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Faculteit der Geneeskunde

How I wish, how I wish you were here

To my mother

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General introduction

GENERAL INTRODUCTION

Anatomy of the intestinal tract

The mammalian intestinal tract is a highly organized organ and its principal function is to digest and absorb nutrients from food. Anatomically, it can be divided into two main sections: the small bowel and large bowel. The small bowel is where the larger part of digestion occurs; it is further subdivided into the duodenum, jejunum and ileum. The large bowel consists of the colon and rectum and its main function is to extract salt and water from the solid wastes.

The entire intestinal tube is composed of a number of distinct layers, from outside to inside: serosa, muscularis externa, submucosa, muscularis mucosa and mucosa (Fig. 1A). The mucosa is lined with a single sheet of polarized columnar epithelial cells which forms a barrier between the luminal environment and the body. These specialized cells are directly responsible for protection, secretion and absorption. The most obvious difference between the small bowel and large bowel is the presence of small fingerlike projections in the small bowel called villi (Fig. 1B), greatly increasing the surface area for nutrient absorption.

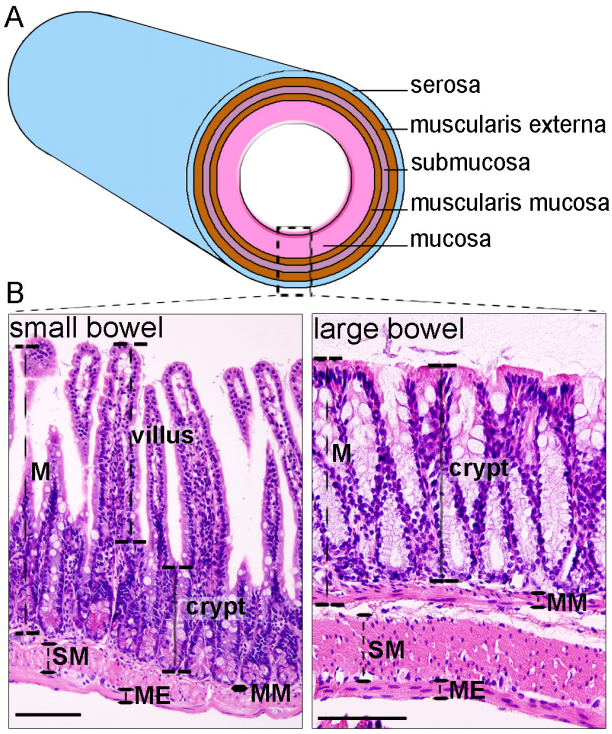

Figure 1. The intestinal mucosa. **A**) Architecture of the layered intestinal tube. From out- to inside: serosa, muscularis externa, submucosa, muscularis mucosa and mucosa. **B**) HE-stained cross-sections of the mouse small and large bowel. The mucosal layer of the small bowel is marked by the presence of protruding villi and deep intestinal crypts (indicated by vertical bars), whereas the large bowel lacks villi. ME, muscularis externa; MM, muscularis mucosa; SM, submucosa; M, mucosa.

Situated between the villi of the small bowel and invaginating from the surface of the colon are the crypts of Lieberkühn, named after the 18th-century German anatomist Johann Nathanael Lieberkühn. These repetitive test-tube shaped units serve as a niche for intestinal epithelial stem and progenitor cells that sustain the self-renewal of the epithelial barrier.

Self-renewal and stem cells in the intestinal epithelium

Epithelial cell proliferation is restricted to crypts of Lieberkühn. This is best illustrated in the crypts of the small bowel where actively dividing epithelial cells occupy a well-defined position (Fig. 2A). The basal part of the crypt compartment accommodates a population of relatively small wedge shaped cells (Fig. 2B). These so-called crypt base columnar cells are marked by expression of Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5). Lineage tracing studies have identified these as cycling intestinal stem cells

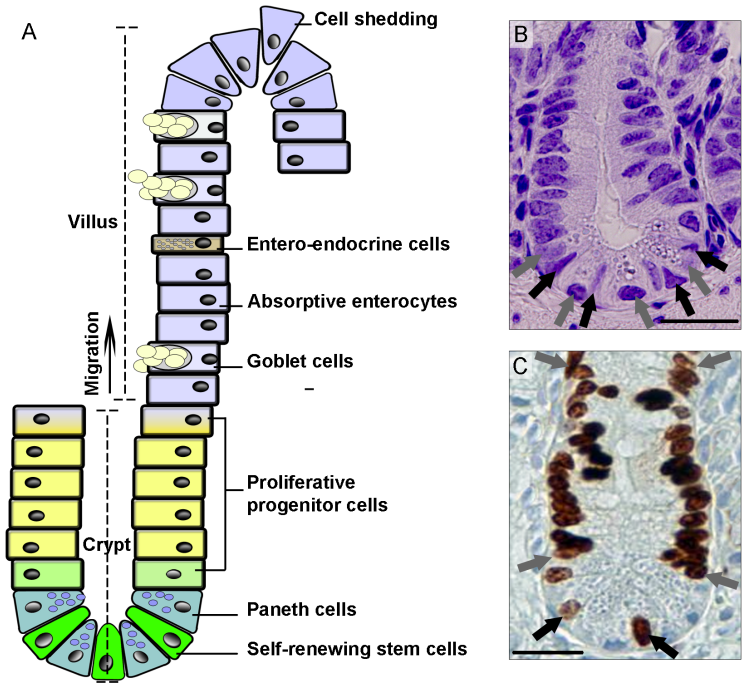

Figure 2. Epithelial renewal and maturation along the crypt-villus axis. **A**) Schematic representation of the crypt-villus axis. Cycling intestinal stem cells reside at the base of the crypts and their progeny, also known as proliferative progenitor cells, give rise to the specialized differentiated cells. Depicted are enterocytes, goblet cells, entero-endocrine cells, and Paneth cells. Paneth cells migrate into the crypt whereas all other differentiated epithelial cells migrate onto the villus where they are eventually shed into the lumen. **B**) HE-stained section of a mouse small intestinal crypt. Paneth cells (grey arrows) can be identified by their oval basal nucleus and large granules that occupy most of the cytoplasm. Inconspicuously hidden between the Paneth cells are the intestinal stem cells (black arrows) which can be identified by their wedge-shaped nucleus and narrow apical cytoplasm. **C**) Example of cell proliferation in the mouse intestinal crypt visualized by anti-Ki67 staining. Transit-amplifying cells (between grey arrows) and cycling crypt base cells (black arrows) are visible in the crypt compartment while terminally differentiated Paneth cells are negative for Ki67 staining (bars, 25 µm).

(ISCs) [1]. Lgr5-postive ISCs divide daily and daughter cells constitute a transit-amplifying compartment of rapidly dividing progenitors (Fig. 2C). These, in turn, differentiate into functionally mature cells while migrating upwards and, at the end of their journey, are shed into the intestinal lumen. This hierarchy allows for the generation of approximately 300 cells per crypt every day [2], ensuring a complete renewal of the intestinal epithelial barrier within a few days**.**

Two main lineages of differentiated cell types can be distinguished within the intestinal epithelium: the absorptive enterocyte lineage and the secretory lineage. The latter encompasses mucin secreting goblet cells, hormone producing entero-endocrine cells and bactericidal protein-secreting Paneth cells. Paneth cells are found only in the small intestine, but not in the colon, and reside at the crypt-base region intermingled with ISCs. Paneth cells contain secretory granules containing antimicrobial factors such as peptides and lysozyme. In addition, these cells secrete essential growth factors and regulatory molecules which help to sustain and modulate adjacent ISCs [3]. The intestinal epithelium also contains relatively small quantities of other cell types implicated in immunity, such as tuft cells and Peyer's patch-associated M (or microfold) cells [4,5].

Signaling pathways in the intestine

The intestinal crypt compartment is maintained through the concerted actions of different signaling pathways, including Wnt, Bone Morphogenic Protein (BMP), Hedgehog, Notch, and Receptor Tyrosine Kinase (RTK) signaling (Fig. 3). Crypt homeostasis is sustained by a pool of multipotent ISCs that are in particular responsive to Wnt signals. These signals are the dominant force in controlling intestinal epithelial cell fate driving the activation of a target-gene program that is required for ISC self-renewal [6,7].

Wnt glycoproteins constitute a large family of secreted proteins that are involved in intercellular signaling and play a key role in regulating homeostatic self-renewal in a variety of adult tissues. In the proliferative crypt compartment several Wnt ligands are expressed, including *Wnt-3, Wnt-6, Wnt-9b, and Wnt-11* [8,9]. ISCs are highly reliant on Wnts produced by surrounding cells in their microenvironment, which include Paneth cells [3], subepithelial telocytes that line the crypt base [10,11], and stromal myofibroblasts [12]. It is important to note that the ISC pool is highly dynamic and follows from neutral competition between dividing stem cells for limited niche access [13]. Therefore, stem-cell fate above all is determined by the availability of nice factors, such as Wnt ligands. For instance, a reduction of Wnt signaling in the basal crypt compartment results in lower ISC numbers, while the remaining stem cells are retained only at the center-base of the crypts, which appears to be the optimal niche for the maintenance and propagation of *Lgr5*-expressing ISCs [14].

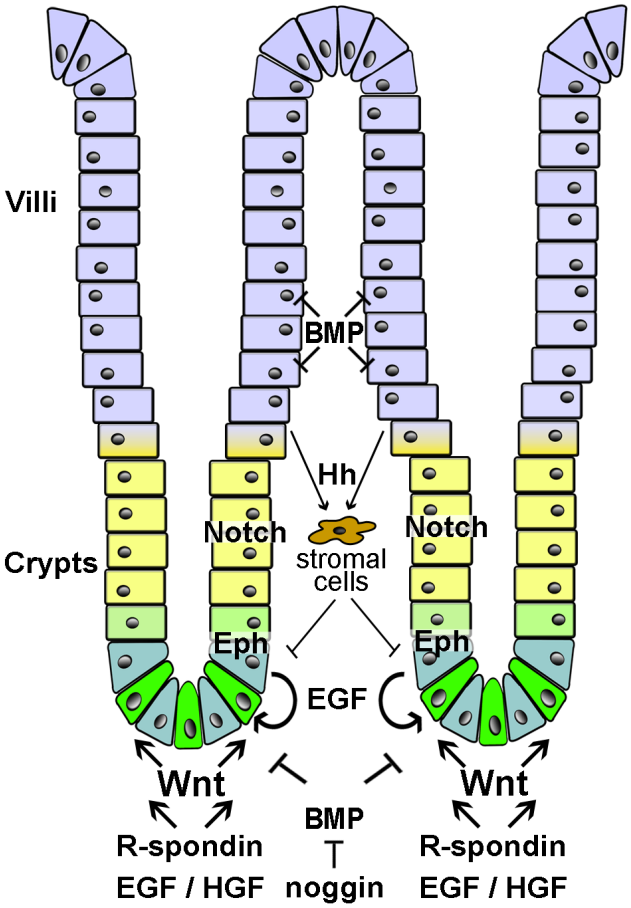

Figure 3. Signaling pathways in the intestine. Cellular organization of the intestinal epithelium is controlled by the concerted action of multiple signaling pathways. Crypt base columnar cells, the proposed intestinal stem cells (green), are maintained by their surrounding niche for precise regulation of self-renewal and differentiation under homeostatic conditions. Paneth cells are intermingled with ISCs and provide these cells with signaling factors such as EGF and Wnt3. R-spondin proteins act as activators of Wnt signaling in the stem cell compartment. BMP and Hedgehog (Hh) signaling act as negative regulators of proliferation. Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are potent mitogens in the crypt compartment.

Canonical Wnt signaling is initiated when Wnt ligands engage receptors of the Frizzled (Fzd) and low-density lipoprotein receptor-related protein (LRP) families on the cell surface (Fig. 4). The central player in the Wnt cascade is cytoplasmic β-catenin, the stability of which is regulated by a multimeric “destruction” complex comprised of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1α

(CK1α). Wnt ligand induced receptor signaling inactivates this destruction complex and, as a consequence, β-catenin accumulates and translocates to the nucleus where it interacts with TCF/LEF transcription factors (Fig. 4, *right panel*). This interaction controls the transcription of a set of Wnt-responsive target genes involved in maintaining the proliferative crypt compartment [15–17]. Signaling is enhanced by soluble R-spondin ligands, which bind to their cognate receptors, the Lgr5 homologues. This complex neutralizes Rnf43 and Znrf3, two transmembrane ubiquitin ligases that function as negative regulators of Wnt signaling by targeting Wnt receptors at the cell surface for degradation [18,19]. Thus, the selective expression of *Lgr5* in ISCs provides these cells with a cooperative molecular mechanism that allows for a strong activation of the Wnt pathway. Indeed both R-spondin and Wnt ligands are required for ISC maintenance [19]. In accordance, elevated levels of Wnt signaling in the crypt compartment results in excessive stem cell division [14,20]. As discussed in more detail below, dysregulation of the Wnt signaling pathway is the major driving force in the pathogenesis of colorectal cancer [21] and, consequently, Wnt target genes represent potential candidates for anti-cancer therapy.

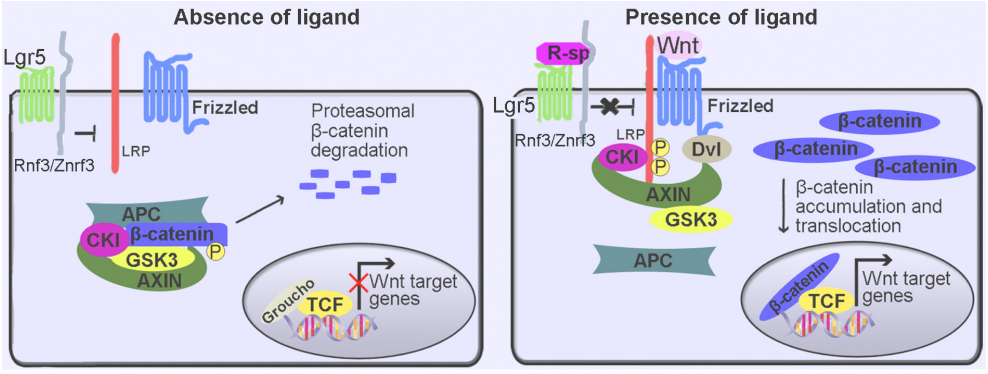

Figure 4. Canonical Wnt signaling in the intestine. Left panel: In the absence of a Wnt signal, β-catenin is bound by APC and Axin to form a degradation complex. Rnf43 (and Znrf3) are transmembrane ubiquitin ligases that have emerged as critical regulators of the Wnt pathway by regulating Wnt receptor turnover thereby determining the responsiveness of cells to Wnt ligand. These interactions facilitate the phosphorylation of serine and threonine residues at the amino terminus of β-catenin by the kinases CK1 and GSK3β, leading to ubiquitination and proteasomal degradation of β-catenin. Due to the lack of free β-catenin the TCF/LEF transcription factor family represses target genes by recruiting transcriptional co-repressors (Groucho/TLE) to their promoters and/or enhancers.
Right panel: In the presence of Wnt ligand, Wnt forms a complex with Frizzled and LRP5/6, which triggers the phosphorylation of LRP by CK1. This induces the relocation of AXIN to the membrane and inactivation of the destruction complex, leading to the accumulation of β-catenin. Cells are further sensitized to Wnt signaling by R-spondin (R-sp) ligand. The Lgr5/R-spondin complex acts by neutralizing the negative Wnt regulators Rnf43/Znrf3. Upon translocation to the nucleus, β-catenin converts the TCF proteins into potent transcriptional activators by displacing Groucho/TLE proteins and recruiting coactivator proteins, driving the activation of a target-gene program that is required for ISC self-renewal and progenitor proliferation. In cancer, Wnt signaling can be aberrantly activated by mutations in components of the pathway, allowing for accumulation of β-catenin. The uncontrolled formation of TCF–β-catenin complexes in the nucleus causes chronic activation of the Wnt target gene program, driving the formation and progression of intestinal tumors.

In addition to Wnt signaling, undifferentiated intestinal epithelial cells also require other signals for self-renewal or differentiation. The BMP signaling pathway functions as an important negative regulator of Wnt signaling and ISC proliferation [22]. BMPs are part of the transforming growth factor beta (TGF-b) superfamily. Signals transduce to the nucleus through receptor-mediated phosphorylation of SMAD transcription factors. BMP2 and BMP4 are expressed in subepithelial stromal cells adjacent to the crypts and their receptors are expressed by epithelial crypt cells [23–25]. BMP signaling inhibits ISC expansion by transcriptional repression of a large number of stem-cell signature genes, including *Lgr5* [26]. In contrast to Wnt signaling, BMP signals are suppressed toward the base of the crypt, where their effects are antagonized by Noggin [23,24]. A conditional deletion of the BMP receptor type IA (Bmpr1a) in epithelial crypt cells results in hyperproliferation and duplication of ISCs [25,26]. Thus, BMP signaling serves to counteract and balance Wnt signaling in the intestinal crypt compartment.

Hedgehog signaling is involved in organizing crypt growth and cell patterning and represents an intricate example of epithelial-stromal interaction. Hedgehog signaling ligands Sonic hedgehog (Shh) and Indian hedgehog (Ihh) are expressed by differentiated epithelial cells, while the expression of the hedgehog signal transducers, Patched and Smoothened, is restricted to mesenchymal stromal cells. Pathway activation results in a negative feedback signal, restricting proliferation in the basal crypt region [27]. Inhibition of the hedgehog pathway results in a disturbed crypt-villus architecture and the persistence of highly proliferative epithelium [28].

Notch signaling plays a critical role in the cell-fate decision between the absorptive enterocyte and secretory cell lineages [29,30]. Notch proteins are single-pass transmembrane glycoproteins that interact with membrane-bound ligands of the Delta and Serrate/Jagged subfamilies on adjacent cells. Pathway inhibition induces a massive conversion of proliferating epithelial cells into differentiated goblet cells [29,31,32]. Conversely, increased notch signaling activity depletes the epithelium of all secretory cells [33]. In addition, notch signaling co-operates with the Wnt pathway to enhance proliferation in the ISC compartment [34].

There is also considerable crosstalk between Wnt signaling and several RTK-mediated pathways in the crypt compartment. RTKs bind extracellular growth-factor ligands with high specificity and affinity and, following engagement, emit intracellular signals that modulate epithelial cell survival, proliferation, differentiation, and migration [reviewed by Lemmon and Schlessinger [35]]. Examples of RTKs in the intestinal mucosa include the erythropoietin-producing hepatocyte kinase B receptors (EphB), the epidermal growth factor receptor (EGFR), and MET the receptor for hepatocyte growth factor. Expression of EphB2 and EphB3 in the intestinal mucosa is controlled by Wnt activity [36] and ISCs are marked by high expression of the EphB2 receptor, which becomes gradually silenced away from the crypt base [37,38]. EphB receptors bind membrane-bound ephrin ligands on neighboring cells. Subsequent

downstream signaling generates cell-to-cell repulsive forces that drive compartmentalization in the crypt compartment [39]. Consequently, disruption of EphB receptor signaling causes a stop in epithelial cell segregation, which is illustrated by the scattering of Paneth cells and proliferating cells along the entire crypt-villus axis [36].

The EGFR is a member of human epidermal growth factor receptor (HER)-ErbB family of RTKs. EGFR expression is restricted to epithelial cells within the crypt [40], and its primary ligand EGF is expressed in the ISC niche by both stromal and Paneth cells [3,41]. Upon ligand binding, the EGFR (ErbB1/HER1) forms homodimers or heterodimers with its family members ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). Receptor dimerization induces autophosphorylation of the intracellular domain through intrinsic tyrosine kinase activity and subsequent activation of downstream signaling cascades. These include the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways, which regulate proliferation, differentiation and survival [42]. Importantly, EGF/EGF receptor-mediated signaling provides the necessary receptor tyrosine kinase signals regulating ISC homeostasis [3,37,43].

Whereas EGFR signaling is generally acknowledged to play a major part in intestinal epithelial homeostasis (e.g. mediating ISC self-renewal), the role of MET receptor tyrosine kinase signaling in the intestinal mucosa has remained more elusive. Interestingly, however, MET has been identified as a prominent target of Wnt/β-catenin signaling in the intestinal mucosa [17,44] suggesting a functional role in intestinal homeostasis. MET activation by its unique ligand HGF induces receptor dimerization and transphosphorylation of regulatory tyrosines in the cytoplasmic domains. Its ligand HGF is prominently expressed in intestinal subepithelial myofibroblasts and is associated with augmented Wnt signaling activity in intestinal epithelial cells [45–47]. Similar to the EGFR, MET interacts in the cytoplasm with growth factor receptor bound protein 2 (Grb2), sarcoma (Src) homology-2-containing (Shc), and Grb2-associated binding protein 1 (Gab1) [reviewed by Mahtouk *et al*. [48]]. Consequently, MET and EGFR activation initiate similar downstream signaling cascades involved in regulation of cell proliferation and survival, including the RAS/MAPK and PI3K/Akt pathways [49], which suggests at least a partial redundancy or a possible synergism in cell fate control.

Wnt signaling and intestinal tumorigenesis

As stated above, aberrant activation of the Wnt/β-catenin signaling pathway is closely related to intestinal tumorigenesis. Germline “loss-of-function” mutations in the APC gene were first identified to be the essential genetic event responsible for Familial Adenomatous Polyposis (FAP), a hereditary disorder characterized by the development of numerous adenomatous polyps along the intestinal tract [50,51]. Soon after, it became clear that somatic APC mutations are also present in the fast majority of sporadic colorectal tumors, even at the earliest stages of tumor formation [52]. Later is was recognized that aberrant Wnt signaling in

ISCs causes adenoma formation with high efficiency [20]. This suggests that ISCs are the cell-of-origin of intestinal cancer. Thus, aberrant Wnt pathway activation is a key early event in the development of intestinal cancer. The molecular mechanisms underlying loss of functional APC protein include a truncating mutation in one allele and loss of the second allele (loss-of-heterozygosity) [53]. This second hit represents a rate-limiting step in tumor formation. Next to APC, mutations in genes encoding other components of the Wnt pathway, such as AXIN2 [54,55] and β-catenin [56], have been identified in intestinal tumors.

The earliest identifiable neoplastic lesions in the human colonic mucosa are dysplastic aberrant crypt foci (ACF) [57], which may develop into an adenoma through clonal expansion. Tumor progression and malignant behavior results from specific mutations in critical growth regulatory genes such as *K-RAS, SMAD4* and *p53*, as well as epigenetic changes and defects in chromosomal stability or DNA repair [58] (Fig. 5). Aberrant crypts, caused by loss of functional APC, typically show enhanced levels of nuclear β-catenin [59], an important indicator of target gene transactivation. Several studies have identified individual target genes in various cellular systems (reviewed in https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). Target genes in the intestinal crypt compartment include *c-MYC* [60], *EphB* [36], *Axin-2* [61], *LEF1* [62], *Lgr5* [1], *MET* [44] *and CD44* [63], and many targets are involved in controlling epithelial proliferation and/or cell fate decisions. CD44 is of particular interest because of its marked expression in ISCs (chapter 2 and 3) [17,64]. Strikingly, CD44 is one of

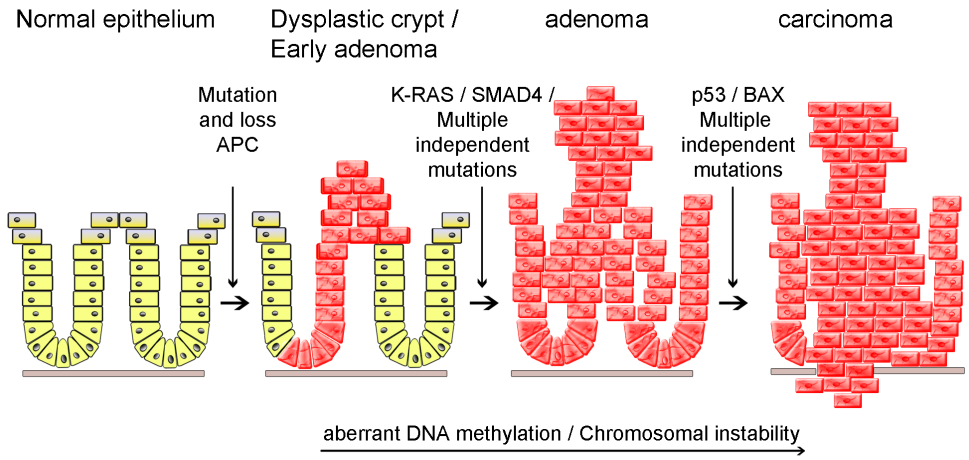

Figure 5. Development of intestinal cancer. Intestinal cancer develops in a stepwise matter and each stage of progression is associated with key genetic changes. The first oncogenic hit typically targets the APC tumor suppressor gene, leading to hyper-proliferation and the formation of dysplastic crypts in the intestinal mucosa. Activating K-RAS mutations are associated with adenoma outgrowth and progression towards carcinoma. The level of chromosomal instability increases with the following steps, which include reduced expression of SMAD4, inactivating mutations in the tumor suppressor gene encoding p53, or in the gene encoding the apoptosis inducer BAX (p53 independent). In this way, successive molecular changes promote the transformation of normal epithelial cells to invasive cancer.

the most prominent Wnt-responsive genes in the intestinal mucosa [15,17,65]. In accordance, CD44 overexpression in intestinal epithelial cells is repressed by dominant negative TCF4, confirming a direct regulation by Wnt/β-catenin signaling [15]. Furthermore, multipotent cells derived from colon tumors endowed with the ability to self-renew and propagate tumor growth are marked by high levels of CD44, indicating a key role in the regulation of long-lived tumorigenic intestinal epithelial cells [38,66]. As stated above, Wnt target gene products may represent attractive pharmacological targets for intestinal cancer therapy. However, the functional contribution of individual Wnt targets, such as the cell-surface adhesion molecule CD44, remains poorly defined. Therefore, this thesis focuses on CD44 in the intestinal mucosa and its contribution to intestinal tumor formation and progression.

A brief history of CD44

The CD44 cell-surface glycoprotein was first identified in the early 80's of the previous century as a p80 glycoprotein expressed on human [67] and murine leukocytes [68]. Various names were used by different research groups, including Pgp-1/Ly-24 [69], In(Lu)-related protein p80 [70], Hermes antigen [71], ECMRIII [72], hyaluronate receptor [73] and HUTCH-1 [74]. Finally it was recognized that these were identical or closely related members of one protein family [75–78]. The designation CD44 (Cluster of Differentiation 44) was ascertained by the Third International Workshop on Human Leukocyte Differentiation [79]. Besides being broadly expressed on leukocytes, it was quickly recognized that CD44 antigen is ubiquitously present on various cell types, including epithelial cells [69,73,76]. Biochemical and biological characterization revealed that CD44 comprises a family of extensively N- and O-glycosylated single-pass transmembrane proteins involved in cell-cell and cell-matrix adhesion [75,80,81]. CD44 is capable of binding several components of the extracellular matrix (ECM), and is currently recognized as the principal cell surface receptor for hyaluronan (also called hyaluronate, hyaluronic acid, or HA) [82,83]. In addition, CD44 was found to be modified by glycosaminoglycan (GAG) addition, making it a "facultative" cell-surface proteoglycan [84–87]. All members of the CD44 family were found to be encoded by a single conserved gene located on chromosome 11p13 in human [88] and chromosome 2 in mouse [89], spanning approximately 50 kb of genomic DNA. Molecular cloning and sequencing of *CD44* cDNAs revealed extensive alternative pre-mRNA splicing, as will be discussed below [90–95]. To date, hundreds of publications involving CD44 structure and function have been published, establishing CD44 as a highly polymorphic molecule involved in many biological processes. CD44 isoforms were found to be prominently expressed on different types of solid tumors, including gastrointestinal cancer [96–102]. Furthermore, CD44 has drawn attention as a marker for highly tumorigenic cancer stem cells (CSC) in breast [103], pancreatic [104], prostate [105] and colorectal cancer [66]. In the next paragraphs, the currently available data on CD44 form and function will be discussed, in particular focusing on its role in intestinal cancer.

CD44 transcripts and proteins

CD44 is remarkable for its ability to generate multiple alternatively spliced forms. The CD44 gene locus contains 20 exons (Fig. 6A) of which the first 5 exons are constant. These nonvariant exons encode the extracellular part of CD44, which contains an N-terminal signal sequence (exon 1), a Link-homology domain containing ligand-binding motifs (exons 2 and 3, residues 32–123), and part of the extracellular stem structure (exons 4 and 5). The next 10 exons are subjected to alternative splicing (from exon 6 to 15, also called variant exons v1–v10). This allows for the generation of mRNA transcripts encoding CD44 isoforms with a variable extracellular membrane-proximal stem domain (CD44v). Note that exon v1 is not expressed in humans [93]. Exon 16 and 17 are again nonvariant and encode the constant part of the stem domain. A single-pass 21 amino acid (aa) transmembrane domain is encoded by exon 18 and 72 aa cytoplasmic tail is encoded by exon 20. Inclusion of exon 19 gives rise to a protein bearing a short 5 aa cytoplasmic tail. However, nearly all cDNAs isolated lack this sequence [75].

The shortest transcribed CD44 isoform, lacking all variable exons, is called CD44 standard (CD44s) (Fig. 6B) and is the most common form expressed by hematopoietic cells [106]. Inclusion of all variant domains occurs downstream of aa 222 and adds up to 381 aa's to the 361 aa core of human CD44 and 423 aa's to the 363 aa core of mouse CD44. Theoretically, hundreds of different CD44 transcripts can be generated by differential utilization of the variant exons. However, only a limited number of splice products have been identified to date. PCR amplification of cDNA of normal epithelial cells has revealed expression of various CD44v isoforms. For example: CD44v8-10 was found to be commonly expressed by different types of epithelium [106–108] and was therefore initially referred to as epithelial CD44. Other examples of variants expressed by epithelial cells are CD44v4-10, CD44v4-7, CD44v6-7 and CD44v6-10 [91,109,110]. In general, expression of CD44v is a characteristic feature of proliferating epithelial cells [110], suggesting a link between CD44 and mitotic signaling mechanisms. In addition, it was recognized that expression of CD44v can be dramatically increased by neoplastic transformation [90], as will be discussed in more detail below. Many studies describe CD44v protein expression by making use of immunohistochemical (IHC) analysis using monoclonal antibodies (mAbs) directed against epitopes in the variant-specific regions of CD44. However, this approach does not provide information about the full length amino acid sequence. Likewise, antibodies specific for the CD44 constant region do not distinguish between CD44s and CD44v. A combined approach using immunohistochemistry and RT-PCR amplification can provide insight into the expression pattern of CD44 variants.

Post-translational modifications further add to the structural diversity of CD44 isoforms. Extensive N-linked and O-linked glycosylation, as well as modification by glycosaminoglycan chains doubles the molecular mass of the CD44s core protein from

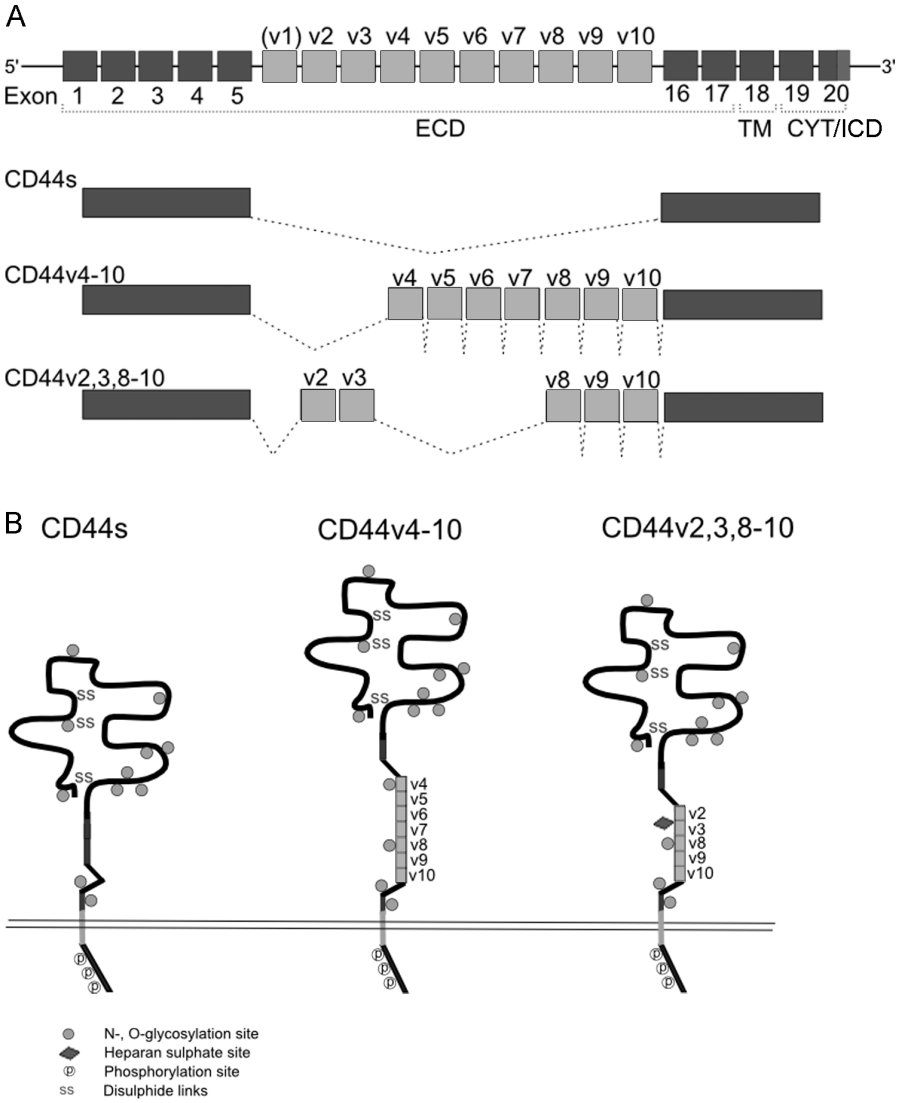

Figure 6. CD44 transcripts and proteins. A) *CD44* pre-mRNA is encoded by 20 exons. The 10 variant exons, denoted by v, are subject to extensive alternative splicing. In contrast to the v1 exon of the mouse, the human v1 exon contains a stop codon. The many splicing possibilities of the variable exons of CD44 could in theory give rise to a vast number of CD44 variants. Expressed CD44 isoforms include CD44 standard (CD44s), CD44v4-10, and CD44v2,3,8-10. **B**) Three examples of CD44 proteins. Alternative splicing affects the extracellular, membrane-proximal stem structure of CD44 proteins. CD44 can undergo post-translational modifications, comprising the attachment of carbohydrates to N- and O-linked glycosylation sites of the extracellular domain (circles), and of glycosaminoglycans (GAGs), such as heparan sulfate, on exon v3. The figure further indicates disulfide bonds (S-S), and intracellular phosphorylation sites (P). The N-terminal distal extracellular domain encoded by the first 5 exons contains the hyaluronan -binding site, while the C-terminal domain is capable of interacting with several intracellular components. The CD44 variants containing the exons v6 and v7 bind osteopontin.

37 kDa to 80-95 kDa. The molecular mass of the different CD44v isoforms ranges from 80 kDa to 250 kDa. Most N-linked glycosylation sites (Asn-residues) are found in the globular extracellular domain, while O-linked glycosylation sites (Ser/Thr-residues) are mostly found in the stem domain of CD44, including the variable domains [72,84,92]. Modification by chondroitin sulfate GAG, which occurs at serines that are followed by glycines (SG-region), is also confined to the stem structure of CD44 [111]. Post-translational modifications can differ between cell-type, differentiation stage or cellular activation state and changes in glycosylation modulate ligand-binding affinity and/or specificity [112–116]. Notably, the SGSG sequence encoded by exon v3 can be modified by heparan-sulphate (HS), which converts CD44 into a heparan-sulfate proteoglycan [84,87,117]. These isoforms can bind growth factors via their HS side chain and target these to nearby high affinity signal transducing receptors [117–121].

The transmembrane and intracellular cytoplasmic domains of CD44 are highly conserved between mammalian species, with 80-90% inter-species homology [122], indicating that these are crucial for the functioning of CD44. Two Cys residues in the transmembrane domain can be acylated (palmitoylation) [123,124], which is associated with increased hyaluronan endocytosis and CD44 turnover from cell surface [125]. The cytoplasmic tail of CD44 contains five inter-species conserved potential phosphorylation sites; Ser291, Ser316, Ser323, Ser325 and Ser337 in human. Direct phosphorylation of Ser323 and Ser325 is in part mediated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [126,127], while activation of protein kinase C (PKC) indirectly results in dephosphorylation at ser325 and phosphorylation of other serine residues such as ser291 and ser316 [128]. Overall changes in phosphorylation motifs are associated with ligand binding properties as well as with reorganization of the cytoskeleton and CD44-mediated signal transduction. Given the ubiquitous expression of CD44 and its ligands, it is likely that regulation of CD44 activation is necessary in order to prevent unnecessary adhesion or signal transduction. The cytoplasmic domain of CD44 directly associates with ezrin, radixin and moesin (ERM) proteins, which link CD44 to the actin cytoskeleton [129–132]. These ERM proteins provide a scaffold enabling CD44 to interact with other transmembrane proteins and signaling molecules, including PI3K and the Src kinase family members Lck, Lyn and Fyn [133–137]. For example, the association between CD44 and ezrin is regulated by phosphorylation and this interaction controls directional cell motility, which plays a role in processes such as tissue development, wound repair, and cancer metastasis [138]. Taken together, CD44 can be considered structurally as one of the most variable cell surface molecules and this remarkable versatility forms the basis of the pleiotropic effects elicited by CD44 in various physiological and pathological processes. This is further illustrated by the fact that expression of CD44v is profoundly associated with malignancy, including the neoplastic transformation of intestinal epithelium.

