Published in final edited form as:

Gastroenterology. 2009 December ; 137(6): 2052–2062. doi:10.1053/j.gastro.2009.08.059.

Foxa1 and Foxa2 Control the Differentiation of Goblet and Enteroendocrine L- and D-Cells in Mice

Diana Z. Ye and Klaus H. Kaestner^{\$}

Department of Genetics and Institute for Diabetes, Obesity, and Metabolism, University of Pennsylvania, Philadelphia, PA 19104

Abstract

Background & Aims—The winged helix transcription factors Foxa1 and Foxa2 are expressed in all epithelia of the gastrointestinal tract from its embryonic origin into adulthood. *In vitro* studies have shown that *Foxa1/a2* can transactivate the promoters of *Mucin 2* (*Muc2*), which is expressed in goblet cells, and of *preproglucagon*, which is expressed in enteroendocrine cells. These findings suggest *Foxa1/a2* as critical factors in the differentiation of gut epithelial cells.

Methods—Mice with intestine-specific simultaneous deletion of *Foxa1* and *Foxa2* were derived using the Cre-loxP system and analyzed using histological and molecular means.

Results—Both *Foxa1* and *Foxa2* were successfully deleted in the epithelia of the small intestine and colon using Villin-Cre mice. Immunohistochemical staining showed that *Foxa1/a2* mutants lack glucagon-like peptide-1 and 2 expressing cells (L-cells), and have reduced numbers of somatostatin (D-cells) and peptide YY (PYY) expressing cells (L-cells). Preproglucagon, somatostatin and PYY mRNA levels were also significantly reduced in *Foxa1/a2* mutants. Thus, Foxa1 and Foxa2 are essential regulators of these enteroendocrine lineages *in vivo*. The mRNA levels of transcription factors Islet-1 and Pax6 were significantly reduced in the small intestine, demonstrating that Foxa1 and Foxa2 impact on a transcription factor network in the enteroendocrine lineage. In addition, deletion of *Foxa1/a2* caused a reduction in goblet cell number with altered expression of the secretory mucins *Muc2*, *Mucin5b*, *Mucin5ac* and *Mucin 6*.

Conclusion—The winged helix factors Foxa1 and Foxa2 are essential members of the transcription factor network that governs secretory cell differentiation in the mammalian gastrointestinal tract.

INTRODUCTION

During embryogenesis, the gastro-intestinal epithelia are derived from the definitive endoderm through a series of complex developmental steps. Several transcription factors, including the bHLH transcription factors Math1 and Beta2, neurogenin-3 (Ngn3), the paired box transcription factors Pax4 and Pax6, the zinc-finger transcription factor Krüppel-like factor 4

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^{\$}Corresponding author, kaestner@mail.med.upenn.edu, Klaus H. Kaestner, PhD, Department of Genetics, University of Pennsylvania, Philadelphia, PA 19104, USA, Phone: 215-898-8759, Fax: 215-573-5892.

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Author contribution

DZY: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript

KHK: study concept and design; critical revision of the manuscript for important intellectual content; obtained funding; study supervision

The authors declare that no conflict of interest exists.

(Klf4) and insulinoma associated-1 (Insm-1 or IA-1), and the homeodomain transcription factor Nkx2.2 have been shown to play critical roles in the differentiation of different types of epithelial cells of the gastrointestinal tract [1–9]. Secretory cell lineages, which include goblet, Paneth and enteroendocrine cells, are derived from a common Math1-expressing progenitor, whereas enterocytes are Math1 independent [6]. Ngn3 controls enteroendocrine cell fate commitment of Math1⁺ secretory progenitors [1,7]. Beta2 acts downstream of Ngn3 and is required specifically for the differentiation of cholecystokinin and secretin-producing cells [1,2]. The differentiation of goblet cells, however, is Ngn3 independent. In fact, Ngn3^{-/-} mice have an increased number of goblet cells in the small intestine, possibly due to the failure of stem cells to differentiate along the enteroendocrine lineage [1]. *Klf4* is another transcription factor that is required for the terminal differentiation of goblet cells in the colon [10]. Nkx2.2 has been shown to be important for the differentiation of several enteroendocrine cells such as CCK, GIP, gastrin, glucagon and somatostatin [8]. Insm-1 is essential for the differentiation of serotonin, CCK and PYY [9].

