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Foxa2 is required for the differentiation of pancreatic α-cells

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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 *Foxa3*Cre transgene to delete the winged helix transcription factor *Foxa2* (formerly HNF-3β) in the pancreatic primordium during midgestation. The resulting *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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.
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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

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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 definite 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 K_{ATP} 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 $Foxa2^{loxP}$ 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

$Foxa2^{loxP/loxP}$ 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:

Foxa2:	5′CCC-CTG-AGT-TGG-CGG-TGG-T-3′
	5′ TTG-CTC-ACG-GAA-GAG-TAG-CC-3′
Cre:	5′ GCG-GCA-TGG-TGC-AAG-TTG-AAT-3′
	5′ CGT-TCA-CCG-GCA-TCA-ACG-TTT-3′

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 $K_3(Fe(CN))_6$, 5 mM $K_4(Fe(CN))_6$, 2 mM $MgCl_2$, 0.02% NP40, 0.01% sodium deoxycholate, and 1 mg/ml Bluo-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):

Hprt (143bp):	5′-GGCCAGACTTTGTTGGATTTG-3′
	5′-TGCGCTCATCTTAGGCTTTGT-3′
Preproglucagon (128bp):	5′-TGAGATGAGCACCATTCTGGA-3′
	5′-TCCGCAGAGATGTTGTGAAGA-3′
Brn 4 (108bp):	5′-GGCTGATTCATCCACAGGAAG-3′
	5′-TTCCAGTACGCCCTTGACACT-3′
Pax 6 (117bp):	5′-AAACAAACGCCCTAGCTCTCC-3′
	5′-CCGCCCTTGGTTAAAGTCCTC-3′
Arx (149bp):	5′-TCCGGATACCCCACTTAGCTT-3′
	5′-GACGCCCCTTTCCTTTAAGTG-3′

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-conjugate 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* antiserum 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. Digoxygenin 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 *Foxa3*Cre 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 *Foxa3*Cre 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 *Foxa3*Cre transgene reproduced the expression of the endogenous *Foxa3* locus and results in the efficient excision of loxP targets.

