RESEARCH COMMUNICATION

Foxl1 is a mesenchymal Modifier of Min in carcinogenesis of stomach and colon

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Constitutive activation of the Wnt/APC/β-catenin pathway is a frequent initiating event in gastrointestinal carcinogenesis. Mutations in the Adenomatous Polyposis Coli (APC) gene up-regulate Wnt signaling by stabilizing β-catenin and causing activation of targets important in proliferation control. Here we show that loss of the mesenchymal transcription factor Foxl1 leads to a marked increase in tumor multiplicity in the colon of ApcMin mice. ApcMin−/Foxl1−/− mice also develop gastric tumors not observed in ApcMin mice. These effects are caused by earlier tumor initiation due to accelerated loss of heterozygosity [LOH] at the Apc locus. Foxl1 is the first mesenchymal Modifier of Min and plays a key role in gastrointestinal tumorigenesis.

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Gastric and colorectal cancers are common malignant neoplasms and account for >1 million deaths worldwide every year. Constitutive activation of the Wnt/APC/β-catenin pathway is an initiating event in colorectal carcinogenesis and is observed in most sporadic and hereditary colorectal cancers as well as in sporadic gastric cancer (Morin 1999; Smith et al. 1999; Pinto et al. 2003). Intrafamilial variation in phenotype contributes significantly to the complexity of familial adenomatous polyposis (FAP), a hereditary form of colorectal cancer. Despite carrying the same mutation in the APC gene, affected individuals can have dramatic differences in disease expression (Crabtree et al. 2002; Su et al. 2002). Beyond environmental effects or variations in clinical practice, the large phenotypic differences even between same-sex siblings strongly suggest the existence of other genes that modify the tumor risk conferred by a given APC mutation. The genes responsible for this variable expression of the FAP phenotype are still unknown. However, the search for modifier genes was greatly facilitated by the discovery of a cancer-predisposing mutation in mice, termed Multiple Intestinal Neoplasia, or Min, which was shown to be caused by a point mutation in the mouse ortholog of APC (Moser et al. 1990; Shoemaker et al. 1997). Min mice develop numerous polyps, predominantly in the small intestine, and die by 5 mo of age with anemia and intestinal blockage. Tumor multiplicity was found to be dependent on the inbred strain in which the Min mutation was analyzed and led to the identification of the first modifier gene of the Min/APC phenotype (Dietrich et al. 1993; Gould et al. 1996). This locus, termed Modifier of Min1 (Mom1), contains several candidate genes, including the epithelial phospholipase A2 [PLA2S-II or Pla2g2a gene] (Cornier et al. 1997, 2000). However, thus far no functional variants have been found in the human ortholog of Pla2g2a in FAP patients, suggesting that other genetic loci might act as modifiers of the phenotype observed in FAP (Tomlinson et al. 1996, Nimmrich et al. 1997).

Foxl1 is a winged helix transcription factor expressed in the mesenchyme of the gastrointestinal tract (Kaestner et al. 1996, 1997) and an important regulator of the Wnt/APC/β-catenin pathway (Perreault et al. 2001). Mice homozygous for a Foxl1-null mutation demonstrate increased nuclear accumulation of β-catenin in the epithelia of the stomach and intestine (Perreault et al. 2001). However, Foxl1-null mice do not develop adenomas in their gastrointestinal tract, even at 1 yr of age. As both Apc and Foxl1 influence the accumulation of nuclear β-catenin, we hypothesized that the absence of Foxl1 protein in the gut mesenchyme would synergize with the ApcMin mutation in the gut epithelium in tumor initiation and/or progression. We tested the hypothesis that Foxl1 is a potential Modifier of Min using a genetic approach, and demonstrate that loss of Foxl1 increases tumor multiplicity in ApcMin mice in a dramatic fashion. This effect is caused by accelerated loss of heterozygosity [LOH] at the ApcMin locus. These findings underscore the need to consider the contribution of the stroma to the initiation and progression of gastrointestinal adenomas and for additional therapeutic approaches to the treatment of gastrointestinal cancer.

