The ontogeny of the endocrine pancreas in the fetal/newborn baboon

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Abstract

Erratic regulation of glucose metabolism including hyperglycemia is a common condition in premature infants and is associated with increased morbidity and mortality. The objective of this study was to examine histological and ultrastructural differences in the endocrine pancreas in fetal (throughout gestation) and neonatal baboons. Twelve fetal baboons were delivered at 125 days (d) gestational age (GA), 140d GA, or 175d GA. Eight animals were delivered at term (185d GA); half were fed for 5 days. Seventy-three nondiabetic adult baboons were used for comparison. Pancreatic tissue was studied using light microscopy, confocal imaging, and electron microscopy. The fetal and neonatal endocrine pancreas islet architecture became more organized as GA advanced. The percent areas of α - β - δ -cell type were similar within each fetal and newborn GA (NS) but were higher than the adults

($P < 0.05$) regardless of GA. The ratio of β cells within the islet (whole and core) increased with gestation ($P < 0.01$). Neonatal baboons, which survived for 5 days (feeding), had a 2.5-fold increase in pancreas weight compared with their counterparts killed at birth ($P = 0.01$). Endocrine cells were also found in exocrine ductal and acinar cells in 125, 140 and 175d GA fetuses. Subpopulation of tissue that coexpressed trypsin and glucagon/insulin shows the presence of cells with mixed endo-exocrine lineage in fetuses. In summary, the fetal endocrine pancreas has no prevalence of a α - β - δ -cell type with larger endocrine cell percent areas than adults. Cells with mixed endocrine/exocrine phenotype occur during fetal development. Developmental differences may play a role in glucose homeostasis during the neonatal period and may have long-term implications.

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Introduction

Type 1 and type 2 diabetes mellitus affect millions of Americans, leading to long-term consequences that can be severe (Polak *et al.* 2000, Halban *et al.* 2010, Zoungas & Patel 2010). The impaired glucose homeostasis seen in both types of this disease has been linked to loss of β -cell mass, impaired β -cell function, as well as increased glucagon secretion. The potential for β -cell regeneration in humans is an area under active investigation (Dhawan *et al.* 2007, Karaca *et al.* 2009, Collombat *et al.* 2010, Halban *et al.* 2010). In order to design therapies with this goal in mind, it is imperative to elucidate the natural development of the endocrine pancreas during fetal life. Pancreas morphogenesis has been studied in rodent models but has not been completely investigated in the fetal human because of the extremely limited availability of fetal pancreas tissue (Svenstrup *et al.* 2002, Kahan *et al.* 2003,

Piper *et al.* 2004, Sarkar *et al.* 2008). In addition, there are large gaps of knowledge in the current literature, particularly during late gestation.

Hyperglycemia has been reported in up to 80% of extremely premature infants (birth weight <1000 g) and has been associated with intra-ventricular hemorrhage, retinopathy of prematurity, and increased risk of death (Pildes 1986, Garg *et al.* 2003, Hall *et al.* 2004, Blanco *et al.* 2006, Liechty 2010). Extremely low birth weight infants lack normal glucose homeostasis, as evidenced by nearly absent glycogen stores at birth (Mena *et al.* 2001). There are long-term consequences of disrupted glucose metabolism in both term and preterm infants, as demonstrated by the development of adult diseases (including type 2 diabetes) earlier in life in infants born to diabetic mothers, premature infants, and infants of low birth weight (Catalano *et al.* 2003, Darendeliler *et al.* 2008, Whincup *et al.* 2008, Meier 2009,

Pilgaard *et al.* 2010). Hyperglycemia is highly prevalent in the preterm baboon and develops spontaneously with a similar incidence to that reported in extremely premature human infants (Blanco *et al.* 2006, 2010). Furthermore, baboons have close (97%) phylogenetic proximity with humans and can develop insulin resistance and diabetes spontaneously. Conversely, the genomic similarity between humans and mice has been reported to be 80% (Waterston *et al.* 2002). These features distinguish baboons from rodents that are frequently used in diabetes research (Chavez *et al.* 2008). Previously, we have shown that the adult baboon is a pertinent nonhuman primate model to examine the underlying cellular/molecular mechanisms responsible for insulin resistance, β -cell failure with islet of Langerhans remodeling, and expansion of the glucagon cell component (Chavez *et al.* 2008, 2009, Guardado-Mendoza *et al.* 2009a,b, Kamath *et al.* 2011). Preterm baboons have significant gestational differences of key insulin signaling proteins in skeletal muscle, which may also contribute to neonatal hyperglycemia (Blanco *et al.* 2010). Moreover, the human endocrine islet architecture is similar to the rhesus macaque and baboon as opposed to the significant differences found when compared with the rodent model (Brissova *et al.* 2005).