The winged-helix transcription factors Foxa1 and Foxa2 are expressed in the definitive endoderm during embryogenesis [11–13], and in many adult tissues derived from the endoderm such as pancreas, liver, stomach and intestine [12]. *Foxa1* null mice die within the first two weeks of life due to hypoglycemia and mild nephrogenic diabetes insipidus [14–17]. *Foxa2* null embryos do not elaborate an organized node and notochord, and they die after gastrulation with defects in dorsal-ventral patterning of the neural tube [18,19]. Due to the early lethality of both *Foxa1* and *Foxa2* null mice, it has been impossible thus far to determine their role in intestinal epithelial cell differentiation in genetically altered mice. The present study was designed to determine the function of Foxa1 and Foxa2 in intestinal epithelial cell differentiation in adult mice using cell-type specific gene ablation.

MATERIALS AND METHODS

Mice

The derivation of *Foxa1*^{loxP/+} and *Foxa2*^{loxP/+} mice has been described previously [20–23]. After mating *Foxa1*^{loxP/+} heterozygote mice with *Foxa2*^{loxP/loxP} homozygous mice, *Foxa1*^{loxP/+}, *Foxa2*^{loxP/loxP} mice were obtained. The Villin-Cre mouse [24] was mated with *Foxa1*^{loxP/+}, *Foxa2*^{loxP/loxP} mice to produce mice with intestine-specific deletion of *Foxa1* and *Foxa2*. *Foxa1*^{loxP/+}, *Foxa2*^{loxP/loxP} or *Foxa1*^{loxP/loxP}, *Foxa2*^{loxP/loxP} mice were used as controls in all studies. Genotyping was performed by PCR using genomic DNA isolated from the toe tips of newborn mice. Eight to nine-week old adult male or female mice were used in all experiments except for the growth rate determination.

RNA preparation and Quantitative Real-time PCR (qRT-PCR)

Intestines were dissected and divided into duodenum, jejunum, ileum, colon, and rectum. Total RNA was extracted in TRIzol (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcribed using 1 µg oligo(dT) primer, Super-Script II reverse transcriptase, and accompanying reagents (Invitrogen), and qRT-PCR was performed as described [25].

Immunohistochemistry

Intestines were dissected and divided into duodenum, jejunum, ileum, colon, and rectum, and fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut to 6-µm sections and processed for immunohistochemistry as described previously [25,26]. The primary antibodies for Foxa1/2 (Santa Cruz, 1:1000), chromogranin A (Immunostar, 20085, 1:2000), GLP-1 (Santa Cruz, sc-7780, 1:1000), GLP-2 (Santa Cruz, sc-7781, 1:100), somatostatin (Santa Cruz, sc-7819, 1:1000), PYY (Abcam, ab22663, 1:8000), cholecystokinin (Abcam, ab27441, 1:200), secretin (Abcam, ab8495, 1:100), GIP (Santa Cruz, sc-23554, 1:1000),

vasoactive intestinal peptide (Immunostar, 20077), gastrin (Nova Castra, NCL-GASp, 1:5000), and serotonin (Immunostar, 20080, 1:10000), BrdU (US Biologicals, B2850-01) were diluted in PBT (1xPBS with 0.1% Triton x-100 and 2% BSA). Slides were incubated in primary antibody overnight at 4°C, washed in 1xPBS, and then incubated with biotinylated anti-goat antibodies (Vector Laboratories, 1:200) or anti-rabbit antibodies (Vector Laboratories, 1:200) for 30 min at 37°C. Slides were rinsed with 1xPBS and incubated with HRP-conjugated ABC reagent (Vector Elite kit) for 30 min at 37°C. After washing, slides were developed using a DAB substrate kit (Vector Laboratories), and counter-stained with hematoxylin.

Assessment of Body Composition

Eight-week old male mice were injected intraperitoneally with sodium pentobarbital (40 mg/kg) and dual energy x-ray absorptiometry was performed (DEXA; Lunar PIXImus2; General Electric Medical systems, Madison, WI) to determine their body composition [27,28].

Electron Microscopy (EM)

Intestines were dissected and divided into duodenum, jejunum, ileum, colon, and rectum, washed with 1xPBS, and suspended in a fixative solution containing 2.5% cacodylate-buffered glutaraldehyde and 4% paraformaldehyde (pH 7.4) overnight at 4°C [10,20].

Chromatin immunoprecipitation (ChIP)

Foxa1 and Foxa2 ChIP were performed as described [29], using antibodies against Foxa1 (a gift from Dr. Günther Schütz) and Foxa2 (a gift from Dr. Jeffrey Whitsett). The ChIP PCR primers spanned the putative Foxa1 or Foxa2 binding site covering -200/-87 bp of the *Muc2* promoter (forward primer: 5'-ccaagtttaccaggagtcac-3'; reverse primer: 5'-gcatttgccaagttatcaggaa-3').

