The mesenchymal winged helix transcription factor Fkh6 is required for the control of gastrointestinal proliferation and differentiation

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The winged helix transcription factor Fkh6 is expressed in the mesoderm of the gastrointestinal tract directly adjacent to the endoderm-derived epithelium. Homozygous null mice for Fkh6 showed postnatal growth retardation secondary to severe structural abnormalities of the stomach, duodenum, and jejunum. Dysregulation of epithelial cell proliferation in these organs resulted in an approximately fourfold increase in the number of dividing intestinal epithelial cells and marked expansion of the proliferative zone. As a consequence, the tissue architecture of the stomach and small intestine was distorted, with abnormal crypt structure, formation of mucin filled cysts, and lengthening of villi. Changes in the cellular phenotype and composition of the gastric and intestinal epithelia also suggests that epithelial cell-lineage allocation or differentiation may be affected by loss of Fkh6. From the analysis of a number of potential signaling molecules, we found Bmp2 and Bmp4 expression reduced in the gastrointestinal tract of Fkh6 mutant mice, suggesting that Fkh6 directs a signaling cascade that mediates communication between the mesenchyme and endoderm of the gut to regulate cell proliferation.

[Key Words: Winged helix transcription factor; mesenchyme; gastrointestinal proliferation; differentiation]

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The mammalian gut epithelium is a highly organized and dynamic system that requires continuous, controlled proliferation and differentiation throughout life. Slowly dividing stem cells differentiate into four cell types in the intestine (enterocytes, goblet cells, enteroendocrine, and Paneth cells) and three major lineages in the stomach (pit cells, parietal cells, and zymogenic cells; for review, see Gordon and Hermiston 1994). The differentiated epithelial cells have a limited life span and are lost either through exfoliation into the gut lumen or via phagocytosis. To date, a number of transcription factors, growth factors, and their receptors have been found to be expressed in the gastrointestinal epithelium or mesenchyme (for review, see Traber and Wu 1995), however, little is known about their specific functions in gastrointestinal development. For those factors where a mutation has been generated by gene targeting, gastrointestinal development either proceeds normally (TGFα, TGFβ1, IGF1, and IGF1 receptor; Shull et al. 1992; Kulkarni and Karlsson 1993; Liu et al. 1993; Luetke et al. 1993; Mann et al. 1993), or the embryos die too early to allow assessment of the function of the genes in gut development (HNF3β, SF/HGF; Ang and Rossant 1994; Weinstein et al. 1994; Schmidt et al. 1995).

The digestive tract develops from heterogeneous embryonic origins. The musculature and the connective tissue are derived from lateral plate mesoderm and the epithelium is derived from the endoderm. Clear evidence has been obtained from tissue recombination in vivo as well as cell culture experiments that reciprocal interactions between the mesenchyme and the endoderm govern the differentiation of the gastrointestinal tract (Haffen et al. 1989; Birchmeier and Birchmeier 1993; Gordon and Hermiston 1994). However, the molecular nature of these interactions in vertebrates has not been well defined. In Drosophila it has been shown that a signal from the visceral mesoderm, mediated through the extracellular proteins decapentaplegic and wingless, controls the expression of the homeotic gene labial in the endoderm of the midgut (Bienz 1994; Hoppler and Bienz 1995). Thus far, this signaling pathway has not been investigated in vertebrates.

We have characterized previously a member of the winged helix gene family of transcription factors, termed Fkh6 (forkhead homolog 6), which is expressed in em-
bryonic mesenchyme (Kaestner et al. 1996). In the gastrointestinal tract, Fkh6 mRNA is localized in mesenchyme directly adjacent to the endoderm-derived epithelium, making Fkh6 a candidate for a gene involved in mesenchyme to epithelium signaling and the control of gastrointestinal proliferation and differentiation. Here we report the phenotypic consequences of a null mutation in the Fkh6 gene in mice.

