rNAPc2 Inhibits Colorectal Cancer in Mice through Tissue Factor

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Abstract

Purpose: Recombinant nematode anticoagulant protein c2 (rNAPc2) is a specific inhibitor of tissue factor (TF)/factor VIIa complex with novel antithrombotic activity. TF is highly expressed in human colorectal tumors, and levels are positively correlated with disease progression.

Experimental Design: To explore the therapeutic potential and mechanism of action of rNAPc2 during tumor growth and metastasis, we tested rNAPc2 in several experimental colorectal cancer models in mice.

Results: Administration of rNAPc2 inhibited pulmonary metastasis in mice systemically disseminated with CT26 murine colon carcinoma cells in a dose-dependent fashion. Combining rNAPc2 with the cytotoxic agent 5-fluorouracil or bevacizumab (humanized anti-vascular endothelial growth factor monoclonal antibody) resulted in additive growth inhibition and simultaneous reduction of microvessel density in HCT116 human colorectal tumor xenografts in nude mice. Furthermore, rNAPc2 potentiated CPT-11 in inhibiting hepatic metastasis in nude mice with portal vein injection of HCT116 tumor cells. Long-term administration of rNAPc2 significantly suppressed spontaneous formation of intestinal tumors in ApcMin/+ mice. Using a RNA interference approach, we showed that TF expression is necessary for rNAPc2-mediated inhibition of HCT116 human colorectal tumor xenograft growth in nude mice, indicating that the antitumor effect of rNAPc2 may be transduced through TF that is expressed on tumor cells.

Conclusions: rNAPc2 is a potent anticancer agent when used in combination with chemotherapy or antiangiogenic therapy in mouse models of colorectal cancer, and TF positivity appears to be required for its activity.

Recombinant nematode anticoagulant protein c2 (rNAPc2) is a small 85-amino acid protein anticoagulant originally isolated from hematophagous hookworms (1). The potent anticoagulant property of rNAPc2 stems primarily from the inhibition of tissue factor (TF)/factor VIIa (fVIIa), which serves as the initiating enzymatic complex in the blood coagulation cascade (1, 2). In addition, rNAPc2 binds to circulating factor X to form a binary complex before its interaction and inhibition of membrane-bound TF/fVIIa (2, 3).

The relationship between cancer and thrombosis has been well established since it was first described by Trousseau over 100 years ago (4, 5). Tumor TF expression has been shown to be a key mediator during the hypercoagulable state of malignancy (6). In humans, aberrant TF expression has been found in a variety of solid tumors including glioma, breast cancer, lung cancer, leukemia, pancreatic cancer, and colorectal cancer (7–9). TF has been implicated in promoting tumor growth, angiogenesis, and metastasis in various in vivo experimental animal models (10–13). The level of TF has been found to correlate positively with stage of malignancy and negatively with overall survival in patients with colorectal cancer (14). Human tumor and cancer line analysis revealed that TF expression correlates directly with increased vascular density and vascular endothelial growth factor expression (9).

The mechanisms by which TF advances tumor progression likely involve both hemostatic activity and cellular response to TF/fVIIa signaling (9).

In the present study, we tested the antitumor potential of rNAPc2, a potent and specific inhibitor of the TF/fVIIa complex, in several colorectal cancer models in mice. We have shown that rNAPc2 additively inhibits the growth of primary and metastatic tumors when used in combination with chemotherapy or antiangiogenic therapy in mice with xenografted, disseminated, or spontaneous colorectal cancer cells. Furthermore, expression of TF in colorectal cancer cells appears to be necessary for the rNAPc2-mediated tumor-inhibitory effect in mice.

Materials and Methods

Reagents. Both CT26 murine colon carcinoma and HCT116 human colorectal carcinoma cell lines were purchased from the American Type Culture Collection. 5-Fluorouracil (5-FU) was from Sigma, CPT-11 (irinotecan HCl) was from HPCI, and bevacizumab (Avastin) was from Genentech.

