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Genetic determinants of pancreatic ε-cell development

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Abstract

Recently, the expression of the peptide hormone ghrelin was detected in α-cells of the islets of Langerhans as well as in ε-cells, a newly discovered endocrine cell type, but it remains unclear how the latter is related in lineage to the four classical islet cell types, α-, β-, δ-, and PP-cells. Here, we provide further evidence that ghrelin is predominantly produced in the α-cells of mouse islets but also in single hormone ghrelin-secreting ε-cells. We additionally demonstrate that pancreatic ε-cells derive from *Neurogenin3*-expressing precursor cells and their genesis depends on Neurogenin3 activity. Furthermore, our data indicate that the number of ghrelin-producing cells is differentially regulated during pancreas morphogenesis by the homeodomain-containing transcription factors Arx, Pax4, and Pax6. *Arx* mutants lack ghrelin$^+$ glucagon$^+$ α-cells whereas *Pax4* mutants develop an excess of these cells. Importantly, the ghrelin$^+$ glucagon$^-$ ε-cell population is not affected following *Arx* or *Pax4* disruption. In contrast, the loss of *Pax6* provokes an unexpected increase of the ghrelin$^+$ glucagon$^-$ ε-cell number which is not due to increased proliferation. Thus, we demonstrate that the development of ghrelin-producing cells is differentially dependent on Neurogenin3 in different domains of the gastrointestinal tract and that, in the endocrine pancreas, ε-cell genesis does not require Arx or Pax4 activities but is antagonized by Pax6.

Keywords: Ghrelin; Pancreas; Neurogenin3; Arx; Pax4; Pax6; Development; Endocrine; Glucagon

Introduction

Pancreatic endocrine cells are located in clusters known as islets of Langerhans. These islets, classically described as micro-organs, possess four different endocrine subtypes: glucagon-producing α-cells, insulin-expressing β-cells, somatostatin-secreting δ-cells, and pancreatic polypeptide-producing PP-cells. Endocrine cells appear in the developing mouse pancreas at embryonic day (E) 9.5 (Upchurch et al., 1994). Insulin immunoreactive cells differentiate 1 day later, at E10.5, and these frequently co-express glucagon (Teitelman et al., 1993), but lack the expression of a critical transcription factor, Pdx1, normally present in mature β-cells at birth (Jensen et al., 1996, 2000; Ohlsson et al., 1993). Around E13.5, the so-called "secondary transition", leads to a peak of α- and β-cell formation (Pictet and Rutter, 1972) concurrently with the generation of Pdx1$^+$ β-cells. Finally, δ- and PP-cells develop around E15 and E18, respectively (Pictet and Rutter, 1972). Recent reports suggest that ghrelin, a growth hormone-releasing and orexigenic peptide originally characterized in enteroendocrine cells of the G-I tract, is also secreted in the pancreas by a new islet cell type, the ε-cell (Prado et al., 2004; Wierup et al., 2004).

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Numerous transcription factors have been characterized for their involvement in endocrine pancreas development. Among those, the bHLH protein Neurogenin3 (Ngn3) represents a common marker of the enteroendocrine cell lineage required for the formation of all intestinal and pancreatic endocrine cells (Gradwohl et al., 2000; Gu et al., 2002; Herrera, 2002; Jenny et al., 2002). Notably, in gastric tissues, despite its expression in the precursors of the glucagon-, somatostatin-, ghrelin-, histamine-, and serotonin-producing cells, *Ngn3* is required uniquely for the formation of glucagon-, gastrin-, and somatostatin-producing cells (Jenny et al., 2002; Lee et al., 2002). In contrast, Ngn3 function is absolutely necessary for the genesis of intestinal and pancreatic endocrine tissues (Gradwohl et al., 2000; Jenny et al., 2002). Following Ngn3 activation, several downstream factors were characterized for their key role in endocrine subtype specification in the pancreas, intestine, and/or stomach, including the homeodomain-containing proteins Pax4, Pax6, Arx, Nkx2.2, and Nkx6.1 (Collombat et al., 2003; Larsson et al., 1998; Sander et al., 1997, 2000; Sosa-Pineda et al., 1997; St-Onge et al., 1997; Sussel et al., 1998). Pax4 promotes the genesis of virtually all duodenal hormone-secreting cells as well as serotonin$^+$ and somatostatin$^+$ cells of the distal stomach, whereas Pax6 acts in the development of duodenal GIP$^+$ (gastric inhibitory polypeptide) cells, and of gastrin$^+$ and somatostatin$^+$ cells of the distal stomach (Larsson et al., 1998). Additionally, both Pax4 and Pax6 regulate pancreatic endocrine development: *Pax4* mutant mice lack β- and δ-cells but display an increased number of α-cells (Sosa-Pineda et al., 1997) whereas *Pax6*-depleted mice exhibit reduced numbers of all four classical islet cell types and present a diminished transcription of the corresponding hormone genes (Heller et al., 2004; Sander et al., 1997; St-Onge et al., 1997). Similarly, the homeodomain-containing protein Arx was recently demonstrated to act downstream of Ngn3 and to be required for α-cell fate specification (Collombat et al., 2003). *Arx*-deficient mice lack α-cells at birth and display a concomitant increase in β- and δ-cell numbers. Interestingly, *Arx* and *Pax4* mutant animals exhibit opposite phenotypes in relation to α-, β-, and δ-cell development possibly due to mutual cross-repressive interactions between these genes (Collombat et al., 2003). Using a loss-of-function approach, the role of additional homeodomain-containing factors in pancreatic endocrine specification has been unraveled. Notably, mice deficient for *Nkx2.2* lack β-cells and possess fewer α- and PP-cells, while δ-cells are not affected (Sussel et al., 1998). The islets of *Nkx2.2*-deficient mice comprise a large number of cells that do not produce any of the classical islet hormones. Further studies indicate that the distantly related factor Nkx6.1, required for normal β-cell development, acts downstream of Nkx2.2 in the endocrine differentiation processes (Sander et al., 2000).

