Colocalization of the Tetraspanins, CO-029 and CD151, with Integrins in Human Pancreatic Adenocarcinoma: Impact on Cell Motility

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Abstract

Purpose: Patients with pancreatic adenocarcinoma have a poor prognosis due to the extraordinary high invasive capacity of this tumor. Altered integrin and tetraspanin expression is suggested to be an important factor. We recently reported that after protein kinase C activation, colocalization of $\alpha6\beta4$ with the tetraspanin CO-029 strongly supports migration of a rat pancreatic adenocarcinoma. The finding led us to explore whether and which integrin-tetraspanin complexes influence the motility of human pancreatic tumors.

Experimental Design: Integrin and tetraspanin expression of pancreatic and colorectal adenocarcinoma was evaluated with emphasis on colocalization and the impact of integrin-tetraspanin associations on tumor cell motility.

Results: The majority of pancreatic and colorectal tumors expressed the $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, and $\beta4$ integrins and the tetraspanins CD9, CD63, CD81, CD151, and CO-029. Expression of $\alpha6\beta4$ and CO-029 was restricted to tumor cells, whereas $\alpha1$, $\alpha2$, $\alpha3$, $\alpha6$, $\beta1$, and CD9, CD81, CD151 were also expressed by the surrounding stroma. CD63, CD81, and $\beta1$ expression was observed at comparably high levels in healthy pancreatic tissue. $\alpha3\beta1$ frequently colocalized and coimmunoprecipitated with CD9, CD81, and CD151, whereas $\alpha6\beta4$ colocalized and coimmunoprecipitated mostly with CD151 and CO-029. Notably, protein kinase C activation strengthened only the colocalization of CD151 and CO-029 with $\beta4$ and was accompanied by internalization of the integrin-tetraspanin complex, decreased laminin 5 adhesion, and increased cell migration.

Conclusion: $\alpha6\beta4$ is selectively up-regulated in pancreatic and colorectal cancer. The association of $\alpha6\beta4$ with CD151 and CO-029 correlates with increased tumor cell motility.

Pancreatic adenocarcinoma is a leading cause of cancer-related death and the frequency is increasing steadily (1). Patients with pancreatic adenocarcinoma have a very poor prognosis, the 1-year survival rate being <20% and the 5-year survival rate being <1% in most clinical centers. This is partly due to the fact that >80% of patients have massive metastatic spread at the time of diagnosis (1, 2). The early spread is proposed to proceed after settling of tumor cells in the peritoneal cavity and the penetration into blood vessels via so-called peritoneal pores, with formation of metastases preferentially in the liver (3). The mechanisms underlying the extraordinarily high invasive capacity of pancreatic adenocarcinoma are not yet understood. It is suggested that altered expression of adhesion molecules, particularly of integrins, might be an important factor (4).

Changes in expression of integrins, particularly, $\alpha3\beta1$ and $\alpha6\beta4$, are frequently related to the metastatic capacity of tumor cells (5, 6). Reports on the integrin expression profile in pancreatic adenocarcinoma revealed partly contradictory results. It has been described that $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$, αv, $\beta1$, and $\beta4$ are overexpressed in pancreatic adenocarcinoma lines, that do not express the $\alpha1$, $\alpha4$, or $\beta2$ integrins (7). High $\alpha6$ expression on tumor tissue and weak expression on the surrounding tissue together with low expression of $\alpha5$ (8), or high $\alpha6\beta4$ expression together with high laminin 5 secretion may also indicate a poor prognosis (9). In the nude mouse, high $\alpha6$ and $\alpha v\beta5$ expression and low $\alpha2$ expression was associated with metastasis (10). However, other reports indicate that pancreatic adenocarcinoma tissues display weak $\alpha6$ expression, but high $\alpha2$, $\alpha3$, $\alpha4$, αv, and $\beta1$ levels (11). Other studies revealed tumor-related $\alpha2$ and $\alpha6$ up-regulation with a diffuse staining pattern (12). High $\beta4$ expression (7) was confirmed by sophisticated molecular profiling, that took into account the

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strong stromal reaction of pancreatic adenocarcinoma as well as the features of chronic pancreatitis (13).

Tetraspanins are also known to contribute to the metastatic process (14–16). Whereas CD63 expression does not seem to be of major impact (17), high CD9 (18–21), and CD82 (19, 22, 23) expression has been associated with a favorable prognosis in gastrointestinal tumors. In contrast, high CD151 and CO-029 expression seem to promote metastasis formation (18, 24).

Tetraspanins are known to form protein complexes, mostly composed of different tetraspanins and integrins (25, 26), which influence cell motility (27–29). The strongest complexes are formed between CD151 and α3 (26, 28, 30). CD151 also associates with α6β4, notably in hemidesmosomes (31). CD9 mostly associates with α3β1 and may associate with α6β4 outside of hemidesmosomes (32, 33). Whether integrins associate with CO-029 in human tissues has not been explored. The rat homologue of CO-029, D6.1A, associates with α3 and α6β1 (14) and, after protein kinase C (PKC) activation, with α6β4 (34).

We had noted in a rat pancreatic adenocarcinoma that coexpression of α6β4 and the D6.1A tetraspanin, but not overexpression of α6β4 by itself, contributes to the hematogeneous spread of tumor cells (34). In view of the poor prognosis for patients with pancreatic adenocarcinoma, it became of interest to explore integrin-tetraspanin associations and their potential impact on cell motility in human pancreatic adenocarcinoma.

Materials and Methods

Tumors and tumor lines. Pancreatic adenocarcinoma (30), normal pancreatic (5), and chronic pancreatitis (10) tissues were collected during surgery and snap-frozen in liquid nitrogen. Histologic type and grading of the tumors are shown in the supplement (Table S1). Informed consent on tissue collection was obtained from each patient and tissue collection was approved by the University Ethics Review Board. Tissue culture lines were established from the pancreatic tumors P73, P106, and P122. These lines consisted of a mixture of tumor cells and stromal elements, the presence of the stroma being essential for tumor cell survival. The long-term pancreatic adenocarcinoma lines AsPC1, BxPC3, Capan1, Capan2, Colo357, MiaPaca1, Panc1, Panc89, Pt45P1, PancTu1, and 8.18 were maintained in RPMI 1640, supplemented with 10% FCS, nonessential amino acids, and 10 mmol/L sodium pyruvate. The long-term colorectal cancer lines Colo320, Colo320DM, HT29, Lovo, SW480, SW707, SW948, and WIDR were maintained in RPMI 1640 and supplemented with 10% FCS. Lines are described in the supplement (Table S2). All lines grew adherent and, when confluent, were detached with trypsin for subculture.

Antibodies and staining procedures. The following monoclonal antibodies have been used: anti-CD9, anti-CD18 (β2), anti-CD29 (β1), anti-CD49a (α1), anti-CD49b (α2), anti-CD49c (α3), anti-CD49e (α5), anti-CD49f (α6), anti-CD53, anti-CD63, anti-CD81, anti-CD82, anti-CD104 (β4), anti-CD151, anti-CO-029 (see supplement, Table S3 for clone description). Where indicated ascitic fluid or hybridoma culture supernatants were purified by passage over protein G Sepharose and were labeled with biotin, FITC, or rhodamine. Dye-labeled secondary antibodies and streptavidin were obtained commercially (BD/PharMingen and Dianova, Hamburg, Germany).

Table 1. Integrin and tetraspanin profile of human pancreatic and colorectal tumors in tumor lines

	Surface expression (flow cytometry)*																
Tumor line	**α1**	**α2**	**α3**	**α4**	**α5**	**α6**	**αL**	**β1**	**β2**	**β4**	**CD9**	**CD53**	**CD63**	**CD81**	**CD82**	**CD151**	**CO-029**
Pancreatic cancer																	
AsPC1	−−	+++	+++	±	+	+++	−−	++	−−	+++	++	−−	++	+	++	+	+++
BxPC3	+	+++	+++	±	+	+++	−−	++	−−	+++	+	−−	+	+	++	±	±
Capan1	±	++	++	−−	±	+++	−−	++	−−	+++	+	−−	+	+++	+	+	++
Capan2	+	+++	+++	−−	+	+++	−−	++	−−	+++	++	−−	++	+	+	++	+
Colo357	+	++	++	−−	−−	+++	−−	++	−−	+++	++	−−	+	+	+	+	±
MiaPaca1	−−	++	+	−−	+	+++	−−	+	−−	+++	++	−−	++	++	+	++	−−
Panc1	±	++	++	−−	++	+++	−−	++	−−	++	++	−−	++	+++	+++	++	−−
Panc89	+	+++	+++	−−	++	+++	−−	+++	−−	+++	+++	−−	++	+++	++	±	±
Pt45P1	+	++	++	−−	++	++	−−	++	−−	−−	+	−−	++	+++	++	++	−−
8.18	±	++	+	−−	−−	+++	−−	+	−−	++	++	−−	+	+++	+	++	++
Colorectal cancer																	
Colo205	+	++	+	−−	±	+++	−−	+	−−	++	++	−−	+	+	+	+	++
Colo320	+	+++	+	±	+	++	−−	+	−−	++	−−	−−	++	++	+	+	+
Colo320DM	+	+++	+	−−	±	++	−−	+	−−	+++	−−	−−	+	++	+	±	±
HT29	+	+++	++	−−	−−	+++	−−	+	−−	+++	++	−−	++	++	++	+	++
Lovo	±	+++	+	−−	+	++	−−	+	−−	+++	+++	−−	+++	+	+	++	+++
SW480	±	++	++	−−	+	+++	−−	+	−−	++	++	−−	+	++	+++	+++	−−
SW707	+	++	+	−−	+	+++	−−	+	−−	+++	++	−−	+	++	++	++	++
SW948	−−	+	+	−−	+	+++	−−	+	−−	+++	++	−−	+	+	+	+	+
WIDR	±	++	+	−−	−−	+++	−−	+	−−	+++	+	−−	+	+	++	++	++

*Flow cytometry data were analyzed according to the increase in the mean fluorescence intensity as compared with the negative control. No staining, −− (intensity 1.0-1.3 fold); weak staining, ± (intensity >1.3-2.0-fold); distinct staining, + (intensity >2.0-5.0-fold); strong staining, ++ (intensity >5.0-10.0-fold); very strong staining, +++ (intensity >10.0-fold).

