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P-cadherin in adhesion and invasion: opposite roles in colon and bladder carcinoma

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Neoexpression or upregulation of placental cadherin (P-cadherin), a member of the classical cadherin family, has previously been described in several carcinomas, such as colorectal and bladder carcinomas. In this study, we combined two different approaches, immunohistochemistry of tumor samples and *in vitro* knockdown of P-cadherin, to gain a better insight into the role of P-cadherin in these types of cancer. First, we performed immunohistochemistry for P- and E-cadherins in a series of 52 colorectal adenocarcinomas, including well, moderately and poorly differentiated (WD, MD, and PD) tumors. Decrease or loss of P-cadherin neoexpression was significantly associated with a higher tumor grade and could discriminate WD from MD and/or PD tumors ($p < 0.001$). E-cadherin, on the other hand, was strongly expressed at the membrane of most WD (18 of 19) and MD tumors (15 of 19). Downregulation correlated significantly with the PD phenotype ($p \leq 0.001$). In a second approach, we transiently or stably knocked down P-cadherin in HT-29 colon adenocarcinoma cells. This led to decreased intercellular adhesion and to an increased migratory and long-term invasive phenotype compared with control HT-29 cells, suggesting that P-cadherin acts as a proadhesive and anti-invasive/antimigratory molecule in colon carcinoma cells. Contrasting with these results and illustrating the context-specific function of P-cadherin were our results obtained in RT-112 bladder carcinoma cells. Stable knockdown of P-cadherin in RT-112 cells diminished invasion and migration, and promoted intercellular adhesion.

Placental cadherin (P-cadherin) belongs to the family of the classical cadherins and is primarily expressed in the basal part of epithelia throughout the human body.[1] Cadherins are calcium-dependent cell adhesion proteins, which are involved in embryogenesis and in homeostasis of normal epithelia and also play crucial roles in tumorigenesis.[2] Epithelial (E-) cadherin is also a member of the classical, Type 1 subgroup, and its capacity to mediate cellular adhesion and inhibit invasion has been studied extensively.[3,4] Earlier studies suggested that the function of P-cadherin is similar to that of E-cadherin, in particular in stratified epithelia of the upper digestive tract and the skin. *In vivo* data in E-cadherin-targeted mice showed that P-cadherin may operate as a backup molecule.[5]

Key words: P-cadherin, colorectal cancer, bladder cancer, invasion, adhesion

Abbreviations: wt: wild-type; si: small interfering; sh: short hairpin; sico: small interfering control RNA; WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated

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P-cadherin downregulation in malignant tumors correlates with poor prognosis, as was shown in oral and esophageal squamous cell carcinomas and in melanomas.[6–8]

Accumulating data, however, suggest that P-cadherin could also function in an opposite sense to E-cadherin. Correlation studies demonstrated association of P-cadherin expression with adverse outcomes in breast carcinoma[9] and endometrial carcinoma[10] and with a shorter time to disease progression in prostate carcinoma.[11] Gene expression profiling of ovarian tumors revealed a high-grade serous carcinoma subgroup with overexpression of P-cadherin.[12] Similarly, in pancreatic tumors, P-cadherin gene *CDH3* upregulation was documented.[13] *In vitro* studies with cell lines corroborated these findings for breast and pancreatic carcinoma, linking P-cadherin expression to an invasive phenotype.[14,15]

This study aims at further documenting the apparent tumor-/tissue-type specific function of P-cadherin, focusing on colon and bladder carcinoma, topics that are not well covered in literature yet.

In the gastrointestinal tract, P-cadherin is absent in normal colon and is only poorly expressed in normal gastric epithelium.[1,16] In its earliest premalignant stage, in the "aberrant crypt foci," however, colonic epithelia show aberrant P-cadherin expression (neoexpression), which persists in adenomatous polyps[17] and colorectal adenocarcinoma.[18] Shimoyama *et al.*[19] showed P-cadherin upregulation in gastric carcinoma.

Although this neoexpression/upregulation could attribute an invasion promoter role to P-cadherin, immunohistochemical results from Yasui *et al.*[20] suggested that P-cadherin expression is again decreased in late stage gastric carcinomas. Considering the small number of colon carcinoma cases in which P-cadherin expression has been investigated thus far, we wondered if P-cadherin neoexpression could be switched off in a subset of colon tumors, in association with loss of differentiation.

Therefore, in this study, we performed immunohistochemistry in a selected panel of human colon carcinoma specimens, focusing on its relation to tumor differentiation and co-expression of E-cadherin. We also studied the function of P-cadherin in HT-29 colon carcinoma cells by transiently and stably knocking down its expression using small interfering (si) and short hairpin (sh) RNA constructs, respectively. Thus, for the first time, we provide *in vitro* data on the role of P-cadherin in adhesion and invasion of colon cancer.

In bladder, normal epithelium shows strong membranous P-cadherin expression in its basal cell layers. Immunohistochemical studies on bladder carcinoma demonstrated increased or "aberrant" cytoplasmic P-cadherin expression.[21–23] However, *in vitro* evidence for an adverse P-cadherin function in bladder tumors is sparse. Bryan *et al.*[23] showed that P-cadherin mediates defective cell-cell adhesion in RT4 cells. In addition, P-cadherin might stimulate the migratory potential of bladder carcinoma cells as shown in EJ and UM-UC-3/P-cadherin transfectants.[22] Because these studies did not reveal a direct link with invasion, we set up *in vitro* experiments with the RT-112 bladder carcinoma cell line, using a similar approach as for HT-29. We documented the function of P-cadherin regarding invasion and further established the contributory role of P-cadherin to the adhesive phenotype of these cells. RT-112 co-expresses E- and neural (N)-cadherins, and therefore this cell line also served as a model to test the interplay of the classical cadherins. By using cadherin-specific antibodies, we checked if the proadhesive N-cadherin function, earlier described in the T24 bladder cell line,[24] could also be observed in a context of E- and P-cadherin co-expression.

Materials and Methods

Cell lines and cultures

RT-112, a human bladder carcinoma cell line, was originally obtained from Dr. Uwe Frixen, Essen, Germany. HT-29 is a human colorectal adenocarcinoma cell line, which we obtained from Dr. Kris Vleminckx, VIB-Ghent University, Ghent, Belgium.

RT-112, HT-29 and chick embryonic heart fragments were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Merelbeke, Belgium), supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B (all from Invitrogen). In aggregation and Matrigel invasion experiments and during wound healing, serum was omitted from the media. Cell cultures were maintained at 37°C in a water-saturated atmosphere containing 10% CO_2.

Antibodies and transfection

Antibodies used for Western blotting were monoclonal anti-P-cadherin, clone 56 (BD Transduction Laboratories, BD Biosciences, Erembodegem, Belgium), monoclonal anti-E-cadherin, Hecd-1 (Takara Bio, Inc., Fisher Scientific, Pittsburgh), monoclonal anti-N-cadherin, CH19 and monoclonal anti-α-tubulin, B-5-1-2 (Sigma-Aldrich, Bornem, Belgium).

Antibodies for blocking cell adhesion were diluted in serum-free DMEM: anti-E-cadherin antibody Hecd-1 (20 μg/mL); anti-N-cadherin antibody GC-4 1:50 (Sigma-Aldrich); mouse IgG isotype control (20 μg/mL; eBioscience, ImmunoSource, Halle-Zoersel, Belgium).

Antibodies used for immunohistochemistry were monoclonal anti-P-cadherin, clone 56, diluted 1:80 (BD Transduction Laboratories) and monoclonal anti-E-cadherin, Flex Ready-to-use clone NCH-38 (Dako, Glostrup, Denmark). All the cadherin antibodies we used are directed against the respective cadherin ectodomains.

