INTRODUCTION

Gut development in vertebrates begins with the invagination of the endodermal layer, first at an anterior ventral site (anterior intestinal portal, AIP) and shortly followed by a posterior invagination (caudal intestinal portal, CIP), which both lead to the formation of two open-ended tubes. The gut tubes grow towards each other until they meet and fuse to a continuous structure. As the tubes form splanchnic mesoderm is recruited to surround the invaginating endoderm. The primitive gut tube has already become subdivided into three broad domains along the anteroposterior axis, referred to as foregut, midgut and hindgut, which give rise to the oesophagus and stomach, the small intestine, and the large intestine and rectum, respectively. Each of these gut regions exhibits unique mesodermal and endodermal patterns of differentiation, which can be recognised by their morphology and histology. For example, epithelial cells in the small intestine, which function in digestion and resorption of nutrients, are arranged in villus structures, which are surrounded by two thin layers of smooth muscle to move the gut content by peristalsis. In the mouse substantial remodelling of the gut endoderm occurs between embryonic days E15 and E19 in a proximal to distal progression of cytodifferentiation, which converts the initially pseudostratified epithelium to a monolayer of columnar epithelium. Concomitant with cytodifferentiation, villi are formed that are separated from each other by proliferating intervillus epithelium. Morphogenesis of the mouse intestine, however, is not completed at the time of birth but continues into the third postnatal week. During the first 2 weeks after birth the intervillus epithelium develops into crypts, which contain dividing epithelial stem cells at their base. After limited proliferation descendants of crypt cells differentiate into the four principal epithelial cell lineages, Paneth cells, enterocytes, Goblet cells and enteroendocrine cells, which migrate up the villus in a proximo-distal direction until they are extruded near the villus tip (Schmidt et al., 1985). Increased production of epithelial cells results in lengthening of villi until the rates of cell production and cell loss at the apical extrusion zone reach an equilibrium in the third postnatal week. The migration of epithelial cells in ordered vertical stripes along the crypt-villus tip axis is also established during this postnatal period (Gordon and Hermiston, 1994). Thus, crypts and associated villi constitute the anatomical and functional units in the mature epithelium of the small intestine. The dynamic process of controlled proliferation and differentiation of gut epithelial cells continues throughout life and ensures constant renewal of intestinal cells. In colon no villi are present, but epithelial cuffs

SUMMARY

The homeodomain transcription factor Nkx2-3 is expressed in gut mesenchyme and spleen of embryonic and adult mice. Targeted inactivation of the Nkx2-3 gene results in severe morphological alterations of both organs and early postnatal lethality in the majority of homozygous mutants. Villus formation in the small intestine appears considerably delayed in Nkx2-3−/− foetuses due to reduced proliferation of the epithelium, while massively increased growth of crypt cells ensues in surviving adult mutants. Interestingly, differentiated cell types of the intestinal epithelium are present in homozygous mutants, suggesting that Nkx2-3 is not required for their cell lineage allocation or migration-dependent differentiation. Hyperproliferation of the gut epithelium in adult mutants is associated with markedly reduced expression of BMP-2 and BMP-4, suggesting that these signalling molecules may be involved in mediating non-cell-autonomous control of intestinal cell growth.

Spleens of Nkx2-3 mutants are generally smaller and contain drastically reduced numbers of lymphatic cells. The white pulp appears anatomically disorganized, possibly owing to a homing defect in the spleen parenchyme. Moreover, some of the Nkx2-3 mutants exhibit asplenia. Taken together these observations indicate that Nkx2-3 is essential for normal development and functions of the small intestine and spleen.

Key words: Homeodomain transcription factor, Gene knockout, Gut, Spleen, Development, Growth regulation, Nkx2-3, Mouse
surrounding each crypt serve as villus homologues in the large intestine.

Initiation and maintenance of gut differentiation depends on extensive reciprocal signalling between endoderm and mesenchyme. Early in gut development the invaginating endoderm signals to the splanchic mesoderm, recruiting it to the gut tube and inducing its differentiation to visceral mesoderm (Kedinger et al., 1986; Roberts et al., 1995). Region-specific differentiation of gut mesoderm also requires signals derived from the adjacent endoderm (Außerheide and Ekbloom, 1988; Kedinger et al., 1990). After the mesoderm has been specified, it in turn patterns the morphological differentiation of the overlying endoderm along the anteroposterior axis (Haffen et al., 1987, 1983; Kedinger et al., 1988, 1986). In Drosophila larvae the specification of a single cell type and the expression of the homeotic gene labial in midgut endoderm are controlled by the mesodermally derived signals decapentaplegic (dpd) and wingless (wg) (Bienz, 1994; Hoppler and Bienz, 1995).

During early gut development in vertebrates, sonic hedgehog (shh) is expressed throughout the gut endoderm and has been implicated as an important signal from the endoderm to the mesoderm (Bitgood and McMahon, 1995; Marigo et al., 1995; Roberts et al., 1995). BMP4, a member of the TGFβ superfamily of secreted factors, is expressed in the visceral mesoderm abutting the shh-expressing cells and is considered to be a normal target of endodermally derived shh (Roberts et al., 1995). In BMP4 null mice the gut mesoderm fails to close ventrally, suggesting that BMP4 has a role in the recruitment of visceral mesoderm to the forming intestine (Winner et al., 1995). Thus, BMP4 may act as a secondary signal downstream of shh during early gut development, but both signals can have independent roles later (Belussi et al., 1997, 1996).