TF monoclonal antibody (mAb) was from American Diagnostica, Ki-67 mAb was from Vector, CD31 mAb was from BD Pharmingen, and β-actin mAb was from Abcam.
Mice. Six- to 8-week-old female BALB/c and nude mice (nu/nu) were obtained from Charles River Laboratories. Mice were maintained under specific pathogen-free conditions and were acclimated before use. Animal experiments were done in accordance with guidelines established by the NIH Office of Laboratory Animal Welfare. All procedures involving animals were approved by the Nuvelo Institutional Animal Care and Use Committee.

ApcMin/+ mice on C57BL/6 strain background were acquired from The Jackson Laboratory, and the colony was expanded and genotyped according to a published protocol (15).

rNAPc2 administration. rNAPc2 was manufactured as a secreted protein using fermentation in the yeast Pichia pastoria and purified using a sequence of chromatographic and filtration steps (16). rNAPc2 was formulated at stock concentration of 1 mg/mL in PBS-based buffer and stored at -20°C.

rNAPc2 was administered to mice as daily intraperitoneal injections at predetermined doses in a volume of <500 μL.

Experimental lung metastasis in mice. CT26 murine colon carcinoma cells were cultured in RPMI 1640 (Invitrogen) with 10% fetal bovine serum. Harvested CT26 cell suspension (1×10⁶ cells) in a volume of 100 μL was intravenously injected into each syngeneic young adult female BALB/c mouse via the tail vein. Mice were sacrificed after 11 days and lungs were perfused, removed, weighed, and fixed in Bouin’s solution (Sigma) overnight. Visible pulmonary surface metastases were counted under stereoscope as described previously (17).

Human colorectal tumor xenografts in mice. HCT116 human colorectal carcinoma cells were grown in RPMI 1640 GlutaMax (Invitrogen) supplemented with 10% fetal bovine serum and harvested at mid-log growth phase. Ten million HCT116 cells were subcutaneously injected into the left flank of each nude mouse. Mice were randomized into treatment groups when the tumor volume reached 50 to 100 mm³. Tumors were measured with a caliper and tumor volume was calculated using the formula: 0.5×a²×b², where a was the length and b was the width of the tumor. On harvest, HCT116 xenografted tumors were extirpated, weighed, and fixed immediately in 10% neutral buffered formalin (Sigma) and processed histologically for paraffin sections at 5 μm thickness.

Immunohistochemistry. Both Ki-67 and CD31 immunostaining of tumor xenograft sections were done on a Leica AutoStainer XL according to protocols recommended by the manufacturers using a corresponding primary antibody. After incubation with biotinylated secondary antibodies, streptavidin-peroxidase was used with diaminobenzidine as a chromogen to generate a brown signal. Sections were counterstained with hematoxylin. Both water and IgG were used in lieu of primary antibodies as negative controls.

Quantification of tumor cell proliferation and intratumor microvessel density. For counting mitotic cells, the tumor tissue sections were viewed under a microscope at ×400 magnification (×40 objective lens.

Translational Relevance

The hypercoagulable state of malignancy has enabled antithrombotic therapy for cancer. The novel anticoagulant rNAPc2, the TF inhibitor, has been tested in animal models of colorectal cancer. We have found that rNAPC2 is efficacious in inhibiting experimental colorectal tumorigenesis, progression, and metastasis possibly through a TF-dependent mechanism. The additive tumor-inhibitory effect of rNAPc2 in these preclinical colorectal cancer models suggests that rNAPc2 may be tested clinically to potentiate chemotherapy or antiangiogenic therapy in human colorectal cancer patients.

Fig. 1. Dose-dependent effect of rNAPc2 in treating pulmonary metastasis in mice systemically disseminated with CT26 murine colon carcinoma cells. A, representative stereoscopic images of harvested mouse lungs after fixation in Bouin’s solution. a, naive; b, CT26/saline; c, CT26/rNAPc2 (300 μg/kg). Bar, 2 mm. B, mouse lung mass on harvest. *, P < 0.05 (CT26/saline versus naive); #, P < 0.05 (CT26/rNAPc2 groups versus CT26/saline). Mean ± SD (n = 8 mice per group).
and ×10 ocular lens) and the number of Ki-67-positive cells was determined. For microvessel density (MVD) measurement, images of tissue sections were acquired under a microscope at ×200 magnification (×20 objective lens and ×10 ocular lens), and the CD31+ microvessels were identified and counted according to the method documented earlier (18).

