L1-Mediated Colon Cancer Cell Metastasis Does Not Require Changes in EMT and Cancer Stem Cell Markers

Nancy Gavert1, Alessia Vivanti1, John Hazin1, Thomas Brabletz2, and Avri Ben-Zeev1

Abstract

Aberrant activation of Wnt/β-catenin signaling is common in most sporadic and inherited colorectal cancer (CRC) cells leading to elevated β-catenin/TCF transactivation. We previously identified the neural cell adhesion molecule L1 as a target gene of β-catenin/TCF in CRC cells. Forced expression of L1 confers increased cell motility, invasion, and tumorigenesis, and the induction of human CRC cell metastasis to the liver. In human CRC tissue, L1 is exclusively localized at the invasive front of such tumors in a subpopulation of cells displaying nuclear β-catenin. We determined whether L1 expression confers metastatic capacities by inducing an epithelial to mesenchymal transition (EMT) and whether L1 cosegregates with cancer stem cell (CSC) markers. We found that changes in L1 levels do not affect the organization or expression of E-cadherin in cell lines, or in invading CRC tissue cells, and no changes in other epithelial or mesenchymal markers were detected after L1 transfection. The introduction of major EMT regulators (Slug and Twist) into CRC cell lines reduced the levels of E-cadherin and induced fibronectin and vimentin, but unlike L1, Slug and Twist expression was insufficient for conferring metastasis. In CRC cells L1 did not specifically cosegregate with CSC markers including CD133, CD44, and EpCAM. L1-mediated metastasis required NF-κB signaling in cells harboring either high or low levels of endogenous E-cadherin. The results suggest that L1-mediated metastasis of CRC cells does not require changes in EMT and CSC markers and operates by activating NF-κB signaling, Mol Cancer Res; 9(1); 14–24. ©2010 AACR.

Introduction

Aberrant activation of Wnt/β-catenin target genes plays a key role both during early and later phases of human colorectal cancer (CRC) development (1, 2). Members of the immunoglobulin-like cell adhesion receptors (NrCAM and L1), mostly known for their presence in nerve cells (3, 4) but also in many cancer cell types (5), were recently identified as target genes of β-catenin/TCF signaling in CRC cells (6, 7). L1 was detected in a small subpopulation of cells at the invasive front of CRC tissue showing nuclear β-catenin (7). Moreover, the expression of L1 in human CRC cells lacking L1 confers enhanced motility and metastasis to the liver (8). A large number of genes induced by L1 in such cultured cells are also shared by human CRC tissue samples, as indicated by gene expression microarray profiles (8). Analysis of the signaling pathways that are involved in L1-mediated CRC cell metastasis indicated that the NF-κB pathway and ezrin are both required for conferring metastatic capacities in these cells (9). A more motile phenotype consistent with an epithelial to mesenchymal transition (EMT)-like process was suggested to be induced by L1 in MCF7 breast cancer cells transfected with L1 (10). EMT was suggested to act in breast cancer progression, by producing cells with CRC stem cell (CSC) characteristics (11, 12). In a recent study where CSCs were isolated from human glioma tissue, based on CD133 expression, L1 was identified as a cosegregating molecule that was highly enriched in CD133+ cells (13). Moreover, suppression of L1 levels in such cells inhibited glioma stem cell proliferation and blocked their metastatic capacities suggesting that L1 can represent a CSC therapeutic target (13). The presence of CD133 on the membrane of CRC cells also enabled the isolation of human CSCs (14, 15). We investigated the possibility that L1 confers its metastatic capacity in CRC cells by inducing EMT-like properties and by possibly cosegregating with cells expressing CSC markers. When L1 levels were increased or suppressed in CRC cells in which the levels of endogenous L1 and/or E-cadherin were dramatically different, no detectable changes in E-cadherin, or in other EMT markers were detected. Moreover, when the expression of Slug and Twist (major EMT regulators; ref. 16) were elevated in CRC cells, this was insufficient to induce metastatic capacity in these cells. On isolation of CD133+ and CD133-CRC cell populations

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from some CRC cell lines, no cosegregation between L1 and CD133 and other CSC markers (CD44 and EpCAM) was observed. In invasive human CRC tissue, both L1 and E-cadherin were expressed in the same cells. Taken together, our results suggest that the L1-mediated induction of CRC cell metastasis does not require an EMT and/or changes in CSC markers.