Results

Gene targeting of the Fkh6 gene

To investigate the potential role of the winged helix transcription factor Fkh6 in gastrointestinal development, we generated mice lacking a functional product of this gene by homologous recombination. The Fkh6 locus had been cloned previously from a mouse strain 129 genomic library (Kaestner et al. 1996). We constructed a targeting vector that deleted most of the winged helix DNA-binding domain and the entire carboxy-terminal region of the protein. Care was taken not to delete any adjacent untranslated regions, to avoid interfering with the regulation of the winged helix gene MFH1, which is located within 8 kb of Fkh6 (Kaestner et al. 1996). In situ hybridization of embryonic day 9.5 (E9.5) wild-type and Fkh6−/− embryos revealed no differences in the MFH1 expression pattern (data not shown). The complete targeting strategy is depicted in Figure 1A. Of 340 stably transfected embryonic stem (ES) cell clones, one had untransfected wild-type ES cells. Genomic ES-cell DNA was digested with the restriction endonucleases indicated, size-fractionated by agarose gel electrophoresis, blotted onto nylon membrane, and hybridized with the probes indicated.

Figure 1. Targeting strategy for Fkh6 inactivation. (A, top line) Gene structure of the MFH1/Fkh6 locus. (Middle line) Targeting vector used for homologous recombination in ES cells. (Bottom line) Gene structure of the targeted allele. Probes A, B, and C are referred to in the text. [A] Asp718; [E] EcoRI; [H] HindIII; [N] NcoI; [Nt] NotI. (B) Southern blot analysis of the correctly targeted ES-cell clone. Genomic ES-cell DNA was digested with the restriction endonucleases indicated, size-fractionated by agarose gel electrophoresis, blotted onto nylon membrane, and hybridized with the probes indicated.

Alteration of intestinal development and architecture in Fkh6−/− mice

From E14.5 to E18.5 the mouse small intestine develops from a stratified epithelium into a simple columnar epithelium with the formation of nascent villi in a proximal to distal progression (Gordon and Hermiston 1994; Tra-
Figure 2. Fkh6 mRNA is absent from Fkh6−/− mice. (A) RNase protection analysis of 20 μg total RNA of stomach and small intestine isolated from adult wild-type (+/+) and mutant (−/−) mice. The probe used for Fkh6 was probe B indicated in Fig. 1, a probe for TATA-box binding protein (TBP) was included as a control. [B, C] In situ hybridization analysis of day-14.5 mouse embryos (section through the midgut) with an antisense probe to Fkh6. [B] Wild type. [C] Fkh6 mutant embryo.

Figure 3. Fkh6−/− mice are growth retarded postnatally. Growth curves for a representative litter obtained from an intercross between heterozygous mice. One of the Fkh6−/− mice died on day 8 after birth.

Postnatal development and architecture of the adult intestinal epithelium

By postnatal day 3, jejunum from normal mice contained...
Figure 4. Fetal development of the gastrointestinal tract in Fkh6−/− mice. Paraffin section of the proximal midgut from wild-type [A, C, E, G, I] or Fkh6−/− [B, D, F, H, J] mice were stained with hematoxylin and eosin [A–F], alcian blue [G, H] or an antibody to proliferating cell antigen Ki67 [L, J]. (A, B) E14.5. The invagination of the mesenchyme to form epithelial ridges is delayed in the Fkh6−/− mice. (C, D) E16.5. Fewer and less defined villi are formed in the mutant mice. (E–J) E18.5. The villi of the Fkh6−/− mice continue to be poorly formed, shorter, and fewer in number. Proliferating cells are localized to the intervillus regions in the wild-type intestine [I], but are spread throughout the villi in the Fkh6−/− mice [J]. Magnification, A–H, 200×; I, J, 400×.

well-formed villi and intervillus regions [Fig. 5A]. As expected from the changes found in the fetal intestine, the jejunum of Fkh6−/− mice had fewer villi that were shorter and wider than those found in normal mice [Fig. 5B]. By postnatal day 12, crypts were evident in both the normal [Fig. 5C] and Fkh6−/− mice, although the crypts in the Fkh6−/− mice appeared to be expanded and exhibited a branched morphology [Fig. 5D]. These mucosal abnormalities were more prominent in adult animals. The crypt compartment was markedly expanded and disorganized in 50-day-old Fkh6−/− mice, with branched crypts and cell-lined cystic structures [Fig. 5F, H]. Abnormalities in the crypt architecture were most pronounced in the proximal intestine [Fig. 5F]. When the intestine was examined further distally, the crypt distortion and the number of cystic structures diminished, with only mild abnormalities observed in the ileum [Fig. 5H]. The colon of the Fkh6−/− mice appeared histologically normal [data not shown].

