Introduction

Both type 1 and type 2 diabetes are characterised by a deficit in β-cell mass, presumably caused by β-cell apoptosis (1, 2). Therefore, restoration of endogenous insulin secretion mass through targeted regeneration of β-cells is an area under active investigation for the future treatment of diabetes (3, 4). In order to establish novel treatment regimens aiming to augment β-cell mass, it is crucial to gain detailed insights into the natural dynamics and mechanisms controlling islet development (5). However, as yet the patterns and dynamics of prenatal β-cell development have not been elucidated in detail.

During embryonic development, the pancreas evolves from a dorsal and ventral protrusion of the primitive gut epithelium, which subsequently fuses to form the definitive pancreas (6–8). In mice, glucagon- and pancreatic polypeptide-containing cells can be found by embryonic day 10.5 (E10.5), whereas insulin expression first appears by E11.5 (6, 9).

These endocrine cells first occur alongside the epithelium of the pancreatic ducts and can subsequently be found as cell clusters within the pancreatic interstitium, where they finally grow to form the mature islets. These newly differentiated cells have been shown to be mitotically quiescent and do not proliferate until birth (6, 10, 11). In contrast, replication of existing β-cells seems to be an important mechanism contributing to the postnatal expansion of β-cell mass in mice and rats (12, 13). Recent studies in transgenic mice also suggested a close interaction between vascular endothelial cells and endocrine cells during islet development (14).

In humans, the dynamics and origins of prenatal β-cell development are less well understood. Bouwens et al. reported a peak in β-cell replication (~3%) around gestational week 14–16, whereas β-cell replication was reported to occur infrequently from week 24 onwards (0.1–0.2%) (15). They also showed the appearance of single endocrine cells or small islet cell clusters alongside the ductal tree during early foetal development.
Along the same lines, Polak et al. demonstrated high frequencies of replication in foetal human pancreas between gestational week 7 and 11 (16). Recently, a series of elegant studies using immunohistochemistry and gene expression profiling has suggested the presence of an undifferentiated islet precursor cell population in the developing human pancreas (17). However, so far, most studies on foetal pancreas development have been carried out in relatively small numbers of cases, and the growth patterns of the endocrine pancreas have not yet been analysed throughout the entire prenatal period (15, 16, 18, 19). In particular, the quantitative expansion of β-cell mass during the embryonic and foetal period has not yet been characterised.

In the present studies, we analysed a series of 65 human embryos and foetuses collected in the Division of Clinical and Functional Anatomy, Innsbruck. To our knowledge, this represents the largest and most complete collection of prenatal human pancreas characterised so far. Using this resource, we applied both qualitative and quantitative analyses to address the following questions in human prenatal pancreatic development: i) what is the timing of β-cell growth? ii) What are the dynamics of β-cell turnover (replication and apoptosis)? iii) Is there morphological evidence for an interaction between α- and β-cells, or a role of vascular endothelial cells in islet development?

### Materials and methods

#### Cases

Pancreatic tissue was obtained from 63 human embryos and foetuses between the 8th and 38th week post conception (p.c.) as well as from two newborn cases. The gender could be determined in 62 cases, and was male in 24 and female in 38 cases. Specimens were provided by the collections of the Institute of Pathology and the Division of Clinical and Functional Anatomy, Medical University of Innsbruck. They were obtained from miscarriage and legal abortions including parental consent and in compliance with the local governmental and institutional guidelines. The specimens were categorized as described (20) by their anatomical age, based upon the crown-rump length or upon the estimated gynaecological age. Cases were included only if careful histological examination of a trained pathologist excluded the absence of pancreatic dysplasia.

#### Tissue preparation

For routine histological staining, specimens were fixed in 7% formalin in PBS (0.2 M) and rinsed in the same buffer. Subsequently, the specimens were embedded in paraffin in a routine histological infiltration processor (Miles Scientific Inc., Naperville, IL, USA). Serial sections were cut at 4 μm with a Microm ERGO Star Rotations microtome (Microm, Walldorf, Germany), mounted on SuperFrost Plus slides, deparaffinized with xylene and dehydrated in graded alcohol series.

