Expression of L1-CAM and ADAM10 in Human Colon Cancer Cells Induces Metastasis

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Abstract

L1-CAM, a neuronal cell adhesion receptor, is also expressed in a variety of cancer cells. Recent studies identified *L1-CAM* as a target gene of β-catenin-T-cell factor (TCF) signaling expressed at the invasive front of human colon cancer tissue. We found that L1-CAM expression in colon cancer cells lacking L1-CAM confers metastatic capacity, and mice injected in their spleen with such cells form liver metastases. We identified *ADAM10*, a metalloproteinase that cleaves the L1-CAM extracellular domain, as a novel target gene of β-catenin-TCF signaling. ADAM10 overexpression in colon cancer cells displaying endogenous L1-CAM enhanced L1-CAM cleavage and induced liver metastasis, and ADAM10 also enhanced metastasis in colon cancer cells stably transfected with L1-CAM. DNA microarray analysis of genes induced by L1-CAM in colon cancer cells identified a cluster of genes also elevated in a large set of human colon carcinoma tissue samples. Expression of these genes in normal colon epithelium was low. These results indicate that there is a gene program induced by L1-CAM in colon cancer cells that is also present in colorectal cancer tissue and suggest that L1-CAM can serve as target for colon cancer therapy. [Cancer Res 2007;67(16):7703–12]

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers in the Western world with ~150,000 new cases and 60,000 deaths each year in the United States alone (1). CRC is thought to evolve from adenomatous polyps, a precursor lesion that, when accompanied by acquired mutations in various genes, can advance to invasive cancer (2). Because evolution of the adenoma-carcinoma sequence takes between 5 and 15 years, there is ample opportunity for early intervention. Studies on both sporadic and inherited CRC revealed that, in the great majority of such cancer patients, the primary mutation targets a single signaling pathway, the Wnt pathway (3–6). A key component in the Wnt pathway is β-catenin that plays a dual role in the cell: it is a major structural component of cell-cell adhesions. In addition, it can function as an activator of gene transcription in the nucleus together with T-cell factor (TCF)/lymphocyte enhancer factor (LEF) transcription factors (7, 8). When inappropriately activated, as is the case in CRC, β-catenin is an oncogene, constitutively activating genes that contribute to CRC development.

A key question in β-catenin–mediated oncogenesis is the identification of β-catenin target genes that are induced during invasive CRC development. Earlier studies showed that such genes include *cyclin D1* (9, 10) and *c-MYC* (11) that can confer growth advantage in cancer cells. Interestingly, the accumulation of β-catenin in the nuclei of cancer cells continues with the progression of the disease, and it is most prominent at the invasive edge of colon cancer tumor tissue (12), indicating that β-catenin signaling also activates genes involved in later stages of CRC development. β-Catenin-TCF target genes characteristic of these stages, promoting tumor cell motility and invasion, have been identified and include metalloproteinases (13–16), ECM components (16–18), and cell adhesion receptors, such as CD44 (19) and urokinase-type plasminogen activator receptor (20). Wnt/β-catenin signaling also regulates the EphB/EphrinB cell sorting receptors and their ligands in normal intestinal epithelial cells (21) and upregulates their expression in early adenomas, but their expression is lost at later stages of CRC progression (22), implying a complex regulation of these receptors during CRC oncogenesis.

In recent studies, we identified genes coding for members of the neuronal L1-CAM family of immunoglobulin-like cell adhesion receptors (L1-CAM and Nr-CAM) as targets of β-catenin/TCF signaling in colon cancer cells (23, 24) and detected L1-CAM and the metalloproteinase ADAM10, which cleaves the L1-CAM ectodomain, in a subpopulation of CRC cells at the invasive front of tumors (24). In the present study, we investigated whether L1-CAM and ADAM10 expression in human CRC cells promotes the metastatic capacity of these cells and compared the gene expression pattern elicited by L1-CAM expression in cultured CRC cells to changes in the profile of genes expressed in human CRC tissue. We found that L1-CAM and ADAM10 expression confers metastatic capacity in CRC cells to the liver, and the genes induced by L1-CAM in cultured CRC cells are higher in tumor tissue than in normal tissue as tested in a large set of human CRC and normal tissue samples.

Materials and Methods

Cell lines, cell culture, and transfections. 293T, SW480, and HCT116 cells were maintained in DME with 10% bovine calf serum. When the cell culture medium was collected, cells were plated in RPMI 1640 with 10% serum, which was replaced the following day with serum-free RPMI 1640,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-0991

and proteins from the medium, collected after 48 h of culture, were precipitated with 0.2 mol/L NaCl and 3 volumes of 100% ethanol at 4°C overnight. For cell growth rate, 5×10^3 cells per well were plated into 24-well dishes and their number was determined every 24 h for 6 days in triplicates.

Transient transfection of 293T cells was done using calcium phosphate. Ls174T and HCT116 cells were transfected using LipofectAMINE 2000 (Invitrogen). In transactivation assays, 0.25 μg of β-galactosidase plasmid was cotransfected with 1 μg reporter plasmid and 2 μg of either β-catenin, ΔNTCF4, or E-cadherin cytoplasmic tail constructs (9). Cells were plated in duplicates and lysed after 48 h, and luciferase and β-galactosidase levels were determined by enzyme assay kits from Promega. Luciferase activity was normalized to β-galactosidase activity as internal transfection control. Fold induction of the *ADAM10* gene promoter constructs was calculated using empty reporter plasmid (pGL3). Ls174T cells stably expressing human L1-CAM [full-length and truncated green fluorescent protein (GFP)-tagged forms] were established by transfection using LipofectAMINE 2000 followed by selection to neomycin resistance (500 μg/mL). Cotransfection with human ADAM10-HA in Ls174T cells expressing L1-CAM was done with LipofectAMINE 2000 followed by selection with puromycin (10 μg/mL). Ls174T cells stably expressing the Tet repressor, selected in blasticidin (10 μg/mL), were transfected with a mouse Twist cDNA construct and selected in zeocin (500 μg/mL). The expression of Twist was induced by adding doxycycline (1 μg/mL) to the culture medium.

Plasmids. GFP-tagged L1-CAM cDNA constructs were as described (25). ΔNTCF4 was provided by M. van de Wetering and H. Clevers (Hubrecht Institute, Utrecht, the Netherlands), as were the Topflash and Fopflash reporter plasmids, and β-catenin (S33Y) was described previously (26). The ADAM10 promoter reporter plasmid was prepared as follows: The sequence was determined from the National Center for Biotechnology Information (NCBI) database and confirmed a published sequence of the promoter (27). Primers for PCR from a bacterial artificial chromosome clone were as follows: forward, GTTAACAGCAGCACATCGATCC; reverse for the shorter promoter, TATCCGCTGTCTCGCCA; and reverse for the longer promoter, GATGGCAGAATCTCACGAATG. The PCR products were cloned into the pGL3 reporter plasmid at *Mlu*I/*Xho*I sites. Twist constructs were prepared by cloning the Twist cDNA into the pcDNA4/TO tetracycline-inducible T-Rex system (Invitrogen) at the *Eco*R1/*Bam*H1 sites.

