

Ghrelin-producing epsilon cells in the developing and adult human pancreas

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Abstract

Aims/hypothesis While the mechanisms of specification and the reciprocal relationships of the four types of endocrine cell (alpha, beta, delta and pancreatic polypeptide cells) within the human endocrine pancreas are well described in adults

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and during fetal development, ghrelin-immunoreactive cells (epsilon cells) remain poorly understood.

Methods We studied epsilon cells in 24 human fetal pancreases between 11 and 39 weeks of development and in 32 pancreases from adult organ donors.

Results We observed single epsilon cells scattered in primitive exocrine tissue from gestational week 13 in developing pancreas. Later in the developmental process, epsilon cells started to aggregate into clusters. From gestational week 21, epsilon cells were observed located around developing islets, forming an almost continuous layer at the peripheral rim of the islets. They remain localised on the mantle of the islets, although at different amounts, in the adult pancreas. Co-production of ghrelin with insulin, glucagon or somatostatin was not detected during fetal development. Co-production with pancreatic polypeptide was evident sporadically. Epsilon cells co-produced NK2 homeobox 2 and ISL LIM homeobox 1, but not NK6 homeobox 1 and paired box 6. A quantitative analysis was performed in the adult pancreas: there was an average of 1.17 ± 1.17 epsilon cells per islet, the relative epsilon cell volume was $0.14 \pm 0.16\%$ and the epsilon cell mass was 0.13 ± 0.15 g. Neither sex nor age affected the epsilon cell mass, although there was a significant inverse correlation with BMI.

Conclusions/interpretation During fetal development epsilon cells show an ontogenetic and morphogenetic pattern that is distinct from that of alpha and beta cells.

Keywords Co-production · Development · Endocrine · Fetal pancreas · Ghrelin · Insulin · Transcription factors

Abbreviations

ISL1 ISL LIM homeobox 1
NKX2-2 NK2 homeobox 2
NKX6-1 NK6 homeobox 1

PAX6 Paired box 6
 PP Pancreatic polypeptide

Introduction

The pancreatic islets are comprised of four major endocrine cell types that produce the hormones necessary for maintaining appropriate levels of circulating glucose. The alpha cells synthesise glucagon, beta cells synthesise insulin, delta cells synthesise somatostatin and pancreatic polypeptide (PP) cells synthesise PP [1–3]. A few years ago, a fifth mammalian islet cell type, the ghrelin-producing epsilon cell, was described [4, 5].

To date, knowledge of the epsilon cell is largely derived from studies on rodents, in which gene regulation can be widely manipulated to provide information about signalling pathways and cell lineages [6]. From these studies we know that epsilon cells can be detected in the mouse pancreas as early as embryonic day 10.5 and are the major source of ghrelin during fetal life [7]. The number of epsilon cells increases during fetal development, and close to birth they begin to localise at the periphery of the developing islets [4, 5]. Generally, the number of epsilon cells is increased in mouse models of beta cell deficiency. In fact, it has been shown that the loss of functional *Nkx2-2* [5, 8], *Pax6* [9] and *Pax4* [10] produces an overabundance of epsilon cells in the pancreas. The origin of epsilon cells in the rodent and human pancreas remains controversial. Ghrelin has variably been reported to be present in alpha cells in rats and humans [11], in beta cells in humans [12] or in separate cell types [4, 13, 14]. In human and rodent pancreases of neonates and adults, a few epsilon cells remain visible in marginal areas of the islets [4, 5, 13]. An insulinostatic function of endogenous ghrelin within islets has been suggested [15–17]. In fact, pharmacological, immunological and genetic blockade of ghrelin or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release [15–17].

There is little information on epsilon cells during fetal development of the human pancreas [4]. Like the mouse pancreas, the human pancreas develops from two endodermal diverticula, the dorsal and ventral [1], which fuse at about 56 days of development post coitum [18]. However, the morphogenesis of the endocrine tissue is unlikely to be comparable between species, given the differences in gestation and the larger relative volume of the human pancreas [19]. Extrapolation of rodent-derived knowledge of epsilon cells and pancreatic development to humans is therefore problematic. We investigated the ontogenetic and morphogenetic pattern of epsilon cells during fetal development of the human pancreas. We undertook quantitative and qualitative studies in order to describe ongoing changes

in ghrelin production during pancreas development. To understand how epsilon cells are related in lineage to the four classical islet cell types, i.e. alpha, beta, delta and PP cells, we investigated co-production of ghrelin with the main islet hormones (insulin, glucagon, somatostatin, PP) and with transcription factors involved in pancreas development (NK2 homeobox 2 [NKX2-2], NK6 homeobox 1 [NKX6-1], paired box 6 [PAX6], ISL LIM homeobox 1 [ISL1]).