#### Staining procedures

Pancreatic sections were stained as follows: i) insulin and Ki67 for light microscopy; ii) insulin and glucagon for light microscopy; iii) insulin and CD31 for light microscopy; iv) insulin, TUNEL and 4',6-diamidino-2-phenylindol (DAPI) for immunofluorescence, and v) insulin, glucagon and DAPI for immunofluorescence. The validity of the double-staining procedures for α- and β-cells was confirmed by a co-staining for α- and β-cells using a different set of antibodies and by the simultaneous staining for insulin and C-peptide (data not shown).

#### Immunohistochemistry for light microscopy

To detect α- and β-cells, double labelling was performed with the sequential sections on a Discovery XT staining system (Ventana, Strasbourg, France). After dewaxing of the sections, the protocol comprised a heat-induced epitope/antigen retrieval step with Tris–EDTA–Borate buffer at pH 7.8 for 1 h at 95 °C. A guinea pig anti-swine insulin antibody (Dako, Glostrup, Denmark, dilution of 1:200) was used to label β-cells. This step was followed by application of a biotinylated secondary antibody. Subsequently, sections were incubated with a streptavidin (SA–HRP) complex, and visualization was obtained with a diaminobenzidine (DAB) detection kit. For the labelling of α-cells, the sections were finally incubated with a ready to use rabbit glucagon antibody (Diagnostic Biosystems, Plasanton, Canada). Anti-rabbit ultra-map alkaline phosphatase and fast red were used for the detection of the target antigen.

A ready to use Escherichia coli anti-human Ki67 MAB (Linaris, Histoprime, Wertheim-Bettingen, Germany) was used for the double staining of insulin and Ki67. The slides were incubated with a biotinylated secondary antibody followed by application of the SA–HRP complex. Visualization of the antigen–antibody complex was achieved by using chromogenic detection with DAB. Subsequently, counterstaining of the sections with haematoxylin was performed.

The procedure of double labelling for pancreatic β-cells and endothelial cells was carried out in the same way. The endothelial cells were labelled with a ready to use monoclonal mouse antibody CD31 (PECAM-1), clone JC/70A (Innovative Diagnostic Systems (DCS), Hamburg, Germany).

All control incubations without application of the primary antibodies yielded no labelling.

#### Immunohistochemistry for fluorescence microscopy

For the co-staining of insulin and glucagon,
pancreatic sections were incubated with a guinea pig anti-swine insulin antibody (Dako, dilution of 1:200). This step was followed by application of a secondary antibody labelled with Cy3 (Jackson Laboratories, BarHarbor, ME, USA; #106-165-003, lot-no. 67623; dilution 1:800). Subsequently, a rabbit anti-glucagon antibody (Dako, cat-no. A 0565, lot-no. 4500 A, dilution 1:100) was applied. Finally, slides were incubated with a FITC-labelled secondary antibody (Santa Cruz Biotechnology, Heidelburg, Germany; #sc-2090, lot-no. l271, dilution of 1:100). To check the signal specificity in the double-staining experiments, either one or both primary antibodies were omitted in the presence of both secondary antibodies.

The validity of the double staining for α- and β-cells was confirmed by two additional staining procedures: first, co-staining for β- and α-cells using a different set of antibodies yielded a staining pattern comparable to the antibody combination described above. In this case, an anti-C-peptide antibody (Acris, Herford, Germany BM270 1:200) was detected by a FITC-labelled secondary antibody (Jackson Laboratories 715-095-150 1:100), and the anti-glucagon antibody (Acris DP 051 1:50) was detected by a Cy3-labelled secondary antibody (Cy3: Jackson Laboratories 711-165-152 1:100). Second, we performed a simultaneous staining for insulin (primary antibody: Dako A0564 1:800; secondary antibody Jackson Laboratories 106-165-003 1:800) and C-peptide (primary antibody: Acris BM270 1:200, secondary antibody FITC; Jackson Laboratories 715-095-150 1:100) that yielded a congruent staining pattern for both antigens (data not shown).