Specific cellular components in the epithelium were characterized using a number of specific stains. Each of the four epithelial cell types (enterocytes, enteroendocrine cells, Paneth, and goblet cells) were present in the mutant intestine. However, goblet cells, which are normally found in an anterior–posterior gradient in the intestine [highest in the colon], were found in increased numbers in the duodenum and jejunum of Fkh6−/− mice, indicating that cell lineage allocation or differentiation might be affected in these animals. Goblet cells and associated mucin were assessed using affinity staining with the lectin UEA 1 [Ulex europaeus I; Fig. 6A, B] (Falk et al. 1994) and histochemistry for alcian blue [acid mucin; Fig. 6C, D]. Goblet cells in the normal intestine were distributed throughout the villus with few found in the crypt compartment [Fig. 6A, B]. In contrast, goblet cells in Fkh6−/− mice were clustered within crypts as well as on villi. Moreover, the cystic structures in the crypt compartment contained both neutral and acid mucin indicating that goblet cells were emptying their contents into these spaces.

Other cell types in the epithelium were present in the Fkh6−/− mice in approximately the same number and distribution as in normal mice. Paneth cells were present in the base of crypts as indicated by UEA 1 staining [Fig. 6A, B, arrowheads]. Serotonin-positive cells demonstrated that at least one enteroendocrine cell type was produced in the Fkh6−/− mice [Fig. 6E, F]. Villus enterocytes in Fkh6−/− mice expressed sucrase-isomaltase on the brush border in a similar distribution as in normal mice [Fig. 6E, F].

In addition to an expansion of the crypt zone, there was an increase in the length of the villi in Fkh6−/− mice 50 days or older when compared with normal animals [Figs. 5E, F and 6A–D, G, H]. This effect was seen only in mice 50 days or older where the length of the villi had reached a steady state, while in Fkh6−/− newborn or fetal mice the villi were shorter, attributable to the delay in villi formation as documented above. In steady state, the length of a villus is determined by the production rate of crypt cells and the subsequent loss of cells from the villus tip (Gordon and Hermiston 1994). Therefore, the increased size of villi in Fkh6−/− mice was possibly attributable to an increased production of cells in the crypts.
and a normal rate of extrusion of senescent cells into the lumen, although other possibilities cannot be ruled out at present (see Discussion).

We speculated that the observed expansion of the jejunal crypt zone in the Fkh6−/− mice (Fig. 5D,F) might be caused in part by an increase in the number of proliferating cells. To test this hypothesis, proliferating cells were identified by staining for the nuclear antigen Ki67 (Schluter et al. 1993). The zone of proliferating cells in the Fkh6−/− mice was greatly expanded, corresponding to the expanded crypt compartment (Fig. 7 A,B). Cells containing labeled nuclei were counted in wild-type and mutant litter mates and revealed an approximately fourfold increase in the number of dividing cells (Fig. 7C) in the jejunal epithelium, however, there was no significant increase in the number of Ki67-positive cells in the mesenchyme (data not shown).

We were interested in determining whether all the proliferating cells in the jejunal epithelium of the Fkh6 mutant mice migrate up the villi for differentiation into mature epithelial cells or whether some were lost attributable to increased apoptosis, especially because some cells appeared to be trapped in the mucin-filled cysts. We therefore identified cells undergoing apoptosis by extension of nicks in the DNA in the presence of biotinylated nucleotides (Wijsman et al. 1993). As is shown in Figure 8, there was an increase in the number of apoptotic cells in the crypts of Fkh6 mutant jejunum as compared with wild-type littermates. The apoptotic cells seemed to be clustered around the mucin-filled cysts.

**Development and adult architecture of the gastric mucosa**

Development of the stomach mucosa also begins in the late fetal stage and is completed in the postnatal period. Columnar epithelial cells invaginate into mesenchyme to form straight tubular glands with multiple cell types arranged in a precise organization (Gordon and Hermiston 1994). The pit region of the gastric gland contains surface mucous cells and a few parietal cells. The isthmus of the gland contains the precursor cells of parietal and pit cells and some mature parietal cells. The neck region of the gland is composed of mucous neck cells and some parietal cells. The base region of the gland contains mainly zymogenic cells (chief cells) but does include a minority of parietal cells (Gordon and Hermiston 1994). The forestomach of the mouse is normally lined with stratified squamous epithelium.

