**Foxa2** is required for the differentiation of pancreatic α-cells

Catherine S. Lee, Newman J. Sund, Rüdiger Behr, Pedro L. Herrera, Klaus H. Kaestner

**Abstract**

The differentiation of insulin-producing β-cells has been investigated in great detail; however, little is known about the factors that delineate the second-most abundant endocrine lineage, the glucagon-producing α-cell. Here we utilize a novel YAC-based Foxa3Cre transgene to delete the winged helix transcription factor Foxa2 (formerly HNF-3β) in the pancreatic primordium during midgestation. The resulting Foxa2loxP/loxP; Foxa3Cre mice are severely hypoglycemic and die within the first week of life. Mutant mice are hypoglucagonemic secondary to a 90% reduction of glucagon expression. While the number of mature glucagon-positive α-cells is dramatically reduced, specification of α-cell progenitors is not affected by Foxa2 deficiency. By marker gene analysis, we show that the expression of the α-cell transcription factors Arx, Pax6, and Brn4 does not require Foxa2 in the transcriptional hierarchy governing α-cell differentiation.

**Keywords:** Foxa2; Hepatocyte nuclear factor 3-β (HNF3-β); Glucagon; α-cells

**Introduction**

During mouse development, dorsal and ventral pancreatic primordia first appear as evaginations of the foregut endoderm on embryonic day (E) 9.0 (Slack, 1995). The induction of the pancreatic phenotype in the dorsal pancreatic bud was thought to be dependent upon a permissive signal from the adjacent notochord (Kim and Melton, 1998; Kim et al., 1997; Slack, 1995), but more recent evidence suggests that this signal originates from blood vessels (Lammert et al., 2001, 2003). In the mouse, the two pancreatic buds rotate and merge to form the organ seen in adult animals that comprises exocrine and endocrine compartments. Development of the endocrine pancreas is a complex process that requires coordinated interactions among multiple transcription factors during different stages of development. It is the precise regulation of transcription factors that directs endocrine progenitors to give rise to five distinct hormone-producing cell types: α, β, δ, PP, and ε cells, which secrete glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. At later stages of development, several of these factors are also required to define and maintain individual mature cell types (Edlund, 2001; Prado et al., 2004; Slack, 1995; Wilson et al., 2003).

Several transcription factors have been identified based on their temporally and spatially restricted expression during pancreatic development (Edlund, 1998; Wilson et al., 2003). In addition, analysis of mice with targeted mutations of the genes that encode these factors has furthered our understanding of islet differentiation. Deletion of Pdx1, a homeobox gene expressed in pancreatic buds, leads to an arrest of pancreatic differentiation at a very early stage (Guz et al., 1995; Jonsson et al., 1994, 1995; Offield et al., 1996). Mice lacking Neurogenin 3 (Ngn3), a bHLH transcription factor expressed in the endocrine progenitor cells, suffer from diabetes due to the absence of all islet cells (Gradwohl et al., 2000; Gu et al., 2002; Herrera et al., 2002; Lee et al., 2002a; Schwitzgebel et al., 2000). Furthermore, the winged-helix transcription factor, Foxa2 [formerly...
hepatocyte nuclear factor 3-β (HNF-3β), is required for normal β-cell function as its β-cell-specific ablation results in hyperinsulinemic hypoglycemia (Lantz et al., 2004; Sund et al., 2001).

In addition to Foxa2, the related winged helix family members Foxa1 and Foxa3 are expressed at the onset of definitive endoderm development, with Foxa2 being activated first, followed by Foxa1, and finally Foxa3 (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993). Expression of the Foxa genes persists into adulthood (Cockell et al., 1995; Wu et al., 1997) and Foxa binding sites have been identified in the promoters or enhancers of several genes expressed in the pancreas, including Pdx1 and preproglucagon (Ben-Shushan et al., 2001; Marshak et al., 2001; Philippe et al., 1994; Sharma et al., 1997; Wu et al., 1997). The binding of Foxa proteins to these promoters is of functional importance in vivo as α-cells lacking Foxa1 express reduced levels of preproglucagon mRNA (Kaestner et al., 1999; Shih et al., 1999) and Foxa2-deficient β-cells cannot fully activate transcription of the Pdx1 gene (Lee et al., 2002b). The Foxa genes are not equivalent, as deletion of Foxa3 has no apparent consequences for pancreatic gene expression, in contrast to the situation described for Foxa1 and Foxa2 (Kaestner et al., 1998).

