c-Kit Is Suppressed in Human Colon Cancer Tissue and Contributes to L1-Mediated Metastasis

Nancy Gavert, Anna Shvab, Michal Sheffer, Amir Ben-Shmuel, Gal Haase, Eszter Bakos, Eytan Domany, and Avri Ben-Ze’ev

Abstract

The transmembrane neural cell adhesion receptor L1 is a Wnt/β-catenin target gene expressed in many tumor types. In human colorectal cancer, L1 localizes preferentially to the invasive front of tumors and when overexpressed in colorectal cancer cells, it facilitates their metastasis to the liver. In this study, we investigated genes that are regulated in human colorectal cancer and by the L1-NF-κB pathway that has been implicated in liver metastasis. c-Kit was the most highly suppressed gene in both colorectal cancer tissue and the L1-NF-κB pathway. c-Kit suppression that resulted from L1-mediated signaling relied upon NF-κB, which directly inhibited the transcription of SPI1, a major activator of the c-Kit gene promoter. Reconstituting c-Kit expression in L1-transfected cells blocked the biological effects conferred by L1 overexpression in driving motility and liver metastasis. We found that c-Kit expression in colorectal cancer cells is associated with a more pronounced epithelial morphology, along with increased expression of E-cadherin and decreased expression of Slug. Although c-Kit overexpression inhibited the motility and metastasis of L1-expressing colorectal cancer cells, it enhanced colorectal cancer cell proliferation and tumorigenesis, arguing that separate pathways mediate tumorigenicity and metastasis by c-Kit. Our findings provide insights into how colorectal cancer metastasizes to the liver, the most common site of dissemination in this cancer. Cancer Res; 73(18); 5754–63. ©2013 AACR.

Introduction

Aberrant activation of Wnt/β-catenin signaling is a hallmark in the majority of human colorectal cancers (1–3). A central question in human colorectal cancer development is the identification and role of downstream β-catenin-T-cell factor (TCF) target genes. We identified members of the neural immunoglobulin-like cell adhesion receptors L1 and Nr-CAM (4, 5) as β-catenin-TCF target genes in colorectal cancer cells and showed that L1 is exclusively expressed in cells at the invasive front of human colorectal cancer tissue (5). Overexpression of L1 in human colorectal cancer cells conferred enhanced motility and metastasis to the liver in a mouse metastasis model (6). We further showed that the mechanisms whereby L1 confers such metastatic capacities involve an association of the L1 juxtamembrane cytoplasmic domain with the cytoskeletal linker protein ezrin and with the inhibitor of κB (IκB) component of the NF-κB signaling pathway (7). This association results in ezrin phosphorylation/activation by Rho-associated protein kinase and an enhanced phosphorylation and proteasomal degradation of IκB leading to the release, nuclear localization, and increased activation of NF-κB-mediated transcription (8). In this study we wished to determine the target genes of the L1-NF-κB pathway that are also regulated in a large set of human colorectal cancer tissue samples compared with normal colon tissue. Surprisingly, we found that the expression of the tyrosine kinase growth factor receptor c-Kit is suppressed in human colorectal cancer tissue when compared with normal colon tissue, as well as in L1-mediated NF-κB signaling that promotes colorectal cancer cell metastasis. We report on the mechanisms whereby the L1-NF-κB pathway inhibits c-Kit expression and the means by which the metastasis suppressive effects are conferred by c-Kit in colorectal cancer cells.

Materials and Methods

Cell culture, proliferation, artificial wound closure, and transfections

HEK 293T, Ls174T, and DLD-1 cells were grown as described (6). Ls174T-L1, Ls174T-p65, and Ls174T-control cells were maintained in medium containing neomycin (800 μg/mL), Ls174T-L1+shp65, Ls174-L1+IκB-SR, and Ls174T-L1+c-Kit cells in medium with both neomycin (800 μg/mL) and puromycin (10 μg/mL). For cell growth assays, 104 cells/well were seeded in 96-well dishes and cell number determined in triplicates for 5 days. An artificial wound was introduced into confluent cell cultures using a micropipette. The medium was replaced with fresh medium containing 0.35 μg/mL mitomycin-C to inhibit cell proliferation. Pictures were taken at 0 and...
24 hours after introducing the wound and percent wound closure was determined. Transient transfection of HEK 293T cells was conducted using the calcium-phosphate method. Ls174T and DLD-1 cells were transfected using Lipofectamine 2000 (Invitrogen). To measure the phosphorylation of AKT, the cells were starved overnight and then medium with 10% FCS was added for serum activation. Inhibition of phosphoinositide 3-kinase (PI3K) was achieved using 50 μmol/L LY294002 (Sigma-Aldrich) added 12 hours after plating cells and further incubation of the cells for 36 hours before harvesting. The cell lines were obtained from American Type Culture Collection and passaged for less than 6 months after harvest.

