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, entero-endocrine, 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 (TGF α , TGF β 1, 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 (HNF3 β , 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-

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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 nontranslated 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 undergone homologous recombination (Fig. 1B). Germ-line chimeras and mice heterozygous for the *Fkh6* mutation were obtained as described in Materials and Methods. To ascertain that we had generated a null allele of the *Fkh6* gene, we analyzed *Fkh6* transcripts in embryos and tissues derived from adult mice genotyped as wild-type or homozygous mutant. As shown in Figure 2, *Fkh6* mRNA

was absent from stomach and intestine in adult mutant animals (Fig. 2A), as well as from 14.5-d.p.c. (days post-coitum) embryonic midgut (Fig. 2C). Figure 2B illustrates the expression of *Fkh6* mRNA in the mesenchyme just adjacent to the epithelium. No differences were observed between heterozygous and wild-type animals in overall development, growth characteristics, histology, or gene expression (see below). When heterozygous animals of the original mixed background (129Sv × C57BL6) were crossed and the offspring genotyped at 4 weeks of age, a marked deficiency of homozygous mutant animals was observed (+/+, 31%; +/-, 61%; -/-, 8%), with an even distribution of the homozygotes between both sexes. Most mutant mice died before weaning between 1 and 3 weeks of age. Although the birthweight of the mutant animals was normal, they exhibited a dramatic reduction in postnatal growth as shown for a representative litter (of 15 analyzed) in Figure 3. Whereas one homozygous mutant died 9 days after birth, the other, once past the critical weaning period, gained weight very quickly and attained the size of its littermates by ~5 months. Differences between individuals of the same genotype have been observed in other gene-targeting experiments as well and have been attributed to variation in the genetic background. We surmised that the growth retardation of the *Fkh6*^{-/-} mice might be caused by an impaired development of the gastrointestinal tract, as this is the major site of *Fkh6* expression, and investigated the gastrointestinal morphology in detail.

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 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.

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.

ber and Wu 1995). The villi are separated by an intervillus epithelium that gives rise to the crypt compartment postnatally. The earliest sign of this epithelial transition is the invagination and condensation of mesenchyme and subsequent formation of epithelial ridges. The timing and organization of this developmental transition in the intestine is altered in the *Fkh6* null mice when compared with normal and heterozygous littermates. In the remainder of the analysis, the *Fkh6*^{-/-} mice will be compared with normal littermates as there was no abnormality noted in the *Fkh6*^{+/-} mice at any time point. The

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.

intestine of both normal and *Fkh6*^{-/-} mice at E14.5 contained a stratified epithelium, however, the mesenchyme in the *Fkh6*^{-/-} mice had not yet begun to invaginate to form epithelial ridges (Fig. 4A,B). Subsequently, there was a delay in formation of villi and fewer, poorly formed villi were present at E16.5 and E18.5 in the *Fkh6*^{-/-} mice (Fig. 4C-H). Although the nascent villi in the *Fkh6*^{-/-} mice were covered with a single cell layer of columnar epithelium, the intervillus regions continued to contain stratified epithelium to a greater extent than comparable regions of the intestine from their normal littermates. At E18.5, the villi in normal mice were elongated and increased in number. In contrast, the villi of *Fkh6*^{-/-} mice continued to be poorly formed, shorter, and fewer in number (Fig. 4G,H). Goblet cells were evident in approximately similar numbers in both the normal and *Fkh6*^{-/-} mice (blue staining cells in Fig. 4G,H). In addition to the changes in formation of villi, the mesenchyme in *Fkh6*^{-/-} mice from E14.5 to E18.5 was less condensed in comparison with the normal controls (Fig. 4A-H).

The pattern of proliferating cells, assessed by staining for Ki67 (Schluter et al. 1993), showed a marked difference between normal and *Fkh6*^{-/-} mice. In the normal epithelium, proliferating cells were localized to the intervillus region of the epithelium (Fig. 4I). In contrast, proliferating cells in the *Fkh6*^{-/-} mice were found in the intervillus regions as well as scattered throughout the villi (Fig. 4J). Therefore, the spatial control of proliferation was distorted very early in intestinal epithelial development in the *Fkh6*^{-/-} mice.

