

# Activation of Wnt signaling in the intestinal mucosa of *Apc*<sup>+/-min</sup> mice does not cause overexpression of the receptor tyrosine kinase Met

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The receptor tyrosine kinase MET is overexpressed in human colorectal adenomas and carcinomas, suggesting an instrumental role for MET signaling in the onset and progression of colorectal cancer. To corroborate this role, animal models are needed. To study the expression of Met in the normal and neoplastic mouse intestine, we generated an Armenian hamster monoclonal antibody against mouse Met. By using this antibody in immunohistochemical studies, we observed strong Met expression in fetal mouse intestinal epithelial cells. In contrast, in the intestines of adult mice, Met expression was very low whereas the protein was undetectable on the neoplastic epithelium of intestinal adenomas in *Apc*<sup>+/-min</sup> mice. By immunoblotting, we were also unable to detect Met in intestinal adenomas, whereas Met mRNA levels in microdissected adenomas were very low. The absence of detectable Met protein expression in adenomas of *Apc*<sup>+/-min</sup> mice contrasts sharply with the vast overexpression of the protein in adenomas of humans with familial adenomatous polyposis or sporadic colorectal carcinomas. Our results imply that deregulation of Wnt signaling in mouse – unlike in human – intestinal epithelium does not result in Met overexpression. Our findings thus reveal important interspecies differences in the regulation of Met expression during intestinal tumorigenesis. (*Cancer Sci* 2006; 97: 710–715)

## Introduction

Proper functioning of the receptor tyrosine kinase MET is of critical importance during both embryogenesis and adult life.<sup>(1,2)</sup> During embryogenesis, the tight spatiotemporal regulation of activation of MET by its ligand, HGF, results in cell type-dependent mitogenesis, morphogenesis or motogenesis required for formation of the trophoblast, liver and subsets of skeletal muscles.<sup>(3–5)</sup> During adult life, MET plays an important role in tissue homeostasis, remodeling and repair.<sup>(2,6)</sup>

In addition to its physiological roles during development and adult life, MET signaling has been implicated in the pathogenesis of a variety of neoplasms, including glioma, lymphoma, renal cell carcinoma and CRC.<sup>(2,6)</sup> In these malignancies, *MET* is either mutated or overexpressed, resulting in uncontrolled activation of the MET signaling pathway. In CRC, *MET* is overexpressed at all stages of the adenoma–carcinoma sequence. At the early stages of disease, *MET* overexpression appears to be caused mainly by transcriptional

activation through the WNT signaling pathway.<sup>(7,8)</sup> However, a study by Di Renzo *et al.* demonstrated amplification of the *MET* gene in a small subset of primary colorectal carcinomas and in the majority of liver metastases studied,<sup>(9)</sup> suggesting that the transcriptional overexpression present during the initial stages of the disease becomes ‘fixed’ during tumor progression. Taken together, these findings suggest that MET signaling is not only involved in the onset of CRC, but also in disease progression and in the formation of metastases.

Mouse models have been of great value in unraveling the pathogenesis of CRC,<sup>(10,11)</sup> but the role of Met in intestinal tumorigenesis in mice has thus far not been explored. Based on mRNA *in situ* hybridization studies, Met expression in normal embryonic and adult mouse tissues appears to be confined largely to epithelial cells, including those of the intestine.<sup>(12,13)</sup> However, due to a lack of antibodies suitable for immunohistochemistry, data on Met protein expression in the normal intestine and intestinal adenomas of mice are currently lacking. This prompted us to generate an Armenian hamster mAb against mouse Met. By using this antibody, we demonstrate that Met, while expressed at high levels in the embryonic mouse intestine, is hardly or not detectable in the normal adult mouse intestine and is not overexpressed in intestinal adenomas of *Apc*<sup>+/-min</sup> mice.