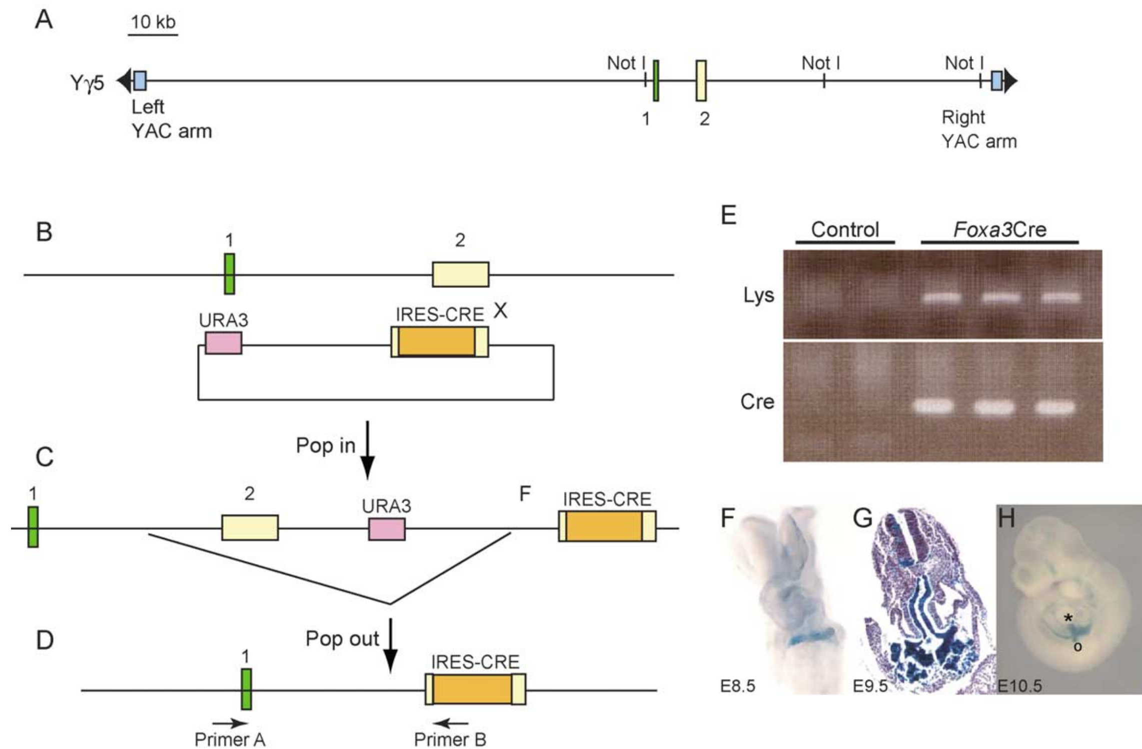

Fig. 1. Derivation of the *Foxa3*Cre YAC and validation of the *Foxa3*Cre mice. (A) Map of the 170-kb *Foxa3* YAC (termed Yγ5). Exon 1 and Exon 2 are represented as green and yellow boxes, respectively. The YAC telomeres are shown as arrows. (B) The targeting plasmid used for the modification of the YAC and the site of recombination (not drawn to scale). The IRES–Cre cassette was placed within exon 2 of *Foxa3*. Selection for the presence of the Ura3 marker ("pop-in") was achieved by growing transfected yeast cells on plates lacking uracil. (C) After homologous recombination, the *Foxa3* YAC contains a partial duplication of *Foxa3* sequences and the IRES–CRE. Selection for the absence of Ura3 is achieved by plating on 5-fluoroorotic acid. Both the desired clones (D) as well as the parental clone (B) were obtained and differentiated by PCR with primers A and B. (D) Map of the *Foxa3*Cre YAC that was used for the generation of transgenic mice. (E) *Foxa3*Cre-transgenic mice were PCR genotyped for the presence of the Cre–cDNA and the *LYS2* selection cassette present in the right arm of the YAC. (F–H) *Foxa3*Cre mice were crossed to the Rosa26R reporter line (Soriano, 1999). E8.5, E9.5, and E10.5 embryos carrying both the *Foxa3*Cre transgene and the Rosa26R allele were identified by PCR and stained for β-galactosidase. Blue staining indicates Cre-recombinase activity. The *Foxa3*Cre transgene is active in all epithelial cells of the developing gut. (F) In whole-mount E8.5 embryos, *Foxa3*Cre activity was localized to the anterior intestinal portal. (G) In sectioned E9.5 embryos, the developing gut (green arrow) as well as the hepatic primordium (red arrow) were stained. (H) In the E10.5 embryo, the gut tube as well as the dorsal (○) and ventral (*) pancreatic buds were positive for LacZ staining.

Derivation of the endoderm-specific Foxa2 knockout mouse

Endoderm-specific *Foxa2* knockout animals (*Foxa2*$^{loxP/loxP}$; *Foxa3*Cre) were obtained by breeding *Foxa3*Cre-transgenic mice with *Foxa2*$^{loxP/loxP}$ mice (Sund et al., 2000). The *Foxa2*$^{loxP/+}$; *Foxa3*Cre offspring were mated to *Foxa2*$^{loxP/loxP}$ homozygous mice to obtain the four possible genotypes: *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and three littermate control groups: *Foxa2*$^{loxP/+}$; *Foxa3*Cre, *Foxa2*$^{loxP/+}$, and *Foxa2*$^{loxP/loxP}$.