**Plasmids**

The wt L1, p65, and ixB-SR cDNA were previously described (7). c-Kit cDNA was obtained from Dr. Françoise Moreau-Gachelin (Institute Curie, Paris, France). The c-Kit responsive promoter reporter plasmid was provided by Dr. Menashe Bar-Eli (University of Texas MD Anderson Cancer Center, Houston, TX). SP1 cDNA was provided by Dr. Jonathan M. Horowitz (North Carolina State University, Chapel Hill, NC) and Dr. Grace Gill (Tufts University Medical School, Boston, MA). p65 shRNA was prepared in pSUPER.puro according to the manufacturer’s instructions (pSUPER.puro RNAi System; OligoEngine) using the target sequences shown in Supplementary Table S1.

**Luciferase reporter assays**

HEK 293T cells were transiently cotransfected in triplicate plates with 0.1 μg L1, p65, or pcDNA3 expression vector, together with 0.25 μg/mL β-galactosidase plasmid and 0.25 μg c-Kit promoter reporter plasmids in pGL3, or with 0.25 μg/mL of empty pGL3. Cells were lysed 24 hours after transfection, and luciferase and β-galactosidase levels were determined by the luciferase assay system (Promega). Luciferase activity was normalized to β-galactosidase activity for transfection efficiency.

**RT-PCR**

RNA was isolated using the EZ-RNA kit (Biological Industries). PCR was conducted using the sequences shown in Supplementary Table S1. Relative gene expression was calculated by quantitative real-time PCR (qRT-PCR) with primers designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), E-cadherin, AP-2, and SP1 (see Supplementary Table S1). qRT-PCR was conducted on the ABgene thermocycler with the ABSolute SYBR Green ROX Mix (ABgene). Triplicates of 1 ng cDNA template and 500 nmol/L gene-specific primers were used. The GAPDH gene served to normalize for RNA levels. Primers were examined for efficiency, displaying an amplification slope of −3.33 ± 0.3 and r² > 0.98. qRT-PCR was started by incubating the samples at 95°C for 10 minutes followed by PCR amplification cycles (95°C for 20 seconds and 60°C for 1 minute for 40 cycles). Data analysis was conducted with the ΔΔCt method with the ABgene thermocycler software. For semiquantitative RT-PCR, the products were analyzed by agarose gel electrophoresis.

**Chromatin immunoprecipitation assays**

Rabbit anti-p65 (sc-109; Santa Cruz Biotechnology, Inc.) and SP1 (pep2; sc-59; Santa Cruz Biotechnology, Inc.) were used for the immunoprecipitation and rabbit anti-immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, Inc.) as control antibody. Chromatin immunoprecipitation (ChIP) was carried out as described (8), with the exception that the DNA was purified using the PCR Purification Kit (Promega) and subjected to PCR with the specific primers shown in Supplementary Table S1.

