Functional Role of Cell Surface CUB Domain-Containing Protein 1 in Tumor Cell Dissemination

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Abstract
The function of CUB domain-containing protein 1 (CDCP1), a recently described transmembrane protein expressed on the surface of hematopoietic stem cells and normal and malignant cells of different tissue origin, is not well defined. The contribution of CDCP1 to tumor metastasis was analyzed by using HeLa carcinoma cells overexpressing CDCP1 (HeLa-CDCP1) and a high-disseminating variant of prostate carcinoma PC-3 naturally expressing high levels of CDCP1 (PC3-hi/diss). CDCP1 expression rendered HeLa cells more aggressive in experimental metastasis in immunodeficient mice. Metastatic colonization by HeLa-CDCP1 was effectively inhibited with subtractive immunization-generated, CDCP1-specific monoclonal antibody (mAb) 41-2, suggesting that CDCP1 facilitates relatively late stages of the metastatic cascade. In the chick embryo model, time- and dose-dependent inhibition of HeLa-CDCP1 colonization by mAb 41-2 was assessed to determine when and where CDCP1 functions during metastasis. Quantitative PCR and immunohistochemical analyses indicated that CDCP1 facilitated tumour cell survival soon after vascular arrest. Live cell imaging showed that the function-blocking mechanism of mAb 41-2 involved enhancement of tumor cell apoptosis, confirmed by attenuation of mAb 41-2-mediated effects with the caspase inhibitor z-VAD-fmk. Under proapoptotic conditions in vitro, CDCP1 expression conferred HeLa-CDCP1 cells with resistance to doxorubicin-induced apoptosis, whereas ligation of CDCP1 with mAb 41-2 caused additional enhancement of the apoptotic response. The functional role of naturally expressed CDCP1 was shown by mAb 41-2-mediated inhibition of both experimental and spontaneous metastasis of PC3-hi/diss. These findings confirm that CDCP1 functions as an antia apoptotic molecule and indicate that during metastasis CDCP1 facilitates tumor cell survival likely during or soon after extravasation. (Mol Cancer Res 2009;7(8):1197–211)

Introduction
Recent years brought about a significant degree of interest in CUB domain-containing protein 1 (CDCP1), identified as a novel human tumor-associated gene by differential cDNA analysis (1). The gene structure of CDCP1 suggested that the putative corresponding protein likely would be involved in cell interactions with the extracellular matrix (ECM). Functional importance of CDCP1 protein in vivo was initially indicated by the demonstration of differentially enhanced levels of CDCP1 in highly metastatic human tumor cells by the monoclonal antibody (mAb) 41-2, generated via subtractive immunization (2). The novel 135-kDa protein precipitated by mAb 41-2 was characterized as a transmembrane CUB domain-containing molecule and confirmed by amino acid sequencing to be CDCP1. The intracellular COOH terminus of CDCP1 harbors several tyrosine residues and is phosphorylated by Src family kinases (2). Phosphorylation of the COOH terminus of CDCP1 by Src kinases along with evidence of CDCP1-mediated activation of several other kinases suggested functional involvement of CDCP1 in outside-in signal transduction as a kinase docking molecule (3, 4). This conception was further affirmed by coprecipitation of protein kinase Cδ, a member of the protein kinase C family, with CDCP1 (5). It has been also proposed that CDCP1 is involved in homotypic complex formation via its extracellular CUB domain (4); however, no such molecular interactions have been shown directly. Recent findings also indicate that overexpression of CDCP1 leads to cell rounding and a loss of adhesion phenotype (3). In addition, CDCP1 expression renders anchorage-independent growth and resistance to anoikis of lung and gastric carcinoma cells (6, 7).

CDCP1 is expressed in many normal human tissues and cells, including hematopoietic stem and progenitor cells (2, 8, 9). Increased levels of CDCP1 were shown in some aggressive epithelial cancers, correlating with poor prognosis, higher relapse rate and occurrence of metastases, and unfavorable overall survival of patients (10, 11). Therefore, CDCP1 emerges as a potential prognostic marker in several types of carcinomas as well as a possible target in cancer therapy. Thus, down-regulation of CDCP1 by RNA interference in lung and gastric carcinoma cells resulted in suppressed invasion and experimental metastasis (6, 7). Treatment with anti-CDCP1 mAb 25A11 coupled with the cytotoxin saporin resulted in an inhibition of prostate cancer
cell metastasis in a mouse xenograft model (12). However, the latter approach based on targeting of CDCP1-positive cancer cells is limited at least by two major considerations. First, the use of a toxin-conjugated antibody recognizing the cell surface molecule that is lacking in a xenogeneic host would kill CDCP1-expressing human cells by a general, likely toxin-antibody-internalization mechanism, not related to the natural functions of CDCP1. Second, in cancer patients, the toxin-conjugated anti-CDCP1 antibodies may harm or kill normal cells due to almost ubiquitous expression of CDCP1 among human tissues. Thus, it seems that delivering of CDCP1-aimed therapeutics would require more focused, time-restricted, or tissue-dependent approaches. In this regard, it becomes essential to mechanistically address specific aspects of CDCP1 functionality such as when and where in the metastatic cascade CDCP1 might function as a prometastatic molecule.

To characterize a prometastatic role of CDCP1, we generated carcinoma cells expressing high levels of CDCP1 by transfecting the CDCP1 cdna into HeLa cells intrinsically lacking CDCP1 expression. In parallel, we have selected a highly disseminating variant of prostate carcinoma PC-3 cells naturally expressing high levels of CDCP1. By using these CDCP1-expressing cells and the CDCP1 function-blocking mAb 41-2 in quantitative experimental metastasis models, we have shown that CDCP1 functions following cell arrest in the vasculature. Our findings also indicate that mAb 41-2–mediated inhibition of metastatic colonization is initiated during or soon after extravasation and is associated with a pronounced enhancement of tumor cell apoptosis involving caspase activation.

Results

Generation of HeLa-CDCP1 Cells

HeLa cells overexpressing CDCP1 protein were generated from the parental carcinoma cells, previously shown to express no detectable CDCP1 (2). Flow cytometry confirmed high levels of cell surface CDCP1 in HeLa-CDCP1 cells and the corresponding lack of CDCP1 in control HeLa-neo cells (Fig. 1A). Analysis by Western blotting indicated that HeLa-CDCP1 cells manifested the 135-kDa full-length and a truncated 70-kDa species of CDCP1 (Fig. 1B).