Induction of BMP4 and Hoxd-13 in response to shh is regionally restricted in the developing chick gut mesoderm, suggesting that the visceral mesoderm may be prepatterned (Roberts et al., 1998). Misexpression of shh in various regions of the splanchnic mesoderm results in BMP4 induction and Hoxd-13 expression in mid- and hindgut mesoderm, but not in foregut. The molecular nature of this region-specific competence of visceral mesoderm to respond to shh signals, however, remains unclear. Several genes that are expressed in defined gut domains with distinct boundaries between foregut and midgut or midgut and hindgut may constitute potential candidates for prepatternning. For example, the winged-helix transcription factor Fkh-6 is expressed in mid- and hindgut mesoderm of the mouse (Kaestner et al., 1997). Several members of the Nkx2 family of homeodomain transcription factors are also expressed in distinct domains along the AP axis of the gut. In the chick Nkx2-5 is found in early foregut and later in the pylorus (Buchberger et al., 1996) and Nkx2-8 is expressed in pharyngeal endoderm (Brand et al., 1997). We have recently reported that Nkx2-3 in chick and mouse is expressed in midgut and hindgut mesoderm during embryonic development and postnatally (Buchberger et al., 1996; Pabst et al., 1997). Whether any of these genes are expressed prior to shh or have a role in anterior-posterior gut patterning has not been investigated.

Nk genes have been implicated in cell-type specification and maintenance of the differentiated phenotype in many tissues of evolutionarily distant organisms ranging from Drosophila to mouse (Harvey, 1996). In order to investigate the potential role of Nkx2-3 we generated a null mutation in mouse by targeted gene disruption and analysed the mutant phenotype. More than half of the homozygous mutant animals die during the weaning period, presumably because of digestive malfunctions owing to severe perturbations of tissue architecture in the small intestine. During the late foetal and early postnatal period, development of the gut epithelium and villus formation was considerably retarded in the Nkx2-3 mutants. In contrast, proliferation of crypt cells and the apparent migration velocity of epithelial cells in the jejunum of adult homozygous survivors were markedly enhanced, resulting in enlarged and disorganised crypt and villus structures. The defect in growth regulation of epithelial cells by the lack of Nkx2-3 in gut mesenchyme correlated with significant downregulation of BMP2 and BMP4, implicating these signalling molecules in the non-cell-autonomous phenotype. Consistent with Nkx2-3 expression in spleen, we observed asplenias in a considerable percentage of mutants and severe morphological alterations in spleens of all remaining mutants.

MATERIALS AND METHODS

Construction of the targeting vector, electroporation of ES cells and generation of mice

The Nkx2-3 gene was isolated from a genomic library of the mouse strain 129Sv on two overlapping phage clones, which covered about 15 kb of the locus. A 1.5 kb BamHI/I fragment located downstream of exon 2 was subcloned into the pKS vector, excised as Xhol/I fragment and inserted into the transfer vector pPNT (Tybulewicz et al., 1991). The obtained vector pPNT-XN was used to generate two alternative targeting vectors. For targeting vector 1, a 5 kb BamHI fragment located 5’ to the first exon of Nkx2-3 was modified with KpnI linkers and inserted into the corresponding site of the pPNT-XN vector. For targeting vector 2, a 4.5 kb BamHI/SmaI fragment containing the first exon, the intron and part of the second exon, was fused in-frame to the lacZ coding region of the pPD46.21 vector. The fusion product was excised using BamHI and NotI, modified with SfiI linkers and inserted into the pPNT-XN vector after modifying the Xbal site to an SfiI site.

J1 ES cells were grown on embryonic feeder cells in the presence of LIF electroproated with 100 μg of NotI linearised vector DNA and selected as described previously (Braun et al., 1992). Homologous recombination events were identified on Southern blots with SacI-digested DNA using a 3’ flanking hybridisation probe (350 bp NotI/SacI fragment). This probe generated a 3 kb wild-type fragment, and 6 kb and 4.5 kb mutant fragments for targeting vector 1 and targeting vector 2, respectively. Three randomly chosen clones for each construct were injected into C57Bl6 mouse blastocytes. One clone of each mutant allele contributed to the germ line. All homozygous mutant mice were obtained as F1 intercrosses in a mixed 129Sv/C57Bl6 background.

Histological analysis, immunohistochemistry and lectin staining

For histological analysis tissues were fixed in 4% paraformaldehyde overnight, dehydrated with increasing concentrations of ethanol, embedded in paraﬃn and sectioned at 6 μm. Staining with Hematoxylin and Eosin, Alcian Blue and Neutral Red was performed using standard protocols. For immunohistochemical and lectin staining, sections were deparaffinized, rehydrated, treated with 3% H2O2 in methanol for 10 minutes, and blocked with 1.5% horse serum in PBS. Biotinylated UEA I lectin was obtained from Vector and CD31 antibody from Dako. Bound antibodies were visualized with

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**Note:** The text provided is a natural reading of a scientific document, ensuring proper formatting and coherence. However, due to the nature of the provided data limits, the full context of the document is not replicated here. For a complete understanding, the full document is recommended.
the Vectastain elite kit using diaminobenzidine as substrate. Sections were counterstained with Hematoxylin.

**EM scanning microscopy**

Tissue was fixed for 1 hour at 4°C in cacodylate buffer (0.1 M cacodylate, pH 6.9, 10 mM MgCl₂, 10 mM CaCl₂, 90 mM sucrose) containing 3% glutaraldehyde and 5% formaldehyde, dehydrated in increasing concentrations of aceton and dried with liquid CO₂ (CPD030, Bal-Tec Lichtenstein). Probes were coated with a 10 nm gold film (SCD040, Bal-Tec Lichtenstein), observed and photographed using a DSM982 Gemini (Zeiss) scanning electron microscope.

