass at birth (or during the late fetal period) after exposure to intrauterine caloric or selective protein restriction. β-Cell replication at birth was reported to be unchanged in intrauterine calorically restricted pups compared with controls (31, 32); however, it was noted to be significantly decreased in a model of maternal protein restriction (34). In 21-d-old rats, β-cell mass was reduced by approximately 40% in rats exposed to intrauterine caloric restriction fol-

lowed by exposure to normal postnatal nutrient availability and by approximately 70% in rats exposed to both intrauterine and postnatal caloric restriction (31, 32). Interestingly in these studies, a decline in β-cell mass was not associated with changes in β-cell proliferation when assessed by BrdU incorporation. These findings led the authors to postulate that diminished β-cell mass may be due to impaired β-cell differentiation/neogenesis (31, 32).

In concert with previously published work, our studies in intrauterine calorically restricted pups at birth demonstrated an approximately 30% decrease in β-cell mass (albeit it just failed to reach significance; $P = 0.06$) and an approximately 50 and 80% decrease in β-cell mass at 21 d in rats exposed to either normal or restricted postnatal nutrient availability, respectively. On the other hand, in contrast to previously published studies (31, 32), we observed significant reductions in the frequency of β-cell replication (measured by immunofluorescent colocalization of Ki-67 and insulin) in 21-d-old intrauterine calorie-restricted rats exposed to either normal or restricted postnatal nutrient environment. This apparent discrepancy may be attributed to methodological difference in 1) determination of β-cell replication (BrdU by immunohistochemistry *vs.* Ki-67 by immunofluorescence), 2) duration of intrauterine caloric restriction (d 15–21 *vs.* d 11–21), and 3) rat strain differences (Wistar *vs.* Sprague Dawley) (31, 32). Further studies are needed to address the contribution of β-cell replication to the observed reduction in β-cell mass that is associated with IUGR along with molecular mechanisms responsible for these observations.

In the current study, we also examined the effect of selective postnatal nutrient restriction upon β-cell mass development and turnover. As expected, postnatal caloric restriction resulted in decreased body and pancreatic weight; however, β-cell fractional area and β-cell mass (adjusted for body weight) were unexpectedly increased compared with control. These data suggest that selective PNGR primarily impacts pancreatic weight, whereas selective prenatal nutrient and growth restriction (IUGR) largely determines endocrine cell development. Despite maintaining normal weight-adjusted β-cell mass PNGR animals demonstrated a significantly diminished rate of β-cell replication/neogenesis compared with control rats. However, it is important to emphasize that PNGR animals are exposed to a 50% curtailment in postnatal nutrient availability. This restriction likely contributes to a decrease in availability of nutrient factors purported to mediate the rate of postnatal β-cell formation (*e.g.* glucose, insulin, free fatty acids, glucagon-like peptide-1, and GH) (36–38). Thus, although β-cell replication/neogenesis in PNGR is reduced compared with control, the rate of β-cell formation in PNGR group perhaps reflects restricted availability of postnatal nutrients and is presumably appropriate for maintaining body weight-adjusted β-cell mass.

Interestingly, a recent study examining the seasonality of birth in adult T2DM patients reported that individuals who experienced fetal life during months of nutrient deprivation and postnatal months with abundant nutrition (IUGR group) had the highest rate of T2DM (27). On the other hand, individuals who underwent fetal development during a nutritionally abundant period and postnatal development exposed to months of nutrient deprivation (PNGR group) demonstrated decreased risk for development of T2DM (27). Although many factors may contribute to the development of T2DM in IUGR and PNGR humans, it is intriguing to hypothesize that variance in β-cell mass formation relative to body weight may be one factor underlying differences in predisposition to T2DM in IUGR and PNGR humans.

In recent years, molecular mechanisms responsible for impaired β-cell mass formation after IUGR have come under investigation. First, it has been proposed that IUGR can result in a reduction of the embryonic β-cell progenitor pool leading to inappropriate postnatal β-cell formation. Stanger *et al.* (15) demonstrated that selective genetic reduction in the size of PDX-1^+ pancreatic progenitors during the fetal period results in impaired β-cell formation during the postnatal period with consequent development of glucose intolerance during adulthood. Consistent with this, maternal food restriction leads to a significant reduction in PDX-1^+ and neurogenin-3^+ pancreatic precursors during embryonic development in rats, diminished postnatal β-cell formation, and inability to expand β-cell mass in response to pregnancy (31, 39). Although it is unknown whether exposure to IUGR leads to a diminished pancreatic progenitor pool in humans, fetal pancreatic tissue taken from fetuses with severe IUGR is characterized by a reduction in endocrine cell mass (40).