**Immunoblotting and immunofluorescence**

Immunoblotting was carried out as described (7) using the following antibodies: mouse anti-1xB/β/MAD-3 (BD Biosciences at 1:1,000), goat Ab against NF-xB p65 (sc-109; Santa Cruz Biotechnology, Inc. at 1:1,000), rabbit anti-c-Kit (Cell Signaling Technologies, Inc. at 1:1,000), mouse anti-E-cadherin (BD Biosciences at 1:1,000), rabbit anti-L1 (gift from Dr. Vance Lemmon, University of Miami, Miami, FL at 1:5,000), rabbit anti-total AKT (Sigma-Aldrich at 1:10,000), mouse anti- phospho-AKT (Ser473; Cell Signaling Technologies, Inc. at 1:5,000), mouse anti-SP1 (pep2; Santa Cruz Biotechnology, Inc. at 1:10,000), rabbit anti-histone H2B (Merck-Millipore at 1:5,000), and mouse anti-α-tubulin (Sigma-Aldrich at 1:200,000). Western blots were developed using the ECL method (Amersham Biosciences). For immunofluorescence, cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% PFA. Immunostaining for E-cadherin was conducted with cells fixed in methanol for 5 minutes at 4°C. The secondary antibodies were Alexa-488-conjugated goat antimouse, anti-rabbit IgG (Invitrogen), and Cy3-labeled goat antimouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Images were acquired by using the Zeiss LSM710 confocal microscope with objective 60×/1.4 NA.

**DNA microarrays**

Ls174T clones expressing L1 were compared with Ls174T cells expressing L1+ixB-SR, p65, or the empty vector. Two individually isolated clones were used in each group. Microarray analysis with RNA extracted from these cells was conducted at the Weizmann Institute Microarray Facility on Affymetrix L0st GeneChips. The chips were processed and analyzed as follows: the data were preprocessed using robust multiairay average (RMA) followed by cyclic Lowess correction algorithm (9). Intensity-dependent variance was estimated for each probe set, using the distribution of the differences between repeats of probe sets having similar mean intensity (10). To look for differentially expressed probe sets between the different conditions, 3 comparisons were made: L1 versus control (denoted C1), p65 versus control (C2), and L1+ixB-SR versus L1 (C3). Each comparison was made between 2 corresponding pairs of replicates using a filtering step that included probe sets that were above threshold (t = 6) in at least one of the conditions of each comparison. Probe sets with unknown gene symbol were removed from the analysis. Modified Fisher inverse χ² approach (11) was used to calculate a meta P-value from the comparisons described earlier. For each

www.aacrjournals.org Cancer Res; 73(18) September 15, 2013

5755
Tumor growth and metastasis assays

Tumor growth was induced by injecting s.c. 2.5 × 10^6 cells in 200 μL PBS into the flanks of nude mice (5 mice per group). Control cells were injected into the opposite flank of the same mice and tumors were removed and compared after 2 to 4 weeks. For metastasis assays, groups of 10 to 15 mice were injected with 1 × 10^6 cells in 20 μL PBS into the distal tip of the spleen. After 6 to 7 weeks the spleens and livers were removed and assigned a new uniform distribution based on their rank and assigned a new uniform distribution based on their rank.

Statistics

Statistical significance was determined by the Fisher exact test for mouse metastasis experiments. Tumor mass was compared and significance determined by ANOVA. The significance of qRT-PCR comparisons for RNA levels was determined by ANOVA. In wound closure and luciferase reporter assay studies the significance was determined by ANOVA. A P value of < 0.05 was considered significant. Standard deviation for phosphorylated AKT as a percentage of total AKT was calculated from the results of 3 experiments. The rate of increase in phosphorylated AKT was calculated after assigning a linear function to each curve: L1+c-Kit: \( y = 15.19x + 49.213; R^2 = 0.99 \) and L1: \( y = 8.81x + 31.748; R^2 = 0.98 \).

Results

L1 suppresses c-Kit expression in colorectal cancer cells by activating NF-κB signaling

In recent studies, we showed that the neural transmembrane adhesion receptor L1 promotes the metastasis of human colorectal cancer cells by activating NF-κB signaling (7). We wished to identify genes that are involved in L1-NF-κB-mediated promotion of colorectal cancer metastasis in human colorectal cancer tissue. For this, gene expression profiles of human colorectal cancer cells overexpressing L1 were compared with those of cells overexpressing L1 together with the IκB-super repressor (IκB-SR) inhibiting NF-κB activation (Fig. 1A). To identify only those genes that are L1 and NF-κB dependent, we further compared this gene expression pattern with that of cells overexpressing the p65 NF-κB subunit (Fig. 1A, relevant genes). We detected both genes that were upregulated and genes that were downregulated in these comparisons (Supplementary Tables S2 and S3). To select only those genes that have in vivo biological significance, we compared the gene expression patterns of Supplementary Tables S2 and S3 with genes that were regulated in 212 human colorectal cancer tissue samples and compared metastases with 52 normal colon tissue samples. About 20% of the genes that were downregulated by L1 and NF-κB were also expressed at lower levels in human tumors as compared with normal colon tissue (Fig. 1C and Supplementary Table S4). Surprisingly, among these genes, c-Kit was on top of the list of genes whose expression was most robustly reduced by both L1, NF-κB and in human colorectal cancer tissue samples (Fig. 1C). c-Kit is most commonly associated with oncogenesis (13), but according to the data in Fig. 1C c-Kit seemed to be among the genes that are suppressed during tumorigenesis. We were therefore interested in investigating how, in L1-NF-κB colorectal cancer cell signaling, c-Kit may act to suppress tumor progression.