Methods

Tissue collection and preparation All tissues examined were collected from the surgical pathology department of San Raffaele Hospital (Milan, Italy) according to the rules of the local institutional review boards. Twenty-four well-preserved pancreatic specimens from fetuses obtained from spontaneous abortion due to maternal causes were used in this study. Tissue preservation was evaluated using histological examination including immunohistochemistry, which showed that staining quality was excellent. In addition to the fetal specimens, we also sectioned a pancreatic block obtained during autopsy of a newborn child ($n=1$). Sections from the body of the pancreas were obtained from 32 human donor pancreases before islet isolation. Before pancreas digestion, a small piece of tissue was removed from the organ and fixed for quality control. The donors (20 men, 12 women) were 48+13 years old with a BMI of 25.2+3.3 kg/m². The study received ethics committee approval.

Immunocytochemistry Paraffin-embedded tissues were cut into 6 µm sections, which were placed on poly-L-lysine-coated slides, dried for 12 h at 37°C and kept at room temperature. The paraffin was removed and the sections rehydrated. Antigenic sites were unmasked by pre-treatment for 3×5 min in 0.01 mol/l citrate buffer (pH 6.0) in a microwave oven. After cooling at room temperature, the sections were washed in PBS (pH 7.4). The slides were then washed in sterile water and incubated for 10 min with 3% H₂O₂ (vol./vol.), for 10 min with BSA 10% (wt/vol.), for 1 h with specific primary antibodies (Table 1), for 30 min with a biotinylated complex super enhancer (Biogenex, San Ramon, CA, USA) and for 30 min with an enzyme label (Super Sensitive; Biogenex). Immunoreactivity was revealed using 3,3'-diaminobenzidine as the chromogen (code: K3468; Dako North America, Carpinteria, CA, USA), counterstained with haematoxylin solution (code: 51275; Sigma-Aldrich, Taufkirchen, Germany). The specificity of immunohistochemical staining was checked by omitting either the primary or the secondary antibody, or by using only the chromogen. These tests produced no staining. Since cross-reactivity between anti-PP antibody and peptide YY has

Table 1 Details of antibodies used for immunohistochemistry

Antigen	Code	Raised in	Dilution used	Source
Ghrelin	SC-10368	Goat	1:2,000	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Insulin	A0564	Guinea pig	1:100	Dako, Carpinteria, CA, USA
Glucagon	4030-01	Rabbit	1:4	Linco Research, St Charles, MO, USA
Somatostatin	A566	Rabbit	1:600	Dako
PP	7100-0659	Rabbit	1:400	Gentaur, Kampenhout, Belgium
PAX6	AB5409	Rabbit	1:1,500	CHEMICON International, Temecula, CA, USA
ISL1	39.4D5	Mouse	1:200	Columbia University, New York, NY, USA
NKX2-2	SC-25404	Rabbit	1:100	Santa Cruz Biotechnology
Synaptophysin	Ab14692	Rabbit	1:1,200	Abcam, Cambridge, UK

been reported, control experiments on paraffin sections of human pancreas were carried out to assess the specificity of the labelling obtained with the anti-PP antibodies. For antigen absorption, optimally diluted anti-PP antibody was incubated overnight with excess amounts of PP and peptide YY (20 µg/ml; Sigma-Aldrich). No positive labelling was seen when the sections were incubated with the primary antibodies pre-absorbed with specific antigen PP, whereas a strong reaction was observed in sections incubated with the primary antibody that were pre-incubated with peptide YY.

Double immunocytochemistry This was performed as described above (see Immunocytochemistry) until application of the first chromogen. Sections prepared in this way then underwent another antigen retrieval treatment. No blocking steps were applied during the second staining. The slides were incubated with specific antibodies and staining visualised by first applying biotinylated antibodies, then streptavidin-coupled alkaline phosphatase antibodies, and finally Vector Red as the chromogen (Vector Red Substrate kit, code: SK-5100; Vector Laboratories, Burlingame, CA, USA). The specificity of immunohistochemical staining was checked as described above.