Materials and Methods

Cell lines, cell culture, and transfections

293T, MDCK, NIH3T3, HCT116, SW480, and SW620 cells were maintained in DME with 10% bovine calf serum. Ls174T cells were grown in RPMI with 10% fetal calf serum. Transient transfection of 293T cells was performed using calcium phosphate. Ls174T, SW480, SW620, and HCT116 cells were transfected using LipofectamineTM 2000 (Invitrogen). Ls174T or HCT116 cells stably expressing human L1 were established by selection to neomycin resistance (500 μg/mL), as described (7). Ls174T cells expressing L1 were cotransfected with shRNA targeted against p65 followed by selection with puromycin (10 μg/mL). In SW620 cells, shRNA targeted against L1 was transfected followed by selection to neomycin resistance (500 μg/mL). Cells inducibly expressing Twist or L1 were obtained by transfecting Ls174T4R cells (expressing the tetracycline receptor) with pcDNA4/TO Twist (or pcDNA4/TO L1), or with the pcDNA4/TO empty vector followed by selection with 800 μg/mL zeocin. HCT116 cells stably expressing inducible Slug were obtained in a similar manner. In transactivation assays, a β-galactosidase plasmid was cotransfected with the reporter plasmid containing 3 copies of an NF-κB-responsive promoter sequence linked to luciferase (3xκB.luc). Cells were plated in triplicates, lysed after 48 hours, and luciferase and β-galactosidase levels were determined by enzyme assay kits from Promega. For transfection control, luciferase activity was normalized to β-galactosidase activity. Fold induction of the NF-κB reporter plasmid was calculated using an empty reporter plasmid (pGL3).

Plasmids

The L1 and Twist cDNA constructs were described (7, 9). Drs. D. Wallach and A. Kovalenko (Weizmann Institute of Science, Rehovot, Israel) provided the NF-κB-responsive reporter plasmid (3xκB.luc). shRNA against p65 was prepared in pSuper-puro according to the manufacturer’s protocol. Primers for L1 were ACGGGCAACATGGCTAAGATCAATGGCTA-5′, CTCAAGATCTGCCGAGTGA-3′, and the scrambled sequence 5′-GGATGGTGTC-3′. Ls174T cells were transfected with the pcDNA4/TO L1, or with the pcDNA4/TO empty vector followed by selection with 800 μg/mL zeocin. HCT116 cells stably expressing inducible Slug were obtained in a similar manner. In transactivation assays, a β-galactosidase plasmid was cotransfected with the reporter plasmid containing 3 copies of an NF-κB-responsive promoter sequence linked to luciferase (3xκB.luc). Cells were plated in triplicates, lysed after 48 hours, and luciferase and β-galactosidase levels were determined by enzyme assay kits from Promega. For transfection control, luciferase activity was normalized to β-galactosidase activity. Fold induction of the NF-κB reporter plasmid was calculated using an empty reporter plasmid (pGL3).

Immunofluorescence

Cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% PFA in PBS. The coverslips were incubated with pAb and mAb against L1 (provided by V. Lemmon, University of Miami, Miami, FL) and against E-cadherin, mAb HECD-1 (Invitrogen). The secondary antibodies were Alexa 488-conjugated goat anti-mouse or anti-rabbit IgG (Invitrogen), and Cy3-labeled goat anti-mouse, or anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Images were acquired using Eclipse E1000; Nikon; objectives ×60/1.4 NA equipped with a camera (ORCA-ER; Hamamatsu) and using Volocity acquisition software (Improvement) and figures were mounted using Photoshop CS3 software.

Western blotting

The Western blots were developed using the ECL method (Amersham Biosciences) using the antibodies described earlier in the text, and a mAb against CD133 (Miltenyi Biotec), mAb against CD44v6 (provided by M. Zoeller, DKFZ, Heidelberg, Germany), mAb against EpCAM (provided by G. Moldenhauer, DKFZ, Heidelberg, Germany), mAb against E-cadherin (Transductions Laboratories, Sigma-Israeil), and mAb against tubulin (Sigma-Israeil).

