Impairment of gastric acid secretion and increase of embryonic lethality in Foxq1-deficient mice

W. Goering a I.M. Adham a B. Pasche a J. Männ er b M. Ochs c W. Engel a
B. Zoll a

a Institute of Human Genetics, and b Department of Anatomy and Embryology, University of Göttingen, Göttingen (Germany); c Institute of Anatomy, University of Bern, Bern (Switzerland)

Accepted in revised form for publication by M. Schmid, 18 January 2008.

Abstract. The mouse Foxq1 gene, also known as Hfh1, encodes a winged helix/forkhead transcription factor. In adult mice, Foxq1 is highly expressed in kidney and stomach. Here, we report that Foxq1 is expressed during prenatal and postnatal stomach development and the transcripts are restricted to acid secreting parietal cells. Mice homozygous for a deletion of the Foxq1 locus on a 129/Sv × C57BL/6J hybrid genetic background display variable phenotypes consistent with requirement of the gene during embryogenesis. Approximately 50% of Foxq1−/− embryos die in utero. Surviving homozygous mutants are normal and fertile, and have a silky shiny coat. Although the parietal cell development is not affected in the absence of Foxq1, there is a lack of gastric acid secretion in response to various secretagogue stimuli. Ultrastructural analysis suggests that the gastric acid secretion defect in Foxq1-deficient mice might be due to impairment in the fusion of cytoplasmic tubulovesicles to the apical membrane of secretory canaliculi.

Gastric acid secretion is required for normal digestion and nutrient absorption in human as well as for the sterilization of fluids and the prevention of bacterial overgrowth (Champagne, 1989). Gastric acid is secreted from parietal cells, which is one of the most abundant cell types in the corpus and antrum of the stomach. Histamine, gastrin and acetylcholine are the major physiological stimuli of acid secretion, which is mediated via binding of these ligands to their receptors located in the basolateral plasma membrane of parietal cells (Fukushima et al., 1999). Activation of the receptors results in initial elevation of intracellular Ca2+ and/or cAMP levels followed by activation of a cAMP-dependent kinase cascade that regulates membrane trafficking (Chew, 1985). Parietal cells contain abundant intracellular membrane compartments, known as tubulovesicles that contain H+,K+-ATPase proton pumps, located inside the tubulovesicular membrane. Upon stimulation, the tubulovesicles fuse with the apical membrane to form the extended secretory canaliculus. Exposition of the H+,K+-ATPase to the gastric lumen enables gastric acid secretion (Urushidani and Forte, 1997).

The mammalian forkhead box (FOX) family of transcription factors is divided into subclasses of proteins that share high homology in the winged helix DNA binding domain (Clark et al., 1993; Kaestner et al., 2000). Members of this evolutionarily conserved family are known to regulate cell fate, proliferation and tissue-specific expression in different organisms (Kaufmann and Knochel, 1996). We have characterized a member of a new subfamily of Fox genes, Foxq1, and found that Foxq1 is highly expressed in kidney and stomach (Frank and Zoll, 1998). In vitro studies showed that Foxq1 represses telokin promoter activity when overexpressed in the A10 vascular smooth muscle cells (Hoggatt et
The role of FOXQ1 for mammalian hair follicle development was revealed by the study of satln mutant mice (sa), in which a mutation in the Foxq1 gene has been found (Hong et al., 2001). These mice have a silky shiny coat-appearance, attributable to aberrant differentiation of the hair shaft. However, a role of FOXQ1 in development and function of stomach and kidney has not been reported.

Here, we show that the specific expression of Foxq1 in stomach is restricted to parietal cells. To address the function of Foxq1, we used homologue recombination in ES cells to delete the protein-coding region of the gene. On a 129/Sv × C57BL/6j hybrid genetic background, 50% of the homozygous null embryos die in utero. Surviving Foxq1+/− pups appear normal at birth and develop a silky coat. Analysis of gastric physiology in the mutant animals demonstrates that FOXQ1 is required for the function of the acid secretory system. These variable phenotypes provide evidence for multiple roles of FOXQ1 during embryogenesis and adult life.