Little is known regarding the transcription factors that govern formation of ghrelin-producing cells in the islets. In human pancreas, ghrelin has been noted for its co-detection with either glucagon (Date et al., 2002) or insulin (Volante et al., 2002), whereas other investigators were unable to detect ghrelin expression within any of the four classical islet cell types (Wierup et al., 2002), indicating that a fifth islet cell type might exist. Sussel and co-workers demonstrated that in mice, ghrelin is produced by α-cells as well as by ε-cells (Prado et al., 2004). Moreover, this study provides evidence that in animals lacking *Nkx2.2* (Sussel et al., 1998) and *Pax4* (Sosa-Pineda et al., 1997), ε-cells are formed in place of β-cells.

In the present report, we characterized pancreatic ghrelin-producing cells throughout pancreas morphogenesis in relation to other endocrine hormones and lineage-associated transcription factors. Specifically, the function of the *Ngn3*, *Arx*, *Pax4*, and *Pax6* genes in the ghrelin-producing cell specification processes was genetically examined. A short-term lineage tracing demonstrates that ε-cells derive from *Ngn3*-expressing precursors whereas the analysis of *Ngn3*-deficient mice indicates that the development of ε-cells depends on Ngn3 activity. Finally, our study reveals that the *Arx* and *Pax4* genes do not regulate ε-cell numbers while Pax6 antagonize the formation of ε-cells.

Materials and methods

Tissue preparation

Mouse pancreata were isolated from embryonic days 10, 12, 14, 18, P5, P10, and 3-, 6- and 16-week-old animals and immediately fixed in cold 4% PFA overnight. Tissue was embedded in paraffin and 4-μm sections were cut onto glass slides.

Ghrelin monoclonal antibody production

A synthetic peptide carrying an octanoyl acid on ser 3 (GSSFLSPEHQRVQQRKES) corresponding to 18 residues located in the N-terminal domain of the human ghrelin peptide was used as antigen to raise the monoclonal antibody 1D7. An additional C-terminal cysteine was added for the coupling to ovalbumin. The method used for antibody production was as previously described (Moog-Lutz et al., 1997). The antibody recognizes both octanoylated and non-octanoylated forms of ghrelin on Western blot and by competition studies it displayed a higher affinity for the non-modified form (data not shown).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Collombat et al., 2003; Gradwohl et al., 2000; Jensen et al., 1996). Briefly, paraffin sections were dewaxed in xylene and rehydrated through descending ethanol series. Antigen retrieval was accomplished through microwave treatment (600 W for 4 min followed by 15 min at 250 W in 10 mM Tris–EGTA buffer, pH 9.0) followed by three

washes in PBS. The tyramide amplification system was used: briefly, the sections were blocked for non-specific binding of the antisera during 30 min at room temperature using TNB buffer, followed by 10% donkey non-immune serum for 10 min. Primary antisera (see Table for dilutions) were added overnight at room temperature followed by three washes in PBS. To test the specificity of the ghrelin antiserum, tissues were preincubated with 5 μg of ghrelin peptide or an irrelevant peptide derived from Notch-1. An anti-rabbit biotinylated secondary antibody (Zymed) was next added for 30 min, followed by three washes in PBS. The sections were then incubated with a streptavidin-peroxidase antiserum (Zymed) for 15 min and rinsed three times in PBS. Tyramide-Cy3 (Perkin and Elmer) was diluted 1:100 and added for exactly 10 min, and followed by three washes in PBS. Finally, the secondary anti-Cy-2, -Cy-3, or -Cy-5 antiserum (Jackson ImmunoResearch) were incubated with the tissues for 30 min. Following three washes in PBS, the slides were mounted with Fluorescent mounting media (Kirkegaard and Perry). Images were collected with an Olympus BX51 microscope, captured using a Hamamatsu C5810 cooled CCD camera and Image Pro Plus 4.5 software interfaced with a PC computer or by confocal microscopy using a Zeiss LSM510 Meta.