CDCP1-Mediated Colonization in the Mouse Experimental Metastasis Model

To determine how expression of CDCP1 affected the colonization potential of HeLa cells in an experimental metastasis model, severe combined immunodeficient (SCID) mice were injected i.v. with either control HeLa-neo or CDCP1-transfected HeLa cells (Fig. 1C). High levels of metastatic colonization were shown 4 weeks later by human-specific quantitative PCR (qPCR) in lungs, brain, and ovaries for HeLa-CDCP1 cells (Fig. 1C, gray columns). Control HeLa-neo cells displayed low levels of lung colonization and essentially lacked the ability to efficiently metastasize/colonize the brain or ovaries (Fig. 1C, black columns). To confirm that the high metastatic potential of HeLa-CDCP1 cells was attributed to CDCP1-mediated functions, the cells were inoculated i.v. into mice along with function-blocking anti-CDCP1 mAb 41-2 or control mouse IgG. As determined by qPCR 3 weeks later, treatment with mAb 41-2 substantially reduced colonization of host organs (Fig. 1D), indicating that ligation of CDCP1 by mAb 41-2 negated the enhanced metastatic potential of HeLa-CDCP1 cells.

CDCP1-Mediated Colonization in the Chick Embryo Experimental Metastasis Model

To examine mechanistically when and where during metastatic colonization CDCP1 exerts its prometastatic functions, we took advantage of our function-blocking anti-CDCP1 antibodies in combination with the chick embryo experimental metastasis model, allowing for efficient morphologic and quantitative analysis of tumor cell metastasis. HeLa-CDCP1 cells were injected i.v. into day 12 chick embryos with 50 μg control mouse IgG or increasing doses of mAb 41-2 (Fig. 2A). Five days later, portions of the CAM, a highly vascularized tissue serving as a preferential site for arrest, extravasation, and expansion of tumor cells, were harvested and analyzed by qPCR to determine actual numbers of human cells in the host tissues. Metastatic colonization of HeLa-CDCP1 cells was diminished by mAb 41-2 in a dose-dependent manner and was sensitive to as low as 5 μg of antibody per embryo (Fig. 2A). Thus, the inhibitory effect of anti-CDCP1 mAb 41-2 on experimental metastasis observed in the mouse system was recapitulated in the quantitative chick embryo model. In addition to mAb 41-2, HeLa-CDCP1 colonization was efficiently inhibited by another CDCP1-specific mAb 10-D7, which similarly to mAb 41-2 was also generated by subtractive immunization. In HeLa-CDCP1 cells, mAb 10-D7 identified a protein band of the same 135 kDa molecular weight as mAb 41-2 (Fig. 2B, inset) and exhibited identical cell surface binding (data not shown). Both anti-CDCP1 mAbs 41-2 and 10-D7 efficiently inhibited colonization of HeLa-CDCP1 cells on average by 74% and 72% of control levels, respectively (Fig. 2B).

Tyrosine Phosphorylation of CDCP1 and mAb 41-2–Mediated Inhibition of Experimental Metastasis

Functional activity of CDCP1 has been assumed to be closely associated with the COOH-terminal tyrosine phosphorylation. Therefore, we generated HeLa-CDCP1 cells where tyrosine residue Y734 was substituted for phenylalanine (Y734F), previously shown to completely abrogate tyrosine phosphorylation of the COOH terminus (5). Similar levels of CDCP1 expression in HeLa cells expressing CDCP1-WT and CDCP1-Y734F constructs were shown by flow cytometry (Fig. 3A), whereas Western blotting indicated that the 135- and 70-kDa species were both constitutively phosphorylated in HeLa-CDCP1-WT cells but not in the HeLa-CDCP1-Y734F mutant (Fig. 3B).

HeLa-CDCP1-Y734F and HeLa-CDCP1-WT cells were injected i.v. into chick embryos along with mAb 41-2 or mouse IgG and analyzed 5 days later. As shown in Fig. 3C, HeLa-CDCP1-Y734F cells exhibited slightly enhanced levels of metastasis compared with HeLa-CDCP1-WT cells. Importantly, HeLa-CDCP1-Y734F cell colonization was >50% inhibited by mAb 41-2, indicating that efficient inhibition of metastasis by mAb 41-2–mediated ligation of CDCP1 apparently does not require CDCP1 tyrosine phosphorylation.
To elucidate the mechanisms underlying the inhibitory effects of CDCP1 ligation by mAb 41-2 on metastasis, we did immunohistochemical analysis of tumor cells in the CAM tissue 5 days after i.v. inoculation of HeLa-CDCP1 cells along with mAb 41-2 or control IgG. The analysis indicated that treatment with mAb 41-2 resulted in reduction of metastatic microfoci formation (Fig. 4A, top). Quantitative analysis confirmed a substantial decrease (~80%) in the foci density in the embryos treated with mAb 41-2 (Fig. 4A, top bar graph). Quantitation of the relative number of tumor cells per individual metastatic focus indicated comparable cell numbers regardless of the antibody treatment (Fig. 4A, bottom and bar graph). These findings suggested that the inhibitory mechanisms of mAb 41-2 involve targeting single CDCP1-expressing tumor cells soon after their inoculation. The 10% to 20% of tumor cells that escaped initial 41-2 targeting generated near-normal size metastatic foci.

The contention that CDCP1 is functionally important at early stages of experimental metastasis was further verified in kinetic experiments where HeLa-CDCP1 cells were injected along with mAb 41-2 or control IgG and actual numbers of human cells within CAM tissue were determined at different time points following inoculations. As a negative control, HeLa-neo cells were also coinjected along with mAb 41-2 or control IgG and analyzed at the early (2 hours) and latest (5 days) time points. The qPCR analysis of CAM tissue (Fig. 4B) showed similar numbers for both HeLa cell types at a 2-hour time point regardless of antibody treatment, indicating that CDCP1 ligation by the mAb 41-2 did not affect the initial arrest of HeLa-CDCP1 cells in the vasculature. However, the number of HeLa-CDCP1 cells was significantly diminished at 1, 2, and 3 days in the embryos treated with mAb 41-2, leading to...
a significant delay in tumor cell colonization (Fig. 4B, black columns) compared with IgG control (Fig. 4B, gray columns). By day 5, this delay manifested itself as the 3- to 5-fold differential in colonization by HeLa-CDCP1 cells inoculated with mAb 41-2 versus control IgG. This analysis also indicated a lack of inhibitory effects of mAb 41-2 on HeLa-neo cells, further showing the specificity of this function-blocking mAb. Additionally, the overall enhanced metastasis of HeLa-CDCP1 cells compared with their CDCP1-negative counterparts was confirmed in these kinetic experiments by using an independent approach (Fig. 4B, gray columns versus white columns at 5 days).

To determine when injected HeLa-CDCP1 cells were most sensitive to ligation of CDCP1 by mAb 41-2, we varied the time of antibody injections (Fig. 4C). This kinetic analysis indicated that mAb 41-2 significantly blocked colonization of HeLa-CDCP1 cells within a narrow time frame during the first 24 hours following cell injection. More precisely, the most pronounced inhibition of metastasis occurred when mAb 41-2 was administered 1 hour before and within 4 hours after cell injections. The mAb 41-2-mediated inhibition of tumor cell colonization gradually diminished after 8 hours following cell inoculations until at 48 to 72 hours mAb 41-2 became completely ineffective (Fig. 4C), affirming that cell surface CDCP1 functions in the early stages of metastatic colonization following initial cell arrest in the vasculature.