Materials and methods

Generation of Foxq1-deficient mice

A lambda phage clone carrying the complete Foxq1 gene was isolated from a 129/Sv genomic mouse library (Frank and Zoll, 1998). The genomic clone was characterised by restriction enzyme mapping and sequencing. The 10-kb NotI-Xhol and 2.5-kb Srf1 fragment genomic segments were subcloned into the Qua1Tox vector (Qiogene). The 2.5-kb Srf1 fragment containing the 3′-flanking region was isolated and ligated with Xhol/ EcoRI-digested pNPT vector (Tybulewicz et al., 1991) after treating with Klenow enzyme (clone Foxq1/1). The 10-kb NotI-Xhol fragment was used as a 5′-flanking region and inserted into the NotI-Xhol restricted clone Foxq1/1. The resulting targeting vector (Fig. 2A) was linearised with NotI and transfected into R1 embryonic stem (ES) cells, and clones resistant to G418 were isolated (Wurst and Joyner, 1993). Genomic DNA extracted from drug-resistant ES clones was digested with BamHI, electrophoresed and blotted onto nitrocellulose membrane. The 453-bp Srf1 fragment located externally 3′ of the targeting vector was radioactively labelled and used to probe the Southern blots. Hybridization was carried out at 65°C overnight in RapidHyb buffer (Amersham Bioscience) following the manufacturer’s protocol. Filters were washed twice at 65°C at a final stringency of 0.2× SSC, 0.1% SDS. Cells from two recombinant ES clones were injected into C57BL/6j blastocysts, and these were transferred into DBA/BL6 pseudo-heritable strains. Germ line-transmitting chimeric males obtained from both lines were backcrossed to C57BL/6j and 129/Sv females. Germ line-transmitting chimeric males obtained from adults that had been fasted overnight. For Northern blot analysis, 20 μg of RNA was electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose membranes (Amersham Bioscience) and hybridized with 12P-labeled probe at the same conditions as used for Southern blot hybridization.

For semi-quantitative RT-PCR analysis, total RNA (2 μg) was reverse transcribed into cDNA at 42°C for 50 min using a poly (dT)-oligodUro nucleotide and the Superscript Reverse Transcriptase kit (Invitrogen). One microliter of the cDNA was then subjected to 35 cycles of PCR. Aliquots of amplified products were taken after the PCR-cycles 28, 30, 32 and 35, and analyzed in 1.5% agarose gels. Primer sequences to amplify cholecystokinin receptor (Cckbr; Sos2), histamine H2 receptor (H2r), ezrin (Vil2), telokin (Mykl) and Hprt were 5′-GATGTGATATAATGACAGCGAGA-3′ and 5′-AGGTGATGCTTCAGCAGAAGTTGAF-3′, 5′-TCTCCTACACCCTCTACTTCC-3′ and 5′-ATCCATT-CCACCAGTCCATAA-3′, 5′-CGAGAAGAGGCGAGAGA-3′ and 5′-TGTAGAGCCATAGGCTCTCT-3′, 5′-CGAGAACATCATGTGTGTC-3′ and 5′-CCATGTCTCTGTCTTTTGTAT-3′, 5′-CGCTGTTGATTTGAGATATG-3′ and 5′-TATGTCCCCCCTTGACTTATG-3′, respectively.

Measurements of gastric pH and output of acid-base equivalents

A measurement of gastric acid output was performed by modification of the method described by Wood and Dubois (1983). Briefly, 3-month-old mice were fasted overnight with free access to water. Each mouse was injected intraperitoneally with a sterile solution of histamine HCl (10 μg/g body weight, Sigma) or pentagastrin (1μg/g body weight, Sigma) in 0.9% NaCl solution. At different time points after injection, mice were sacrificed and the abdomen was immediately opened. After clamping the gastroesophageal and pyloric junctions, the stomach was then immersed in 2 ml oxygen-saturated normal saline solution and opened along the greater curvature to release the gastric contents. The stomach was then removed, blotted dry with absorbent paper, and weighed. The gastric content was centrifuged at 500 g for 5 min, and the supernatant was used for measuring the pH value. After determination of the pH, the supernatant was titrated with 0.01 N NaOH or 0.01 N HCl. Results are expressed as microequivalents of H+ or OH− per gram of stomach wet weight.

Biochemical analysis of blood

Trunk blood was collected into heparinised tubes for analysis on an AVL OMN19 (Roche, Mannheim, Germany) blood gas analyzer.

