The islet ghrelin cell

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Abstract

The islets of Langerhans are key regulators of glucose homeostasis and have been known as a structure for almost one and a half centuries. During the twentieth century several different cell types were described in the islets of different species and at different developmental stages. Six cell types with identified hormonal product have been described so far by the use of histochemical staining methods, transmission electron microscopy, and immunohistochemistry. Thus, glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP-cells, serotonin-producing enterochromaffin-cells, and gastrin-producing G-cells have all been found in the mammalian pancreas at least at some developmental stage. Species differences are at hand and age-related differences are also to be considered. Eleven years ago a novel cell type, the ghrelin cell, was discovered in the human islets. Subsequent studies have shown the presence of islet ghrelin cells in several animals, including mouse, rat, gerbils, and fish. The developmental regulation of ghrelin cells in the islets of mice has gained a lot of interest and several studies have added important pieces to the puzzle of molecular mechanisms and the genetic regulation that lead to differentiation into mature ghrelin cells. A body of evidence has shown that ghrelin is an insulinostatic hormone, and the potential for blockade of ghrelin signalling as a therapeutic avenue for type 2 diabetes is intriguing. Furthermore, ghrelin-expressing pancreatic tumours have been reported and ghrelin needs to be taken into account when diagnosing pancreatic tumours. In this review article, we summarise the knowledge about islet ghrelin cells obtained so far.

Introduction

The pancreatic islets were first described by Langerhans (1869). He noticed clusters of cells, 100–200 μm in diameter, that were interspersed in the exocrine tissue. He named these clusters Zellhaufen. Langerhans also described that these clusters were more richly innervated than the surrounding pancreatic tissue.

In 1893, Laguesse proposed the name 'islets of Langerhans' in honour of their discoverer (Biedl (1913); for review see Laguesse (1893) and Falkmer (1995)). During the almost one and a half centuries that have passed since

the islets were first discovered, several islet cell types have been described in different mammalian species and at different stages of development. In adult humans, the islets have until recently been thought to harbour four endocrine cell types characterised by respect to their principal hormone content, i.e. the insulin-producing β-cells, the glucagon-producing α-cells, the somatostatin-producing δ-cells and the pancreatic polypeptide-producing PP-cells. This concept was based on the findings made during the twentieth century using mainly three

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different approaches, i.e. histochemical staining methods, transmission electron microscopy and immunohisto-chemistry. Evidence for the existence of a novel islet cell type, the islet ghrelin cell, has been presented and confirmed by several laboratories. This cell is the fifth cell type to be described in the adult human islets. Further, it is the seventh islet cell with known hormonal content when counting all mammalian species and all developmental stages. Species differences with respect to islet ghrelin cells occur and, further complicating studies of the role of ghrelin cells in islets, the ghrelin cells display a develop-mental regulation. This review aims at summarising what is known so far about ghrelin cells in the islets of different species and at different developmental stages.

Discovery of the islet cell types

β-cells

Based on studies showing that islet cells harboured granules distinct from the exocrine zymogen granules, Lane demonstrated in 1907 that the islets contain at least two different cell types (Lane 1907). He showed that the granules displayed different properties in different cells and that the cells we now know as β-cells stained basophilic with certain staining methods, while the other cells did not (Samols 1991). The non-B-cells were called A-cells. The principal hormone, insulin, of the B-cells was discovered by Banting & Best (1922). Later immunohistochemical studies provided evidence for insulin as the hormonal content of the B-cells (Lacy & Davies 1957). The B-cell was ultrastructurally charac-terised by several groups; e.g. Lacy (1957) described the B-cell ultrastructure in several mammals and Like (1967) made a corresponding description of B-cells of normal human pancreas. The granules of the B-cells are charac-terised by a dense core of crystalline structure in humans and round to ovoid in rodents, surrounded by an electron lucent halo between the membrane and the core.

α-cells

Several decades after the studies by Lane, it became evident that the A-cells, defined as non-B-cells, were in fact two cell populations; one that was argyrophil, the A1 cells, and another that was not argyrophil, the A2-cells, with the contemporary silver stainings (Hellman *et al.* 1962). Later the α-cells (A2) could be specifically distin-guished from the other cells by virtue of their argyrophilia using, e.g. the Grimelius (1968) silver impregnation

technique. Based on the finding that the ventral part of pancreas is poorly supplied with A-cells and also virtually lacking glucagon in dogs and cats, the work of Bencosme & Liepa (1955) and Bencosme *et al.* (1955) provided indirect evidence that glucagon is the hormonal product of A-cells. This notion was confirmed in 1962 when Baum *et al.* (1962) and Mikami & Ono (1962) showed by the use of immunohistochemical techniques that the A-cells store glucagon. As for the B-cells, the ultrastructure of the A-cells was described by several groups (Lacy 1957, Like 1967, Sundler & Håkanson 1988). The A-cells is charac-terised by highly electron dense, uniformly sized and spherical dense cores of the granules. Further, the granule membrane is separated from the dense cores by a distinct but narrow electron lucent halo.

δ-cells

The third cell type, the cells we now know as the δ-cells were originally described by Bloom (1931). He described a cell type, which he named the D-cell, that stained differently than the A and B-cells described by Lane, when using the Mallory-azan staining (Bloom 1931). It needs to be mentioned here that a C-cell was described earlier by Bensley; this cell lacks granules and has later been regarded as non-endocrine cell (Bloom 1931). Probably, this was the reason for Bloom to name the cell D-cell, instead of C-cell, which would have caused a more streamlined nomenclature for the islet cells when looking in retrospect. More than 30 years after the initial studies by Bloom, the δ-cells were rediscovered as a sub-population of the A-cells, the so-called A1-cells, when it became evident that they could be identified for their argyrophilia using a novel silver impregnation method, the Hellerström–Hellman silver method (Hellerström & Hellman 1960, 1962, Hellman *et al.* 1962). Fujita (1968) proposed that the D-cells described by Bloom were actually identical to the (A1) cell described by Hellerstöm & Hellman. Efforts of several laboratories, and the use of immunocytochemistry for somatostatin led to the discovery of the hormonal content of the δ-cell (Hokfelt *et al.* 1975, Orci *et al.* 1975, Polak *et al.* 1975, Alumets *et al.* 1977). The δ-cells are ultrastructurally characterised by round fairly electron lucent granules (Alumets *et al.* 1977, Sundler & Håkanson 1988).