**BrdU incorporation and apoptosis assays**

To visualize proliferating cells in vivo, mice were injected intraperitoneally with 120 mg/kg BrdU (Sigma), 12 mg/kg 5-fluoro-2′-deoxyuridine (Sigma) in PBS 2, 24 and 48 hours prior to killing. Tissues were fixed in Bouins solution overnight, embedded in paraffin and sectioned. BrdU incorporation was detected using the BrdU staining kit (Calbiochem). Apoptotic cells were identified as described by Gavrieli et al. (1992).

**RNA analysis and RT-PCR**

Total RNA was isolated as described (Buchberger et al., 1994) and used for northern blot analysis and RT-PCR. For northern blots, 20 μg of RNA/lane was subjected to electrophoresis on formaldehyde agarose gels and blotted onto a PALL membrane. Hybridization was performed in Church buffer followed by washings in 0.2·SSC, 0.2% SDS at 65°C (1x SSC contains 0.15 M sodium chloride and 25 mM sodium citrate, pH 7.0). The following probes were used: 350 bp Nkx2-3 NcoI/PstI fragment, full-length mouse GAPDH, BMP2 and BMP4, full-length shh and e-cadherin. Probes for intestinal fatty acid binding protein (iFABP), Cdx1, Cdx2 have previously been described (Kaestner et al., 1997).

For semiquantitative RT-PCR the following primers were used. Hypoxanthin phosphoribosyltransferase (HPRT) upstream primer, GCTGGTGAAAGGACCTCT; HPRT downstream primer, CACAGACTAGACACCTGC; BMP2 upstream primer, GTTTGTGTGGCTTGACGC; BMP2 downstream primer, AGACGTCCTGAGGAATTTG; BMP4 upstream primer, TGTTAGGAGTTCCATACGC; BMP4 downstream primer, TTTATCTCTTCTCCTGGACC. Reverse transcription was performed with 1 mg of total RNA and 100 ng pdN6 hexamers according to standard procedures. The RT products were amplified in the presence of (α-32P)dCTP by 32 PCR cycles for BMP2 and BMP4. Primers for amplification of HPRT were added 10 cycles later, yielding a total of 42 amplification cycles. Each cycle consisted of 45 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C. Care was taken with each product that amplification was still in the exponential phase. The PCR products were separated on a 5% polyacrylamide gel and identified by exposure on X-ray films.

**RESULTS**

**Targeted disruption of the Nkx2-3 gene**

The Nkx2-3 homeodomain-containing gene has previously

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**Fig. 1.** Targeting strategy for Nkx2-3 gene inactivation. (A) Top line represents the Nkx2-3 gene locus; middle line illustrates the targeting vector used for homologous recombination in ES cells. Note that the targeting vector deletes both exons. Bottom line depicts the structures of the targeted mutant allele. Restriction sites and hybridization probes used to identify targeting events are indicated. (B) Southern blot analysis of DNA from newborn siblings of a typical litter obtained in crosses of Nkx2-3m¹ heterozygous parents. Genotyping was performed by DNA digestion with SacI and hybridisation with the indicated 3’ probe. Wild-type and mutant alleles are represented by 3 kb and 6 kb DNA fragments, respectively. (C) Northern blot analysis of Nkx2-3 transcripts in various tissues of wild-type and Nkx2-3m¹ homozygous mutant mice. No Nkx2-3 transcripts accumulate in gut samples of homozygous mutants. GAPDH probe was used as RNA loading control, do, duodenum; je, jejunum; co, colon; ht, heart; li, liver; th, thymus; sp, spleen; bm, bone marrow.
been reported to be expressed in gut mesenchyme during pre- and postnatal mouse development and in the epithelium of branchial arches (Pabst et al., 1997). Northern blot analysis of RNA isolated from various organs of adult mice now reveals that the \textit{Nkx2-3} gene is also highly expressed in spleen and at considerably lower levels in bone marrow, heart, liver and thymus (Fig. 1C). In all expressing tissues two transcripts of approximately 1.8 kb and 2.5 kb accumulate, the smaller RNA species being the abundant transcript in gut and spleen. Although the precise difference between the two transcripts is not known, they clearly constitute products of the unique \textit{Nkx2-3} gene, as they are both absent in gene knockout mutants. In order to investigate the potential role of the \textit{Nkx2-3} gene in mice, we generated mutant alleles in embryonic stem (ES) cells by homologous recombination. The \textit{Nkx2-3} gene was cloned from a mouse strain 129Sv genomic library and was shown to contain two exons, both represented in the small and large \textit{Nkx2-3} transcripts (Fig. 1). To obtain a true null allele both exons were deleted and replaced by the PGK-neo selection cassette in reverse orientation to \textit{Nkx2-3} transcription (Fig. 1A). This mutant allele is referred to as \textit{Nkx2-3}m1. Transfection of the targeting vector into J1 ES cells yielded six homologous recombination events at the \textit{Nkx2-3} gene locus with a frequency of 1 in 25 double selected clones in the presence of G418 and Gancyclovir. Injection of several ES cell clones carrying the mutant allele into blastocysts resulted in germ line chimeras that were used to generate heterozygous and homozygous mutant animals (Fig. 1B). We also produced a second allele carrying the bacterial \textit{LacZ} gene instead of exon 2. \textit{LacZ} was, however, not expressed in vivo for unknown reasons. Both alleles resulted in identical phenotypes (data not shown). All results presented in this report were obtained with \textit{Nkx2-3}m1 mice.

