

Wnt Signaling Regulates Expression of the Receptor Tyrosine Kinase Met in Colorectal Cancer¹

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Abstract

Overexpression of the receptor tyrosine kinase Met is an early event in the colorectal adenoma-carcinoma sequence. This suggests a link with disruption of adenomatous polyposis coli-controlled regulation of β -catenin/T-cell factor (TCF)-mediated transcriptional activation, which is crucial in initiating tumorigenesis. Indeed, in intestinal biopsies from patients with familial adenomatous polyposis, we find Met already overexpressed in dysplastic aberrant crypt foci, the earliest neoplastic lesions of colorectal cancer (CRC). Moreover, in CRC cells, induction of dominant-negative TCF proteins and the consequent abrogation of β -catenin/TCF-mediated transcriptional activation lead to a strong down-regulation of Met expression. Our results indicate that Met expression is part of a genetic program controlled by the Wnt pathway and suggest a role for Met in controlling the turnover and differentiation of intestinal epithelium.

Introduction

CRC³ is the second leading cause of cancer-related death in the Western world. It evolves through a series of morphologically recognizable stages known as the adenoma-carcinoma sequence (1). Along these stages complex genetic alterations occur, of which mutations involving components of the Wnt signaling cascade, such as APC, β -catenin, and axin/conductin, are mandatory for the initial neoplastic transformation of intestinal epithelium. These mutations result in stabilization of β -catenin, which translocates to the nucleus and acts as a transcriptional coactivator of lymphoid enhancer factor/TCF transcription factors (reviewed in Refs. 2–4). Sustained complex formation between β -catenin and TCF4 in the nucleus of intestinal epithelial cells leads to constitutively activated transcription of TCF target genes, causing a disruption of the normal balance between cell division, differentiation, and apoptosis. Thus far, the TCF target genes instrumental for colorectal tumorigenesis have only been partly identified. Studies from our own and other laboratories have shown that the tyrosine kinase Met, the receptor for the epithelial growth, motility, and survival factor HGF/scatter factor, is overexpressed in CRC (5–7). The cause of this overexpression is unknown, but the fact that Met is already up-regulated at the adenoma stage suggests regulation by the Wnt signaling cascade. To explore this hypothesis, we studied Met expression in ACF of FAP patients, which lack functional APC. Furthermore, we analyzed the expression of Met in CRC cell lines with inducible expression of N-truncated TCF constructs (8). These dnTCF proteins inhibit the formation of β -catenin/TCF complexes and, in this way,

prevent the transcription of TCF target genes. Our studies identify Met as a target of the Wnt signaling cascade.

Materials and Methods

Immunohistochemistry. Colon mucosa biopsies from 12 patients, taken at routine colonoscopy, were obtained from the archives of the Department of Pathology, Academic Medical Center, University of Amsterdam (Amsterdam, the Netherlands). Five- μ m sections were prepared and stained with the primary antibodies VFF18 (a mouse mAb against human CD44v6; Bender Co., Vienna, Austria) and Do24 (a mouse mAb against human Met; Upstate Biotechnology, Lake Placid, NY), followed by avidin-biotinylated horseradish peroxidase complex (Dakopatts, Glostrup, Denmark). The peroxidase was visualized with 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO).

Cell Culture and Transfections. CRC cells were grown in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS (Integro, Leuvenheim, the Netherlands), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin (all from Life Technologies, Inc.). The T-Rex system (Invitrogen, Paisley, United Kingdom) was used according to the manufacturer's instructions to generate CRC cell lines with inducible dnTCF1 or dnTCF4 (8). After selection on zeocin (500 μ g/ml) and blasticidin (10 μ g/ml), resistant clones were tested for dnTCF induction. Cells were transiently transfected by LipofectAMINE Plus reagent (Invitrogen) with 5 μ g of either TOPflash or FOPflash TCF reporter plasmids (Upstate Biotechnology). After transfection, cells were grown with or without doxycyclin (1 μ g/ml) for 24 h. Cells were lysed in lysis buffer (luciferase assay system; Promega, Madison, NY), and 20 μ l of each lysate were monitored for luciferase activity using luciferase assay substrate buffer (Promega). Light units were recorded using a luminometer.