The morphology of the adult pancreas has been well defined through numerous studies on humans and several animal models (Orci 1982, Brissova *et al.* 2005, Guardado *et al.* 2009a,b). However, information is relatively sparse regarding the morphogenesis of the pancreas throughout gestation, in particular late gestation (Lukinius *et al.* 1992, Bouwens *et al.* 1997, Polak *et al.* 2000, Svenstrup *et al.* 2002, Bock *et al.* 2003, Kahan *et al.* 2003, Sarkar *et al.* 2008, Meier *et al.* 2010). With the increased survival of premature infants and the high incidence of diabetes in the pregnant population, studying the fetal pancreas during this vulnerable period is of utmost importance. This could have important implications for future targeted therapies in adult diseases. One of the many advantage of using baboons is that they are long-lived animals and therefore, it is possible to study the potential long-term effects of disrupted glucose metabolism *in utero* and at early stages of development.

In this study, we hypothesized that maturational differences in the endocrine cell compartment were present among different gestational ages (GAs) with morphological changes approaching those of adults as gestation increased. We analyzed the pancreases of 12 fetal (four animals per group at three different GAs) and eight newborn baboons (four exposed to enteral feeds, four nonexposed) and used adult baboon specimens (73 control animals) for comparisons.

Materials and Methods

Animals

Fetuses Twelve fetal baboons (seven males, five females) at the Texas Biomedical Research Institute (TBRI) in

San Antonio, Texas were delivered prematurely via c-section under general anesthesia from healthy, nondiabetic mothers at 125 days (d) GA, 140d GA, or near term at 175d GA (full term = 185d GA) and killed immediately after birth. Four animals were delivered in each GA group.

Newborns Animals were born at either TBRI or Baboon Research Resources at the University of Oklahoma Health Sciences Center in Oklahoma City, Oklahoma (UOHSC). Four term baboons (all female) were delivered via spontaneous vaginal delivery from healthy, nondiabetic mothers and killed shortly after birth. Four additional animals (one male, three females) were born at term, fed *ad libitum* by their mothers, and subsequently by veterinary staff with infant formula (Similac, Abbott); these animals are referred to as neonatal throughout the paper. At 4 ± 2 d of life, these animals underwent general anesthesia for 6 h and were exposed to a euglycemic-hyperinsulinemic clamp for 2 h followed by killing to collect data with regards to endogenous glucose production.

Adults Seventy-three adult, nondiabetic animals were used as controls. Pancreas tissues from these animals were used as normal adult pancreas (Guardado-Mendoza *et al.* 2009a).

Blood samples were obtained from the umbilical cord in fetal baboons (shortly after birth) and before being killed by venous puncture in term animals. Plasma glucose was measured using the Analox GM9 analyzer (Analox Instruments, London, UK). Plasma insulin was measured by ELISA (Alpco Diagnostics, Salem, NH, USA).

All studies were approved by the Institutional Animal Care Committee at the TBRI and at the University of Texas Health Science Center, San Antonio. Administrative approval was obtained from UOHSC. Animal experiments were conducted in accordance with accepted standards of humane animal care.

Tissue preparation

Fetal, term, neonatal, and adult pancreatic tissues were obtained postmortem for routine histological staining. The weight of each animal and of the pancreatic tissue was recorded at necropsy and pancreas as a percentage of body weight was calculated. The majority of the pancreas was fixed in 10% neutral buffered formalin and stored in 70% EtOH solution. The specimens were then processed using a Tissue-Tek VIP5 (Sakura Finetek, Torrance, CA, USA) and embedded in paraffin using the Tissue-Tek Embedding Console System (Sakura Finetek). Serial sections were cut at 4 μ m using the HM325 Rotary Microtome (Thermo Scientific, Austin, TX, USA) and mounted on coated Plus slides (Fisher Scientific, Pittsburg, PA, USA).