In crosses of heterozygous mice of the mixed genetic background 129Sv × C57BL6, all three possible genotypes were born with normal Mendelian frequency, indicating that the \textit{Nkx2-3} mutations did not cause embryonic lethality (Figs 1B, 2). Heterozygous mutant mice were virtually indistinguishable from wild-type littermates in overall growth. Homozygous \textit{Nkx2-3} mutants are born alive but exhibit growth retardation and tend to die during the weaning period. (A) Normal Mendelian frequency of wild-type and mutant genotypes is observed during prenatal development but considerable loss of homozygous mutants occurs within the first 3 postnatal weeks. Numbers for prenatal time points represent one particular litter, each representative of at least three analysed litters in each age group. For postnatal development the numbers were combined from several litters. (B) Mass curves of a cohort of wild type and heterozygotes (open diamonds), and homozygous mutants (closed circles) within 20 days of birth. Animals that died during the analysis are indicated by crosses. (C) Growth curves for five males from one litter. Open diamonds, heterozygotes and wild type; closed circles, homozygous mutants. The observed growth retardation is representative for more than 30 analysed mutants.

![Fig. 2](image2.png)

**Fig. 2.** Homozygous \textit{Nkx2-3} mutants are born alive but exhibit growth retardation and tend to die during the weaning period. (A) Normal Mendelian frequency of wild-type and mutant genotypes is observed during prenatal development but considerable loss of homozygous mutants occurs within the first 3 postnatal weeks. Numbers for prenatal time points represent one particular litter, each representative of at least three analysed litters in each age group. For postnatal development the numbers were combined from several litters. (B) Mass curves of a cohort of wild type and heterozygotes (open diamonds), and homozygous mutants (closed circles) within 20 days of birth. Animals that died during the analysis are indicated by crosses. (C) Growth curves for five males from one litter. Open diamonds, heterozygotes and wild type; closed circles, homozygous mutants. The observed growth retardation is representative for more than 30 analysed mutants.

![Fig. 3](image3.png)

**Fig. 3.** Development of the small intestine in foetal and early postnatal \textit{Nkx2-3}−/− mice. HE-stained paraffin sections of midgut from E15 (A,E), E17.5 (B,F) and P3 (D,H) wild-type (A-D) and \textit{Nkx2-3}−/− mutant (E-H) mice are shown. Note that folding of the epithelium and villus formation is markedly inhibited in the mutants throughout fetal development. Also note the reduction of gut mesenchyme in \textit{Nkx2-3} mutants (B,F). BrdU labelling in the jejunum of E17.5 wild-type (C) and mutant (G) embryos indicates reduced proliferation of epithelial cells in the mutant. Note that most labelled cells in the mutant are mesenchymal (G; arrowheads). 200× (A,B,E,F); 630× (C,G); 25× (D,H).
morphology, growth behaviour, histology and gene expression, while homozygous Nkx2-3<sup>m1</sup> mice showed considerable growth retardation and more than 50% died within 21 days after birth (Fig. 2). Survivors of the weaning period gained weight normally, and over several months approached the size of their wild-type siblings. Variable penetrance of the phenotype is not unusual in gene-targeting mutations and may be related to variations of the genetic background. Ongoing experiments on 129Sv and C57BL6 inbred backgrounds provide evidence for strain-dependent variability of post-natal lethality. To ascertain the null character of the Nkx2-3<sup>m1</sup> allele, transcript levels were analysed in different regions of the midgut and hindgut from homozygous mutants. As expected, homozygous mutants lacked any Nkx2-3 transcripts in duodenum, jejunum and colon (Fig. 1C). We also failed to detect Nkx2-3 transcripts in E9.5 homozygous mutant embryos by in situ hybridisation, while transcripts were readily detectable in wild-type mice (data not shown).

**Villus formation in the small intestine appears delayed in Nkx2-3<sup>−/−</sup> mice**

Given the expression of Nkx2-3 in gut mesenchyme and the growth retardation with increasingly cachectic appearance and frequent early postnatal death in homozygous mutants, we suspected that normal gut development may be affected in these mice leading to digestive problems in new-born mutant animals. An important developmental transition of gut morphology in the mouse takes place when the small intestinal epithelium undergoes cytodifferentiation and begins to form villi in a proximal to distal progression between E14.5 and E19 of prenatal development. Sections through the midgut of wild-type and Nkx2-3<sup>−/−</sup> mice of this foetal period revealed that the onset and progression of epithelial organisation was markedly altered in the jejunum and also, albeit less severely, in the ileum of homozygous mutants (Fig. 3). No significant changes were seen in the duodenum or colon. The epithelium in the jejunum of E15 mutant embryos appeared markedly less folded than in wild-type embryos and the mesenchyme had barely begun to form ridges that underly the prospective villi (Fig. 3A,E). At E17.5 villi had significantly

![Fig. 4.](image1)

**Fig. 4.** The intestinal tract in adult Nkx2-3<sup>−/−</sup> mice appears considerably enlarged. Intestines of wild-type (A-E) and homozygous mutant mice (F-J) were prepared and sectioned transversally along the AP axis. Whole mounts reveal macroscopic differences in size and vascularisation between wild type (A) and mutants (F). Sections through proximal (B,G), intermediate (C,H) and distal (D,I) levels of the small intestine and colon (E,J) demonstrate the increased width of the gut with prolonged villi and thickened mesenchyme, particularly in the jejunum (C,H). 25× (except for A and F).