PCR

RNA was extracted from cells using EZ-RNA Total RNA Isolation Kit (Biological Industries) according to the manufacturer’s protocol. After obtaining cDNA, PCR was performed using Red Load Taq Master (Larova) according to the manufacturer’s protocol. Primers for L1 were ACGGGCAACATGGCTAAGATCAATGGCTA-5′, CTCAAGATCTGCCGAGTGA-3′, 5′-GGATGGTGTC-3′. Ls174T cells were transfected with the pcDNA4/TO L1, or with the pcDNA4/TO empty vector followed by selection with 800 μg/mL zeocin. HCT116 cells stably expressing inducible Slug were obtained in a similar manner. In transactivation assays, a β-galactosidase plasmid was cotransfected with the reporter plasmid containing 3 copies of an NF-κB-responsive promoter sequence linked to luciferase (3xκB.luc). Cells were plated in triplicates, lysed after 48 hours, and luciferase and β-galactosidase levels were determined by enzyme assay kits from Promega. For transfection control, luciferase activity was normalized to β-galactosidase activity. Fold induction of the NF-κB reporter plasmid was calculated using an empty reporter plasmid (pGL3).

Metastasis assays

Groups of 5 mice were anesthetized by peritoneal injection of xylazine (20 μg/mL) and ketamine (100 μg/mL). Through a 1-cm incision in the upper left lateral abdomen, the spleen was delivered into the wound, and 10⁶ cells in 20 μL of PBS were injected, using a Hamilton syringe, into the distal tip of the spleen that was replaced in the abdomen and the incision closed with staples. After 5 to 6 weeks, the animals were sacrificed and the spleen and liver removed for examination.

Artificial wound healing

A round “wound” was introduced into a confluent monolayer of cells, in 24-well dishes, with the tip of a micropipette using suction to remove the cells. The culture medium was replaced with fresh medium, and 0.1 μg/mL of Mitomycin C was added to inhibit cell proliferation. Wounds (in quadruplicate) were photographed every hour for 18 to 24 hours and the percentage of wound closure was calculated from the photographs taken at the start of the experiment and after 18 to 24 hours by using the Photoshop CS3 analyzer to measure the wound area not closed compared with the area of the wound at the beginning of the experiment.
Fluorescence-activated cell sorting and analysis

Human CRC cell lines were subjected to fluorescence-activated cell sorting (FACS). Human-specific anti-CD133 (293C3) conjugated to phycoerythrin (PE; Milyteni Biotec) was used for FACS analysis. The cells were pelleted and resuspended in FACS buffer (PBS pH 7.2, 0.5% bovine serum albumin, 2 mmol/L EDTA) containing the anti-CD133 antibody (1:100). After incubation for 20 minutes at 4°C, the cells were washed and the samples analyzed using a Becton Dickinson (BD) FACSVantage SE flow cytometer. The BD Cell Quest software was used for gating and analysis.

Immunohistochemistry

Immunohistochemistry was carried out on 25 paraffin-embedded human colorectal adenocarcinomas as described (17). In brief, antigen retrieval included pretreatment in citrate buffer pH 6.0 in a pressure cooker for 30 minutes. For L1 and E-cadherin detection, polyclonal rabbit anti-L1 antisera (18), diluted 1:1,000, and a monoclonal antibody against E-cadherin (clone 36; BD Transduction; diluted 1:200) were used for overnight staining at 4°C. The streptomycin/AB system (Dako) was used for detection according to the manufacturer’s protocol. Sections were counterstained with hemalaun (Merck).

Statistics

Statistical significance was determined by Fisher’s exact test (19) for mouse metastasis experiments. In wound closure, significance was determined using non-paired Student’s t-test. P < 0.05 was considered significant.

