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The Cell Adhesion Nectin-Like Molecules (Necl) 1 and 4 Suppress the Growth and Tumorigenic Ability of Colon Cancer Cells

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ABSTRACT

A key step in human colon cancer development includes the hyperactivation of Wnt/β-catenin signaling and the induction of β-catenin-TCF target genes that participate in colon cancer progression. Recent studies identified members of the immunoglobulin-like cell adhesion molecules (IgCAM) of the L1CAM family (L1 and Nr-CAM) as targets of β-catenin-TCF signaling in colon cancer cells. L1 was detected at the invasive front of colon cancer tissue and confers metastasis when overexpressed in cells. In contrast to L1, we did not detect in colon cancer cells significant levels of another IgCAM family of molecules, the nectin-like (Necl) receptors Necl1 and Necl4, while Necl4 was previously found in the normal small intestine and colon tissues. We studied the properties of colon cancer cells in which Necl4 and Necl1 were expressed either alone, or in combination, and found that such cells display a wide range of properties associated with tumor suppression. Expression of both Necl1 and Necl4 was the most efficient in suppressing the tumorigenicity of colon cancer cells. This was associated with enhanced rates of apoptosis and change in several apoptosis-related markers. In contrast to its capacity to suppress tumorigenesis, Necl4 was unable to affect the highly malignant and metastatic capacities of colon cancer cells in which L1 was overexpressed. Our results suggest that various IgCAM receptor families play different roles in affecting the tumorigenic function of the same cells, and that Necl1 and Necl4 can fulfill a tumor suppressive role. J. Cell. Biochem. 108: 326–336, 2009.

KEY WORDS: Necls; CELL ADHESION; COLON CANCER; TUMORIGENESIS; METASTASIS

Aberrant activation of Wnt/β-catenin signaling and consequently, the induction of β-catenin-TCF target genes are key factors in the initiation and development of colon cancer in most human patients [Clevers, 2006; Polakis, 2007]. Recent studies identified members of the immunoglobulin-like cell adhesion molecules (IgCAMs) of the L1CAM family (Nr-CAM and L1) as targets of β-catenin-TCF signaling in colon cancer cells that confer enhanced motility and tumorigenesis [Conacci-Sorrell et al., 2002; Gavert et al., 2005]. While these IgCAMs are mostly known for their function in nerve cells, playing a role during brain development [Kamiguchi et al., 1998; Kenwrick et al., 2000], the expression of L1 in colon cancer cells induces metastasis to the liver, and L1 was detected at the invasive front of human colon cancer tissue [Gavert et al., 2007]. Another family of IgCAM receptors functioning mostly in nerve cell adhesion and recognition is the nectin-like family of molecules (Necl, also known as SynCAM/IGSF4/CADM) that fulfill important roles in the nervous system in processes like myelination [Maurel et al., 2007; Spiegel et al., 2007; Takai et al., 2008] and synapse formation [Biederer et al., 2002]. Members of this family, especially Necl2 (also known as TSLC1/SynCAM1), were implicated in tumorigenesis displaying a tumor suppressive function in lung and various other types of human cancer [Fukuhara et al., 2001; Kuramochi et al., 2001; Ito et al., 2003; Steenbergen et al., 2004; Murakami, 2005; Lung et al., 2006; Williams et al., 2006; Heller et al., 2007]. Recently, Necl4 (TSLL2) was suggested to act as a tumor suppressor in prostate cancer cells [Williams et al., 2006]. In contrast, Necl5 (also known as PVR/CD155), a more distant member of the Necl family possessing a very different cytoplasmic domain, plays an oncogenic role [Chadeneau et al., 1996; Masson et al., 2001; Morimoto et al., 2008; Takai et al., 2008]. In the present study, we investigated the effects of Necl1 and Necl4 expression in colon cancer cells and found that these IgCAMs confer a variety of cellular properties compatible with tumor suppressive functions in colon cancer cells.

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MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

LS174T and HCT116 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS). HT29 and SW480 cells (and all other cell lines) were grown in DMEM with 10% FCS, and SW620 cells in Leibowitz's L-15 medium with 10% FCS, 1% glutamine, 1% sodium bicarbonate and 1% Hepes buffer. LS174T-Necl4 cells were maintained in the presence of neomycin (800 μg/ml), and LS174T Necl4 + Necl1 and LS174T L1 + Necl4 in the presence of both neomycin (800 μg/ml) and puromycin (10 μg/ml). Primary rat Schwann cells (RSC) were grown on Primeria plates (Falcon) in DMEM (high glucose and pyruvate) with 3% FCS, 5% NDF-β-conditioned medium and 2 μM forskolin (Sigma). NDF-β-conditioned medium was obtained from CHO cells overexpressing NDF-β by growing the cells in DMEM/F12 containing 1% FCS, and collecting the conditioned medium.

PLASMIDS, EXPRESSION VECTORS, AND TRANSFECTIONS

The pcDNA3-Necl4, pcDNA3-Necl1, pSX-Necl1, pSX-Necl2, pCX-Necl3, pCX-Necl4, and pCX-Necl5 expression vectors were prepared as described [Spiegel et al., 2007]. The pcDNA3-puro^r-Necl1 plasmid was generated by cloning the Necl1 cDNA from the pcDNA3-Necl1 plasmid into the pcDNA3 puro^r vector at the *Hin*dIII/*Xba*I sites. The pBabe-puro^r-Necl4 plasmid was generated by amplifying Necl4 from the pcDNA3-Necl4 vector using Necl4-SnaBI forward (ACTTTACGTAAAGCTTGCCACCATGGGCCG) and Necl4-SalI reverse (ACGCGTCGACGTAGGGTCAAATGAAGAATT) primers and cloning the Necl4 amplified fragment into pBabe-puro^r at the *Sal*I/*Sna*BI cloning sites. Transfection of LS174T cells was performed with Lipofectamine^{TM} 2000 (Invitrogen). Retroviral infections were conducted using the pBabe-puro^r and pBabe-Necl4 vectors. Retroviruses were produced by transient transfection of Phoenix-eco cells with the retroviral vectors together with the ecotropic packaging vector pSV-Ψ-E-MLV, and virus-containing medium was collected 48 h after transfection and used for infecting B16F10 cells in the presence of polybrene (8 μg/ml). LS174T cells stably expressing Necl4 were obtained by transfecting 5 μg pcDNA3-Necl4, using Lipofectamine^{TM} 2000, followed by selection with 800 μg/ml neomycin. LS174T expressing both Necl4 and Necl1 were obtained by transfecting LS174T-Necl4 cells with pcDNA3-puro-Necl1 and selection with 10 μg/ml puromycin. LS174T cells stably expressing both L1 and Necl4 were isolated by transfecting LS174T cells expressing L1 with the pcDNA3-Necl4 and selection with 10 μg/ml puromycin.

SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted from cells using the EZ-RNA kit (Biological Industries), reverse-transcribed using the SuperScriptII reverse transcriptase (Gibco), and analyzed for Necl4 and Necl1 expression by PCR (Necl4 forward: GTACGTGCTGGATGTGCAGT, Necl4 reverse: AACCGACGTCTGAGCCTCTA, Necl1 forward: ATCTTCAC-TATGCCTGTGCG, Necl1 reverse: AGAGATTCATGGTTCACAGA). GAPDH was amplified as a control (GAPDH forward: ACCACAGTC-CATGCCATCAC, GAPDH reverse: TCCACCCTGTTGCTGTA). PCR products were analyzed by 1% agarose gel electrophoresis.

WESTERN BLOT ANALYSIS

Cells were washed and harvested with ice-cold PBS, pelleted by centrifugation (at 4°C) and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS) supplemented with the Complete Protease Inhibitor Cocktail (Roche) and phosphatase inhibitors cocktails 1 and 2 (Sigma–Aldrich). The cell lysates were incubated on ice and centrifuged at 4°C. Protein concentration in the supernatants was determined using the BCA^{TM} Protein Assay Kit (Thermo Scientific). Proteins (50 μg/lane) were separated by 10% SDS–PAGE, transferred to membranes, incubated with the relevant antibodies and the signals were visualized by the ECL method.