PP-cells

The fourth islet cell type, the PP-cell, was discovered using immunocytochemistry in 1974: first in chicken

(Larsson *et al*. 1974), thereafter in humans in 1975 (Larsson *et al*. 1975) and in several mammals in 1976 (Larsson *et al*. 1976*b*). PP turned out to be the hormonal content of one population of the cells with small granules that had been identified with electron microscopy earlier (Ekblad & Sundler 2002). In rats, the PP-cells are recognised by their small moderately electron dense granules that are distinguishable from those of other small granular cells due to their irregular shape (Larsson *et al*. 1976*a*). In cats and dogs, the PP-cells turned out to be identical to the previously described F-cells (Ekblad & Sundler 2002). Further adding to the complexity, PP-cells in human pancreas seem to be of two types: one small-granular and the other one with large granules, reminiscent of feline and canine PP-cells (Solcia *et al*. 1985, Sundler & Håkanson 1988, Ekblad & Sundler 2002).

Enterochromaffin-cells and G-cells

In addition, less frequently occurring, sometimes transiently present during embryonic development and species-specific islet cell types have been described. Thus, serotonin-producing enterochromaffin (EC)-cells, thought to correspond to the EC-cells of the gut, are regularly seen dispersed in the pancreas in certain species, e.g. the pig (Capella *et al*. 1978, Alumets *et al*. 1983) and gastrin-producing G-cells have been demonstrated in the rat pancreas during neonatal development, while such cells are present, albeit in small numbers, in the pancreas of adult cat and dog (Larsson *et al*. 1976*a*). Recently, G-cells have also been found to be a distinct islet cell type in mice during development (Suissa *et al*. 2013).

Morphologically characterised cells without known hormonal content

A few less well-defined cell types have been described in the islets in different species and at different stages of ontogenetic development. Hence, cells with small granules, such as the P-cell (Capella *et al*. 1978) and the D1-cell (Larsson *et al*. 1976*b*), collectively referred to as P/D1-cells (Rindi *et al*. 2002*a*), are present in the human islets. One report also described small granule cells in human pancreas as type VI cells (Jirasek & Kubes 1972). These cells were all characterised merely with respect to ultrastructure and the hormone product has remained unknown for more than three decades (Larsson *et al*. 1976*b*, Capella *et al*. 1978, Bordi *et al*. 1986, Solcia *et al*. 1987, Sundler & Håkanson 1988).

The novel hormone ghrelin

In their search for an endogenous ligand of the previously characterised orphan growth hormone secretagogue receptor (GHS-R; Howard *et al*. 1996), Kojima *et al*. (1999) isolated a novel 28 amino acid peptide from rat stomach, which activated GHS-R signalling in a cell line transfected with GHS-R1a. The peptide was given the name ghrelin, for its ability to stimulate growth hormone (GH) secretion (GH-relin). Independently, searching for genes with expression restricted to the gastric epithelium, Tomasetto *et al*. (2000) discovered an mRNA in the mouse stomach, which encodes a novel protein of 117 amino acid residues with distant chemical relationship to the motilin precursor; due to its resemblance with prepro-motilin it was named motilin-related peptide (MTLRP). It was soon acknowledged that MTLRP is, in fact, the mouse homologue of preproghrelin (Peeters 2005). Kojima *et al*. (1999) found a difference between postulated and actual molecular weight of ghrelin. The explanation for this turned out to be a previously unknown post-translational modification, the octanoylation of the N-terminally third serine residue. This octanoylation has been claimed to be essential for the biological activity of ghrelin. Other forms of ghrelin, including non-octanoylated ghrelin as well as a variant lacking one amino acid, have been reported as reviewed in Korbonits *et al*. (2004).

Sources of circulating ghrelin

The initial studies by Kojima *et al*. (1999) and Tomasetto *et al*. (2000) localised ghrelin to the stomach. Later studies showed that the gastric source of ghrelin was the previously ultrastructurally characterised and 'hormone-less' A-like cells in rats and the P/D1-cells in humans (Date *et al*. 2000, Dornonville de la Cour *et al*. 2001, Rindi *et al*. 2002*a*). Predominantly in rodents, gut ghrelin cells are most abundant in the oxyntic mucosa, less frequent in the gastric antrum and still fewer in the small intestine (Date *et al*. 2000, Dornonville de la Cour *et al*. 2001). As the stomach is the richest source of ghrelin in adults, other sources of the hormone have been given less attention. Studies on the rat have given much attention to the gastric fundus and ruled out the production of ghrelin from other sites. Thus, in rats only 20% of circulating ghrelin remains after fundectomy (Dornonville de la Cour *et al*. 2001). The situation is, however, different in humans where 35–45% remains after total gastrectomy (Ariyasu *et al*. 2001, Popovic *et al*. 2005).

Thus, although the stomach is the major source of circulating ghrelin, other sources such as the pancreas and intestine (Wierup *et al.* 2007) clearly contribute.