Detection of apoptosis in goblet and enteroendocrine cells

Apoptosis of goblet and enteroendocrine cells was detected by using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon, S7100), and ApopTag® Red In Situ Apoptosis Detection Kit (Chemicon, S7165), following manufacturer's instructions. Briefly, intestinal sections were deparaffinized by xylene followed by 95% and 70% ethanol. The sections were then pretreated with 20 µg/mL proteinase K. After quenching endogenous peroxidase with 3% hydrogen peroxide, the sections were incubated with equilibration buffer. Terminal deoxynucleotidyl transferase (TdT) was then applied to the sections and incubated for 1 hr at 37°C, after which the stop/wash buffer was applied to the sections for 10 min at room temperature. After incubation of the sections with anti-digoxigenin conjugate, sections were incubated in Acian blue solution for 20 min. For detection of enteroendocrine cells, sections were incubated with chromogranin A antibody overnight at 4°C before the application of anti-digoxigenin conjugate (rhodamine).

Results

Derivation of mice with intestine-specific deletion of *Foxa1* and *Foxa2*

Foxa1 and Foxa2 play important roles in the formation of the definitive endoderm during gastrulation and in the differentiation of endoderm-derived tissues [18,19]. In adult mice, *Foxa1* and *Foxa2* are expressed in all intestinal epithelia [12]. Since both Foxa1 and Foxa2 are present in the crypts where the stem cells reside, we hypothesized that Foxa1 and Foxa2 control epithelial cell differentiation. We derived mice deficient for *Foxa1* and *Foxa2* in the intestine using the Cre-loxP system as illustrated in Figure 1A. Immunohistochemical staining demonstrated that both Foxa1 and Foxa2 proteins were absent from the small intestine (Fig. 1E), colon and rectum (see supplemental Fig. 1) of *Foxa1*^{loxP/loxP}, *Foxa2*^{loxP/loxP}; Villin-Cre

mice (termed “*Foxa1/a2* mutants” in the following). Because the third member of the FoxA gene family, *Foxa3*, is also expressed in the intestine [13,30,31], we considered the possibility that this gene might be upregulated in *Foxa1/a2* mutants to compensate for the loss of *Foxa1* and *Foxa2*. Instead, we found *Foxa3* mRNA levels were significantly reduced in the *Foxa1/a2* mutants’ jejunum but not in the duodenum (Fig. 1B). Thus, there is no compensatory upregulation of *Foxa3* expression in *Foxa1/a2* mutants.

Foxa1/a2 mutants were born at the expected Mendelian ratio and their body weights were similar to control mice at birth (data not shown). However, the growth rate of both male and female *Foxa1/a2* mutants was reduced compared to that of controls from postnatal day 3 onward (Fig. 1C, supplemental Fig. 2). At 8 weeks of age, *Foxa1/a2* mutants were significantly smaller (26.8%) and shorter (7.3%) than littermate controls (Fig. 1D). DEXA Scan analysis determined that bone mineral content (BMC), lean muscle and fat weight were reduced by 16.8%, 22.3% and 42.4%, respectively (Fig. 1D). The percentage of whole body fat was not significantly different from controls (Fig. 1D).

Defective goblet cell differentiation in *Foxa1* and *Foxa2*-deficient mice

Examination of gross morphology showed that the overall anterior/posterior patterning of the intestine was normal in 8-week old *Foxa1/a2* mutants. To determine whether deletion of *Foxa1* and *Foxa2* affects epithelial cell proliferation, BrdU was given to 8-wk old mice at 1 hr or 48 hr before organ harvest by intraperitoneal injection. The intestinal sections were stained with anti-BrdU antibody. At 1 hr, the BrdU positive cells were present at the bottom of the crypts for both controls and mutants (supplemental Fig. 3). After 48 hr, the BrdU positive cells had migrated comparable distances up the crypt-villus axis in both controls and mutants (supplemental Fig. 3). These data demonstrate that epithelial cell proliferation is not affected by intestine-specific deletion of *Foxa1* and *Foxa2*.