Mouse strains

Ngn3, *Arx*, and *Pax4* mutant mice were bred and maintained as previously described (Collombat et al., 2003; Gradwohl et al., 2000; Sosa-Pineda et al., 1997). Pancreata from day E15, E18, and post-natal day 1 mice were fixed in 4% PFA at 4°C for 2 h, rinsed and equilibrated in 30% sucrose in PBS at 4°C for 12 h, mounted in Tissue-Tek (Sakura), frozen on dry ice, cut, and processed for immunocytochemistry. Small eye (*Pax6* mutant) mice (Hill et al., 1991) were obtained from Dr. Veronica van Heyningen (Edinburgh, Scotland) and were bred at M&B A/S, Ry, Denmark.

Antigen	Species	Dilution	Source
β-galactosidase	Mouse	1:7500	Promega
Brn4	Rabbit	1:2000	M. Geoff Rosenfeld, UCSD, San Diego, CA
Pdx1 253	Rabbit	1:5000	Joel Habener, MGH, Boston, MA
Pax6	Rabbit	1:2000	Babco, Richmond, CA
Nkx2.2	Rabbit	1:2000	Thomas Jessell, Columbia Univ. New York
Nkx6.1	Rabbit	1:5000	Jensen et al., 1996
ISL-1	Rabbit	1:1000	Helena Edlund, Umeå, Sweden
Insulin	Mouse	1:75	Novo Nordisk A/S, Denmark
Insulin	Guinea pig	1:200	DAKO, Glostrup, Denmark
Glucagon	Mouse	1:50	Novo Nordisk A/S, Denmark
PP	Rabbit	1:500	DAKO, Glostrup, Denmark
Somatostatin	Mouse	1:10	Novo Nordisk A/S, Denmark
Ghrelin	Goat	1:500	Santa Cruz Biotechnology, CA
Ghrelin	Mouse	1:1500	Catherine Tomasetto, IGBMC, Strasbourg
Ghrelin1882	Rabbit	1:1500	Tomasetto et al., 2000

Cell quantitation and data analysis

Double immunostaining for ghrelin and glucagon was performed on cryosections. The number of single ghrelin-positive cells and ghrelin-glucagon-double-positive cells was counted and expressed as a percentage of the total ghrelin-positive cells on the sections at different embryonic stages. The quantitation of the number of ghrelin cells in the *Pax6* mutant mice was performed as follows. The total pancreatic area and total number of ghrelin cells were counted on at least 6 sections from three different animals. The data are expressed as the number of ghrelin cells/mm^2 pancreas tissue. Ghrelin cells in the *Arx* and *Pax4* mice were quantitated as previously described (Collombat et al., 2003). Results are expressed as mean ± SEM. Mean changes were compared using the Students *t* test. $P < 0.05$ was considered to be statistically significant.

Results

Ghrelin is expressed in α-cells but also as a unique hormone-producing cell type in the mouse islets

To determine whether the ghrelin hormone is expressed in one or more of the four classical endocrine cell subtypes in the mouse pancreas, we performed double immunohistochemical stainings on E18.5 pancreatic sections using an antiserum raised against ghrelin, combined with antisera against glucagon, insulin, somatostatin, or pancreatic polypeptide, labeling α-, β-, δ-, or PP-cells, respectively. Consistent with previous findings (Prado et al., 2004), our results demonstrate a clear co-localization of ghrelin with glucagon, but also uncover the presence of cells uniquely immunoreactive for ghrelin (Fig. 1A). The absence of co-localization of ghrelin with insulin (Fig. 1B), somatostatin (Fig. 1C), or PP (Fig. 1D) indicates that these ghrelin$^+$ glucagon$^-$ cells represent a distinct pancreatic endocrine cell type. Identical results were obtained using three different ghrelin antisera (data not shown). To further demonstrate the presence of ghrelin$^+$ cells lacking the four classical islet hormones, a dual labeling was performed using a ghrelin antiserum in combination with glucagon, insulin, somatostatin, and PP antisera. Consistent with our previous results, several ghrelin immunoreactive cells negative for all four other hormones were observed (Fig. 1E). Double immunocytochemistry for ghrelin and gastrin or PYY showed that these two did not co-localize at E15 (data not shown). At 2 weeks of age and later, ghrelin immunoreactivity was not detected in mouse pancreas (data not shown). Hence, our data confirm and extend the findings of Prado et al. that in the mouse endocrine pancreas, ghrelin peptide expression is detectable in α-cells but also in a single-hormone ghrelin-secreting cell subtype, which we refer to as ε-cells.