Discovery of the islet ghrelin cell

Ghrelin cells in humans

Soon after the discovery of ghrelin, we investigated the possibility of ghrelin expression in the human pancreas using *in situ* hybridisation (ISH) and immunocytochemistry. We also included foetal specimens as previous studies have shown that many islet peptides are transiently overexpressed during foetal development (Wierup & Sundler 2008). Immunostainings for ghrelin revealed that ghrelin-immunoreactive cells were found in all the human pancreatic specimens studied. The cells were, in fact, numerous in foetal and neonatal pancreas, while fewer in number in the pancreas of adults. Double- or triple-immunostainings for ghrelin and the four main islet hormones (glucagon, insulin, somatostatin and PP) revealed that the ghrelin-immunoreactive cells were consistently devoid of the hormones produced by the well-characterised islet cell types (α-, β-, δ- and PP-cells) (Wierup *et al.* 2002; Fig. 1). *In situ* hybridisation for ghrelin mRNA confirmed the ICC data (Wierup *et al.* 2002). Thus, in foetal and neonatal specimens numerous cells with labelling for ghrelin mRNA were seen at the islet periphery, whereas in adult islets only a few cells with such labelling were seen (Wierup *et al.* 2002). Further ISH for ghrelin mRNA and the different islet hormones on consecutive islet sections demonstrated the lack of ghrelin mRNA in, e.g. α-cells or β-cell, and consequently, the lack of insulin or glucagon mRNA in ghrelin cells (Wierup 2005). These data led us to the conclusion that the ghrelin-immunoreactive cells constitute a separate, previously unrecognised, islet cell population. We named this novel cell type simply 'the ghrelin cell'. The cells were usually round or ovoid in shape and sometimes issued cytoplasmic extensions. As a rule, the ghrelin cells were located at the peripheral rim of the islets and sometimes, in foetal and neonatal islets, formed a continuous cell layer embracing the other islet cells. A few ghrelin IR cells were also seen in the duct epithelium or as single, extra-islet cells dispersed among exocrine cells. When comparing densities of ghrelin cells in pancreata of different gestational stages and in pancreas from adult humans, we found that the ghrelin cells displayed a characteristic developmental regulation. Thus, in foetal (15–26 weeks of gestation) and neonatal specimens, the
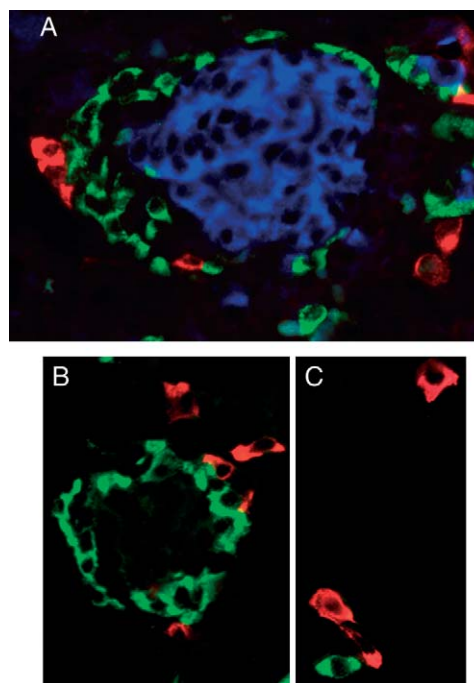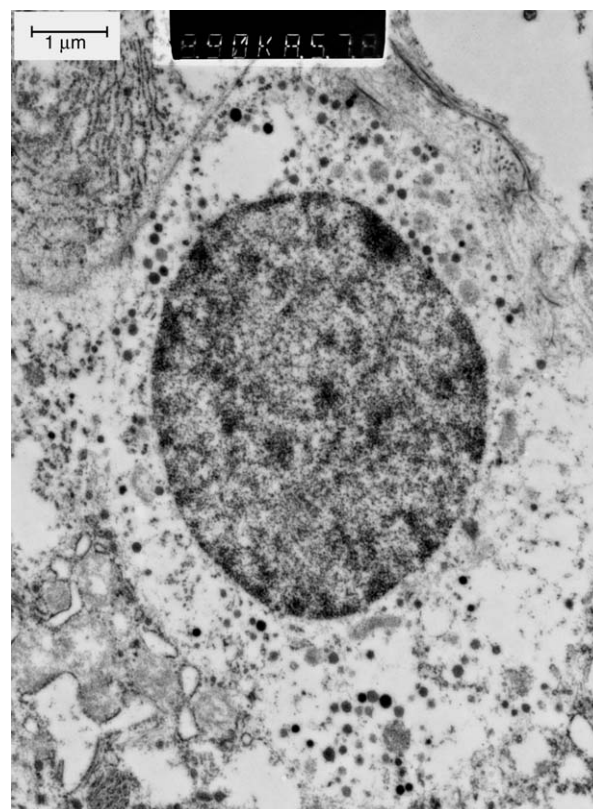

Figure 1
Double- or triple-immunostained foetal human islets (22 weeks of gestation). Ghrelin red in all images. (A) Insulin (blue) and somatostatin (green). (B) Glucagon (green). (C) PP (green). Note that ghrelin is not colocalised with any of the four major islets hormones. For antibody details see Wierup *et al.* (2002).

ghrelin cells constitute ∼10% of all islet cells; the corresponding figure in adults is ∼1%. This developmental pattern of ghrelin-immunoreactive cells in human pancreas was also in part shown by Rindi *et al.* (2002*a*). They reported that cells with immunoreactivity for ghrelin were 'fairly represented' in the human foetal pancreas, but were unable to detect ghrelin immunoreactivity in the adult human pancreas. They did not further characterise the ghrelin-immunoreactive cells. A more recent study has showed a similar pattern for ghrelin mRNA (D'Amour *et al.* 2006). Thus, peak levels of ghrelin mRNA were found at week 14 of gestation, while lower levels was seen in pancreas from adults (D'Amour *et al.* 2006). The same group also showed, when aiming at producing endocrine cells from human embryonic stem cells, that ghrelin cells appear as a separate cell type distinct from α-, β-, δ- and PP-cells after certain procedures (D'Amour *et al.* 2006, Kroon *et al.* 2008). It was also revealed that while many cells expressed more than one islet hormone, e.g. insulin and glucagon, ghrelin was not coexpressed with other islet hormones to the same extent (D'Amour *et al.* 2006). Our studies in addition showed that there is no difference

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in ghrelin cell density between the head, tail and body of the pancreas. This is different from PP- and α-cells. PP-cells predominate in the head-portion, while they are few in the tail portion (Larsson *et al.* 1976*b*). The α-cells display the opposite distribution, i.e. they are more common in the tail portion than in the head portion (Bencosme & Liepa 1955).

When ghrelin cell density in foetal pancreas was compared with that in the foetal stomach (the main site of ghrelin production in adults), we found that ghrelin cells were numerous in pancreas, while very few in the stomach. Interestingly, a study of circulating ghrelin in foetal blood showed that the human foetus has endogenous ghrelin production from 20 weeks of gestation to term. This conclusion was based on data showing a lack of correlation between maternal blood levels of ghrelin and foetal blood ghrelin levels, as well as a lack of difference in ghrelin concentration between umbilical vein and artery blood (Cortelazzi *et al.* 2003). They concluded that the foetus is a source of ghrelin production, and as no other major source was reported, we suggested that ghrelin production in the foetal pancreas is the explanation (Wierup & Sundler 2004*a*). Taken together, these data suggest that the pancreas may be the main source of circulating ghrelin during foetal development. These data are supported by similar observations in rodents (Wierup *et al.* 2004*b*).