Table 2. Integrin and tetraspanin profile of human pancreatic and colorectal tumors in tumor tissue

	Mean intensity of expression and percentage of distinctly positive tissues (immunohistology*; *P* values)†												
Tumor tissue	**α1**	**α2**	**α3**	**α5**	**α6**	**β1**	**β4**	**CD9**	**CD63**	**CD81**	**CD82**	**CD151**	**CO-029**
Normal pancreatic gland (4)													
Exocrine tissue	−−	−−	−−	−−	−−	+	−−	+	++	++	−−	−−	−−
Ducti	±	±	+	−−	±	+	±	+	++	++	±	+	+
Chronic pancreatitis (10)													
Exocrine tissue	−−	±	±	−−	++	++	+	+	++	++	−−	+	−−
Ducti	±	±	++	±	++	++	++	+	++	++	±	++	++
	basal	basal	basal		basal	basal	basal‡						
Pancreatic cancer (30)													
(%) Distinctly positive tumors	50.0	63.3	63.3	46.7	93.3	63.3	83.3	63.3	96.7	100.0	46.7	96.7	80.0
Overall mean intensity	+	++	++	±	++	++	++	++	++	+++	++	+++	++
(%) Strongly stained tissues	16.7	36.7	40.0	10.0	60.0	36.7	43.3	33.3	86.7	90.0	36.7	66.7	66.7
(%) Distinctly stained tissues	33.3	26.7	23.3	26.7	33.3	26.7	40.0	30.0	10.0	10.0	10.0	30.0	13.3
(%) Weakly stained tissues	16.7	16.7	16.7	43.3	6.7	16.7	13.3	23.3	3.3	0	36.7	3.3	6.7
P		0.019			0.038			0.010	0.007		0.002	0.0003	0.0003

*Immunohistological stainings were scored as: −−, negative; ±, very weak; ±, weak; +, distinct; ++, strong; +++, very strong.
†Statistical significance was calculated by the two-sided Wilcoxon exact test for differences between normal pancreatic and pancreatic carcinoma tissue.
‡Basal, basal staining.

Flow cytometry followed routine procedures using 1 to 3 × 10^5 tumor cells per sample. Trypsinized cells were allowed to recover for 2 hours at 37°C in RPMI 1640, 10% FCS. Samples were analyzed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Immunohistology. Cryostat sections (5 μm) of snap-frozen tissue were fixed in chloroform/acetone (1:1) for 4 minutes. Tissues were incubated for 1 hour with the first antibody, washed, and exposed to the biotinylated secondary antibodies (30 minutes) and alkaline phosphatase–conjugated avidin-biotin complex (Vector Laboratories, Grünberg, Germany) solutions (5-20 minutes). Tissue sections were counterstained with Mayer's hematoxylin. The primary antibody was replaced with normal mouse, rat, or rabbit IgG for negative controls.

For immunofluorescence microscopy, cells were seeded on laminin 5-coated cover slides. Where indicated, cells had been starved and pretreated with 10^{-8} mol/L phorbolmyristate acetate (PMA). After spreading, slides were washed, cells were fixed in 4% paraformaldehyde

Table 3. Coordination between pancreatic cancer staging/grading and integrin-tetraspanin expression

	Mean intensity of expression* (*P* values)†												
Pancreatic tissue	**α1**	**α2**	**α3**	**α5**	**α6**	**β1**	**β4**	**CD9**	**CD63**	**CD81**	**CD82**	**CD151**	**CO-029**
Pancreatic cancer (30)	1.15	1.24	1.50	0.92	1.75	1.37	1.27	1.47	2.59	2.57	1.28	1.98	2.40
Ductal (9)	1.28	1.50	1.39	0.84	1.89	1.42	1.00	1.72	2.89	2.89	1.78	2.25	2.56
Papillary (4)	1.38	1.00	2.25	1.25	1.62	1.75	1.25	2.00	2.50	2.50	1.00	1.50	2.50
Grade 2 (18)	1.15	1.16	1.32	0.93	1.50	0.89	1.29	1.12	2.40	2.53	1.24	2.20	2.38
Grade 3 (11)	0.83	1.00	1.33	0.63	1.92	1.50*	1.08	1.75**	2.83	2.33	1.25	1.33*	2.25
N_0 (9)	0.79	0.76	1.07	0.64	1.50	1.11	0.79	1.57	2.04	2.14	1.50	2.00	2.86
N_1 (20)	1.13	1.32*	1.50	0.90	1.74	1.23	1.32*	1.34*	2.71*	2.63*	1.26	2.00	2.14*
M_0 (10)	0.83	0.70	1.22	0.61	1.39	0.98	1.50	1.39	2.20	2.11	0.89	2.14	2.44
M_1 (4)	1.50*	1.50*	1.75*	1.17	2.00*	1.83***	1.17	1.83***	3.00*	3.00*	1.25	1.83	2.00
Primary (25)	1.10	1.28	1.52	0.90	1.79	1.25	1.31	1.44	2.49	2.46	1.40	2.07	2.43
Recurrency (3)	1.00	0.88	0.63	0.88	1.38	1.25	0.88	1.13	3.00	3.00	1.13	1.33	2.13
Metastasis (2)	**2.00**	1.50	**3.00**	1.25	2.00	**3.00**	1.50	**2.50**	3.00	3.00	**0.25**	2.00	2.50

NOTE: Immunohistological stainings were scored as: 0.25, very weak; 0.5, weak; 1, distinct; 2, strong; 3, very strong. Mean values are shown.
†Statistical significance was calculated by the two-sided Wilcoxon exact test for ductal versus papillary, GII versus GIII; N0 versus N1, and M0 versus M1. *P* values: *, <0.1; **, <0.01; ***, <0.001. Strong differences between primary tumors and metastasis, which due to low numbers could not be evaluated statistically, are printed in boldface.

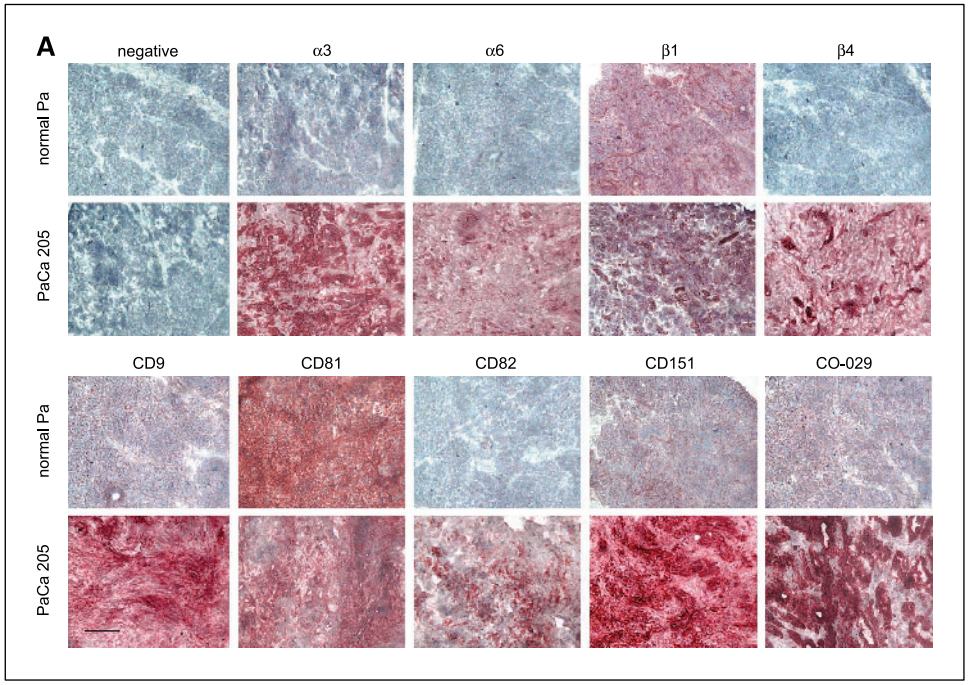

Fig. 1. Comparative evaluation of integrin and tetraspanin expression on pancreatic carcinoma tissue. *A*, cryostat sections (5 μm) of a normal pancreatic and a pancreatic adenocarcinoma tissue were stained with anti-α3, -α6, -β1, -β4, -CD9, -CD81, -CD82, -CD151, and -CO-029 and counterstained with methylene blue; bar, 50 μm.

(w/v in PBS) and, where indicated, were permeabilized (4 minutes, 0.1% Triton X-100). After washing and blocking (0.2% gelatin, 0.5% bovine serum albumin in PBS), cells were incubated with the primary antibody (2-10 μg/mL) in PBS/bovine serum albumin for 60 minutes at 4°C. Slides were rinsed and subsequently incubated for 60 minutes at 4°C with a fluorochrome-conjugated secondary antibody. After washing, free binding sites were blocked by incubation with an excess of unlabeled mouse or rat IgG. Unlabeled mouse or rat IgG was also added during incubation with the second, directly labeled antibody (60 minutes, 4°C). For cross-linking, cells were incubated at 37°C for 15 minutes with the primary antibody and for 20 minutes with an excess (10 μg/mL) of the secondary, dye-labeled antibody. Cells were washed with ice-cold PBS and all consecutive steps were done at 4°C. After washing, slides were mounted in Elvanol. Digitized images were generated using a Leica DMRBE microscope equipped with a SPOT CCD camera from Diagnostic Instruments, Inc. and Software SPOT2.1.2.