Another mechanism proposed to explain reduced β-cell formation after IUGR is related to prenatal glucocorticoid exposure. Maternal undernutrition (similar to the one employed in the current study) significantly increased both fetal and maternal corticosterone concentrations in rats (41). Subsequently, maternal and/or fetal overexposure to glucocorticoids (via administration of dexamethasone) impairs both fetal and postnatal β-cell formation in rodents and nonhuman primates (41–44). Blondeau and colleagues (41) have shown that fetal corticosterone concentrations are inversely correlated with fetal insulin content and postnatal β-cell formation in rats.

Evidence suggests that glucocorticoids can exert a direct effect on the developing fetal pancreas via transcriptional modulation of transcription factors involved in

β-cell formation and differentiation (42). Glucocorticoid receptors are present in the pancreas during embryonic development of rodents and humans (42). Specifically, glucocorticoids can bind to the PDX-1 promoter and thus suppress fetal endocrine cell differentiation (42). For example, glucocorticoid treatment has been shown to significantly reduce fetal expression of key endocrine transcription factors such as PDX-1 and Pax-6 but simultaneously increase expression of transcription factors that regulate development of the exocrine pancreas (44). In concert with these findings, another hypoxic-ischemic model of IUGR (due to bilateral uterine artery ligation) is also characterized by a permanent decrease in islet *PDX-1* mRNA expression. This decrease has recently been shown to be due to progressive epigenetic silencing of the *PDX-1* gene locus secondary to proximal promoter methylation (45, 46). Because PDX-1 expression has been shown to be critical for proper β-cell mass formation and expansion in adulthood, the apparent reduction in PDX-1 expression in IUGR may be responsible for the decreased rate of β-cell replication and inappropriate postnatal β-cell mass development (46, 47).

In summary, prenatal nutrient restriction in both male and female rats led to an inappropriate postnatal β-cell mass formation attributed to a decrease in the rate of β-cell replication and a potential decrease in β-cell neogenesis. In contrast, male and female rats exposed to postnatal nutrient restriction alone (with normal prenatal nutrient exposure) were characterized by decreased pancreatic and body weight, but with weight-adjusted β-cell mass higher than control animals. These data are consistent with the hypothesis that impaired prenatal nutritional environment can negatively influence postnatal development of β-cell mass and thus predispose individuals for developing T2DM later in life. Additionally, our studies provide a possible mechanism behind the divergence in predisposition to T2DM in IUGR and PNGR humans.