CD44 isoform expression in intestinal cancer

CD44 is prominently expressed on proliferating cells within the lower crypt compartment of the intestinal epithelium [63,73,139], and as shown in chapter 2 and 3, on intestinal stem cells. It is noteworthy that CD44 isoform expression in normal crypts is not limited to ISCs since transit amplifying cells also express CD44. However, CD44 variant isoform expression is most prominent on ISCs (chapter 2 and 3), indicating involvement in ISC biology and associated pathogenic pathways. Initial interest regarding a role for CD44v in solid tumors originates from the observation that CD44 v6-containing isoforms conferred metastatic potential to a non-metastasizing rat pancreatic carcinoma cell line [91]. Soon after it became apparent that CD44v is highly expressed in adenomas and adenomatous polyps of the colon [99,140,141]. In addition, strikingly increased CD44v expression is already observed during the earliest stages of transformation, diagnosed as dysplastic aberrant crypt foci (Fig. 7A), implicating CD44 variant isoforms in the initial stages of the pathogenesis of colorectal cancer [99,141–143]. In accordance, it was established that the *CD44* gene is a direct Wnt/β-catenin target in the intestinal mucosa [63]. The constitutive activation of β-catenin/TCF-mediated transcription in adenomatous lesions in the mucosa of FAP patients and tumor prone $Apc^{Min/+}$ mice results in a strongly enhanced expression of CD44. By contrast, disruption of β-catenin/TCF-mediated transcription in the intestinal crypt compartment in *Tcf4* knockout mice leads to a complete loss of CD44 expression [63]. Furthermore, regulation of CD44 expression by β-catenin/TCF-mediated transcription is evident in human colorectal adenocarcinoma cells [15], and specific TCF4-binding sites located in and around the CD44 gene have been identified [144]. Unlike many other important Wnt target genes, including *EphB* [36], *LEF1* [62], and *AXIN2* [61], the regulation of CD44 in intestinal epithelial cells is independent of the transcription factor c-MYC [65].

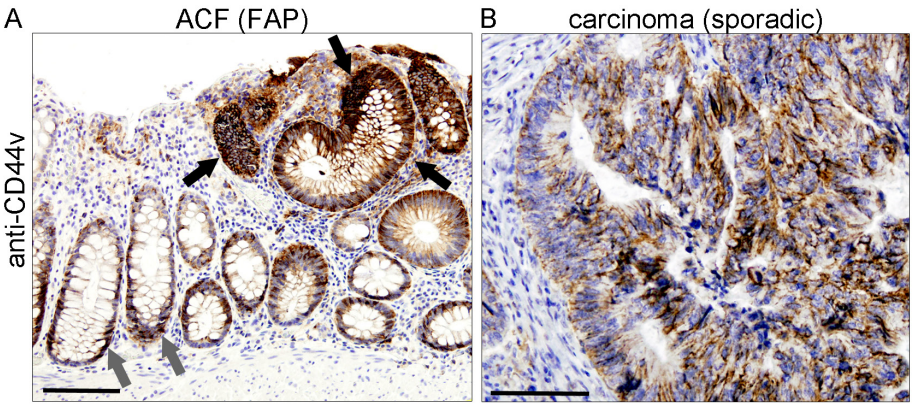

Figure 7. CD44v expression in aberrant crypt foci and in colon cancer. **A**) Immunohistochemical staining for CD44v6 in human colon tissue from a patient with familial adenomatous polyposis (FAP). Grey arrows indicate CD44v-positive cells in the base of non-neoplastic crypts. Black arrows indicate overexpression of CD44v in a microadenoma, the earliest stage of intestinal tumor formation. **B**) Aberrant CD44v expression in colorectal cancer.

Aberrant CD44v expression is frequently observed in advanced stages of colorectal cancer (Fig. 7B) and several studies have identified CD44v in primary tumor tissues as an independent prognostic factor associated with poor survival in patients [102,145–151]. In addition, CD44v expression is associated with tumor stage (metastatic dissemination) [99,143146–148,152,153]. Alternative *CD44* pre-mRNA splicing in epithelial cells is part of a larger alternative splicing program regulated by Epithelial Splicing Regulatory Proteins 1 and 2 (ESRP1 and ESRP2) [154–156]. It is important to point out that *CD44* v-exon inclusion is associated with activation of RAS/MAPK signaling [157,158], a major pathway frequently dysregulated in colorectal cancer [159]. It is now evident that within a tumor CD44 isoforms mark a subpopulation of cells selectively endowed with tumor-initiating capacity [66]. These so-called colorectal cancer stem cells (CoCSC) are considered to drive tumor development and are capable of generating a xenograft tumor with properties of the primary tumor. Interestingly, ISCs and CoCSCs seem to be profoundly related to each other. The latter cells contain an ISC-specific gene expression signature, which can be used to predict disease relapse in colorectal cancer patients [38,160], showing that CoCSCs require ISC-specific functions to regenerate a tumor. In human intestinal tumors, CD44 expression is associated with an ISC gene expression signature [38]. Typically, these ISC-like tumor cells reside at sides where there is a close interaction with cells of the surrounding stroma, including myofibroblasts [161]. This suggests that stemness can be dictated by the microenvironment during tumor development. CD44 v6-containing isoforms are thought to play a pivotal role in the crosstalk between CoCSCs and the tumor microenvironment [83,162,163]. Thus, dysregulated CD44 expression in intestinal cancer is closely associated with stemness, niche interactions, tumor progression, dissemination and disease recurrence. Therefore, there is a need to identify the distinct isoforms and the underlying signaling mechanisms involved, as these may represent attractive targets for therapeutic intervention.

Extracellular functions of CD44

CD44 is an important receptor that mediates cell-cell adhesion and adhesion to extracellular matrix (ECM) components, with the major ligand being HA [82]. HA is a non-sulfated and relatively simple non-branching glycosaminoglycan that is distributed ubiquitously in the extracellular space and, in particular, is enriched in the pericellular matrix of proliferating and migrating cells [164]. Cell-cell adhesion mediated by CD44 is achieved via multivalent HA binding on adjacent cells or via inter-CD44 binding via attached glycosylation moieties [165]. In addition, certain glycosylated CD44 isoforms mediate cell-cell adhesion through binding to selectin family members on the surface of adjacent cells [166]. The ability of CD44 to bind HA is tightly regulated and depends on post-translational modifications such as phosphorylation, sulfation and glycosylation [82,112,113,127,167–171]. Binding of HA can induce intracellular signaling events thereby affecting cell migration, differentiation, survival and proliferation [82,172–174]. The CD44 intracellular domain is also required for HA binding,

indicating a reversible regulatory mechanism [175,176]. As a receptor for HA, CD44 is involved in its uptake and degradation, which plays a role in cell migration and tissue remodeling [177,178]. In addition, CD44-mediated HA binding can also modulate GAG-dependent growth factor signaling [104,179–181]. It appears that the ability of CD44 to bind HA is reduced by the inclusion of variant exons [113,182]. However, it should be noted that these responses can be cell type specific and thus should not be generalized per se. Other extracellular ligands that are capable of interacting with CD44 are collagen [72,85], chondroitin [82,183], fibronectin and laminin [86], although at present there is insufficient evidence for a significant physiological role of these interactions on intestinal epithelial cells. However, an ECM protein ligand of CD44 of potential interest is osteopontin [184]. This extracellular component is produced by fibroblasts and immune cells and has been implicated in tumorigenesis and intestinal tumor cell proliferation *in situ* [161,162]. Osteopontin specifically binds to CD44 variants containing exon v6 or v7, but not to CD44s [185]. However, the molecular mechanisms underlying these observations need to be further explored.

The function of CD44 on hematopoietic cells has been studied extensively, showing crucial functions in lymphocyte activation, lymph node homing, myelopoiesis and lymphopoiesis [186–189]. These findings have demonstrated that many types of normal hematopoietic cells, including erythrocytes, and T and B lymphocytes, preferentially express CD44s. Expression of CD44v was found on activated lymphocytes and on hematopoietic neoplasms such as lymphomas, indicating their involvement in these processes [190–194]. For an overview on CD44 in hematological neoplasia, see Hertweck *et al*. [195]. The adhesive and migratory functions of CD44 isoforms on hematopoietic cells are thought to be similar to those used by epithelial tumor cells to facilitate motility, tissue invasion and metastasis.

CD44 may also interact with several other extracellular molecules such as growth factors and matrix metalloproteinases (MMPs). For example, CD44 provides a docking site for MMP9, which is involved in the degradation of ECM components [196,197]. In addition, CD44 bound MMP9 can activate latent transforming growth factor-β (TGF-β) on the cell surface [198]. Likewise, interactions with other proteases, such as MMP7 [121] and MMP14 [199] have been described, suggesting an important function of CD44 in the extracellular environment. ADAM metalloproteinases (a disintegrin and metalloproteinase (ADAM)) play a central role in the cleavage of CD44 itself [200,201], resulting in a soluble extracellular protein capable of antagonizing membrane bound CD44 [202]. Growth factor binding is mainly mediated by CD44 v3-containing isoforms which, through their HS side chains, can present these growth factors to their high affinity receptors. A few examples of heparin binding growth factors known to interact with CD44 v3-containing isoforms are CCL4/MIP-Iβ [118], heparin-binding EGF-like growth factor (HB-EGF), basic fibroblast growth factor [117], b-FGF2, vascular endothelial growth factor (VEGF) [203], and hepatocyte growth factor (HGF) [120]. Importantly,

structural modifications of HS side chains determine their specificity for a given heparin-binding growth factor, creating a mechanism for cell or tissue selective growth factor binding [reviewed by Kjellén and Lindahl [204]]. Taken together, these observations show that CD44 acts as a multifunctional ligand-binding receptor by interacting with ECM and other extracellular components.

Signal transduction via CD44

CD44 itself lacks catalytic activity but it is closely involved in the formation of signaling complexes composed of multiple proteins, mediating the transduction of signals from the cell surface to cytoplasmic and nuclear effectors. It is now widely appreciated that several families of adhesion molecules participate in various signaling functions [reviewed by Cavallaro and Dejana [205], and CD44 in particular is an excellent example of this function. The intracellular domain (ICD) of CD44 plays a central role in transducing extracellular stimuli to different signaling cascades. First of all, CD44 may undergo proteolytic cleavage resulting in the release of CD44-ICD, which subsequently translocates to the nucleus were it regulates gene transcription [206]. One of the CD44-ICD interactors is the transcription factor CREB thereby increasing expression of the proliferation regulatory protein Cyclin D1 in thyroid cancer cells [207]. Further research is needed to address the cell biological relevance of this signaling mechanism in the intestinal epithelium.

Furthermore, as mentioned previously, the cytoplasmic domain of CD44 contains binding sites for the adaptors ankyrin and ERM proteins, which operate as molecular linkers between CD44 and the actin cytoskeleton. These interactions are regulated by phosphorylation of the CD44-ICD [208]. Ankyrin is a membrane-associated protein, linking CD44 to the cytoskeletal component spectrin. HA-induced interaction between CD44 and ankyrin has been associated with inositol 1,4,5-trisphosphate (IP3) receptor-dependent Ca^{2+} mobilization, which in turn results in the activation of calmodulin-dependent signal transduction pathways involved in diverse cellular reactions [127,201,209,210]. CD44-ERM interactions facilitate the assembly of intracellular signaling complexes, including Src, the Src-like protein tyrosine kinases Lck, Lyn and Fyn [133,134], Rho GTPase family members (e.g., RhoA, Rac1 and Cdc42), Rho-kinase/ROK/ROCK (Rho-Associated Coiled-Coil-Containing Protein Kinase) and Rho GDP-dissociation inhibitor (GDI) [200,210–212], T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1) [213], guanine nucleotide exchange factor VAV2 [214], GRB2-associated-binding protein 1 (Gab1) [215], GTPase activating protein 1 (IQGAP1) [216], and PKC [138]. Some of them are constitutively bound to CD44-ICD [217]. These interactions demonstrate that CD44 is closely involved in the transduction of signals from the ECM to intracellular effectors and support a model in which the selective interaction between certain CD44 isoforms and specialized transmembrane receptor proteins modulates downstream signaling events.

CD44 isoforms function as co-receptor for receptor tyrosine kinases

Over the past decade, it has been established that CD44 isoforms are able to act in conjunction with other transmembrane signaling proteins. Perhaps even the most important function of CD44 isoforms on epithelial cells is their ability to collaborate with different types of receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR)/ ErbB family members [121,179,218–220] and the HGF receptor MET [120,221]. The ErbB family of RTKs consists of four members: EGFR/ErbB1, ErbB2/Neu, ErbB3, and ErbB4, which are activated by members of the EGF family of peptide growth factors [reviewed by Arteaga and Engelman [222]]. Ligand binding induces receptor homo- and heterodimerization, activation of intrinsic tyrosine kinase activity, and subsequent trans-phosphorylation of the partnered receptor, resulting in downstream signaling. ErbB3 lacks intrinsic kinase activity but contains multiple docking sites for PI3K while ErbB2 lacks ligand binding capacity but functions as binding partner for other ErbB family members. Immunoprecipitation experiments have shown that CD44 is closely associated with all four ErbB family members [121,179,218–220,223]. HA-bound CD44, in particular, triggers ligand-induced ErbB receptor signaling [223–226]. The association of CD44 with cytoskeletal complexes is regulated by dynamic interactions with the ERM adaptor proteins and, as stated above, this complex formation activates a phosphorylation cascade of protein kinases (Fig. 8A). Downstream signaling cascades include the PI3K/AKT pathway and the MAPK pathway that coordinately regulate cell proliferation, differentiation, motility, and survival. To date, a systematic analysis of the specific CD44 isoforms responsible for mediating ErbB signaling and their precise downstream targets has not been carried out. Whereas a direct association between CD44s and ErbB members has been described [219], it could be that CD44 v3-containing isoforms, via their HS side chain, participate as co-receptor in growth factor signaling.

Of interest, also CD44 v6-containining isoforms are implicated in activation of RTK downstream signaling pathways, as their expression is closely associated with MAPK pathway activation and increased cell proliferation [91,158,227]. For example, CD44 v6-containing isoforms promote receptor activation by vascular endothelial growth factor (VEGF) on endothelial cells [228], and activation of the HGF receptor MET on colon cancer cell lines [221]. The latter being of interest as HGF is a major cytokine produced in the tumor microenvironment [47,229]. Indeed, more recent findings implicate a functional involvement of CD44v6 and MET on CoCSCs promoting metastatic growth and increased Wnt signaling activity [162]. This interplay is of great functional relevance as both Wnt signaling and RTK activation are required for both normal ISC homeostasis and maintenance of a tumorigenic CSC phenotype in tumors. We therefore set out to investigate the role of CD44-mediated RTK signaling on crypt homeostasis and intestinal tumorigenesis in (chapter 4).

The MET RTK and its unique ligand HGF play a pleiotropic role in many biological processes, including cell proliferation, apoptosis, differentiation, and migration [reviewed by Mahtouk

et al. [48]. MET is a heterodimer resulting from the cleavage of a 170-kDa-precursor protein into a 50-kDa α-chain and a 140-kDa β-chain linked by disulfide bonds [230]. Its intracellular domain harbors four key tyrosine residues that either regulate its enzymatic activity or form docking sites for several signal transducers and adaptor proteins. For instance, the Gab1 adaptor protein forms a platform for the assembly of a multiprotein signaling complex [231]. HGF binding to MET induces receptor dimerization, tyrosine transphosphorylation and activation of downstream signaling. The co-operation between CD44, MET and HGF is blocked by administering mAbs directed against the v6-domain of CD44, resulting in impaired downstream signaling [221]. The role of CD44 v6-containing isoforms in MET signaling seems to be two-fold (Fig. 8B). First, the extracellular domain of CD44v interacts with the extracellular domain of MET, leading to the formation of a ternary complex with HGF and

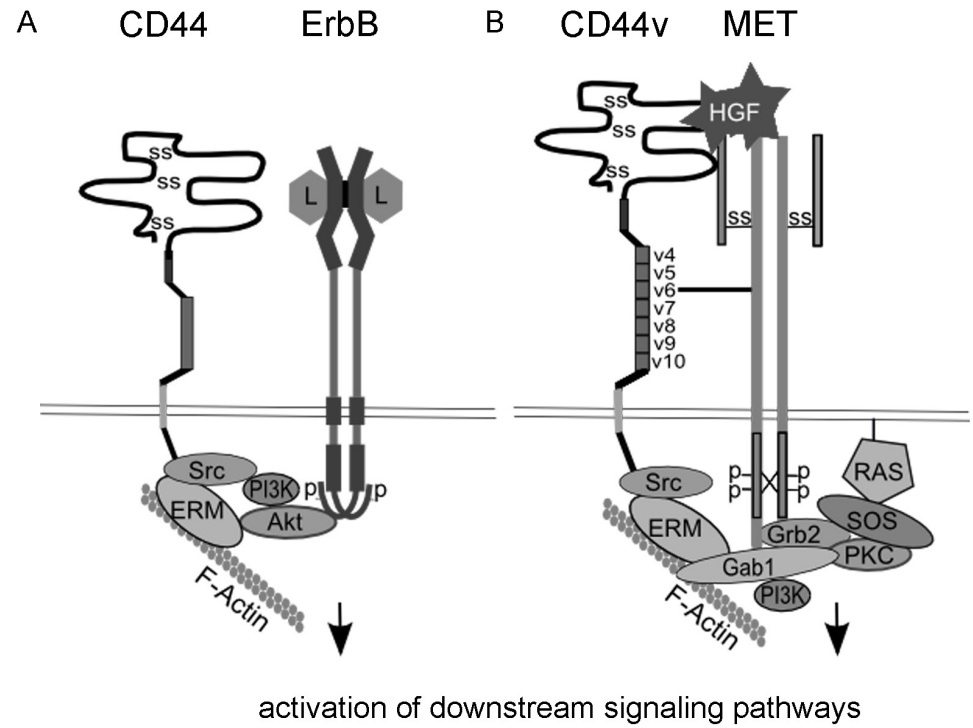

Figure 8. CD44 promotes signaling by acting as a co-receptor for receptor tyrosine kinases (RTKs.) A) CD44-mediated activation of ErbB family signaling. Ligand (L) binding causes RTK receptor dimerization and subsequent autophosphorylation (p) of intracellular tyrosine residues. CD44 complexes with SRC kinases and ERM proteins, and with the cytoskeleton via the ERM actin-binding domains. These interactions augment activation of PI3K and AKT and subsequent downstream signaling. **B**) CD44v6-containing isoforms play a dual role in HGF/MET-mediated signaling. The extracellular domain of CD44v6 facilitates HGF-induced MET activation. In addition, the cytoplasmic domain of CD44v6, via interactions with the cytoskeleton and adaptor protein Gab1 allows for the formation of a signaling complex that organizes the activation of the GTPase RAS by SOS. Downstream signaling involves activation of the MAPK signaling cascade

subsequent MET activation [221,232,233]. Second, the ICD of CD44, through its interaction with ERM proteins, the cytoskeleton and adaptor proteins such as Gab1, allows for the activation of RAS and/or PI3K and subsequent downstream signaling. This intimate relation between a cell adhesion molecule and a RTK renders CD44 a regulator of the translation of extracellular cues into intracellular signaling events.

CD44 and apoptosis in intestinal cancer

A tight regulation of programmed cell death, or apoptosis, is vital for the maintenance of normal crypt homeostasis [reviewed by Booth and Potten [234]]. Resistance to apoptosis represents one of the hallmarks of cancer initiation and progression [235]. Cell death occurs through diverse mechanisms and apoptotic cells can be distinguished morphologically by the condensation of nuclear chromatin and biochemically by the activation of cysteine proteases (caspases). The BCL-2 protein family and associated kinase-signaling pathways play a pivotal role in regulation of the balance between cell death and survival. This family consist of anti-apoptotic factors, such as BCL-2 and BCL-X_L, and pro-apoptotic effectors such as BAX, BAK and BOK [Reviewed by Adams and Cory [236]]. Strikingly, epithelial cells in the stem cell compartment express relatively high levels of pro- and anti-apoptotic proteins when compared to differentiated cells higher up the crypt [237,238] (Chapter 5). This suggest a tight control of apoptosis in the ISC compartment. Disruption of apoptosis is considered an early event in intestinal tumorigenesis [234]. Notably, enhanced Wnt signaling can predispose intestinal epithelial cells to undergo apoptosis [16,65,239]. Ultimately, it is the balance between all pro- and anti-apoptotic signals that determines whether newly transformed cells live or die. This balance is regulated at several levels and a number of signaling pathways act in an integrated way to promote or inhibit apoptosis in response to various cues [240]. For example, activation of transcription factor NF-κB confers resistance to apoptosis in the intestinal epithelium of the mouse via induction of the anti-apoptotic *Bcl-2* gene [238]. In this study, the simultaneous inactivation of *Bcl-2* and *Apc* impaired adenoma formation in vivo. These results demonstrate that APC-mutant cells in the intestinal epithelium are dependent on anti-apoptotic BCL-2 family members for their survival and expansion.

The p53 tumor-suppressor protein is a critical damage sensor in the apoptosis-inducing circuitry. Mutation of the *p53* gene is a common feature of CRC and occurs in about 40%-60% of the cases, although *p53* mutations are identified as late events in adenoma-carcinoma progression [241]. The p53 protein is a transcriptional regulator of many target genes that control cell-cycle progression or cell death. Transcriptional targets include the cell cycle inhibitor *CDKN1A (p21)*, and the apoptosis effector BAX [reviewed by Fulda and Pervais [240]]. In mammary epithelial cells, CD44 expression was found to be directly repressed by binding of p53 to its promoter region [242]. In addition, CD44-mediated signaling in these cells resulted in resistance to p53-induced apoptosis in response to cytotoxic stress. This study demonstrates that CD44 can promote resistance to apoptosis and shows opposing effects of

CD44 and p53 on epithelial cell survival.

Indeed, CD44, and especially CD44v, has been implicated in the suppression of apoptosis in several systems and through diverse mechanisms. As discussed earlier, CD44v-mediated signaling is associated with RTK-PI3K-AKT pathway activation, which promotes survival signaling [137,162,208,220,243]. Overactivation of PI3K-AKT signaling is a frequent event in CRC, either by RTK activation, PI3K mutation or amplification, AKT mutation, or by loss of function of the negative regulator PTEN [reviewed by Danielsen *et al* [244]]. Activation of PI3K-AKT signaling enhances cell survival by phosphorylation and inactivation of several target proteins, such as the apoptosis initiator protein BAD [245], and caspase-9 [246]. However, there are well over a hundred AKT substrates reported in literature, implicating a diverse range of action. Interactions between CD44 and HA have been shown to be important for signal transduction as well. Interfering with CD44-HA interaction by using HA oligosaccharides disrupted PI3K-AKT signaling and induced apoptosis in human CRC cells *in vitro* [247]. Overall, the underlying mechanisms by which CD44-mediated PI3K-AKT activation can elevate apoptosis in the intestinal epithelium are far from clear.

Besides the PI3K/AKT signaling pathway, CD44 signaling can confer apoptosis resistance via other mechanisms. For example, CD44-RTK interactions can promote the induction of cyclooxygenase 2 followed by the activation of prostaglandin E2 signaling and increased intestinal epithelial cell survival [248]. CD44 also contributes to cell survival via regulating the death receptor FAS. HA binding to CD44 reduced FAS expression and FAS-mediated apoptosis [249–251]. Notably, knock-down of CD44 in human CRC cells increased their sensitivity to apoptosis which was accompanied by increased levels of pro-apoptotic BAX and decreased levels of the pro-survival guardian proteins BCL-2 and BCL-X_L [252]. Taken together, CD44 seems to be able to promote the apoptotic response of epithelial cells at multiple levels. The exploration of these mechanisms may yield novel treatment approaches for CRC. In addition, therapeutic strategies that modulate the balance between survival and cell death in the intestinal crypt may postpone disease development, and thus benefit patients that are at high risk for developing tumors.

AIMS AND OUTLINE OF THIS THESIS

This thesis investigates the contribution of CD44, in particularly CD44v, to intestinal tumorigenesis. Individual pathways involved in the control of intestinal epithelial proliferation and survival are explored, such as the Wnt pathway, apoptotic signaling, including p53, and signaling through RTKs. **Chapter 1** introduces CD44 and the vast amount of indirect evidence for its involvement in the pathobiology of colorectal cancer. These observations underlie the studies presented in the thesis. **Chapter 2** demonstrates an important role for CD44 in intestinal tumorigenesis. We demonstrate that CD44 is prominently expressed in crypt-base columnar cells, which are the stem cells of the intestine. *Apc*$^{Min/+}$ mice lacking CD44 were generated and these show a strong reduction in the number of intestinal adenomas, compared to their wild-type counterparts. This reduction is primarily caused by a decrease in the formation of aberrant crypts, implying the involvement of CD44 in tumor initiation. **Chapter 3** shows that intestinal stem cells (ISCs) display a specific set of CD44 variant isoforms, but remarkably lack the CD44 standard isoform. By employing knock-in mice expressing either CD44v4-10, being the largest isoform detected in ISCs, or as control CD44s, we identify CD44v as a component of the ISC program critical for intestinal tumorigenesis. **Chapter 4** demonstrates that CD44v cooperates with the hepatocyte growth factor (HGF)-MET receptor tyrosine kinase on intestinal epithelial cells. HGF/MET signaling can substitute for EGFR signals in intestinal organoid cultures and enhance the growth of adenoma spheroid cultures. These activities of MET are promoted by the ISC-specific CD44 isoform CD44v4-10. MET signaling is dispensable for normal intestinal homeostasis *in vivo*. However, MET is involved in intestinal epithelial regeneration after damage. In addition, MET deletion attenuates adenoma formation *in vivo*, which is accompanied by increased numbers of apoptotic cells in *Met*-deficient microadenomas. Together, these data suggest a scenario in which CD44v, through selective interaction with MET, controls signaling that regulates ISC fate and tumor growth. **Chapter 5** explores the relation between apoptosis sensitivity and Wnt signaling in the crypt compartment. This study shows that intestinal adenoma formation, caused by loss of functional APC, is accompanied by strongly elevated levels of apoptosis. Expression profiling demonstrates the involvement of β-catenin/TCF-4-mediated signaling in the regulation of *BOK* and *BAX*, encoding for two pro-apoptotic Bcl-2 family proteins. **Chapter 6** shows that expression of CD44 in CRC is independent of p53 status. Although the CD44 promoter has been identified as a target of p53-mediated transcriptional repression in breast epithelial cells, this chapter reveals that p53 does not repress CD44 in colon cancer cells and in normal intestinal epithelium. **Chapter 7** summarizes and discusses the results presented in this thesis.

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Chapter 2

Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis

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Deletion of the WNT Target and Cancer Stem Cell Marker CD44 in Apc(Min/+) Mice Attenuates Intestinal Tumorigenesis

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Abstract

Mutation of the genes encoding the WNT signaling components adenomatous polyposis coli or β-catenin plays a critical role in the initiation of colorectal cancer. These mutations cause constitutively active β-catenin/TCF–mediated transcription, driving the transformation of intestinal crypts to colorectal cancer precursor lesions, called dysplastic aberrant crypt foci. CD44 is a prominent WNT signaling target in the intestine and is selectively expressed on the renewing epithelial cells lining the crypts. The expression of CD44 is dramatically increased in aberrant crypt foci in both humans and tumor-susceptible *Apc*$^{Min/+}$ mice, suggesting a role for CD44 in intestinal tumorigenesis. To study this role, we crossed C57BL/6J-*Cd44*$^{-/-}$ mice with C57BL/6J-*Apc*$^{Min/+}$ mice. Compared with C57BL/6J-*Cd44*$^{+/+}$/*Apc*$^{Min/+}$ mice, C57BL/6J-*Cd44*$^{-/-}$/*Apc*$^{Min/+}$ mice showed an almost 50% reduction in the number of intestinal adenomas. This reduction was primarily caused by a decrease in the formation of aberrant crypts, implying the involvement of CD44 in tumor initiation. The absence of CD44 in the normal (nonneoplastic) crypts of *Cd44*$^{-/-}$/*Apc*$^{Min/+}$ mice did not alter the proliferative capacity and size of the intestinal stem cell and transit-amplifying compartments. However, compared with *Cd44*$^{+/+}$/*Apc*$^{Min/+}$ mice, *Cd44*$^{-/-}$/*Apc*$^{Min/+}$ showed an increase in the number of apoptotic epithelial cells at the base of the crypt which correlated with an increased expression of the proapoptotic genes *Bok* and *Dr6*. Our results show an important role for CD44 in intestinal tumorigenesis and suggest that CD44 does not affect proliferation but is involved in the control of the balance between survival and apoptosis in the intestinal crypt. [Cancer Res 2008;68(10):3655–61]

Introduction

The renewal of the intestinal epithelium requires a tight control of the proliferation, differentiation, and migration of epithelial cells along the crypt-villus axis. Crypt base columnar (CBC) cells, recently identified as intestinal stem cells residing at the base of the crypt, give rise to a transient population of rapidly dividing epithelial progenitor cells (1). These so-called "transit-amplifying" cells differentiate and migrate until they reach the tip of the villus where they are shed by exfoliation. In this way, the epithelial cell lining of the intestine is repopulated every 3 to 5 days. WNT signaling is one of the key pathways involved in maintaining crypt homeostasis. Mutation of the genes encoding the WNT signaling components adenomatous polyposis coli or β-catenin cause constitutively active β-catenin/TCF4-mediated transcription, leading to the transformation of normal crypts into the earliest colorectal cancer precursor lesions, called dysplastic aberrant crypt foci [reviewed by Radtke and Clevers (2)]. Thus far, the WNT signaling target genes mediating the neoplastic transformation of the intestinal crypts have only been partially identified.

A prominent target of WNT signaling in the intestinal mucosa is CD44 (3, 4). CD44 comprises a family of transmembrane glycoproteins generated from a single gene by alternative splicing and differential glycosylation (5, 6). Members of the CD44 family have been implicated in a number of important biological processes including lymphocyte homing, apoptosis, tumor progression, and metastasis (7–12). In these processes, CD44 functions as a cell adhesion and signaling receptor, linking extracellular matrix molecules, specifically hyaluronate, to the cell and cytoskeleton (10). Furthermore, CD44 isoforms decorated with heparan sulfate side chains can bind growth factors and modulate growth factor receptor–mediated signaling (13, 14). CD44 is selectively expressed on the renewing epithelial cells lining the intestinal crypts. Disruption of β-catenin/TCF–mediated transcription in the intestinal crypt compartment in *Tcf4* knockout mice leads to a complete loss of CD44 expression (3). By contrast, the constitutive activation of β-catenin/TCF–mediated transcription in human colorectal cancer and in the intestinal adenomas of *Apc*$^{Min/+}$ mice results in a strongly enhanced expression of CD44 (3). This CD44 overexpression can be repressed by dominant-negative TCF4, confirming regulation by β-catenin/TCF–mediated transcription (15). Unlike many other important WNT target genes, including *EphB* (16), *LEF1* (17), and *AXIN2* (18), the regulation of CD44 in the intestinal epithelium is independent of c-MYC (19).

The identification of CD44 as a major WNT signaling target, displaying strong expression in normal and neoplastic crypts as well as in advanced adenomas, implies that CD44 could be instrumental in intestinal tumorigenesis. Consistent with this hypothesis, CD44 has recently been identified as a marker for colorectal cancer stem cells (CSC; ref. 20), whereas its expression in invasive colorectal carcinomas is associated with an unfavorable prognosis (4, 21). To explore the role of CD44 in intestinal tumorigenesis, we studied the effect of CD44 deletion on intestinal adenoma formation in tumor-prone *Apc*$^{Min/+}$ mice.

Materials and Methods

Scoring and classification of adenomas. C57BL/6J (*Cd44*$^{+/+}$) mice and C57BL/6J-*Cd44*$^{-/-}$ (*Cd44*$^{-/-}$) mice (22) were crossed with C57BL/6J-Min/+ mice (*Apc*$^{Min/+}$; the Jackson Laboratory). The latter mice harbor a truncating mutation at codon 850 of the *Apc* gene (23). For macroscopic assessment of adenoma formation, mice were sacrificed at 16 weeks of age (n = 11 per

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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group) and the colon, as well as the small intestine, were isolated. The small intestine was subdivided into three equal segments of ~12-cm-denominated duodenum, jejunum, and ileum. Intestinal segments were flushed with PBS, opened longitudinally, and examined using an operation binocular. Adenomas were counted, classified, and measured according to standard criteria (24). All results were statistically analyzed using Student's *t* test. Aberrant crypt formation was assessed in sections from 8- and 16-week-old mice (n = 4 per group). Intestinal segments were fixed in 4% formalin and embedded in paraffin. H&E-stained tissue sections were analyzed by two experienced independent observers. Neoplastic lesions were categorized into three subclasses; aberrant crypts (<0.25 mm), small adenomas (0.25–1 mm), and large adenomas (>1 mm).

Microarray analysis. $Cd44^{+/+}$ and $Cd44^{-/-}$ mice were sacrificed at 8 weeks. Duodenal sections of 4 mm were taken 3 to 5 cm distal from the pylorus. Any blood vessels or pancreas tissue were carefully removed and tissue was snap-frozen in liquid nitrogen until further processing. Total RNA was isolated using Trizol extraction (Invitrogen), further purified using isopropanol precipitation followed by DNase treatment, and concentrated using the RNeasy MinElute Cleanup Kit (Qiagen). The quantity of total RNA was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and the RNA quality was examined using the Agilent 2100 bioanalyzer (Agilent). Only high-quality total RNA was selected for further processing (ServiceXS). Briefly, Cy3-labeled (green) cDNA was synthesized from each sample using the Fluorescent Direct Label kit (Agilent) together with an equal amount of a Cy5-labeled (red) common reference cDNA pool (Universal Mouse Reference RNA; Stratagene). These were hybridized under standard procedures to Agilent's mouse G4121A microarrays containing 60-mer probes for >20,000 genes. After hybridization, the arrays were scanned and processed using Agilent's feature extraction software resulting in an array data set. Eight samples were analyzed resulting in four "$Cd44^{+/+}$ versus reference" arrays and four "$Cd44^{-/-}$ versus reference" arrays. The array data set was analyzed in the Rosetta Resolver system using the re-ratio option. For each gene, P values were calculated by the error-weighted ANOVA test on the generated re-ratio data. Genes with a P value of <0.05, an absolute fold change of >3.0, and intensity at >200 were considered to be differentially expressed. Annotation was further enhanced by coupling the accession numbers of differentially expressed genes to the corresponding Gene Ontology terms (25).

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded tissue sections. Monoclonal antibodies used were rat anti-mouse CD44, IM7 (PharMingen), and rabbit anti-Ki67, SP6 (Lab Vision). Polyclonal antibody used was rabbit anti-cleaved caspase-3, Asp175 (Cell Signaling Technology). After deparaffination, antigen was retrieved by boiling slides for 10 min in 10 mmol/L of Tris with 1 mmol/L of EDTA (pH 9). Endogenous peroxidase was blocked, and prior to incubation, slides were blocked with 5% normal goat serum. IM7 staining was followed by biotinylated rabbit anti-rat IgG (Dako) and streptavidin ABC complex/horseradish peroxidase (Dako). Anti-Ki67 and anti–cleaved caspase-3 staining was followed by PowerVision poly-HRP anti-rabbit IgG (Immunologic, Klinipath). Peroxidase activity was detected using 3,3-diaminobenzidine tetrachloride (Sigma). Slides were counterstained with hematoxylin.

Immunoblotting. For immunoblot analysis, rat anti-mouse CD44 monoclonal antibody KM114 (PharMingen) was used. Secondary antibody used was horseradish peroxidase–conjugated goat anti-rat IgGs (Dako). Immunocomplexes were visualized with a standard enhanced chemiluminescence protocol (Amersham Biosciences).

Real-time reverse transcription-PCR. Epithelial cells from more than 200 duodenal crypts per mouse were isolated using the Veritas Microdissection System (Molecular Devices Corporation). For RNA isolation, the PicoPure RNA Isolation Kit (Molecular Devices Corporation) was used according to the manufacturer's protocol. Total RNA (250 ng) was reverse-transcribed in a total volume of 25 μL with Moloney murine leukemia virus reverse transcriptase (Life Technologies), and 10 ng of this reaction mixture was used per reaction. The quantitative reverse transcription-PCR (qRT-PCR) runs were performed on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche). Results were analyzed using LinReg PCR analysis software (version 7.5; ref. 26). Expression was normalized over β-actin expression. Primers were chosen on different exons in order to prevent amplification from any residual DNA. Primers are in 5′ to 3′. *Cd44*, "GAATGTAACCTGCCGCTACG" (sense) and "GGAGGTGTTGGACGTGAC" (antisense) with an amplicon of 268 bp (bp); *Dr6*, "TCTTCGTGGACGAGTCAGAG" (sense) and "GCAAGTCACAGGGGTCCAG" (antisense), 144 bp; *Bok*, "CTCAGCCTGCCATGGTTC" (sense) and "GCTGACCACACACTTGAGG" (antisense) 119 bp; *Lgr5*, "CTACTTGACTTTGAGGAAGACC" (sense) and "AGGAAAGCGCCAGTACTGC" (antisense) 145 bp; and *β-actin*, "GGATGCAGAAGGAGATTACTG" (sense) and "CCGATCCACACAGAGTACTTG" (antisense), 91 bp. Student's *t* test was used for statistical analysis.

