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Suppressing neutrophil-dependent angiogenesis abrogates resistance to anti-VEGF antibody in a genetic model of colorectal cancer

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We tested cis-Apc Δ716/Smad4Δ−/− and cis-Apc Δ716/Smad4Δ−/− KrasG12D mice, which recapitulate key genetic abnormalities accumulating during colorectal cancer (CRC) tumorigenesis in humans, for responsiveness to anti-VEGF therapy. We found that even tumors in cis-Apc Δ716/Smad4Δ−/− KrasG12D mice, although highly aggressive, were suppressed by anti-VEGF treatment. We tested the hypothesis that inflammation, a major risk factor and trigger for CRC, may affect responsiveness to anti-VEGF. Chemically induced colitis (CIC) in cis-Apc Δ716/Smad4Δ−/− and cis-Apc Δ716/Smad4Δ−/− KrasG12D mice promoted development of colon tumors that were largely resistant to anti-VEGF treatment. The myeloid growth factor G-CSF was markedly increased in the serum after induction of colitis. Antibodies blocking G-CSF, or its target Bv8/PROK2, suppressed tumor progression and myeloid cell infiltration when combined with anti-VEGF in CIC-associated CRC and in anti-VEGF-resistant CRC liver metastasis models. In a series of CRC specimens, tumor-infiltrating neutrophils strongly expressed Bv8/PROK2. CRC patients had significantly higher plasma Bv8/PROK2 levels than healthy volunteers and high plasma Bv8/PROK2 levels were inversely correlated with overall survival. Our findings establish Bv8/PROK2 as a translational target in CRC, in combination with anti-VEGF agents.

Significance

Using mouse models that recapitulate key genetic abnormalities accumulating during colorectal cancer (CRC) tumorigenesis, we report that chemically induced colitis promoted development of colon tumors that were largely resistant to anti-VEGF antibody treatment. Serum G-CSF levels were markedly elevated after induction of colitis. Inhibition of G-CSF or Bv8/PROK2 increased the efficacy of anti-VEGF antibody and prevented onset of resistance. To verify the potential clinical relevance of these findings, we examined a series of CRC specimens and found that tumor-infiltrating neutrophils strongly expressed Bv8/PROK2. CRC patients had significantly higher plasma Bv8/PROK2 levels than healthy volunteers and high plasma Bv8/PROK2 levels were inversely correlated with overall survival. These findings establish Bv8/PROK2 as a translational target in CRC, in combination with anti-VEGF agents.

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Competing interest statement: J.R. is an employee of Genentech, Inc. P.H. is a former employee of Genentech, Inc. and a current employee of Foundation Medicine, Inc.

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is tumor specific and may profoundly affect therapeutic responses to anticancer agents (9). Indeed, the various cell types communicate through cytokine/chemokine networks that may suppress or promote tumor progression (10). In CRC, it has been proposed that the density of infiltrating lymphocytes inside tumor tissues or “immune score” can predict clinical outcomes (11). These findings led to the hypothesis that activating T cell function by blocking programmed death ligand 1 (PD-L1) or programmed death 1 (PD-1) signaling may be an effective treatment for advanced CRC patients. However, studies with anti-PD-L1 or PD-1 antibodies so far have not demonstrated significant benefit to CRC patients, although these agents conferred durable benefits on subsets of patients with other solid tumor types (12). Recently, it was reported that microsatellite instability (MSI) in CRC predicts responses to immunotherapy (13). However, MSI-high patients account for only 15% of total CRC patients (14).

Here we report that treatment with an anti-VEGF antibody significantly suppressed tumor angiogenesis and growth in mice harboring key genetic mutations associated with human intestinal cancer. Since inflammation is involved in sporadic and heritable CRC, as well as in ulcerative colitis-associated cancers (15), we tested the hypothesis that colitis may affect responsiveness to anti-VEGF. Indeed, chemically induced colitis (CIC) caused by dextran sulfate sodium (DSS) promoted the development of CRC in mice that were largely unresponsive to anti-VEGF antibody, although small intestinal tumors in the same animal were still responsive. We sought to dissect the mechanisms of such resistance to anti-VEGF therapy.

Results

Anti-VEGF Treatment Suppresses Tumor Formation and Prolongs Survival in Intestinal Cancer Genetic Engineered Mouse Models (GEMMs). It has been reported that treatment with an anti-VEGF antibody suppresses tumor growth and prolongs survival of adenomatous polyposis coli (Apc<sup>min</sup>) mice (16), which carry a heterozygous truncation allele at codon 850 of Apc and serve as a model of familial adenomatous polyposis (FAP) (17). However, these findings do not predict whether malignant cancer responds to anti-VEGF antibody treatment because Apc mutations alone lead to benign adenomas with few malignant adenocarcinomas (18). Namely, additional mutations in Kras, p53, or loss of heterozygosity at chromosome 18q (including Smad2/4) appear to be involved in adenoma-carcinoma progression. To determine whether the cross-species reactive anti-VEGF-A neutralizing monoclonal antibody B20-4.1 (19) (anti-VEGF hereafter) has inhibitory effects on intestinal cancer, we took advantage of a GEMM carrying cis-compound mutations in Apc and Smad4 (cis-Apc/Smad4) that develops spontaneous invasive intestinal cancers (18), compared with the intestinal adenoma model with Apc<sup>min</sup> mutation.

In agreement with earlier findings (16), tumor formation and growth were significantly suppressed in Apc<sup>min</sup>/mice treated with anti-VEGF (tumor numbers 7.6 ± 2.6 vs. 13.4 ± 2.2, P < 0.01; tumor area, 3.8 ± 1.6 vs. 28.7 ± 11.5 [mm<sup>2</sup>], P < 0.01, Student’s t test) (SI Appendix, Fig. S1 A–D). Tumor growth was also suppressed in cis-Apc/Smad4 mice (Fig. 1 A and B and SI Appendix, Fig. S1E). Namely, when cis-Apc/Smad4 mice were treated with anti-VEGF for 6 wk or 9 wk from 13 wk old on, both tumor numbers and total tumor areas were significantly reduced (tumor numbers of 6-wk treatment 14.1 ± 4.2 vs. 17.7 ± 2.8, P < 0.05; tumor area of 6-wk treatment, 8.9 ± 3.6 vs. 69.8 ± 25.9 [mm<sup>2</sup>], P < 0.01; tumor numbers of 9-wk treatment 12.0 ± 3.9 vs. 21.4 ± 9.1, P < 0.05; tumor area of 9-wk treatment, 9.1 ± 5.0 vs. 91.3 ± 38.9 [mm<sup>2</sup>], P < 0.01, Student’s t test) (Fig. 1B). Mice with intestinal tumors showed pale paws due to anemia caused by mucosal bleeding, which often resulted in death. Anti-VEGF maintained hematocrit levels in a normal range (6-wk treatment 54.3 ± 2.9 vs. 35.0 ± 8.3 [%], P < 0.01; 9-wk treatment 57.3 ± 2.6 vs. 30.3 ± 4.0 [%], P < 0.01, Student’s t test) (SI Appendix, Fig. S1F). Anti-VEGF also suppressed tumor invasion and progression in tumor malignancy grades (Fig. 1C). Moreover, anti-VEGF improved mouse survival in a dose-dependent manner (isotype IgG 186 ± 11.9, anti-VEGF 10 mg/kg/week 250 ± 18.4, anti-VEGF 20 mg/kg/week 333 ± 24.4 [days], P < 0.01, log-rank test) (Fig. 1D).