Substrate. Laminin 5 was a kind gift from K. Miyazaki (Division of Cell Biology, Yokahama City University, Yokohama, Japan; ref. 35). Plates were coated with 0.3 μg/mL laminin 5 and free binding sites were blocked by incubation with PBS/1% bovine serum albumin.

Immunoprecipitation. Cells (1×10^7) were lysed (4 hours, 4°C) in 4 mL ice-cold lysis buffer [25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$ (pH 7.2)] containing 1% Brij58 or 1% Brij96. Lysis buffers contained a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and 2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation for 30 minutes at 15,000 rpm, lysates (1 mL) were precleared by incubation with 1/10 volume protein G Sepharose and protease inhibitor cocktail (2 hours, 4°C). Precleared lysates were incubated overnight at 4°C with 1 μg of antibody or control IgG. Protein G Sepharose was added for an additional 2 hours. Immune complexes were washed four to six times with lysis buffer and precipitated proteins were eluted with 50 μL of 100 mmol/L glycine (pH 2.7). Immunoprecipitated proteins were analyzed by SDS-PAGE, followed by Western blotting.

Western blotting. Lysates and immunoprecipitated proteins were resolved on 12% or 15% SDS-PAGE under nonreducing conditions and the proteins were transferred to Hybond enhanced chemiluminescence at 30 V overnight. After blocking (5% fat-free milk powder), immunoblotting was done with the indicated antibodies, followed by rabbit anti-mouse horseradish peroxidase. Blots were developed with the enhanced chemiluminescence detection system. Densitometric analysis was done with NIH Image 1.60 software.

Adhesion and migration. In adhesion assays, cells were incubated with [^{3}H]thymidine for 16 hours, washed, and seeded in triplicate on laminin 5-coated flat-bottomed 96-well plates. After incubation (120 minutes, 37°C) and vigorous washing, remaining adherent cells were detached with 0.2% trypsin. Cells were harvested and counted in a β-counter. Where indicated, 10 μg/mL antibodies were added during incubation.

Cell migration was evaluated using a scratch assay or a modification thereof, where Petri dishes were coated with laminin 5 as described above. Thereafter, the central area of the Petri dishes was covered with a cover slide (6 mm diameter). When tumor cells seeded on laminin 5-coated Petri dishes reached near-confluence, the cover slide was removed, medium was aspirated, plates were washed, and RPMI supplemented with 1% FCS and, where indicated, 10^{-8} mol/L PMA was added. Mean values and SDs of the number of cells migrating in the originally cell-free area were evaluated by counting 10 fields of 1 mm^2 at the boundary towards the originally cell-free area using an inverted microscope at 24 and 48 hours after removal of the cover slide. Values represent the mean of three independently performed experiments. Alternatively, subconfluent monolayers were scratched with a blunt-edged needle. Plates were incubated for 48 hours, washed, fixed, and stained with H&E.

Statistics. Significance of differences was calculated by the two-tailed Student's *t* test or the two-sided Wilcoxon exact test with *P* values adjusted according to Bonferroni-Holm.

Results

Clinical studies provide evidence for a significant acceleration of metastasis of pancreatic tumors after isolated tumor cells have settled in the peritoneal cavity (36). Furthermore, the association of integrins with tetraspanins has been suggested to promote cell motility (27–29, 37). Therefore, we investigated whether pancreatic adenocarcinoma and colorectal cancer

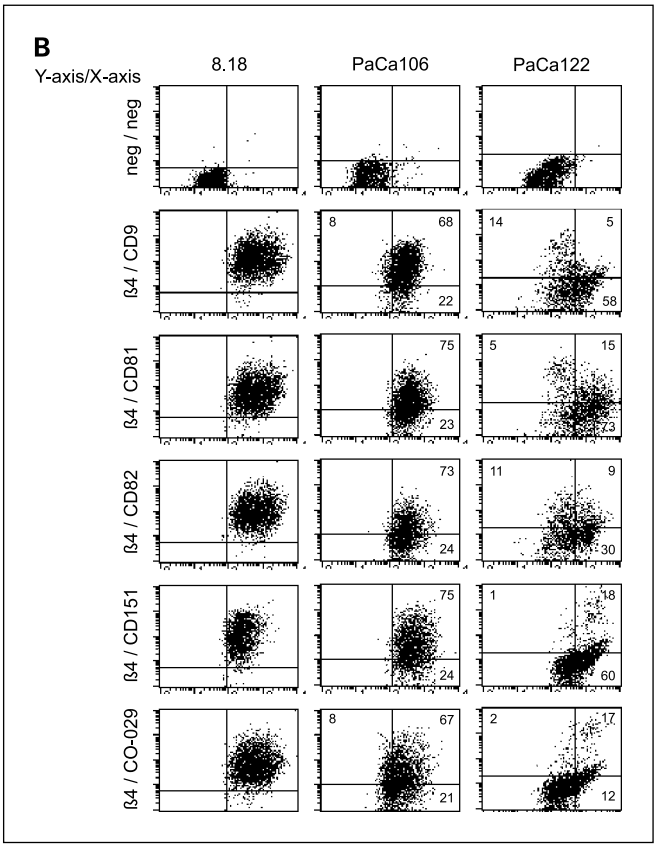

Fig. 1 continued. *B*, P106 and P122 cells are derived from the second or third *in vitro* passage of two pancreatic adenocarcinomas. Tumor cell survival depends on the presence of stroma cells, which are dominant in the P122 cultures. Double staining of both lines in comparison to the long-term 8.18 line with anti-β4 and anti-tetraspanins is shown. The PaCa122 line obviously contains two populations of cells (a minority of tumor cells and a majority of fibroblasts), which differ in β4, CO-029 (mostly tumor cells), and CD9 expression (stronger on tumor fibroblasts). CD151, CD81, and CD82 are expressed by both tumor cells and tumor stroma, although not by all cells.

express defined integrin and tetraspanin patterns, whether and which integrins associate with tetraspanins, and whether the association is accompanied by altered cell motility.

Integrin and tetraspanin expression. Expression of α1 to α6, αL, β1, β2, and β4 and of CD9, CD53, CD63, CD81, CD82, CD151, and CO-029 was evaluated by flow cytometry in 10 pancreatic adenocarcinoma and 9 colorectal cancer lines (Table 1). All tumor lines expressed α2, α3, α6 and β1, and 18 of 19 tumor lines expressed β4. β4 expression mostly exceeded that of β1. Weak α1 and α4 expression was only rarely observed. The tumor lines did not express αL, β2, or CD53. CD63, CD81, and CD82 were expressed by all tumor lines. CD151 was expressed to some extent on all cell lines, and strongly on 70% of pancreatic adenocarcinoma and 89% of colorectal cancer lines. With the exception of two colorectal cancer lines, cells expressed CD9. CO-029 was moderately expressed in 79% of colorectal cancer and 40% of pancreatic adenocarcinoma lines.

To exclude an *in vitro* artifact due to long-term culture, integrin and tetraspanin expression was evaluated on primary tumor tissue sections (Table 2). The expression profile on tissue samples did not differ significantly from that of tumor lines. Over 90% and 80% of pancreatic adenocarcinoma tissue moderately expressed α6 and β4, respectively. CD9, CD63, CD81, and CD151 were expressed by nearly all pancreatic adenocarcinoma tissues. CO-029 expression was higher in pancreatic adenocarcinoma tissues (>80%) than in lines. α1, α3, α6, β1, and β4 were also expressed by normal pancreatic tissue, albeit at a low level, and by chronic pancreatitis tissues, where α3, α6, β1, and β4 expression was distinctly up-regulated. Normal pancreatic tissue expressed CD9, CD63, CD81. Ductal cells expressed moderately CD151 and CO-029 and, at a very low level, CD82. Expression of CD151 and CO-029 on ductal cells was up-regulated in chronic pancreatitis as compared with normal pancreatic tissue.

When tumor tissues were grouped according to grading and staging, statistical analysis provided some significant differences despite the great variability between different tumors. No significant differences were seen between papillary and ductal pancreatic adenocarcinoma. There were no grade 1 pancreatic adenocarcinomas. Grade 3 pancreatic adenocarcinoma expressed β1 and CD9 at a higher level, and CD151 at a lower level than grade 2 tumors, the difference in CD9 expression

Table 4. Integrin and tetraspanin profile of freshly explanted human pancreatic tumor tissue

	Percentage of stained cells and mean intensity of expression (flow cytometry)*															
	α1		α2		α3		α4		α5		α6		β1		β4	
Tumor line	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean
P106 (T)	59	+	55	+	83	+++	0	−−	0	−−	76	++	47	++	73	+
P122 (F)	52	+	50	+	59	+++	10	±	21	+	34	++	50	++	19	+

	CD9		CD63		CD81		CD82		CD151		CO-029	
	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean	(%)	Mean
P106 (T)	89	+	52	+	97	+	96	+	98	++	88	+
P122 (F)	63	++	73	++	88	+	39	+	78	++	29	±

*Flow cytometry data were analyzed according to the increase in the mean fluorescence intensity as compared with the negative control. No staining, −− (intensity 1.0-1.3 fold); weak staining, ± (intensity >1.3-2.0-fold); distinct staining, + (intensity >2.0-5.0-fold); strong staining, ++ (intensity >5.0-10.0-fold); very strong staining, +++ (intensity >10.0-fold).

being highly significant. Highly significant differences ($P < 0.01$) were also seen in β1 and CD9 expression of pancreatic adenocarcinoma tissues from patients with liver metastasis. CD9, β1, and α3 expression was also high in the two metastatic tissues, which hardly expressed CD82. However, the low number of metastatic tissues prohibited a statistical evaluation (Table 3).