RNA reverse transcription-PCR. The primers used were TGTCTCGCTCCTTGAAACA and GGGTTTTATCCAGCCCAAT for isopentenyl-diphosphate delta isomerase 1, GGTACCCTGGAAACCACCTT and TTCAGAAGGGAATGGGAGTG for squalene exposidase, TGCCTCTGCCTTTTCTGTCT and GCACTTGGTCCCTTGTTTGT for Rho GDP dissociation inhibitor a, and TGGAAATTCCTGGTGAGGAG and CTCAGGTGGGGTCAGTAGGA for ferredoxin reductase using the following amplification program: 94°C for 1 min, 60°C for 30 s, 72°C for 30 s, for 24 cycles. Primers were chosen using an online program.[8]

Immunofluorescence. Cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% paraformaldehyde in PBS. The coverslips were incubated with polyclonal antibody against L1-CAM, recognizing both the extracellular and intracellular domains of L1-CAM (provided by V. Lemmon, University of Miami, Miami, FL). Monoclonal antibody (mAb) against Twist was from I. Gitelman (Ben-Gurion University, Beer Sheva, Israel). mAb against β-catenin was from Transduction Laboratories and against HA from Babco Laboratories. The secondary antibodies were Alexa Fluor 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 the Delta Vision System (Applied Precision) equipped with a microscope (model Axiovert 100; Carl Zeiss MicroImaging) and Photometrics 300 series scientific-grade cooled charged-coupled device camera, reading 12-bit images, and using the 63×/1.4 numerical aperture Plan-Neofluar objective. Adjustments of brightness, contrast, color balance, and final size of images were processed using Adobe Photoshop 5.5.

Western blotting. The Western blots were developed using the enhanced chemiluminescence method (Amersham Biosciences). Protein was extracted from the liver tissue of mice by splicing small sections, adding Laemmli's sample buffer, and boiling for 5 min before SDS-PAGE and transfer to nitrocellulose filters.

Metastatic capacity assays. Metastatic capacity was determined by injecting 10^6 cells per mouse into the spleen of nude mice. 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 10^6 cells in 20 μL were injected, using a Hamilton syringe, into the distal tip of the spleen that was replaced in the abdomen and the incision was closed with staples. When cells expressing inducible Twist were injected into the spleen, the mice received doxycycline (200 μg/mL) in their drinking water. After 4 to 5 weeks, the animals were sacrificed and the spleen and liver were removed for examination.

Tissue immunohistochemistry. Immunohistochemistry was done on 3-μm serial sections from 25 formalin-fixed, paraffin-embedded spleens and livers of mice. Samples were deparaffinized, rehydrated, and pretreated for antigen retrieval by microwave treatment for 20 min in 10 mmol/L of citrate buffer (pH 6.0). To detect L1-CAM, the antibodies described above were used. Fluorescent rabbit anti-goat antiserum was used as secondary antibody, and specimens were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The formalin-fixed, paraffin-embedded specimens of the spleen and liver were stained with H&E.

DNA microarrays. Two individually isolated Ls174T clones stably transfected with L1-CAM were compared with control, neo^r Ls174T cells lacking L1-CAM. RNA was extracted using the Qiagen RNA Extraction kit. Microarray analysis was done using Affymetrix U133A GeneChips containing 22,777 genes and expressed sequence tags. The chips were processed and scaled using Affymetrix MAS5 following the preprocessing steps: (*a*) genes that were marked by Affymetrix as "present" in all samples were kept, leaving a group of 11,331 probe sets; (*b*) thresholding to 1 (i.e., any expression value below 1 was changed to 1); (*c*) $\log_2$ transformation; and (*d*) for visualization, the data were centered and normalized. To analyze the differences between the two experimental chips (for each Ls174T cell clone expressing L1-CAM) and the neo^r control cell clone, a generalization of the standard fold change approach was applied: because the differences between the log expression values of two microarrays diminish with increasing intensities, a single fold change cutoff cannot be used to determine which probe sets are significantly different from the control. The main assumption was that most genes are not differentially expressed and thus can be used to estimate the distribution width of the log of fold change for a gene with a given average expression (estimated using all genes whose average expression in the two conditions lies within a window). Using this distribution, one can identify genes that behave differently (but would be missed by the fold change approach). Each probe set is given a *P* value estimating how different is its expression in the "experiment" from the control. The null hypothesis is that there is no difference. False discovery rate of 0.05 was used to select the probe sets that were overexpressed. This test was applied to compare each of the two experiments to the control. Probe sets significantly different from the control, in both experiments, were analyzed using a second set of data obtained with U133A Affymetrix GeneChips from colon cancer patients after obtaining consent from each subject. This analysis included samples from 170 human colon carcinomas and 43 normal colon epithelial tissues, obtained in the framework of a collaborative National Cancer Institute/NIH program project grant. The expression matrix of the overexpressed probe sets was two-way sorted by sorting points into neighborhoods (SPIN; once for the genes and once for the samples; ref. 28). Functional annotation was determined using the NCBI program DAVID.[9]

Statistical analysis. The statistical significance of metastatic tumor formation in mice was determined by Fisher's exact test, comparing mice injected with L1-CAM–expressing cells and control cells. A P value of <0.05 was considered significant.

[8] http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

[9] http://david.abcc.ncifcrf.gov/

Results

Expression of L1-CAM in colon cancer cells induces liver metastasis. In a previous study, L1-CAM expression in human colon cancer cells was shown to enhance their growth and motility, and L1-CAM was localized in a subpopulation of tumor cells at the invasive front of human colon cancer tissue (24). Metastasis to the liver of colon cancer cells is a characteristic step in the development of CRC. To examine whether L1-CAM expression in human colon cancer cells promotes their metastasis to the liver, we isolated two independent clones of Ls174T cells in which we stably expressed L1-CAM. Ls174T cells, in contrast to some other colon cancer cells, including SW480 and HCT116, do not normally express L1-CAM (Fig. 1*B*; ref. 24). To analyze for the metastatic capacity of colon cancer cells, we adopted an experimental model that consists of injecting human tumor cells into the spleen of nude mice and followed their ability to invade, via the portal vein, into the liver and form there metastatic lesions. When 10^6 L1-CAM–expressing Ls174T human colon cancer cells were injected into the distal tip of the spleen of nude mice, they formed, within 4 to 6 weeks, very extensive metastases in the liver of 84% (n = 20 of 24) of the injected mice, whereas cells transfected with the empty vector (neo^r) did not promote metastasis (n = 0 of 27; P < 0.001, Fisher's exact test; Fig. 1*A*). Both the neo^r control and L1-CAM–expressing Ls174T cells grew locally in the spleen at the site of injection to form a primary tumor (Fig. 1*A, arrows*), but there was no direct correlation between the size of these primary tumors and the extent of liver metastasis (Fig. 1*A* and *C*). Western blot (Fig. 1*B*) and immunohistochemical analyses of the tumors confirmed that the tumor cells that invaded the liver express L1-CAM, mostly at cell-cell contact sites (Fig. 1*D*), consistent with the known function of L1-CAM as a transmembrane cell adhesion receptor mediating homophilic interactions. We also established Ls174T cells expressing the transcription factor Twist under an inducible promoter (Fig. 1*B*). Twist, a potent modulator of epithelium to mesenchyme transition, was recently implicated in breast cancer cell metastasis (29). Injection of Ls174T colon cancer cell clones expressing an inducible Twist construct (Fig. 1*B*) into the spleen of mice revealed that these cells only formed tumors at the site of injection, but no