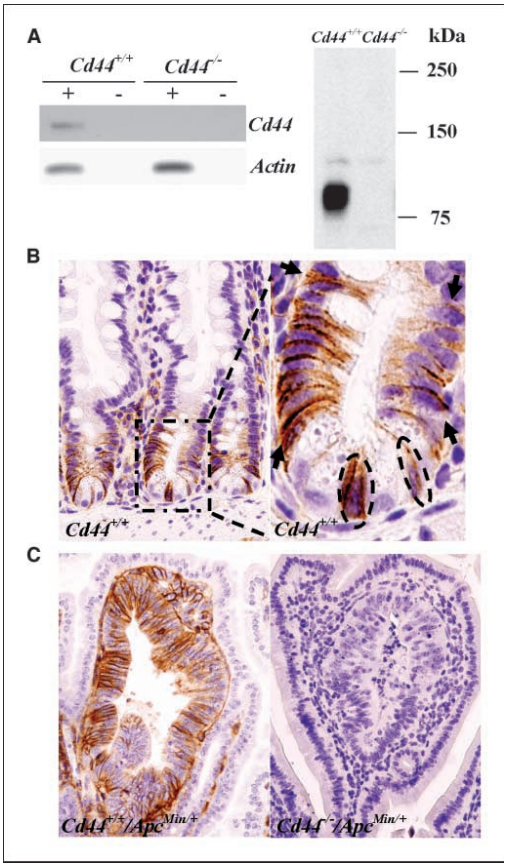

Figure 1. CD44 expression in $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice. *A*, $Cd44^{-/-}$ mice lack *Cd44* mRNA expression. *Left*, RT-PCR analysis of duodenal mucosa of $Cd44^{+/+}$ and $Cd44^{-/-}$ mice using primers for *Cd44* exon-2 and exon-3, with (+) or without (−) reverse transcription. *Right*, $Cd44^{-/-}$ mice do not express CD44 protein. Total spleen lysates were analyzed by immunoblotting for CD44 expression using the anti-CD44 antibody KM114. *B*, CD44 is expressed on transit-amplifying cells and CBC cells in the intestinal crypt. Immunohistochemical staining of a duodenal crypt from a $Cd44^{+/+}$ mouse using the anti-CD44 antibody IM7. The high magnification (*right*) of the crypt base displays CD44 expression on transit-amplifying cells (*arrows*) and CBC cells (*circled*). *C*, CD44 expression in $Apc^{Min/+}$ small intestinal adenomas. IM7 staining of $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ small intestinal adenomas. CD44 is strongly expressed in the $Cd44^{+/+}$ adenoma and absent in the $Cd44^{-/-}$ adenoma.

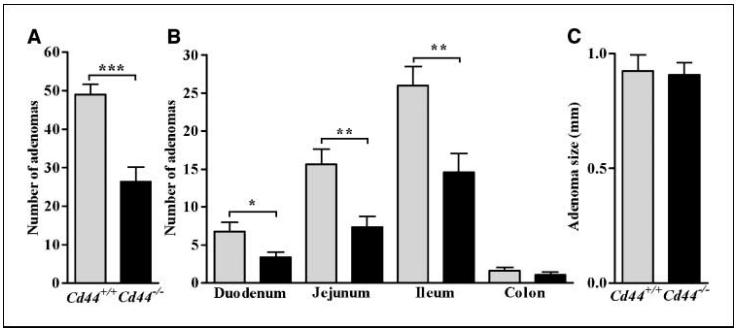

Figure 2. The absence of CD44 reduces intestinal adenoma formation in $Apc^{Min/+}$ mice. *A,* number of adenomas in the intestinal tract of $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice. Adenoma numbers were counted by operation binocular at 16 wk (*columns,* mean; *bars,* SE; $n = 11$ per group; ***, $P < 0.001$). *B,* the distribution of intestinal adenomas along the mouse intestine was not altered by CD44. Adenoma numbers in the duodenum, jejunum, ileum, and colon of $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice (*, $P < 0.05$; **, $P < 0.01$). *C,* the average adenoma size was not affected by CD44. The size of intestinal adenomas (mm) was measured in both groups of mice (*columns,* mean; *bars,* SE).

Nonradioactive *in situ* hibridization. Digoxigenin-labeled probes were made according to the manufacturer's specifications, using Dig-UTP (Roche). Specific PCR primers were used to create a probe for full-length *Bok*. Primers used were "ATTTAGGTGACACTATA-G-AAGGGTCTA-GACCGCCG" (SP6, sense) and "AGT-TAATACGACTCACTATA-GG-GTCA-CAGAGGCCAGCTCC" (T7 antisense). *In situ* hibridization on 6 μm paraffin-embedded duodenal sections was performed as described previously (27). Briefly, sections were digested for 12 min at 37°C with 20 mg/mL of proteinase K dissolved in PBS, followed by a 5-min rinse in 0.2% glycine/PBS and two 5-min rinses in PBS. Sections were then re-fixed for 20 min in 4% formaldehyde/0.2% glutaraldehyde dissolved in PBS to ensure firm attachment of the sections to the microscope slides, and washed twice in PBS for 5 min. The hybridization mix was composed of 50% formamide, 5× SSC, 1% blocking solution (Roche), 5 mmol/L of EDTA, 0.1% Tween 20, 0.1% CHAPS (Sigma), 0.1 mg/mL of heparin (Becton Dickinson), and 1 mg/mL of yeast total RNA (Roche). Sections were prehybridized in hybridization mix without probe for 1 h at 70°C and then hybridized with 200 ng/mL of probe at 70°C overnight.

Results

Attenuated intestinal adenoma formation in $Apc^{Min/+}$ mice lacking CD44. To explore the role of CD44 in intestinal adenoma formation, $Apc^{Min/+}$ mice lacking CD44 were generated by crossing $Cd44^{-/-}$ mice (22) with $Apc^{Min/+}$ mice. RT-PCR and immunoblotting confirmed the CD44 expression status of the mice (Fig. 1*A*). In line with our previous observations (3, 4), immunohistochemical staining of tissue sections of the small intestines of $Cd44^{+/+}/Apc^{Min/+}$ mice showed CD44 expression on the epithelial cells occupying the crypt compartment as well as on stromal cells, lymphocytes, and plasma cells in the intestinal lamina propria (Fig. 1*B*). Within the epithelial compartment, prominent CD44 expression was observed at the basolateral surface of transit-amplifying cells (*arrows*). Moreover, CBC cells, which have recently been shown to represent intestinal stem cells (1), also showed strong CD44 expression (Fig. 1*B, circled*). In addition to expression on normal crypt epithelium, $Cd44^{+/+}/Apc^{Min/+}$ mice displayed strong CD44 expression on the neoplastic epithelium of intestinal adenomas (Fig. 1*C*). As expected, CD44 expression was completely absent in the normal and neoplastic intestinal epithelium of the $Cd44^{-/-}/Apc^{Min/+}$ mice.

To follow the development and progression of intestinal tumors, mutant mice were monitored over time. At the time when $Apc^{Min/+}$ mice with wild-type CD44 developed adenomas, $Apc^{Min/+}$ mutant animals lacking CD44 were also sacrificed (after 16 weeks, or earlier if moribund), and the number and size of the adenomas in the entire small and large intestine was determined. All adenomas in the intestine were of the (tubulo)villous type. The number of adenomas in $Apc^{Min/+}$ mice with CD44 expression was 49.0 ± 2.8 (mean ± SE), which compares well with data obtained in other studies (23, 28). Targeted disruption of the *Cd44* locus and consequential absence of CD44 expression resulted in a mean adenoma number of 26.4 ± 4.0, a reduction of almost 50% ($n = 11$ per group, $P < 0.001$; Fig. 2*A*). Comparison of the distribution of adenomas in the different segments of the intestinal tract revealed a similar reduction in adenoma number in each separate part (Fig. 2*B*). The mean adenoma diameter was ~0.9 mm in both groups and did not differ significantly (Fig. 2*C*). These observations strongly suggest that *Cd44* deletion suppresses adenoma initiation, rather than the outgrowth of the tumors.

To verify that CD44 indeed does not influence tumor progression, we compared the number of aberrant crypts, small adenomas, and large adenomas (see Materials and Methods for classification) in the intestines of 8- and 16-week-old $Cd44^{+/+}/Apc^{Min/+}$ versus $Cd44^{-/-}/Apc^{Min/+}$ mice (Fig. 3). The number of aberrant crypts in $Cd44^{+/+}$ mice was 12.0 ± 1.4 at 8 weeks and 10.7 ± 1.5 at 16 weeks. The $Cd44^{-/-}$ mice displayed a strongly reduced number of aberrant crypts both at 8 weeks (3.1 ± 1.0) and at 16 weeks (4.3 ± 1.0), confirming a diminished tumor initiation. In the $Cd44^{+/+}$ mice as well as in the $Cd44^{-/-}$ mice, the number of small and large adenomas showed an increase over time, implying that progression from aberrant crypts into small and eventually large adenomas can take place independent of CD44.

CD44 deletion does not affect cellularity and proliferation in the transit-amplifying and stem cell compartments. As shown above, deletion of CD44 inhibits adenoma initiation in $Apc^{Min/+}$ mice but does not prevent tumor outgrowth. This observation, as well as the previous finding that $Cd44^{-/-}$ mice show no gross intestinal abnormalities (3, 22), suggests that CD44 is not required for maintaining the proliferative capacity of (neoplastic) intestinal epithelial cells. Indeed, in tissue sections of the small intestine of $Cd44^{-/-}/Apc^{Min/+}$ mice, the pattern of expression of the proliferation marker Ki67 was indistinguishable from that in $Apc^{Min/+}$ with wild-type CD44 and the size and cellularity of the transit-amplifying compartments were not different (Fig. 4*A*). We also compared the number of intestinal stem cells in $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice. These CBC cells are broad-based with a wedge-shaped nucleus and scarce organelles, and reside at the crypt base interspersed between Paneth cells. Their cell cycle time is in the order of 1 day and the cells are typically Ki67-positive (1). Duodenal crypts from both $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice showed Ki67-positive CBC cells at the crypt base (Fig. 4*A,*

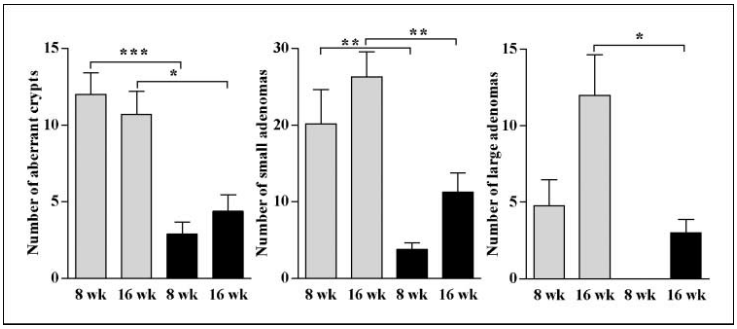

Figure 3. Reduction of aberrant crypt and adenoma numbers, but not adenoma outgrowth, in mice lacking CD44. The number of aberrant crypts (<0.25 mm), small adenomas (0.25–1 mm), and large adenomas (>1 mm) in intestinal sections (Swiss rolls) of $Cd44^{+/+}/Apc^{Min/+}$ (*gray column*) and $Cd44^{-/-}/Apc^{Min/+}$ (*black column*) mice at 8 and 16 wk ($n = 4$ per group; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

circled). Quantification of Ki67-positive cells at the crypt base revealed that the number of cycling CBC cells did not differ between $Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{+/+}/Apc^{Min/+}$, or $Cd44^{-/-}/Apc^{Min/+}$ mice (Fig. 4*B*), implying that the size of the intestinal stem cell compartment was not altered by CD44 deletion (or introduction of an Apc^{Min} allele). In line with this conclusion, the mRNA expression levels of the stem cell marker Lgr5 did not significantly differ between the different mouse strains (Fig. 4*B*).

Increased apoptosis in the intestinal crypts of $Cd44^{-/-}/Apc^{Min/+}$ mice. Because CD44 has been implicated in the regulation of sensitivity to apoptosis in several systems (9, 11, 12, 29, 30), we assayed the effect of CD44 deletion on apoptosis in the intestinal crypt compartment. Apoptotic crypt epithelial cells were visualized by immunohistochemical staining with an antibody against cleaved caspase-3. As shown in Fig. 5*A*, the crypt compartments of $Cd44^{+/+}$, $Cd44^{-/-}$, and $Apc^{Min/+}$ mice contained similar numbers of apoptotic cells, indicating that neither the absence of CD44 nor the presence of an Apc^{Min} allele per se affect crypt cell apoptosis. Interestingly, however, in $Cd44^{-/-}/Apc^{Min/+}$ mice, the number of apoptotic cells was significantly increased, suggesting a role for CD44 in controlling apoptosis resistance of intestinal crypt epithelium of the $Apc^{Min/+}$ mice. The majority of these active caspase-3–positive cells were localized below the transit-amplifying zone in the basal crypt compartment, between Paneth cells (Fig. 5*B*). Although we cannot exclude a contribution of Paneth cells, this localization suggests that these apoptotic cells represented CBC cells.

To identify possible effectors of CD44-mediated apoptosis resistance, the gene expression profiles of duodenal mucosa of $Cd44^{+/+}$ and $Cd44^{-/-}$ mice were analyzed for differential expression of a subset of genes involved in apoptosis (Supplementary Table S1). Interestingly, two proapoptotic genes were identified that showed a more than 3-fold up-regulation in the $Cd44^{-/-}$ mice, i.e., BCL-2–related ovarian killer protein (*Bok*; 3.2×) and tumor necrosis factor receptor superfamily member 21 (*Tnfrsf21*), also known as death receptor 6 (*Dr6*; 3.4×). qRT-PCR on cDNA derived from duodenal crypts isolated by using laser capture microdissection from $Cd44^{+/+}$ and $Cd44^{-/-}$ mice confirmed the up-regulation of *Bok* and *Dr6* expression in the crypt compartment of both $Cd44^{-/-}$ mice and $Cd44^{-/-}/Apc^{Min/+}$ mice (Fig. 6*A*). Furthermore, mRNA *in situ* hybridization studies showed that *Bok* is specifically expressed in the epithelial cells of the intestinal crypts, i.e., the compartment that also expresses *Cd44* (Fig. 6*B*). It is noteworthy that no changes in cell cycle–regulating genes or Wnt-signaling target genes including *cMyc, cyclin D1, EphB, Lef1*, and *Axin2* were found (data not shown). These data suggest a possible role for the proapoptotic proteins BOK and DR6 in the maintenance of the balance between apoptosis and survival in the epithelial cells lining the intestinal crypt.

Discussion

The intestinal crypt compartment is one of the most dynamic cell systems of the body. Its lining is continuously being renewed from a stem cell niche residing at the crypt bottom (1), which gives rise to a transit-amplifying cell compartment of rapidly dividing epithelial cells. This architectural hierarchy allows for the generation of a vast progeny of terminally differentiated cell types. Proliferation as well as migration of these cells is kept under tight control by different signaling pathways, including WNT, hedgehog, bone morphogenic protein (BMP), and Eph/ephrin signaling [reviewed by Crosnier et al. (31)]. Aberrant WNT signaling initiates malignant transformation of intestinal epithelium in both humans and mice (2), perturbing the proliferation, migration, differentiation, and apoptosis of progenitor cells in intestinal crypts (32). In the present study, we show that the WNT signaling target CD44 is an important regulator of adenoma formation in $Apc^{Min/+}$ mice, affecting tumor initiation and the balance between survival and apoptosis in the intestinal crypts.

We observed that adenoma formation in tumor-prone $Apc^{Min/+}$ mice was reduced by almost 50% in the absence of CD44 (Fig. 2*A*), whereas the distribution of adenomas along the intestinal tract and the average size of the adenomas were not affected (Fig. 2*B* and *C*). These findings suggest that CD44 affects tumor initiation rather than outgrowth. Consistent with this hypothesis, we found a similar progression from aberrant crypt to small and eventually large adenoma over time, independent of CD44 (Fig. 3). This observation, as well as the previous finding that CD44 knockout mice show no gross intestinal abnormalities (3, 22), suggests that CD44 is not required for maintaining the proliferative capacity of (neoplastic) intestinal epithelial cells. Indeed, in tissue sections of the small intestine of either $Apc^{+/+}$ or $Apc^{Min/+}$ mice lacking CD44, the size and cellularity of the intestinal stem cell and transit-amplifying compartments, which show strong CD44 expression in normal mice (Fig. 1*B*), were not different from that in $Apc^{+/+}$ or $Apc^{Min/+}$ with wild-type CD44 (Fig. 4*A* and *B*). Also, our cDNA microarray studies of the intestinal mucosa of wild-type CD44 and CD44-deficient mice did not reveal differential expression of cell cycle genes.[1]

[1] Unpublished observation.

Interestingly, the $Apc^{Min/+}$ mice lacking CD44 showed a significant increase in apoptotic cell numbers in the intestinal crypts. These apoptotic cells were primarily situated at the crypt base between the "0" and the "+4" position, which represents the

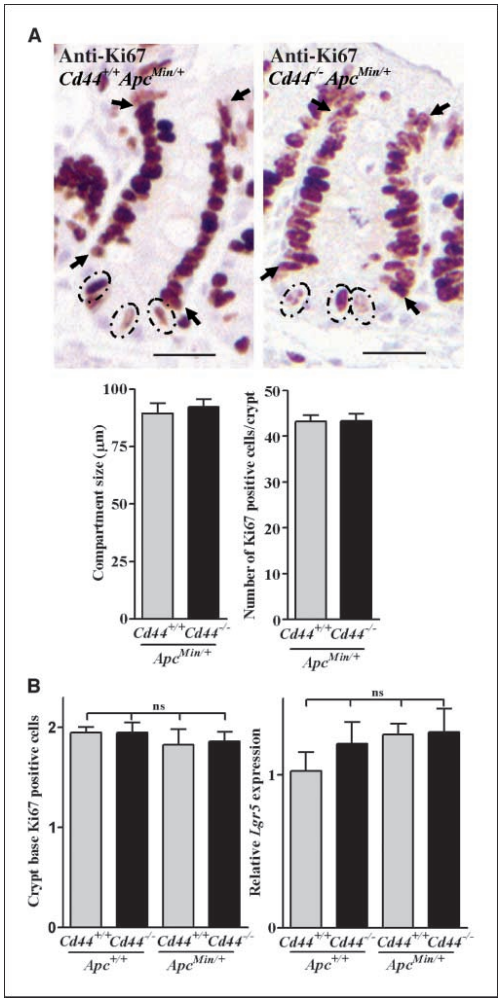

Figure 4. The absence of CD44 does not affect the proliferation of transit-amplifying and CBC cells. *A,* CD44 does not affect the proliferation of transit-amplifying and CBC cells. *Top,* examples of cell proliferation in the duodenal crypt of $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice, visualized by anti-Ki67 staining. Transit-amplifying cells (*arrows*) and cycling CBC cells (*circled*) are visible in both types of mice (*bars,* 25 μm). *Bottom,* quantification of the duodenal transit-amplifying compartment size in $Cd44^{+/+}/Apc^{Min/+}$ (*gray column*) and $Cd44^{-/-}/Apc^{Min/+}$ (*black column*) mice and cell proliferation in the transit-amplifying compartment. Ki67-positive cells in the transit-amplifying compartment were counted in both groups of mice (n = 4 per group, at least 20 crypts per mouse were scored). *B,* CBC cell cycling and *Lgr5* expression was not affected by the absence of CD44. *Left,* quantification of Ki67-positive CBC cells in $Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{+/+}/Apc^{Min/+}$, and $Cd44^{-/-}/Apc^{Min/+}$ mice. *Right,* relative expression of the stem cell marker *Lgr5* in the four groups of mice.

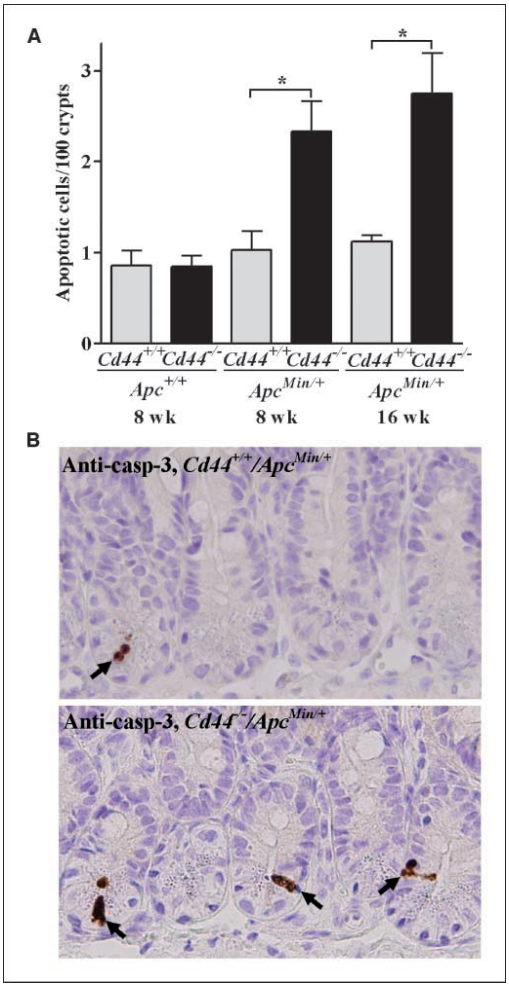

Figure 5. Increased apoptosis in the intestinal crypts of $Cd44^{-/-}/Apc^{Min/+}$ mice. *A,* intestinal crypts of $Cd44^{-/-}/Apc^{Min/+}$ show increased numbers of apoptotic epithelial cells. Quantification of apoptotic cells in the small intestinal crypts of $Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{+/+}/Apc^{Min/+}$, and $Cd44^{-/-}/Apc^{Min/+}$ mice at 8 and 16 wks (*, $P < 0.05$; n = 4 per group). *B,* apoptotic cells are located in the basal crypt compartment. Examples of apoptotic epithelial cells (*black arrows*) in the crypts of both $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice, visualized by using an antibody against cleaved caspase-3.

border of the transit-amplifying zone (Fig. 5*B*). Apart from Paneth cells, this area contains the CBC cells, which have recently been identified as intestinal stem cells (1). A small but constant rate of cell death within the basal crypt compartment has previously been observed in wild-type C57BL/6J mice and is most likely initiated by DNA damage (33, 34). This preference for "altruistic" apoptotic cell death, rather than activation of DNA repair, has been suggested to play a role in the protection against neoplastic transformation

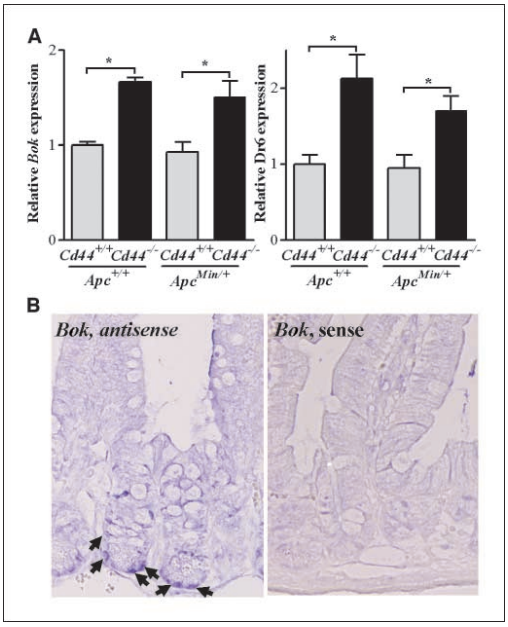

Figure 6. Increased expression of the proapoptotic genes *Bok* and *Dr6* in the crypts of $Cd44^{-/-}$ and $Cd44^{-/-}/Apc^{Min/+}$ mice. *A*, increased *Bok* and *Dr6* expression in the crypts of $Cd44^{-/-}$ mice. qRT-PCR of *Bok* and *Dr6* expression in the duodenal crypts of $Cd44^{+/+}/Apc^{Min/+}$ and $Cd44^{-/-}Apc^{Min/+}$ mice ($n = 4$ per group; *, $P < 0.05$). *B*, *Bok* is specifically expressed in the basal crypt compartment. *Left*, *Bok* mRNA expression in a duodenal section of a $Cd44^{+/+}$ mouse (*black arrows*). *Right, in situ* hybridization sense control.

during the rapid turnover of the intestinal epithelium (35). Whether a damaged cell dies or is allowed to progress through the transit-amplifying compartment is determined by the balance between proapoptotic and antiapoptotic proteins, setting a survival threshold (36). CD44 may affect this threshold in various ways: Engagement of its ligand hyaluronan or other extracellular matrix components may activate survival pathways, e.g., the phosphoinositide-3-kinase/AKT pathway (9, 29, 30, 37). Also, its function as a regulator of signaling and coreceptor in multiprotein complexes, involving interactions with receptor tyrosine kinases such as MET (10, 14) and ERBB1–4 (38, 39), the Src family kinases LCK and FYN (40), and actin cytoskeleton regulators like the ERM proteins (41, 42), could be instrumental in promoting cell survival. CD44 may suppress apoptosis induction via FAS, either by modulating FAS expression or by interfering with death receptor signaling (11, 12, 43). In human colorectal cancer cell lines, CD44 can mediate resistance to stress-induced apoptosis by affecting both death receptor signaling and the mitochondrial pathway (29, 44), whereas increased sensitivity to radiation-induced apoptosis in $Cd44^{-/-}$ mice involves the mitochondrial pathway (45). Our current microarray studies suggest that the control of the apoptotic balance in the intestinal crypts by CD44 may also involve regulation of proapoptotic genes like *Bok* (46) and *Dr6* (ref. 47; Supplementary Table S1; Fig. 6). Whether these proapoptotic molecules indeed sensitize *Apc*-deficient crypt cells to apoptosis, and thereby affect adenoma formation, needs further exploration.

Two recent findings are of great interest to our current study, that is, the finding that CD44 is a marker for CSC (20) and the identification of the CBC cell as the normal intestinal stem cell (1). Importantly, we observed a strong expression of CD44 on cells which, by localization, morphology, and Ki67 expression, were identified as CBC cells (Figs. 1*B* and 4*A*, *circled*). This implies that normal intestinal stem cells express high levels of CD44. On the other hand, our data also clearly show that CD44 expression is not restricted to the intestinal stem cell compartment, but that CD44 is also present on the cells of the transit-amplifying zone (Fig. 1*B*). This indicates that CD44 is not a stem cell marker per se, but rather, marks cycling cells in the intestinal crypt. In view of the recent identification of CD44 as an important marker for CSCs in colorectal cancer as well as in several other solid cancers and leukemia (48–50), this observation is of great interest as it addresses the relation between normal (intestinal) stem cells and CSCs. Our findings suggest that the CD44-positive colorectal CSCs, as described by Clarke and coworkers (20), could either be derived from intestinal stem cells or from transit-amplifying cells that have undergone dedifferentiation while migrating up the crypt-villus axis. Both these populations represent highly proliferative cells, which upon transformation, could be endowed with tumorigenic potential. Additional markers besides CD44, in particular, highly specific markers for intestinal stem cells like Lgr5, may help to further delineate the relation of CSCs to the normal intestinal stem cell compartment. However, independent of the precise relation between normal and cancer stem cells, our current findings show that CD44 is instrumental in adenoma formation in $Apc^{Min/+}$ mice and affects tumor initiation, possibly by controlling apoptosis in the intestinal stem cell compartment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Chapter 3

Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling

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Commentary by W. Guo and P.S. Frenette

Alternative CD44 splicing in intestinal stem cells and tumorigenesis

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SHORT COMMUNICATION

Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling

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A gene signature specific for intestinal stem cells (ISCs) has recently been shown to predict relapse in colorectal cancer (CRC) but the tumorigenic role of individual signature genes remains poorly defined. A prominent ISC-signature gene is the cancer stem cell marker CD44, which encodes various splice variants comprising a diverse repertoire of adhesion and signaling molecules. Using Lgr5 as ISC marker, we have fluorescence-activated cell sorting-purified ISCs to define their CD44 repertoire. ISCs display a specific set of CD44 variant isoforms (CD44v), but remarkably lack the CD44 standard (CD44s) isoform. These CD44v also stand-out in transformed human ISCs isolated from microadenomas of familial adenomatous polyposis patients. By employing knock-in mice expressing either CD44v4-10 or CD44s, we demonstrate that the CD44v isoform, but not CD44s, promotes adenoma initiation in $Apc^{Min/+}$ mice. Our data identify CD44v as component of the ISCs program critical for tumor initiation, and as potential treatment target in CRC.

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Keywords: CD44; colorectal cancer; stem cell; Wnt signaling

INTRODUCTION

The self-renewal of the intestinal epithelium is a tightly controlled dynamic process. Intestinal stem cells (ISCs) residing at the crypt base divide once a day and their daughter cells form a transit-amplifying (TA) compartment of rapidly replicating progenitor cells, which differentiate into enterocytes, goblet-, paneth-, or enteroendocrine cells.[1] Wnt signaling has a key role in maintaining the ISC compartment,[2,3] while the vast majority of colorectal cancers (CRCs) are initiated by mutations in the Wnt pathway[4] causing constitutive transcriptional activation of Wnt/TCF target genes.[5–7] Microarray based gene-expression studies have defined a core target-gene set activated by Wnt/TCF in intestinal epithelial cells;[8–10] a subset of these genes showed expression restricted to ISCs.[11] Activating Wnt pathway mutations in ISCs give rise to intestinal adenomas with a much higher efficacy than in other intestinal epithelial cell populations,[7] while CRC cells with an ISC-like signature are endowed with high-tumor initiating potential as well as long-term self-renewal capacity,[12] implying an instrumental role for ISC signature genes in tumorigenesis.

A prominent component of the ISC signature is *CD44*, a gene that has first been associated with CRC metastasis and relapse almost two decades ago.[13–15] CD44 comprises a family of transmembrane glycoproteins generated from a single gene by extensive alternative splicing and differential glycosylation.[16] These proteins operate as cell adhesion and signaling (co)receptors, linking extracellular matrix (ECM) components, specifically hyaluronan and osteopontin, to the cell and cytoskeleton.[16] By modulating growth factor receptor signaling[16–18] they exert pleiotropic effects on important biological processes including cell migration, epithelial to mesenchymal transition, cancer metastasis and apoptosis resistance.[16,18,19] In the intestinal mucosa, *CD44* is a major direct target of TCF/β-catenin mediated transcription[9,10,20] (Figure 1a), while several lines of evidence suggest an important role in intestinal cancer. Dalerba and coworkers demonstrated that CD44 marks human CRC cells with cancer stem cell properties[21] and our own recent studies have shown that deletion of CD44 in $Apc^{Min/+}$ mice strongly attenuates intestinal tumor initiation.[22] Furthermore, in clinical CRC samples, high expression levels of CD44 variant isoforms (CD44v) on the primary tumor have been associated with tumor recidives at distant sites and poor prognosis.[23] Collectively, these studies indicate that CD44 has an important role in intestinal tumorigenesis, which is associated with ISC fate. However, to date, the identity and contribution of different CD44 isoforms, which present potential treatment targets in CRC patients remains unexplored.[16] Here, we report the characterization of the CD44 expression repertoire of ISCs. By generating $Apc^{Min/+}$ mice exclusively expressing CD44s or CD44v, we identify a specific CD44v isoform as component of the ISC signature instrumental in tumor initiation.

RESULTS AND DISCUSSION

To identify the variant isoforms that constitute the CD44 repertoire of ISCs, epithelial cells were isolated from the small intestinal crypts of Lgr5-GFP mice[24] (Figure 1b). Single cells were sorted by fluorescence-activated cell sorting into three distinct subpopulations: GFP-high, GFP-medium and GFP-low (Figure 1c), representing pure ISCs, an intermediate fraction, and TA cells, respectively.[11,24] *CD44* pre-mRNA is encoded by 20 exons, 10 of which are subject to extensive alternative splicing (variant exons v1 through v10, Figure 1d), generating multiple differentially spliced mRNA transcripts. The composition of these transcripts was resolved by using exon-specific real-time PCR.[25] This analysis

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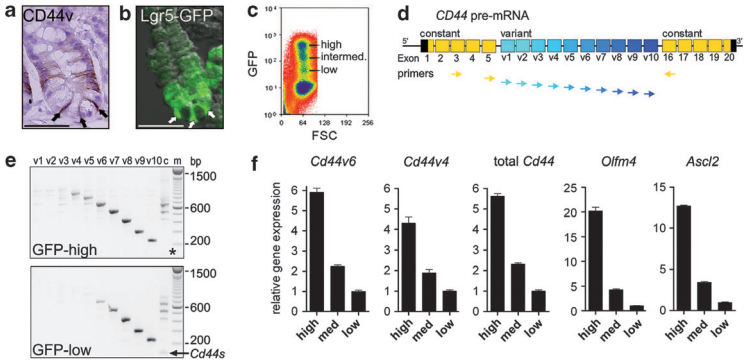

Figure 1. CD44v is part of the intestinal stem cell signature. (**a**) Immunohistochemical staining of a mouse small intestinal crypt using anti-CD44v6 mAb (9A4; Bender MedSystems GmbH, Vienna, Austria). Black arrows indicate CD44v-positive crypt-base columnar cells intercalated between Paneth cells. (**b**) Confocal image of an isolated crypt from an Lgr5-GFP mouse. Crypts and GFP-expressing cells were isolated as described previously.[11] White arrows indicate Lgr5-positive stem cells (scale bar = 50 μm). (**c**) After dissociation, three positive cell populations, GFP-high, GFP-intermediate and GFP-low, are discriminated. (**d**) *CD44* pre-mRNA is encoded by 20 exons. The variant exons (v1–v10) are depicted in blue. PCR primer sites are indicated by arrows and sequences are listed in Supplementary Table S1. (**e**) Exon-specific real-time PCR analysis for the expression of murine *CD44* variant exons in GFP-high (top panel) and GFP-low cells (lower panel). The PCR protocol was analogous to that from van Weering *et al.*[25] The lane indicated by 'c' shows PCR products obtained by using primers spanning all variant exons. The arrow indicates *Cd44s* product and no visible band for *Cd44s* is indicated by an asterisk. Lane 'm' shows a 100-bp DNA ladder. (**f**) Quantitative real-time PCR results showing relative gene expression levels in each subpopulation for respectively, *Cd44v6*, *Cd44v4*, and total *Cd44*, as well as *Olfm4* and *Ascl2*. Quantitative real-time PCR data were obtained on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche Molecular Systems, Inc, CA, USA) and was analyzed using LinReg software.[36] Results were normalized to β-actin. Error bars indicate s.e.m. Primers are listed in Supplementary Table S1.

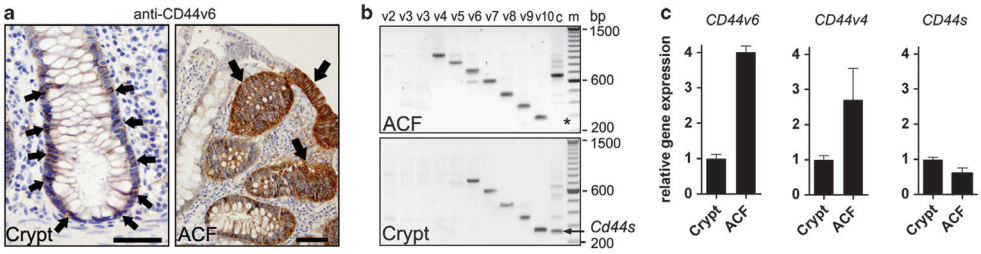

Figure 2. CD44v expression is induced in neoplastic epithelial cells from microadenomas of familial adenomatous polyposis patients. (**a**) Immunohistochemical staining of familial adenomatous polyposis colon using anti-CD44v6 mAb VFF-18 (BMS125; Bender MedSystems GmbH). Left panel, normal crypt; arrows indicate CD44v-positive cells. Right panel, microadenomas (ACF) indicated by arrows (scale bar = 50 μm). This study was performed in accordance with the ethical standards and approved by the research code committee on human experimentation of our institute. (**b**) Exon-specific real-time PCR analysis of epithelial cells isolated from microadenomas (top panel) and from normal crypts (lower panel) of familial adenomatous polyposis patients. The arrow indicates *CD44s* product and no visible band for *CD44s* is indicated by an asterisk. Note that exon v1 is non-functional in human cells and two primers directed against human exon v3 were used. (**c**) Quantitative real-time PCR results showing relative gene expression levels in each subpopulation for respectively, *Cd44v6*, *Cd44v4* and *Cd44s*. Quantitative real-time PCR data were obtained on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche Molecular Systems, Inc) and was analyzed using LinReg software. Primers are listed in Supplementary Table S1.

revealed that the TA and intermediate cell fractions express mRNAs encoding CD44v6-10, CD44v7-10 and CD44v8-10, whereas the GFP-high ISC fraction expressed an additional isoform identified as CD44v4-10 (Figure 1e). Unexpectedly, the GFP-high ISCs did not express the standard form of CD44 (CD44s), a common isoform reported to be widely expressed by many tissues/cell types including primary CRCs and CRC cell lines.[13,20] Quantitative analysis by quantitative real-time PCR, revealed that the GFP-high ISC cell fraction expresses five to sixfold higher levels of *CD44v6*, *CD44v4,* and total *CD44* than the GFP-low (TA) fraction (Figure 1f). Exon spanning primers specific for *CD44s* failed to generate an amplification product (data not shown), confirming the absence of CD44s expression. Expression of the stem cell signature genes *Olfm4* and *Ascl2* was almost exclusively restricted to the GFP-high cell fraction (Figure 1f), corroborating that these cells represent ISCs. These findings identify a limited and distinct repertoire of CD44v in ISCs, the largest isoform being CD44v4-10.

