Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production

Mattias Ormestad1, Jeanette Astorga1, Henrik Landgren1, Tao Wang2, Bengt R. Johansson3, Naoyuki Miura2 and Peter Carlsson1,*

Development of the vertebrate gut is controlled by paracrine crosstalk between the endodermal epithelium and the associated splanchic mesoderm. In the adult, the same types of signals control epithelial proliferation and survival, which account for the importance of the stroma in colon carcinoma progression. Here, we show that targeting murine Foxf1 and Foxf2, encoding forkhead transcription factors, has pleiotropic effects on intestinal paracrine signaling. Inactivation of both Foxf2 alleles, or one allele each of Foxf1 and Foxf2, cause a range of defects, including mega colon, colorectal muscle hypoplasia and agangliosis. Foxf expression in the splanchnic mesoderm is activated by Indian and sonic hedgehog secreted by the epithelium. In Foxf mutants, mesenchymal expression of Bmp4 is reduced, whereas Wnt5a expression is increased. Activation of the canonical Wnt pathway—with nuclear localization of β-catenin in epithelial cells—is associated with over-proliferation and resistance to apoptosis. Extracellular matrix, particularly collagens, is severely reduced in Foxf mutant intestine, which causes epithelial depolarization and tissue disintegration. Thus, Foxf proteins are mesenchymal factors that control epithelial proliferation and survival, and link hedgehog to Bmp and Wnt signaling.

KEY WORDS: Forkhead, Intestine, Hedgehog, Wnt, Bmp
interfere with the ability of the newborn to breathe and suckle, and to be the immediate cause of death. Here, we investigate the role of murine Foxf genes in gut development and its relation to paracrine signaling by analyzing the intestinal phenotype of Foxf2−/− and Foxf1−/−; Foxf2−/− compound heterozygote mutants.

MATERIALS AND METHODS

Mouse strains

Targeted mutants of Foxf1 (Mahlapuu et al., 2001a; Mahlapuu et al., 2001b), Foxf2 (Wang et al., 2003) and the Bmp4∆z knock-in (Lawson et al., 1999) have been described elsewhere. All mutants were maintained on a C57Bl/6J background.

Histology, immunohistochemistry and in situ hybridization

Tissues were fixed in 4% paraformaldehyde and processed either for routine paraffin or cryosectioning. The following antibodies were used for immunohistochemistry: collagen I (Biorex), collagen IV (Biomex), laminin (Abcam), smooth muscle α-actin (SMA, Sigma, Clone 1A4, Alkaline Phosphatase conjugate), neurofilament M (Chemicon International), E-cadherin (Zymed, Clone ECD-2), β-catenin (Transduction Laboratories), PCNA (Dako, Clone PC10), Enactin (NeoMarkers, Clone ELM1), Syndecan 1 (Research Diagnostics, Clone 281-2) and Perlecain (Chemicon International, Clone A7L6). For detection, biotinylated secondary antibodies were used with HRP-streptavidine and amplification with TSA Biotin System (NEN Life Science Products). TUNEL assay was performed as previously described (Blixt et al., 2000) and X-gal staining according to Hogan et al. (Hogan et al., 1994). Automated whole-mount in situ hybridization with digoxigenin-labeled RNA probes was performed on an InsituPro instrument (Intavis AG, Germany). The following in situ probes were used: Foxf1 (Mahlapuu et al., 2001b), Foxf2 (Ormestad et al., 2004), Wnt5a (IMAGE 3487288), Wnt4 (IMAGE 445179), Wnt11 (IMAGE 349486), Sfrp5 (IMAGE 1395864), Pch1 (Goodrich et al., 1996). Histological staining and electron microscopy followed standard procedures.

Transfection of primary intestinal fibroblasts

Primary fibroblasts were prepared by trypsin dissociation of the mesenchyme from E18.5 intestine, plated on glass slides and transfected (Lipofectamine, Invitrogen) with a plasmid encoding a dominant-negative FoxF protein (DNA-binding domain only) fused to GFP (Hellqvist et al., 1998). Cells were fixed (0.5% formalin in PBS for 5 minutes at room temperature followed by 100% methanol for 1 minute at −20°C) and stained with collagen I antiserum (visualized with Alexafluor 568, Molecular Probes) 24 hours post-transfection. Transfected cells were identified by their nuclear GFP fluorescence and nuclei by DAPI staining.