Immunofluorescence Immunofluorescence was performed on paraffin-embedded tissue. Primary antibodies included rabbit anti-glucagon, mouse anti-insulin and goat anti-ghrelin (for details see Table 1), and were used at twice the dilutions used for immunohistochemistry. Secondary antibodies were FITC and tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) (1:400; Jackson ImmunoResearch, West Grove, PA, USA). Images were obtained with a Leica DM5000 microscope (Leica Microsystems, Wetzlar, Germany), a colour camera (Evolution MP; Leica) and image management software (Leica).

Quantitative analysis All immunoreactive cells were analysed using a Leica DMIRE2 microscope equipped with a colour video camera connected to a computer (Hewlett

Packard, Cernusco sul Naviglio, Italy) and quantified using Axio Vision 4.4 (Carl Zeiss, Oberkochen, Germany). For immunofluorescence, we used a Leica IM50 Image Manager. The ghrelin, insulin and glucagon cell numbers were normalised by the total islet cell number of corresponding islets and expressed as its percentage. Between five (minimum) and 11 islets per case were calculated. Islet size was calculated with the image analysis of single measured islets and expressed as area (µm²).

The relative beta cell and epsilon cell volumes were calculated by convention as the ratio of beta cell area: exocrine area and epsilon cell area:exocrine area. To measure this ratio, slides immunostained for insulin or ghrelin were analysed using Axio Vision 4.4 software (Carl Zeiss). The slide was scanned using an objective with ×4 magnification. A representative area of pancreas section was chosen for analysis and the coordinates entered into the program. The image analysis quantified total tissue area within this region, followed by the insulin- or ghrelin-positive areas, in order to determine the ratio of hormone staining to total pancreas area. Sensitivity for the hormone-positive areas was high, so all hormone-positive areas were included irrespective of staining intensity. Between 40 (minimum) and 60 fields per case were calculated. Beta cell and epsilon cell mass were calculated using the formula: hormone cell mass=relative hormone cell volume×pancreas weight. Results are reported as means±SD.

Results

Characterisation of ghrelin-producing epsilon cells during fetal development of the human pancreas We evaluated human fetal pancreas from weeks 13 to 39 of gestational age. A progressive increase in the endocrine cell (synaptophysin-immunoreactive cell) population was evident from the earliest time point (13 weeks), reflecting the progressive expansion of the pancreatic epithelium and an increase in

the density of endocrine cells within the epithelium (Electronic supplementary material [ESM] Fig. 1). Evidence of formation of endocrine cell clusters was observed with increasing fetal age, especially from 15 weeks onwards, although solitary endocrine cells were still present at all time points. At early time points, endocrine cell clusters generally displayed a homotypic association of either insulin- or glucagon-positive cells. At later time points, heterotypic endocrine cell clustering and the appearance of typical islet-like structures were characteristic. In samples from week 24 until birth we observed localisation of endocrine cells in a way that was typical for mature, adult islets. Thus, the centre of the islet was mainly occupied by insulin-immunoreactive cells, whereas somatostatin- and glucagon-immunoreactive cells were localised on the mantle of the islet and PP cells were rarely present, appearing on the islet periphery (ESM Fig. 2).