The high expression of CD9 and (α3)β1 in grade 3 and advanced tumors was unexpected, since both markers have been associated with good prognosis (5, 6, 18–21). However, pancreatic adenocarcinomas are characterized by the induction of a strong stromal reaction and tumor surrounding fibroblasts differed from fibroblasts in normal pancreatic tissue in as much as they frequently and strongly expressed CD9 and CD151 (Fig. 1A). In view of the apparent influence of pancreatic adenocarcinoma on their tissue environment, it became important to evaluate integrin and tetraspanin expression in freshly explanted pancreatic adenocarcinoma tissue, which required surrounding fibroblasts for survival. Tumor cells dominated in the PaCa106 line, whereas in the PaCa122 line, few tumor cells formed clusters between abundant fibroblasts (data not shown). The majority of cells in both lines expressed α1, α2, α3, β1, CD9, CD63, CD81, and CD151, with higher CD9 and CD63 expression in PaCa122 than in PaCa106 cells. In contrast, β4, CD82 and CO-029 expression was higher in PaCa106 than PaCa122 cells (Table 4). Double fluorescence analysis (Fig. 1B) confirmed that CD9 and CD63 were preferentially expressed on stromal cells, whereas β4 was preferentially expressed by tumor cells, i.e., in the PaCa122 line, which contains few tumor cells, β4 was minimally expressed by CD9+ cells, whereas the majority of β4-expressing cells were CD151+ and CO-029+. CD151 was expressed by tumor cells and stromal cells and CO-029 was expressed predominantly by tumor cells. The result was not as clear-cut for CD81 and CD82, inasmuch as some, but not all, CD81+ and CD82+ cells were β4+. Nonetheless, the analysis of these tumor/stroma cell lines confirmed that β4 and CO-029 are expressed predominantly by tumor cells, whereas CD9 and CD63 are expressed more strongly by tumor-associated fibroblasts than the tumor cells.

Taken together, tumor lines and tissues mostly express α3 and β1 at an intermediate, and α6 and β4 at a high level, although not all tumors express β4. Expression of CD9, CD63, CD81, CD82, CD151, and CO-029 is frequently high. CD9 and CD63 are also strongly expressed on the tumor stroma. CD82 is rather tumor-specific, but is not expressed by all tumors.

Colocalization and association of integrins and tetraspanins. Tetraspanins frequently associate with integrins (38). Since expression of several integrins and tetraspanins was up-regulated in pancreatic adenocarcinoma and colorectal cancer,

Table 5. Colocalization of integrins with tetraspanins

	Colocalization*													
	α3 with			β1 with			α6 with				β4 with			
Tumor line	CD9	CD81	CD151	CD9	CD81	CD151	CD9	CD81	CD151	CO-029	CD9	CD81	CD151	CO-029
Pancreatic cancer														
AsPC1	+	+	nt	+	+	nt	+	– –	+	++	– –	– –	+	+
BxPC3	+	++	nt	++	++	nt	+	– –	+	±	++	– –	+	±
Capan1	+	++	nt	+	++	nt	– –	– –	±	±	– –	– –	±	±
Capan2	++	+	nt	+	+	nt	+	++	±	– –	++	+	±	±
Colo357	+	±	nt	++	±	nt	– –	– –	±	±	– –	– –	±	±
MiaPaca1	±	+	++	±	+	++	±	±	±	na	+	±	±	na
Panc1	±	++	++	±	++	+	±	+	+	na	±	– –	+	na
Panc89	+	++	nt	+	+	nt	±	±	+	±	±	±	+	+
Pt45P1	+	+	++	+	+	++	±	– –	±	na	na	na	na	na
PancTu1	+	+	nt	+	+	nt	+	+	±	+	±	+	+	+
8.18	+	+	++	+	++	+	±	+	+	+	– –	– –	+	+
P73	±	±	nt	±	±	nt	+	+	±	±	±	±	±	±
P106	– –	– –	nt	– –	– –	nt	±	±	±	±	±	+	+	+
P122	±	±	nt	– –	– –	nt	+	±	++	+	+	+	+	+
Colorectal cancer														
Colo320	na	+	nt	na	+	nt	na	+	– –	±	na	– –	– –	±
Colo320DM	na	+	nt	na	+	nt	na	+	– –	±	na	±	– –	±
HT29	+	+	nt	+	+	nt	±	±	++	++	±	±	++	++
Lovo	±	+	+	±	+	+	±	– –	+	±	±	– –	±	±
SW480	+	±	++	+	+	+	±	±	+	na	– –	– –	+	na
SW707	±	±	+	±	±	+	+	±	+	+	+	±	++	±
SW948	±	±	nt	±	±	nt	+	±	±	±	– –	– –	±	+
WIDR	±	+	nt	+	+	nt	±	±	+	+	– –	– –	+	+

Abbreviations: nt, not tested; na, not applicable (cell lines with no, very weak, or weak expression were excluded).
*Degree of colocalization: ± , very weak; ±, weak; +, distinct; ++, strong.

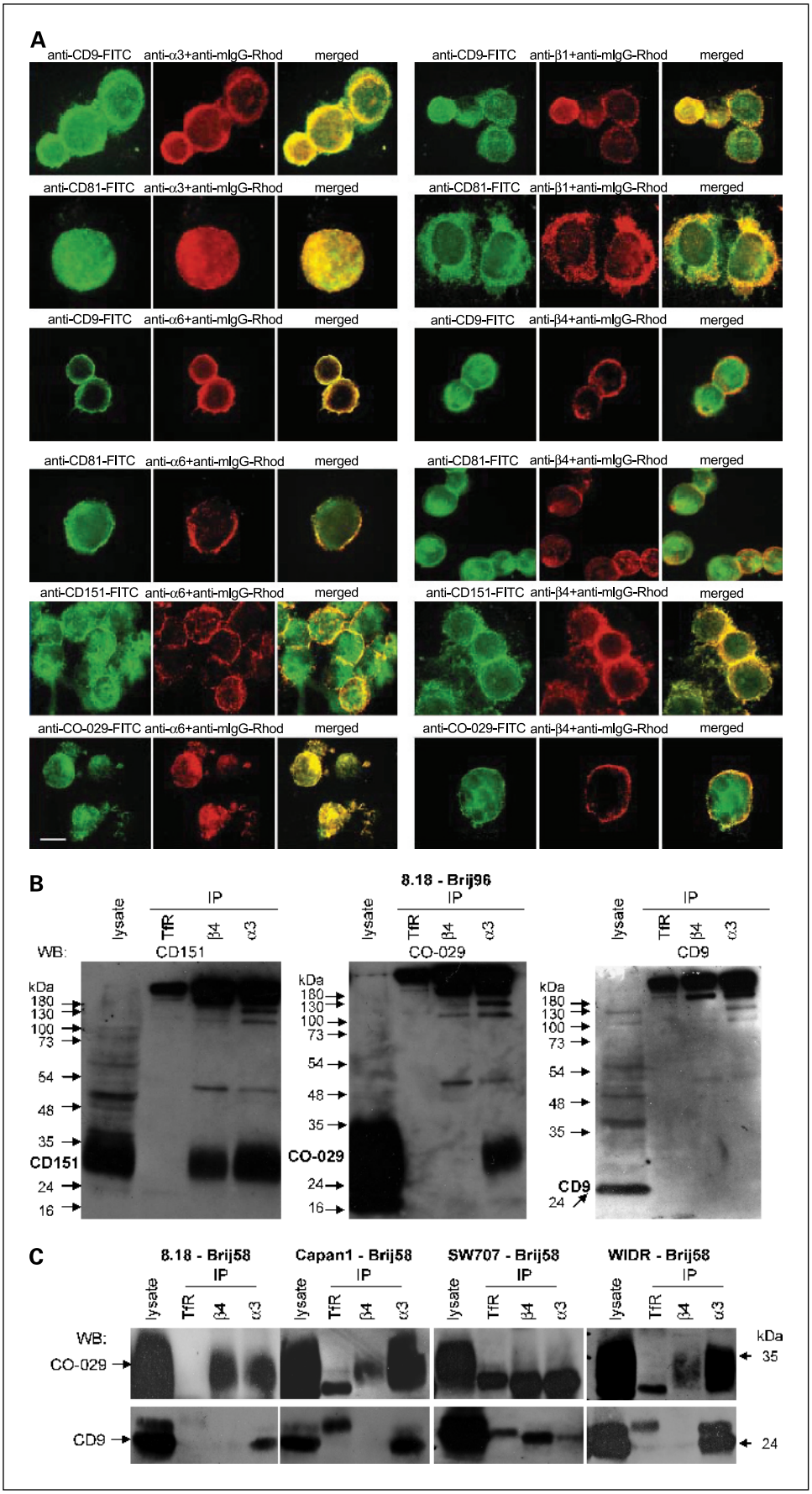

Fig. 2. Integrin and tetraspanin colocalization and coimmunoprecipitation. *A*, 8.18 cells were seeded on cover slides and incubated with the first antibody (15 minutes, 37°C) and the secondary, rhodamine-labeled antibody (30 minutes, 37°C). Cover slides were placed on ice, washed, blocked, and stained with the second, FITC-labeled antibody (1 hour, 4°C). After washing, cells were embedded in Elvanol. Digitized images were generated. Single stainings and merged overlays are shown. Colocalization is indicated by yellow staining; bar, 50 μm. *B*, 8.18 cells were lysed in Brij96 and lysates were precipitated with anti-α3 or anti-β4 or anti-transferrin receptor as control. After SDS-PAGE and transfer, membranes were blotted with anti-CD151, anti-CO-029, and anti-CD9. CD151 was detected in α3 and β4 precipitates and CO-029 in α3 precipitates. CD9 was not detected in either α3 or in β4 precipitates; the latter also did not contain CO-029. *C*, 8.18, Capan1, SW707, and WIDR cells were lysed in Brij58. Lysates were precipitated with anti-α3 and anti-β4. After SDS-PAGE and transfer, membranes were blotted with anti-CD9 and anti-CO-029. α3 precipitates contained CD9 and CO-029, β4 precipitates contained only CO-029. From 10 tested lines (only 4 lines are shown), only the SW707 line differed in as much as anti-β4 precipitated CD9, but not CO-029, whereas α3 precipitates contained CD9 and CO-029. All experiments were repeated at least thrice. A representative example is shown.