CELL GROWTH, CELL CYCLE, COLONY FORMATION, CELL ATTACHMENT, AGGREGATION, WOUND HEALING, ANNEXIN V AND NECL-Fc BINDING ASSAYS

Cell growth was determined by seeding 10^4 cells into 24-well plates in triplicates, in the presence of 10% or 0.5% serum. Cell number in triplicates was determined every day. In some experiments, cell growth was determined by the MTT assay (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) that determines the number of viable cells. Cell cycle analysis was conducted with propidium iodide-stained cells using the FACScan and the CellQuest program. Colony formation capacity was examined by seeding 8×10^3 cells in 24-well plates, in triplicates. Cells were grown for 2 weeks and then stained with Giemsa. Cell aggregation was determined in cells dissociated with a solution containing 0.4% Trypsin XI and 1 mM EDTA in Hepes buffered Ca^{2+}- and Mg^{2+}-free PBS. The dissociated cells were seeded on plates precoated with 2% BSA and rotated on a gyrator shaker at 37°C at 70 rpm. The number of aggregates containing four or more cells was counted every 10 min. Initial cell attachment to the culture dish, after seeding and incubating cells at 37°C for the indicated time periods, was determined by removing the medium and unattached cells, rinsing the cells and counting the number of cells that remained attached to the dish.

The wound-healing assay was performed by first introducing an artificial wound into a confluent cell culture using a micropipette. Then, the cell culture medium was replaced with fresh medium containing 0.1 μg/ml Mitomycin C to inhibit cell proliferation. Pictures were taken at 0 time and 24 h after introducing the wound, and the extent of wound closure was determined. For Annexin V staining, the cells were harvested with trypsin and stained with FITC-conjugated Annexin V and propidium iodide using the Phosphatidylserine Detection^{TM} kit (IQ products) according to the manufacturer's protocol. Detection of cells labeled with Annexin V by fluorescence-microscopy was carried out using the Annexin V-FLUOS Staining kit (Roche).

Medium containing the Necl Fc-fusion proteins was obtained by transfecting 293T cells with pSX-Necl1, pSX-Necl2, pCX-Necl3, pCX-Necl4, or pCX-Necl5. Binding assays were conducted by incubating cells expressing Necl4 (in the dark) with medium containing the Fc-fusion proteins that were pre-incubated with anti-human Fc-Cy3 (Jackson Laboratories). Unbound proteins were removed by washes and the cells were permeabilized and fixed with 0.5% Triton X-100 and 3% paraformaldehyde in PBS.

TUMOR FORMATION AND METASTATIC CAPACITY

Tumor formation was determined by injecting 2×10^6 cells subcutaneously into both flanks of 4-weeks old male CD1-nude mice, experimental cells into one flank, and control cells into the other flank. Mice were sacrificed after 2 weeks and tumor weight was determined. In metastasis assays, the ability of cells to metastasize to the liver was determined by injecting 10^6 cells in 20 μl PBS into the distal tip of the spleen of 6-weeks old male mice. Mice were anesthetized by peritoneal injection of 1 μl/mg Xylazine (20 mg/ml) and 1 μl/mg Ketamine (100 mg/ml). Animals were sacrificed after

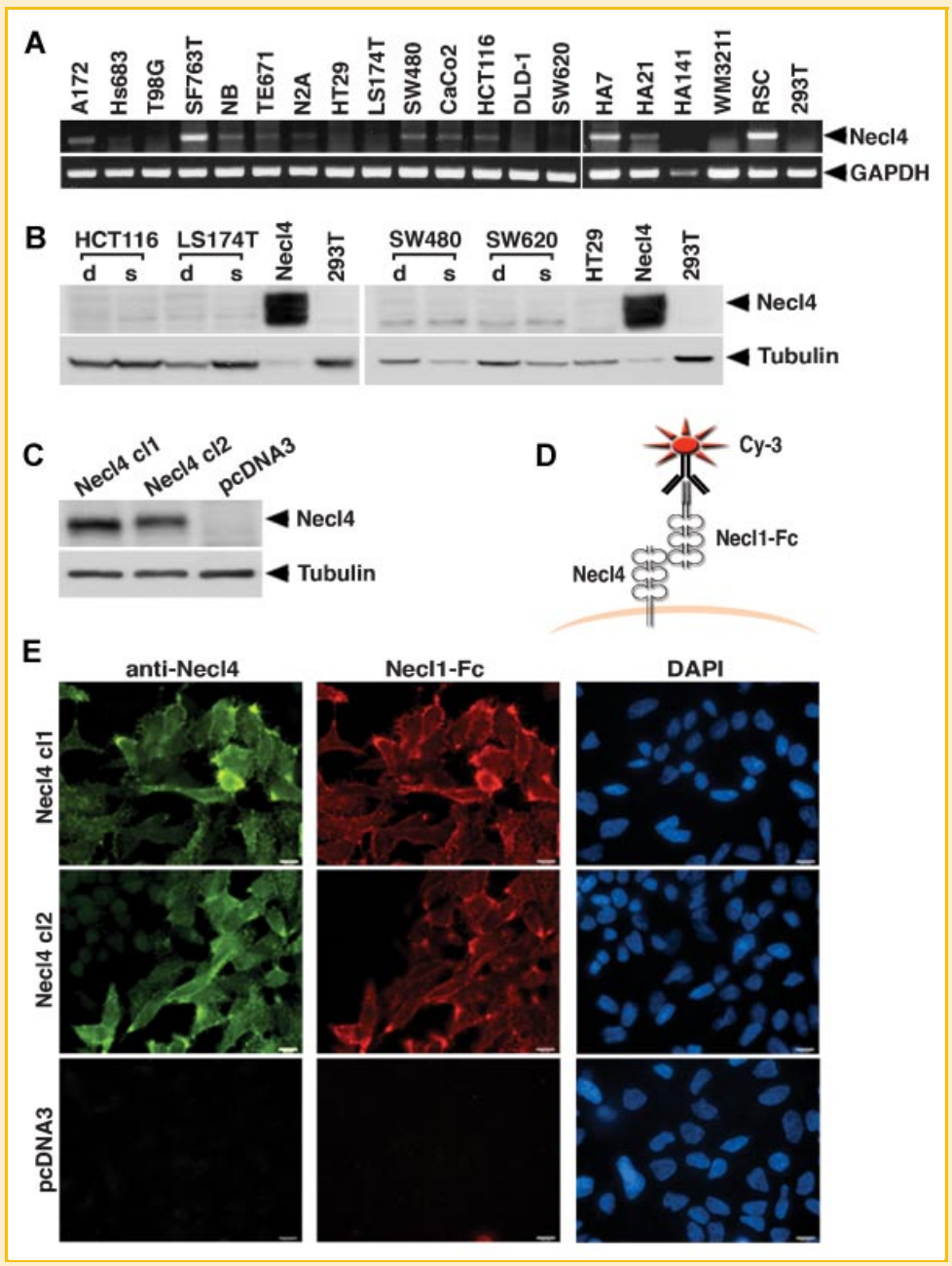

Fig. 1. Necl4 expression in colon cancer cells. A: Necl4 RNA levels in different cell lines of human melanoma (HA7, HA21, HA141, WM3211), neuroblastoma (NB, N2A), glioblastoma (A172, T985, Hs683, SF763T), medulloblastoma (TE671) and colon cancer (HT29, LS174T, SW480, CaCo2, HCT116, DLD-1, SW620) was determined by RT-PCR. Rat Schwann cells (RSC) and 293T cells were used as positive and negative controls for Necl4 expression, respectively. GAPDH served as a control for RNA levels. B: Necl4 protein levels in the colon cancer cell lines HCT116, LS174T, SW480, SW620, seeded as sparse (s) and dense (d) cultures, and in HT29 cells, were determined by Western blot analysis using a polyclonal antibody against Necl4. 293T cells transiently transfected with Necl4 served as positive control and tubulin as loading control. C: LS174T cells were stably transfected with a Necl4 expression plasmid, or the empty vector, and the levels of Necl4 in two independent cell clones, and a control cell clone (expressing the empty pcDNA3) were determined by Western analysis. D: Diagram describing the binding assay of Necl1-Fc to Necl4 expressing cells. The binding of Necl1-Fc was visualized with anti-Fc-Cy3 antibody. E: Immunofluorescence staining of Necl4 in LS174T-Necl4 clones and control cells expressing the empty vector using anti-Necl4 antibody (green) and Necl1-Fc binding (red). Nuclei were stained with DAPI (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

5 weeks, and tumor formation at the site of injection in the spleen and metastasis development in the liver were determined.

