Diabetes Publish Ahead of Print, published online March 11, 2008

Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans

Juris J. Meier[1], Alexandra E. Butler[1], Yoshifumi Saisho[1], Travis Monchamp[2], Ryan Galasso[1], Anil Bhushan[1], Robert A. Rizza[3], Peter C. Butler[1]

1: Larry Hillblom Islet Research Center, UCLA David Geffen School of Medicine, Los Angeles, USA
2: Division of Endocrinology, Diabetes and Hypertension, UCLA David Geffen School of Medicine, Los Angeles, USA
3: Division of Endocrinology, Diabetes, Metabolism and Nutrition, Mayo Clinic, Rochester, Minnesota, USA

Corresponding Author:
Peter C. Butler
Larry Hillblom Islet Research Center
UCLA David Geffen School of Medicine
24-130 Warren Hall
900 Veteran Avenue
Los Angeles
CA 90095-7073
pbutler@mednet.ucla.edu

Received for publication 26 September 2007 and accepted in revised form 26 February 2008.

ABSTRACT

Objective: Little is known about the capacity, mechanisms or timing of growth in beta cell mass in humans. We sought to establish if the predominant expansion of beta cell mass in humans occurs in early childhood, and if, as in rodents, this coincides with relatively abundant beta cell replication. We also sought to establish if there is a secondary growth in beta cell mass coincident with the accelerated somatic growth in adolescence.

Research Design and Methods: To address these questions, pancreas volume was determined from abdominal computer tomographies in 135 children aged 4 weeks to 20 years, and morphometric analyses were performed in human pancreatic tissue obtained at autopsy from 46 children aged 2 weeks to 21 years.

Results: We report that (1) beta-cell mass expands by several-fold from birth to adulthood, (2) islets grow in size rather than in number during this transition, (3) the relative rate of beta-cell growth is highest in infancy and gradually declines thereafter to adulthood with no secondary accelerated growth phase during adolescence, (4) beta-cell mass (and presumably growth) is highly variable between individuals, (5) a high rate of beta cell replication is coincident with the major postnatal expansion of beta-cell mass.

Conclusions: These data imply that regulation of beta cell replication during infancy plays a major role in beta cell mass in adult humans.

Glucose homeostasis is regulated by the secretion of insulin from pancreatic beta-cells (1). Sufficient beta-cells are therefore required to maintain normal glucose concentrations, as demonstrated by development of diabetes if beta cell mass is decreased surgically or by beta cell toxins (2-4). A deficit in the number of beta-cells (collectively referred to as the beta cell mass) contributes to the development of both type 1 and 2 diabetes (5-8). Correction of hyperglycemia by replenishment of beta cell mass in both type 1 and 2 diabetes further emphasizes a central role of beta cell deficiency in diabetes. However beta cell replacement by transplantation (whole pancreas or islet transplantation) has limitations as a therapy for diabetes (9, 10). First, the incidence of diabetes far exceeds the supply of donor organs. Second, the side effects of chronic exposure to immunosuppression includes increased risk for lymphoproliferative disease as well as renal and islet failure (9). Third, there is relatively rapid progressive graft failure in the case of islet transplantation, although this is less problematic for whole pancreas transplantation (11).

Alternative approaches have been explored to restore an effective beta cell mass as a therapy for diabetes. One approach is to create beta cell surrogates by gene manipulation of a variety of cell lines, but to date these efforts have not developed glucose responsive cells that secrete sufficient insulin to be clinically useful (12). Another approach is to foster differentiation of embryonic stem cells into beta cells. There has been some success in this regard (13, 14), but such approaches are still far from being applicable to humans.

An alternative strategy is to foster beta cell regeneration from endogenous sources. Cellular regeneration is most readily accomplished in tissue with a high basal rate of turnover (for example gut epithelium) or a marked capacity for new cell formation after cell loss (for example liver). There is indirect evidence of beta cell turnover in adult humans (7). Beta cell mass can be adaptively increased in humans, for example in obesity (8, 15), but the extent of this adaptive increase is modest compared to that in obese rodents (~0.5 fold versus ~10 fold) (8, 16). We reasoned that the most significant period of new beta cell formation in humans occurs between birth and the fully grown young adult. Effective beta cell regeneration in established type 1 diabetes would need to provide a comparable degree of beta cell formation.

There are two phases of rapid growth in childhood, the first three years of life and adolescence. There is an increased incidence of type 1 and 2 diabetes in adolescence. There is an increasing incidence of type 1 diabetes in young childhood. These clinical observations draw particular attention to the pattern of beta cell growth during these two periods of childhood.

Therefore our first aim in this study was to establish by use of pancreas obtained at autopsy the extent and timing of the expansion of beta cell numbers during human childhood from infancy to fully grown young adults. Our second aim was to establish the predominant source of newly forming beta cell cells. Based on partial pancreatectomy studies in rats, it has been suggested that beta-cell regeneration may occur in part through the formation of new islets from endocrine progenitor cells residing in the exocrine ductal epithelium (17). Other investigators have proposed that bone marrow stem cells may give rise to beta cells in mice (18), although this has subsequently been challenged in murine and human studies (19, 20).

Documentation of beta cell formation from stem cells requires lineage studies, an

approach that is impracticable in humans. We therefore reasoned that having identified the period of maximal expansion of beta cell numbers in humans, we would seek to establish if this is accompanied by an increased frequency of beta cell replication. We also reasoned that if the expansion of beta cell mass during childhood is accomplished primarily by beta cell replication, then there should be a concurrent expansion in the numbers of beta cells per islet. On the other hand, if the predominant source of new beta cells during the expansion of beta cell mass in childhood is through the source of new islet formation from pancreatic stem cells, then there should be a substantial increase in the number of islets during the most rapid growth of beta cell mass.

Therefore in the present studies, we sought to establish the extent, timing and predominant source of beta cell formation during normal growth in humans. To accomplish these goals we obtained pancreas at autopsy from 46 individuals aged 2 weeks to 21 years of age, and examined these pancreases for fractional beta cell area and beta cell turnover by immunohistochemistry and morphometric analysis. By use of population pancreas volume data obtained by CT scan we then computed growth of beta cell mass during growth from infancy to adulthood in humans.

METHODS

Assessment of pancreas volume. To measure the pancreatic volume in different age groups, abdominal computer tomography scans from 135 (male 77, female 58) subjects aged 20 years and under were used. These data have already been published in a previous report focused on the changes in pancreatic volume over time (21).

Autopsy Cases. Human pancreatic tissue was obtained at autopsy from 46 individuals (34 males, 12 females), aged 2 weeks to 21 years [means ± SD: 9.7 ± 7.5 years]. Potential cases were identified by retrospective analysis of the Mayo Clinic autopsy database. To be included, cases were required to have 1) had a full autopsy within 24 h of death, 2) pancreatic tissue stored that was of adequate size and quality. Cases were excluded if pancreatic tissue had undergone autolysis or showed evidence of acute pancreatitis. None of the individuals had a history of diabetes or any other diseases affecting the pancreas. The characteristics of the cases and diagnoses leading to death are presented in table 1.

Pancreatic tissue processing. Pancreas was fixed in formaldehyde and embedded in paraffin for subsequent analysis as previously described (8). Sequential 5 µm sections were stained as follows (1) for insulin (peroxidase staining) and hematoxylin for light microscopy, (2) Insulin and TUNEL combined (peroxidase staining), (3) insulin, Ki67 and DAPI combined (immunofluorescence).

For immunohistochemical staining the following primary antibodies were used: guinea pig anti-insulin, 1:200 (DAKO); mouse Ki67, 1: 200 (MIB-1, Dako). Secondary antibodies labelled with Cy3, FITC, and AMCA were obtained from Jackson Laboratories and used at dilutions of 1:100 – 1:200. For TUNEL staining, in situ cell death detection KIT AP from Roche diagnostic was used.

Morphometric analysis. For the determination of the fractional beta-cell area, the entire pancreatic section was imaged at 40 x magnification (4 x objective). The ratio of the beta-cell area/exocrine area was digitally quantified as previously described (8) using Image Pro Plus software. The mean areas of the pancreatic sections included in these analyses was 117 ± 47 mm^2

For the determination of beta-cell replication and of the extent to which beta-cells were associated with exocrine ducts, 10 random locations in each section stained for insulin, Ki67 and DAPI were imaged at 200 x

magnification (20 x objective) and analyzed in detail. The total number of Ki67-positive cells per section, as well as the number of beta-cells co-staining with Ki67 were quantified, and the number of replicating beta-cells was expressed as percentage of the total number of beta-cells per case. By these means, an average number of 678 ± 88 beta-cells per case were counted. Furthermore, the number of islets adjacent to (i.e. closer than 5 nuclei width) exocrine ducts was quantified in each image. For these analyses, exocrine ducts were identified based on their typical cell shape and orientation.