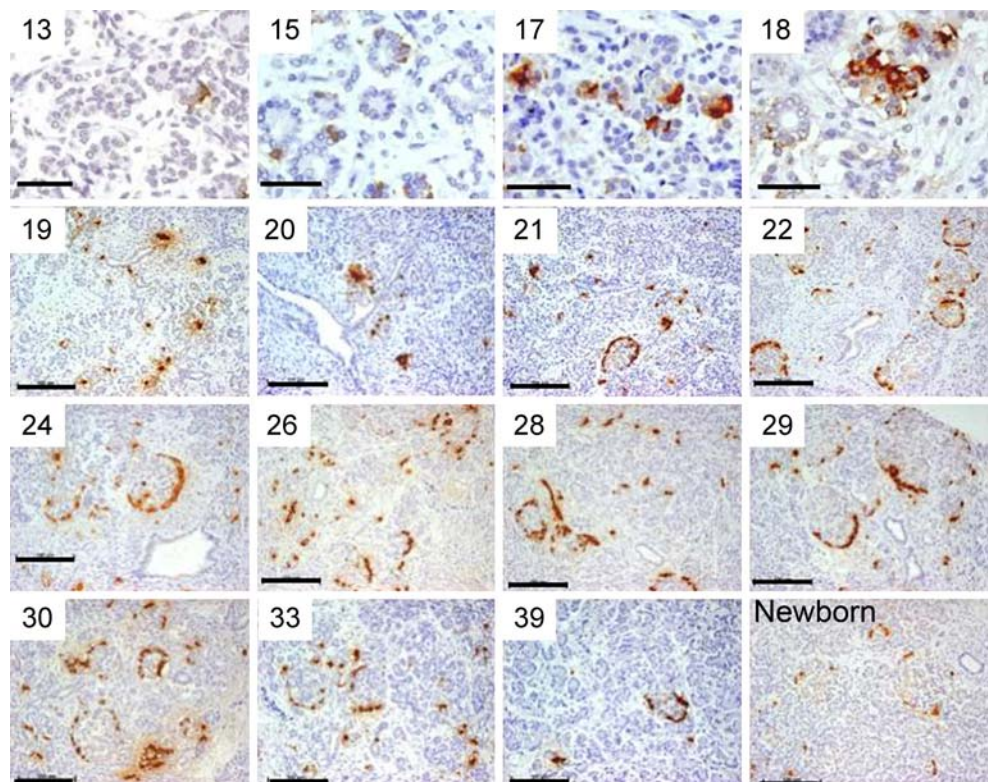
We observed single epsilon cells scattered in the primitive exocrine tissue in pancreas obtained as early as week 13 of gestation (Fig. 1). Later (weeks 17–20), epsilon cells started to aggregate into clusters, but unlike insulin- and glucagon-reactive cells, which are predominantly localised to clusters at this age (ESM Fig. 3, ESM Fig. 4), epsilon cells were still frequently seen as single cells randomly localised in the surrounding mesenchyme. In pancreas from week 21 and through to late gestation, epsilon cells were located around developing islets, forming an almost continuous layer at the peripheral rim of the

islets, with a crescent-like structure. We also observed immunoreactivity for ghrelin in duct cells at all periods investigated.

The percentage of ghrelin-, insulin- and glucagon-immunoreactive cells in islets was evaluated (Fig. 2). We were able to distinguish newly formed islets from gestational week 17. At this time, the average islet size was $998 \pm 418 \mu\text{m}^2$. The size increased progressively reaching $13,460 \pm 1,500 \mu\text{m}^2$ at birth and $15,321 \pm 1,753 \mu\text{m}^2$ in adult life. Epsilon cells in the islets were observed in pancreases from week 21 of gestation. At this time, epsilon cells represented $14.8 \pm 10.2\%$ of the total islet cell number. The highest percentage was observed in samples from week 23 of gestation ($29.9 \pm 5.8\%$). Thereafter, the relative proportion of epsilon cells abruptly decreased until birth ($4.3 \pm 1.5\%$), with epsilon cells being rare in adult pancreases ($0.74 \pm 0.5\%$). The percentage of insulin-immunoreactive cells in islets was less than 50% until gestational week 23 ($39.2 \pm 11.1\%$), but rose progressively to reach $71.9 \pm 8.6\%$ in adult pancreases. The percentage of glucagon-immunoreactive cells was about 33% until gestational week 26, subsequently decreasing to $24.4 \pm 8.2\%$ in adult samples.

We did not observe co-localisation of ghrelin with insulin, glucagon or somatostatin at any stage of development (Fig. 3). We did, however, sporadically see occasional co-production of ghrelin with PP in samples from late gestation (ESM Fig. 5). All epsilon cells in fetal pancreas were positive for transcription factor ISL1 (Fig. 4); co-

Fig. 1 Immunohistochemical staining for ghrelin in developing human pancreatic region at indicated weeks of gestation. Beginning from single scattered cells embedded in the primitive pancreas (weeks 13 to 15), cells start to aggregate into clusters after week 17 of fetal development. In week 21 ghrelin-positive cells surround primary islets with a cell monolayer. Ghrelin-positive cells are most evident in week 23, tending in later stages to disaggregate and decrease in number. Scale bars: 25 μm for weeks 13–18; 100 μm for weeks 19 onwards