**Figure 5.** Postnatal development of the small intestine in Fkh6−/− mice. Intestinal samples from wild-type [A,C,E,G] and mutant [B,D,F,H] littermates from postnatal day 3 [A,B], 12 [C,D], and 50 [E−H] were sectioned and stained with hematoxylin and eosin. Note the expanded crypt zone in the jejunum of day-3 and day-12 mice [B,D], as well as the elongated villi and mucin-filled cyst in the duodenum of 50-day-old Fkh6−/− mice [F]. The morphology of the ileum [G,H] is almost normal. Magnification, E,F, 100×; all others 200×.
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Figure 7. Increased epithelial proliferation in the jejunum of Fkh6-/- mice. Paraffin sections of proximal jejunum from 50-day-old wild-type [A] and mutant [B] littermates were stained with an antibody against the nuclear antigen Ki67, which is specific to proliferating cells. Magnification, 200x. (C) Quantification of Ki67 positive cells. Stained nuclei in 10 crypts each from age matched mouse were counted and expressed as dividing cells per crypt +/- SEM. [Blue bar] Wild type; [purple bar] homozygous Fkh6-/- mutant.

Figure 8. The jejunal epithelium of Fkh6-/- mice exhibits an increased number of apoptotic cells. Wild-type [A] and mutant [B] jejunum from 50-day-old littermates was labeled by nick translation to reveal the presence of apoptotic cells. Although in wild-type crypts, only occasional apoptotic cells are found, a much higher number is present in the crypt region of the mutant mouse. Magnification, 100x.

At day E14.5, there was little difference between the stomachs of normal and Fkh6-/- mice [data not shown]. By postnatal day 3, the epithelium of the stomach body in normal mice was arranged in short glandular structures (Fig. 9A). In contrast, the stomach mucosa in newborn Fkh6-/- mice was markedly abnormal [Fig. 9B]. The glandular epithelium was expanded and the architecture was distorted. Instead of orderly glands, there was vacuolization of surface cells and branching of the glands within the gastric mucosa. In addition, the subepithelial mesenchymal tissue was less dense and expanded in the Fkh6-/- mice compared with normal mice. The abnormalities within the gastric glandular epithelium became more pronounced with age. In adult Fkh6-/- mice the gastric mucosa was as much as sixfold thicker than in normal mice [Fig. 9E,F]. In addition, large cell-lined cysts and distorted mucosal glands were evident. As in the neonatal animals, the submucosal mesenchyme of Fkh6-/- mice was less condensed than normal controls through day 50. At each stage of development differences between normal and Fkh6-/- mice could not be detected in the squamous epithelium of the forestomach.
Figure 9. Perturbed stomach development in Fkh6−/− mice. Paraffin sections from stomachs obtained from wild-type (A,C,E) and mutant (B,D,F) littersmates 3 (A,B), 12 (C,D), or 50 (E,F) days old were stained with hematoxylin and eosin. Note the loose appearance of the mesenchyme in B, the vacuolization of the surface cells and branching of the glands in D. The thickness of the stomach epithelium is greatly expanded in 50-day-old mice (cf. F and E, both at the same magnification). There are also a large number of cysts in the stomach of the Fkh6−/− mice. Magnification, A-D, 200x; E,F, 100x.

To characterize the cell lineages of the gastric mucosa, the stomachs of adult (day 50) animals were stained with DBA (Dolichos biflorus) and UEA I lectins. In the stomach, DBA binds to parietal cells and UEA I binds strongly to surface or pit mucous cells and weakly to neck mucous cells and parietal cells [Falk et al. 1994]. In the normal stomach, the parietal cells were found in orderly columns primarily within the isthmus and neck region of the glandular epithelium (Fig. 10A). Parietal cells were also identified in Fkh6−/− mice, however the distribution was disorganized [Fig. 10B]. UEA I staining of normal stomach was strongly positive in the pit region (Fig. 10C). The Fkh6−/− mice had a distinctly different staining pattern, with intense staining from the surface to the base of the glandular epithelium (Fig. 10D). The parietal cells were weakly positive in the Fkh6−/− mice, as they were in the normal controls, therefore the strongly positive cells are possibly surface mucous cells that have aberrantly migrated throughout the gastric epithelium.