We also added an oncogenic Kras mutation G12D, controlled by loxP stop cassette (LSL-Kras<sup>G12D/+</sup>) (20) and villin-Cre transgene (21) to the cis-Apc/Smad4 mutation mice (cis-Apc/Smad4 Kras<sup>G12D/villin</sup>). Expression of intestinal epithelial cell-specific Cre in villin-Cre mice was confirmed by mating with Gt(Rosa26)<sup>ACGT-ufTomato-EGFP</sup> mice (SI Appendix, Fig. S2 A and B). As anticipated, cis-Apc/Smad4 Kras<sup>G12D/villin</sup> mice had a short lifetime of about 8 to 14 wk after birth, due to highly invasive intestinal tumors (SI Appendix, Fig. S2C). Nevertheless, cis-Apc/Smad4 Kras<sup>G12D/villin</sup> mice treated with anti-VEGF had smaller tumor areas (40.7 ± 23.2 vs. 79.1 ± 22.2 [mm<sup>2</sup>], P < 0.01, Student’s t test), showed longer survival (95.0 ± 33.7 vs. 73.0 ± 4.3 [days], P < 0.01, log-rank test), and the tumors were less invasive, although tumor numbers were not much affected (29.0 ± 13.6 vs. 35.0 ± 11.8, P = 0.33) (Fig. 1 E–G). Collectively, these results indicated that anti-VEGF treatment suppressed tumor growth and invasion in mouse models for spontaneous intestinal cancer, leading to increased survival.

Colorectal Tumors Associated with CIC in cis-Apc/Smad4 and cis-Apc/ Smad4 Kras<sup>G12D(CDX2)</sup> Mice Are Resistant to Anti-VEGF Therapy. Inflammation is one of the key events in the pathogenesis of cancer (22) and CRC is strongly associated with inflammation (15). Although polyps in patients with hereditary CRC such as FAP or Lynch syndrome rarely show clear signs of chronic inflammation, tumorigenesis can be prevented or suppressed by long-term administration of antiinflammatory drugs (23). It has been reported that CIC induced by short-term DSS feeding causes colorectal tumor formation in <sup>apo</sup><sup>min</sup> mice (24). cis-Apc/Smad4 mutant mice, as well as Apc<sup>min</sup> mice, also showed tumor formation in the colorectum after only 1-wk administration of DSS (tumor number 11.1 ± 5.0 vs. 1.5 ± 0.8, P < 0.01; tumor area 86.8 ± 40.0 vs. 12.9 ± 7.8 [mm<sup>2</sup>], P < 0.01, Student’s t test (Fig. 2 A–C and SI Appendix, Fig. S3 A and C), which recapitulated invasive human CRC with Apc and Smad4 mutations (Fig. 2 D and SI Appendix, Fig. S3 C and D). In this model, we did not observe any differences in tumor formation in the small intestine (tumor number 16.5 ± 6.1 vs. 16.5 ± 3.4, P = 0.62; tumor area 35.7 ± 22.6 vs. 56.9 ± 26.8 [mm<sup>2</sup>], P = 0.07, Student’s t test) (Fig. 2C). We treated cis-Apc/ Smad4 mice with anti-VEGF for 6 wk following the induction of colorectal tumors with DSS, and found that they were refractory to anti-VEGF treatment (tumor number 9.5 ± 4.1 vs. 11.1 ± 5.0, P = 0.45; tumor area 66.0 ± 40.2 vs. 86.8 ± 39.8 [mm<sup>2</sup>], P = 0.25, Student’s t test) (Fig. 2C). On the other hand, small intestinal tumors still responded even after DSS feeding (tumor area 13.6 ± 7.6 vs. 35.7 ± 22.6 [mm<sup>2</sup>], P < 0.01, Student’s t test) (Fig. 2C). Since we could not design the DSS feeding in CIC mouse model (24), we added mice carrying CDX2P-CreERT<sup>2</sup> transgenic mice due to their short lifespan of 100 d or less, we bred mice carrying CDX2P-CreERT<sup>2</sup> transgene (25) instead of villin-Cre in order to induce Cre activation by tamoxifen administration (4 consecutive days at 9 wk of age intraperitoneally) only in colorectal epithelial cells (SI Appendix, Fig. S2 A and B) and added it to cis-Apc/Smad4 mice to generate cis-Apc/ Smad4 Kras<sup>G12D(CDX2)</sup> mice. DSS administration following Cre activation by tamoxifen significantly promoted colorectal tumor formation in cis-Apc/Smad4 Kras<sup>G12D(CDX2)</sup> mice (Fig. 2 A–C and SI Appendix, Fig. S3 E). We treated cis-Apc/Smad4 Kras<sup>G12D(CDX2)</sup> mice with anti-VEGF for 3 wk following the induction of colorectal tumors with tamoxifen and DSS and found that they were refractory to anti-VEGF treatment as well (tumor area 281 ± 40 vs. 242 ± 63 [mm<sup>2</sup>], P = 0.21, Student’s t test) (Fig. 2C).
Higher Serum Granulocyte-Colony Stimulating Factor (G-CSF) Levels in Mice Harboring Anti-VEGF Resistant Colorectal Tumors. To investigate the differences between the small intestinal and colorectal tumors in DSS-treated cis-Apc/Smad4 mice, we performed immunohistochemistry (IHC) for β-Catenin and Smad4. However, both small intestinal and colorectal tumors of ApcΔ716 mice showed nuclear accumulation of β-Catenin and Smad4, whereas those of cis-Apc/Smad4 mice had only β-Catenin accumulation in the nuclei lacking Smad4 expression in tumor epithelial cells (SI Appendix, Fig. S3 C and D). Although it has been reported that DSS causes some inflammation in the small intestine, clearly its major effects are in the colon and rectum (26) (SI Appendix, Figs. S3B and S4A). Thus, we examined a panel of inflammation-related genes in the colorectal tumor tissue of DSS-treated mice as compared with small intestinal tumors, and found that most of them were indeed up-regulated (SI Appendix, Fig. S4B).