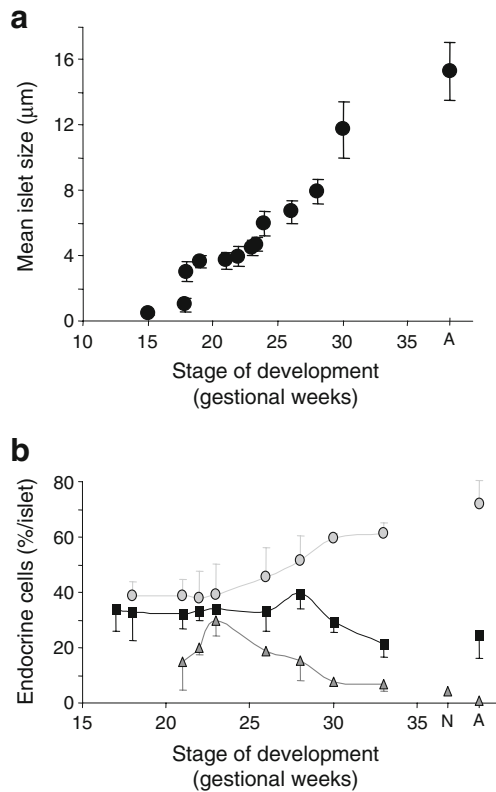


Fig. 2 Dynamics of endocrine mass evolution during human pancreas development. **a** Islet size during development. Islet size was calculated as area. Calculation was based on five (minimum) to 60 islets each time. **b** Percentage of islet cells immunoreactive for ghrelin (triangles), insulin (circles) and glucagon (squares) during development. The hormone-positive cell number was normalised by total islet cell number of corresponding islet and expressed as percentage. Calculation was based on five (minimum) to 11 islets each time. Data are expressed as mean±SEM. A, adult; N, newborn

localisation of ghrelin with NKX2-2 was also observed. We did not detect any co-production of ghrelin with PAX6 or NKX6-1.

Characterisation of ghrelin-producing epsilon cells in adult human pancreas Sections from pancreases were obtained from 32 human donor pancreases and were then used for islet isolation. Epsilon cells were usually round or ovoid in shape and were often located at the periphery of the islets, either as single cells or small clusters of cells. We also occasionally observed immunoreactivity for ghrelin in duct cells (Fig. 5a,b). No co-localisation of ghrelin with insulin or glucagon was observed (Fig. 5c,d).

Analysis of serial sections revealed an average of 1.2 ± 1.2 epsilon cells per islet, with some islets lacking demonstrable ghrelin-immunoreactive cells. The relative beta cell volume (ratio of beta cell area:exocrine area [20]) was $2.5 \pm 1.94\%$. The relative epsilon cell volume (ratio of epsilon cell area:exocrine area) was $0.14 \pm 0.16\%$. As the total pancreas weights were available (100 ± 21 g), it was possible to estimate the total beta and epsilon cell masses (2.4 ± 1.9 and 0.13 ± 0.15 g, respectively). Sex and age did not affect the number of epsilon cells per islet, the relative epsilon cell volume or epsilon cell mass, while a significant inverse correlation with BMI was evident, suggesting a selective loss of pancreatic epsilon cells with an increase in body weight (Fig. 5e–h).

Discussion

The aim of our study was to investigate the ontogenetic and morphogenetic pattern of epsilon cells during the development of human pancreas. Our findings show that epsilon

Fig. 3 Double immunofluorescence of islet hormones in developing human pancreas. Ghrelin (red) (**a**, **b**), insulin (green) (**a**) and glucagon (green) (**b**) are shown. Week of gestation are indicated. Original magnification $\times 400$