it was important to determine whether and which integrins would colocalize and/or associate with tetraspanins. Colocalization was evaluated by fluorescence microscopy (Table 5, Fig. 2A). In most pancreatic adenocarcinoma and colorectal cancer lines, α3 and β1 distinctly colocalized with CD9 (12 of 20) and CD81 (14 and 16 of 20). Colocalization of α3 and β1 with CD151 (evaluated for 7 lines) was seen in all instances. The α6 integrin colocalized distinctly with CD9 in only 8 of 20 and with CD81 in 7 of 22 tumor lines. Colocalization of β4 with CD9 (5 of 19) and CD81 (4 of 21) was rare, indicating that CD9 and CD81 preferentially colocalized with α3β1 rather than α6β1 integrins. Colocalization of α6 with CD151 and CO-029 (11 of 22 and 7 of 18) mostly coincided with colocalization of β4 with CD151 (11 of 21) and CO-029 (9 of 18). The latter colocalization was also seen in the freshly explanted P106 and P122 cells. Thus, α3β1 frequently colocalized with CD9, CD81, and CD151. In contrast, α6β4 colocalized mostly with CD151 and CO-029.

To see whether colocalization corresponds to protein association, coimmunoprecipitation of α3(β1) and (α6) β4 with CD9, CD151, and CO-029 was evaluated (Fig. 2B and C). CD151 coimmunoprecipitated with α3 and β4 after stringent lysis in Brij96. α3 precipitates, but not β4 precipitates, contained CO-029. CD9 was not detected in α3 or β4 precipitates after Brij96 lysis. In contrast, after mild lysis in Brij58, α3 precipitates from all tested lines contained CD9 and, with the exception of the SW707 line, CO-029. Also with the exception of the SW707 line, β4 coprecipitated CO-029, but not CD9 (Table 6).

Thus, coimmunoprecipitation confirmed the associations of CD151 and CD9 with α3β1 (28, 39) and of CD151 with α6β4 (31). CO-029 associates with α3β1 and under mild lysis conditions with α6β4. Notably, with the exception of one colorectal cancer line, α6β4 did not associate with CD9. High α3β1 (5, 6) and CD9 (18–21) expression are frequently associated with a good prognosis, whereas high α6β4 (9), CD151, and CO-029 (18, 24) expression indicate a poor prognosis. Taking into account that α6β4 associated only with CD151 and CO-029, we speculated that this association may have an impact on tumor cell motility.

The impact of integrin-tetraspanin complexes on tumor cell migration. The α3β1 and α6β4 integrins both bind to laminin 5 (40) and all tested lines bound more strongly to laminin 5 than to bovine serum albumin. Laminin 5 binding was reduced in most lines in the presence of blocking anti-α3 and anti-β4. Panc89 and Colo357 were exceptions in that laminin 5 binding of Colo357 cells was not inhibited by anti-α3 and Panc89 binding was not inhibited by anti-α3 and β4. PKC activation can support cell migration by inducing internalization of tetraspanin-integrin complexes (41). In fact, laminin 5 adhesion was strongly reduced in AsPC1, Capan1, and HT29 cells, distinctly in Colo357 and 8.18 cells and weakly in WIDR cells after PMA treatment (Fig. 3A). Furthermore, reduced laminin 5 adhesion after PKC activation correlated mostly with increased cell motility (Fig. 3B). Migration of cells out of a semiconfluent monolayer into a cell-free area, which had been protected by a cover slide, was evaluated after 24 hours. Whereas the number of migrating Panc89 and WIDR cells was independent of the presence of PMA, AsPC1, Capan1, Colo357, 8.18, and HT29 cells migrated more readily. The finding was confirmed when a monolayer was stained 48 hours after wounding with a blunt-edged needle (Fig. 3C). Wound closure of AsPC1, Capan1, Colo357, 8.18, and HT29 cells was more advanced in the presence than in the absence of PMA. Thus, PKC activation was accompanied by reduced laminin 5 adhesion in those lines that gained in motility. The two lines where PMA treatment had no impact migrated very rapidly even without PKC activation.

As increased migration after PMA treatment correlated with reduced laminin 5 adhesion, we speculated that PMA treatment might be accompanied by redistribution and/or changes in integrin-tetraspanin colocalization. We hypothesized that the association of α6β4 with CD151 and CO-029, rather than the association of α3β1 with CD9, might account for increased tumor cell motility. Therefore, we compared the impact of PMA treatment on the α3β1-CD9, α6β4-CD151, and α6β4-CO-029 association.

Cells were seeded on laminin 5-coated plates and treated for 1 or 2 hours with PMA. In most lines, α6β4 colocalized more readily with CD151 and CO-029 after PMA treatment (Table 7). Quantification of coimmunoprecipitates revealed that anti-α3 precipitated less CD9, CO-029, and CD151, whereas anti-β4 precipitated increased amounts of CO-029 and CD151 (Table 8). α6β4-CD151 and α6β4-CO-029 complexes had disappeared from the cell membrane after 1 hour of PMA-treatment and became enriched in the perinuclear region. After 2 hours of PMA-treatment, the codistribution of α6β4-CD151 and α6β4-CO-029 differed in the individual lines. In Capan1 cells, whose motility was strongly influenced by PMA treatment, α6β4-CD151 and α6β4-CO-029 complexes remained diffusely dispersed and were not enriched at the membrane. In HT29, whose migratory activity was less

Table 6. Coimmunoprecipitation of CD9, CD151, and CO-029 with α3 and β4 in human pancreatic and colorectal tumor lines

		Coimmunoprecipitation*					
Tumor line	Immuno-precipitation	α3	β4	α3	β4	α3	β4
	Western blot	CD9		CO-029		CD151	
Pancreatic cancer							
AsPC1		+	– –	+	+	++	+
Capan1		++	– –	++	±	++	++
8.18		+++	– –	++	++	+++	++
BxPC3		+	– –	na	na	na	na
MiaPaca1		++	– –	na	na	++	++
Colorectal cancer							
WIDR		++	– –	++	+	+++	++
SW707		±	+	±	– –	++	+

Abbreviations: nt, not tested; na, not applicable (cell lines with no, very weak, or weak expression were excluded).
*Coimmunoprecipitation was evaluated after lysis in Brij58 (Western blot, CD9 and CO-029) or Brij96 (Western blot, CD151); the amount of coprecipitating protein was estimated by the densitometric ratio of precipitate to lysate; negative, ⟨0.1; ±, ⟩0.1-0.2; +, ⟩0.2-0.4; ++, ⟩0.4-0.6; +++, ⟩0.6.

