Dual inhibition of VEGFR and EGFR signaling reduces the incidence and size of intestinal adenomas in ApcMin/+ mice

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Abstract
Both the epidermal growth factor (EGF) and the vascular endothelial growth factor (VEGF) pathways are associated with intestinal cancer, and therapeutic approaches targeting either EGF receptor (EGFR) or VEGF receptor (VEGFR) signaling have recently been approved for patients with advanced colorectal cancer. The ApcMin/+ mouse is a well-characterized in vivo model of intestinal tumorigenesis, and animals with this genetic mutation develop macroscopically detectable adenomas from ~6 weeks of age. Previous work in the ApcMin/+ mouse has shown that therapeutic approaches targeting either VEGFR or EGFR signaling affect predominantly the size or number of adenomas, respectively. In this study, we have assessed the effect of inhibiting both these key pathways simultaneously using ZD6474 (Vandetanib, ZACTIMA), a selective inhibitor of VEGFR and EGFR tyrosine kinases. To assess the effects of ZD6474 on early- and later-stage disease, treatment was initiated in 6- and 10-week-old ApcMin/+ mice for 28 days. ZD6474 markedly reduced both the number and the size of polyps when administered at either an early or a later stage of polyp development. This reduction in both adenoma number and size resulted in a total reduction in tumor burden in the small intestine of nearly 75% in both studies (P < 0.01). The current data build on the concept that EGFR-dependent tumor cell proliferation and VEGF/VEGFR2-dependent angiogenesis and survival are distinct key mechanisms in polyp development. Pharmacologic inhibition of both signaling pathways has significant antitumor effects at both early and late stages of polyp development. Therefore, targeting both VEGFR- and EGFR-dependent signaling may be a beneficial strategy in early intestinal cancer. [Mol Cancer Ther 2008;7(3):590–8]

Introduction
The third most common cause of cancer-related death in western countries is colorectal cancer, which accounts for 550,000 annual deaths worldwide (1). Tumor development in colorectal cancer is characterized by cells within the normal mucosa acquiring multiple genetic mutations. Targets for somatic genetic “hits” during the early stages of colorectal cancer tumorigenesis include the oncogene KRAS and the tumor suppressor genes adenomatous polyposis coli (APC), SMAD4, and TP53 (2, 3). Somatic mutations in the APC gene occur in the majority of sporadic colorectal cancers; in addition, germ-line mutations in the APC gene result in the hereditary cancer syndrome of familial adenomatous polyposis (4).

The ApcMin/+ mouse was derived by chemical mutagenesis with ethylnitrosourea (5) and is heterozygous with somatic genetic “hits” during the early stages of colorectal cancer tumorigenesis. The current data build on the concept that VEGF/VEGFR2-dependent angiogenesis and survival are distinct key mechanisms in polyp development. Pharmacologic inhibition of both signaling pathways has significant antitumor effects at both early and late stages of polyp development. Therefore, targeting both VEGFR- and EGFR-dependent signaling may be a beneficial strategy in early intestinal cancer. [Mol Cancer Ther 2008;7(3):590–8]

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Hanrahan et al. detected a correlation between tumor grade and expression of several members of the VEGF family. These data suggested VEGF signaling may be important in early tumorigenesis, at the stage of adenoma formation (14). In another study of human colon cancer tissue, Easwaran et al. showed a direct correlation between activation of β-catenin signaling and up-regulation of VEGF, implying that β-catenin signaling may be involved in the regulation of angiogenesis in colon cancer (15). When the ApcMin/+ mouse was crossed with a mouse null for the endogenous angiogenic protein thrombospondin 1, the resulting offspring (ApcMin+/+TSP1−/−) developed more polyps, with larger diameters, compared with controls (16). Data obtained from another mouse model of intestinal adenomas (Apc1716) are also consistent with an association between VEGF and polyp development, where VEGF levels were elevated in polyps compared with epithelial cells from control gastrointestinal tracts (17).