Explant cultures

Intestine and stomach were dissected from E12.5 and E13.5 embryos, and cultured on filters as previously described (Mahlapuu et al., 2001a). Beads (Affi-gel blue, BioRad) soaked in Bmp4 (10 ng/μl), Noggin (100 ng/μl) (both from R&D Systems) or BSA (as control) were grafted into the mesenchyme of the explants. For inhibition of hedgehog signaling, cycloamine was used in the culture medium at a concentration of 20 μM. After in vitro culture for 24 hours, explants were fixed briefly in 4% paraformaldehyde and analyzed by whole-mount in situ hybridization with probes for Wnt5a, Pch1, Foxf1 and Foxf2.

RESULTS

Foxf1 and Foxf2 have overlapping functions in gut development

To investigate the role of Foxf genes in gut development we examined the intestines of embryos with various combinations of mutations in Foxf1 and Foxf2. Neither heterozygote (Foxf1−/+ and Foxf2−/+ ) had any obvious intestinal defects and Foxf1−/− embryos were resorbed by E10, before gut morphogenesis had begun (Mahlapuu et al., 2001b). All Foxf2−/+ and an estimated 94% of Foxf1−/+; Foxf2−/+ compound heterozygotes died shortly after birth (three out of 154 surviving offspring from Foxf1−/+×Foxf2−/+ crosses were compound heterozygotes and survived for up to a few weeks). Foxf1−/+ heterozygotes have increased perinatal mortality – on some genetic backgrounds exceeding 90% – owing to lung and foregut malformations (Kaliniychenko et al., 2001; Mahlapuu et al., 2001a). However, the C57Bl/6J strain used here is particularly resistant to Foxf1 haploinsufficiency and heterozygotes have approximately the same survival rate and life expectancy as wild type (Mahlapuu et al., 2001a). The inviability of compound heterozygotes therefore represents non-allelic non-complementation and indicates a functional overlap between Foxf1 and Foxf2. Examination of E18.5 fetuses revealed defects and malformations in the intestine of Foxf2−/− and Foxf1−/+; Foxf2−/+ , whereas cleft palate was only observed in Foxf2−/− (Wang et al., 2003). The two mutant genotypes had similar intestinal abnormalities and will be collectively referred to as Foxf mutants. In cases where consistent phenotypic differences between them were observed, this will be specifically commented on.

Reduced Foxf gene dosage causes aganglionic megacolon

At E18.5 the mesodermal component of the murine intestine has given rise to fibroblasts in a subepithelial mesenchyme, longitudinal and circular muscular layers, and a mesothelium that delimits the gut from the coelom (Fig. 1J). In most Foxf mutant fetuses, the distal colon was thinned and dilated (megacolon; Fig. 1B,E,F,H,I), and in some Foxf2−/− ended in a blind sac (intestinal atresia or imperforate anus; Fig. 1F). Congenital megacolon in human infants is caused by a defective innervation of the colon (aganglionic colon or Hirschsprung’s disease) (Carrasquillo et al., 2002) and we therefore examined the distribution of enteric neurons. Immunostaining for the neuronal markers neurofilament (Fig. 1L,M) and PGP9.5 (not shown) showed that the innervation of distended parts of the distal colon in Foxf mutants was weak and patchy. The reduction in enteric neurons correlated with the degree of colon dilation; in the worst affected regions, neurons were virtually absent.

The distended distal colon of mutants typically had a flat epithelium and lacked well-developed fibroblasts or mesothelium (Fig. 1K). The mesodermal component instead consisted of a lax disorganized assembly of cells with the appearance of incompletely differentiated smooth muscle cells (SMCs, Fig. 1K). The poor development of the musculature in colon and rectum was confirmed by a faint and incoherent immunostaining for smooth muscle α-actin (SMA; Fig. 1N,O). Proximal, non-dilated, parts of the colon maintained a more normal histology, but generally had a smaller diameter than wild type.