We used Ls174T human colon cancer cell lines in which either L1, NF-κB (p65), or a combination of L1 and IκB-SR were overexpressed (7) to examine the levels of c-Kit. RT-PCR analysis revealed that Ls174T cells overexpressing L1 (Fig. 2A, lane 2) displayed a marked reduction in c-Kit RNA (Fig. 2A, lane 2 compare to lane 1) and protein levels as compared with control (Fig. 2B, lanes 3 and 4 compare to lanes 1 and 2). When NF-κB signaling was inhibited in L1-transfected cells by the stable expression of IκB-SR (Fig. 2A, lanes 3 and 4), or with shRNA to the p65 NF-κB subunit (Fig. 2A, lanes 5 and 6), c-Kit RNA levels were partially restored in 2 independently isolated cell clones in each case (Fig. 2A, lanes 3–6). Furthermore, 2 clones of colorectal cancer cells overexpressing the p65 subunit of NF-κB (but not L1) also downregulated c-Kit RNA (Fig. 2A, lanes 7 and 8 compare to lane 1) and protein levels (Fig. 2B, lanes 5 and 6 compare to lanes 1 and 2), similar to L1 (Fig. 2A, lanes 2: IκB-SR, or a combination of L1 and IκB-SR). Together, these results indicate that L1 and the p65 subunit of NF-κB inhibited the activation of the c-Kit gene promoter reporter plasmid (Fig. 2D). Moreover, the inhibition of NF-κB activity, by IκB-SR in L1-expressing cells, restored the activity of the c-Kit promoter (Fig. 2D, compare pcDNA3 to L1+IκB-SR). Together, these results indicate that L1 reduces the transcription of c-Kit through the activation of NF-κB signaling.

L1 and NF-κB are suppressing c-Kit transcription via Sp1 regulation

We next wished to determine the mechanism whereby L1, via NF-κB activation, downregulates c-Kit transcription. Because previous reports indicated that c-Kit gene expression is regulated by the AP-2 transcription factor (14), we determined the levels of AP-2 in Ls174T cells stably expressing L1, the p65 NF-κB subunit, or the empty vector, but did not find...
significant differences in AP-2 RNA levels in such cells (Supplementary Fig. S1). Next, we identified 3 putative NF-κB binding sites within the promoter region of c-Kit (Supplementary Fig. 2A) and conducted ChIP analyses to examine whether NF-κB directly binds to these sequences to inhibit c-Kit transcription. However, we did not detect a binding of the p65 NF-κB subunit to these sequences in the c-Kit promoter (Supplementary Fig. S2B). Another known transcriptional regulator of c-Kit expression is the transcription factor SP1 (15). Quantitative RT-PCR analysis detected a significant decrease in SP1 RNA levels in both L1 and even more in p65 overexpressing colorectal cancer cell clones compared with control (Fig. 3A), in accordance with the reduction in c-Kit protein level by L1 and p65 (Fig. 2B). A marked decrease in SP1 levels was also detected in p65 overexpressing cell clones (Fig. 3B, lanes 7 and 8). Moreover, we found that L1-expressing cells displayed a dramatic reduction in the activity of the c-Kit gene promoter as compared with control cells and re-introduction of SP1 into these cells (with or without L1) activated the c-Kit promoter (Fig. 3C). By ChIP experiments we also showed that SP1 binds to the c-Kit promoter in Ls174T colorectal cancer cells (Fig. 3D). Because a previous study showed that NF-κB can reduce SP1 transcription directly by its binding to SP1 promoter sequences (16), we conducted ChIP experiments to examine whether in Ls174T colorectal cancer cells NF-κB binds to the SP1 promoter and found that this is indeed the case (Fig. 3E). We therefore conclude that changes in SP1 levels are involved in reducing c-Kit transcription, via an NF-κB-mediated inhibition of the SP1 promoter, suggesting that by this mechanism NF-κB downregulates c-Kit transcription in L1-expressing cells (Fig. 3F).