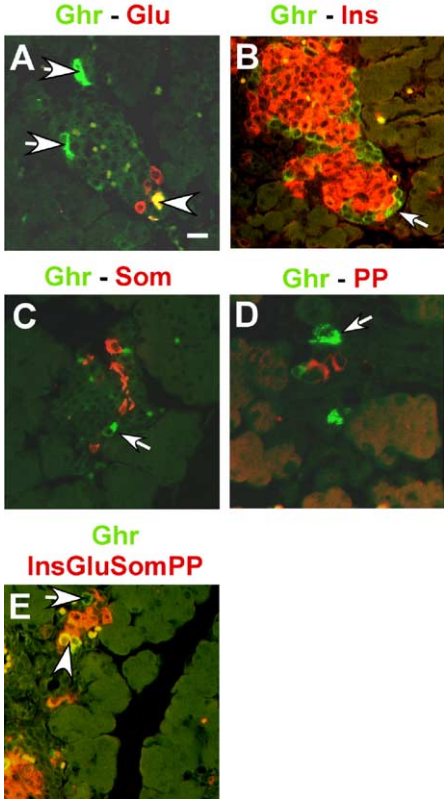

Fig. 1. Analysis of ghrelin immunoreactivity in E18.5 mouse pancreas. (A) The glucagon and ghrelin hormones are co-detected in a subset of islet cells. (B–E) Ghrelin is not co-secreted with the insulin (B), somatostatin (C) or pancreatic polypeptide (D) hormones. (E) Ghrelin$^+$ cells devoid of any of the classical islet hormones are clearly detectable. Arrows point to examples of ghrelin single positive cells and arrowheads designate examples of cell co-expressing ghrelin and the indicated marker. Scale bar is 30 μm.

ε-cells develop in an Ngn3-dependent fashion and derive from Ngn3-expressing precursors

To decipher the molecular mechanisms regulating the genesis of ghrelin- and glucagon-secreting cells in embryonic and early post-natal pancreas, we examined both cell types in wild-type and *Ngn3*-deficient mice (Gradwohl et al., 2000). In control animals, our results confirm that throughout normal embryonic and early post-natal development (from E10.5 to P1), ghrelin is often co-detected with glucagon, but also clearly establish the presence of ghrelin$^+$ glucagon$^-$ ε-cells (Fig. 2; Table 1). Importantly, the analysis of *Ngn3*-deficient mice reveals that ghrelin expression in the pancreas requires Ngn3 function at all stages examined (Fig. 2), indicating that ghrelin$^+$ ε-cells in the pancreas share a common lineage with the four classical endocrine cells. To shed light upon the relationship between *Ngn3*-expressing progenitors and ε-cells, we performed short-term lineage tracing experiments using mice expressing the GFP marker under the control of *Ngn3* regulatory sequences created in the same way as the Ngn-3-YFP mice (Mellitzer et al., 2004). Since mature hormone-expressing cells do not express Ngn3 and Ngn3-positive cells are not observed to express hormones (Gradwohl et al., 2000; Jensen et al., 2000), detection of co-localized GFP and ghrelin will indicate that ghrelin$^+$ cells are derived from cells previously expressing *ngn3* but with GFP still being present due to the longer half-life of GFP compared to NGN3. Triple staining