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 (I,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 I (*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 I 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 four-fold 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 $\times$; all others 200 $\times$.

Figure 6. The jejunal epithelium of *Fkh6*^{-/-} mice contains cells of all major lineages. Wild-type (A,C,E) or mutant (B,D,F) jejunum from 50-day-old littermates were processed for lectin staining (UEA I; A,B), alcian blue staining (C,D) or immunohistochemistry with antisera to sucrase isomaltase (E,F). UEA I stains goblet cells, associated mucins as well as Paneth cells (arrowheads in A and B). Alcian blue reveals goblet cells and acid mucins. Sucrase-isomaltase antibody staining of the brush border appears normal in the *Fkh6*^{-/-} mice. This antibody also detects serotonin-producing enteroendocrine cells. Magnification, A,B, 100 $\times$; all others 200 $\times$.

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 new-

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, 200 $\times$. (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 $\pm$ SEM. (Blue bar) Wild type; (purple bar) homozygous *Fkh6*^{-/-} mutant.

born *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 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, 100 $\times$.

Figure 9. Perturbed stomach development in *Fkh6*^{-/-} mice. Paraffin sections from stomachs obtained from wild-type (A,C,E) and mutant (B,D,F) littermates 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, 200 $\times$; E,F, 100 $\times$.

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 nor-

mal 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 HNF3 α and HNF3 β are expressed in the entire gastrointestinal epithelium, and HNF3 γ 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 (TGF α , TGF β 1, TGF β 2, and TGF β 3), neuregulin and scatter factor/hepatocyte growth factor (SF/HGF). Of particular interest was TGF α , 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 TGF α is not altered in *Fkh6* mutant mice, as demonstrated by RNase protection analysis (Fig. 11C). We also analyzed the expression of TGF β 1, TGF β 2, TGF β 3, 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 $\times$; B,D,F,H, 100 $\times$.

Figure 11. Marker gene expression in *Fkh6*^{-/-} mice. (A) RNase protection analysis of 20 µg total RNA isolated from the stomach, proximal intestine (P. Int) and medial intestine (M. Int) of wild-type (+/+) and *Fkh6* mutant (-/-) mice. The probes used were *cdx1* and *cdx2*, as well as TBP as control. (B) Northern blot analysis of 10 µg total RNA isolated from the stomach (Sto.), proximal intestine (P. Int), medial intestine (M. Int.), and colon of wild-type (+/+) and *Fkh6* mutant (-/-) mice. The probes used were ileal fatty acid-binding protein (iFABP), liver fatty acid-binding protein (IFABP), and glyceraldehyde phosphate dehydrogenase (GAPDH) as control. (C) RNase protection analysis of 20 µg total RNA isolated from the stomach, proximal intestine (P. Int), medial intestine (M. Int), and colon of wild-type (+/+) and *Fkh6* mutant (-/-) mice. The probes used were TGFα and TBP as control.

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-

Figure 12. *Bmp2* and *Bmp4* gene expression is reduced in *Fkh6*^{-/-} mice. (A) RNase protection analysis of 20 µg total RNA isolated from the duodenum of postnatal-day-30 wild-type (+/+) and *Fkh6* mutant (-/-) mice. The probes used were *Bmp2* and *Bmp4* as well as TBP as control. (B, C) RNase protection analysis was carried out with RNA from stomach, duodenum, proximal jejunum (p. jej.), and distal jejunum (d. jej.). The signals obtained were quantified with a PhosphorImager and the mRNA levels expressed as the ratios of the *Bmp2* (B) or *Bmp4* (C) signal to that of TBP. (Hatched bars) Wild type; (open bars) *Fkh6*^{-/-} mice. Values are means ± standard error (*n* = 4). (Asterisk) Statistically significant differences (*P* < 0.01; student's *t*-test) between wild-type and -/- mRNA levels.

ganized 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 *Drosophila* 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 (Bellusci 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