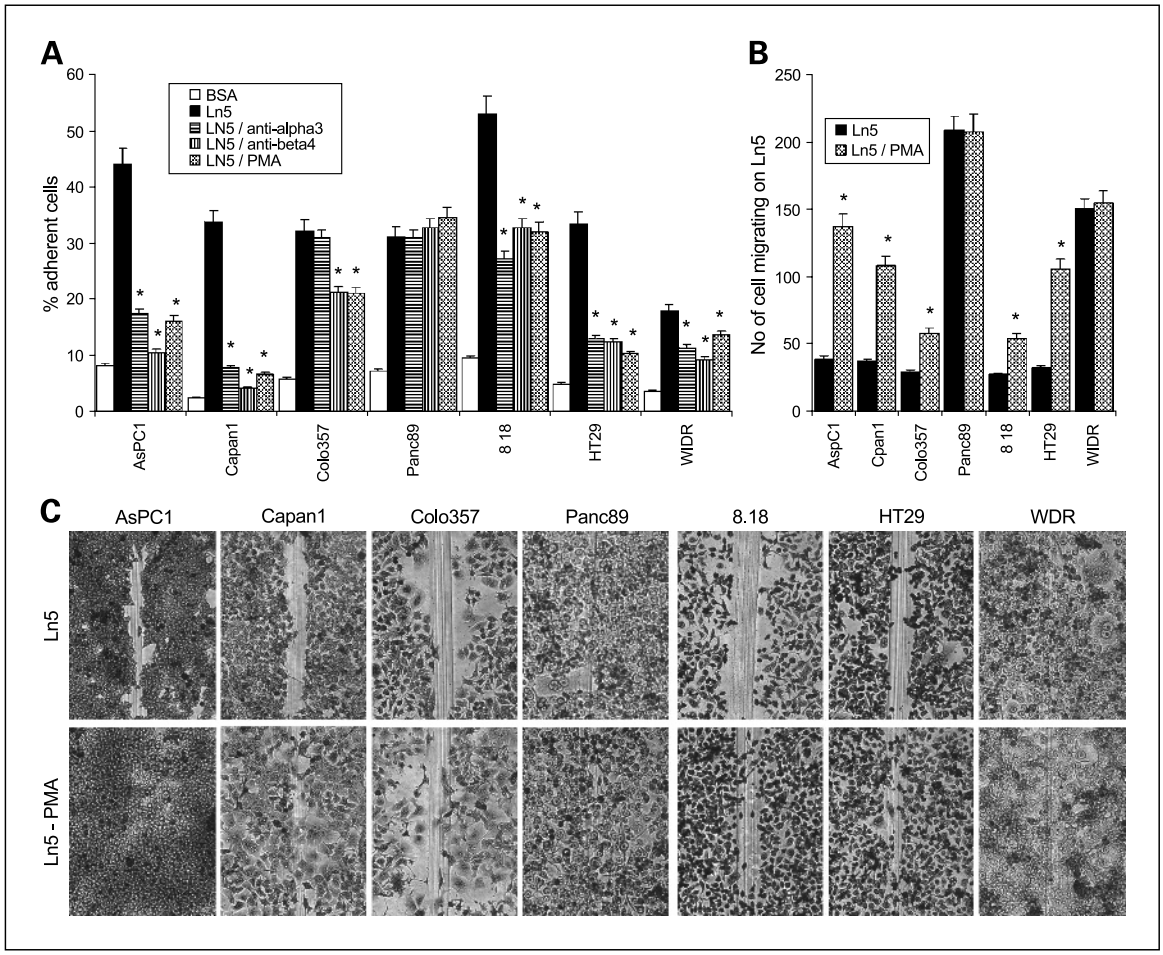

Fig. 3. Adhesion and migration of pancreatic adenocarcinoma and colorectal cancer tumor lines on laminin 5. *A*, AsPC1, Capan1, Colo357, Panc89, 8.18, HT29, and WIDR were labeled overnight with [^{3}H]thymidine. During the last 2 hours of culture, medium was replaced by fresh medium without FCS, containing 10^{-8} mol/L PMA, where indicated. After washing, cells were seeded in bovine serum albumin- or laminin 5-coated flat-bottomed 96-well plates in the absence or presence of anti-α3 or anti-β4 (10 μg/mL). After incubation (2 hours, 37°C) and stringent washing, adherent cells were detached by trypsin. Cells were harvested and [^{3}H]thymidine uptake was determined in a β-counter. The percentage of adherent cells (mean ± SD of triplicates) is shown; *, significant differences ($P < 0.01$). *B*, tumor cells were seeded on laminin 5-coated Petri dishes, where the central area had been covered by a cover slide. When cells reached subconfluency, the cover slide was removed and medium was exchanged. Where indicated, the added medium containing 10^{-8} mol/L PMA. Cells, which had moved into the area protected by the cover slide, were counted after 24 hours. Columns, mean; bars, ± SD of three plates; *, significant differences ($P < 0.01$). *C*, Tumor cells were seeded on laminin 5-coated Petri dishes. When cells reached subconfluency, the monolayer was wounded with a blunt tweezer and medium was exchanged as in (*B*). Cells were cultured for an additional 48 hours in the presence or absence of 10^{-8} mol/L PMA. Petri dishes were washed and stained with H&E. Migration into the wounded area in the presence or absence of PMA is shown; bar, 50 μm. Experiments were repeated at least thrice.

increased, and in WIDR cells, which moved independently of PMA treatment, the complexes were enriched or exclusively located at the cell membrane. In WIDR cells, CO-029 and β4 colocalized at the migratory front of the leading lamella (Fig. 4B and C). After 4 hours of PMA treatment, CO-029 and CD151 colocalized with β4 in the leading lamella of all three lines (data not shown). We did not observe a similar coincidence of increased motility, increased colocalization and internalization for α3 and CD9 in PMA-treated tumor cells. Without PMA treatment, α3β1-CD9 colocalization was most pronounced at cell-cell contact sites, where α6β4-CD151 and α6β4-CO-029 rarely colocalized. This can best be seen with WIDR cells, which tend to grow in clusters. After PMA-treatment, α3-CD9 colocalization was mostly seen in the cytoplasm of all lines (Fig. 4A).

Thus, PKC activation supported the association of α6β4 with CD151 and CO-029. α6β4-CD151/CO-029 complexes were transiently enriched in the cytosol, mostly in the perinuclear region, but reappeared—with a cell line-dependent kinetic—at the leading lamella. A3β1-CD9 colocalization was most pronounced at cell-cell contact sites. Upon PMA treatment, cell-cell contacts became loose, α3β1-CD9 complexes became diffusely distributed in the cytoplasm and, in contrast to α6β4-CD151/CO-029, less α3β1 associated with tetraspanins. Thus, only the α6β4-CD151/CO-029 associations were strengthened by PKC activation and increased colocalization correlated with increased cell motility.

Discussion

Pancreatic adenocarcinomas have a poor prognosis due to early and massive spread into the peritoneal cavity and settlement in the liver (1, 3, 4). Several reports indicate a contribution of α3β1 and α6β4 (5, 42). We noted in a highly metastatic rat pancreatic adenocarcinoma line that α6β4 associated with CO-029 after PKC activation. The complex

Table 7. Protein kinase C activation and colocalization

	Colocalization after PMA treatment*					
	β4/CD151		β4/CO-029		α3/CD9	
PMA	− −	+	− −	+	− −	+
Tumor line						
AsPC1	+ (s)†	+ (s)	+ (s)	+ (s)	+ (s)	+ (i)
Capan1	± (s)	+ (i)	± (s)	+ (i)	+ (s)	+ (i)
Colo357	± (s)	+ (s + i)	± (s)	+ (s + i)	+ (s)	+ (i)
PancTu1	+ (s)	+ (s)	+ (s)	+ (s)	+ (s)	+ (i)
Panc89	+ (s)	+ (s)	+ (s)	+ (s)	+ (s)	+ (i)
8.18	+ (s)	++ (i)	+ (s)	++ (s + i)	+ (s)	+ (s + i)
HT29	++ (s)	++ (s)	+ (s)	++ (s + i)	+ (s)	+ (s + i)
WIDR	+ (s)	++ (s)	+ (s)	++ (s)	+ (s)	+ (s + i)

*Cells were cultured for 2 hours in the absence of FCS in medium containing 10^{-8}M PMA; degree of colocalization: ±, weak; +, distinct; ++, strong.
†s, mostly surface staining; i, mostly internalized; s + i, equally distributed.

became internalized and cells changed from a sessile towards a motile phenotype (34). In view of these observations, we speculated that coexpression of integrins, particularly of α6β4, and tetraspanins, may be a key element in the metastatic behavior of pancreatic adenocarcinoma. Here, we show high expression of several integrins and tetraspanins in pancreatic adenocarcinoma tissues and lines in comparison to normal pancreatic and chronic pancreatitis tissues. Furthermore, laminin 5-binding integrins were found in association with tetraspanins. However, only the α6β4-CD151/CO-029 associations were strengthened by PKC activation, which was frequently accompanied by increased motility. We suggest that α6β4-CD151/CO-029 complexes might promote the massive metastatic spread of pancreatic adenocarcinoma.

Integrin and tetraspanin expression in tumors and tumor stroma. A recent study on molecular profiling of pancreatic adenocarcinoma defined α2, α3, and β4 significantly up-regulated in comparison to normal and chronic pancreatitis tissue (13). This is well in line with our findings, which show, in addition, strongly up-regulated α6 and β1 expression in chronic pancreatitis. However, expression remained restricted to the basal membrane, whereas the orientation was lost in pancreatic adenocarcinoma. Furthermore, in freshly explanted pancreatic adenocarcinoma tissue, where islets of tumor cells could be clearly distinguished morphologically from the tumor stroma, α1, α2 and α3 were expressed at a high level. As those integrins are weakly expressed in normal pancreatic tissue, it is likely that their high expression on the tumor stroma may contribute to tumor cell survival. The strong stromal reaction of many pancreatic adenocarcinomas may also account for the reported differences in integrin expression of pancreatic adenocarcinoma (6, 43). Taken together, pancreatic adenocarcinoma and colorectal cancer do not differ significantly, but differ from healthy and inflamed tissue by α1, α2, α3, and β4 overexpression. Tumor stroma is characterized by α1, α2, α3, and β1 up-regulation.

In relation to the impact of tetraspanins on pancreatic adenocarcinoma progression, CD82 and CD9 mRNA levels have been reported to correlate inversely with histopathologic grading and survival time, whereas CD63 mRNA levels appeared to be independent of grading and staging (20). We also noted that tetraspanin expression varies between healthy, chronically inflamed, and cancerous tissue. CD63 expression was slightly increased in chronic pancreatitis and pancreatic adenocarcinoma as compared with healthy tissue. As reported (44), CD9 expression on tumor stroma exceeded expression

Table 8. Protein kinase C activation and coimmunoprecipitation

		Densitometric ratio of coprecipitate/lysate*					
		Immunoprecipitation					
		α3			β4		
		PMA					
	Western blot	− −	+	Ratio (±)	− −	+	Ratio (±)
Tumor line							
Capan1	CD151	nt	nt		0.65	0.90	1.38
8.18	CD151	0.63	0.42	0.67	0.48	0.65	1.35
Capan1	CD9	0.53	0.46	0.87	− −	− −	
MiaPaca1	CD9	0.46	0.24	0.52	− −	− −	
8.18	CD9	0.76	0.47	0.62	− −	− −	
WIDR	CD9	0.52	0.36	0.69	− −	− −	
Capan1	CO-029	0.55	0.50	0.92	0.14	0.38	2.71
8.18	CO-029	0.58	0.29	0.50	0.41	0.56	1.38
WIDR	CO-029	0.57	0.36	0.63	0.31	0.42	1.35

Abbreviations: nt, not tested.
*Coimmunoprecipitation was evaluated after lysis in Brij58 (Western blot, CD9 and CO-029) or Brij96 (Western blot, CD151); the amount of coprecipitating protein was estimated by the densitometric ratio of precipitate to lysate. Mean values of three to five experiments are shown.