Histological analysis, immunohistochemistry and in situ hybridization

Embryos were collected in PBS, fixed in Bouin’s fixative, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Stomachs were opened and washed three times in PBS to remove stomach content. Stomachs were fixed in Bouin’s fixative or pinned on cork and then fixed in 4% paraformaldehyde. After embedding in paraffin, and sectioning, at 5–7 μm, Bouin’s fixed sections were used for immunohistochemical analysis. Paraformaldehyde-fixed sections were subjected to immunohistochemical analysis and in situ hybridization.

For immunohistochemistry, paraformaldehyde-fixed sections were preincubated for 1 h with 5% normal goat serum in 0.05% Triton X-100/PBS and incubated overnight at 4°C with the primary antibodies, washed three times in PBS and incubated for 1 h with secondary antibodies, washed three times with PBS, and then stained with DAPI (4’,6-diamidino-2-phenylindole, Vector). Slides were examined with a BX60 microscope (Olympus, Hamburg, Germany) with fluorescence equipment and analysis software program (Soft Imaging System, Münster, Germany). Monoclonal anti-H-, K+-ATPase β-subunit was diluted at 1:1000 (Acris Antibodies, Hiddenhausen, Germany). Secondary antibodies were goat anti-mouse fluorescein isothiocyanate (Sigma).

The digoxigenin-UTP-labeled riboprobes were synthesized using the DIG RNA labelling Kit (Roche, Germany) from linearized plasmid templates containing the 5′ region of Foxq1 cDNA. Antisense and sense RNA probes were then used to hybridize the stomach sections. Prehybridization, hybridization and washing was performed according to the manufacturer’s manual of DIG Nucleic Acid Detection Kit (Roche). Slides were imaged as described above.
Results

Foxq1 is expressed in the parietal cells of gastric mucosa

Expression analyses of Foxq1 in adult mouse tissues revealed that Foxq1 is highly expressed in the stomach (Frank and Zoll, 1998). To evaluate the expression of Foxq1 during prenatal development and stomach maturation, we performed RT-PCR and Northern blot analyses. As shown in Fig. 1A, Foxq1 transcripts were detected by RT-PCR at different stages (from embryonic days 8.5 to 12.5). Northern blot analysis showed a high expression of Foxq1 in the stomach at embryonic day 15.5 and the expression levels were maintained through neonatal stages and adulthood (Fig. 1B). To ascertain whether a specific compartment of the stomach shows high expression of Foxq1, in situ hybridization was undertaken on sections of adult stomach. The antisense Foxq1 probe hybridized with cells located in the glandular epithelium (Fig. 1C). Immunohistochemical staining of equivalent serial sections with antibodies to human elongation factor-1 revealed the integrity of RNA loading.

Targeted disruption of Foxq1

To investigate the physiological function of Foxq1 in the mouse, we generated Foxq1-deficient mice by gene targeting. A Foxq1 targeting construct was designed to replace a 2.0-kb genomic fragment containing the entire coding region of Foxq1 with a pgk-neo selection cassette (NEO). The 3′ external probe used and the predicted length of BamHI restriction fragments in Southern blot analysis are shown. Abbreviations: TK, Thymidine kinase cassette; N, NotI; B, BamHI; X, Xhol; S, SstI; K, KpnI. (B) Southern blot analysis of recombinant ES cell clones. Genomic DNA extracted from ES cell clones was restricted with BamHI and probed with the 3′ probe shown in panel A. The wild-type allele generated a 10-kb BamHI fragment, whereas the targeted allele yielded a 5-kb BamHI fragment, as indicated in panel A. (C) Northern blot analysis with total RNA isolated from stomach of Foxq1+/+, Foxq1+/− and Foxq1−/− adult mice was hybridized with a Foxq1-specific cDNA probe. Rehybridization of blot with human elongation factor-1 (EF1) revealed the integrity of RNA loading.