For the co-staining of insulin and TUNEL, the in situ cell death detection KIT (Roche Diagnostics GmbH; cat-no. 11 684 809 910, lot-no. 13184900) was used according to the manufacturer’s recommendations. Tissue samples were excluded from the determination of apoptosis if the pancreas sections exhibited areas of tissue damage or necrosis caused by autolysis.

**Tissue analyses**

For the qualitative morphological evaluation of pancreatic development, cases were categorised into three developmental stages: i) the late embryonic and early foetal period (weeks 8–12 p.c.), ii) the mid-foetal period (weeks 13–25 p.c.), and iii) the late foetal period (weeks 26 p.c.–birth).

**Morphometric analyses** All sections were examined with an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and photographed as colour images using a Zeiss axiovision software running on a Pentium 4 (Intel Inc., Santa Cruz, CA, USA) working on a Windows XP platform (Microsoft Inc). To ensure colocalisation between different fluorescent markers, ultra-thin (0.5 μm) optical layers were generated using an apotome microscope system (Zeiss).

![Figure 1](https://www.eje-online.org)

**Figure 1** Left panels: sections through the gut showing the dorsal pancreatic evaginations in two foetuses collected at weeks 8 (a and b) and 9 (c and d) p.c. Images were acquired at 100× magnification. Right panels: high power images (400×) from the same sections. Upper sections (a and b) were stained for glucagon (brown), and lower sections were stained for insulin (brown) and CD31 (red). Red arrows point to single endocrine cells adjacent to exocrine ducts.
The relative β-cell area per pancreatic section was quantified in all 65 cases by scanning the tissue area using ×10 objective magnification as described (21). The total tissue area within this region was quantified, followed by the insulin-positive area to generate the ratio of insulin staining to total pancreas area using Axiovision software. One pancreatic section per case was analysed to measure β-cell mass.

To measure the frequency of replication in β-cells and exocrine cells, ten random fields per slide stained for insulin and Ki67 were imaged at 20× objective magnification \((n=33\text{ cases})\). The respective cell numbers were counted manually, and the relative expression frequency of Ki67 was determined in each cell type. The mean number of β-cells counted to measure β-cell replication was \(585 \pm 293\) (range 159–1410).

To measure the frequency of β-cell apoptosis, ten random islets per slide stained for insulin, TUNEL and DAPI were imaged at 20× objective magnification \((n=30\text{ cases})\). The number of cells co-staining for insulin and TUNEL was quantified and expressed in relation to the total number of insulin-positive cells. The mean number of β-cells counted to measure β-cell apoptosis was \(706 \pm 314\) (range 87–1364).

**Data analysis**

Results are presented as means \(\pm\) S.E.M. Statistical analyses were carried out by Student’s \(t\)-test using GraphPad Prism, La Jolla, USA, version 3.0. Correlations were carried out by linear regression analysis.

**Results**

**Islet morphology**

**Late embryonic and early foetal period (weeks 8–12 p.c.)** Pancreas development originated from the ventral and dorsal protrusions of the primitive gut (Fig. 1). Expression of glucagon was detectable already by gestational week 8. In contrast, expression of insulin was absent by week 8 p.c. The appearance of β-cells was first noted at week 9 p.c. (Fig. 1), and abundant expression of insulin was found in the later stages. During embryonic and early foetal development, both α- and β-cells typically appeared as small clusters of single cells scattered along or residing inside the ductal epithelium (Fig. 1). The exocrine pancreas was primarily composed of primitive ductal formations giving rise to the subsequent development of acinar secretory units. The ductal formations as well as the early endocrine cell clusters were surrounded by a loose mesenchymal stroma. Notably, a small subset \((\sim 1–2\%)\) of endocrine cells co-expressed both insulin and glucagon.

