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# Ghrelin is dispensable for embryonic pancreatic islet development and differentiation

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ABSTRACT

Ghrelin is a peptide hormone that has been implicated in the regulation of food intake and energy homeostasis. Ghrelin is predominantly produced in the stomach, but is also expressed in many other tissues where its functions are not well characterized. In the rodent and human pancreas, ghrelin levels peak at late gestation and gradually decline postnatally. Several studies have suggested that ghrelin regulates beta cell function during embryonic development and in the adult. In addition, in a number of mouse models, ghrelin cells appear to replace insulin- and glucagon-producing cells in the islet. In this analysis, we investigated whether the absence or overexpression of ghrelin influenced the development and differentiation of the pancreatic islet during embryonic development. These studies revealed that ghrelin is dispensable for normal pancreas development during gestation. Conversely, we demonstrated that elevated ghrelin in the Nkx2.2 null islets is not responsible for the absence of insulin- and glucagon-producing cells. Finally, we have also determined that in the absence of insulin, ghrelin cells form in their normal numbers and ghrelin is expressed at wild type levels.

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## 1. Introduction

The adult endocrine pancreas consists of four hormone-producing cell types organized in islets of Langerhans. In the rodent, islets are comprised of a large core of insulin-producing beta cells surrounded by smaller numbers of glucagon-producing alpha cells, somatostatin-producing delta cells, and pancreatic polypeptide-producing PP cells. During embryonic development, the epsilon cell population and a subset of alpha cells produce a fifth hormone, ghrelin [1–4].

Ghrelin is a 28-amino acid peptide hormone that was originally isolated as an activator of the growth hormone secretagogue receptor (GHSR1a) [5]. In adults, serum ghrelin levels are primarily dependent on secretion by the X/A-like cells in the posterior stomach [6,7]. Serum ghrelin levels peak shortly before normal feeding times and is higher in fasting animals. Cerebral administration of ghrelin in rats acutely stimulated food intake and increased long-term weight gain [8–11]. IV injection of ghrelin into human patients also resulted in increased food intake [12]. Surprisingly, ghrelin null mice show few significant differences with respect to bone mineral density, fat content, body weight, food intake, and serum leptin or glucose concentration before or after fasting [13,14]. Therefore, it is possible that ghrelin is sufficient, but not necessary to stimulate appetite.

In addition to the stomach, ghrelin expression has been reported in a variety of tissues including the small intestine, lymphocytes, placenta, lung, kidney, brain, and gonads [15,16]. In many of these tissues, ghrelin exerts a variety of endocrine and nonendocrine effects. For example, ghrelin expression in the testes has been shown to function as a paracrine signal to control the replication of immature Leydig cells, steroidogenesis, and stem cell factor (SCF) expression in sertoli cells [17,18]. Furthermore, there is emerging evidence to suggest that ghrelin modulates cell proliferation and/or differentiation of osteoblasts, adipocytes and neuronal cells [19–22]. Therefore, ghrelin may play an important role in regulating the development of specific tissues.

In the developing mouse pancreas, ghrelin can be detected as early as e9.5 and continues to be expressed throughout gestation, with levels peaking shortly before birth [23]; (supplemental figure 1). Postnatally, ghrelin levels gradually decline until little pancreatic ghrelin can be detected in the adult islet [24]. Interestingly, the number of ghrelin-positive cells is significantly increased in the Pax4, Pax6 and Nkx2.2 knockout models at the expense of many of the other hormone-producing cell populations [1,25]. Given the potential role for ghrelin in regulating cell differentiation in other tissues, it is

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possible that ghrelin may influence the development and differentiation of the pancreatic endocrine cell populations during development. In this study we analyzed the ghrelin null phenotype in the embryonic pancreas at various stages of development. Our data show that the number of insulin- and glucagon-producing cells is unchanged in ghrelin null mice. Furthermore, RNA levels of insulin, glucagon, somatostatin and pancreatic polypeptide as well as the transcription factors Nkx2.2 and Pdx1 are unaffected. We also demonstrate that the upregulation of ghrelin is not responsible for the loss of insulin and glucagon in Nkx2.2 null embryos. Overall, this study suggests that ghrelin is dispensable for the development and differentiation of the pancreas during embryogenesis.

## 2. Materials and methods

### 2.1. Animals

Nkx2.2 and ghrelin heterozygous mice were generated by homologous recombination as previously described [14,26]. Nkx2.2+/− and ghrelin +/− or −/− animals were maintained in a Swiss Black (Taconic) background. Double heterozygous mice were mated to obtain Nkx2.2−/−; ghrelin−/− (DKO) mice. Genotyping of mice and embryos was performed by PCR analysis as described [14,26]. Animals were housed and treated according to UCHSC and Columbia University Institutional Care and Animal Use Committee approval protocols. Ins1/Ins2 DKO embryos and their wild type controls were provided by Dr. A. Pugliese for these studies [27].