**Morphologic Analysis of Inhibitory Effects of mAb 41-2 on HeLa-CDCP1 Experimental Metastasis**

HeLa-CDCP1 cells were prelabeled with green fluorescent CellTracker and injected into the chick embryos along with mAb 41-2 or control IgG. At different time points, non-fixed CAM was analyzed by fluorescence microscopy after highlighting the vasculature with rhodamine-conjugated lens culinaris agglutinin (Fig. 5). As soon as 10 minutes after inoculation, HeLa-CDCP1 cells arrive in the terminal capillaries of the CAM (Fig. 5A, dotted ovals). The cells are clearly intravascular, approaching the tips of terminal capillaries (Fig. 5A, bottom). By 2 hours, most of the HeLa-CDCP1 cells have reached the ends of terminal capillaries and seem arrested in narrow spaces of the capillary plexus (Fig. 5B). There were no apparent effects of mAb 41-2 on HeLa-CDCP1 cells at these early time points (Fig. 5B, left and right), affirming the nearly identical 2-hour quantitative cell arrest data presented in Fig. 4B.

By 12 hours after injection with control IgG, the HeLa-CDCP1 cells seem to scatter from the terminal capillaries, presenting cell protrusions characteristic of motile behavior (Fig. 5C, left). However, in the embryos injected with mAb 41-2, HeLa-CDCP1 cells are still positioned close to the ends of terminal capillaries (Fig. 5C, middle), indicative of their inefficient outward motility. More importantly, many of mAb 41-2-treated HeLa-CDCP1 cells displayed morphology typical of HeLa cells that underwent apoptosis (13), with cell retraction, plasma membrane blebbing, and condensed and fragmented nuclei (i.e., resembling apoptotic bodies; Fig. 5C, middle, arrows). At the 24-hour time point, HeLa-CDCP1 cells in control embryos continue their motile scattering into the areas between arteriolar and venous capillaries (Fig. 5D, left), whereas in the embryos treated with mAb 41-2 the majority of tumor cells seem to be halted at the tips of the terminal capillaries with many of them having undergone blebbing and/or fragmentation (Fig. 5D, right, arrows).
Quantitative analysis of the percentage of fragmented cells (apoptotic bodies) was done on multiple digital images of the CAM at different time points (Fig. 5E). This kinetic analysis showed a slow, time-dependent increase of cell fragmentation in embryos injected with control IgG, reaching ~8% by 48 hours after cell inoculations. However, treatment with mAb 41-2 caused a steep 20% to 25% increase in cell fragmentation at 12 and 24 hours, thereby constituting a substantial 3- to 5-fold differential over IgG control.

To confirm that fragmented cells actually represented apoptotic bodies and that the increase in their numbers mediated by mAb 41-2 was indeed attributed to actual apoptosis, HeLa-CDCP1 cells were first pretreated with the caspase inhibitor z-VAD-fmk and then injected into embryos along with mAb 41-2 and the inhibitor. Treatment with z-VAD-fmk resulted in a dramatic decrease in the percentage of fragmented cells in the CAM tissue below control levels (Fig. 5E, black column), consistent with inhibition of apoptosis and release of apoptotic bodies by blocking caspase activation (14). Morphologically, z-VAD-fmk-treatment reverted appearance of HeLa-CDCP1 cells injected with mAb 41-2 to that observed in the presence of control antibody manifesting very few fragmented cells (Fig. 5C, right versus left). Sensitivity of HeLa-CDCP1 cell fragmentation to z-VAD-fmk indicates a general involvement of caspase activation and suggests that at the early stages of experimental metastasis, inoculated cells undergo naturally occurring apoptosis, the levels of which are substantially enhanced by ligation with anti-CDCP1 mAb 41-2.

Effects of mAb 41-2 on Functions of HeLa-CDCP1 Cells

In vitro

z-VAD-fmk–sensitive fragmentation of HeLa-CDCP1 cells, enhanced by mAb 41-2 as the cells exit the vasculature, suggested that this function-blocking antibody might induce anoikis events due to inhibition of CDCP1-mediated cell-cell adhesion or cell adhesion to ECM proteins. This suggestion was analyzed in a series of in vitro assays addressing different aspects of cell functionality. First, we analyzed whether CDCP1 expression rendered HeLa cells with enhanced levels of homotypic interactions. As shown in Fig. 6A, the expression of CDCP1 did not enhance homotypic adherence of HeLa-CDCP1 cells. Adherence of HeLa-CDCP1 cells was equal to HeLa-neo and HeLa-CDCP1 cells both 2 hours following cell plating when cells had just attached or later, after 6 hours, when plated cells had firmly adhered and spread on the underlying cellular layers. We next investigated the possibility whether CDCP1 enhanced heterotypic adherence to vascular endothelial cells and mesenchymal fibroblasts and whether these heterotypic interactions were sensitive to mAb 41-2. The expression of CDCP1 did not confer HeLa-CDCP1 cells with enhanced capability of heterotypic adhesion to endothelial cells (Fig. 6B) or fibroblasts (Fig. 6C) compared with HeLa-neo control. In addition, mAb 41-2 did not affect HeLa-CDCP1 cell adhesion to endothelial cells but slightly inhibited adhesion to fibroblasts (Fig. 6B and C, black columns). In ECM-mediated adhesion, HeLa-CDCP1 cells showed significantly less adhesion to type I collagen, fibronectin, and Matrigel compared with HeLa-neo cells.

**FIGURE 3.** Inhibition of experimental metastasis of HeLa-CDCP1 cells by mAb 41-2 does not depend on tyrosine phosphorylation of CDCP1 protein. A. Flow cytometry analysis of HeLa cells expressing Y734F tyrosine mutant of CDCP1. HeLa cells transfected with an empty vector (control), wild-type CDCP1 (CDCP1-WT), or CDCP1-Y734F mutant were analyzed in a FACScan for cell surface expression of CDCP1 after staining with mAb 41-2. B. Western blot analysis of tyrosine phosphorylation of CDCP1. CDCP1 was precipitated from HeLa-CDCP1-WT, HeLa-CDCP1-Y734F, and HeLa-neo cells with mAb 41-2 and probed with anti-phosphotyrosine mAb 4G10. Left, position of molecular weight standards (in kDa). Solid and open arrows, CDCP1 bands with approximate molecular weight of 135 and 70 kDa, respectively. C. Inhibition of experimental metastasis of HeLa-CDCP1-Y734F cells by mAb 41-2. HeLa-CDCP1-WT (WT) and HeLa-CDCP1-Y734F (Y734F) cells were injected i.v. into chick embryos along with control IgG or mAb 41-2. Levels of metastatic colonization in CAM were determined 5 d later. Data are presented as percentage of control colonization (HeLa-CDCP1-WT cells, mouse IgG) determined from pooled data obtained in four independent experiments using from 21 to 47 embryos per variable. Columns, mean; bars, SE. *, P < 0.05; ***, P < 0.0005, compared with IgG control.
(Fig. 6D-F), but the levels of cell adhesion were not significantly inhibited by mAb 41-2 with the exception of a slight inhibition of attachment to fibronectin (Fig. 6D-F, black columns). Similarly, HeLa-CDCP1 cells did not show significant levels of chemotactic invasion in Matrigel assays compared with HeLa-neo control, and HeLa-CDCP1 invasion levels were not inhibited by mAb 41-2 (Fig. 6G). Finally, ligation of CDCP1 by mAb 41-2 did not affect significantly the motility of HeLa-CDCP1 cells in Transwell assays where haemotactic migration was mediated by type I collagen, fibronectin, or Matrigel, and chemotactic migration was induced by serum (data not shown).