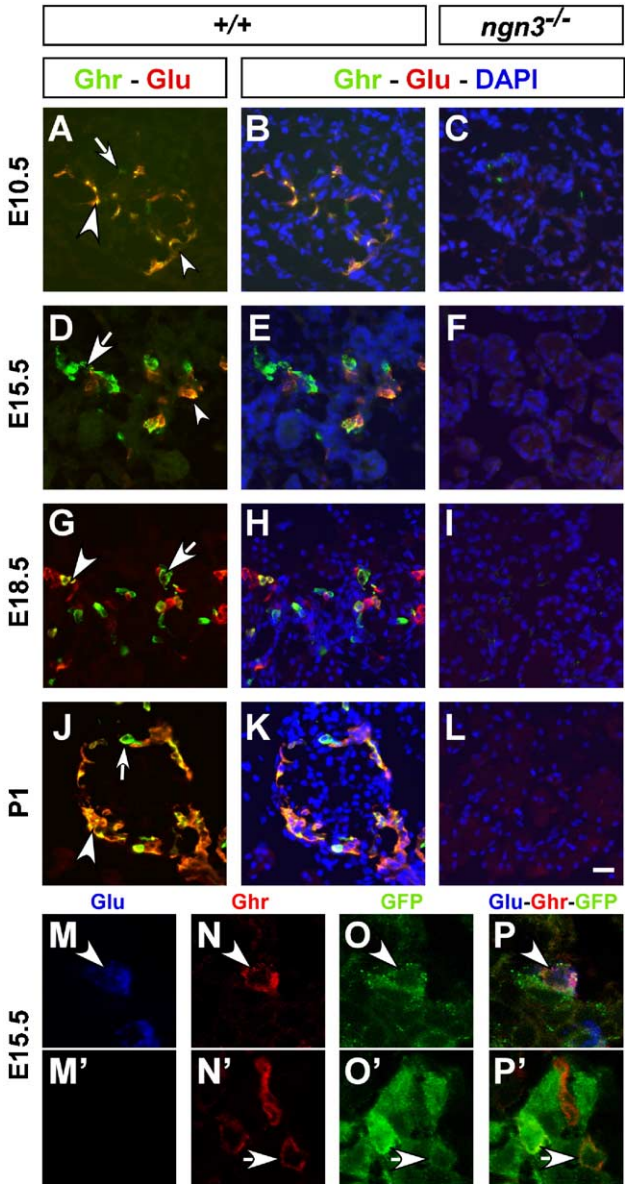

Fig. 2. *Ngn3* is required for pancreatic ghrelin expression in mice. (A–C) At E10.5, most ghrelin-secreting cells produce glucagon in control animals (A, B) whereas these cells are lacking in *Ngn3* mutant mice (C). (D–F) At E15.5, a majority of ghrelin immunoreactive cells do not express glucagon, while most glucagon-expressing cells are immunoreactive for ghrelin in wild-type mice (D–E); both cell types are deficient in *Ngn3* mutant mice (F). (G–L) At E18.5 (G–I) and P1 (J–L), three populations of cells in wild-type mice are detectable: cells uniquely expressing either ghrelin or glucagon and cells producing both hormones (G, H, J, K). None of these populations are found in *Ngn3* mutants (I, L). A lineage analysis of ghrelin and glucagon-producing cells indicates that both populations derive from cells expressing the GFP marker under the control of the *Ngn3* promoter (M–P). Arrowhead points to an example of a GFP/ghrelin/glucagon triple positive α-cell and the arrow (M′–P′) designates an example of an ε-cell co-expressing GFP and ghrelin but not glucagon. Scale bar is 30 μm.

Table 1
Relative proportions of ghrelin-producing cells throughout pancreas development

	% Single Ghr$^+$ cells/total Ghr$^+$ cells	% Ghr$^+$ Glu$^+$ cells/total Ghr$^+$ cells
E10.5 (n = 38)	5	95
E15.5 (n = 211)	30	70
E18.5 (n = 260)	25	75
P1 (n = 114)	8	92

Numbers are provided as percent of single-hormone ghrelin$^+$ cells and double-hormone ghrelin$^+$ glucagon$^+$ cells in relation to the total population of ghrelin$^+$ immunoreactive cells.

for ghrelin, glucagon, and GFP at E15 noticeably demonstrates the existence of both GFP$^+$ ghrelin$^-$ glucagon$^+$ and GFP$^+$ ghrelin$^+$ glucagon$^+$ α-cells (Figs. 2M–P), as well as GFP$^+$ ghrelin$^+$ glucagon$^-$ ε-cells (Figs. 2M′–P′), providing further evidence that ε-cells, like the other four islet cell types, arise from *Ngn3*-expressing precursors. Not all ghrelin$^+$ cells contained GFP consistent with the notion that only short-term lineage tracing is possible using this system.

Arx and Pax4 differentially regulate ghrelin expression

The *Arx* and *Pax4* genes have recently been shown to play opposite roles in specifying α- versus β- and δ-cell fates during pancreas ontogeny (Collombat et al., 2003). In order to determine whether the *Arx* deficiency, resulting in the lack of mature α-cells, would also promote the loss of ghrelin expression, *Arx* loss-of-function and control mice were examined for the presence of ghrelin- and glucagon-producing cells. Our findings demonstrate that the ghrelin$^+$ glucagon$^+$ α-cell population is drastically reduced in *Arx* mutants (Figs. 3A, C, E, G, I and Table 2). However, ghrelin$^+$ glucagon$^-$ ε-cells are still present in Arx mutant pancreas (Figs. 3E, I) and the quantitative data indicate that the number of these cells is not statistically different from the amount of ghrelin$^+$ glucagon$^-$ ε-cells observed in control mice, suggesting that ε-cells are not affected by the *Arx* deletion (Table 2). To verify this assumption, *Pax4* mutant pancreas, characterized by an increase of the *Arx* transcript content associated with a loss of β- and δ-cells concomitantly with an augmentation of the α-cell population, were also assayed (Collombat et al., 2003; Sosa-Pineda et al., 1997). A striking increase of the ghrelin$^+$ glucagon$^+$ α-cell population was detected as compared to controls while the number of ghrelin$^+$ glucagon$^-$ ε-cells was again found unchanged (Figs. 3B, D, F, H, J, and Table 2). Hence, these data are consistent with the notion that Arx and Pax4 are both required for the proper α-cell fate specification but not for the ε-cell destiny.