To assess the onset and efficiency of Cre-mediated gene deletion in *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*Cre mice. However, *Foxa2*-positive cells are reduced in the foregut and completely absent in the midgut of E9.5 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre embryos (Figs. 2B and D). This indicates that *Foxa3*Cre 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*$^{+}$/*Foxa2*$^{+}$ cells (orange) and *Pdx1*$^{-}$/*Foxa2*$^{+}$ cells (green) (Fig. 2E). The presence of *Pdx1*$^{-}$/*Foxa2*$^{+}$ 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*$^{+}$ and almost completely deleted in *Pdx1*$^{-}$ cells in the pancreatic primordium of *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre 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*$^{\mathrm{loxP/loxP}}$;*Foxa3*Cre mice (data not shown). As a control, expression of *Foxa2* in the lung is still present in *Foxa2*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre mice since *Foxa3* is not expressed in the lung (data not shown; Monaghan et al., 1993).

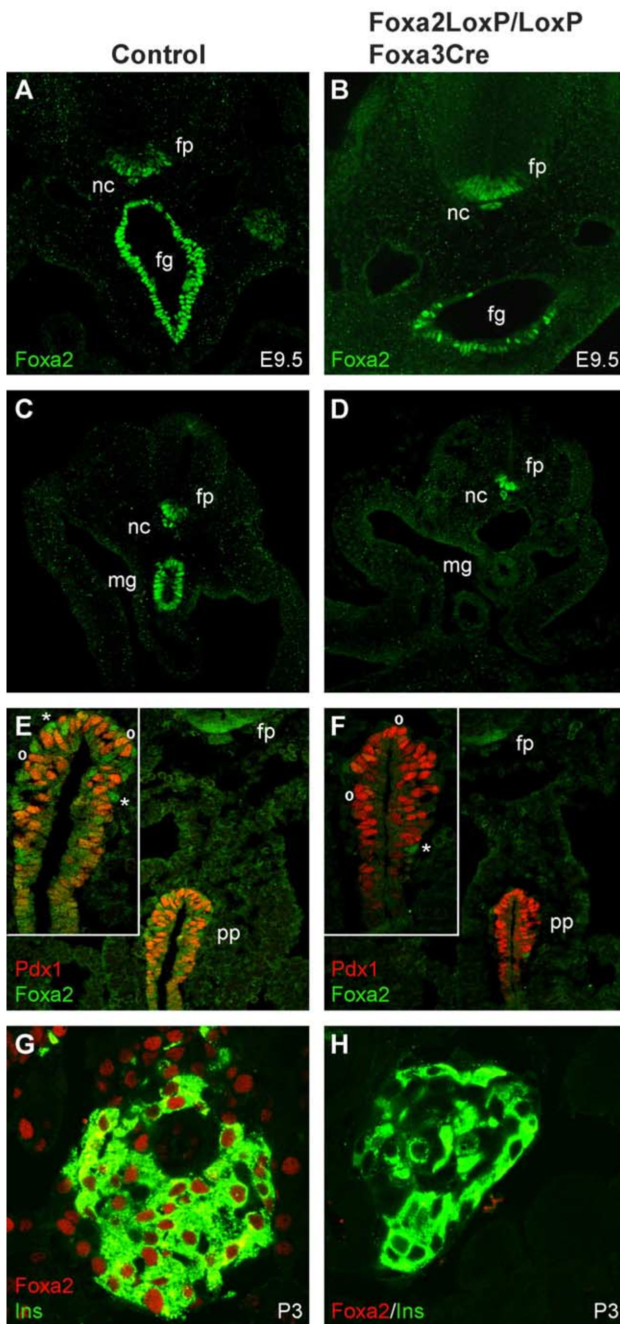

Fig. 2. Deletion of *Foxa2* in *Foxa2*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre mice. Efficiency and timing of *Foxa2* deletion by *Foxa3*Cre were evaluated by immunofluorescence. Whole-mount immunofluorescence on E9.5 embryos using anti-*Foxa2* antibody was performed followed by vibratome sectioning. (A and 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 "*") 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 "*"). (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*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre pancreas (H). Abbreviations: floorplate (fp), notochord (nc), foregut (fg), midgut (mg), pancreatic primordium (pp).

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*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre 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*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre 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$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre 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*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre mouse has survived beyond P5. *Foxa2*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre mice appeared dehydrated as evidenced by reduced skin turgor; however, *Foxa2*$^{\mathrm{loxP/loxP}}$; *Foxa3*Cre mice had milk in their stomachs, suggesting normal suckling behavior.