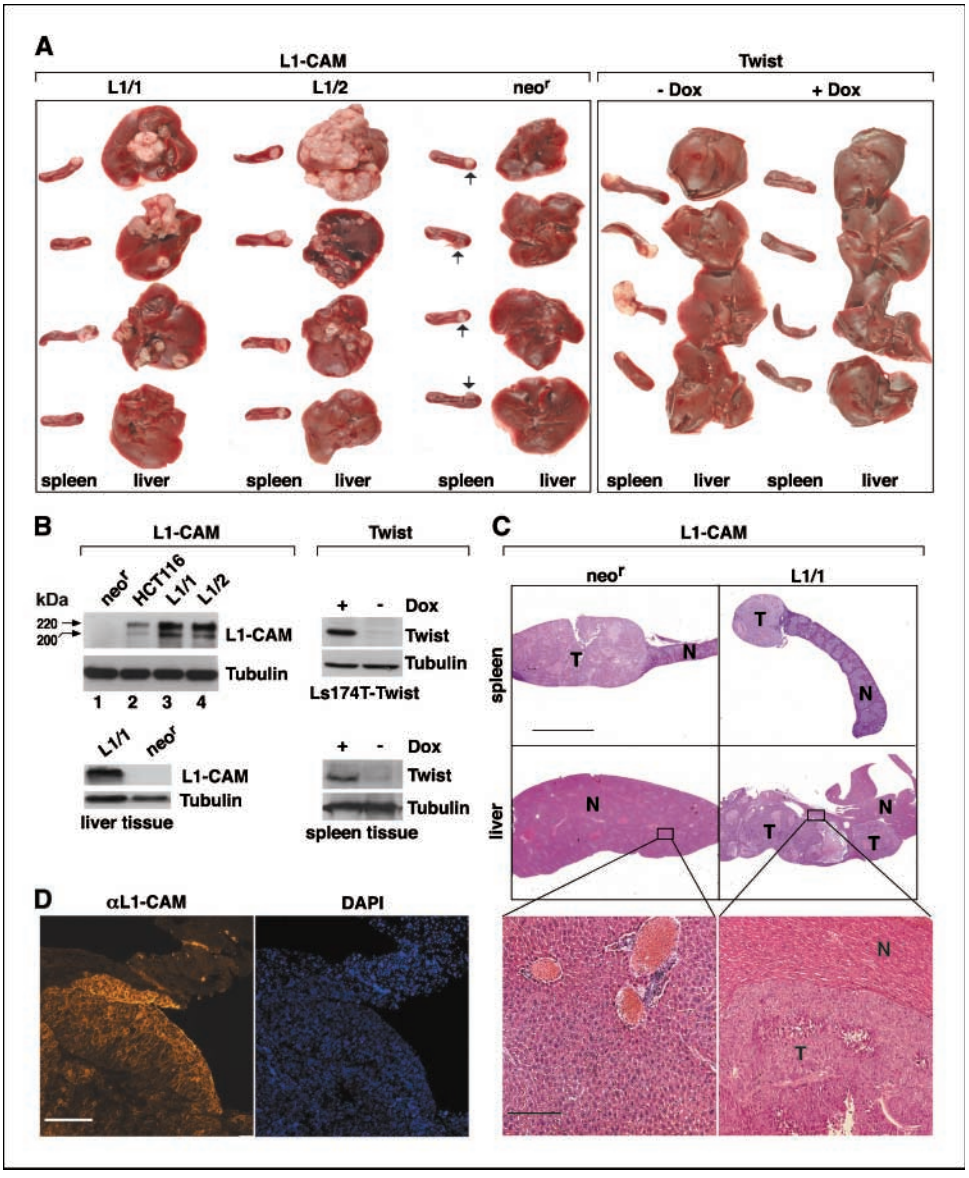

Figure 1. Expression of L1-CAM in colon cancer cells confers metastatic capacity. *A,* Ls174T human colon cancer cells were stably transfected with L1-CAM or with doxycycline (*Dox*)-inducible Twist, and two independent clones expressing L1-CAM (*L1/1* and *L1/2*), a neo^r control clone, and Ls174T cells expressing doxycycline-inducible Twist were injected into the distal tip of the spleen in groups of five mice with 10^6 cells per mouse. For Twist induction, the mice received 200 μg/mL doxycycline in their drinking water. After 5 wks, the spleens and livers were excised and photographed. The *arrows* point to tumor cells growing at the site of injection. *B,* Western blot analysis of L1-CAM expression in Ls174T cell clones and in HCT116 colon cancer cells expressing endogenous L1-CAM (220 kDa molecular weight is the full-length molecule and the 200 kDa marks the shed ectodomain). Twist was induced in Ls174T cells with doxycycline. Tubulin expression was used as loading control. Levels of L1-CAM and Twist in liver (*L1-CAM*) and spleen tissue (*Twist*) from mice injected with the cells described in (*A*) were determined by Western blotting. *C,* histochemical (H&E) tissue staining of paraffin sections of spleen and liver from mice injected with control and L1-CAM–expressing colon cancer cells. *T,* tumor tissue; *N,* normal tissue. *D,* immunohistochemical staining with anti-L1-CAM mAb and FITC-labeled secondary antibody of liver sections from mice injected with L1-CAM–expressing cells and counterstained with DAPI to visualize nuclei. The experiment in (*A*) was conducted four times with similar results. Bars, 2.5 mm (*C, top*), 100 μm (*C, bottom*), and 100 μm (*D*).

liver metastases were apparent (Fig. 1*A*). Ls174T clones stably expressing Twist (producing a much lower level of Twist protein than the inducible clones) also did not form liver metastases (data not shown). These results suggest that L1-CAM expression in human colon cancer cells can confer the capacity to form liver metastases, whereas Twist, in contrast to its role in breast cancer cells (29), is unable to induce such property in Ls174T cells.

Full-length L1-CAM is required to confer enhanced growth and metastasis in colon cancer cells. Recent studies investigated the role of various molecular domains of L1-CAM, a key cell adhesion molecule in nerve cells, in conveying signals that promote neuronal cell branching and outgrowth (30). To begin to understand the mechanisms by which L1-CAM induces metastatic capacity in human colon cancer cells, we used several truncation mutants of L1-CAM that were GFP tagged at their COOH terminus (Fig. 2*A*) and stably expressed them in Ls174T cells. Cells expressing comparable levels of full-length L1-CAM and deletion mutations that removed either the cytoplasmic domain of L1-CAM (Fig. 2*B, ΔICD*), or its ectodomain and transmembrane segment (Fig. 2*B, ICD*), were isolated. Expression of the ectodomain of L1-CAM as part of the full-length or the truncated version of the L1-CAM molecule in Ls174T cells resulted in shedding of a considerable amount of L1-CAM into the culture medium (Fig. 2*B, right*). The full-length L1-CAM and a mutant lacking the L1-CAM cytodomain, but which had the transmembrane and ectodomain (Fig. 2*A, ΔICD*), were both localized at cell-cell contact sites in the membrane (Fig. 2*C*), whereas L1-CAM that only possesses the cytodomain (ICD), but lacked the transmembrane domain, was

