E-Cadherin/p120-Catenin and Tetraspanin Co-029 Cooperate for Cell Motility Control in Human Colon Carcinoma

Céline Greco1,2,4, Marie-Pierre Bralet4,5, Naouel Ailane1,2, Anne Dubart-Kupperschmitt2,3, Eric Rubinstein1,2, François Le Naour1,2, and Claude Boucheix1,2

Abstract

Tumor invasion and metastasis are major obstacles to clinical treatment that rely on cell migration. Here, we elucidate a mechanism of colon carcinoma cell migration that is supported by the cell-surface tetraspanin Co-029 (tspan8), which is known to favor tumor progression and metastasis. This mechanism is unmasked by silencing of E-cadherin or its associated adapter molecule p120-catenin (p120ctn), and it involves a switch in signaling between the collagen-binding integrins α1β1 and α2β1. Direct interaction between E-cadherin and Co-029 was documented by chemical cross-linking and immunohistologic analysis of colon carcinomas. High expression of Co-029 and cytoplasmic delocalization of p120ctn were each associated with poor prognosis. Cell motility was reduced severely by antibody-mediated disruption of Co-029 only when p120ctn was silenced, suggesting that tumor progression may be hindered by Co-029 targeting. Our findings define a function for tetraspanin Co-029 as a modifier of cancer cell motility and reveal an adhesion signaling network implicated in progression and metastasis. Cancer Res; 70(19): 7674–83. ©2010 AACR.

Introduction

Metastasis and tumor invasion remain the overwhelming causes of death for cancer patients. To establish effective therapeutic methods, new targets implied in these processes have to be identified. A relationship between the expression of some tetraspanins and tumor progression makes these molecules good candidates for such type of approach. Ample clinical and experimental evidences show that tumor progression and prognosis are related to the level of tetraspanin expression in several types of tumors (1, 2). For instance, CD82 has been described as a metastasis suppressor gene (3) and associated with favorable prognosis by inhibiting cell motility and invasiveness. High CD9 expression has also mostly been associated with a favorable prognosis for patients and reduced metastasis in animal models (2, 4). Interestingly, intratracheal administration of adenovirus encoding either MRP-1/CD9 or KAI1/CD82 into lung tumor-bearing mice reduced metastasis to the mediastinal lymph node (5). Moreover, the reduction of expression of CD9 and CD82 acts in synergy toward an increased metastatic potential in lung carcinoma (6).

By contrast, two tetraspanins, CD151 and Co-029 (2), are associated with tumor progression. CD151 overexpression has been reported to enhance Rac and cdc42 activation (7) and to contribute to integrin-mediated tumor cell motility via focal adhesion kinase (FAK) activation (8).

Over the years, it has been evidenced that tetraspanins organize a network of molecular interactions, referred to as the “tetraspanin web” or tetraspanin-enriched microdomains, and implying tetraspanins in membrane compartmentalization (1–14). This “web” is highly organized, with a particular tetraspanin targeting-specific nontetraspanin partner molecules (to which they associate directly) to these domains. The importance of cholesterol and lipid modifications (palmitoylation) of tetraspanins for the maintenance of these interactions has been shown (15, 16).

As tetraspanins are able to connect membrane proteins involved in different signaling pathways, it is thought that they can also modulate the motility and the metastatic potential of tumor cells through compartmentalization and subsequent modulation of the function of these associated molecules (1, 2, 11, 17). For instance, an effect on cell migration could be mediated by integrin modulation of adhesion and signaling or by regulation of growth factor receptor signaling.

Our laboratory has performed extensive analysis of tetraspanin microdomains by mass spectrometry (18, 19). Colon carcinoma cell lines derived from the metastasis and the primary tumor of the same patients were used for these analyses. They led to the identification of >30 proteins associated to tetraspanin complexes, functionally related to adhesion, signalization, proteolysis, traffic, and fusion. We found that some of these proteins were differentially expressed between the primary tumors and their metastasis cell lines. For
instance, in the Isreco cell line model (20), a strong expression of the tetraspanin Co-029 was detected in the metastasis cell lines of one patient, whereas it was absent from the primary tumor cell line (19).