Electron microscopy

Mice were injected with pentagastrin as described above. After 60 min of gastrin treatment the stomach was dissected and fixed in freshly prepared 1.5% paraformaldehyde, 1.5% glutaraldehyde in 0.15 M HEPES buffer. Tissue blocks were osmicated, stained in 1.5% aqueous uranyl acetate overnight, dehydrated in acetone, and finally embedded in longitudinal and transversal orientation in araldite as described previously (Fehrenbach and Ochs, 1998).
detected a 10-kb BamHI wild-type fragment and a 5-kb BamHI recombinant fragment (Fig. 2A and B). Two Foxq1+/– ES cell lines with a correctly targeted allele were identified and cells were injected into C57BL/6J blastocysts. The resulting male chimeras were crossed with C57BL/6J or 129/Sv females to establish the Foxq1-disrupted allele on a C57BL/6J × 129/Sv hybrid and on a 129/Sv inbred genetic background. Both ES cell lines transmitted the mutation into the germline. The heterozygous animals appeared normal and were intercrossed to obtain homozygous null animals. Foxq1–/– animals exhibited normal growth and survival compared with wild-type littermates. Both male and female Foxq1–/– mice were fertile. However, Foxq1–/– mice display satin hair similar to satin mutant mice, which have a spontaneous mutation in the Foxq1 locus (Hong et al., 2001).

To confirm the inactivation of Foxq1 we performed Northern blot analyses. Total RNA from stomach tissues was isolated from 3-month-old wild-type, Foxq1+/– and Foxq1–/– mice. Northern blot analysis failed to detect Foxq1 mRNA in the Foxq1–/– stomach (Fig. 2C).

**Foxq1-deficient mice exhibit increased embryonic lethality**

Expression of Foxq1 in early embryonic development led us to determine the consequence of Foxq1 deficiency for embryonic development. A total of 191 F2 live-born progeny at three weeks was genotyped and we found 31.7% Foxq1+/+, 53.3% Foxq1+/– and 15.0% Foxq1–/– mice. Litter size of Foxq1–/– intercrosses was significantly smaller (average litter size, 4.5 ± 2.2 [n = 15]; average wild-type litter size, 9.1 ± 1.3 [n = 15]). These results prompted us to determine the embryonic stage where the reduction of Foxq1–/– embryos occurs. Embryos from timed wild-type and Foxq1+/– mating were analysed at different days of gestation (Fig. 3A). At E8.5, no deviation in the number of embryos was observed between these groups. At E10.5, the number of Foxq1–/– embryos decreased to approximately 85%, dropped to 69% at E12.5 and 44% at E15.5. The wild-type control group exhibits a decrease of approximately 6% between E8.5 and E15.5. Morphological examinations revealed that around 50% of Foxq1-deficient embryos at E10.5 display head abnormalities. Head malformation was characterized by shrinkage of the brain vesicle possibly due to reduced cerebrospinal fluid volume (Fig. 3D and E). However, normal developed Foxq1+/– embryos did not differ histologically from wild-type littermates (Fig. 3B and C). No head malformation was observed in Foxq1-deficient embryos at E12.5. However, a notable number of resorbed embryos were recovered from litters examined at E12.5 indicating the lethality of malformed Foxq1–/– embryos between E10.5 and E12.5. In all examined Foxq1–/– embryos, we observed normal organogenesis of fetal liver, heart and extraembryonic placenta tissue when compared with Foxq1+/– and Foxq1+/– littermates (data not shown). Therefore, the exact cause of embryonic lethality, although undetermined, cannot be attributed to a placental defect. The observed embryonic lethality of Foxq1–/– is restricted to the C57BL/6J × 129/Sv mixed genetic background.

**Loss of acid secretion in Foxq1-deficient mice**

Histological analysis was performed with adult kidney and stomach, in which Foxq1 is predominantly expressed. Despite the high levels of Foxq1 in both tissues, no significant histopathology was observed in these tissues of Foxq1–null mice (Fig. 4A and B, and data not shown).

To determine whether the absence of Foxq1 in kidney might cause disturbance of acid-base or ion homeostasis, we analysed blood gases, pH and electrolyte concentration under normal conditions. Blood pH, HCO3, and Na+, Ca2+ and K+ concentration were virtually identical in both groups of wild-type and Foxq1–null mice (data not shown) indicating that the lack of Foxq1 caused no significant perturbation of acid-base or ion homeostasis.
To determine the consequence of Foxq1 deficiency on the development of parietal cells, we performed immunohistochemistry using a parietal specific marker, H+K+-ATPase β-subunit (Fig. 4 and D). Cell counts of H+K+-ATPase β-subunit expressing cells per gastric unit of Foxq1+/+ and Foxq1−/− mice showed the presence of roughly equivalent numbers of parietal cells in the wild-type (12.8 ± 1.9) and Foxq1−/− mice (12.0 ± 1.5) mice (P > 0.05; n = 50 glands). This result indicates that the Foxq1 deficiency does not impair the development of parietal cells.