For small interfering RNA (siRNA) knockdown, predesigned siRNA targeting P-cadherin, accession number NM_001793 (ON-TARGETplus SMARTpool siRNA) and control siRNA (ON-TARGETplus nontargeting siRNA) were purchased from Dharmacon (Thermo Fisher Scientific, Erembodgem, Belgium). Cells were transiently transfected by electroporation, using an Amaxa Nucleofector® kit (Amaxa; Lonza Cologne AG, Cologne, Germany). HT-29 cells at 70% to 80% confluence were detached by trypsinization, and 1×10^7 cells were pelleted and collected in a Nucleofector-certified cuvette by gentle suspension in Nucleofector solution R. After adding 3 μg siRNA, cells were electroporated in a Nucleofector device (program W-17) and seeded in different plates for Western blotting and invasion assays or brought in suspension for testing aggregation.

Plasmids, cDNA constructs, retroviral transduction and cell sorting

For stable knockdown of P-cadherin, target sequences were cloned into pLV-TH.[25] Briefly, a PCR fragment consisting of part of the H1 promoter, the target sequence (sense), loop and target sequence (antisense) was generated by two subsequent PCRs. The first PCR product, with pSuper as a template, was generated using the forward primer (5′)CTGC AG**GAATTC**GAACGCTGACGTCATCAA(3′) (with the *Eco*RI site in bold) and as reverse primer a primer with as formula (5′)[seq (s17→s19)-*TCTCTTGAA*-seq(as19→as1)-GGGGATC TGTGGTCTCATACAGAACTTATAA](3′). The part of the H1 promotor is underlined; the loop sequence is indicated in italics; s1→s19 and as19→as1 represent the "sense" and "antisense" sequences present in *CDH3*, respectively, with the respective sense sequences (s1→s19) for constructs 995 and 3125 being ACAGGCTGGTTGTTGTTGA and CTATGAG

TCTGACGTTAGA. A 1000-fold dilution of this PCR product was used as a template for a second PCR, with the same forward primer and as a reverse primer a primer with as formula (5′)[CC**ATCGAT**TTCCAAAAA-seq(s1→s19)-*TCTCTTGAA*-SEQ (as19→as16)](3′), with the *Cla*I site indicated in bold. The PCR program consisted of 3 min initial denaturation at 94°C, followed by 20 cycles of 1 min denaturation at 94°C, 55 sec annealing at 55°C and 55 sec elongation at 72°C, and final elongation at 72°C for 7 min. This product was subjected to agarose gel electrophoresis, followed by gel extraction (Qiagen), *Eco*RI/*Cla*I digestion, purification (Qiagen) and ligation into *Eco*RI/*Cla*I digested, dephosphorylated, pLVTH (also purified from gel). After transformation of competent DH5α bacteria with the ligated product, ampicillin-resistant colonies were screened by PCR using primers complementary to sequences of the pLVTH vector surrounding the insert. All constructs were verified by sequencing. Production of lentiviral particles and transduction of cells was essentially as described before.[26] Transduced cells were sorted (based on EGFP positivity) using an Epics Altra cell sorter (Beckman Coulter).

Aggregation assays

In suspension: 50 mL Erlenmeyer flasks containing 6×10^5 bladder or colon carcinoma cells in 6 mL medium were placed on a continuously gassed (5–10% CO_2) Gyrotory shaker at 72 rpm (New Brunswick Scientific, Co., New Brunswick, NJ). After incubation for 24 or 48 hr, aggregates were transferred to a 6-well plate and fixed in 10% phosphate-buffered formaldehyde. HT-29 cells transfected with the respective siRNA pools were incubated for 72 hr, allowing the cells to recover from transfection. After fixation, the shortest (a) and largest (b) diameters of aggregates in at least 10 randomly chosen 10× microscopy fields were measured by phase-contrast microscopy using Axiovision software (Carl Zeiss, Göttingen, Germany). The estimated aggregate volumes were calculated with the equation: $0.4 \times a^2 \times b$.[27] This experiment was done twice, and representative data are presented.

On semisolid agar: 2×10^4 cells in 200 μl serum-free culture medium were seeded on a solidified agar in a 96-well plate. In the siRNA experiments, HT-29 cells were seeded 24 hr after transfection. For cadherin-blocking experiments, antibodies or IgG control were added.[28]

Wound healing assay

Cell cultures were grown on 6-well plate dishes until confluent. Artificial wounds were created in the monolayers by gently scratching with a plastic tip. To remove detached cells, cultures were washed three times with PBS and covered with 1 mL serum-free medium. The migratory capacity of the cells was assessed by measuring in randomly selected areas the widths of the wounds with an inverted microscope at the start of the experiment ($t = 0$) and at different time intervals. All experiments were done at least twice.

Boyden chamber invasion assay

Transwell® Permeable Supports consisting of polycarbonate membrane (8.0-μm pore size) inserted in a 24-well plate (Costar®; Cole-Parmer, London, United Kingdom) were coated with 40 μg (in 20 μL) Matrigel® (BD Biosciences). 1×10^5 cells in 0.1 mL were added in the Transwell chamber, which was placed in a 24-well plate culture dish, containing 0.6 mL conditioned medium of the human lung fibroblasts MRC5 as a chemoattractant. After incubation for 16 to 24 hr, cells that had invaded into the Matrigel and through the filter were fixed in methanol and stained with DAPI (Sigma). The sum of the total number of invaded cells from two Transwell chambers (tested in duplex) was counted using a fluorescence microscope. Each experiment was repeated at least three times.

Chick heart invasion assay

This assay investigates the capacity of tumor cells to invade into embryonic heart fragments, composed of fibroblasts, myoblasts, endothelial cells and extracellular matrix, as earlier described in detail.[29] Briefly, precultured heart fragments (PHF) of 9-day-old chick embryos were allowed to attach to aggregates or monolayer fragments of tumor cells on semisolid agar. After 24-hr incubation, these confronting cultures were brought in suspension under continuous Gyrotory shaking for 3.5 or 7 days. The aggregates were fixed in 10% formaldehyde, embedded in paraffin, serially sectioned and stained with hematoxylin-eosin. Invasion was scored as follows: 0 if only PHF is found and no confronting cells attached to it can be observed; 1 if the confronting cells are attached to the PHF and do not occupy the heart tissue; 2 if occupation of the PHF is limited to the outer fibroblast-like and myoblast cell layers; 3 if the confronting cells have occupied the PHF but have left more than half of the original volume of heart tissue intact; and 4 if the confronting cells have occupied more than half of the original volume of the PHF.

Western blotting

Immunoblotting and quantification of the bands were done as detailed in an earlier report.[30] Only cell cultures at less than 80% to 90% confluence were lysed. Equal total amounts of protein were separated on a freshly prepared 8% polyacrylamide gel. Experiments were done at least in duplex; representative blots are shown.

Colon carcinoma specimen selection and immunohistochemistry

Paraffin blocks from surgical (colo)rectal resection specimens were selected from the archive of the Department of Pathology, Antwerp University Hospital, according to local ethical guidelines for the use of archived tissue samples. A random selection was made from files covering the last 3 years, taking care that blocks were representative for the whole tumor, contained enough material and that comparable numbers of

Table 1. Immunohistochemical analysis of membranous P- and E-cadherin (neo)expression in colorectal adenocarcinomas, in accordance with tumor grade

	E-cadherin								
	Well differentiated			Moderately differentiated			Poorly differentiated		
	Strong	Moderate	Weak/negative	Strong	Moderate	Weak/negative	Strong	Moderate	Weak/negative
P-cadherin									
Strong	14	0	0	1	1	0	0	2	0
Moderate	3	1	0	13	2	0	0	3	0
Weak/negative	1	0	0	1	1	0	2	3	4

No. tumors after scoring E- and P-cadherin immunoreactivity and grouping—strong: homogeneous, strong membranous cadherin (neo)expression in >60% of tumor cells; moderate: heterogeneous membranous cadherin (neo)expression, see Materials and Methods; weak/negative: predominantly (>60%) negative or cytoplasmic (neo)expression.

cases represented each tumor grade. The tissue samples in these paraffin blocks had been routinely fixed in 10% neutral buffered formalin and embedded in paraffin for histopathologic examination. The paraffin sections of all tumors were revised by a pathologist (V.V.M.), and the tumors were graded according to the WHO recommendations.[31]

Consecutive 5 μm sections were made from a representative tumor block containing adenocarcinoma and adjacent normal colon mucosa. Deparaffinization, rehydration and antigen retrieval using Target retrieval solution high pH (Dako) were automated in a PT-Link pretreatment system (Dako). Blocking of endogenous peroxidase activity, incubation with primary antibody, signal enhancement of P-cadherin staining with Envision Flex+ Mouse (Linker) and revealing immunoreactivity were performed in an Autostainer Link 48 (Dako) using an EnVision Flex high pH (Link) kit. Incubation times for the primary antibodies were 10 min (E-cadherin) and 20 min (P-cadherin). Immunoreactivity was revealed with EnVision Flex/HRP and DAB (Dako). Slides were counterstained with hematoxylin using an automatic slide stainer Varistain® Gemini ES (Thermo Fisher Scientific).