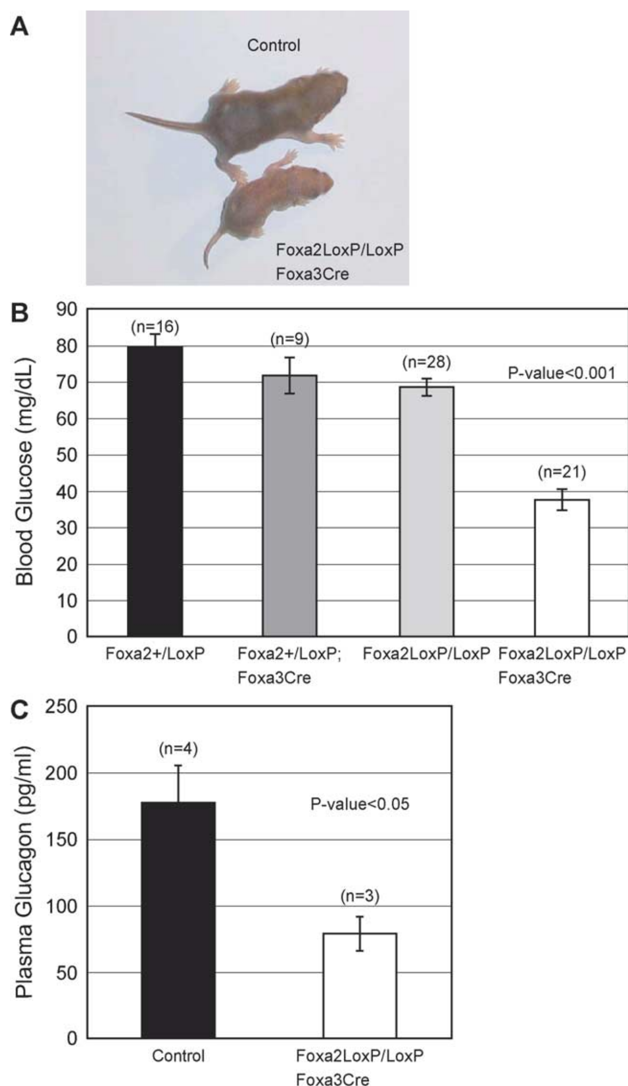

Fig. 3. *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre mice die shortly after birth and are hypoglycemic. (A) Five-day-old (P5) *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre mouse (bottom) and its control sibling (top). (B) P1–P3 mice of all possible genotypes were sacrificed and blood glucose was measured. (C) Plasma glucagon of pooled samples (~10 pups) from *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and control mice were measured by radioimmunoassay.

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$^{loxP/loxP}$*; Foxa3Cre 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. 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.,