c-Kit suppresses L1-mediated motility and metastasis in colorectal cancer cells

Because c-Kit was among the genes whose expression is suppressed by L1 during human colorectal cancer progression
(Fig. 1C), we wished to determine whether the reduction in c-Kit contributes to L1-mediated promotion of metastasis (6, 7). We reintroduced c-Kit into L1 overexpressing cells by stable transfection (Fig. 4A) and found that cells expressing both L1 and c-Kit are less motile than cells expressing L1 alone (Fig. 4B). In fact, these cells were only as motile as control cells lacking L1 (Fig. 4C). Most importantly, the metastasis of L1 transfected cells in which c-Kit expression was reconstituted was dramatically reduced (Fig. 4D and E). Fewer than 10% of mice developed liver metastasis after splenic injection compared with almost 90% in L1-expressing colorectal cancer cells (Fig. 4E). These cells continued to express L1 in vivo as showed by analysis of the splenic primary tumor and, in the case of isolated liver metastases, also in the liver metastatic tissue (Fig. 4F). We conclude that without the suppression of c- Kit, L1 expression in colon cancer cells is insufficient to promote metastasis, and the loss of c-Kit is an essential step in the mechanism of L1-mediated metastasis in colorectal cancer.

Interestingly, when we analyzed the proliferative and tumorigenic abilities of the L1+c-Kit expressing colorectal cancer cells, we found that c-Kit expression enhanced their proliferation both in medium containing 10% of serum and in 0.5% of serum (Supplementary Fig. S3A and S3B). Moreover, upon subcutaneous injection into nude mice, the L1+c-Kit cells formed larger tumors than cells only expressing L1 (Supplementary Fig. S3C–S3E), indicating that they are more tumorigenic. We conclude that overexpression of the oncogene c-Kit, while promoting the tumorigenic capacity of these cells, inhibits their metastatic spread.

c-Kit promotes an epithelial phenotype by suppressing Slug and increasing E-cadherin via AKT activation

To begin to understand the changes that colorectal cancer cells undergo as a result of c-Kit expression, we examined the colorectal cancer cell lines described in Fig. 4A for morphological and molecular characteristics. L1-expressing colorectal cancer cells are characterized by the spontaneous formation of three-dimensional aggregates in culture, even when only moderately dense (Fig. 5A; ref. 5). In sharp contrast, these L1-transfected cells, when reconstituted to also express c-Kit, formed flat colonies of well-spread cells with cell–cell contacts and clear margins resembling an epithelial morphology even at moderate density (Fig. 5B). This phenotype was associated with an increase in E-cadherin RNA and protein levels in L1+c-Kit expressing cells (Fig. 5C–E). The change in neoplastic properties from metastatic to nonmetastatic, as

Figure 2. L1 suppresses c-Kit expression in colorectal cancer cells by activating NF-κB signaling. A, RNA was extracted from individual clones isolated from stably transfected LS174T cells expressing L1, control plasmid (pcDNA3), L1 together with hκB-SR or shRNA against p65, and p65 alone. Semiquantitative RT-PCR was conducted using primers for L1, c-Kit, and GAPDH for loading control. B, Western blot analysis for many of the cell lines shown in A. C, the DLD-1 colon cancer cell line was stably transfected with L1, 3 individual clones were isolated, and the levels of L1 and c-Kit were determined by Western blot analysis. D, the c-Kit promoter reporter plasmid was transfected together with pSV β-galactosidase control vector (for transfection efficiency normalization) into Ls174T colorectal cancer cells stably transfected with L1 or the p65 subunit of NF-κB (p65) or L1 together with the κB super repressor (L1+hκB-SR) and a control clone (pcDNA3). Fold c-Kit promoter activation was determined after dividing luciferase activity by the values obtained with the empty reporter plasmid.
well as the morphological reversion from highly transformed three-dimensional aggregates to flat epithelial colonies is reminiscent of mesenchymal-to-epithelial transformation (MET). However, further analysis of mesenchymal and epithelial markers did not reveal differences in the expression of vimentin or cytokeratins 8 and 18, regardless of whether c-Kit was expressed in these cells (Fig. 5D). In addition, the analysis of several colonic epithelial cell markers (17) did not show