Four fields per tissue section were randomly analyzed, excluding peripheral surrounding connective tissue and central necrotic tissue areas. Microscopic slides were blindly coded during the assessment.

Liver metastasis model in mice. Female nude mice were anesthetized by ketamine. The portal vein was exposed through an upper midline incision in the abdomen. Two million HCT116 human colorectal carcinoma cells (suspended in 200 μL PBS) were injected slowly into the portal vein as described previously (19). Absorbable gelatin sponge (Gelfoam; Pfizer) was placed over the point of injection on the portal vein to prevent bleeding and potential leaking of HCT116 cells. The mouse abdomen was closed with two layers of suture.

After 6 to 8 weeks, the livers were removed, weighed, examined stereoscopically, and divided into anatomic lobes. Each liver lobe was fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections of the maximal cut surface of liver tissue were generated and stained with H&E. Images of liver sections were captured by a Leica digital microscope. The extent of liver metastasis was calculated as percentage of the sum of tumor areas in the total liver.

Spontaneous intestinal tumorigenesis in ApcMin/+ mice. Sex- and age-matched ApcMin/+ mice were randomized into treatment groups on weaning. Necropsy was done for ApcMin/+ mice at age 16 weeks. The entire intestinal tract was removed, cut open longitudinally, and washed with ice-cold PBS. The small intestine was divided into three segments of equal length, the proximal, middle, and distal sections. Tumor nodules were visualized and counted under the dissecting scope for both small intestine and colon. The smallest scorable tumors were –0.5 mm in diameter (20).

“Swiss-roll” sections were generated from harvested intestinal tissues and stained with H&E. Tumors were identified, enumerated, and photographed under a digital microscope. Using image measurement software (ImagePro Discovery; Media Cybernetics), the tumor diameter was obtained by measuring its widest point. Tumor areas were calculated under the assumption that all tumors were perfect circles. Total tumor area was the average of the sums of the tumor areas for each ApcMin/+ mouse. Each tumor nodule was also categorized as adenoma (benign) or adenocarcinoma (malignant) based on established criteria (21). The tumor histometry was done in blinded fashion.

TF knockdown by short hairpin RNA. HCT116 human colorectal carcinoma cells were transfected with human TF short hairpin RNA (shRNA) vector shRNAmir (Open Biosystems) by targeting sequence CGCACATAACATGCTTTAGATT. Nontargeting shRNAmir control vector was also used. Stable TF shRNA clones were selected in RPMI 1640 GlutaMax containing 0.5 μg/mL puromycin. Down-regulation of endogenous TF expression in HCT116 cells was assessed by TF Western

Fig. 2. Combined use of rNAPc2 with either 5-FU or bevacizumab (anti-vascular endothelial growth factor mAb) in treating HCT116 human colorectal tumor xenograft growth in nude mice. A, xenografted HCT116 tumor volume in nude mice over the experimental period. B, xenografted HCT116 tumor volume and weight on harvest. *, P < 0.05 (rNAPc2 versus vehicle control; 5-FU versus vehicle control; bevacizumab versus vehicle control; rNAPc2 + 5-FU versus vehicle control; rNAPc2 + bevacizumab versus vehicle control; rNAPc2, rNAPc2 + 5-FU, rNAPc2 + bevacizumab versus rNAPc2); **, P < 0.05 (rNAPc2 + 5-FU versus 5-FU); ##, P < 0.05 (rNAPc2 + bevacizumab versus bevacizumab). Assessment of cell proliferation (C) and MVD (D) in harvested HCT116 human colorectal tumor xenografts. *, P < 0.05 (rNAPc2 versus vehicle control; 5-FU versus vehicle control; bevacizumab versus vehicle control; rNAPc2 + bevacizumab versus vehicle control; rNAPc2 + 5-FU, rNAPc2 + bevacizumab versus rNAPc2); **, P < 0.05 (rNAPc2 + 5-FU versus 5-FU; rNAPc2 + bevacizumab versus rNAPc2); ***, P < 0.05 (rNAPc2 + bevacizumab versus bevacizumab). Mean ± SD (n = 6 mice per group). BEV, bevacizumab.
analysis, TF flow cytometry, and Actichrome TF activity assay (American Diagnostica).