When using transmission electron microscopy and immunogold labelling techniques, it became evident that human ghrelin cells have unique ultrastructural features compared with α-, β-, δ- and PP-cells (Wierup & Sundler 2005). The ghrelin cell secretory granules are comparatively small and spherical, and the granule cores displayed varying electron density, from dense to rather electron-lucent. Further, the limiting membrane is tightly applied to the dense core of most of the granules (Fig. 2). The ghrelin cells are readily distinguished from β-cells, which are, in the foetus, recognised by the characteristic irregular crystalline dense core of their granules (for references see Klöppel & Heitz (1984)). Further, ghrelin cells are clearly distinct from δ-cells which are recognised by their large, uniformly electron lucent granules. Ghrelin cells are also readily separated from PP-cells which harbour small and often irregularly shaped, moderately electron dense granules (Sundler & Håkanson 1988). Nevertheless, although clearly different from those of the other islet cell types, the secretory granules of the ghrelin cells are most similar to those of the α-cells; however, careful morphometrical analysis of the diameters of the dense cores of the granules revealed that the ghrelin cells have
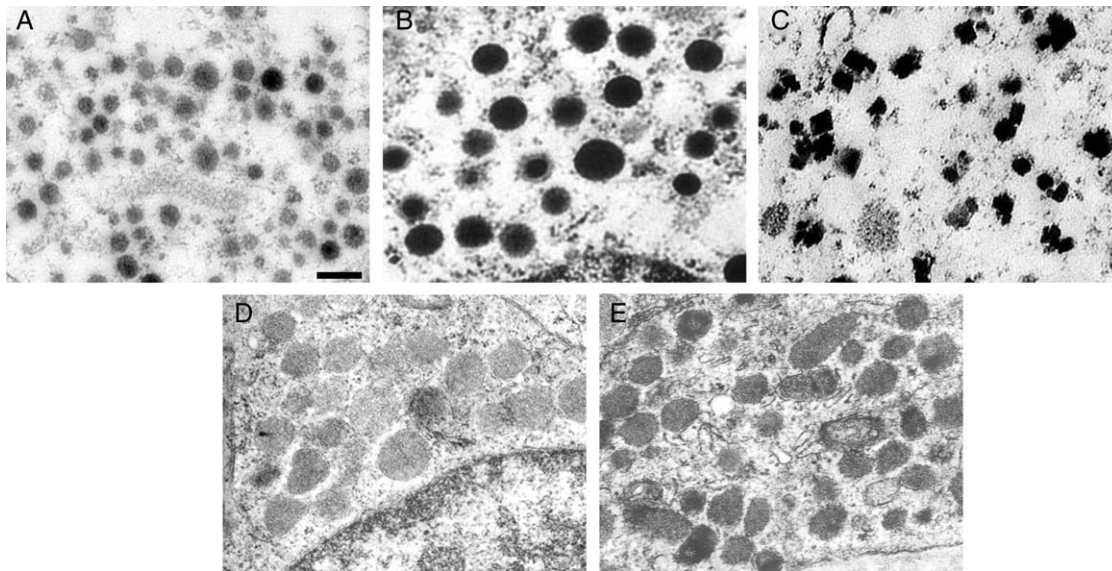

Figure 2
TEM-micrograph of a ghrelin cell in the foetal human pancreas at mid-gestation. Note that the secretory granules are small, spherical and display varying electron density. For technical details see Wierup & Sundler (2005).

substantially smaller secretory granules, (110 ± 3 nm) than the α-cells (185 ± 7 nm). Granules of ghrelin cells and the other islet cell types are shown in Fig. 3.

With this knowledge in mind, it became clear that the ghrelin cells shared a lot of features with the previously described, ultrastructurally characterised 'small-granular' islet cell types. Thus, the pattern of developmental regulation (Wierup *et al.* 2002) and granule properties (Wierup & Sundler 2005) for ghrelin cells fits well with the previously described 'hormoneless' pancreatic P/D1-cells (Larsson *et al.* 1976*b*, Capella *et al.* 1978, Bordi *et al.* 1986, Sundler & Håkanson 1988). Larsson *et al.* (1976*b*) described that the granules of D1-cells had fairly low electron density, this is in agreement with our more recent observations for ghrelin cells (Wierup & Sundler 2005). Capella *et al.* (1978) reported that P/D1-cells were numerous in the foetal pancreas, but rare in the adult pancreas. They also showed that the cells are numerous in foetal pancreas, while few in the stomach (Capella *et al.* 1978). This pattern is strikingly similar to that of the

Figure 3

Transmission electron micrographs of islet cell secretory granules adapted from Wierup & Sundler (2005). Secretory granules from islet cells. (A) Ghrelin cell. (B) Glucagon cell. (C) Insulin cell. (D) Somatostatin cell. (E) PP-cell. Note that ghrelin cell granules are distinct from other islet cell granules. Scale bar = 250 nm, in A for all images.

ghrelin cells (Wierup *et al*. 2002), suggesting that the islet ghrelin cells in fact are P/D1-cells. Remarkably, Jirasek & Kubes (1972) described in 1972 a new cell type, the so-called 'type VI cells', in the human islets. They showed that this cell type had granules that were smaller (120–240 nm diameter) than those of D-cells (290–620 nm), and that the type VI-cell was less frequently found than the D-cell, but more frequently found than the pancreatic G-cells (gastrin) and EC-cells. Their data for the type VI cell also fit well with what we today know about ghrelin cells, and we suggest that type VI cells are also in fact identical to ghrelin cells and P/D1–cells. When the P/D1/typeVI-cells were first described with transmission electron microscopy during the seventies, it was not possible to identify the hormonal product, ghrelin, as it was isolated almost 30 years later (Kojima *et al*. 1999, Tomasetto *et al*. 2000).

Ghrelin cells in mice

The development of gene-targeting techniques has favoured the mouse as an animal model for developmental studies. Genetic and transcriptional programming of pancreatic development have gained a lot of interest during the last few decades and studies on mice with disruption of key transcription factors that orchestrate cellular differentiation have provided important information about the molecular events that regulate islet cell fate (reviewed in Edlund (2002) and Oliver-Krasinski & Stoffers (2008)). When studying mice with a null mutation in the gene for *Nkx2.2*, a homeobox transcription factor expressed in CNS and pancreatic islets (Rudnick *et al*. 1994), Sussel *et al*. (1998) found that *Nkx2.2* null mutant mice display a large number of islet endocrine cells that are devoid of the four major islet hormones. They also found that the mutant mice develop severe hyperglycaemia, due to lack of β-cells, and die shortly after birth. Based on normal expression of the β-cell markers, islet amyloid polypeptide (IAPP) and PDX1 in the mutant islets, they suggested that the unidentified endocrine cell population was incompletely differentiated β-cells. Later, Prado *et al*. (2004) showed that these cells were in fact ghrelin cells. Importantly, ghrelin cells were also found to exist in developing islets of normal mice. Independently, Wierup & Sundler (2004*b*) and Heller *et al*. (2005) made similar observations in the mouse. Prado *et al*. (2004) found that ghrelin cells were present in normal mice from E10.5 and that the density of cells peaked by E15.5. In fact, we have found that ghrelin cells appear already at E8.5 (Fig. 4). On the other hand, only low levels of ghrelin were found in the pancreas of adult mice. In agreement, Wierup & Sundler (2004*b*) and Heller *et al*. (2005) (Wierup N & Sundler F 2004, Scott-Heller R 2005, unpublished observations) have been unable to find ghrelin cells after 2 weeks of age. Making the story about ghrelin cells in