To explore whether human ISCs display a comparable CD44 repertoire, we analyzed epithelial cells from dysplastic aberrant crypt foci of familial adenomatous polyposis patients. Due to mutational activation of the Wnt pathway, these microadenomas retain a stem cell program.[10] In accordance, immunohistochemical staining using monoclonal anti-CD44v6 antibody showed a strong induction of CD44v in almost all epithelial cells of aberrant crypt foci (Figure 2a). Epithelial cells from the basal crypt region of normal colon and from aberrant crypt foci were isolated by using

laser-capture microdissection (Supplementary Figure S1). Similar to murine ISCs, exon-specific real-time PCR and quantitative real-time PCR analysis of these microadenomas revealed a prominent expression of mRNA encoding CD44v, including CD44v4-10 (Figures 2b and c), suggesting involvement at early stages of human CRC.

The above findings strongly suggest an instrumental role for ISC specific CD44v isoforms in intestinal cancer formation. To explore this role, we generated knock-in mice exclusively expressing either CD44v4-10, being the largest isoform detected in ISCs, or as control, CD44s. Two gene replacement targeting vectors containing a partial genomic *Cd44* clone were constructed (Figure 3a) and homologs recombination in embryonic stem cells resulted in mice solely expressing either CD44v4-10 (*Cd44*$^{v4\text{-}10/v4\text{-}10}$ mice) or CD44s (*Cd44*$^{s/s}$ mice; Figure 3b and c). The *Cd44* knock-in mice were fertile and showed no gross abnormalities. Flow cytometric analysis of purified crypt cells using the antipan CD44 mAb IM7 showed comparable expression of CD44 in the intestinal epithelium of adult mice (Figure 3d), while immunohistochemical staining of intestinal tissue sections confirmed that the CD44 proteins were expressed in the stem cell compartments (Figure 3e). A detailed analysis of the intestines revealed no abnormalities in crypt/villus length ratio's, number of cycling crypt-base columnar and TA cells, or apoptotic cell numbers in the crypt compartment (Supplementary Figure S2). The latter findings are consistent with the previously reported absence of intestinal abnormalities in CD44-deficient mice.[22]

To study the effect of CD44v on intestinal tumorigenesis, *Cd44*$^{v4\text{-}10/v4\text{-}10}$ and *Cd44*$^{s/s}$ mice were intercrossed with *Apc*$^{Min/+}$ mice to generate *Cd44*$^{v4\text{-}v10/v4\text{-}v10}$/*Apc*$^{Min/+}$ and *Cd44*$^{s/s}$/*Apc*$^{Min/+}$ mice. In addition, *Apc*$^{Min/+}$ mice expressing both CD44v4-10 and CD44s (*Cd44*$^{v4\text{-}10/s}$/*Apc*$^{Min/+}$) or completely lacking CD44 (*Cd44*$^{-/-}$/*Apc*$^{Min/+}$) were also employed. Mice were sacrificed at 8 weeks of age ($n = 14$ per group) and the number of adenomas in the intestinal tracts was assessed by operation binoculars. In addition, micro-adenomas were counted in hematoxylin and eosin stained tissue sections of intestine embedded as 'Swiss rolls'. All intestinal adenomas were of the (tubulo)villous type and a similar anatomical distribution, with a preferential localization in the distal part of the small intestine, was found for all genotypes

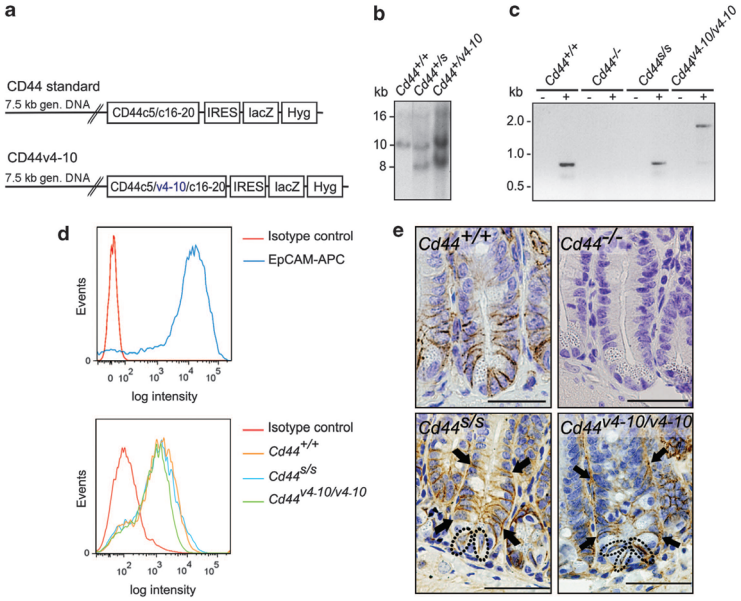

Figure 3. Generation of CD44 variant knock-in mice. (**a**) Targeting constructs for *Cd44*$^{s/s}$ (top) and *Cd44*$^{v4\text{-}10/v4\text{-}10}$ (bottom) knock-in mice. A vector was constructed containing 7.5 kb of genomic DNA (cloned from a λFIX-II 129/Sv library; Stratagene, CA, USA) upstream from the translation initiation codon up to the ClaI site in exon 5. This fragment was fused to a partial complementary DNA encoding the relevant c-terminal domains and furthermore an independent ribosomal entry site (IRES), a β-galactosidase encoding gene (lacZ) and a hygromycin cassette (Hyg). Genomic fragments and cassettes are not drawn to scale. Constant exons are designated as 'c' and variant exons as 'v'. All mouse studies were approved by the animal ethical committee (DEC) of the Academic Medical Center, University of Amsterdam. (**b**) Southern blot analysis of ES cells that have undergone a homologous recombination event. ES cells were cultured, electroporated and analyzed as described earlier.[37] Founders were crossed with C57BL/6J/*Cd44*$^{-/-}$ females[38] to obtain compound heterozygous offspring (that is, *Cd44*$^{s/-}$, *Cd44*$^{v4\text{-}10/-}$). (**c**) *CD44* mRNA analysis in spleen cells of the variant knock-in mice using real-time PCR. (−, without reverse transcription; +, with reverse transcription.) Germline transmission was determined by PCR on genomic DNA. Primers are listed in Supplementary Table S2. Animals with the CD44 knock-in allele were backcrossed to C57B/6J-Ico (B6) for more than 10 generations. Homozygous offspring on a C57BL/6J background was obtained by a final interbreeding. (**d**) CD44 expression profiles in the intestinal epithelium of *Cd44*$^{+/+}$, *Cd44*$^{s/s}$, and *Cd44*$^{v4\text{-}10/v4\text{-}10}$mice. Single crypt cells were isolated as described previously[11] and were analyzed by flow cytometry. Pan-epithelial surface antigen EpCAM was used to confirm epithelial cell populations (>99% purity). Pan-CD44 mAb IM7 (BD Pharmingen, CA, USA) was used tot determine the cell surface expression of CD44. (**e**) CD44 protein expression in the crypt compartments of *Cd44*$^{+/+}$, *Cd44*$^{-/-}$, *Cd44*$^{s/s}$, and *Cd44*$^{v4\text{-}10/v4\text{-}10}$mice. Total CD44 expression was detected by immunohistochemical staining using the anti-pan CD44 mAb IM7. TA-cells (arrows) and crypt-base columnar (CBC) cells (circled) are indicated in the CD44 variant knock-in mice (scale bar = 50 μm).

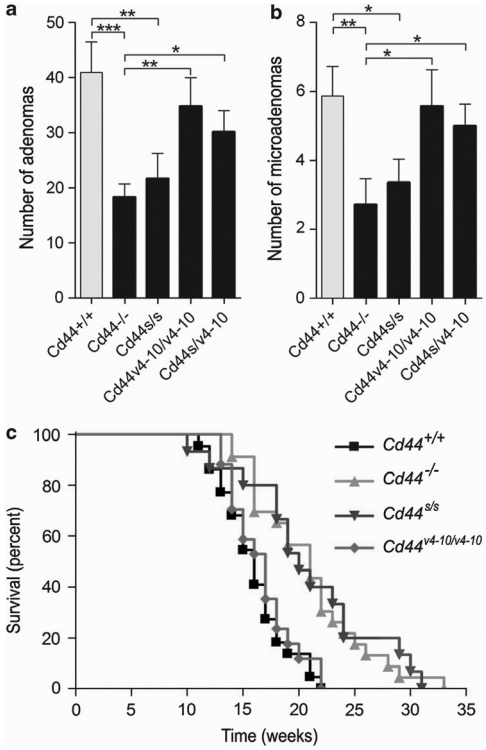

Figure 4. CD44v is instrumental in intestinal tumorigenesis. (**a**) Adenoma numbers in the intestinal tracts of $Cd44^{+/+}$-, $Cd44^{-/-}$-, $Cd44^{s/s}$-, $Cd44^{v4\text{-}10/v4\text{-}10}$- and $Cd44^{s/v4\text{-}10}$-$Apc^{Min/+}$ mice at 8 weeks of age ($n=14$ per group). (**b**) Microadenoma ($<250\,\mu m$) numbers assessed in hematoxylin and eosin stain stained intestinal sections at 8-weeks (mean ± s.e.m.; $n=14$ per group; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$). Small intestine and colon were flushed with phosphate-buffered saline, opened longitudinally and examined using an operation binocular. Adenomas were assessed by two independent experienced observers and were classified according to standard criteria.[26] For statistical analysis, the Mann − Whitney test was used. (**c**) Kaplan − Meier curves showing the relation between *Cd44* genotype and survival in $Apc^{Min/+}$ mice. Survival of mice lacking *Cd44v* ($Cd44^{-/-}$ and $Cd44^{s/s}$) was significantly prolonged compared with mice having *CD44v* ($Cd44^{+/+}$ and $Cd44^{v4\text{-}10/v4\text{-}10}$) ($P<0.01$). Mice were inspected daily and sacrificed when sign of illness appeared (rectal prolapse, weight loss, anemia). $Cd44^{+/+}$- ($n=23$), $Cd44^{-/-}$- ($n=22$), $Cd44^{s/s}$- ($n=16$) and $Cd44^{v4\text{-}10/v4\text{-}10}$- ($n=17$) $Apc^{Min/+}$ mice. Curves were compared by log-rank test. All mice strains were determined to be *Mom-1* and *Mom-2* susceptible, both loci are major modifiers of the $Apc^{Min/+}$ phenotype in C57BL/6 J mice.[39]

(data not shown). The total number of adenomas in the $Cd44^{+/+}/Apc^{Min/+}$ mice was 41.0 ± 5.5 (mean ± s.e.m.; Figure 4a), comparable to adenoma numbers reported in earlier studies.[22,26] The $Cd44^{-/-}/Apc^{Min/+}$ animals showed an over twofold reduction in adenoma formation (18.3 ± 2.5, $P<0.001$), confirming our previous finding that CD44 deletion strongly attenuates adenoma initiation.[22] In the $Cd44^{s/s}/Apc^{Min/+}$ knock-in mice adenoma numbers were not different from that in CD44-deficient mice (21.6 ± 4.6, $P=0.53$). In marked contrast, however, adenoma formation in the $Cd44^{v4\text{-}v10/v4\text{-}v10}/Apc^{Min/+}$ knock-in mice was enhanced significantly compared with that in CD44-deficient mice and was similar to that in $Apc^{Min/+}$ animals with wild-type CD44 (34.5 ± 5.9, $P<0.05$ compared with $Cd44^{-/-}/Apc^{Min/+}$ mice and $P=0.43$ compared with $Cd44^{+/+}/Apc^{Min/+}$ mice). Also in mice heterozygous for CD44v4-10, significantly enhanced adenoma formation as compared with $Cd44^{-/-}/Apc^{Min/+}$ mice was found (30 ± 3.7, $P<0.05$). These results show that expression of CD44v4-10, but not CD44s, restores the wild-type phenotype of adenoma formation, implying that this stem-cell signature associated CD44v isoform is functionally involved in intestinal tumorigenesis. Like the formation of (macro)adenomas, microadenoma formation was also significantly impaired in CD44-deficient and CD44s mice, and could be completely restored by CD44v4-10 (Figure 4b), suggesting a role for CD44v in tumor initiation rather than in adenoma outgrowth. Interestingly, survival analysis performed in a separate group of mice revealed that the attenuated adenoma formation in $Apc^{Min/+}$ lacking CD44v results in a significant survival advantage (Figure 4c).

Our previous observations suggested that, CD44 controls intestinal tumor formation by affecting apoptosis resistance in the crypt compartments of $Apc^{Min/+}$ mice.[22] Specifically, $Apc^{Min/+}$ mice lacking CD44 showed an increased number of apoptotic cell numbers in the intestinal crypts compared with $Cd44^{+/+}/Apc^{Min/+}$ mice, but interestingly, also with $Cd44^{-/-}$ mice expressing wild-type *Apc* ($Apc^{+/+}$), indicating that neither the absence of CD44 nor the presence of an Apc^{Min} allele *per se* affect crypt cell apoptosis. Assaying apoptosis within the basal crypt compartments of the Apc^{Min} mutant animals revealed no differences between the $Cd44^{+/+}$-, $CD44^{s/s}$-, $CD44^{v4\text{-}10/v4\text{-}10}$-expressing groups, however, similar to that previously reported, $Cd44^{-/-}$-$Apc^{Min/+}$ mice exhibited an increase in apoptotic cell numbers (Supplementary Figure S3). No growth or cell cycle abnormalities were observed. These results indicate that all CD44 isoforms control cell survival in the intestinal crypt epithelium to a similar degree and suggest that the regulation of apoptosis by CD44 does not contribute to intestinal tumorigenesis.

Our study demonstrates an instrumental role for CD44v in intestinal tumorigenesis. CD44v4-10 represents the longest isoform identified in ISCs and the fact that this ISC CD44 isoform promotes adenoma formation, presumably by acting on tumor initiation, supports the concept that ISCs are the cells of origin of intestinal cancer.[7] Importantly, CD44 variants, including CD44v4-10, were also prominently expressed in neoplastic epithelial cells from early lesions of familial adenomatous polyposis patients, suggesting that these isoforms are also implicated in human CRC. By contrast, murine ISCs and their human neoplastic counterparts are CD44s negative/low and our *in vivo* studies demonstrate that CD44s cannot compensate for the negative effect of CD44 deficiency on adenoma initiation in $Apc^{Min/+}$ mice. The results of earlier studies which reported expression of CD44s in primary CRC as well as in CRC cell lines[13,20,27] presumably reflect the presence of more differentiated (non-ISC) cell populations within the tumor.

Although the vast complexity of CD44 prohibits definitive mechanistic conclusions, CD44 has several biological functions that are of potential value for stem cells and could promote tumor initiation. As a mediator of bidirectional signaling between the ECM and the cytoskeleton, CD44 controls crosstalk between transmembrane and cytoplasmic molecules. In these interactions, it functions as a potent signal amplifier, specifically of receptor tyrosine kinase signaling.[16] For example, CD44v isoforms are required for effective hepatocyte growth factor (HGF)-induced signal transduction through the receptor tyrosine kinase MET in several cell types including colon cancer cells (CSCs).[18,27] HGF is a potent mitogen and survival factor as well as a key mediator of epithelial to mesenchymal transition.[28] In colon cancer,

MET is frequently overexpressed by the tumor cells while HGF is expressed by myofibroblasts at the invasive front of the tumor.[29,30] Like CD44 expression, expression of MET is controlled by the Wnt pathway, and MET overexpression at the initial stage of CRC results from enhanced Wnt signaling.[10,31] Interestingly, a recent study by Vermeulen *et al.*[32] suggests a pivotal role for HGF in determining stemness of CSCs: High Wnt activity functionally designates CSCs and can be regulated by myofibroblast-secreted HGF, indicating that stemness of CSCs is in part orchestrated by the microenvironment. CD44v could be a crucial player in this scenario as it is critical for MET signaling in HT-29 CRC cells.[27] Indeed, we have recently obtained evidence for functional collaboration between CD44v and MET in the mouse intestinal stem cell compartment and in human colorectal CSCs (unpublished observations). Apart from affecting tyrosine kinase *viz.* HGF/MET signaling, CD44v might also modulate cellular functions by modulating interaction with ECM. CD44 splicing can regulate interaction with its main ligand, hyaluronan.[17,33] As ISCs reside in a niche that abounds in ECM molecules, including hyaluronate,[34] alterations in adhesion might affect stemness, proliferation and apoptosis resistance. Furthermore, CD44-ECM interaction could affect intestinal stromal cell function and this can contribute to malignant transformation in an indirect manner, for instance, by regulating sensitivity for inflammation.[35] Irrespective of the precise mechanism(s), however, our current findings demonstrate that CD44v marks ISCs and is instrumental in intestinal adenoma formation. These findings are of great interest as they specify and functionally underpin a number of recent studies which have identified CD44 as an important marker for colon carcinoma cells with tumor-initiating properties,[21] as well as for tumor initiating cells in several other solid cancers and leukemia.[16] In contrast to the standard form of CD44, which is ubiquitously expressed by many different cell types, expression of CD44v is highly restricted in normal tissue.[16] Thus, the current findings may also provide a novel handle for targeted treatment aiming to suppress tumorigenic and metastatic properties in cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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COMMENTARY

Alternative CD44 splicing in intestinal stem cells and tumorigenesis

W Guo and PS Frenette

As an important player in stem cells and cancer, CD44 is expressed in multiple isoforms via alternative mRNA splicing. Whether, and if so, how various isoforms play distinct roles in normal stem cells and tumorigenesis remains unclear. In this issue of *Oncogene*, Zeilstra *et al.* report studies showing that intestinal stem cells express a specific CD44 variant that promotes intestinal tumorigenesis induced by the activation of Wnt signaling, whereas the more commonly expressed standard CD44 isoform is not expressed by stem cells and does not promote tumor formation. This finding demonstrates an isoform-specific function of CD44 in intestinal stem cells and tumorigenesis.

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CD44, a class I transmembrane glycoprotein that binds to hyaluronan, has attracted much interest due to its role in cancer and stem cells. Initial studies have suggested that CD44 promotes tumor metastasis.[1] This function is supported by further studies demonstrating the association of CD44 expression with poor patient survival in multiple cancer types, including colon cancers.[2] More recently, CD44 emerged as a useful marker in certain solid tumors for enriching cancer stem cells (CSCs), the highly tumorigenic cancer cell population within a tumor mass. CSCs in both breast and colon cancers were indeed shown to express CD44.[3,4] It is worth noting that CD44 expression is not limited to CSCs, as many non-CSCs can also express CD44.[5] Thus, specific identification of CSCs requires combination of CD44 and other markers. However, CD44 is functionally involved in the maintenance and homing of CSCs as studies in breast and prostate cancer cell lines have shown that CD44 is required for tumorigenicity, and its expression is repressed by tumor suppressors p53 and microRNA-34a.[6,7] In addition, CD44 participates in leukemia stem cell homing to their niches.[8,9] This effect may be mediated in part by interactions with hyaluronan and also through specific fucosylation of CD44 standard isoform (CD44s) that confers E-selectin ligand binding activity and ability to interact more closely with bone marrow endothelium.[10]

In addition to its multifunctional cell adhesion properties, CD44 signaling also connects extracellular matrixes with intracellular signaling networks.[2,5] The diverse functions of CD44 are enabled, in part, by the expression of multiple isoforms through alternative mRNA splicing. CD44s, the shortest CD44 molecule, is encoded by 10 exons. Between the standard exons 5 and 6, up to 10 variant exons can be inserted in various combinations to form CD44 variants (CD44v).[2,5] These extra exons allow CD44 to interact with multiple signaling molecules in addition to its main ligand hyaluronan. For example, CD44v3 can associate with heparin-binding proteins such as fibroblast growth factor 2, whereas CD44v6 binds to hepatocytes growth factor and vascular endothelial growth factor.[2] These distinct properties of CD44 isoforms suggest they may have specific physiological functions *in vivo*. However, the exact function of CD44 isoforms remains largely undefined.

The report by Zeilstra *et al.*[11] sheds light on distinct roles of CD44s and CD44v isoforms in intestinal stem cells and in intestinal tumor formation. As a target of Wnt signaling pathway, CD44 is expressed in intestinal crypts and overexpressed in colon cancers, which often have hyperactivation of the Wnt signaling pathway.[11] Using a well-defined intestinal stem cell marker Lgr5,[12] Zeilstra *et al.*[11] separated mouse intestinal stem cells and transit-amplifying cells and performed isoform-specific reverse transcription–PCR. This analysis revealed that intestinal stem cells do not express CD44s, but rather CD44v isoforms, including a long isoform that contains variant exons 4–10 (CD44v4–10) that is only expressed in the stem cell compartment. In contrast, transit-amplifying cells express CD44s and other shorter CD44v isoforms, but not CD44v4-10. Human cryptic foci, which are microadenomas retaining a stem cell program, similarly express CD44v4-10 but not CD44s.

To dissect the function of CD44v4–10 and CD44s *in vivo,* Zeilstra *et al.*[11] generated mice that solely express either CD44v4–10 or CD44s by knocking in specific cDNAs into the *Cd44* locus. Interestingly, the animals that are deficient in either CD44s or CD44v developed a normal intestinal stem cell compartment, which is consistent with the earlier finding that pan-CD44 knockout mice develop intestine crypts normally.[11] However, when bred into the $Apc^{min/+}$ background, CD44s-expressing mice have significantly reduced tumor burden compared with wild-type mice. Similar defects were previously observed in pan-CD44 knockout mice.[11] In contrast, mice expressing CD44v4–10 have a tumor burden similar to wild-type mice. This shows that CD44v4–10, but not CD44s, is the functional CD44 isoform that contributes to intestinal tumor formation.

These studies provide convincing genetic evidence of differential functions of CD44 isoforms in intestinal tumorigenesis. However, the precise mechanism by which CD44v promotes intestinal tumorigenesis remains to be elucidated. Deficiency of CD44v does not affect cell proliferation or survival in the intestinal

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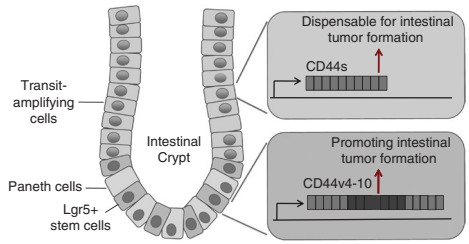

Figure 1. Alternative CD44 splicing in intestine. Intestinal stem cells and transit-amplifying cells express distinct CD44 isoforms. The stem cell isoform CD44v4–10, but not CD44s, contributes to the tumor formation.

crypt of $Apc^{min/+}$ mice, suggesting that other mechanism(s) are involved. Given the important role of intestinal stem cells in adenomatous polyposis coli mutation-induced tumor formation[13] and the specific expression of CD44v4–10 in intestinal stem cells, it would be interesting to investigate whether CD44 regulates the self-renewal and differentiation of stem cells. Although the intestinal stem cell compartment appears phenotypically normal in CD44s knock-in mice, it would be worthwhile to determine whether there is any defect in stem cell activity, especially under stress or wounding conditions or upon oncogenic insult.

Intestinal stem cells express a few other shorter CD44 variants in addition to v4–10. Can any of these shorter isoforms replace CD44v4–10 in intestinal tumor initiation? Identifying the exact exon(s) that are required for tumor formation may help pinpoint the specific CD44-mediated signaling pathway(s) that are involved in tumorigenesis. Another intriguing question for future investigation is whether CD44s plays any role in intestinal tumor progression at all. Interestingly, CD44s was shown to be required for epithelial–mesenchymal transition.[14] Given the contribution of epithelial–mesenchymal transition in tumor progression and metastasis, the function of CD44s in late-stage tumor progression needs to be further investigated. Because the $Apc^{min/+}$ model forms mostly adenomas, other tumor models recapitulating invasive and metastatic intestinal cancers would be required to address this question.

Overall, the study by Zeilstra *et al.*[11] highlights interesting aspects of isoform-specific functions of CD44 (Figure 1). The CD44 isoform-specific knock-in models will likely become a valuable genetic tool for dissecting the functions of CD44s and CD44v in various normal physiological and pathological settings. Such studies have been performed previously by interfering with CD44 function with variant-specific blocking antibodies. Although this approach is somewhat useful in the hematopoietic system, it is not as effective in solid tissues where it most likely does not completely block CD44 function.[2] The present study also raises an intriguing possibility that normal and CSCs of other tissues may express specific CD44v isoforms rather than CD44s. Thus far, most of CSC studies that defined CD44 as a CSC marker have used pan-CD44 antibodies. Because CD44s is widely expressed by various cell types, pan-CD44 antibodies do not faithfully distinguish CSCs and non-CSCs. Thus, it is worth to explore if CD44 variant-specific antibody can detect normal and CSCs more specifically. Finally, it is now recognized that CD44 is part of a larger alternative splicing program regulated by ESRP1 and ESRP2.[15] Thus, it is conceivable that other genes may also be alternatively spliced between intestinal stem cells and non-stem cells. Identifying these genes by global profiling approaches, such as RNA-seq, may uncover novel stem cell markers and regulators.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter 4

MET signaling mediates intestinal crypt-villus development, regeneration, and adenoma formation and is promoted by stem cell CD44 isoforms

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Commentary by A. T. Chan and C. S. Williams

MET Signaling in Intestinal Homeostasis and Cancer

MET Signaling Mediates Intestinal Crypt-Villus Development, Regeneration, and Adenoma Formation and Is Promoted by Stem Cell CD44 Isoforms

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BACKGROUND & AIMS: Resistance of metastatic human colorectal cancer cells to drugs that block epidermal growth factor (EGF) receptor signaling could be caused by aberrant activity of other receptor tyrosine kinases, activating overlapping signaling pathways. One of these receptor tyrosine kinases could be MET, the receptor for hepatocyte growth factor (HGF). We investigated how MET signaling, and its interaction with CD44 (a putative MET coreceptor regulated by Wnt signaling and highly expressed by intestinal stem cells [ISCs] and adenomas) affects intestinal homeostasis, regeneration, and adenoma formation in mini-gut organoids and mice. **METHODS:** We established organoid cultures from ISCs stimulated with HGF or EGF and assessed intestinal differentiation by immunohistochemistry. Mice with total epithelial disruption of MET ($Ah^{Cre}/Met^{fl/fl}/LacZ$) or ISC-specific disruption of MET ($Lgr5^{Creert2}/Met^{fl/fl}/LacZ$) and control mice ($Ah^{Cre}/Met^{+/+}/LacZ$, $Lgr5^{Creert2}/Met^{+/+}/LacZ$) were exposed to 10 Gy total body irradiation; intestinal tissues were collected, and homeostasis and regeneration were assessed by immunohistochemistry. We investigated adenoma organoid expansion stimulated by HGF or EGF using adenomas derived from $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ and $Lgr5^{Creert2}/Met^{+/+}/Apc^{fl/fl}$ mice. The same mice were evaluated for adenoma prevalence and size. We also quantified adenomas in $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ mice compared with $Ah^{Cre}/Met^{+/+}/Apc^{fl/+}$ control mice. We studied expansion of organoids generated from crypts and adenomas, stimulated by HGF or EGF, that were derived from mice expressing different CD44 splice variants ($Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{s/s}$, or $Cd44^{v4-10/v4-10}$ mice). **RESULTS:** Crypts incubated with EGF or HGF expanded into self-organizing mini-guts with similar levels of efficacy and contained all differentiated cell lineages. MET-deficient mice did not have defects in intestinal homeostasis. Total body irradiation reduced numbers of proliferating crypts in $Ah^{Cre}/Met^{fl/fl}/LacZ$ mice. $Lgr5^{Creert2}/Met^{fl/fl}/LacZ$ mice had impaired regeneration of MET-deficient ISCs. Adenoma organoids stimulated with EGF or HGF expanded to almost twice the size of nonstimulated organoids. MET-deficient adenoma organoids did not respond to HGF stimulation, but did respond to EGF. ISC-specific disruption of *Met* ($Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ mice) caused a twofold increase in apoptosis in microadenomas, resulting in an approximately 50% reduction of microadenoma numbers and significantly reduced average adenoma size. Total epithelial disruption of *Met* ($Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ mice) resulted in an approximate 50% reduction in (micro)adenoma numbers. Intestinal crypts from $Cd44^{-/-}$ mice did not expand to the same extent as crypts from $Cd44^{+/+}$ mice on stimulation with HGF, but had the same response to EGF. The negative effect on HGF-mediated growth was overcome by expression of CD44v4–10, but not by CD44s. Similarly, HGF-mediated expansion of adenoma organoids required CD44v4–10. **CONCLUSIONS:** In studies of intestinal organoid cultures and mice with inducible deletion of MET, we found HGF receptor signaling to regulate intestinal homeostasis and regeneration, as well as adenoma formation. These activities of MET are promoted by the stem cell CD44 isoform CD44v4–10. Our findings provide rationale for targeting signaling via MET and CD44 during anti-EGF receptor therapy of patients with colorectal cancer or in patients resistant to EGF receptor inhibitors.

Keywords: Colon Cancer; Hepatocyte Growth Factor; Colorectal Carcinogenesis; WNT.

Intestinal epithelial self-renewal is a continuous and tightly controlled process. Intestinal stem cells (ISCs), marked by leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) expression, reside at the crypt base between Paneth cells and give rise to a population of rapidly dividing progenitor cells that terminally differentiate into all cell types of the gut.[1,2] Wnt signaling plays a key role in maintaining the intestinal crypt compartment by driving the activation of a target-gene program that is required for ISC self-renewal and progenitor proliferation.[3–8] Aberrant Wnt-pathway activation in ISCs, resulting in constitutive Wnt target-gene transcription, leads to malignant transformation.[9] Thus, Wnt targets are crucial in the regulation of intestinal epithelial self-renewal as well as in tumorigenesis.

Abbreviations used in this paper: **β-gal, β-galactosidase; CRC, colorectal cancer; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; HGF, hepatocyte growth factor; ISC, intestinal stem cell; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5; mCRC, metastatic human colorectal cancer; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinase; TBI, total body irradiation.**

EDITOR'S NOTES

BACKGROUND AND CONTEXT

Resistance of human colorectal cancer cells to drugs that block epidermal growth factor receptor (EGFR) signaling could be caused by aberrant activity of other receptor tyrosine kinases, activating overlapping signaling pathway.

NEW FINDINGS

MET signaling regulates intestinal homeostasis, regeneration, and adenoma formation. These activities of MET are promoted by stem-cell CD44 isoforms.

LIMITATIONS

The study did not measure the effect of simultaneous deletion or inhibition of the EGFR and MET.

IMPACT

These results provide rationale for targeting signaling via MET and CD44 in patients with colorectal cancer during anti-EGR receptor treatment or patients resistant to EGFR inhibitors.

Interestingly, in the presence of the stem cell niche factors Wnt, R-spondin, Noggin, and epidermal growth factor (EGF), both mouse and human ISCs can be cultured ex vivo over long periods as epithelial organoid "mini-guts" containing all differentiated intestinal cell lineages.[10] Although EGF/EGF receptor (EGFR) signaling is generally credited for providing the necessary receptor tyrosine kinase (RTK) signals regulating ISC homeostasis, this notion is largely based on experimental evidence obtained in *Drosophila,* an organism with a limited RTK repertoire,[11] and on stem cell–specific deletion of *Lrig-1* a regulator of multiple tyrosine kinases, including both the EGFR and MET.[12] Hence, although capable of supporting ISCs in intestinal organoid cultures, the specific role of EGFR signaling in ISC homeostasis and intestinal tumorigenesis in vivo and possible contribution of other RTKs and their ligands, remains incompletely defined. Indeed, although an impaired regenerative response and reduced adenoma formation were reported in *Egfr* mutant mouse models, these models did not display spontaneous intestinal defects,[13–16] indicating a redundant role of EGFR signals under homeostatic conditions. Furthermore, studies with EGFR-blocking antibodies in metastatic human colorectal cancer (mCRC) suggest that therapy resistance can be caused by aberrant activity of alternative RTKs.[17,18] A potential candidate in this context is MET, which can be activated by either *MET* amplification, or by overexpression of MET and/or the MET ligand hepatocyte growth factor (HGF).[17,19–23] Interestingly, apart from mediating growth and survival, HGF, also known as "scatter factor," is a potent inducer of tumor dissemination and invasive growth,[24,25] whereas *Met* is a transcriptional target of Wnt signaling[26] and is strongly upregulated in colorectal cancers (CRCs) and their precursors.[24,27] Together, these studies suggest that HGF/MET signaling contributes to ISC fate, and that mCRCs may escape from addiction to EGFR signaling by co-opting HGF/MET signaling.[17,19–23]

In this study, we therefore explored the role of MET in ISC homeostasis and intestinal tumorigenesis. We report that HGF/MET signaling can fully substitute EGFR signals in intestinal organoid cultures and moreover promote expansion of adenoma spheroids. Furthermore, by using inducible *Met*-deficient mouse models, we show that MET is involved in the regenerative response of ISCs to irradiation, as well as in intestinal adenoma formation. Finally, we show that ISC-specific CD44v-isoform(s) regulate HGF/MET-dependent expansion of both normal and neoplastic intestinal organoids.

Methods

Crypt and ISC Culture

The isolation and culture of crypts and LGR5-expressing cells has been described previously.[10,28] In short, duodenal cells and crypts were isolated from adult mice at 7 to 12 weeks of age, after repeated washing with phosphate-buffered saline (PBS) followed by incubation with 2 to 5 mM EDTA. Isolated crypts were dissociated to single cells by means of a second round of incubation with EDTA (2 mM) in serum-free medium containing 50% Wnt3a conditioned medium and 10 μM Y-27632 (Sigma-Aldrich, St Louis, MO) to prevent cell death. For each experiment, crypts or cells were pooled from 3 intestines. Approximately 200 purified crypts or 500 green fluorescent protein (GFP)-high LGR5-expressing cells were mixed with 50 μL Matrigel (BD Biosciences, San Jose, CA) and plated in 24-well plates using at least 3 wells for each condition. Cultures were incubated with serum-free Advanced Dulbecco's modified Eagle's medium M/F12 (Invitrogen, Carlsbad, CA) supplemented with 100 ng/mL Noggin (Peprotech, Rocky Hill, NJ), 500 ng/mL R-spondin1 (R&D Systems, Minneapolis, MN), N2 and B27 supplements (Invitrogen) and EGF (Peprotech), and/or HGF (Reliatech, Hamburg, Germany) at indicated concentrations. Growth factors were added every other day, and the entire medium was changed every 4 days. Epithelial structures were photographed using an Olympus (CKX41; Tokyo, Japan) microscope with a Coolsnap CF camera (Photometrics, Tucson, AZ) and surface area was quantified by use of ImageJ software (National Institutes of Health, Bethesda, MD).

Mice

All mouse studies were approved by the animal ethical committee of the Academic Medical Center, Amsterdam. *Lgr5-EGFP-ires-CreERT2 (Lgr5^{Creert2})* and *Apc$^{fl/fl}$* mice have been previously described.[1,29] *Met$^{fl/fl}$* mice were a generous gift of C. Birchmeier (MGI:3050810).[30] *Rosa^{LacZ} (LacZ)*[31] reporter mice were obtained from Jackson Laboratories (Bar Harbor, ME) and *Ah^{Cre}* mice were obtained from D. Winton.[32] *Cd44$^{-/-}$*, *Cd44$^{s/s}$*, and *Cd44$^{v4-10/v4-10}$* were described previously,[33,34] and backcrossed to C57Bl/6J for more than 10 generations. Homozygous offspring on a C57Bl/6J background were obtained by a final interbreeding. *Apc$^{Min/+}$* mice expressing specific CD44 splice variants were generated by crossings with C57Bl/6J-min/+ (*Apc$^{Min/+}$*) mice (Jackson Laboratory). Recombination in vivo was induced by 2 single intraperitoneal injections of 250 μL 10 mg/mL Tamoxifen (Sigma) with a 2-day interval for *Lgr5^{Creert2}* mice. Recombination in *Ah^{Cre}* mice was induced by 5 daily

injections of 300 mg/kg betanaphtoflavone (Sigma) intraperitoneally according to Ireland et al.[32] Three daily betanaphtoflavone injections were used to establish optimal recombination for mice in radiation experiments. Mice received 10 Gy total body irradiation (TBI), 0.85 Gy/min using a 137Cs IBL 637 (CIS Bio International, Saclay, France) and were killed after 72 hours. The intestines were removed and flushed with PBS, opened longitudinally, and processed to Swiss rolls that were fixated in formalin and embedded in paraffin. All control mice were littermates and all mice in studies with Cre-mediated recombination contained a *LacZ* reporter. Of all mice used, a DNA sample was taken to confirm the mouse genotypes.