During the invasive process, the balance between cell-matrix adhesion—due to the association of integrins to their substrate, allowing the agglomeration of proteins constituting focal adhesion and their ligation to the actin cytoskeleton—and intercellular adhesion maintained by cadherin-based adherens junctions (mainly E-cadherin for epithelial cells) is a key factor for allowing cell migration (21).

As cell motility is considered as a critical factor for invasion and metastatic diffusion (22, 23), and because of the restricted expression of Co-029 in the metastasis cell lines, we investigated whether this tetraspanin could play a determinant role in this process. For that purpose, we expressed the tetraspanin Co-029 in the cell line Isreco1 derived from the primary tumor of a patient with metastatic colon carcinoma. We carried out analysis at single-cell level and observed that Co-029 regulates cell motility on collagen I through its cooperation with the E-cadherin/p120-catenin (p120ctn) complex, induces the selective recruitment of the α3β1 integrin pathways, and interferes with small GTPase regulation.

Materials and Methods

Cells and cell culture

The cell lines Isreco1, Isreco2, and Isreco3 were initially derived from a primary human colon cancer (Duke’s C, class III) surgical specimen (Isreco1) and its corresponding liver and peritoneal metastases (20). These cells, transferred to our laboratory in 2002 by Dr. B. Sordat (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland), were cultured in DMEM supplemented with 10% FCS, glutamax, and antibiotics (all from Invitrogen). For experiments, tissue culture plastics were coated with rat tail collagen I (BD Biosciences) for 1 hour at 50 μg/mL and rinsed with PBS. Phenotypic characteristics of the Isreco cell lines (morphology and surface markers) were reported previously (19); they are checked after each thawing and before a set of experiments to avoid contamination between cell lines.

Lentiviral vectors

The human Co-029 and CD81 cDNA coding sequence were inserted in the TRIPΔ3-EF1α vectors (24). Vector particles were produced by cotransfection of 293T cells by the TRIPΔ3-EF1α Co029 and TRIPΔ3-EF1α-CD81 plasmids together with an encapsidation and an envelope (vesicular stomatitis virus) expression plasmids. Isreco1 cells were transduced once or twice with concentrated lentiviral particles.

Antibodies

For the production of TS29, which was not reported before, BALB/c mice were injected i.p. twice with a mixture of 107 Isreco3 and Lovo cells, and a final boost was performed 3 weeks later with CD9-containing complexes collected by immunoprecipitation from a Brij 97 lysate of 109 Isreco3 cells. Spleen cells were fused with P3X63AG8 mouse myeloma cells (5 × 107 and 3 × 107, respectively) according to standard techniques and distributed into 96-well tissue culture plates. After 2 weeks, hybridoma culture supernatants were harvested and tested for Isreco1 and Is1-Co029 staining by indirect immunofluorescence. Positive supernatants were then further characterized by immunoprecipitation and by comparison with the Co-029 monoclonal antibody (mAb) AZM22 (see Supplementary Materials and Methods).

Immunofluorescence

For flow cytometry analysis of surface molecules, cells were detached using a nonenzymatic solution (Invitrogen). For intracellular cytometry of phosphoproteins, sparse cells were detached by scraping in PBS at 4°C, then fixed with 1% formaldehyde, and permeabilized in 100% methanol for 10 minutes at –20°C. For in situ labeling, cells cultured in LabTek chambers were immunostained by fixing with 4% formaldehyde for 10 minutes followed by permeabilization with Triton X-100 at 1% for 10 minutes.

RNA silencing

See Supplementary Materials and Methods.

Cross-linking of cell surface proteins

For cross-linking, cells were incubated for 30 minutes at 4°C in the culture flask with 0.5 mmol/L water-soluble BS3 (Pierce) in 1× PBS. They were washed three times in 1× PBS before lysis in 1% Triton X-100 at 4°C and immunoprecipitation (19).

