[CANCER RESEARCH 63, 3145–3153, June 15, 2003]

β-Catenin Regulates Vascular Endothelial Growth Factor Expression in Colon Cancer

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ABSTRACT

To evaluate whether β-catenin signaling has a role in the regulation of angiogenesis in colon cancer, a series of angiogenesis-related gene promoters was analyzed for β-catenin/TCF binding sites. Strikingly, the gene promoter of human vascular endothelial growth factor (*VEGF*, or *VEGF-A*) contains seven consensus binding sites for β-catenin/TCF. Analysis of laser capture microdissected human colon cancer tissue indicated a direct correlation between up-regulation of *VEGF-A* expression and adenomatous polyposis coli (*APC*) mutational status (activation of β-catenin signaling) in primary tumors. In metastases, this correlation was not observed. Analysis by immunohistochemistry of intestinal polyps in mice heterozygous for the multiple intestinal neoplasia gene (*Min*/+) at 5 months revealed an increase and redistribution of VEGF-A in proximity to those cells expressing nuclear β-catenin with a corresponding increase in vessel density. Transfection of normal colon epithelial cells with activated β-catenin up-regulated levels of *VEGF-A* mRNA and protein by 250–300%. When colon cancer cells with elevated β-catenin levels were treated with β-*catenin* antisense oligodeoxynucleotides, *VEGF-A* expression was reduced by more than 50%. Taken together, our observations indicate a close link between β-catenin signaling and the regulation of VEGF-A expression in colon cancer.

INTRODUCTION

Tumor growth and metastasis are highly dependent upon neoangiogenesis, the formation of capillary sprouts. These may arise from either from preexisting blood vessels, circulating endothelial cells, or bone marrow-derived endothelial precursor cells (1). VEGF[3] (or VEGF-A) is one of the principal regulators of pathological and physiological angiogenesis (1–5). VEGF-A expression is up-regulated in numerous solid tumors, including primary and metastatic colorectal carcinoma (6–10).

Originally discovered as a stimulator of vascular permeability, VEGF-A was subsequently found to be a mitogen and survival factor for endothelial cells through its interaction with two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (4, 11–13). The survival function is mediated through activation of the phosphatidylinositol 3′-kinase-Akt signaling pathway and up-regulation of Bcl-2 (14–16) as well as through up-regulation of inhibitors of apoptosis (IAP) family members (17). VEGF-A expression is regulated at the level of transcription and translation in a complex fashion by numerous oncogenic and tumor suppressor pathways, conditions of hypoxia, and estradiol-receptor complexes (18–29).

In colon cancer, evidence from preclinical and clinical studies has shown the necessity of angiogenesis for tumor growth and metastasis. Furthermore, these studies implicate VEGF-A as the principal angiogenic factor (8, 30). The elegant work of Fearon and Vogelstein (31) has described the stepwise progression of the genetic lesions that are found in early adenoma and culminate in carcinoma and metastasis in colon cancer. In both the sporadic and familial adenomatosis polyposis forms of colorectal cancer, inactivation of the *APC* tumor suppressor gene initiates tumor formation. APC is a component of the Wnt signaling pathway and negatively regulates levels of β-catenin, a critical mediator of the Wnt transcriptional response (32).

Absence of functional APC results in the posttranslational stabilization of β-catenin protein, its translocation into the nucleus, interaction with TCF/Lef family transcription factors, and changes in target gene expression including *c-Myc* (33), *cyclin D1* (34), *matrilysin* (35), *pArf* (36), *Connexin* 43 (37), *uPAR*, *c-jun* and *fra-1* (38), *WISP*, (39), *fibronectin* (40), *PPARδ* (41), and *Naked* (42).[4] These genes contribute to the growth-promoting activities mediated by β-catenin signaling.

Recent work suggests that Wnt/β-catenin signaling regulates vessel development in normal and pathological conditions. Several studies have demonstrated the expression of Wnt ligands, Wnt receptors [frizzleds (Fz)], and Wnt inhibitors in vascular cells (43). Moreover, mice deficient for *Wnt-2* or *Fz-5* have severe vascular defects, and mutations in human *Fz-4* have been linked to familial exudative retinopathy, a hereditary disorder in which retinal angiogenesis is severely impaired (43–45). β-Catenin accumulation in the cytosol and nucleus has been observed in proliferating vessels of the human placenta and has been frequently observed in vascular cells during pathological angiogenesis (43). A recent report described *VEGF-A* as a β-catenin target gene in HeLa cells and colon cancer cells (46).

To further explore the relationship between the Wnt/β-catenin pathway and angiogenesis, we searched the promoters of genes encoding the proteins that participate in vessel formation for nucleotide sequences that recognize TCF/Lef. Notably, in the promoter for *VEGF-A*, we found seven TCF/Lef binding elements. This prompted us to investigate the relationship between *VEGF-A* levels and *APC* mutational status in human colon cancer samples and cancer cell lines and also to evaluate this relationship in the *Min*/+ mouse model of colorectal cancer. In human colon cancer cell lines, β-catenin induced *VEGF-A* mRNA and protein expression. Our experiments thus confirm and expand the report that *VEGF-A* is a β-catenin target gene (46), emphasizing the importance of β-catenin in the early and stepwise events of colon cancer neoangiogenesis.

MATERIALS AND METHODS

Cell Culture and Transfections. SW620, HCT116, and HEK293 cell lines were acquired from the American Type Culture Collection and maintained in DMEM (Life Technologies, Inc., Bethesda, MD) supplemented with 10% fetal

Received 8/14/02; accepted 4/11/03.

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; APC, adenomatous polyposis coli; q-PCR, quantitative reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bFGF, basic fibroblast growth factor; TBE, TCF binding element; Min, multiple intestinal neoplasia; vWF, von Willebrand factor; ODN, oligodeoxynucleotide; Lef, lymphoid enhancer factor.