RESULTS

ISOLATION OF NECL4 OVEREXPRESSING COLON CANCER CELLS

Since Necl4 was previously detected in normal small intestine and colon tissues [Fukuhara et al., 2001], we wished to determine the levels of Necl4 protein in a variety of colon cancer cell lines (SW480, SW620, HCT116, LS174T, and HT29) under varying conditions of cell density. These conditions were previously shown to modulate in such cells the expression of other IgCAMs (i.e., L1) [Gavert et al., 2005] and cell adhesion receptors such as E-cadherin [Conacci-Sorrell et al., 2003]. The results shown in Figure 1B demonstrate that we could not detect significant levels of Necl4 protein in any of the colon cancer cell lines analyzed (Fig. 1B). Similar results were obtained when the levels of Necl4 RNA in colon cancer and a variety of other types of cancer cell lines derived from the nervous system were analyzed (Fig. 1A). This suggested that Necl4 expression is downregulated in these cancer cells from the nervous system, as also observed in prostate cancer cells [Williams et al., 2006]. To analyze the possible functions of Necl4 in human colon cancer cells, we isolated LS174T colon cancer cell clones stably expressing a transfected full length Necl4 cDNA (Fig. 1C). The cell surface localization of Necl4 in the transfected cells was verified based on the known specific interaction of Necl1 with Necl4 [Maurel et al., 2007; Spiegel et al., 2007], by adding Necl1-Fc fusion protein to Necl4-transfected cells for a short time, followed by immunofluorescent staining of cells with antibody against the Fc-fusion domain (Fig. 1D). The same cells were also immunostained with an antibody against the cytoplasmic domain of Necl4 (Fig. 1E). The co-staining of Necl4 by both methods (Fig. 1E) indicated that the transfected Necl4 molecules are localized, as expected, on the cell surface. We also studied the capacity of Necl4 expressing cells to bind other Necl family member Fc-fusion proteins and found, in agreement with previous studies with cells of the nervous system, that in addition to Necl1, Necl4 expressing cells effectively bound Necl3, but not Necl2, Necl4, or Necl5 (Fig. 2) [Maurel et al., 2007; Spiegel et al., 2007; Takai et al., 2008]. None of the Fc-fusion Necl molecules bound to colon cancer cells expressing the empty vector (data not shown).

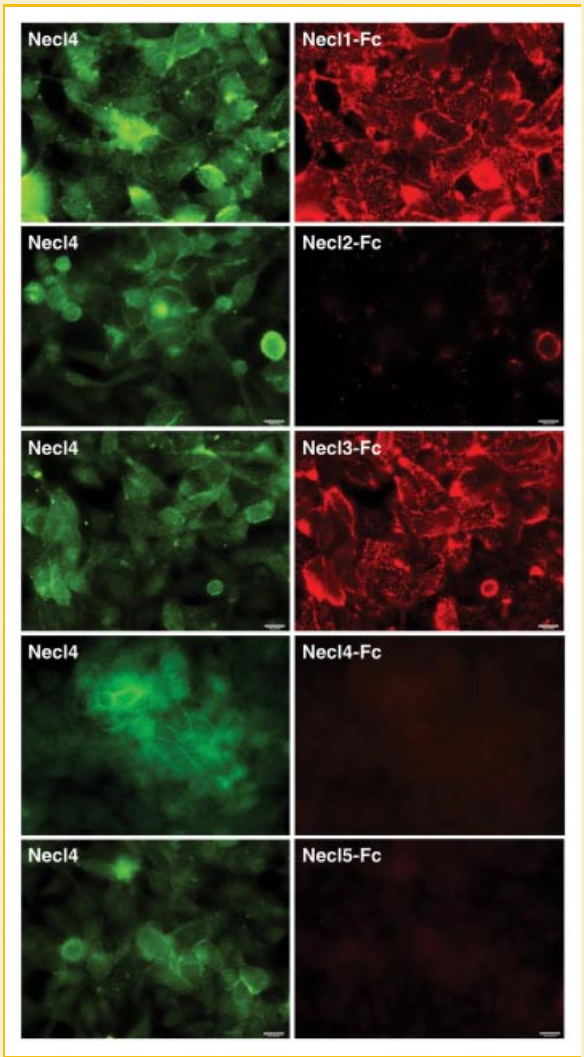

Fig. 2. Necl4 expressed in colon cancer cells forms heterophilic interactions with Necl1 and Necl3, but not homophilic interactions. The binding of Necl4 to other Necl family members was visualized by the binding of Fc-fusion proteins to LS174T-Necl4 cells using a secondary antibody against human-Fc (red). Cells were also stained for Necl4 using anti-Necl4 antibody (green). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TRANSFECTION OF NECL4 INHIBITS CELL GROWTH, COLONY FORMATION, INITIAL CELL ADHESION, AGGREGATION AND MOTILITY

We wished to determine the effects of Necl4 expression in colon cancer cells on cellular properties associated with tumorigenesis. Analysis of cell growth rates in cells cultured under stressful conditions (in the presence of 0.5% serum) revealed that cell clones stably expressing Necl4 grew more slowly than their control counterparts (Fig. 3A). The Necl4 containing colon cancer cells also less efficiently formed colonies when seeded at low (clonal) density compared to cells transfected with the empty vector (Fig. 3B). The reduced capacity to form cell colonies after Necl4 transfection was also observed with a different cell type, the highly metastatic B16F10 mouse melanoma cell line (Fig. 3C). When these cells were transduced with Necl4, using a retroviral promoter-based plasmid (pBabe-Necl4), cells expressing Necl4 were almost sevenfold less efficient in forming colonies compared to control transfected cells (Fig. 3C), implying a more general capacity of Necl4 to confer reduced colony formation. Next, we analyzed the motile capacity of the cells by measuring their ability to close an artificial wound introduced in a confluent monolayer, and found that two independently isolated Necl4 expressing cell clones were slower in closing such "wounds" compared to control cells (Fig. 3D). The ability of single cells in suspension to rapidly form multicellular aggregates under rotating conditions is considered a characteristic property of many cancer cells. As shown in Figure 3E, this aggregation capacity was significantly reduced in cells transfected with Necl4. Finally, cancer cells rapidly adhere to the culture dish when seeded as single cells, while untransformed cells are slower in