Results

Overexpression of L1 in CRC cells containing high or low levels of E-cadherin increases cell motility and induces liver metastasis

The expression of L1 in Ls174T CRC cells that lack L1 (7), and only possess very low levels of E-cadherin (Fig. 1A, lane 2), confers enhanced motility and the injection of such cells into the spleen of nude mice causes liver metastasis (8, 9). To examine whether overexpression of L1 in CRC cells displaying high levels of E-cadherin will cause similar responses and whether L1 will affect the organization and levels of E-cadherin, L1 was transfected into HCT116 CRC cells that display low levels of endogenous L1 and high levels of E-cadherin (Fig. 1A, lane 3). Two independent cell clones overexpressing L1 were isolated (Fig. 1B, lanes 2 and 3) and these CRC cells displayed a more than 2-fold increase in motility as measured by their ability to close an artificial wound in a monolayer (Fig. 1C, left and right). Moreover, on injection of these HCT116 cell clones overexpressing L1 into the spleen of nude mice, they formed liver metastases (Fig. 1D, C1 and C2, arrows). HCT116 cells transfected with the empty vector only formed primary tumors at the site of injection after 6 weeks (P < 0.05; Fig. 1D, control, arrowheads). As with Ls174T cells, there was no correlation between the sizes of tumors and liver metastases. A, the levels of L1 and E-cadherin were determined by Western blotting in human CRC cell lines Ls174T, HCT116, and SW620. NIH3T3 cells served as a negative control and MDCK as a positive control for E-cadherin expression. Tubulin served as loading control. B, L1 was stably transfected into HCT116 cells and the level of L1 in 2 individual cell clones (L1/C1 and L1/C2) was determined. C, the capacity of HCT116-L1 cells (described in B) to close an artificial wound was compared with that of control HCT116 cells after 24 hours. D, left, the motility of HCT116-L1/C1 and C2 was compared with that of control HCT116 cells by determining simultaneously the closure of 4 wounds for each cell line. The same areas were photographed immediately after wounding (0 hours) and 24 hours later (C, right). D, the cell clones described in B were injected into the spleen of nude mice and tumor growth at the site of injection (spleen) and formation of metastases (liver) were determined. The arrowheads point to tumors formed in the spleen and the arrows to large macrometastases in the liver.
formed at the site of injection and the metastatic capacity (8, 9). This indicates that the low level of endogenous L1 in HCT116 cells is insufficient to cause liver metastasis within this time period (6 weeks), and only at much later times (3.5–4 months) liver metastases are observed when control HCT116 cells were injected (data not shown).

Overexpression of L1 induces NF-κB activation in HCT116 cells and suppression of p65 levels blocks the capacity to confer liver metastasis

The transfection of L1 into Ls174T CRC cells was recently reported to induce NF-κB signaling, and the activated NF-κB subunit p65-P was shown to colocalize in cells at the invasive CRC tissue front together with L1 (9). We found that L1 transfection into HCT116 cells similarly induced the transcriptional activity of an NF-κB reporter plasmid (Fig. 2A), and the stable suppression of endogenous p65 levels by shRNA to p65 (Fig. 2B) resulted in the suppression of NF-κB activity (Fig. 2C) and blocked the metastatic capacity of these cells (P < 0.05; Fig. 2D). This suggests that L1 overexpression in both Ls174T and HCT116 CRC cells confers liver metastasis by a mechanism requiring the activation of NF-κB signaling.

Overexpression of L1 in CRC cells does not affect the levels of E-cadherin or other EMT markers

L1 was detected in a small subpopulation of CRC tissue cells at the invasive tumor front (7) that often lose E-cadherin expression (20), indicating the possibility that L1 may induce cellular properties compatible with an EMT. Indeed, a recent study suggested that in MCF7 breast cancer cells the transfection of L1 enhances invasive cell motility by affecting the organization of E-cadherin, reminiscent of an EMT-like process (10). We therefore examined the levels of E-cadherin and other epithelial and mesenchymal markers in CRC cell lines in which the levels of L1 were either increased or suppressed. As shown in Figure 3A, the stable transfection of L1 into Ls174T cells (Fig. 3A, lane 3) did not influence the level of endogenous E-cadherin, which remained low as in control Ls174T cells (Fig. 3A, compare lane 3 with lane 2). The high levels of E-cadherin also remained unaffected in HCT116 cells stably transfected with L1 (Fig. 3A, compare lane 5 with lane 4). In SW620 CRC cells that possess endogenous L1 and high levels of E-cadherin (Fig. 1A, lane 4; Fig. 3A, lane 6), suppression of L1 levels by shRNA to L1 had no significant effect on E-cadherin levels (Fig. 3A, compare lane 7 with lane 6). It is noteworthy that the suppression of L1 in SW620 reduced their tumorigenic capacities in nude mice (9). In addition to E-cadherin, no changes were observed in the epithelial cell specific cytokeratin 18, and an increase in the levels of the mesenchymal markers N-cadherin or vimentin was not apparent when L1 levels were manipulated (Fig. 3A).