These in vitro results indicate that mAb 41-2-mediated inhibition of HeLa-CDCP1 colonization apparently does not involve significant abrogation of cell-cell or cell-ECM interactions, which in turn could elicit anoikis. Whether ligation of CDCP1 would increase cell death under anoikis-inducing conditions was verified in a series of in vitro experiments where HeLa-CDCP1 cells were incubated with or without mAb 41-2: Neither cell growth nor percentage of dead cells (varying from 1.5% to 6% for mouse IgG control and from 3.4% to 4.5% for mAb 41-2-treated cells) was significantly affected under adhesion-free conditions in ultralow adhesion plates (Fig. 6H). Similarly, mAb 41-2 did not significantly affect HeLa-CDCP1 colony formation in soft agar cultures (data not shown).

**Opposing Effects of CDCP1 Expression and mAb 41-2 Treatment on Drug-Induced Apoptosis**

As indicated in the in vivo live cell imaging experiments (Fig. 5), anoikis or apoptosis might occur under conditions where mAb 41-2–treated CDCP1–positive cells encounter stress or a proapoptotic environment. Therefore, we further analyzed the in vitro effects of mAb 41-2 on HeLa-CDCP1 cells under proapoptotic conditions, which were induced by incubation with doxorubicin, an inducer of caspase activation–mediated cell death (15-18). Drug-induced apoptosis causes cell retraction, rounding, and cell detachment (13), and therefore, the numbers of detached cells served as a measurement of apoptotic response. Treatment of either HeLa-CDCP1 cells or their control HeLa-neo counterparts with doxorubicin caused apoptosis accompanied by detachment of dead cells (Fig. 7A).

As expected, the doxorubicin-treated cells exhibited caspase activation confirmed by Western blotting (Fig. 7B) and nuclei fragmentation visualized by 4′,6-diamidino-2-phenylindole staining (Fig. 7C). Importantly, the levels of doxorubicin-induced apoptosis were ~50% lower in HeLa-CDCP1 cells compared with identically treated HeLa-neo control, thus showing that the expression of CDCP1 provided significant resistance to apoptosis (Fig. 7A). Doxorubicin-induced apoptosis was abrogated by pretreatment of the cells with z-VAD-fmk, confirming the involvement of caspase activation (Fig. 7A, hatched columns).

We next verified whether addition of mAb 41-2 to HeLa-CDCP1 cells cultured in proapoptotic conditions enhanced drug-induced cell death and detachment (Fig. 7D). Whereas mAb 41-2 did not induce any negative effects in the absence of doxorubicin, treatment with mAb 41-2 in the presence of the drug resulted in an additional 2-fold increase in the number of detached cells compared with IgG control, and this differential was essentially abrogated by z-VAD-fmk (Fig. 7D, hatched columns). The specificity of the mAb 41-2–induced differential was shown by the complete lack of any increase in doxorubicin-induced cell detachment in the presence of another HeLa-CDCP1 cell surface–reacting mAb 29-7 recognizing CD44 (data not shown).

**CDCP1 Naturally Expressed in Tumor Cells Facilitates Experimental and Spontaneous Metastasis**

To expand our findings to tumor cells endogenously expressing CDCP1, we used a recently selected variant of the CDCP1–positive human prostate PC-3 carcinoma cell line, PC3-hi/diss, which is capable of high levels of dissemination in both spontaneous and experimental metastasis models. Cell surface staining showed relatively high levels of CDCP1 expression in PC3-hi/diss cells (Fig. 8A), whereas Western blotting confirmed that, similar to CDCP1 overexpressed in HeLa cells, the naturally expressed CDCP1 was also represented mainly by the 135-kDa species and a 65- to 70-kDa fragment (Fig. 8B).

In the experimental metastasis model, PC3-hi/diss cells were injected into chick embryos along with one or two injections of mAb 41-2 or control IgG. Single and double treatments with mAb 41-2 resulted in a more than 50% and 80% inhibition of experimental metastasis, respectively (Fig. 8C). Thus, similar to the CDCP1 molecule overexpressed in transfected cells, naturally expressed CDCP1 was also sensitive in vivo to the function-blocking mAb 41-2. We also carried out a kinetic analysis of inhibition of PC3-hi/diss colonization by timed additions of mAb 41-2 and showed that inoculated PC3-hi/diss cells were sensitive to mAb 41-2 during the first 4 hours following injection (Fig. 8D). Importantly, this kinetics of inhibition by mAb 41-2 confirmed our findings observed in the HeLa-CDCP1 model system (Fig. 4C) and clearly pointed to a specific time frame available for targeting CDCP1 soon after vascular arrest and initial extravasation of tumor cells.

We also determined whether CDCP1-mediated functions can be blocked by mAb 41-2 in a spontaneous metastasis model. When grafted on the CAM of the chick embryos, PC3-hi/diss cells form primary tumors and spontaneously disseminate to the distal CAM and internal organs. Administration of function-blocking mAb 41-2 did not significantly affect primary tumor formation by PC3-hi/diss cells (Fig. 8E, bottom) but almost completely abrogated their spontaneous dissemination (Fig. 8E, top). Therefore, yet another functional feature of endogenously expressed CDCP1 was verified in the PC3-hi/diss model, confirming that CDCP1 plays an important mechanistic role in tumor cell dissemination.

**Discussion**

High expression levels of CDCP1 on the cell surface of hematopoietic stem cells/cell progenitors (8, 9) and malignant cells of different tissue origin suggested a functional importance of the antigen in cell differentiation, homing, and dissemination. However, the precise role of CDCP1 in these processes is still
not well defined. The involvement of CDCP1 in cancer has been investigated in several studies, indicating that CDCP1 has a functional role in tumor cell metastasis. Different aspects of CDCP1 functionality have been highlighted, including elevated expression in several metastatic cancers (1, 2, 8-11, 19), CDCP1-mediated signaling (3-7, 20), and inhibition of CDCP1-mediated metastasis by toxin-conjugated anti-CDCP1 mAb (12). Nevertheless, the question whether CDCP1 expression facilitates early stages of tumor cell dissemination, such as primary tumor formation, tumor cell escape, and intravasation, or later stages, such as vascular survival, arrest, extravasation, and metastatic foci formation, remained largely unexplored.