TF flow cytometry was done by labeling HCT116 cells with phycoerythrin-conjugated TF mAb in a BD FACSCalibur system. Stable TF shRNA clone 3-2 was verified for its TF expression knockdown and was used in the current study.

Western blot analysis. TF Western analysis was done in a similar manner on HCT116 cell lysates using TF mAb as reported previously (22). The blots were also reprobed with β-actin mAb after stripping to ensure equal loading.

Statistical analysis. All experiments were done multiple times with similar results obtained within repetitive studies. Data points are presented as mean ± SD with n ≥ 6 unless stated otherwise. Statistical differences were determined by one-way ANOVA. Statistical significance was established at P < 0.05.

Results

rNAPc2 decreases pulmonary metastasis in syngeneic mice with CT26 murine colon carcinoma cell dissemination. To study the antimetastatic activity of rNAPc2, we injected CT26 murine colon carcinoma cells into the tail vein of syngeneic mice. rNAPc2 was given as daily intraperitoneal injections to mice immediately after CT26 cell dissemination until the time of lung tissue harvest. Representative stereoscopic lung images from various treatment groups are shown in Fig. 1A. Lung wet weight was measured as an indicator of tumor burden (Fig. 1B). Whereas CT26 dissemination in mice enhanced lung mass from 428 mg in naive controls to 688 mg (P < 0.05),
rNAPc2 administration dose-dependently lowered lung mass to 612 \( (P < 0.05) \), 569 \( (P < 0.05) \), 539 \( (P < 0.05) \), 517 \( (P < 0.05) \), and 512 \( (P < 0.05) \) mg at doses of 10, 30, 100, 300, and 1,000 \( \mu g/kg \), respectively (Fig. 1B). An average of 204 stereoscopically visible tumor nodules were found on lungs in CT26 cell-injected mice in the vehicle-treated control group, whereas mice treated with rNAPc2 exhibited a substantial reduction in the number of surface metastases to 124, 89 \( (P < 0.05) \), 81 \( (P < 0.05) \), 64 \( (P < 0.05) \), and 65 \( (P < 0.05) \) at doses of 10, 30, 100, 300, and 1,000 \( \mu g/kg \), respectively (Supplementary Fig. S1). These results suggest that rNAPc2 significantly reduces lung metastasis in syngeneic mice with systemic dissemination of CT26 mouse colon carcinoma cells.

\[ \text{rNAPc2 additively inhibits HCT116 human colorectal tumor xenograft growth in nude mice treated with 5-FU or bevacizumab.} \]

To further study the anticancer activity of rNAPc2, we used a HCT116 human colorectal tumor xenograft model to evaluate rNAPc2 in combination with either chemotherapy or antiangiogenic therapy for modulating primary tumor growth. Whereas administration of either rNAPc2 or 5-FU alone reduced HCT116 tumor xenograft volume by 32.7% \( (P < 0.05) \) or 53.7% \( (P < 0.05) \), respectively, combining rNAPc2 and 5-FU additively reduced HCT116 tumor xenograft growth by 81.3% \( (P < 0.05) \) in nude mice (Fig. 2A). Similarly, bevacizumab (humanized anti-vascular endothelial growth factor mAb) treatment alone inhibited HCT116 tumor xenograft growth by 61.1% \( (P < 0.05) \) in nude mice, whereas combining rNAPc2 and bevacizumab additively suppressed HCT116 tumor xenograft growth by 87.7% \( (P < 0.05); \text{Fig. 2A}). \]

Consistent with tumor volume measurement, combining rNAPc2 and 5-FU or bevacizumab additively inhibited HCT116 tumor xenograft growth in nude mice based on extirpated tumor weight on harvest (Fig. 2B).