Ablation of Foxa2 in the pancreatic β-cell using Cre recombinase driven by the rat insulin promoter (Ins.Cre) led to the surprising finding that Foxa2 is required for the normal function of β-cells and the expression of the two subunits of the KATP channel (Lantz et al., 2004; Sund et al., 2001). While it was demonstrated that Foxa2 contributes to the maintenance of mature β-cells, it is possible that deletion of the Foxa2loxP target effected by the Ins.Cre transgene occurred too late to demonstrate the full effect of Foxa2 on the initiation of pancreatic development (Sund et al., 2001). To overcome this limitation, we have derived a new Cre line, the first to direct Cre expression to the gut endoderm before the onset of pancreatic differentiation. We have used this new tool to delete Foxa2 in the embryonic gut and to uncover a previously unrecognized role for Foxa2 in pancreatic α-cell development.

Materials and methods

Targeting vector construction

A yeast artificial chromosome (YAC) encompassing the mouse Foxa3 (formerly HNF3γ or Hnf3γ) gene was modified as described (Hiemisch et al., 1997), with the exception that we used the Cre coding sequence to replace exon 2 of Foxa3.

Animals and genotype analysis

Foxa2loxP/loxP mice were derived as previously described (Sund et al., 2000). All mice were kept on a mixed outbred-CD1 background. Genotyping was performed by PCR analysis using genomic DNA isolated from the tail of newborn mice (Sund et al., 2000). Genotyping primers were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>Foxa2</td>
<td>5’-CCCC-CTG-AGT-TGG-CGG-TGG-T-3’</td>
<td>5’-TTG-CTC-ACG-GAA-GAG-TAG-CC-3’</td>
</tr>
<tr>
<td>Cre</td>
<td>5’-GGC-GCA-TGG-TGC-AAG-TTG-AAT-3’</td>
<td>5’-CGT-TCA-CCG-GCA-TCA-ACG-TT-3’</td>
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Analytical procedures

Blood glucose values were determined from whole venous blood using a glucose monitor (Glucometer Elite, Bayer). To prepare plasma, mice were killed by decapitation on P1–P3, and plasma obtained by centrifugation. Plasma glucagon was measured using RIA (University of Pennsylvania Diabetes Center). Since 100 μl of plasma was required for this assay, plasma from multiple animals of the same genotype were pooled.

β-Galactosidase detection

Embryos were fixed in 4% paraformaldehyde (PFA) at 4°C for 1 h. After fixation, embryos were washed three times for 10 min in PBS and incubated overnight (O/N) in staining solution [5 mM K3(Fe(CN))6, 5 mM K4(Fe(CN))6, 2 mM MgCl2, 0.02% NP40, 0.01% sodium deoxycholate, and 1 mg/ml Blue-Gal (Gibco) BRL in PBS]. Subsequently, embryos were washed three times for 10 min in PBS and postfixed in 4% PFA O/N at 4°C. Embryos were washed in PBS and photographed.

RNA analysis

Total RNA from E18.5 and P1 pancreas was isolated after homogenization and processed using Trizol (LTI). Reverse transcription PCR (RT-PCR) analysis was performed as described (Duncan et al., 1997). A Stratagene Mx4000 Real-Time PCR machine was used for the quantitative PCR analysis. Conditions and primer concentrations suggested by the SYBR Green Assay protocol were followed. The following forward and reverse primers were used for amplification (size in bp):

<table>
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<tr>
<th>Gene</th>
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<th>Reverse Primer</th>
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<tr>
<td>Hprt (143bp)</td>
<td>5’-GCCAGCAGTTGTTGGATTTG-3’</td>
<td>5’-TGCCGCATCATCTAGGCTTTTGT-3’</td>
</tr>
<tr>
<td>Preproglucagon (128bp)</td>
<td>5’-TGAGATGAGCACCATCCTGGGGA-3’</td>
<td>5’-TGGCAAGAGATGTTGTTGAAA-3’</td>
</tr>
<tr>
<td>Braf 4 (108bp)</td>
<td>5’-GGGTATTACATCCACAGGAAG-3’</td>
<td>5’-TTCCAGATAGCGCTTCTGACACT-3’</td>
</tr>
<tr>
<td>Pax 6 6 (117bp)</td>
<td>5’-AAAAAAGCCTGACTCTGCC-3’</td>
<td>5’-CCGGCTTTGGTTAAAGGCTTCTCC-3’</td>
</tr>
<tr>
<td>Arx 149bp</td>
<td>5’-TCCGGATTCCACTACATTGGT-3’</td>
<td>5’-GAGGGCACTTTCCCTTTAAATG-3’</td>
</tr>
</tbody>
</table>
Immunofluorescence