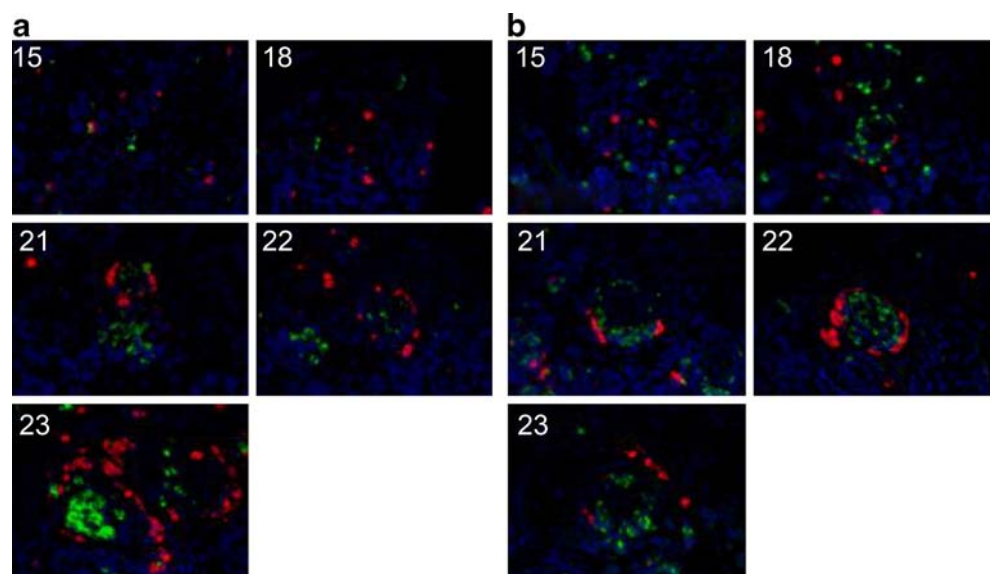
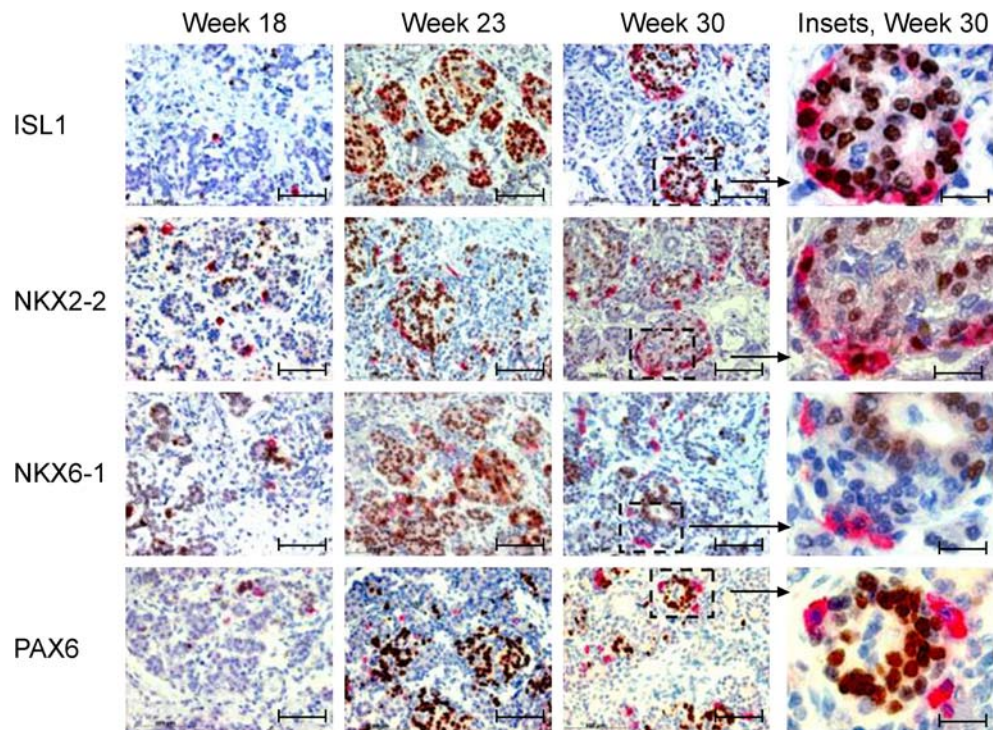


Fig. 4 Ghrelin-immunoreactive cells (red) double-stained with transcription factors (brown) as indicated, at weeks 18, 23 and 30 of development. Scale bars, 100 μm (20 μm on insets). Original magnification $\times 400$



cells have developmental features that are distinct from those of beta and alpha cells.