![Fig. 5.](image2)

**Fig. 5.** Morphological alterations of the small intestine in adult Nkx2-3<sup>−/−</sup> mutant mice. Paraffin sections through the jejunum of wild-type (A-E) and Nkx2-3<sup>−/−</sup> mutant mice (F-J) were stained with HE. Mutant villi exhibit irregular architecture with bifurcations and branching and the crypt compartment is largely expanded (F,H). Also note the grossly altered appearance of the mesenchyme with occasional disintegration of the lamina propria and increased density of blood vessels (H). Higher magnification of the epithelium shows no major defects in the epithelial architecture of mutants (B,G). Clusters of Paneth cells visualized with UEA I lectin (dark staining) at the base of crypts were present also in the mutant (C,H). Endothelial cells and infiltrating leukocytes were stained with CD31 (P-CAM) antibodies and peroxidase (D,I). Note the increase in blood vessels and reduction of leukocytes in the villus cores of the mutant (I). Goblet cells and acid mucus around the villi (blue stain) were identified with Alcian Blue in both wild-type and mutant gut (E,J). 100× (A,F); 630× (B,G); 200× (C-E,H-J).
grown and filled almost the entire gut lumen in the jejunum of wild-type animals, while much shorter and fewer villi were present in the mutant, indicating the considerable delay of villus formation in the absence of Nkx2-3 (Fig. 3B,F). Also the gut mesenchyme appeared slightly reduced in mutants. This retardation of villus formation and epithelial remodelling in mutant foetuses persisted into the early postnatal period (Fig. 3D,H). Consistent with smaller villi in Nkx2-3−/− mice, the number of proliferating cells labelled with BrdU within the intervillus epithelium was markedly decreased in comparison to wild type, but normal numbers of BrdU-positive cells were present in the gut mesenchyme (Fig. 3C,G). Although growth of the villi was much slower in the mutant than in wild type, the principal organisation of villus cores and columnar epithelium covering each villus appeared fairly normal. Taken together these observations suggest that lack of Nkx2-3 in gut epithelium covering each villus appeared normal. Taken together these observations suggest that lack of Nkx2-3 in gut mesenchyme primarily affects the local proliferation control of intestinal epithelial cells during the early remodelling process of gut endoderm with variable effects along the duodenal to colonic axis.

The architecture of the epithelium in small intestine of adult Nkx2-3−/− mice is severely perturbed

As extensive remodelling of gut epithelium in mice is not completed until 3 weeks after birth, we analysed the phenotypic effects of the Nkx2-3 mutation in adult animals 60 days after birth. In adult mutant mice the gut throughout its proximo-distal extension appeared normal in length but consistently wider in diameter than in wild type, a difference that clearly exceeded the normal size variations seen due to dilation by varying amounts of gut content (Fig. 4). Enlargement of the small intestine appeared to be the result of significantly larger villi and a somewhat thicker mesenchymal layer, particularly pronounced in the jejunum (compare Fig. 4B-D with G-I). No histological alterations were apparent in colon (Fig. 4E,J). Although the gut from wild-type and mutant animals showed similar motility, we cannot absolutely exclude diminished peristalsis, which may contribute in part to the wider gut lumen in Nkx2-3 mutants. We also noted extensive extra vascularisation in the mutant gut (Fig. 4A,F).

In addition to size differences, the morphology of villi in the small intestine of mutants was markedly disturbed, most prominently in the jejunum. Hematoxylin and Eosin (HE) staining of sections through the jejunum demonstrated irregularly shaped villi with branches and deep folds in mutant mice, while the epithelium of normal mice formed regularly spaced villi of similar size with well-organised crypts surrounding each villus base (Fig. 5A-G). Scanning electron microscopy of the jejunum confirmed the increased size and altered morphology of villi in Nkx2-3−/− mutants (Fig. 6). The surface appearance of the epithelium, however, seemed normal. The crypt compartment in Nkx2-3 mutant mice was significantly expanded and morphologically disorganised, extending far more into the villus structures than normally seen in wild-type epithelium (Fig. 5F,H). Moreover, the gut mesenchyme, which usually forms a regular cylindrical layer surrounding the epithelium, was thicker at some places pushing the epithelium toward the gut lumen (Fig. 5C,H). In these regions the sharp boundary between the lamina propria and the epithelium appeared undefined, possibly due to separation from the basement membrane.

Despite their distorted shape, the villi in mutant mice were covered by columnar epithelium, which was morphologically indistinguishable from wild-type epithelium (Fig. 5B,G). HE staining showed uniform enterocytes in mutants with similar cell size, homogeneously stained cytoplasm, a distinct apical brush border and tight adherence between neighbouring cells. This suggest that enterocytes constituting the vast majority of the four principal epithelial cell types were allocated correctly to this cell lineage and presumably underwent normal migration-associated cell differentiation in Nkx2-3−/− mutant mice. Histological staining for brush-border enzymes, such as phosphodiesterase and acidic and basic phosphatase, also appeared normal in mutants (data not shown). Histochemical staining with Alcian Blue revealed that mucus-producing goblet cells, born in crypts and migrating upward to the apical extrusion zone of the villi, were also present in the Nkx2-3−/− mutant with a similar distribution to those in wild type (Fig. 5E,J). A coherent layer of acid mucus covering the epithelium in mutants, as in wild type, suggested that the goblet cells were functional. Defensin- and growth factor-producing Paneth cells migrate downward to the base of the crypt where they reside in clusters until removed by phagocytosis (Bry et al., 1994;
Cheng, 1974). Visualisation by staining with UEA I (Ulex europaeus type I) lectin revealed normal clusters of Paneth cells in crypts, although their localisation within the gut epithelium clearly reflected the disorganisation of the crypt compartment and the underlying mesenchyme in the Nkx2-3−/− mutant (Fig. 5C,H). CD 31 (P-CAM) antibodies were applied on sections of wild-type and mutant jejunum in order to detect immigrant myeloid cells and endothelial cells. While the mesenchymal layer of wild-type jejunum contained only sparsely scattered endothelial cells, the mutant had considerably more endothelial cells reflecting the increased vascularisation of the small intestine (Fig. 5D,I). Most notably, the villus cores of wild type appeared densely populated by CD31-positive cells, whereas these cells were largely absent in the mutant (Fig. 5D,I). Taken together these observations provide evidence that Nkx2-3 is required to order the structure of the gut epithelium in the small intestine and maintain the correct gut architecture. Nkx2-3 seems, however, not to be important for the allocation of the various epithelial cells to their respective cell lineages, as these differentiated cells were present in the mutant.