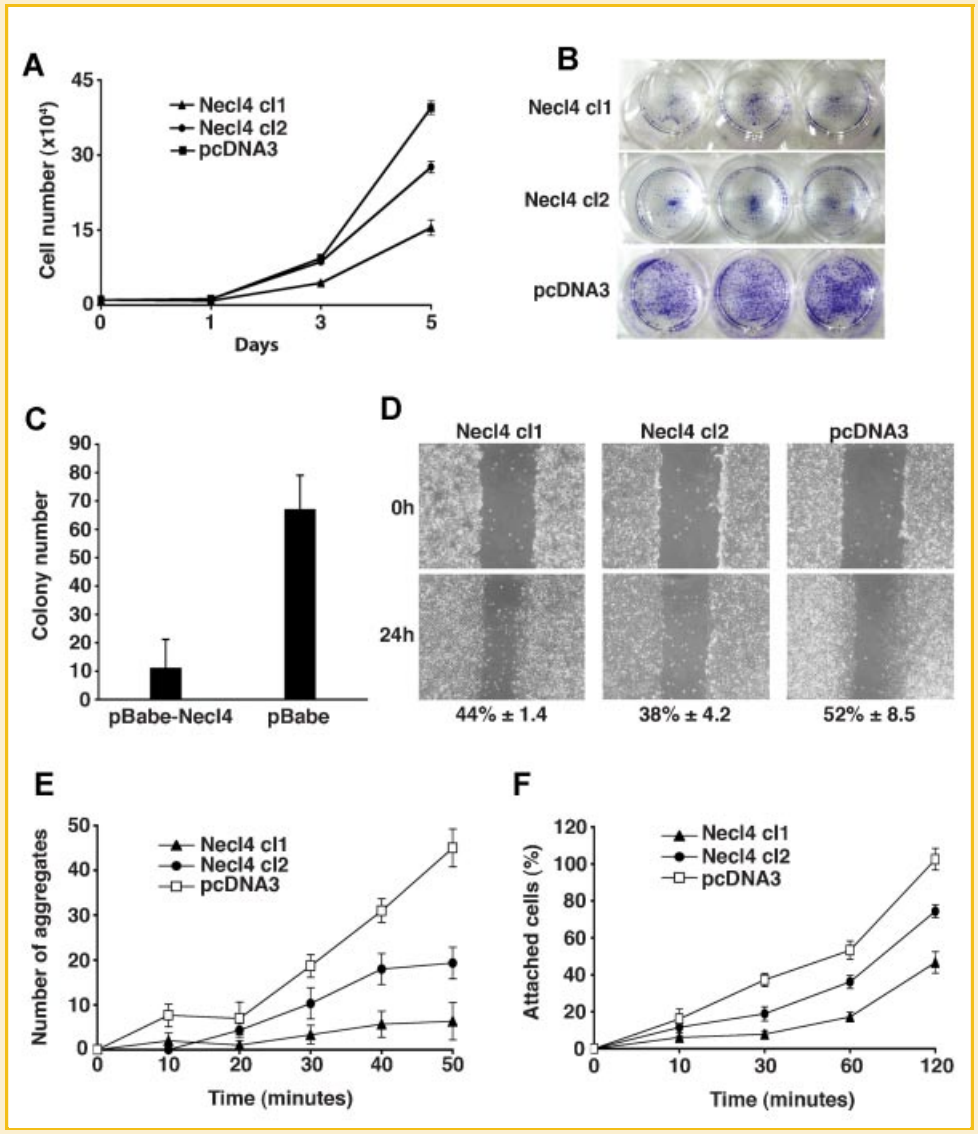

Fig. 3. Necl4 overexpression inhibits cell proliferation, migration, initial cell–cell aggregation and cell-substrate adhesion. A: Growth rates of Necl4 overexpressing cells in the presence of low serum (0.5%) containing medium. B: Colony formation capacity of LS174T-Necl4 clones seeded at clonal density in triplicates, and stained after 2 weeks with Giemsa. C: Colony formation by B16F10 cells and B16F10-Necl4 cells produced by retroviral transduction (pBabe-puro^r) under selection with 0.25 μg/ml of puromycin. D: The ability of LS174T-Necl4 clones and LS174T-pcDNA3 control cells to close an artificial wound was determined. Representative wounds and percent wound closure from three independent experiments are shown. Pictures of the same fields were taken at 0 and 24 h after wounding. E: Suspended LS174T-Necl4 cells and LS174T-pcDNA3 control cells were examined for their ability to form aggregates of four or more cells under gentle shaking conditions. F: Initial adhesion to the substrate was determined by plating LS174T-Necl4 and LS174T-pcDNA3 control cell clones on dishes, and counting the number of attached cells at different times after seeding the cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

this ability. The studies summarized in Figure 3F demonstrate a reduced initial cell to substrate adhesion rate in Necl4 expressing cells at early times after their seeding. Together, these studies indicate that the expression of Necl4 in colon cancer cells reduces their potential to display properties characteristic of tumor cells.

NECL4 SUPPRESSES THE TUMORIGENIC CAPACITY OF COLON CANCER CELLS IN MICE

The results described above support the hypothesis that the introduction of Necl4 into colon cancer cells might confer tumor suppressive effects. To test this hypothesis more directly, nude mice were injected subcutaneously with both control cells, on one flank, and with a similar number of Necl4-expressing cells on the other flank. The results summarized in Figure 4 show that in each injected mouse the size of the tumors formed by cells expressing Necl4 is smaller compared to tumors formed by the control cells (Fig. 4A). A similar result was obtained with two independently isolated clones of Necl4-expressing LS174T cells (Fig. 4B), demonstrating a decrease of about fourfold in total tumor weight with Necl4-expressing cells.

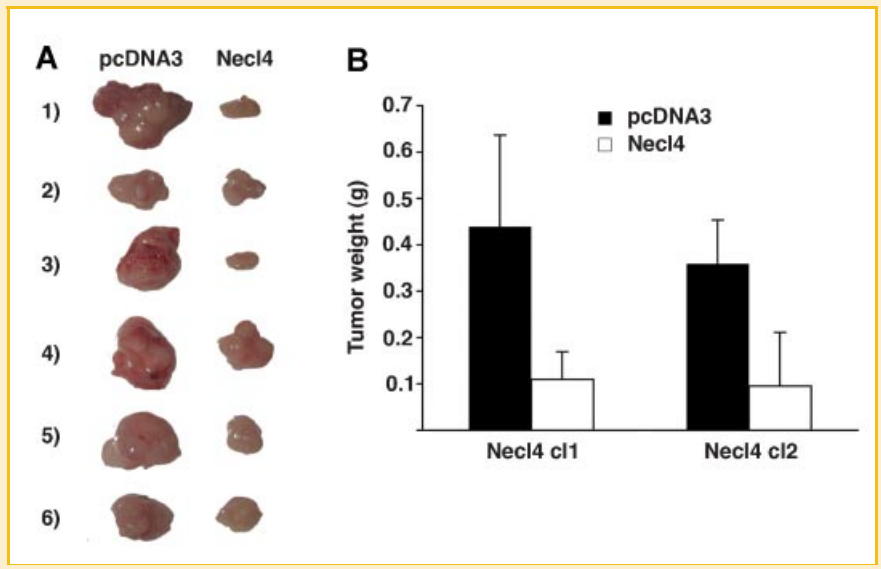

Fig. 4. Necl4 inhibits the growth of tumors formed by colon cancer cells in nude mice. A: LS174T-Necl4 and LS174T-pcDNA3 control cells were injected into groups of six mice: in one flank, the LS174T-Ncl4 cells and in the other flank, of the same mice, the control cells. The mice were sacrificed after 2 weeks, the tumors excised and photographed, and tumor weight was determined (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NECL1 AND NECL4 ADDITIVELY SUPPRESS THE TUMORIGENESIS OF COLON CANCER CELLS

Necl4 was shown to operate in the nervous system mostly via a heterophilic interaction with Necl1 [Maurel et al., 2007; Spiegel et al., 2007]. Since we found that LS174T-Necl4 cells can bind Necl1-Fc (Fig. 1), we wished to determine whether Necl1 has an effect in colon cancer cells. We started by analyzing the levels of Necl1 in a variety of colon cancer cell lines under varying conditions of cell density. The results summarized in Figure 5B, demonstrated that Necl1 protein could not be detected in any of the colon cancer cell lines examined. Semi-quantitative RT-PCR also did not identify Necl1 RNA in any of these colon cancer cell lines, nor in a variety of cells derived from brain tumors (Fig. 5A). We wished to determine whether Necl1 could affect the tumorigenic properties of colon cancer cells when expressed alone, or in combination with Necl4 in such cells. Individual LS174T colon cancer cell clones expressing either Necl4 (Fig. 1C), or Necl 1 (Fig. 5C), and cells expressing both Necl1 and Necl4 (Fig. 5C) were isolated. Immunofluorescence analysis with antibodies specific against Necl1 and Necl4 demonstrated that these cells express the expected molecules in the majority of the cells, often at cell–cell adhesion sites (Fig. 5E, arrows). Next, we analyzed the growth properties of these cells in 0.5% serum containing medium and found that cell clones expressing both Necl1 and Necl4 display a more dramatic inhibitory effect on cell proliferation compared to cells expressing either Necl1 or Necl4 alone (Fig. 6A). These Necl1 and Necl4 expressing cells were also much more adhesive to each other and remained adherent in small clusters, even after their removal from the culture plates with trypsin (Fig. 5D), indicating increased cell–cell adhesion. Necl1 and Necl4 were additive in their capacity to suppress the growth of tumors produced by cells injected subcutaneously as was demonstrated in nude mice injected with cells transfected with Necl1, Necl4, or both Necl1 and Necl4. Colon cancer cells expressing both Necls were almost 10 times less tumorigenic than control cells (Fig. 6B). Cells expressing Necl1 alone were the least effective in suppressing tumor growth and the tumors formed by such cells grew to almost the same size as those produce by control colon cancer cells (Fig. 6B).