Because EMT is considered to be a very transient process, both during embryogenesis and tumor progression (21), we prepared Ls174T CRC cells expressing a doxycycline-inducible L1 and followed the expression of L1 and E-cadherin at various times after treating the cells with doxycycline (Fig. 3B). L1 was already partially induced after 6 hours (Fig. 3B, compare lane 6 with lane 1) and continued to accumulate reaching much higher levels between 12 and 72 hours after doxycycline treatment (Fig. 3B, lanes 7–10). As previously shown (22), the levels of E-cadherin increase in CRC cell lines as cell density increases, and accordingly an increase in E-cadherin at later times of cells in culture, especially after 72 hours, was noted (Fig. 3B, lanes 5 and 10). However, this density dependent increase in E-cadherin was the same in cells lacking L1 and in cells where high levels of L1 were induced (Fig. 3B, lanes 5 and 10).

Because Ls174T CRC cells are among the few CRC cell lines that express wt p53, we also examined the possibility that induction of L1 will elicit a response from the p53 pathway. As shown in Figure 3B, the levels of p53 remained largely unchanged during the 3 days of L1 induction, whereas L1 levels increased dramatically. These results imply that when L1 levels are extensively modulated in CRC cells by stable shRNA-mediated suppression, overexpression, or induction by a doxycycline-responsive system, there were no detectable changes in the expression of key EMT markers in such cells.

Overexpression of Slug or Twist in CRC cells reduces E-cadherin levels but does not induce metastasis by these cells

Next, we addressed the possibility that the induction of an EMT-like process in CRC cells by the overexpression of major EMT transcription regulators, such as Twist and Slug, will be sufficient to cause liver metastasis by these cells. Two Ls174T cell clones overexpressing Twist were isolated and the level of E-cadherin in such cells was determined when the cells were either grown as sparse or dense cultures for 3 days (Fig. 4A). As shown previously (22), E-cadherin levels in control cells are higher in dense cultures where extensive cell–cell contacts are established (Fig. 3B; Fig. 4A, compare lane 5 with lane 6). The transfection of Twist into Ls174T cells dramatically reduced the levels of E-cadherin even in dense cell cultures (Fig. 4A, compare lanes 1 and 3 with control, lane 5). Interestingly, a dramatic shift in fibronectin expression (a mesenchymal marker) from a very high level in sparse cultures to almost undetectable levels in dense cultures could be observed (Fig. 4A, compare lanes 1, 3, and 5 with lanes 2, 4, and 6). However, this shift in fibronectin expression by cell culture density was not affected by Twist expression. In addition, Ls174T cells expressing a doxycycline-inducible Twist construct were isolated (8) and the cells were injected into the spleen of nude mice and the expression of Twist in these mice was kept under either uninduced or induced conditions (with doxycycline). The expression of Twist in the injected cells, however, was insufficient to confer metastatic capacity in these cells (Table 1; Fig. 4D, top and bottom). Slug is another major regulator of EMT whose expression is modulated in CRC cells (22). The overexpression of Slug by stable transfection into Ls174T cells (Fig. 4B, left) or
under a doxycycline-inducible promoter in HCT116 cells (Fig. 4B, right) reduced the levels of E-cadherin in both sparse and dense cell cultures compared with untransfected, or uninduced cells (Fig. 4B, left, compare lane 1 with lane 3; Fig. 4B, right, compare lane 1 with 3 and 2 with 4). Slug also induced the expression of mesenchymal markers (vimentin and fibronectin; Fig. 4C, left and right). HCT116 cells expressing inducible Slug were injected into the spleen of nude mice, and while forming tumors at the site of injection, Slug expression was insufficient to confer metastatic capacities in these cells (Fig. 4B, right, compare lane 1 with 3 and 2 with 4). Slug also induced the expression of mesenchymal markers (vimentin and fibronectin; Fig. 4C, left and right).