Results and Discussion

Loss of Foxl1 increases gastrointestinal tumor load in ApcMin mice

To evaluate whether Foxl1 is a Modifier of Min, we crossed ApcMin mice with mice carrying a null mutation in Foxl1 on the defined, inbred C57Bl6 background (Kaestner et al. 1997). We found no polyps in the stomachs of Apc+/Min; Foxl1−/− [Fig. 1A], or Apc−/Min; Foxl1−/− mice [data not shown] following up to 90 d. However, Apc+/Min; Foxl1−/− mice developed an average of 5.5 polyps in the stomach by 3 mo of age, demonstrating a major impact of Foxl1 on gastric tumor initiation [Fig. 1B,E] (P < 0.002). Foxl1 also had a dramatic effect on tumor load in the colon. While Apc+/Min; Foxl1−/− and Apc−/Min, Foxl1−/− mice [Fig. 1C,F] had an average of 3.2 and 3.7 colonic polyps, respectively, Apc+/Min; Foxl1−/− mice [Fig.

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ANOVA; \( n < 0.002 \) for the stomach and with an average of 5.5 polyps in ∼ normally develop were found in the stomach of \( Apc^{+/Min/Foxl1} \) mice. These results suggest that significantly affected by the loss of \( Apc \) act cooperatively to regulate tumor multiplicity in the stomach and colon.

Figure 1. Loss of \( Foxl1 \) in \( Apc^{+/Min} \) mice increases tumor multiplicity. Macroscopic evaluation of stomach (A,B) and colon (C,D) demonstrated increased tumor multiplicity in 70- to 90-d-old \( Min \) mice with a homozygous deletion of \( Foxl1 \) [B,D]. No polyps (labeled as p) were found in the stomach of \( Apc^{-/Min/Foxl1^{-/-}} \) mice [A] compared with an average of 5.5 polyps in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice [B]. Loss of \( Foxl1 \) in \( Apc^{-/Min} \) mice also had a dramatic effect on tumor number in the colon. \( Apc^{-/-Min/Foxl1^{-/-}} \) mice [C] had an average of 3.2 colonic polyps compared with an average of 27.8 polyps in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice [D]. Thus, in both the stomach and colon [F], the loss of \( Foxl1 \) had a significant effect on tumor multiplicity in \( Apc^{-/Min} \) mice (\( P < 0.002 \) for the stomach and \( P < 0.005 \) for the colon by one-way ANOVA; \( n = 4 \) per genotype analyzed).

1D,F) developed an average of 27.8 polyps per animal, a 7.7-fold increase over \( Apc^{-/-Min/Foxl1^{-/-}} \) mice [Fig. 1F] (\( P < 0.005 \)). In the small intestine, \( Apc^{+/Min} \) mice normally develop ∼50 polyps per mouse (Shoemaker et al. 1997), tumor multiplicity up to 90 d was not significantly affected by the loss of \( Foxl1 \) (data not shown). These results suggest that \( Foxl1 \) and \( Apc \) act cooperatively to regulate tumor multiplicity in the stomach and colon.

No gastric adenomas were found in the stomachs of \( Apc^{-/-Min/Foxl1^{-/-}} \) or \( Foxl1^{-/-} \) mice (Fig. 2A,C), consistent with our previous findings that \( Foxl1^{-/-} \) mice do not develop tumors even at 1 yr of age (Kaestner et al. 1997). By contrast, \( Apc^{-/-Min/Foxl1^{-/-}} \) mice developed adenomatous polyps in the stomach, with disturbed glandular architecture, increased nuclear to cytoplasmic ratio, and nuclear atypia [Fig. 2E]. To confirm that these lesions are truly polyps of proliferating epithelial cells, we performed immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Adenomatous polyps in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice displayed a high frequency of PCNA-positive epithelial cells [Fig. 2F], in contrast to \( Apc^{-/-Min/Foxl1^{-/-}} \), in which proliferating cells are confined to the neck region [Fig. 2B], and \( Foxl1^{-/-} \) mice with a more scattered distribution of PCNA-positive cells [Fig. 2C]. We also considered the possibility that decreased apoptosis might contribute to the increased number of epithelial cells in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice. However, there was no change in the rate of apoptosis in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice compared with controls as determined by TUNEL assay (data not shown). In the colon, \( Apc^{-/-Min/Foxl1^{-/-}} \) mice formed adenomas that were histologically similar to the polyps found in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice (data not shown). There was no evidence of invasion or metastases in any of the tumors. Thus, loss of \( Foxl1 \) promotes gastric and colonic tumorigenesis in the \( Apc^{-/Min} \) mice, but does not affect tumor grade or stage in either the stomach or the colon up to 90 d of age.