First, epsilon cells show a distinct change in localisation and number during pancreas development. Wierup et al.

reported on the production of ghrelin at human mid-gestation (18–22 weeks) [4] and at adult life, but with a limited number of cases. To date, however, the dynamics of pancreatic ghrelin during fetal life have not been investigated. We have

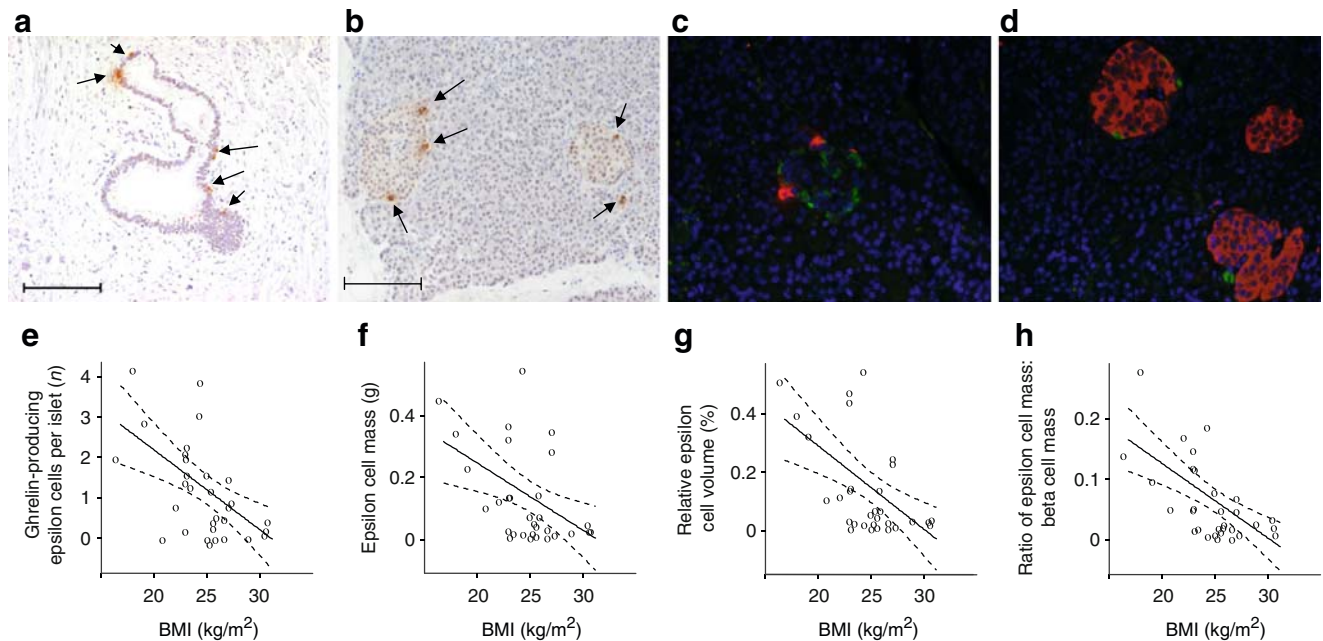


Fig. 5 Ghrelin in adult human pancreas. Immunohistochemical staining of ghrelin-immunoreactive cells in adult pancreas islet (a) and duct (b) (black arrows). Double immunofluorescence of ghrelin (red)/glucagon (green) (c) and ghrelin (green)/insulin (red) (d) in adult human islets. Scale bars, 100 μm . Original magnification $\times 400$. Ghrelin-producing epsilon cells per islet (n) ($R=0.565$; $p=0.001$) (e),

epsilon cell mass (g) ($R=0.482$; $p=0.006$) (f), relative epsilon cell volume (%) ($R=0.57$; $p=0.001$) (g) and ratio of epsilon cell mass:beta cell mass ($R=0.627$; $p<0.001$) (h) in relation to donor BMI (white circles), $n=32$. Solid line, linear regression; dashed lines, 95% mean prediction interval

now shown that epsilon cells remain as single scattered cells in the primitive exocrine tissue until gestational week 21. At this time, they constitute 15% of cells in developing islets, reaching 30% at week 23 and then decreasing to less than 5% at birth and less than 1% in the adult pancreas. Of note is the crescent-like pattern of epsilon cells from 21 weeks to birth, suggesting a polarisation phenomenon in developing islets. A potential limitation of the study is that we were able to examine relatively few islets in the pancreas during development, and quantitative results could differ if more or other islets were examined.