Pax6-deficient mice develop an excess of ghrelin-producing ε-cells

Nkx2.2-deficient mice were recently reported to have increased numbers of ghrelin$^+$ cells (Prado et al., 2004). The reduced *Pax6* expression levels exhibited by these mutant

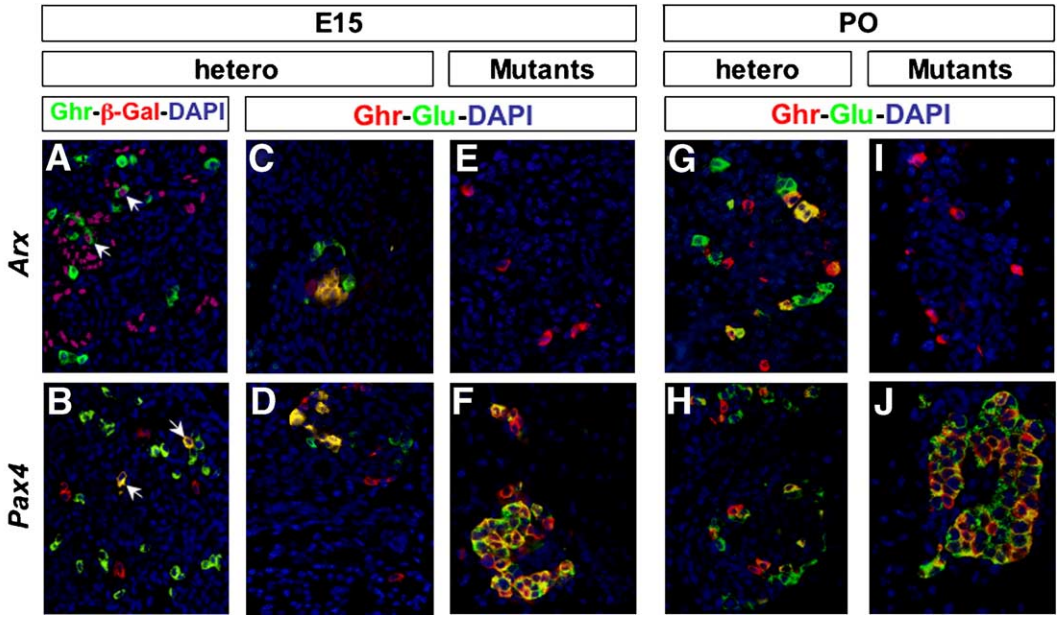

Fig. 3. Arx and Pax4 exhibit differential roles in ghrelin-producing cell generation. Examination of E15 (A–F) and P0 (G–J) sections stained with anti-ghrelin (A–J) and anti-β-galactosidase antisera (A, B) and counterstained with DAPI (A–J) reveals that the ghrelin peptide is co-expressed with the *β-galactosidase* reporter gene in the *Pax4* or *Arx* gene locus present both in *Arx* and *Pax4* heterozygous mice (arrows in panels A and B, respectively). In the case of Arx, these data were further confirmed using an anti-Arx antibody (data not shown). *Arx* deficiency is characterized by an early-onset loss of ghrelin$^+$ glucagon$^+$ cells (E and I), although ghrelin$^+$ glucagon$^-$ cells remain. The loss of a *Pax4* functional allele produces the opposite phenotype leading to an early increase of the ghrelin$^+$ glucagon$^+$ cell population without affecting the ghrelin$^+$ glucagon$^-$ population (F and J). Each picture is representative of 3–6 animals from different litters. Scale bar is 30 μm.