Figure 2. Loss of \( Foxl1 \) in \( Apc^{-/Min} \) mice leads to gastric polyp formation. Histological analyses of the stomach confirmed the presence of gastric tumors in 70- to 90-d-old \( Apc^{-/-Min/Foxl1^{-/-}} \) animals. Hematoxylin and cosin staining was performed on paraffin sections of stomach from either \( Apc^{-/-Min/Foxl1^{-/-}} \) [A], \( Foxl1^{-/-} \) [C], or \( Apc^{-/-Min/Foxl1^{-/-}} \) [E]. The gastric mucosa from the \( Apc^{-/-Min/Foxl1^{-/-}} \) [A] showed no polyp formation. Although the morphology of the gastric epithelia in the \( Foxl1^{-/-} \) mice [C] was abnormal with branching of the glands and the presence of mucin filled cysts, no polyps were observed. \( Apc^{-/-Min/Foxl1^{-/-}} \) mice [E] showed a dramatic induction in polyp formation. Immunohistochemical detection of PCNA shows proliferating cells confined to the neck region of the glandular stomach in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice, scattering of proliferating cells in \( Foxl1^{-/-} \) mice, and a dramatic accumulation of proliferating epithelial cells in the polyps of \( Apc^{-/-Min/Foxl1^{-/-}} \) mice. [B,D,F] Immunohistochemical detection of PCNA. [B] Proliferating cells are confined to the neck region in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice. [D] Proliferating cells are spread throughout the epithelium of \( Foxl1^{-/-} \) mice. [F] Adenomatous polyps in \( Apc^{-/-Min/Foxl1^{-/-}} \) mice display a high frequency of proliferating cells. Magnification is indicated on the individual panels.
Fox11 deficiency accelerates tumor initiation in APC<sup>Min</sup> mice

Previous reports have suggested that the majority of polyps in the Apc<sup>Min</sup> mouse arise by 3 mo of age (Moser et al. 1990, 1992). At this age, Apc<sup>Min</sup>;Fox11<sup>−/−</sup> mice already developed an increased number of colonic polyps compared with Apc<sup>Min</sup>;Fox11<sup>+/−</sup> mice. We therefore hypothesized that loss of Fox11 affects tumor initiation, not tumor progression. To investigate this notion, we analyzed 30-d-old mice for evidence of early tumor initiation. Colon sections were removed and embedded in paraffin in a Swiss roll orientation such that their entire length could be evaluated on a single section. The sections were then analyzed and scored for the formation of adenomas [Fig. 3]. As expected, no adenomas were found in young Apc<sup>Min</sup>;Fox11<sup>+/−</sup> animals, and their colonic crypts displayed normal architecture and cell morphology [Fig. 3A, B]. In Apc<sup>Min</sup>;Fox11<sup>−/−</sup> mice, however, we observed a number of well-developed adenomas at 30 d of age [Fig. 3D, E]. The epithelial cells in these polyps were dysplastic and disordered with nuclear hyperchromasia and atypia [Fig. 3D, E]. These adenomas also exhibited increased BrdU labeling, indicative of increased DNA synthesis [Fig. 3F], while in the normal-appearing colon of 30-d-old Apc<sup>+/Min</sup>;Fox11<sup>−/−</sup> mice, cells in S phase are confined to the bottom third of the crypts [Fig. 3C]. The increased proliferation rate in adenomas of 30-d-old Apc<sup>+/Min</sup>;Fox11<sup>−/−</sup> mice was also confirmed by PCNA staining [Fig. 3, cf. I and G, H].