**Mid-foetal period (weeks 13–25 p.c.)** During this intermediate stage of prenatal pancreas development, the appearance of islets became increasingly apparent (Fig. 2). These newly forming islets already exhibited

![Figure 2](image-url) Pancreatic sections obtained from human foetuses at weeks 12 (upper panel), 22 (middle panel) and 34 (lower panel) stained for insulin (brown) and Ki67 (red). These figures illustrate the lobular organisation of the pancreas in the early prenatal stages as well as the increase in islet size throughout the prenatal development. Images were acquired at 200× magnification.
a rich vascular supply with large arterioles and capillaries branching into the immature islet structures (Fig. 3). While in the earlier cases, the abundance of α-cells exceeded the respective number of β-cells, β-cell mass gradually increased during that time period to become the predominant endocrine cell type. A considerable percentage of β-cells (up to 8.7% at week 13) were found to co-express insulin and glucose.
glucagon (Fig. 4). While in the earliest cases, the developing islets were typically comprised by 3–5 endocrine cells only, the characteristic islet architecture with a predominant expression of β-cells in the centre and a ring of α-cells in the islet periphery become increasingly apparent during that stage of development (Fig. 4). At the same time, the size of the immature islets constantly increased. Furthermore, the abundance of exocrine secretory units progressively increased, whereas the proportion of the pancreas comprised of mesenchymal tissue gradually declined. During the early and intermediate stages of prenatal pancreas development, the lobular organisation of the pancreas was much more pronounced than typically observed in adult human pancreas (Fig. 2).

**Late foetal period (weeks 26 p.c. – birth)** During this late stage of prenatal development, the islet architecture was already largely reminiscent of that seen in postnatal human pancreas (Fig. 3). Thus, expression of insulin was primarily, but not exclusively, found in the islet core, whereas glucagon expression was found more abundantly in the islet periphery. However, this characteristic pattern of micro-architecture was far less pronounced than typically observed in mice or rats. Insulin/glucagon co-expressing cells were rarely found during this period. During all stages of prenatal pancreas development, the islets were accompanied by a rich supply of capillaries and arterioles, consistent with an interaction between endocrine and endothelial cells (Fig. 2).

**Pancreas morphometry**

**Fractional β-cell area** The fractional β-cell area of the prenatal pancreas was heterogeneous and subject to considerable inter-individual variation (range: 0.14–5.67%). There was a linear increase in fractional β-cell area during prenatal pancreas development, with the fractional insulin-positive area increasing from 0% at gestational week 8 to ~ 3% around birth (r = 0.60, P < 0.0001; Fig. 5). β-cell area was 2.8 and 3.0% in the two newborn cases examined.

**β-cell replication**

β-cell replication was readily detectable already at the onset of β-cell differentiation (week 9), with an average of 2.9 ± 0.4% of β-cells expressing the replication marker Ki67 throughout all stages of prenatal development (Figs 6 and 7). There was a trend towards higher frequencies of β-cell replication at the earlier stages of development (Fig. 7). Of note, Ki67 was almost exclusively expressed in β-cells that were not adjacent to exocrine ducts, but never occurred in single β-cells residing inside the ducts. The frequency of replication in exocrine cells was significantly higher than in β-cells (10.7 ± 1.7%; P < 0.0001). Furthermore, the frequency of replication in the exocrine cells decreased in an exponential fashion with increasing gestational age (r = 0.84, Fig. 7).

**β-cell apoptosis**

The overall frequency β-cell apoptosis was 1.5 ± 0.3% throughout all age groups, suggesting a relatively high level of β-cell turnover and renewal (Figs 8 and 9). There were no differences in the frequency of β-cell
apoptosis between the age groups. In particular, there was no evidence for a rise in β-cell apoptosis prior to birth.

**Discussion**

The present studies were undertaken to examine the dynamics of β-cell growth and to quantify the extent of β-cell replication and apoptosis in prenatal human pancreas. We report that a) endocrine cells start to develop around day 8 p.c., b) pancreatic β-cell area expands by several fold during prenatal development, c) β-cell replication is present at high frequencies throughout all stages of prenatal life, and d) β-cell apoptosis is occurring at relatively high levels until birth.