Tissues were fixed in 4% PFA overnight at 4°C, embedded in paraffin, cut to 6 μm sections, and applied to Probe-on Plus slides (Fisher Scientific). Slides were deparaffinized in xylene and rehydrated through a series of ethanol washes. Slides were subjected to microwave antigen retrieval by boiling for 15 min in 10 mM citric acid buffer (pH 6.0) and allowed to cool for 10 min at room temperature (RT). All slides were washed in PBS, then blocked with protein blocking reagent (Immunotech) for 20 min at RT. The primary antibodies were diluted in PBS containing 0.1% BSA and 0.2% Triton X-100 (PBT) unless noted otherwise and incubated with the sections overnight at 4°C. Slides were washed in PBS and incubated with the appropriate secondary antibodies diluted in PBT for 2 h at RT. Slides were washed in PBS, mounted, and examined using confocal microscopy (Leica). The following primary antibodies were used at the indicated dilutions: rabbit anti-Foxa2 (K2 1:2000; a gift from Dr. T. Jessel), goat anti-Foxa2 (1:200, AbCam), rabbit anti-Pdx1 (1:5000; a gift from Dr. D. Stoffers), goat anti-Somatostatin (1:5000; Linco), rabbit anti-Somatostatin (1:50 in Antibody Diluent Solution; Zymed), rabbit anti-Glucagon (Zymed), guinea pig anti-Insulin (1:2000; Linco), goat anti-Ghrelin (1:200; Santa Cruz), rabbit anti-PP (1:50; Zymed), rabbit anti-Pax6 (1:200; BabCO), goat anti-Amylase (1:200; Santa Cruz), and rabbit anti-PC2 (1:100; Chemicon). The following secondary antibodies were used: Cy3-conjugated donkey anti-rabbit IgG (1:750; Jackson), Cy2-conjugated donkey anti-guinea pig IgG (1:200; Jackson), Cy3-conjugated donkey anti-goat (1:300; Jackson), and Cy2-conjugated donkey anti-goat (1:300; Jackson).

Whole-mount immunostaining

Embryos at E9.5 were fixed in 4% PFA at RT for 30 min and 4°C for 1 h. Embryos were washed in PBS/0.1% Triton X (PT) for 30 min at RT, then blocked in PT/5%BSA at 4°C O/N. Rabbit anti-Foxa2 antiseraum was added at 1:1000 in PT/5%BSA and the embryos were incubated O/N at 4°C. Embryos were washed in PT/1%BSA for 1.5 h at RT. Goat anti-Rabbit-Alexa 488 (Molecular Probe) was added at 1:400 in PT/5%BSA for 2 h at RT. Embryos were washed in PT/1%BSA for 1.5 h at RT, sectioned with vibratome, and examined using confocal microscopy.

Whole-mount RNA in situ hybridization

Embryos were dissected from plugged females for E9.5 and E10.5. Whole-mount RNA in situ hybridization procedure was described in Collombat et al. (2003). In brief, embryos were fixed in 4% PFA and dehydrated through a methanol series. Embryos were treated with 10 μg/ml proteinase K and prehybridized at 70°C for 1 h in solution containing 50% formamide, 5× SSC pH4.5, 50 μg/ml yeast RNA, 1% SDS, and 50 μg/ml heparin. Digoxigenin glucagon probe was added to the same solution at 1 μg/ml and incubated overnight at 70°C. After series of washes, anti-DIG antibody was added overnight at 4°C and the BM purple AP substrate (Roche) was used for color detection.

Results and discussion

Derivation of Foxa3Cre-transgenic mice

Targeting Cre-mediated gene deletion to the primitive gut tube before the onset of liver and pancreas specification has thus far been hampered by the lack of suitable promoter or enhancer fragments. Commonly used Cre lines such as the Albumin–Cre and Pdx1–Cre have an onset of expression after liver and pancreas have been specified (Gu et al., 2002; Herrera, 2000; Postic et al., 1999; Sund et al., 2000). To overcome this limitation, we developed a new Cre-transgenic line that is active in the endoderm of the developing gut before the onset of organogenesis. Foxa3 is one of the few genes expressed early in the endoderm but not in other tissues (Monaghan et al., 1993). We have previously characterized the cis-regulatory elements essential for the expression of Foxa3 in vivo and have shown that a 170-kb yeast artificial chromosome (YAC Yγ5) transgene mimics the endogenous Foxa3 expression (Hiemisch et al., 1997). A simplified map of the Yγ5 YAC is shown in Fig. 1A. In this YAC, the Foxa3 gene is flanked by ~100 kb upstream and 60 kb downstream sequences. We have generated a construct placing the Cre-recombinase cDNA into exon 2 of the Foxa3 gene, preceded by an internal ribosome entry site (Fig. 1). The modified Foxa3Cre YAC construct was used for the derivation of transgenic mice by pronuclear injection (Schedl et al., 1993). The presence of the transgene was assessed by PCR analysis for the Cre cDNA (Fig. 1E). To ascertain that the transgene had integrated intact into the genome of the transgenic mice, we probed for the presence of the yeast selectable markers LYS2 and TRP, which are positioned in the right and left arm of the YAC, respectively (Fig. 1E and data not shown).

