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 ApcMin/+ mice, suggesting a role for CD44 in intestinal tumorigenesis. To study this role, we crossed C57BL/6J-Cd44+/− mice with C57BL/6J-ApcMin/+ mice. Compared with C57BL/6J-Cd44+/−/ApcMin/+ mice, C57BL/6J-Cd44+/−/ApcMin/+ 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+/−/ApcMin/+ mice did not alter the proliferative capacity and size of the intestinal stem cell and transit-amplifying compartments. However, compared with Cd44+/−/ApcMin/+ mice, Cd44+/−/ApcMin/+ 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.

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 ApcMin/+ 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 ApcMin/+ 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 (ApcMin/+; 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...
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 fixed 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 MiniElute 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 deparaffinization, 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 PowerVision poly-HRP anti-rabbit IgG (ImmunoLogic, Klinpath). 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 (Amer sham 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 1 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, “GAATGAACTGCGCGCTAG” (sense) and “GGAGGTGGTGGACGCTGAC” (antisense) with an amplicon of 268 bp (bp); Dn6, “CTCTCTGGGAGAGCTCAGAG” (sense) and “GCAAGTCACAGGG-TCCAG” (antisense), 144 bp; Bok, “CTGACCCTGCCGATGGTCC” (sense) and “GGCAGCACACACTTGGAGTG” (antisense) 119 bp; Lgr5, “CTACCTGACTTT-GAGGAAGAC” (sense) and “AGGAAAGGCCGAGTACTGGC” (antisense) 145 bp; and β-actin, “GGATGCAGAAGGAGATCTG” (sense) and “CCGATCCACAGAGTACTGG” (antisense), 91 bp. Student’s t test was used for statistical analysis.

Figure 1. CD44 expression in Cd44+/+ApcMin/− and Cd44−/−ApcMin/− 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 ApcMin/− small intestinal adenomas. IM7 staining of Cd44+/−ApcMin/− and Cd44−/−ApcMin/− 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<sup>Min</sup>/mice. A, number of adenomas in the intestinal tract of C44<sup>+/+/ApcMin/+</sup> and C44<sup>++/ApcMin/+</sup> 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 C44<sup>++/Apc<sup>Min</sup></sup> and C44<sup>++/Apc<sup>Min</sup></sup> 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 hybridization. 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 Cdk6. Primers used were "ATTAGGTGACATATA-G-AGGGTCAGACGCGC" (S6, sense) and "AGTAAATAGCTACTAAGTGTACGACAGCCGCAGCCTAC" (T7 antisense). In situ hybridization 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), 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<sup>Min</sup>/mice lacking CD44. To explore the role of CD44 in intestinal adenoma formation, Apc<sup>Min</sup>/mice lacking CD44 were generated by crossing C44<sup>++/ApcMin/+</sup> mice with Apc<sup>Min</sup>/mice. RT-PCR and immunoblotting confirmed the C44 expression status of the mice (Fig. 1A). A line with our previous observations (3, 4), immunohistochemical staining of tissue sections of the small intestines of Apc<sup>Min</sup>/mice (22) with Apc<sup>Min</sup>/mice, the number of aberrant crypts, small adenomas, lymphocytes, and plasma cells in the intestinal lamina propria (Fig. 1B). Within the epithelial compartment, prominent C44 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 C44 expression (Fig. 1B, circled). In addition to expression on normal crypt epithelium, C44<sup>++/Apc<sup>Min</sup></sup> mice displayed strong C44 expression on the neoplastic epithelium of intestinal adenomas (Fig. 1C). As expected, C44 expression was completely absent in the normal and neoplastic intestinal epithelium of the C44<sup>++/Apc<sup>Min</sup></sup> mice.

To follow the development and progression of intestinal tumors, mutant mice were monitored over time. At the time when Apc<sup>Min</sup>/mice with wild-type CD44 developed adenomas, Apc<sup>Min</sup>/mice 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 either of the (tubulo) villous type. The number of adenomas in Apc<sup>Min</sup>/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 C44 locus and consequential absence of C44 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. 2A). 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. 2B). The mean adenoma diameter was ~0.9 mm in both groups and did not differ significantly (Fig. 2C). These observations strongly suggest that C44 deletion suppresses adenoma initiation, rather than the outgrowth of the tumors.