Adenoma Culture

Adenomas were dissected from the proximal small intestine of chemically induced $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ and $Lgr5^{Creert2}/Met^{+/+}/Apc^{fl/fl}$ mice and from $Cd44^{+/+}$, $Cd44^{s/s}$, or $Cd44^{v4-10/v4-10}$-$Apc^{Min/+}$ mice and dissociated by trypsin treatment together with 2000 U/mL DNase (Sigma) for 30 minutes at 37°C. Dissociated fragments were passed through a 70-mesh filter (BD Biosciences) and washed with PBS. Fractions were cultured in Matrigel with serum-free Advanced Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) in the absence of Noggin/R-spondin for 7 days, after which adenoma organoids were collected, washed, and dissociated into subfractions. Fractions were embedded into Matrigel and covered with medium supplemented with 100 ng/mL HGF or 50 ng/mL EGF after which spheroids were allowed to expand over a 4-day period. Adenoma organoids were daily photographed using an Olympus (CKX41) microscope with a Coolsnap CF camera and surface area was quantified by use of ImageJ software.

Met^{fl} Recombination Analysis

Efficient recombination of the Met^{fl} allele was determined by means of quantitative POLYMERASE CHAIN REACTION analysis using DNA isolated from whole tissue sections of Ah^{Cre} or $Lgr5^{Creert2}$ x $Met^{fl/fl}$ or $Met^{fl/+}$ using primers flanking and overlapping the recombination site. Primers used for the recombined *Metd* allele were 5′-CACAGAGGATAGCCCCATACC-3′ and 5′-AGCAAATATGTGAGCTCTGTCAA-3′ and for Exon16 as control primers 5′-CCCCTATCCTGACAAGTGGA-3′ and 5′-AATTTCGCA GATCTCCATGC-3′. Recombination status of adenoma derived from Ah^{Cre} or $Lgr5^{Creert2}$ x $Met^{fl/f}/Apc^{fl/fl}$ or $Met^{+/+}/Apc^{fl/fl}$ was determined using the following primers: 3′LoxP 5′-ATG CAACGTGTCCCCTTTTA-3′ and 5′-AGCAAATATGTGAGCTCTGT CAA-3′, *MetΔ* 5′-AGCCTAGTGGAATTCTCTGTAAG-3′ and 5′-AGCAAATATGTGAGCTCTGTCAA-3′, Exon16 5′-CCCCTATCCT GACAAGTGGA-3′ and 5′-CTACGTGCTGAACTGCTTGG-3′, and Exon18 5′-CATGAAAGACTTCAGCCATCC-3′ and 5′-AATTTCGCA GATCTCCATGC-3′.

Immunohistochemistry

Sections of 5 μm were cut from paraffin-embedded Swiss rolls, or whole organoids were isolated from Matrigel and immunohistochemical stainings were performed for cell proliferation using Rabbit-anti-Ki67 (SP6; Thermo Fisher Scientific, Waltham, MA), for apoptosis using Rabbit-anti-cleaved Caspase-3 (Asp175; Cell Signaling, Danvers, MA), for Rabbit-anti-β-gal (A11132; Life Technologies, Carlsbad, CA) as readout for recombination as well for the differentiation markers, Rabbit-anti-Synapthofysin for enteroendocrine cells (SP11; Thermo Fisher Scientific), Rabbit-anti-Dclk1 for Tuft cells (ab31704; Abcam, Cambridge, UK), and Rabbit-anti-Lysozyme for Paneth cells (EC3.2.1.17; Dako, Santa Clara, CA). Alcian blue or Pas-D and alkaline phosphatase were used to visualize Goblet cells and enterocytes, respectively. Analysis of Ki67-positive proliferating cells in crypts was performed unilaterally. Swiss roll sections were scanned using a Philips IntelliSite UFS (Philips, Cambridge, MA) and further processed and analyzed using Philips Digital Pathology Solutions and ImageJ software.

Statistical Analysis

Student *t* test was used to test for statistical significance. Error bars represent mean ± SEM.

Results

HGF/MET Signaling Can Mediate Outgrowth of ISCs into "Mini-gut" Organoids, Substituting EGFR Signals

RTK signaling is essential for proliferation of normal and neoplastic intestinal epithelium. The established RTK stimulus for proliferation in Matrigel-based intestinal organoid cultures is EGF,[10] but the fact that *Egfr* deletion has no effect on intestinal homeostasis in vivo[13] suggests redundancy among RTKs. A prime candidate is MET: EGFR and MET activate largely overlapping intracellular signaling cascades. Moreover, in neoplastic intestinal epithelium, MET signaling can protect cells from EGFR inhibition.[17,19,35] However, whether or not HGF, like EGF, indeed can mediate ISC expansion and/or crypt-villus development has not been explored. To address this question, we established murine intestinal crypt cultures, which under defined conditions develop into organoids comprising crypt-villus units in situ ("mini-guts").[10] Crypts from wild-type C57BL/6J mice were seeded in Matrigel with appropriate nutrients,[10] supplemented with EGF, HGF, EGF and HGF, or medium alone. As expected, in the presence of EGF, crypts expanded into self-organizing mini-guts containing multiple crypt-like structures (Figure 1*A*), whereas crypts cultured in the absence of RTK stimuli remained dormant (Figure 1*A* and *C*). Interestingly, we observed that HGF was able to fully replace EGF in mini-gut formation with similar organoid expansion rates (Figure 1*A* and *C*; Supplementary Figure 1*A*). Stimulation with a combination of EGF and HGF further enhanced organoid expansion (Figure 1*B*). Importantly, ISC-specific *Met* deletion in crypt cells derived from *Lgr5-EGFP-ires-CreERT2*($Lgr5^{Creert2}$)/$Met^{fl/fl}$ mice resulted in >50% reduction in HGF-mediated organoid expansion (Figure 1*D*), but did not influence EGF-dependent crypt expansion (Figure 1*E*), confirming the involvement of MET signaling. In addition, ISC-specific *Met* deletion resulted in a strong reduction of GFP-expressing organoids (Supplementary Figure 1*B*). Notably, the observed partial growth inhibition can be explained by the variegated Cre recombinase activation in $Lgr5^{Creert2}$ mice.[36] These results show that HGF,

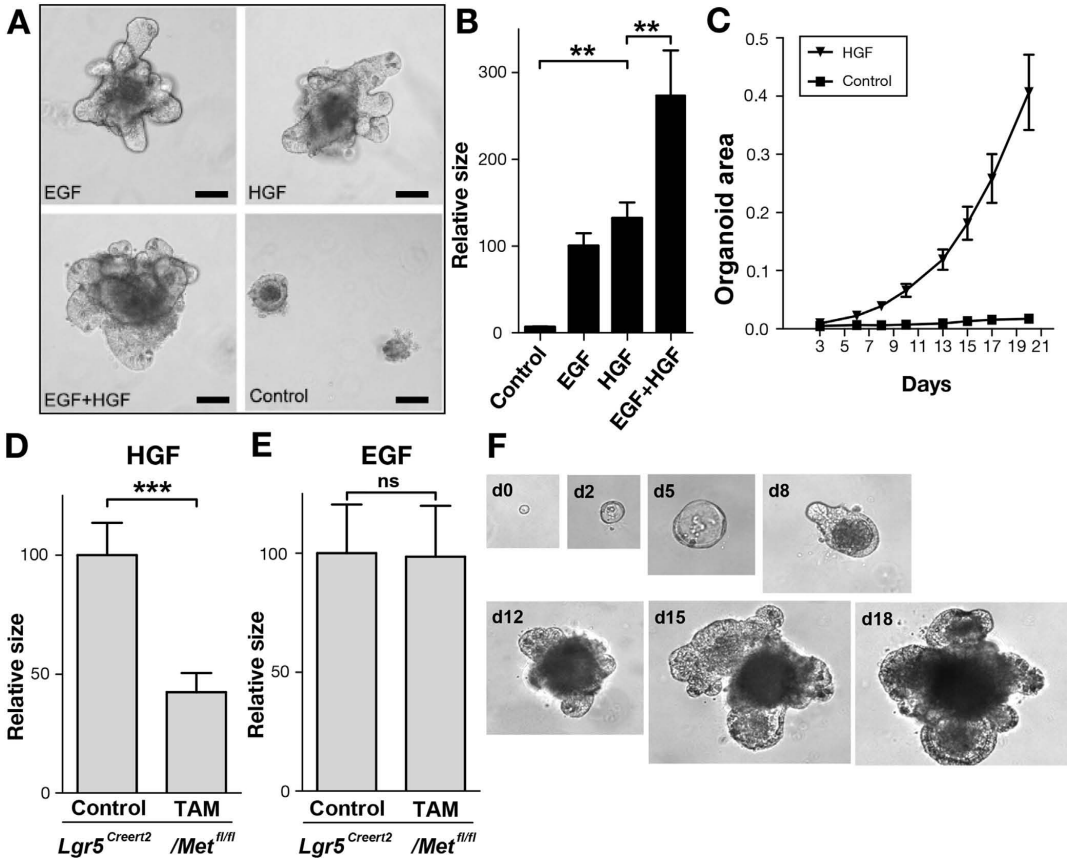

Figure 1. HGF/MET signaling can mediate outgrowth of ISCs into mini-gut organoids, substituting EGFR signals. (*A*) Development of mini-gut organoids from intestinal crypts after 7 days of culture in medium supplemented with either EGF, HGF, EGF + HGF, or control (no growth factor). (*B*) Relative mini-gut size at day 10 after plating (n = 10; $^{**}P < .01$). (*C*) Time kinetics of organoid expansion stimulated in the presence of HGF-containing or control medium. (*D*, *E*) Relative expansion of crypts from $Lgr5^{Creert2}/Met^{fl/fl}$ mice stimulated with HGF or EGF in the presence or absence of 4-hydroxytamoxifen (TAM) (n = 10; $^{***}P < .001$). (*F*) Mini-gut formation starting with sorted LGR5+ stem cells from *Lgr5-EGFP-ires-creERT2* small intestines in culture medium supplemented with HGF. Crypt-like structures start to form 1 week after plating.

like EGF, can provide the RTK signals required for crypt expansion and mini-gut formation.

To establish whether HGF can also mediate expansion of ISCs and support differentiation into mini-guts, LGR5-expressing cells were sorted from $Lgr5^{Creert2}$ mice based on GFP expression[1,37] and cultured in the presence of HGF. Approximately 0.10% of the cells formed rounded cysts within 1 week, which subsequently developed into mini-guts (Figure 1*F*) containing all differentiated epithelial lineages (Supplementary Figure 1*C*). Wild-type $Lgr5^{Creert2}$-derived ISCs cultured without HGF (or EGF) and tamoxifen-treated ISCs from $Lgr5^{Creert2}/Met^{fl/fl}$ mice stimulated with HGF showed no signs of expansion (data not shown). These results demonstrate that HGF/MET signaling can support mini-gut formation from a single ISC onward.

MET Is Dispensable for Normal Intestinal Homeostasis

The previous findings demonstrate that HGF/MET signaling can mediate the development of ISCs into mini-gut organoids, suggesting an important role for MET signaling in intestinal homeostasis and, conceivably, also in intestinal tumorigenesis. However, no data on the in vivo role of MET signaling in the intestine were available. To study the role of MET signaling in crypt homeostasis, we generated 2 inducible mouse models (ie, $Lgr5^{Creert2}/Met^{fl/fl}/LacZ$ and $Ah^{Cre}/Met^{fl/fl}/LacZ$ mice). In the $Lgr5^{Creert2}$ model, activation of Cre leads to ISC-specific deletion of *Met*, whereas in the Ah^{Cre} model, Cre activation induces *Met* deletion throughout the intestinal epithelium. These 2 models thus allow to distinguish between the consequences of ISC-specific and

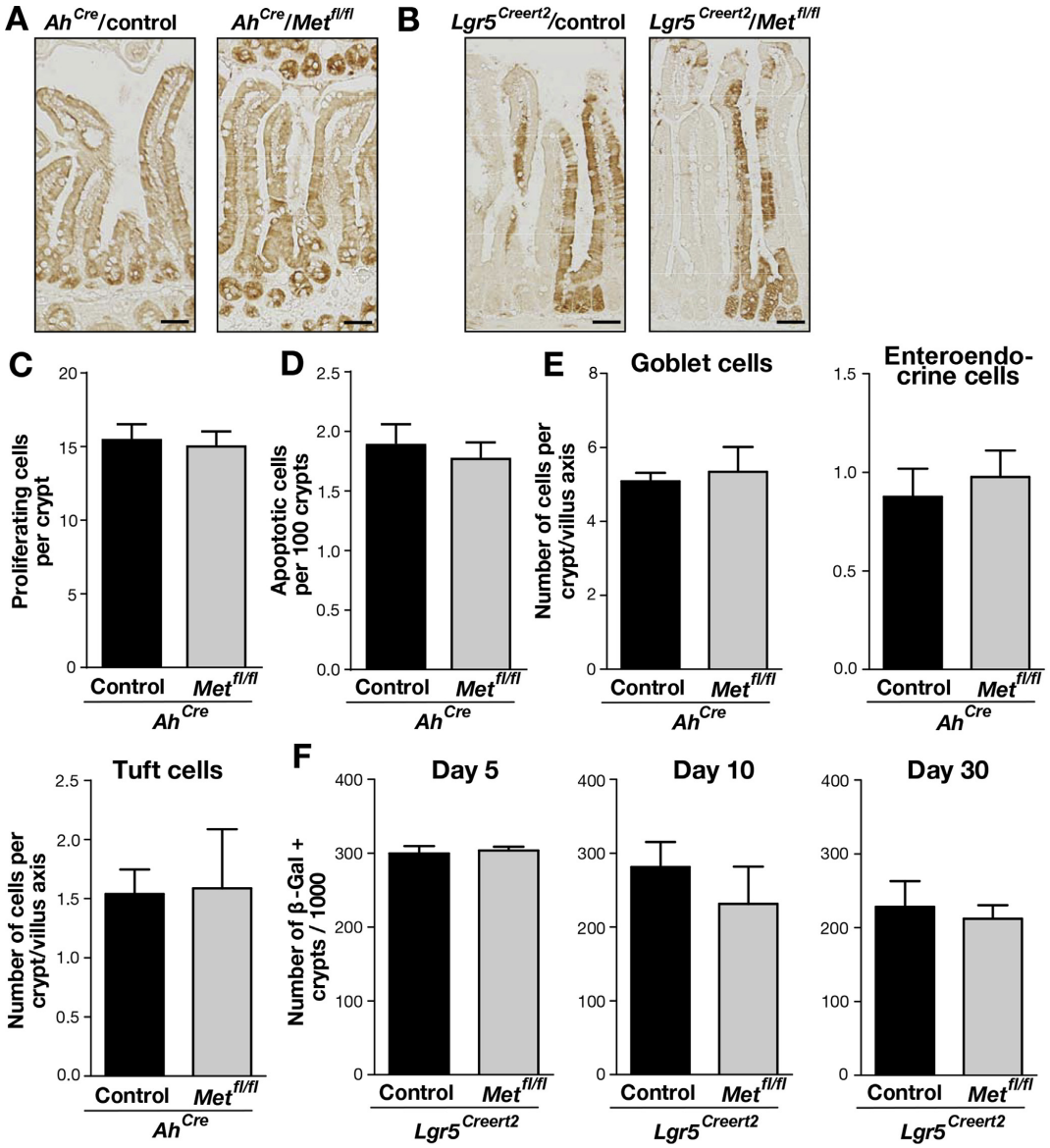

Figure 2. MET expression is dispensable for normal intestinal homeostasis. (*A*, *B*) β-Gal stain on Ah^{Cre} (*A*) and $Lgr5^{Creert2}$ (*B*) $Met^{fl/fl}$ and $Met^{+/+}$ control intestines 7 days after Cre-mediated recombination. (*C*, *D*) Quantification of Ki67+ cells and Caspase-3+ cells per crypt ($n \geq 3$) in $Ah^{Cre}/Met^{fl/fl}$ and $Ah^{Cre}/Met^{+/+}$ control mice. (*E*) Quantification of intestinal cell populations for Goblet, Enteroendocrine, and Tuft cells ($n \geq 3$) in $Ah^{Cre}/Met^{fl/fl}$ and $Ah^{Cre}/Met^{+/+}$ control mice. (*F*) Number of β-gal+ crypts per 1000 crypts in $Lgr5^{Creert2}/Met^{fl/fl}$ and $Lgr5^{Creert2}/Met^{+/+}$ littermate control mice at day 5, 10, or 30 after induction of Cre-mediated recombination ($n \geq 3$).

complete epithelial *Met* deletion. In both models, immunohistochemical staining for β-galactosidase (β-gal) expression was used to monitor Cre-mediated recombination (Figure 2*A* and *B*). Induction of recombination in the $Lgr5^{Creert2}$ model resulted in a variegated β-gal expression pattern (Figure 2*B*), indicating partial *Met* deletion as reported for this model.[36] By contrast, in the Ah^{Cre} model, all epithelial cells became β-gal–positive on Cre activation,

indicating highly efficient *Met* deletion (Figure 2*A*). Quantitative POLYMERASE CHAIN REACTION analysis of tissue sections of $Lgr5^{Creert2}$ and Ah^{Cre} mice underscored the efficient Met^{fl} recombination (Supplementary Figure 2*A*). Importantly, in neither of the 2 models were gross alterations in intestinal integrity or architecture observed on *Met* deletion (Figure 2*A* and *B*). In the Ah^{Cre} model, the MET-deficient epithelial lining was intact, whereas the MET-deficient ISCs of the $Lgr5^{Creert2}$ mouse model proliferated and gave rise to β-gal–positive crypt-villus units.

To investigate whether *Met* deficiency alters proliferation, apoptosis at the crypt base, or differentiation along the crypt-villus axis, we performed stainings on $Ah^{Cre}/Met^{fl/fl}$ or control $Ah^{Cre}/Met^{+/+}$ intestines to identify cycling and apoptotic cells as well as the various differentiated intestinal cell types, including Paneth cells, enterocytes, Goblet cells, and Enteroendocrine and Tuft cells. No significant changes in numbers of proliferating or apoptotic cells (Figure 2*C* and *D*; Supplementary Figure 3*A* and *B*) or in subset prevalence or distribution of differentiated cell types were observed (Figure 2*E*; Supplementary Figure 3*C*), indicating that *Met* deficiency does not affect intestinal epithelial homeostasis.

Notably, the partial recombination in $Lgr5^{Creert2}$ mouse model results in the presence of *Met*-deficient and *Met*-proficient stem cells, side-by-side within a single crypt. Competition between ISCs in this model may therefore reveal possible effects of *Met* deletion on stem cell fitness. As shown in Figure 2*F*, in both $Met^{fl/fl}$ and $Met^{+/+}$ control mice 30% to 35% of the crypts showed β-gal expression at 5 days after induction of Cre activity, a percentage that slowly declined over time as a result of neutral drift. Hence, there was no evidence for a competition advantage of *Met*-proficient over *Met*-deficient stem cells. Taken together, our data show that MET is dispensable for crypt-cell proliferation, survival, and differentiation under homeostatic conditions.

MET Deficiency Attenuates Intestinal Regeneration After Radiation-Induced Damage

MET signaling has been reported to play an essential role in tissue repair, including liver regeneration and wound healing.[30,38] To assess whether MET plays a role in intestinal regeneration, we used a radiation-induced damage model in which mice are exposed to 10 Gy TBI. This results in intestinal damage with a maximal regenerative response at 72 hours.[39] Regeneration can be assessed by quantifying the proliferative activity of the crypts by measuring Ki67 expression in tissue sections (Figure 3*A*). In MET-deficient $Ah^{Cre}/Met^{fl/fl}/LacZ$ mice, the crypt-proliferative response to TBI was significantly attenuated as compared with MET-proficient $Ah^{Cre}/Met^{+/+}/LacZ$ littermates (Figure 3*B*), indicating that *Met* deficiency results in impaired intestinal epithelial recovery.

To specifically study the role of ISC-expressed MET in intestinal regeneration, we explored the effect of *Met* deletion in the ISC-specific $Lgr5^{Creert2}$ model. Similar to the results obtained in the $Ah^{Cre}/Met^{fl/fl}/LacZ$ mice, irradiated $Lgr5^{Creert2}/Met^{fl/fl}/LacZ$ mice also showed reduced numbers of Ki67-expressing crypt cells compared with their wild-type littermates. This reduction was approximately one-third of that in $Ah^{Cre}/Met^{fl/fl}/LacZ$ mice (Figure 3*B*),

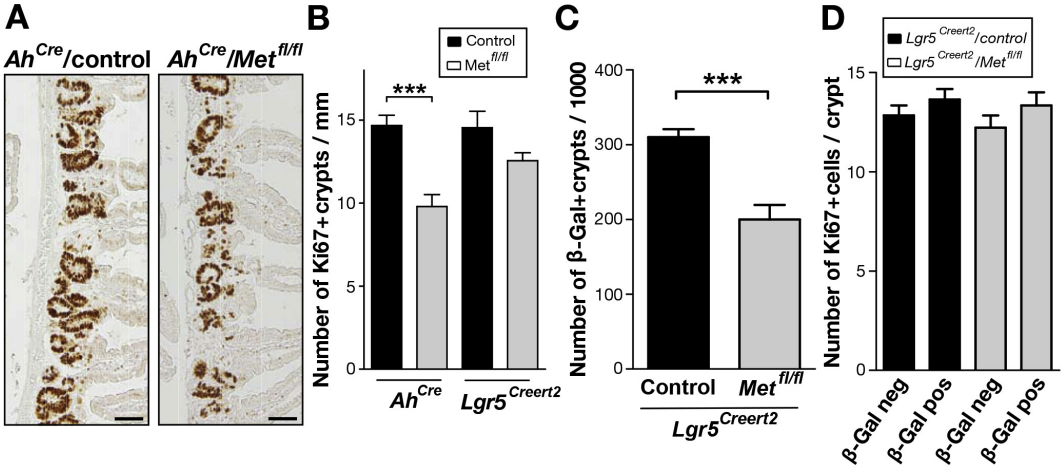

Figure 3. *Met* deficiency leads to attenuated repair of radiation-induced damage. (*A*) Cycling crypt cells in $Ah^{Cre}/Met^{fl/fl}$ vs $Ah^{Cre}/Met^{+/+}$ after Cre-mediated recombination of *Met*. Representative picture of Ki67 expression at 72 hours after 10 Gy radiation. (*B*) Quantification of cycling crypts in $Ah^{Cre}/Met^{fl/fl}$ vs $Ah^{Cre}/Met^{+/+}$ mice and in $Lgr5^{Creert2}/Met^{fl/fl}$ vs $Lgr5^{Creert2}/Met^{+/+}$ after Cre-mediated recombination of *Met*. Number of Ki67+ crypts/mm measured at 72 hours after 10 Gy radiation (n = 5; ***$P < .001$). (*C*) Number of β-gal+ crypts per 1000 crypts in $Lgr5^{Creert2}/Met^{fl/fl}$ *vs* $Lgr5^{Creert2}/Met^{+/+}$ control littermates at 72 hours after 10 Gy radiation (n = 10; ***$P < .001$). (*D*) Number of Ki67+ cells per β-gal–positive or –negative crypt at 72 hours after 10 Gy radiation.

reflecting the lower recombination efficiency in the $Lgr5^{Creert2}$ model. This partial recombination in the model creates a potentially competitive setting between *Met*-deficient and *Met*-proficient ISCs within a single crypt. To determine whether *Met* deletion influences the regenerative capacity of ISCs exposed to irradiation, we quantified the percentage of β-gal–positive crypt-villus units in the $Lgr5^{Creert2}/Met^{fl/fl}/LacZ$ mice and their control $Lgr5^{Creert2}/Met^{+/+}/LacZ$ littermates. Interestingly, in the *Met*-deficient $Lgr5^{Creert2}/Met^{fl/fl}/LacZ$ mice, a significant reduction in β-gal–positive crypts was found (Figure 3*C*). This reduction was in line with the (moderate) reduction in Ki67-positive crypts (Figure 3*B*). These results indicate that *Met*-deficient ISCs have a competitive disadvantage during regeneration from radiation-induced damage. Interestingly, on β-gal/Ki67 double staining, equal numbers of cycling cells were found in β-gal–positive and –negative crypts (Figure 3*D*). This indicates that the *Met*-deficient ISCs that survive irradiation are as capable of forming offspring as their wild-type counterparts. Together, these data show that MET is involved in intestinal epithelial regeneration after TBI, presumably by enhancing stem cell fitness.

MET Signaling Promotes Expansion of Adenoma Organoids

The signaling events during intestinal regeneration and tumorigenesis show marked similarities. Both processes are driven by Wnt-pathway activation, including activation of the downstream targets like MYC and AKT,[40] key mediators of tumorigenesis, metastasis, and relapse. To study the impact of HGF/MET signaling on adenoma growth, adenomas derived from $Apc^{Min/+}$ mice were dissociated and cultured in Matrigel, where they form rounded adenoma organoids (Figure 4*A*). These spheroids were grown in the presence of either HGF-containing or control medium and their expansion was quantified for 4 days. HGF-stimulated adenoma spheroids expanded to almost twice the size of nonstimulated spheroids (Figure 4*B*). To further investigate HGF-stimulated expansion, we generated adenomas from $Lgr5^{Creert2}/Apc^{fl/fl}/Met^{fl/fl}$ and

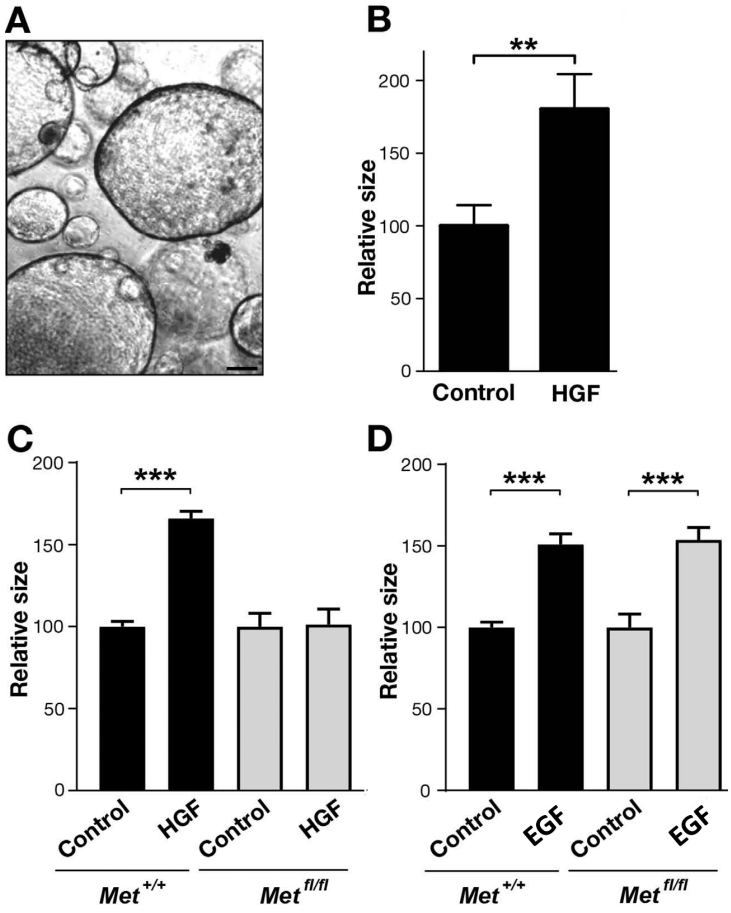

Figure 4. MET signaling promotes expansion of adenoma organoids. (*A*) Example of an adenoma spheroid culture derived from $Apc^{Min/+}$ mice 1 week after reseeding. (*B*) Relative expansion of $Apc^{Min/+}$ derived adenoma organoids stimulated with HGF-containing or control medium (no growth factor) (n = 30; $^{**}P < .01$). (*C*, *D*) Relative expansion of tamoxifen induced $Lgr5^{Creert2}/Apc^{fl/fl}$-derived adenoma organoids stimulated with HGF- or EGF-containing or control medium (no growth factor) (n = 30; $^{***}P < .001$).

$Lgr5^{Creert2}/Apc^{fl/fl}/Met^{+/+}$ organoids by administration of tamoxifen, yielding *Met*-proficient and *Met*-deficient adenoma cultures. These were subsequently replated in medium containing HGF, EGF, or control medium, and growth was monitored for 4 days. *Met*-proficient, but not *Met*-deficient, adenomas responded to HGF with accelerated growth (Figure 4*C*). By contrast, EGF accelerated expansion in both models (Figure 4*D*). These experiments show that HGF/MET signaling results in accelerated expansion of adenoma organoids.

Met *Deletion Attenuates Adenoma Formation In Vivo*

We next explored the possible role of HGF/MET signaling in adenoma formation in vivo. Initially, we compared adenoma formation in $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ and $Lgr5^{Creert2}/Met^{+/+}/Apc^{fl/fl}$ littermates. In this model, Cre-mediated deletion of both *Apc* alleles, with or without concomitant deletion of *Met*, leads to a vast induction of Wnt signaling in ISCs, resulting in the formation of multiple adenomas within 3 weeks.[41] Upon Cre activation, we indeed observed massive adenoma formation throughout the intestinal mucosa (data not shown). This adenoma formation occurred in both the *Met*-deficient $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ and *Met*-proficient $Lgr5^{Creert2}/Met^{+/+}/Apc^{fl/fl}$ littermates, but the effect of *Met* deletion could not be accurately assessed because the multiplicity and confluence of the adenomatous changes prohibited reliable macroscopic adenoma enumeration. However, histologic examination of this mouse model revealed a highly significant reduction of the number of microadenoma in *Met*-deficient mice (Figure 5*A*). This was predominantly caused by a reduction of microadenomas in the ileum and colon (Supplementary Figure 4*A*). Equal numbers of further developed adenomas were observed (Figure 5*B*), but, interestingly, *Met*-proficient adenomas had expanded to a larger average size compared with *Met*-deficient adenomas (Figure 5*C*). Indeed, further subdivision by adenoma size revealed that *Met*-proficient mice had developed significantly more adenomas of >1 mm and significantly fewer small adenomas (<0.5 mm) (Figure 5*D*). No differences in adenoma distribution were found (Supplementary Figure 4*B*).

To clarify the previous findings, we compared cell proliferation and apoptosis in *Met*-proficient and *Met*-deficient microadenomas and adenomas. No differences in the Ki67 expression were found (Figure 5*E* and *F*). Interestingly, however, the number of apoptotic, Caspase-3 positive cells, in *Met*-deficient microadenomas was approximately twofold higher than in *Met*-proficient microadenomas (Figure 5*G*). This elevated apoptotic rate was not observed in full-blown adenomas (Figure 5*H*). These findings may explain the observed reduced microadenoma numbers and average adenoma size in the absence of MET (Figure 5*A* and *C*).

To study the role of MET in adenoma formation in a more physiological setting, we subsequently used $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/+}$ and $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ mice and control littermates. Similar to intestinal adenoma formation in human individuals with familial adenomatous polyposis and in $Apc^{Min/+}$ mice, stochastic loss of the second *Apc* allele is required for adenoma formation in these models. In the ISC-specific $Lgr5^{Creert2}/Apc^{fl/+}$ model, very low numbers of adenomas were obtained (1.3 ± SD 2.6 for *Met*-proficient mice), preventing assessment of the expected moderate impact of *Met* deletion. Interestingly, however, in the $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ model, total intestinal epithelial *Met* deficiency resulted in an approximately 50% reduction in adenoma numbers (Figure 5*I*) and in a significant reduction of microadenoma formation (Figure 5*J*). *Met*-proficient $Ah^{Cre}/Met^{+/+}/Apc^{fl/+}$ mice developed more adenomas in the duodenum and jejunum compared with *Met*-deficient $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ mice (Supplementary Figure 4*C*), but no differences in adenoma size were observed (Supplementary Figure 4*D*). Analysis of DNA extracted from tissue sections demonstrated that adenomas originated from recombined cells (Supplementary Figure 2*B*). Taken together, these results demonstrate a functional role of MET in adenoma formation in vivo.

Intestinal Stem Cell CD44v Isoforms Support HGF-Dependent Mini-gut Formation and Adenoma Growth

CD44 is a prime transcriptional target of Wnt signaling and, consequently, is strongly expressed in ISCs and adenomas.[37,42] We recently demonstrated that a specific CD44 variant (CD44v) isoform is abundantly expressed in ISCs and promotes intestinal adenoma formation in vivo.[34,42] By contrast, adenoma formation was not supported by the CD44s isoform. Because previous studies in cell lines, including human CRC cells, have provided evidence for CD44v involvement in RTK, and particularly in MET, signaling,[43–45] we explored if CD44v indeed functionally collaborates with MET and EGFR signaling in normal and neoplastic intestinal epithelium, by stimulating crypts from $Cd44^{+/+}$ and $Cd44^{-/-}$ mice with EGF- or HGF-supplemented medium.

In the presence of EGF, $Cd44^{-/-}$ crypts showed a similar expansion compared with $Cd44^{+/+}$ crypts (Figure 6*A*). However, HGF-stimulated *Cd44*-deficient crypts showed a strongly reduced expansion as compared with $Cd44^{+/+}$ crypts (Figure 6*B* and *C*). These findings demonstrate a functional role of CD44 in HGF-mediated expansion of crypts into mini-guts.

To investigate whether CD44v or CD44s mediates HGF-induced intestinal epithelial expansion, we isolated crypts from *Cd44* splice variant knockin mice, which selectively express CD44v4–10 (the largest isoform in ISCs) or CD44s.[34] Interestingly, in the presence of HGF, crypts expressing CD44s showed a strongly reduced expansion comparable to $Cd44^{v4-10/v4-10}$ mice (Figure 6*B* and *C*). By contrast, the expansion of CD44v4–10 crypts was comparable to $Cd44^{+/+}$ mice (Figure 6*A* and *B*). These findings demonstrate that CD44v, but not CD44s, controls HGF-driven intestinal epithelial expansion into mini-gut organoids. On EGF stimulation, all crypts showed a similar expansion rate (Figure 6*A*), demonstrating that EGF/EGFR-driven crypt expansion is CD44 independent and that the growth potential of the crypts from the distinct CD44 isoform knockin mice were comparable.

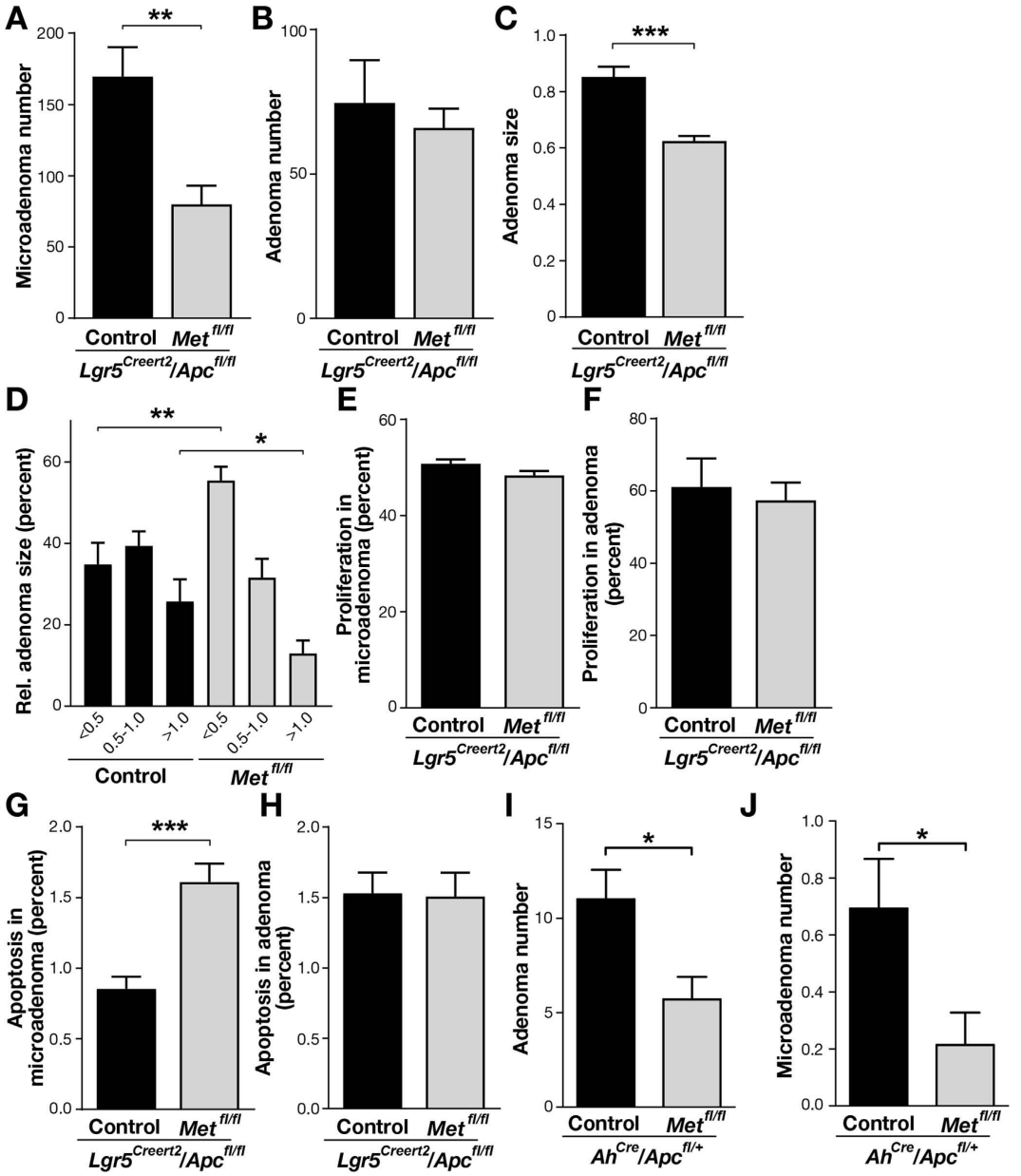

Figure 5. *Met* deletion attenuates adenoma formation in vivo in both $Lgr5^{Creert2}$ and Ah^{Cre} mouse models. (*A*, *B*) Number of microadenomas and adenomas present in a single Ki67-stained Swiss roll section of $Lgr5^{Creert2}/Apc^{fl/fl}/Met^{fl/fl}$ or $Lgr5^{Creert2}/Apc^{fl/fl}/Met^{+/+}$ control mice ($n \geq 6$; $^{**}P < .01$). (*C*) Adenoma size of (*B*) ($^{***}P < .001$). (*D*) Size distribution of adenomas as percentage of adenomas per group ($^{*}P < .05$; $^{**}P < .01$). (*E*, *F*) Quantification of Ki67+ cells in (micro)adenoma in $Ah^{Cre}/Met^{fl/fl}$ and $Ah^{Cre}/Met^{+/+}$ control mice ($n \geq 6$). (*G*, *H*) Quantification of Caspase3+ cells in (micro)adenoma in $Ah^{Cre}/Met^{fl/fl}$ and $Ah^{Cre}/Met^{+/+}$ control mice ($n \geq 6$; $^{***}P < .001$). (*I*) Number of intestinal adenomas in $Ah^{Cre}/Apc^{fl/+}/Met^{fl/fl}$ vs $Ah^{Cre}/Apc^{fl/+}/Met^{+/+}$ littermates 16 weeks after Cre-induction ($n = 14$; $^{*}P < .05$). (*J*) Number of microadenoma in $Ah^{Cre}/Apc^{fl/+}/Met^{fl/fl}$ vs $Ah^{Cre}/Apc^{fl/+}/Met^{+/+}$ scored on Ki67-stained tissue sections ($n = 14$; $^{*}P < .05$).