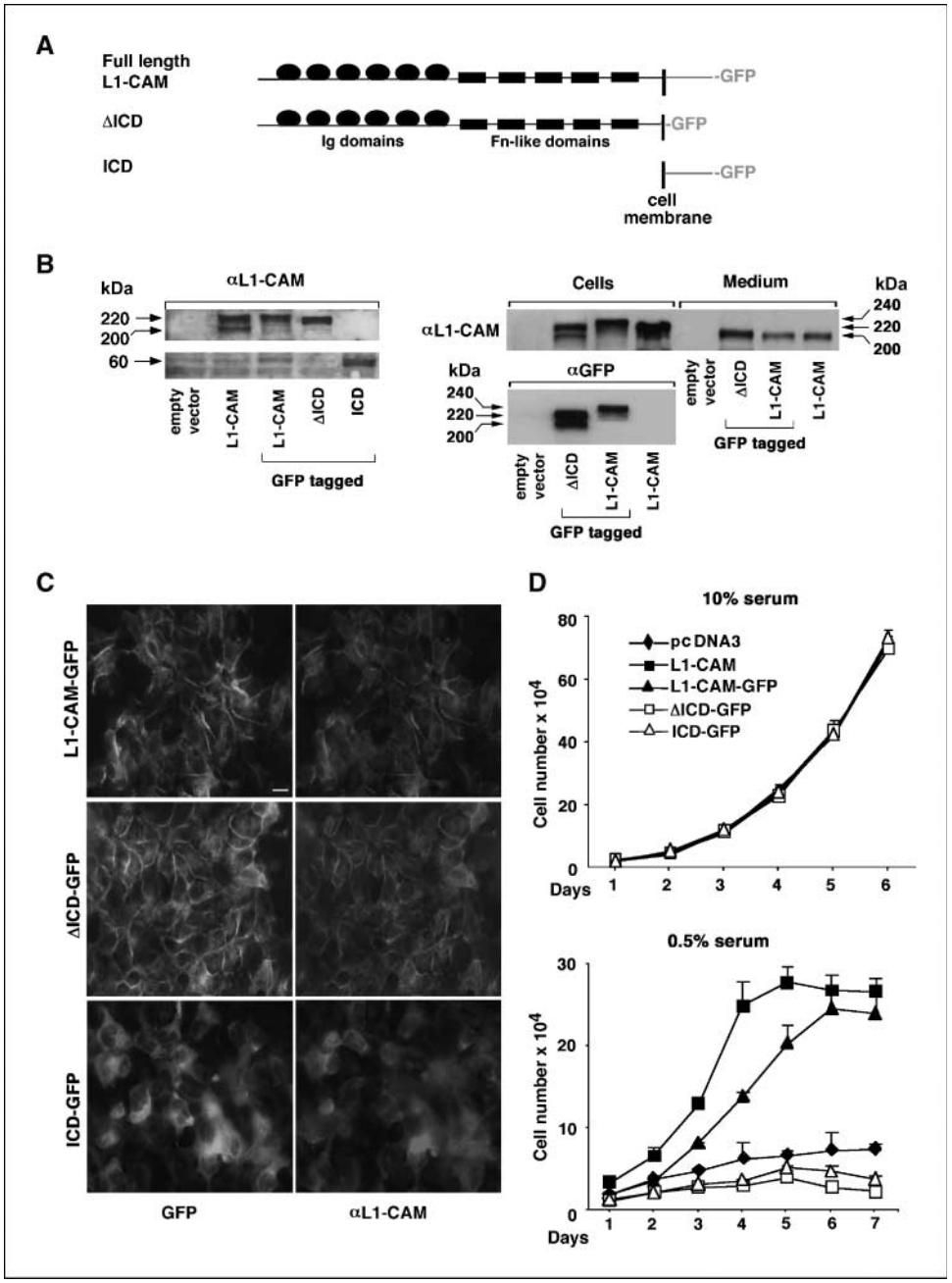

Figure 2. Full-length L1-CAM but not deletion mutants of the extracellular or intracellular domain confers growth advantage in colon cancer cells. *A,* Ls174T cells stably expressing GFP-tagged full-length L1-CAM, or constructs lacking the cytoplasmic domain (*ΔICD*), or the extracellular domain (*ICD*) were isolated. *B,* expression of the various L1-constructs was identified by Western blotting using a polyclonal L1-CAM antibody recognizing both the extracellular and intracellular domains of the molecule or by an antibody to GFP. *Right,* analysis of full-length and deletion mutant L1-CAM expression in the cell layer (*Cells*) and the cleaved/shed ectodomain of L1-CAM in the culture medium (*Medium*) using a mAb against the L1-CAM ectodomain. *Bottom,* expression of L1-CAM constructs in the cell layer was also identified in immunoblots with anti-GFP antibody. The full-length GFP-tagged L1-CAM has an apparent molecular weight of 240 kDa compared with 220 kDa of the untagged molecule. The cleaved ectodomain ran as a 200-kDa molecular weight protein, and the cytoplasmic domain at ~60 kDa. *C,* fluorescence images of cells expressing the various L1-CAM constructs visualized by the GFP tag and double stained with polyclonal L1-CAM antibodies and Cy5-labeled secondary antibody. Bar, 10 μm. *D,* 5×10^3 cells were seeded per well, in duplicates, in 24-well dishes and the growth of cells in 10% and 0.5% of serum was determined by counting cell number for 7 d. *Bars,* SD. These experiments were repeated thrice with similar results.