## Materials and Methods

### Generation of a monoclonal antibody against mouse Met

The Armenian hamster fibroblast line ARHO12 (kindly provided by Dr Jannie Borst, NKI, Amsterdam, the Netherlands) was cultured as described previously.<sup>(14)</sup> In brief, cells were grown in flasks (Greiner Bio-One BV, Alphen a/d Rijn, the Netherlands) in IMDM medium supplemented with 10% fetal calf serum, 20 mM L-glutamine (both from Gibco BRL/Life Technologies, Paisley, UK), 1 mg/mL gentamicin (Invitrogen, Paisley, UK) and 2-mercaptoethanol (Sigma, St Louis, MO, USA). ARHO12 cells were transfected with

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Abbreviations: ACF, aberrant crypt foci; CRC, colorectal cancer; DIG, digoxigenin; E15, embryonic day 15; HGF, hepatocyte growth factor; IMDM, Iscove's Modified Dulbecco's Media; mAb, monoclonal antibody; nt, nucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PFA, para formaldehyde; RT, reverse transcription; SDS, sodium dodecylsulfate; TBP, TATA box binding protein.

pcDNA3.1/Neo(+) containing murine *Met* cDNA, using Lipofectamine Plus (Gibco BRL/Life Technologies) and were selected with geneticin (G418 sulfate; Invitrogen) at 0.1 mg/mL. Resulting clones were tested for *Met* expression by immunoblotting with the polyclonal anti-*Met* antibody SP260 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An Armenian hamster (*Cricetulus migratorius*; Cytogen, West Roxbury, MA, USA) was injected intraperitoneally five times at weekly intervals with  $10^7$  4% PFA-fixed ARHO12 cells expressing *Met* stably. Three weeks after the fifth injection, the hamster was boosted intraperitoneally with  $10^7$  cells. Three days later, hamster spleen cells were fused with mouse myeloma SP2/0 cells by standard hybridoma technology. Binding of hybridoma supernatants to the ARHO12 clone stably expressing *Met* was tested by flow cytometry as well as by immunohistochemistry. As a negative control, binding of hybridoma supernatants to non-transfected ARHO12 cells was tested. Hybridomas binding specifically to the ARHO12-*Met* cells were subcloned until they were monoclonal and stable. The hybridoma, designated We-1, was grown in large amounts and immunoglobulin was purified using Protein A bound to Sepharose CL-4B (Sigma). Purified antibody was conjugated with DIG (Roche Diagnostics, Almere, the Netherlands).

### SDS-PAGE

The isotype of We-1 was determined by SDS-PAGE. The antibody was run on both a reduced (10% SDS) and a non-reduced (7% SDS) polyacrylamide gel. Immunoglobulin bands were detected using Coomassie brilliant blue staining.

### Immunohistochemistry

Staining with the mAb We-1-DIG was carried out on ARHO12 cells (transfected with or without *Met* and fixed with 4% PFA) and on cytospins of mouse fibroblast cells (NIH/3T3 cells and NIH/3T3-*Met* cells; a kind gift from Dr G. F. Vande Woude). Furthermore, different organs from adult C57BL6/J mice and *Apc<sup>+/-min</sup>* mice (ages ranging from 9 to 20 weeks) (The Jackson Laboratory, Bar Harbor, ME, USA) were isolated and mounted into tissue-tek (Miles Scientific, Naperville, IL, USA). Also, whole C57BL6/J embryos were embedded into tissue-tek. Sections (5  $\mu$ m) were prepared and stained with the primary antibodies, We-1-DIG, goat-antimouse macrophage stimulating protein receptor (MSPR) (R&D Systems, Abingdon, UK), goat-antihuman/mouse/rat- $\beta$ -catenin (R&D Systems) and 9A4 (against mouse Cd44v6), followed by an avidinbiotinylated horse radish-peroxidase complex (Dakopatts, Glostrup, Denmark). The peroxidase was visualized with 3-amino-9-ethylcarbazole (Sigma).