Taking advantage of our unique function-blocking mAbs, 41-2 and 10-D7, specifically targeting cell surface CDCP1, herein we sought to identify specific steps of the metastatic cascade in which CDCP1 functions as a prometastatic molecule as well as to define the mechanisms by which ligation of cell surface CDCP1 with a specific anti-CDCP1 mAb inhibits metastasis. To facilitate these studies, tumor cells overexpressing CDCP1 were generated from the parental CDCP1-negative HeLa carcinoma.

CDCP1 expression in HeLa cells seemed to confer these relatively nonaggressive cells with higher efficiency in experimental metastasis in mice, where substantial colonization by HeLa-CDCP1 cells was shown in the lungs, brain, and ovaries. Importantly, HeLa-CDCP1 metastatic colonization in mice was extremely sensitive to early treatment with function-blocking mAb 41-2, suggesting that CDCP1 functions soon after tumor cell inoculation into the vasculature. To further investigate which cellular steps of experimental metastasis are promoted by...
CDCP1, we took advantage of the chick embryo metastasis model, which facilitates detailed kinetic studies and microscopic analyses of live tumor cells disseminating \textit{in vivo}. We confirmed that HeLa cells overexpressing CDCP1 were more efficient in metastatic colonization in chick embryos and that similar to the mouse model, this colonization was sensitive to anti-CDCP1 mAb 41-2. In addition to mAb 41-2, another anti-CDCP1 mAb, 10-D7, also blocked colonization of HeLa-CDCP1 cells.
Interestingly, both 41-2 and 10-D7 mAbs were generated independently via subtractive immunization, an approach that seems to result in high-frequency production of function-blocking antibodies exhibiting exceptional affinity toward their respective antigens (2, 21-24).

Binding of mAb 41-2 to HeLa-CDCP1 cells resulting in ligation of cell surface CDCP1 could trigger antigen clustering and signal transduction. The existence of several tyrosine phosphorylation sites in the COOH terminus of CDCP1 originally suggested outside-in signaling through phosphorylation by tyrosine kinases (2), which was ascribed later to members of the src kinase family (4, 5) and then to tumor cell functions such as anoikis resistance and metastatic potential of lung and gastric carcinoma cells (6, 7). To address these relevant issues, we generated HeLa cells expressing a critical CDCP1 mutant, HeLa-CDCP1-Y734F. In contrast to HeLa cells expressing wild-type of CDCP1, their Y734F counterpart completely lacks tyrosine phosphorylation, confirming the singular importance of Y734 for phosphorylation of the whole native molecule (5, 6). However, despite complete lack of tyrosine phosphorylation, HeLa-CDCP1-Y734F mutant exhibited high levels of metastatic colonization as well as sensitivity to mAb 41-2. Therefore, with regard to the inhibitory mechanisms of the anti-CDCP1 mAb 41-2, blocking of CDCP1 functions in our in vivo models apparently does not involve outside-in signaling mediated via tyrosine phosphorylation of CDCP1.

To delineate the in vivo mechanisms by which mAb 41-2 inhibits experimental metastasis of tumor cells expressing CDCP1, we did quantitative analyses of metastatic colonization by HeLa-CDCP1 cells in the presence or absence of mAb 41-2 over a detailed time course. First, immunohistological studies indicated that mAb 41-2 targeted individual HeLa-CDCP1 cells following inoculation rather than inhibited outgrowth of established metastatic foci. This conclusion was corroborated by qPCR analysis showing a clear delay in colonization kinetics produced by mAb 41-2 versus control mouse IgG. Furthermore, mAb 41-2 injections done at different time intervals after HeLa-CDCP1 cell inoculations clearly pointed to the narrow, ~8-hour window open for the inhibition of CDCP1-dependent experimental metastasis. Importantly, this finding was confirmed by kinetic studies done with the prostate carcinoma PC3-hi/diss cells naturally expressing CDCP1. Altogether, these findings for the first time indicated that CDCP1 is likely involved in early stages of metastatic colonization (i.e., after initial cell arrest in the vasculature and before the beginning of multicellular foci formation).

Microscopy studies of live HeLa-CDCP1 cells were done to address the question of which precise steps in the early stages of experimental metastasis were affected by mAb 41-2. The findings showed that mAb 41-2 induced enhanced fragmentation of HeLa-CDCP1 cells soon after their extravasation from terminal capillaries, where the cells initially arrest after inoculation. These fragmented HeLa-CDCP1 cells, the percentage of which increased substantially on in vivo mAb 41-2 treatment, closely resembled apoptotic bodies characteristic of cells that underwent apoptosis (14, 25) and appeared as cells with blebbed cytoplasm and condensed, disintegrated nuclei. These morphologic characteristics are the same features observed in HeLa cells that were induced by drugs to undergo apoptosis in vitro (13). Early stages of experimental metastasis in mice are associated with a substantial clearing of inoculated tumor cells, partially due to apoptotic events involving caspase activation in ~10% of cells that reached the lung vasculature (26). Similarly, natural apoptotic fragmentation of HeLa-CDCP1 cells was observed in vivo in our chick embryo experimental metastasis model where quantification of apoptotic bodies showed their gradual increase to ~10% within 48 hours after cell inoculation with control IgG. However, if HeLa-CDCP1 cells were cojected with mAb 41-2, the percentage of in vivo fragmented cells increased ~6-fold by 12 hours. Importantly, this increase was completely abrogated with z-VAD-fmk, which brought the percentage of apoptotic bodies to levels even lower than those observed in control. Our results are consistent with findings showing that prevention of caspase activation during chemotherapeutic agent–induced apoptosis in vitro leads to the inhibition of apoptotic body formation (14). Furthermore, metastatic colonization in mice, which is normally accompanied by 5% to 10% levels of apoptosis of i.v. inoculated cells, could be substantially inhibited by an additional 2- to 2.5-fold increase of tumor cell apoptosis (26), providing a clear precedent for a direct link between enhanced apoptosis and diminished metastasis.

Inhibition of experimental metastasis by mAb 41-2 is fundamentally different from the recently described, not unexpected inhibition of prostate carcinoma metastasis by the highly cytotoxic saporin conjugated to human CDCP1-specific mAb 25A11 (12). In the case of function-blocking mAb 41-2, inhibition of tumor cell metastasis is CDCP1 specific and likely enhances the apoptotic rate of tumor cells during or soon after extravasation. Enhancing the apoptotic rate of tumor cells that reached the bloodstream and arrested in the vasculature seems to constitute an important approach to regulate the outcome of metastatic events because extravasation was recently shown to be rate limiting in the metastatic cascade (27). The contention that mAb 41-2 can enhance apoptosis in CDCP1-positive cells experiencing stress was further validated in vitro under proapoptotic conditions induced by doxorubicin where