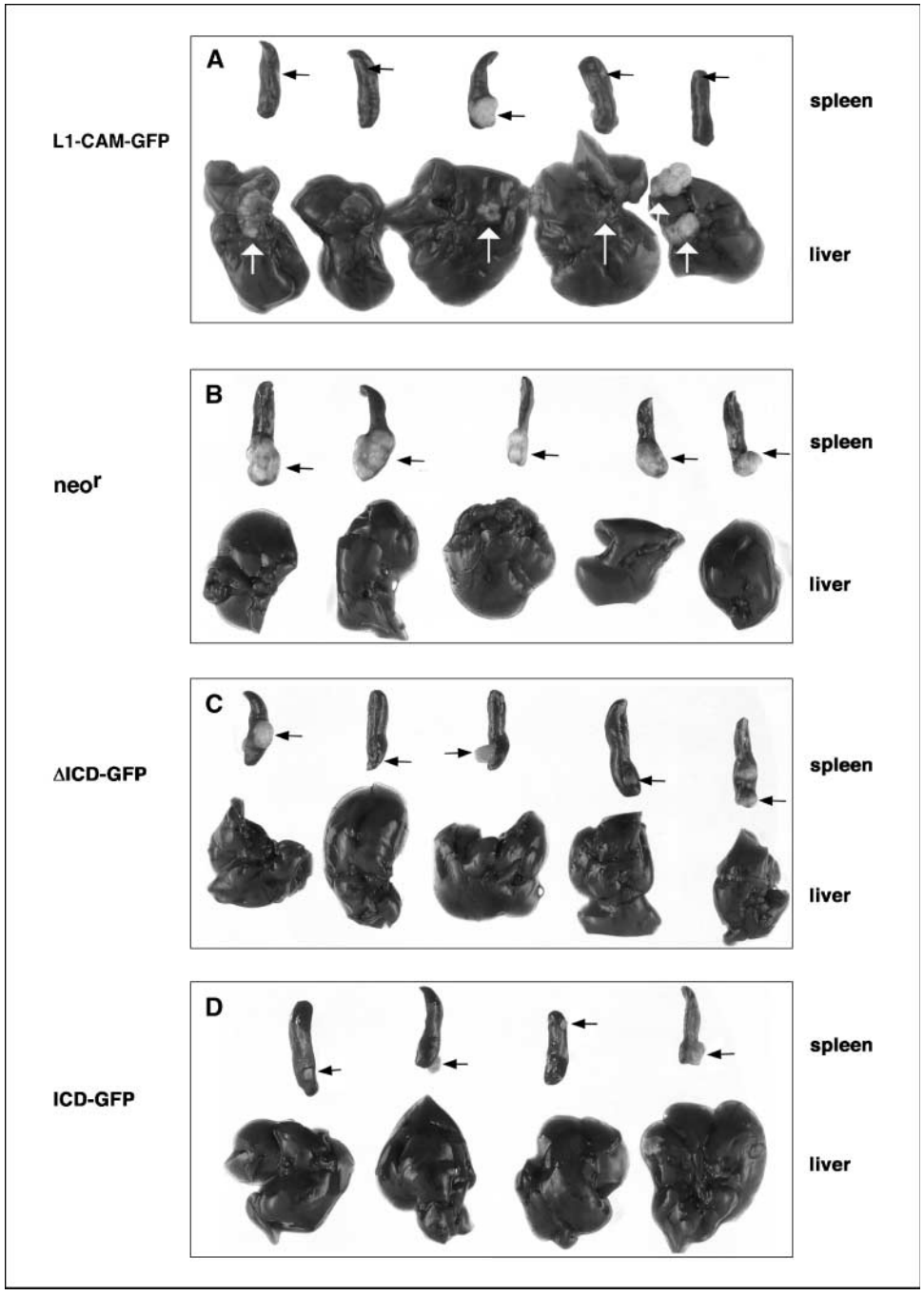

Figure 3. Full-length but not deletion mutants of L1-CAM confers metastatic potential in human colon cancer cells. LS174T cells expressing full-length, GFP-tagged L1-CAM (*A*); control, neo^r (*B*); L1-CAM lacking the cytodomain (ΔICD) and GFP-tagged at the COOH terminus (*C*); and L1-CAM that only contains the cytodomain (ICD) tagged with GFP (*D*) were injected into the spleen of nude mice and the spleen and livers were excised 5 wks after injection of the cells. The *white arrows* point to liver metastases, whereas the *black arrows* mark the local tumor growth in the spleen at the site of injection. This experiment was repeated thrice with similar results. Note that only full-length L1-CAM expression in colon cancer cells can confer growth in the liver.

mostly detected in the cytoplasm (Fig. 2*C*). These constructs were detected in cells both by their GFP tag and by anti-L1-CAM antibody (Fig. 2*C*).

In the presence of 10% serum, control Ls174T cells and cells transfected with wild-type L1-CAM and with its various mutant forms proliferated equally well (Fig. 2*D*). In the presence of 0.5% of serum, however, cells expressing full-length L1-CAM had a significant growth advantage over control cells and over cells expressing truncated mutant forms of L1-CAM (Fig. 2*D, bottom*). Similarly, only colon cancer cells expressing the full-length L1-CAM were capable of forming liver metastases (77.8%, $n = 7$ of 9) when injected into the spleen of nude mice (Fig. 3*A, white arrows*) but not the cells expressing the mutant L1-CAM forms (Fig. 3*B–D*). However, all L1-CAM–expressing Ls174T cell clones and the neo^r control colon cancer cells proliferated and formed primary tumors in the spleen ($n = 0$ of 20, for L1-CAM clones, and $n = 0$ of 9, for neo^r controls; $P = 0.001$; Fig. 3*D, black arrows*). As seen above (Fig. 1*A*), there was no direct correlation between tumor size in the spleen, at the site of injection, and the ability to form liver metastases by these mutant L1-CAM–expressing cells. We conclude that full-length L1-CAM expression is required for conferring both growth advantage under stressful conditions (in 0.5% serum) and for inducing the capacity to form liver metastases by colon cancer cells.