For the determination of apoptosis, 200 islets per case were analyzed, and the number of apoptoic beta-cells was expressed in relation to the respective beta-cell area.

For the determination of islet size and density, pancreatic sections stained for insulin (peroxidase) and hematoxylin were analysed. After careful assessment of the entire pancreatic section, 5 representative islets were identified. For each islet, first the total islet size was measured using Image Pro Plus version 4.5.1 (Media Cybernetics, Silver Spring, Maryland) and then the insulin positive area of each islet was measured. Islet density was quantified by measuring the total area of the pancreas section using Image Pro Plus, and then counting the number of islets contained within that pancreatic area. An islet was defined as a cluster of 4 or more insulin positive cells.

To measure the nuclear and whole cell diameter, insulin stained sections of pancreas (peroxidase) counterstained with hematoxylin were used. 5 islets per case selected at random were photographed at x 200 magnification on an Olympus IX70 inverted system microscope (Olympus America, Melville, NY). These islets were then examined to identify 5 representative beta-cell nuclei each. Selection criteria included: clear presence of the nucleus within a beta-cell, the ability to clearly visualize nuclear boundaries, circular shape (similar dimensions in all directions) and the appearance to the observer that the nucleus had been sectioned through its maximum diameter. Once the identified nucleus was encircled, measurement of 180 nuclear diameters per nucleus was made using Image Pro Plus software version 4. 5.1 (Media Cybernetics, Silver Springs, MD) which quantified these 180 diameters at 2° angles throughout the circumference of the nucleus. Thus, the mean of 4,500 single measurements per case was used to compute the mean nuclear diameter per case. For the determination of the mean cell diameter, 5 distances between two adjacent beta-cell nuclei (including one of the nuclei) were measured in each of the 5 islets. The mean intra-individual coefficients of variations for the determinations of beta-cell nuclear size and beta-cell size were 11 and 10 %, respectively.

Assessment of beta-cell mass. Since the pancreas weight is not readily determined at autopsy, we sought to establish the growth pattern of the pancreas volume in a population-based fashion. These measures of pancreas volume were then used to estimate beta-cell mass in each age group. Thus, respective values for beta-cell mass in each case were derived from the actual measurement data of the fractional beta-cell area and the population-based average volume of the pancreas in the respective age group. Accordingly, beta-cell mass was calculated in each of the 46 autopsy cases as a product of the fractional beta-cell area as determined by immunohistochemical staining, and the pancreatic parenchymal volume for the respective age, as calculated on the basis of the CT scans. These calculations were based on the assumption that in aqueous organs, such as the pancreas, 1 g of weight equals 1 cm^3 of volume. Thus, it is theoretically possible that the actual determinations of beta-cell mass were influenced by subtle differences in the volume density between

different organs. However, since only normal pancreases without overt signs of fibrosis were included, there is no reason to expect major variations in the actual beta-cell mass calculations implied by this factor.

The mean beta cell area per case was calculated from the measured mean cell diameter in each autopsy case assuming a spherical shape of beta-cells. The beta-cell number was then computed by dividing the total beta-cell area by the individual area per beta-cell. The total number of islets per case was calculated from the product of the measured number of islets per cm^3 times the pancreatic volume.

Calculation of somatic and beta-cell growth. To calculate the absolute somatic growth rate (in kg), the actual body weight in each case was subtracted by the mean body weight of the respective lower age quintile and expressed in relation to the respective time interval (in years). The youngest case available (2 week old male) was chosen as the baseline for the youngest quintile. The relative growth (in percent per year) was computed in a similar way by expressing the actual body weight as a percentage of the mean weight of the lower quintile subtracted by 100. The absolute and relative increases in beta-cell mass and beta-cell number were calculated accordingly.

Statistical Analysis. Subject characteristics are reported as mean ± SD, results are presented as mean ± SEM. For statistical comparisons, the 46 autopsy cases aged 11 weeks to 21 years were grouped into quintiles according to their age. Statistical calculations were carried out by one-way-ANOVA and Tukey's *post hoc* test using GraphPad Prism 4, San Diego, USA. A p-value < 0.05 was taken to indicate significant differences. Regression analyses were carried-out using GraphPad Prism 4.

RESULTS

Pancreas volume. As described in detail elsewhere (21) the parenchymal volume of pancreas in human childhood increases in a linear manner with age (Fig. 1; $r= 0.9$, $p< 0.0001$) described by the equation $y^p = 4.3 + 2.25x$, were y^p is the parenchymal pancreas volume (cm^3), and x is age (years).

Pancreas morphology. Histological evaluation of the pancreas demonstrated a more pronounced lobular organization in the youngest cases (Fig. 2, 3). Within these lobules in early infancy a high proportion of beta cells were present as single cells (Fig. 2 B, C) or as small clusters of beta cells (Fig 2 A). In later infancy, the scattered beta cells were present in more pronounced and larger islets, in which beta cells were often distributed at one pole of the islet (Fig. 2 D, F). The density of these newly formed islets was heterogeneous with highly packed islets (Fig. 2 D, E) in some areas, and other areas of pancreas still more represented by scattered single or small clusters of beta cells. Although there was no obvious relationship between densely packed groups of islets and ductal tissue, there was an occasional ductal tree decorated with islets (Fig. 2E).

The fractional pancreatic area positive for insulin was highest in the youngest age group and decreased during childhood ($p < 0.05$; fig. 4A) and there was a gradual decline in islet density during childhood ($p < 0.0001$; Fig. 4B). In contrast, mean islet size as well as the mean insulin-positive islet area increased throughout childhood ($p < 0.001$; fig. 4C, D). Since neither beta cell nuclear diameter or beta cell diameter changed during childhood, the increased insulin positive area per islet reflected increased numbers of beta cells rather than larger beta cells (Fig. 5).

There was a tendency towards a decline in the percentage of ductal cells positive for insulin and number of islets immediately adjacent to exocrine ducts with ageing (fig. 6A, B). This decline in the number of duct-

associated islets was parallel to the decline in islet density (Fig. 6C, D) and was opposite to pancreatic acinar growth (21). Therefore the decline in numbers of islets adjacent to ducts with aging appeared to be most reflective of greater exocrine than endocrine growth after infancy.

Consistent with the impression that beta cell numbers increased within islets rather than by formation of new islets after infancy, the computed total number of islets remained stable through childhood after infancy ($p = 0.22$; Fig. 7). Overall, islet number increased by 3.6-fold between the youngest case (2 weeks) and the oldest age group, whereas beta-cell mass increased by 30.5-fold during the same time period (Fig. 8C). Based on these numbers, the contribution of new islet formation to the overall increase in beta-cell mass from birth to adolescence was 11.7 %, whereas 88.3 % of new beta-cell formation was manifest as an increase in islet size, apparently from beta cell replication (see below).

Body weight and height and beta cell mass and beta-cell number. One of our questions was to establish if there was a secondary increase in the rate of growth of beta cell numbers during adolescence when there is a well documented acceleration in somatic growth and insulin resistance mediated by increased sex hormones and growth hormone. As expected, body weight and height increased with ageing ($p < 0.0001$; fig. 8A, B). The increase in somatic growth was greatest in the youngest quintile with a second peak around puberty (fig. 8). Beta-cell mass was calculated as 37 mg in the youngest case (male, 2 weeks) and increased gradually to 1,125 ± 170 mg in the oldest age group ($p < 0.001$; fig. 8C). This corresponded to a total beta-cell number of 49 million in the youngest case and 1,553 ± 256 million in the oldest cases ($p < 0.001$). The rate of increase in beta-cell mass (and numbers) was most pronounced in the youngest age group and gradually declined thereafter (Fig. 8). There was no evidence of a secondary acceleration in growth of beta-cell mass around puberty despite a corresponding acceleration of somatic growth. The variance in beta cell mass and presumably growth rate of beta cell mass became increasingly more apparent after approximately 5 years of age (Fig. 8C).

Beta-cell apoptosis. Beta-cell apoptosis was very low in all age groups (mean ± SEM: 0.0025 ± 0.0025 cells per mm^2 beta-cell area). This equates to no detectable beta cell apoptosis in most pancreas samples. There were no differences in the frequency of beta-cell apoptosis between the age groups ($p = 0.37$). Of note, we did not observe any evidence for increased beta-cell apoptosis in the youngest cases.

Beta-cell replication. The frequency of beta-cell replication was highest in the youngest case (2.6 % beta-cells positive for Ki67) and substantially decreased in a hyperbola-like fashion thereafter (Fig. 9, 10). In the youngest cases both scattered individual beta cells and small islets contained relatively few beta cells, and had Ki67 positive nuclei at a much higher frequency than was present in older children or young adults. However, there was a large degree of variability in beta-cell replication between individuals, especially in the oldest quintile (range: 0 to 1.2 % beta-cells positive for Ki67). Interestingly, the curve for the frequency of beta-cell replication seemed to follow a similar pattern as the calculated relative increase in beta-cell mass in the respective age groups (Fig. 8).