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References

1. **Saenger P, Czernichow P, Hughes I, Reiter EO** 2007 Small for gestational age: short stature and beyond. Endocr Rev 28:219–251
2. **Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM** 1993 Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia 36:62–67
3. **Barker DJ, Osmond C** 1986 Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1:1077–1081
4. **Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD** 1991 Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303:1019–1022
5. **Whincup PH, Kaye SJ, Owen CG, Huxley R, Cook DG, Anazawa S, Barrett-Connor E, Bhargava SK, Birgisdottir BE, Carlsson S, de Rooij SR, Dyck RF, Eriksson JG, Falkner B, Fall C, Forsén T, Grill V, Gudnason V, Hulman S, Hyppönen E, Jeffreys M, Lawlor DA, Leon DA, Minami J, Mishra G, Osmond C, Power C, Rich-Edwards JW, Roseboom TJ, Sachdev HS, Syddall H, Thorsdottir I, Vanhala M, Wadsworth M, Yarbrough DE** 2008 Birth weight and risk of type 2 diabetes: a systematic review. JAMA 300:2886–2897
6. **Jones RH, Ozanne SE** 2007 Intra-uterine origins of type 2 diabetes. Arch Physiol Biochem 113:25–29
7. **Harris MI, Klein R, Welborn TA, Knuiman MW** 1992 Onset of NIDDM occurs at least 4–7 yr before clinical diagnosis. Diabetes Care 15:815–819
8. **Defronzo RA** 2009 Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. Diabetes 58:773–795
9. **Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC** 2003 β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110
10. **Florez JC** 2008 Newly identified loci highlight β-cell dysfunction as a key cause of type 2 diabetes: where are the insulin resistance genes? Diabetologia 51:1100–1110
11. **Oak SA, Tran C, Pan G, Thamotharan M, Devaskar SU** 2006 Perturbed skeletal muscle insulin signaling in the adult female intrauterine growth-restricted rat. Am J Physiol Endocrinol Metab 290: E1321–E1330
12. **Phillips DI, Barker DJ, Hales CN, Hirst S, Osmond C** 1994 Thinness at birth and insulin resistance in adult life. Diabetologia 37:150–154
13. **Thamotharan M, Garg M, Oak S, Rogers LM, Pan G, Sangiorgi F, Lee PW, Devaskar SU** 2007 Transgenerational inheritance of the insulin-resistant phenotype in embryo-transferred intrauterine growth-restricted adult female rat offspring. Am J Physiol Endocrinol Metab 292:E1270–E1279
14. **Rahier J, Wallon J, Henquin JC** 1981 Cell populations in the endocrine pancreas of human neonates and infants. Diabetologia 20: 540–546
15. **Stanger BZ, Tanaka AJ, Melton DA** 2007 Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. Nature 445:886–891
16. **Simmons RA, Templeton LJ, Gertz SJ** 2001 Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes 50:2279–2286
17. **Brufani C, Grossi A, Fintini D, Tozzi A, Nocerino V, Patera PI, Ubertini G, Porzio O, Barbetti F, Cappa M** 2009 Obese children with low birth weight demonstrate impaired β-cell function during oral glucose tolerance test. J Clin Endocrinol Metab 94:4448–4452