We also tried to determine whether there are any differences in CIC colorectal tumors among genetic backgrounds by comparing tumor tissues from ApcΔ716, cis-Apc/Smad4, and cis-Apc/Smad4 KrasG12D(villin) mice treated with DSS. However, we found only minor differences in terms of inflammatory and angiogenic factor expression among the various backgrounds (SI Appendix, Fig. S4C). It is worth noting that release of cytokines/chemokines into the systemic circulation is essential for an effective communication between the tumor and bone marrow. Therefore, we compared serum cytokine profiles between DSS-treated and nontreated cis-Apc/Smad4 mice using cytokine protein arrays (SI Appendix, Fig. S5 A–D). The results showed increased levels of...
C-X-C motif chemokine ligand (CXCL)13 and G-CSF (among 40 cytokine/chemokines) in DSS-fed mice, with or without anti-VEGF treatment. To analyze these cytokines in a more quantitative manner, we collected serum samples chronologically and performed ELISA. Serum G-CSF levels increased in a biphasic manner; shortly after DSS treatment (initial peak), and after recovery within 2 wk, they gradually increased again, coincident with colorectal tumor growth (secondary peak). The G-CSF levels in cis-Apc/Smad4 mice were elevated significantly 6 wk after DSS feeding (water with isotype 0.16 ± 0.05, water with anti-VEGF 0.36 ± 0.09, DSS with isotype 1.42 ± 0.85, DSS with anti-VEGF 1.33 ± 0.74 [ng/mL]), whereas they stayed at the baseline in wild-type mice (water on wild-type 0.09 ± 0.05, DSS on wild-type 0.10 ± 0.06, P = 0.66 [Student’s t test] and P = 0.60 [Mann–Whitney u test]) (Fig. 2 E, Right and Fig. 2 F, Right). On the other hand, CXCL13 levels did not show any transient up-regulation shortly after DSS treatment, but they increased gradually throughout the observation period (Fig. 2 E, Left and Fig. 2 F, Left). Interestingly, serum CXCL13 levels also increased even in tumor-free wild-type mice after DSS treatment (water on wild-type 0.45 ± 0.15, DSS on wild-type 0.72 ± 0.16 [ng/mL], P < 0.01 [Student’s t test and Mann–Whitney u test]), suggesting that this change was caused by the recovery from acute colonic inflammation, rather than by the tumor growth.

G-CSF Causes Neutrophil Infiltration into Tumor Stroma to Up-Regulate Bv8/Prokineticin 2 (PROK2) and Promote Angiogenesis in Colorectal Tumors Refractory to Anti-VEGF Antibody. Next we tried to determine the source(s) of G-CSF and CXCL13. We determined their mRNA levels in the colon tumors and adjacent normal tissues. Csf3 (encoding G-CSF) and its receptor Csf3r were up-regulated dramatically in colorectal tumor tissues from cis-Apc/Smad4 mice treated with DSS (Fig. 3 A). On the other hand, Cxcl13 and its receptor Cxcr5 were expressed mainly in the normal colon tissues (Fig. 3 A). Among the cytokines that are highly expressed in colorectal tumors of DSS-treated cis-Apc/Smad4 mice (SI Appendix, Fig. S4B), IL17a is known to stimulate G-CSF expression in the tumor microenvironment (27). Taking advantage of a recently described method (28), we cultured colorectal cancer stem cells...
Fig. 3. G-CSF mRNA was expressed in tumor tissues, but G-CSF does not directly affect tumor cell growth. (A) Relative mRNA expression of Csf3r/G-CSF, its receptor Csf3r, Cxcr1, and IL17a mRNA was collected from colorectal tumor and adjacent normal colon tissues of DSS-treated mice, with or without anti-VEGF treatment. Experiments were repeated three times. **P < 0.01, Student’s t test. (B) Immunocyto-staining of primary spheroid culture cells from colorectal tumors of DSS-fed mice. All spheroid cells expressed E-cadherin, whereas no cells expressed α-SMA. (Scale bar, 50 μm.) (C) Csf3r/G-CSF mRNA expression and its protein expression from tumor spheroid culture. Experiments were repeated three times. **P < 0.01, Student’s t test. (D) IHC for Bv8/PROK2 and MPO in tumor tissues of cis-Apc/Smad4 mice with DSS. Red arrowheads, polynuclear neutrophils. (Scale bar, 50 μm.) (E) A representative “score 3” (see also SI Appendix, Fig. S6G) Bv8/PROK2 IHC from a human CRC specimen. (Scale bar, 40 μm.) (F) Relative expression of Bv8/PROK2 mRNA from peripheral neutrophils with or without treatment with 10 ng/mL of G-CSF for 4 h in Hank’s balanced salt solution (HBSS) buffer with 0.5% bovine serum albumin (BSA). Experiments were repeated three times. Triplicate experiments, repeated three times. **P < 0.01, Student’s t test. (G) Cell proliferation assay (WST-1 reagent) in primary spheroid cells from colorectal tumors of DSS-treated mice. Fetal bovine serum and EGF were used as positive controls. Experiments were repeated three times; *P < 0.05, Student’s t test.

as spheroids and treated them with recombinant IL17a (Fig. 3 B and C). Both mRNA and protein levels of G-CSF were significantly up-regulated in the IL17a-treated colorectal tumor spheroids (Fig. 3C). These results suggest that IL17a induced in the tumor microenvironment may stimulate G-CSF production in colorectal tumor cells.