DISCUSSION

In the present studies we sought to elucidate the extent, pattern and predominant mechanisms of beta-cell growth from birth to adulthood in humans. Based on a combination of abdominal CT scans and morphometric analysis of human pancreatic tissue, we report that (1) beta-cell mass expands by several-fold from birth to adulthood, (2) this is

accomplished by an increase in numbers of beta cells per islet with a concomitant expansion of islet size rather than an increase in numbers of islets, (3) the relative rate of beta-cell formation is greatest in infancy and then declines from youth to adulthood, notably not increasing during adolescence when somatic growth accelerates, (4) replication of existing beta-cells is an important mechanism subserving the postnatal expansion of beta-cell numbers, being most evident in infancy when beta cell numbers are increasing most rapidly.

As with all studies the present study has important limitations. The barriers confronting those who seek to study growth of beta cell mass and beta cell turnover in human childhood are formidable and explain the relative scarcity of available data (22, 23). Human pancreas in childhood is difficult to access. Pancreas obtained at autopsy might be influenced by the disease leading to death, by post mortem changes (particularly autolysis) and by ascertainment bias of cases attending a major tertiary medical center. Finally, the variation in beta-cell area and turnover between different pancreas samples is considerable thereby necessitating a sufficient number of cases to be included in such studies. We sought to minimize these limitations by restricting pancreas to that with no autolysis and where possible to cases with relatively sudden death to avoid the confounding effects of a chronic final illness. By definition, these studies are cross sectional since there is no opportunity to measure beta cell mass (or turnover) in vivo in humans on multiple occasions. The normal growth pattern (height and weight, figure 8) in our group of cases suggest that the cases were for the most part representative of healthy children until death. Another limitation of these studies is the fact that we examined only tail of the pancreas, not the whole pancreas. To the extent that the islet development and beta cell turnover in a sample of the tail of the pancreas is not representative of the whole organ, our studies are subject to bias. This limitation is unavoidable since only the tail of the pancreas is removed at autopsy. A prior study in humans suggests that use of the tail of pancreas only gives reasonable representation of the whole pancreas with regard to beta cell numbers, although that study was restricted to adults (24). In order to compensate for the lack of a measured pancreas volume to compute beta cell mass we previously generated a population curve by CT scan of pancreas volume growth during childhood (21). Pancreatic growth was quite well predicted by the resulting equation although there is of course individual variation which increases after ten years of age. By including a relatively large sample of autopsy pancreas cases (n=46) and pancreas CT scans (N=135), we have sought to limit the bias of these potential sampling errors.

In agreement with Kassem and colleagues we observed a high frequency of beta cell replication in young children (23). We report that this is the period of most rapid expansion of beta cell numbers in human childhood, with the growth rate of beta cell numbers and beta cell replication declining after early childhood. In this regard the postnatal expansion of beta cells in humans is seen to follow the same pattern as that seen in mice (25). The period of most rapid growth in beta cell mass is driven by increased numbers of beta cells per islet but not increased numbers of islets. Indeed these data provide a cautionary note on the use of periductal islets or intraductal beta cells as method to indirectly quantify the putative formation of new islets (26). We observe beta cells within exocrine ducts and in small clusters adjacent to ducts in infancy that appear to simply grow in number in parallel with the overall expansion of beta cell numbers in islets, apparently through the predominant mechanism of beta cell replication. Therefore it is plausible that periductal islets observed in

adult humans represent the periductal clusters of beta cells present at birth that subsequently expanded at this site through replication of beta cells. Of course it is not possible to rule out formation of new beta cells through transdifferentiation of periductal precursors in humans, but on the other hand the appearance of periductal islets is insufficient evidence to support the existence of this alternative pathway. From a pragmatic point of view, the existence of an alternative pathway of beta cell formation independent of duplication of existing beta cells is attractive as a potential tool for beta cell regeneration. Patients with long standing type 1 diabetes with minimal beta cells would be unlikely to benefit from a strategy dependent on fostering duplication of beta cells while they might be more readily amenable to a therapy that promoted beta cell formation from an alternative pathway such as from a stem cell pool.

Beta cell mass in individuals in each quintile after five years of age showed considerable variation between individuals, as previously noted in adult humans (27). Likewise, the frequency of beta-cell replication was subject to great inter-individual variation. In humans this variation might reflect a sampling artifact based on the study of a portion of pancreas rather than the whole pancreas, and the use of a population pancreas volume. Furthermore, the observed high variation in the frequency of beta-cell replication might have been partly due to the limited number of beta-cells counted for this quantification (678 cells per case). As such it is important that we have relatively large groups in each quintile to generate the mean data for each group. Alternatively it is possible that there is a wide range of growth rates of beta cell mass during childhood. This possibility is supported by similar variance in measured beta cell mass in adult rodents (28) where the whole pancreas is available.

Previous studies in rats and pigs have reported a wave of beta-cell apoptosis after birth (29-31). In the present cohort, beta-cell apoptosis was low in all cases studied, and we did not observe increased beta-cell apoptosis in the youngest cases. This finding is consistent with previous studies in humans, where most remodeling of the endocrine pancreas was shown to occur prior to or around birth (32). Since the youngest case included in this study was 2 weeks old, this would have occurred before the observation period in the present study.

The incidence of both type 1 and type 2 diabetes is increased in adolescence (33, 34). The incidence of type 1 diabetes is also increasing in early childhood (less than three years of age)(33). We report that beta cell mass increases by the greatest increment before 2 years of age (figure 8F), and precedes the corresponding greatest increase in growth of body weight (age 15-18, figure 8D) which coincides with increased insulin demand associated with puberty (35). The discordance in this pattern of growth is actually quite well illustrated in figure 8. Collectively these observations imply that early childhood is characterized by a relative surplus of beta cells, but that by the time of puberty, beta cell numbers are more closely matched to their requirement providing insight into the peak in incidence of both type 1 and 2 diabetes in relation to puberty. Our stated hypothesis that there would be a secondary growth phase in beta cell mass in relation to puberty is therefore rejected.

Another implication of the rapid rate of beta cell replication during infancy is that it likely has a relatively major impact on the final adult beta cell mass. Recent genome wide studies have reported several genes that are involved in cell cycle regulation are linked to risk for type 2 diabetes (37). Intrauterine growth retardation results in a decrease in beta cell mass and increased risk for diabetes (38) (39). Collectively these data suggest that the rapid period of increased beta cell mass during gestation and in infancy might be

vulnerable to nutritional, environmental and genetic factors that influence the ultimate adult beta cell mass, and in turn the risk for subsequent development of diabetes.

We conclude that in humans recognizable islets form early in infancy from scattered beta cells present at birth. Thereafter beta-cell mass expands by several fold by adulthood, this being accomplished through increased islet size (and beta cells per islet) rather than increased numbers of islets. The greatest rate of increase in beta cell mass is in infancy with no obvious secondary phase of increased beta cell mass coincident with the accelerated rate of somatic growth of adolescence. The frequency of beta cell replication parallels the rate of increase in beta cell mass. These data support the notion that postnatal beta-cell mass is dynamic, likely effected predominantly through beta cell replication and highly variable between individuals. Establishing the mechanisms that promote replication of beta cells in infancy may offer insights into therapeutic approaches to drive beta cell replication as a therapy in early diabetes. Establishing the mechanisms underlying the wide variance of beta cell growth rates between individuals may shed light on the predisposition to diabetes.

ACKNOWLEDGEMENTS

These studies were supported by funding from the NIH (DK 59579, DK61539), the JDRF, the Larry L. Hillblom Foundation and the Deutsche Forschungsgemeinschaft (Me 2096/2-1).