λ 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 *NcoI*-*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 10⁷ 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' (CGCGTCGAGCCCCCGCAGAAG); *Fkh6* 3' (CCCCTTCTCACGTG-GCACCTTC); and *lacZ* (CGCCATTCGCCATTCAGGCTGC). 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). Hybond N filters (Amersham) were hybridized in 50% formamide, 5× SSC, 50 mM Na phosphate at pH 6.5, 8× Denhardt's solution, 1% SDS, and 0.5 mg/ml of total yeast RNA according to Ruppert et al. (1990). RNase protection analysis was carried out as described previously (Kaestner et al. 1989). The probes used for HNF3α, HNF3β, and HNF3γ were described previously (Kaestner et al. 1994a). The probes for *cdx1* and *cdx2* (Duprey et al. 1988; James and Kazenwadel 1991) corresponded to base pairs 662–913 and 437–942, respectively. For the analysis of *Wnt2*, *Wnt5a*, *shh*, *ihh*, *Bmp2*, and *Bmp4* mRNAs, antisense probes were synthesized of plasmids kindly provided by A. McMahon (Harvard University, Cambridge, MA) and B. Hogan (Vanderbilt University, Nashville, TN). The rat liver and intestinal fatty acid-binding protein probes were described previously (Gordon et al. 1983; Alpers et al. 1984).

In situ hybridization

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 (pH 7.2) overnight, dehydrated through an ethanol series, cleared in toluene and embedded in paraffin. Six-micron sections were cut and hybridizations carried out as described in (Wilkinson 1992). The antisense probes used for *Fkh6* were as described (Kaestner et al. 1996), the probes for *c-met*, neuregulin, and SF/HGF (Sonnenberg et al. 1993; Meyer and Birchmeier 1994) were kindly provided by C. Birchmeier. A 290-bp fragment of mouse TGFα (Vaughan et al. 1992) was amplified by reverse transcriptase (RT)-PCR and subcloned into Bluescript (Stratagene).

Immunohistochemistry and apoptosis assay

Tissues were fixed in either Bouin's solution or 4% paraformaldehyde, embedded in paraffin, and 5-μm sections were applied to Probe-on Plus slides (Fisher Scientific). The primary antibodies used included rabbit anti-serotonin (1:2000, Incstar), rabbit anti-rat SI (1:500, gift from Dr. K. Yeh, Louisiana State University, Shreveport, LA), and rabbit anti-Ki67 (1:500, Novocastra). The antibodies were visualized with goat anti-rabbit biotinylated secondary antiserum, an avidin/biotin detection system (Vectastain ABC system, Vector Laboratories), and DAB devel-

oper (Sigma or Research Genetics, Inc.). Deparaffinized and rehydrated slides were incubated with 6% hydrogen peroxide in methanol for 20 min. For Ki67 staining, before hydrogen peroxide and methanol, the slides were immersed in 0.01 M citrate 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 1× automation buffer (Biomedica Corp.) with 1% BSA. The tissues were incubated for 20 min at 37°C with blocking buffer (1× 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 1× 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 Wijsman 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). Deparaffinized 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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References