FIGURE 5. Kinetic analysis of live HeLa-CDCP1 cells during experimental metastasis. A to D, HeLa-CDCP1 cells were prelabeled with green fluorescent CellTracker and injected iv into chick embryos with control IgG or mAb 41-2. The vasculature was highlighted with rhodamine-conjugated lens culinaris agglutinin. At indicated time points, the nonfixed CAM tissue was visualized in a fluorescent AxioVision microscope. Images were taken at original magnifications of x100 (top, bar, 50 μm) and x400 (bottom, bar, 20 μm) at 10 min (A), 2 h (B), 12 h (C), and 24 h (D) after cell injection. Dotted ovals in A delineate the tips of terminal capillaries with arrested HeLa cells. In C, a group of embryos was inoculated with the cells pretreated with z-VAD-fmk. Arrows in C and D point to fragmented HeLa cells. E, Fragmented cells were counted among all green fluorescent cells in the images taken with 20x objective. White columns, control IgG; gray columns, mAb 41-2; black columns, pretreatment with z-VAD-fmk followed by injection with mAb 41-2. Data are presented as the percentage of fragmented cells from total number of analyzed cells. Columns, mean calculated from up to 30 images pooled from three to five independent experiments per time point each using from three to five embryos per condition; bars, SE. *P < 0.05; **P < 0.01; ***P < 0.005, compared with IgG control for corresponding time point.
mAb 41-2 specifically increased 2 to 2.5 times the percentage of detached apoptotic cells, whereas another mAb, specifically binding to cell surface CD44, did not produce such effect. Conversely, CDCP1 expression was shown to confer HeLa cells with the ability to better withstand apoptosis-inducing conditions. Although proapoptotic conditions were drug induced in this in vitro system, it allowed us to highlight anti-apoptotic properties of CDCP1. Furthermore, these results
supported our suggestion that ligation of CDCP1 with function-blocking mAb 41-2, resulting in the inhibition of experimental metastasis, is linked to events involved in natural apoptosis triggered in vivo by stress during the traumatic extravasation step, as was indicated by our live cell imaging kinetic studies.

The mechanisms of antibody-mediated inhibition of metastasis can involve blocking of functionally active adhesion sites in cell-associated matrices. Thus, anti-laminin single-chain antibody was shown to effectively inhibit establishment and growth of s.c. tumors in mice (28). Therefore, in search of alternative mechanisms involved in the inhibition of tumor cell metastasis by CDCP1 antibody ligation, we investigated whether mAb 41-2 is capable of blocking tumor cell adhesion to cells and ECM proteins. However, our in vitro findings indicated that mAb 41-2–mediated inhibition of tumor cell metastasis apparently did not involve blocking interactions between the cell surface–expressed CDCP1 and putative ligands on endothelial cells and mesenchymal fibroblasts (i.e., the two major cell types that would be encountered by tumor cells during vascular arrest and extravasation). Our data indicate that strong CDCP1–specific receptors are apparently not present on the surface of HeLa-CDCP1 cells, endothelial cells, or fibroblasts. The data also indicate that type I collagen and the components of Matrigel do not present strong lamin-like motifs to cell surface–expressed CDCP1. However, slight but statistically significant inhibition of HeLa-CDCP1 cell adhesion to fibronectin in a short in vitro assay may indicate that ligation of CDCP1 in vivo during much longer time frame of experimental metastasis could be associated with abrogation of ligand binding. This abrogation could be due to diminishment of cell surface CDCP1 due to antibody-induced internalization, which in turn could elicit anoikis-like mechanisms, ultimately leading to cell death at early stages of experimental metastasis. In addition to the established mechanism of antibody-induced apoptosis via binding to death receptors (25), destabilization of actin cytoskeleton also has been implemented as one of the possible mechanisms of antibody-mediated apoptosis (29). Induction of death via a classic apoptotic pathway has been recently shown for mAb SC104 raised against a cell surface antigen expressed in colorectal tumors (30). Interestingly, mAb SC104 showed additive tumor cell killing when used in combination with cytotoxic drugs. Direct apoptosis-inducing abilities have also been ascribed for mAb Pro 1.5 generated by subtractive immunization against prostate cancer cells (24). The precise intracellular mechanisms triggered by ligation of cell surface CDCP1 with mAb 41-2 and ultimately leading to inhibition of experimental metastasis are not yet established but are under active investigation.

Our novel findings implicating CDCP1 in the extravasation step of tumor metastasis were further extended to tumor cells naturally expressing CDCP1 (i.e., PC3-hi/diss cells). Similarly to HeLa-CDCP1 cells, experimental metastasis of PC3-hi/diss cells was efficiently inhibited by mAb 41-2 during a narrow time frame, again indicating the likely functional importance of CDCP1 during tumor cell extravasation. In addition to experimental metastasis in the chick embryo model system, PC3-hi/diss cell forms sizable tumors and spontaneously intra-vasate and disseminate into secondary organs. Therefore, this model system allowed us to address whether ligation of CDCP1 with mAb 41-2 also inhibited natural spontaneous metastasis of tumor cells. Remarkably, one i.v. inoculation of mAb 41-2 dramatically reduced dissemination of PC3-hi/diss cells from primary tumors. In conjunction with the likely role of CDCP1 in extravasation, indicated by our experimental metastasis data, kinetic analysis of spontaneous dissemination by live cell imaging microscopy would allow us in the future to determine whether ligation of CDCP1 by mAb 41-2 also prevents intra-vasation of CDCP1-expressing tumor cells, one of the earliest and possibly rate-limiting steps in the metastatic cascade. By using quantitative and kinetic in vivo analyses, our analytic probing for the function of CDCP1 in cancer progression now provides new insights as to where and when the cell surface CDCP1 functions as a prosurvival molecule in the metastatic cascade.

Materials and Methods
Cell Lines and Culture Conditions
The HeLa and PC-3 cells were purchased from the American Type Culture Collection and maintained in DMEM-10% FCS (D10; HyClone). HeLa cells were transfected using Lipofectamine 2000 with CDCP1 cDNA (2) in the pcDNA3.1-neo vector (Invitrogen Corp.). Several CDCP1–positive clones were combined to generate a HeLa-CDCP1 cell line. Control HeLa-neo cells were generated by transfection of parental cells with the empty vector. HeLa cells stably expressing tyrosine-to-phenylalanine mutant of CDCP1 (CDCP1-Y734F) were generated by transfection with Flag-tagged construct created by site-directed mutagenesis of