We subsequently analyzed whether rNAPc2 regulates cell proliferation and neovascularization in tumor tissue. Harvested HCT116 tumor xenograft sections were immunostained with Ki-67 antibody for tumor cell proliferation and CD31 antibody for intratumoral MVD assessment (Supplementary Fig. S2). Morphometric analysis of both mitotic cell index (Fig. 2C) and MVD (Fig. 2D) was done. Whereas rNAPc2 alone moderately reduced HCT116 cell proliferation \( (P < 0.05) \), cotreatment with rNAPc2 and either 5-FU or bevacizumab resulted in additive cell growth inhibition in HCT116 tumor xenografts \( (P < 0.05); \text{Fig. 2C}). \]

Likewise, whereas rNAPc2 alone down-regulated MVD in HCT116 tumor xenografts \( (P < 0.05); \text{Fig. 2C})\), combined administration of rNAPc2 with either 5-FU or bevacizumab additively inhibited MVD in HCT116 tumor xenografts \( (P < 0.05); \text{Fig. 2D})\). These data indicate that the antitumor activity of rNAPc2 is most likely mediated through suppression of both tumor cell mitosis and simultaneous formation of microvessels.

Combining rNAPc2 with CPT-11 additively reduces hepatic metastasis in nude mice with portal vein injection of HCT116 tumor cells. An experimental liver metastasis model was used to further explore the efficacy of rNAPc2 in regulating colorectal cancer metastasis in mice. The liver is the most common site

Table 1. Evaluation of rNAPc2 in modulating tumor burden in small intestine in Apc\(^{Min/+}\) mice

<table>
<thead>
<tr>
<th>Microscopic measurements</th>
<th>Apc(^{Min/+}) mice</th>
<th>Saline control</th>
<th>rNAPc2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor no.</td>
<td>23.1 ± 7.5 (100)</td>
<td>10.5 ± 2.7* (46)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>0.59 ± 0.09 (100)</td>
<td>0.48 ± 0.05* (90)</td>
<td></td>
</tr>
<tr>
<td>Total tumor area (mm(^2))</td>
<td>7.73 ± 0.89 (100)</td>
<td>2.54 ± 0.49* (33)</td>
<td></td>
</tr>
<tr>
<td>% of Adenoma</td>
<td>79.6 ± 3.2</td>
<td>89.7 ± 4.2*</td>
<td></td>
</tr>
<tr>
<td>% of Adenocarcinoma</td>
<td>20.4 ± 3.2</td>
<td>10.3 ± 4.2*</td>
<td></td>
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NOTE: Percentage data (in parentheses) are arbitrarily normalized to saline control. * \( P < 0.05 \) (rNAPc2 versus saline control). Mean ± SD \( (n = 6 \text{ mice per group}) \).
of metastasis in colorectal cancer patients and we thus chose a mouse model of liver metastasis to resemble the clinical setting of advanced colorectal cancer (19). Cultured HCT116 cells were injected directly into portal vein of nude mice to induce metastasis in liver lobes. Treatment with rNAPc2 and/or CPT-11 was administered immediately after HCT116 cell injection and continued for 4 weeks. On harvest, mouse livers were weighed (Fig. 3A) and examined under a stereoscope (Fig. 3B, a-e). The liver wet weight in HCT116 cell-injected mice increased from 1.21 g in naive mice to 1.69 g ($P < 0.05$), indicating the metastatic tumor burden developed by HCT116 cells (Fig. 3A). Treatment with rNAPc2 or CPT-11 reduced liver wet weight to 1.43 ($P < 0.05$) or 1.34 ($P < 0.05$) g, respectively (Fig. 3A). Furthermore, combined use of rNAPc2 and CPT-11 additively decreased liver wet weight to 1.21 g ($P < 0.05$; Fig. 3A). The antitumor effect of rNAPc2 and/or CPT-11 was also visible by the macroscopic appearance of mouse livers (Fig. 3B, a-e).

Histometric analysis of H&E-stained mouse liver sections confirmed the metastasis-inhibitory role of rNAPc2 and/or CPT-11 (Fig. 3B, f-j). Tumor areas were readily discernible on microscopic H&E-stained liver sections; however, immunocytochemistry of carcinoembryonic antigen was also used to aid in identifying metastatic tumor regions in mouse liver (data not shown) as described previously (23). The extent of liver metastasis, as indicated by percentage of liver tumor area, was 51.6% in HCT116 cell-injected mice with control vehicle treatment, which was reduced to 23.2% ($P < 0.05$) or 7.1% ($P < 0.05$) by administration of rNAPc2 or CPT-11, respectively (Fig. 3C). Moreover, combined administration of rNAPc2 and CPT-11 further reduced liver metastasis to a residual 0.97% ($P < 0.05$), indicating the additive effect of using both rNAPc2 and CPT-11 in inhibiting liver metastasis in mice (Fig. 3C). These data indicate that rNAPc2 is an effective anticancer agent in inhibiting mouse liver metastasis when used in combination with CPT-11.