- Alpers, D.H., A.W. Strauss, R.K. Ockner, N.M. Bass, and J.I. Gordon. 1984. Cloning of a cDNA encoding rat intestinal fatty acid binding protein. *Proc. Natl. Acad. Sci.* **81**: 313–317.
- Alwine, J.C., D.J. Kemp, and G.R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzylomethy-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci.* **74**: 5350–5354.
- Ang, S.L. and J. Rossant. 1994. HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**: 561–574.
- Bellusci, S., R. Henderson, G. Winnier, T. Oikawa, and B.L.M. Hogan. 1996. Evidence from normal expression and targeted misexpression that Bone Morphogenetic Protein-4 (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* **122**: 1693–1702.
- Bienz, M. 1994. Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Genet.* **10**: 22–26.
- Birchmeier, C. and W. Birchmeier. 1993. Molecular aspects of mesenchymal-epithelial interactions. *Annu. Rev. Cell Biol.* **9**: 511–540.
- Bitgood, M.J. and A.P. McMahon. 1995. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Dev. Biol.* **172**: 126–138.
- Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation. *Anal. Biochem.* **162**: 156–159.
- Dempsey, P.J., J.R. Goldenring, C.J. Soroka, I.M. Modlin, R.W. McClure, C.D. Lind, D.A. Ahlquist, M.R. Pittelkow, D.C. Lee, E.P. Sandgreem, D.L. Page, and R.J. Coffey. 1992. Possible role of transforming growth factor alpha in the pathogenesis of Menetrier's disease: Supportive evidence from humans and transgenic mice. *Gastroenterology* **103**: 1950–1963.
- Duprey, P., K. Chowdhury, G.R. Dressler, R. Balling, D. Dimon, J.-L. Guenet, and P. Gruus. 1988. A mouse gene homologous to the *Drosophila* gene *caudal* is expressed in epithelial cells from the embryonic intestine. *Genes & Dev.* **2**: 1647–1654.
- Falk, P., K.A. Roth, and J.I. Gordon. 1994. Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am. J. Physiol.* **266**: 987–1003.
- Galili, N., R.J. Davis, W.J. Fredericks, S. Mukhopadhyay, F. Rauscher, B.S. Emanuel, G. Rovera, and F.G. Barr. 1993. Fusion of a fork head domain gene to PAX3 in the solid tumor alveolar rhabdomyosarcoma. *Nature Genet.* **5**: 230–235.
- Gordon, J.I. and M.L. Hermiston. 1994. Differentiation and self-renewal in the mouse gastrointestinal epithelium. *Curr. Opin. Cell Biol.* **6**: 795–803.
- Gordon, J.I., D.H. Alpers, R.K. Ockner, and A.W. Strauss. 1983. The nucleotide sequence of rat liver fatty acid binding protein mRNA. *J. Biol. Chem.* **258**: 3356–3363.
- Haffen, K., M. Keding, and P. Simon-Assmann. 1989. Cell contact dependent regulation of enterocytic differentiation. In *Human gastrointestinal development* (ed. E. Lebenthal), pp. 19–39. Raven Press, New York, NY.
- Hatini, V., S.O. Huh, D. Herzlinger, V.C. Soares, and E. Lai. 1996. Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. *Genes & Dev.* **10**: 1467–1478.
- Hoppler, S. and M. Bienz. 1995. Two different thresholds of wingless signalling with distinct developmental consequences in the *Drosophila* midgut. *EMBO J.* **14**: 5016–5026.
- James, R. and J. Kazenwadel. 1991. Homeobox gene expression in intestinal epithelium of adult mice. *J. Biol. Chem.* **266**: 3246–3251.
- Kaestner, K.H., S.C. Bleckmann, A.P. Monaghan, J. Schlöndorff, A. Mincheva, P. Lichter, and G. Schütz. 1996. Clustered arrangement of winged helix genes *fkh-6* and *MFH-1*: Possible implications for mesoderm development. *Development* **122**: 1751–1758.
- Kaestner, K.H., J.M. Ntambi, T.J. Kelly, Jr., and M.D. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes. A second differentially expressed gene encoding stearoyl-CoA desaturase. *J. Biol. Chem.* **264**: 14755–14761.
- Kaestner, K.H., K.H. Lee, J. Schlöndorff, H. Hiemisch, A.P. Monaghan, and G. Schutz. 1993. Six members of the mouse forkhead gene family are developmentally regulated. *Proc. Natl. Acad. Sci.* **90**: 7628–7631.
- Kaestner, K.H., H. Hiemisch, B. Luckow, and G. Schutz. 1994a. The HNF-3 gene family of transcription factors in mice: Gene structure, cDNA sequence, and mRNA distribution. *Genomics* **20**: 377–385.
- Kaestner, K.H., L. Montoliu, H. Kern, M. Thulke, and G. Schutz. 1994b. Universal beta-galactosidase cloning vectors for promoter analysis and gene targeting. *Gene* **148**: 67–70.
- Kühn, R., K. Rajewski, and W. Müller. 1991. Generation and analysis of interleukin-4 deficient mice. *Science* **254**: 707–710.
- Kulkarni, A.B. and S. Karlsson. 1993. Transforming growth factor beta-1 knockout mice: A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am. J. Pathol.* **143**: 3–8.
- Li, J. and P.K. Vogt. 1993. The retroviral oncogene *qin* belongs to the transcription factor family that includes the homeotic gene fork head. *Proc. Natl. Acad. Sci.* **90**: 4490–4494.
- Liu, J.-P., J. Baker, A.S. Perkins, E.J. Robertson, and A. Efstradiadis. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 igf receptor (Igf 1r). *Cell* **75**: 59–72.
- Luetke, N.C., T.H. Qiu, R.L. Peiffer, P. Oliver, O. Smithies, and D.C. Lee. 1993. TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73**: 263–278.
- Mann, G.B., K.J. Fowler, A. Gabriel, E.C. Nice, R.L. Williams, and A.R. Dunn. 1993. Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* **73**: 249–261.
- Meyer, D. and C. Birchmeier. 1994. Distinct isoforms of neuroregulin are expressed in mesenchymal and neuronal cells during mouse development. *Proc. Natl. Acad. Sci.* **91**: 1064–1068.
- Monaghan, A.P., K.H. Kaestner, E. Grau, and G. Shutz. 1993. Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 alpha, beta and gamma genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119**: 567–578.
- Parr, B.A. and A.P. McMahon. 1994. Wnt genes and vertebrate development. *Curr. Opin. Genet. Dev.* **4**: 523–528.
- Ruppert, S., M. Boshart, F.X. Bosch, W. Schmid, R.E.K. Fournier, and G. Schütz. 1990. Two genetically defined trans-acting loci coordinately regulate overlapping sets of liver-specific genes. *Cell* **61**: 895–904.
- Schluter, C., M. Duchrow, C. Wohlenberg, M.H. Becker, G. Key,