REFERENCES

1. Meier, J.J., and Butler, P.C. 2005. Insulin secretion. In *Endocrinology*. L.J. DeGroot, and J.L. Jameson, editors. Philadelphia: Elsevier Saunders. 961-973.
2. Goodner, C.J., Koerker, D.J., Weigle, D.S., and McCulloch, D.K. 1989. Decreased insulin- and glucagon-pulse amplitude accompanying beta-cell deficiency induced by streptozocin in baboons. *Diabetes* 38:925-931.
3. Kjems, L.L., Kirby, B.M., Welsh, E.M., Veldhuis, J.D., Straume, M., McIntyre, S.S., Yang, D., Lefebvre, P., and Butler, P.C. 2001. Decrease in beta-cell mass leads to impaired pulsatile insulin secretion, reduced postprandial hepatic insulin clearance, and relative hyperglucagonemia in the minipig. *Diabetes* 50:2001-2012.
4. Leahy, J.L., Bonner-Weir, S., and Weir, G.C. 1984. Abnormal glucose regulation of insulin secretion in models of reduced B-cell mass. *Diabetes.* 33:667-673.
5. Gepts, W. 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619-633.
6. Pipeleers, D., and Ling, Z. 1992. Pancreatic beta cells in insulin-dependent diabetes. *Diabetes Metab Rev* 8:209-227.
7. Meier, J.J., Bhushan, A., Butler, A.E., Rizza, R.A., and Butler, P.C. 2005. Sustained beta-cell apoptosis in patients with long-standing type 1 diabetes: Indirect evidence for islet regeneration? *Diabetologia* 48:2221-2228.
8. Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., and Butler, P.C. 2003. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110.
9. Robertson, R.P. 2004. Islet transplantation as a treatment for diabetes - a work in progress. *N Engl J Med* 350:694-705.
10. Robertson, R.P., Davis, C., Larsen, J., Stratta, R., and Sutherland, D.E. 2000. Pancreas and islet transplantation for patients with diabetes. *Diabetes Care.* 23:112-116.
11. Ryan, E.A., Paty, B.W., Senior, P.A., Bigam, D., Alfadhli, E., Kneteman, N.M., Lakey, J.R., and Shapiro, A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.
12. Meier, J.J., Bhushan, A., and Butler, P.C. 2006. The Potential for Stem Cell Therapy in Diabetes. *Pediatr Res* 59:65R-73R.
13. Segev, H., Fishman, B., Ziskind, A., Shulman, M., and Itskovitz-Eldor, J. 2004. Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* 22:265-274.
14. Brolen, G.K., Heins, N., Edsbagge, J., and Semb, H. 2005. Signals From the Embryonic Mouse Pancreas Induce Differentiation of Human Embryonic Stem Cells Into Insulin-Producing {beta}-Cell-Like Cells. *Diabetes* 54:2867-2874.
15. Kloppel, G., Lohr, M., Habich, K., Oberholzer, M., and Heitz, P.U. 1985. Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Surv Synth Pathol Res* 4:110-125.
16. Butler, A.E., Janson, J., Soeller, W.C., and Butler, P.C. 2003. Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52:2304-2314.

17. Bonner-Weir, S., Baxter, L.A., Schuppin, G.T., and Smith, F.E. 1993. A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. *Diabetes* 42:1715-1720.
18. Ianus, A., Holz, G.G., Theise, N.D., and Hussain, M.A. 2003. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest* 111:843-850.
19. Lechner, A., Yang, Y.G., Blacken, R.A., Wang, L., Nolan, A.L., and Habener, J.F. 2004. No evidence for significant transdifferentiation of bone marrow into pancreatic beta-cells in vivo. *Diabetes* 53:616-623.
20. Butler, A.E., Huang, A., Rao, P.N., Bhushan, A., Hogan, W.J., Rizza, R.A., and Butler, P.C. 2007. Hematopoietic stem cells derived from adult donors are not a source of pancreatic beta-cells in adult nondiabetic humans. *Diabetes.* 56:1810-1816. Epub 2007 Apr 1824.
21. Saisho, Y., Butler, A.E., Meier, J.J., Monchamp, T., Allen-Auerbach, M., Rizza, R.A., and Butler, P.C. In Press, 2007. Pancreas Volumes in Humans from Birth to Age One Hundred taking into account Sex, Obesity and presence of Type 2 Diabetes. *Clinical Anatomy*.
22. Ogilvie, R.F. 1937. A quantitative estimation of the pancreatic islet tissue. *Quarterly Journal of Medicine* 6:287-300.
23. Kassem, S.A., Ariel, I., Thornton, P.S., Scheimberg, I., and Glaser, B. 2000. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. *Diabetes* 49:1325-1333.
24. Yoon, K.H., Ko, S.H., Cho, J.H., Lee, J.M., Ahn, Y.B., Song, K.H., Yoo, S.J., Kang, M.I., Cha, B.Y., Lee, K.W., et al. 2003. Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab.* 88:2300-2308.
25. Georgia, S., and Bhushan, A. 2004. Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 114:963-968.
26. Bonner-Weir, S. 2001. beta-cell turnover: its assessment and implications. *Diabetes* 50 Suppl 1:S20-24.
27. Ritzel, R.A., Butler, A.E., Rizza, R.A., Veldhuis, J.D., and Butler, P.C. 2006. Relationship between beta-cell mass and fasting blood glucose concentration in humans. *Diabetes Care* 29:717-718.
28. Matveyenko, A.V., and Butler, P.C. 2006. Beta-cell deficit due to increased apoptosis in the human islet amyloid polypeptide transgenic (HIP) rat recapitulates the metabolic defects present in type 2 diabetes. *Diabetes* 55:2106-2114.
29. Scaglia, L., Cahill, C.J., Finegood, D.T., and Bonner-Weir, S. 1997. Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. *Endocrinology.* 138:1736-1741.
30. Finegood, D.T., Scaglia, L., and Bonner-Weir, S. 1995. Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. *Diabetes* 44:249-256.
31. Bock, T., Kyhnel, A., Pakkenberg, B., and Buschard, K. 2003. The postnatal growth of the beta-cell mass in pigs. *J Endocrinol.* 179:245-252.

32. Kassem, S.A., Ariel, I., Thornton, P.S., Scheimberg, I., and Glaser, B. 2000. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. *Diabetes* 49:1325-1333.
33. Pundziute-Lycka, A., Dahlquist, G., Nystrom, L., Arnqvist, H., Bjork, E., Blohme, G., Bolinder, J., Eriksson, J.W., Sundkvist, G., and Ostman, J. 2002. The incidence of Type I diabetes has not increased but shifted to a younger age at diagnosis in the 0-34 years group in Sweden 1983-1998. *Diabetologia* 45:783-791.
34. Dabelea, D., Hanson, R.L., Bennett, P.H., Roumain, J., Knowler, W.C., and Pettitt, D.J. 1998. Increasing prevalence of Type II diabetes in American Indian children. *Diabetologia.* 41:904-910.
35. Moran, A., Jacobs, D.R., Jr., Steinberger, J., Hong, C.P., Prineas, R., Luepker, R., and Sinaiko, A.R. 1999. Insulin resistance during puberty: results from clamp studies in 357 children. *Diabetes.* 48:2039-2044.
36. Meier, J.J., Butler, A.E., Galasso, R., Rizza, R.A., and Butler, P.C. 2006. Increased islet beta cell replication adjacent to intrapancreatic gastrinomas in humans. *Diabetologia* 49:2689-2696.
37. Zeggini, E., Weedon, M.N., Lindgren, C.M., Frayling, T.M., Elliott, K.S., Lango, H., Timpson, N.J., Perry, J.R., Rayner, N.W., Freathy, R.M., et al. 2007. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science* 316:1336-1341.
38. Harder, T., Rodekamp, E., Schellong, K., Dudenhausen, J.W., and Plagemann, A. 2007. Birth weight and subsequent risk of type 2 diabetes: a meta-analysis. *Am J Epidemiol.* 165:849-857. Epub 2007 Jan 2010.
39. Simmons, R.A., Templeton, L.J., and Gertz, S.J. 2001. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes.* 50:2279-2286.

TABLE 1. Characteristics of 46 autopsy cases included.

Case no.	Age (years)	Gender (male/female)	BMI (kg/m^2)	Diagnoses leading to death
1	0,05	M	13,5	Respiratory distress; Down's syndrome
2	0,21	M	14,3	Complete transposition of great arteries
3	0,58	F	17,3	Tetrology of Fallot
4	0,58	F	14,9	Ventricular septal Defect; encephalopathy
5	1,07	M	16,5	Apnea and cardiac arrest following meningitis
6	1,54	F	12,5	Subdural and epidural hematoma
7	1,85	M	14,8	Congenital heart disease
8	1,87	M	15,2	Intractable seizures
9	1,99	M	14,5	Idiopathic restrictive cardiomyopathy with pulmonary venous hypertension
10	2,05	M	17,2	Tetralogy of Fallot
11	2,07	M	13,1	Collision with motor vehicle leading to subdural hematoma, hypoxic encephalopathy
12	2,8	M	14,4	Ingestion of alkalai (liquid dairy farm cleaner), leading to aspiration and hypoxiecencephalopathy
13	2,91	M	11,8	Acute thrombolitic occlusion of a gortex shunt following cardiac surgery for congenital heart disease
14	3,66	F	13,9	Unable to wean from bypass for congenital heart disease
15	3,66	F	14	Cerebral venous malformation leading to pontine hemorrhage
16	4,17	F	12,5	Unable to wean from bypass for congenital heart disease
17	4,53	M	15,1	Cerebellar meduloblastoma
18	5,42	M	15,7	Reye's Syndrome, encephalopathy
19	5,52	M	14,7	Massive head trauma following collision with a tractor
20	6,27	F	16,5	Polytrauma following collision with pickup truck
21	6,32	F	10,6	Skull fracture following collision with truck.
22	6,5	M	20,6	Down's syndrome leading to respiratory failure
23	8,72	F	16,1	Car accident
24	9,23	F	14,9	Congenital heart disease leading to cardiac failure
25	9,49	M	14,3	Head trauma following motor vehicle accident
26	11,62	M	21,4	Intracranial hemorrhage following accidental hit by a golfball
27	13,87	M	24	Ventricular fibrillation post appendectomy
28	14,6	M	12,8	Tetrology of Fallot leading with pulmonary venous hypertension
29	15,15	M	21,6	Skull fracture following motor vehicle accident
30	15,46	M	23,4	Polytrauma following motor vehicle accident
31	16,36	M	16,7	Polytrauma following bike accident
32	17,13	M	23,3	Polytrauma following fall from a hotel roof
33	17,16	M	25,5	Polytrauma following bike accident
34	17,56	M	20,5	Subdural hematoma and edema of the right hemisphere following football injury
35	17,92	M	21,3	Multiple traumatic injuries following motorcycle accident
36	18,25	M	25,5	Profound hypothermia (12 hours) after accidental cryoexposure
37	18,34	M	19,6	Pulmonary aspiration of gastric contents, leading to cardio-respiratory arrest following motorcycle accident
38	18,57	M	18,7	End stage plexogenic pulmonary arteriopathy