To assess the activity of Cre recombinase under the control of the cis-regulatory elements of Foxa3, we crossed the Foxa3Cre mouse to a lacZ reporter mouse (Rosa26), which indicates Cre activity by β-galactosidase staining (Soriano, 1999). We expected to detect the expression of β-galactosidase in all cells that normally express Foxa3. When staining embryos for the activity, we found expression in the anterior intestinal portal at E8.5 (Fig. 1F), in the foregut endoderm and its derivatives (hepatic cords) at E9.5 (Fig. 1G), and in the dorsal and ventral pancreatic buds at E10.5 (Fig. 1H), demonstrating that the Foxa3Cre transgene reproduced the expression of the endogenous Foxa3 locus and results in the efficient excision of loxP targets.
Derivation of the endoderm-specific Foxa2 knockout mouse

Endoderm-specific Foxa2 knockout animals (Foxa2<sup>loxP/loxP</sup>; Foxa3Cre) were obtained by breeding Foxa3Cre-transgenic mice with Foxa2<sup>loxP/loxP</sup> mice (Sund et al., 2000). The Foxa2<sup>loxP/+</sup>; Foxa3Cre offspring were mated to Foxa2<sup>loxP/loxP</sup> homozygous mice to obtain the four possible genotypes: Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and three littermate control groups: Foxa2<sup>loxP/+</sup>; Foxa3Cre, Foxa2<sup>loxP/+</sup>, and Foxa2<sup>loxP/loxP</sup>.

To assess the onset and efficiency of Cre-mediated gene deletion in Foxa2<sup>loxP/loxP</sup>; Foxa3Cre animals, Foxa2 protein expression was examined in E9.5 embryos (Figs. 2A–F) and postnatal day 1 (P1) pancreas (Figs. 2G–H) by immunofluorescence. During early embryogenesis, Foxa2 expression is detected in the notochord, floorplate, and entire gut tube of wild type E9.5 embryos (Figs. 2A and C). Similar to control embryos, Foxa2 expression is present in both the notochord and floorplate of Foxa2<sup>loxP/loxP</sup>; Foxa3Cre mice. However, Foxa2-positive cells are reduced in the foregut and completely absent in the midgut of E9.5 Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryos (Figs. 2B and D). This indicates that Foxa3Cre specifically deletes Foxa2 in the endoderm without affecting Foxa2 expression in the notochord and floorplate.

In order to assess whether Foxa2 is efficiently deleted in the pancreatic primordium, we performed dual label immunofluorescence with Foxa2 and Pdx1 antibodies on E9.5 embryos. Pdx1 is one of the earliest markers of pancreas development (Ahlgren et al., 1996). In the control embryo, two populations of staining were observed: the Pdx1<sup>+</sup>/Foxa2<sup>+</sup> cells (orange) and Pdx1<sup>−</sup>/Foxa2<sup>+</sup> cells (green) (Fig. 2E). The presence of Pdx1<sup>−</sup>/Foxa2<sup>−</sup> cells is not surprising since it has been documented that not all epithelial cells in the pancreatic bud are Pdx1 positive and that these cells are still present in Pdx1-deficient animals (Ahlgren et al., 1996). In contrast, Foxa2 expression is almost completely absent in Pdx1<sup>−</sup> and almost completely deleted in Pdx1<sup>−</sup>/Foxa2<sup>−</sup> cells in the pancreatic primordium of Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryos (Fig. 2F). This demonstrates that Foxa2 expression is efficiently deleted in the pancreatic primordium as early as E9.5.
To confirm that the Foxa2 gene is efficiently deleted in the pancreas at later stages, we also examined Foxa2 protein expression in P3 pancreas by immunofluorescence. In the control pancreas, Foxa2 is found in both acini and islets (Fig. 2G) whereas Foxa2 protein is not detected in these tissues of Foxa2-loxP/loxP; Foxa3Cre animals (Fig. 2H). This demonstrates that the Foxa2 gene is completely absent in all cell types of the pancreas by P3. Since both Foxa2 and Foxa3 are also expressed in other endodermally derived organs, we have also verified deletion of Foxa2 in liver, stomach, and intestine in Foxa2-loxP/loxP; Foxa3Cre mice (data not shown). As a control, expression of Foxa2 in the lung is still present in Foxa2-loxP/loxP; Foxa3Cre mice since Foxa3 is not expressed in the lung (data not shown; Monaghan et al., 1993).