Ligand-dependent activation of the epidermal growth factor (EGF) receptor (EGFR) signaling pathway is also a key process in the development and progression of many human tumors, including colorectal cancer (18). EGFR activity is an important regulator of epithelial cell proliferation, survival, and invasiveness (19) as well as the expression of proangiogenic factors such as VEGF (20). Indeed, inhibition of EGFR signaling is a promising therapeutic approach in colorectal cancer (21, 22). In ApcMin/+ mice, there was increased activation of the EGFR-phosphatidylinositol 3-kinase-Akt signaling pathway in adenomas relative to normal enterocytes (23) and pharmacologic inhibition of EGFR tyrosine kinase activity significantly reduced polyp number (21, 24). The potential importance of EGFR signaling was also shown by back-crossing ApcMin/+ mice with mice bearing an EGFR mutation (EGFR<sup>max2</sup>) that results in a marked reduction in EGFR tyrosine kinase activity (24). The offspring had a marked (90%) reduction in intestinal polyp number compared with ApcMin/+ mice expressing wild-type EGFR.

The aim of the current study was to investigate the therapeutic potential of inhibiting both VEGFR- and EGFR-dependent signaling pathways in the ApcMin/+ mouse model of intestinal tumorigenesis. We used ZD6474, a potent, orally bioavailable, small-molecule inhibitor of VEGFR2 and EGFR tyrosine kinase activity (25). ZD6474 has in vitro efficacy against a historically diverse range of human xenograft models, including tumors of colorectal origin (25, 26).

**Materials and Methods**

Chemicals were purchased from Sigma unless otherwise stated. ZD6474 [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl) methoxy]quinazolin-4-amine was synthesized by AstraZeneca Pharmaceuticals as described previously (27).

**Animals**

ApcMin/+ heterozygous mice were originally obtained as a gift from Amy R. Moser, (McArdle Laboratory for Cancer Research, University of Wisconsin-Madison). Male mice were backcrossed to female C57BL/6J and the resultant embryos were transferred by aseptic hysterectomy to foster mothers in specific pathogen-free isolators. All breeding was subsequently by brother-sister mating with the C57BL/6J colony at Cancer Research UK. All procedures, including mutant and transgenic breeding of ApcMin/+ mice, were approved by the Cancer Research UK and Imperial College School of Medicine Animal Ethics Committees and covered by the appropriate licenses under the Home Office Animal Procedures Act 1986.

**Genotyping**

DNA was extracted from mice tail-snips using a DNeasy Tissue kit (Qiagen) according to the manufacturer’s instructions, and PCR was used to identify mice carrying the ApcMin/+ mutation. The general PCR conditions, including primer pairs and amplification, have been described previously for detection of the Apc mutation (28).

**Experimental Protocol**

**Early Intervention Study.** Recently weaned (6-week-old) ApcMin/+ mice were given ZD6474 p.o. (12.5, 25, or 50 mg/kg/d) or vehicle only.

**Late Intervention Study.** Ten-week-old ApcMin/+ mice were given ZD6474 (50 mg/kg/d) or vehicle. Each study included 12 animals per group (mixed sexes) and ZD6474 was given once daily at 0.1 mL/10 g body weight for 28 days by oral gavage. For separate histologic evaluation, satellite groups of 6- and 10-week-old mice received 50 mg/kg/d ZD6474 or vehicle (n = 4-6 per group). Twenty-four hours after the last day of ZD6474 dosing, mice were injected i.p. with vincristine sulfate (1 mg/kg; Mayne Pharma Plc) to induce metaphase arrest 2 h before being killed humanely and with bromoectoxyuridine (BrdUrd; to label DNA-synthesizing cells at 50 mg/kg i.p.) 1 h before being killed humanely.