The loss of the wild-type Apc allele in Apc<sup>Min</sup> mice (LOH) is accelerated in absence of Fox11

Next, we investigated the molecular mechanism by which the mesenchymal factor Fox11 impacts on the Wnt/APC/β-catenin pathway to accelerate tumor initiation. We previously established that loss of Fox11 alone leads to augmented nuclear localization of β-catenin by increasing the amount of heparan sulfate proteoglycans, known coreceptors for Wnt signaling, in the extracellular matrix (Perreault et al. 2001). Therefore, we considered the possibility that enhanced signaling via the Wnt/APC/β-catenin pathway caused by the Apc<sup>Min</sup> mutation, combined with the increased Wnt signaling operative in the absence of Fox11, might be sufficient to cause tumor initiation even without the loss of the second, wild-type, Apc<sup>−/−</sup> allele. However, when we determined nuclear β-catenin levels in the stomach and colon of 30-d-old mice of all relevant genotypes, we found these not significantly different between Fox11<sup>−/−</sup> and Apc<sup>+/Min</sup>/Fox11<sup>−/−</sup> mice (Fig. 4A). Thus, the synergism between Fox11 and Apc mutations described above is not the result of passing the threshold of nuclear β-catenin required for tumorigenesis. Therefore, we investigated whether the loss of the second, wild-type Apc allele might be accelerated in the absence of Fox11. LOH is thought to be a stochastic event that is dependent on the age of the individual or, more specifically, the number of times the genome undergoes replication. To determine whether LOH had occurred, we isolated small numbers of cells from either normal colonic mucosa or adenomas (only present in Apc<sup>+/Min</sup>; Fox11<sup>−/−</sup> mice) [Fig. 3] from 30-d-old mice by laser capture microdissection. LOH at the Apc locus can be analyzed by taking advantage of a restriction fragment length polymorphism (Yamada et al. 2002). As shown in Figure 4B, in 30-d-old Apc<sup>+/Min</sup>;Fox11<sup>−/−</sup>, which lack adenomas, no LOH was observed, consistent with previous findings (Yamada et al. 2002). In contrast, 30-d-old Apc<sup>+/Min</sup>;Fox11<sup>−/−</sup> mice have lost the wild-type Apc allele in their polyps, demonstrating that LOH is accelerated in the absence of Fox11, providing a molecular explanation for the increased tumor load in these mice [Fig. 4]. Thus, LOH at the Apc locus is clearly accelerated in the absence of Fox11. We propose that due to the higher turnover of the epithelial cell caused by loss of Fox11 (Perreault et al. 2001), epithelial stem cells or their descendants have undergone three to four times the number of S phases than a Fox11<sup>−/−</sup> mouse by 1 mo of age, increasing the likelihood of somatic recombination at the Apc locus and thus LOH by the
same magnitude. This increased rate of recombination then results in the earlier onset of adenoma formation in Foxl1+/−;ApcMin mice. We hypothesize that other genes, or even environmental conditions, that favor increased turnover of gastrointestinal epithelial cells will likewise accelerate tumor formation in ApcMin mice or FAP patients. In summary, we have identified Foxl1 as an important regulator of the Wnt/β-catenin pathway and the first mesenchymal Modifier of Min.

Materials and methods

Mice

C57BL/6j-ApcMin mice were obtained from Jackson Laboratory. For studies of early tumor initiation, ApcMin/+;Fox1+/−, and ApcMin;Fox1−/− mice on a C57BL/6j background were sacrificed at 30 d of age. For other studies, age-matched ApcMin;Fox1−/−, ApcMin;Fox1−/−, and Apc−/− Fox1−/− mice on a C57BL/6j background were sacrificed between 70 and 90 d of age. Mice were genotyped for ApcMin and Fox1 alleles as reported previously [Dietrich et al. 1993; Kaestner et al. 1997].

Histology

Tissues were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, cut to 5-μm sections, and applied to Probe-on Plus slides [Fisher Scientific]. Tissues were incubated after rehydration in Hema-toxylin for 2.5 min, rinsed in water, dipped quickly in 0.5% acid alcohol, and washed in water. Tissues were then immersed in 0.2% NaHCO3, rinsed in water, dipped in eosin for 15 sec, and briefly rinsed in water before dehydration and mounting.