NECL4 AND NECL1 DO NOT AFFECT THE METASTATIC POTENTIAL OF COLON CANCER CELLS

The results described above suggested that the combined presence of Necl4 and Necl1 was more effective in suppressing the tumorigenic capacity of colon cancer cells than when each molecule was expressed alone. We have also examined the metastatic abilities of such colon cancer cells by applying a model consisting of injecting the cells into the tip of the spleen and following their metastasis to the liver. Using LS174T colon cancer cells, we have previously observed a dramatic induction in their metastatic capacity after transfection with L1 (another IgCAM family member) [Gavert et al., 2007]. The results summarized in Figure 6C show that neither Necl1 or Necl4, nor a combination of both molecules, confer the formation of liver metastases in these cells, contrary to the very effective capacity of L1 to induce metastasis. As reported previously [Gavert et al., 2007], the size of tumor growth at the injection site in the spleen did not correlate with their metastatic potential.

Next, we wished to determine whether Necl4 can suppress not only the tumorigenic capacity of colon cancer cells (Fig. 4), but whether it can reduce the metastatic potential of colon cancer cells to the liver. We employed LS174T cells stably expressing the IgCAM L1 molecule that effectively induces metastasis to the liver [(Fig. 6C), and Gavert et al., 2007], and isolated cell clones stably expressing both L1 and Necl4 (Fig. 7A). The presence of Necl4 on the surface of these cells was demonstrated by their binding of Necl1-Fc fusion protein (Fig. 7B). The proliferation of cells expressing both L1 and Necl4 in 0.5% serum was only slightly reduced compared to cells

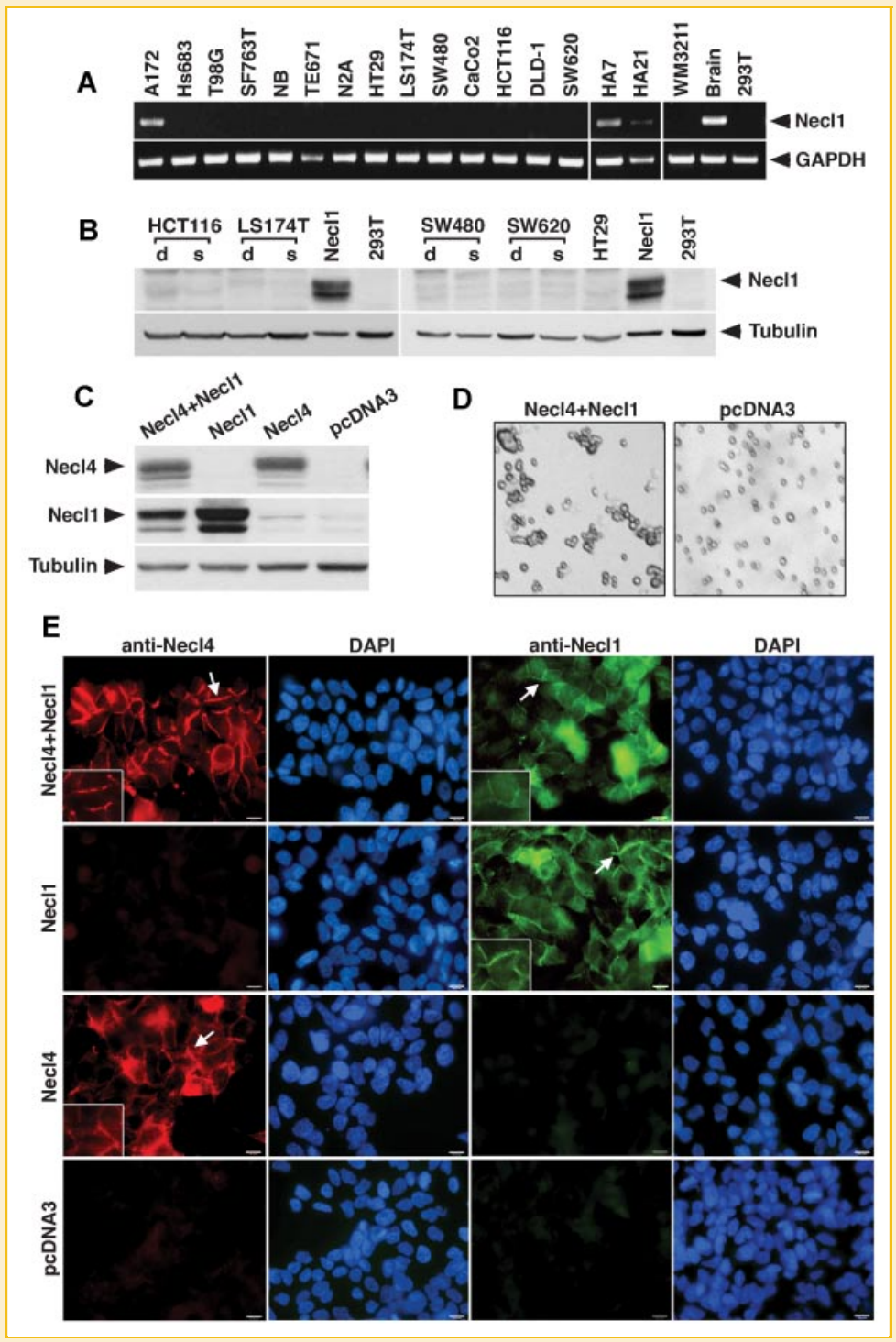

Fig. 5. Expression of Necl4 and Necl1 in LS174T cell clones. A: Necl1 RNA levels in different cell lines of melanoma (HA7, HA21, WM3211), neuroblastoma (NB, N2A), glioblastoma (A172, T985, Hs683, SF763T), medulloblastoma (TE671) and colon cancer (HT29, LS174T, SW480, CaCo2, HCT116, DLD-1, SW620) were determined by RT-PCR. Mouse brain and 293T cells were used as positive and negative controls for Necl1 expression, respectively. GAPDH served as control for equal amount of RNA. B: Necl1 protein levels in colon cancer cell lines HCT116, LS174T, SW480, SW620, seeded as dense (d) and sparse (s) cultures, and in HT29 cells, were determined by Western blot analysis using a polyclonal antibody against Necl1. 293T cells transiently transfected with Necl1 served as positive control for Necl1, and tubulin as loading control. C: LS174T cells stably transfected with Necl1, Necl4, and both Necl4 and Necl1 (Necl4 + Necl1), or with the empty vectors (pcDNA3) were subjected to Western blot analysis for Necl4 and Necl1 expression. D: Cells expressing both Necl1 and Necl4 form cell clusters after trypsinization. E: Cells were immunostained for Necl4 (with anti-Necl4 antibody, red) and for Necl1 (with anti-Necl1 antibody, green). Nuclei were stained with DAPI (blue). Arrows point to areas where Necls are found at cell–cell contact sites. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