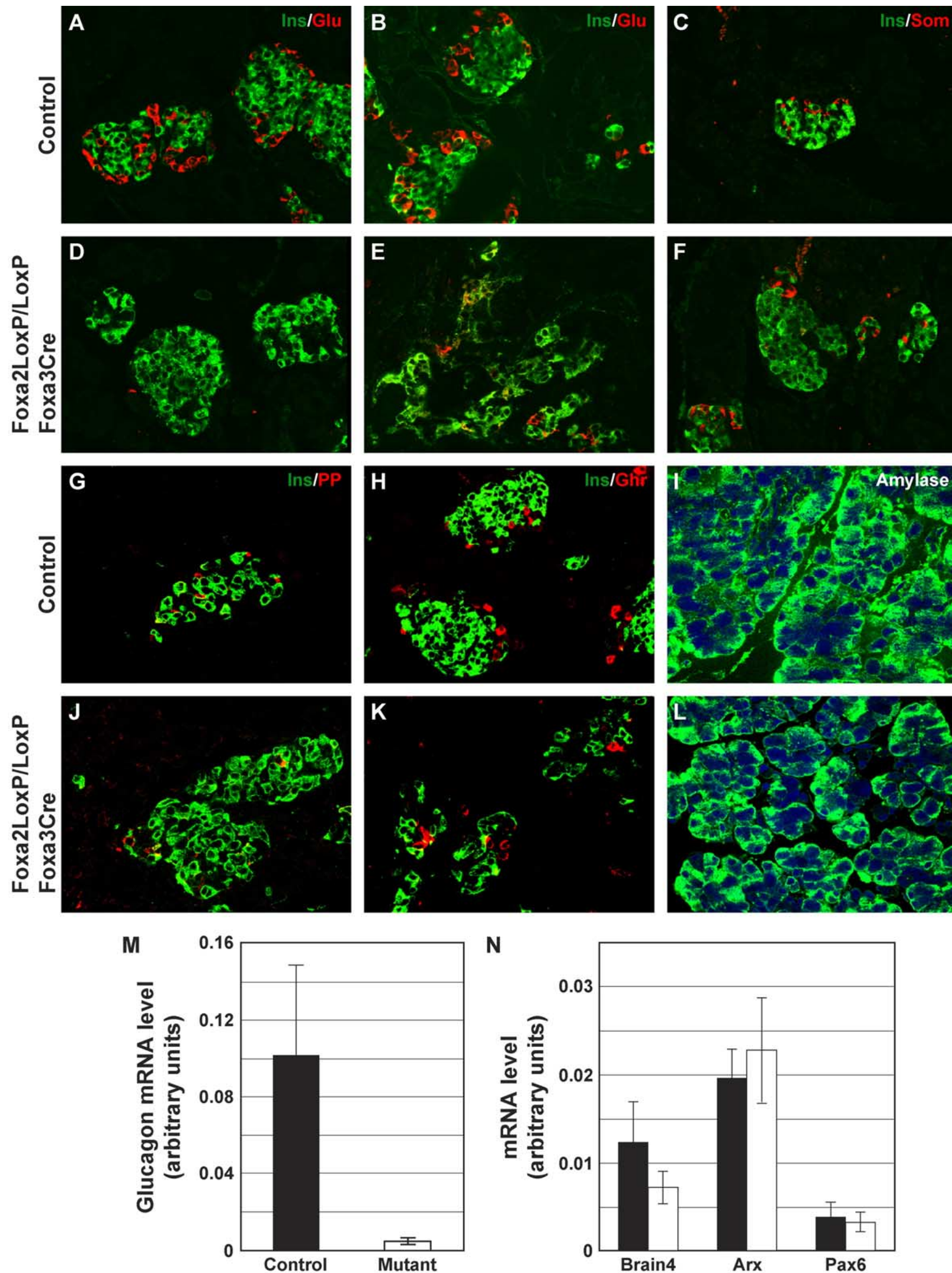

Fig. 4. *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre mice have few glucagon-positive cells and *Foxa2* is required for the transcription of glucagon. Pancreas sections from P1 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and control mice were immunostained for islet and acinar cell markers. The images were captured by confocal 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) *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre (F, J, and K). Amylase expression is also found normal in the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre (white bars) and control (black bars) pancreata. HPRT was used as an internal control. n = 4 for both control and *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre animals.

2002). Ghrelin expression was examined by immunofluorescence in the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*Cre pancreas, we examined amylase expression and found similar expression the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and control mice (Figs. 4I and L).

Foxa2$^{loxP/loxP}$; *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 preα-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*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*-Cre 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*$^{loxP/loxP}$; *Foxa3*Cre 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.