39	19,2	F	22,6	Polytrauma following motor vehicle accident
40	19,2	M	34,1	Polytrauma following motor vehicle accident
41	19,21	M	24,6	Polytrauma following motor vehicle accident
42	19,25	M	20,5	Polytrauma following motor vehicle accident
43	19,45	M	14,7	Polytrauma following motor vehicle accident
44	19,5	M	29,3	Bowel perforation leading to peritonitis
45	21,04	F	17,5	Rheumatic heart disease
46	21,46	M	25,7	Gun shot wound to head

TABLE 2. Measures of beta-cell mass in 46 children aged 2 weeks to 21 years grouped into quintiles

Parameter	0.2–2.05	2.06–5.6	5.7– 4.6	14.7-18.4	18.5-21.5	p-value
Fractional beta-cell area [%]	2.6 ± 0.5	2.1 ± 0.3	1.7 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	0.023
Mean islet area [μm^2]	2589 ± 476	4325 ± 729	5010 ± 594	7493 ± 1393	9377 ± 1163	< 0.0001
Insulin-positive islet area [μm^2]	1533 ± 231	2842 ± 500	3374 ± 454	5030 ± 900	6296 ± 639	< 0.0001
Islet density [islets /mm^2]	27.9 ± 3.5	18.3 ± 2.6	11.9 ± 1.0	6.6 ± 0.7	7.3 ± 1.2	0.011
Islet number [millions]	0.98 ± 0.17	1.42 ± 0.25	1.91 ± 0.43	1.31 ± 0.20	1.89 ± 0.50	0.22
Beta-cell nuclear diameter [µm]	7.35 ± 0.15	6.91 ± 0.12	7.18 ± 0.13	7.43 ± 0.25	7.27 ± 0.28	0.40
Beta-cell diameter [µm]	11.15 ± 0.10	10.77 ± 0.09	11.03 ± 0.10	11.37 ± 0.15	11.19 ± 0.21	0.0502

Means ± SEM, p-values were calculated by ANOVA.

FIGURE 1

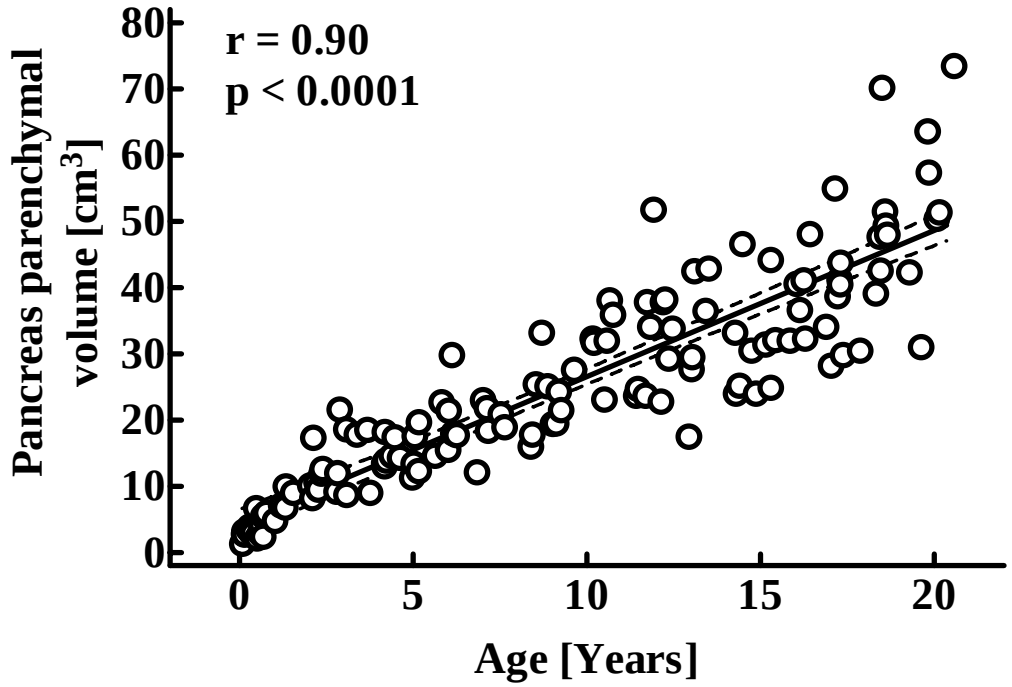

FIGURE 1. Parenchymal volume of the pancreas in 135 children aged 30 days to 20 years. Dashed lines denote the respective upper and lower 95% confidence intervals. r- and p-values were calculated by linear regression analysis.

FIGURE 2

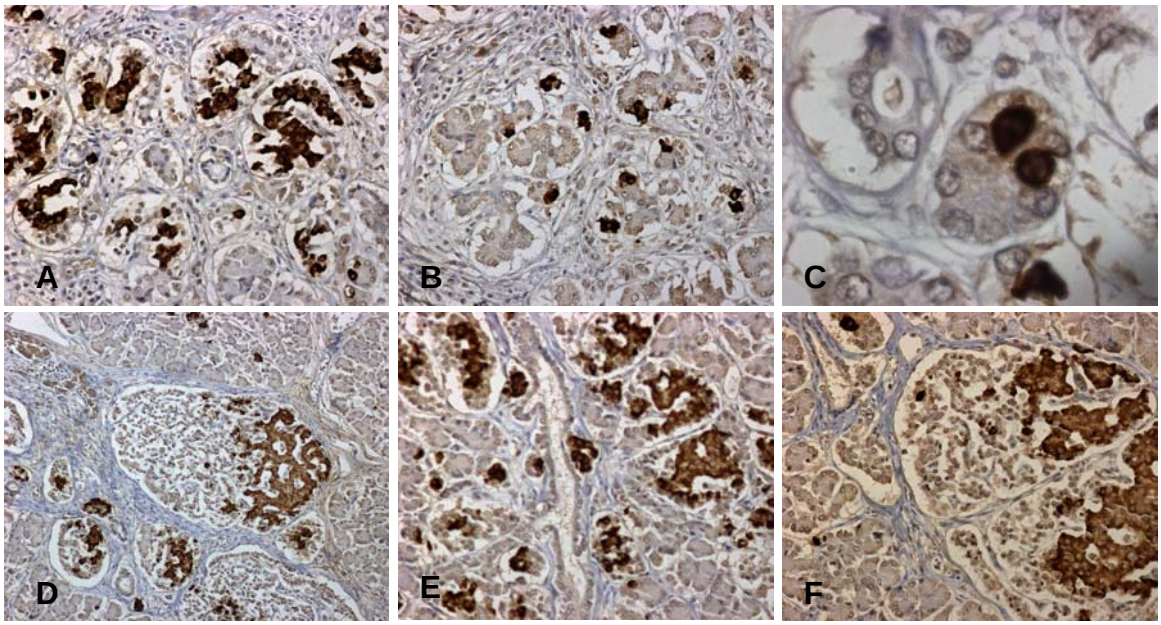

Figure 2. Representative pancreatic sections stained for insulin (brown) and hematoxylin from case 1, aged 2.5 weeks (table 1) (A,B,C) and case 2, aged 10 weeks (D, E, F). In early infancy beta cells were abundant as small clusters (A, objective x20) surrounded by a single cell layer non fibrous capsule. In other areas beta cells were mostly present as single cells (B objective x 20), shown in high power in C (objective x100). By 10 weeks of age the small clusters of beta cells had grown in size and typically occupied an encapsulated islet like structure with the beta cells being predominantly polar within the islet as previously described (D, objective x 10, F objective x 20). The capsule by 10 weeks had some fibrous tissue. At 10 weeks of age islets were densely located throughout the pancreas but in higher density in some locations (E objective x20), in this case decorating a ductal tree.