Foxf genes are important for extracellular matrix production by intestinal fibroblasts

Throughout the intestine, Foxf mutants showed signs of poor adhesion between cells, a defect that was more pronounced in Foxf2−/− than in Foxf1−/+; Foxf2−/−. The epithelium, the mesenchyme and the two muscular layers all separated from each other, and in the worst affected parts each layer dissolved into individual cells. Spontaneous disintegration and increased susceptibility to mechanical stress suggested a deficiency in the extracellular matrix (ECM). When viewed by electron microscopy (TEM), cells of the colon from Foxf1−/+; Foxf2−/+ mutants appeared loosely assembled (Fig. 2). The basal laminae surrounding SMCs as well as the basement membrane underneath the epithelium were indistinct and frequently replaced by gaps of extracellular space (Fig. 2D). Immunohistochemistry revealed a striking deficiency of fibrillar (type I) as well as sheet-forming (type IV) collagens throughout the...
intestines of E18.5 Foxf2−/− mutants (Fig. 3). Laminin staining was also weaker, although the reduction was not as dramatic (not shown). In Foxf1+/++; Foxf2−/− mutants the ECM staining was reduced (Fig. 3D), but to a lesser degree than in Foxf2−/−. The compound heterozygote also had ectopic expression of smooth muscle α-actin in intravillus mesenchyme (Fig. 3M).

Mesodermal cells of Foxf1+/++; Foxf2−/− colon had poorly developed endoplasmic reticulum (ER; Fig. 2D,F), suggestive of a lower production of secreted proteins. To investigate if inhibition of Foxf activity in fibroblasts would reduce secretion of ECM proteins, we transfected primary fibroblasts from wild-type E18.5 small intestine with a plasmid expressing a dominant-negative Foxf protein fused to GFP (Hellqvist et al., 1998) (Fig. 3P,Q). Immunostaining showed abundant collagen I in ER vesicles of untransfected cells, as well as in fibers on the growth surface underneath. Transfected cells – identified by their GFP positive nuclei – contained almost no collagen I and similar results were obtained for collagen IV (not shown). Expression of GFP had no effect on collagen production (Fig. 3N,O). These results suggest that inhibiting transcriptional regulation of Foxf target genes leads to loss of collagen synthesis and indicates a cell-autonomous requirement for Foxf proteins in ECM production by fibroblasts.
ECM deficiency leads to epithelial depolarization and inter-villus adhesion, but surprisingly little apoptosis

Polarization of epithelial cells is induced by interaction with the basement membrane through integrin receptors (Kedinger et al., 2000) and an expected consequence of the ECM deficiency in Foxf mutants was therefore loss of polarity. Indeed, epithelial cells in Foxf mutants showed typical signs of depolarization: rounded in shape with central, rather than basal, nuclei. This anomaly was apparent also in areas where the physical integrity was not yet affected. The subcellular distribution of E-cadherin, a component of adherence junctions normally confined to lateral membranes of epithelial cells,
expanded into the basal and apical membranes, and in the most depolarized cells was circumferentially distributed (Fig. 4D-F). A similar shift from lateral to more or less ubiquitous membrane staining was also observed for β-catenin, another component of adherence junctions. One consequence of cell adhesion proteins being exposed on the apical surface is adhesion between epithelial cells from separate villi. In areas with overt depolarization the result was extensive inter-villus cross-linking, which lead to complete luminal obstruction (Fig. 4C).

The integrin-mediated attachment to the basement membrane, and the resulting polarization, are also essential for epithelial cell survival (Frisch and Ruoslahti, 1997). This is illustrated by the presence of apoptotic cells in sites of poor epithelio-mesenchymal contact of wild-type intestine (Fig. 4K). Surprisingly, TUNEL assay did not reveal the expected massive apoptosis in Foxf2−/− mutants, in spite of the severe deficiency of several ECM components (Fig. 4L). Loosely attached, completely depolarized cells failed to

Fig. 3. Tissue disintegration due to ECM deficiency in E18.5 Foxf2−/− intestine. (A,B) Hematoxylin and Eosin stained sections of colon from wild type (A) and Foxf2−/− (B). The mutant has a distended distal colon and the mesodermal layers separate from each other and from the epithelium (top in close-up) because of poor cell adhesion. (C-K) Immunostaining with antisera against a sheet forming collagen (type IV), a fibrillar collagen (type I) and SMA. Both collagens are reduced throughout the length of the intestine (from duodenum to rectum) in Foxf2−/−. The mutant intestinal wall (I) is flimsy in appearance compared with the wild type (H), owing to lack of collagen fibers. Both the small intestine (I) and colon (E; here from a proximal, non-distended part) has a smaller diameter in the mutant than in wild-type littermates (C,H). Foxf1−/−; Foxf2−/− (D; proximal colon) also has reduced amounts of ECM components, but less extreme than in Foxf2−/−.