Examination of the morphology of enterocytes or colonocytes showed that these cells displayed normal columnar morphology (supplemental Fig. 3). Paneth cells were also differentiated properly (data not shown). Next, we studied the effects of *Foxa1* and *Foxa2* deletion on goblet cell differentiation. We stained intestinal sections with Alcian blue, which labels the glycoproteins in the secretory granules of goblet cells. The number of Alcian blue positive cells was reduced throughout the intestinal tract (Fig. 2A), and quantification of goblet cells showed a significant decrease of goblet cell numbers in the duodenum, jejunum and ileum in the mutants compared to controls (Fig. 2B). In both colon and rectum, partially or completely empty vacuoles were observed in goblet cells of *Foxa1/a2* mutants by ultrastructural imaging (Fig. 3). Electron microscopy also showed reduced overall granule density, indicating fewer mucins contained within each granule in *Foxa1/a2* mutants compared to controls (Fig. 3).

The major secretory products of goblet cells are the mucin glycoproteins, among which Muc2 is the main component. *In vitro* studies by van der Sluis and colleagues had shown that *Foxa1* and *Foxa2* can bind to and transactivate the *Muc2* promoter [32]. We found *Muc2* mRNA levels decreased in the colon by 87%, and in the rectum by 86% in *Foxa1/a2* mutants but not significantly changed in the small intestine (Fig. 4A). Besides Muc2, Mucin 5b (Muc5b), Mucin 5ac (Muc5ac), and Mucin 6 (Muc6) are also present in the intestine. Both Muc5b and Muc5ac mRNA levels were significantly increased in the colon and rectum of *Foxa1/a2* mutants (Fig. 4B and 4C). In contrast, Muc6 mRNA levels were decreased in the colon by 99% and in the rectum by 69% in *Foxa1/a2* mutants compared to controls (Fig. 4D). Thus, the mucin expression profile is dramatically altered in the absence of *Foxa1* and *Foxa2*.

Trefoil factor 3 (Tff3), a protein secreted by goblet cells, plays an important role in the maintenance and repair of the intestinal mucosa [33]. The expression of Tff3 was dramatically reduced in the colon by 75% and rectum by 96% in *Foxa1/a2* mutants compared to controls

(supplemental Fig. 5A). Because *Klf4* had been shown previously to affect goblet cell differentiation in the colon, we determined its expression levels by qRT-PCR. *Klf4* expression was not altered in the colon but significantly reduced in the rectum (supplemental Fig. 5B).

To determine whether Foxa1 or Foxa2 can bind to the *Muc2* promoter *in vivo*, we performed ChIP assay with chromatin isolated from the small intestinal epithelium. Shown in Fig. 4E is the putative Foxa binding site as predicted by the JASPAR database, and the chromatin immunoprecipitation experiment demonstrating occupancy of this site by Foxa1 *in vivo*. Quantification of the ChIP experiment revealed that the *Muc2* promoter sequence was enriched in chromatin precipitated with the Foxa1-, but not the Foxa2- specific antibody (Fig. 4E and F). When we performed ChIP on *Foxa1/a2* mutant tissue, no enrichment to the *Muc2* promoter remained, demonstrating that our antibodies and ChIP conditions were specific for Foxa1 (Fig. 4E and F). Our data indicate that Foxa1 is the preferred transcription factor in binding and activating of the *Muc2* promoter. Consistent with our findings, previous *in vitro* studies also showed that Foxa1 is more efficient than Foxa2 in activating the *Muc2* promoter in co-transfection assays [32].

To determine whether the decrease in goblet cells was caused by increased apoptosis, intestinal sections were co-stained with Alcian blue and TUNEL. Very few goblet cells were TUNEL-positive in either controls or mutant tissue, indicating similar apoptosis rates of these two groups (supplemental Fig. 4A). These data suggest that decreased goblet cells number is more likely due to decreased goblet cell differentiation rather than increased apoptosis.

Enteroendocrine cell differentiation depends on Foxa1 and Foxa2

Foxa2 is an essential transcriptional regulator for the differentiation of pancreatic endocrine cells [21,26]. Moreover, both Foxa1 and Foxa2 can bind to and activate the preproglucagon promoter *in vitro* [17,34]. Two protein products from the preproglucagon gene, Glp-1 and Glp-2, are expressed in intestinal enteroendocrine L-cells. In adult mice, Foxa1 and Foxa2 localize to the intestinal crypts which contain stem cells and transit amplifying cells that produce all epithelial cells (Supplemental Fig 1, [12]). Therefore, we hypothesized that Foxa1 and Foxa2 contribute to enteroendocrine cell differentiation. In order to determine whether enteroendocrine cells were present in the mutant intestinal epithelium, we performed immunohistochemical analysis with antibodies against chromogranin A, a major component of the secretory granules of all enteroendocrine cells. Chromogranin A positive cells were scattered throughout the epithelium of the small intestine of control mice, while only a few chromogranin A positive cells were present in the *Foxa1/a2* mutants (Fig. 5). To determine whether the decrease in enteroendocrine cell number was due to increased apoptosis, intestinal sections were co-stained with chromogranin A and TUNEL. We observed very few chromogranin A/TUNEL double positive cells in either controls or mutants (supplemental Fig. 4B), suggesting that decreased enteroendocrine cell differentiation rather than increased apoptosis was the cause of reduced enteroendocrine cell number in *Foxa1/a2* mutant mice.