Glucagon **Ghrelin**

PDX-1 **Glu-Ghr- PDX-1**
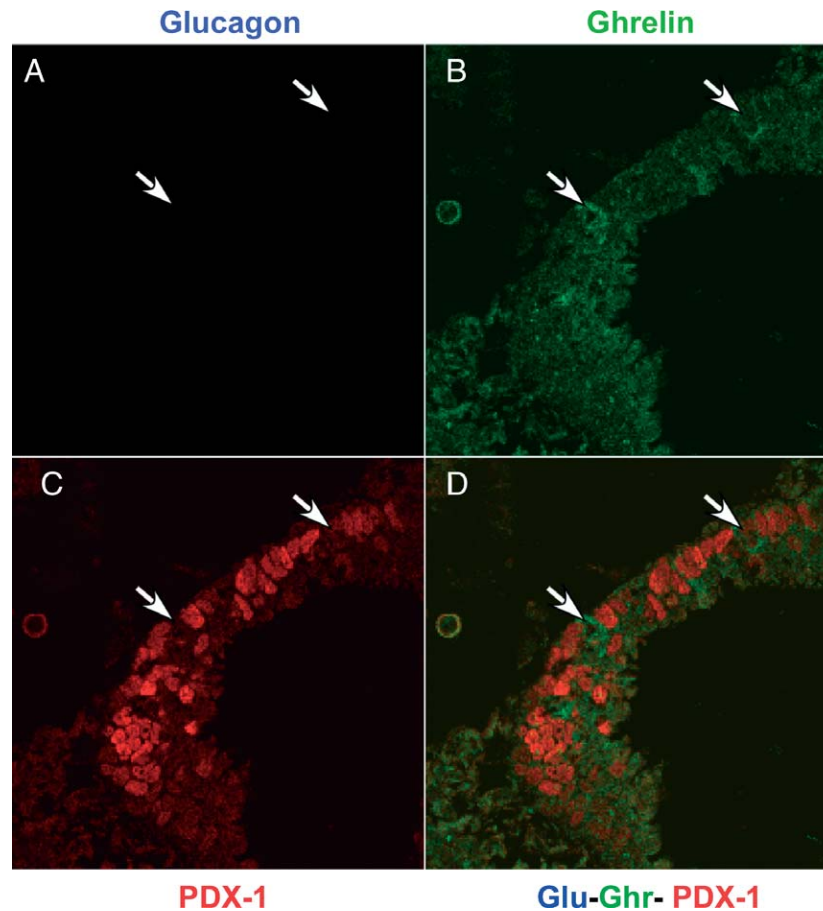
Figure 4

Expression of glucagon and ghrelin$^+$ cells at E8.5 in the mouse. The early pancreatic epithelium is localised in red by Pdx1 immuno-reactivity. Two arrows denote cells expressing ghrelin (green) but not glucagon (staining absent) at this early developmental time point. For antibody details see Heller *et al*. (2005). All images are 400× magnification.

mouse islets more complicated, Prado *et al*. found in neonatal mice that one-third of the ghrelin IR cells coexpressed glucagon, whereas two-thirds of the ghrelin cells in normal mice constituted a separate cell population. On the other hand, Heller *et al*. (2005) showed that from E10.5 to P1, 70–95% of the ghrelin IR cells also express glucagon. Thus, although it is a fact that ghrelin cells constitute a separate cell population in mouse islets, a great proportion of the ghrelin cells harbour glucagon (Prado *et al*. 2004, Wierup & Sundler 2004*b*, Heller *et al*. 2005). Therefore, in the developing mouse pancreas it is necessary to distinguish between ghrelin$^+$glucagon$^+$ cells and ghrelin$^+$glucagon$^-$ cells. In this respect, the mouse differs from the rat, in which glucagon and ghrelin only occasionally are coexpressed around birth (Wierup *et al*. 2004*b*). It is also different from islet ghrelin cells in humans, in which no glucagon expression has been reported at any stage of development.

The large degree of coexpression of ghrelin and glucagon observed in the mouse could be interpreted as a relationship with respect to cell lineage between ghrelin cells and α-cells. On the other hand, Prado *et al*. (2004) proposed that ghrelin cells share lineage with the β-cells, based on the expansion of the ghrelin cell population in the absence of transcription factors crucial for β-cell development (Prado *et al*. 2004). Thus, *Nkx2.2* mutant mice displayed dramatically increased number of ghrelin$^+$ glucagon$^-$ cells. They also showed that *Pax4* mutant mice had an expanded ghrelin cell population, but did not examine whether these cells coexpressed glucagon or not. Interestingly, a subpopulation of the ghrelin$^+$ cells in WT mice harboured *Pax6* (although it is not clear whether these express glucagon or not), and PAX6 protein is absent in *Nkx2.2* mutant mice (Heller *et al*. 2004, Prado *et al*. 2004). *Arx* is necessary for the upregulation of ghrelin mRNA levels in *Nkx2.2* mutant epsilon cells

(Mastracci *et al*. 2011) and in addition, *Nkx2.2*/*Arx* double mutants result in ghrelin[+]/somatostatin^{+} cells, which are never observed in nature. Recent work has clearly demonstrated a very important role for *Nkx2.2* in repressing the expression of other endocrine hormones in β-cells, where ghrelin expression cells are formed at the expense of β-cells in *Nkx2.2* knockout mice (Papizan *et al*. 2011).