Northern Blotting and Expression Analysis. Cells were grown with or without doxycyclin (1 μ g/ml) for 24 h. Total RNA was isolated using Trizol (Life Technologies, Inc.). Total RNA (15 μ g/lane) was electrophoresed on a 1% formaldehyde-containing agarose gel, transferred onto a nylon membrane (Hybond N(+); Amersham Pharmacia Biotech Inc., Aylesbury, United Kingdom), and hybridized with a ³²P-labeled 394-bp human Met cDNA probe (amplified from human Met cDNA, nucleotides 3917–4311) and subsequently with a ³²P-labeled 357-bp human CD44 probe (amplified from human CD44 cDNA, nucleotides 341–698). Blots were reprobbed with a ³²P-labeled human β -actin probe (Invitrogen) to correct for loading. Intensities were quantified using a phosphorimager in combination with AIDA Image Analyzer software (Isotopenme β geräte GmbH, Staubenhardt, Germany). Each sample was corrected for loading by comparison with the appropriate β -actin signal. Within the same clone, the corrected Met or CD44 signals were then compared before and after the addition of doxycyclin.

FACS Analysis. For FACS analysis, cells were stimulated with doxycyclin as described above. Cells were washed with FACS buffer (1% BSA in PBS). Then, cells were incubated with the primary mouse anti-Met Do24 antibody or with an isotype-matched IgG2a control antibody for 1 h, washed, and incubated with RPE-conjugated secondary antibodies. Incubations were performed in FACS buffer at 4°C, and cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

Results

ACF with Dysplasia in FAP Patients Overexpress Met. Previous studies in human CRC have shown that Met overexpression is present in colorectal adenomas as well as in invasive carcinomas (5–7). This prompted us to explore whether dysplastic ACF, the earliest neoplastic

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³ The abbreviations used are: CRC, colorectal cancer; TCF, T-cell factor; dnTCF, dominant-negative TCF; ACF, aberrant crypt foci; HGF, hepatocyte growth factor; FAP, familial adenomatous polyposis; mAb, monoclonal antibody; FACS, fluorescence-activated cell-sorting; APC, adenomatous polyposis coli; RPE, R-phycoerythrin.