### 2.2. Immunofluorescence

Immunofluorescence was performed on cryopreserved tissue fixed with 4% paraformaldehyde for 3 h (Pdx1 and Nkx6.1) or overnight, as previously described [28]. Staining was performed on 10 μm sections. Antibodies used were rabbit anti-amylase (1:1000, Sigma), rabbit anti-ghrelin (1:200, Phoenix peptide), guinea pig anti-glucagon (1:3000, Linco), rabbit anti-glucagon (1:1000, Phoenix peptide), guinea pig anti-insulin (1:1000, Linco), mouse anti-insulin (1:500, Sigma), rabbit anti-Nkx6.1 (1:800, Beta Cell Biology Consortium (BCBC)), rabbit anti-Pdx1 (1:1000, Chemicon), guinea pig anti-pancreatic polypeptide (1:500, Linco), and rabbit anti-somatostatin (1:200, Phoenix peptide). Secondary antibodies used were Alexafluor-488 anti-rabbit (1:400), Alexafluor-488 anti-guinea pig (1:400), Alexafluor-488 anti-mouse (1:200), Alexafluor-594 anti-guinea pig (1:400), Alexafluor-594 anti-mouse (1:200), Alexafluor-594 anti-rabbit (1:400) and Cy5-anti-rabbit. Confocal images were processed with a Zeiss Microscope, LSM 510 META at 25× magnification. Fluorescent images were obtained with a Nikon Eclipse 80i microscope, Q-Image camera and ImagePro software from Media Cybernetics.

### 2.3. Morphometric analysis

Wild type and ghrelin null embryos were processed as above. Every fifth section (e13.5) or every tenth section (e15.5 and e18.5) was collected and stained by immunofluorescence for insulin and glucagon. DAPI staining was used to visualize the nuclei to aid in cell counting. The entire pancreas from each wild type and mutant embryos $n=4$ (e13.5), $n=4$ (e15.5), $n=3$ (e18.5) was used to obtain a representative number of hormone positive cells per embryo. The respective immunofluorescent-positive cells on glucagon+, insulin+ sections were counted. Total islet area was calculated using Image Pro Plus 5.1 software using amylase immunofluorescence staining to delineate the islet. Islet area was unchanged in the ghrelin null embryos (data not shown).

### 2.4. Quantification of mRNA

Total RNA was extracted from pancreatic tissue and prepared using a Qiagen RNeasy Micro kit. cDNAs were prepared with random hexamer primers and Superscript III (Invitrogen). Real-time PCR was performed using Taqman probes (ABI Assays on Demand) for Gapdh (4352932E), ghrelin (Mm00445450_m1), glucagon (Mm00801712_m1), insulin 2 (Mm00731595_gH), somatostatin (Mm00436671_m1), pancreatic polypeptide (Mm00435889_m1), and Pdx1 (Mm00435565_m1), on the ABI 7000. Taqman probes and primers were designed for Nkx2.2 (FAM-CCATTGACTCTGCCCCATCGCTTCT, Forward: CTCCCCGAGTGGCAGAT, Reverse: GAGTTCTATCCTCTCCAAAAGTTCAAA). Taqman probes and primers were designed for Cyclophilin B (FAM -TGGTACGGAAGGTGGAG, Forward: GCAAAGTTCTAGAGGGCATGGA, Reverse: CCCGGCTGTCTGTCTTGGT). Each gene was normalized to Gapdh (Fig. 5). Ghrelin levels in Supplemental figure 1 were normalized to cyclophilin B. Samples were quantified with

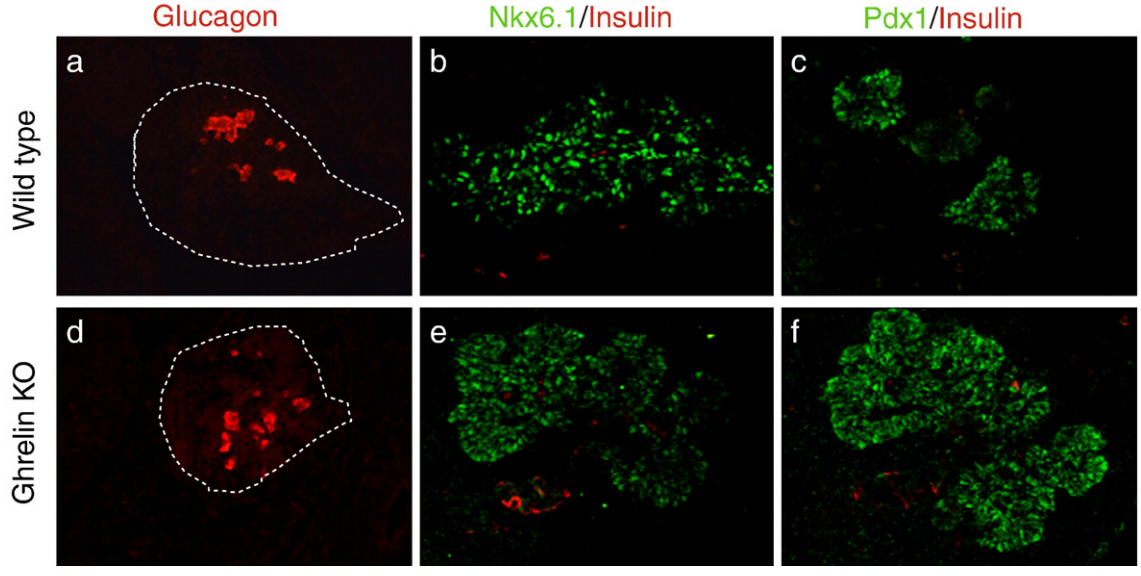


**Fig. 1.** Absence of ghrelin does not affect early endocrine cell differentiation. Immunofluorescence staining of embryonic pancreata in e13.5 wild type (a–c) and ghrelin null (d–f) mice. Nkx6.1 (b and e, green) and Pdx1 (c and f, green) are broadly expressed throughout the pancreatic epithelium in the ghrelin null embryos and the wild type littermates. Glucagon (a and d, red) and insulin (b, c, e and f, red) cells are evident in the ghrelin null embryos in numbers comparable to wild type. Magnification: 40× (a and d) and 25× (b, c, e and f). Confocal microscopy was used for b, c, e and f.

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