The pattern of mucin expression in the stomach was examined using alcian blue histochemistry. Alcian blue normally does not stain in the stomach, however in the Fkh6−/− mice alcian blue stained the surface and the material in some of the cyst-like structures (Fig. 10E,F). This would indicate that the gastric cells ectopically produced acid mucin. This finding, in addition to the changes observed in the gastric mucous cells, clearly indicates that the normal differentiation program of the gastric epithelium is perturbed in Fkh6−/− mice.

The zone of proliferating cells in the Fkh6−/− mice, normally limited to the region of the neck and isthmus [Fig. 10G], was markedly expanded, extending throughout the thickness of the mucosa [Fig. 10H]. Similar to the findings in the small intestine, the mucous-filled cystic areas were lined with proliferating cells.

Gene expression in Fkh6 mutant mice: possible targets of Fkh6

To assess whether the dramatic changes observed in gastrointestinal morphology observed in Fkh6 mutant mice were related to changes in gene expression in the gut epithelium, we measured steady-state mRNA levels for known epithelial transcription factors and other epithelial marker genes. The murine homologs of the Drosophila homeobox gene caudal, cdx1, and cdx2 had been shown previously to be expressed in the epithelia of the small intestine and colon [Duprey et al. 1988; James and Kazenwadel 1991; Suh et al. 1994], whereas the winged helix transcription factors HNF3α and HNF3β are expressed in the entire gastrointestinal epithelium, and HNF3γ from the glandular stomach to the colon [Monaghan et al. 1993]. RNase protection analysis of adult stomach and intestine demonstrated that cdx1 and cdx2 expression is not altered in the Fkh6 mutant animals [Fig. 11A], the same was found for the HNF3 mRNAs (data not shown). Similarly, the levels and distribution patterns of the fatty acid-binding proteins were unchanged in the Fkh6 mutant mice. (Fig. 11B). Therefore, the dramatic changes in the proliferation of the gastrointestinal epithelium as well as the alterations observed in the cellular differentiation by histological means do not appear to affect the anterior-posterior expression pattern of these marker genes.

As Fkh6 is expressed in the mesenchyme of the gut during development, we hypothesized that an important
function of Fkh6 might be the activation or repression of mesenchymal growth or differentiation factors that act on the gut epithelium to control proliferation and differentiation. Several candidates for such mesenchymal signal mediators have been described (for review, see Traber and Wu 1995), among them transforming growth factor α and β (TGFα, TGFβ1, TGFβ2, and TGFβ3), neuregulin and scatter factor/hepatocyte growth factor (SF/HGF). Of particular interest was TGFα, as overexpression of this protein in the stomach of transgenic mice led to hypertrophic alterations of the epithelium, some of which are reminiscent of the changes seen in the Fkh6−/− mice [Dempsey et al. 1992; Tgaki et al. 1992]. However, expression of TGFα is not altered in Fkh6 mutant mice, as demonstrated by RNase protection analysis [Fig. 11C]. We also analyzed the expression of TGFβ1, TGFβ2, TGFβ3, neuregulin, and SF/HGF in Fkh6−/− embryos [E14.5–16.5] by in situ hybridization and RNase protection analysis, and found no differences from control animals (data not shown).

Several classes of secreted polypeptide factors that have been shown to have an important role in cell–cell signaling, and that are expressed either in the mesenchyme or the epithelium of the gastrointestinal tract, are encoded by the Wnt genes (for review, see Parr and McMahon 1994), the decapentaplegic homologs Bmp2 and Bmp4 [Bitgood and McMahon 1995] and the hedgehog homologous shh and ihh [Bitgood and McMahon 1995]. We therefore analyzed the expression levels of Wnt2, Wnt5a, Bmp2, Bmp4, shh, and ihh by in situ hybridization [E15.5] and RNase protection assay [E15.5, postnatal day 4 (P4), P12, and P30] in wild-type and mutant gastrointestinal tract. Although no differences were observed in the expression levels of the Wnt and hedgehog genes, a significant reduction (30% to 40%) was found in the expression levels of Bmp2 in the stomach, duodenum, and jejunum, and for Bmp4 in the stomach [Fig. 12A–C], indicating Bmp2 and Bmp4 as downstream (direct or indirect) targets of Fkh6 action. No differences were found in the ileum and colon, correlating well with the lack of morphological abnormalities in the distal gut described above.