### Micro-dissection and RT-PCR

Using laser microdissection, tissue from normal mouse intestines and adenomas was isolated. cDNA was synthesized by a standard RT reaction, using Moloney leukemia virus reverse transcriptase (GibcoBRL/Life Technologies) and pd(N)<sub>6</sub> random hexamer primers (Amersham Pharmacia Biotech, Aylesbury, UK). RT-PCR was carried out using *Taq* DNA Polymerase (GibcoBRL/Life Technologies), 200  $\mu$ M of each dNTPs (Amersham Pharmacia Biotech) and 1.5 mM MgCl<sub>2</sub> in 1 $\times$  PCR buffer (GibcoBRL/Life Technologies). Primers used were: *Met* forward, GTC CAC AAC AAG ACG GGT GC (nt

3703); *Met* reverse, GGA GAC CAG TTC GGA AAA GG (nt 3999); *Ron* forward, GTG TCC TAC AGG TGG AGA TA (nt 1670); *Ron* reverse, TGC TCC TCA TTG ACC TGT TC (nt 2450); and as a loading control, *TBP* forward, CAG GAG CCA AGA GTG AAG AAC (nt 794); and *TBP* reverse, AAT TCT GGC TCA TAG CTA CT (nt 995). PCR was started with a 5 min denaturation step at 95°C and followed by amplification in 39 cycles of denaturation at 95°C for 30 s, annealing at the appropriate melting temperature for 20 s and elongation at 72°C for 30 s. After a final elongation step at 72°C for 7 min, samples were cooled on ice and analyzed by electrophoresis.

### Western blot analysis

Protein extracts were prepared from different organs by mincing into lysis buffer (10 mM Tris pH 8.0, 15 mM NaCl, 1% NP-40, 10% glycerol, 0.4 mg/mL sodium orthovanadate). Protein extracts (50  $\mu$ g) were separated by SDS-PAGE and blotted onto immobilon-P transfer membranes (Millipore, Bedford, MA, USA). Membranes were blocked in Tris-buffered saline (100 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 0.1% Tween (Sigma) and 5% non-fat dry milk, and probed with mAb AC-15 against  $\beta$ -actin (Sigma) or polyclonal antibody SP260 against *Met* (Santa Cruz Biotechnology). Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Dakopatts) and a standard chemiluminescence immunoblotting protocol (ECL, Amersham Pharmacia Biotech). All signals were normalized for loading by comparison with the appropriate  $\beta$ -actin signal.

## Results

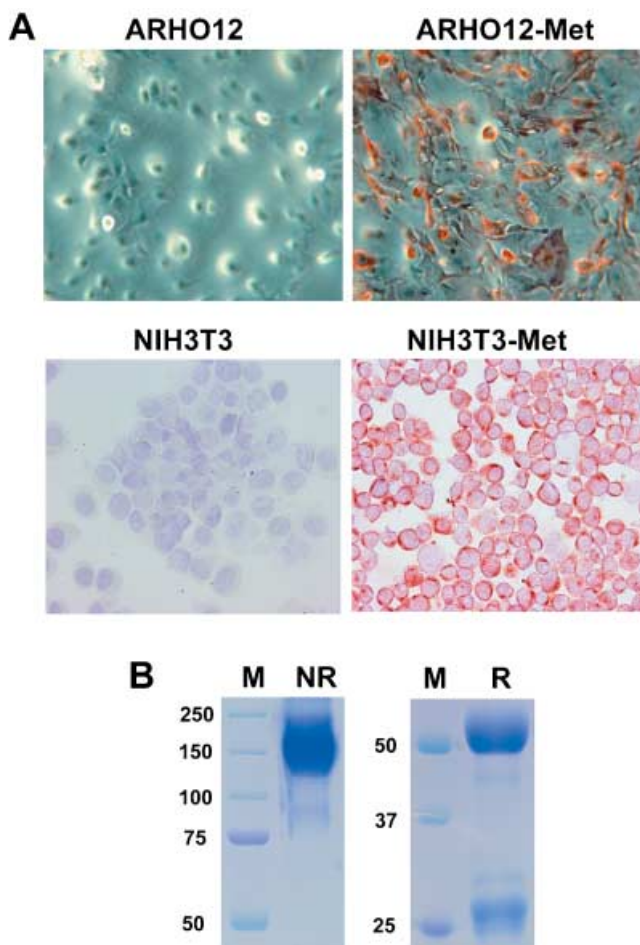
### Generation of a monoclonal antibody against mouse *Met*

To study the expression of *Met* in the normal mouse intestine and in intestinal adenomas, we generated an antimouse *Met* mAb. To this end, the complete coding sequence of mouse *Met* was cloned into pcDNA3.1 and transfected stably into ARHO12 cells. These *Met* transfectants were used to immunize Armenian hamsters, and hybridomas were generated. By using this approach, a mAb specific for *Met* was obtained. This antibody, designated We-1, strongly stained the plasma membranes of ARHO12 and NIH/3T3 cells transfected with mouse *Met*, but not that of non-transfected control cells (Fig. 1A).