Foxa2 is not required at the onset of pancreatic development

It has been demonstrated both in vitro and in vivo that Foxa2 controls Pdx1 gene expression in pancreatic β-cells (Ben-Shushan et al., 2001; Cockell et al., 1995; Lee et al., 2002b; Lin et al., 2002; Marshak et al., 2001; Sharma et al., 1997; Wu et al., 1997). However, its role during early formation of the pancreatic primordium, as well as the regulation of Pdx1 expression in vivo, has not yet been investigated due to the lack of suitable mouse models. To address this question, we have examined Pdx1 expression in the Foxa2-loxP/loxP; Foxa3Cre embryo at E9.5 (Figs. 2E–F). Since Foxa2 is expressed in the foregut before the formation of the pancreatic primordium, we expected to see a change in Pdx1 expression. However, not only is Pdx1 expression not affected in the Foxa2-loxP/loxP; Foxa3Cre embryo, the pancreatic primordia are formed properly (Fig. 2F). This indicates that Foxa2 is not required at the onset of pancreatic development for Pdx1 expression. This may reflect functional compensation for the loss of Foxa2 expression by Foxa1 or Foxa3.

Endoderm-specific deletion of Foxa2 leads to early lethality and severe hypoglycemia

Foxa2-loxP/loxP; Foxa3Cre mice were born alive with reduced size when compared to their littermate control groups (Fig. 3A) and most died by P3, with a few surviving to P5. Of more than 200 offspring analyzed, no Foxa2-loxP/loxP; Foxa3Cre mouse has survived beyond P5. Foxa2-loxP/loxP; Foxa3Cre mice appeared dehydrated as evidenced by reduced skin turgor; however, Foxa2-loxP/loxP; Foxa3Cre mice had milk in their stomachs, suggesting normal suckling behavior.

Fig. 2. Deletion of Foxa2 in Foxa2-loxP/loxP; Foxa3Cre mice. Efficiency and timing of Foxa2 deletion by Foxa3Cre were evaluated by immunofluorescence. Whole-mount immunofluorescence on E9.5 embryos using anti-Foxa2 antibody was performed followed by vibratome sectioning. (A and C) Foxa2 protein (green) was found in the floorplate, notochord, foregut, and midgut of control embryos. (B and D) Expression of Foxa2 was still detected in the floorplate and notochord. However, many of the cells in the foregut were negative for Foxa2 staining (B) and Foxa2 expression was completely deleted in the midgut (D). (E) Co-immunofluorescence staining for Pdx1 (red) and Foxa2 (green) shows Foxa2 expression in both Pdx1⁺ (orange denoted by “o”) and Pdx1⁻ (green denoted by “w”) cells in the pancreatic primordium of E9.5 control embryos. (F) Foxa2 expression is deleted in both Pdx1⁺ (denoted by “o”) and Pdx1⁻ cells except for one cell (denoted by “w”). (G–H) Expression of Foxa2 was also assessed by immunofluorescence in the P3 pancreas. Insulin (green) and Foxa2 (red) were found in the control pancreas (G), while Foxa2 protein was completely deleted in both islets and acini in the Foxa2-loxP/loxP; Foxa3Cre pancreas (H). Abbreviations: floorplate (fp), notochord (nc), foregut (fg), midgut (mg), pancreatic primordium (pp).
The essential role of pancreatic endocrine cells in regulating glucose homeostasis led us to examine glucose levels in Foxa2^loxP/loxP; Foxa3^Cre mice and their control littermates. Blood glucose levels were dramatically reduced in Foxa2^loxP/loxP; Foxa3^Cre animals (Fig. 3B). Normally, hypoglycemia triggers counter-regulatory glucagon secretion; however, we observed a 50% reduction of plasma glucagon when compared to control littermates (Fig. 3C). This inappropriate reduction in circulating glucagon levels in the face of significant hypoglycemia suggests a defect in the production of this hormone due to reduced α-cell number, glucagon biosynthesis, or glucagon secretion. We have previously detected defects in glucose metabolism in Foxa1- and Foxa3-deficient mice. Mice homozygous for a null mutation in Foxa1 are hypoglycemic with reduced levels of plasma glucagons, despite possessing the normal complement of α-cells (Kaestner et al., 1999). In addition, Foxa3-deficient mice exhibit moderate hypoglycemia after a prolonged fast, which is mediated by a dramatic decrease in the expression of the glucose transporter GLUT 2 (Shen et al., 2001). Thus, a common role for the Foxa genes is the protection of the organism from hypoglycemia.