The tissue sections were scored semiquantitatively by two pathologists (V.V.M. and G.V.D.E.), assessing the extent and cellular localization, and for membranous E-cadherin also the staining intensity. All sections were completely screened. First, the predominant staining pattern was noted, being present in more than 60% of the tumor cells (usually at least 80% of tumor cells). In addition, a secondary staining pattern was taken into account if present in more than 10% of tumor cells.

Each staining pattern was scored as "membranous," "cytoplasmic" when there was a concomitant loss in membranous staining or "negative." Membranous E-cadherin staining was scored on a three-tiered scale as strong (equal to that of normal colonic epithelium), moderate or weak.

Focusing on membranous expression, tumors were segregated into the following groups:

P-cadherin neoexpression—Strong: homogeneous membranous staining pattern; Moderate: predominant membranous with secondary cytoplasmic or negative staining pattern; Weak/negative: predominant cytoplasmic staining pattern or no neoexpression.

E-cadherin expression—Strong: strong membranous ± secondary moderately strong membranous staining pattern; Moderate: homogeneous moderately strong membranous staining pattern or strong membranous with secondary cytoplasmic, weak membranous or negative staining pattern; Weak/negative: predominant weak membranous or cytoplasmic staining pattern.

Statistical analysis

The statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL). Normality of continuous data (aggregation, wound healing and Matrigel invasion assays) was tested with the Kolmogorov-Smirnov test, assuming normality if $p > 0.1$. For all other statistical analyses $p \leq 0.05$ was considered significant. If normally distributed, equality of means was tested with the Student's t test. If not normally distributed, medians were compared using a Mann-Whitney U test.

Associations between categorical variables (immunohistochemical expression of P-cadherin and E-cadherin and differentiation grade) were analyzed with a Pearson's χ^2 test or, when the assumptions of the χ^2 test were not met, a Fisher's exact test. Categorical variables were dichotomized as study groups were otherwise too small.

Results

Cadherin expression in colorectal adenocarcinoma

We hypothesized that in colorectal carcinoma membranous, P-cadherin expression correlates with differentiation, as it has been reported for E-cadherin.[32] Therefore, P- and E-cadherin expression was assessed by immunohistochemistry in serial sections of 52 primary colorectal tumors, 19 well differentiated (WD, Grade 1), 19 moderately (MD, Grade 2) and 14 poorly differentiated (PD, Grade 3–4). Table 1 depicts the results after scoring (see Materials and Methods).

All but two carcinomas expressed P-cadherin. However, only 18 tumors showed homogeneous membranous neoexpression ("strong"), 14 of which were WD. P-cadherin in these cases is localized at the lateral intercellular junctions, yielding a honey-comb pattern on transverse sectioning (Fig. 1*a*). Heterogeneous, moderate staining was observed in 22 tumors, either with shifts from membranous to cytoplasmic staining (n = 16) or with foci of complete absence of neoexpression (n = 6; Fig. 1*b*). Moderate P-cadherin neoexpression was the most prevalent immunostaining pattern in the MD group (15/19, 79%) and seemed to be associated with loss of cell polarity and disturbed glandular formation, which are histological criteria for loss of differentiation[31] (Fig. 1*b*, insert). A third group of tumors (n = 12) was defined as showing only minor or no membranous P-cadherin neoexpression ("weak/negative"). In 10 cases, P-cadherin immunostaining was either negative in most part of the tumor (Fig. 1*c*) or predominantly localized in the cytoplasm (Fig. 1*f*). Two PD tumors, which showed almost complete absence of glandular differentiation, were scored as completely negative for P-cadherin (Figs. 1*d* and 1*k*). Sixty-four percent of the PD tumors (9 of 14), in contrast to only 5% of the WD (1 of 19) and 11% of the MD (2 of 19) adenocarcinomas, showed this weak/negative expression pattern.

As expected, E-cadherin was strongly expressed at the membrane in nearly all WD (18 of 19) and most MD (15 of 19) adenocarcinomas, whereas all PD tumors except two showed moderate (8 of 14), weak or no expression (4 of 14). Although tumors with loss of E-cadherin (cytoplasmic expression or predominantly negative) did also show absence of P-cadherin neoexpression (Figs. 1*e* and 1*f*), there was no association between E- and P-cadherin expression (Table 1). Twenty tumors showed strong membranous E-cadherin expression along with a moderate (n = 16) or weak/negative P-cadherin neoexpression pattern (n = 4; Fig. 1*g* and 1*h*), and the 6 remaining tumors with few P-cadherin at the membrane were still moderately positive for E-cadherin (Figs. 1*i* and 1*j*). Only 3 of the 52 tumors showed less E- than P-cadherin expression.

Statistical analysis was done on dichotomized groups, as detailed in Table 2. With respect to P-cadherin neoexpression, WD tumors differed significantly from their MD and/or PD counterparts (strong membranous *vs.* moderate or weak/negative; $p < 0.001$). However, the extent of P-cadherin neoexpression did not discriminate between PD and MD tumors ($p = 1.0$). When comparing PD *vs.* the total group of MD+WD tumors in this respect, only borderline significance was reached ($p = 0.04$). Membranous E-cadherin expression, on the other hand, was significantly associated with tumor differentiation ($p \leq 0.001$), except for the comparison of WD *vs.* MD tumors ($p = 0.34$), reflecting the rather retained "stability" of E-cadherin expression in the MD group.

Together, we showed that the membranous P-cadherin neoexpression in colon adenocarcinoma is significantly lost with increasing tumor grade and seems to occur at an earlier stage in the dedifferentiation process than loss of E-cadherin.

P-cadherin downregulation in HT-29 colon carcinoma cells affects cell aggregation and promotes motility and invasion

At least part of the high-grade tumors we studied by immunohistochemistry showed an infiltrative growth pattern with loss of tumor cell adhesion and P-cadherin neoexpression (*e.g.*, Figs. 1*f* and 1*j*). To verify whether P-cadherin might determine the adhesive and/or invasive phenotype of colon carcinoma cells, the P-cadherin-expressing HT-29 colorectal adenocarcinoma cell line was selected for further *in vitro* studies.