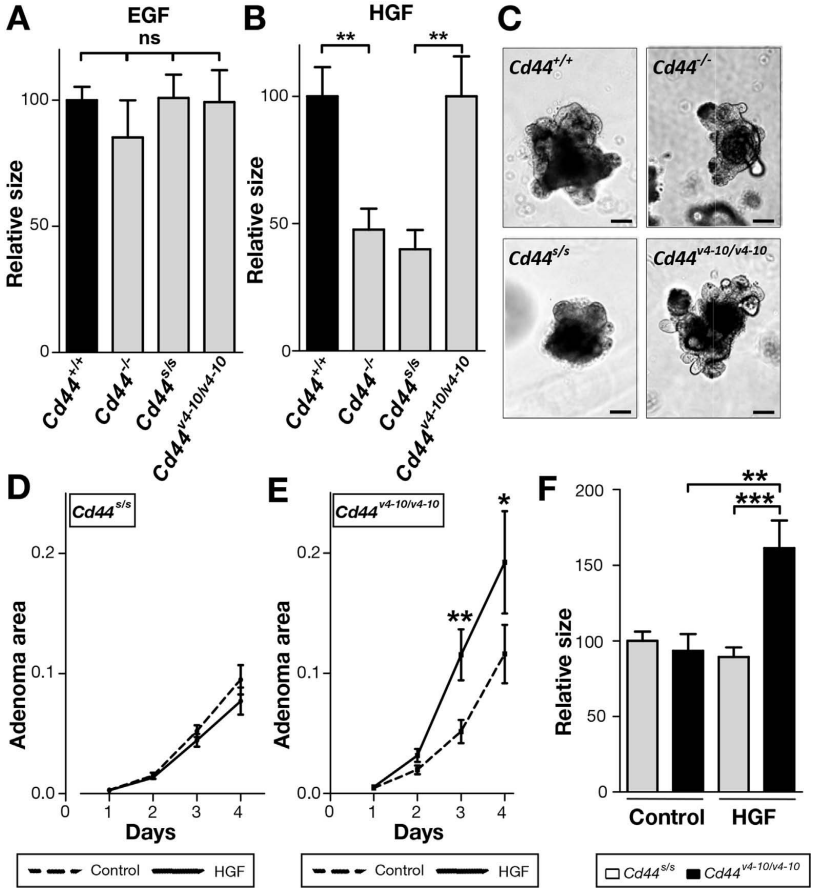

Figure 6. Intestinal stem cell CD44v isoforms supports HGF-dependent mini-gut formation and adenoma growth. (*A*) Relative size of small intestinal organoids from crypts of $Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{s/s}$, and $Cd44^{v4-10/v4-10}$ mice cultured for 10 days in medium supplemented with EGF (50 ng/mL). (*B*) Organoids from the same batches of crypts as in (*A*) cultured for 10 days in medium supplemented with HGF (100 ng/mL) (n = 15; $^{**}P < .01$). (*C*) Representative pictures of small intestinal organoids from crypts of $Cd44^{+/+}$, $Cd44^{-/-}$, $Cd44^{s/s}$, and $Cd44^{v4-10/v4-10}$ mice cultured in medium supplemented with HGF (100 ng/mL). (*D*, *E*) Expansion kinetics of $Cd44^{s/s}$ and $Cd44^{v4-10/v4-10}$ adenoma spheroids cultured with either HGF-containing or control medium (n = 15; $^{**}P < .01$). (*F*) Quantification of $Cd44^{s/s}$ and $Cd44^{v4-10/v4-10}$ spheroid size at day 3 after plating in either HGF-containing or control medium (n = 15; $^{**}P < .01$; $^{***}P < .001$).

CD44v Controls HGF-Dependent Intestinal Adenoma Expansion

Constitutive activation of β-catenin/TCF-mediated transcription by Wnt-pathway-activating mutations in human colorectal adenomas and adenomas of $Apc^{Min/+}$ mice results in a strongly enhanced expression of CD44v isoforms,[26,46] including CD44v4–10.[34] Furthermore, this Wnt-pathway activation also includes upregulation of MET,[26,37] resulting in MET overexpression at early stages of CRC development.[26] These data suggest that CD44v and MET may play a collaborative role in adenoma growth. To explore this hypothesis, intestinal adenomas were harvested from $Cd44^{v4-10/v4-10}/Apc^{Min/+}$ or $Cd44^{s/s}/Apc^{Min/+}$ mice. Established tumor spheroids were re-seeded in the presence of HGF-containing or control medium and spheroid expansion was measured for 4 days. HGF-stimulated CD44s-adenoma spheroids expanded at a similar rate compared with those on control medium (Figure 6*D* and *F*). In contrast, HGF-stimulated spheroids expressing CD44v4–10 showed an enhanced expansion compared with those cultured with control medium (Figure 6*E* and *F*). These results demonstrate that HGF stimulation can accelerate adenoma expansion in vitro and that this accelerated growth requires CD44v isoforms.

Discussion

Whereas EGFR signaling is generally acknowledged to play a major role in intestinal epithelial homeostasis, mediating ISC self-renewal,[3,47] the role of HGF/MET signaling in the intestinal mucosa has remained largely unexplored. Our current results show that HGF, comparable to EGF, can mediate the outgrowth of LGR5+ ISCs to "mini-gut" organoids containing all differentiated epithelial lineages (Figure 1). This suggests that the EGF/EGFR and HGF/MET pathways have, at least partly, redundant functions in the intestinal mucosa. Indeed, EGF and HGF are known to activate largely overlapping intracellular signaling cascades, including the RAS-MAPK and the PI3K-AKT pathways,

mediating multiple biological responses, including cell proliferation and survival.[25,48–50] Redundancy between EGFR and MET signaling in the intestine could explain the remarkable observation that neither *Egfr* nor *Met* epithelial inducible knockout mice display intestinal mucosal defects under homeostatic conditions[13] (Figure 2). Under conditions of stress, however, *Met* deletion causes an impaired regenerative response to radiation-induced damage (Figure 3). A comparable attenuated regeneration response also has been reported in $Egfr^{Wa2}$ mice, which harbor an *Egfr* mutation impairing ligand-dependent receptor activation.[14] Interestingly, we found that *Met*-deficient LGR5+ ISCs have a competitive disadvantage during regeneration (Figure 3). Notably, although LGR5+ cells are dispensable for intestinal homeostasis and can be replaced by BMI1-expressing stem cells,[51] they are crucial for radiation-induced intestinal regeneration.[52] Hence, our findings indicate that *Met* deficiency diminishes the fitness of LGR5+ ICSs. It was recently demonstrated that recovery of intestinal epithelium from radiation damage depends on reprogramming of LGR5+ ICSs by YAP, a downstream transcriptional effector of Hippo signaling, and involves YAP-mediated EGFR pathway activation.[53] It will be of interest to determine whether YAP also affects HGF/MET signaling during intestinal regeneration.

Although multiple lines of evidence, including genomic, gene expression, and functional data, indicate an important pathogenetic role for HGF/MET signaling in human CRC,[17,19–23] direct in vivo evidence for a role of HGF/MET signaling in Wnt-pathway driven intestinal tumorigenesis was thus far missing. We addressed this role by exploring the functional effects of HGF stimulation on adenoma expansion in vitro and found that it promotes adenoma growth to a similar degree as stimulation with EGF (Figure 4*B*). Furthermore, by interrogating conditional *Met*/*Apc* double-knockout mouse models, we found that MET supports adenoma formation in vivo. In the $Lgr5^{Creert2}/Met^{fl/fl}/Apc^{fl/fl}$ model, ISC-specific deletion of both *Apc* alleles results in vast induction of WNT signaling leading to massive adenoma formation throughout the intestinal mucosa. Histologic analysis revealed a strong reduction of microadenomas and a reduced adenoma size in the absence of *Met* (Figure 5*A*, *C*, and *D*). This reduction in microadenoma number and adenoma size may be explained by the observed increased apoptotic rate in *Met*-deficient microadenoma (Figure 5*G*), which suggests an attenuated fitness of adenoma-initiating cells and may decelerate the outgrowth of microadenomas into adenomas. In addition, in the more physiological $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ model, *Met* deficiency resulted in an approximately 50% reduction in adenoma formation (Figure 5*I*) as well as a significant reduction in the number of microadenomas (Figure 5*J*). Like in the formation of intestinal adenomas in humans and in $Apc^{Min/+}$ mice, in this $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ model loss of heterozygosity at the *Apc* locus must take place before adenomas can arise. A similar reduction in adenoma load was previously reported in $Apc^{Min/+}$ mice bearing mutant $Egfr^{Wa2}$ or $Egfr^{Wa5}$, which both result in strongly reduced EGFR signaling.[15,54]

Interestingly, the reduction in adenoma formation we observed in $Ah^{Cre}/Met^{fl/fl}/Apc^{fl/+}$ was comparable to our previously observed reduction in adenoma formation on *Cd44* deletion in $Apc^{Min/+}$ mice.[34,42] A potentially important function of CD44 in the biology of the normal intestinal mucosa and in adenoma formation is its ability to associate with RTKs, in particular with EGFR family members and MET.[55] CD44 has been implicated in EGFR signaling in ovarian, melanoma, and breast cancer cell lines[56–58]; however, we found that CD44 deletion does not affect EGF-mediated intestinal mini-gut formation (Figure 6*A*). By contrast, HGF-mediated formation of these organoids was significantly attenuated by CD44 deletion, indicating collaboration between CD44 and MET (Figure 6*B*). This negative effect of CD44 deletion on HGF/MET signaling and organoid formation could not be compensated by knockin of the CD44s isoform, but was fully annihilated by knockin of CD44v4–10 (Figure 6*B*). Hence, CD44 domains encoded by variant exons that are highly expressed by ISCs are crucial for CD44-MET cooperation during mini-gut formation. Our results also imply that interaction with hyaluronic acid, a major CD44 ligand abundantly present in Matrigel that binds the extracellular constant domain of CD44, does not affect organoid outgrowth.

In addition to supporting mini-gut expansion, CD44v also promoted HGF-mediated growth of adenomatous epithelium from *Apc* mutant mice (Figure 6*E* and *F*), in contrast to the CD44s isoform (Figure 6*D* and *F*). These results are fully in line with the observed reduced adenoma initiation in $Cd44^{-/-}/Apc^{Min/+}$ mice, which can be completely rescued by introduction of CD44v4–10, but not by CD44s.[34] Consistent with these tumor-growth–promoting effects, CD44v isoforms have previously been reported to complex with HGF and MET and enhance MET signaling in human CRC and pancreatic cancer cell lines.[44,45,59] Importantly, both CD44 and MET are targets of Wnt signaling in the intestinal mucosa.[26,46,60] Because of constitutively active Wnt signaling, neoplastic intestinal epithelium retains an ISC-like molecular signature of which CD44v and MET are important components.[26,37,46] Indeed, expression of CD44v and MET in human CRC correlates with a stem cell–like expression profile, which is associated with an unfavorable prognosis.[61,62] Interestingly, HGF is abundantly expressed by cancer-associated fibroblasts and induces Wnt signaling and CD44v expression in CRC-initiating cells, indicating a role in the maintenance of the cancer stem cell niche.[62,63] Together, these data suggest a scenario in which CD44v isoforms through selective interaction with MET promote the proliferation of normal and neoplastic ICS, thereby fueling the self-renewal of the intestinal mucosa and promoting tumor growth.

Our finding that EGFR and MET signaling play overlapping, partially redundant, roles in the intestinal mucosa is of great potential clinical relevance. Most patients with mCRC show EGFR overexpression and treatment targeting this receptor has proven to be of clinical benefit in cancers with wild-type *RAS*.[18,64] However, these initially sensitive tumors almost invariably become therapy resistant due to selection of subclones carrying (epi)genetic alterations that confer EGFR-independent growth. These include mutations

in *KRAS*, *NRAS*, and *BRAF* downstream of the EGFR, but can also consist of aberrant RTK activation by cell-autonomous or microenvironmental cues, including amplification of *MET* or overexpression of HGF,[20,65,66] accompanied by CD44v overexpression. Taken together, the results of our current study strongly support the notion that mCRCs may escape from addiction to EGFR signaling by co-opting HGF/MET signaling. These findings could help in understanding how growth factor signaling contributes to resistance to EGFR inhibition and may contribute to the design of precision therapies for patients with mCRC targeting HGF/MET/CD44v signaling.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2017.07.008.

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Conflicts of interest
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MET SIGNALING IN INTESTINAL HOMEOSTASIS AND CANCER

HGF/MET signaling supports the intestinal stem cell niche and may function as a compensatory pathway in promoting *metastatic colorectal cancer* dissemination in the setting of epidermal growth factor receptor blockade.

The intestinal crypt–villus unit is repopulated by the stem cell activities organized in a hierarchy of engagement. Wnt signaling regulates expression of cellular programs critical to intestinal stem cell survival and inappropriately sustained Wnt activity contributes to malignant transformation of the gut. Intestinal crypts can be maintained ex vivo as minigut cultures if the proper cocktail of stem cell–promoting factors, in the presence of ill-defined extracellular matrix components, is provided. Critical factors include Wnt3A, R-spondin, Noggin, and epidermal growth factor (EGF). The addition of EGF is required to activate receptor tyrosine kinase pathways important to stem cell maintenance. Although EGF receptor (EGFR) activation can sustain minigut cultures and its activation contributes to intestinal tumorigenesis, it is possible that other receptor tyrosine kinases also contribute to these processes. Indeed, loss of efficacy with the therapeutic targeting of EGFR in metastatic colorectal cancer (CRC) may be due to the activation of other compensatory RTKs.

In this issue of *Gastroenterology*, Joosten et al demonstrate that MET signaling mediates intestinal crypt villus development, regeneration, and adenoma formation. MET is the receptor for hepatocyte growth factor (HGF). Also known as “scatter factor,” HGF is pro-proliferative and increases cell survival as well as induces tumor invasion and dissemination, and thus activation of this pathway may promote metastatic CRC. This group demonstrates that HGF-mediated MET signaling can compensate for the loss of EGFR in the maintenance of intestinal organoid cultures and enhance the growth of adenoma spheroid cultures. MET-null mice were used to demonstrate MET dependency on intestinal regeneration after radiation-induced enteritis to determine a critical requirement in adenoma formation. CD44v is a Wnt transcriptional target. Expressed in ISCs, it also can promote adenoma formation. In this report, the authors determine that CD44v regulates HGF-dependent intestinal organoid growth and adenoma expansion. In sum, HGF/MET signaling contributes to intestinal stem cell survival and adenoma formation and perhaps metastasis. It is an intriguing hypothesis that HFG/MET may contribute to metastatic CRC escape from EGFR blockade. Thus, targeting both pathways may be required in the treatment of a subset of CRC.

Chapter 5

WNT signaling controls expression of pro-apoptotic BOK and BAX in intestinal cancer

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WNT signaling controls expression of pro-apoptotic *BOK* and *BAX* in intestinal cancer

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ABSTRACT

In a majority of cases, colorectal cancer is initiated by aberrant activation of the WNT signaling pathway. Mutation of the genes encoding the WNT signaling components adenomatous polyposis coli or β-catenin causes constitutively active β-catenin/TCF-mediated transcription, driving the transformation of intestinal crypts to cancer precursor lesions, called dysplastic aberrant crypt foci. Deregulated apoptosis is a hallmark of adenomatous colon tissue. However, the contribution of WNT signaling to this process is not fully understood. We addressed this role by analyzing the rate of epithelial apoptosis in aberrant crypts and adenomas of the $Apc^{Min/+}$ mouse model. In comparison with normal crypts and adenomas, aberrant crypts displayed a dramatically increased rate of apoptotic cell death. Expression profiling of apoptosis-related genes along the crypt-villus axis and in *Apc* mutant adenomas revealed increased expression of two pro-apoptotic Bcl-2 family members in intestinal adenomas, *Bok* and *Bax*. Analysis of the colon of familial adenomatous polyposis (FAP) patients along the crypt-to-surface axis, and of dysplastic crypts, corroborated this expression pattern. Disruption of β-catenin/TCF-4-mediated signaling in the colorectal cancer cell line Ls174T significantly decreased *BOK* and *BAX* expression, confirming WNT-dependent regulation in intestinal epithelial cells. Our results suggest a feedback mechanism by which uncontrolled epithelial cell proliferation in the stem cell compartment can be counterbalanced by an increased propensity to undergo cell death.

1. Introduction

The continuous self-renewal of the intestinal epithelium is a tightly controlled dynamic process. Crypt base columnar cells in the intestinal crypts, recently identified as intestinal stem cells, divide daily and their daughter cells constitute a transit-amplifying compartment of rapidly dividing epithelial progenitors. These, in turn, differentiate while migrating upwards and are eventually shed into the lumen [reviewed by van der Flier [1]]. The WNT signaling pathway plays a critical role in the maintenance of the stem cell and transit-amplifying compartments [2]. Active WNT signaling promotes the nuclear translocation of cytoplasmic β-catenin, the stability of which is regulated by a destruction complex containing adenomatous polyposis coli (APC), AXIN and GSK3β. When translocated to the nucleus, β-catenin activates the transcription of target genes through its interactions with TCF/LEF-family transcription factors. The requirement for active WNT signaling in the crypt compartment is illustrated by the absence of stem cells and proliferative crypts in mice lacking either Tcf-4 [3] or β-catenin [4]. In contrast, mutation of the genes encoding APC or β-catenin causes constitutively active β-catenin/TCF-mediated transcription and initiates the formation of aberrant crypts or microadenoma (MA), the earliest identifiable colorectal cancer precursor lesions [5,6].

Besides promoting cell proliferation and tissue expansion, WNT signaling has also been linked to the regulation of apoptotic cell death. For instance, active WNT signaling drives apoptosis of neuronal cells in the retina of *Drosophila* [7] and a similar relation between active WNT signaling and apoptosis has also been reported in the intestinal crypt compartment. For example, epithelial crypt cells expressing a constitutively active LEF1/β-catenin fusion protein were highly sensitive to apoptosis [8]. Moreover, the conditional deletion of APC in all intestinal epithelial cells of adult mice strongly increased the rate of apoptotic cell death in the proliferating epithelial cell fraction [9,10]. In addition, in comparison to cells of the basal crypt compartment, the epithelial cells lining the villi of the small intestine and intercrypt tables of the colon, where WNT signaling is low, were relatively resistant to apoptosis [11]. The Bcl-2 family of pro- and anti-apoptotic proteins regulates the critical balance between survival and controlled cell death in the intestinal mucosa [reviewed by Watson [12]]. Interestingly,

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inactivation of the tumor suppressor protein p53, a key regulator of apoptosis in epithelial cells, had no effect on the rate of apoptosis in crypts and adenomas [13,14], suggesting p53-independent pro-apoptotic signaling upon neoplastic transformation of intestinal crypts. These findings indicate a causal relationship between (aberrant) activation of WNT signaling and increased apoptosis sensitivity. Despite these findings, however, the effects of WNT/β-catenin signaling on the apoptotic program have remained largely unclear. In the current study, we explored this relationship by analyzing apoptotic cell death at the earliest stages of WNT-driven intestinal tumorigenesis in tumor prone $Apc^{Min/+}$ mice and FAP patients. Our results demonstrate an important function of WNT signaling in controlling the expression levels of two pro-apoptotic Bcl-2 family members, *BOK* and *BAX*, in intestinal epithelial cells during tumorigenesis and suggest a feedback mechanism by which uncontrolled epithelial cell proliferation in the stem cell compartment can be counterbalanced by an increased propensity to undergo cell death.

2. Materials and methods

2.1. Immunohistochemistry and quantification of apoptotic cells

$Apc^{Min/+}$ mice were obtained from the Jackson Laboratory and were sacrificed at 8 weeks of age. Small intestine was isolated and immunohistochemistry was performed on paraffin-embedded tissue sections as described in Zeilstra et al. [15]. The antibody used was rabbit anti-cleaved caspase-3, Asp175 (Cell Signaling Technology). Multiple images of crypts, microadenomas (<0.25 mm) and adenomas (>1 mm) were acquired and total epithelial cell numbers as well as apoptotic epithelial cells were quantified using ImageJ software [16]. Clusters of apoptotic fragments were scored as one cell death event and Apoptotic Index (AI), defined as the proportion of active caspase-3 positive cells per 100 epithelial cells, was determined as previously described [11]. Student's *t* test was used for statistical analysis. Samples of human colon were obtained according to standard medical ethical procedures of the Academic Medical Center. Paraffin-embedded colonic sections were stained using primary mAb mouse anti-human BAX, Ab-1 (Thermo Fisher Scientific). Antibody binding was visualized using the Powervision poly-HRP detection system (ImmunoVision Technologies) and DAB+ (Dako).

2.2. Laser-aided microdissection

Patients were diagnosed with FAP with 100 or more colorectal adenomas (two males, two females, median age: 30 years, range: 22–44 years). Sections of snap-frozen tissue were counter-stained with hematoxylin and digitally scanned with a Veritas Microdissection System (Molecular Devices Corporation). Epithelial cells were cut and collected into Capsure Macro LCM caps. RNA was isolated using the PicoPure RNA Isolation Kit (Molecular Devices Corporation) according to the manufacturer's protocol. RT-PRC primers used are listed in Supplementary Table 1. Student's *t* test was used for statistical analysis.

2.3. RT-MLPA

MLPA method was described by Schouten et al. [17]. RT-MLPA was performed as described earlier by Eldering et al. [18]. Apoptosis related gene expression was analyzed using the SALSA RT-MLPA kit R011 (MRC-Holland). Amplicons were quantified using an ABI 3100 Avant capillary electrophoresis system (Applied Biosystems) after addition of the GeneScan-500 ROX size standard. Data were analyzed using Genotype and GeneScan software (Applied Biosystems). The sum of all data values was set at 100% to correct for fluctuations in total signal between samples, and individual values were calculated relative to the 100% value. Results were statistically analyzed using Student's *t* test and *P*-values were corrected for multiple comparisons according to the method of Benjamini and Hocherg. Values less than .05 were considered statistically significant.

2.4. Cell culture

Inducible dominant-negative TCF-4 Ls174T cells were a generous gift of Clevers and van de Wetering [19]. Cells were cultured in RPMI (10% FCS) and expression of dnTCF-4 was induced with 1 μg/ml doxycyclin for 24 h or vehicle as control.

2.5. Real-time reverse transcription-PCR

Quantitative reverse transcription-PCR (qRT-PCR) runs were performed on a Roche LightCycler 1.5 using FastStart DNA Master SYBR Green I kit (Roche Molecular Systems). Results were analyzed using LinReg PCR analysis software (version 7.5; [20]). Expression was normalized over *β-actin* expression.

3. Results

3.1. Intestinal microadenomas display increased sensitivity to apoptosis after loss of functional APC

To investigate the rate of apoptosis in microadenomas and adenomas initiated by APC mutations we made use of the $Apc^{Min/+}$ model. These mice carry an *Apc* allele with a truncating mutation at codon 850 and, similar to FAP patients, spontaneously develop multiple intestinal lesions throughout their intestinal tract [21]. Apoptotic intestinal epithelial cells were visualized by immunohistochemical staining of small intestinal tissue sections of 8 weeks old $Apc^{Min/+}$ mice using an antibody which recognizes active caspase-3 (Fig. 1A–C). Examination of the normal crypt compartment revealed the presence of low numbers of apoptotic enterocytes (Apoptotic Index (AI): 0.023 ± 0.002 (mean ± SE, $n = 14$). This small but constant rate of apoptosis is in line with previously reported data [22]. Strikingly, intestinal microadenomas displayed an over 150-fold increase in apoptosis compared to normal crypts (AI of 3.78 ± 0.35, $P < 0.001$; Fig. 1D). The AI of full size intestinal adenomas was 0.72 ± 0.06 (mean ± SE; $n = 50$), a more than 30-fold increase compared to that of normal crypts ($P < 0.001$) but, interestingly, a more than 5-fold reduction when compared to the AI of microadenomas ($P < 0.001$; Fig. 1D). We conclude that intestinal adenoma formation, caused by loss of functional APC, is accompanied by strongly elevated levels of apoptosis. Hence, these findings suggest that enhanced WNT signaling potentiates mitochondrial apoptosis in the intestinal epithelium during intestinal tumorigenesis.

3.2. Increased gene expression of pro-apoptotic Bok and Bax in Apc mutant adenomas

We next set out to identify putative WNT-regulated apoptosis-related genes in the intestinal mucosa. This was addressed by performing gene expression profiling of laser-capture microdissected intestinal epithelial cells isolated from normal duodenal crypts, adjacent villi, and from distant duodenal adenomas of 8 weeks old $Apc^{Min/+}$ mice ($n = 4$). For this purpose, we employed RT-Multiplex Ligation-dependent Probe Amplification (RT-MLPA), a PCR-based method that allows for the relative quantification of over 35 apoptosis-related genes in small amounts of RNA [18]. (For a complete list of genes and results: see Supplementary Table 2).

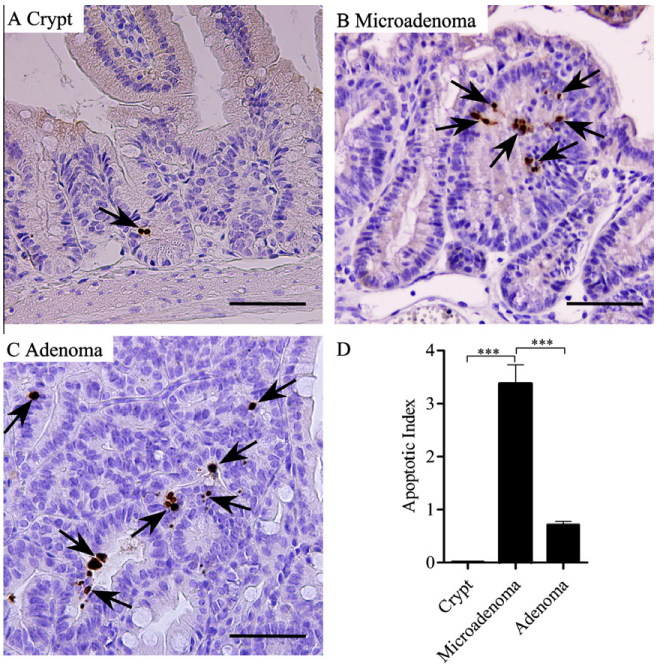

Fig. 1. Increased apoptosis in intestinal microadenomas of $Apc^{Min/+}$ mice. Apoptotic epithelial cells in the intestinal mucosa visualized by immunohistochemical staining for active caspase-3 (arrows). (A) Normal small intestinal crypt. (B) Microadenoma. (C) Adenoma. (Scale bars represent 50 μm). (D) Apoptotic index of crypts ($n = 14$), microadenomas ($n = 32$) and adenomas ($n = 50$) ($***P < 0.001$).

We identified 10 genes with a significant ($P < 0.05$), and at least 2-fold change in expression level in villi (low WNT signaling) compared with crypts (active WNT signaling) (Table 1). The top down-regulated gene along the crypt-villus axis was *Birc5* (also known as *Survivin*) (>14-fold, $P < 0.001$), a member of the inhibitory of apoptosis (IAP) gene family with an essential function in cell division [23]. *Birc5* is a known WNT target gene in the intestinal mucosa and expression of this gene is characteristic for the proliferating crypt compartment [24]. Other genes with decreased expression in the villi were the pro-apoptotic *Bok*, *Htra2*, *Moap1*, *Diablo* and *Bax*. By contrast, as expected, *Cdkn1a* expression was increased in the villi. This gene encodes the cell cycle inhibitor $p21^{cip1/waf1}$ and is induced as a result of decreased levels of the WNT target c-MYC [19]. Other genes with increased expression in the villi were *Noxa, Bid* and *Rambo*.

We subsequently correlated the levels of these 10 putative WNT-regulated genes to the apoptosis-prone phenotype observed in adenomatous intestinal epithelium. This analysis revealed four genes with significantly altered expression in the *Apc* mutant adenomas compared to normal crypts (Fig. 2). *Cdkn1a* expression was found to be upregulated in comparison to normal crypts (2.1-fold, $P < 0.05$; Fig. 2A), while the cell division gene *Birc5* was found to be downregulated (2.8-fold, $P < 0.05$; Fig. 2B). Upregulated pro-apoptotic genes were *Bok* (4.0-fold, $P < 0.001$; Fig. 2C) and *Bax* (1.3-fold, $P < 0.05$; Fig. 2D). The other selected genes did not show this correlation. Our findings demonstrate a shift in the balance between the expression of pro- and anti-apoptotic genes in *Apc* mutant adenomas and suggest a novel link between aberrantly activated WNT signaling and regulation of the pro-apoptotic Bcl-2 family members *Bok* and *Bax* in the intestinal mucosa.

Table 1
Differentially expressed apoptosis-related genes in villi compared with crypts.

Gene symbol(s)	Gene name	Function	Fold change	*P*-value
Birc5/Survivin	Baculoviral IAP repeat-containing 5	Anti-apoptotic	−14.6	9.00E-04
Bok/Mtd	Bcl2-related ovarian killer protein	Pro-apoptotic	−5.2	4.97E-02
Htra2/Prss25	HtrA serine peptidase 2	Pro-apoptotic	−4.7	2.01E-02
Moap1/Map-1	Modulator of apoptosis 1	Pro-apoptotic	−3.9	2.45E-03
Diablo/mac	Diablo homolog (*Drosophila*)	Pro-apoptotic	−2.8	4.99E-03
Bax	Bcl2-associated X protein	Pro-apoptotic	−2.0	1.44E-03
Rambo/Bcl2l13	Bcl2-like 13 (apoptosis facilitator)	Pro-apoptotic	3.3	9.60E-05
Bid	BH3-interacting domain death agonist	Pro-apoptotic	4.9	1.43E-02
Cdkn1a/p21	Cyclin-dependent kinase inhibitor 1	Cell-cycle arrest	6.4	1.65E-03
Noxa/Pmaip1	PMA-induced protein 1	Pro-apoptotic	6.5	4.84E-03

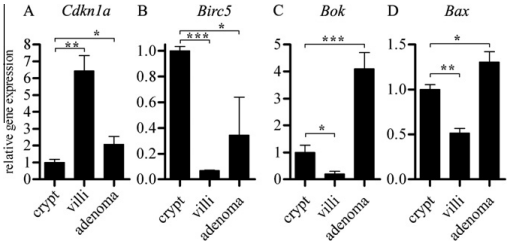

Fig. 2. Selected apoptosis related genes differentially expressed in the intestinal mucosa of $Apc^{Min/+}$ mice. (A–D) RT-MLPA results showing relative gene expression levels for, respectively, *Cdkn1a*, *Birc5*, *Bok* and *Bax* in intestinal crypts, villi and *Apc* mutant adenomas ($n = 4$ per group; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

3.3. BOK, BAX and BIRC5 expression in human colonic dysplastic crypts

The above results demonstrate a correlation between loss of APC function and altered expression of apoptosis related genes in mouse intestinal adenomas. In order to establish the expression levels of *BOK*, *BAX*, and *BIRC5* in the equivalent human disease, especially in the earliest identifiable colonic lesions initiated by aberrantly activated WNT signaling, we set out to analyze the colon mucosa of FAP patients. Pure epithelial cell fractions were isolated from dysplastic crypts, as well as from the base and tables of normal crypts distant from adenomatous tissue, by using laser-aided microdissection (see Supplementary Fig. 1 for details). Gene expression was analyzed by real-time qRT-PCR on cDNA derived from these cells. Expression of *CDKN1A* and the WNT target genes *CD44* [25] and *c-MYC* [26] was studied as control.

Compared to expression in the epithelium at base of human colon crypts, expression levels of *CD44* and *c-MYC* were decreased in the epithelial cells derived from the crypt surface and strongly increased in the dysplastic epithelial cells (Fig. 3A and B), confirming the expected WNT signaling activity in the different cell fractions. In addition, compared to the $Apc^{Min/+}$ mice, we also observed a similar *CDKN1A* expression pattern (Fig. 3C) and, as expected, the *BIRC5* expression levels showed a significant decrease along the crypt-to-surface axis (4.5-fold, $P < 0.001$; Fig. 3D). However, in contrast to the other two established WNT target genes *CD44* and *c-MYC*, *BIRC5* expression was not further increased in the human dysplastic crypts and showed a relatively more variable expression between the individual samples. Notably, this expression pattern was also observed in the $Apc^{Min/+}$ mouse adenomas (Fig. 2B). Examination of *BOK* expression revealed decreased levels at the crypt surface (2.2-fold, $P < 0.05$; Fig. 3E), while expression levels at the colonic crypt base and in dysplastic crypts were similar ($P > 0.05$). *BAX* levels were slightly, but not significantly, decreased at the crypt surface (1.6-fold, $P > 0.05$; Fig. 3F) and, interestingly, almost 2-fold increased in dysplastic crypts ($P < 0.05$). These findings demonstrate that *BOK* and *BAX* are predominantly expressed at the colonic crypt base and in early dysplastic lesions characterized by aberrantly activated WNT signaling.

3.4. APC mutant human colonic dysplastic crypts display increased BAX immunoreactivity

To confirm increased BAX levels in *APC* mutant aberrant crypt foci we studied BAX protein expression in colonic tissue sections derived from the FAP patients by immunohistochemistry. BOK protein expression could not be studied in tissue sections because of

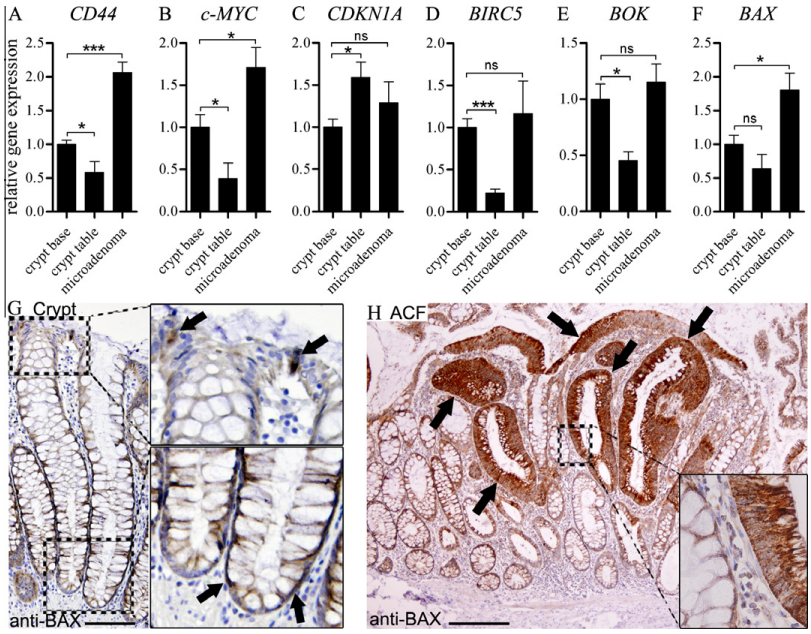

Fig. 3. Pro-apoptotic *BOK* and *BAX* expressions in the intestinal mucosa of FAP patients correlate with β-catenin/TCF target gene expression. (A–F) qRT-PCR results showing relative gene expression levels for, respectively, *CD44*, *c-MYC*, *CDKN1A* (*p21*$^{CIP1/WAF1}$), *BIRC5*, *BOK* and *BAX* ($n = 4$ per group; $*P < 0.05$; $***P < 0.001$; ns, not significant). (G) BAX immunoreactivity in normal colonic mucosa. Epithelial cells at the intercrypt tables (upper panel) and at the crypt base (lower panel) are positive for BAX immunostaining (arrows; scale bar = 50 μm). (H) Representative picture of a colonic ACF from a FAP patient stained with anti-BAX antibody (arrows; scale bar = 100 μm).

the lack of available high-quality specific antibodies. Histologically normal colorectal mucosa displayed BAX immunostaining in the cytoplasm of epithelial cells at the base of the crypts as well as in epithelial cells higher up the crypt axis and at the crypt tables (Fig. 3G). Compared to normal mucosa, aberrant crypt foci showed strongly increased BAX immunoreactivity (Fig. 3H). These results were consistent with the RT-PCR results and demonstrate high levels of BAX protein in the earliest identifiable neoplastic lesions in human colon.