Quantification of micro- and macroadenomas

To evaluate macroadenomas in adult mice, the gastrointestinal tract from stomach to rectum was removed en bloc. The small intestine was cut into thirds, and the stomach, cecum, and colon were separated. Segments were cut longitudinally and washed gently with PBS to remove fecal material. Tissues were placed in a petri dish, and the mucosa was overlaid with 1% methylene blue. A dissection microscope was used to count the polyps. For quantification of macroadenomas in 30-d-old mice, the entire colon was removed, fixed in 4% paraformaldehyde overnight, and embedded in paraffin in Swiss roll orientation such that the entire length of the colon could be identified on a single section. Five-micron sections were stained with Hematoxylin and eosin as described above. The sections were then analyzed and scored for presence of adenomas.

Proliferation and apoptosis assays

Mice were injected intraperitoneally with 10 mL/kg BrdU reagent [ZYMED] 90 min prior to sacrifice. For immunofluorescence, tissues were fixed, sectioned, and processed as described above. Slides were subjected to microwave antigen retrieval by boiling for 6 min in 10 mM citric acid buffer (pH 6.0). Slides were washed in PBS and blocked with protein blocking reagent [Immunotech] for 15 min at room temperature. Mouse monoclonal anti-BrdU (1:500, Roche) was diluted in PBS containing 0.1% BSA and 0.2% Triton X-100 (PBT) and incubated with the sections overnight at 4°C. Slides were washed in PBS, followed by incubation with FITC-conjugated goat anti-mouse antibody (1:25, Roche), diluted in PBT, for 2 h at room temperature. Slides were washed in PBS, counterstained in 0.01% Evan's blue solution for 20 sec, rinsed in PBS, mounted with Vectashield [Vector], and examined using confocal fluorescence microscopy. For PCNA immunohistochemistry, endogenous peroxidases were quenched by treating slides in 1.5% hydrogen peroxide for 15 min. Tissues were blocked with Avidin D and Biotin blocking reagents for 15 min each, followed by blocking in 1.5% normal goat serum. Slides were probed with anti-PCNA (1:9000, mouse monoclonal, DAKO). Sections were washed three times for 10 min in 1x PBS and incubated with biotinylated anti-mouse secondary for 30 min at room temperature. Sections were washed three times for 10 min in 1x PBS, followed by incubation with HRP-conjugated ABC reagent from Vecta Elite Kit [Vector] for 30 min at room temperature. The signal was developed using DAB Substrate Kit [Vector].

Apoptosis was analyzed using the in situ cell death detection kit-AP [Roche Molecular Biochemicals]. Fifty microliters of TUNEL reaction mixture were added to the samples and incubated in a humidified chamber for 1 h at 37°C. Following washes in 1x PBS, slides were incubated with anti-fluorescin antibody-AP [Roche] for 30 min at 37°C. The signal was developed using NBT [100 mg/mL] and BCIP [50 mg/mL] [Boehringer] for 2–5 min. Positive cells were identified by blue nuclear staining.

Western blot analysis

The amounts of β-catenin and YY1 as loading control in nuclear extracts from colon of 30-d-old mice was determined as described previously [Perreault et al. 2001].

Allelic loss analysis (LOH studies)

Fox1−/−;ApcMin, Fox1−/−;Apc−/−, and Fox1+/−;Apc−/− mice on a C57BL/6j background were sacrificed at 30 and 70 d of age. To identify areas of dysplasia, the stomach and colons were removed, the colons opened longitudinally, and fixed in 4% paraformaldehyde overnight at 4°C overnight, embedded in paraffin, cut into 5-μm sections, applied to membrane slides for laser-microdissection [Molecular Machines and Industries], and examined by using an en face preparation. To identify areas of dysplasia, slides were stained in Hematoxylin and eosin. For each
for 10 min. The Allelic Discrimination SNP Real-time PCR conditions were initial denaturation formed using a Stratagene Mx4000.

67/H11032 and mutant, 5′-TAC-3/H11032. Dual-labeled probes were designed using the following criteria: point mutation, as follows: wild type, 5′-GAGAAAG-3′; and antisense, 5′-H11032-ATGGTAAGCACTGAGGCCAA

products were digested with HindIII and separated by electrophoresis on a 2.5% agarose gel and stained with SYBRgreen.

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