HT-29 cells were transiently transfected with a pool of siRNAs directed against P-cadherin (siPcad) or with control siRNA (sico; see Materials and Methods). Western blotting of cell lysates 3 to 4 days after transfection showed a more than 95% decrease in P-cadherin levels in HT-29 siPcad cells as compared with HT-29 sico. E-cadherin expression was somewhat decreased, the exact reason of which is unknown, but might be related to some off-target effect or to less efficient intercellular adhesion, imposed by the strong decrease in P-cadherin levels (Fig. 2*a*). Aggregation assays were performed in parallel. First, cells were allowed to aggregate on agar. Figure 2*b* shows that wild-type HT-29 cells were localized at the centre of the wells, forming interconnected strands (24 hr, top left) that evolve into a single dense aggregate (72 hr, bottom left). HT-29 cells that had been treated with control siRNA behaved similarly (Fig. 2*b*, middle). Following transfection with siRNA against P-cadherin, however, the HT-29 cell clusters were more dispersed toward the periphery of the wells, yielding more, yet smaller aggregates at longer time intervals (Fig. 2*b*, right). Secondly, HT-29 cell cultures were placed on a Gyrotory shaker, inducing aggregate formation in suspension. As depicted by Figure 2*c*, aggregation of HT-29 siPcad cells is impaired. The mean estimated volume of the aggregates, calculated from the diameters (see Materials and Methods), was 50% to 75% smaller than in HT-29 sico cultures ($p < 0.001$).

HT-29 cell cultures were also tested in wound healing and Matrigel (Boyden chamber) invasion assays. HT-29 sico and siPcad cells both showed low levels of migration and invasion (data not shown); no differences in the migratory or invasive phenotype were observed. It has to be kept in mind that P-cadherin downregulation by siRNA was obtained by transiently transfecting HT-29 cells, which possibly may not lead to initiation of a proinvasive program potent enough to counteract the anti-invasive effect of E-cadherin.

To overcome possible shortcomings inherent to transient transfections, we repeated our experiments using HT-29 cells with stable knockdown of P-cadherin. HT-29 cells were transduced with two different short hairpin P-cadherin constructs (shPcad995 or shPcad3125) and sorted to more than 95% EGFP- (and thus shPcad-) positive populations (data

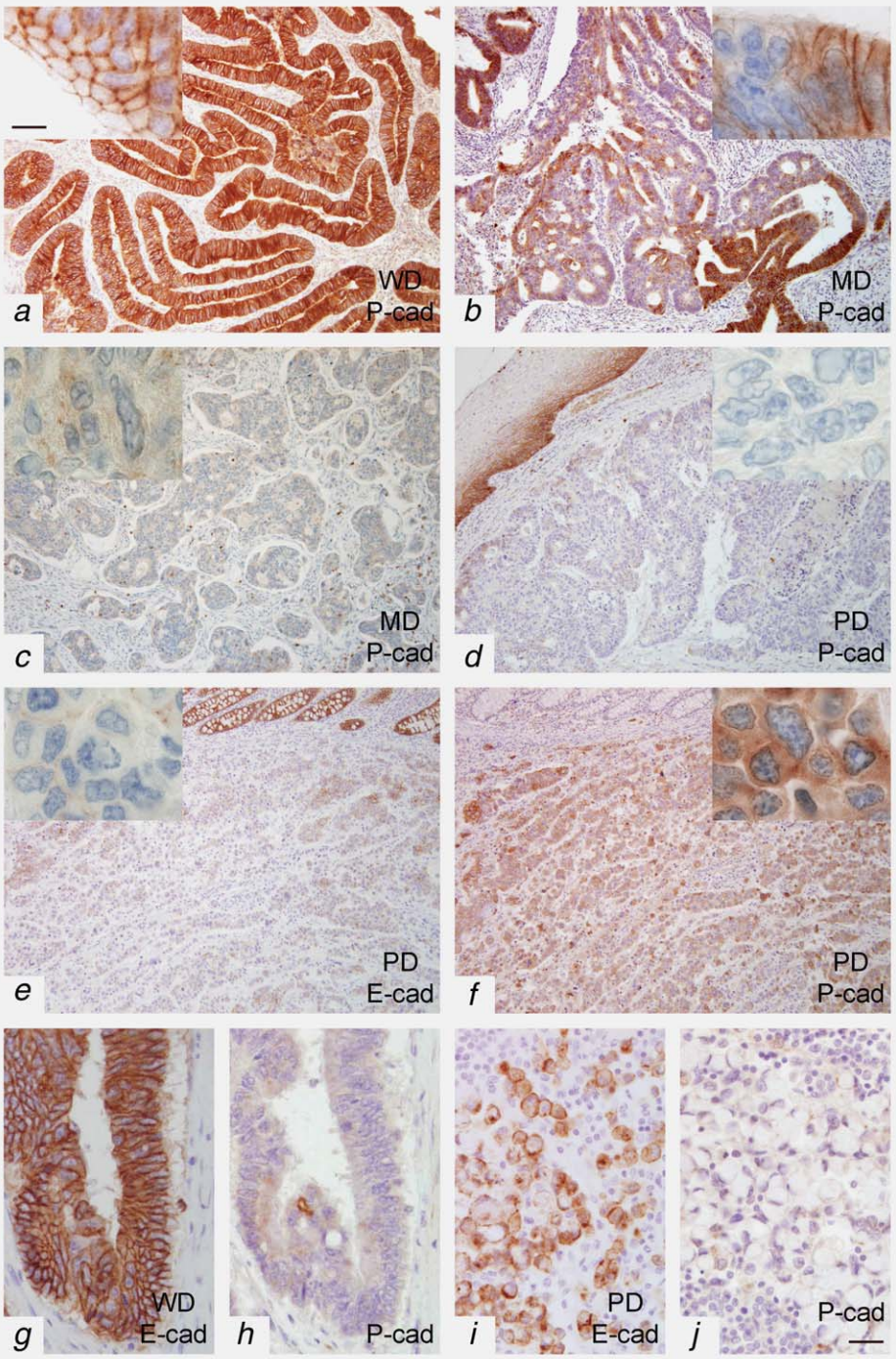

Figure 1. Representative micrographs of colorectal adenocarcinomas, immunohistochemically stained for P-cadherin (P-cad) or E-cadherin (E-cad) as indicated, showing variability in staining pattern. The inserts show the predominant staining pattern at high magnification. (*a*) Homogeneous, strong membranous P-cadherin neoexpression, showing a honey-comb pattern. (*b*) Focal absence of P-cadherin neoexpression in association with areas of loss of cell polarity. (*c*) Predominantly negative staining with few tumors cells showing cytoplasmic P-cadherin neoexpression. (*d*) Absence of P-cadherin neoexpression in a rectal tumor with limited glandular differentiation infiltrating underneath the squamous epithelium of the anal canal (positive internal control). (*e*, *f*) Loss of membranous E-cadherin in most tumor cells and a predominant cytoplasmic P-cadherin staining pattern. As expected, the normal colon epithelium (upper part) is strongly positive for E-cadherin, but negative for P-cadherin. (*g–j*) Examples of tumors with discordant cadherin staining pattern. In contrast to the strong membranous (*g*) or mixed membranous/cytoplasmic (*i*) E-cadherin staining pattern, P-cadherin neoexpression is largely (*h*) or completely lost (*j*). Note the signet-ring phenotype of the latter tumor. Bars: 100 μm: *a–f*; 25 μm: *g–j*; 10 μm: inserts. WD: well differentiated, MD: moderately differentiated, PD: poorly differentiated.

Table 2. Statistical analysis of the association of P-cadherin neoexpression and E-cadherin expression with tumor grade in dichotomized groups: p of Pearson χ^2 or Fisher's exact test

		Strong *vs.* moderate or weak/negative (neo)expression	
Tumor differentiation		P-cad	E-cad
WD	MD	<0.001	0.34
WD	PD	<0.001	<0.001
WD	MD + PD	<0.001	0.001
WD + MD	PD	0.04	<0.001
MD	PD	1.000	<0.001

Abbreviations: P-cad: P-cadherin; E-cad: E-cadherin; WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated.

not shown). Downregulation of P-cadherin, together with unaltered E-cadherin and N-cadherin expression, was confirmed by Western blotting. P-cadherin expression levels were decreased with 94% and 56% in HT-29 shPcad995 and shPcad3125, respectively (Fig. 2*d*), which allows scoring of a "dose-dependent" knockdown effect.