3.5. WNT signaling controls gene expression of pro-apoptotic BOK and BAX

To test whether gene expression of *BOK* and *BAX* is indeed controlled by β-catenin/TCF-4-mediated signaling we made use of human Ls174T colorectal cancer cells carrying a doxycycline inducible expression plasmid encoding an N-terminally truncated version of TCF-4 [19]. This dominant negative form of TCF-4 is unable to bind β-catenin and strongly inhibits WNT signaling activity. Ls174T cells express mutant β-catenin protein and carry wild-type alleles of *p53* and *APC* [19]. Cells were treated with doxycycline for 24 h and gene expression was analyzed using real-time qRT-PCR. Parental cells expressing the Tet-repressor alone were used as control. As positive control for the effect of transcriptional disruption by dnTCF-4, we measured the relative expression levels of *CD44*, *c-MYC*, *CDKN1A* and *BIRC5*. As expected, induction of dnTCF-4 inhibited the expression of CD44, *c-MYC* and *BIRC5*, and strongly induced the expression of *CDKN1A* (Fig. 4A–D). Quantification of *BOK* mRNA levels revealed a reduction of almost 50% after dnTCF-4 induction ($P < 0.001$; Fig. 4E), which was comparable to the reduction of *c-MYC* and *BIRC5* levels. Analysis of *BAX* gene expression revealed a similar down-regulation ($P < 0.01$; Fig. 4F). These results imply that expression of pro-apoptotic *BOK* and *BAK* in the intestinal epithelium is controlled by β-catenin/TCF-4 mediated transcription.

4. Discussion

Dysregulated WNT signaling, caused by loss of functional APC is the driving force behind the initiation of dysplastic aberrant crypts in FAP patients and $Apc^{Min/+}$ mice and has been observed in the majority of sporadic colorectal tumors [5,6]. In the current study, we demonstrate that loss of APC function is associated with a strong initial increase in the rate of epithelial cell apoptosis in the adenomatous lesions of $Apc^{Min/+}$ mice. This finding is in line with previous reports showing increased apoptosis in the intestinal mucosa after conditional deletion of *Apc* [9,10]. The sharp increase in apoptosis supports a relation between WNT signaling and the expression of apoptosis-related genes in the intestinal mucosa. Indeed, gene expression analysis along the normal crypt-villus axis and in intestinal adenomas of $Apc^{Min/+}$ mice revealed the differential expression of two pro-apoptotic Bcl-2 family members, *Bok* and *Bax* (Fig. 2). BAX is the prototypic apoptosis-promoting member of the Bcl-2 family and is directly involved in the execution of apoptosis by inducing the release of mitochondrial cytochrome c [27]. Interestingly, inactivating *BAX* mutations have been found in over 50% of human microsatellite instable colon tumors, suggesting a suppressor role for the wild-type *BAX* gene in a p53-independent pathway for colorectal tumorigenesis [28]. Similar to BAX, BOK contains the conserved Bcl-2 homology (BH) domains 1–3 and, likewise, exerts its pro-apoptotic function through the mitochondrial pathway [29,30]. *Bok* expression was previously demonstrated in the basal crypt compartment of mice and, importantly, increased expression was associated with increased epithelial apoptosis at this position [15]. We demonstrate herein that *BOK* and *BAX* are prominently expressed in the human colonic basal crypt compartment as well as in the dysplastic crypts of FAP patients. Furthermore, the expression of *BAX* was almost 2-fold increased in these dysplastic lesions (Fig. 3F), which was confirmed by immunohistochemistry (Fig. 3H). These results demonstrate that increased *BOK* and *BAX* expression is an early event in

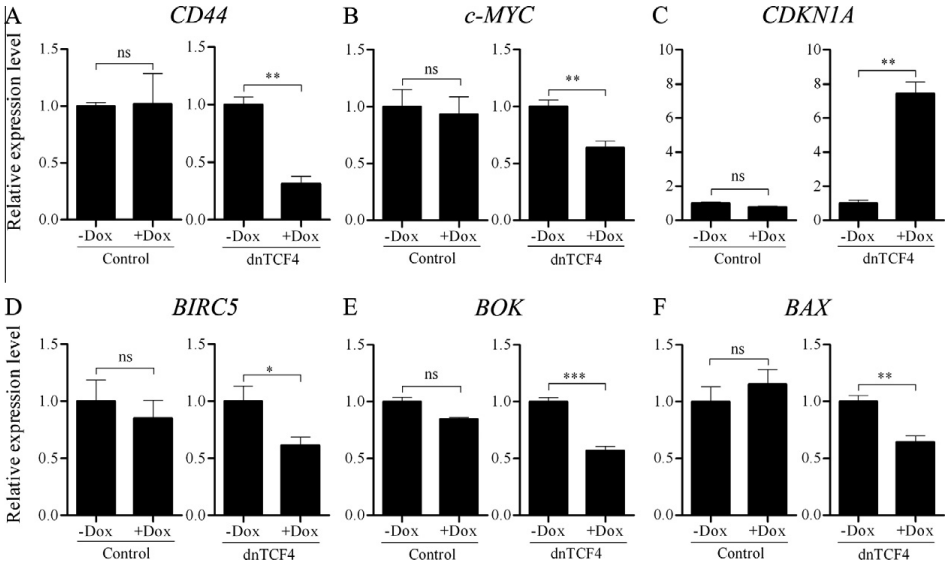

Fig. 4. WNT signaling regulates gene expression of pro-apoptotic *BOK* and *BAX* in human epithelial colon cells. Relative gene expression levels measured by qRT-PCR in Ls174T cells carrying doxycycline-inducible TCF-4 or control cells. Cells were incubated with or without doxycycline (Dox) for 24 h. (A and B) Relative expression levels of the WNT target genes *CD44* and *c-MYC*. C, *CDKN1A* ($p21^{CIP1/WAF1}$). (D) *BIRC5*. (E and F) *BOK* and *BAX* ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant).

intestinal tumorigenesis and suggest that their expression is dependent on WNT signaling. Indeed, experiments employing human colorectal cancer cells carrying inducible dnTCF-4 constructs corroborated the involvement of β-catenin/TCF-4-mediated signaling in the regulation of *BOK* and *BAX* (Fig. 4). Relevant to these findings is the recent identification of c-MYC as a key downstream mediator of WNT regulated gene expression following APC loss [10,31]. Deregulated c-MYC expression is associated with an increased sensitivity to apoptosis [32], and it has been reported that *BAX* is a c-MYC target gene [33]. Further studies are needed to elucidate the role for c-MYC in the regulation of apoptotic signaling in the context of colorectal cancer.

Of interest, although we and other groups have demonstrated that *BIRC5* is a WNT target gene in intestinal epithelial cells [19,24], *BIRC5* expression levels were not further increased in the mouse and human adenomatous lesions. Moreover, expression profiling of normal mucosa showed that *BIRC5* expression was highly restricted to the proliferating crypt compartment (Figs. 2B and 3 D). These results are in agreement with earlier studies demonstrating that BIRC5/Survivin primarily functions as a mitotic regulator [23], and suggest an essential role for this protein in the proliferation of intestinal epithelial cells. It should be noted that the dynamics of apoptosis in conditions of enhanced proliferation depends on many factors: pro-apoptotic BOK and BAX can interact with anti-apoptotic Bcl-2 family members, such as, BCL-2, BCL-xL, BCL-W and MCL-1 [29,34], and the balance between all these different pro- and anti-apoptotic components defines the threshold of responsiveness to apoptotic signals. This decision making process is further influenced by the inhibitor of apoptosis (IAP) family of proteins [35]. The increased apoptosis observed in intestinal epithelial cells lacking APC demonstrates a functional role of WNT signaling in shifting this balance, however, whether this indeed affects adenoma formation and progression needs further exploration.

Taken together, our findings demonstrate a novel link between dysregulated WNT signaling and the transcription of pro-apoptotic Bcl-2 family members at the early stages of WNT-driven intestinal tumorigenesis. Accordingly, exploitation of this relationship might prove useful in the prevention and treatment of intestinal cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.070.

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Chapter 6

CD44 expression in intestinal epithelium and colorectal cancer is independent of p53 status

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CD44 Expression in Intestinal Epithelium and Colorectal Cancer Is Independent of p53 Status

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Abstract

CD44 marks stem cell-like cells in a number of tumour types, including colorectal cancer (CRC), while aberrant CD44 expression conveys increased tumourigenic, invasive, and metastatic potential. Previous data indicate that CD44 is a direct target of p53-mediated transcriptional repression in breast cancer. Since inactivating *p53* mutations are frequent genetic events in CRC these could unleash expression of CD44. In the present study, we therefore explored the relation between *p53* mutational status and CD44 expression in a cohort of 90 localized primary CRCs and studied the effect of radiation-induced p53 activation on CD44 expression. Interestingly, we observed that, in contrast to breast cancer, loss of function *p53* mutations were not associated with elevated CD44 expression in colon cancer. Moreover, DNA-damage induced p53 activation did not result in repression of CD44 expression, neither in colon cancer cells nor in normal intestinal epithelial cells. Our data demonstrate that CD44 expression in normal and malignant intestinal epithelial cells is not regulated by p53, implying that regulation of this potentially important therapeutic target is tissue and cancer-type specific.

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Introduction

CD44 comprises a family of cell adhesion and signalling molecules that exert pleiotropic effects on important biological processes including proliferation, survival, migration, epithelial to mesenchymal transition (EMT), and cancer metastasis (reviewed by Zöller [1]). In the intestinal mucosa, CD44 is a major direct target of Wnt signalling and is prominently expressed on intestinal stem cells [2–4]. There is accumulating evidence that CD44 is involved in the initiation and progression of intestinal tumours and the development of metastasis [1,3,5–9]. In addition, prominent expression of CD44 is a hallmark of highly tumourigenic CRC cells [10]. Accordingly, it was recently demonstrated that *CD44* is part of an intestinal stem cell gene signature that predicts disease relapse in CRC patients [11]. This signature was specifically associated with CRC cells endowed with high-tumour initiating potential as well as long-term self-renewal capacity. Hence, CD44 represents a potential therapeutic target for the treatment of CRC and it is therefore important to understand the different mechanisms that underlie the regulation of CD44. In the majority of cases of CRC, expression of CD44 is increased as a result of dysregulated Wnt/β-catenin signalling [2,12]. However, there is ample evidence that other not-yet identified pathways and mechanisms contribute to the regulation of Wnt/β-catenin target gene expression in intestinal tumours [13]. The tumour suppressor protein p53 is a transcription factor that plays a critical role in the suppression of cancer. In response to oncogenic stress, such as DNA damage, activated p53 protein binds to sequence-specific DNA sites, thereby regulating the transcription of a wide range of target genes involved in cell cycle control and survival signalling [14]. Mutational inactivation of the *p53* gene is a frequent genetic event in the progression of many types of human tumours, including breast cancer and colorectal cancer (CRC) [15]. It was recently demonstrated that p53 transcriptionally represses *CD44* expression in both normal and tumour-derived mammary epithelial cells by direct binding to the *CD44* promoter [16]. This p53-dependent regulation of CD44 was observed in both human and mouse mammary glands, indicating an evolutionary conserved function. Importantly, down-regulation of CD44 expression was found to be a prerequisite for p53-dependent growth regulation and induction of apoptosis in mammary epithelium [16]. A similar functional interplay between p53 and CD44 might also take place in intestinal epithelial cells and tumours. To explore whether CD44 expression is controlled by p53 protein in CRC, we analysed a cohort of primary colon carcinomas for *p53* mutational status and CD44 expression. Our study reveals that loss of p53 function is not associated with elevated CD44 expression in CRC. Furthermore, we demonstrate that activation of wild-type p53 is unable to repress CD44 expression in human colon cancer cells as well as in primary cultures of mouse intestinal crypt-villus organoids.

Materials and Methods

Ethical Statement

The study involving human biopsy samples was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee of The University of Amsterdam, AIEC (Algemene Instellingsgebonden Ethische Commissie). Patients gave written informed consent for the sample collection.

Tumour Samples, p53 Mutation Analysis and Gene Expression Assay

The study cohort consisted of 90 AJCC stage II CRC patients that underwent intentionally curative surgery in the Academic Medical Center (AMC) in Amsterdam, The Netherlands, in the years 1997–2006 [17]. Representative fresh frozen tumour tissue was cut into 20 µm-thick sections that were immediately placed in TRIzol reagent (Invitrogen Life Technologies, Breda, the Netherlands), after which total RNA was extracted. Tumour load was examined routinely by an experienced pathologist. *p53* mutational status was determined using RT-PCR. In short, 2 µg of total RNA was reverse-transcribed in 25 µl reaction volume using pdN6 (Amersham Biosciences, Roosendaal, the Netherlands) and MMLV transcriptase (Gibco BRL, Breda, the Netherlands). PCR was performed on 1 µl of cDNA template using platinum Taq polymerase (Invitrogen Life Technologies). Oligo primers are listed in Table 1. PCR products were amplified by 35 cycles of 45 s at 95°C, 45 s at 60°C, and 1 min and 30 s at 72°C, and were sequenced directly using Big Dye Terminator Kit (Amersham) together with either sense or anti-sense oligo primer. Sequences were analysed using CodonCode Aligner software (CodonCode Corp., Dedham, MA). Gene expression levels in the tumours were assessed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 array platform (Affymetrix, Santa Clara, CA). Purified RNA was processed, hybridized, and scanned according to the manufacturer's protocol. Data was analysed using the software package R2 (http://r2.amc.nl), a web-based microarray analysis application developed by J.K. Data was MAS5-normalized and expression values were Log2 transformed. Statistical significance was assessed using one-way analysis of variance (ANOVA). Probe sets assayed were: *CDKN1A* (*p21*), ID: 202284_s_at; *MDM2*, 229711_s_at; and *CD44*, 209835_x_at. Other probe sets assaying *CD44* produced similar results, for example; 204489_s_at, $P<0.01$ and 210916_a_at, $P<0.01$).

Immunohistochemistry

Paraffin-embedded tumour tissue was stained using primary mAb mouse anti-human p53 (Dako, Glostrup, Denmark) and primary mAb mouse anti-human CD44 (VFF18) that recognizes CD44v6 [18]. Antibody binding was visualised using the Powervision poly-HRP detection system (ImmunoVision Technologies, Daly City, CA) and DAB+ (Dako). The intensity (I) of the staining was scored on semiquantitative scales as follows: "0", no reaction; "1", weak reaction; "2", moderate reaction; and "3", strong reaction. The extent of the signal was scored as percentage of positive cells (P). Overall staining score was calculated by multiplying the intensity by the percentage of positive cells (Score = P * I; maximum = 300). Fisher's exact test was used for statistical analysis ($P<0.001$).

Table 1. Oligo primers used for *p53* mutation analysis.

Target	*Orientation*	*Sequence (5′ to 3′)*
exon 1	sense	GCTTTCCACGACGGTGACA
exon 5	anti-sense	TTGTTGAGGGCAGGGGAGTA
exon 4	sense	TGTCATCTTCTGTCCCTTCC
exon 7	anti-sense	GATGGTGGTACAGTCAGAGC
exon 6	sense	TTGCGTGTGGAGTA
exon 11	anti-sense	GCAAGCAAGGGTTCAAAGACC

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Cell Culture, Immunoblotting and Real-time Reverse Transcription-PCR

RKO cells were cultured in McCoy's 5A medium supplementented with 10% FCS until subconfluent. Mouse small intestinal crypts were isolated in accordance with protocols approved by the local animal ethics committee of The University of Amsterdam, DEC (Dier Ethische Commissie) and cultured for one week as described by *Sato et al.* [19]. Cultures were exposed to a single dose of 10 Gy from a ^{137}Cs γ-ray radiation source at a dose rate of 0.8 Gy/min or incubated with 500 ng/mL neocarzinostatin (NCS) either in combination with 10 µM nutlin or not. Cells were harvested in lysis buffer at the indicated time points. Antibodies used for immunoblotting were anti-pan CD44 mAb Hermes-3 [20], anti-p21 mAb sx118 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p53 mAb DO-1 (Santa Cruz Biotechnology). β-actin was used as loading control. In parallel, total RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience, Mountain View, CA) and real-time qRT-PCR was performed as described previously [3,21]. One way-analysis of variance (ANOVA) was used to determine significant changes ($P<0.05$) in time.

Results

Loss of Function Mutation of p53 is Not Associated with Elevated CD44 Expression in Colon Cancer

The recent identification of p53 as a transcriptional repressor of *CD44* in breast cancer and mammary epithelium [16], prompted us to explore whether a similar functional relation exists in colon cancer and intestinal epithelium. We therefore examined the relation between p53 mutational status and *CD44* mRNA levels in a cohort of 90 colorectal carcinomas. All tumours included in this study were adenocarcinomas with invasion through the muscularis propria, but without lymph node or distant metastasis (Dukes B, AJCC Stage II). Mutational status was assessed by cDNA sequencing of the entire coding region of the *p53* gene, spanning exons 1 to 11. Sequence analysis identified 25 tumours (28%) with a mutation, resulting in a transcriptionally inactive p53 protein according to the definition of Soussi et al. [22] (Table 2). Comparison between the groups with wild-type and mutant *p53* revealed a significantly decreased mRNA expression of two canonical p53 transcriptional targets, *CDKN1A* (*p21*) ($P<0.01$) [23] and *MDM2* ($P<0.001$) [24] in the tumours with *p53* loss of function mutations (Figure 1A and B). Interestingly, in contrast to mammary tumours in which loss of p53 function was found to be significantly correlated with elevated *CD44* expression [16], *p53* mutation in colon carcinomas was correlated with decreased *CD44* mRNA expression levels ($P<0.01$; Figure 1C). These results imply that p53 does not act as a transcriptional repressor of *CD44* expression in CRC.

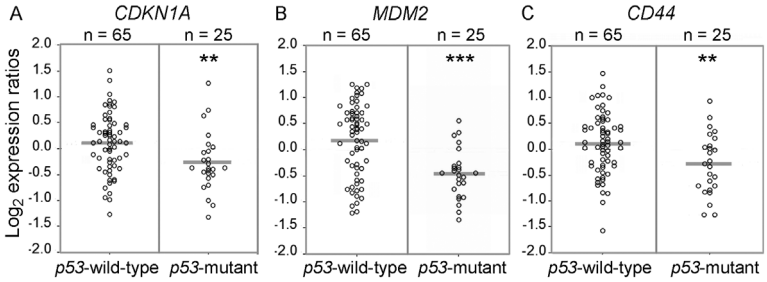

Figure 1. Loss of function mutation of *p53* is not associated with elevated *CD44* expression in colon cancer. Relative gene expression levels in *p53* mutant and *p53* wild-type adenocarcinomas for (A) *CDKN1A* (*p21*) (**, P<0.01), (B) *MDM2* (***, *P*<0.001), (C) *CD44,* (**, *P*<0.01).
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CD44 Protein Expression is not Increased in Colon Carcinomas with *p53* Mutation

In order to confirm that *p53* mutational status and mRNA levels of *CD44* in primary colon cancer specimens reflect protein levels, we examined p53 and CD44 expression by immunohistochemistry in a subset of the tumours (n = 15/group). Mutations in *p53* often result in an inappropriate stabilization of the protein and nuclear accumulation [25]. In accordance, whereas tumours harbouring only wild-type *p53* gene sequences showed either no staining for p53 protein or nuclear staining in scattered cells, tumours containing a *p53* mutant gene showed a strong nuclear staining of the majority of the malignant cells (*P*<0.001, Figure 2A and B). CD44 expression was observed on the cell membrane of the vast majority tumours with either unmutated *p53* (14 out of 15) or mutated *p53* (14 out of 15) (Fig. 2A). Importantly, there was no significant difference in the CD44 staining score between tumours of both groups (*P*>0.05; Figure 2B). These findings demonstrate

Table 2. *p53* mutations detected in colon adenocarcinomas.

#	Sample	Gender	Age	Location	Mutation	Transactivation class (*)
1	COL01	M	41	descending colon	Y205D	non-functional
2	COL06	M	76	transverse colon	R273P	non-functional
3	COL09	F	92	ascending colon	R273H	non-functional
4	COL10	F	54	cecum	P250L	non-functional
5	COL17	F	64	sigmoid colon	R273H	non-functional
6	COL35	M	67	cecum	R267W	non-functional
7	COL38	F	95	sigmoid colon	R282W	non-functional
8	COL44	M	78	transverse colon	R267P	non-functional
9	COL45	M	75	sigmoid colon	K132N	non-functional
10	COL48	F	90	hepatic flexure	T284P	non-functional
11	COL50	F	68	cecum	deletion Exon-9	non-functional
12	COL55	F	78	transverse colon	R175H	non-functional
13	COL59	F	34	cecum	F134C	non-functional
14	COL60	F	79	sigmoid colon	N235S & R249M	non-functional
15	COL61	M	78	cecum	deletion(AT) Codon 237	non-functional
16	COL62	F	80	sigmoid colon	R175H	non-functional
17	COL65	M	63	sigmoid colon	R175H	non-functional
18	COL68	M	74	sigmoid colon	R273H	non-functional
19	COL69	F	55	sigmoid colon	deletion(T) Codon 275	non-functional
20	COL73	M	80	descending colon	R175H	non-functional
21	COL74	M	74	sigmoid colon	C176Y	non-functional
22	COL76	M	69	sigmoid colon	R248Q	non-functional
23	COL79	M	72	ascending colon	R175H	non-functional
24	COL83	F	76	sigmoid colon	R342 Stop	non-functional
25	COL94	M	87	sigmoid colon	deletion Exon 7 Exon 8	non-functional

*p53 transactivation function according to Soussi T et al. [22].
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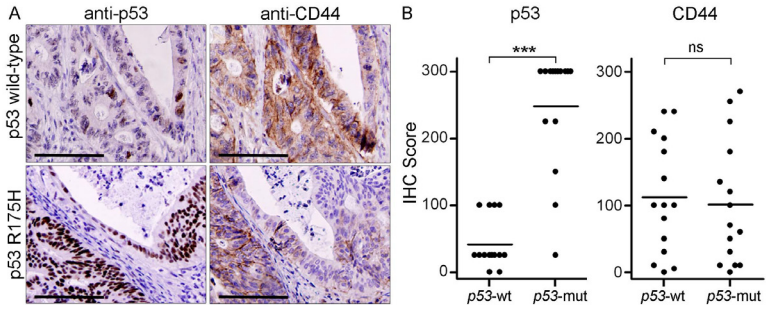

Figure 2. Loss of function mutation of *p53* is not associated with elevated CD44 protein expression in colon cancer. (A) Serial sections of a colon carcinoma without and with a p53 loss of function mutation (*i.e.*, R175H, Table 2), stained for p53 and CD44 protein (bars indicate 50 μm). (B). Immunohistochemistry (IHC) score of p53 and CD44 protein expression, respectively (***, $P<0.001$, ns = not significant).
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that, other than in breast cancer, loss of p53 function in colon cancer is not connected with increased CD44 protein expression.

p53 does not Repress *CD44* in Colon Cancer Cells and Normal Intestinal Epithelium

The above findings do not exclude the possibility that wild-type p53 may (partially) suppress CD44 expression in normal and neoplastic intestinal epithelium upon activation by genotoxic stress. To address this possibility, we determined the effects of DNA damage-induced p53 activation on CD44 levels in human RKO colon cancer cells. These cells express wild-type p53 and K-Ras, and are diploid [26]. Of particular interest, RKO cells also contain wild type *APC* and *CTNNB1* genes and lack constitutive β-catenin/TCF-4-mediated transcription [27]. This is of importance since the transcriptional regulation of *CD44* by p53 might be masked by constitutive Wnt pathway activation, leading to β-catenin/TCF-4-mediated *CD44* expression. RKO cells were exposed to 10 Gy of γ-radiation after which expression of *CDKN1A (p21)* and *CD44* and were analysed by real-time qRT-PCR. Expression of *c-MYC*, a direct Wnt target gene [28,29] was also assayed to control for the maintenance of a steady state of β-catenin/TCF-4-driven transcriptional activity. In addition, p53, p21, and CD44 protein levels were analysed by immunoblotting. As expected, ionizing radiation-induced DNA damage resulted in p53 stabilization (Figure 3A) and the consequent transactivation of p21 was observed at all time points (Figure 3A and B). However, *CD44* gene expression and CD44 protein levels did not decrease over time (Figure 3A and B), while *c-MYC* mRNA levels remained stable (Figure 3B). These data indicate that p53 is unable to repress *CD44* expression in human colon cancer cells.

To extend our observations to normal intestinal epithelium, we next investigated the CD44 response to p53 activation in epithelial cells lining the crypt-villus axis of mouse small intestines. For this purpose, we employed *in vitro* cultured mouse intestinal epithelial crypts-villus organoids [19]. Organoids comprising multiple crypt domains (Figure 4A) were exposed to 10 Gy of γ-radiation after which *Cdkn1a*, *Cd44*, and *c-Myc* mRNA expression levels were analysed by real-time qRT-PCR. Similar to RKO cells, *Cdkn1a* mRNA levels were increased in the organoids in response to ionizing radiation (Figure 4B). These results are consistent with previous studies on radiation-induced p53 activation in the mouse crypt compartment [30]. *Cd44* mRNA expression was not significantly changed after radiation exposure, while expression levels *c-Myc* remained stable (Figure 4B). Similarly, chemical induction of p53 activation using NCS also resulted in increased levels of *Cdkn1a* mRNA. Simultaneous incubation with the p53 stabilizing agent nutlin further elevated *Cdkn1a* mRNA levels. In both conditions *Cd44* mRNA expression was not significantly altered, while *c-Myc* expression levels remained stable (Figure 4C).These results confirm our findings in the human RKO cells and in primary colon carcinomas, and demonstrate that *CD44* gene expression is not regulated by p53 in both normal and transformed intestinal epithelial cells.

Discussion

The identification of p53 as a transcriptional repressor of CD44 expression in breast cancer [16] prompted us to investigate the relation between *p53* mutational status and CD44 expression in colon cancer. We demonstrate that, other than in breast cancer, *CD44* mRNA and protein levels are not increased in colon carcinomas with loss of functional p53, compared to tumours without *p53* mutations (Figure 1C, 2B). In addition, *CD44* expression in both normal and neoplastic intestinal epithelium was not affected by chemical or radiation-mediated activation of p53, indicating that p53 does not function as a transcriptional repressor of *CD44* in intestinal epithelial cells.

The observed tissue specific difference between breast and colon in transcriptional regulation of *CD44* might be explained by the complexity of p53 function. At least two features of the p53 protein are required for its gene regulatory function: p53 needs to recognize and bind a specific DNA sequences in the promoter of the target gene and p53 must recruit several transcriptional co-regulators (reviewed by Laptenko and Prives [14]). The *CD44* promoter contains a non-canonical p53 binding sequence [16], however, multiple interactions with co-activators and co-repressors as well as with the components of the general transcriptional machinery dictate its ability to direct promoter activation [14]. For example, interactions with ASPP1, BRCA1 or PTEN, or the coordinated activity of both p63 and p73, have been identified as determinants that direct specific responses [14,31]. Differences in the expression and activity of these co-regulators between breast and intestinal epithelium could therefore contribute to a divergent role for p53 in the transcriptional control of the *CD44* gene in breast and colon epithelium and cancer cells. In addition, p53 can undergo several types of post-translational modification, including phosphorylation, acetylation and ubiquitination [32], which can direct promoter selection [33]. Hence, p53 function depends on a complex and tight regulation, and cell-specific modifications or

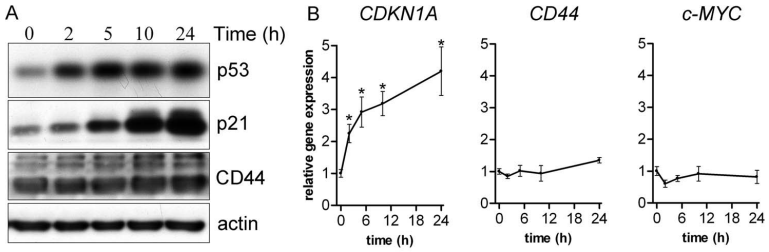

Figure 3. p53 protein does not repress CD44 expression in colon cancer cells. (A) Immunoblotting analysis of p53, p21 and CD44 protein levels in RKO colon cancer cells treated with ionizing radiation. Actin was used as loading control. (B) qRT-PCR results showing relative gene expression levels for *CDKN1*A (*p21*), *CD44,* and *c-MYC*. Data represent mean ± SEM of duplicate experiments;.(*, $P<0.05$ compared with t = 0).
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interactions may explain its inability to repress CD44 in intestinal epithelial cells. Our finding that CD44 expression in normal intestinal epithelium and colon carcinomas is independent of p53 expression and p53 mutational status is of significance for understanding the pathogenesis of CRC and may have important therapeutic implications. Aberrant CD44 expression is advantageous for the growth, survival, and dissemination of tumour cells [1]. In CRC these biological functions of CD44 extend beyond its ability to antagonize the pro-apoptotic and cytostatic functions of p53 [16,34]. This may, at least partly, explain the limited role of p53 in modulating the immediate phenotype of newly formed intestinal adenomas [35]. Furthermore, several studies have demonstrated that CD44 is a robust marker with functional importance for colon cancer stem cells [10,11,36–38]. These cells are believed to be relatively resistant to therapy and responsible for tumour-propagation, which makes CD44 an attractive target for cancer stem cell directed treatment, independent of p53.

Author Contributions

Conceived and designed the experiments: JZ SPJJ MS STP. Performed the experiments: JZ SPJJ LV JK. Analyzed the data: JZ SJ LV JK JPM RV MS STP. Contributed reagents/materials/analysis tools: LV JK JPM RV. Wrote the paper: JZ SPJJ MS STP.

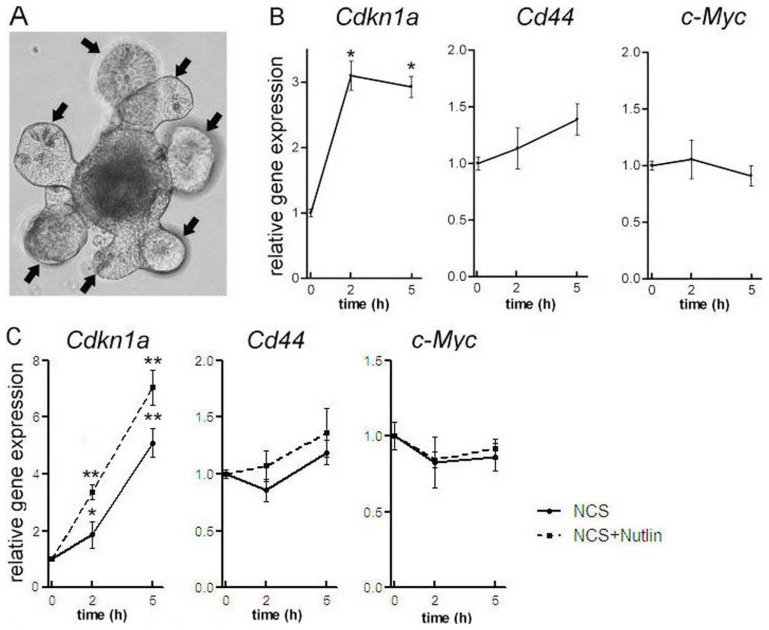

Figure 4. p53 is unable to repress *CD44* in the normal intestinal mucosa. (A) Crypt-villus organoid after one week of culture. Arrows indicate crypt-like compartments (B) qRT-PCR results showing relative gene expression levels after radiation treatment for *Cdkn1a*, *Cd44,* and *c-Myc*. Data represent mean ± SEM of duplicate experiments; (*, $P<0.05$ compared with t = 0). (C) qRT-PCR results showing relative gene expression levels after treatment with NCS alone or NCS plus nutlin for *Cdkn1a*, *Cd44* and *c-Myc* (*, $P<0.05$, **, $P<0.01$ compared with t = 0).
doi:10.1371/journal.pone.0072849.g004

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Chapter 7

General discussion

GENERAL DISCUSSION

Cell adhesion molecules, such as CD44, were once thought to function primarily in tethering cells to extracellular ligands, thereby simply serving as "molecular glue" [1]. However, it is now evident that many adhesion molecules provide biochemical and physical signals, which regulate a range of diverse functions such as cell proliferation, differentiation, apoptosis, migration and gene expression [2], making these molecules, and in particular CD44, highly versatile. Various CD44 isoforms are expressed by a wide range of tissues and their expression pattern can vary widely across different cell types and contexts. The prominent expression of CD44 by different types of adult stem cells, including mesenchymal [3], neural [4] and breast stem cells [5], strongly suggest a functional role in stem cell biology. In chapter 2, we demonstrate, by using immunohistochemistry, that crypt base columnar cells in mice, which are pluripotent intestinal stem cells (ISCs), prominently express CD44. Activating Wnt pathway mutations in ISCs give rise to intestinal adenomas with a much higher efficacy than in more differentiated intestinal epithelial cells [6], thus associating CD44 expression with the oncogenic transformation of ISCs. By crossing CD44 knock-out mice with tumor-prone *Apc*$^{Min/+}$ mice, we demonstrate that CD44 is an important regulator of adenoma formation (Chapter 2). Compared to controls, mice lacking CD44 showed a significant reduction in the number of intestinal adenomas. This reduction is primarily caused by a decrease in the formation of aberrant crypts. Analysis of *CD44* mRNA species using exon-specific RT-PCR shows that epithelial cells at the crypt base express a distinct and limited set of CD44 variant isoforms, which includes CD44v6-10, CD44v7-10, and CD44v8-10. One isoform in particular, CD44v4-10, was exclusively detected in Lgr5-high ISCs (Chapter 3), suggesting involvement in stem cell functioning. Further support for a link between CD44v and ISC biology comes from the finding that *CD44v* mRNA levels were found to correlate with expression of the ISC signature genes *Olfm4* and *Ascl2*. It is important to note that this expression pattern is essentially conserved between mouse and human as neoplastic epithelial cells from early lesions of FAP patients display prominent expression of *CD44v4-10* as well. Compared to expression in ISCs, expression of *CD44v* mRNA was drastically decreased in the transit amplifying compartment. Taken together, these findings demonstrate that CD44v4-v10 is part of the ISC program and indicate a role for this specific isoform in the malignant transformation of ISCs in both mouse and human. In contrast, expression of the standard form of CD44 (CD44s) was not observed in mouse ISCs, nor in FAP lesions (chapter 3). Notably, CD44s expression has been reported in a variety of tissues/cell types, including colorectal cancer cells [7,8]. Furthermore, assessment of CD44 expression in a set of commonly used human colorectal cancer cell lines, including *DLD-1*, HCT116 and HT29, has revealed expression of CD44s, in addition to that of CD44v4-10 (unpublished results). Expression of CD44s by these cell lines is presumably caused by aberrant regulation of pre-mRNA splicing, possibly by downregulation of the epithelial cell type-specific regulator of *CD44* variant exon inclusion ESRP1 [9].

To dissect the function of the identified variant domains of CD44 *in vivo*, we crossed knock-in mice exclusively expressing either CD44v4-10 or CD44s with tumor-prone *Apc*$^{Min/+}$ mice (chapter 3). This study revealed a critical role for CD44v4-10, but not CD44s, in the malignant transformation of intestinal epithelial cells. These results demonstrate that ISC-associated CD44v4-v10 contributes to intestinal tumor formation and imply that the variant domains of CD44 are involved in oncogenic signaling in intestinal epithelial cells. This is not only relevant for tumor initiation. The prominent expression of variant isoforms by tumorigenic colon cancer cells also suggests a functional role at later stages of the disease [10]. CD44 is an established surface marker of colon cancer stem cells (CoCSCs) with tumor-initiating capacity [11–13] and numerous studies point to a detrimental role for CD44 variant isoforms in advanced CRC [For a recent review see Ma *et al.* [14]]. For example, a study by Merlos-Suárez *et al.* demonstrated that an ISC gene-expression signature in primary tumors predicts disease relapse in CRC patients. This signature includes *CD44* and is specifically associated with CoCSCs, yet distinct from a proliferative gene signature [15]. We conclude that CD44v expression, including expression of CD44v4-v10, is a general feature of ISCs and newly transformed intestinal tumor cells and these variant domains, but not CD44s, promote oncogenic signaling.