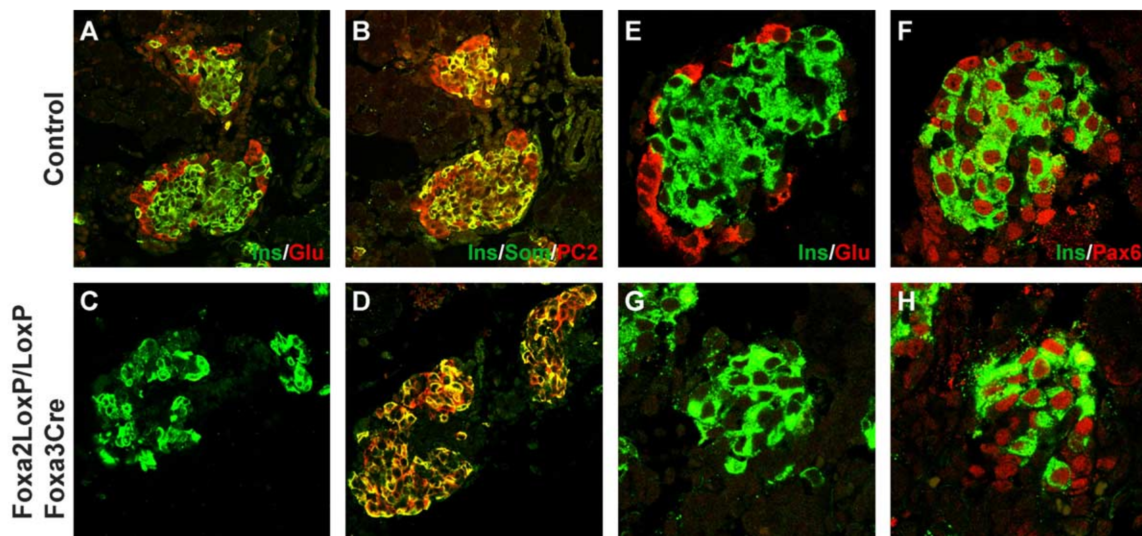

Fig. 5. α-Cell progenitors are specified in the pancreas of the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre mice. Adjacent sections of P1 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and control pancreata were stained for insulin/glucagon (A, C, E, and G), insulin/somatostatin/PC2 (B and D), or insulin/Pax6 (F and H). Confocal images of insulin (green) and glucagon (red) of control (A and E) and *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*Cre pancreas (D). The numbers of insulin-negative and Pax6-positive cells were comparable between the control (F) and *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre pancreas (H). Magnification for A–D is 400× and E–H is 1000×.

Foxa2 is not required for maintaining the expression of Brn4, Pax6, and Arx during α-cell differentiation

Three transcription factors have been shown to be involved in different stages of α-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 α-cells (St-Onge et al., 1997). In addition, mutation of the *Aristaless related homeobox* gene (*Arx*) results in an early onset loss of mature pancreatic α-cells with an increase in β- and δ-cell numbers (Collombat et al., 2003). To place *Foxa2* in the α-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*$^{loxP/loxP}$; *Foxa3*Cre and control pancreas (Fig. 4N). Since *Brn4* in the pancreas is specific to α-cells, this finding supports our notion that α-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*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*Cre and control islets. Expression of Pax6 appears similar in both insulin-positive and insulin-negative endocrine cells between *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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 α-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*$^{loxP/loxP}$; *Foxa3*Cre embryo (Figs. 6A–B). When E10.5 embryos were examined, we observed down-regulation of glucagon expression in the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*.