Figure 10. Abnormal epithelial architecture in Fkh6−/− mice. Wild-type [A,C,E,G] and mutant [B,D,F,H] stomach stained with lectins [A,B, DBA; C,D, UEA I], alcian blue [E,F] or an antibody to proliferating cell antigen Ki67 [G,H]. Parietal cells are labeled green by DBA. Although the parietal cells are localized to columns within the neck and isthmus region of the wild-type stomach [A], the staining pattern is disorganized in the Fkh6−/− stomach [B]. Surface mucous cells are labeled strongly by UEA I binding in the wild-type sample [C], but UEA I-positive cells are spread throughout the epithelium in the Fkh6−/− mice [D]. Alcian blue, which detects acid mucins, normally does not stain the stomach [E], but detects ectopic acid mucins in the Fkh6 mutant [F]. Proliferation is localized to the neck and isthmus region in wild-type stomach [G]. In the homozygous mutant stomach, proliferating cells are detectable throughout the epithelium [H]. Magnification, A,C,E,G, 200×; B,D,F,H, 100×.
Discussion

Mice lacking the winged helix transcription factor Fkh6 show profound alterations in the gastric and intestinal epithelia from the late fetal period extending into adulthood. Phenotypic consequences in the intestine of Fkh6−/− mice in the late fetal period are the pronounced delay in the transition from stratified epithelium to columnar epithelium and retarded formation and growth of the nascent villi. This retarded development results in villi that are shorter than those of littermates until postnatal day 12. However, once the length of the villi has reached steady state in adulthood, the villi in the mutant intestine are longer than normal. This is accompanied by an increase in the number of proliferating cells and an expansion of the crypt zone. At present we cannot rule out the possibility that an increase in the residence time [decrease in the rate of cell migration toward the intestinal lumen] contributes to the lengthening of the villi as well.

Intestinal abnormalities are most prominent in the proximal intestine with few changes in the ileum and no discernible changes in the colon. The architectural changes in the intestinal epithelium suggest that the abnormal structure results from delayed villus development, enhanced epithelial cell proliferation, and disor-
organized crypt morphogenesis, which might be affected by the dysregulation of cellular proliferation. An alteration in the precise spatial assignment for the initiation of cell proliferation might lead to crypt branching and distortion. The distorted crypt architecture might then lead to the trapping of mucin in the crypts and subsequent formation of cystic structures. In addition, there appears to be an alteration in the cell-lineage allocation or differentiation in the Fkh6-/- mice as indicated by the increased number of goblet cells.

The abnormalities in the stomach of Fkh6 null mice are very similar in many respects to those found in the small intestine. In adult animals, the gastric mucosa is expanded and there is a marked increase in the number of proliferating cells. The glandular architecture is distorted and there are multiple mucin-filled cysts. Also, there is a marked increase in mucin-producing cells throughout the mucosa indicating an alteration in the types of cells present in different glandular compartments. One puzzling aspect of the observed phenotype is the fact that the morphology of the ileum and colon appear normal, despite the fact that Fkh6 is expressed in the gut mesenchyme all the way through the colon.

The findings presented here indicate Fkh6 as a negative regulator of epithelial cell proliferation. Other members of the winged helix gene family have been implicated in growth control, such as the avian oncogene Qin [Li and Vogt 1993], which is a close relative of the rodent transcription factor BF1 (Tao and Lai 1992). Interestingly, a null mutation in BF1 led to a decrease in the size of the cerebral hemispheres through a reduction in cellular proliferation, indicating BF1 as a positive regulator of proliferation [Xuan et al. 1995]. The related winged helix protein BF2 was shown to have an essential role in kidney morphogenesis, as mice with a null mutation for this gene exhibit a reduced rate of growth and branching of the ureter and collecting duct system [Hatini et al. 1996]. In addition, the oncogene in alveolar rhabdomyosarcoma is a fusion protein between the paired box transcription factor Pax3 and another winged helix gene, termed FKHR [Galili et al. 1993]. In the future, the Fkh6 mutant mice will allow us to identify the mechanism by which Fkh6 regulates proliferation in the gastrointestinal tract.