Long-term administration of rNAPc2 suppresses spontaneous intestinal tumorigenesis and progression in Apc$^{Min/+}$ mice. Mutation in the tumor suppressor gene Apc is implicated in $>70\%$ of all human colorectal cancers (24). Intestinal tumors form spontaneously in Apc$^{Min/+}$ mutant mice (15, 20), providing the most rigorous test for rNAPc2 as an antitumor agent in vivo. Weanling Apc$^{Min/+}$ mice were injected daily with rNAPc2 (100 μg/kg) until they reached age 16 weeks. The

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Requirement of TF for rNAPc2-mediated antitumor effect. A, Western analysis of TF from cultured cell lysates of parental, control shRNA-transfected, or TF shRNA-transfected HCT116 human colorectal carcinoma cells (a) and flow cytometry analysis of TF on the surface of HCT116 cells following TF shRNA transfection (b). B, effect of rNAPc2 in modulating tumor xenograft growth in nude mice implanted with parental or TF shRNA-transfected HCT116 human colorectal carcinoma cells. C, tumor volume and tumor weight on mouse harvest. *, $P < 0.05$ (parental/rNAPc2 versus parental/saline). Mean ± SD ($n = 6$ mice per group). KD, knockdown.
whole intestinal tracts including both small intestine and colon were harvested from Apc<sup>Min</sup>/+ mice and the number of tumor nodules was counted stereoscopically (Fig. 4A). Compared with saline vehicle treatment, long-term treatment with rNAPc2 substantially reduced the number of intestinal tumors in Apc<sup>Min</sup>/+ mice, including proximal, middle, and distal segments (P < 0.05; Fig. 4B). Administration of rNAPc2 led to a 53.2% (P < 0.05) and 69.6% (P < 0.05) reduction, respectively, in the number of tumors formed in small intestine and colon (Fig. 4B).

Bowel tissues from Apc<sup>Min</sup>/+ mice were processed histologically and then subjected to morphometric analysis (Table 1). Apc<sup>Min</sup>/+ mice administered with rNAPc2 yielded a 54% (P < 0.05) reduction in tumor number, a 20% (P < 0.05) reduction in tumor size, and a 67% (P < 0.05) reduction in total tumor area in small intestine in comparison with saline vehicle-treated Apc<sup>Min</sup>/+ mice (Table 1). Furthermore, rNAPc2 injection significantly decreased the percentage of adenocarcinomas in small intestine in Apc<sup>Min</sup>/+ mice (P < 0.05; Table 1). A similar rNAPc2-mediated antitumor effect was also seen in colon in Apc<sup>Min</sup>/+ mice (Supplementary Table S1). Taken together, long-term treatment with rNAPc2 significantly reduced tumor number, tumor size, total tumor burden, and tumor malignancy in both small intestine and colon in Apc<sup>Min</sup>/+ mice.

**TF is necessary for rNAPc2-mediated growth-inhibitory effect in HCT116 tumor xenograft-bearing nude mice.** To understand whether the anticancer activity of rNAPc2 is mediated by TF expression on the surface of tumor cells, we used HCT116 cells lines lacking TF. We employed TF-specific RNA interference strategy to knockdown its expression in HCT116 cells. HCT116 cell clones were selected and analyzed for TF expression by Western blot (Fig. 5A, a), flow cytometry (Fig. 5A, b), and TF procoagulant activity assay (data not shown) following stable transfection with TF shRNA. Parental, control shRNA-transfected, and TF shRNA-transfected HCT116 cells proliferated at a similar rate in cell culture (Supplementary Fig. S3). However, TF-suppressed HCT116 xenografts grew relatively slowly in nude mice compared with those of parental HCT116, whereas control vector-transfected and parental HCT116 xenografts showed similar growth rates (Supplementary Fig. S4), suggesting an in vivo growth advantage of colorectal tumors with TF positivity as reported previously (22).