**Foxa2^loxP/loxP; Foxa3^Cre animals have reduced numbers of glucagon-positive cells**

Since glucagon is synthesized in pancreatic α-cells, we next examined glucagon expression in Foxa2^loxP/loxP; Foxa3^Cre and control pancreas by immunofluorescence (Fig. 4). In control islets, numerous glucagon-positive cells were found in the islet mantle (Figs. 4A,B and 5A,E), whereas very few or no glucagon-positive cells were present in the Foxa2^loxP/loxP; Foxa3^Cre islets (Figs. 4D,E and 5C,G). Next, we examined preproglucagon mRNA levels in Foxa2^loxP/loxP; Foxa3^Cre and control pancreas by real-time PCR analysis (Fig. 4M). Consistent with the absence of glucagon immunoreactivity, preproglucagon transcripts were reduced by 90% in the Foxa2^loxP/loxP; Foxa3^Cre pancreas (Fig. 4M). Thus, Foxa2 is required for the differentiation of mature glucagon-producing α-cells in the pancreas. Thus, despite the expression of all three Foxa genes in the endocrine pancreas, deletion of Foxa2 alone results in perturbed pancreatic α-cell differentiation. While in vitro all three Foxa genes can activate similar target genes, our data suggest that they play different roles during α-cell development in vivo. The differentiation of α-cells requires the presence of Foxa2 as evidenced by reduced levels of preproglucagon mRNA and protein in the Foxa2^loxP/loxP; Foxa3^Cre pancreas (Fig. 4M). Thus, Foxa2 is required for the differentiation of mature glucagon-producing α-cells in the pancreas. Although Foxa2 has been shown to activate preproglucagon transcription in vitro (Kaestner et al., 1999), it is likely that the lack of glucagon production in the Foxa2^loxP/loxP; Foxa3^Cre pancreas is secondary to a defect in α-cell maturation, as both Foxa1 and Foxa3 can also activate the preproglucagon promoter (Kaestner et al., 1999).

Foxa2 is not required to specify the insulin, somatostatin, pancreatic polypeptide, ghrelin, or acinar lineages

We investigated whether endocrine lineages other than α-cells are dependent on Foxa2. To this end, we performed immunostaining for insulin, somatostatin, pancreatic polypeptide, and ghrelin. Immunofluorescent staining for insulin demonstrated that β-cells were specified in the absence of Foxa2 (Figs. 4A–C, G–H and 5A–B, E–F). Somatostatin- and pancreatic polypeptide-positive cells were found with equal frequency in islets from Foxa2^loxP/loxP; Foxa3^Cre and control mice (Figs. 4C, F, G, and J). Ghrelin is a hormone that has been recently described to be expressed in the ε cells in the adult pancreas (Prado et al., 2004; Wierup et al.,...
Foxa2loxP/loxP; Foxa3Cre mice have few glucagon-positive cells and Foxa2 is required for the transcription of glucagon. Pancreas sections from P1 Foxa2loxP/loxP; Foxa3Cre and control mice were immunostained for islet and acinar cell markers. The images were captured by confocal microscopy. The markers for endocrine pancreas were glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells), pancreatic polypeptide (PP-cells), and ghrelin (ε-cells). The marker for exocrine pancreas was amylase. (A and B) Normal insulin and glucagon expression were seen in the control pancreas. (D and E) Foxa2loxP/loxP; Foxa3Cre mice have few or no glucagon-positive cells. Note that while some islets look normal (D), a majority of the islets are misshapen (E). δ-, PP-, and ε-cells appear normal in both control (C, G, and H) and Foxa2loxP/loxP; Foxa3Cre (F, J, and K). Amylase expression is also found normal in the Foxa2loxP/loxP; Foxa3Cre animal (I and L). Magnification is 400×. (M) Quantitative RT-PCR analysis demonstrated a 90% reduction in glucagon expression. Bars represent mean ± SEM with P value < 0.05 by two-tailed Student’s t test. (N) mRNA levels of Brn4, Arx, and Pax6 are not different between Foxa2loxP/loxP; Foxa3Cre (white bars) and control (black bars) pancreata. HPRT was used as an internal control. n = 4 for both control and Foxa2loxP/loxP; Foxa3Cre animals.
Ghrelin expression was examined by immunofluorescence in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control pancreas. We found similar numbers of ghrelin-positive cells surrounding the insulin-positive cells in each genotype (Figs. 4H and K), suggesting that the ghrelin lineage is independent of Foxa2. To determine whether the exocrine cell lineage is affected in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas, we examined amylase expression and found similar expression to the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control mice (Figs. 4I and L).