FIGURE 6. Effects of CDCP1 expression and mAb 41-2 treatment on functional characteristics of HeLa cells in vitro. A. Homotypic adhesion of HeLa-CDCP1 cells to HeLa-neo or HeLa-CDCP1 cells. Columns, mean of percentage of cells adhered after 2- and 6-h incubation periods; bars, SE. Presented is one of two independent experiments done in triplicate. B and C. Heterotypic adhesion of HeLa-neo and HeLa-CDCP1 cells to human endothelial (B) or chicken fibroblasts (C) in the presence of control IgG or mAb 41-2. Presented is one of five (B) and three (C) independent experiments done in triplicate. Data are percent of HeLa-neo cell adhesion in the presence of control IgG. Columns, mean; bars, SE. **, P < 0.005, compared with neo control; †, P < 0.05, compared with adhesion of HeLa-CDCP1 cells in the presence of control IgG. D to F. Haptotactic adhesion of HeLa-neo and HeLa-CDCP1 cells. Cells were allowed to adhere for 45 min to type I collagen (D), fibronectin (E), or Matrigel (F) in the presence of control IgG or mAb 41-2. Presented is one from four independent experiments done in triplicate. Data are percent of control HeLa-neo cell adhesion in the presence of mouse IgG. Columns, mean; bars, SE. ***, P < 0.0001, compared with HeLa-neo control; †, P < 0.05, compared with adhesion of HeLa-CDCP1 cells in the presence of mouse IgG. G. Chemotactic migration of HeLa-neo and HeLa-CDCP1 cells. Cells were plated in SF-DMEM into Transwell inserts precoated with Matrigel and stimulated to migrate for 48 h toward 5% FCS placed into the outer chamber with control mouse IgG or mAb 41-2. Data are presented as percentage of migration observed with HeLa-neo cells. Columns, mean; bars, SE. Presented is one from three independent experiments done in triplicate. H. HeLa-CDCP1 growth under adhesion-free conditions. Cells were plated into ultrawall adhesive plates in D10 supplemented with mouse IgG or mAb 41-2 and counted at 48 and 72 h. Presented is one from four independent experiments done in triplicate. Columns, mean of total cell numbers per well; bars, SE.
full-length wild-type CDCP1 (CDCP1-WT) using PfuUltra (Stratagene). A high-disseminating variant of the prostate carcinoma PC-3 cell line (PC3-hi/diss) was generated by serial passaging of primary tumors developed on the CAM of chick embryos. Subconfluent cell cultures were briefly treated with trypsin-EDTA, washed with D10 and serum-free DMEM (SF-DMEM), and resuspended in SF-DMEM.

**Antibodies**

The following CDCP1-specific antibodies were used in the study: mAb 41-2, generated by subtractive immunization with a nonmetastatic population versus a metastatic population of epidermoid HEp-3 carcinoma (2); mAb 10-D7, also generated by subtractive immunization but using HeLa-neo and HeLa-CDCP1 cells as tolerogen and immunogen, respectively; and

![Graph showing CDCP1 expression protection from doxorubicin-induced apoptosis](image1)

**FIGURE 7.** mAb 41-2 enhances drug-induced apoptosis of HeLa-CDCP1 cells. A. CDCP1 expression protects HeLa cells from doxorubicin-induced apoptosis. HeLa-neo and HeLa-CDCP1 cells were treated with doxorubicin (+) or corresponding volume of diluent (−). Where indicated, the cells were pretreated for 2 h with z-VAD-fmk (+) before addition of doxorubicin. Data are presented as the number of nonadherent cells representing detached apoptotic cells counted 48 h later. B. Doxorubicin-induced activation of caspase-3. HeLa-CDCP1 cells were treated with doxorubicin for indicated time (h). Both adherent and nonadherent cells were harvested and caspase activation status was analyzed by Western blotting. Left, positions of pro-caspase-3 and activated cleaved caspase-3; right, position of molecular weight markers (kDa). C. Nuclei fragmentation caused by doxorubicin treatment. HeLa-CDCP1 cells were treated with vehicle for 24 h, fixed, and stained with 4′,6-diamidino-2-phenylindole. Bar, 10 μm. D. mAb 41-2 enhanced doxorubicin-induced apoptosis. HeLa-CDCP1 cells were treated with vehicle (−) or doxorubicin (+) in the presence of control IgG or mAb 41-2. Hatched columns, where indicated, HeLa-CDCP1 cells were pretreated with z-VAD-fmk (+) before adding doxorubicin. Following 48-h incubation, nonadherent apoptotic cells from individual wells were counted. Columns, mean from one of four independent experiments done in triplicate; bars, SE. *, P < 0.05, compared with HeLa-neo cells; **, P < 0.05, compared with HeLa-CDCP1 cells incubated in doxorubicin and IgG.
goat antibody ab1377, generated against the COOH-terminal peptide of CDCP1 (Abcam). Murine anti-phosphotyrosine mAb 4G10 and rabbit antibody 9662 recognizing the proform and activated species of caspase-3 were purchased from Cell Signaling.

**Flow Cytometry**

Cells were incubated first with 2 μg/mL of mAb 41-2 or 10-D7 and then with FITC-conjugated goat anti-mouse antibody (Sigma) and analyzed in a flow cytometer (Becton Dickinson).

**Experimental Metastasis in Mice**

Six- to 8-wk-old female immunodeficient SCID or nonobese diabetic SCID mice (TSRI breeding facility) were injected i.v. with 1 × 10^6 HeLa-CDCP1 or HeLa-neo cells. Where indicated, 100 μg of mAb 41-2 or mouse IgG (Jackson ImmunoResearch Laboratories) were inoculated with cell suspensions. Additional antibody injections were done i.p. on days 2 and 3 following cell inoculations. Three weeks (nonobese diabetic SCID) or 4 wk (SCID) later, the mice were sacrificed and internal organs were harvested to determine numbers of human tumor cells.

**Experimental and Spontaneous Metastasis in Chick Embryos**

Experimental metastasis was done in chick embryos developed from fertilized SPAFAS White Leghorn eggs (Charles River). On day 12 of incubation, the embryos were injected i.v. with 5 × 10^4 or 1 × 10^5 cells, as described (31). CDCP1-specific mAbs 41-2 or 10-D7 or control IgG was inoculated i.v. at 50 μg per embryo at indicated time points. On day 5, portions of the CAM were harvested to determine numbers of human tumor cells.

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**FIGURE 8.** Functional analysis of CDCP1 naturally expressed in prostate PC-3 carcinoma variant. A, Flow cytometry analysis of CDCP1 expression in PC3-hi/diss variant. PC3-hi/diss cells were incubated with control IgG (open histogram) or mAb 41-2 (closed histogram). MFI, mean fluorescence intensity. B, Western blot analysis of CDCP1 expression in PC3-hi/diss and HeLa-CDCP1 cells was done by probing SDS-PAGE-separated lysates with goat anti-CDCP1 antibody. Solid and open arrows, 135- and 70-kDa CDCP1 protein bands, respectively. Right, position of molecular weight markers (in kDa). C, Inhibition of PC3-hi/diss experimental metastasis with mAb 41-2. PC3-hi/diss cells were injected i.v. into day 12 chick embryos along with mouse IgG (MoIgG) or mAb 41-2. The following day, a group of embryos received an additional injection of mAb 41-2. Colonization by PC3-hi/diss cells is presented as mean ± SE calculated from pooled data obtained in two independent experiments involving from 6 to 12 embryos per antibody treatment. *, P < 0.05; **, P < 0.005, compared with IgG control. D, Kinetic analysis of the sensitivity of PC3-hi/diss experimental metastasis to mAb 41-2. PC3-hi/diss cells were injected i.v. into chick embryos with no antibody (No Ab) or with mAb 41-2 injected at the indicated time points. Colonization by PC3-hi/diss cells, determined 5 d after cell injection, is presented as numbers of human cells in one of two independent experiments using from 4 to 12 embryos per time point. Columns, mean; bars, SE. P < 0.05 in two-tailed (*) and one-tailed (**) t tests compared with the no antibody control. E, Inhibition of spontaneous metastasis of PC3-hi/diss cells with mAb 41-2. PC3-hi/diss cells were grafted on the CAM of day 10 chick embryos. The following day, the embryos were injected i.v. with mouse IgG (Mo IgG) or mAb 41-2. Columns, mean of tumor weights (top) and numbers of disseminated human cells (bottom) determined 7 d after cell inoculations by qPCR; bars, SE. ***, P < 0.005, compared with control IgG in Mann-Whitney test.
Spontaneous metastasis in chick embryos was done as described (31). On day 10 of incubation, 2 × 10^6 PC3-hi/diss cells were grafted on the CAM. The following day, the embryos were injected i.v. with 50 μg mAb 41-2 or control IgG. On day 7 after cell grafting, the primary tumors were excised and weighed, and portions of the CAM were harvested and analyzed for numbers of human cells.