We previously reported that Bv8/PROK2 is expressed in CD11b+Gr-1+ myeloid cells, and that it regulates myeloid cell-dependent tumor angiogenesis (29). In the present study, we found that Bv8/PROK2 and its receptors Prokr1 and Prokr2 (30) were also highly up-regulated in the colorectal tumor tissues of DSS-treated cis-Apc/Smad4 mice (Fig. 3A), suggesting that G-CSF from colorectal tumors stimulated production of Bv8/PROK2 within the tumor. In order to confirm expression of Bv8/PROK2 within the colorectal tumor tissues, we performed immunohistochemistry for Bv8/PROK2 and myeloperoxidase (MPO). We found that polynuclear granulocytes in the tumors of DSS-treated mice expressed both antigens (Fig. 3D and SI Appendix, Fig. S6A), as well as human CRC tissues (Fig. 3E), consistent with the finding that tumor tissues had higher expression of Bv8/PROK2 mRNA compared with adjacent normal tissue (Fig. 3A). To further validate Bv8/PROK2 expression in granulocytes/neutrophils, we isolated peripheral neutrophils from tumor-bearing mice with anti-Ly-6G beads (SI Appendix, Fig. S6B), and treated them with recombinant mouse G-CSF. After stimulation with 10 ng/mL G-CSF, peripheral neutrophils from DSS-treated cis-Apc/Smad4 mice showed significant up-regulation of Bv8/PROK2 expression (Fig. 3F). We also tested the possibility that the molecules that we targeted may have direct effects on tumor cell proliferation, we incubated tumor spheroids with IL17a, G-CSF, CXCL13, Bv8/PROK2, endocrine gland-derived vascular endothelial growth factor (EG-VEGF) (structurally related to Bv8/PROK2) or VEGF-A and found that none of these molecules had stimulatory effects (Fig. 3G).

We next investigated whether G-CSF expression in tumor tissues affected neutrophil infiltration into the tumor stroma by immunofluorescent staining for Ly-6G (a marker of mouse neutrophils). Anti-VEGF treatment alone promoted neutrophil infiltrations into the stroma of small intestine tumors in cis-Apc/Smad4 mouse, even without CIC (SI Appendix, Fig. S6 C and E). DSS-induced CIC dramatically recruited neutrophil infiltration into the stroma of colorectal tumors, but not in small intestinal tumors (SI Appendix, Fig. S6 C and E).

We determined vascular densities in CIC-associated colorectal tumors by anti-CD34 immunohistochemistry (SI Appendix, Fig. S6 D and F). When treated with anti-VEGF, the tumor microvessel density of mice without DSS treatment was very low. These results are consistent with those that anti-VEGF alone almost completely suppressed tumor formation of cis-Apc/Smad4 mice without DSS (Fig. 1 A and B). In contrast, CIC-associated colorectal tumors contained abundant microvessels even in mice treated with anti-VEGF (SI Appendix, Fig. S6 D and F).
suggesting that anti-VEGF antibody alone was unable to suppress tumor angiogenesis, and that other angiogenic factors such as Bv8/PROK2 contributed to the resistance against anti-angiogenic agents in this mouse model.

**Plasma Bv8/PROK2 Levels Predicted Patient Overall Survival in Human Unresectable CRC in Response to Iritotecan, 5-Fluorouracil, Leucovorin (IFL).** It has been reported that serum G-CSF levels are correlated with stages in human CRC (31). In order to further validate the findings of our mouse studies, we sought to assess Bv8/PROK2 expression in human CRC specimens by IHC using tissue microarrays (Fig. 3E and SI Appendix, Fig. S6G). IHC results were scored in the range of 0 to 3 based on the number of cells detected in the tumor area (SI Appendix, Fig. S6G). Fig. 3E illustrates a representative sample with score 3. As shown in SI Appendix, Fig. S6H, most of the CRC patients had the highest score of Bv8/PROK2-positive cells. Since Bv8/PROK2 is a secreted protein, we sought to determine whether its plasma levels have any correlation with the presence of CRC. Indeed, we found that Bv8/PROK2 levels were significantly higher in CRC patients compared with age-matched healthy donors (Fig. 4A). We also sought to assess whether there is a correlation between plasma Bv8/PROK2 levels and survival of CRC patients. Plasma samples were available from a total of 120 patients from the IFL (irinotecan 5-fluorouracil, leucovorin) regimen arm of trial AVF2107 (NCT0109070) (4). As shown in Fig. 4B, plasma Bv8/PROK2 was associated with poor overall survival (OS). Namely, patients with higher than the median value of 38.1 pg/mL plasma Bv8/PROK2 level (n = 68) had a median OS of 13 mo, whereas those with lower (n = 52) had 17.2 mo, leading to a hazard ratio of 1.77 (95% confidence interval 1.23 to 2.55, unstratified log-rank P < 0.01).

**In Combination with Anti-VEGF Antibody, Anti-IL17a, Anti-G-CSF, or Anti-Bv8/PROK2 Suppressed Growth of Refractory Tumors, but Not anti-CTXCL13 or Anti-PD-L1.** To further validate the significance of G-CSF, Bv8/PROK2, and CXCL13 on anti-VEGF therapy in refractory CRC, we treated mice with anti-IL17a, anti-G-CSF, anti-Bv8/PROK2, or anti-CXCL13 antibody, in the absence or presence of anti-VEGF, for 3 wk after CIC (Fig. 4C). We also administered anti-CD163 antibody as an additional treatment group. This is because tumor-associated neutrophils (TANS) and/or granulocytic myeloid-derived suppressor cells (MDSCs) have been reported to suppress antitumor immunity through expression of PD-L1 (33, 34). In fact, expression of tumor immune factors such as Pdl1, Pdl1, Cld4, Foxp3, and Arg1 in CIC-associated colorectal tumor tissues from cis-Apc/Smad4 mice was up-regulated compared to small intestinal tumor tissues, whereas CD8α expression was down-regulated (SI Appendix, Fig. S7A). As anticipated, IHC revealed infiltration of Foxp3-positive cells in CIC-associated colorectal tumors, whereas CD8α-positive cell infiltration was observed in small intestinal tumor tissues from the same mice (SI Appendix, Fig. S7B).