Histology and immunohistochemistry for light microscopy

Consecutive sections from the pancreatic body region were stained with hematoxylin and eosin (H&E) and immunostained for insulin, glucagon, and somatostatin for light microscopy.

Slides from each paraffin block were processed for immunohistochemistry using the Benchmark XT automatic slide preparation system (Ventana Medical Systems, Tucson, AZ, USA). After dewaxing, the sections underwent a heat-induced antigen retrieval step with a high pH antigen retrieval solution (Cell Conditioning 1 Solution, Ventana Medical Systems) for 30 min. An anti-insulin polyclonal guinea pig antibody (Cell Marque, Hot Springs, AZ, USA) was used to label β cells, an anti-glucagon polyclonal rabbit antibody (Leica Biosystems, Bannockburn, IL, USA) for the labeling of the α cells, and an anti-somatostatin polyclonal rabbit antibody (Cell Marque) for the labeling of δ cells. This step was followed by the application of a biotinylated secondary antibody (all antibodies were prediluted from manufacturers). Sections were subsequently incubated with a streptavidin–HRP complex. Visualization was obtained with a diaminobenzidine detection kit (Ventana Medical Systems). All control incubation without application of the primary antibodies yielded no labeling. Finally, the specificity of the antibody staining for α -, β -, and δ -cells was also validated by competitive displacement experiments, in which the primary antibodies to insulin, glucagon, and somatostatin were preincubated overnight at 4 °C with chemical-grade insulin, glucagon, and somatostatin at a final concentration of 500 nmol. Immunocytochemistry experiments were then completed as described earlier (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Immunofluorescence staining and confocal image analysis

Pancreas specimens were immunostained as previously described (Federici *et al.* 2001). After heat-induced antigen retrieval (2×5 min microwave treatment in 10 mM citrate buffer, pH 6.0), the sections were incubated overnight with the primary antibodies. The following primary antibodies were used: anti-insulin polyclonal guinea pig (Roche; diluted 1:300), anti-glucagon polyclonal rabbit (R&D Systems, Minneapolis, MN, USA; diluted 1:150), and anti-trypsin monoclonal mouse (Chemicon, Temecula, CA, USA; diluted 1:100). Staining with primary antibody was followed by incubation for 2 h with FITC (diluted 1:200), TRITC (diluted 1:150), or CY5 (diluted 1:200)-conjugated secondary antibodies (Jackson ImmunoResearch, Baltimore, MD, USA).

Image acquisition Islets were imaged using a Bio-Rad MRC 1024 confocal laser-scanning microscope (Bio-Rad). To reduce the bleed-through from below, confocal images were acquired sequentially, using the LaserSharp2000 software with a low iris diameter (1–2). The fluorophores (FITC, TRITC, and CY5) are all commonly used for triple immunostaining and the bleed-through for these fluorophores is negligible when sequential scanning is employed. Identical parameters (laser power, iris diameter, and gain) were maintained to acquire images from all sections. Background signal due to nonspecific binding was subtracted from a ‘test’

image. The ‘test’ image is the image obtained from a pancreas section processed exactly as the experimental samples but with secondary antibodies only (no primary antibody).

Image analysis and quantification Quantifications of red and blue fluorescence on digital images were performed using the Image-Pro 3D Analyser 5.1 Image Software (Media Cybernetics, Bethesda, MD, USA). Briefly, single-stain confocal images were merged and enhanced, and then a macro was created in order to automatically quantify the green- and red-stained areas in defined islet regions. Areas labeled for glucagon (green=a) and for insulin (red=b) were quantified in the whole islet, in the islet mantle, and in the islet core. The mantle region is defined as the region of 20 μ m deep that follows the external perimeter of the islet (Bosco *et al.* 2010). The core region is the total islet area minus the 20 μ m deep mantle area. Data are expressed as a ratio between single hormone and stained areas (insulin + glucagon-stained areas). Fifteen islets per section were analyzed from three different animals for each category. Experiments were performed in duplicate. Differences between means were assessed by one-way ANOVA, followed by Kruskal–Wallis test. A *P* value <0.05 was considered statistically significant.