FIGURE 3

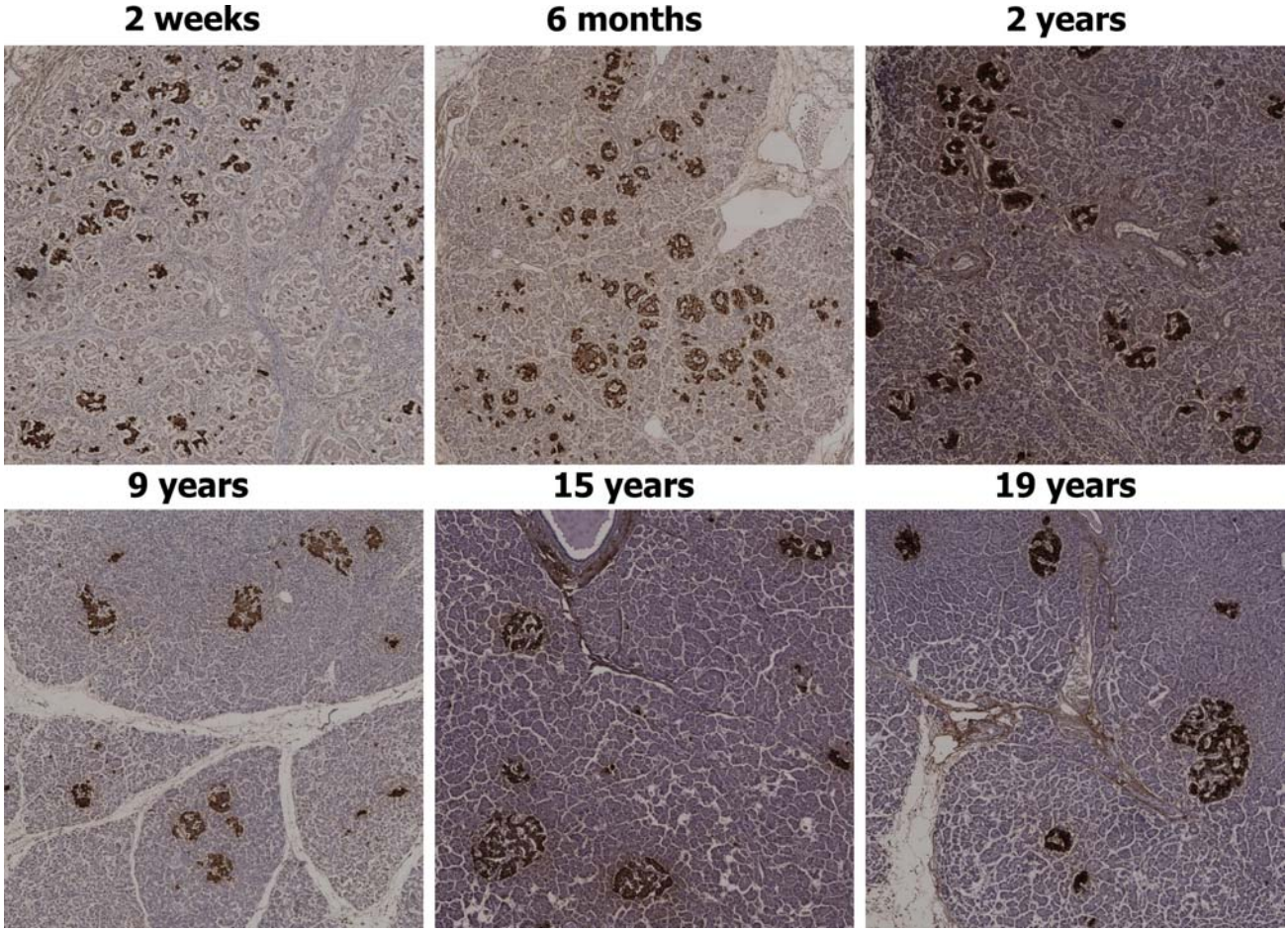

Figure 3: Representative pancreatic sections stained for insulin (brown) and hematoxylin from 6 children aged 2 weeks to 19 years. Images were taken at 100 x magnification (10x objective).

FIGURE 4

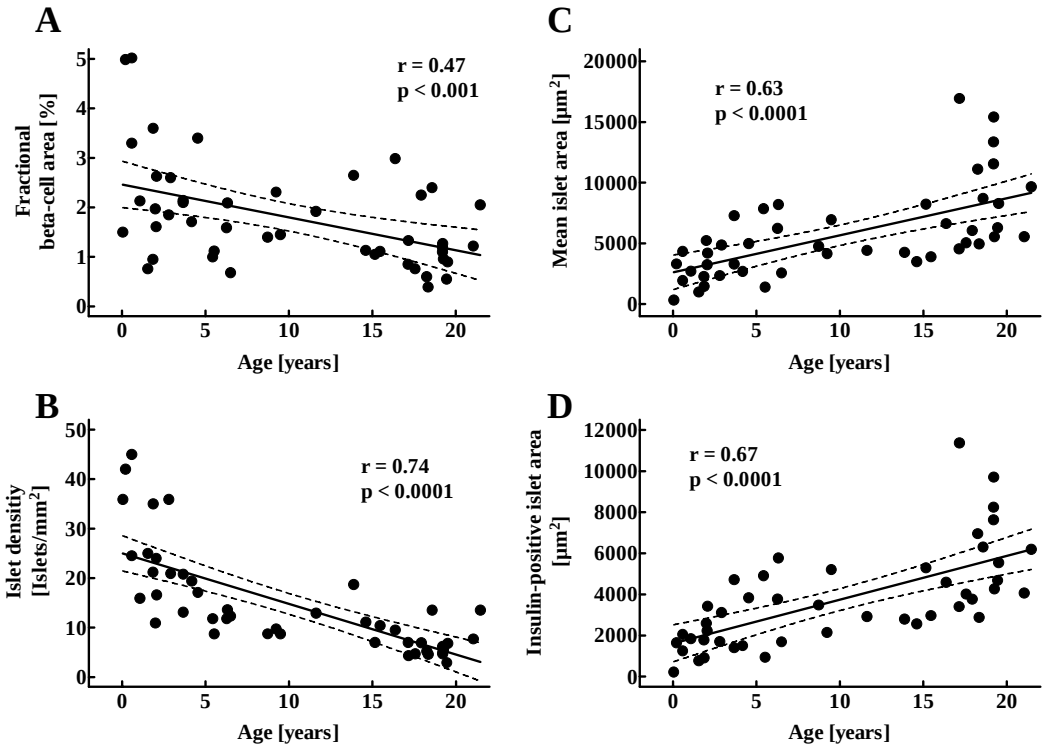

Figure 4. Fractional pancreatic area positive for insulin (A), mean islet density (number of islets per mm^2 pancreatic tissue; B), as well as mean islet area (C) and mean insulin-positive islet area (D) in 46 children aged 2 weeks to 21 years. Solid lines indicate the regression lines, dashed lines denote the respective upper and lower 95% confidence intervals. r- and p-values were calculated by linear regression analysis.

FIGURE 5

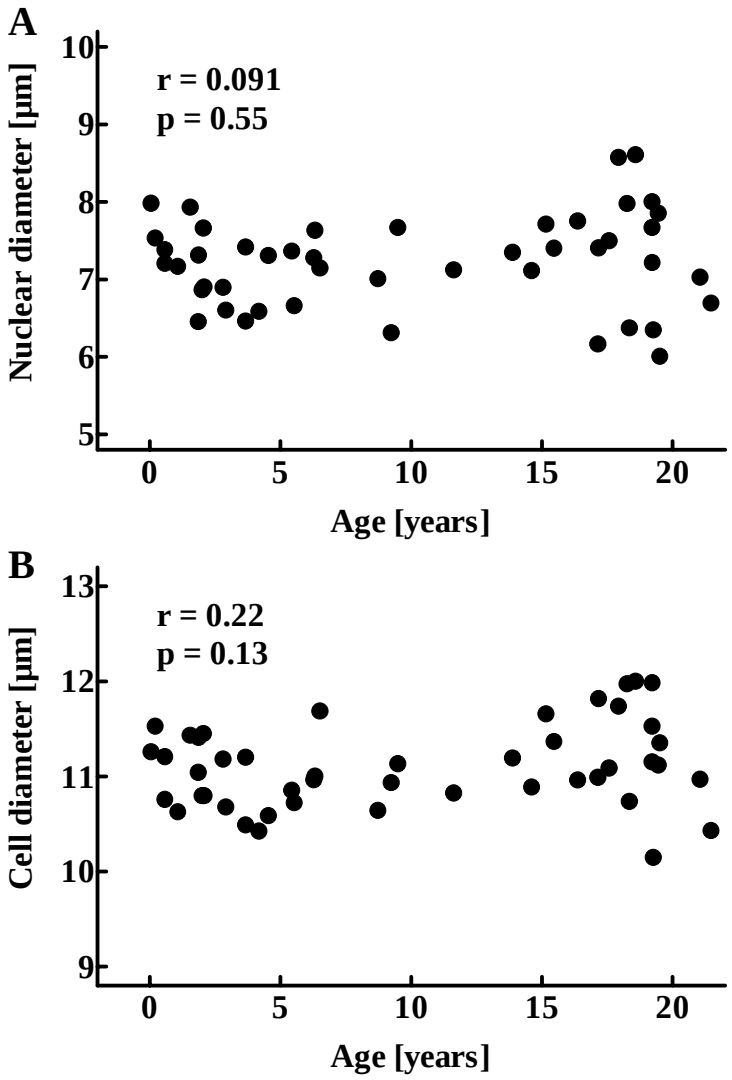

Figure 5. Nuclear diameter (A) and total diameter (B) of beta-cells in 46 children aged 2 weeks to 21 years. r- and p-values were calculated by linear regression analysis.