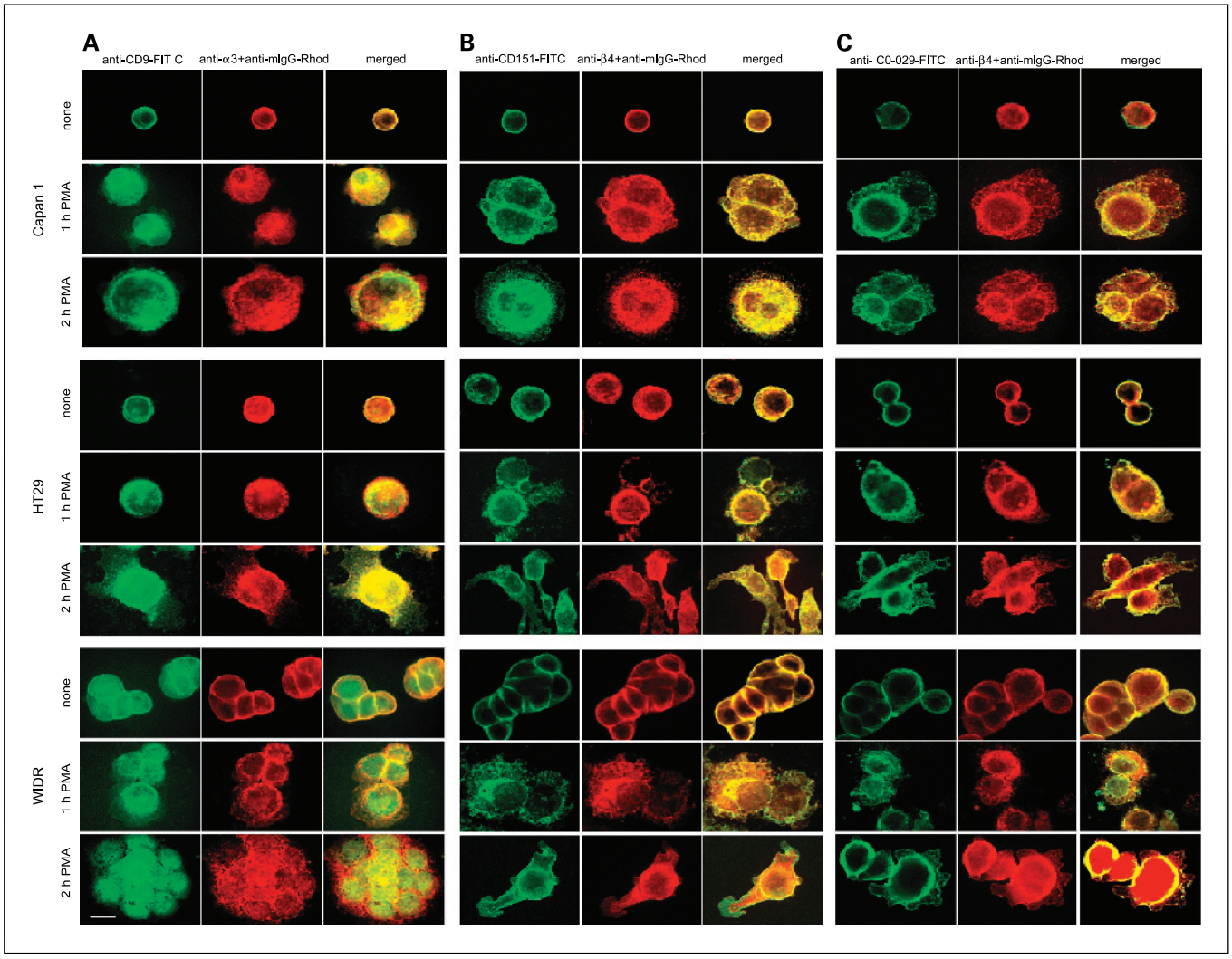

Fig. 4. Colocalization and redistribution of α3-CD9 and β4-CD151/CO-029 after PKC stimulation. Capan1, HT29, and WIDR cells were seeded on cover slides, starved, and cultured for 1 or 2 hours in the presence of 10^{-8} mol/L PMA. Cells were fixed, permeabilized, and stained with the first antibody and a rhodamine-labeled secondary antibody. After washing and blocking, cells were stained with the second, FITC-labeled antibody. Digitized images were generated. Single stainings and merged overlays are shown for (*A*) α3 and CD9, (*B*) β4 and CD151, (*C*) β4 and CO-029; bar, 10 μm.

on tumor cells. However, and possibly hidden by the stromal expression, we did not observe low CD9 expression in progressed pancreatic adenocarcinoma tissue (19–21). CD81 expression was distinctly increased in pancreatic adenocarcinoma. CD151 was weakly expressed in healthy tissue, moderately in chronic pancreatitis, and strongly in most pancreatic adenocarcinoma. CO-029 was moderately expressed on ductal cells, expression was up-regulated in chronic pancreatitis and was strong in pancreatic adenocarcinoma, although not in all tumors. Tumor stroma did not express CO-029. CD82 was the only tetraspanin not expressed by normal pancreatic tissue and very weakly by chronic pancreatitis tissue, but strongly by nearly 50% of pancreatic adenocarcinoma. Thus, CD82 can be considered as a distinct tumor marker. The finding was unexpected, because CD82 has been described repeatedly to inhibit metastasis formation (19, 21, 23, 43, 45) and pancreatic adenocarcinoma are highly metastatic. Correlating CD82 expression with tumor grading and disease state, indeed, revealed very low CD82 expression on local recurrences and liver metastases. However, the number of recurrent/metastatic tissues was too low for a statistical evaluation. Thus, further studies are required to unravel whether this metastasis-inhibitory molecule becomes down-regulated only at a late stage of pancreatic adenocarcinoma progression, which actually has been observed in prostate cancer (46).

Integrin-tetraspanin colocalization in pancreatic and colorectal cancer. Tetraspanins form multimolecular complexes that frequently include integrins (25–28). The tetraspanin CD151 strongly associates with α3β1 (28, 47) and α6β4 (31). CD9 associates preferentially with α3β1 and α6β1 (39, 48). CD81 forms complexes with α3β1, α4β1, and other integrins (39, 49). We described an association of the rat CO-029 homologue with α6β1, α3β1 (14) and, outside of hemidesmosomes, with α6β4 (34). This colocalization pattern largely resembles that of CD151 with laminin-binding integrins (31). Our findings on pancreatic adenocarcinoma and colorectal cancer are in line with the described features and confirm that the α6β4-CD151,

but not the α6β4 and CO-029 complex resists lysis in detergents of intermediate strength (31). As tetraspanins can mutually associate (50), we hypothesize that CO-029 associates rather with CD151 than with the integrin. Several observations support the hypothesis: the coimmunoprecipitation pattern of CO-029 with α3β1, α6β1, and α6β4 overlaps with the coimmunoprecipitation pattern of CD151 (31); colocalization of α3β1 with CD9 was most strong at cell-cell contact sites (37), where α6β4-CD151/CO-029 colocalization was less prominent; PMA treatment strengthened the α6β4-CD151/CO-029, but weakened the CD9-α3β1 association; PMA-induced internalization of CD151, CO-029, and β4 was similar, but was different from that of CD9 and α3.

α3β1-CD151 complexes resist strong detergents and are observed at a high stoichiometry (50). Our findings of a preferential association of CD151 with α6β4 could well be explained by the studies of Sterk et al. (31), which showed efficient competition of α6β4 for CD151 within hemidesmosomes such that α3β1 was dislodged towards focal adhesion sites and only part of CD151 remained associated with α3β1. Sterk et al. (31) also described exclusion of CD9 from CD151-α6β4 complexes and we noted that this also accounts for CD151-CO-029-α6β4 complexes with the exception of SW707 cells. Thus, we would argue that in the absence of α6β4 or laminin 5, α3β1 is found in clusters with CD151, CD9, and CO-029. In contrast, in CD151-α6β4 clusters, CO-029 remains preferentially associated with α6β4 via CD151, whereas CD9 remains associated with α3β1. The reason for this supposed "reorganization" of tetraspanin-integrin complexes remains to be explored. It could be due to a competition of the integrins for laminin 5 or to differences in the cytoplasmic regions of the tetraspanins. Thus, CO-029 and CD151, but not CD9, have a tyrosine-based internalization motif (38), which could account for CO-029-CD151-α6β4 complex guidance into recycling vesicles.

The impact of tetraspanin-integrin complexes on tumor cell motility. Tetraspanin-integrin complexes contribute to cell-cell adhesion (19, 27, 37, 38), relocation of tetraspanin complexes to actin-based structures (51) and are proposed to account for turnover and sorting of associated integrins (19, 27, 37, 38). Because we observed (*a*) that α3β1 preferentially colocalized with CD9 at cell-cell contact sites, where colocalization of CD151 and CO-029 with α6β4 was hardly detected; and (*b*) that re-expression after PMA-induced internalization of CD151-CO-029-α6β4 differed from that of α3β1-CD9 complexes, we wondered whether these tetraspanin-integrin complexes might have a different impact on tumor cell motility.

In fact, tumor lines which displayed reduced adhesion in the presence of anti-β4, were also poorly adhesive after PMA treatment and showed improved migration. In contrast, anti-β4 did not affect the adhesiveness of Panc89 cells, which did not increase migration after PMA treatment. Furthermore, the PMA-induced increase in colocalization of CD151-CO-029-α6β4 as well as the strength of internalization correlated with increased motility and decreased adhesiveness. Finally, AsPC1, Capan1, Colo357, 8.18, and HT29 cells, which gained in motility as a result of PKC activation, have been described as (highly) metastatic, whereas WIDR and Panc89 are defined as low or nonmetastatic (see supplement, Table S2). Thus, stimulation-induced internalization of CD151/CO-029 and α6β4 may enhance tumor cell motility and contribute to the high metastatic potential of pancreatic adenocarcinoma and colorectal cancer after dissemination in the peritoneal cavity, which can provide a stimulatory milieu.