18. **Georgia S, Bhushan A** 2004 β-Cell replication is the primary mechanism for maintaining postnatal β cell mass. J Clin Invest 114:963–968
19. **Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC** 2008 β-Cell replication is the primary mechanism subserving the postnatal expansion of β-cell mass in humans. Diabetes 57:1584–1594
20. **Tsurusawa M, Ito M, Zha Z, Kawai S, Takasaki Y, Fujimoto T** 1992 Cell-cycle-associated expressions of proliferating cell nuclear antigen and Ki-67 reactive antigen of bone marrow blast cells in childhood acute leukemia. Leukemia 6:669–674
21. **Köhler CU, Kreuter A, Rozynkowski MC, Rahmel T, Uhl W, Tannapfel A, Schmidt WE, Meier JJ** 2010 Validation of different replication markers for the detection of β-cell proliferation in human pancreatic tissue. Regul Pept 162:115–121
22. **Taupin P** 2007 BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198–214
23. **Bonner-Weir S, Toschi E, Inada A, Reitz P, Fonseca SY, Aye T, Sharma A** 2004 The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatr Diabetes 5(Suppl 2):16–22
24. **Villar J, Carroli G, Wojdyla D, Abalos E, Giordano D, Ba'aqeel H, Farnot U, Bergsjø P, Bakketeig L, Lumbiganon P, Campodónico L, Al-Mazrou Y, Lindheimer M, Kramer M** 2006 Preeclampsia, gestational hypertension and intrauterine growth restriction, related or independent conditions? Am J Obstet Gynecol 194:921–931
25. **Eriksson JG, Osmond C, Kajantie E, Forsén TJ, Barker DJ** 2006 Patterns of growth among children who later develop type 2 diabetes or its risk factors. Diabetologia 49:2853–2858
26. **Vaiserman AM, Carstensen B, Voitenko VP, Tronko MD, Kravchenko VI, Khalangot MD, Mechova LV** 2007 Seasonality of birth in children and young adults (0–29 years) with type 1 diabetes in Ukraine. Diabetologia 50:32–35
27. **Vaiserman AM, Khalangot MD, Carstensen B, Tronko MD, Kravchenko VI, Voitenko VP, Mechova LV, Koshel NM, Grigoriev PE** 2009 Seasonality of birth in adult type 2 diabetic patients in three Ukrainian regions. Diabetologia 52:2665–2667
28. **van den Akker CH, Vlaardingerbroek H, van Goudoever JB** 2010 Nutritional support for extremely low-birth weight infants: abandoning catabolism in the neonatal intensive care unit. Curr Opin Clin Nutr Metab Care 13:327–335
29. **Inada A, Nienaber C, Katsuta H, Fujitani Y, Levine J, Morita R, Sharma A, Bonner-Weir S** 2008 Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci USA 105:19915–19919
30. **Solar M, Cardalda C, Houbracken I, Martín M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L, Ferrer J** 2009 Pancreatic exocrine duct cells give rise to insulin-producing β-cells during embryogenesis but not after birth. Dev Cell 17:849–860
31. **Garofano A, Czernichow P, Bréant B** 1998 β-Cell mass and proliferation following late fetal and early postnatal malnutrition in the rat. Diabetologia 41:1114–1120
32. **Garofano A, Czernichow P, Bréant B** 1997 In utero undernutrition impairs rat β-cell development. Diabetologia 40:1231–1234
33. **Alvarez C, Martín MA, Goya L, Bertin E, Portha B, Pascual-Leone AM** 1997 Contrasted impact of maternal rat food restriction on the fetal endocrine pancreas. Endocrinology 138:2267–2273
34. **Dumortier O, Blondeau B, Duvillié B, Reusens B, Bréant B, Remacle C** 2007 Different mechanisms operating during different critical time-windows reduce rat fetal β cell mass due to a maternal low-protein or low-energy diet. Diabetologia 50:2495–2503
35. **Petrik J, Reusens B, Arany E, Remacle C, Coelho C, Hoet JJ, Hill DJ** 1999 A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. Endocrinology 140:4861–4873
36. **Bonner-Weir S** 2000 Life and death of the pancreatic β-cells. Trends Endocrinol Metab 11:375–378
37. **Heit JJ, Karnik SK, Kim SK** 2006 Intrinsic regulators of pancreatic β-cell proliferation. Annu Rev Cell Dev Biol 22:311–338
38. **Steil GM, Trivedi N, Jonas JC, Hasenkamp WM, Sharma A, Bonner-Weir S, Weir GC** 2001 Adaptation of β-cell mass to substrate oversupply: enhanced function with normal gene expression. Am J Physiol Endocrinol Metab 280:E788–E796
39. **Blondeau B, Avril I, Duchene B, Bréant B** 2002 Endocrine pancreas development is altered in foetuses from rats previously showing intra-uterine growth retardation in response to malnutrition. Diabetologia 45:394–401
40. **van Assche FA, Aerts L** 1979 The fetal endocrine pancreas. Contrib Gynecol Obstet 5:44–57
41. **Blondeau B, Lesage J, Czernichow P, Dupouy JP, Bréant B** 2001 Glucocorticoids impair fetal β-cell development in rats. Am J Physiol Endocrinol Metab 281:E592–E599
42. **Breant B, Gesina E, Blondeau B** 2006 Nutrition, glucocorticoids and pancreas development. Horm Res 65(Suppl 3):98–104
43. **de Vries A, Holmes MC, Heijnis A, Seier JV, Heerden J, Louw J, Wolfe-Coote S, Meaney MJ, Levitt NS, Seckl JR** 2007 Prenatal dexamethasone exposure induces changes in nonhuman primate offspring cardiometabolic and hypothalamic-pituitary-adrenal axis function. J Clin Invest 117:1058–1067
44. **Gesina E, Tronche F, Herrera P, Duchene B, Tales W, Czernichow P, Breant B** 2004 Dissecting the role of glucocorticoids on pancreas development. Diabetes 53:2322–2329
45. **Park JH, Stoffers DA, Nicholls RD, Simmons RA** 2008 Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest 118:2316–2324
46. **Stoffers DA, Desai BM, DeLeon DD, Simmons RA** 2003 Neonatal exendin-4 prevents the development of diabetes in the intrauterine growth retarded rat. Diabetes 52:734–740
47. **Kulkarni RN, Jhala US, Winnay JN, Krajewski S, Montminy M, Kahn CR** 2004 PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. J Clin Invest 114:828–836