HCT116 cells expressing inducible Slug were injected into the spleen of nude mice, and while forming tumors at the site of injection, Slug expression was insufficient to confer metastatic capacities in these cells (Table 2). These results imply that whereas the major regulators of EMT, Twist and Slug, reduced the levels of E-cadherin and induced mesenchymal markers in CRC cells, their overexpression (unlike that of L1) is insufficient to confer metastasis by these cells in nude mice.

Changes in L1 levels do not result in a different organization of E-cadherin in CRC cells and tumor tissue

In MCF7 breast cancer cells, the transfection of L1 was suggested to result in a dramatic loss of E-cadherin from adherens junctions and its replacement by L1 and the induction of a motile, EMT-like phenotype in such cells (10). We therefore examined the organization of L1 and E-cadherin in CRC cells in which the levels of L1 were modulated. The organization of endogenous L1 and E-cadherin in SW620 CRC cells was mostly detected at cell–cell contact sites, but with rather little overlap (Fig. 5A–C). The complete suppression of L1 levels in these cells by shRNA to L1 (Fig. 5E) did not result in a detectable change in E-cadherin organization at cell–cell contacts (Fig. 5D).

The overexpression of L1 in HCT116 cells (Fig. 1B) produced cells expressing L1 at cell–cell contact sites (Fig. 5H) and E-cadherin localized in adherens junctions (Fig. 5G). In such cells, there was also rather little overlap between the localization of these 2 molecules in the cell membrane (Fig. 5I). Note that the single untransfected cell marked in the image shown in Figure 5G–I (arrowheads) displayed a similar organization of E-cadherin like the neighboring HCT116 cells that overexpressed the transfected L1.

We have also studied the organization of L1 and E-cadherin in cells at the invasive front of CRC tissue (in 25
tumor samples), where most of the L1 molecules are localized in CRC (7, 9). The loss of differentiation in small clusters of invasive cells was characterized by reduced or cytoplasmic expression of E-cadherin and nuclear β-catenin localization in all cases (Fig. 6B, C, E, and F). Approximately 75% (20 out of 25) of the cases displayed variable expression of L1 in invading tumor cells (Fig. 6A). Thus, cytoplasmic localization of E-cadherin was not restricted to only L1-expressing tumor cells (Fig. 6B), but was also detectable in L1-negative cells (Fig. 6E). Note the membranal localization of E-cadherin in the more central and differentiated areas of the tumor that lack L1 (Fig. 6H). The arrowheads (Fig. 6D) indicate normal L1 expression in nerve bundles of the submucosal layer where invading CRC cells are found (7). Taken together, in both cultured human CRC cell lines and in CRC tissue cells the presence of L1 does not appear to have a detectable effect on the organization of E-cadherin in these cells.

Expression of CRC stem cell markers and L1 in CRC cells

L1 is most abundant in nerve cells and in tumors of the nervous system (3). A recent study reported on the isolation of CSCs from human glioma and found that L1 cosegregates with glioma stem cells expressing the CD133 marker (13). Suppression of L1 levels in such cells blocked brain tumor formation in mice indicating that L1 could function as a glioma CSC target for therapy. Because CD133 positivity was also suggested to be a key marker for human CRC stem cells (14, 15), we wished to determine whether L1 expression in CRC cells cosegregates with CD133 and with other human CRC stem cell markers. FACS analysis was employed to separate from the HCT116 cell line, cell populations that either express or lack CD133 (Fig. 7A, left). Approximately 94% (in different experiments) of the cells in the HCT116 cell line displayed CD133. CD133 + and CD133−/C0 HCT116 cells were separated by 2 cycles of purification using FACS analysis and when equal amounts of protein were analyzed by Western blotting for CD133, an effective separation between CD133 + and CD133− cells could also be confirmed by this method (Fig. 7A, right, second panel). No enrichment for L1 in CD133 + cells was observed compared with CD133−/C0 cells and both cell populations expressed similar amounts of L1 (Fig. 7A, right, top panel). The analysis of other CRC stem cell markers, including CD44 and EpCAM, did not indicate a specific enrichment in CD133 + cells (Fig. 7A, right, second panel). No enrichment for L1 in CD133−/C0 cells was observed compared with CD133− cells and both cell populations expressed similar amounts of L1 (Fig. 7A, right, top panel). The analysis of other CRC stem cell markers, including CD44 and EpCAM, did not indicate a specific enrichment in CD133 + cells (Fig. 7A, right, second panel). No enrichment for L1 in CD133−/C0 cells was observed compared with CD133− cells and both cell populations expressed similar amounts of L1 (Fig. 7A, right, top panel).
expression or shRNA-mediated suppression (Fig. 7C). Here again, the level of CD133 RNA (Fig. 7C, left) and protein (Fig. 7C, right) remained unchanged (or did not correlate with L1) in the individual cell clones in which the level of L1 was dramatically altered. Taken together, these studies imply that in human CRC cell lines expressing endogenous L1 there is no specific cosegregation of L1 with cell populations expressing CRC stem cell markers.