Next, we performed immunohistochemical staining with antibodies specific to the dominant hormone produced by each subtype. Glp-1 and Glp-2 positive cells were absent from the small intestine of *Foxa1/a2* mutants (Fig. 6). *Foxa1/a2* mutants also had fewer somatostatin and PYY positive cells (Fig. 6). In contrast, staining for other principal intestinal hormones including cholecystokinin (CCK), secretin (SEC), gastrin (GAS), serotonin (SER), GIP, and vasoactive intestinal polypeptide (VIP) showed that expression of these enteroendocrine hormones is *Foxa1/a2* independent (data not shown). In addition, preproglucagon mRNA levels were undetectable in the intestine of *Foxa1/a2* mutants by qRT-PCR (Fig. 7A). Compared with control mice, somatostatin mRNA levels in *Foxa1/a2* mutant mice were reduced by 71% and 83% in the duodenum and jejunum, respectively (Fig. 7B). *PYY* expression is restricted to the ileum and was significantly decreased (80%) in *Foxa1/a2* mutants compared to controls (Fig.

7C). Thus, *Foxa1* and *Foxa2* are required for L-cell (Glp-1, Glp-2, PYY) and D-cell (somatostatin) differentiation in the mouse small intestine.

Foxa1 and Foxa2 act upstream of Isl-1 and Pax6 to control L- and D-cell differentiation

Ngn3, Isl-1, Beta2, Pax4, Pax6, Nkx2.2 and Insm-1 are transcription factors required for normal enteroendocrine cell differentiation [1–4,8,9,35]. Ngn3 is necessary for the elaboration of all intestinal enteroendocrine cell types and controls enteroendocrine cell fate commitment of Math1⁺ secretory lineage progenitors [1]. Beta2 acts downstream of Ngn3 to regulate cholecystokinin and secretin-producing cells [1]. To determine at which stage of enteroendocrine cell differentiation Foxa1 and Foxa2 are required, we examined the expression of *Ngn3*, *Isl-1*, *Beta2*, *Pax4*, *Pax6*, *Nkx2.2* and *Insm-1* by qRT-PCR in *Foxa1/a2* mutants and controls. *Ngn3* and *Beta2* expression was unchanged in the *Foxa1/a2* mutants jejunum compared to controls (Fig. 7D), suggesting that Foxa1 and Foxa2 act downstream of Ngn3 in the enteroendocrine cell lineage (Fig. 8). In contrast, *Isl-1* and *Pax6* expression levels were reduced by 59% and 69%, respectively (Fig. 7D and E), while *Pax4* expression was unchanged in the ileum of *Foxa1/a2* mutants (Fig. 7E). The expression of Nkx2.2 and Insm-1 was unchanged as well (supplemental Fig. 5). Isl-1 and Pax6 are transcription factors that bind to and transactivate the preproglucagon promoter [36,37]. We propose that Isl-1 and Pax6 act downstream of and coordinately with Foxa1/Foxa2 to regulate L-cell differentiation (Fig. 8). Moreover, Isl-1 can also bind to and transactivate somatostatin [38] promoter. Thus, Foxa1/Foxa2 and Isl-1 cooperate to control D-cell differentiation in a feed-forward loop. Although Isl-1, Pax6 and PYY expression were reduced in *Foxa1/a2* mutants, ChIP experiments using Foxa1 or Foxa2 antibodies did not reveal direct binding of Foxa1 or Foxa2 to the Isl-1, Pax6 or PYY promoters (supplemental Fig. 6).

Discussion

The intestinal epithelium renews every three to four days in mice through the differentiation of transit amplifying cells emerging from the crypts. Multiple transcription factors are involved in this process, including Math1, Ngn3, Isl-1, Pax4 and Pax6, NeuroD, Nkx2.2 and Insm-1. Here, we propose that Foxa1 and Foxa2 are additional transcription factors important for proper differentiation of goblet and enteroendocrine cells. Goblet cells function to produce mucus to protect the gut, and alteration in mucin expression has been implicated in intestinal inflammation and carcinogenesis [39–41]. We observed decreased numbers of goblet cells throughout the intestinal tract of *Foxa1/a2* mutants. *Foxa1/a2* mutants also had significantly reduced Muc2 expression in the colon and rectum at age of 8 weeks, while the entire mucin pattern was perturbed (Fig. 4). Taken together, Foxa1 and Foxa2 are important factors for goblet cell differentiation, and possibly the maintenance of intestinal health.