As discussed above, there is a growing body of evidence indicating that CD44v expressing CRC cells exhibit increased tumorigenicity. A further understanding of the molecular mechanisms of action is needed in order to advance therapeutic treatment options involving CD44v. The finding that CD44v, and in particular variant isoforms containing exon v6, serve as co-receptor for the receptor tyrosine kinase MET [16,17], the principal receptor involved in HGF signaling, is of great interest for understanding the function of CD44v in the intestinal mucosa. The interaction between CD44 and MET is vital in mouse embryonic development, as MET-haploinsufficient CD44 knockout mice die at birth as a result of defects in the nervous system [17]. HGF/MET signaling has been implicated in a variety of biological responses, including adhesion, motility, proliferation, survival, and morphogenesis, while uncontrolled activation of MET is oncogenic in several human epithelial tissues, including kidney and lung. Mouse models that express the receptor or ligand as a transgene develop various types of tumor [Reviewed by Gherardi [18]]. Intestinal epithelial cells express MET [19], whereas HGF is produced by mesenchymal cells in the stroma adjacent to the epithelium [19,20]. Importantly, both *MET* and *CD44* are targets of Wnt signaling in human intestinal epithelial cells [8,21], suggesting a functional collaboration already at the initiation stage of CRC. Further evidence for a functional relation between CD44v and HGF/MET signaling comes from the observation that HGF/MET-mediated Ras activation promotes the inclusion of CD44 variant exons, providing a positive feedback loop [22]. Together, these findings support a molecular interplay between CD44v and MET in ICSs as molecular mechanism explaining the oncogenic function of CD44v.

In chapter 4, we demonstrate that single *Lgr5*-expressing mouse ISCs respond to HGF stimulation to initiate mini-gut formation *ex vivo.* In addition, we show that APC-deficient intestinal adenomas can utilize HGF, as an alternative to EGF, for expansion. Deletion of *MET* in the ISC compartment impairs HGF-driven epithelial expansion. These experiments demonstrate that HGF is a potent mitogen for the murine intestinal stem cell compartment. Notably however, ISC specific MET deletion *in vivo* does not affect the crypt phenotype, nor is there a selection for ISCs expressing MET as a result of neutral drift, thus showing that MET is dispensable for normal intestinal homeostasis. By examining the intestinal epithelium *in vivo* following radiation-induced injury, we established that ISCs depend on HGF/MET signaling during epithelial regeneration. This shows that HGF-mediated signaling is crucial in facilitating a regenerative response, permitting accelerated proliferation of the epithelial barrier. Most importantly, we observed that deletion of MET in the intestinal crypt compartment attenuates adenoma formation *in vivo*. This is accompanied by increased numbers of apoptotic cells in MET-deficient intestinal microadenomas. These findings indicate that HGF/MET-signaling promotes tumorigenesis by altering the balance between proliferation and apoptosis in the ISC compartment upon epithelial cell stress. Importantly, the effects of HGF on intestinal epithelial cells requires the presence of the correct CD44 isoform. We demonstrate that ISC-associated CD44v4-v10 and MET collaborate in regulating HGF-dependent mini-gut formation and tumor growth but this collaborative function cannot be fulfilled by CD44s. Thus, a principle function of CD44v on ISCs is to facilitate HGF/MET signaling and this “ménage à trois” between CD44v, MET and HGF likely supports adenoma formation *in vivo*.

Interestingly, the signaling events during intestinal epithelial regeneration after injury and during tumorigenesis show great similarity as both processes are marked by enhanced Wnt signaling, PI3K/AKT pathway activation, and MYC-mediated transcription [23]. These pathways are closely linked to both sporadic CRC and inflammatory-associated cancer development [24,25]. The niche adaptations that maintain ISC activity during homeostatic renewal and injury-induced intestinal regeneration have been reviewed by Santos [26]. As stated above, we identify a functional role of CD44v/MET signaling in niche-driven intestinal regeneration, but not in intestinal homeostasis. This highlights that CD44v/MET is part of an intestinal regenerative program that is distinct form normal intestinal homeostasis and capable of regulating the balance between proliferation and survival under stress conditions. Similar mechanisms in the intestinal crypt compartment have been reported by other research groups. For example, activation of SCF/c-Kit signaling and subsequent downstream PI3K/AKT signaling triggers dedifferentiation of Paneth cells and subsequent regeneration of the intestinal stem cell compartment after inflammatory insult [27]. This study emphasizes a great cellular plasticity in response to ISC damage and the contribution of niche signals that support stem cell fate decisions. Likewise, the

HIPPO/YAP signaling pathway is critical in reprogramming ISCs after epithelial damage, thereby promoting cell survival and EGF-mediated proliferation. Of relevance to our findings, interfering with HIPPO signaling strongly reduces adenoma formation in tumor prone *Apc*$^{Min/+}$ mice, and impairs adenoma growth *ex vivo* while inducing apoptosis [28]. These latter results are strikingly similar to those described in chapter 4 and illustrate that the intestinal regeneration program can be hijacked by tumor initiating cells to promote adenoma formation. Another example of a mediator of intestinal transformation without affecting homeostasis is the anti-apoptotic Bcl-2 protein. Work by van der Heijden and coworkers shows that this protein is specifically expressed in ISCs in both mouse and human. Interestingly, deletion of *Bcl-2* along with *Apc* in the intestinal epithelium of mice results in impaired adenoma formation when compared to controls. Loss of *Apc* in the ISC compartment causes cellular stress that leads to increased apoptotic cell death. Bcl-2 contributes to efficient transformation by alleviating apoptosis following loss of *Apc* [29]. This work points to a close connection between levels of pro- and anti-apoptotic proteins in ISCs and tumorigenesis. A clear link between Wnt signaling and intestinal epithelial cell survival is provided in chapter 5. In this chapter, we demonstrate that uncontrolled Wnt signaling drives expression of pro-apoptotic Bcl-2 family members BOK and BAX during the early stages of intestinal tumorigenesis. These results are in line with previous reports describing a specific degree of β-catenin signaling optimal for tumorigenesis [30,31]. APC interacts with cytoplasmic β-catenin through three 15-amino-acid repeats and seven 20-amino-acid repeats, thereby promoting its phosphorylation and subsequent degradation [32,33]. Typically, human colorectal adenomas retain expression of one truncated APC protein containing one or two of its original seven 20-amino-acid repeats, allowing some residual β-catenin downregulating activity. This “just-right signaling” model implies that a complete loss of APC-mediated downregulation of β-catenin activity triggers the induction of an apoptotic response thereby preventing tumor formation [30]. In accordance, β-catenin overexpression induces cell death in several cell lines [34]. The findings described in chapter 5 are consistent with this model and suggests a negative-feedback mechanism by which uncontrolled epithelial cell proliferation in the stem cell compartment can be counterbalanced. It should be noted that apoptotic cell clearance depends on many factors: Pro-apoptotic BOK and BAX can interact with several anti-apoptotic Bcl-2 family members such as, BCL-2, BCL-X_L, BCL-W and MCL-1. A dynamic balance between the many different pro- and anti-apoptotic components defines the threshold of responsiveness to intrinsic and extrinsic cues. For example, the use of the non-steroidal anti-inflammatory drug sulindac induces BAX-mediated apoptosis in intestinal epithelial cells independent of p53 [35,36]. This decision making process is further influenced by the inhibitor of apoptosis (IAP) family of proteins [37]. For a recent review on apoptosis, see Adams and Cory [38]. As stated in chapter 6, further exploitation of p53-independent pro-apoptotic signaling in

colorectal cancer may prove critical to finding more effective therapies in advanced disease. In particular, the extent by which BOK and BAX may modulate intestinal tumor formation and outgrowth in mouse and humans needs further investigation. The identification of targets that explicitly initiate apoptosis in intestinal epithelial cells could provide new approaches to prevent tumorigenesis with minimal toxicity. One suggestion is the use of sulforaphane in individuals susceptible to malignancy. This putative chemopreventive compound is capable of inducing apoptosis by altering the BAX/BCL-2 ratio in human CRC cells [39,40].

Whether CD44v/HGF/MET signaling is involved in modulating epithelial cell death and/or reprogramming of intestinal epithelial cells in humans needs further investigation. First, however, these findings need to be validated in human primary cell cultures. Nevertheless, our studies demonstrate a fundamental role for CD44v/MET in niche-dependent intestinal epithelial regeneration. This regulation of cell fate is most likely preserved in human CRC as stromal cells in primary carcinomas display prominent expression of HGF protein [19], which is connected with cancer-associated fibroblast that represent key players in the tumor cell microenvironment [41]. Intriguingly, CD44v mediated HGF/MET-signaling may also contribute to tumor cell dissemination, because HGF is a major growth factor expressed at the primary sites of metastatic spread, which are the lymph nodes and liver [42,43]. This notion is supported by recent work showing a critical role for CD44v6-dependent HGF/MET-signaling in driving the migration and metastasis of CoCSCs. Targeting both MET and CD44v6 prevented invasive capability of tumorigenic CRC cells. Intriguingly, these cells were shown to depend on PI3K/AKT-mediated activation of CD44v6 for viability and migration [10]. Combined, these observations show coordinate signaling by CD44v and MET in highly tumorigenic colon cancer cells and relevance in patients with advanced disease.

CONCLUSIONS AND PERSPECTIVES

From its initial identification as a transmembrane hyaluronan receptor mediating cell adhesion, the list of functions ascribed to CD44 has increased dramatically. These insights make CD44 an exemplary family of molecules, demonstrating how a single gene locus can be involved in the regulation of multiple physiological and pathophysiological processes. Alternative pre-mRNA splicing plays a major role in regulating cell-type specific functions, which are further diversified by post-translational modification. It is clear that CD44s and CD44v have distinct biological functions and the variant isoforms of CD44 probably have both overlapping as well as distinct functions on intestinal epithelial cells. This thesis describes a specific function of CD44v4-v10 in the intestinal epithelium. The extracellular variant domains of CD44 play a key role in regulating HGF/MET signaling, leading to the activation of downstream cascades that are associated with ISC fate and tumorigenesis (Fig. 1). Downstream signaling possibly stimulates CD44 variant exon inclusion [22,44], so this potentially creates a positive-feedback loop, augmenting both proliferative and survival signaling mechanisms. In contrast to the standard form of CD44, which is ubiquitously

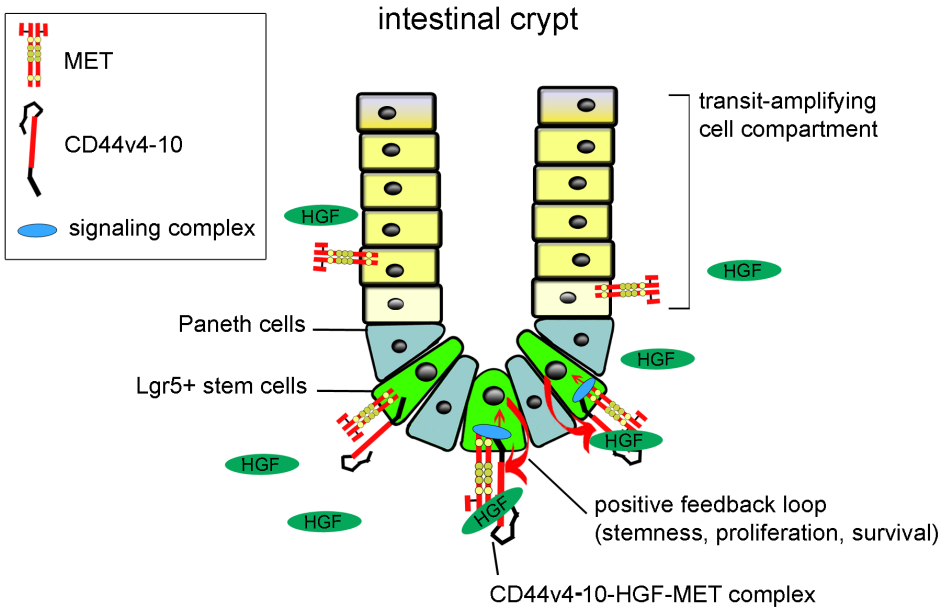

Figure 1. Mechanism by which CD44v4-10 and MET cooperate in signaling. CD44v4-10 acts as co-receptor for MET on Lgr5+ ISCs. CD44v4-10/MET signaling via binding of stromal-derived HGF potentially affects the RAS/MAPK and PI3K/AKT signaling pathways through an effector complex linked to the actin cytoskeleton. Signaling can augment ISC proliferation and survival. In addition, active RAS/MAPK signaling may sustain the inclusion of variant exons in CD44 mRNA, thereby creating a positive feedback loop. This model highlights a mechanism in which alternative splicing of CD44 can modify cellular response. A mechanism that can be essential during epithelial regeneration following injury or inflammation, but also increases susceptibility to tumorigenesis. In a similar way, enhanced downstream signaling by CD44v4-10/HGF/MET could provide an advantage to transformed cells during tumor progression and metastasis.

expressed by many different cell types, expression of CD44v is restricted in normal tissue [45]. The notion that CD44v/MET signaling is not required for intestinal homeostasis provides opportunities for therapeutic intervention while minimizing adverse side effects. Pharmacological inhibition of signalosome formation may be beneficial as a preventive strategy against the development of CRC in high risk populations, such as patients with a hereditary predisposition. This could possibly be achieved by an allosteric and selective inhibitor of CD44v6 capable of blocking MET activation and subsequent survival signaling. One possible candidate is the peptide AMC303 which has recently been used in advanced cancer patients [46]. Apart from this, blocking CD44v-MET signaling in advanced CRC may also benefit patients who are treated with EGFR tyrosine kinase inhibitors. Studies with EGFR-blocking antibodies in metastatic human CRC show that therapy resistance can be explained by aberrant activity of MET [47–49]. Consequently, combination therapy targeting both EGFR and HGF/MET is considered a promising approach for these patients [50]. Thus, targeting the CD44v/MET signalosome may improve response to EGFR inhibitors in CRC. More strategies targeting CD44v6 are being explored. These include the development of anti-CD44v6 mAbs [51,52] and CD44v6 specific peptides and aptamers that interfere with protein-protein interactions [46,53–55]. Combination therapy using these agents may provide an opportunity to improve treatment for CRC patients.

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Appendix

Summary

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SUMMARY

Cancer accounts for millions of deaths worldwide each year. After lung and breast cancer, colorectal cancer (CRC) is the most common cause of death from malignant disease in the Western world. CRC derives from epithelial cells lining the crypts and develops through a series of genetic modifications that transforms normal epithelium to an adenoma and then ultimately adenocarcinoma. Somatic mutations that activate the Wnt/β-catenin signaling pathway in the intestinal crypt compartment constitute a principle route to tumorigenesis. Active Wnt signaling is crucial to maintain a proliferating and multipotent pool of intestinal stem cells (ISCs). *CD44* is one of the most prominent Wnt-responsive genes in the intestinal mucosa and, as a consequence, CD44 protein expression is abundant on ISCs and their transit amplifying progeny. CD44 comprises a versatile family of transmembrane adhesion receptors capable of binding extracellular matrix components. The occurrence of variable exons, mainly involving the extracellular domain, allows for the expression of various isoforms with presumably both overlapping and unique functions. Importantly, certain CD44 isoforms function as a coreceptor for growth factors that are present in the ISC microenvironment. It's involvement in sensing, integrating and transducing microenvironmental signals makes CD44 a putative factor in regulating ISC fate decisions and in promoting intestinal tumorigenesis. These concepts are strengthened by the sustained expression of CD44 on colon cancer cells with stem-cell like properties. Aberrant CD44 expression in invasive CRC is associated with increased tumorigenic and metastatic potential, indicating a functional role throughout disease. The elucidation of tumor-specific CD44 isoforms, or signaling pathways affected by these isoforms, may contribute to the development of targeted treatment strategies. However, the functional contribution of CD44, and in particular individual splice variants of CD44, to the onset and progression of intestinal cancer has remained elusive. This thesis investigates the contribution of CD44 and variant isoforms to intestinal tumorigenesis and focuses on its role in affecting signaling cascades that regulate cell fate, cancer growth and progression.

Chapter 1 gives a general overview of the intestinal tract. Epithelial homeostasis is maintained by *Lgr5*-expressing stem cells that reside at the base of intestinal crypts and that are surrounded by a supportive stem cell niche. Self-renewal and differentiation is tightly controlled by the concerted action of a multitude of signals, including Wnt signaling. *CD44* is a major Wnt target gene in the intestine. The *CD44* gene locus encodes for a variety of CD44 variant isoforms that are subject to further post-translational modification. CD44 is the major cell receptor for the extracellular glycosaminoglycan hyaluronic acid, but is also capable of interacting with other ligands. By interacting with cell-surface associated growth factors, enzymes and cytokines CD44 can stimulate signaling. Notably, CD44 can serve as a co-receptor for RTKs. The cytoplasmic domain of CD44 plays an essential role in signal transduction by interacting with cytoplasmic effector proteins. In this way CD44

can affect signaling cascades that modulate cell proliferation and survival. Expression of CD44 variant isoforms is associated with the initial stages of intestinal cancer and its expression on tumor cells is associated with tumor-initiating capacity at distant sites and worse overall patient survival. **Chapter 2** addresses the role of CD44 in intestinal tumor formation. We show that CD44 is prominently expressed by crypt-base columnar cells, which are the stem cells in the intestine. Deletion of CD44 in the $Apc^{Min/+}$ mouse model for intestinal tumorigenesis results in a strong decrease in aberrant crypt formation, implying the involvement of CD44 in intestinal tumor formation. The deletion of CD44 in $Apc^{Min/+}$ mice is associated with increased apoptosis in the basal crypt compartment. This chapter demonstrates that CD44 affects the earliest stages of tumor formation. In **chapter 3** we show that ISCs display a specific set of CD44 variant isoforms, but lack the CD44 standard isoform. We identify CD44v4-v10 as the largest variant isoform being present on mouse ISCs and on the dysplastic aberrant crypt foci of familial adenomatous polyposis patients. This suggest that this isoform is implicated in murine and human intestinal tumorigenesis. To address the contribution of this specific isoform to tumor formation, mice exclusively expressing either CD44v4-10 or, as control, CD44s were intercrossed with $Apc^{Min/+}$ mice. This study demonstrates that the variant domains of CD44 are involved in augmenting intestinal tumor formation, since mice lacking CD44 or mice exclusively expressing the CD44 standard isoform display a strongly reduced number of microadenomas in the intestinal tract when compared to mice with either wild type CD44 or mice exclusively expressing CD44v4-v10. These findings identify CD44v as a component of the ISC program critical for intestinal tumorigenesis. In **chapter 4** we explore the role of MET RTK and CD44v in controlling ISC homeostasis, tumorigenesis and epithelial regeneration after injury. We employed *in vitro* cultured mouse intestinal crypts, which develop into “mini-guts” under defined growth conditions, which include EGF stimulation. These experiments demonstrated that HGF/MET signaling can fully substitute EGFR signals in intestinal organoid cultures. In addition, deletion of *Met* impaired HGF-mediated crypt expansion without disrupting EGF-induced growth. In a similar manner, we show that HGF/MET signaling can stimulate the expansion of *Apc*-mutant adenomas in culture. In addition, we demonstrate that CD44v4-10, but not CD44s, mediates HGF-induced epithelial expansion in both crypt organoids as well as in cultured *Apc*-mutant tumor spheroids. These results show that HGF, like EGF, can provide the RTK signals required for both crypt and adenoma expansion, and that HGF-dependent growth requires the presence of CD44v. Conditional *in vivo* disruption of *Met* in the mouse intestinal epithelium revealed that MET is dispensable for crypt-cell proliferation, survival and differentiation under homeostatic conditions, but that MET is involved in intestinal epithelial regeneration after radiation injury. *Met* disruption in conditional *Apc*-mutant mice revealed a significant reduction in adenoma burden when compared to *Met* wild-type mice, demonstrating a functional role of MET in adenoma formation *in vivo*. Loss of MET

function in *Apc*-mutant intestinal adenomas was associated with higher apoptotic rates. This study sets forth a theoretical model in which CD44v and MET collaborate in the onset and progression of human CRC. A finding that can be of importance to clinical medicine, since amplification of MET receptor signaling drives resistance to anti-EGFR therapies in colorectal cancer. **Chapter 5** addresses the relation between Wnt signaling and apoptosis related gene expression in intestinal epithelial cells. This study demonstrates strongly elevated apoptotic rates in *Apc*-mutant adenomas. Apoptotic gene expression analysis along the murine crypt-villus axis and in adenomas of $Apc^{min/+}$ mice revealed increased expression of two pro-apoptotic Bcl-2 family members in intestinal adenomas, *Bok* and *Bax*. In addition, we corroborate the restricted expression pattern of *Birc5*/Survivin, a member of the inhibitory of apoptosis gene family and a known Wnt target gene in the intestine. Gene expression analysis along the crypt-to-surface axis and of dysplastic crypts of the colon of familial adenomatous polyposis patients revealed a similar expression pattern in the human colonic mucosa. Inhibition of Wnt signaling in a human CRC cell line significantly decreased *BOK* and *BAX* expression, confirming Wnt-dependent regulation. These findings demonstrate a link between dysregulated Wnt-signaling and the induction of pro-apoptotic Bcl-2 family members at the early stage of Wnt-driven intestinal tumor formation. A relation between CD44 and the tumor suppressor protein p53 is investigated in **chapter 6**. In breast cancer, *CD44* expression is directly repressed by p53. This restrained expression can be released by loss of p53 function, which unleashes the pro-tumor functions of CD44. Since inactivating *p53* mutations are frequent genetic events in CRC, we hypothesized a similar correlation between *p53* mutational status and *CD44* expression in the intestinal mucosa. Analysis of *p53* mutational status and *CD44* mRNA expression in a cohort of 90 colorectal adenocarcinomas revealed that loss of function mutation of *p53* is not associated with elevated *CD44* expression. These results were corroborated with immunohistochemistry. In addition, *CD44* was not found to be regulated by DNA damage-induced p53 activation in both murine crypt-villous organoid cultures and in CRC cells with wild-type p53 and intact Wnt signaling. This excludes the possibility that wild-type p53 can suppress *CD44* expression upon activation by genotoxic stress. These findings demonstrate a tissue specific difference between breast and colon in the transcriptional regulation of *CD44* by p53.

NEDERLANDSE SAMENVATTING

In Nederland krijgen jaarlijks meer dan 14.000 mensen kanker aan hun spijsverteringskanaal. Het overgrote deel van deze mensen krijgt dikkedarmkanker. Er overlijden ieder jaar meer dan 5.000 mensen aan darmkanker. Daarmee is dit de derde meest voorkomende kankersoort in Nederland. Een tumor in de darm ontstaat uit epitheelweefsel. In de gezonde darm is dit een enkele laag van cilindrische cellen die een selectieve barrière vormt tussen de darmholte (lumen) en het interne van het lichaam. Darmepitheel laat de opname van voedingsstoffen toe en beschermt het menselijk lichaam tegen micro-organismen. Deze epitheliale laag vernieuwd zichzelf continu vanuit vele kleine instulpingen (crypten) waaruit nieuw gevormde cellen omhoog bewegen. Eenmaal bovenin gekomen sterven de epitheelcellen af door gecontroleerde celdood en worden de celrestanten afgestoten in het lumen. Op deze manier wordt de bekleding van de darm iedere paar dagen volledig vernieuwd. Diep onder in de crypten zitten de darmstamcellen die verantwoordelijk zijn voor de continue productie van nieuwe epitheliale cellen. Deze relatief kleine cellen met weinig cytoplasma kunnen herkend worden aan de hand van *Lgr5* genexpressie. Iets hoger in de crypt bevinden zich ongedifferentieerde dochter cellen (progenitors) die nog een aantal celdelingen ondergaan, waarna deze differentiëren tot gespecialiseerde darmepitheelcellen. Dit zijn onder andere de enterocyten die betrokken zijn bij de opname en het transport van voedingsstoffen en de slijmbekercellen cellen (goblet cellen) die beschermende eiwitten maken. Cellen die differentiëren tot antimicrobiële Paneth cellen blijven onder in de crypt waar ze mengen met de stamcellen. Een ongedifferentieerde darmepitheelcel bevat verschillende signaaltransductieroutes die celdeling, differentiatie, en geprogrammeerde celdood reguleren. Wnt/β-catenine signalering speelt een centrale rol bij deze regulatie, en bij het ontstaan van kanker in de darm. Het APC eiwit, het product van het *APC*-gen, regisseert de Wnt-signaalroute door de hoeveelheid β-catenine in het cytoplasma te regelen. Het APC eiwit is onderdeel van een “vernietigingscomplex” in de cel dat er voor zorgt dat β-catenine wordt afgebroken. De detectie van Wnt-signalen aan de buitenkant van de cel remt deze afbraak waardoor de hoeveelheid β-catenine in het cytoplasma zal stijgen. Uiteindelijk zal β-catenine de celkern ingaan en daar bepaalde genen aan- en uitzetten. De Wnt-signalering kan worden ontregeld door een mutatie in het *APC* gen. Er ontstaat dan een beschadigd APC eiwit waardoor het vernietigingscomplex de hoeveelheid β-catenine niet meer kan verminderen. De celkern krijgt dan een grote hoeveelheid β-catenine wat leidt tot veranderingen in de uitgroei van het darmepitheel en het ontstaan van een goedaardig gezwel (poliep) in de darm. Een mutatie in het *APC*-gen is de eerste stap in 80% van alle gevallen van darmkanker. In de resterende gevallen is er vaak een mutatie in het gen voor β-catenine. Hoe meer verschillende genen gemuteerd raken, hoe afwijkender de epitheelcellen groeien. Uiteindelijk kan er een kwaadaardig gezwel ontstaan dat door de darmwand heen groeit en buiten de darm verder kan groeien.

De genen die door β-catenine worden gestimuleerd spelen een belangrijke rol bij het ontstaan en de ontwikkeling van darmkanker. Deze bieden dan ook aanknopingspunten voor preventie en behandeling. Eén van de genen die aangezet wordt door Wnt-signalering onder in de crypten codeert voor het CD44 eiwit. CD44 is een familie van transmembraan eiwitten met verschillende functies. Het *CD44*-gen bevat een aantal variabele delen (exonen) in het coderende DNA. Na vertaling van het coderende DNA in preRNA worden een aantal van deze variabele delen verwijderd door deze er uit te knippen (splicing). Het mRNA dat op deze manier ontstaat wordt verder vertaald in eiwit. Vanwege alternatieve splicing kunnen er meerdere eiwitvarianten (isovormen) worden gevormd die verschillen in de lengte van het extracellulaire domein. De langere varianten worden aangeduid met CD44v, en de variant van CD44 zonder deze variabele delen wordt aangeduid met CD44s (CD44 standaard). Het is waarschijnlijk dat de verschillende CD44 eiwitvarianten zowel overlappende als unieke functies hebben. CD44 is onder andere betrokken bij het detecteren van signalen aan de buitenkant van de cel en het doorgeven van signalen van de membraan naar de celkern. Dit maakt het waarschijnlijk dat CD44 betrokken is bij de regulatie van darmstamcelfunctie en bij het ontstaan van darmkanker. Dit concept wordt versterkt door de versterkte en aanhoudende expressie van CD44 in de verschillende stadia van darmkanker wat tevens duidt op een cruciale rol in de progressie. Centraal in dit proefschrift staat de vraag in hoeverre CD44, en in het bijzonder de langere varianten van CD44, bijdragen aan het ontstaan van darmkanker en wat de functie is van deze eiwit varianten. Dit kan leiden tot een beter inzicht in de moleculaire mechanismen die ten grondslag liggen aan het ontstaan en de progressie van darmkanker en uiteindelijk tot het ontwikkelen van nieuwe behandelingen gericht op CD44 of de specifieke signaaltransductie routes die door CD44 worden geactiveerd.

In **hoofdstuk 2** laten we zien dat CD44 betrokken is bij het ontstaan van darmkanker in genetisch gemodificeerde muizen. We laten zien dat het celmembraan van darmstamcellen relatief veel CD44 eiwit bevat. Bij deze studie zijn muizen gebruikt waarbij één allel van het *Apc*-gen gemuteerd is als model voor het spontaan ontstaan van darmtumoren ($Apc^{Min/+}$ muizen). Wanneer deze $Apc^{Min/+}$ muizen het CD44 eiwit niet meer tot expressie brachten ontwikkelden deze beduidend minder tumoren in de darm vergeleken met $Apc^{Min/+}$ muizen met CD44. Onder in de crypt van de $Apc^{Min/+}$ muizen zonder CD44 eiwit bevonden zich meer stervende cellen, wat duidt op een relatie tussen CD44 en afremmen van gecontroleerde celdood.

In **hoofdstuk 3** hebben we onderzocht welke varianten van CD44 door darmstamcellen in de muis tot expressie worden gebracht. Tevens hebben we deze analyse gedaan op menselijke darmepitheelcellen in het vroegste stadium tumorontwikkeling. Darmstamcellen in de muis en darmtumorcellen in de mens bleken voornamelijk langere CD44 varianten te bevatten, terwijl de standaard vorm van CD44 niet te detecteren was. In zowel het menselijke en het

muizen darmepitheel was *CD44* mRNA te vinden met hierin de variabele exonen v4 t.m. v10. Dit duidt er op dat deze specifieke isovorm betrokken is bij het ontstaan van darmkanker in zowel de muis als de mens. Dit hebben we onderzocht door muizen met enkel expressie van één CD44 variant te kruisen met de $Apc^{Min/+}$ muizen. Deze studie liet zien dat muizen die specifiek CD44v4-v10 tot expressie brengen evenveel tumoren ontwikkelden als muizen met wild-type CD44, terwijl er in de muizen met enkel CD44s of in muizen zonder CD44 beduidend minder darmtumoren waren ontstaan. Deze bevindingen tonen aan dat een langere stamcel specifieke variant van CD44 betrokken is bij tumorvorming in de darm.

In **hoofdstuk 4** hebben wij de rol van de MET-signaleringsroute en CD44 bij het ontstaan en de groei van darmkanker bestudeerd. Het transmembraan receptor tyrosine kinase eiwit MET lijkt een belangrijke rol te spelen bij het ontstaan en de uitgroei van darmkanker. Net als CD44 kan ook de expressie van dit eiwit worden gereguleerd door Wnt-signalering. Tevens komt MET tot expressie in alle stadia van dikkedarmkanker, wat duidt op betrokkenheid bij tumorontwikkeling en progressie. MET-signalering wordt geactiveerd door hepatocyt groeifactor (HGF) en uit eerdere studies is gebleken dat langere varianten van CD44 hierbij betrokken kunnen zijn. Bij dit onderzoek hebben we gebruik gemaakt van gekweekte minidarmen die onder bepaalde groeicondities kunnen uitgroeien vanuit één stamcel uit de muizendarm. Onder normale omstandigheden hebben ongedifferentieerde darmepitheelcellen de groeifactor EGF nodig om te kunnen uitgroeien tot minidarmen. Wij hebben hier laten zien dat deze cellen in afwezigheid van EGF als alternatief HGF/MET-signalering kunnen gebruiken voor uitgroei. Deze HGF-afhankelijke uitgroei is niet meer mogelijk wanneer het *Met*-gen wordt verstoord in deze cellen. Bovenal, deze HGF-geïnduceerde groei bleek afhankelijk te zijn van de aanwezigheid van de juiste CD44 isovorm op deze cellen. Gekweekte darmcellen zonder CD44 of met enkel CD44s expressie vertoonden een sterk verminderde uitgroei na stimulatie met HGF vergeleken met cellen die wild-type CD44 of specifiek CD44v4-v10 tot expressie brachten. De betrokkenheid van MET-signalering bij het ontstaan van darmtumoren bleek uit experimenten met muizen waarin zowel het *Apc*-gen als het *Met*-gen verstoord konden worden. Muizen zonder functioneel MET ontwikkelden beduidend minder tumoren in de darm, wat vergelijkbaar was met het CD44 muizenmodel uit hoofdstuk 3. De mogelijkheid om uit te groeien met behulp van HGF in plaats van EGF kan ook worden gebruikt door gekweekte darmtumorcellen uit *Apc*-mutant muizendarmen. Net als bij de gezonde darmcellen, bleek bij deze tumorcellen de HGF-geïnduceerde uitgroei afhankelijk van de aanwezigheid van CD44v. Deze resultaten tonen aan dat HGF-signalering een volledig alternatief is voor EGF-afhankelijke darmepitheelgroei, mits de juiste CD44 variant aanwezig is op de cellen. Bovendien lijkt het er op dat CD44v/HGF/MET-signalering de uitgroei van EGF-gestimuleerde cellen versterkt. Dit mechanisme kan van belang zijn bij herstel na beschadiging van het darmepitheel. Echter, deze versterkte

signaleringscascade zou ook “misbruikt” kunnen worden tijdens de ontwikkeling en uitgroei van darmtumoren. Omdat CD44v/HGF/MET-signalering onder normale omstandigheden niet van belang lijkt voor het onderhouden van gezond epitheelweefsel is deze signaleringsroute geschikt als nieuw mikpunt voor therapie.

Hoofdstuk 5 gaat dieper in op de relatie tussen Wnt-signalering en gecontroleerde celdood in het darmepitheel. Deze studie heeft laten zien dat verlies van functioneel APC in darmepitheel leidt tot een sterke activatie van gecontroleerde celdood. We hebben gezocht naar de betrokken eiwitten door genexpressie profielen te vergelijken van ongedifferentieerd, gedifferentieerd en APC-mutant epitheelweefsel. Deze experimenten toonden aan dat ongedifferentieerde cryptcellen een relatief sterke expressie hebben van een aantal genen die coderen voor eiwitten die betrokken zijn bij het reguleren van gecontroleerde celdood. Deze resultaten wijzen erop dat natuurlijke celdood een belangrijke functie heeft in het proces van epitheliale vernieuwing vanuit het stamcelcompartiment. Twee specifieke genen die nauw betrokken zijn bij de uitvoering van gecontroleerde celdood, *Bok* en *Bax*, bleken versterkt tot expressie te komen in het vroegste stadium van tumorontwikkeling. De Wnt-afhankelijke regulatie van deze genen hebben we bevestigd in menselijke darmkankercellen. Dit onderzoek bevestigd een relatie tussen verstoorde Wnt-signalering en de activatie van gecontroleerde celdood in het vroegste stadium van tumorontwikkeling. Dit biedt verdere aanknopingspunten om ongebreidelde celgroei in darmtumoren tegen te gaan.

Uit eerder onderzoek op borstkankercellen is gebleken dat het tumoronderdrukkend eiwit p53 in staat is om *CD44* genexpressie te beteugelen. Het p53 eiwit kan dit doen om zo de tumor stimulerende effecten van CD44 tegen te gaan. Omdat p53 inactiverende mutaties vaak voorkomen bij darmkankerpatiënten hebben wij in **hoofdstuk 6** onderzocht of er een vergelijkbare correlatie is tussen p53-functie en CD44-expressie in darmepitheelcellen. Analyse van *p53*-mutatiestatus en *CD44*-mRNA-expressie in een cohort van darmcarcinomen toonde aan dat verlies van p53 functie niet geassocieerd is met verhoogde *CD44*-genexpressie. Deze resultaten hebben we bevestigd door eiwitkleuringen uit te voeren op patiëntmateriaal. Bovendien bleek *CD44*-genexpressie in muizendarm onafhankelijk van de activatie van p53. Deze bevindingen hebben uitgesloten dat wild-type p53 de *CD44*-genexpressie kan onderdrukken in de darm en wijzen op weefselspecifieke verschillen in *CD44* genregulatie.

De resultaten beschreven in dit proefschrift tonen aan dat stamcel geassocieerde CD44v varianten nauw betrokken zijn bij het ontstaan en de uitgroei van darmtumoren. Eén van de meest waarschijnlijke functies van CD44v op darmepitheelcellen is de regulatie van MET-signalering. Deze signaaltransductieroute is nauw betrokken bij tumorgroei en de overleving van cellen. Deze studies impliceren dat CD44v een belangrijke rol speelt in het ontstaan en de ontwikkeling van darmkanker. Hierom lijkt CD44v, evenals een onderliggende signaaltransductieroute, geschikt als mikpunt voor therapie.

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CURRICULUM VITAE

Jurrit Zeilstra is geboren op 23 april 1976, te Hilversum. In 1996 behaalde hij zijn VWO diploma aan het Comenius College te Hilversum. In september van dat jaar begon hij aan de studie Moleculaire Wetenschappen in Wageningen (Wageningen University & Research). Tijdens deze studie liep hij stage bij de leerstoelgroep Microbiologie onder supervisie van dr. Leon D. Kluskens en prof. dr. John van der Oost. Hier werkte hij aan het kloneren en karakteriseren van thermostabiele enzymen uit anaerobe bacteriën. Hier merkte hij dat hij geïnteresseerd was in de veranderingen van erfelijk materiaal die er voor kunnen zorgen dat cellen zich aanpassen. Tijdens zijn stage op de Vrije Universiteit Amsterdam bij de Moleculaire en Cellulaire Neurobiologie onder begeleiding van dr. K. W. Li werkte hij aan een proteomics project waar hij onderzoek deed naar signaaltransductie in de hersenen met behulp van 2D elektroforese en massaspectrometrie. Tijdens dit werk kon hij zijn kennis over genetica en biochemie verder verdiepen. In 2002 nam hij zijn ingenieurs diploma in ontvangst. In 2003 is hij begonnen aan zijn promotieonderzoek bij de afdeling Pathologie van het Academisch Medisch Centrum in Amsterdam onder supervisie van prof. dr. Steven T. Pals. Het onderzoek dat hier werd verricht staat grotendeels beschreven in dit proefschrift. In 2010 ontdekte hij dat hij plezier beleefde aan het overbrengen van kennis aan een nieuwe generatie talent. Hierom is hij in 2012 begonnen met lesgeven in het voortgezet onderwijs. In 2014 heeft hij zijn Master Leraar Voorbereidend Hoger Onderwijs in Scheikunde behaald aan de Universiteit van Amsterdam. Momenteel werkt hij als docent bij het Hervormd Lyceum Zuid te Amsterdam.

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