Aggregation assays performed on agar confirmed the results from the transient transfection experiments. As shown for the 24-hr time point in Figure 2*e*, HT-29 shPcad995 and, to a lesser extent, HT-29 shPcad3125 aggregates were dispersed and smaller than wild-type (wt) HT-29 aggregates.

Next, we performed motility and invasion assays. In the wound healing assay, HT-29 shPcad cells showed a motile phenotype, in contrast to the wt HT-29 cell cultures. Migration into the wounded areas occurred at a significantly higher speed in shPcad-transduced HT-29 than in wt HT-29 cell cultures (shPcad995: $p = 0.005$ and 0.001, shPcad3125: $p < 0.001$ and <0.001 at time intervals 12 hr and 60 hr, respectively; Fig. 2*f*). To test the effect of P-cadherin on invasiveness, we first studied invasion through Matrigel-coated membranes in a Boyden chamber. Although in some experiments HT-29 shPcad cells, in particular shPcad995 cells, were more invasive than wt HT-29, no statistical significance was reached ($p > 0.05$; Fig. 2*g*). The results of this short-term assay suggest that P-cadherin downregulation in HT-29 cells is insufficient to have a significant impact on the net invasiveness, perhaps because of a dominant anti-invasive effect exerted by E-cadherin, which is still present in these cells. Therefore, we also tested the HT-29 cell lines in the chick heart invasion assay, in which tumor cells are, for longer time intervals (3.5 or 7 days), induced to interact with fibroblastic and myoblastic cells from precultured embryonic chick heart fragments.[29] As summarized in Figure 2*h*, shPcad transduced HT-29 cells adopted an invasive phenotype, which became most apparent after 7 days of incubation, yielding invasion score 3 in 3 of 6 (sh995) and 3 of 6 (sh3125) *vs.* 0 of 4 (wt) confronting cultures (see Materials and Methods for scoring method). Representative pictures from 7-day-old confronting cultures show that fine tumor strands or isolated HT-29 shPcad995 and shPcad3125 cells were present in the chick heart fragments (arrowheads), whereas the parental HT-29 cells remained at the periphery, without invading the chick heart (Fig. 2*i*).

In summary, we showed that knockdown of P-cadherin expression in HT-29 cells results in reduced cellular aggregation and, in longer-term experiments, may induce an invasive phenotype. In view of our immunohistochemistry findings, these data support the functional relevance of loss of aberrant P-cadherin expression during the dedifferentiation process of colorectal adenocarcinoma

P-cadherin downregulation in RT-112 bladder carcinoma cells results in increased cell aggregation and abolished migration and invasion

The above-described findings in colon cancer cells are opposite to the invasion-promoter function of P-cadherin that we and others reported earlier for breast and pancreatic tumors.[14,15] To further document this apparent cell-/tumor-type discrepancy, we selected the RT-112 bladder carcinoma cell line,[33] which expresses P-cadherin along with E- and N-cadherins (Fig. 3*a*). In analogy with HT-29, RT-112 cells were virally transduced with the same shPcad constructs and were sorted for EGFP positivity, yielding stable cell populations of more than 90% positive cells (Fig. 3*a*, top). Western blotting of total cell lysates revealed strongly reduced P-cadherin levels in RT-112 shPcad995 and shPcad3125 cells, being 1.2% and 7.6% of those in the nontransduced cells, respectively. There was no concomitant decrease in E- or N-cadherin expression (Fig. 3*a*, bottom panels).

In three different *in vitro* assays, P-cadherin downregulation imposed decreased motility and invasiveness to RT-112, contrary to what was observed in P-cadherin knocked down HT-29 cells. First, in the wound healing assay, there was almost no cell migration in both shPcad-transduced RT-112 cell cultures, in contrast to wt RT-112 (Fig. 3*b*; data not shown for shPcad3125). The velocity of cell migration was significantly lower in RT-112 shPcad995 ($p < 0.001$) and shPcad3125 ($p < 0.001$) than in wild-type RT-112 cell cultures (Fig. 3*c*). Second, in the Matrigel invasion assay, the number of tumor cells that had invaded the Matrigel-coated membranes dropped significantly on P-cadherin downregulation (shPcad995: $p = 0.01$; shPcad3125: $p = 0.03$; Fig. 3*d*). Third, RT-112 cell lines were tested in the chick heart invasion assay. Few wt RT-112 cells had invaded into the chick heart fragments after 3.5 days (score 3 in 2 of 10). In the majority of the 7-day-old wt RT-112 confronting cultures, however, invasion was observed by means of nests of tumor cells amidst the chick heart tissue (score 3 in 6 of 9; Fig. 3*e* and 3*f*). In contrast, none of the RT-112 shPcad confronting cultures yielded an invasive phenotype (19 of 19 score 0; Fig. 3*e*); the RT-112 shPcad995 and shPcad3125 cells seemed to be incapable of adhering to the chick heart fragments and, thus, remained as separate aggregates (Fig. 3*f*, middle and right).

Next, we checked the effect of P-cadherin downregulation on RT-112 cell adhesion and aggregation. In suspension, the parental RT-112 cells remained single cell or showed clusters of only a few cells, whereas RT-112 shPcad995 and shPcad3125 cultures contained well-formed aggregates after 24 hr (data not shown) and 48 hr (Fig. 4*a*). On agar, the aggregates of the parental RT-112 cells were also small and formed a loosely interconnected two-dimensional network, even after more than 2 days (Fig. 4*b*, left). RT-112 cells transduced with shPcad995 or shPcad3125, however, mainly formed one large aggregate that clustered into a compact, sharply outlined structure with longer incubation (Fig. 4*b*, middle and right). In the presence of the E-cadherin-blocking antibody Hecd-1, RT-112 shPcad cell aggregation was poor,