**Histologic Evaluation**

**Macroscopic Assessment of Tumor Burden**

At necropsy, organs and intestinal tract was dissected and processed as described previously (29). Briefly, the small intestine was divided into three equal sections: proximal, middle, and distal. These sections, as well as the entire colon, were then dissected longitudinally using a recently described cutting guide (30) and fixed in Carnoy’s fluid for 2 h and stored in 70% ethanol. For macroscopic polyp counts assessed with a dissecting microscope (at ×20 magnification), the number and size (mean of two largest diameters measured with digital calipers) of polyps in the small and large intestines were determined. Polyp volume was derived from polyp diameter; consistent with their histologic appearance, a hemispherical shape was assumed in the small bowel polyps and a spherical shape for the polyps in the colon. Tumor burden was calculated as the product of polyp number and polyp volume (31).
blocks in a standard manner. Step sections (3 μm) were stained with H&E and scored morphologically for micro-polyps. Micro-polyps were assigned to a category from 1 to 4: polyps that remained within the limits of a single villus were classified as category 1; those within the space of >1 to 5 villi were classified as category 2, >5 to 10 villi as category 3, and >10 villi as category 4 (32).

**von Willebrand Factor (Factor 8).** von Willebrand factor (vWF) was detected in Carnoy's fixed tissue (n = 6 per group) using a rabbit anti-human vWF polyclonal primary antibody (DakoCytomation) and developed in 3,3'-diaminobenzidine. After counterstaining with hematoxylin, a quantitative “point counting” method was used. Ten high-power fields (five from both normal and tumor tissue) from each of the six mice per group (control and treated) were scored for ‘hits’ using a 50-point Weibel 2 graticule, with the number of hits being proportional to the volume fraction (33).

**Bromodeoxyuridine.** BrdUrd was detected in Carnoy’s fixed tissues (n = 6 per group) using sequential incubation with a mouse anti-BrdUrd monoclonal primary antibody (Abcam), a biotin-conjugated rabbit anti-mouse secondary antibody, and streptavidin-peroxidase system was developed in 3,3'-diaminobenzidine. Thirty well-oriented crypts per animal were scored, and each crypt was scored from the crypt base to either the villus junction or the top of the colonic crypt. The presence of labeled and metaphase cells and the crypt length (in cells) were recorded (34). All tissues were scored “blind,” in that the person reading the slides was unaware of the group to which the slides belonged.

**β-Catenin.** β-Catenin was detected in Carnoy’s fixed tissue (n = 6) with a mouse anti-β-catenin monoclonal primary antibody (DakoCytomation) and a standard streptavidin-biotin system visualized with 3,3'-diaminobenzidine. Ten high-power fields (×40) were assigned a score for the proportion and intensity of staining in both the cytoplasm and the nuclei: For proportion: 0, no staining; 1, 0-33% stained; 2, >33-66% stained; 3, >66-100% stained. For intensity: 0, no staining; 1, 0-33% intensely stained (that is, remainder are weakly stained or not stained); 2, >33-66% intensely stained; 3, >66-100% intensely stained.

**CD31.** Immunostaining was done with 4-μm sections of formalin-fixed paraffin-embedded intestinal tissue from the late intervention study (n = 4; ref. 29). Epitope retrieval was carried out using DakoCytomation antigen retrieval buffer in a 2100-retriever pressure cooker (PickCell Laboratories). Staining for CD31 used anti-mouse CD31 (Santa Cruz) along with an avidin-biotin block (DakoCytomation). Detection was achieved using a standard streptavidin-biotin system (Vectastain ABC-Elite solution) visualized with 3,3'-diaminobenzidine (DakoCytomation). CD31 analysis was done blind using a Automated Cellular Imaging System (ChromaVision Medical Systems) to measure CD31¹ vessel number and CD31 staining area for each tissue region selected. Briefly, each tissue section was scanned into the Automated Cellular Imaging System at ×10 magnification. An analysis threshold was set and applied to all sections within the study. Tumor regions (n = 3-7 depending on whether treated or control samples) and normal regions (n = 7) within each section were selected and analyzed.

**Phospho-EGFR.** Immunostaining was done on formalin-fixed paraffin-embedded intestinal sections from the late intervention study. Antigen retrieval was carried out with high pH target retrieval solution (DakoCytomation) using the a 2100-retriever pressure cooker (PickCell Laboratories). Endogenous peroxidase activity was blocked using a peroxidase block (DakoCytomation) and then incubated with phospho-EGFR (Tyr992) in 0.05% TBS-Tween at 4°C overnight. Isotype controls were also included. Subsequent staining followed with an anti-rabbit peroxidase-labeled polymer visualized in 3,3'-diaminobenzidine (DakoCytomation).