A striking feature of the Fkh6 phenotype is the fact that lack of a mesenchymal transcription factor leads to dramatic changes in the gastrointestinal epithelium. Therefore the likely role of Fkh6 resides in the interaction between mesenchymal and epithelial cells. Fkh6 may regulate the expression of one or several mesenchymal genes that in turn regulate the proliferation of epithelial cells and possibly promote cellular differentiation programs. Potential classes of such regulatory target genes include secreted growth factors, cell matrix proteins, or cell surface receptors that may interact with epithelial cells or the extracellular matrix. In the Drosohila midgut, two secreted polypeptides expressed in the visceral mesoderm, wingless [wg] and decapentaplegic [dpp], control the expression of the homeotic gene labial in the midgut [Bienz 1994; Hoppler and Bienz 1995]. We have therefore investigated the expression of several mammalian homologs of wg and dpp in Fkh6-/- mice. Although the expression levels of TGFβ1, TGFβ2, and TGFβ3, as well as Wnt2 and Wnt5a were not changed, we have identified Bmp2 and Bmp4 as downstream targets of Fkh6 by their reduced mRNA level in the stomach and intestine of Fkh6-/- mice. Bmp2 was shown to be expressed in the endoderm-derived epithelium of the developing gut, whereas Bmp4 expression is localized to the mesenchyme [Bitgood and McMahon 1995]. As the Bmps belong to a family of secreted polypeptides implicated in a large number of signaling processes, they are good candidates to be part of a signal transduction pathway from the mesenchyme to the epithelium. As Bmp2 and Fkh6 are expressed in adjacent cell layers, Bmp2 can only be an indirect target of Fkh6. In contrast, Bmp4 and Fkh6 are coexpressed in the gastrointestinal mesenchyme, allowing for the possibility that Fkh6 is a direct activator of Bmp4. In the context of the proliferation defect in the Fkh6-/- mice it is interesting to note that overexpression of Bmp4 in the epithelium of the lung led to inhibition of epithelial proliferation [Belluscì et al. 1996], whereas in the stomach of Fkh6-/- mice, reduced expression of Bmp4 correlates with increased proliferation of the epithelium.

Although to date several genes have been targeted that are also expressed in the mammalian gut, the Fkh6-/- mice represent the first example of a mutation in a mesenchymal transcription factor that profoundly affects the proliferation and the cytoarchitecture of the gastrointestinal epithelium. The noncell autonomous nature of the defect indicates a signal transduction cascade, in which the mesenchymal transcription factor Fkh6 regulates effectors of cellular proliferation of the gastrointestinal epithelium. We will use the Fkh6-/- mice in the future as a tool to elucidate the intermediary steps in this signaling pathway.

Materials and methods

Gene targeting

A Phage clones containing the murine Fkh6 gene had been isolated from a mouse ES cell strain 129 library [Kaestner et al. 1993]. A gene targeting vector was constructed in the β-galactosidase containing plasmid pHM2 [Kaestner et al. 1994b]. In an 8.4-kb EcoRI subclone containing the entire Fkh6-coding sequence, an 860-bp Ncol–NruI fragment, which encodes 80% of the winged helix DNA-binding domain and the entire carboxyl terminus of the protein, was replaced by the lacZ and neomycin resistance cassette of pHM2. Thereby an in-frame fusion was created between the amino-terminal 60 amino acids of the Fkh6 protein and the β-galactosidase protein. The targeting vector was linearized with NotI and 20 μg of DNA electroporated into 10^7 E14-1 ES cells [Kühn et al. 1991]. Stably transfected cells were isolated after selection in 350 μg/ml G418 (Gibco) and 340 clones analyzed by Southern blot for homologous recombination. A 1.5-kb HindIII–EcoRI fragment (probe A in Fig. 1) located 5' to the Fkh6 gene was used as an external probe for Southern analysis of DNA digested with Asp718. Positively targeted clones were confirmed with a probe fragment encoding the neomycin phosphotransferase gene (probe C in Fig. 1) and with
three additional restriction digests (data not shown). ES cells from the correctly targeted clone were injected into blastocysts derived from C57BL/6 mice. Blastocysts were transferred to pseudopregnant NMRI females and chimeric offspring were identified by the presence of agouti hair. Chimeric males were mated to C57BL/6 females to obtain ES-derived offspring that were analyzed by Southern blot of tail DNA to identify the heterozygous Fkh6−/− mutants. Heterozygotes were mated inter se to generate mutant (+/−) mice. Embryos and mice were also genotyped by PCR using 3 primers: Fkh6 5′ (CGCCGT-GAGCCCCGGCAGAA), Fkh6 3′ (CCCCCTTCACCTGTC-GAATTC), and lacZ (GCCCATTCGACCATTGC-GC). PCR reactions were carried out for 30 cycles (94°C, 30 sec; 67°C, 40 sec; 72°C, 60 sec) in a buffer containing 1.5 mM MgCl2. The wild-type allele produced a band of 197 bp and the targeted allele a band of 317 bp.