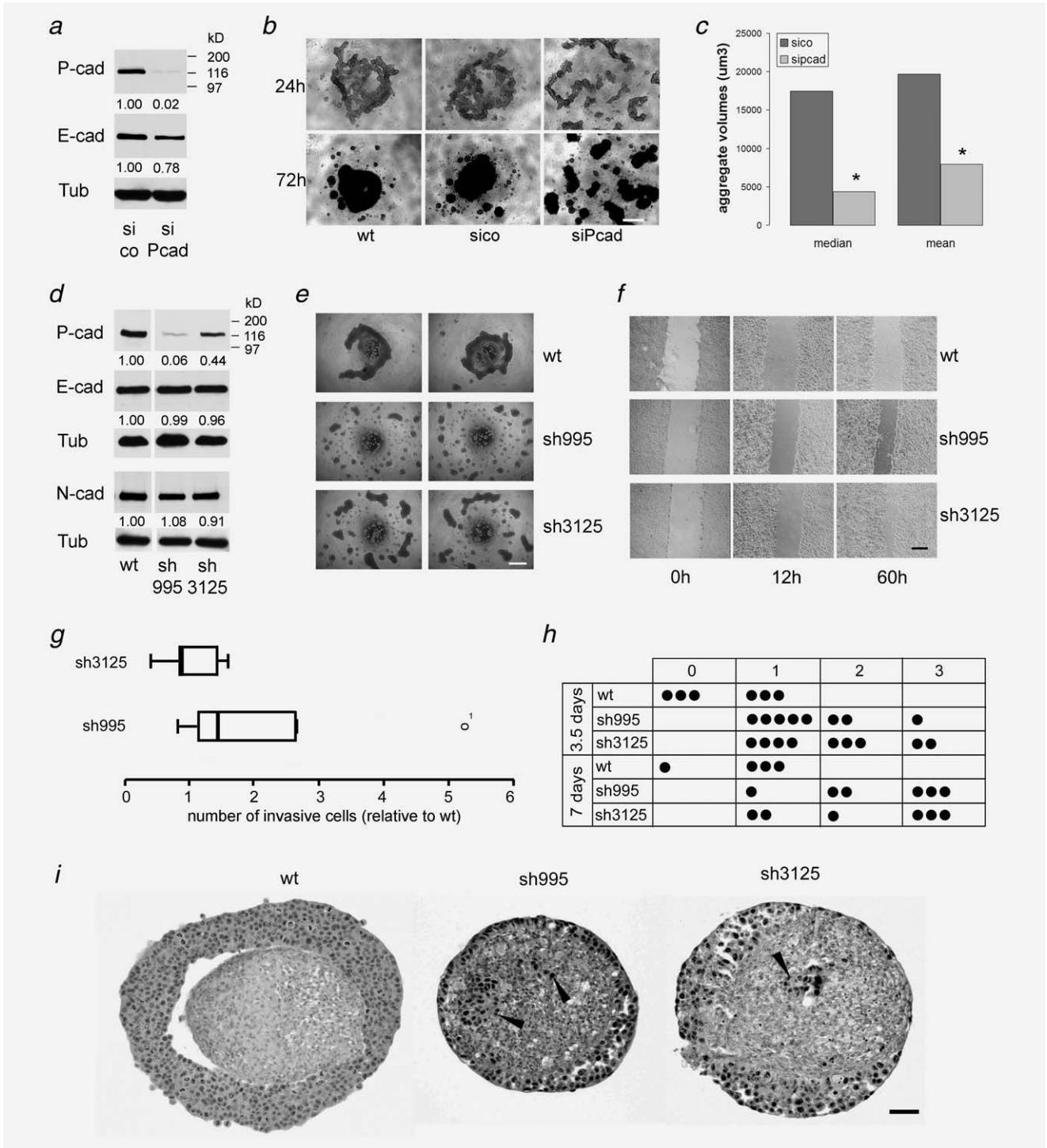

Figure 2.

yielding much smaller, loosely arranged clusters (Fig. 4*c*, second row). Contrarily, incubation with the N-cadherin blocking antibody GC4 resulted in even more compaction of RT-112 shPcad cells. GC4-treated parental RT-112 showed no differences in aggregation as compared with IgG control-treated cells (Fig. 4*c*, first and third row). Interestingly, treatment with both Hecd-1 and GC4 resulted in poor aggregation of parental and shPcad transduced cells, very similar to that after treatment with Hecd-1 alone (Fig. 4*c*, fourth row).

In summary, our *in vitro* results on RT-112 suggest that E-cadherin is the major player in aggregation of bladder carcinoma cells and that its proadhesive function is counteracted by P-cadherin and, to a lesser extent, by N-cadherin. In keeping with this, we also showed that P-cadherin may function as a motility factor and an invasion promoter in bladder carcinoma. These findings are opposite to those in the HT-29 colon carcinoma cell line, which corroborates the concept of context-dependent functioning of P-cadherin.

Discussion

In this study, we first documented P-cadherin expression in colorectal carcinoma. Recent reports showed that the P-cadherin neoexpression, which was observed in precursor lesions,[17] persists in colorectal cancer and is associated with a demethylated status of the *CDH3* promoter.[18,34] The apparent role of P-cadherin in tumorigenesis could be tumor cell proliferation rather than promotion of invasion. Favoring this hypothesis, Milicic *et al.*[18] showed increased crypt proliferation in transgenic mice with gastrointestinal tract-targeted P-cadherin overexpression. We wondered if switching off P-cadherin neoexpression could occur in a subset of high-grade colorectal tumors, as a possible mechanism of tumor progression. Therefore, we performed immunohistochemistry on colorectal cancer tissue samples, taking tumor grade into account. The results of our study confirm the appearance of P-cadherin in colorectal adenocarcinoma cells, but also show that, for the first time, its extent and cellular localization is variable. Decrease or loss of neoexpressed membranous P-cadherin was significantly associated with a higher tumor grade and could discriminate WD from MD and/or PD tumors ($p \leq 0.01$). The inverse association of E-cadherin with increasing tumor grade that we observed is in line with earlier reports in literature.[32,35–37]

Interestingly, P- and E-cadherin expression seemed unrelated, as less than half of the cases we studied (25 of 52) showed concordant immunohistochemical scores (Table 1). P-cadherin neoexpression was more often moderate or weak/negative in Grade 1 (5 of 19) and especially Grade 2 (17 of 19) tumors than E-cadherin expression (moderate in 1 of 19 and 4 of 19 cases, respectively). Consequently, unlike P-cadherin, strong membranous E-cadherin expression did not allow to separate WD from MD tumors ($P = 0.34$). In the PD tumor group, on the other hand, the E-cadherin expression pattern was most often moderate, and not as frequently weak/negative, when compared with P-cadherin. Thus, strong E-cadherin expression was significantly associated with the MD (*vs.* PD) tumor grade ($p < 0.001$), whereas this was not the case for neoexpressed P-cadherin ($p = 1.000$). The extent of E-cadherin downregulation in colon carcinoma reported in literature is variable,[38] ranging from 12.5% overall downregulation[39] to a 52%/67%[40] and 89%/100%[32] decrease in MD/PD tumors, respectively. For the MD group (21%) in particular, we obtained lower rates. This could be due to the more sensitive immunohistochemical detection technique used or to a more stringent definition of the MD *vs.* PD phenotype.

Our data suggest that P-cadherin might be superior to E-cadherin in detecting more subtle changes in the differentiation status of colon carcinoma. Alternatively, loss of the neoexpressed P-cadherin could reflect other, yet unknown, biological events that occur early in tumor progression.

From these results, one might consider P-cadherin to be some kind of "differentiation marker" or, alternatively, an

Figure 2. (*a*) Western blotting for P- and E-cadherin in total cell lysates from HT-29 cells, transfected with siRNA against P-cadherin (siPcad) or with control siRNA (sico). Immunostaining for β-tubulin was included as a loading control. The values indicate the intensities of the cadherin bands normalized for tubulin and relative to control. (*b*) Phase-contrast micrographs of HT-29 cells in the aggregation assay on agar at the indicated time points. Compared with wild-type HT-29 (wt) and HT-29 sico (sico), HT-29 siPcad (siPcad) aggregation is impaired; bar = 50 μm. (*c*) Median (left) and mean (right) volume of HT-29 aggregates, 48 hr after transfection with siPcad RNA (siPcad) or control siRNA (sico), showing that P-cadherin downregulation results in significantly decreased aggregation. *$p < 0.001$ for siPcad *vs*. sico. (*d*) Western blotting for P-, E- and N-cadherins in total cell lysates from HT-29 cells, wild-type (wt) or transduced with shPcad (sh995 and sh3125). Quantification of cadherin band intensities is relative to wild-type and normalized for tubulin. (*e*) Phase-contrast micrographs of HT-29 (-derived) cells in the aggregation assay on agar (24 hr), showing decreased aggregation in P-cadherin downregulated HT-29 cells; bar = 50 μm. (*f*) Phase-contrast micrographs of HT-29 (-derived) cell cultures in the wound healing assay at the indicated time points after wounding. HT-29 shPcad cells show increased wound closure compared with wild-type HT-29; bar = 400 μm. (*g*) Total number of HT-29-derived invasive cells in the Matrigel invasion assay after 16 to 24 hr, relative to wild-type. Although in some experiments shPcad transduced HT-29 cells were more invasive than wild-type HT-29 (ratio > 1.0), differences were not statistically significant—0^1: outlier. (*h*) Table depicting the invasion scores of the HT-29 cell lines in the chick heart invasion assay. Each dot represents one independent confronting culture. Whereas wild-type HT-29 cells were not invasive, on transduction with shPcad, up to half of the co-cultures showed an invasive phenotype (score 3+). (*i*) Light micrographs of representative 7-day-old co-cultures processed for histology, illustrating that HT-29 shPcad tumor cells invade into the chick heart tissue in contrast to wild-type HT-29 (HE-staining); bar = 50μm.