Videomicroscopy

Cell motility analysis was performed by time-lapse videomicroscopy on an inverted microscope equipped with a 37°C chamber with 5% CO2. Stacks of phase-contrast images were collected every 15 minutes for 24 hours at ×200 magnification. Cell migration was quantified using the manual tracking plug-in of ImageJ. Data were transferred to Excel for calculations and statistics. For each position, at least 10 cells were analyzed. For all differences reported, P ≤ 0.0001 (Mann-Whitney test).

Immunohistochemistry

Antibody labeling was performed on tumor tissues from 52 patients diagnosed and operated in 2002 to 2003 at Hôpital Européen Georges Pompidou. These patients had not received treatment before surgical removal of the tumor, and at least 12 lymph nodes were removed during surgery for correct assessment of tumor stage. Formalin-fixed, paraffin-embedded tissue blocks containing carcinoma specimens were retrieved from the archives of the Department of Pathology. Blocks displaying simultaneously normal mucosal areas and carcinoma were identified on corresponding H&E-stained slide. Each paraffin section was placed on a positively charged glass slide (Superfrost). More information can be found in Supplementary Materials and Methods.
Results

Generation of Isreco1 colon carcinoma cell lines expressing Co-029

The tetraspanin Co-029 is absent from Isreco1 cells and strongly expressed on the metastasis-derived cells Isreco2 and Isreco3 (19). For functional studies, the Co-029-negative Isreco1 cells were transduced with lentiviral vectors to obtain an expression of Co-029 similar to the metastatic cell line Isreco2 (Supplementary Fig. S1). Two Co-029—expressing Isreco1 cell lines were used: Is1-Co029 (with a high expression of Co-029) and Is1-Co029low. Isreco1 and Is1-CD81 (transduced with the tetraspanin CD81) were used as controls.

Relation between E-cadherin and Co-029 in cell motility

We explored by videomicroscopy whether cell motility could be altered by the forced expression of Co-029. As Isreco1 cells do not move on tissue culture plastic, we tested different matrices (Matrigel, laminin 5, and collagen I). The cells migrate randomly (Supplementary Movie S1), with the highest velocity on collagen I that was retained for the rest of the study. Sparse Isreco cells are intensely motile through cell-matrix interactions because the blocking mAb anti-β1 integrin chain 4B4, which inhibits the collagen I receptors α1β1 and α2β1 (25), completely stopped the cells (Supplementary Movie S2). On collagen I, Isreco1 and Is1-Co029 sparse cells formed focal adhesions containing vinculin, paxillin, and FAK with anchored actin fibers (Supplementary Fig. S2). The integrin α2β1 clustered at focal adhesions (Supplementary Fig. S3), whereas α1β1 expression was punctuated and diffuse (Supplementary Fig. S4).

Migration is arrested when cell density increases, favoring cell contacts that are stabilized by the formation of adherens junctions (Supplementary Fig. S5), but surprisingly, E-cadherin was well expressed on motile sparse cells (Fig. 1A) with a diffuse membrane distribution, raising the question of a possible role of free E-cadherin in cell motility. Whereas velocity of Isreco1 and Is1-Co029 sparse cells was not significantly different, silencing of E-cadherin effectively increased the motility of Is1-Co029 cells (Fig. 1B), an effect confirmed with four different siRNAs to Co-029 (b) Is1-Co029low cells were less accelerated by p120ctn silencing than Is1-Co029 cells, and (c) neither Isreco1 nor Is1-CD81 cells accelerated after p120ctn silencing (Fig. 2D). These data are compatible with the hypothesis that p120ctn mediates the effect of E-cadherin on cell migration. The fact that both silencing and delocalization to the nucleocytoplasmic area (through E-cadherin silencing) produce the same effect suggests that both cell migration and delocalization to the nucleocytoplasmic area are important in the migration process. The effect of p120ctn silencing on motility was clearly linked to the presence of Co-029 because (a) it was not observed after simultaneous p120ctn and Co-029 silencing (Fig. 2D), as confirmed with four different siRNAs to Co-029; (b) Is1-Co029low cells were less accelerated by p120ctn silencing than Is1-Co029 cells; and (c) neither Isreco1 nor Is1-CD81 cells accelerated after p120ctn silencing (Fig. 2D). These data are compatible with the hypothesis that p120ctn mediates the effect of E-cadherin on cell migration. The fact that both silencing and delocalization to the nucleocytoplasmic area (through E-cadherin silencing) produce the same effect suggests that both cell migration and delocalization to the nucleocytoplasmic area are important in the migration process.