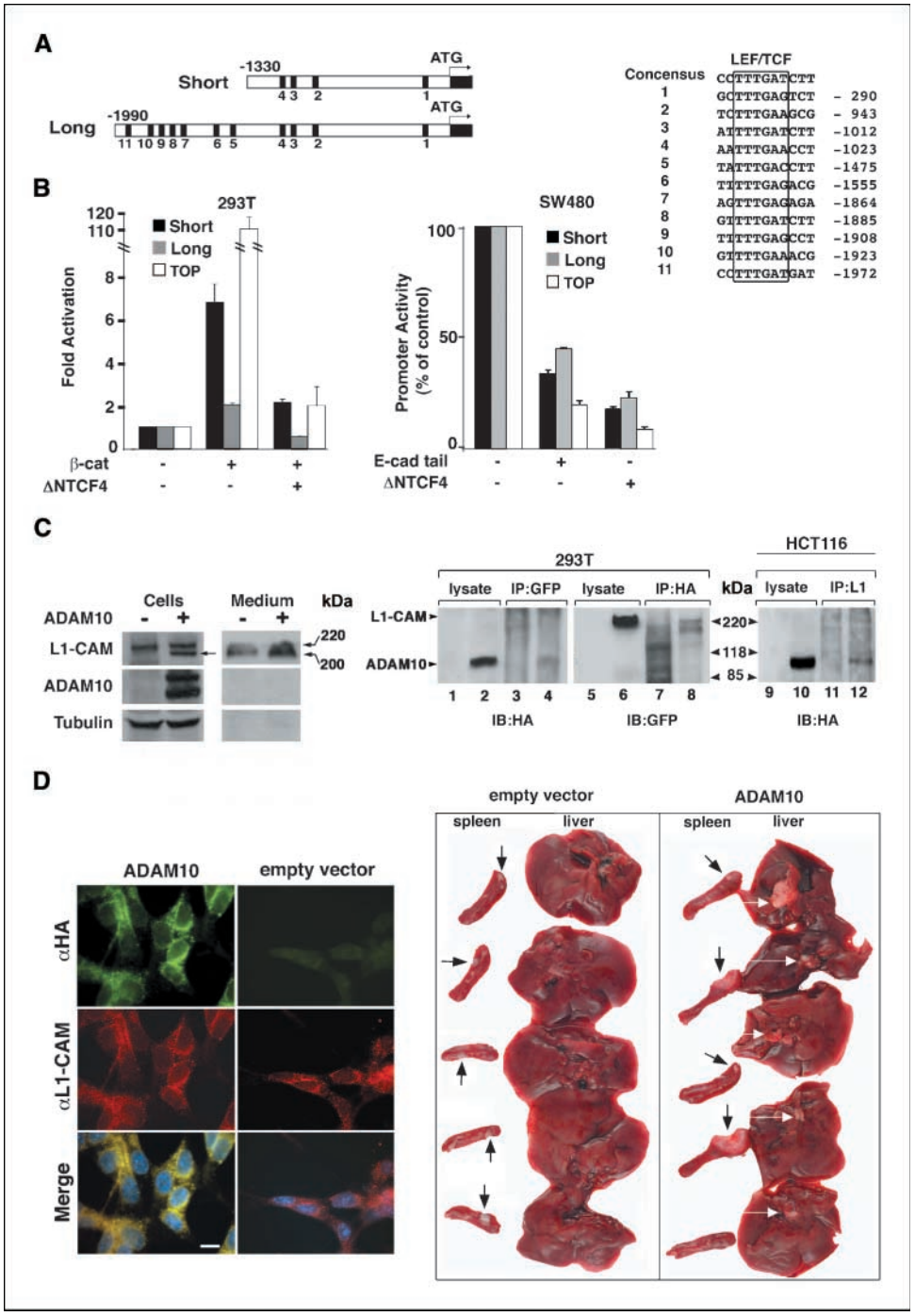

Figure 4. The *ADAM10* gene promoter is a novel target of β-catenin-TCF signaling and ADAM10 expression enhances L1-CAM cleavage and confers liver metastasis in HCT116 cells. *A,* shorter (1.33 kb) and longer (1.99 kb) reporter plasmids of the *ADAM10* gene promoter were constructed and potential LEF/TCF-binding nucleotide sequences in the promoter were identified. *B,* the short and long reporter plasmids and a synthetic TCF reporter plasmid, Topflash (*TOP*), were transiently transfected into 293T cells together with either an activated form of β-catenin (*β-cat*) or a dominant-negative TCF construct (*ΔNTCF4, left*) and also into SW480 colon cancer cells (*right*) without β-catenin transfection but with a construct expressing the cytoplasmic tail of E-cadherin (*E-cad tail*). Promoter activity was normalized to that obtained with empty plasmid transfection and to β-galactosidase expression for transfection efficiency in SW480 cells and to a mutant synthetic TCF reporter plasmid (Fopflash) in 293T cells. The experiments were carried out in duplicates and repeated with similar results between three and five times for each cell line. *C,* Western blot analysis of HCT116 cells stably transfected with HA-tagged ADAM10. Note that cells overexpressing ADAM10 display both full-length endogenous L1-CAM and a lower molecular weight cleaved L1-CAM product (*small arrow*) and also an increase in shed L1-CAM in the culture medium. Transfected ADAM10 appears as two bands representing a processed (mature) and an unprocessed form of the molecule. *Right,* 293T cells cotransfected with GFP-L1-CAM and HA-ADAM10 showed that the two molecules coprecipitated in a complex by either anti-GFP (*lane 4*) or anti-HA antibodies (*lane 8*). *Lane 12,* in HCT116 cells, anti-endogenous L1-CAM antibody coprecipitated HA-ADAM10. *D,* endogenous L1-CAM and transfected ADAM10 also colocalized in HCT116 cells as seen by double immunofluorescence analysis with anti-L1-CAM and anti-HA-tag antibodies. *Right,* HCT116 cells stably transfected with the empty neo^r vector, or with ADAM10, when injected into the spleen of nude mice formed liver metastases. This is a representative experiment of three independent experiments. Bar, 10 μm (*D, bottom*).

***ADAM10* is a novel target gene of β-catenin/TCF signaling in colon cancer cells.** In a recent study, expression of the metalloproteinase ADAM10 that cleaves and induces the shedding of L1-CAM (31), and of other cell adhesion receptors (32–34), was shown to be coregulated in colon cancer cells (by cell culture density) together with L1-CAM expression (24). Both molecules were also detected at the invasive front of CRC tissue (24). We wished to determine whether the *ADAM10* gene is a target of β-catenin/TCF signaling similar to the *L1-CAM* gene promoter. For this, two sequences of the *ADAM10* gene promoter (a shorter and a longer sequence; Fig. 4*A*) were isolated and reporter plasmids containing these promoter sequences were prepared. Analysis of *ADAM10* promoter sequences indicated that they contain a large number of putative TCF-binding sites (Fig. 4*A, right*). Reporter plasmid constructs of the short and long promoter sequences, driving the expression of luciferase, showed that the *ADAM10* gene promoter is activated by β-catenin in 293T cells, and this activation is inhibited by a dominant-negative TCF construct (ΔNTCF4), suggesting that the *ADAM10* gene is also a target of β-catenin-TCF signaling (Fig. 4*B*). Note that the longer promoter construct was less active than the shorter one, indicative of sequences that enable the binding of negative transcriptional regulators in the longer

ADAM10 promoter. These *ADAM10* promoter constructs were also activated by the endogenous β-catenin-TCF complex in SW480 colon cancer cells (similar to the synthetic Topflash reporter plasmid; Fig. 4*B, TOP*), and were inhibited by the E-cadherin cytoplasmic tail (Fig. 4*B, E-cad tail*) that sequesters away β-catenin from TCF, and by a dominant-negative TCF construct (Fig. 4*B, ΔNTCF4*).

ADAM10 enhances the metastatic capacity of colon cancer cells expressing L1-CAM. We wished to determine whether transfection of ADAM10 into HCT116 human colon cancer cells expressing endogenous L1-CAM (Fig. 1*B, lane 2*) affects the ability of these cells to form liver metastases. HCT116 cells transfected with ADAM10 display colocalization of L1-CAM and ADAM10 mostly in cytoplasmic vesicles and in the cell membrane (Fig. 4*D*). A molecular association between L1-CAM and ADAM10 could also be shown by coimmunoprecipitation using 293 cells transfected with both molecules and in HCT116 cells expressing endogenous L1-CAM (Fig. 4*C, right*). In addition, cells transfected with ADAM10 displayed an increase in cell-associated cleaved L1-CAM (Fig. 4*C, arrowhead*) and enhanced shedding of L1-CAM into the culture medium (Fig. 4*C, Medium*), indicative of increased ADAM10-mediated L1-CAM cleavage in such cells. Mice injected in their spleen with HCT116 cells expressing the empty vector did not form (in this experimental model) detectable metastases (n = 0 of 9), although they formed local tumors in the spleen (Fig. 4*D, right, black arrows*). This results from the longer incubation time and injection of higher cell number required for metastasis (five times more cells and 2 months are required to detect metastases), probably because the expression of endogenous L1-CAM in HCT116 cells is lower that in Ls174T cells stably transfected with L1-CAM (Fig. 1*B*). However, HCT116 cells transfected with ADAM10 formed metastases in the livers of all injected animals (n = 5 of 5; P < 0.001; Fig. 4*D, white arrows*), in addition to primary tumor growth seen in the spleen at the site of injection. We conclude that ADAM10 and L1-CAM can form a molecular complex, and ADAM10 expression in HCT116 cells enhances L1-CAM cleavage and shedding and increases the capacity to form liver metastases.

Next, we wished to determine whether ADAM10 enhances the metastatic capacity also in human colon cancer cells that already overexpress a transfected L1-CAM. For this, we stably transfected ADAM10 into Ls174T cells previously transfected with L1-CAM (Fig. 5*C*). Injection of these Ls174T cell clones into the spleen of mice revealed that cells expressing both L1-CAM and ADAM10 not only formed metastases in the liver (n = 5 of 5) but these were larger than those formed by cells transfected with L1-CAM alone (n = 5 of 5, P < 0.001, when compared with control cells, n = 0 of 4; Fig. 5*A, B*, and *D*). In such doubly transfected cells, we observed an increase in L1-CAM shedding into the culture medium (Fig. 5*C, top, lane 3*). The cells of such cultures contained mostly the cleaved L1-CAM form with an apparent lower molecular weight (Fig. 5*C, lane 3, white arrowhead*). Together, these results suggest that ADAM10 expression enhances the cleavage of L1-CAM and the metastatic capacity of colon cancer cells expressing endogenous L1-CAM and also the metastasis of colon cancer cells transfected with L1-CAM.