FIGURE 6

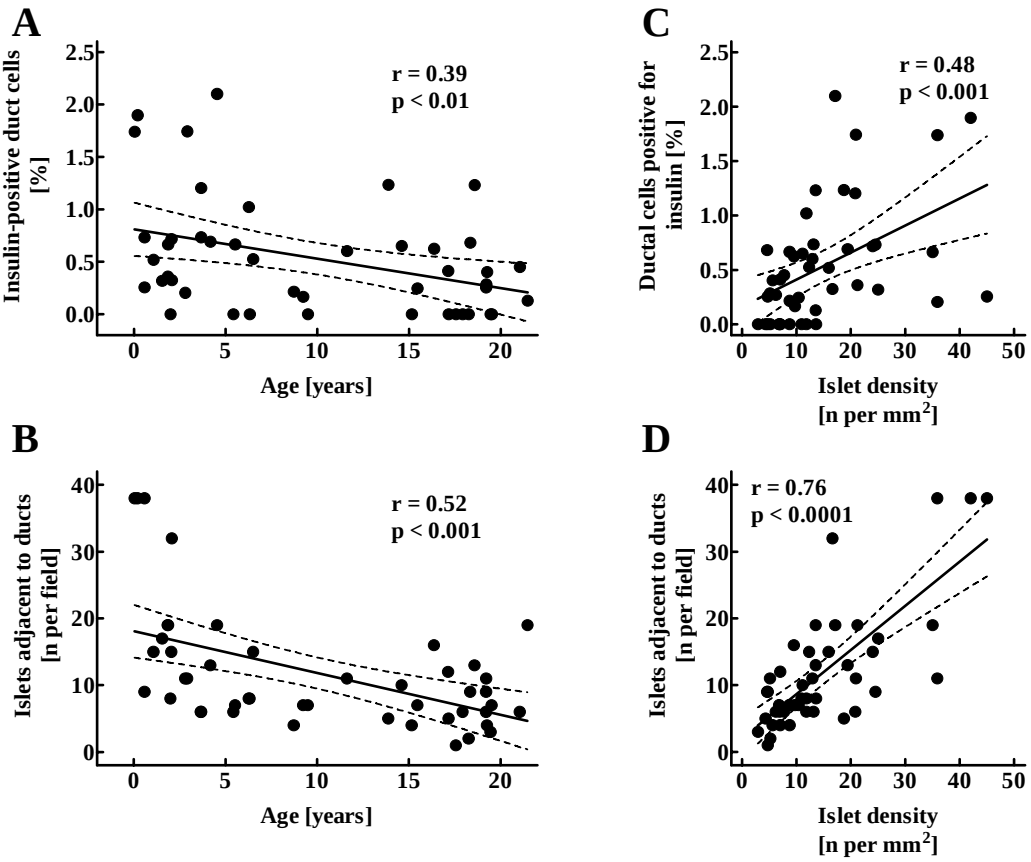

Figure 6. Percentage of exocrine ductal cells positive for Insulin (A), mean number of islets adjacent to (= 5 or less nuclei away) exocrine ducts (B) in 46 children aged 2 weeks to 21 years, as well as correlation between the percentage of ductal cells positive for Insulin (C) and the number of islets adjacent to ducts (D) and the mean islet density. Solid lines indicate the regression lines, dashed lines denote the respective upper and lower 95% confidence intervals. r- and p-values were calculated by linear regression analysis.

FIGURE 7

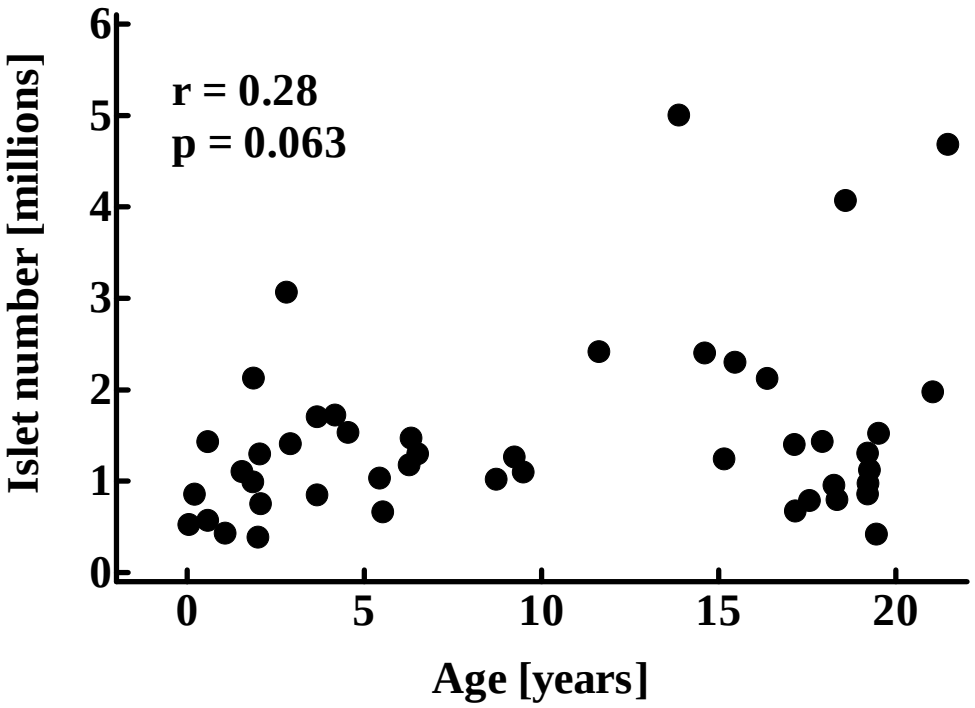

Figure 7. Total calculated number of islets in 46 children aged 2 weeks to 21 years. Data were computed from evaluation of islet density in pancreas samples and population pancreatic volumes (Fig 1). Data are presented as individual data points. r- and p-values were calculated by linear regression analysis.