Cell signaling in p120ctn-silenced cells—role of small G proteins

We then searched the mechanisms mediating acceleration of Is1-Co029 cells on p120ctn silencing by analyzing the contribution of α1β1 and α2β1 integrins in the motility process. It was firstly observed using RNAi and function-blocking antibodies that whereas Isreco1 and Is1-CD81 cell motility relied preferentially on α1β1 integrin, the motility of Is1-Co029 cells relied mainly on α2β1 integrin (Fig. 3A). This observation shows that on expression of Co-029, signaling from the α2β1 integrin receptor is recruited. Furthermore, we observed that the p120ctn silencing acts also on integrin (26–29) and has been reported to regulate Bho and Rac functions in cell adhesion and motility (30, 31). On E-cadherin RNAi, a major part of p120ctn relocated to the nucleocytoplasmic area in Isreco1 (data not shown) and Is1-Co029 cells (Fig. 2A). Flow cytometry analysis of permeabilized cells after E-cadherin RNAi shows that there is no change in total cellular content of p120ctn (Fig. 2B), confirming the absence of rapid degradation of cytoplasmic p120ctn reported earlier (32). The role of p120ctn in mediating E-cadherin effects on cell migration was explored using RNAi. The level of p120ctn was reduced by 85% after silencing and accompanied by a 50% decrease in E-cadherin surface expression (Fig. 2B), consistent with the ability of p120ctn to stabilize E-cadherin at the cell surface (29, 33). The silencing of p120ctn dramatically increased the motility of Is1-Co029 cells by ~80% (Fig. 2C and D; Supplementary Movie S3). The same effect was obtained with two different siRNAs. Again, the effect of p120ctn silencing on motility was clearly linked to the presence of Co-029 because (a) it was not observed after simultaneous p120ctn and Co-029 silencing (Fig. 2D), as confirmed with four different siRNAs to Co-029; (b) Is1-Co029low cells were less accelerated by p120ctn silencing than Is1-Co029 cells; and (c) neither Isreco1 nor Is1-CD81 cells accelerated after p120ctn silencing (Fig. 2D). These data are compatible with the hypothesis that p120ctn mediates the effect of E-cadherin on cell migration. The fact that both silencing and delocalization to the nucleocytoplasmic area (through E-cadherin silencing) produce the same effect suggests that in our model, the membrane-bound p120ctn can deliver a negative signal for the migration process.
signaling because the increased velocity of Is1-Co029 required signaling from both \( \alpha_1\beta_1 \) and \( \alpha_2\beta_1 \) integrins as shown by RNAi (Fig. 3B; Supplementary Movie S5).

We investigated then the pathways reported to be involved in cell motility following integrin stimulation (34). Inhibitors of the mitogen-activated protein kinase (MAPK) pathways extracellular signal-regulated kinase 1/2 (ERK1/2), p38-MAPK, and c-Jun NH2-terminal kinase (JNK) reduced the motility of Is1-Co029 cells particularly after p120ctn silencing (Fig. 4A). However, the effect on Isreco1 was marginal or absent. The same results were obtained by Akt1/2 silencing. The complete blockade of motility by LY294002 showed that,

**Figure 1.** Co-029 is functionally and physically linked to E-cadherin. A, surface expression of E-cadherin (Ecadh) shows low variation of E-cadherin levels on sparse cells versus confluent cells. M.F.I., mean fluorescence intensity. B, silencing of E-cadherin (si-Ecad) induces an increased motility restricted to Is1-Co029 cells and suppressed by co-silencing of Co-029 (si-Co029). Averaged distance traveled by cells. Columns, mean; bars, SE. *, \( P \leq 0.0001 \).