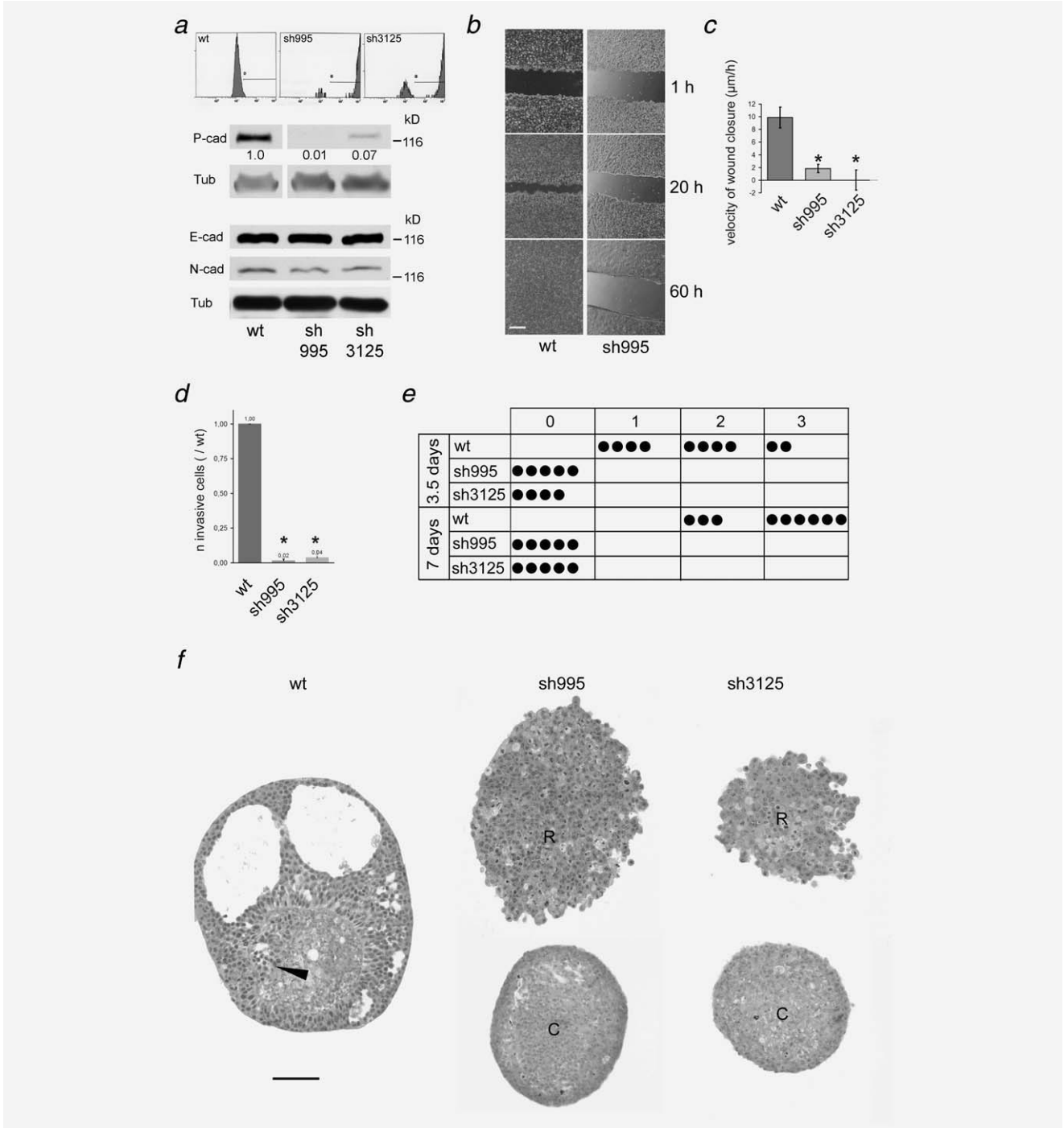

Figure 3. (*a*) Top: flow cytometric evaluation of EGFP expression in RT-112 cells, wild-type (wt) and transduced with short hairpin RNA constructs targeting P-cadherin (sh995, sh3125); bottom: Western blotting for P-, E- or N-cadherin of total cell lysates from RT-112 cells and their transduced derivatives. Immunostaining for β-tubulin was included as a loading control. The values indicate the intensities of the P-cadherin bands normalized for tubulin and relative to wild-type. RT-112 shPcad995 and shPcad3125 show strongly decreased P-cadherin expression levels. (*b*) Phase-contrast micrographs from a representative wound-healing experiment showing confluent RT-112 cell cultures at the indicated time intervals after wounding. RT-112 cells transduced with shPcad995 (sh995) did not migrate into the wounded area as opposed to wild-type RT-112 cells (wt); bar = 400µm. (*c*) Mean velocity of cell migration (µm/hr) into the wounded area after 24 hr (mean of all measurements of a representative experiment). The RT-112 cell lines transduced with shPcad show less (sh995) or no detectable (sh3125) migration compared with RT-112 wild-type (wt); $*p < 0.001$ for control *vs.* shPcad995 or shPcad3125. (*d*) Total number of cells invasive through a Matrigel-coated filter after 16 hr, relative to wild-type (wt) (mean of three independent experiments performed in duplex). Invasion was strongly abolished in RT-112 shPcad cell lines; $*p = 0.01$ and 0.03 for control *vs.* shPcad995 and shPcad3125, respectively. (*e*) Table depicting the invasion scores of RT-112 cell lines in the chick heart invasion assay, each dot representing one confronting culture. RT-112 wild-type cells (wt) showed in 2 of 10 and in 6 of 9 confronting cultures an invasive phenotype (score 3) after 3.5 and 7 days of incubation, respectively. In contrast, all RT-112 cells transduced with shPcad (sh995 and sh3125) were noninvasive (score 0). (*f*) Light micrographs of HE-stained paraffin sections of representative confronting cultures after 7 days of incubation in the chick heart invasion assay. RT-112 wild-type cells (wt, left) surround the embryonic chick heart tissue fragment and invade it (arrowhead), whereas the RT-112 shPcad cells (R) do not show any tendency to adhere to the chick heart fragment (C); bar = 100µm.