**Figure 3.** L1 and NF-κB suppress c-Kit expression via SP1 regulation. A, RNA levels of SP1 were determined using qRT-PCR in Ls174T cells stably transfected with either empty vector (pcDNA3) or L1 or the p65 subunit of NF-κB (2 clones, p65 C1 and p65 C2); \( P = 6.61 \times 10^{-5} \). B, Western blot analysis for Sp1, L1, p65, histone H2B, and tubulin from equivalent cell volumes of nuclear and cytoplasmic extracts from the colorectal cancer cell clones described in A. Quantitation of SP1 and p65 in the nuclear fraction was determined by densitometry. C, the c-Kit reporter plasmid was transfected together with pSV β-galactosidase control vector (for transfection efficiency normalization) into Ls174T cells stably expressing L1 or a control clone (pcDNA3). Fold c-Kit promoter activation was determined after dividing luciferase activity by the values obtained with an empty reporter plasmid. For L1, \( P = 0.0002 \); for pcDNA3, \( P = 0.0047 \). D, CHIP-based PCR analysis using nuclear lysates from Ls174T cells. A primer sequence that amplifies the SP1 binding site (scheme) or a control (NS) irrelevant sequence was used. Rabbit anti-SP1 and nonimmune IgG were used. E, CHIP-based PCR analysis using nuclear lysates from Ls174T cells stably transfected with L1. A primer set that amplifies the NF-κB-binding sequence (scheme) or a control (NS) irrelevant sequence was used. Binding of p65 to the NF-κB-binding sequence in the IκB gene was used as positive control. DNA fragments conjugated with nuclear proteins were immunoprecipitated with rabbit anti-NF-κB or nonimmune goat IgG and amplified by PCR. F, scheme depicting L1 that promotes the phosphorylation of IκB and the release of NF-κB to act in the cell nucleus (see ref. 7) to inhibit the transcription of SP1 that results in the reduction of c-Kit transcription.
changes in their expression in cells overexpressing c-Kit and/or L1 (Supplementary Fig. S4). To understand how E-cadherin expression is regulated in these cells, we analyzed the levels of some classic inhibitors of E-cadherin transcription, Snail, and Slug. Although Snail RNA levels remained unchanged, Slug RNA was reduced in c-Kit-expressing cells as compared with cells only expressing L1 (Fig. 5F), providing a likely mechanism whereby c-Kit can enhance E-cadherin protein levels.

Next, we wished to explore the mechanism/s whereby c-Kit enhances E-cadherin expression. Because c-Kit exerts many of its effects by activating the PI3K/AKT signaling pathway (18–20), we determined whether the activation of AKT increases in L1–c-Kit overexpressing cells compared with L1 cells. We found that after an overnight serum starvation, L1–c-Kit cells expressed more phosphorylated AKT (p-AKT) than L1 cells (Fig. 6A, lane 5 compare to lane 1). Furthermore, upon serum activation, L1–c-Kit cells reacted with increased AKT phosphorylation as compared with L1-expressing cells (Fig. 6A and B). The LY294002 inhibitor reduced not only the levels of p-AKT, but also of E-cadherin protein in both L1 and L1–c-Kit transfected cells (Fig. 6C, lane 4 compare to lane 3), indicating that E-cadherin expression in these cells is modulated by the PI3K/AKT pathway.