C, double labeling with anti-E-cadherin (biotin-labeled rabbit polyclonal) and TS29 (mouse mAb to Co-029) antigen shows a partial colocalization at adherens junctions or a more diffuse membrane colocalization in sparse cells. Scale bar, 10 \( \mu \)m. D, the association of E-cadherin and Co-029 is suggested by experiments with the water-soluble cross-linker BS3. Each lane represents one sixth of one pooled 150-cm² flasks with confluent cells or the same amount of cells in five 150-cm² flasks (sparse). Triton X-100 extracts were immunoprecipitated (I.P.) by the Ecadh mAb Hecd1, the Co-029 mAb TS29, or the CD9 mAb TS9. Western blots (W.B.) performed with biotinylated antibodies (biot) revealed the presence of a band (arrowheads) above E-cadherin (arrows) that could correspond to a Co-029/E-cadherin complex. This band is found in sparse and confluent Is1-Co029 cells in which it is reduced following E-cadherin RNAi (70% reduction of E-cadherin). It is absent from the Isreco1 cell line. A corresponding band cannot be observed in the cross-linked immunoprecipitated Co-029 because it may be masked by several cross-linked proteins of high molecular weight, revealed by the TS29 antibody, which remain to be identified. Note crosslinked Co-029 is marked in the upper blot by an asterisk. Gel loading for paired samples was normalized by electrophoresis and Hsp70 quantification on cellular extracts before performing immunoblots. When indicated, blots were incubated successively with TS29 and E-cadherin antibodies.
in this system, motility is highly dependent on phosphatidylinositol 3-kinase (PI3K), which is due not only to its Akt effector but also perhaps through spatial patterns of 3’-phosphoinositide lipid products at the membrane that control the optimal localization of signaling molecules. Particularly, in addition to its action on the Akt pathway, PI3K regulates the binding to the membrane through the plekstrin homology domain of guanine exchange factors that are key modulators of Rho family G proteins. Treatment with C3 exo-transferase, which inhibits Rho-GTPases, or silencing of RhoA, but not RhoC, accelerated Is1-Co029 cells to the same extent as p120ctn silencing (the effects were not cumulative),

Figure 2. p120ctn regulates effects of Co-029 on cell motility. A, the labeling of Is1-Co029 cells treated with control siRNA (si-ctr) shows a colocalization of p120ctn and E-cadherin at adherens junctions. On silencing by si-Ecad, E-cadherin has nearly disappeared (same exposure and contrast), whereas p120ctn remains expressed but mainly concentrated around and over the nucleus area. A similar aspect was observed in both cell lines. Scale bar, 20 μm. B, Is1-Co029 cells cultured on collagen I were detached and permeabilized for analysis of p120ctn by flow cytometry 48 h after p120ctn or E-cadherin silencing. The mean fluorescence intensity values are indicated below each condition. This experiment shows that si-p120 reduces the amount of p120ctn by 80%, whereas si-Ecad has no effect on p120ctn expression. Surface E-cadherin of Is1-Co029 cells cultured on collagen I was analyzed by flow cytometry after silencing by si-Ecad or si-p120. We observed a 75% and 50% reduction of surface E-cadherin on si-Ecad and si-p120 treatments, respectively, showing the requirement of p120ctn for E-cadherin stability at the membrane. C, the motility of p120ctn-silenced cells is increased when Co-029 is expressed. Gray lines represent cumulative distance traveled by individual cells versus time. D, the si-p120 effect on Is1-Co029 cell motility is reversed by si-Co029. The x symbol indicates conditions that were not tested in this experiment. Columns, mean; bars, SE. *, P ≤ 0.0001.

Figure 3. Switch from α1 to α2 collagen receptor recruitment in Is1-Co029 cells. A, RNAi and function-blocking antibodies show that Is1-Co029 cells are dependent on α2 integrin signaling for motility. B, RNAi to α1 and α2 integrins shows that Is1-Co029 silenced for p120ctn can be slowed down by both siRNAs and cells treated with the three siRNAs are stopped. NQ, not quantifiable. Columns, mean; bars, SE. *, P ≤ 0.0001.
indicating that RhoA inhibits motility of Is1-Co029 cells (Fig. 4B). On the other hand, C3 exotransferase and RhoA silencing slowed down Isreco1 cells, suggesting that in these cells some RhoA signaling is necessary for full velocity.