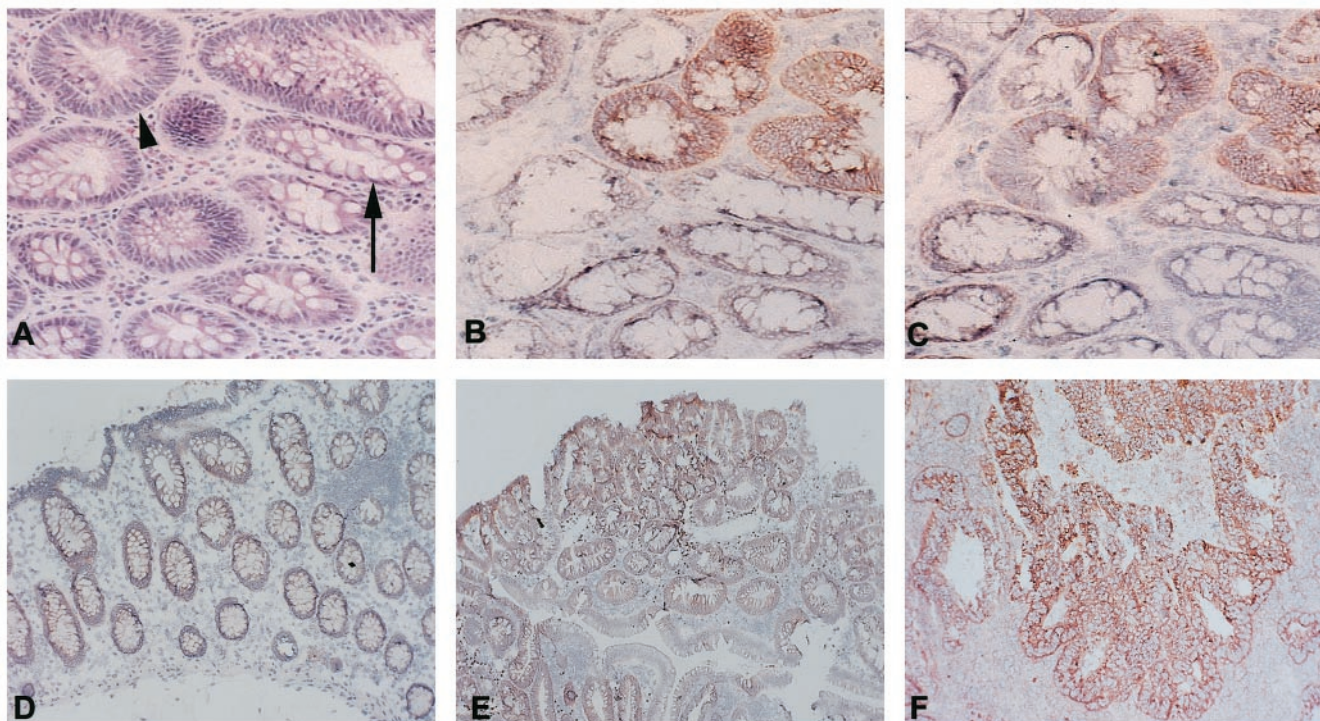


Fig. 1. ACF with dysplasia, adenomas, and carcinomas in FAP patients overexpress Met. Serial sections of colon mucosa with ACF were stained with either (A) H&E, (B) mAb Do24 against Met, or (C) mAb VFF18 against CD44v6, and (D) normal colon mucosa, (E) adenoma, and (F) invasive carcinoma were stained with mAb Do24 against Met. Arrow, a normal crypt; arrowhead, an ACF.

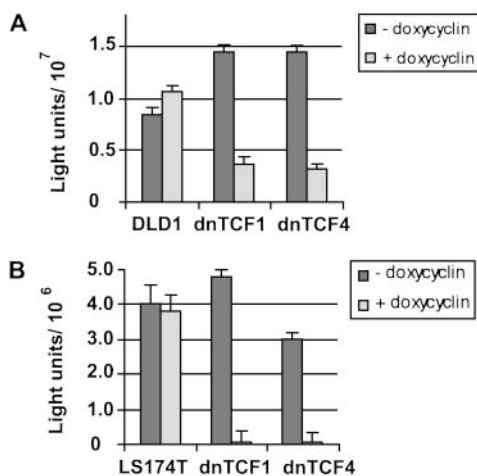


Fig. 2. TCF reporter activity is abrogated by induction of dnTCF in CRC cells. (A) DLD-1 or (B) LS174T cells were transfected with a TCF reporter plasmid (TOPflash) or control (FOPflash). Reporter activities were measured after doxycyclin induction of dnTCFs for 24 h. FOPflash activities were <30,000 light units for all cell types and constructs tested.

lesions of CRC, also overexpress Met. ACF are found at high frequency in colon biopsies from patients with FAP and are believed to arise as a direct consequence of the loss of APC tumor suppressor protein function. Serial sections were stained from 12 independent patients (ACF of 4 patients, adenomas of 4 patients, and carcinomas of 4 patients). We observed a strong up-regulation of Met in ACFs (Fig. 1B), which was accompanied by up-regulation of CD44 (Fig. 1C), a previously identified target of the Wnt signaling cascade (9). In accordance with previous observations, Met overexpression is also present at the adenoma stage and in invasive carcinomas (Fig. 1, E and F). These *in vivo* findings imply a close link between the initiation of CRC and Met deregulation and support our hypothesis that Met expression is regulated by Wnt signaling.