Electron microscopy and immunoelectron microscopy

For ultrastructural study, pancreatic tissues were fixed for 2 h at 4 °C in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M (pH 7.3) cacodylate buffer, postfixed in 1% osmium tetroxide for 1 h at room temperature, dehydrated in ethanol, and embedded in epon–araldite.

For electron microscopy immunocytochemistry, pancreatic tissues were fixed for 2 h at 4 °C in a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M (pH 7.3) cacodylate buffer and embedded in London White Resin (Polysciences, Warrington, PA, USA). Thin sections, after pretreatment with ovalbumin 1% for 5 min, were incubated for 24 h at 4 °C with the following primary antibodies: guinea pig polyclonal anti-insulin antibody (Dako, Carpinteria, CA, USA; diluted 1:50), rabbit polyclonal anti-glucagon antibody (Milab, Malmö, Sweden; diluted 1:1000), and mouse monoclonal anti-trypsin antibody (Chemicon; diluted 1:50). Then, sections were incubated with 12 nm Colloidal Gold–AffiniPure donkey antirabbit (Jackson ImmunoResearch Laboratories) diluted 1:50, or 18 nm Gold–AffiniPure (Jackson ImmunoResearch Laboratories) goat anti-mouse diluted 1:20, or 12 nm Colloidal Gold–AffiniPure donkey anti-guinea pig (Jackson ImmunoResearch Laboratories) diluted 1:20 for 1 h at room temperature. Finally, after counterstaining with uranyl acetate and lead citrate, all thin sections were examined with a Philips (Morgagni 268 D) electron microscope. Specificity controls consisted of omission of the first layer, use of tissues with or without pertinent antigens, and substitution of the primary antibody with normal serum as previously described (Van Noorden 1986).

The pancreas from four fetal baboons (67% gestation) and seven adult baboons were analyzed. For each case, we studied two sections evaluating 200 cells per section, for a total of 400 cells per case.

Tissue analysis

For the morphological assessment of pancreatic development, cases were grouped into six development categories: i) 67% gestation (125d GA); ii) 75% gestation (140d GA); iii) near term, 95% gestation (175d GA); iv) term, 100% gestation (~185d GA); v) term gestation exposed to enteral feeds (185d GA + 5 days); and vi) adults.

Morphometric analysis

Morphometric analysis was performed using the Computer Assisted Stereology Toolbox (CAST) 2.0 system (Visiopharm, Ballerup, Denmark) coupled with an Olympus BX61 microscope (Olympus Corporation of the Americas, Center Valley, PA, USA). By using the stereology fundamentals previously described on pancreatic sections randomly collected (Mandarim-de-Lacerda 2003), we calculated the percent areas of the different tissue structures through the use of lines and points. Four serial sections per animal (H&E, insulin, glucagon, and somatostatin) were taken from the pancreas to quantify endocrine cells. Each field was selected randomly from the whole section using the CAST sampling. On average, we analyzed 48 fields per slide; in each field, point counting of total pancreatic tissue, islets, and individual endocrine cells was performed at the magnification of 40× to calculate the percent area of each component; 180 points were inserted on each field and an average of 8028 points were used per slide. The quantification of the cell percent areas within the total pancreas was calculated by using the following formula: (CP/TP) × 100, where CP, points that hit specified endocrine cells and TP, total pancreas points (Guardado et al. 2009a).

Statistical analysis

Statistical calculations were performed with SPSS for Windows (Version 16.5, SPSS, Inc., Chicago, IL, USA).

Power calculation was based on pancreas weight from term + 5 days animals (0.941 ± 0.24 g); with an expected 33% decrease in pancreas weight in fetal animals, a one-sided *t*-test, α of 0.05 and 80% power, a total of four animals per group were calculated for significance. Differences between groups were analyzed using ANOVA after arcsine data transformation. A *P* < 0.05 was considered to be statistically significant. Confocal data analysis was performed with GraphPad Prism, one-way ANOVA, Kruskal–Wallis test, and Dunn’s multiple comparisons tests.