Because the amount of biological material issued from sparse cells was scarce, we monitored by flow cytometry single-cell events with phospho-specific antibodies for activated forms of signaling molecules (35). On p120ctn silencing, there was a reproducible increase of phosphorylated JNK (pJNK) and phosphorylated Akt (pAkt) in Is1-Co029 cells (Fig. 4C). Altogether, in Is1-Co029 cells, the potent effect of RhoA inhibition and the activation by si-p120 of JNK, which is downstream of Rac, support a role for small G proteins in the mechanisms triggered by Co-029 expression. Because the balance between Rac and RhoA is regulated by p120ctn, this suggests that p120ctn associated to surface E-cadherin could control the motility of Is1-Co029 cells, at least partially, through local regulation of small G proteins.

**p120ctn delocalization and Co-029 high expression are adverse prognostic factors in human colon carcinoma**

These results led us to test the relation of Co-029 expression with prognosis by analyzing the primary tumors of 52 patients with colon carcinoma. We compared the results with p120ctn delocalization (Fig. 5; Table 1) that is related to aggressive disease (36). Conditions were optimized for...
antigenic revelation of Co-029 in formalin-fixed, paraffin-embedded tissues, giving a labeling pattern identical to that found previously in frozen tissue sections (19). Because quantification of labeling is difficult on tissue sections, we took advantage of the expression of Co-029 on normal epithelial cells to use it as a reference for grading the tumor labeling. The comparison of labeling in normal epithelium and in tumor areas was performed on the same sections.

Of the 24 patients who died or relapsed in the 3 years following tumor removal, 71% (17 of 24) had p120ctn delocalization and 87% (21 of 24) had high Co-029 expression, whereas it was 25% (7 of 28; \( P = 0.0019 \)) and 57% (16 of 28; \( P = 0.029 \)), respectively, for the patient group in complete remission at 3 years. The pattern of p120ctn expression was also significantly related to initial staging of the tumor, whereas it was not significant for Co-029. The pattern of p120ctn expression was not significantly correlated to the level of Co-029 (\( P = 0.269 \)), indicating that these two markers may vary independently. When the two criteria were combined into three groups with either no adverse factor, one adverse factor, or two adverse factors, the prognosis significance was even greater (\( P = 0.0012 \)).

**Table 1.**

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NOTE: A. Outcome and staging of 52 patients according to p120ctn localization and Co-029 expression in primary tumor. Correlations between p120ctn localization and Co-029 expression level and either evolution or staging of the 52 colon carcinoma patients analyzed for p120ctn localization and intensity of Co-029 expression in primary tumor. Cytoplasmic localization of p120ctn and high Co-029 expression are statistically significant adverse outcome factors (top part of the table). The localization of p120ctn is linked to staging, whereas the association is not significant for Co-029 expression. B. Patients were grouped according to the two adverse factors: p120ctn delocalization and high Co-029 expression. Group 1 corresponds to patients without adverse prognosis factors, group 2 includes patients with a single adverse factor, and group 3 includes patients who have two adverse factors. Abbreviation: CR, complete remission.

Of the 24 patients who died or relapsed in the 3 years following tumor removal, 71% (17 of 24) had p120ctn delocalization and 87% (21 of 24) had high Co-029 expression, whereas it was 25% (7 of 28; \( P = 0.0019 \)) and 57% (16 of 28; \( P = 0.029 \)), respectively, for the patient group in complete remission at 3 years. The pattern of p120ctn expression was also significantly related to initial staging of the tumor, whereas it was not significant for Co-029. The pattern of p120ctn expression was not significantly correlated to the level of Co-029 (\( P = 0.269 \)), indicating that these two markers may vary independently. When the two criteria were combined into three groups with either no adverse factor, one adverse factor, or two adverse factors, the prognosis significance was even greater (\( P = 0.0012 \)).