### RNA analysis

Total RNA from embryonic and adult tissues was isolated after homogenization in guanidinium thiocyanate (Chomczynski and Sacchi 1987). RNA was separated in formaldehyde containing agarose gels for Northern analysis as described by Alwine et al. (1977). Hybridization of the mRNA of interest (expressed in the various tissues) was performed by standard methods. Oligonucleotide probes were described previously (Kaestner et al. 1989). The probes used for HNF3c¢, HNF3 B, and HNF3~ were described in (Gordon et al. 1983; Alpers et al. 1984).

### Immunohistochemistry and apoptosis assay

Mouse embryos were obtained from matings between heterozygous (Fkh6−/+) mice. By convention, the day of the vaginal plug was counted as day 0.5. Embryos were fixed in 4% paraformaldehyde in PBS at pH 7.2 overnight, dehydrated through an ethanol series, cleared in toluene and embedded in paraﬁn. Six-micron sections were cut and hybridizations carried out as described in (Wilkinson 1992). The antisense probes were hybridized in Agilent Biostar and prehybridization buffer (pH 6.0) and incubated for 25 min at 90°C in a steam bath. The following steps used the Microprobe system (Fisher Scientiﬁc) for incubation of slides with solutions. Slides were washed with PBS, followed by 1x automation buffer (BioMedica Corp.) with 1% BSA. The tissues were incubated for 20 min at 37°C with blocking buffer (1x automation buffer, 1% BSA, and 1.3% goat serum). Primary antibody was applied and the tissues incubated for 45 min to 1 hr at 37°C. The sections were washed in PBS followed by 1x automation buffer and 1% BSA. Biotinylated secondary antibody was applied to the tissue and incubated for 30 min at 37°C. The slides were developed using the avidin-biotin complex and DAB (as per the manufacturer). The tissue was counterstained lightly with hematoxylin (Lerner II hematoxylin) and eosin Y (Fisher Scientiﬁc). For quantiﬁcation of Ki67-positive nuclei, care was taken to only evaluate those crypts that were sectioned longitudinally.

Cells that had initiated apoptosis were identiﬁed as described by Wijisman et al. [1993]. Briefly, formalin-ﬁxed and parafﬁn-embedded tissues were sectioned (5 μm), treated with proteinase K, and incubated with Klenow DNA polymerase in the presence of biotinylated nucleotides. After extensive washing, the incorporated nucleotides were detected with streptavidin-conjugated horseradish peroxidase and chromogenic substrates.

### Lectin staining and histochemistry

Tissues were ﬁxed in Bouin’s solution, embedded in parafﬁn, and 5-μm sections were applied to Probe-on Plus (Fisher Scientiﬁc) slides to stain for periodic acid Schiff (PAS) and alcian blue. For PAS deparafﬁnized and rehydrated slides were placed in 0.5% aqueous periodic acid for 5 min, rinsed and placed in Schiff reagent for 15 min. After washing in water, the slides were placed in Harris hematoxylin for 2 min, washed in water, and placed in 0.5% acid alcohol. The slides were washed in water and placed brieﬂy in saturated lithium carbonate, washed again in water, and dehydrated and mounted. To stain with alcian blue, deparafﬁnized and rehydrated slides were placed in acetic acid 3% for 3 min and then directly into alcian blue 1% in 3% acetic acid (pH 2.5) for 30 min. The slides were washed and counterstained in nuclear fast red 0.1% for 5 min, washed, dehydrated, and mounted.

For lectin staining, tissues were incubated with either DBA conjugated to FITC (Sigma) or UEA I conjugated to TRITC (Sigma). Deyparafﬁnized and rehydrated slides were placed in PBS containing 1% BSA and 0.3% Triton X-100 for 5 min. The lectins were incubated at a concentration of 10 mg/ml for 1 hr at room temperature. The slides were washed in PBS, mounted, and viewed by ﬂuorescent microscopy.

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