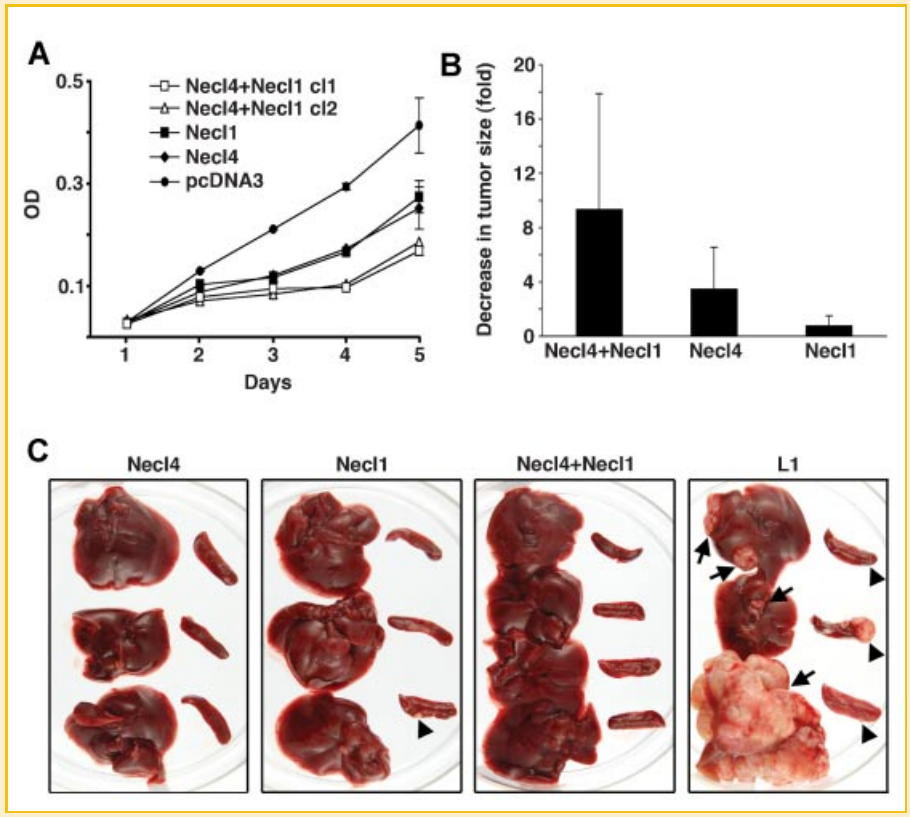

Fig. 6. Necl4 and Necl1 cooperate in the inhibition of cell proliferation and tumor growth. A: Cell proliferation in 0.5% serum was determined by the MTT assay in cells expressing Necl4, Necl1, and both Necl4 and Necl1 (Necl4 + Necl1, clones 1 and 2), or the empty vector (pcDNA3). B: Tumor formation was analyzed by injecting cells expressing Necls as described in (A) with control cells (pcDNA3) injected in one flank and Necl expressing cells in the other flank of nude mice. The data represent the ratio between tumor weight obtained with cells expressing different combinations of Necls and control cells. C: CD-1 nude mice were injected in their spleen with cells expressing different Necls, or the IgCAM molecule L1. Tumor formation in the spleen (arrowheads) and metastasis to the liver (arrows) are shown after 5 weeks. Mice injected with LS174T cells expressing L1 served as positive control for liver metastasis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expressing L1 alone (Fig. 7C), and their ability to close an artificial wound remained largely unchanged compared to cells transfected with L1 (Fig. 7D). This indicates that in the highly tumorigenic and metastatic cells produced by L1 expression, Necl4 has only a very limited tumor suppressive effect. Analysis of the metastatic potential of colon cancer cells expressing either L1, or L1 in combination with Necl4 showed that Necl4 expression had no significant effect on the capacity of L1 expressing cells to form metastases in the liver (Fig. 7E). This suggests that Necl4 expression in colon cancer cells transfected with L1 is unable to suppress the increase in cell motility and metastatic capacity conferred by L1.

NECL4 AND NECL1 EXPRESSION INCREASES APOPTOSIS IN COLON CANCER CELLS

To begin investigating the molecular mechanisms by which Necl4 and Necl1 confer tumor suppressive effects in colon cancer cells, we first determined whether their expression increases the fraction of apoptotic cells in the total cell population. We hypothesized that the decrease in the rate of cell growth under low serum conditions and the reduced capacity of cells to form colonies at clonal (low) density (Fig. 2) might indicate that cells expressing Necl1 or Necl4 are more prone to apoptosis. The results summarized in Figure 8 are compatible with this hypothesis, since we found that Necl1, Necl4 or both Necl1 and Necl4 expressing cells contained a higher proportion of the cell population in the sub G1 fraction compared to control cells (Fig. 8A), and also displayed a higher percentage of Annexin V-positive cells (Fig. 8B and C). Moreover, analysis of additional molecular markers known to be associated with apoptosis, such as the increased cleavage of PARP (Fig. 8D) and the downregulation of the antiapoptotic gene products Bcl-2 and Bcl-xL, was also observed in cells expressing Necl4 (Fig. 8E). These results suggest that Necl4 and Necl1 expression cause the decrease in cell proliferation under stress (thereby suppressing the tumorigenic capacity of colon cancer cells) by increasing the rate of apoptosis in these cells.

DISCUSSION

Previous studies have detected Necl4 RNA expression in normal human small intestine and colon tissues [Fukuhara et al., 2001], but we did not find detectable levels of Necl4 protein in a variety of human colon cancer cell lines. In this study, we have shown that the expression of Necl4 in human colon cancer cells induces a variety of