**p120 silencing renders cells sensitive to a motility-inhibitory effect of Co-029 mAb**

In an attempt to target functional properties of tumor cell, we measured cell motility of cells exposed to antibodies. Co-029 mAb TS29 had a unique effect in provoking a 75% motility reduction of Is1-Co029 cells silenced for p120ctn (Supplementary Fig. S10; Supplementary Movie S6), whereas no effect of mAbs to tetraspanins CD9, Co-029, and CD81 were noted on the different untreated cell lines. This indicates that in conditions where p120ctn expression is delocalized and Co-029 is well expressed, as it happens frequently in pathologic situations, motility of the cells could be significantly downregulated.

**Discussion**

This study describes a new mechanism leading to increased cell motility by connecting the tetraspanin Co-029 and the E-cadherin/p120ctn complex. It shows that E-cadherin, frequently described as the major component of epithelial cell junctions, could be a key regulator of cell motility. It also points to important prognosis markers in colon carcinoma, where p120ctn delocalization and strong...
expression of Co-029 are frequently associated and linked to adverse outcome.

The importance of assessing single-cell motility has been elegantly studied in in vivo models (37, 38). Recently, a switch from cohesive movement to single-cell motility induced by transforming growth factor-β was shown to promote hematogenous metastasis (39). In the in vitro system used in our work, the remarkable stability of the velocity of Isreo cells on collagen I indicates that this process is tightly regulated. Motility signals are transduced by the integrins α1β1 and α2β1, that are both collagen receptors but which display marked differences in downstream signaling (40). Surprisingly, if the motility of Isreo1 cells on collagen I was similar whether Co-029 was expressed or not, we observed that Isreo1 and Is1-Co029 used different signaling pathways for migration. Indeed, Isreo1 cells were rendered motile on collagen I by signals elicited through integrin α1β1, whereas the expression of Co-029 induced a switch to integrin α2β1. This switch did not change the final result in terms of velocity as long as the E-cadherin/p120ctn complex remained unaltered at cell surface. We show using RNAi that reduction of membrane expression of the complex and more specifically of p120ctn relieves the control exerted by this complex on cell motility of sparse cells; however, when E-cadherin is suppressed, an effect of delocalized intracellular p120ctn cannot be dismissed. We observed that silencing of p120ctn induced the reactivation of the integrin α1β1 pathway in Is1-Co029, allowing the cells to use both receptors, thereby increasing their motility, whereas Isreo1 cell motility remained unaffected.

Because the global effect of the E-cadherin/p120ctn complex is to maintain cell motility of sparse Is1-Co029 cells at the level of the control Isreo1 cells, it behaves like a motility “rheostat” that prevents the acceleration observed on silencing of E-cadherin or p120ctn. This suggests a new role for a form of E-cadherin that is nonjunctional and nontransadhering as a sensor of motility and a regulator through its associated protein p120ctn. The question of nonjunctional outside-in signaling by E-cadherins has been suggested by studies on differentiation and proliferation of keratinocytes (41).

More generally, the fact that in the Isreo model, p120ctn silencing increases only the motility of cells expressing Co-029 could be one of the reasons for discrepancies between results in the literature about p120ctn-suppressive effects on cell motility and metastasis, where Co-029 expression was not measured (26, 27, 30).

The mechanism that determines which of the two collagen receptors (α1 and α2) of the integrin issued signals should be translated into motility remains to be elucidated and needs the in-depth analysis of the cross talk between p120ctn and integrin signaling. A direct activation of α2β1 integrin by Co-029 is not supported by the localization of these two molecules because tetraspanins are not found in focal adhesions, but does not exclude an indirect effect involving proteins associated with tetraspanins in the tetraspanin web. Of course, the interaction between Co-029 and E-cadherin provides a hint toward a possible mechanism because the E-cadherin/p120ctn complex can interfere with many signaling pathways (26, 41, 42) in relation to cell proliferation and cell motility.