On the other hand, Heller *et al*. (2005) found that neither *Pax4* nor the glucagon-cell specific transcription factor *Arx* (Collombat *et al*. 2003) impacts on the ghrelin[+]glucagon[−] cell fate. It should be mentioned that *Arx* null mutant mice had reduced the number of ghrelin[+]glucagon^{+} cells, while *Pax4* mutants had increased the number of such cells, the latter reminiscent of the findings by Prado *et al*. (2004). A recent report has confirmed the expansion of the population of ghrelin[+]glucagon^{+} cells in *Pax4* deficient mice (Wang *et al*. 2008). It was, however, not studied how *Pax4* deficiency affects the ghrelin[+]glucagon[−] cell population. The same report provided evidence for Arx expression in ghrelin^{+} cells. Whether these cells express glucagon or not is not reported. The diminished expression of Pax6 in *Nkx2.2* mutant mice is interesting in view of the expanded ghrelin[+]glucagon[−] cell population in these mice. Interestingly, therefore, Heller *et al*. (2005) showed that the ghrelin[+]glucagon[−] cell population is expanded in *Pax6* mutant mice. Furthermore, *Pax4* mutant mice, which have a normal ghrelin[+]glucagon[−] cell number, also display normal levels of Pax6 (Wang *et al*. 2004). The potential for Pax6 as a lineage divider between ghrelin[+]glucagon^{−} cells and ghrelin[+]glucagon^{+} cells is intriguing and needs further investigation. Notably, ghrelin[+]glucagon^{−} cells are devoid of Pax6, whereas ghrelin[+]glucagon^{+} cells express Pax6 (Fig. 5). Heller *et al*. (2005) also found that the ghrelin[+]glucagon^{−} cells, like the four major islet cell types, are dependent on the basic helix–loop–helix transcription factor Ngn3 (Gradwohl *et al*. 2000), because ghrelin-immunoreactive cells are absent in Ngn3 deficient mice during development. Thus it can be concluded that ghrelin cells at least share lineage with all the other four cell types at the early stages of islet cell development. Furthermore, like the majority of all islet cells, ghrelin cells also express Isl1 (Heller *et al*. 2005). It should be mentioned that, although mice with mutated *Foxa2* display 90% lower glucagon expression than normal mice (Lee *et al*. 2005), they have a normal number of ghrelin-immunoreactive cells. Whether these cells coexpress glucagon is not known. The transcription factor Brn4 has been shown to be important for the development of α-cells (Hussain *et al*. 1997). Although Brn4 is seen in subpopulations of the ghrelin cells (Jenny *et al*. 2004, Wierup & Sundler 2004*b*), it does not seem to be essential for ghrelin cell development as *Brn4* (*Pou3f4*) null mutant mice display both scattered ghrelin[+] glucagon^{−} cells and ghrelin[+]glucagon^{+} cells (Heller *et al*. 2004). Finally, mice with a loss of function mutation in microphthalmia transcription factor were recently found to exhibit lower ghrelin mRNA levels (Mazur *et al*. 2013).

Despite the fact that ghrelin cells are affected by mutations in transcription factors necessary for β-cell development, it still remains a fact that ghrelin cells in humans, rats and the ghrelin[+]glucagon^{−} cells in WT mice do not at any stage of development examined so far express insulin or the β-cell specific transcription factors PDX1 or Nkx6.1 (Wierup *et al*. 2002, 2004*b*, Prado *et al*. 2004, Wierup & Sundler 2004*b*, Heller *et al*. 2005, Walia *et al*. 2008). As ghrelin is to a varying extent instead coexpressed with glucagon, PP- and the α-cell specific transcription factor Brn4 (Wierup *et al*. 2004*b*, Heller *et al*. 2005, Walia *et al*. 2008), we rather believe that the ghrelin cells are embryogenetically more closely related to α-cells. Very recent work in lineage tracing ghrelin cells during development has demonstrated that they might give rise to a broad range of cell types including α, PP cells and rare β-cells. In addition, the authors observed that a few ghrelin cells are expressed at P0 but are absent during development, and these ghrelin-expressing cells represent a unique Ngn3 independent cell lineage with the ability to become both endocrine as well as duct and exocrine cells (Arnes *et al*. 2012).

Transmission electron microscopy is the gold standard technique for characterisation of cellular identity. Studies of the ultrastructure of mouse islet ghrelin-immunoreactive cells are, however, lacking. Information about potential differences in ultrastructural properties between ghrelin[+]glucagon^{+} cells and ghrelin[+]glucagon^{−} cells would add important pieces to the hitherto unresolved cell lineage puzzle.

Ghrelin cells in rats

Ghrelin cells are also present in rat pancreas during foetal and neonatal development (Wierup *et al*. 2004*b*, Fak *et al*. 2007, Walia *et al*. 2008). As in the human (Wierup *et al*. 2002, Wierup & Sundler 2005) and mouse (Prado *et al*. 2004, Heller *et al*. 2005) pancreas, the rat ghrelin cells display a developmental regulation (Wierup *et al*. 2004*b*). Thus, ghrelin cells are readily seen from midgestation to 1 month postnatally, with the density of the cells peaking
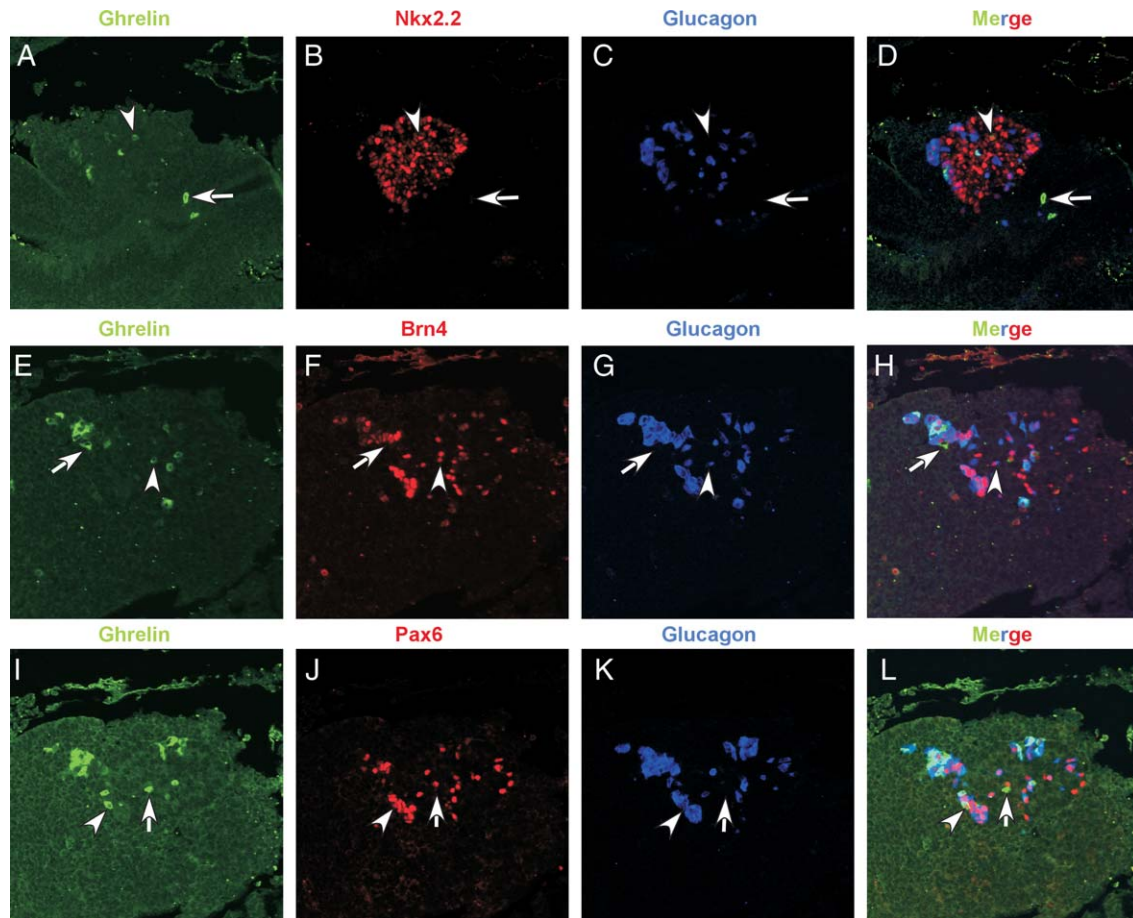
Figure 5