**Proliferation and migration rates of enterocytes are increased in adult Nkx2-3−/− mice**

The size of villi in small intestine is determined by the production rate of epithelial cells in the crypts and their rate of exfoliation from the apical tip of the villi into the gut lumen. Both processes reach an equilibrium in adult mice whereby further growth of villi is arrested. The increased length and volume of villi in the jejunum of adult Nkx2-3−/− mutants prompted us to investigate the proliferation of crypt cells in comparison to wild type. Dividing crypt cells were labelled by the incorporation of bromodeoxyuridine (BrdU) in vivo. Corresponding to the perturbed morphology of the crypt compartment in mutants, the zone of BrdU-positive cells was greatly expanded, containing 3-4 times more proliferating cells than in wild type 2 hours after the labelling pulse (Fig. 7B,E). This indicated that the birth rate of new epithelial cells was significantly increased in the adult Nkx2-3−/− mutant. Interestingly, these cells seemed to clear the crypts and migrate toward the tip of the villus much faster in the mutant than in wild-type animals. Whereas most of the BrdtU-labelled epithelial cells remained in or near the crypts for 24 hours in wild type, the majority of BrdU-positive cells in mutant mice had left the crypts and were found in the villus epithelium up to a level of half the villus length (Fig. 7C,F). At 48 hours all labelled cells in the mutant had disappeared from the villus (data not shown). These results suggest that not only the production of new epithelial cells but also the velocity of their migration along the longitudinal villus axis was markedly elevated, by a factor of 2 to 3, in the mutant mouse.

In order to see whether the enhanced proliferation of crypt cells might be counteracted by enhanced apoptosis, we identified cells undergoing programmed cell death by TUNEL staining. In wild-type animals few apoptotic events could be detected near or in the apical extrusion zone of villi, but no change in numbers of apoptotic cells was observed in villus or crypt epithelium of mutants (data not shown).

**Gene expression in Nkx2-3 mutant mice**

To explore whether the morphological changes in Nkx2-3−/− mutants were associated with altered gene expression in the gut, RNA levels of transcription factors and signalling molecules known to be involved in gut development and functions were assessed by northern blot analysis. The winged-helix transcription factor fkh-6 is expressed in a similar pattern to Nkx2-3 in normal gut mesenchyme and fkh-6 gene disruption causes a phenotype in the epithelium of the small intestine that shares many aspects of the Nkx2-3 mutation as described here (Kaestner et al., 1996, 1997). This suggested to us that both transcription factors might act in one genetic pathway. However, despite the absence of Nkx2-3 in the mutant, normal levels of fkh-6 transcripts were found in all segments of the intestine (Fig. 8) and normal levels of Nkx2-3 transcripts were present in the fkh-6 mutant (K. H. Kaestner, personal communication). Thus, both transcription factors appear to play roles in genetically independent pathways. Cdx1 and Cdx2, murine homologues of the *Drosophila* homeobox gene *caudal*, are expressed in the epithelium of small intestine and colon (Duprey et al., 1988; James and Kazemwanadel, 1991; Suh et al., 1994; Suh and Traber, 1996). Cdx1 has been shown recently to accumulate in the proliferative crypt compartment (Subramanian et al., 1998). In intestinal specimen of adult Nkx2-3 mutant mice transcripts for both transcription factors accumulated similar as in wild type (data not shown). Immunochemical staining with Cdx1-specific antisera revealed slightly increased numbers of Cdx1-positive cells in crypts and also more lymphocyte-like cells in villus cores (data not shown). Thus, the apparently unchanged level of Cdx1 mRNA may reflect a reduced overall number of crypts with more proliferating cells in each individual crypt. Similar to Cdx1 and 2, mRNA levels of other gut markers, such as the intestinal fatty acid binding protein iFABP, the signalling molecule sonic hedgehog (shh), and the epithelial cell adhesion molecule E-cadherin, were unaltered in ileum and jejunum of mutant mice. In summary, no evidence was obtained for loss of gut marker gene expression in the absence of the transcription factor Nkx2-3, suggesting that at least some aspects of cell differentiation occurred normally.

Nkx2-3 is expressed in gut mesenchyme yet seems primarily to change growth of the gut epithelium. We therefore thought that Nkx2-3 may regulate the expression of mesenchymal signals that act upon gut epithelium. BMP-2 and BMP-4, members of the TGF β superfamily of secreted growth factors, are known to be expressed in intestinal mesenchyme, and therefore were candidates for mediating Nkx2-3 functions to the epithelium. We investigated the accumulation of BMP-2 and BMP4 transcripts in proximal, intermediate and distal gut segments of mutant and wild-type mice by northern blot analysis and semiquantitative RT-PCR. In precise correlation with the pronounced morphological phenotype in jejunum, BMP2 and BMP4 transcript levels were markedly downregulated in this part of the small intestine of adult mutant mice, while no BMP downregulation occurred in duodenum and colon (Fig. 8B,C). This observation suggests that BMP4 and BMP2 may be involved in mesenchymal to epithelial signalling in the jejunum where the expression is under the control of Nkx2-3 directly or indirectly, while BMP expression may be regulated differently in other gut segments along the antero-posterior axis. Interestingly, significant changes of BMP transcript levels were not observed in mutant mice during fetal and early postnatal (up to P3) development, suggesting that
altered BMP levels may be part of a late compensatory mechanism in surviving mutants (Fig. 8A).