Results

Gross findings

Demographics for the fetal and neonatal animals studied are shown in Table 1. As expected, the weights of the baboons at term or near-term are significantly higher than at lower GAs (*P* < 0.05). The pancreas weights were similar between fetal baboons before term, regardless of GA. Surprisingly, for those baboons born at term, the pancreas weight increased by greater than twofold at 5 days after birth when compared with their term counterparts killed immediately after birth (*P* = 0.02).

Qualitative analysis/morphology

Immunoreactivity for insulin, glucagon, and somatostatin was found throughout all GAs (Fig. 1). The percent area of endocrine cellular staining for insulin, glucagon, and somatostatin was consistent throughout the fetal GAs, regardless of the hormone studied (Fig. 2 and Supplementary Figure 2, see section on supplementary data given at the end of this article). Additionally, scattered endocrine-stained cells were observed among exocrine cells in fetal pancreas samples (Fig. 3). Although fetal islets resembled the postnatal (term and neonatal) baboon islets, the architectural structure became more organized as GA advanced. Regardless of GA, baboon islet architecture was composed of centralized β -cells with α - and δ -cells mainly peripherally located. The displacement experiments confirmed the specificity of the staining techniques as antigen/hormone specific (Supplementary Figure 1).

Table 1 Demographic characteristics

GA	Weight (g)	Pancreas weight (g)	Pancreas % of body weight	Gender	Fasting serum insulin (mIU/dl)	Fasting serum glucose (mg/dl)
125 d (n=4)	421.5 ± 69.6	0.353 ± 0.26	0.08	3 Male/1 female	2.6 ± 2.2	40.6 ± 4.7
140 d (n=4)	493 ± 40.9	0.247 ± 0.10	0.05	2 Male/2 female	2.9 ± 1.1	37 ± 7.9
175 d (n=4)	1046.5 ± 163.1	0.352 ± 0.25	0.03	2 Male/2 female	2.4 ± 2.7	31.4 ± 4.7
185 d (n=4)	862 ± 74.5	0.394 ± 0.178	0.04	0 Male/4 female	NA	NA
185 + 5 d (n=4)	935.5 ± 94.9	0.941 ± 0.24 [†]	0.10*	0 Male/4 female	8.7 ± 6.1	59.2 ± 10.6
Adult (19 years)	27 000 ± 9000	17.93 ± 1.98 ^a	0.07	29 Male/44 female	32.1 ± 41.0	87.0 ± 16.6

**P* < 0.05 when compared to all GAs for pancreas % of body weight. [†]*P* = 0.01 when compared to 185 d for pancreas weight. ^aAverage adult pancreas weight as previously reported by Mahaney et al. (1993).

Figure 1 Hormone expression in the developing baboon pancreas. GA in fetal baboons is shown at 125, 140, 175, and 185 d gestation (full term = 185 d). 185 + 5 d represent baboons that survived 5 d after natural delivery, adult baboons fed normal diets are shown. Immunohistochemistry panels for insulin, glucagon, and somatostatin for each time point. Bar = 50 µm; all images were taken at the same magnification.

Figure 2 Percent area of islet by cell type within the entire pancreas at different GAs. The volume of α -cells is represented by the black bars, β -cells by gray bars, and δ -cells by white bars. * $P < 0.05$ for each adult cell type compared with all other GAs.

Quantitative analysis/morphometry/immunoelectron microscopy

The percent area of α -, β -, and δ -cells within the endocrine pancreas was similar between and within each fetal and neonatal gestations (NS); however, the percent area of each cell type (α , β , and δ) was 15-, 5-, and 94-fold higher than adult animals, respectively, $P < 0.05$ (Fig. 2). Representative immunostains from each cell type from fetal and adult animals are depicted in [Supplementary Figure 2](#).

Confocal microscopy The organization of fetal, neonatal, and adult pancreas was analyzed by confocal microscopy after immunostaining with specific hormones as markers of the endocrine pancreas and trypsin as a marker of acinar cells (Fig. 4A, B and C). In adult pancreases, well-defined islets were surrounded by trypsin-positive acinar cells. In neonatal pancreases, islets were clearly recognizable and separated from the exocrine tissue, while in fetal pancreases, islet morphology was less well defined. In the adult islet, the

majority of cells was insulin positive; fetal and neonatal islets had a more even mixture of insulin-, glucagon-, and somatostatin-positive cells. The islet architecture was also analyzed using triple staining with insulin, glucagon, and trypsin (Fig. 4D, E, F, G, and H). In adult and neonatal pancreases, glucagon-positive cells had a prevalent peripheral localization, while insulin-positive cells were mainly detected in the islet core. In fetal samples, insulin-positive cells were surrounded by cells slightly positive for glucagon. At the islet periphery, the margin between the endocrine and the exocrine tissue was not always clearly defined.