**In situ Hybridization for VEGFR2.** VEGFR2 mRNA from formalin-fixed tissue (distal small bowel, n = 4 per group) was specifically localized using an antisense ribo-probe that was synthesized with T7 RNA polymerase using [35S]UTP (~800 Ci/mmol; Amersham-GE) and a plasmid prepared from IMAGE clone 5359101 (MRC Geneservice) and consecutive procedure as described before (35). Signal above the muscular layer (that is, mucosa, muscularis mucosa, and submucosa) was scored in a blinded fashion and semiquantitative analysis was used to rank sections in order of staining intensity.

**Statistical Analysis**

All results are presented as group mean ± SE. One-way ANOVA was used to test for any effect of treatment, and if appropriate, Dunnett’s test was applied. All statistics were done using Minitab Statistical Software Release 10.5 Xtra (Minitab).

**Results**

**Macroscopic and Histologic Characteristics of the ApcMin/+ Mouse**

ApcMin/+ mice develop numerous spontaneous adenomas that were predominantly located in the upper gastrointestinal tract (Fig. 1). Histologic analysis of polyps using an antibody for vWF revealed strong staining in both small (categories 1 and 2) and large (categories 3 and 4) micro-polyps near the luminal surface, showing that angiogenesis occurs during micro-polyp formation in ApcMin/+ mice (data not shown).

**Early Intervention Study**

In vehicle-treated animals, macroscopic analysis showed a large number of polyps in the small bowel with fewer, but larger, polyps in the colon (Fig. 2A). In the small bowel, ZD6474 treatment resulted in significantly fewer polyps per animal [mean ± SE polyp number, 90.9 ± 20.7 versus 49.5 ± 12.9 in control and (50 mg/kg/d) treated, respectively; P = 0.033]. Mean polyp diameter was also significantly lower at all doses of ZD6474 tested (12.5-50 mg/kg/d). These effects of ZD6474 on polyp number and/or diameter produced a significant lower polyp burden throughout the small bowel across the dose range, including a 75%
polyps in the small bowel (62.6 ± 0.02% (of body weight), the 12.5 mg/kg was not possible to unequivocally rule out an effect of ZD6474 on β-catenin staining and intracellular distribution in nonadenoma tissue.

The number of cells per polyp was significantly lower in animals treated with ZD6474 compared with vehicle-treated controls. Metaphase and BrdUrd labeling indices were lower in adenomas than in nontumor crypts, and these were unaffected by ZD6474 treatment despite the smaller tumor size. Microvessel density (measured by either vWF or CD31 staining) was similar in adenoma and nontumor tissue, and this was not affected by ZD6474 treatment (Table 1). The intensity of nuclear β-catenin staining and the proportion of cells with nuclear β-catenin staining were markedly higher in adenoma tissue compared with nonadenoma tissue (Table 1). In ZD6474-treated animals, the nuclear β-catenin staining in adenomas was significantly lower than in vehicle-treated controls (Fig. 4; Table 1).

Membrane-associated phospho-EGFR staining was apparent in adenomas of vehicle-treated animals (Fig. 5). In contrast, animals treated for 4 weeks with ZD6474 (50 mg/kg/d) showed no evidence of membrane staining of phospho-EGFR, although there was still some residual staining of the basement membrane.

Discussion

The results of this study show that pharmacologic inhibition of VEGFR and EGFR tyrosine kinase activity with ZD6474 reduces macropolypl number, size, and burden in the small bowel in the Apc^Min/+ mouse model of familial adenomatous polyposis. Micropolyps, the putative precursors to macropolyps, were also decreased in number and size by ZD6474 treatment. Qualitatively similar results were obtained when ZD6474 was given as once-daily oral administration for 28 days to 6-week-old

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mice (early intervention) or 10-week-old mice (late intervention). In the early intervention study, ZD6474 also significantly inhibited polyp number in the colon, a finding that is notable given the initial low polyp count in the colon.