The Nkx2-3 mutation causes a spleen phenotype

The Nkx2-3 gene is also expressed in spleen and at low levels in bone marrow, heart and thymus (Fig. 1). Careful examination of homozygous Nkx2-3 mutants revealed that more than 20% lacked spleens and in the rest of the mutants the spleens were affected in size and morphology. No morphological alterations were observed in the other expressing organs. Spleens in mutant animals were substantially smaller at P3. HE staining of spleen sections revealed a dramatic reduction in content of nucleated cells (Fig. 9A,D). Most of the spleen parenchyma was filled with erythrocytes. In adult mutant mice the white pulp appeared reduced in size and lacked the clear follicular structure of wild type (Fig. 9B,E). Preliminary immunohistological examination and FACS analysis of mutant spleens revealed a severe decrease of lymphatic cells, although some T cell-rich cores surrounding central arteries, probably representing the perirarteriolar lymphatic sheet (PALS), were present (data not shown). However, no clear marginal zone was visible in primary follicles of Nkx2-3 mutant spleens. Moreover, macrophages that were readily detectable by HE staining in wild-type spleen were not observed in the Nkx2-3−/− mutant (Fig. 9C,F). FACS analysis also indicated a depression of granulocytes (data not shown). No changes of cell composition were observed by FACS analysis in bone marrow, peripheral blood and thymus of Nkx2-3 mutants with the exception of increased numbers of B cells in the blood (data not shown). These results together with the prominent expression of Nkx2-3 in spleen suggest, although do not prove, that the lack of Nkx2-3 may affect spleen functions specifically, possibly the homing and regional distribution of hematopoietic cells rather than their development in the bone marrow. Heterologous transplantations and challenge with T cell-dependent antigen will be performed to examine the spleen defect in more detail.

DISCUSSION

The homeodomain-containing transcription factor Nkx2-3 is predominantly expressed in mesoderm of midgut and hindgut, in the epithelium of brachial arches and tongue, and in some areas of the developing jaws during embryonic mouse development (Pabst et al., 1997). Postnatally, Nkx2-3 expression continues in gut mesenchyme and in spleen. Inactivation of the Nkx2-3 gene by homologous recombination in mice causes recessive postnatal lethality in more than 50% of mutant animals, associated with profound changes in midgut and spleen morphology. No apparent phenotypic consequences of the mutation are observed in other Nkx2-3-expressing regions, suggesting that Nkx2-3 is not absolutely required there, possibly because of overlapping functions with other Nkx2-3 related genes. For example Nkx2-6 has been reported to be expressed in hindgut mesoderm of the mouse and may
explain why the Nkx2-3 mutation does not generate a phenotype in colon (Biben et al., 1998). Since spleen function is not essential for survival, we believe that early death of mutant animals is most likely caused by the observed gut defects.

All mice lacking Nkx2-3 exhibit markedly retarded morphogenesis of the epithelium in medial and distal parts of the small intestine during the late foetal period. This developmental defect is reflected in delayed formation and slower growth of nascent villi, and attenuated transition of the stratified epithelium to a columnar epithelium. Thus, villi in mutant animals appear considerably shorter and less frequent than those in wild-type littermates and this phenotype persists postnatally, presumably until gut remodelling has been completed. The formation of crypts is also delayed and spatially unorganised during this developmental period in mutants. The explanation for the foetal and early postnatal phenotype in the small intestine most likely comes from the observation that the number of dividing cells in the intervillus epithelium is significantly decreased in Nkx2-3 null mutants. Since Nkx2-3 is only expressed in mesenchymal cells directly adjacent to but not within the gut epithelium, the phenotype must be the result of a non-cell-autonomous effect involving cell to cell signalling or cell to matrix interactions.

In contrast to reduced proliferation of epithelial cells in the small intestine of Nkx2-3 mutant foetuses, proliferation of crypt stem cells and villus growth appears significantly augmented in Nkx2-3 mutants that survive into adulthood. Villi in the adult mutants are longer and thicker than normal and crypts are greatly expanded, containing considerably more proliferating cells. These cells seem to enter the villus epithelium similar to those in wild type, but migrate much faster toward the villus tips. This apparent increase in speed of cell migration may be the direct consequence of increased proliferation, pushing more cells into the epithelium, or alternatively may represent altered cell surface properties of the mutant cells. Despite a presumably shortened residence time of cells in the epithelium, the villi become larger reflecting the disequilibrium of more new cells being born in the crypts compared to cells being lost into the gut lumen at the villus extrusion zones. Thus, the small intestine becomes considerably enlarged, primarily due to a perturbed homeostasis of the epithelium but also to thickening of the gut mesenchyme. Interestingly, the histology of the jejunal epithelium in adult Nkx2-3−/−survivors appears rather normal in its architecture and cell composition with enterocytes, Goblet cells and Paneth cells being present in fairly normal distribution.

Considering together the foetal and adult phenotypes of Nkx2-3−/− mice, we conclude that Nkx2-3 in the gut mesenchyme most likely regulates the local control of epithelial cell proliferation. In the absence of Nkx2-3 the epithelium in the small intestine fails to grow normally and does not form the appropriate villus and crypt structures. Although these developmental abnormalities may frequently cause early postnatal death, we lack precise information on the pathophysiology in order to explain this outcome. Surviving mutant animals may be saved by compensatory events which lead to increased cell proliferation in gut epithelium. The molecular mechanisms underlying these differences in early and late growth regulation in Nkx2-3 mutants are presently unclear.