Administration of rNAPc2 resulted in inhibition of tumor xenograft growth with either parental HCT116 cells (Fig. 5B) or HCT116 cells transfected with control shRNA vector (data not shown). On harvest following 27 days of rNAPc2 treatment, rNAPc2 showed a 43.7% (P < 0.05) inhibition by volume or a 50.1% (P < 0.05) inhibition by weight of parental HCT116 tumor xenograft growth in nude mice (Fig. 5B and C). In contrast, tumor xenograft growth of HCT116 cell line lacking TF was not further suppressed by rNAPc2 treatment (108.8% by volume and 97.4% by weight; Fig. 5B and C). Similar data were obtained with previously described TF knockdown HCT116 line (SI-3; provided by Janusz Rak, McGill University) xenografted in nude mice (Supplementary Fig. S5), suggesting that the loss of rNAPc2 antitumor efficacy without TF expression is not restricted to a single TF knockdown HCT116 cell clone. Collectively, these results indicate that the anticancer activity of rNAPc2 is mediated by TF expression in HCT116 tumor xenografts in mice.

### Discussion

In the current study, we have shown that the anticoagulant TF inhibitor rNAPc2 acts as an anticancer agent in several cancer models, including HCT116 human colorectal tumor xenograft growth and liver metastasis in immunodeficient mice. In addition, rNAPc2 inhibits the metastasis of CT26 murine colon carcinoma to mouse lungs and substantially reduces the overall tumor burden that occurs spontaneously along the intestinal tract in Apc<sup>Min</sup>/+ mice. Moreover, we have shown that rNAPc2 activity is mediated through TF expression on cancer cells, implying a tumor-suppressive effect through TF-mediated mechanism.

Consistent with previous findings (25), daily intraperitoneal dose of rNAPc2 <10 mg/kg to mice for 2 weeks were well tolerated without any sign of overt bleeding and other abnormalities (Supplementary Table S2). Thus, the rNAPc2 dose range used in the current report (30 µg/kg/d to 1 mg/kg/d) was both safe (tolerated up to a 3-month dosing period) to recipient mice and efficient in inhibiting tumor growth and metastasis.

Cancer patients are highly susceptible to thrombotic complications, which account for a significant portion of the morbidity and mortality of the disease (26, 27). Antithrombotic agents including warfarin and low molecular weight heparin have been used to lower the risk of deep vein thrombosis and pulmonary embolism in cancer patients (28). Besides preventing venous thromboembolism, antithrombotic therapy may also have direct antitumor benefits (29). In patients with small cell lung carcinoma, both warfarin and unfractionated heparin prolonged survival (29, 30). A similar survival advantage for low molecular weight heparin was also seen in studies of deep vein thrombosis in patients with solid tumor malignancies (31). Hirudin, a specific inhibitor of thrombin, was shown to inhibit experimental lung metastasis in syngeneic mice injected with B16-F10 melanoma cells (32). Another anticoagulant peptide isolated from hookworms, a potent inhibitor of factor Xa, also attenuated pulmonary metastasis in severe combined immunodeficient mice systemically disseminated with human melanoma cells (33).