[4] http://www.stanford.edu/~rnusse/pathways/targets.html.

bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. The NCM460 cell line, acquired from Incell Corp. (San Antonio, TX), is an epithelial cell line derived from the normal human colon mucosa (47). It is not infected or transfected with any exogenous genetic information, it does not grow in soft agar, and it is nontumorigenic. The NCM460 cells were maintained in M3:10 medium (Incell Corp). Plasmid transfections were performed using LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's instructions. Antisense oligonucleotides complementary to human *β-catenin* mRNA or reverse control oligonucleotides (48) were transfected into SW620 and HCT116 cells at a final concentration of 100 nM using a cationic peptoid reagent (49).

Patient Samples. Tissue samples were taken at surgery, with the informed consent of the patients, and frozen for subsequent cryosectioning and laser capture microdissection (50). All Institutional Review Board requirements were met.

Animals. C57BL/6 *Min*/+ and C57BL6 wild-type littermates were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed 5 mice/cage in microisolator cages in an American Association of Laboratory Animal Care-accredited animal facility. Mice were fed special Purina laboratory animal diets (5K20). Mice received food and water *ad libitum*.

q-PCR. Total RNA was prepared from cell lysates using the RNeasy total RNA isolation kit (Qiagen Inc., Chatsworth, CA). Using a reverse transcription reaction kit (Perkin-Elmer, Boston, MA), 1 μg of total RNA was reverse transcribed. Ten percent of the reverse transcription reaction was amplified by q-PCR using the LightCycler System (Roche, Indianapolis, IN), and the product was quantified using a standard curve that calculated each cycle number at which the amplification of the product was in the linear phase. This value was normalized to the value of the internal standard *GAPDH* for each analysis. In the case of patient samples, RNA was extracted from laser capture microdissected cells from matched tumor and normal biopsies and amplified using established procedures to accurately retain relative abundance of mRNAs (51). Normalization was to *glucuronidase synthase* as described previously (42). For the *VEGF-A* mRNA, q-PCR was performed using primers as described by Liu *et al.* (52).

Mutational Status of APC, β-Catenin, and Ras in Patient Samples. The mutational status of *β-catenin* and *K-Ras* was measured by single strand conformational polymorphism using total tumor RNA, as described previously (42). This gel analysis allows mutant RNA to be separated from wild type and is thus more sensitive than PCR and sequencing for detecting small percentages of mutant RNA in the tumor sample. Two hundred ng of total RNA from colon cancer patient samples were reverse transcribed and PCR amplified using the primers described previously for *β-catenin* (42) and the forward (*K-Ras-F*, 5′-AGAGGCCTGCTGAAAATG-3′) and reverse (*K-Ras-R*, 5′-TTCCTGTAGGAATCCTCTATTGTT-3′) primers for *K-Ras*.

To detect truncations in *APC*, 200 ng of total tumor RNA from colon tissue samples were reverse-transcribed with primer RT-*APC* (5′-GTATGGTTACAGATGAGGTTTTTCC-3′) and amplified (Advantage GC-rich kit; CLONTECH) with primers as described previously (42). The PCR products were transcribed *in vitro* and translated with the T7-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine and [^{35}S]cysteine to detect translation products after SDS-PAGE and autoradiography (42, 53).

ELISA. VEGF-A and bFGF protein in tissues and cell lysates was quantified using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). Protein concentration of each sample was determined using Bio-Rad (Hercules, CA) protein assay reagent. Preparation of small intestine homogenates from *Min*/+ and wild-type mice involved freezing the dissected tissue in liquid nitrogen followed by homogenization in ice-cold lysis buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS with a protease inhibitor mixture).

Immunohistochemistry. Small intestine and colon from *Min*/+ mice and normal litter mates were cleaned of feces with saline, cut open, and laid flat, marking the proximal and distal ends of the organs. Samples were rolled, placed into a histology cassette, and fixed for 4–6 h in 10% neutral buffered formalin. Samples were then processed and embedded in paraffin blocks.

Sections (3–4 μm) sections were cut and floated onto glass slides, baked and deparaffinized in xylene, and rehydrated in graded alcohols. Antigen retrieval was performed using Citrate Plus buffer (Biogenex, San Ramon, CA) in a Biocare steamer. Cooled samples were stained using a Ventana Medical Systems NEXUS automated stainer. Slides were treated for 32 min with appropriate primary antibody [VEGF-A-20 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:100 dilution, β-catenin antibody (B19022; Chemicon, Temecula, CA) at 1:500 dilution, or rabbit antibody to vWF (DAKO Carpinteria, CA) at 1:1000 dilution]. Samples were treated for 32 min with secondary antibody [goat antirabbit F(ab′)$_2$ biotinylated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at 1:100 dilution]. The signal was resolved with a streptavidin-horseradish peroxidase conjugate, 3,3′-diaminobenzidine, and heavy metal enhancement with copper sulfate (Ventana DAB detection reagents; Ventana Medical Systems, Tucson, AZ). Slides were counterstained with hematoxylin and bluing reagent (Ventana Medical Systems). Negative control samples were exposed to rabbit γ-globulin (Jackson ImmunoResearch Laboratories Inc.) at concentrations matching concentrations of primary antibodies and stained as described. All antibodies were diluted in 1× PBS/0.5% BSA (IgG-free fraction)/0.01% thiomersol (pH 7.4).

Cell Fractionation and Western Blotting. For the analysis of cytosolic β-catenin, HCT116 cells were lysed in hypotonic buffer with protein inhibitors and passed through a 27G1/2-guage needle. After centrifugation at 6,000 rpm for 10 min, supernatants were further clarified in an ultracentrifuge (Optima TLX; Beckman) at 10,000 rpm for 35 min at 4°C. After normalization for protein, aliquots of lysate were subjected to Western analysis with a β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY).