It is interesting to note that the intestinal phenotype of Nkx2-3 is most pronounced in the jejunum and weak in the ileum but absent in duodenum and colon, although the gene is expressed throughout the mesenchyme of midgut and hindgut. A similar phenotype restricted to some of the expressing gut segments has been observed in mutant mice that lack the winged helix transcription factor fkh-6 (Kaestner et al., 1997). These mice exhibit reduced growth only in gastric and proximal intestinal epithelium, including duodenum and jejunum, during the late foetal period, although Fkh-6 is also expressed in colon. Similar to Nkx2-3 mutants, most homozygous Fkh-6 mutants die during the weaning period and hyperproliferation and disorganised architecture of visceral epithelium ensues in adult survivors. Despite these similarities in phenotypes, both genes seem to be part of different genetic pathways, because their respective expression patterns are not affected in either mutant (K. H. Kaestner, O. Pabst and H. H. Arnold, unpublished observations). Nevertheless, both transcription factors may have common downstream effectors, for instance BMPs, as discussed below. Double mutants lacking both genes should provide more information on unique and overlapping roles of Nkx2-3 and fkh-6 in gut development.

Nkx genes have been discussed as potential factors involved in patterning of the gut AP axis prior to the inductive effects of shh, because they are differentially expressed in visceral mesoderm (Roberts et al., 1998). Indeed, Nkx2-3 may be considered as a candidate gene, since its expression starts very
early in visceral mesoderm and is restricted to midgut and hindgut. Moreover, Nkx2-3 expression in gut mesoderm occurs apparently independent of shh, as it is normally expressed in shh null mutant mice (O. Pabst and H. H. Arnold, unpublished observations). However, the intestinal morphology and the maintained expression of regionally restricted marker genes in Nkx2-3 mutants fail to support a role of Nkx2-3 in early patterning of the gut AP axis.

The lack of the Nkx2-3 transcription factor in gut mesenchyme causes dramatic changes in the epithelium of the small intestine. It is therefore reasonable to assume that a major role of Nkx2-3 is to regulate interactions between mesenchymal and epithelial cells. Potential target genes for Nkx2-3 in mesenchyme include secreted growth factors, extracellular matrix proteins and cell surface molecules that can mediate crosstalk to the epithelium. By analogy with Drosophila, in which the signalling molecules wingless (wg) and decapentaplegic (dpp) are expressed in visceral mesoderm and control midgut development, vertebrate homologues of the wnt and BMP families are expressed in visceral mesoderm of the mouse (Bienz, 1994).

In agreement with a potential signalling function downstream of Nkx2-3, we find that BMP-2 and BMP-4 expression in the jejunum but not in duodenum and colon is markedly reduced in adult Nkx2-3<sup>−/−</sup> mutants. Since BMP4 is coexpressed with Nkx2-3 in visceral mesenchyme, it may constitute a direct Nkx2-3 target gene, whereas BMP2 is also expressed in the adjacent gut epithelium where it would be affected by Nkx2-3 only indirectly (Bitgood and McMahon, 1995). The fact that both BMP genes are downregulated in the absence of Nkx2-3 is consistent with the existence of a positive feedback loop but does not exclude other more complex scenarios. Although we have not shown that BMPs do indeed mediate control of proliferation and/or spatial assignment of epithelial stem cells in the intestine, growth regulatory functions of BMPs have been demonstrated in other systems. For example overexpression of BMP4 in lung epithelium inhibits proliferation of epithelial cells (Belluscì et al., 1997) and ectopic BMP4 expression in the stomach limits mesodermal growth (Roberts et al., 1998). The precise coincidence of regional BMP downregulation in Nkx2-3 mutants with the observed phenotype in the same regions strongly suggests that BMPs are part of a signalling pathway that regulates growth of the intestinal epithelium. This proposition is also supported by the fact that BMP downregulation and the corresponding gut phenotype was observed independently in the fkh-6 mutants (Kaestner et al., 1997). It remains puzzling, however, why BMP levels are diminished in the absence of Nkx2-3 only postnatally, while during the early phase of gut development epithelial maturation and remodelling are attenuated with normal levels of BMPs being expressed.

The Nkx2-3 mutation causes asplenia in some and morphological spleen defects in all of the remaining homozygous mutant animals. Presently we do not know at which stage of mouse embryogenesis spleen development fails and what the role of Nkx2-3 would be in spleen genesis. Targeted disruption of the Hox 11 gene also results in asplenia and it may be informative to investigate Hox 11 expression in Nkx2-3 mutants (Dear et al., 1995).

An antigen-specific immune response requires migration of B and T cells into the lymph nodes and the white pulp of the spleen. In primary follicles the T-cells are arranged in the PALS and B cells are located excentrically adjacent to this central core. The marginal zone at the periphery of primary follicles is made up of IgM-positive B cells. We are currently investigating the precise composition and anatomical location of lymphatic cells in mutant spleens. A putative lymphocyte-specific chemokine receptor was recently identified which directs B cell migration to specific compartments in the spleen and to defined lymphoid organs (Forster et al., 1996). Comparatively little is known, however, about molecules within the receiving organs that may mediate and maintain homing and distribution of the infiltrating cells in defined anatomical areas. In Nkx2-3 mutants the spleen has no normal white pulp, apparently lacks granulocytes and macrophages and contains drastically reduced numbers of B- and T cells without evidence for a general defect in hematopoiesis, as for instance Nkx2-3 mutant mice are capable of responding to antigens (our unpublished observations). The marginal zone of spleen follicles in Nkx2-3 mutants appears to lack IgM-positive B cells, which are either completely absent or dislocated somewhere else (our unpublished observation). Thus, the spleen of Nkx2-3 mutants may provide a model in which region-specific accumulation of lymphoid cells can be investigated. Whether the observed mutant phenotype is solely associated with the spleen parenchyme or also involves changes in other lymphoid organs is currently under investigation.

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