To determine the isotype of We-1, the antibody was run on a SDS-PAGE gel (Fig. 1B), which was stained with Coomassie Brilliant blue. On a non-reduced gel this yielded a 150 kDa band, indicating that We-1 is of immunoglobulin G isotype (Fig. 1B, left panel). Under reducing conditions, bands of 50 kDa and 25 kDa were present, representing the immunoglobulin heavy and light chains of the mAb, respectively (Fig. 1B, right panel).

### Expression of *Met* in normal intestinal epithelium and in adenomas

We subsequently used We-1 to assess the expression of *Met* protein in the normal murine intestinal mucosa and in intestinal adenomas. In the crypt and villus epithelium of the normal small and large intestine, we detected hardly any *Met* expression. In adenomas of the small and large intestine of *Apc<sup>+/-min</sup>* mice, *Met* expression was low or absent (Fig. 2A,



**Fig. 1.** Characterization of a hamster monoclonal antibody, We-1, against mouse Met. (A) We-1 antibody was used to stain parental ARHO12 and NIH3T3 cells (left panel) or ARHO12 and NIH3T3 cells transfected with Met (right panel). (B) The isotype of the antibody We-1 was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis gel on reduced (R) and non-reduced (NR) gels and staining with Coomassie brilliant blue. The immunoglobulin bands are visible next to the marker (M).

upper panel). In contrast, in the mouse kidney (Fig. 2A, lower right panel) and liver (data not shown), strong Met expression was present. In the kidney, tubular epithelial cells, glomerula and vessels stained positive for Met. In the liver, Met expression was detected on hepatocytes, bile ducts and vessels (data not shown). Our observation that mouse intestinal adenomas lack Met expression was unexpected as human intestinal tumors overexpress MET from the earliest stage (i.e. the ACF) onwards.<sup>(8)</sup> This overexpression has been linked to deregulation of the WNT signaling pathway,<sup>(8)</sup> which also underlies adenoma formation in *Apc*<sup>+/*min*</sup> mice. As has been shown by others,<sup>(15)</sup> nuclear  $\beta$ -catenin expression was detected in intestinal polyps of *Apc* <sup>$\pm$ /*Min*</sup> mice, indicating that activated Wnt signaling was present (Fig. 2A, lower left panel). Activated Wnt signaling in these polyps, however, did not result in Met overexpression.

To confirm that Met is indeed not expressed in mouse intestinal tumors, Met expression was also analyzed by

immunoblotting using a polyclonal antibody against mouse Met (SP260).<sup>(16-18)</sup> Using this approach, the Met protein was also not detected in the neoplastic (and normal) mouse intestine, although it was detected readily in kidney lysates (Fig. 2B). By using laser microdissection, we isolated adenomatous and adjacent normal intestinal tissue from *Apc*<sup>+/*min*</sup> mice and determined the Met mRNA levels by semiquantitative RT-PCR. Although we did detect Met mRNA in both the normal intestine and in intestinal polyps, the expression levels were low compared to that in the kidney (Fig. 2C).