The Apc<sup>Min</sup> mouse is one of several animal models of human gastrointestinal cancer, and it is a widely used model for the study of various factors on the early stages of intestinal cancer (8, 36). From the age of 6 weeks, Apc<sup>Min</sup> mice have macroscopically detectable adenomas (5). Similarly, in man, early inactivation of the Apc tumor suppressor gene usually occurs in both sporadic colorectal cancer and familial adenomatous polyposis (37). However, the differences between animal models and human disease should be considered when interpreting data. The predominant site of polyp formation in Apc<sup>Min</sup> mice is the small intestine, whereas polyp formation in Apc-deficient humans is mainly confined to the colon. Nevertheless, the types of adenomas (intravillous adenoma) found in the small bowel are remarkably similar in mice and men (38).

Figure 2. Polyp number (macropolyp), diameter, and burden in small intestine and colon of the (A) early and (B) late intervention studies. *, P < 0.05; **, P < 0.01 versus the vehicle-treated control.
The aim of conducting 6- and 10-week intervention studies was to assess the effect of ZD6474 on early and more established tumors, respectively. A comparison of micropolyp formation in these two studies confirms that the \textit{Apc}^\text{Min/+} model is one of progressive tumorigenesis (Figs. 2 and 3; Table 1). The small bowels of older mice had more micropolyps, and a greater proportion of these were classified as category 3 or 4. However, ZD6474 showed profound antitumor effects in both studies. This suggests that the development of both nascent and established adenomas depends, at least in part, on aberrant signaling pathway(s) that are sensitive to ZD6474.

The relative contribution of anti-VEGFR and anti-EGFR mechanisms to the antitumor activity of ZD6474 observed in this model has not been determined. However, the data are consistent with a mode of action that is antiangiogenic, at least in part. Tumor angiogenesis is stimulated when tumors grow beyond a certain size, and this angiogenic switch is common to both benign adenomas and malignant adenocarcinomas (39).

The angiogenic switch occurs at an early, premalignant stage of tumor development and VEGF signaling is thought to play a key role in the process (40). Recent data (41) have shown that highly selective inhibition of VEGF-A signaling prevents the angiogenic switch in \textit{Apc}^\text{Min/+} mice and that this is associated with a reduction in polyp diameter but with no effect on polyp number. This is consistent with the hypothesis that inhibition of VEGF-A signaling is sufficient to inhibit growth of established intestinal adenomas through inhibition of angiogenesis but that VEGF-A signaling is not important in the establishment of early adenomas before the angiogenic switch (41). Interestingly, an elegant genetic study of \textit{Apc}^\text{Min/+} mice with wild-type or reduced EGFR activity showed that the establishment of intestinal tumors, but not the rate of polyp growth, was dependent on EGFR activity (24). This finding was

<table>
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<th>Table 1. Tissue analyses from late intervention study (mean ± SE)</th>
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<td>Treatment group</td>
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<tr>
<td>Control</td>
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<tr>
<td>ZD6474 (50 mg/kg/d)</td>
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<td>Nonadenoma</td>
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<td>Cells per crypt</td>
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<td>Metaphases per crypt</td>
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<td>Metaphase index (%)</td>
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<td>BrdUrd per crypt</td>
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<td>Vessel density (vWF)</td>
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<td>( \beta )-catenin nuclear intensity</td>
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<td>VEGFR2 \textit{in situ} hybridization cells per villus</td>
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<td>Adenoma</td>
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supported by pharmacologic data from 12-week-old ani-
mals that showed a reduction in polyp number, but no
effect on polyp diameter, following treatment with an
EGFR tyrosine kinase inhibitor. It has been shown that APC
deficiency is associated with an increase in EGFR activity
(23) and that EGFR signaling is required for microadenoma
formation following azoxymethane treatment (42), implic-
ating this signaling pathway in the earliest stages of colon
carcinogenesis. EGFR is a transmembrane receptor tyrosine
kinase characterized by a “cobblestone” appearance on
immunohistochemical sections due to its localization to the
plasma membrane. Intracellular staining of EGFR may also
be detected to variable extents in tumor and normal tissues,
possibly reflecting receptor recycling and/or intracellular
compartmentalization. In the current study, strong phos-
pho-EGFR activity was seen on the cell membrane and was
dramatically reduced by ZD6474. Because of the technical
limitations of the available antibodies, it is currently only
possible to determine phospho-VEGFR2 levels by immu-
nohistochemistry in situations where there is a high level of
activity of the receptor, such as the lung, where inhibition
of phospho-VEGFR2 by ZD6474 has been shown (43). The
expression levels of VEGFR2 and pVEGFR2 in the intestinal
epithelia are relatively low (41).