Statistical Analysis. Data are reported as mean ± SD of multiple experiments. Differences between the laser microdissected, matched normal and malignant specimens were compared using paired Student's *t* tests. The unpaired Student's *t* test was used to compare *VEGF-A* mRNA levels in liver metastases with a pool of normal colon tissue and in the *Min*/+ and wild-type (control) mouse experiments. Correlations between *APC* and *K-Ras* mutational status and *VEGF-A* or *VEGF-D* gene expression were estimated using a dichotomous model and analyzed by the χ^2 test. An arbitrary ratio cutoff of >2.0 for tumor tissue/normal tissue mRNA was selected (Figs. 2 and 3). All experiments were repeated a minimum of three times unless otherwise stated. Two-tailed tests with $P < 0.05$ were accepted as significant.

RESULTS

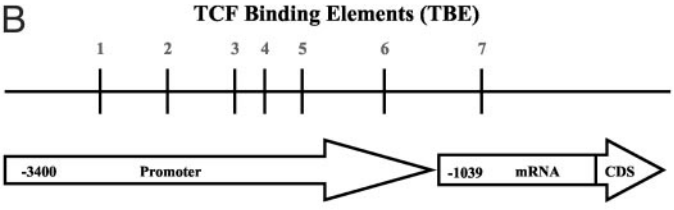
Analysis of Angiogenesis-related Gene Promoters for β-Catenin/TBEs. A search of the GenBank nucleotide database revealed the presence of β-catenin/TCF binding sites (TBE: T/A T/A CAA T/A G) in the promoters of several genes known to regulate angiogenesis (Fig. 1*A*; Refs. 34, 35, and 54). Thus, whereas the probability of uncovering a β-catenin/TCF binding consensus sequence by chance is 1 for every 2048 nucleotides, the promoter regions of *VEGF-A* and *KGF* each contained at least three such binding sites. Of these, the human *VEGF-A* gene was particularly noteworthy; it contains four consensus binding sites (sites 1, 2, 5, and 7) and an additional three putative, partial sites (sites 3, 4, and 6) over a 3500-nucleotide stretch that includes the promoter and 5′-untranslated region (Fig. 1*B*). These findings strongly suggest that *VEGF-A* is a β-catenin target gene.

VEGF-A Expression Correlates with APC Mutational Status and Activation of β-Catenin Signaling in Human Colon Cancer. *VEGF-A* is a transcriptional target of the Ras-Raf- mitogen-activated protein kinase pathway, which is frequently up-regulated in many cancers (20, 22). In colon cancer, activation of K-Ras is a genetic event that usually succeeds activation of the β-catenin pathway (55). We therefore determined whether correlations existed, *in vivo*, between *VEGF-A* expression and the *APC*, *β-catenin*, and/or *K-Ras* mutational status in both primary tumors and liver metastases from colon cancer patients. Using laser capture microdissection, epithelial cells from malignant and matched autologous (*i.e.*, from the same patient) histologically normal colon were isolated from 20 different subjects for RNA extraction. Quantitative real-time PCR showed that whereas *VEGF-D* mRNA levels did not differ between primary tumor and autologous normal tissue (0.87 ± 0.40-fold; $P = 0.182$, paired *t* test), *VEGF-A* mRNA levels were significantly greater in the malignant tissue (2.60 ± 1.93-fold; $P = 0.002$, paired *t* test; Fig. 2*A*). Of the 20 primary lesions investigated, 17 harbored *APC* gene deletions, and

A

Gene Promoter	TCF Binding Elements (TBE)
VEGF-A	7
VEGF-D	1
KDR	1
FLT-1	1
PlGF	NA
EGF	2
KGF	3
FGF-2	1
FGF-BP	2
Pleiotrophin	0
VRF	0
TEK	1
Plasminogen	1
Thrombospondin 2	1+
ANG 1,2	NA
Urokinase	0
UPAR	0
Integrin αV	0
Integrin β3	0
Integrin β5	NA
c-myc	2

B



Human VEGF-A gene (GenBank Acc.# AF095785)

TBE	Sequence	Orientation
1	tacaaag	forward
2	ctttgtt	reverse
3	ggtttgaa	reverse
4	aacaaggg	forward
5	ctttgat	reverse
6	ttcaat	forward
7	cattgat	reverse

Fig. 1. β-Catenin/TBEs and angiogenic factors. *NA* denotes that the sequence of the promoter was not available. *A*, binding elements identified from a search of National Center for Biotechnology Information GenBank database in promoter sequences of genes involved in angiogenesis (complete T/A T/A CAA T/A G and partial T/A CAA T/A G sites are included). *B*, location of TBEs within the promoter for *VEGF-A* (GenBank accession number AF095785). Insert describes the sequence of each TBE and indicates whether it is in the forward or reverse complement orientation. *CDS* denotes coding sequence.

in all samples, *β-catenin* was wild type (Fig. 2*B*). Of these, 11 expressed elevated levels of *VEGF-A* mRNA when normalized to *glucuronidase synthase* mRNA (as an internal control) and using an arbitrary cutoff ratio for tumor tissue/normal tissue mRNA of >2.0. By this analysis, none of the three lesions with wild-type *APC* was positive for elevated *VEGF-A* levels. Using a conservative χ^2 analysis and acknowledging that there are only three samples with wild-type *APC*, the results indicate that lesions harboring APC mutations have statistically higher levels of *VEGF-A* gene expression than lesions with wild-type *APC* ($\chi^2 = 4.31$; $P = 0.038$). Of the five primary tumors showing mutations in both *APC* and *K-Ras*, there was no evidence of a significant cumulative effect on *VEGF-A* mRNA levels (Fig. 2*A*; $\chi^2 = 0.61$; $P = 0.436$). The explanation for these latter data awaits analysis of more patient specimens.