TF is the primary cellular initiator of blood coagulation and its inactivation in mice leads to embryonic lethality due to hemorrhaging, implying that TF plays a critical role during vasculature development (34–36). In addition to its function during hemostasis, accumulating evidence has suggested that TF participates in the regulation of tumor growth, angiogenesis, and metastasis (37). TF expression on tumor cells appears to be positively correlated with the stage and aggressiveness of prostate, lung, breast, and colorectal cancers (14). The cytoplasmic domain signaling of TF has recently been shown to be involved in regulating protease activated receptor-2-dependent angiogenesis in mice (10). In support of that, we and others (22) have found that loss of endogenous TF expression delayed human colorectal tumor xenograft growth in nude mice, indicating that TF positivity provides growth advantage to tumors in vivo. Moreover, both human TF pathway inhibitor and TF pathway inhibitor-2 markedly reduce growth and metastasis of experimental tumors in mice (38, 39), indicating that TF blockers may be effective agents to interfere with tumor growth. Thus, TF abrogation may provide an opportunity to develop novel antithrombotic cancer therapeutics other than conventional cytotoxic agents.
Administration of rNAPc2 has been shown to reduce B16 murine melanoma xenograft growth in mice and inhibit pulmonary tumor metastasis in mice with tail vein injection of mouse Lewis lung carcinoma cells (25). It was also shown that rNAPc2 can down-regulate fibroblast growth factor-2-induced angiogenesis in the Matrigel plug assay in mice (25). In the current report, we evaluated rNAPc2 as a potent inhibitor of TF/VIIa in regulating tumorigenesis and progression by selecting in vivo models that use colon cancer cell lines with relatively high levels of TF expression (14). We have shown that rNAPc2 further inhibits the primary tumor growth of HCT116 xenografts in nude mice receiving chemotherapy using 5-FU or antiangiogenic therapy using bevacizumab most likely through down-regulation of both tumor cell growth and tumor tissue neovascularization. Our finding thus provides preclinical proof-of-concept data to support clinical testing of rNAPc2 in combination with either chemotherapy or antiangiogenic therapy to treat human colorectal cancer patients.

Besides inhibiting primary tumor growth, we have also shown that rNAPc2 reduces lung tumor metastasis in mice systemically disseminated with CT26 murine colon carcinoma cells in a dose-dependent fashion. Because liver is the most prominent site of metastasis in human colorectal cancer patients (19), we therefore further investigated the antimetastatic potential of rNAPc2 using a clinically relevant liver metastasis model in mice. Administration of rNAPc2 exhibited antimetastatic effects in nude mice with portal vein injection of HCT116 human colorectal carcinoma cells by itself or in combination with the chemotherapeutic agent CPT-11, suggesting that either rNAPc2 alone or rNAPc2 potentiates CPT-11 in reducing hepatic metastasis in a mouse model of colorectal cancer. Use of a patient-like orthotopic metastatic model of human cancer may also be chosen to further establish the role of rNAPc2 in regulating spontaneous metastasis during colorectal cancer development (40).

Mutation of tumor suppressor gene Apc is responsible for colorectal tumor formation in humans with sporadic adenoma and familial adenomatous polyposis (41). To fully understand the tumor-inhibitory activity of rNAPc2, we have used germ-line mutant ApcMin/+ mice that develop spontaneous intestinal tumorigenesis. TF expression was found to be significantly up-regulated in ApcMin/+ tumor nodules in gene profiling analysis using oligonucleotide microarrays (42), suggesting the relevance of testing TF inhibitors such as rNAPc2 in modulating intestinal tumor growth in ApcMin/+ mice. Our results show that rNAPc2 reduces the tumor number, tumor size, and tumor burden in both small intestine and colon in ApcMin/+ mice. Furthermore, daily injection of rNAPc2 during the experimental period did not display any detectable adverse effect in ApcMin/+ mice, including the incidence of hemorrhage. These results established the safety profile of long-term administration of rNAPc2 in ApcMin/+ mice while efficaciously reducing intestinal tumor burden. However, in contrast to human colorectal cancer, the majority of the tumor polyps developed in the small intestine rather than in the colon in ApcMin/+ mice with relatively few invasive adenocarcinomas observed (15, 20). In addition, tumors in ApcMin/+ mice do not appear to metastasize (15, 20). Therefore, genetic models, such as ApcMin/+Smad3−/− and ApcMin/+Ephb3−/− mice in which tumorigenesis is accelerated in the colon and rectum, may represent a clinically more relevant colorectal cancer model to further elaborate the antitumor activity of rNAPc2 (43, 44).

In conclusion, we have shown that the TF/VIIa inhibitor rNAPc2 is efficacious in inhibiting experimental colorectal tumorigenesis, progression, and metastasis in mice possibly through a TF-dependent mechanism. Furthermore, the additive tumor-inhibitory activity of rNAPc2 in preclinical colorectal cancer models indicates that rNAPc2 may be a useful "add-on" antitumor agent in treating human colorectal cancer patients to potentiate chemotherapy or antiangiogenic therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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