### Met protein is expressed strongly by embryonic intestinal epithelial cells

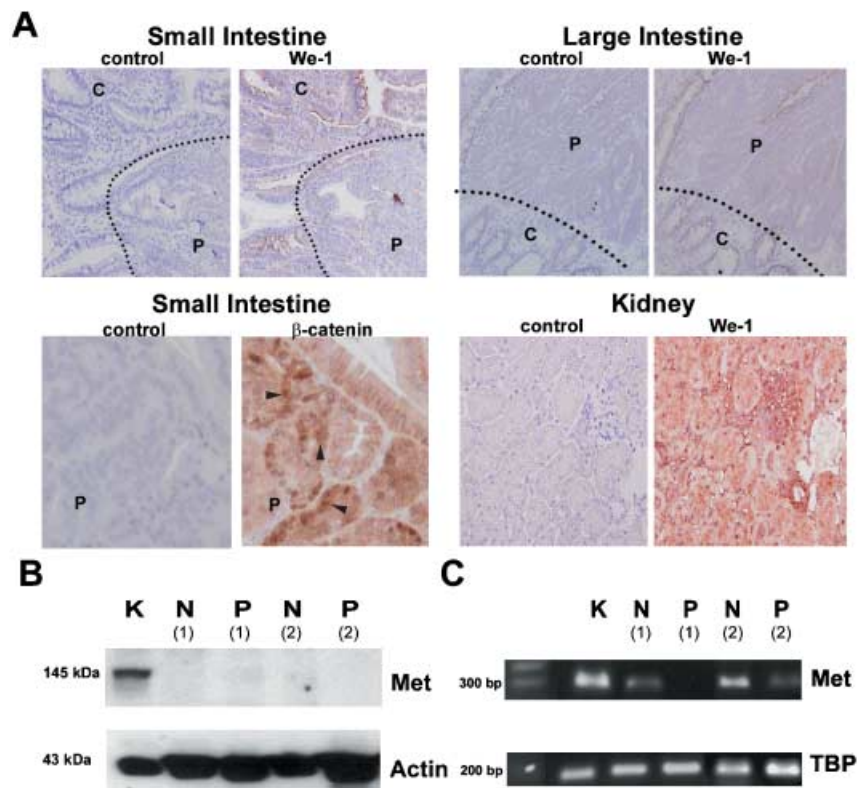
Using *in situ* hybridization and Northern blot analysis, Sonnenberg and colleagues<sup>(12)</sup> demonstrated that *Met* mRNA is expressed in the developing intestine, peaking at embryonic day 15 (E15). To explore whether Met protein expression can also be detected by We-1 at this stage of development, we carried out immunohistochemical studies. Indeed, we observed that the intestinal epithelium of E15 mouse embryos stained strongly with We-1 (Fig. 3A). Also, immunoblotting of E15 intestinal tissue demonstrated expression of Met protein. Taken together, these data demonstrate that Met is expressed in intestinal epithelial cells during development but hardly or not at all in the normal adult mouse intestine or in intestinal tumors of *Apc*<sup>+/*min*</sup> mice (Fig. 3B).

### Met family member Ron is not expressed in intestinal adenomas of *Apc*<sup>+/*min*</sup> mice

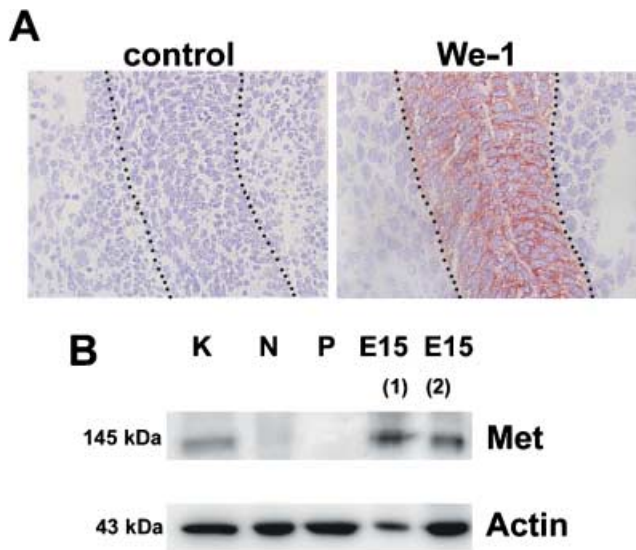
Our observation that Met, although strongly overexpressed in human colorectal adenomas, is not expressed in adenomas of *Apc*<sup>+/*min*</sup> mice implies that Met is either not instrumental in intestinal tumor formation or that its role in intestinal tumorigenesis in the mouse is substituted for by a functional homolog. To explore the latter possibility, we studied the expression of Ron, which shows a high degree of homology to Met (75% amino acid identity) and represents the receptor for the only other member of the plasminogen-like growth factor family (i.e. MSP). We observed that Ron is not expressed in intestinal adenomas, but only in the surrounding normal mucosa, specifically in the epithelial cells of the intestinal villi (Fig. 4A). The expression patterns of both Ron and Met are in sharp contrast to the expression of the Wnt target CD44v6,<sup>(19)</sup> which is expressed in the epithelial cells of the crypts (Fig. 4A). Isolating adenomatous and normal intestinal tissue by laser microdissection corroborated the immunohistochemical data. In these tissues Ron mRNA was detected in the lysate of normal, but not neoplastic, intestinal cells (Fig. 4B).