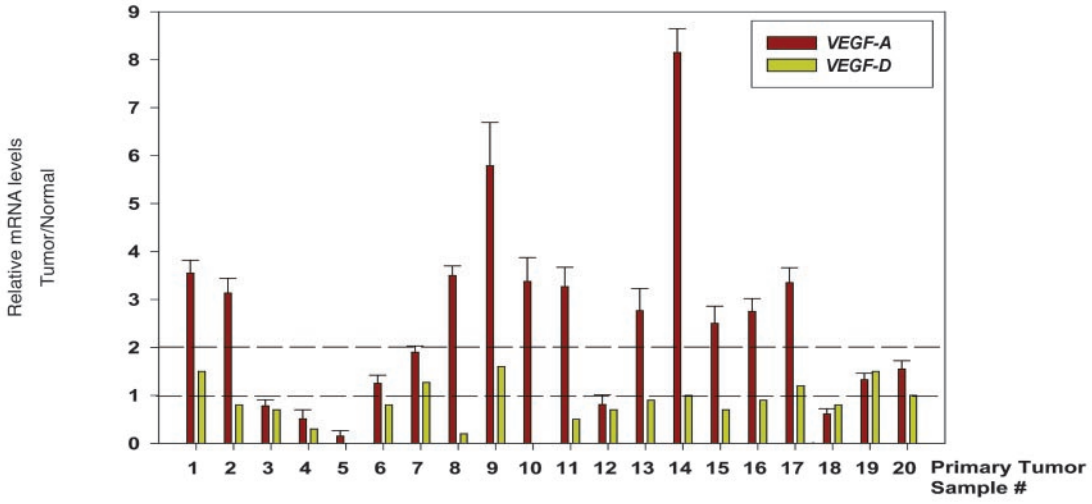
However, in liver metastases, *VEGF-A* mRNA levels were elevated in 20 independent patients when compared with a pool of normal colon tissue from 20 patients [autologous tissue was not available (4.70 ± 2.03-fold; $P = 0.0001$, unpaired *t* test)], with no correlations to the *APC* status of the patient (Fig. 3, *A* and *B*). It should be noted that in none of the samples analyzed (primary tumors and metastases) was the β-catenin gene mutated.

β-Catenin Regulates VEGF-A in the APC Min/+ Mouse Model. To determine the role of β-catenin in the regulation of VEGF-A during colon cancer progression, we used the Min mouse model (56). The *Min*/+ mouse strain, heterozygous for a nonsense mutation in the murine *APC* gene at codon 850 (57), permits the study of factors that regulate the transition between precancerous and neoplastic growth. VEGF-A protein levels were quantified from small intestine and serum of *Min*/+ mice and wild-type littermates by ELISA assays. VEGF-A protein levels were increased in samples from Min/+ mice relative to wild-type littermates at 5 months but not at 1 month of age (Fig. 4*A*). Moreover, levels of bFGF were not augmented at 5 months in the *Min*/+ mice (data not shown).

Given the increase in VEGF-A detected by ELISA seen at 5 months of age in the *Min*−/+ mice, immunohistochemistry was implemented to visualize the distribution of β-catenin, VEGF-A, and the endothelial cell marker vWF in the small intestine of *Min*/+ mice and wild-type littermates. Overall, significant differences were seen in the staining pattern for all three antibodies in normal *versus* mutant tissues. In wild-type mice, β-catenin staining was observed at the plasma membrane, consistent with its participation in stabilizing the cadherin cell adhesion complex (Ref. 58; Fig. 4*B*). In these samples, no nuclear β-catenin was detected. In sharp contrast, in 5-month-old *Min*/+ mice, β-catenin was detected in both the nucleus and cytoplasm (Fig. 4*C*). VEGF-A immunohistochemistry in normal mice revealed low-intensity, uniform expression in enterocytes as well as in specific regions of the crypts with somewhat higher intensity (Fig. 4*B*). Immunolocalization of VEGF-A in the *Min*/+ mouse was more diffusely distributed, with some enterocytes showing very intense staining, particularly within polyps. Overall, the level of VEGF-A appeared greater in the *Min*/+ mice compared with wild-type mice, corroborating the ELISA data. Furthermore, VEGF-A appeared to infiltrate the entire polyp, perhaps contributing to the genesis of a more expansive vasculature (Fig. 4*C*). Staining of normal tissue with vWF showed the expected sporadic occurrence of well-defined blood vessels in the cellular lamina propria of the villi, adjacent to some crypts, with some larger vessels being present in the surrounding connective tissue including the muscularis mucosa (Fig. 4*B*). In 5-month-old *Min*/+ mice, the intensity of vWF staining was increased compared with the wild-type controls. The tight, focused staining that typified vessels in normal animals was seen to be more diffuse, indicating a distinct change in vessel morphology of *Min*/+ mice (Fig. 4, *B* and *C*).

β-Catenin Induces VEGF-A Expression in Normal Colon Epithelial Cells. To test whether β-catenin can directly induce VEGF-A expression, an early-passage human colon epithelial cell line, NCM460, derived from normal colon mucosa (47), was transfected with cDNA encoding a mutant form of *β-catenin*, S37A. This mutant is resistant to phosphorylation-dependent ubiquitination, resulting in

A)



B)