Discussion

The major finding in this study is that a decrease in the expression of c-Kit is associated with colorectal cancer development in a large set of human colorectal cancer tissue samples compared with normal colon tissue, and that the suppression of c-Kit is necessary for L1-mediated colorectal cancer metastasis to the liver in a mouse model of metastasis. The mechanisms involved in the suppression of c-Kit include the L1-mediated activation of NF-kB signaling (7, 8) and the suppression of SP1 transcription by NF-kB, by the direct binding of NF-kB to SP1 promoter sequences. Because SP1 is a major activator of c-Kit transcription (21), the inhibition of SP1 expression resulted in reduced c-Kit transcription and consequently, in reduced c-Kit protein levels in L1-expressing cells.
cells (via NF-κB signaling). This also occurred in the absence of L1 when NF-κB signaling was enhanced. Although NF-κB is considered mainly as an activator of transcription in cancer and inflammation (22, 23), previous studies have already reported on the inhibition of SPI transcription by NF-κB, including in colon cancer cell lines (16). The reconstitution of c-Kit expression by its transfection into L1-expressing colorectal cancer cells inhibited all the effects conferred by

![Figure 5](image-url)  
*Figure 5.* c-Kit promotes an epithelial morphology and increases E-cadherin expression. Phase contrast images of live Ls174T cells stably expressing L1 (A) or L1 + c-Kit (B). Scale bar, 75 μm. C, the cells from A and B were doubly immunostained with anti-E-cadherin and anti-L1 antibodies. Scale bar, 20 μm. D, Western blot analysis of Ls174T cells stably expressing L1 (2 individual clones) or L1 + c-Kit (2 individual clones) for L1, c-Kit, E-cadherin, cytokeratins 8 and 18 (CK), vimentin, and tubulin. E, mRNA levels of E-cadherin were measured using qRT-PCR in Ls174T cells stably transfected as detailed in A–C (*P* = 0.00234). F, RNA was extracted from individual cell clones as described in D and semiquantitative RT-PCR was conducted using primers for Slug, Snail, and GAPDH followed by agarose gel electrophoresis.

![Figure 6](image-url)  
*Figure 6.* Increased AKT phosphorylation in c-Kit-expressing cells increases E-cadherin levels. A, Ls174T cells stably transfected with L1 or L1 + c-Kit were starved overnight and then serum stimulated for various times. Western blot analysis was conducted for the levels of L1, c-Kit, p-AKT, and total AKT. B, graphic representation of 3 separate experiments as described in A. Densitometric measurements of films for determining the amount of p-AKT as compared with total AKT in the same sample for each time point were conducted as described in Materials and Methods. C, the cells described in A were treated with the LY 294002 PI3K inhibitor or with DMSO and collected after 48 hours. The levels of L1, c-Kit, E-cadherin, p-AKT, and total AKT were determined by Western blot analysis.

L1 in colorectal cancer cells, and included a reduction in cell motility and, most importantly, blocked the metastatic capacity of such cells. In addition, c-Kit expression also altered
the cell phenotype to a more epithelial organization into colonies with clear borders, increased E-cadherin at cell–cell junctions, and a reduction in Slug. Although this phenotype is consistent with an MET, many other markers of EMT do not show a change in their levels and this was also the case for differentiation markers of colonic epithelial cells.

Our findings seem to be in contrast to numerous reports on the potential oncogenic role of the tyrosine kinase c-Kit receptor (13, 24, 25). The fact that loss of c-Kit can promote a more aggressive cancer phenotype was already reported in a number of cancers, including melanoma (14), where c-Kit expression in a highly metastatic cell line resulted in suppression of its metastatic capacity (14). In addition, studies in breast (25–27) and some other types of cancer (28) also reported on a decrease in c-Kit at more advanced stages of the disease.

Several immunohistochemical studies in colorectal cancer also detected a reduction in c-Kit that is associated with later stages of tumor development (29, 30). However, studies on c-Kit expression in normal human colon and colon cancer tissue showed contradicting results: originally, c-Kit was detected throughout human adult and fetal tissues and in the colonic mucosa, submucosa, and intestinal smooth muscle layers (31). More recently, c-Kit was detected in stem cells of colonic crypts (32), whereas in colon cancer, c-Kit was found in 1.6% to as many as 25% of human colon cancer specimens (33, 34). Although c-Kit immunostaining is uncommon in primary human colon tumor specimens, c-Kit is expressed at lower levels in more advanced cancer as compared with a higher expression in adenomas. This implies a loss of c-Kit in later stages of colorectal cancer progression in agreement with our study.