**Table 1.** Tumor growth and liver metastasis in Twist-expressing Ls174T cells

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<th>Cells</th>
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<th>Liver metastasis</th>
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<tr>
<td>L1</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Ls174T-inducible Twist</td>
<td>4/4</td>
<td>0/4</td>
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<tr>
<td>Doxycycline +</td>
<td>3/4</td>
<td>0/4</td>
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<td>Doxycycline -</td>
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NOTE: Half of the mice were fed doxycycline to induce Twist. Tumor growth and liver metastasis were determined as described in Figure 1D.

**Table 2.** Tumor growth and liver metastasis in Slug-expressing HCT116 cells

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<th>Liver metastasis</th>
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<td>Doxycycline +</td>
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NOTE: Half of the mice were fed doxycycline to induce Slug. Tumor growth and liver metastasis were determined as described in Figure 1D.
Discussion

EMT is thought to endow cancer cells with migratory, invasive, and stem cell properties by activating a variety of signaling pathways including the Wnt/β-catenin and NF-κB pathways (11, 21, 23–25) and their target genes. Because the expression of L1 (a β-catenin/TCF target gene) in human CRC cells enhances cell motility and invasion (7), and confers metastatic capacity to the liver in nude mice (8) by activating NF-κB signaling (9), in this study we determined whether these properties conferred by L1 are associated with changes in EMT and CSC markers.

This study showed that L1 expression induced NF-κB signaling in CRC cells and is necessary for conferring enhanced motility and liver metastasis in both CRC cells that express very low levels (Ls174T) or high levels (HCT116) of endogenous E-cadherin. A recent study showed that NF-κB is also induced by L1 in pancreatic ductal carcinoma cells (26). The forced expression of L1 (in Ls174T and HCT116), or the shRNA-mediated suppression of endogenous L1 in SW620 CRC cells that had profound effects on the tumorigenic capacity of these cells (9), was not associated with changes in the expression and organization of E-cadherin. The analysis of E-cadherin localization in small clusters of invasive CRC cells

Figure 5. Changes in L1 levels do not result in a different organization of E-cadherin in CRC cells. A–F, SW620 cell clones stably transfected with shRNA to L1, or with control shRNA were doubly immunostained with anti-E-cadherin (A and D) and anti-L1 (B and E) antibodies. Nuclei were stained with DAPI (F). C, merged image of A and B. G–I, HCT116 cells stably transfected with L1 were immunostained with anti E-cadherin (G) and anti L1 (H) antibodies. A cell that does not overexpress L1 is marked by arrowheads (G–I). The scale bar represents 20 μm.
at the tumor front showed a similar organization and expression of E-cadherin in tumor cells displaying or lacking L1, implying that the regulation of L1 and E-cadherin levels are unrelated to each other in CRC cells. The levels of other epithelial or mesenchymal markers also remained unchanged in CRC cells when L1 expression was altered.

The reversible plasticity in the expression of EMT markers by epithelial cells and in the induction of β-catenin/TCF signaling can readily be shown in both nontumorigenic (27, 28) and CRC cells (22) by changing the cell culture density for short time periods, as also shown in this study. Nevertheless, the stable suppression of E-cadherin levels together with the induction of mesenchymal markers by the major EMT regulators, Twist and Slug, was insufficient to endow these CRC cells with metastatic capacity, suggesting that L1 confers metastatic abilities in these cells by other mechanisms not involving changes in EMT markers.