Expression of homeodomain transcription factors Pax6, Nkx2.2 and Brn4 in pancreatic ghrelin⁺ cells at E15.5. Single panels for the triple stainings are presented along with a merge for all transcription factors. (A, B, C and D) Pax6 is expressed in ghrelin⁺glucagon⁺ double immunoreactive α-cells but not in ghrelin⁺glucagon⁻ ε-cells (E, F, G and H) Variable expression of Nkx2.2 is observed independent of whether the cells coexpress glucagon or not. (I, J, K and L) Bnr4 immunoreactivity is observed only in ghrelin cells immunoreactive for glucagon. Arrows point to examples of ghrelin single positive cells and arrowheads designate examples of cell coexpressing ghrelin and the indicated marker. For antibody details see Heller *et al*. (2005). All images are 400× magnification.

around birth. Forty days after birth ghrelin cells are only occasionally seen, and in adult rat islets ghrelin cells are very rare. Similar to human ghrelin cells (Wierup *et al*. 2002, 2004*b*), the rat pancreatic ghrelin cells precede the gastric ghrelin cells during pre- and postnatal development. Furthermore, the decrease in pancreatic ghrelin cell density is matched in time by an increase in gastric ghrelin cell density, indicating an inverse relationship between the two cell populations (Wierup *et al*. 2004*b*). This concept was supported by Chanoine & Wong (2004) and Chanoine *et al*. (2006), who showed that the ghrelin gene expression and ghrelin peptide content in the foetal pancreas are markedly higher than that in the foetal stomach of rats. Thus the pancreas could be the main source of circulating ghrelin during late prenatal and early

postnatal development when the gastric ghrelin cell density is still low. This pattern is reminiscent of gastrin-producing G-cells in rats, which are present in the pancreatic islets well before birth, but in the stomach only postnatally (Larsson *et al*. 1976*a*). Furthermore, although the number of gastric ghrelin cells is maintained at lower level by delayed weaning of rat pups, the islet ghrelin cell population is unaffected (Fak *et al*. 2007). The importance of this potential difference between the two cell populations in response to different nutrients needs further investigation. Very little is known about the developmental regulation of ghrelin cells in the rat. The data obtained so far indicate that rat ghrelin cells do not at any stage of development express insulin, PDX1 or somatostatin. On the other hand, occasional cells

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(<1% of all ghrelin cells) express glucagon during a period around birth (Wierup *et al.* 2004*b*, Walia *et al.* 2008). Occasionally ghrelin cells also coexpress PP (Wierup *et al.* 2004*b*) (such cells are more common than those co-expressing ghrelin and glucagon) during a period around birth. This is indicating a developmental relationship between ghrelin cells, α-cells and PP-cells. In addition, a subpopulation of the ghrelin cells harbour Brn4, a proposed α-cell specific transcription factor (Hussain *et al.* 1997), further emphasising a lineage relationship between the α-cells and the ghrelin cells. All ghrelin cells are devoid of glucagon and PP at later stages of development than postnatal day 5. Interestingly, the ghrelin cells are consistently devoid of CART peptide immunoreactivity (Wierup *et al.* 2004*a*, Wierup & Sundler 2006). In this respect, the ghrelin cell differs from α-, β-, δ- and PP-cells, which all to a varying extent express CART during foetal and neonatal development. The importance of this observation needs further investigation. It is, however, intriguing in view of the fact that all the CART-containing cell types remain to adulthood, while the ghrelin cells virtually disappear within weeks of birth. The observation that some ghrelin cells express cytokeratin 20, a marker of duct cells and islet precursor cells, indicates that the ghrelin cells originate from ductal epithelium, an acknowledged origin for islet cells in general (Bouwens *et al.* 1994). Some ghrelin cells also stain positive for proliferation markers, indicating that the ghrelin cells proliferate at a mature state. Hence, rat ghrelin cells seem to be generated both by duct cell differentiation and by cell replication. Further, Wang *et al.* reported that a great proportion of the ghrelin$^+$ glucagon$^+$ cells in mice also expressed IAPP to a high extent; this is different from the rat where IAPP expression in ghrelin cells is a rare phenomenon (Wierup *et al.* 2004*b*).

Ghrelin cells in islets of other species

The phylogeny of the ghrelin cells has not been thoroughly examined so far. Important pieces to the puzzle have, however, been added recently. Vedtofte *et al.* (2007) reported that the islets of the desert gerbil (*Psammomys obesus*) also harbour ghrelin cells during development. The desert gerbil is closely related to the mouse and the data are largely in line with previous studies in mice (Prado *et al.* 2004, Heller *et al.* 2005). The same group reported, in accordance with adult mice and rats, that the islets of adult African ice rat (*Otomys slogetti robertsi*) are lacking ghrelin cells (Gustavsen *et al.* 2008). We have been unable to detect ghrelin cells in the islets

of adult pigs, cats, dogs and guinea-pigs (Wierup N & Sundler F, 2004, unpublished observations). Very recently ghrelin-immunoreactive cells have been found in the developing pancreas, but not in the adult pancreas, of the tammar wallaby (*Macropus eugenii*) (Menzies *et al.* 2008). We found ghrelin-immunoreactive cells in the pancreas of the Xenopous frog, while ghrelin cells are few in the pancreas of chickens (Wierup N & Sundler F, 2004, unpublished observations). Importantly, ghrelin mRNA is expressed in pancreas of cat fish (Kaiya *et al.* 2005) and ghrelin cells have been found in the zebrafish (Pauls *et al.* 2007). Thus, although it is clear that the ghrelin cell is highly evolutionarily conserved, further studies are needed to elucidate the phylogeny for the ghrelin cell.

Ghrelin expression in other islet cell types?