The endocrine cells of the gastrointestinal tract form the largest endocrine system of the body. Hormones produced by enteroendocrine cells control various functions such as glucose metabolism, exocrine pancreatic secretion, the growth and repair of the intestinal epithelium, and the motility of the intestinal wall [35]. Several transcription factors such as Isl-1, Pax6, Pax4, Beta2, Nkx2.2 and Insm-1 are not only important for the expression of gut hormones but also involved in the differentiation of these enteroendocrine cells. The present study shows that Foxa1 and Foxa2 control the differentiation of enteroendocrine L- and D-cells via regulating expression of several important transcription factors including Isl-1 and Pax6 (Fig. 7 D and E). The current model of the differentiation of the secretory cell lineages in the mammalian intestine is summarized in Figure 8.

The principal hormones expressed in L-cells, Glp-1 and Glp-2, were absent from the intestine of *Foxa1/a2* mutants (Fig. 6). Transcription of the preproglucagon gene is controlled by numerous transcription factors, including Isl-1, Pax6, and Foxa1 or Foxa2 [17,26,34,36,42].

Preproglucagon promoter activity is regulated through G1, G2, G3, and G4 elements. Both Foxa1 and Foxa2 can interact with similar affinities to either the G1 or G2 element of preproglucagon promoter *in vitro* [17,34]. Recently, an additional conserved Foxa binding site located close to the transcription start site of the preproglucagon promoter has been identified and shown to bind preferentially to Foxa1 in a glucagon-producing pancreatic islet α -cell line [43]. Moreover, *Foxa1* null mice have markedly reduced levels of plasma glucagon [17]. Consistent with prior work in the pancreas, our study shows that deletion of both Foxa1 and Foxa2 ablates preproglucagon transcripts in the small intestine (Fig. 7A).

Pax6 is a critical regulator of pancreatic α -cell differentiation during development [42]. It is a transcription factor of preproglucagon gene and inhibition of Pax6 led to decreased endogenous glucagon expression in pancreatic α -cell lines [44]. Mice expressing a dominant-negative Pax6 mutant have reduced preproglucagon mRNA levels and lack Glp-1 and Glp-2 positive cells in the small and large intestine [37]. Pax6 activates the preproglucagon promoter through G1 and G3 elements [45]. *In vitro*, Pax6 binds to an overlapping site on G1 element with a greater affinity as Foxa1 or Foxa2 [34]. Binding of Foxa1 and Foxa2 impairs Pax6-mediated transactivation of the preproglucagon promoter via G1 and G3 elements in co-transfection assays, suggesting an antagonism between Pax6 and the Foxa factors in the regulation of this gene [34]. We show here that intestine-specific deletion of *Foxa1* and *Foxa2* causes decreased Pax6, Glp-1 and Glp-2 expression (Fig. 6 and Fig 7). Thus, our data indicate that in small intestine Foxa1 and Foxa2 control Pax6 transcription, and Pax6 acts downstream of and in concert with Foxa1 and Foxa2 to regulate preproglucagon gene transcription in a feed-forward loop.

Isl-1 is another important transcription factor of preproglucagon gene transcription, and *Isl-1* null mice exhibit defective islet development and α -cell formation [36,46]. In early embryogenesis, Isl-1 can be detected in the foregut, implicating its possible role in the gut development [47]. Our data showed that *Isl-1* expression is significantly reduced in mice with intestine specific deletion of *Foxa1* and *Foxa2* (Fig. 7D), suggesting that Foxa1 and Foxa2 may regulate *Isl-1* gene transcription and Isl-1 lie downstream of *Foxa1/a2*-mediated enteroendocrine lineage. Reduction of *Isl-1* expression could contribute to the significant decrease of preproglucagon gene expression in *Foxa1/a2* mutants.