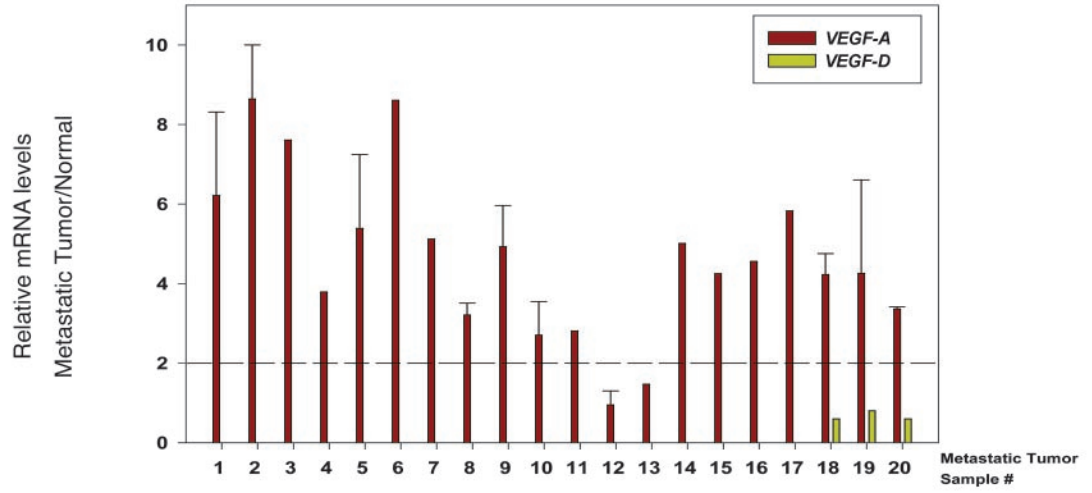
Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
APC	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	WT	WT	WT
$\beta_{catenin}$	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
K-Ras	Δ	Δ	Δ	Δ	Δ	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

Fig. 2. *VEGF-A* mRNA and mutational status of primary colon tumors. *a*, relative mRNA levels for *VEGF-A* and *VEGF-D* in primary tumor *versus* normal tissue as measured by q-PCR from laser capture microdissected tissue. *B*, mutational status for *APC*, *β-catenin*, and *K-Ras* for the tumors analyzed in *A*.

its stabilized expression (59). Forty-eight h after transfection of a *S37A-β-catenin* cDNA plasmid, *VEGF-A* mRNA levels were quantified in the NCM460 cells using real-time q-PCR. In a representative experiment, after transfection of *S37A-β-catenin*, a 280% increase in *VEGF-A* mRNA and a 240% increase in *cyclin D1* mRNA (a known β-catenin target gene; Ref. 34) were observed (Fig. 5, *A* and *B*). Fig. 5*A* is the quantitation of the PCR products from the Light cycler using the standard curve. The products of the reaction are shown on the gel in Fig. 5*B*. Overexpression of β-catenin also produced an increase in cellular VEGF-A protein, comparable with the change seen in *VEGF-A* mRNA in the same experiment (Fig. 5*C*). These observations indicate that in normal colon epithelial cells, transcriptional control of VEGF-A appears to be rate limiting, a situation comparable with estradiol-stimulated expression of VEGF-A in endometrial cells (29).

β-Catenin Antisense ODNs Down-Regulate VEGF-A Expression in Colon Cancer Cells. To confirm a functional link between β-catenin and regulation of VEGF-A expression, the HCT116 and SW620 colon cancer cell lines expressing stabilized β-catenin [a consequence of mutated *β-catenin* or inactivation of *APC* function,

A)



B)

Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
APC	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	WT	WT	WT
$\beta_{catenin}$	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
K-Ras	Δ	Δ	Δ	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT

Fig. 3. *VEGF-A* mRNA and mutational status of liver metastases. *A*, relative mRNA levels of *VEGF-A* and *VEGF-D* for liver metastases *versus* a pool of normal tissue from 20 patients as measured by q-PCR from laser capture microdissected tissue. *B*, samples were genotyped as described in Fig. 2.