Reports on various colorectal cancer cell lines showed that c-Kit is expressed in some cell lines including HT29 (35) and DLD-1 (36), but not in many others (37), in agreement with our studies showing c-Kit in DLD-1 and LS174T cells, but not in others (such as SW480 and HCT116, data not shown). c-Kit inhibitors were proposed as therapeutic agents in the treatment of colorectal cancer, and ST1571 (imatinib mesylate) reduced HT29 colorectal cancer cell proliferation and tumor growth in mice (38). These findings are in line with our studies showing that c-Kit promotes colorectal cancer cell proliferation both in vitro and in vivo. An additional study (39) with this inhibitor showed a reduction in the proliferation and transwell migration of LS180 cells in contrast to our findings on the effect of c-Kit on cell motility. Because EGF was used to stimulate the migration of LS180 cells, this could conceivably activate signaling pathways that are inhibited by imatinib (such as the Brca/Ab1 pathway), independently of c-Kit activity.

Although the metastasis to the liver mediated by L1 in colorectal cancer cells was suppressed by c-Kit, the proliferation of such cells in culture and their tumorigenic capacity upon subcutaneous injection into mice was enhanced, as expected from a strong proto-oncogene such as c-Kit. Interestingly, a recent study also reported on such dichotomic effect when the oncogene c-Myc was transfected into breast cancer cell lines resulting in enhanced tumorigenesis, but a decrease in their metastatic capacity (40). These studies support the notion that the promotion of earlier stages in tumor development, including cell proliferation and tumorigenesis, do not necessarily also promote metastasis and may in some cases interfere with later stages of the disease, including metastasis. Recent studies have also shown such dichotomic behavior for EMT that was amply shown to be associated with tumor dissemination (41). These studies (42, 43) showed, using transgenic mice expressing an inducible form of Twist 1 (another EMT transcriptional regulator), that although EMT is important during the early stages of tumor development (tumor cell dissemination), a switch to MET (i.e., a reversion of EMT) is required for efficient colonization and macrometastasis (43) and when EMT continued, no effective metastasis occurred.

Our study emphasizes the differential role of proto-oncogenes such as c-Kit in earlier stages of tumor development and their inhibitory effect (most probably by promoting a less motile epithelial phenotype) at later stages of colorectal cancer metastasis, as showed here for the L1-NF-kB signaling pathway.

Although some studies have shown that pharmacologic inhibition of c-Kit reduces both cell and tumor proliferation (37, 38) and have recommended their use in the treatment of colorectal cancer, we believe that a more conservative approach is required. Unlike intestinal stromal tumors where c-Kit overexpression is well documented and primary tumors shrink upon treatment to operable size, neoadjuvant therapy before surgery is much less common. Because ours is the first study showing a relationship between c-Kit suppression and metastasis development in colorectal cancer, further studies on the mechanisms governing c-Kit suppression and the promotion of metastasis are needed before recommending changes in the use of c-Kit inhibitors in tumor therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Ben-Ze’ev, N. Gavert, E. Bakos
Development of methodology: A. Ben-Ze’ev, N. Gavert, E. Domany
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Ben-Ze’ev, N. Gavert
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): A. Ben-Ze’ev, N. Gavert, A. Shvab, M. Sheffer, A. Ben-Shmuel, G. Haase, E. Domany
Writing, review, and/or revision of the manuscript: A. Ben-Ze’ev, N. Gavert, A. Shvab, A. Ben-Shmuel, G. Haase
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Ben-Ze’ev, N. Gavert
Study supervision: A. Ben-Ze’ev, N. Gavert, A. Shvab

Grant Support

This study was supported by grants from the Israel Cancer Research Fund (ICRF) and from the Israel Science Foundation (ISF) and by the Leir Charitable Foundation (E. Domany and M. Sheffer).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 27, 2013; revised July 3, 2013; accepted July 17, 2013; published OnlineFirst September 5, 2013.
References

c-Kit Is Suppressed in Human Colon Cancer Tissue and Contributes to L1-Mediated Metastasis

Nancy Gavert, Anna Shvab, Michal Sheffer, et al.


**Updated version**
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-0576

**Cited articles**
This article cites 42 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/18/5754.full#ref-list-1

**Citing articles**
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/18/5754.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/73/18/5754.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.