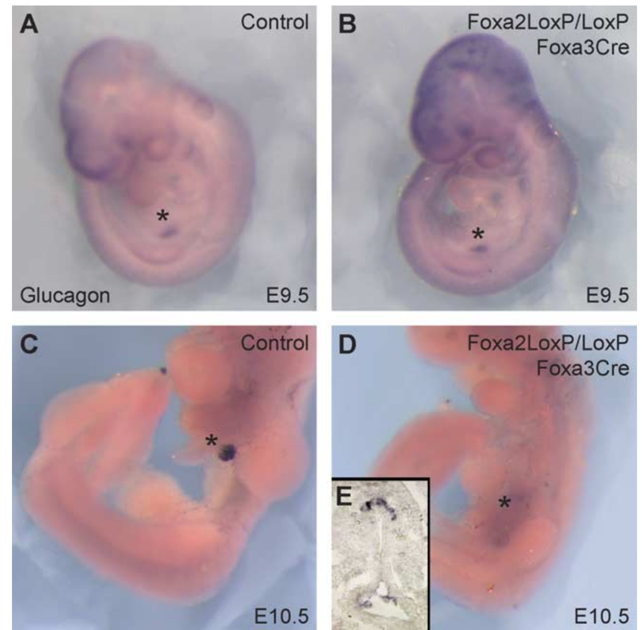

Fig. 6. *Foxa2* is required for the maintenance but not the induction of first wave glucagon-positive cells. RNA whole-mount in situ hybridization of E9.5–E10.5 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre and control embryos. (A–B) Expression of glucagon mRNA in the E9.5 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre embryo is similar to that of the control embryo. (C–D) Glucagon mRNA is reduced in the E10.5 *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre embryo when compared to the control. (E) A transverse section of the *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre embryo at the pancreatic primordium plane showed residual glucagon mRNA. Dorsal pancreatic bud is on the top. Pancreatic primordia are denoted by "*."

The shape and size of the islets are altered in the Foxa2$^{loxP/loxP}$*; Foxa3Cre animals*

In addition to the low number of terminally differentiated α-cells, we also observed perturbed islet architecture in *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre pancreas (Fig. 4E). Control pancreas exhibited roughly spherical islets (Figs. 4A–C and 5A–B, E–F), but islets from *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre animals were often irregularly shaped (Figs. 4E, K). Furthermore, *Foxa2*$^{loxP/loxP}$; *Foxa3*Cre 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*$^{loxP/loxP}$; *Foxa3*Cre 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 β-cells, as cell-type-specific gene ablation leads to reduced expression of *Pdx1* and of Sur1 and Kir6.2, the subunits of the K_{ATP} channel, and perturbed insulin secretion (Lantz et al., 2004; Lee et al., 2002b; Sund et al.,

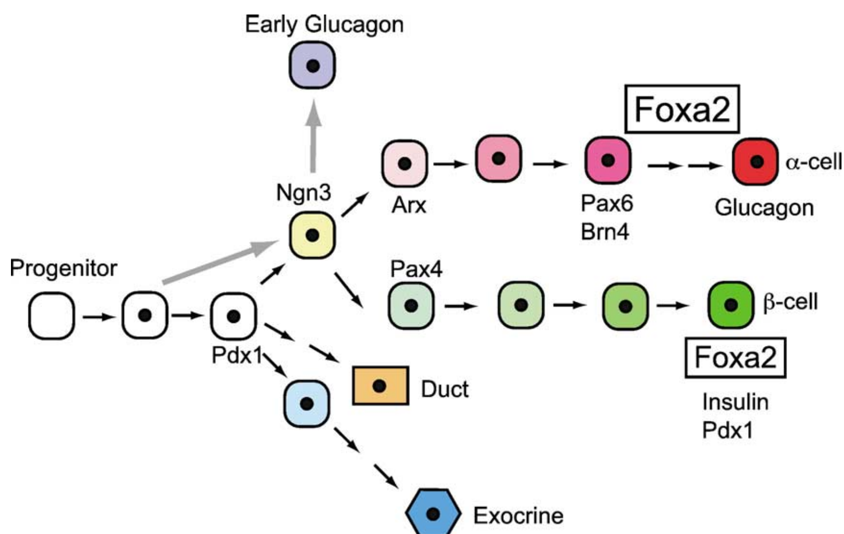

Fig. 7. Model for the role of *Foxa2* during pancreas differentiation. *Foxa2* plays multiple roles during pancreas development. *Foxa2* cooperates with an as yet unidentified factor in the maintenance of *Pdx1* in pancreatic β-cells. As shown here, the generation of early glucagon-containing cells is only partially dependent on *Foxa2*. After the secondary transition, the terminal differentiation of α-cells does not occur in the absence of *Foxa2*.

2001). 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 (*Foxa3*Cre) that targets the earliest stage of gut development and thus represents an important new tool for the developmental genetic analysis of the definite 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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