Identification of genes induced *in vitro* by L1-CAM in colon cancer cells and measurement of their expression patterns in human colon carcinoma tissue. To begin elucidating the mechanisms by which L1-CAM confers metastatic potential in colon cancer cells, and the relevance of such mechanisms to

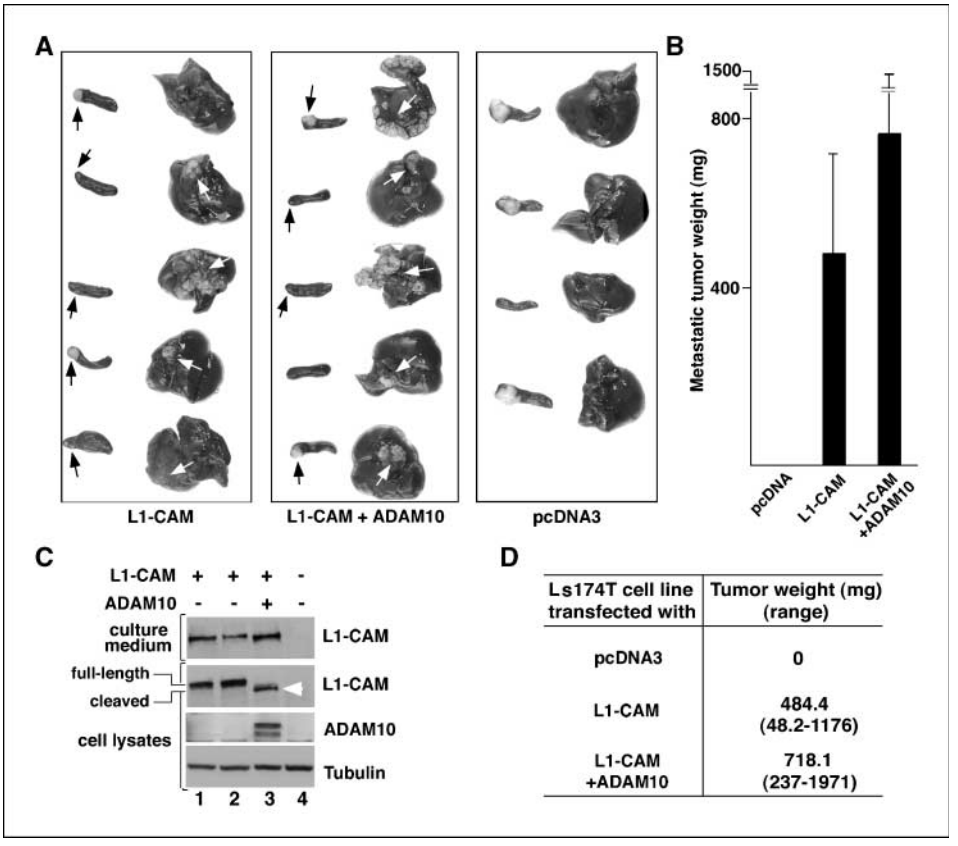

Figure 5. ADAM10 expression in Ls174T cells transfected with L1-CAM enhances their metastatic capacity. *A,* Ls174T cells stably transfected to express HA-tagged ADAM10 and L1-CAM were injected into the spleens of nude mice and their capacity to form liver metastases was compared with Ls174T cells expressing the empty vector (pcDNA) as detailed in Fig. 1. *B,* average of total tumor weight in the liver of each injected mouse group with the different cell types. *Columns,* mean; *bars,* SD (they differ by P < 0.05). *C,* Western blot analysis showed enhanced shedding of L1-CAM in cells cotransfected with L1-CAM and ADAM10 (*lane 3*) and the production of a lower molecular weight cleaved L1-CAM product that was associated with the cell layer (*lane 3, white arrowhead*). *Lanes 1* and *2,* two Ls174T cell clones L1/1 and L1/2 shown in Fig. 1. *D,* variations in metastatic nodule weight in each mouse and the average weight of metastases in groups of mice injected with the different cell types.

human colon cancer development *in vivo*, we did DNA microarray analysis in L1-CAM–overexpressing colon cancer cells using U133A Affymetrix GeneChips. We searched for genes that were significantly overexpressed in two independent clones of L1-CAM–transfected Ls174T cells (Fig. 1*A, L1/1* and *L1/2*) compared with empty vector–transfected Ls174T cells. We identified 160 unique genes (Supplementary Table S1) that were significantly ($P < 0.05$, see Materials and Methods) overexpressed in both L1-CAM–expressing clones compared with control neo^r-expressing cells. Next, we studied the expression profiles of these 160 genes (represented by 202 probe sets) in a data set of 213 human colon tissue specimens that included 43 normal colon epithelial tissue samples and 170 carcinomas at all tumor-node-metastasis stages (I–IV) of colon cancer progression. The expression matrix of the 202 probe sets (that represent the genes induced in Ls174T cells by L1-CAM), as measured by the same type of DNA microarray, is shown in pseudocolor in Fig. 6*C*, where the red color represents high expression levels and the blue color represents low expression levels. Both the genes and tissue samples were ordered by the SPIN algorithm (28). The resulting distance matrix of the genes (Fig. 6*A*) clearly shows two well-defined gene clusters: a smaller cluster with higher expression levels in normal colon tissue and a larger cluster (marked by the *black box* in Fig. 6*A*) with lower expression in normal tissue samples. The latter cluster contains 68 unique genes (Fig. 6*B*; *yellow highlights* in Supplementary Table S1), known for their involvement in nucleotide and DNA metabolism and repair, lipid and steroid metabolism, and protein synthesis. Expression levels of the genes in this cluster are low in normal colon epithelial samples (Fig. 6*C, orange* in the color bar below the expression matrix). We conducted semiquantitative reverse transcription-PCR (RT-PCR) analysis to validate changes in RNA levels for several of the genes in this cluster (labeled in *italics* and *boxed* in Fig. 6*B*) and found that their expression was induced in both L1-CAM–expressing Ls174T cell clones when compared with the control Ls174T neo^r clone (Fig. 6*D*). These results suggest that L1-CAM expression in human colon cancer cells induces the expression of a group of genes also induced in human colon carcinoma tissue, indicative of the physiologic relevance of L1-CAM overexpression in the gene patterns characteristic of human colon cancer tissue.

Discussion

The present study showed that expression of the neuronal cell adhesion receptor L1-CAM in human colon cancer cells confers metastatic capacity to the liver in mice injected with tumor cells into their spleen. Transfection of ADAM10, a metalloproteinase that cleaves and causes L1-CAM ectodomain shedding (31, 35), also induced metastatic capabilities in colon cancer cells. In addition, we showed that *ADAM10*, similar to L1-CAM (24), is a target gene of β-catenin-TCF signaling. Because both L1-CAM and ADAM10 were detected at the invasive front of human colon cancer tissue (24), and ADAM10 expression was recently correlated in a colon cancer tissue array analysis with higher tumor stage (36), our studies support the view that these β-catenin target genes