## Discussion

To explore the role of Met in intestinal tumorigenesis in *Apc*<sup>+/*min*</sup> mice, we generated an Armenian hamster mAb against mouse Met. By using this antibody, designated We-1, we detected strong expression of the Met protein in the intestinal epithelium of mouse embryos, a cell type that has previously been shown to express *Met* by mRNA *in situ* hybridization studies.<sup>(12)</sup> In contrast, in the normal intestine of adult mice as well as in intestinal adenomas of *Apc*<sup>+/*min*</sup> mice, expression of



**Fig. 2.** Expression of Met in normal intestinal epithelium and in adenomas of *Apc<sup>+/-min</sup>* mice. (A) We-1 antibody was used to stain normal intestinal tissue (C, crypts) and polyps (P) in the small and large intestine (upper panels). Dashed line depicts the boundary between normal intestinal tissue and adenomatous tissue. In addition, kidneys were stained with We-1 (lower right panels). Also, intestinal tissue of the small intestine was stained for  $\beta$ -catenin expression (lower left panel). Arrowheads indicate nuclear  $\beta$ -catenin expression. (B) Met expression was detected by western blotting in lysates of kidney (K), normal intestinal tissue (N) and polyps (P) ( $n = 3$ ).  $\beta$ -Actin was used as a loading control. (C) Using laser microdissection we isolated normal intestinal tissue and tissue from polyps and determined Met mRNA levels using reverse transcription–polymerase chain reaction. Met mRNA levels were detected in the kidney, normal intestine or polyps ( $n = 3$ ). As a loading control TATA box binding protein (TBP) levels were determined. In (B) and (C) the results of two different mice are shown (1 + 2).

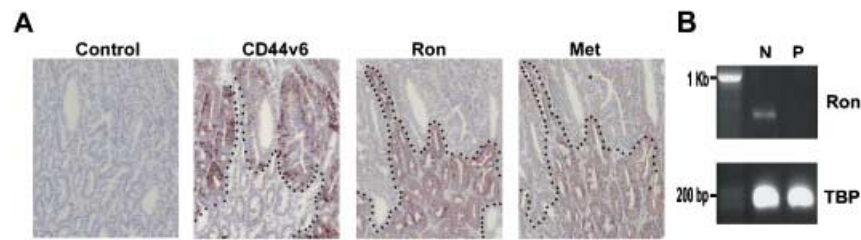


**Fig. 3.** Met expression in the intestinal tract of mice at embryonic day 15 (E15). (A) The intestinal tract of embryos was stained with We-1. Dashed line depicts the boundary between epithelial cells and surrounding cells. (B) Using immunoblotting Met protein expression was determined in lysates of kidney (K), adult intestinal tissue (N, normal; P, polyp) and intestinal tissue of two different E15 embryos (1 + 2).  $\beta$ -Actin was used as a loading control.

the Met protein was very low. These observations suggest a role for the Hgf/Met signaling pathway in development of the mouse intestine, but also imply that Met signaling does not contribute to intestinal tumorigenesis in *Apc<sup>+/-min</sup>* mice.

We demonstrated that the mAb We-1 specifically stains ARHO12 and NIH/3T3 cells transfected with mouse *Met*, but not untransfected control cells (Fig. 1A). In addition, in immunohistochemical studies, We-1 strongly stains kidney tubular and glomerular cells, which have previously been shown to express *Met* mRNA *in situ* hybridization.<sup>(12)</sup> We-1 is of the immunoglobulin G isotype (Fig. 1B), and as the mAb did not detect Met by immunoblotting (data not shown), presumably it is directed against a conformational epitope on the Met protein.