A few reports have shown ghrelin immunoreactivity in other islet cells than the ghrelin cells of adult humans or rodents. Thus, Volante *et al.* (2002) showed ghrelin immunoreactivity in human β-cells, while Date *et al.* (2002) showed ghrelin immunoreactivity in human α-cells. Date *et al.* (2002) also showed ghrelin immunoreactivity in the α-cells of rats. We could not confirm any of these observations. Importantly, our ISH for ghrelin mRNA do not show any expression of ghrelin mRNA in α- or β-cells (Wierup *et al.* 2002, 2004*b*). The reason for this discrepancy still remains to be elucidated. Although some reports show undetectable (Chanoine & Wong 2004, Wierup *et al.* 2004*b*, Walia *et al.* 2008) and others only very low (Dornonville de la Cour *et al.* 2001, Chanoine *et al.* 2006) levels of ghrelin in adult rat pancreas, it cannot be excluded that cell types other than ghrelin cells express ghrelin at very low levels, e.g. below the detection level of immuno-cytochemistry. Another possible explanation for detect-able levels of ghrelin in the extracts of adult rat pancreas, which is devoid of islet ghrelin cells, could be the presence of ghrelin cells in association with pancreatic ducts (Wierup N & Sundler F, 2004, unpublished observations).

Ghrelin and islet hormone secretion

A body of evidence shows that ghrelin is an insulinostatic agent, and that GHS-R is expressed in β-cells (Wierup *et al.* 2004*b*, Dezaki *et al.* 2008). Thus, ghrelin inhibits insulin release in humans, rats, mice, as well as from clonal β-cells (Broglio *et al.* 2001, 2003, Egido *et al.* 2002, Colombo *et al.* 2003, Reimer *et al.* 2003, Wierup *et al.* 2004*b*). These data indicate that ghrelin released from ghrelin cells within the islets could act as a paracrine inhibitor of insulin secretion.

Although reports showing that ghrelin inhibits insulin secretion predominate, it should be mentioned that a few reports have shown stimulatory effects (Date *et al.* 2002, Lee *et al.* 2002, van der Lely *et al.* 2004, Salehi *et al.* 2004, Cummings *et al.* 2005). The divergent data may reflect species differences or differences in methodology, such as ghrelin and glucose concentrations and time of observation, *in vitro* vs *in vivo* experiments, etc. Illustrating this complexity, Salehi *et al.* demonstrated a dose-dependent, biphasic action of ghrelin on GSIS from isolated mouse islets. Thus, in low concentrations ghrelin suppressed insulin secretion while in high concentrations ghrelin increased insulin secretion (Salehi *et al.* 2004). Another fact that has to be accounted for is the constitutive, i.e. ligand-independent receptor activity of GHS-R (Holst *et al.* 2003). This is an intriguing finding that needs further investigation in the context of the physiological regulation of insulin release caused by ghrelin. From a physiological point of view, it seems reasonable that the inhibitory effect is the most likely. The fact that ghrelin is increased by fasting (a state with a low need for insulin) and suppressed post-prandially (Tschop *et al.* 2000, Dornonville de la Cour *et al.* 2001, Toshinai *et al.* 2001) (a state of high insulin demand), together with the documented reciprocal relationship between ghrelin and insulin in plasma (Korbonits *et al.* 2004, Dezaki *et al.* 2008), do not favour a role for ghrelin as a stimulator of insulin secretion. It is tempting to hypothesise that the role for ghrelin is to prevent hypoglycaemia by suppressing inappropriate insulin secretion between meals. The effect of ghrelin on islet hormones other than insulin is less studied. One report showed inhibitory effects of ghrelin on somatostatin secretion, while no effects were seen on glucagon secretion from perfused rat pancreas (Egido *et al.* 2002). On the other hand, a stimulatory effect of ghrelin on glucagon secretion was observed on isolated islets but not in intact mice (Salehi *et al.* 2004). Another report showed a stimulatory effect of ghrelin on both somatostatin and PP-secretion in humans (Arosio *et al.* 2003). Taken together, ghrelin is an insulinostatic hormone, and a role for ghrelin regulation of glucagon, somatostatin and PP seems likely, although the exact function needs further investigation.

Clinical importance of islet ghrelin

The importance of the finding of a new human cell type, secreting a novel hormone at a novel site is hard to overestimate. Ghrelin cells are present in most islets in the human adult pancreas. On average three to five ghrelin cells are found in each islet. The human pancreas harbours approximately one million islets. Hence between three and five million ghrelin cells are present in each adult human pancreas. In humans 35–45% of the circulating ghrelin remains when the stomach, which is regarded as the dominating site of ghrelin production, is surgically removed (Ariyasu *et al.* 2001, Popovic *et al.* 2005). It is highly likely that the islet ghrelin cells together substantially contribute to a substantial part of the remaining circulating ghrelin.

A body of evidence has shown that ghrelin inhibits insulin secretion (Dezaki *et al.* 2008). Therefore blocking of ghrelin signalling is a novel strategy for the treatment for type 2 diabetes. Interestingly, therefore, oral administration of a GHS-R antagonist improved glucose homeostasis in rodents (Esler *et al.* 2007). In combination with the appetite stimulatory effects of ghrelin, the potential for such agents to treat also obesity is intriguing and needs further investigation (Dezaki *et al.* 2008).

Ghrelin cells appear as a separate cell type distinct from α-, β-, δ- and PP-cells when culturing human embryonic stem cells (D'Amour *et al.* 2006, Kroon *et al.* 2008). Hence, developmental studies and studies aimed at engineering new β-cells from human embryonic stem cells for transplantation to cure type 1 diabetes should benefit from increased knowledge about the different terminal fates of the islets cell types.

Several reports of pancreatic tumors with ghrelin expression (Rindi *et al.* 2002*b*, Volante *et al.* 2002, Leontiou *et al.* 2007) suggest that ghrelin could be a potential tumour marker to search for in the blood samples, although one report was unable to detect differences in plasma ghrelin between patients with ghrelin-expressing tumours and controls (Corbetta *et al.* 2003), it has been suggested that patients with malignant gastric endocrine tumours should be investigated for ghrelin production (Tsolakis *et al.* 2004). The potential for ghrelin as a novel tool for detecting previously silent tumours is intriguing and needs further investigation.

Concluding remarks

The ghrelin cell is a novel islet cell type in humans, rats, mice and fish. The ghrelin cell is thus highly conserved during evolution. Man is the only species so far known to have substantial numbers of ghrelin cells in the islets as adults. This makes studies of ghrelin cells and their role in the pancreas of adults difficult, because there are no known animal models with ghrelin cells in adults. The information gained on developing animals must be

interpreted with caution as they poorly reflect the situation in man. Important information about the regulation of ghrelin cell development has been presented recently, but the transcriptional events that determine ghrelin cell fate need further investigation. Ghrelin secreted from ghrelin cells within the islets could act as a paracrine inhibitor of insulin secretion, and blockade of ghrelin signalling could be a therapeutic strategy for type 2 diabetes.

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