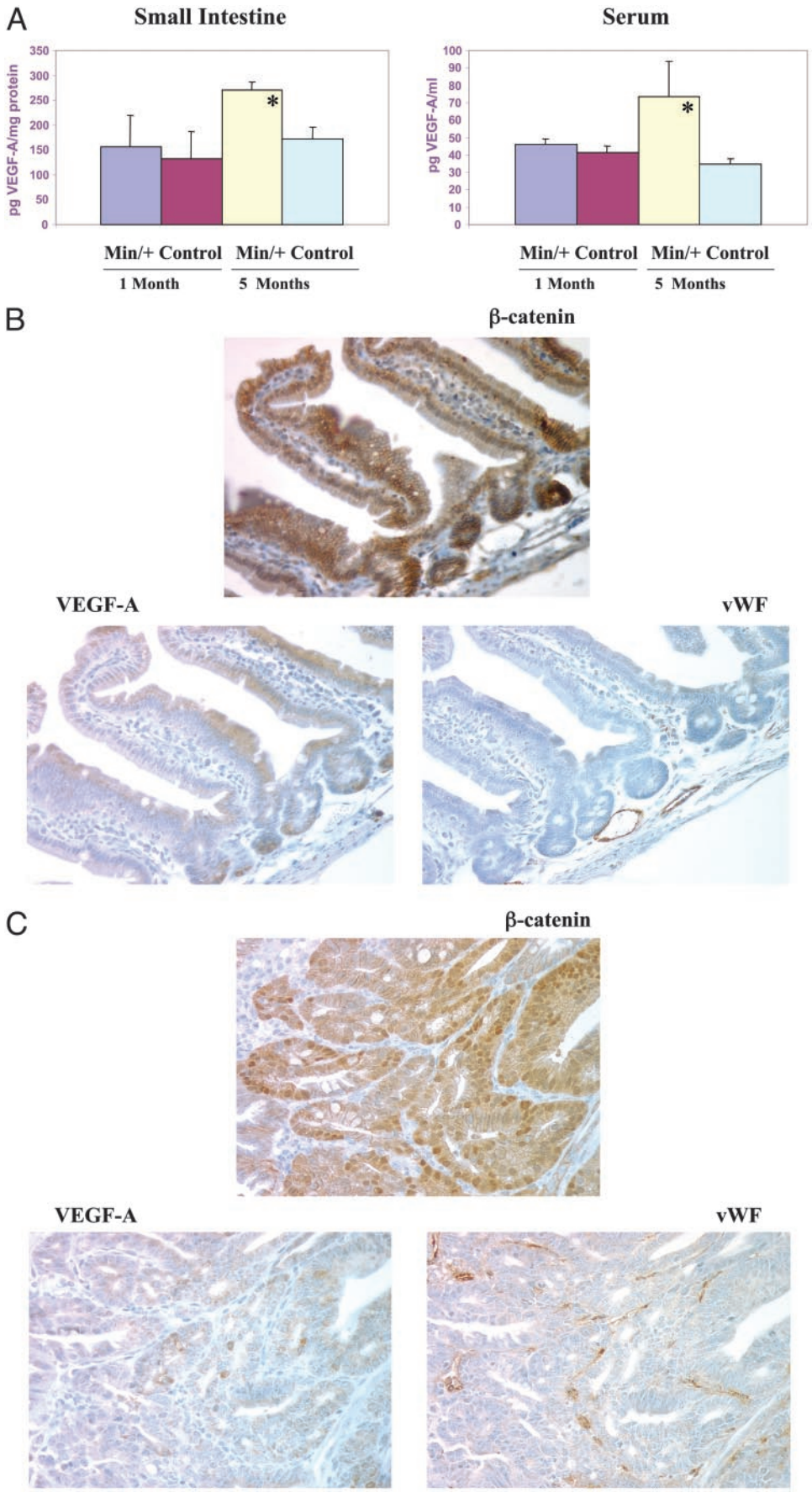


Fig. 4. *VEGF-A* levels are increased in small intestine and serum of Min/+ mice. *A*, VEGF-A protein levels quantified by ELISA analysis in homogenized intestine or serum, respectively, from *Min/+* mice and wild-type littermates. Each *bar* represents samples from between five and eight mice, with each measurement performed in triplicate. This experiment was repeated twice. *Asterisks* indicate that values differed significantly from control by unpaired Student's *t* test ($P < 0.05$). *B* and *C*, tissue distribution and intensity of β-catenin, VEGF-A, and vWF-staining cells in *Min/+* mice. Immunohistochemical analysis at 5 months using antibodies to β-catenin, VEGF-A, and vWF. Intestine from wild-type mice (*B*) compared with intestine/polyps from *Min/+* mice (*C*).

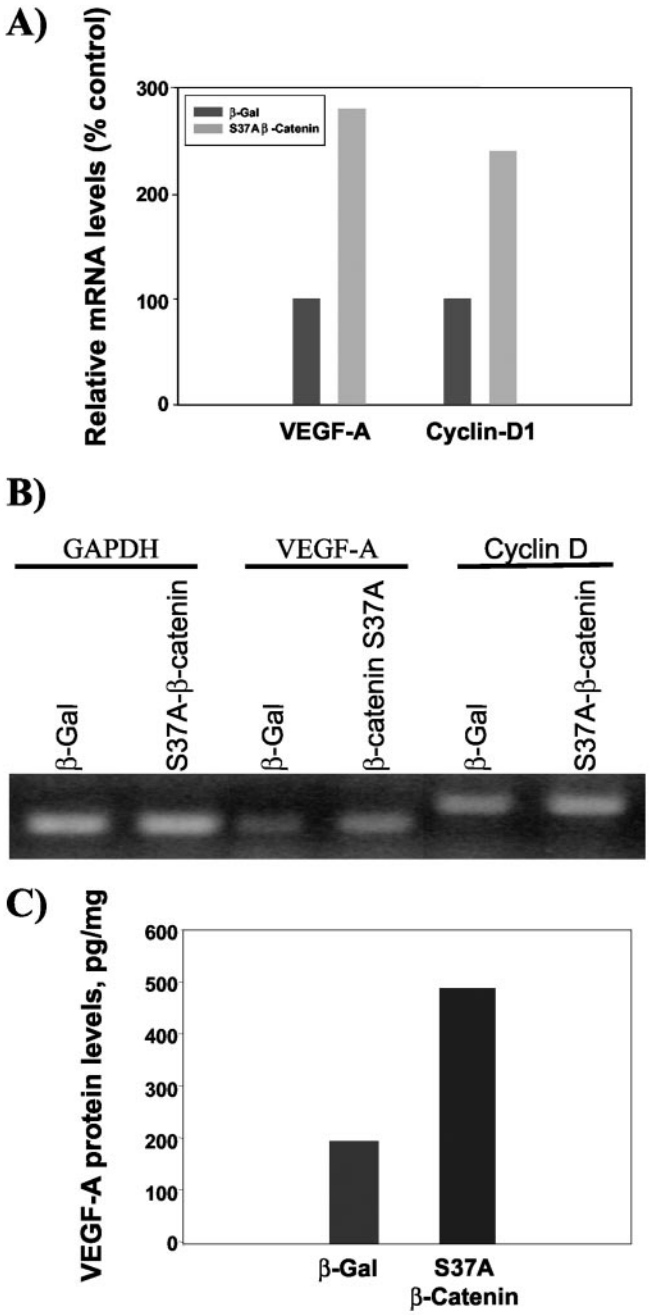


Fig. 5. Transfection of a normal colon mucosal cell line (NCM460) with *S37A β-catenin* mediates an increase in *VEGF-A* mRNA and protein. *A* and *B*, *VEGF-A* and *cyclin D* mRNA (control) levels were determined by q-PCR, normalized to *GAPDH*, and expressed as a change relative to their levels in cells transfected with a *β-galactosidase* plasmid. *B*, representative q-PCR products used for the analysis in *A*. *C*, VEGF-A protein levels from NCM460 cell lysates transfected with *S37A-β-catenin* determined by ELISA.

respectively (60, 61)] and VEGF-A (62) were treated with antisense ODNs to β-catenin (48). β-Catenin antisense ODN transfection of cell lines resulted in reduction of *β-catenin* mRNA levels by >90% (Fig. 6*A*) with a concurrent reduction in β-catenin signaling monitored using a β-catenin reporter assay (data not shown). A significant reduction in β-catenin protein was also seen by Western analysis in the *β-catenin* antisense ODN-treated HCT116 cells, relative to cells transfected with reverse control ODN (Fig. 6*B*). In an earlier report, transfection of cells with the same *β-catenin* antisense ODN used here inhibited expression of axin 2 and human Nkd, whereas the reverse control ODN failed to inhibit their expression (42). *β-Catenin* antisense ODN transfection of HCT116 cells significantly down-regulated *VEGF-A* mRNA (60 ± 6%; $P < 0.05$), as measured by quantitative PCR (Fig. 6*A*). c-Myc, another known β-catenin target gene, was also down-regulated by the *β-catenin* antisense ODN treatment (33). Similar results were obtained in SW620 colon cancer cells (data not shown).