Anti-G-CSF treatment, with or without anti-VEGF, suppressed neutrophil counts in peripheral blood (SI Appendix, Fig. S8A), whereas other antibodies did not. Consistent with the above findings, 3-wk treatment with anti-VEGF alone did not reduce colorectal tumor formation in cis-Apc/Smad4 mice with CIC (tumor number, isotype 7.2 ± 3.2 vs. anti-VEGF 6.9 ± 3.0, P = 0.85; tumor area, isotype 36.3 ± 15.9 vs. anti-VEGF 33.6 ± 17.4 [mm²], P = 0.73, Student t test) (Fig. 4 C–E). Anti-IL17a, anti-G-CSF, anti-Bv8/PROK2, or anti-CXCL13, or anti-PD-L1 did not show any significant effects when tested as monotherapy in both cis-Apc/Smad4 mice (tumor number, anti-IL17a 7.9 ± 3.0, anti-G-CSF 5.6 ± 2.2, anti-Bv8/PROK2 8.6 ± 3.0, anti-CXCL13 9.7 ± 5.2, anti-PD-L1 5.9 ± 2.0; tumor area, anti-IL17a 37.8 ± 20.6, anti-G-CSF 35.1 ± 14.6, anti-Bv8/PROK2 47.5 ± 15.2, anti-CXCL13 53.4 ± 32.4, anti-PD-L1 34.9 ± 16.9 [mm²]) and cis-Apc/Smad4 KrasG12D(DCX2) mice (tumor area, anti-G-CSF 286.5 ± 57.2, anti-Bv8/PROK2 295.1 ± 49.0, anti-PD-L1 237.2 ± 54.8 [mm²]) with CIC (Fig. 4 C–E and SI Appendix, Fig. S8 D and E). However, when tested in combination with anti-VEGF to DSS-fed mice, anti-IL17a, anti-G-CSF or anti-Bv8/PROK2, but not anti-PD-L1, antibody significantly reduced the lesion areas and/or numbers of colorectal tumors cis-Apc/Smad4 mice with CIC (tumor number, anti-VEGF + anti-IL17a 4.9 ± 3.8, vs. isotype P = 0.21, anti-VEGF + anti-G-CSF 3.4 ± 2.0, vs. isotype P < 0.01, anti-VEGF + anti-Bv8/PROK2 4.1 ± 2.5, vs. isotype P < 0.01; tumor area, anti-VEGF + anti-IL17a 17.9 ± 16.2 [mm²], vs. isotype P < 0.05, anti-VEGF + anti-G-CSF 12.0 ± 9.0 [mm²], vs. isotype P < 0.01, anti-VEGF + anti-Bv8/PROK2 13.6 ± 8.9 [mm²], vs. isotype P < 0.01, Student’s t test) (Fig. 4 C–E). We also confirmed that anti-G-CSF (or anti-Bv8/PROK2) neutralization in combination with anti-VEGF significantly suppressed colorectal tumor formation in cis-Apc/Smad4 KrasG12D(DCX2) mice (tumor area, anti-VEGF + anti-G-CSF 165 ± 60 [mm²], vs. isotype P < 0.01, anti-VEGF + anti-Bv8/PROK2 168 ± 37 [mm²], vs. isotype P < 0.01, Student’s t test) (SI Appendix, Fig. S8 D and E). These results suggest that neutralization of both VEGF and IL17a or VEGF and G-CSF/Bv8/PROK2 is required to suppress tumor formation after CIC in cis-Apc/Smad4 mice and cis-Apc/Smad4 KrasG12D(DCX2) mice. Treatment of cis-Apc/Smad4 mice with anti-CXCL13 resulted in a trend toward increased colorectal tumor formation. Although we did not fully reach statistical significance (tumor numbers, anti-CXCL13 9.7 ± 5.2, vs. isotype P = 0.22; anti-VEGF + anti-CXCL13 12.0 ± 4.9, vs. isotype P = 0.02, tumor area, anti-CXCL13 53.4 ± 32.4 [mm²], vs. isotype P = 0.15; anti-VEGF + anti-CXCL13 62.2 ± 35.6 [mm²], vs. isotype P = 0.05, Student’s t test), we noted that some of the largest tumors in this study were in the anti-CXCL13 groups (Fig. 4 C–E). Remarkably, in small intestinal tumors of cis-Apc/Smad4 mice and cis-Apc/Smad4 KrasG12D(DCX2) mice with CIC, anti-VEGF monotherapy achieved significant tumor suppression (SI Appendix, Fig. S8 B and C).

In DSS-treated cis-Apc/Smad4 mice receiving these combination therapies, we also confirmed neutrophil infiltration and angiogenesis in colorectal tumors by immunofluorescent staining (Fig. 5 A and C). Anti-G-CSF treatment, with or without anti-VEGF, significantly reduced neutrophil infiltration inside the tumor microenvironment, consistent with the peripheral white blood cell counts (Fig. 5 A and C and SI Appendix, Fig. S84). Anti-IL17a and anti-Bv8/PROK2 treatment also had similar effects, suppressing local neutrophil infiltration, without significantly affecting peripheral neutrophil counts (Fig. 5 A and C and SI Appendix, Fig. S84). Angiogenesis in the colorectal tumors was dramatically suppressed when DSS-fed cis-Apc/Smad4 mice were treated with anti-IL17a, anti-G-CSF, or anti-Bv8/PROK2 antibody in combination with anti-VEGF (Fig. 5 B and D). In contrast, none of the monotherapies with anti-IL17a, anti-G-CSF, or anti-Bv8/PROK2 antibody caused such angiogenesis-suppressive effects inside colorectal tumors, which is consistent with the results of tumor suppression described above.

**G-CSF Expression Confers Resistance to Anti-VEGF Therapy in a Mouse Allograft Model of CRC Liver Metastasis.** CRC becomes lethal when it metastasizes to distant organs, the liver being the most common. Therefore, we sought to verify the above results using allograft models of CRC liver metastasis. Anti-VEGF therapy was effective when MC38 mouse CRC cells were grown in the liver of C57BL/6 syngeneic mice following injection in the spleen, as assessed by liver weight (isotype 3.5 ± 2.0 vs. anti-VEGF 1.2 ± 0.4 g, P < 0.05, Student’s t test) (SI Appendix, Fig. S9 A and B). We transduced Gcsf into MC38 cells (SI Appendix, Fig. S9C). Lentiviral transduction of Gcsf significantly increased G-CSF expression from cancer cells, leading to increased serum G-CSF.
levels and recruitment of polynuclear neutrophils expressing Bv8 in liver metastatic foci (Fig. 6B and SI Appendix, Fig. S9D). Importantly, G-CSF expressing MC38 cells acquired resistance to anti-VEGF (MC38-empty 1.1 ± 0.17 vs. MC38-Gcsf 1.54 ± 0.58 [g], P < 0.05, Student’s t test) (Fig. 6A), and neutralization of G-CSF/Bv8 cascade with anti-G-CSF/anti-Bv8 antibodies in combination with anti-VEGF abrogated the acquired resistance in G-CSF-expressing tumors (isotype 1.9 ± 0.93 [g], anti-VEGF + anti-G-CSF 1.0 ± 0.25, vs. isotype P < 0.05; anti-VEGF + anti-Bv8 0.97 ± 0.18, vs. isotype P < 0.05, Student’s t test) (Fig. 6C). The treatments including anti-G-CSF antibody suppressed peripheral neutrophil counts in this model, whereas other treatments did not (Fig. 6D). Another CRC metastasis allograft model with CT26 cells derived from BALB/c mouse showed essentially the same results (SI Appendix, Fig. S9E–H).