Interestingly, a subpopulation of cells showed both an endocrine and acinar phenotype as shown by colocalization of trypsin and glucagon in fetal baboons (Fig. 4G) whereas neonatal baboons did not (Fig. 4H). The above findings were confirmed by immunoelectron microscopy, which demonstrated 2% of fetal cells with both glucagon-immunoreactive α -type secretory granules and trypsin-positive zymogen granules in the cytoplasm. Additionally, 1% of fetal cells contained both insulin-immunoreactive β -type secretory granules and trypsin-positive zymogen granules in the cytoplasm (Fig. 5). Conversely, this colocalization was not observed in adult cells ([Supplementary Figure 3](#), see section on [supplementary data](#) given at the end of this article).

In the adult pancreas, the segregated cell type distribution was clearly evident with β cells prevalently localized in the mantle and in the core islet (Fig. 6). This observation was confirmed by a quantitative analysis. Areas labeled for glucagon (Fig. 6A, green) and for insulin (Fig. 6A, red) were quantified in the whole islet, in the islet mantle, and in the islet core. The mantle region was defined as the region of 20 μ m deep that followed the external perimeter of the islet ([Bosco et al. 2010](#)). The core region was the total islet area minus the mantle area. The results in a mature islet (adult) are expressed as $b/(a+b)$ (a = glucagon, b = insulin). β -cells were 0.46 ± 0.02 within the mantle and 0.78 ± 0.03 within the core, demonstrating that β -cells are prevalently segregated in

Figure 3 Hormonal expression in ductal (thin arrows) and acinar (thick arrows) components of the fetal exocrine pancreas (A=insulin stain, B=glucagon stain, C=somatostatin) and term newborn pancreas (D=insulin stain, E=glucagon stain, F=somatostatin). Bar=50 μ m.

Figure 4 Confocal microscopy of fetal, neonatal, and adult baboon pancreas. Islets of Langerhans are clearly recognizable and well organized in the adult and neonatal pancreas samples. (A, B, and C) Double immunostaining with trypsin (green) and insulin (red). Insulin-positive cells are interspersed in the exocrine pancreas of the fetal sample only. (D, E, and F) Triple immunostaining with trypsin (green), insulin (red), and glucagon (blue). Glucagon staining (arrowhead) was detected among trypsin-positive cells in the fetal pancreas only. (G and H) Enlarged images of the triple immunostaining. Bar = 30 µm.

the core region in the mature islet (Fig. 6A and B). Pancreatic islets from fetal (125 and 175 days) and neonatal baboons were analyzed in the same fashion. The segregation of β -cells in the core region in the most immature fetal islets ($b/(a+b)$) were 0.52 ± 0.03 and 0.30 ± 0.02 in the core and mantle respectively; it progressively increased where neonatal islet ($b/(a+b)$) β -cells were 0.62 ± 0.03 and 0.38 ± 0.03 in the core and mantle, respectively. However, the total β -cell ratios ($b/(a+b)$) within the islet were significantly lower in fetal and neonatal islets compared with adult islets and the ratios increased as GA increased (0.42 ± 0.02 , 0.46 ± 0.02 , 0.53 ± 0.02 , and 0.68 ± 0.02 in the fetal 125 d, fetal 145 d, neonatal, and adult respectively. $P < 0.001$).

Discussion

In this study, we demonstrated that fetal endocrine pancreas has no prevalence of a single cell type during fetal development and the early newborn period. The true timing of endocrine differentiation is not well understood, but it is

theorized that massive differentiation is contributory towards the increased percentage of endocrine pancreas seen during fetal development (Polak *et al.* 2000). Although fetal islets resembled postnatal baboon islets, the architectural structure became progressively more organized as GA advanced. Studies on humans have shown similar results, but these studies have involved fetuses from miscarriages and terminations of pregnancies; therefore the number of samples studied have been small and scattered through gestation (Lukinius *et al.* 1992, Bouwens *et al.* 1997, Polak *et al.* 2000, Piper *et al.* 2004, Brissova *et al.* 2005, Meier *et al.* 2010).