Quantitation of Human Tumor Cells by Real-time qPCR
Numbers of human cells within murine or chick embryo tissues were determined by qPCR done exactly as described (31) using a standard curve generated by serial dilutions of human tumor cells within a constant number (10^6) of chick embryo or mouse fibroblasts.

Immunohistochemistry
CAM samples were fixed in zinc-formalin and embedded in paraffin. HeLa-CDCP1 cells were visualized by immunostaining with anti-human pan-cytokeratin mAbs (C 2562; Sigma) essentially as described (31). Digital images were captured using Olympus BX60 microscope equipped with a digital DVC video camera and processed with SC3 Adobe Photoshop software (Adobe Systems, Inc.).

Live Cell Imaging
HeLa cells were labeled with 2 μmol/L green CellTracker CMFDA and injected i.v. at 1 × 10^6 per embryo with 50 μg mAb 41-2 or control mouse IgG. Where indicated, HeLa-CDCP1 cells were preincubated for 2 h in 50 μmol/L z-VAD-fmk (R&D Systems). To analyze haptotactic adhesion, HeLa-neo and HeLa-CDCP1 cells were plated with green fluorescent CellTracker CMFDA (Invitrogen) and/or doxorubicin. The cells that had undergone apoptosis in the presence or absence of 5 μmol/L doxorubicin (Sigma).

Cell-Cell Interactions and Cell Adhesion
To analyze homotypic adhesion of HeLa-CDCP1 cells, confluent layers of HeLa-neo or HeLa-CDCP1 cells grown in D10 in 24-well clusters were washed in SF-DMEM and overlaid with 1 × 10^5 HeLa-CDCP1 cells prelabeled with green fluorescent CellTracker CMFDA (Invitrogen). To analyze heterotypic cell adhesion, HeLa-neo and HeLa-CDCP1 cells were preincubated with green fluorescent CellTracker and plated in SF-DMEM at 5 × 10^5 per well onto confluent layers of human lung microvascular endothelial cells (Cambrex) or chicken CAM fibroblasts in the presence of 50 μg/mL of control mouse IgG (Jackson ImmunoResearch Laboratories) or mAb 41-2. Following 2- or 6-h incubation, nonadherent cells were gently washed out and remaining cells were detached with trypsin/EDTA and analyzed in a flow cytometer to determine the percentage of green fluorescent cells among total detached cells.

To analyze haptotactic adhesion, HeLa-neo and HeLa-CDCP1 cells were resuspended in SF-DMEM supplemented with 50 μg/mL of control mouse IgG or mAb 41-2 and plated onto layers of type I collagen, fibronectin, or growth factor–reduced Matrigel, each precoated at 5, 10, and 10 μg/mL, respectively. Following incubation for 45 min, nonadherent cells were washed out and adherent cells were fixed and stained with 0.2% crystal violet solution in 10% ethanol. After washing with PBS, incorporated dye was extracted from the cells with sodium phosphate (100 mmol/L, pH 4.5) in 50% ethanol and absorbance was measured at 560 nm.

For haptotactic migration, Transwell filter inserts of 6.5-mm diameter and 8-μm pore size (Corning) were coated on the underside with type I collagen, fibronectin, or growth factor–reduced Matrigel (Becton Dickinson) in PBS at 5, 10, and 10 μg/mL, respectively, overnight at 4°C. HeLa-neo and HeLa-CDCP1 cells were plated at 1 × 10^6 per Transwell insert and allowed to migrate in SF-DMEM to the precoated surfaces for 24 h. For chemotactic migration, HeLa cells were placed into uncoated Transwell inserts in SF-DMEM and stimulated to migrate for 48 h toward 5% FCS placed into the outer Transwell chamber in the presence of 25 μg/mL of control mouse IgG or mAb 41-2. The transmigrated cells were detached with trypsin/EDTA and counted. Matrigel invasion was done exactly as described for chemotactic migration, but Transwell inserts were precoated each with 2 μg of low growth factor Matrigel.

Growth of HeLa-CDCP1 cells under nonadherent conditions was evaluated 48 and 72 h after plating 5 × 10^6 cells in 1 mL D10 per well of a 12-well ultralow adhesion cluster (Nunc) in the presence of 25 μg/mL mouse IgG or mAb 41-2. Single-cell suspensions were prepared by treatment with trypsin/EDTA, cell viability was determined by trypan blue exclusion.

Induction of Apoptosis In vitro
HeLa cells were seeded at 0.5 × 10^6 per well of a 12-well plate. After overnight incubation, the D10 was changed to SF-DMEM supplemented with indicated antibodies (50 μg/mL) in the presence or absence of 5 μmol/L doxorubicin (Sigma). Where indicated, HeLa-CDCP1 cells were preincubated for 2 h with 50 μmol/L z-VAD-fmk before addition of mAb 41-2 and/or doxorubicin. The cells that had undergone apoptosis detach from the bottom of the plate (13), and therefore, the
percentage of nonadherent cells was used as a measure of apoptosis. The detached apoptotic cells were harvested and counted after 48-h incubation. To confirm apoptotic status of doxorubicin-treated cells, caspase activation was verified by Western blotting and nuclei fragmentation by fluorescent microscopy after staining of nonadherent methanol-fixed cells with 1 μg/ml 4′,6-diamidino-2-phenylindole (Invitrogen).

Data Analysis and Statistics
Data processing and statistical analysis were done using GraphPad Prism (GraphPad Software). All experiments were done at least twice and the numbers of experiments, animals, and samples are indicated in the text or figure legends. Data are presented as mean ± SE calculated from numerical data from a representative or pooled experiments. Where indicated, fold differences were determined from pooled data as the ratios of numerical values for individual embryos over a mean of the control in the corresponding experiment. Student’s two-tailed and one-tailed t tests and Mann-Whitney test were used to determine P value of differences between the means of experimental data sets; P < 0.05 was considered as statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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