Secondary messengers of E-cadherin/p120 signaling for cell acceleration are not definitely shown, but the fact that, in our study, RhoA inhibition has the same effect as p120ctn silencing, and that there is no additive effect when both are silenced, suggests that RhoA could play this role. This would imply that specifically in Is1-Co029 cells, activation of RhoA is required to maintain cell motility below a certain threshold. On the contrary, an inhibition of RhoA by p120ctn has been reported but it was not analyzed in view of its membrane location (26, 43). For instance, in confluent keratinocytes, transadhering E-cadherin stabilized by p120ctn was reported to activate RhoA (36, 44). In addition, the requirement for a membrane location of N-cadherin–associated p120ctn for regulation of Rho proteins was already reported (45). The versatility of p120ctn is further substantiated by the observation that its effects on tumor growth are inverse depending on E-cadherin expression (46). Thus, the effect of p120ctn can vary according to the cellular system and the functional effect that is explored, partly due to its membrane location and the isoform expressed. Rac1 involvement is also suggested by the increase of pJNK on p120ctn silencing in Is1-Co029 cells. Because Rac1 is upstream of JNK, membrane p120ctn, in Is1-Co029 cells, may inhibit this pathway.

Finally, these results show that functional properties of the tetraspanin Co-029 are tightly controlled by p120ctn and require delocalization of the E-cadherin/p120ctn complex or absence of p120ctn to be revealed.

About RhoC silencing, contrary to RhoA, it reduced cell velocity. It was previously reported that the effects of these two small G proteins may vary in opposite. For instance, in colon carcinoma. RhoA seems to inhibit epithelial-mesenchymal transition, whereas RhoC promotes it. RhoC expression is also associated with a worse prognosis in this disease (47).

Few studies addressed the question of p120ctn as prognostic factor in colorectal carcinoma with some inconsistency in the results (48), but in a large study including 557 patients, delocalization or loss of p120ctn was associated with poor survival (36). In our work, the cooperative effect on motility consecutive to Co-029 expression and membrane delocalization of p120ctn (due to E-cadherin silencing) or p120ctn silencing may find its correspondence in clinical situation where high Co-029 expression and p120ctn delocalization are both adverse prognosis factors, an effect amplified by the combined presence of the two patterns of expression. Low p120ctn at the membrane could result from reduced expression of p120ctn or from a default of p120ctn trapping to the membrane consecutive itself to a defect of E-cadherin expression or mutations hampering E-cadherin correct addressing (21, 49).

A consequence of our observations is that the tetraspanin Co-029 could be a potential target for cancer treatment. Interestingly, its expression is more restricted compared with other tetraspanins such as CD9, CD82, or CD151, which are frequently expressed on tumor cells but have also a wide tissue distribution. According to transcriptional data (50),
it is found mainly in the digestive tract including colorectal epithelium (19), liver, and pancreas and also in the trachea and some central nervous system substructures.

Anti-tetraspanin mAbs have been widely reported to alter cell motility, but with great variability according to experimental conditions (1, 10). The mechanism remains unknown, but an effect mediated by tetraspanin-associated proteins may be implicated through membrane reorganization, internalization, or even conformational changes of associated molecules. In our model, the effect on cell motility is specific for the tetraspanin Co-029, as opposed to what is observed usually with tetraspanin antibodies (1). In addition, knockdown of p120ctn is required, indicating that the E-cadherin/p120ctn rheostat overcomes the effect of the antibody. The specific effect of the Co-029 mAb on cell motility will require extensive investigation of molecular interactions and downstream signaling in the context of antibody binding as well as in vitro testing to determine if it could reduce tumor invasion and/or metastasis. The interest of using an anti-tetraspanin antibody directed to CD151 that inhibits cell migration in vitro and in vivo was shown in a mouse model in which the mice were injected with the human tumor cells HT1080 and Hep3 (51).

On the whole, this work describes the encounter of a facilitator of metastasis, Co-029, and of the E-cadherin/p120ctn complex that, by controlling adhesion and motility, determines tumor fate. Co-029 could be the Achilles’ heel of aggressive tumors underexpressing the membrane E-cadherin/p120ctn complex because it can be functionally targeted.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Céline Greco, Marie-Pierre Bralet, Naouel Ailane, et al.

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