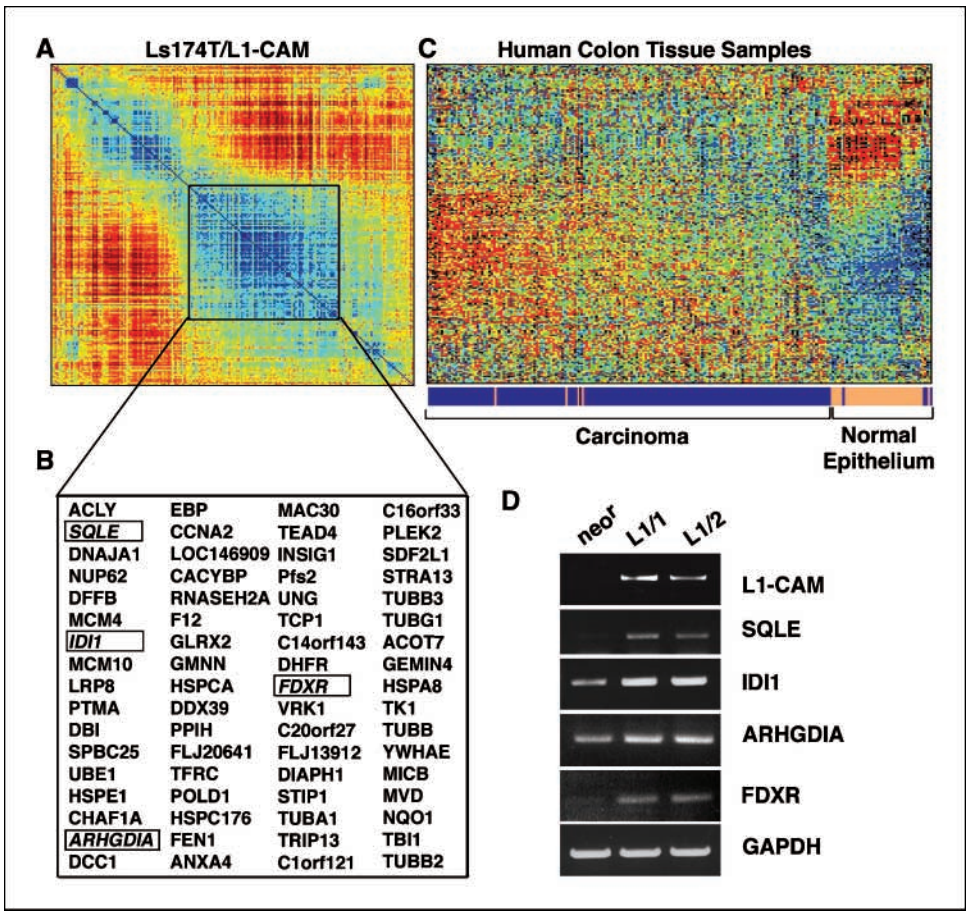

Figure 6. L1-CAM expression in Ls174T cells increases the levels of a cluster of genes also elevated in human colon carcinoma tissue. U133A Affymetrix GeneChips were used to analyze the expression of genes induced in two independently derived Ls174T cell lines (L1/1 and L1/2) and compared with control neo^r Ls174T cells. In the list of genes that differ by $P < 0.05$ from control, 160 unique genes (represented by 202 probe sets on the microarray) were identified and clustered (see Supplementary Table S1). The expression levels of these probe sets in a data set of U133A microarrays, consisting of 170 human colon carcinoma and 43 normal colon epithelial tissue samples, are shown in (*C*), in pseudocolor where *red* is high and *blue* is low expression level. Each *row* corresponds to a probe set (gene) and each *column* to a sample. The tissue type is identified by the color bar below the expression matrix, with *dark blue* and *orange* representing carcinoma and normal tissue, respectively. *Rows* and *columns* were ordered by the SPIN algorithm. The resulting distance matrix of the probe sets shown in (*A*) reveals gene clusters with different expression profiles. A group of 68 genes (*blue color, boxed area*), listed in (*B*), which were overexpressed in L1-CAM–transfected Ls174T cells, was found to also be elevated in a significant number of human carcinoma patients, but its expression was low in normal colon epithelium. *D,* semiquantitative RT-PCR for some of the genes elevated in this cluster (individually boxed in *B*) was carried out using samples of the two Ls174T cell lines (*L1/1* and *L1/2*) expressing L1-CAM and compared with that in neo^r control Ls174T cells.

significantly contribute to the development of the invasive stage of colon cancer. Interestingly, although Twist was shown to be a target of Wnt/β-catenin signaling (37), and a role for Twist in breast cancer metastasis was suggested in a recent study (29), its expression in colon cancer cells did not promote metastasis in our animal model system.

Although both L1-CAM and ADAM10 are target genes of the Wnt/β-catenin pathway, analysis of their role in transgenic mouse models with hyperactive Wnt signaling is not expected to be informative because such mice do not recapitulate the late stages in colon cancer development (especially metastasis to the liver; ref. 38). In contrast, metastasis to the liver of colon cancer cells is a key step in the pathogenesis of CRC in humans. The experimental model we used, consisting of injecting CRC cells into the distal tip of the spleen to form a primary tumor followed by metastasis of the cells into the liver, measures the more general capacity of these cells to metastasize. In several previous studies, this system was presented as a model for measuring hepatic metastasis (39, 40). Because the cells were not injected directly into the bloodstream (in contrast to many other models for metastasis), but into the spleen, the cells first formed a primary tumor, which, in most cases, did not result in further spread. Only at later stages, with some cell lines, the cells metastasized to the liver via hematogenous spread via the splenic vein to the portal system and to the liver where they grew in the liver parenchyma. This model, therefore, could measure liver metastasis by a more limited definition.

We also compared, by gene expression arrays, the genes induced by L1-CAM in colon cancer cells to those elevated in a large set of human colon carcinoma tissue samples. This analysis identified a large cluster of genes shared by L1-CAM–expressing colon cancer cells and carcinoma tissue samples but not by normal colon epithelial tissue. Together, our results suggest that L1-CAM contributes significantly to the pathophysiologic aspects of CRC development by inducing genes that are relevant to colon carcinoma progression and which can serve as novel markers for diagnosis and targets for therapy.

We began addressing the molecular mechanisms involved in these complex processes conferred by L1-CAM in colon cancer cells that endow them with metastatic potential by using deletion mutants of L1-CAM that remove the extracellular or intracellular domain of the molecule. Our observations indicated that the capacity to both enable growth in low serum and induce metastasis required the full-length L1-CAM molecule. These results agree with a recent study where these L1-CAM mutants were expressed in human breast cancer cells, and in such cells again, only full-length L1-CAM increased cell motility and scattering (25). In addition, ADAM10 expression enhanced the cleavage and shedding of both the endogenous L1-CAM ectodomain in HCT116 human colon cancer cells and conferred metastasis and also enhanced both L1-CAM shedding and the metastatic capacity of Ls174T colon cancer cells stably transfected to overexpress L1-CAM. Because ADAM10 was reported to cleave a wide variety of other cell surface receptors, including members of the cadherin family (32–34), it remains to be determined whether its metastasis-promoting effect in CRC cells is solely mediated by L1-CAM cleavage.

Recent studies have used a fine mutational analysis of L1-CAM, including point mutations in the cytodomain of the molecule to unravel some of the L1-CAM cytoplasmic partners by which L1-CAM affects neuronal cell extension and branching (30). Further studies using such and other L1-CAM mutants will shed more light on the molecular mechanisms by which L1-CAM induces metastasis in CRC cells.

Acknowledgments

Received 3/15/2007; revised 5/20/2007; accepted 6/1/2007.

Grant support: Israel Science Foundation, German-Israeli Foundation for Scientific Research and Development, Yad Abraham Center for Cancer Diagnosis and Therapy, Delores and Eugene M. Zemsky Weizmann-Johns Hopkins Research Program (A. Ben-Ze'ev), Ridgefield Foundation, and National Cancer Institute under Program Project Grant 5 P01 CA 565930-06.

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We thank V. Lemmon, M. van de Wetering, and I. Gitelman for reagents; S. Saban and R. Eilam-Alstadler for help with DNA microarray analysis and tissue immunohistochemistry; and W.L. Gerald for providing the RNA samples for the expression measurements of the cancer and normal colon tissues.

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