A previous study with MCF7 breast cancer cells transfected with L1 suggested that L1 disrupts E-cadherin containing adherens junctions and induces cell scattering and an EMT-like conversion (10). However, no changes in E-cadherin levels and mesenchymal markers or in key EMT regulators (Twist, Slug, Snail) were provided to support this conclusion in MCF7 cells (10). The current studies with CRC cells while further supporting an increase in cell motility induced by L1, did not observe concomitant changes in E-cadherin in a variety of CRC cell lines, or in the more motile and invasive CRC cells of the human tumor tissue front. In Caenorhabditis elegans, the L1 homologue SAX-7 and E-cadherin play a redundant role during development and can substitute for each other (29). These 2 molecules, however, do not completely colocalize and their organization and expression is not affected when either one of the molecules is genetically eliminated (29). Our results are consistent with this independence in the 2 adhesive systems mediated by L1 and E-cadherin and the lack of influence on their expression when the level of either of the molecules is altered.

Recent studies have suggested that CSCs can be isolated from a variety of cancer tissues using the cell surface marker CD133, including CSCs from CRC tissue (14, 15). EMT is believed to act in tumor development by producing cells with stem cell characteristics (11, 12) and L1 was found to cosegregate with CD133+ glioma stem cells and determine their metastatic capacity (13). We therefore investigated whether the L1-mediated increase in motility and metastatic capacity in CRC cells is associated with changes in the levels of CSC and of other markers associated with the more advanced stages in tumor progression (CD133, CD44, and EpCAM). Our results did not support a specific enrichment for L1, or cosegregation of CD133 with L1 when employing a variety of CRC cell lines that expressed mixed populations of CD133+ and CD133− cells. Recent studies have challenged the use of
more tumorigenic than their CD133+ counterparts (31). Moreover, the expression of colon CSC properties (including the activation of Wnt signaling) are modulated by cells and factors in the microenvironment that can reverse the more differentiated cancer cells to display stem cell properties (25). The analysis of CD133 presence in human CRC tissue indicated that CD133 is not detected in the invasive cells at the tumor front, but in the more differentiated areas of the tumor in a subpopulation of glandular cells that do not display nuclear β-catenin (32). Because L1 is only detected in the invasive front in cells displaying nuclear β-catenin (8), our results indicating no L1-CD133 cosegregation are neither unexpected nor surprising.

Although L1 expression in CRC cells did not confer an EMT signature, overexpression of Ras in CRC cells was shown to result (by gene expression profiling) in the induction of an EMT-like signature (33, 34). Because L1-mediated metastasis in CRC cells requires NF-κB signaling in cells expressing different E-cadherin levels (this study and ref. 9), and active NF-κB colocalizes with L1 in cells at the invasive tumor front (9), future studies of gene array patterns induced by L1 and NF-κB should provide more information on the mechanisms by which L1 confers enhanced motility and metastatic abilities in CRC cells. Such studies could aid in both CRC diagnosis and therapeutics.

Figure 7. Expression of CRC stem cell markers and L1 in CRC cells. A, HCT116 cells were sorted by FACS using antibody to CD133 (left), and analyzed by Western blotting for the expression of L1, CD133, CD44, and EpCAM (right). Tubulin was used as loading control. B, SW620 cells were separated by FACS into CD133+ and CD133− cell populations as in A (left), and analyzed by Western blotting for the expression of E-cadherin and EpCAM (right). The levels of CD133 and L1 RNA (C, left) and protein (C, right) were determined in single cell clones of HCT116 and SW620 CRC cells in which the levels of L1 were modulated by overexpression (HCT116), or were suppressed by L1 shRNA (SW620). GAPDH served as control for RNA levels and tubulin as protein loading control.

CD133 as a specific marker for CSCs and found a wide range of epithelial tissues expressing CD133 (30). Another study showed that CD133− populations of tumor cells are even

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L1-Mediated Colon Cancer Cell Metastasis Does Not Require Changes in EMT and Cancer Stem Cell Markers

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