Second, epsilon cells do not produce other pancreatic hormones (insulin, glucagon, somatostatin) at any time during development. Date et al. [11] presented data indicating that ghrelin is co-localised with glucagon in adult human and rat pancreas, whereas results from Volante et al. [12] indicated that ghrelin is co-localised with insulin in human. We were not able to confirm either of these results; instead, our data indicate that through to adult life ghrelin peptide production is confined to a distinct cell type [4, 5, 13, 21]. The reason for this discrepancy is not clear. The absence of co-localisation of ghrelin with other hormones in humans is in contrast to the findings in mice by Prado et al. [5] and Heller et al. [9], where ghrelin and glucagon were found together early but not later in development. According to Polak et al. [22], most endocrine cells in the human pancreas are polyhormonal at about gestational week 8, but lose this characteristic by week 9. We cannot rule out the possibility that ghrelin co-localises with other hormones in stages of human pancreas development before gestational week 13.

Third, we studied the production of some critical transcription factors in epsilon cells. PAX6 was not co-produced with ghrelin during development. This finding corresponds closely with the results in the mouse of Heller et al. [9], who demonstrated that epsilon cell genesis is not under PAX6 control, but is antagonised by PAX6 transcription factor. This line of evidence, together with the finding that epsilon cells do not co-produce glucagon during development, indicates that alpha and epsilon cells represent distinct endocrine cell types subjected to different regulatory signals. NKX6-1 was also not co-produced with ghrelin during development, while all epsilon cells co-produced NKX2-2. Consistent with our results, one study found that there was no observable modification of ghrelin cells in the islets of *Nkx6-1* knockout mouse [5]. The role of NKX2-2 appears to be more complex. In fact, *Nkx2-2* [5] plays a key role in determining fate of the ghrelin cells by endocrine precursors in the mouse, since its ablation promotes a striking augmentation of epsilon cells [5, 8]. Together, the results in mutant mice and our evidence of NKX2-2 and ghrelin co-production in humans suggests that NKX2-2 may not be necessary to specify epsilon cells, but, being co-produced in the same cell type, may play a role in

determining the transition of those cells into beta cell-type cells. Finally, all epsilon cells co-produced ISL1, a transcription factor required for development of the mesenchyme of the dorsal pancreatic bud and also for differentiation of the dorsal pancreatic epithelium into endocrine cells [23, 24].

Fourth, and for the first time, we estimated the epsilon cell mass in human adult pancreas (about 0.13 g) and showed a significant inverse correlation of epsilon cell mass with BMI. The estimate is based upon relative volume calculations in a small portion of pancreas and should therefore be considered preliminary. Moreover, the range of the donors' BMI was rather narrow and the mean was at the higher end of what is normal or even above the limit for being overweight. Nevertheless, the relationship with BMI is consistent with reports of an inverse correlation between plasma ghrelin and BMI [25, 26].

Little is known about the role of ghrelin within the pancreas, but the hypothesis that ghrelin could play a paracrine/autocrine role in the regulation of beta cell survival and function [27] appears reasonable. The production of ghrelin in a prominent endocrine cell population in the fetal pancreas suggests a potential role in islet development. Interestingly, in this context, ghrelin has recently been reported to promote proliferation and cell growth, and also to inhibit apoptosis of pancreatic beta cells and human islets [27, 28]. Therefore, one function of ghrelin in the developing pancreas could be to promote cell growth and maturation locally.

An insulinostatic function of endogenous ghrelin within islets has been suggested [13, 15, 17, 29–31]. It was also recently shown to exert inhibitory effects on expression and production of inflammatory cytokines [32] and to attenuate the development of acute pancreatitis in rats by reducing inflammatory infiltrates of pancreatic tissues [33]. On this basis, we can speculate that the loss of epsilon cell mass associated with increased BMI could produce increased insulin secretion, but also an enhanced susceptibility to apoptosis and inflammation damage in pancreatic islets.

In conclusion, we investigated the endocrine mass during human pancreas development, with a special emphasis on the dynamics of changes in epsilon cells. We were able to show that large numbers of epsilon cells are present in human pancreas during fetal life and that the number and localisation of these cells is developmentally regulated. Epsilon cells were monohormonal and co-produced NKX2-2 and ISL1, but not NKS6-1 and PAX6. The exact role of ghrelin in endocrine pancreas maturation during fetal life and in pancreas reorganisation in adulthood is still unknown, but results obtained in our study suggest a significant impact of epsilon cells in early islet biology.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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