The morphological characteristics of the prenatal human pancreas have been examined in a number of previous studies (15–17, 19, 22–24). Also, high frequencies of β-cell replication have previously been noted in prenatal human tissue (15, 16). The present studies extend these findings by for the first time providing a morphological quantification of the increase in β-cell area during prenatal human development. Furthermore, in the present study, the frequencies of β-cell replication and apoptosis have been quantified in a large group of human foetuses comprising all stages of prenatal development.
The potential for β-cell regeneration in humans is an area under active investigation, as the importance of β-cell mass has become increasingly evident over the past years (3, 25, 26). Consequently, extensive efforts have been undertaken to develop therapeutic strategies to enhance β-cell regeneration and to prevent the excessive loss of β-cells via β-cell apoptosis. Indeed, a regenerative potential has been ascribed to incretin-based therapies, i.e. the glucagon-like peptide (GLP)-1 receptor agonists and dipeptidyl-peptidase (DPP)-4 inhibitors, as well as other treatment options, such as glitazones and metformin (25). However, the clinical interpretation of these findings is limited by the fact that so far studies in the field of β-cell regeneration have almost exclusively been carried out in rodent models of diabetes or under in vitro conditions, whereas the knowledge on new β-cell formation in humans is relatively sparse.

The origins of β-cell formation have been widely debated. While it is undisputed that endocrine cells arise from the differentiation of pancreatic and duodenal homeobox (PDX)-1-positive precursors in the embryonic and foetal pancreas (6, 8, 17), the relative contributions of β-cell neogenesis (i.e. differentiation) and replication are still a matter of vivid discussion. In the present study, we noted high frequencies of β-cell replication throughout all stages of prenatal pancreas development. At the same time, a co-localisation of β-cells and exocrine ducts was apparent, and this relationship was most pronounced in the early foetal period. This may indicate that the first endocrine cells during prenatal life originate from ductal precursor cells, consistent with previous studies (27). However, the high expression frequencies of KI67 throughout all developmental stages demonstrate that β-cell replication contributes substantially to the increase in β-cell mass until birth.

The present data also demonstrate that β-cell mass expands significantly during prenatal human development, and that the fractional insulin-positive area of the pancreas increases in a linear fashion between gestational week 9 and birth. Thus, by the time of delivery, ~3–4% of the entire pancreatic area was covered by β-cells. This is in line with previous studies in postnatal humans, where fractional β-cell area was calculated to be ~5% immediately after birth and subsequently declined to ~1.5% over the following two decades (28).

Most likely, this postnatal reduction in fractional β-cell area is due to a more rapid growth of the exocrine versus the endocrine pancreas, consistent with higher frequencies of replication in acinar cells than in islet β-cells. This suggests that the growth patterns of the endocrine and exocrine pancreas are disproportionate and likely controlled by distinct factors.

Of note, pancreatic β-cell area was subjected to large inter-individual variation, with an ~40-fold difference in β-cell area between individuals at similar stages of development. This finding is consistent with other studies in postnatal human pancreas (28, 29) and demonstrates that β-cell mass can vary extensively between different individuals. Although some of this variation can certainly be attributed to sampling error and the variability of the immunohistochemical and morphometric methods, this suggests a great inter-individual heterogeneity in the kinetics of β-cell growth, which might be related to genetic factors as well as individual intraterine conditions (30–32). It is also well conceivable that the failure to adequately expand β-cell mass during the prenatal period predisposes the development of diabetes in some individuals.