We established a spontaneous liver metastasis model in accordance with a previous report, with minor modifications (35). As expected, mice with Kras<sup>+/LSL-G12D</sup> and Pten<sup>flx/flx</sup> transgene driven by villin-Cre (Kras<sup>G12D</sup> Pten<sup>flx/flx</sup>) showed both intestinal and liver tumors (Fig. 6E–G). We confirmed that liver tumors in this mouse model derived from intestinal epithelial cells by adding Gt(ROSA26)<sup>ACTB-tdTomato-EGFP</sup> gene (Fig. 6F and G and SI Appendix, Fig. S9I). As reported, both intestinal primary tumor and liver metastasis tumors showed Wnt and Erk activation in cancer cells, which is a common feature of advanced CRC (SI Appendix, Fig. S9J) (35). Metastatic liver tumors in this model demonstrated prominent up-regulation of Csf3 and Prok2 expression, and histological examination revealed significant recruitment of polynuclear neutrophils with Bv8/PROK2 expression in the liver tumors (Fig. 6H and I). Collectively, these results demonstrate that the G-CSF/Bv8/PROK2 axis played an important role in eliciting resistance to anti-VEGF therapy for CRC liver metastasis, and that blockade of G-CSF/Bv8/PROK2 can become a therapeutic target for such tumors.

Fig. 4. Plasma Bv8/PROK2 levels and survival correlations and treatment of anti-IL17a/G-CSF/Bv8/PROK2 axis in combination with anti-VEGF in cis-Apc/Smad4 mice with DSS. (A) Plasma Bv8/PROK2 levels of CRC patients and age-matched healthy volunteers. **P < 0.01, Mann–Whitney u test. (B) Kaplan–Meier estimates of the subpopulation from the cohort of AVF2107 trial (4, 32). Patients from the IFL arm were divided into two groups, one with plasma Bv8/PROK2 levels higher than median value (= 38.1 pg/mL) and the other with lower value. **P < 0.01, log-rank test. (C) Representative images of colorectum from wild-type or cis-Apc/Smad4 mice, with or without DSS administration, followed by the indicated antibody treatments. (Scale bar, 5 mm.) Red arrowhead, colorectal tumors. (D and E) Comparison of tumor numbers (D) and total tumor areas (E) of cis-Apc/Smad4 mice with DSS, followed by indicated antibody treatments. n = 8, 8, 10, 9, 11, 8, 10, 12, 10, 8, 7, 11, 11, 9, and 7 in group from the Left. *P < 0.05; ns, not significant, Student’s t test.
Discussion

We investigated the mechanisms of resistance to anti-VEGF in GEMMs of CRC and evaluated their relevance to clinical outcomes in human patients. In our mouse models, administration of anti-Bv8/PROK2, anti-G-CSF, or anti-IL17a antibody in combination with anti-VEGF effectively suppressed colorectal tumor formation on GEMM with CIC, although anti-IL17a antibody appeared slightly less effective than the other treatment groups. In the clinical setting, bevacizumab is typically used in combination with cytotoxic chemotherapy agents that typically cause neutropenia. Anti-G-CSF antibody may exacerbate chemotherapy-induced neutropenia, which may cause critical infectious diseases. Moreover, in addition to G-CSF, GM-CSF and IL10 can also stimulate Bv8/PROK2 expression in human neutrophils/monocytes (36). On the other hand, Bv8/PROK2 is a downstream target of G-CSF (30), and anti-Bv8/PROK2 treatment did not cause neutropenia in our mouse model (Fig. 6D and SI Appendix, Fig. S8A). Therefore, targeting Bv8/PROK2 may be a safer approach.

We found a biphasic increase in serum G-CSF levels after treatment with DSS in cis-Apc/Smad4 mice (Fig. 2F). The initial peak was observed almost immediately after DSS exposure, both in cis-Apc/Smad4 and wild-type mice, which may have been caused by the acute inflammation. However, DSS-treated cis-Apc/Smad4, but not DSS-treated wild-type mice, showed a gradual increase in serum G-CSF levels, with or without anti-VEGF treatment. These increased G-CSF levels were accompanied by colorectal tumor growth (Fig. 2F). The main source of this secondary G-CSF peak was the tumor tissue itself (Fig. 3A), suggesting that tumor-derived G-CSF played an important role in recruiting neutrophils (TAN and/or granulocytic-MDSC) to the tumor site. The spontaneous liver metastasis model with KrasG12D Ptenfloxflox/flox also revealed that G-CSF expression from metastatic tumors was associated with recruitment of neutrophils at the metastatic sites. It is believed that neutrophils play an important role in tumorigenesis for many types of cancers, although their role is complex and possibly tumor dependent and may result in tumor promotion or suppression (37, 38). For example, a high preoperative neutrophil-to-lymphocyte ratio (NLR) from peripheral blood cell counts has been correlated with overall survival in CRC (39) or in other types of cancers (40, 41). Recent studies reported that NLR may predict chemotherapy outcomes in advanced cancers (42, 43). NLR has been also reported to predict benefit from bevacizumab therapy in patients with advanced CRC (44, 45). In addition to systemic neutrophils as a marker of inflammatory, neutrophilic/granulocytic lineage cells inside the tumor microenvironment such as TAN or their precursors, 
granulocytic MDSCs, are also thought to play a major role in tumor immunity (46). However, it is as yet unclear whether neutrophils inside the tumor microenvironment promote or suppress tumor progression (37). In fact, while some of the previous clinical reports showed that presence of neutrophils inside tumors was an independent poor prognostic factor for several types of cancers (34, 47), others showed that neutrophils can play antitumoral roles (48).