(L,M) Immunostaining with anti-SMA in wild type (L) and Foxf1−/−; Foxf2−/− (M) E18.5 small intestine shows ectopic expression of SMA in intravillus mesenchyme of the mutant. (N-Q) Cell-autonomous requirement for Foxf proteins for collagen expression in intestinal fibroblasts. Primary fibroblasts were prepared from E18.5 wild-type intestine and transected with a plasmid expressing GFP (N,O) or a dominant-negative Foxf protein fused to GFP (P,Q). After 24 hours, cells were fixed and stained with an antiserum against collagen I (red). Collagen staining is seen in cytoplasmic vesicles and fibers between the cell and the glass substrate. In cells expressing the dominant-negative Foxf protein, identified by their green nuclear fluorescence, collagen staining is reduced dramatically. Blue, DAPI nuclear staining.
undergo apoptosis, and only in areas that had reached the final stages of tissue disintegration did the frequency of TUNEL-positive cells increase significantly (Fig. 4M).

**Epithelial overgrowth in small intestine of Foxf1−/−; Foxf2−/− compound heterozygotes**

Cell adhesion and ECM production was less affected in the compound heterozygotes than in Foxf2 knockouts, and tissue layers of the small intestine did not normally disintegrate. Instead, the villi were large and club-shaped with multilayered epithelia (Fig. 4B). Normal villi are covered by a monolayer of epithelial cells (Fig. 4A) and the continuous addition from the basal, proliferative compartment is balanced by apoptosis and desquamation at the tip of villi (Stappenbeck et al., 1998). The large clusters of epithelial cells in Foxf1−/−; Foxf2−/− small intestine, particularly in the distal parts of villi, therefore suggested overproliferation or loss of normal control of cell survival. Epithelial proliferation in E18.5 small intestine is confined to the intervillus pockets, predecessors of crypts.
of Lieberkühn which do not form until postnatally. Transition from the basal, proliferative to the distal, postmitotic, compartment is indicated by loss of proliferation markers such as PCNA (Fig. 4G). In Foxf mutants, the sharp boundary between proliferating and non-proliferating cells was dissolved and PCNA-positive cells were found throughout the villi (Fig. 4H,I). Persistent cell division therefore appears to account for the observed surplus of epithelial cells.

**Nuclear β-catenin along the entire villus axis indicates ectopic Wnt signaling**

Persistent proliferation and partial resistance to apoptosis in the intestinal epithelium suggested abnormal activation of the Wnt-β-catenin-Tcf/Lef pathway. On sections of E18.5 wild-type small intestine, β-catenin staining was intense in basal epithelium and faded along the villus axis (Fig. 5A). Higher magnification showed nuclear β-catenin at the base of villi, but only membrane associated staining distally (Fig. 5F,H,J), consistent with Wnt signaling emanating from mesenchyme underlying the intervillus pits and stabilizing β-catenin in basal epithelial cells. Sections of intestine from Foxf mutants showed β-catenin staining remaining strong along the villus axis (Fig. 5B) and nuclear localization also at the apices of villi (Fig. 5G,I,K). The multilayered epithelia of Foxf1+/−; Foxf2−/− mutants had a mosaic pattern with clusters of epithelial cells whose β-catenin staining was significantly weaker (Fig. 5B-D). TUNEL assay produced a similar mosaic pattern, which suggests that apoptosis is prevalent within low-β-catenin areas (Fig. 5E).