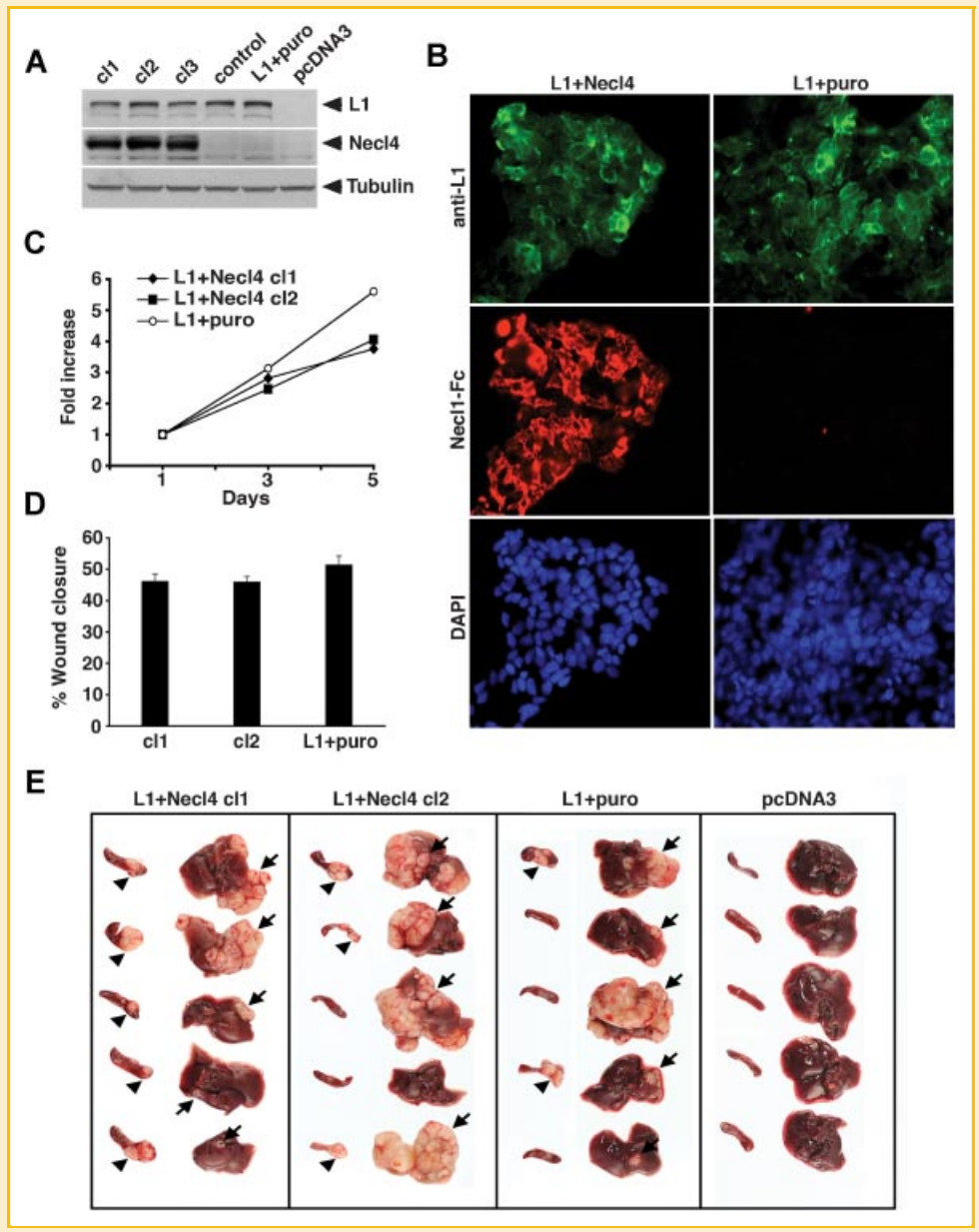

Fig. 7. Necl4 reduces the proliferation of LS174T L1 cells, but does not affect L1-mediated motility and liver metastasis. A: LS174T cells overexpressing L1 were stably transfected with Necl4. The levels of Necl4 and L1 were determined in cell clones expressing L1 alone, or both L1 and Necl4 (clones 1–3) by Western blot analysis using anti-L1 and anti-Necl4 antibodies. B: The cells were immunostained for Necl4 presence (using Necl1-Fc binding, red) and for L1 (with anti-L1 antibody, green). Nuclei were stained with DAPI (blue). C: Cell growth in 0.5% serum was determined by the MTT assay in cells expressing both L1 and Necl4 (L1 + Necl4 cl1 and cl2) or L1 alone (L1 + puro). D: The motile ability of the cell clones described in (C) was determined by the wound closure experiment. E: The metastatic spreading of the cell clones described in (A) to the liver (arrows) was determined 5 weeks after cell injection into the spleen (arrowheads) of nude mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cellular changes compatible with tumor suppression. These changes include a reduction in cell proliferation under stressful (low serum) conditions and in cell plating efficiency at clonal density, decreased rates of initial cell adhesion to the substrate and cell aggregation under rotating conditions in suspension culture, a reduced capacity to close an artificial wound in a monolayer, and a smaller tumor size in mice injected with these cells. In contrast to Necl4, when Necl1 was transfected, it only had a very weak tumor suppressive activity in these cells (Fig. 6), compatible with the restricted expression of Necl1 in brain tissue which probably limits its function to particular nerve cells [Maurel et al., 2007; Spiegel et al., 2007]. However, the tumor suppressive capacities of Necl4 were significantly enhanced in cells when Necl1, its strongest cell–cell adhesion partner, was co-transfected into the same cells. This suggests that the heterophilic adhesive interactions between these Necl adhesion receptors are responsible for their effects on cells. Such cells showed a dramatic increase in cell–cell adhesion, displaying strong immunostaining for Necl1 and Necl4 at cell–cell contact sites (Fig. 5E), and remaining

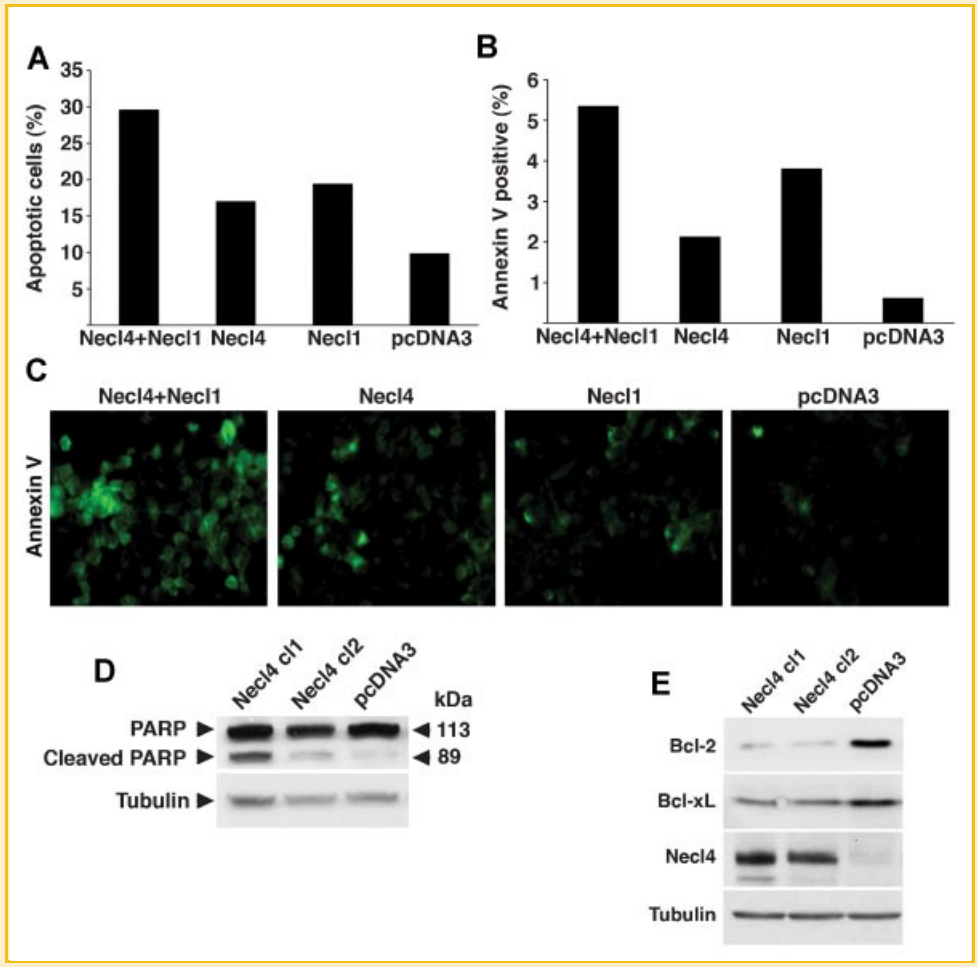

Fig. 8. Necl4 and Necl1 expression induces apoptosis in colon cancer cells. A: Cells expressing Necl4, Necl1, or both Necl4 and Necl1 (Necl4 + Necl1), and the empty vector (pcDNA3), were grown for 3 days in 0.5% serum, stained with propidium iodide and subjected to cell cycle analysis. The percent sub-G1 cell population representing apoptotic cells is presented. B: The cell clones described in (A) grown in 10% serum-containing medium were shifted to 0.5% serum for 24 h, and apoptosis was quantified by flow cytometry after staining with Annexin V and propidium iodide. Percent positive cells for Annexin V and negative for propidium iodide, representing early stages of apoptosis, is presented. C: Cells were stained for Annexin V and analyzed by fluorescent microscopy. D: LS174T-Necl4 clones and LS174T pcDNA3 control cells grown in 0.5% serum were analyzed for PARP cleavage by Western blot analysis using anti-PARP antibody. E: LS174T-Necl4 clones and LS174T pcDNA3 control cells grown in 0.5% serum were analyzed with specific antibodies against Bcl-2, Bcl-xL and Necl4. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

attached to each other even after their removal from the culture plate (Fig. 5D).

Inhibition of the tumorigenic properties conferred by Necl4 in colon cancer cells reported here is in agreement with the tumor suppressive capacity of Necl4 recently demonstrated in prostate cancer cells [Williams et al., 2006]. In addition, several studies on Necl2, another close member of the Necl family, have also shown a strong tumor suppressive function for Necl2 in a variety of human cancers [Kuramochi et al., 2001; Fukami et al., 2003; Fukuhara et al., 2003; Ito et al., 2003; Murakami, 2005]. Since we did not detect significant levels of Necl1 or Necl4 protein in a variety of colon cancer cell lines, and also in human cancer cell lines originating from different nerve cells including neuroblastoma, glioblastoma, astrocytoma, or melanoma (Figs. 1A and 5A), it is conceivable that at least in some of these cells the expression of Necl1 and Necl4 is downregulated during the course of tumor development. Together with the loss of Necl4 expression in human prostate cancers and cell lines, and the loss of the chromosomal region 19q13.2 that includes the locus of the *Necl4* gene [Williams et al., 2006], these results imply that Necl4 has the capacity to act as a tumor suppressor. The increase in the fraction of apoptotic cells induced by Necl1 and Necl4 expression, and in a variety of apoptotic markers (Annexin V, PARP cleavage), and the decrease in Bcl-2 and Bcl-xL levels, indicate that these Necls confer their tumor suppressive activities by enhancing the rate of apoptosis in colon cancer cells.