ZD6474 reduced both polyp number and diameter in
both early and late intervention studies. These data are
consistent with the hypothesis that EGFR signaling is
required during the establishment of intestinal adenomas
but is less important during their subsequent growth.
It is tempting to speculate that the reduction in polyp size
and polyp number seen in the present study following
 treatment with ZD6474 is due to inhibition of both EGFR
and VEGF-R signaling, affecting both the establishment and
the growth of adenomas in the intestine of ApcMin/+ mice.
However, in contrast to the recent data selectively inhibit-
ing VEGF-A signaling (41), ZD6474 has the potential to
inhibit signaling through VEGFR1, VEGFR2, and VEGFR3.
Therefore, it is not possible to rule out an important role
for other VEGF ligands (VEGF-B, VEGF-C, VEGF-D and
placenta growth factor) or signaling through VEGFR3 in
the establishment intestinal adenomas because these would
not be affected by inhibiting VEGF-A signaling alone.
Indeed, in a previous study with a highly selective inhibitor
of VEGFR1, VEGFR2, and VEGFR3 signaling with no
activity against EGFR signaling, there was a reduction in
polyp number and diameter in a 6-week intervention study
but only a reduction in polyp diameter in a 10-week
intervention study, suggesting that VEGF signaling
through ligands other than VEGF-A may play a role in
the development of at least some adenomas in ApcMin/+ mice (29).

In the present study, microvessel density measured
by vWF staining was not affected by ZD6474 treatment,
which was confirmed by CD31 staining. However, in
vehicle-treated ApcMin/+ mice, the microvessel density in
adenomas was not significantly different from that in the
surrounding intestine, so a reduction in microvessel
density in the adenomas of ZD6474-treated mice was not

Figure 4. Effects of ZD6474 on VEGFR2 mRNA and β-catenin
expression. Small intestine showing localization of VEGFR2 mRNA using
in situ hybridization (white areas in dark-field illumination) from control (A)
and ZD6474-treated (B) animals. C and D, corresponding dark-field
images. Note the marked reduction in the signal (silver grains) in the
treated mice and the location of the signal in the lamina propria of the villi.
E to H, as above, but for polyps from control and treated groups, reduction
of signal also occurs in the treated groups. Immunohistochemistry of
β-catenin expression and localization in ZD6474-treated groups (J) was
significantly reduced when compared with control (I). Bar, 100 μm.
ZD6474 offers the potential advantage of inhibiting two key pathways in tumor growth and development. Targeting VEGFR activity in endothelial cells offers potential advantages compared with agents that only target tumor cells directly. These include a reduced probability for the emergence of drug-resistant clones, because normal endothelial cells lack the genetic instability of cancer cells, as well as a possible amplification of any antitumor effect, because each capillary in a tumor supplies many hundreds of tumor cells. ZD6474 is currently in phase III clinical development, and promising efficacy has been observed in recurrent non-small cell lung cancer (48).

In conclusion, the current data suggest that ZD6474 is effective at different stages of adenoma development, with the potential to target EGFR signaling during the establishment of adenomas before the angiogenic switch and to target VEGFR signaling and blood vessel growth in established adenomas. These data provide a scientific rationale for studying the effects of inhibition of these pathways in both early and late stages of intestinal cancer in the clinic. These results are particularly timely as they follow recent reports of clinical efficacy in colorectal cancer with targeted inhibitors of VEGF-A (49) and EGFR (50) signaling.

Acknowledgments

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References

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Molecular Cancer Therapeutics

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