How does a decreased Foxf expression in the mesenchyme lead to β-catenin stabilization in the epithelium? In mice lacking Foxi1, another mesenchymal forkhead gene, accumulation of proteoglycans has been suggested to facilitate Wnt signaling and thereby promote epithelial overgrowth (Kaestner et al., 1997; Katz...
et al., 2004; Perreault et al., 2001). However, in Foxf mutants, the major intestinal proteoglycans were present in normal (perlecan and nidogen/entactin) or reduced (syndecan 1) amounts (Fig. 5L-Q). We therefore investigated if a reduced Foxf gene dose increases the expression of Wnt genes in the mesenchyme. To exclude secondary effects due to interrupted epithelio-mesenchymal signaling, E14.5 embryos were chosen — a stage at which the association between epithelium and mesenchyme is still intact in the mutants. Whole-mount in situ hybridization with candidate Wnt genes (Wnt4, Wnt5a and Wnt11) showed Wnt5a to be upregulated in Foxf2–/– embryos (Fig. 6A). Wnt5a and Foxf2 are co-expressed in several parts of the embryo, such as limbs and genital tubercle, and an increase in Wnt5a mRNA was seen in all these organs of Foxf2–/– null embryos, with intermediate levels in Foxf2+/– heterozygotes (not shown). In fact, the difference in Wnt5a mRNA content between Foxf2–/– and wild type was greater in limbs, where Foxf2 is the only expressed Foxf gene, than in the intestine where Foxf1 and Foxf2 are co-expressed.

Reduced Bmp4 expression contributes to Wnt5a upregulation

Wnt5a is co-expressed with Foxf genes in the mesenchymal cells of the developing intestine and might therefore be a direct transcriptional target. However, in limbs, Wnt5a expression is increased in Foxf2–/– embryos, in spite of the two genes being expressed in adjacent, non-overlapping cell populations; we therefore suspected that the link between Foxf genes and Wnt5a may be indirect also in the gut. A candidate extracellular mediator was Bmp4; it inhibits Wnt5a expression in the genital tubercle (Suzuki et al., 2003) and its expression in the early splanchnic mesoderm requires Foxf1 (Mahlapuu et al., 2001b). We first compared Bmp4 expression in Foxf2–/– and wild-type using a Bmp4lacZ knock in. β-galactosidase activity in the intestine of Foxf2–/–; Bmp4Δin2/embryos (E15.5) was reduced (Fig. 6B,C) in the gut, whereas tissues without Foxf expression, such as eye lids and hair follicles, retained normal Bmp4-driven β-galactosidase activity.
activity (Fig. 6D,E). To investigate if Bmp signaling influences Wnt5a expression in the intestinal mesenchyme, we applied beads soaked in Bmp4 or the Bmp antagonist Noggin to the mesenchyme of gastric or intestinal explants (E12.5 or 13.5), cultured in vitro and analyzed Wnt5a expression by whole-mount in situ hybridization (Fig. 6F-J). Bmp4 inhibited Wnt5a expression in Foxf2–/– explants (Fig. 6H,I), and Noggin induced high levels of Wnt5a mRNA in wild-type explants, from a low basal expression (Fig. 6J). By contrast, Foxf gene expression was not influenced by Bmp4 or Noggin (not shown).

Intestinal Foxf expression is activated by hedgehog signaling

In the early splanchnic mesoderm Foxf1 and Foxf2 are activated by sonic and Indian hedgehog (J.A. and P.C., unpublished), and Foxf1 expression in foregut and lung requires Shh (Mahlapuu et al., 2001a). The overlapping expression of Shh and Ihh, together with the early lethality of the double null mutant, limit the use of genetic methods to evaluate the role of hedgehogs in activation of Foxf genes in the intestine. Instead, we used cyclopamine to inhibit hedgehog signaling in explant cultures. Cyclopamine was found to reduce the expression of both Foxf1 and Foxf2 to the same extent as of a known hedgehog target, *Ptch1* (Fig. 6K–P). We therefore conclude that Shh and Ihh from the epithelium activates mesenchymal transcription of Foxf genes also in the intestine.

DISCUSSION

The data presented here place Foxf proteins at the crossroad of several of the major signaling pathways in gut development. Activated by hedgehog proteins from the epithelium they control expression of Wnt and Bmp in the mesenchyme. The conservation between mammals and *Drosophila* (Zaffran et al., 2001) of a role in gut development implies that this represents an ancient and presumably primeval role of the FoxF class of forkhead transcription factors.