Using We-1, we observed strong Met expression in the intestinal epithelium of E15 mouse embryos, an observation consistent with previous mRNA *in situ* hybridization studies.<sup>(12)</sup> In contrast, Met was hardly detectable or not detectable in the adult mouse intestine and in intestinal adenomas of *Apc<sup>+/-min</sup>* mice. In particular, the latter observation was unexpected as it is in striking contrast to the situation in human colorectal tumors, where strong overexpression of Met is present from the earliest stage of the adenoma–carcinoma sequence (i.e. ACF) onwards.<sup>(7,8)</sup> Like familial adenomatous polyposis patients, *Apc<sup>+/-min</sup>* mice lack one functional *Apc* allele; loss of the second allele leads to activation of the Wnt signaling pathway resulting in uncontrolled Tcf/ $\beta$ -catenin-mediated transcription. Activation of the WNT pathway, either by loss of APC or by mutation of  $\beta$ -catenin, also presents the primary defect in the vast majority of sporadic human colorectal carcinomas. We have previously identified MET as a target gene of the WNT signaling pathway in human CRC.<sup>(8)</sup> Furthermore, previous studies have shown that MET can also activate the Wnt signaling pathway.<sup>(16,20)</sup> The absence of Met overexpression in intestinal adenomas of *Apc<sup>+/-min</sup>* mice thus



**Fig. 4.** Expression of CD44v6, Ron and Met in normal intestinal epithelium and in polyps of *Apc<sup>+/-min</sup>* mice. (A) Parallel sections of intestinal tissue were stained with antibodies against CD44v6, Ron and Met. Dashed line depicts the boundary between normal intestinal tissue and adenomatous tissue. (B) After microdissection Ron mRNA levels were determined in normal intestinal (N) or polyp (P) tissue using reverse transcription–polymerase chain reaction. As a loading control TATA box binding protein (TBP) levels were measured.

implies interspecies differences in the regulation of Met and WNT signaling targets between humans and mice.

The Met promoter region of mice and humans is approximately 70% identical at the nucleotide level. A search for TCF binding sites<sup>(21)</sup> revealed no consensus TCF binding sites in both promoter regions. In human CRC, regulation of MET expression through WNT signaling is therefore probably an indirect effect. Enhancers and other binding sites may also influence Met expression. The SP1-binding sites in the Met promoter are highly conserved between mice and man, implicating that transforming growth factor  $\beta$  (TGF $\beta$ ) signaling may be important in regulating Met expression.

Our current finding that Met is not overexpressed in intestinal adenomas in *Apc<sup>+/-min</sup>* mice is corroborated by a mRNA microarray study showing no regulation of Met in polyps of *Apc<sup>+/-min</sup>* mice compared to normal intestinal epithelium (R. Smits, personal communication). In view of the compelling evidence for a key role of MET in tumor invasion and metastasis, it is tempting to speculate that the absence of Met overexpression in mouse intestinal adenomas could be a factor in their inability to progress to invasive and metastatic cancer.

Our observation that the Met protein, unlike in human adenomas, is not expressed in intestinal adenomas of *Apc<sup>+/-min</sup>* mice

implies that Met is not instrumental in their development. Ron, the receptor for MSP/hepatocyte growth factor line (HGFL),<sup>(22,23)</sup> which is closely related to Met, is also not expressed by mouse intestinal adenomas and hence does not act as a functional substitute for Met. The absence of Ron expression in mouse intestinal tumors contrasts the reported overexpression of RON in human colorectal cancer.<sup>(24,25)</sup> In the mouse intestine, we detected Ron expression on differentiated villus cells.

In conclusion, our findings demonstrate important interspecies differences in the regulation of Met expression during intestinal tumorigenesis. Our current study demonstrates that intestinal adenoma formation in *Apc<sup>+/-min</sup>* mice is not accompanied by Met overexpression, implying that Wnt signaling deregulation in mouse, unlike in human intestinal epithelium, does not result in Met overexpression.

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