Expression of several integrins and tetraspanins is up-regulated in pancreatic adenocarcinoma and colorectal cancer, and α3β1 as well as α6β4 form complexes with CD9, CD151 and/or CO-029. However, only the α6β4-CD151/CO-029 association seems to contribute to cell motility. In metastasizing pancreatic and colorectal carcinoma lines, PKC activation is accompanied by transient internalization of α6β4-CD151/CO-029 complexes, changes in cell shape towards a migratory phenotype and increased motility. The formation of this integrin-tetraspanin complexes could well be a key feature for the high motility of pancreatic adenocarcinoma and colorectal cancer cells and their pronounced metastatic progression after settling in the peritoneal cavity.

References

1. Rosenberg L, Lipsett M. Biotherapeutic approaches to pancreatic cancer. Expert Opin Biol Ther 2003;3:319–7.
2. Brand R. The diagnosis of pancreatic cancer. Cancer J 2001;7:287–97.
3. Kleeff J, Friess H, Berberat PO, Martignoni ME, Z'graggen K, Buchler MW. Pancreatic cancer—new aspects of molecular biology research. Swiss Surg 2000;6:231–4.
4. Perugini RA, McDade TP, Vittimberga FJ Jr, Callery MP. The molecular and cellular biology of pancreatic cancer. Crit Rev Eukaryot Gene Expr 1998;8:377–93.
5. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002;2:91–100.
6. Mercurio AM, Rabinovitz I. Towards a mechanistic understanding of tumor invasion-lessons from the α6β4 integrin. Semin Cancer Biol 2001;11:129–41.
7. Lohr M, Trautmann B, Gottler M, et al. Expression and function of receptors for extracellular matrix proteins in human ductal adenocarcinomas of the pancreas. Pancreas 1996;12:248–59.
8. Sawai H, Funahashi H, Matsuo Y, et al. Expression and prognostic roles of integrins and interleukin-1 receptor type I in patients with ductal adenocarcinoma of the pancreas. Dig Dis Sci 2003;48:1241–50.
9. Katayama M, Sanzen N, Funakoshi A, Sekiguchi K. Laminin γ2-chain fragment in the circulation: a prognostic indicator of epithelial tumor invasion. Cancer Res 2003;63:222–9.
10. Nishimori H, Yasoshima T, Denno R, et al. A new peritoneal dissemination model established from the human pancreatic cancer cell line. Pancreas 2001;22:348–56.
11. Linder S, Castanos-Velez E, von Rosen A, Biberfeld P. Immunohistochemical expression of extracellular matrix proteins and adhesion molecules in pancreatic carcinoma. Hepatogastroenterology 2001;48:1321–7.
12. Weinel RJ, Rosendahl A, Neumann K, et al. Expression and function of VLA-α2, -α3, -α5 and -α6-integrin receptors in pancreatic carcinoma. Int J Cancer 1992;52:827–33.
13. Logsdon CD, Simeone DM, Binkley C, et al. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. Cancer Res 2003;63:2649–57.
14. Claas C, Seiter S, Claas A, Savelyeva L, Schwab M, Zöller M. Association between the rat homologue of CO-029, a metastasis-associated tetraspanin molecule and consumption coagulopathy. J Cell Biol 1998;141:267–80.
15. Odintsova E, Sugiura T, Berditchevski F. Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. Curr Biol 2000;10:1009–12.
16. Testa JE, Brooks PC, Lin JM, Quigle JP. Eukaryotic expression cloning with an antimetastatic monoclonal antibody identifies a tetraspanin (PETA-3/CD151) as an effector of human tumor cell migration and metastasis. Cancer Res 1999;59:3812–20.
17. Sordat I, Decraene C, Silvestre T, et al. Complementary DNA arrays identify CD63 tetraspanin and α3 integrin chain as differentially expressed in low and high metastatic human colon carcinoma cells. Lab Invest 2002;82:1715–24.
18. Hashida H, Takabayashi A, Tokuhara T, et al. Clinical significance of transmembrane 4 superfamily in colon cancer. Br J Cancer 2003;89:158–67.
19. Ono M, Handa K, Withers DA, Hakomori S. Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. Cancer Res 1999;59:2335–9.
20. Sho M, Adachi M, Taki T, et al. Transmembrane 4

superfamily as a prognostic factor in pancreatic cancer. Int J Cancer 1998;79:509–16.

21. Mori M, Mimori K, Shiraishi T, et al. Motility related protein 1 (MRP1/CD9) expression in colon cancer. Clin Cancer Res 1998;4:1507–10.

22. White A, Lamb PW, Barrett JC. Frequent downregulation of the KAI1 (CD82) metastasis suppressor protein in human cancer cell lines. Oncogene 1998;16: 3143–9.

23. Liu L, Wu DH, Li ZG, Yang GZ, Ding YQ. Effects of KAI1/CD82 on biological behavior of human colorectal carcinoma cell line. World J Gastroenterol 2003;9: 1231–6.

24. Kanetaka K, Sakamoto M, Yamamoto Y, et al. Overexpression of tetraspanin CO-029 in hepatocellular carcinoma. J Hepatol 2001;35:637–42.

25. Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J 1997;11: 428–42.

26. Sincock PM, Fitter S, Parton RG, Berndt MC, Gamble JR, Ashman LK. PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function. J Cell Sci 1999;112:833–44.

27. Fitter S, Sincock PM, Jolliffe CN, Ashman LK. Transmembrane 4 superfamily protein CD151 (PETA-3) associates with β1 and αIIb β3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. Biochem J 1999;338:61–70.

28. Yauch RL, Berditchevski F, Harler MB, Reichner J, Hemler ME. Highly stoichiometric, stable, and specific association of integrin α3β1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. Mol Biol Cell 1998;9:2751–65.

29. Hintermann E, Bilban M, Sharabi A, Quaranta V. Inhibitory role of α6β4-associated erbB-2 and phosphoinositide 3-kinase in keratinocyte haptotactic migration dependent on α3β1 integrin. J Cell Biol 2001;153:465–78.

30. Yauch RL, Kazarov AR, Desai B, Lee RT, Hemler ME. Direct extracellular contact between integrin α(3)β(1) and TM4SF protein CD151. J Biol Chem 2000;275: 9230–8.

31. Sterk LM, Geuijen CA, Oomen LC, Calafat J, Janssen H, Sonnenberg A. The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin α6β4 and may regulate the spatial organization of hemidesmosomes. J Cell Biol 2000;149:969–82.

32. Jones PH, Bishop LA, Watt FM. Functional significance of CD9 association with β1 integrins in human epidermal keratinocytes. Cell Adhes Commun 1996; 4:297–305.

33. Baudoux B, Castanares-Zapatero D, Leclercq-Smekens M, Berna N, Poumay Y. The tetraspanin CD9 associates with the integrin α6β4 in cultures human epidermal keratinocytes and is involved in cell motility. Eur J Cell Biol 2000;79:41–51.

34. Herlevsen M, Schmidt DS, Miyazaki K, Zöller M. The association of the tetraspanin D6.1A with the α6β4 integrin supports cell motility and liver metastasis formation. J Cell Sci 2003;116:4373–90.

35. Kariya Y, Ishida K, Tsubota Y, et al. Efficient expression system of human recombinant laminin-5. J Biochem (Tokyo) 2002;132:607–12.

36. Makary MA, Warshaw AL, Centeno BA, Willet CG, Rattner DW, Fernandez-del Castillo C. Implications of peritoneal cytology for pancreatic cancer management. Arch Surg 1998;133:361–5.

37. Penas PF, Garzia-Diez A, Sanchez-Madrid F, Yanez-Mo M. Tetraspanins are localized at motility-related structures and involved in normal human keratinocyte wound healing migration. J Invest Dermatol 2000; 114:1126–35.

38. Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. J Cell Sci 2001; 114:4143–51.

39. Charrin S, LeNaour F, Oualid M, et al. The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. J Biol Chem 2001;276: 14329–37.

40. Belkin AM, Stepp MA. Integrins as receptors for laminins. Microsc Res Tech 2000;51:280–301.

41. Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific β(1) integrins. J Biol Chem 2001;276:25005–13.

42. Bogenrieder T, Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003; 22:6524–36.

43. Lombardi DP, Geradts J, Foley JF, Chiao C, Lamb PW, Barrett JC. Loss of KAI1 expression in the progression of colorectal cancer. Cancer Res 1999; 59:5724–31.

44. Shinohara T, Nishimura N, Hanibuchi M, et al. Transduction of KAI1/CD82 cDNA promotes hematogenous spread of human lung-cancer cells in natural killer cell-depleted SCID mice. Int J Cancer 2001; 94:16–23.

45. Crnogorac-Jurcevic T, Efthimiou E, Capelli P, et al. Gene expression profiles of pancreatic cancer and stromal desmoplasia. Oncogene 2001;20:7437–46.

46. Lijovic M, Somers G, Frauman AG. KAI1/CD82 protein expression in primary prostate cancer and in BPH associated with cancer. Cancer Detect Prev 2002; 26:69–77.

47. Berditchevski F, Gilbert E, Griffiths MR, Fitter S, Ashman L, Jenner SJ. Analysis of the CD151-α3β1 integrin and CD151-tetraspanin interactions by mutagenesis. J Biol Chem 2001;276:41165–74.

48. Kawakami Y, Kawakami K, Steelant WF, et al. Tetraspanin CD9 is a "proteolipid", and its interaction with α3 integrin in microdomain is promoted by GM3 ganglioside, leading to inhibition of laminin-5-dependent cell motility. J Biol Chem 2002;277: 34349–58.

49. Stipp CS, Hemler ME. Transmembrane-4-superfamily proteins CD151 and CD81 associate with α3β1 integrin, and selectively contribute to α3β1-dependent neurite outgrowth. J Cell Sci 2000;113:1871–82.

50. Hemler ME. Specific tetraspanin functions. J Cell Biol 2001;155:1103–7.

51. Yanez-Mo M, Mittelbrunn M, Sanchez-Madrid F. Tetraspanins and intercellular interactions. Microcirculation 2001;8:153–68.