Phenotypic similarity between Foxf2–/– and the Foxf1; Foxf2 compound heterozygotes indicates a functional overlap between the two proteins. Rather than redundancy, the relation between Foxf1 and Foxf2 represents non-allelic non-complementation, as at least three functional alleles are required for normal development.
However, although functionally overlapping, the presence of phenotypic differences between the mutants also indicates non-equivalence, in agreement with the distinct properties of the activation domains shown previously (Hellqvist et al., 1998; Hellqvist et al., 1996; Mahlapuu et al., 1998). The more severe ECM deficiency in Foxf2−/− may explain the strikingly different small intestine histology of the two mutants. Both share an activation of the canonical Wnt pathway, presence of cycling cells in the distal parts of villi and a partial resistance to apoptosis in cells with poor anchorage. In the presence of an intact, although weakened, basement membrane this will allow accumulation of excessive epithelial cells – as seen in the compound heterozygote – whereas loss of contact between epithelium and mesenchyme will impede Wnt signaling, lead to less proliferation and the more severe disintegration typical of Foxf2−/−.

Regions with disintegrated tissues and crosslinked villi can be found next to areas with a grossly normal histology. This rules out difference in genetic background as the sole source of phenotypic variation and instead suggests that reduction of Foxf gene dose creates an unstable situation that will deteriorate rapidly once the defects reach a certain level. Hedgehog signaling from epithelium to mesenchyme requires an intimate contact between cells and will be hampered by loss of ECM. With Foxf genes activated by hedgehog, a degenerative cycle may be initiated once the epithelio-mesenchymal contact is disturbed: lower expression from the remaining Foxf alleles would produce even less ECM and gradually aggravate the tissue disintegration (Fig. 7). Initial small differences in the contact between cells – enough to trigger a degenerative cycle in some areas, but not in others – may be introduced when cell adhesion is challenged by the forces of the commencing peristalsis.

Similarities between the hedgehog and Foxf mutant phenotypes suggest that reduced Foxf expression is responsible for many of the observed defects in hedgehog mutants, such as megacolon (in Ihh−/−), anal atresia (in Shh−/− and Gli2−/− mutants) and smooth muscle hypoplasia (in Ihh−/−) (Mo et al., 2001; Ramalho-Santos et al., 2000). Inhibition of hedgehog signaling by transgenic expression of Hhip mimics the phenotype described here, including activation of the Wnt/β-catenin pathway, epithelial overproliferation and SMA-positive cells in the villus core (Madison et al., 2005). A conserved role for Foxf proteins in intestinal smooth muscle development is further supported by the loss of gut SMCs in Foxf1 morpholino knockdown in Xenopus embryos (Tseng et al., 2004) and the defective visceral musculature in Drosophila binou mutants (Zaffran et al., 2001).

The posterior gut aganglisis, which Foxf mutants share with Ihh−/− (Ramalho-Santos et al., 2000), resembles Hirschsprung’s disease in humans (Carrasquillo et al., 2002). Bmp4 inhibits enteric nerve differentiation (Sugikawa et al., 2000), and premature differentiation of migrating, neural crest-derived neuronal precursors – as a result of decreased Bmp4 expression – could lead to their depletion preferentially in the posterior gut. SMCs produce a neurotrophic factor, neurturin, that stimulates growth of enteric nerves (Tseng et al., 2004) and the defective visceral musculature in Ihh−/− mice (Madison et al., 2005). However, although functionally overlapping, the presence of cycling cells in the subepithelial and intra-villus fibroblasts is consistent with this model.

FoxI1 is expressed in intestinal fibroblasts and – like Foxf genes – controls β-catenin accumulation in epithelial cells, although by a different mechanism (Kaestner et al., 1997; Perreault et al., 2001). Loss of both FoxI1 alleles on an Apc−/− (Min) genetic background leads to a marked increase in tumor multiplicity in the colon, and Apc−/−; FoxI1−/− mice also develop gastric tumors not observed in ApcMin−/− mice (Perreault et al., 2005). The fact that Foxf mutants have an increased intestinal Wnt signaling and that fibroblasts express a marker characteristic of activated tumor stroma raise the possibility that loss of Foxf alleles could contribute to tumor susceptibility.

We thank B. Hogan for the Bmp4 mutant; H. Clevers and E. Batlle for advice on β-catenin immunostaining; and the Swedish Cancer Foundation (grant 3517-B04-11XAC to P.C.) and The Asahi Glass Foundation (to N.M.) for support.

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