IL17a, mainly produced by Th17 helper lymphocytes, is one of the key players of inflammation, including inflammatory bowel disease (IBD). In addition to IBD, IL17a plays a critical role in the pathogenesis of colitis-associated cancers (49). In the present study, we demonstrated that IL17a stimulate G-CSF expression from cancer cells, which recruits neutrophils in the tumor microenvironment. Among several possible explanations of how neutrophils promote tumor growth, angiogenesis represents an attractive possibility (37, 50). We have previously reported that tumor-infiltrating CD11b+Gr-1+ cells with neutrophilic/granulocytic phenotype produced the Bv8/PROK2 protein, which resulted in refractoriness to anti-VEGF therapy (29, 51, 52). However, most of these studies were performed in tumor allograft/xenograft models that may not accurately reflect the complexity of the tumor microenvironment.

In the present study, we used CIC to stimulate colonic tumorigenesis in an intestinal cancer GEMM (cis-Apc/Smad4 and cis-Apc/Smad4 KrasG12D(CDX2)). Colorectal tumors with CIC and metastatic liver tumors in our models contained abundant neutrophils in their stroma, which also reflects human CRC (48). Without DSS, cis-Apc/Smad4 mice showed tumors mainly in small intestine, and they received a marked benefit from anti-VEGF therapy. On the other hand, DSS-treated cis-Apc/Smad4 mice developed tumors also in the colorectum that showed resistance to anti-VEGF antibody, suggesting that CIC triggered escape mechanisms. Interestingly, even in DSS-treated cis-Apc/Smad4 mice and cis-Apc/Smad4 KrasG12D(CDX2) mice, small intestinal tumors were still responsive to anti-VEGF therapy (Fig. 2 C and SI Appendix, Fig. S8 B and C), suggesting that inflammation is important for resistance to antiangiogenesis since DSS causes inflammation primarily in the colon (SI Appendix, Figs. S3B and S4A). These findings raise the possibility that heterogeneity in the tumor responses to anti-VEGF may be, at
least in part, related to different degrees of inflammation in different regions/sites, even within the same tumor. To identify factors potentially implicated in such resistance, we focused our analysis on serum samples because systemically released factors are likely implicated in the recruitment of inflammatory cells from the bone marrow or the systemic circulation into the inflamed colon. Analysis of mouse sera by cytokine arrays demonstrated that only G-CSF and CXCL13 were significantly elevated in DSS-treated wild-type mice that never showed intestinal tumors, arguing against a role of this chemokine in tumor growth (Fig. 2G). CXCL13 is a CXC chemokine originally implicated in the recruitment and development of B cells through interaction with CXCR5 (53). The significance of CXCL13 in tumor biology has been somewhat controversial. Bindea et al. reported that patients with high CXCL13 expression in colorectal cancer show better survival than those with low expression (54) and hypothesized that CXCL13 plays an important role in antitumor immunity. However, other studies have reported a stimulatory role of CXCL13 for various tumor cell types, including colorectal cancer cells (55, 56), raising the possibility that this chemokine may be a therapeutic target in cancer. Our findings seem consistent with the hypothesis that CXCL13 represents a homeostatic mechanism limiting tumor growth (54) since tumors in anti-CXCL13-treated groups tended to be the largest.

Anti-PD-L1 antibody treatment, with or without anti-VEGF, did not cause tumor suppression, which is consistent with the lack of clinical efficacy in CRC patients without MSI (13). Interestingly, combination of an anti-PD-L1 antibody with bevacizumab has yielded promising results in multiple malignancies (57–59), but there are no reports that such combination enhances the efficacy of bevacizumab in CRC.

There are four consensus molecular subtypes (CMSs) of CRC (60). Among these, CMS4, with activation of TGF-β and angiogenesis, predicts the worst prognosis. The CIC CRC mouse models we used here may truly reflect the clinical setting of aggressive CMS4 CRC, because CRC tissues from cis-Apc/Smad4 mice showed up-regulation of Tgif1 and some angiogenic factors such as Bv8/PROK2 and Plgf (SI Appendix, Fig. S4B). In addition, it was reported that serum G-CSF levels are strongly correlated with tumor stage in colorectal cancer patients (31). Further evidence for the CRC mouse models reflecting real clinical setting comes from the high frequency of Bv8/PROK2-positive neutrophils observed in human CRC (Fig. 4A and SI Appendix, Fig. S6F). Moreover, plasma Bv8/PROK2 levels were associated with poor prognosis in this disease, providing a potential strategy to target Bv8/PROK2 as a suitable candidate for therapeutic intervention. We used cis-Apc/Smad4 mice as the CIC CRC model because expression profile from tumor tissues of either ApcΔ716, cis-Apc/Smad4, or cis-Apc/Smad4 KrasG12D(CDX2) essentially showed little difference between each other (SI Appendix, Fig. S4C).

We also found that liver tumors in a spontaneous metastasis model with KrasG12D/Pten−/− driven by villin-Cre transgene showed significant increases in Csf3 and Bv8/PROK2 expression (Fig. 6d). We did not attempt to treat these mice with targeted antibodies since it has been reported that the frequency of liver metastasis is only 25%, and that some of the metastases take over 1 y to be detectable (35). Therefore, it could be challenging to generate statistically significant data in a timely fashion. Nevertheless, this model provides support for our hypothesis as it shows that metastatic tumors express abundant amounts of G-CSF, with recruitment of Bv8-expressing polymuclear granulocytes.

In conclusion, on the basis of the GEMM findings reported in this manuscript, we provide a validation of the potential role for the G-CSF/Bv8/PROK2 axis as a therapeutic target in human colorectal cancer. A challenge in developing anti-Bv8 therapy was the difficulty in identifying an antibody that fully blocked Bv8 function, hence the need to combine two antibodies, in this and in earlier studies (29, 52). Hopefully, our findings will renew interest in developing reagents more suitable for clinical trials.

Materials and Methods

Cytokine Array. Serum samples were diluted 10-fold and applied to the membranes of Mouse Cytokine Array Panel A (Raybiotech), according to the manufacturer’s protocol. After incubation with biotinylated detection antibody mixture, membranes were treated with streptavidin-conjugated IRDye800CW (LI-COR Biosciences), and fluorescent intensity was detected by Odyssey (LI-COR).

Quantitative Reverse Transcript PCR (qRT-PCR). Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocol. cDNA was generated using high Capacity cDNA Reverse Transcription Kit (Applied Biosystems), followed by quantification with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) using VIA 7 (Applied Biosystems). Expression levels for mouse genes listed in SI Appendix, Supplementary Table were normalized by mouse Actb. The ∆∆CT method was used for quantification of gene expression levels.