Despite the anti-tumorigenic capacity of Necl4 in colon cancer cells, the ability of Necl4 to interfere with the tumorigenic and metastatic capacity of another IgCAM family member, L1, that confers strong oncogenic and metastatic capacities in these cells [Gavert et al., 2005, 2007] was very limited (Fig. 7). These data further point to the very different pathways by which various IgCAM receptor protein families may function. In this respect, it is noteworthy that a more distant protein of the Necl family, Necl5, also known as PVR/CD155, and containing a cytodomain that

diverges significantly from those of Necls1-4 [Takai et al., 2008], has a strong oncogenic rather than tumor suppressive activity. Necl5 was shown to enhance cell proliferation and motility, and its level is increased in transformed cells [Chadeneau et al., 1996; Minami et al., 2007; Morimoto et al., 2008; Takai et al., 2008], including human colorectal carcinoma cells [Masson et al., 2001]. Further studies are needed to determine the molecular mechanisms and the signaling pathways by which these different Necl family cell adhesion receptors confer these changes in key cancer cell properties. The restricted tissue type expression of Necl 1 and Necl4 and their cell surface localization could contribute to designing novel ways for diagnosis and interference with tumor progression.

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REFERENCES

Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Sudhof TC. 2002. SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science 297:1525–1531.

Chadeneau C, LeCabellec M, LeMoullac B, Meflah K, Denis MG. 1996. Overexpression of a novel member of the immunoglobulin superfamily in Min mouse intestinal adenomas. Int J Cancer 68:817–821.

Clevers H. 2006. Wnt/beta-catenin signaling in development and disease. Cell 127:469–480.

Conacci-Sorrell ME, Ben-Yedidia T, Shtutman M, Feinstein E, Einat P, Ben-Ze'ev A. 2002. Nr-CAM is a target gene of the beta-catenin/LEF-1 pathway in melanoma and colon cancer and its expression enhances motility and confers tumorigenesis. Genes Dev 16:2058–2072.

Conacci-Sorrell M, Simcha I, Ben-Yedidia T, Blechman J, Savagner P, Ben-Ze'ev A. 2003. Autoregulation of E-cadherin expression by cadherin-cadherin interactions: The roles of beta-catenin signaling, Slug, and MAPK. J Cell Biol 163:847–857.

Fukami T, Fukuhara H, Kuramochi M, Maruyama T, Isogai K, Sakamoto M, Takamoto S, Murakami Y. 2003. Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. Int J Cancer 107:53–59.

Fukuhara H, Kuramochi M, Nobukuni T, Fukami T, Saino M, Maruyama T, Nomura S, Sekiya T, Murakami Y. 2001. Isolation of the TSLL1 and TSLL2 genes, members of the tumor suppressor TSLC1 gene family encoding transmembrane proteins. Oncogene 20:5401–5407.

Fukuhara H, Masuda M, Yageta M, Fukami T, Kuramochi M, Maruyama T, Kitamura T, Murakami Y. 2003. Association of a lung tumor suppressor TSLC1 with MPP3, a human homologue of Drosophila tumor suppressor Dlg. Oncogene 22:6160–6165.

Gavert N, Conacci-Sorrell M, Gast D, Schneider A, Altevogt P, Brabletz T, Ben-Ze'ev A. 2005. L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. J Cell Biol 168:633–642.

Gavert N, Sheffer M, Raveh S, Spaderna S, Shtutman M, Brabletz T, Barany F, Paty P, Notterman D, Domany E, Ben-Ze'ev A. 2007. Expression of L1-CAM and ADAM10 in human colon cancer cells induces metastasis. Cancer Res 67:7703–7712.

Heller G, Geradts J, Ziegler B, Newsham I, Filipits M, Markis-Ritzinger EM, Kandioler D, Berger W, Stiglbauer W, Depisch D, Pirker R, Zielinski CC, Zochbauer-Muller S. 2007. Downregulation of TSLC1 and DAL-1 expression occurs frequently in breast cancer. Breast Cancer Res Treat 103:283–291.

Ito T, Shimada Y, Hashimoto Y, Kaganoi J, Kan T, Watanabe G, Murakami Y, Imamura M. 2003. Involvement of TSLC1 in progression of esophageal squamous cell carcinoma. Cancer Res 63:6320–6326.

Kamiguchi H, Hlavin ML, Lemmon V. 1998. Role of L1 in neural development: What the knockouts tell us. Mol Cell Neurosci 12:48–55.

Kenwrick S, Watkins A, De Angelis E. 2000. Neural cell recognition molecule L1: Relating biological complexity to human diseas mutations. Hum Mol Genet 9:879–886.

Kuramochi M, Fukuhara H, Nobukuni T, Kanbe T, Maruyama T, Ghosh HP, Pletcher M, Isomura M, Onizuka M, Kitamura T, Sekiya T, Reeves RH, Murakami Y. 2001. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. Nat Genet 27:427–430.

Lung HL, Cheung AK, Xie D, Cheng Y, Kwong FM, Murakami Y, Guan XY, Sham JS, Chua D, Protopopov AI, Zabarovsky ER, Tsao SW, Stanbridge EJ, Lung ML. 2006. TSLC1 is a tumor suppressor gene associated with metastasis in nasopharyngeal carcinoma. Cancer Res 66:9385–9392.

Masson D, Jarry A, Baury B, Blanchardie P, Laboisse C, Lustenberger P, Denis MG. 2001. Overexpression of the CD155 gene in human colorectal carcinoma. Gut 49:236–240.

Maurel P, Einheber S, Galinska J, Thaker P, Lam I, Rubin MB, Scherer SS, Murakami Y, Gutmann DH, Salzer JL. 2007. Nectin-like proteins mediate axon Schwann cell interactions along the internode and are essential for myelination. J Cell Biol 178:861–874.

Minami Y, Ikeda W, Kajita M, Fujito T, Monden M, Takai Y. 2007. Involvement of up-regulated Necl-5/Tage4/PVR/CD155 in the loss of contact inhibition in transformed NIH3T3 cells. Biochem Biophys Res Commun 352:856–860.

Morimoto K, Satoh-Yamaguchi K, Hamaguchi A, Inoue Y, Takeuchi M, Okada M, Ikeda W, Takai Y, Imai T. 2008. Interaction of cancer cells with platelets mediated by Necl-5/poliovirus receptor enhances cancer cell metastasis to the lungs. Oncogene 27:264–273.

Murakami Y. 2005. Involvement of a cell adhesion molecule, TSLC1/IGSF4, in human oncogenesis. Cancer Sci 96:543–552.

Polakis P. 2007. The many ways of Wnt in cancer. Curr Opin Genet Dev 17:45–51.

Spiegel I, Adamsky K, Eshed Y, Milo R, Sabanay H, Sarig-Nadir O, Horresh I, Scherer SS, Rasband MN, Peles E. 2007. A central role for Necl4 (SynCAM4) in Schwann cell-axon interaction and myelination. Nat Neurosci 10:861–869.

Steenbergen RD, Kramer D, Braakhuis BJ, Stern PL, Verheijen RH, Meijer CJ, Snijders PJ. 2004. TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. J Natl Cancer Inst 96:294–305.

Takai Y, Miyoshi J, Ikeda W, Ogita H. 2008. Nectins and nectin-like molecules: Roles in contact inhibition of cell movement and proliferation. Nat Rev Mol Cell Biol 9:603–615.

Williams YN, Masuda M, Sakurai-Yageta M, Maruyama T, Shibuya M, Murakami Y. 2006. Cell adhesion and prostate tumor-suppressor activity of TSLL2/IGSF4C, an immunoglobulin superfamily molecule homologous to TSLC1/IGSF4. Oncogene 25:1446–1453.