Foxa1/a2 mutants are smaller and have lower bone mineral content, less lean muscle and body fat than age-matched controls. These phenotypes can be explained, in part, by reduced action of Glp-2, PYY and somatostatin. Glp-2 functions as a key regulator of mucosal integrity, permeability, and nutrient absorption (reviewed in [48]). While we did not observe decreased epithelial cell proliferation in the small intestine of *Foxa1/a2* mutants, the lack of Glp-2 may affect nutrient absorption and thus body weight. PYY is important for water and electrolyte absorption [49], and indeed we observed increased water loss via the feces of *Foxa1/a2* mutants (data not shown). Somatostatin controls gastric emptying, reduces smooth muscle contraction and blood flow within the intestine, and suppresses the release of several gastrointestinal hormones including gastrin, cholecystokinin, secretin, VIP, GIP, Glp-1 and Glp-2 ([50], reviewed in [51,52]). *Foxa1/a2* mutants have relatively normal intestinal gastrin, cholecystokinin, secretin, VIP, and GIP expression as shown by immunohistochemical analysis and qRT-PCR (data not shown), but we cannot exclude that altered somatostatin levels might affect plasma levels for these hormones.

Since Glp-1 is important for glucose metabolism, intestine-specific deletion of *Foxa1* and *Foxa2* may have an impact on glucose homeostasis. Glp-1 stimulates insulin and inhibits glucagon secretion from the endocrine pancreas, thus contributing to postprandial glucose uptake (reviewed in [53,54]). It also inhibits gastrointestinal motility, regulates appetite and food intake (reviewed in [53,54]). In the pancreas, Glp-1 stimulates β -cell proliferation and

neogenesis, and prevents β -cell apoptosis [55–58]. Surprisingly, the resting glucose level was normal in *Foxa1/a2* mutants (data not shown). One possibility for this finding is that deletion by the Villin-Cre transgene is not complete in the proximal duodenum, and that the remaining L-cells present there produce enough Glp-1 to control glucose homeostasis. Another possibility for the normal glucose homeostasis in our model is the fact that GIP expression is normal in *Foxa1/a2* mutants, and both GLP-1 and GIP are insulinotropic peptides. GIP-receptor deficient mice have higher blood glucose levels with impaired initial insulin response in the oral glucose test [59]. GLP-1 receptor null mice had mild glucose intolerance and impaired insulin secretion after intraperitoneal glucose injection [60]. Interestingly, GLP-1 receptor null mice had increased plasma GIP levels, suggesting a compensatory relationship between the two signaling mechanisms [61]. It might be interesting to test the metabolic response of *Foxa1/a2* mutants to a high fat diet, which is often used to uncover metabolic defects, in the future.

In conclusion, we demonstrate that intestine-specific deletion of *Foxa1* and *Foxa2* alters the differentiation of goblet and enteroendocrine L- and D-cells. In goblet cells, *Foxa1* binds to the *Muc2* promoter preferentially over *Foxa2*. In L- and D-cells, *Foxa1* and *Foxa2* control expression of Glp-1/2, somatostatin and PYY both directly and through feed-forward regulation of *Isl-1* and *Pax6*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Gary Wu for critical reading of the manuscript, and Ms. Karrie Brondell for care of the mouse colony. We acknowledge the Penn Diabetes and Endocrinology Research Center Mouse Metabolic Phenotyping Core (P30-DK-19525) for performing mouse body composition and CLAMS studies, and we want to thank Ravindra Dhir and Xiaoyan Yin for excellent technical support. This study was supported by the NIH (DK R01-053839).

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Figure 1. Reduced body weight and slower growth rate in mice with intestine specific deletion of *Foxa1* and *Foxa2*

A) Scheme for generation of mice with intestine specific deletion of *Foxa1* and *Foxa2*. Two loxP sites were inserted to flank exon 2 of *Foxa1* or exon 3 of *Foxa2*. Villin-cre transgenic mice were employed to achieve intestine-specific deletion. B) qRT-PCR demonstrated that *Foxa3* expression was unchanged in the duodenum (D) but decreased in the jejunum (J) of *Foxa1*, *Foxa2*; villin-cre mice (*Foxa1/a2* mutants) (n=4, male). C) Growth rate of *Foxa1/a2* mutants is lower than controls (n=4, male). D) DEXA Scan showed that *Foxa1/a2* mutants have lower bone mineral content (BMC), lean muscle, and whole body fat. (n=4, male). E) Representative image of immunohistochemical analysis showing that *Foxa1* and *Foxa2* were

deleted in the ileum of *Foxa1/a2* mutants (n=4, male). All the qRT-PCR results were normalized to the expression of “house-keeping gene” hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are means $\pm$ SE, *p<0.05, **p<0.01 vs. controls.