The percent areas of α -, β -, and δ -cells did not change over time in fetuses or in term animals after survival with or without exposure to feeds but remained larger than the adults (Fig. 2); pancreas function, expressed as plasma insulin and glucose levels, was similar between gestations and was within the levels reported in preterm and newborn humans (Blanco *et al.* 2008, Committee on Fetus and Newborn 2011). The islet architecture, however, was clearly more organized in those animals that survived beyond birth (Fig. 1). Data in newborn humans are lacking, in particular during the first

Figure 5 Electron microscopy. The cytoplasm of acinar cells shows numerous electron-dense zymogen granules (a), which are positive for trypsin (b). Some cells showed in the cytoplasm, zymogen granules and α -type secretory granules (c), with typical electron-dense core and clear halo (inset). Double label electron microscopy immunocytochemistry (d) demonstrated that α -type secretory granules were glucagon positive (glucagon immunoreactivity identified with 12 nm colloidal gold), while zymogen granules were positive for trypsin (trypsin immunoreactivity is evidenced with 18 nm colloidal gold). Moreover, other cells presented in the cytoplasmic zymogen granules and crystalline β -type secretory granules (some indicated with arrows) (e). Double label electron microscopy immunocytochemistry (f) demonstrated that β -type secretory granules were insulin positive (insulin immunoreactivity identified with 12 nm colloidal gold), while zymogen granules were positive for trypsin (trypsin-immunoreactivity is evidenced with 18 nm colloidal gold). In tissues processed for electron microscopy immunocytochemistry (embedded in London White Resin, without postfixation in 1% osmium tetroxide), zymogen granules appear clearer than that observed in tissue processed for conventional electron microscopy.

week of life. Infant death during this period of life is usually due to sepsis, metabolic disorders, or genetic conditions that would not provide a normal pancreas morphology (Iams & Creasy 2004). Therefore, we suggest that this model may provide appropriate insight into the postnatal changes in pancreas development in a healthy state.

Islet cell organization in the baboon is similar to that seen in humans. We demonstrated prevalent peripheral localization of glucagon-positive cells with a central localization of insulin-positive cells by confocal microscopy (Figs 4 and 6). Interestingly, the ratio of β -cell population within the islet and the percent ratio found in the core increased as GA increased, resembling the organization of mature islets

(adult baboons). Studies of fetal pancreas from rodent and nonhuman primates showed inconsistent results with regards the core-mantle architecture (Bocian *et al.* 1999, Murtaugh 2007, Collombat *et al.* 2010). However, studies of pancreas from humans have demonstrated core-mantle organization, with insulin-producing cells localized centrally beginning in the mid-gestational period (Meier *et al.* 2010). Although some mouse/rat models show a typical core-mantle architecture similar to humans (Brissova *et al.* 2005), genetic similarity to mouse is only 80% (Waterston *et al.* 2002). On the other hand, the genetic similarity between humans and nonhuman primates (Chimpanzees and Baboons) has been demonstrated to be in the range of 94–98% (Cheng *et al.* 2005, Chavez *et al.* 2008). Furthermore, adult baboons also develop insulin resistance and diabetes spontaneously, a rare feature in other animal models, and protein expression of key insulin signaling in baboons has been shown to be 97–98% identical to humans (Chavez *et al.* 2008). Therefore, studying the morphogenesis of the pancreas during critical periods of development in baboons may be key in the development of new treatments to prevent and treat diabetes, but most relevant to the present study is to provide new insights in the understanding of islet differentiation.

Surprisingly, a significant increase of the total pancreas weight was seen postnatally after 5 days of extrauterine life. Since there were no differences in the percent areas of each cell type in those newborns exposed to feeds, this postnatal increase in pancreas weight appears to be due to a large expansion of both endocrine and exocrine pancreas. In the rodent model, the postnatal period between birth and weaning is characterized by a massive expansion in β -cell mass with the increasing physiological demands but also balancing with β -cell death during pancreatic remodeling (Finegood 1995, Georgia & Bhushan 2004, Dhawan *et al.* 2007). Further studies targeting markers of cell death/regeneration will be performed to elucidate the cause/effect of these changes.