FIGURE 8

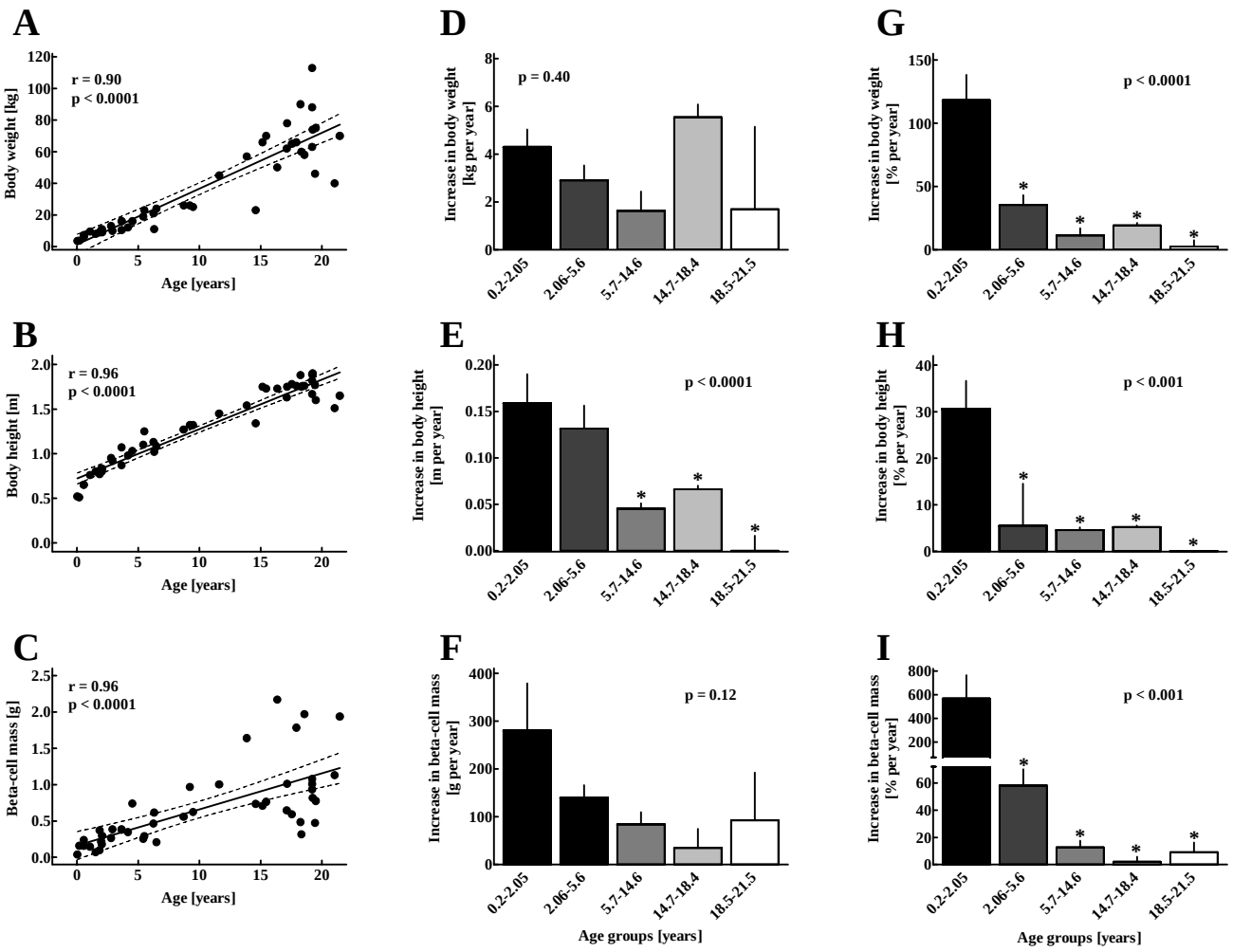

Figure 8. Left panels: Body weight (A), body height (B), and beta-cell mass (C) in 46 children aged 2 weeks to 21 shown as individual data. Solid lines indicate the regression lines, dashed lines denote the respective upper and lower 95% confidence intervals. r- and p-values were calculated by linear regression analysis. The absolute and relative increase in each parameter is shown as mean values ± SEM on the middle and right panels, respectively. For these analyses, the group was divided into equal quintiles. Asterisks indicate significant differences versus the youngest age group, respectively.

FIGURE 9

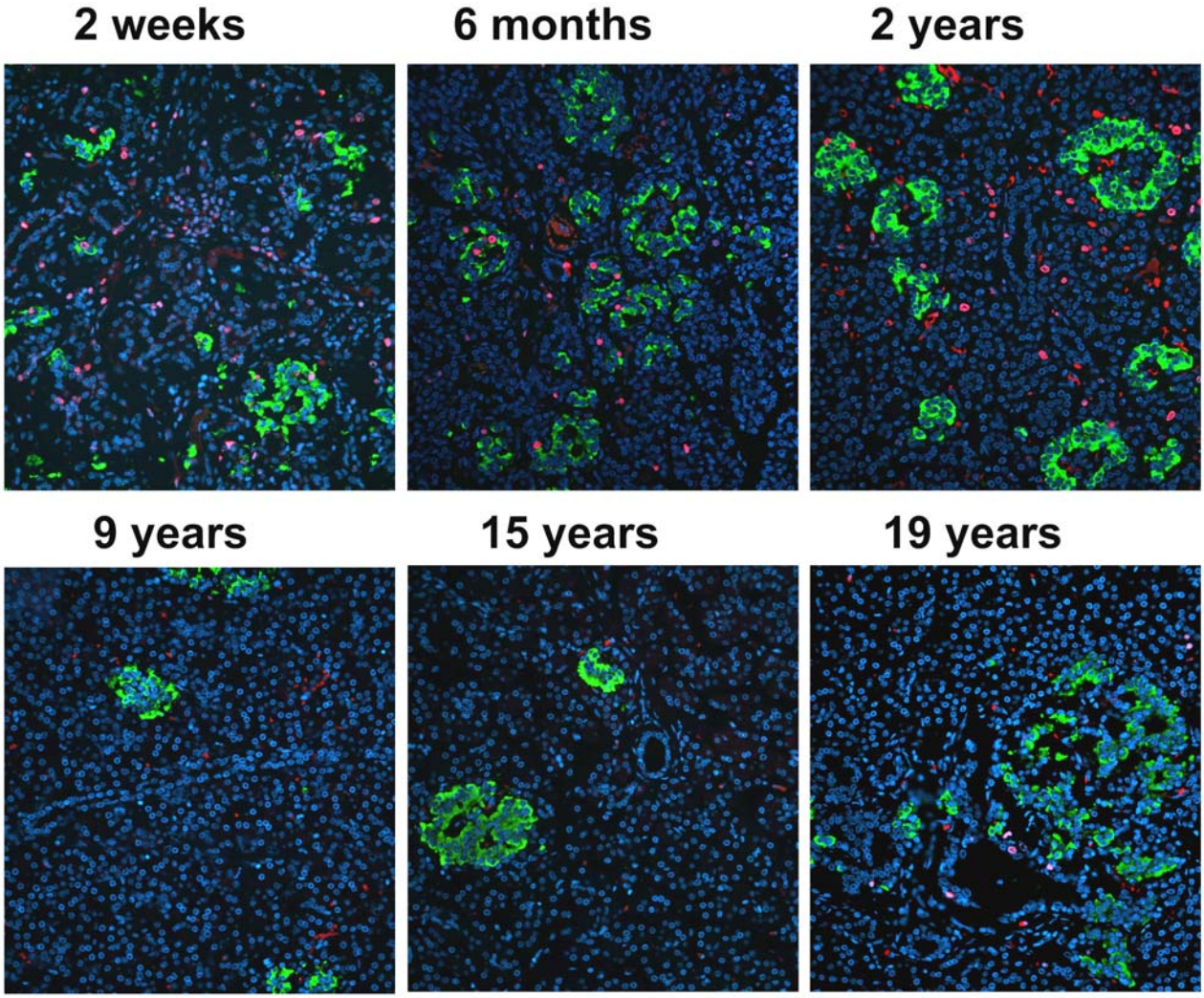

Figure 9. Representative pancreatic sections (same cases as in figure 2) stained for insulin (green), Ki67 (red), and DAPI (blue) from 6 children aged 2 weeks to 19 years. Images were taken at 200 x magnification (20x objective).

FIGURE 10

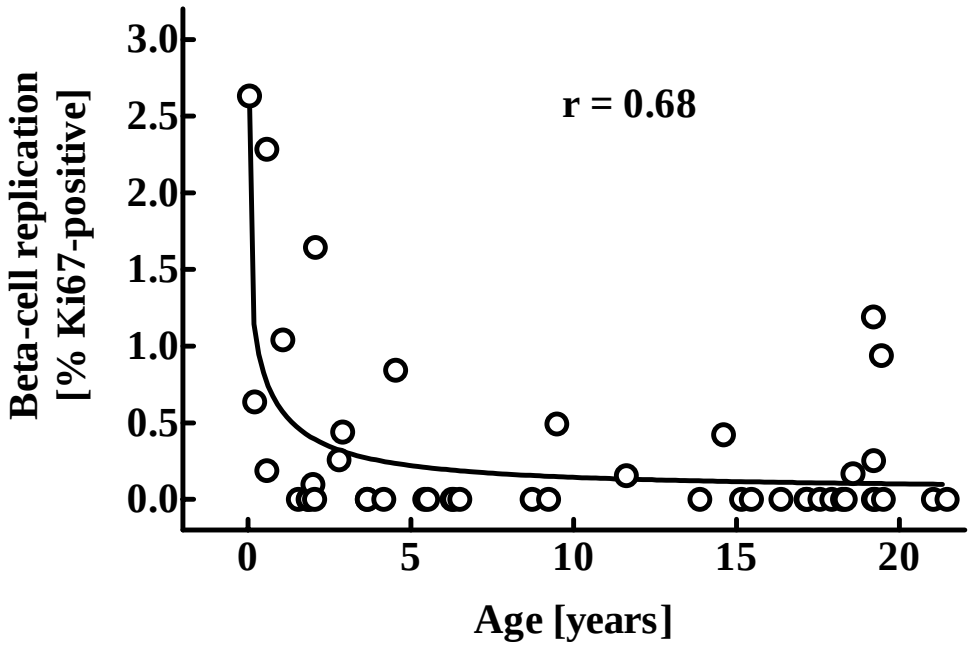

Figure 10. Frequency of beta-cell replication (percent beta-cells positive for Ki67) in 46 children aged 2 weeks to 21 years. The r-value was calculated using non-linear regression analysis.