Figure 2. Defective goblet cell differentiation in *Foxa1/a2* mutants

A) Alcian blue staining of jejunum, colon and rectum. *Foxa1/a2* mutant mice have a progressive decrease of the number of goblet cells (blue) along the small intestine, colon and rectum. B) The number of goblet cells was counted in the duodenum, jejunum and ileum (n=4, male). Data are means $\pm$ SE, *p<0.05 vs. controls.

Figure 3. Reduced mucin granules in goblet cells of *Foxa1/a2* mutants

Representative image of EM analysis showing decreased mucin granules in colon and rectum goblet cells of *Foxa1/a2* mutants (n=2, male). Red arrows indicates vacuoles with complete or partial loss of mucin granules.

Figure 4. Defective goblet cell differentiation in *Foxa1/a2* mutants

qRT-PCR was performed to measure mRNA levels of Muc2 (A), Muc5b (B), Muc5ac (C), and Muc6 (D). Muc2 and Muc6 mRNA levels were significantly reduced in the colon and rectum of *Foxa1/a2* mutants compared to controls. Muc5b and Muc5ac mRNA levels were significantly increased in the colon and rectum of *Foxa1/a2* mutants compared to controls. All qRT-PCR results were normalized to the expression of HPRT. E) Top panel: Putative Foxa binding site in the *Muc2* promoter as predicted by JASPAR. Lower panel: A gel representation of *Muc2* promoter enrichment after ChIP with Foxa1 antibody. The myelin basic protein (MBP) gene was used as a nonspecific control. F) qRT-PCR analysis of ChIP performed using Foxa1 or Foxa2 antibody. Enrichment of the *Muc2* promoter was calculated by using MBP. Foxa1 is

the preferred transcription factor for binding to the *Muc2* promoter. Data are means $\pm$ SE, * $p < 0.05$, ** $p < 0.01$ vs. controls.

Figure 5. Decreased enteroendocrine cell differentiation in *Foxa1* and *Foxa2* mutants
Immunohistochemical analysis of duodenum, jejunum, and ileum with an antibody against chromagranin A. The number of chromagranin A positive cells was decreased in *Foxa1/a2* mutants compared to controls.

Figure 6. Decreased differentiation of L- and D-cells in *Foxa1/a2* mutants

Immunohistochemical staining was performed using antibodies against Glp-1, Glp-2, somatostatin or PYY. Glp-1 and Glp-2 positive cells (brown and arrow) were absent from the ileum of *Foxa1/a2* mutants (20 $\times$). Somatostatin (SST) positive cells (brown and arrow) were reduced in the jejunum of *Foxa1/a2* mutants (20 $\times$). PYY positive cells (brown and arrow) were reduced in the ileum of *Foxa1/a2* mutants (20 $\times$).

Figure 7. Isl-1 and Pax6 act downstream of Foxa1 and Foxa2 in the enteroendocrine cell lineage qRT-PCR was performed to measure mRNA levels of preproglucagon (A), somatostatin (B), PYY (C) (n=4, male). Preproglucagon and somatostatin mRNA levels were significantly decreased in the duodenum and jejunum of *Foxa1/a2* mutants. The PYY mRNA level was significantly decreased in the ileum of *Foxa1/a2* mutants. D) qRT-PCR was performed to measure mRNA levels of Isl-1, Ngn3, and Beta2. Isl-1 expression was significantly reduced while Ngn-3 and Beta2 mRNA levels were not altered. E) qRT-PCR was performed to measure mRNA levels of Pax6 and Pax4. Pax6 mRNA expression was significantly decreased but Pax4 expression was unchanged. (n=4, male) Data are means $\pm$ SE, *p<0.05, **p<0.01 vs. controls.

Figure 8. Proposed model for the role of Foxa1 and Foxa2 during differentiation of the secretory cell lineage in the mammalian intestine

During the process of intestinal epithelial cell regeneration, stem cells located in the crypts divide to generate multiple cell lineages. Secretory cell lineages derive from a common progenitor expressing *Math1* [6]. *Ngn3* controls enteroendocrine cell fate commitment of *Math-1* positive progenitors of the secretory lineage [1]. *Beta2* acts downstream of *Ngn3* and is required specifically for differentiation of cholecystokinin (CCK) and secretin (SEC)-producing cells [1]. The present study showed that Foxa1 and Foxa2 act downstream of *Ngn3* in the enteroendocrine cell lineage. We propose that *Isl-1*, *Pax4* and *Pax6* act downstream of Foxa1/Foxa2 to regulate the L- and D-cell differentiation. Moreover, Foxa1 and Foxa2 are also critical for goblet cell differentiation and function.