Other investigators have reported a 2–5% percent β -cell in humans and other animal models (Beringue *et al.* 2002, Bock *et al.* 2003, Limesand *et al.* 2005, Meier *et al.* 2010). By contrast, in the current study we report a larger β -cell percent area throughout late gestation and early neonatal periods (Fig. 2 and Supplementary Figure 2). Unfortunately, the methodology used for pancreas measurements in different studies is largely variable, the GAs included have a wide range of age, and most have studied postnatal development. Moreover, most human studies have used aborted or infected fetuses and/or neonates who have died due to neonatal disease (Beringue *et al.* 2002, Meier *et al.* 2010); disease processes, particularly those of infectious origin, may have an impact on pancreas development due to potential effects on mitosis/apoptosis rates. Therefore, a nonhuman primate model, where serial, consistent measurements are performed in an otherwise healthy animal, may be more representative of normal development. Another potential explanation for the larger endocrine pancreas found during fetal development in

Figure 6 Distribution of α - and β -cells in islet of Langerhans subregions during development. (A) Double immunofluorescence stainings. Pancreas sections from fetal (125 d), fetal (175 d), neonatal, and adult baboons were double stained with insulin (red) and glucagon (green), and images were analyzed by means of confocal microscopy. Representative images are shown. White lines delimiting the islet mantle and core subregions are shown. Bar: 20 μ m. (B) Analysis of α - and β -cell distribution in islet subregions. Areas labeled for glucagon (a) and insulin (b) were measured in the whole islets (whole) and in different subregions (mantle and core) as described in Materials and Methods section. Results are expressed as insulin/(a+b) or glucagon/(a+b) ratio. Columns are means $\pm$ S.E.M. $n=45$ islets from three pancreas.

this study may be species related differences or gender differences due to small sample size.

It has been established that the endocrine pancreas derives from epithelial (ductal) precursor cells (Bouwens *et al.* 1997). Ductal cell differentiation from putative precursor cells, sometimes referred to as 'neogenesis', has been suggested to occur in neonatal animals (Finegood 1995, Dhawan *et al.* 2007). It has not been determined, however, whether early pancreas cells are pluripotent with the capability of contributing to both endocrine and exocrine lineages, or if there is a subpopulation of cells that may be unipotent stem cells. In these experiments, we demonstrate a subpopulation of cells that show both an endocrine and acinar phenotype in the fetal baboon by immunohistochemistry and by

colocalization of trypsin/glucagon and trypsin/insulin by confocal microscopy and immunoelectron microscopy (Figs 4 and 5). To the best of our knowledge, this is the first report of both exocrine and endocrine phenotypes occurring in the same pancreatic cell. The importance of this finding cannot be understated due to the potential long-term implications of the effects of different interventions in a pancreatic cell that might be pluripotent at a particular period of development. Thus, the baboon provides a unique model where differentiation and different stages of development of endocrine and exocrine pancreas could be followed long term.

With prematurity being one of the two leading causes of perinatal morbidity and mortality and because postnatal adaptation is associated with increasing incidences of type 2 diabetes mellitus, essential hypertension, and coronary artery disease in surviving low birth weight babies (Hack 2006, Hofman *et al.* 2006, Hovi *et al.* 2007, Rotteveel *et al.* 2008, Whincup *et al.* 2008, Bacchetta *et al.* 2009), an increase in knowledge regarding fetal pancreas development will further our understanding of the disruptive glucose metabolism in these infants.

The ontogeny of the endocrine pancreas in the fetal/newborn baboon model provides insight into the normal pancreas development from late gestation through the neonatal period. Furthermore, the presence of pancreatic cells with both endocrine and exocrine phenotypes during late gestation may have long-term implications if born during this critical period of development. A nonhuman primate model that survives prematurity and develops diabetes spontaneously would lead to further studies that might pave the way to the development of novel therapeutic strategies for patients with hyperglycemia and diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-12-0070>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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