DISCUSSION

In the precancerous stage of colon tumor development, the β-catenin pathway is activated through genetic lesions that increase the amount of transcriptionally available β-catenin (32, 61, 63). Furthermore, angiogenesis is essential for tumors to grow beyond a minimal size, and the major regulators of this process are bFGF and VEGF-A, the latter of which has been implicated in colon cancer. Increasing evidence assigns an important role to Wnt/β-catenin signaling in normal and pathogenic angiogenesis. Thus far, several Wnt ligands, receptors, and inhibitors have been shown to be expressed in endothelial and smooth muscle cells. Increased levels of cytosolic and nuclear β-catenin have been seen in proliferating vessels during embryogenesis and have also been seen in tumor vessels in, for example, human glioblastoma multiforme (43, 64). Interestingly, the accumulation of cytoplasmic and nuclear β-catenin has not been observed in adult vessels, whereas the expression of two secreted Wnt inhibitors, FRP-1 and FRP-3, is inversely correlated with the proliferative state of the vasculature (43).

Given that *APC* is a tumor suppressor in human colon cancer and

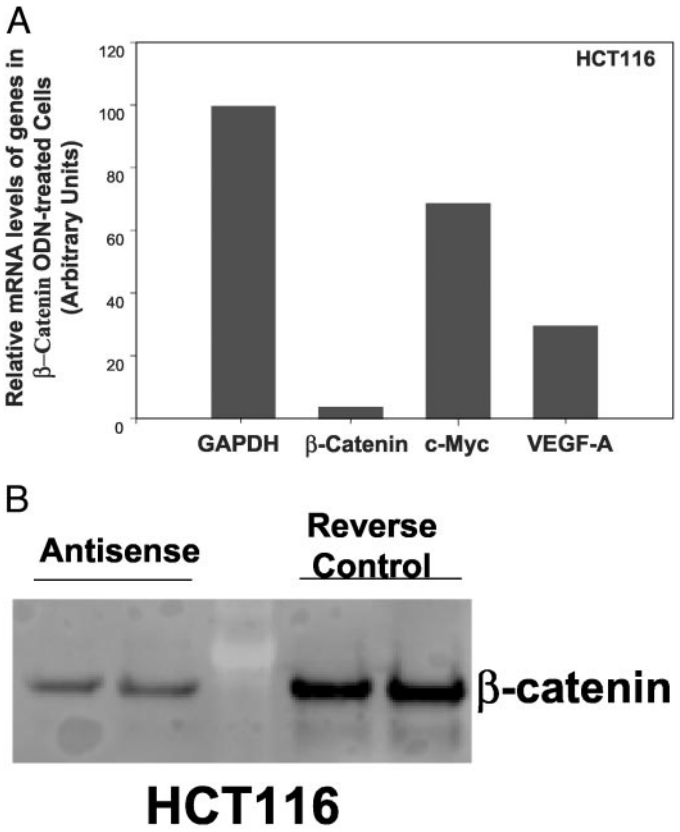


Fig. 6. *A*, *β-catenin* antisense ODN transfection of HCT116 colon cancer cells reduces *VEGF-A* mRNA levels. *VEGF-A* levels were determined 48 h after HCT116 colon cancer cells were transfected with antisense to *β-catenin* ODN or control ODN. Relative *VEGF-A* mRNA ratios were determined after each transfection was normalized to *GAPDH* mRNA. Levels of *c-Myc* mRNA, a known β-catenin target gene, were also reduced in these cells. *B*, lysates from HCT116 cells were analyzed for levels of cytosolic β-catenin 48 h after transfection with β-catenin antisense and control ODNs.

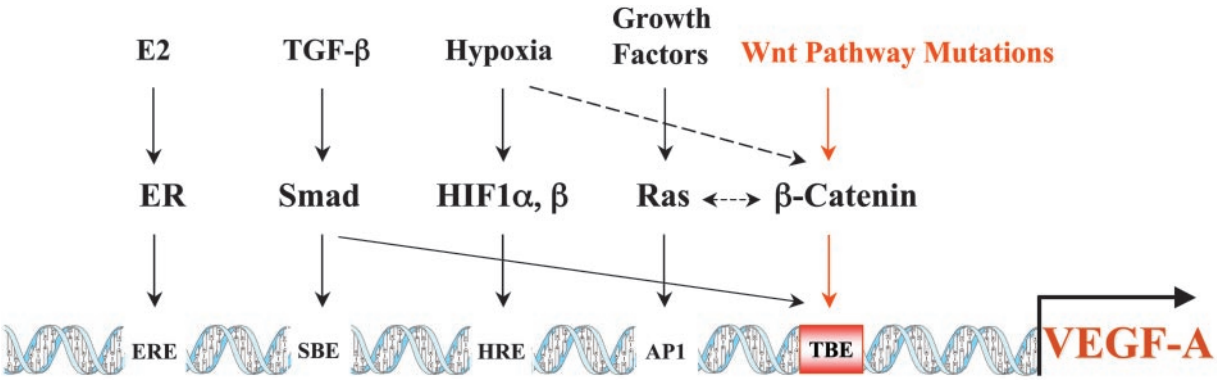


Fig. 7. VEGF-A is a β-catenin target. Diagrammatic representation to show the multiple signaling pathways including β-catenin that regulate VEGF-A.

that its mutation enhances β-catenin-regulated gene expression, we wished to determine whether β-catenin could be involved in the regulation of angiogenesis-related genes. Strikingly, analysis of the promoter for *VEGF-A* revealed the presence of seven potential β-catenin/TCF consensus binding sites, pointing to a direct interaction between two of the recognized players involved in colon carcinogenesis. These observations are consistent with the transcription data reported by Zhang *et al.* (46), who showed that *VEGF-A* promoter activity could be stimulated by oncogenic β-catenin in HeLa cells.