Foxa2<sup>loxP/loxP</sup>; Foxa3Cre animals have few mature α-cells

Endocrine cell differentiation occurs in a serial progression in which pancreatic precursors are first specified to become endocrine progenitors that are marked by expression of Neurogenin 3 (Ngn3) (Apelqvist et al., 1999; Gu et al., 2002; Herrera et al., 2002; Schwitzgebel et al., 2000). Next, combinations of different transcription factors are activated to cause these cells to adopt one of the five known endocrine cell fates (Wilson et al., 2003). Thus, endocrine cells differentiate as development proceeds and reach maturity when they produce and secrete their respective hormones. The phenotype caused by the lack of Foxa2 expression could represent a failure to specify the earliest pre-α-cell lineage, a failure of an intermediate differentiation step, or the lack of terminal differentiation to the mature phenotype.

In order to address whether Foxa2 is required for the initial specification of α-cell precursors, we examined expression of prohormone convertase 2 (PC2). PC2 is an endopeptidase expressed in all endocrine precursors beginning on day 10 of gestation, including those destined to become mature α-cells (Marcinkiewicz et al., 1994). PC2 is the only endopeptidase found in α-cells as early as E10.0, and it is down-regulated in the adult (Marcinkiewicz et al., 1994). Since the majority of pancreatic endocrine cell types are α-, β-, and δ-cells at birth, we examined the presence of these cell types by immunofluorescence using antibodies against insulin and glucagon (Figs. 5A and C) and PC2, insulin and somatostatin (Figs. 5B and D) on serial sections of control and Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas. If Foxa2 is important during α-cell lineage allocation or the survival of the lineage, then we would expect no or few PC2-positive and insulin- and somatostatin-negative cells. However, if Foxa2 is crucial during a later step of α-cell differentiation, then we would expect to find to see numerous PC2-positive/insulin- and somatostatin-negative cells that do not stain for glucagon, suggesting the presence of immature α-cells. In fact, the latter scenario was observed in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre animals. In the control pancreas, normal insulin and glucagon staining was observed (Fig. 5A) and PC2-positive, insulin-negative, and somatostatin-negative cells are either proglucagon- or glucagon-producing α-cells (Fig. 5B). In the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas, only insulin but no glucagon staining was seen (Fig. 5C); however, there were still numerous PC2-positive, insulin-, and somatostatin-negative cells detected, consistent with the presence of immature α-cells (Fig. 5D). Thus, Foxa2 is required for the terminal differentiation of pancreatic α-cells, but not the initiation of the α-cell lineage.

Fig. 5. α-Cell progenitors are specified in the pancreas of the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre mice. Adjacent sections of P1 Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control pancreata were stained for insulin/glucagon (A, C, E, and G), insulin/somatostatin/PC2 (B and D), or insulin/Pax6 (F and H). Confocal images of insulin (green) and glucagon (red) of control (A and E) and Foxa2<sup>loxP/loxP</sup>, Foxa3Cre pancreas (C and G). PC2-positive, insulin- and somatostatin-negative cells (red) and PC2-, insulin-, and somatostatin-positive cells (yellow) were present in similar numbers in both control (B) and Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas (D). The numbers of insulin-negative and Pax6-positive cells were comparable between the control (F) and Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas (H). Magnification for A–D is 400 × and E–H is 1000 ×.
**F**oxa2 is not required for maintaining the expression of Brn4, Pax6, and Arx during a-cell differentiation

Three transcription factors have been shown to be involved in different stages of a-cell development based on their expression patterns and loss-of-function phenotypes. Brain 4 (Brn4), a POU homeodomain-containing protein, is found in the glucagon-expressing cells of the pancreatic buds at E10.0; however, mice lacking Brn4 do not exhibit any pancreatic phenotype (Collombat et al., 2003; Heller et al., 2004; Hussain et al., 1997; Phippard et al., 1999). Pax6, a paired-box gene found in all endocrine cells, is required for the specification of a-cells (St-Onge et al., 1997). In addition, mutation of the Aristal**less related homeobox gene (Arx) results in an early onset loss of mature pancreatic a-cells with an increase in b- and d-cell numbers (Collombat et al., 2003). To place Foxa2 in the a-cell differentiation hierarchy, we examined the expression of Brn4, Pax6, and Arx by real-time PCR analysis. mRNA levels of these genes were unchanged between Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control pancreas (Fig. 4N).

Since Brn4 in the pancreas is specific to a-cells, this finding supports our notion that a-cells are specified in the absence of Foxa2, even though they fail to differentiate terminally. In addition, we examined expression of Pax6 protein by immunofluorescence (Figs. 5E–H). Control and Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas were double stained for insulin and glucagon (Figs. 5E and G) and insulin and Pax6 on adjacent sections (Figs. 5F and H). This allowed us to compare the numbers of Pax6-positive/insulin-negative cells between Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control mice. Expression of Pax6 appears similar in both insulin-positive and insulin-negative endocrine cells between Foxa2<sup>loxP/loxP</sup>; Foxa3Cre and control mice, again indicating that Pax6 expression is not dependent on Foxa2. Thus, we conclude that Foxa2 does not act upstream of Arx, Pax6, and Brn4 in the differentiation of a-cells.