Table 2
Quantification of the alterations in pancreatic ghrelin-producing cell numbers following *Arx* or *Pax4* deletions

	Ghrelin$^+$ glucagon$^-$ ε-cells	Ghrelin$^+$ glucagon$^+$ α-cells
E15.5 WT	15 ± 7	35 ± 12
E15.5 Arx$^{-/-}$	9 ± 5	0
E15.5 Pax4$^{-/-}$	23 ± 12	89 ± 17 (+254%)
P0 WT	26 ± 9	153 ± 19
P0 Arx$^{-/-}$	21 ± 8	0
P0 Pax4$^{-/-}$	35 ± 11	359 ± 26 (+235%)

Ghrelin$^+$ glucagon$^-$ ε-cells and ghrelin$^+$ glucagon$^+$ α-cells were counted on immunostained sections of E15 and P0 serially cut pancreas estimated to be of the same size. Data are shown as the mean number ± standard error of the mean (SEM) of hormone-positive cells per section (every fifth section) from at least three independent pancreata. As early as E15.5, α-cells lack in *Arx* mutant pancreas, whereas twice more are detectable following *Pax4* disruption, as compared to controls. Interestingly, the ε-cell population is not altered following *Arx* or *Pax4* deletion.

mice (Wang et al., 2004) led us to speculate that Pax6 might represent a target of Nkx2.2 inhibiting ghrelin cell genesis. We therefore examined the ghrelin-producing cell population in *Pax6*$^{sey/sey}$ mice which have a spontaneous mutation in the *Pax6* gene (Hill et al., 1991). In accordance with our assumption, E19 *Pax6*$^{sey/sey}$ mutant embryos displayed an excess of ghrelin-expressing cells (Fig. 4) whereas the glucagon-producing α-cell population was drastically reduced as previously reported (Heller et al., 2004; Sander et al., 1997; St-Onge et al., 1997). A quantification of the number of ghrelin$^+$ cells in sections of wild-type and *Pax6* mutant pancreas revealed that wild-type mice possessed 8 ± 6 ghrelin$^+$ cells/mm^2 of pancreas tissue, while *Pax6*$^{sey/sey}$ mutants display 45 ± 13 ghrelin$^+$ cells/mm^2 pancreas tissue ($P < 0.01$, $n = 3$), representing a more than 5-fold increase in the total ghrelin$^+$ cell number. It is important to note that most of the ghrelin$^+$ cells found in E19 *Pax6*$^{sey/sey}$ pancreas do not express glucagon or any of the other three classical islet hormones, suggesting that they correspond to ε-cells (Fig. 4, and data not shown), nor do they express the β-cell markers Pdx1 and Nkx6.1 (Fig. 4). In order to address if the increased number of ghrelin$^+$ glucagon$^-$ cells seen in the Pax6 mutant were due to increased proliferation of these cells, we analyzed the expression of Ki67, a marker of proliferating cells in wild-type and mutant mice. We find that in both wild-type and mutant mice, the ε-cell population is post-mitotic (Figs. 4D, H). Taken together, these data indicate that the lack of a functional *Pax6* allele promotes the formation of ε-cells.

Discussion

Ghrelin, a peptide hormone originally characterized in gastric tissues (Kojima et al., 1999; Tomasetto et al., 2000), is also present in the mouse pancreatic islets. Here, we provide definitive evidence that the ghrelin hormone is synthesized by the glucagon-producing cell population. However, our study also confirmed the presence of single-hormone ghrelin-producing cells devoid of any of the four classical islet hormones, termed ε-cells (Prado et al., 2004). Intriguingly, these findings contrast with those reported by Volante et al. (2002) displaying a co-localization of ghrelin with insulin in human pancreas. The reason for this discrepancy remains unclear but could originate in the different staining procedures. Our results demonstrating the