Expression of Met Is Regulated by the Wnt Signaling Cascade.

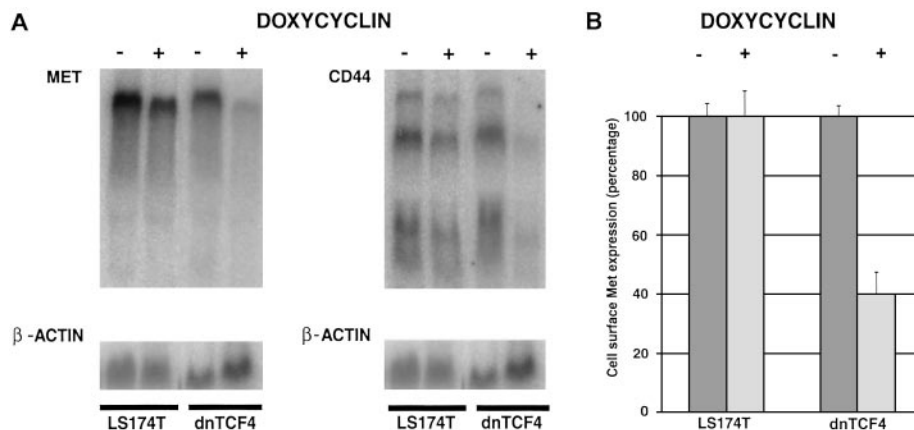
To further explore the role of Wnt signaling in the regulation of Met expression, the human CRC cell lines DLD-1 and LS174T were used. These cell lines, which contain mutated APC and β -catenin, respectively, were stably transfected with inducible dnTCF1 or dnTCF4 constructs (8). In these CRC cell lines, TCF target genes are constitutively expressed. Inhibition of TCF transcriptional activation by dnTCF1 or dnTCF₄ was monitored by transfecting a TCF reporter (pTOPflash) or, as a control, a reporter containing scrambled TCF binding sites (pFOPflash).

Doxycyclin-induced expression of dnTCF1 or dnTCF4 abrogated β -catenin/TCF-activated TOPflash activity in DLD-1 cells as well as in LS174T cells (Fig. 2). In the parental cell lines, which contained no dnTCF constructs, we observed no change in TOPflash activity after doxycyclin stimulation. FOPflash activities were <30,000 light units for all cell types and constructs tested. To determine whether blockade of Wnt signaling by induction of dnTCF proteins affects Met messenger expression, mRNA was isolated from the cells and analyzed using Northern blotting by a Met-specific probe. Signals were quantified by densitometry. Upon induction of dnTCF4, we observed a 2.5–3.2-fold down-regulation of Met mRNA in both DLD-1 and LS174T cells (Table 1, Fig. 3A). dnTCF1 induced an approximately 2.5-fold down-regulation of Met (Table 1). In contrast, the mRNA expression levels of Met were only marginally decreased (~10%) in the parental cell lines after the addition of doxycyclin. In parallel with Met, CD44, a previously identified target of the Wnt signaling cascade, was also down-regulated (Table 1; Ref. 9).