**First wave glucagon-producing cells require Foxa2 for their maintenance**

There are two populations of endocrine cells: the early endocrine cells, which exist before E12.5, and cells that are generated after the secondary transition (Ahlgren et al., 1996). To determine whether loss of Foxa2 would affect the early glucagon-positive cells, we examined glucagon expression in E9.5 and E10.5 embryos by whole-mount RNA in situ hybridization (Figs. 6A–E). At E9.5, normal glucagon expression was detected in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryo (Figs. 6A–B). When E10.5 embryos were examined, we observed down-regulation of glucagon expression in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryo; with only a few glucagon-positive cells present in the dorsal pancreatic bud (Figs. 6C–E). This indicates that the first wave of glucagon-positive cells is partially dependent on Foxa2.

The shape and size of the islets are altered in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre animals

In addition to the low number of terminally differentiated a-cells, we also observed perturbed islet architecture in Foxa2<sup>loxP/loxP</sup>; Foxa3Cre pancreas (Fig. 4E). Control pancreas exhibited roughly spherical islets (Figs. 4A–C and 5A–B, E–F), but islets from Foxa2<sup>loxP/loxP</sup>; Foxa3Cre animals were often irregularly shaped (Figs. 4E, K). Furthermore, Foxa2<sup>loxP/loxP</sup>; Foxa3Cre islets displayed a disorganized architecture with many small clusters of endocrine cells embedded in the exocrine tissue (data not shown). Although Foxa2 is also deleted in the acinar tissue (Fig. 2F), we did not observe any obvious morphological changes and differences in the amylase staining in the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre acini (Figs. 4I and L).

**Foxa2 regulates multiple steps in pancreatic development**

On the basis of the observations described above, we propose a new model for Foxa2 action at multiple stages of pancreatic development (Fig. 7). Previously, we have shown that Foxa2 is an essential regulator of transcription in mature pancreatic b-cells, as cell-type-specific gene ablation leads to reduced expression of Pdx1 and of Sur1 and Kir6.2, the subunits of the K<sub>ATP</sub> channel, and perturbed insulin secretion (Lantz et al., 2004; Lee et al., 2002b; Sund et al., 2003). However, a role for Foxa2 in the specification of a-cells has not been previously demonstrated. This role is supported by data showing that Foxa2 expression is down-regulated in the absence of Foxa3 in the E9.5–E10.5 Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryo similar to that of the control embryo. (C–D) Glucagon mRNA is reduced in the E10.5 Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryo when compared to the control. (E) A transverse section of the Foxa2<sup>loxP/loxP</sup>; Foxa3Cre embryo at the pancreatic primordium plane showed residual glucagon mRNA. Dorsal pancreatic bud is on the top. Pancreatic primordia are denoted by ‘*’.
As shown here, Foxa2 is not required at the onset of pancreatic development to activate Pdx1 expression. This maybe due to compensation by Foxa1 and Foxa3. However, Foxa2 is a critical regulator of α-cell differentiation, acting after the initial specification of the α-cell lineage. Due to the paucity of markers, at present it is not known how many intermediates exist between the Neurogenin 3-positive endocrine precursor and the mature α-cell, or at which step Foxa2 acts. As shown above, Foxa2 is required at a late stage of α-cell development, as expressions of Arx, Brn4, and Pax6 do not depend on Foxa2 (Fig. 7; black arrow). In addition, Foxa2 is not absolutely required during delineation of the first wave of glucagon-positive cells (Fig. 7; grey arrow). Involvement of a transcriptional regulator in several steps of pancreatic development has been shown previously for Pdx1, which is required for the early expansion of the pancreatic primordium and in the mature β-cell (Ahlgren et al., 1998; Jonsson et al., 1994; Offield et al., 1996). We have shown here that similar to Pdx1, Foxa2 also plays multiple roles in different endocrine cell types at different stages.

In conclusion, we have derived a novel endoderm-specific Cre transgene (Foxa3Cre) that targets the earliest stage of gut development and thus represents an important new tool for the developmental genetic analysis of the definitive endoderm and its derivatives. By using this mouse, we were able to delete Foxa2 in the pancreas as early as E9.5 and demonstrate that Foxa2 is required for the terminal differentiation of glucagon-producing α-cells, thereby identifying a novel function of Foxa2.

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