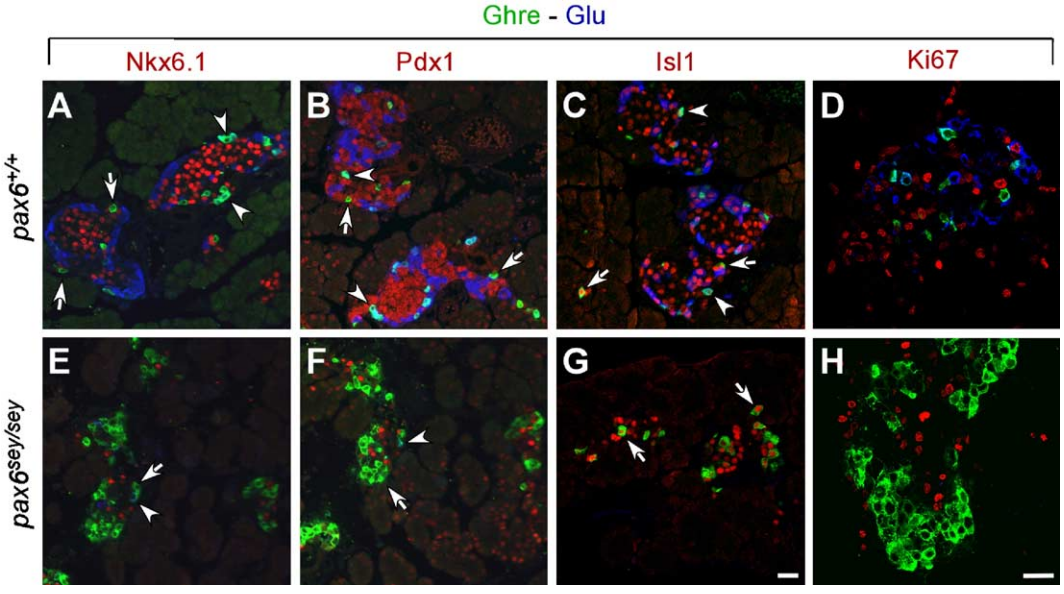

Fig. 4. *Pax6* regulates the generation but not the proliferation of ghrelin-secreting cells in the mouse endocrine pancreas. Nkx6.1 (A, E) and Pdx1 (B, F) expression is lacking in most ghrelin- and glucagon-secreting cells in both wild-type (A, B) and *Pax6* mutant (D, E) mice. (C, G) All hormone immunoreactive cells including the ε-cells are Isl1 positive. (D, H) The ε-cell population is post-mitotic in both wild-type and mutant mice as demonstrated by examination for Ki67, a marker of proliferating cells. Arrows point to examples of ghrelin$^+$ glucagon$^-$ cells and arrowheads indicate ghrelin$^+$ α-cells. Scale bars are 30 μm.

absence of cells co-producing ghrelin with insulin proteins or the β-cell-specific transcription factors Pdx-1 and Nkx6.1 clearly establish that mouse β-cells do not express the ghrelin peptide.

The analysis of mice deficient for *Ngn3* as well as animals driving the expression of the *GFP* reporter gene under the control of the *Ngn3* promoter demonstrates that ε-cells, as other islet cell types, derive from *Ngn3*-expressing precursors and are fully dependant on Ngn3 activity (Gradwohl et al., 2000; Gu et al., 2002; Herrera, 2002). Interestingly, the fact that the ghrelin$^+$ cells in the stomach develop independently of Ngn3 (Jenny et al., 2002) underscores the existence of two independent mechanisms in the G-I tract that leads to ghrelin-producing cells: the first one necessitates Ngn3 activity in the endocrine pancreas while the other in the stomach does not depend on Ngn3. Aiming to further characterize the factors acting in the ε-fate specification, we undertook an analysis of this population in animals deficient for several Ngn3-dependent genes that have been demonstrated as key factors in endocrine cell specification (Collombat et al., 2003; Sander et al., 1997; Sosa-Pineda et al., 1997; St-Onge et al., 1997). Our data demonstrate that in the absence of a functional *Arx* allele, a drastic reduction in the total number of ghrelin$^+$ cells occurs. As Arx is indispensable for the α-cell fate specification and antagonizes β- and δ-cell formation (Collombat et al., 2003), such a decrease in ghrelin$^+$ cells can easily be attributed to the loss of ghrelin$^+$ glucagon$^+$ α-cells. Similarly, the large expansion of the ghrelin$^+$ cells found in *Pax4* mutant mice suggests that the cells normally fated towards a β- and/or δ-cell fate adopt an alternative ghrelin$^+$ glucagon$^+$ α-cell destiny. Importantly, the numbers of pancreatic ghrelin$^+$ glucagon$^-$ ε-cells appear unchanged following Arx or Pax4 depletion as compared to wild-type pancreas, suggesting that unlike α-cells, ε-cells develop independently from both factors. Additionally, it was recently reported that Foxa2 is required for the α- but not the ε-cell lineage (Lee et al., 2005). This latter result also establishes that ε-cells have an α-cell independent origin.

Along the same line of evidence, the increase in the ε-cell content concomitant with the loss of the α-cell population observed in *Pax6* mutants confirms that α- and ε-cells represent distinct endocrine cell types subjected to different regulatory signals.

Taken together, our data indicate that pancreatic ghrelin-producing ε-cells derive from Ngn3-expressing precursors and require Ngn3 function. We demonstrate that both Arx and Pax4 factors regulate the α-cell destiny but do not act on the ε-cell specification. On the contrary, Pax6 appears to play a key role in determining the ε-cell fate for endocrine precursors since its ablation promotes a striking augmentation of the ε-cell population at the expense of the other cell types. Sussel and co-workers have also reported excess numbers of ghrelin-producing cells in mice deficient for *Nkx2.2* (Prado et al., 2004). Based on these observations, together with the loss of *Pax6* expression detected in Nkx2.2$^{-/-}$ animals (Wang et al., 2004) and the unaffected *Nkx2.2* expression levels exhibited by *Pax6*$^{sey/sey}$ mice (Heller et al., 2004), we propose that Pax6 represents a downstream target of Nkx2.2 in pathways leading to the pancreatic ghrelin-secreting ε-cell fate.

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