Another interesting observation of our study is the co-localisation of insulin and glucagon in a subpopulation of endocrine cells during early foetal pancreas development. This finding is consistent with previous studies (9, 17, 33, 34), and has been interpreted as indicating that either all mature β-cells develop from the transdifferentiation of α-cells, or that all islet cell types arise from a common multipotent progenitor cells, which subsequently differentiates into the respective endocrine cell types (34). However, subsequent lineage-tracing studies in mice have challenged this interpretation by demonstrating that glucagon gene expression is not essential for β-cell development (35). Consistent with the latter interpretation, we observed a constant decline of the percentage of these co-expressing cells with increasing gestational age. Since new β-cell formation is ongoing until adulthood (25, 36), it is rather unlikely that these double hormone-positive cells represent an obligatory stage in β-cell lineage.

In adult mice and rats, islet cells are typically segregated into a β-cell containing core and a mantle...
region enriched with α-, δ- and PP-cells (37). This characteristic architecture together with the centrifugal direction of the intra-islet blood flow has been suggested to be important for the coordinated secretion of insulin and glucagon (38–40). However, in humans, the importance of this characteristic islet architecture has recently been challenged by studies employing confocal microscopy (41, 42). In the present studies, insulin- and glucagon-expressing cells were found to be organized in a more random fashion during the earliest stages of islet development, with α-cells appearing ~1 week earlier than β-cells. However, in the later stages of foetal growth, the characteristic intra-islet architecture became predominant and remained apparent until birth (Fig. 6). Thus, even though endocrine islet cell segregation still appears to be more prominent in rodents than in humans, our data do support the concept of a coordinated cytoarchitecture in human islets.

In the present study of prenatal human pancreas, β-cell apoptosis was detectable at relatively high frequencies (~1.5%) throughout all stages of development, with no discernable peak in the immediate prenatal period. Taken together with the high frequencies of β-cell replication, this suggests that β-cells are subject to constant renewal during prenatal life. In contrast, previous studies in rats have implied that islets undergo a period of pronounced remodelling immediately prior to birth and during weaning (43–45). Such increased β-cell apoptosis has been suggested to participate in the segregation of endocrine cells leading to the typical adult-type islet architecture. Our present data are at variance with these studies and suggest that islets undergo constant remodelling until birth with no discernable difference in islet turnover rates around the time of delivery.

The importance of vascular supply for islet development has been highlighted by a series of elegant studies in mice (14). Thus, mature islets exhibit a dense capillary network, which is typically supplied by one to three afferent arterioles and constitutes ~10% of the whole pancreatic blood flow (46). Furthermore, a number of growth factors including hepatocyte growth factor (HGF) or fibroblast growth factor (FGF) are being secreted from endothelial cells, and in turn, islet β-cells induce endothelial cell proliferation in particular through the secretion of vascular endothelial growth factor A (VEGF-A) (14). A recent series of experiments in mice also demonstrated that vascular endothelial cells provide inductive signals for embryonic islet cell development (14). Consistent with these studies, we observed β-cells to primarily develop alongside major blood vessels, and a dense capillary network was obvious already in the earliest stages of islet development. Thus, these data emphasize the importance of endothelial cell signalling for foetal β-cell development in humans.

In conclusion, β-cell differentiation in humans occurs from gestational week 9 onwards, and fractional β-cell area of the pancreas increases in a linear fashion until birth. In early prenatal development, β-cells are associated with the ductal epithelium, but at the same time β-cell replication is detectable at high frequencies until birth. A small subset of endocrine cells co-express insulin and glucagon during early foetal life, and the characteristic intra-islet distribution of α- and β-cells becomes increasingly apparent during late gestation. These results highlight the importance of both β-cell differentiation and replication for the prenatal expansion of β-cell mass in humans. Identifying strategies to re-establish these mechanisms during adult life might pave the way to the development of novel therapeutic strategies for patients with diabetes.

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.

Funding
These studies were supported by the Deutsche Forschungsgemeinschaft (DFG-grant Me 2096/5-1).

Acknowledgements
We are indebted to Elisabeth Richter and Kirsten Mros for excellent technical assistance. We also thank Professor Anne Jörns and Professor Anil Bhushan for their helpful comments.

References
12 Georgia S & Bhushan A. Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. Journal of Clinical Investigation 2004 114 963–968.