- H.D. Flad, and J. Gerdes. 1993. The cell proliferation-associated antigen of antibody Ki-67: A very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. *J. Cell Biol.* **123**: 513–522.
- Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**: 699–702.
- Shull, M.M., I. Ormsby, A.B. Kier, S. Pawlowski, R.J. Diebold, M. Yin, R. Allen, C. Sidman, G. Poetzel, D. Calvin, N. Annunziata, and T. Doetschman. 1992. Targeted disruption of the mouse transforming growth factor beta-1 gene results in multifocal inflammatory disease. *Nature* **359**: 693–699.
- Sonnenberg, E., D. Meyer, K.M. Weidner, and C. Birchmeier. 1993. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J. Cell Biol.* **123**: 223–235.
- Suh, E., L. Chen, J. Taylor, and P.G. Traber. 1994. A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol. Cell. Biol.* **14**: 7340–7351.
- Tao, W. and E. Lai. 1992. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron* **8**: 957–966.
- Tgaki, H., C. Jhappan, R. Sharp, and G. Merlino. 1992. Hyper-trophic gastropathy resembling Menetrier's disease in transgenic mice overexpressing transforming growth factor alpha in the stomach. *J. Clin. Invest.* **90**: 1161–1167.
- Traber, P.G. and G.D. Wu. 1995. Intestinal development and differentiation. In *Gastrointestinal cancers: Biology, diagnosis, and therapy* (ed. A.K. Rustgi), pp. 21–43. Lippincott-Raven, Philadelphia, PA.
- Vaughan, T.J., J.C. Pascall, and K.D. Brown. 1992. Nucleotide sequence and tissue distribution of mouse transforming growth factor-alpha. *Biochem. Biophys. Res. Comm.* **1132**: 322–324.
- Weinstein, D.C., A. Ruiz i Altaba, W.S. Chen, P. Hoodless, V.R. Prezioso, T.M. Jessell, and J.E. Darnell, Jr. 1994. The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78**: 575–588.
- Wijsman, J.H., R.R. Jonker, R. Keijzer, C.J. van de Velde, C.J. Cornelisse, and J.H. van Dierendonck. 1993. A new method to detect apoptosis in paraffin sections: In situ end-labeling of fragmented DNA. *J. Histochem. Cytochem.* **41**: 7–12.
- Wilkinson, D.G. 1992. *In situ hybridization. A practical approach*. Oxford University Press, Oxford, UK.
- Xuan, S., C.A. Baptista, G. Balas, W. Tao, V.C. Soares, and E. Lai. 1995. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14**: 1141–1152.

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References

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