The specific expression of Foxq1 in the parietal cells, which are the acid secreting cells within the stomach, prompted us to assume a role of Foxq1 in the regulation of gastric acid secretion. We first measured intragastric pH in overnight fasted mice. The basal gastric pH in Foxq1-null mice was not significantly different from that in wild-type mice (6.79 ± 0.22 vs. 6.97 ± 0.18; P > 0.05) (Fig. 5A). Acid secretion was then measured at different time points after administration of histamine and gastrin, and found that gastric acid secretion is highly stimulated in wild-type stomach after 30 and 60 min of histamine and gastrin treatment, respectively (data not shown). In stomach of Foxq1−/− mice, acid secretion was undetectable and there was no response to stimulation with histamine or gastrin. In contrast, the acid output induced by histamine and gastrin was increased approximately sevenfold in wild-type mice (40.5 ± 10.9 μEq/g and 39.4 ± 12.9 μEq/g, respectively), as compared to basal level (5.8 ± 5.9 μEq/g) (Fig. 5B and C).

Expression analysis of genes regulating gastric acid secretion

We examined the consequence of Foxq1 deficiency on the expression of several genes, whose inactivation in mice affects gastric acid secretion. Northern blot analysis did not show significant difference in the expression of gastrin (Gast) and H,K ATPase α-subunit (Atp4a) in Foxq1+/+ and Foxq1−/− stomach, whereas the expression of somatostatin was significantly reduced in Foxq1−/− stomach (Fig. 6A). A decreased expression of somatostatin mRNA in stomach...
was not observed in pancreas of Foxq1−/− (data not shown). In addition, semi-quantitative RT-PCR analysis of histamine H2-receptor (Hrh2), gastrin-receptor (Cckbr), ezrin (Vil2) expression did not exhibit marked differences between wild-type and mutant mice (Fig. 6B). Hoggatt et al. (2000) reported that Foxq1 regulates the expression of the smooth muscle protein telokin (Mylk). However, RNA analysis did not show a distinct alteration in Mylk expression in Foxq1−/− stomach (Fig. 6B).

**Defects in the formation/expansion of apical secretory canaliculi in Foxq1−/− parietal cells**

We investigated whether the defects in gastric acid secretion can be correlated with ultrastructural alteration of Foxq1−/− parietal cells in resting state and upon secretagogue stimuli. Foxq1+/+ and Foxq1−/− mice were fasted in order to suppress the secretion of gastric juice. In wild-type and Foxq1-deficient parietal cells, secretory canaliculi with numerous microvilli were readily observed. Tubulovesicles, which are similar in dimension and appearance to canalicul microvilli, were densely located just beneath the canalicula membrane (Fig. 7A and B). To determine the effect of secretagogue stimuli on parietal cells, mice were fasted overnight, and stomachs were collected for fixation 60 min after gastrin treatment. Measurements of pH of gastric juice from wild-type stomach indicated that the drug treatments were effective (data not shown). In gastrin-stimulated wild-type parietal cells, the luminal space of the secretory canaliculi was markedly expanded and tubulovesicles were decreased in number due to a fusion of cytoplasmic tubulovesicular structures to the apical canalicular plasma membrane (Fig. 7C). In contrast, most of Foxq1−/− parietal cells showed the morphological appearance of resting cells. The gastrin-treatment did not induce the expansion of apical secretory canaliculi, leaving the cytoplasm densely packed with tubulovesicles (Fig. 7D). These results indicate that Foxq1−/− parietal cells were less responsive to gastrin, and that this might be the main cause for the impairment of gastric secretion.

**Discussion**

The goal of this study was to determine the role of the transcription factor FOXQ1 in development and function of parietal cells. This study was motivated by the strong, selective expression of Foxq1 in gastric parietal cells of mouse. The expression of Foxq1 in stomach is evolutionarily conserved. Thus, it has been found that Foxq1 is expressed in stomach of Xenopus during prenatal development (Choi et al., 2006).