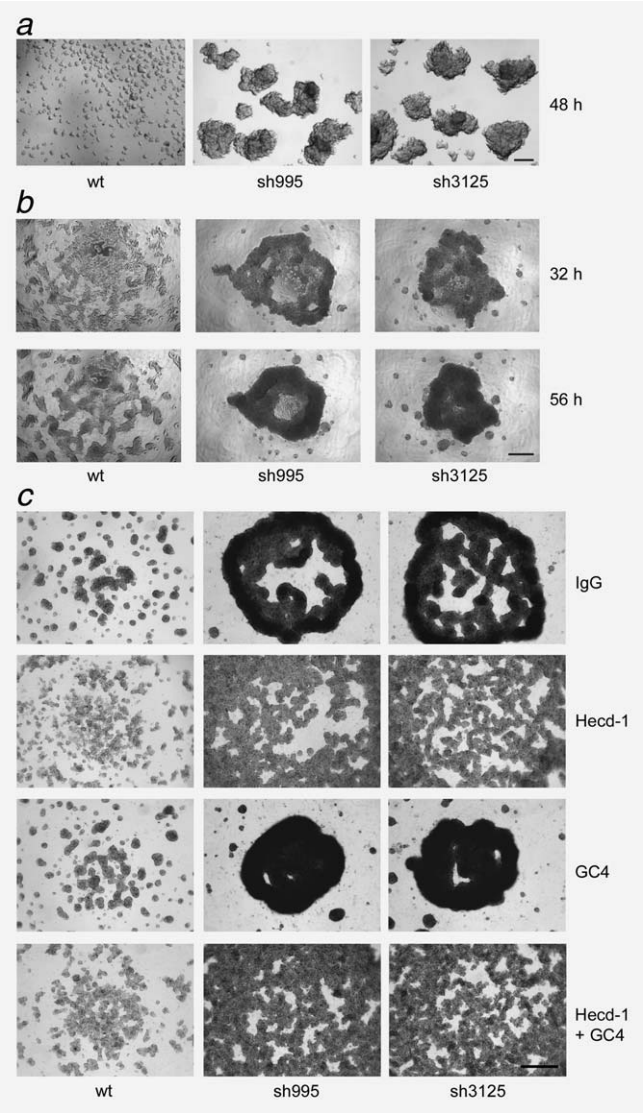

Figure 4. Phase-contrast micrographs of RT-112 (wt) and RT-112 shPcad (sh995 and sh3125) cells cultured for the indicated time in suspension (*a*) and on soft agar (*b*, *c*). (*a*) On P-cadherin downregulation, the RT-112 cells showed increased cell-cell aggregation; bar = 200 μm. (*b*) Whereas wild-type RT-112 display loose cell clustering (left), RT-112 shPcad995 (middle) and shPcad3125 (right) cells showed compact aggregation after 32 hr and 56 hr of incubation; bar = 400 μm. (*c*) RT-112 cell cultures treated for 72 hr with the indicated antibodies. Aggregation of wild-type and shPcad cells was decreased after treatment with Hecd-1 directed against E-cadherin (panels in second row), when compared with IgG control treatment (upper panels). Inhibition of N-cadherin by GC4 had no effect on wild-type RT-112, but an opposite effect on the shPcad cells, inducing even stronger aggregation of shPcad cells (panels in third row). Treatment with both Hecd-1 and GC4 gave similar results as with Hecd-1 alone, suggesting that the effect of E-cadherin is dominant (bottom panels); bar = 400 μm.

active player in processes as adhesion, motility and invasion of colon carcinoma cells. Since no *in vitro* studies have evaluated these hypotheses, we switched to HT-29 cells as a model for colorectal carcinoma. Knocking down P-cadherin in HT-29 colon carcinoma cells[41] by two different methods using siRNA and shRNA resulted in decreased aggregation. In agreement with these findings, a more motile and long-term invasive phenotype was observed in the stably transduced HT-29 shPcad cells. Interestingly, a significant proinvasive effect was not observed in transiently knocked down cells or in short-term invasion experiments, which could be due to the unaltered strong E-cadherin expression from which proadhesive and anti-invasive effects are expected.[38,42]

Our results in HT-29 cells indicate that P-cadherin actively assists E-cadherin in establishing an adhesive phenotype and in exerting a noninvasive effect in colon carcinoma cells. However, the precise role of the appearance of P-cadherin in early colorectal carcinogenesis remains to be investigated.

Although we previously established similar proadhesive and anti-invasive functions to P-cadherin in melanoma cells,[43] our laboratory and others also found an opposite, proinvasive effect in breast[14] and pancreatic carcinoma cells.[15] Based on our results with RT-112 bladder carcinoma cells presented in this study, we add another cell type to the latter list. These findings corroborate and extend literature data, which suggest that P-cadherin promotes a more malignant and invasive phenotype in bladder cancer.[23] Immunohistochemical studies showed an upregulation of P-cadherin in bladder carcinomas with increasing stage and grade.[21,23] In late stage tumors, the P-cadherin expression pattern switched from the normal localization in the basal cell layers to a diffuse expression pattern[21] and was also upregulated at the invasive front.[23] Bryan *et al.* also linked P-cadherin expression to patient outcome and found that increased P-cadherin, but not E-cadherin nor β-catenin, independently and inversely correlates with overall and bladder cancer-specific survival.

Our data clearly show that RT-112 shPcad cells were significantly impaired in their ability to migrate into artificial wounds, which is in agreement with recent observations from P-cadherin transfected EJ and UM-UC-3 bladder carcinoma cells in a Boyden chamber assay.[22] Moreover, we provide for the first time *in vitro* evidence for the putative intrinsic invasion-promoter function of P-cadherin in bladder carcinoma. First, we showed significantly decreased invasion of P-cadherin-silenced RT-112 cells into basement membrane material (Matrigel)-coated filters. Second, in the chick heart invasion assay during 3.5 and 7 days, RT-112 shPcad tumor cell aggregates failed to adhere properly to—let alone invade into—the precultured chick heart fragments. Mandeville *et al.* could not induce alterations in the invasive capacity of EJ and UM-UC-3 cells. This could be due to the fact that forced P-cadherin expression in these cells was mainly cytoplasmic,[22] in contrast to wild-type RT-112 cells, in which P-cadherin is localized at the membrane (data not shown).

RT-112 shPcad cells cultured on plastic were more compact and piled up when reaching confluence compared with parental RT-112 (data not shown), which suggests that downregulation of P-cadherin leads to increased intercellular adhesion. Earlier reports showed effective calcium-dependent adhesion in bladder carcinoma cell lines, amongst which RT-112, but not the individual contribution of each type of classical cadherin to it.[24] The observations of Bryan *et al.*[23] regarding adhesion of RT4 bladder carcinoma cell cultures treated with anti-cadherin antibodies principally revealed that P-cadherin could not backup for E-cadherin and, consequently, might contribute to an invasive phenotype. Our aggregation experiments gave more direct evidence for an antiadhesive effect of P-cadherin expression. Both RT-112 shPcad cell lines showed a stronger capacity to aggregate in two different assays.

Since RT-112 cells co-express E-, P- and N-cadherins, we studied the effect of treatment with function-blocking antibodies against E- and N-cadherins. The compact aggregates on agar of RT-112 shPcad treated with an antibody against N-cadherin (GC4) reflect the E-cadherin-mediated adhesion, whereas the differences in phenotype between the rather dispersed wild-type and the more dense shPcad cell cultures are the P-cadherin effect. These differences were at most when E-cadherin was functionally present (IgG control and GC4-treatment), but could still be seen in the "+ Hecd-1 ± GC4" conditions, suggesting that the P-cadherin-mediated defective adhesion is an active process of "dispersion" (see Fig. 4). Western blotting showed that there was no N-cadherin down- or E-cadherin upregulation that could account for the increased aggregation of RT-112 shPcad cells.

Another important result from the aggregation assays on agar is that N-cadherin partly compensated for the effect of decreased P-cadherin expression. Adding GC4 to RT-112 shPcad cells led to even more compact aggregation than in IgG control conditions. The fact that GC-4 treatment had no or little effect when E-cadherin was blocked or when P-cadherin was present (wild-type) could be explained by the overall low N-cadherin expression levels in RT-112.

An antiadhesive effect of N-cadherin in bladder carcinoma has not yet been described in literature but is in line with *in vitro* studies showing a proinvasive function in bladder cancer[44] and other epithelial tumors.[45] N-cadherin-mediated adhesion was found in T24 bladder carcinoma cells,[24] but this cell line is negative for E-cadherin and P-cadherin. Further studies should be undertaken to clarify the role N-cadherin might play in tumor progression as immunohistochemical reports remain conflicting.[21,23,46,47]

In summary, our results support and extend the literature findings that the role of P-cadherin in tumor cell adhesion and invasion is subject to tumor type and context (tissue architecture and microenvironment). In bladder cancer cells, we showed for the first time evidence of an active process of dispersion conducted by P-cadherin and N-cadherin, which counteracted the adhesive effect of E-cadherin and, possibly linked to this, data supporting an intrinsic migratory and invasion-promoter role for P-cadherin.

In colon carcinoma cells, in contrast, we demonstrated that P-cadherin, like E-cadherin, promotes adhesion and has inhibitory effects on migration and invasion. We also showed that in colorectal carcinoma tissue, loss of P-cadherin neoexpression is significantly associated with tumor grade. Together, these findings suggest that P-cadherin may have an active antimigratory and anti-invasive function, which is lost on tumor progression.

Although these results set off P-cadherin as a potential therapeutic target in bladder cancer, it is probably a favorable prognostic marker in colon cancer. Especially for colon cancer, further studies should be done to substantiate this hypothesis.

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