Table 1. Fold down-regulation of Met and CD44 mRNA levels after induction of dnTCF1 or dnTCF4 in DLD1 and LS174T cells

Cell type/construct	Fold down-regulation of Met mRNA levels	Fold down-regulation of CD44 mRNA levels
DLD1/dnTCF1	2.5	3.0
DLD1/dnTCF4	2.5	4.0
LS174T/dnTCF1	2.4	3.5
LS174T/dnTCF4	3.2	7.6

Fig. 3. Induction of dnTCF4 down-regulates Met mRNA and protein expression levels in LS174T cells transfected with a dnTCF4 construct compared with LS174T parental cells. *A*, down-regulation of Met mRNA by dnTCF4. LS174T cells were stimulated with doxycyclin for 24 h. mRNA was extracted from these cells and analyzed on Northern blot using a Met-specific probe (top left panel). As a positive control, down-regulation of CD44 mRNA is shown (top right panel). Bottom panels, reprobe of Northern blot with a control probe (β -actin). *B*, down-regulation of Met protein by dnTCF4. LS174T cells were stimulated with doxycyclin for 24 h. Met expression was measured by FACS analysis using mAb Do24 against Met followed by RPE-conjugated goat antimouse antibody. Within each clone, the expression of the untreated cells were set at 100%.



In parallel with down-regulated mRNA levels, FACS analysis showed a 2.5-fold down-regulation of surface-expressed Met protein levels in LS174T cells (Fig. 3B).

Discussion

Our current results demonstrate that expression of the HGF receptor Met in intestinal epithelium is controlled by the Wnt cascade. Activation of β -catenin/TCF signaling *in vivo*, as present in ACF in FAP patients, is associated with Met overexpression, whereas blockade of β -catenin/TCF signaling in CRC cell lines by dnTCF leads to down-regulation of Met.

HGF, a member of the plasminogen-like growth factor family, is a pleiotropic cytokine that regulates cell motility, growth, and survival (reviewed in Ref. 10). Apart from being crucial in mammalian development and epithelial morphogenesis (11, 12), the HGF/Met pathway has also been shown to play a pivotal role in tumor growth, invasion, and metastasis (10, 13–15). Overexpression and/or uncontrolled activation of Met has been found in many tumor cell lines as well as in primary human tumors, including carcinomas of the stomach, liver, pancreas, thyroid gland, and colon (5–7, 10). This uncontrolled Met signaling can be caused by several different mechanisms, *e.g.*, translocation of *Met* to the *Tpr* locus resulting in a constitutively active Tpr-Met oncoprotein, *Met* amplification, or the presence of an autocrine HGF/Met loop. Moreover, activating mutations in the kinase domain of Met have been shown to play a causative role in tumorigenesis in patients with hereditary papillary renal carcinoma (16). These mutations, which are present in the germ line of affected members of hereditary papillary renal carcinoma families, were shown to mediate transformation, invasive growth, and protection from apoptosis (17, 18).

In CRC, Met is overexpressed in the vast majority of adenomas, invasive carcinomas, and metastases (5–7), whereas HGF is expressed by both cultured intestinal fibroblasts (10) and fibroblasts within the tumor microenvironment (7). This suggests a role for paracrine HGF/Met interaction in promoting colorectal tumor growth and invasion. Furthermore, HGF is expressed in lymph nodes and liver, the primary sites of metastatic spread in CRC. Met signaling may also contribute to metastasis formation by promoting metastatic outgrowth. Interestingly, Di Renzo *et al.* (6) have previously reported the presence of *Met* amplification in 10% of primary colon tumors but in the majority of liver metastases examined. Based on these findings, they proposed that during colorectal tumorigenesis, an initial phase of “regulatory” Met overexpression is followed by a second phase during which this overexpression has become “fixed” by gene amplification, leading to constitutive Met activation. In this way, the tumor would gain growth factor independence, resulting in a growth and metastasis advantage. Our current study corroborates this hypothesis by showing that Met overexpression in the initial phase of CRC is “regulatory” and results from enhanced Wnt signaling.

In conclusion, our study identifies Met as a target of the Wnt signaling cascade, which initiates colorectal tumorigenesis. Because a vast body of clinical, genetic, and cell biological studies have revealed a key role for the HGF/Met pathway in tumor growth, invasion, and metastasis in a variety of cancers, this finding strongly suggests an instrumental role for Met signaling in colorectal tumorigenesis.

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