Analyses of Foxq1-null mice showed that the Foxq1 deficiency does not impair the differentiation and maintenance of parietal cells, but severely affects the parietal cell function. Basal gastric secretion was abolished and could not be induced by the major acid secretagogues including histamine or gastrin. Expression of gastrin, gastrin-, histamine H2-receptor and H⁺,K⁺-ATPase α-subunit gene, which play a role in acid secretion (Samuelson and Hinkle, 2003), in stomach of wild-type and Foxq1−/− mice did not reveal any obvious change suggesting that FOXQ1 does not regulate these genes. Reduced expression of somatostatin in Foxq1−/− stomach might be not the result of FOXQ1-deficiency in the parietal cells, but could be attributed to reduced acid secretion, which normally exerts an inhibitory effect on the expression of somatostatin in the D-cells (Holst et al., 1992; Pashankar et al., 2001; Yip et al., 2004).

Parietal cells in resting state contain a large amount of tubulovesicles. Upon stimulation of parietal cells to secrete acid, this tubulovesicular membrane is incorporated into the apical plasma membrane to expand the secretory canaliculi. Tubulovesicles are reformed by entocytic retrieval from canalicule membrane as the cell returns to the resting state (Agnew et al., 1999; Yao and Forte, 2003). Ultrastructural data presented here demonstrate that the tubulovesicles are largely abundant in the Foxq1−/− parietal cells, while the secretory canaliculi are not expanded in gastrin-stimulated Foxq1−/− parietal cells. These results suggest that the FOXQ1-regulated genes are not required for genesis of the tubulovesicular compartments, but are necessary for the formation/expansion of canalicular apical membranes in gastric parietal cells.

Various knockout mouse models have shown altered gastric secretory capabilities (Friis-Hansen et al., 1998; Schultheis et al., 1998; Aihara et al., 1999; Kobayashi et al., 2000; Spicer et al., 2000; Gawenis et al., 2004, 2005; Kato et al., 2004; Roepke et al., 2006). The underlying causes for impaired acid secretion in these mutant strains are different. The failure of tubulovesicular membranes to fuse with the apical membranes of canaliculi in Foxq1−/− parietal cells is similar to that seen in ezrin knockdown mice (Tamura et
The cytoskeleton-associated protein ezrin, a member of the ezrin/radixin/moesin (ERM) family, is believed to be one of the critical participants in membrane folding and the modulation of secretory membranes (Forte et al., 1998). Defects in the expansion of apical secretory canaliculi in Foxq1- and ezrin-deficient parietal cells lead us to suggest that Foxq1 might regulate the expression of the ezrin gene. However, the ezrin gene was not found to be downregulated in the stomach of Foxq1-deficient mice. Furthermore, Foxq1 has been found to mediate repressing effects in the regulation of smooth muscle specific genes Mylk, SM22α and γ-actin in nonmuscle cells (Hoggatt et al., 2000). However, RNA analysis did not reveal an alteration in Mylk expression in Foxq1−/− stomach.

We have further observed several striking morphological anomalies associated with Foxq1 deficiency. In the hybrid genetic background, 50% of Foxq1−/− embryos die in utero at about 10.5 to 12.5 days p.c. and display head abnormalities. However, the incidence of embryonic lethality of Foxq1−/− is lower in the inbred 129/Sv background, presumably due to the effect of different combinations of modifier genes. In contrast, the satin hair phenotype is fully penetrant with Foxq1 deficiency in both genetic backgrounds.

On the basis of Foxq1 expression in mouse kidney (Frank and Zoll, 1998), it seems possible that FOXQ1 might play a role in regulation of kidney development and/or function. However, we observed no physiological perturbations that could be attributed to altered renal function. There were no apparent differences in blood pH, HCO3 and Na+ levels between Foxq1−/− and Foxq1+/+ mice.

In summary, our results suggest that Foxq1 plays a crucial role in the formation/expansion of canalicular apical membranes in gastric parietal cells, leading to gastric acid secretion. Therefore, FOXQ1 may be added to the growing list of candidate genes that causes achlorhydria in human.

Acknowledgments

We thank M. Schindler, H. Riedesel and S. Wolf for help in the generation and breeding of knockout mice.
References