ELISA. Mouse sera were diluted fivefold, and G-CSF and CXCL13 levels were measured using specific ELISA kits (MCS00 and MCK130 respectively, R&D Systems). For G-CSF ELISA in conditioned media of mouse CRC cell lines, membranes were treated with streptavidin-conjugated IRDye800CW (LI-COR Biosciences), and fluorescent intensity was detected by Odyssey (LI-COR).

Data Availability. All data are included in the article and supporting information.

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1. N. Ferrara, A. P. Adams, Ten years of anti-vascular endothelial growth factor therapy. Nat. Rev. Drug Discov. 15, 385–403 (2016).
2. R. S. Apte, D. S. Chen, N. Ferrara, VEGF in signaling and disease: Beyond discovery and development. Cell 176, 1248–1264 (2019).
3. G. C. Jayson, R. Kerbel, L. M. Ellis, A. L. Harris, Antiangiogenic therapy in oncology: Current status and future directions. Lancet 388, 518–529 (2016).
4. H. Hurwitz et al., Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer: A randomized phase III trial. J. Natl. Cancer Inst. 94, 776–784 (2002).
5. B. J. Gianniotis et al., Eastern Cooperative Oncology Group Study E3200, Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: Results from the eastern cooperative oncology group study E3200. J. Clin. Oncol. 25, 1539–1544 (2007).
6. J. Benbouma et al., ML18147 Study Investigators, Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): A randomised phase 3 trial. Lancet Oncol. 14, 29–37 (2013).

Itatani et al.
15. J. Terzic, S. Grivennikov, E. Karin, M. Karin, Inflammation and colon cancer. Gastroenterology 138, 2102–2114.e5 (2010).
16. N. Kosiaari et al., Inhibition of VEGF-A prevents the angiogenic switch and results in increased survival of ApcMin mice. Proc. Natl. Acad. Sci. U.S.A. 104, 10625–10630 (2007).
17. L. K. Su et al., Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 256, 668–670 (1992).
18. K. Takakura et al., Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell 92, 645–656 (1998).
19. W. C. Liang et al., Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J. Biol. Chem. 281, 951–961 (2006).
20. D. A. Tuveson et al., Endogenous oncogenic Kras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5, 375–387 (2004).
21. B. B. Madison et al., Six elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J. Biol. Chem. 277, 33275–33283 (2002).
22. A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation. Nature 454, 436–444 (2008).
23. J. Burn et al., CAPP2 Investigators, Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomised controlled trial. Lancet 378, 2081–2087 (2011).
24. H. S. Cooper et al., The role of mutant Apc in the development of dysplasia and cancer in the mouse model of dextran sulfate sodium-induced colitis. Gastroenterology 121, 1407–1416 (2001).
25. Y. Feng et al., S9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivating mutation. Am. J. Pathol. 183, 493–503 (2013).
26. S. Kitajima, S. Takuma, M. Morimoto, Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. Am. J. Pathol. 183, 67–70 (2009).
27. A. S. Chung et al., An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. Nat. Med. 19, 1114–1123 (2013).
28. H. Miyoshi, T. S. Stappenbeck, In vitro expansion and genetic modification of gastrointestinal stem cells in spherooid culture. Nat. Protoc. 8, 2471–2482 (2013).
29. F. Shojaei et al., βv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 450, 825–831 (2007).
30. L. Negri, N. Ferrara, Metastatic growth instructed by neutrophil-derived transferrin. Proc. Natl. Acad. Sci. U.S.A. 115, 11060–11065 (2018).
31. F. Shojaei et al., Role of mutant Apc in the development of dysplasia and cancer in the mouse model of dextran sulfate sodium-induced colitis. Gastroenterology 121, 1497–1411 (2001).
32. K. Takakura et al., Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell 92, 645–656 (1998).
33. W. C. Liang et al., Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J. Biol. Chem. 281, 951–961 (2006).
34. D. A. Tuveson et al., Endogenous oncogenic Kras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5, 375–387 (2004).
35. B. B. Madison et al., Six elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J. Biol. Chem. 277, 33275–33283 (2002).
36. A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation. Nature 454, 436–444 (2008).
37. J. Burn et al., CAPP2 Investigators, Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: An analysis from the CAPP2 randomised controlled trial. Lancet 378, 2081–2087 (2011).
38. H. S. Cooper et al., The role of mutant Apc in the development of dysplasia and cancer in the mouse model of dextran sulfate sodium-induced colitis. Gastroenterology 121, 1407–1416 (2001).
39. Y. Feng et al., S9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivating mutation. Am. J. Pathol. 183, 493–503 (2013).
40. S. Kitajima, S. Takuma, M. Morimoto, Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. Am. J. Pathol. 183, 67–70 (2009).
41. A. S. Chung et al., An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. Nat. Med. 19, 1114–1123 (2013).
42. H. Miyoshi, T. S. Stappenbeck, In vitro expansion and genetic modification of gastrointestinal stem cells in spherooid culture. Nat. Protoc. 8, 2471–2482 (2013).
43. F. Shojaei et al., βv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 450, 825–831 (2007).
44. L. Negri, N. Ferrara, The prokineticins: Neuromodulators and mediators of inflammation. J. Vet. Med. Sci. 66, 656 (1998).
45. F. Shojaei et al., Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. Nat. Biotechnol. 25, 911–920 (2007).
46. K. Kowatani et al., Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc. Natl. Acad. Sci. U.S.A. 107, 21248–21255 (2010).
47. D. F. Legler et al., B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. J. Exp. Med. 187, 655–660 (1998).
48. G. Binde et al., Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity 39, 782–795 (2013).
49. J. Pande et al., Chemokine CXCL13 is overexpressed in the tumour tissue and in the peripheral blood of breast cancer patients. Br. J. Cancer 99, 930–938 (2008).
50. Z. Zhu et al., CXCL13-CXCR5 axis promotes the growth and invasion of colon cancer cells via PI3K/AKT pathway. Mol. Cell. Biochem. 400, 287–295 (2015).
51. J. I. Wallin et al., Atezolizumab in combination with bevacizumab enhances antigen-specific T-cell migration in metastatic renal cell carcinoma. Nat. Commun. 7, 12624 (2016).
52. M. A. Socinski et al., IMpower150 Study Group, Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. N. Engl. J. Med. 378, 2288–2301 (2018).
53. R. S. Finn et al., IMB0005 Investigators, Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N. Engl. J. Med. 382, 1894–1905 (2020).
54. J. Guinan et al., The consensus molecular subtypes of colorectal cancer. Nat. Med. 21, 1350–1356 (2015).