To verify that C44 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 C44<sup>++/Apc<sup>Min</sup></sup> versus C44<sup>++/Apc<sup>Min</sup></sup> mice (Fig. 3). The number of aberrant crypts in C44<sup>++/Apc<sup>Min</sup></sup> mice was 12.0 ± 1.4 at 8 weeks and 10.7 ± 1.5 at 16 weeks. The C44<sup>++/Apc<sup>Min</sup></sup> mice displayed a strongly reduced number of aberrant crypts both at 8 weeks (3.1 ± 0.7) and at 16 weeks (4.3 ± 0.7), confirming a diminished tumor initiation. In the C44<sup>++/Apc<sup>Min</sup></sup> mice as well as in the C44<sup>++/Apc<sup>Min</sup></sup> 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<sup>Min</sup>/mice but does not prevent tumor outgrowth. This observation, as well as the previous finding that C44<sup>++/Apc<sup>Min</sup></sup> 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 C44<sup>++/Apc<sup>Min</sup></sup> mice, the pattern of expression of the proliferation marker Ki67 was indistinguishable from that in Apc<sup>Min</sup>/mice with wild-type CD44 and the size and cellularity of the transit-amplifying compartments were not different (Fig. 4A). We also compared the number of intestinal stem cells in C44<sup>++/Apc<sup>Min</sup></sup> and C44<sup>++/Apc<sup>Min</sup></sup> mice. These CBC cells are broad-based with a wedge-shaped nucleus and sparse 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 C44<sup>++/Apc<sup>Min</sup></sup> and C44<sup>++/Apc<sup>Min</sup></sup> mice showed Ki67-positive CBC cells at the crypt base (Fig. 4A, 4B).
Quantification of Ki67-positive cells at the crypt base revealed that the number of cycling CBC cells did not differ between Cd44+/−, Cd44+/+, Cd44+/−/ApcMin−/+ or Cd44+/−/ApcMin−/− mice (Fig. 4B), implying that the size of the intestinal stem cell compartment was not altered by CD44 deletion (or introduction of an ApcMin 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. 4B).

**Increased apoptosis in the intestinal crypts of Cd44−/−/ApcMin−/+ 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. 5A, the crypt compartments of Cd44+/+, Cd44+/−, and ApcMin−/+ mice contained similar numbers of apoptotic cells, indicating that neither the absence of CD44 nor the presence of an ApcMin allele per se affect crypt cell apoptosis. Interestingly, however, in Cd44−/−/ApcMin−/+ mice, the number of apoptotic cells was significantly increased, suggesting a role for CD44 in controlling apoptosis resistance of intestinal crypt epithelium of the ApcMin−/+ 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. 5B). 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 (Trfrs21), 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−/−/ApcMin−/+ mice (Fig. 6A). 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. 6B). It is noteworthy that no changes in cell cycle-regulating genes or Wnt-signaling target genes including cMyc, cyclin D1, EphiB, Left1, 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 ApcMin−/+ mice, affecting tumor initiation and the balance between apoptosis and survival in the intestinal crypts. We observed that adenoma formation in tumor-prone ApcMin−/+ mice was reduced by almost 50% in the absence of CD44 (Fig. 2A), whereas the distribution of adenomas along the intestinal tract and the average size of the adenomas were not affected (Fig. 2B 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 ApcMin−/+ mice lacking CD44, the size andcellularity of the intestinal stem cell and transit-amplifying compartments, which show strong CD44 expression in normal mice (Fig. 1B), were not different from that in Apc+/+ or ApcMin−/+ with wild-type CD44 (Fig. 4A 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

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1 Unpublished observation.
Interestingly, the ApcMin/+ 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 border of the transit-amplifying zone (Fig. 5B). 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.
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. 1B and 4A, 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. 1B).

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 ApcMin/+ 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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Figure 6. Increased expression of the proapoptotic genes Bok and Dr6 in the crypts of Cd44+/− and Cd44+/−/ApcMin−/− mice. A, increased Bok and Dr6 expression in the crypts of Cd44+/− mice. qRT-PCR of Bok and Dr6 expression in the duodenum of a Cd44+/−/ApcMin+/− mouse (black arrows). Right, in situ hybridization sense control.

Figure 6. Increased expression of the proapoptotic genes Bok and Dr6 in the crypts of Cd44+/− and Cd44+/−/ApcMin−/− mice. A, increased Bok and Dr6 expression in the crypts of Cd44+/− mice. qRT-PCR of Bok and Dr6 expression in the duodenum of a Cd44+/−/ApcMin+/− mouse (black arrows). Right, in situ hybridization sense control.


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