In this report, we extended the studies of Zhang *et al.* (46) that described *VEGF-A* as a β-catenin target gene in cell lines. Our findings indicate that in primary human colon tumors, increased *VEGF-A* expression correlated with mutations in the *APC* tumor suppressor gene that activates β-catenin signaling. Of 17 tumors with a proven *APC* mutation, 11 showed an elevation greater than 2-fold. Laser capture microdissection permitted accurate sampling of malignant and normal control tissue in these tumors. Such a correlation was not observed in laser capture microdissected human liver metastases, in which *VEGF-A* levels were increased regardless of the *APC* status. Perhaps in this latter situation, genetic instability and tumor progression activate other of the multiple pathways that regulate VEGF-A, overriding the contribution made by the β-catenin pathway in primary lesions (see "Introduction" and Fig. 7). Although only a small number of patient tumors were analyzed, laser capture microdissection of malignant and matched autologous normal tissue gave the study an additional level of precision and statistical power. Seventeen of the tumors (85%) tested in this analysis had an *APC* functional deletion, in accord with the published values for colorectal patients in which *APC* is mutated in 85% of sporadic colorectal cancers and is detected as a germ-line mutation in >95% of familial adenomatosis polyposis colorectal patients (55). We did not detect any point mutations in the NH_2-terminal domain of *β-catenin* itself (32). To our knowledge, this is the first report to correlate *VEGF-A* expression and *APC/β-catenin* genotype in the same human colon cancer samples and suggests that β-catenin-regulated VEGF-A production may be pivotal in the early stages of colon cancer. Other pathways that regulate VEGF-A production may have more prominence at later stages of the disease.

The patient data described above were further corroborated by *in vivo* studies in the *Min/+* mouse. The murine homologue of human *APC* is the *Min* gene, and on a sensitive C57BL/6J (B6) background, mice heterozygous for *Min* develop adenomas in the intestinal tract, mimicking the human disease (56). In this animal model, the relationship between the activation of *β-catenin* and *VEGF-A* seen in our patient specimens persisted. Levels of VEGF-A were elevated in both the intestine and serum of *Min/+* mice compared with wild-type littermates as determined by ELISA assay (Fig. 4*A*). It is unclear why the elevation in VEGF-A was apparent at 5 months and not at 1 month, given that the mice harbor the *APC* mutation from birth. Immunohistochemistry of the intestinal polyps from the *Min/+* mice clearly showed β-catenin staining in the nuclei with regions of VEGF-A-positive staining throughout the polyp. This correlated with an expansive vasculature within the polyp that exhibited an altered morphology compared with the vessels seen in intestine from wild-type mice (Fig. 4, *B* and *C*). This is consistent with numerous reports describing tumor vessels as leaky and unstable (65).

In vitro experiments with colon cell lines transfected with a stabilized, cytosolic β-catenin (S37A) resulted in the production of augmented levels of *VEGF-A* mRNA and protein (Fig. 5, *A*−*C*). Similarly, a reduction in these levels was seen when the cell lines were transfected with antisense *β-catenin* ODN (Fig. 6*A*). These results support reporter assays measuring *VEGF-A* promoter activity in response to stabilized cytosolic β-catenin in cancer cell lines (46).

The data described here suggest an intimate link between β-catenin and VEGF-A production in human colon cancer samples and that this early event may initiate angiogenesis. It is relevant to consider the regulation of VEGF-A by β-catenin described here within the context of the temporal progression of colon cancer (31). However, another early event, activation of the cyclooxygenase-2 pathway, also produces an increase in VEGF-A that is β-catenin independent (48, 66). Subsequent to these steps are the activation of Ras and inactivation of p53, events that in combination with hypoxia activate a transcriptional program that includes an increase in VEGF-A (25, 55, 67). Additionally, we have shown that cytoplasmic levels of β-catenin are increased in response to hypoxia.[5] The multitude of pathways that increase VEGF-A levels provide compelling evidence of its importance at every stage during tumor progression.

However, translation of therapeutics targeting the VEGF pathway in the clinic remains a challenge for the future (68, 69). Several possibilities may account for this. One recent report suggests that the genetic make-up of the tumor cells can profoundly influence the response to antiangiogenic therapy. A combination of vinblastine with an antibody directed against VEGFR-2 was less effective in tumor xenografts derived from HCT116 cells lacking *p53* compared with their isogenic counterparts with intact *p53* (70). Furthermore, when the p53 pathway is activated, the oncogenic capabilities of β-catenin are diminished (36, 71). Thus, there is selective pressure for tumors to inactivate p53 to sustain activation of the Wnt/β-catenin pathway. In light of these findings as well as the emerging importance of Wnt/β-catenin signaling in the neovasculature, therapies that focus on disrupting this pathway may be beneficial in the field of oncology.

[5] V. Easwaran and W. J. Fantl, unpublished data.

ACKNOWLEDGMENTS

We greatly appreciate the critical reading of the manuscript by Drs. Maurice Wolin, Steve Harrison, and Sandra Milan. We thank Dr. Christoph Reinhard for the antisense ODNs. We thank Mike Rohan for sequence analysis and Linda M. Saiyad for administrative assistance in preparing the manuscript.

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