

CD24 Expression Causes the Acquisition of Multiple Cellular Properties Associated with Tumor Growth and Metastasis

Petra Baumann,¹ Natascha Cremers,¹ Frans Kroese,² Gertraud Orend,³ Ruth Chiquet-Ehrismann,⁴ Toshi Uede,⁵ Hideo Yagita,⁶ and Jonathan P. Sleeman¹

¹Forschungszentrum Karlsruhe, Institut für Toxikologie und Genetik, Karlsruhe, Germany; ²Department Cell Biology, University of Groningen, Groningen, the Netherlands; ³Institute of Biochemistry and Genetics, University of Basel; ⁴Friedrich Miescher Institute for Biomedical Research, Novartis Forschungsstiftung, Basel, Switzerland; ⁵Division of Molecular Immunology, Institute for Genetic Medicine, Hokkaido University, Kita, Sapporo, Japan; and ⁶Department of Immunology, Juntendo University School of Medicine, Bunkyo, Tokyo, Japan

Abstract

The glycosylphosphatidylinositol-anchored membrane protein CD24 functions as an adhesion molecule for P-selectin and LI and plays a role in B-cell development and neurogenesis. Over the last few years, a large body of literature has also implicated CD24 expression in tumorigenesis and progression. Here, we show that ectopic CD24 expression can be sufficient to promote tumor metastasis in experimental animals. By developing a doxycycline-inducible system for the expression of CD24 in breast cancer cells, we have also analyzed the cellular properties that CD24 expression influences. We found that CD24 expression increased tumor cell proliferation. Furthermore, in addition to promoting binding to P-selectin, CD24 expression also indirectly stimulated cell adhesion to fibronectin, collagens I and IV, and laminin through the activation of $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrin activity. Moreover, CD24 expression supported rapid cell spreading and strongly induced cell motility and invasion. CD24-induced proliferation and motility were integrin independent. Together, these observations implicate CD24 in the regulation of multiple cell properties of direct relevance to tumor growth and metastasis. (Cancer Res 2005; 65(23): 10783-93)

Introduction

CD24, also known as heat-stable antigen in the mouse, is a glycosylphosphatidylinositol-anchored membrane protein of heterogeneous molecular weight ranging from 30 to 70 kDa (1). The mature protein is only 27 to 30 amino acids long, and most of the molecular weight of the protein consists of extensive N- and O-linked glycosylation. CD24 is expressed in cells of the hematopoietic system, such as B-cell precursors and neutrophils, in neuronal tissue, and in certain epithelial cells, such as keratinocytes and renal tubular epithelium.

CD24 is thought to function as an adhesion molecule. It is known to bind to P-selectin, a protein expressed on thrombin-activated platelets and endothelial cells (2), and to LI, a member of the immunoglobulin superfamily that is expressed on neural and lymphoid cells (3, 4). CD24-deficient mice display defects in B-cell development (5) consistent with the reported role for CD24 in cell-cell interactions of B cells (6). CD24-deficient mice also display

increased neurogenesis (7), in agreement with the observation that CD24 inhibits neurite outgrowth (8, 9).

An expanding body of literature points to a role for CD24 in the tumorigenesis and progression of a number of types of cancer. CD24 expression is a prognostic indicator of poor survival in breast cancer (10), non-small cell lung carcinomas (11), and ovarian (12) and prostate tumors (13). Enhanced CD24 expression in comparison to matched nonmalignant tissue has also been reported for a number of other types of cancer, including B-cell lymphoma (14), renal cell carcinoma (15), small cell lung carcinoma (16), nasopharyngeal carcinoma (17), hepatocellular carcinoma (18), Merkel cell carcinoma (19), pancreatic carcinoma (20), and neural tumors (21). Additionally, CD24 has been repeatedly identified in gene expression profiling screens to identify genes whose expression correlates with tumorigenesis and tumor progression (22–26). In cellular and animal assays, CD24 has been reported to support the rolling of tumor cells on endothelial monolayers (27) and to promote tumor cell invasiveness *in vivo* (28).

In previous studies, we isolated CD24 in suppression subtractive hybridization screens to identify genes whose expression is up-regulated in metastatic breast and pancreatic carcinoma cells (23). Here we show that CD24 expression can be sufficient to promote metastasis *in vivo*, and report studies which identify properties that CD24 expression confers on tumor cells. By establishing a system in which we can induce expression of CD24 in mammary carcinoma cells, we show that CD24 expression stimulates tumor cell proliferation, can promote tumor cell binding to P-selectin, fibronectin, and other extracellular matrix components, and also stimulates cell motility and invasion. These properties are highly relevant for tumor growth and progression and suggest that CD24 is a pleiotropic stimulator of these processes.

Materials and Methods

Cell culture, antibodies, and peptides. The rat carcinoma lines IAS and MTLy were cultivated as previously described (23). The anti-rat α_3 integrin antibody was obtained from Chemicon (Temecula, CA). The other anti-rat integrin subunit antibodies (29) and the anti-rat CD24 antibody HIS50 (30) have been described. Anti-focal adhesion kinase (FAK) polyclonal antibodies (C903) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phosphotyrosine antibodies conjugated to horseradish peroxidase (PY20H) were purchased from BD Transduction Laboratories (San Diego, CA). CS-1 and RGD peptides were bought from Bachem (Weil am Rhein, Germany).

Construction of the CD24 expression plasmids. A full-length rat CD24 cDNA corresponding to bases 21 to 320 of Genbank accession no. NM_012752 was amplified by reverse transcription-PCR (RT-PCR) using HF polymerase (Clontech, Palo Alto, CA). The PCR product was cloned into the pTRE2 vector (Clontech) to create the pTRE-CD24 plasmid and into the

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Jonathan P. Sleeman, Forschungszentrum Karlsruhe, Institut für Toxikologie und Genetik, Postfach 3640, 76021 Karlsruhe, Germany. Phone: 49-7247-82-6089; Fax: 49-7247-82-3354; E-mail: sleeman@itg.fzk.de.

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doi:10.1158/0008-5472.CAN-05-0619

pSVT7 vector to create the pSVT7-CD24 expression construct. The sequence of cDNA was verified by sequencing.

Production of IAS cells stably overexpressing CD24. IAS cells were cotransfected with a neomycin resistance plasmid together with either the pSVT7-CD24 expression construct or the empty vector using GenePorter (PeqLab, Erlangen, Germany) as a transfection reagent according to the manufacturer's instructions. Transfectants were selected in medium containing 700 µg/mL neomycin. After selection and isolation of stably transfected clones, the clones were analyzed for CD24 expression using flow cytometry.

Production of CD24-deficient MTLy CD24mut cells. MTLy cells were transfected with a puromycin resistance plasmid using Tfx 50 (Promega, Madison, WI) according to the manufacturer's instructions. Transfectants were selected in medium containing 1.5 µg/mL puromycin. After selection and isolation of stably transfected clones, the clones were analyzed for absence of CD24 expression using flow cytometry.

Production of MTLy cells with doxycycline-inducible CD24. MTLy CD24mut cells were transfected with the pTet-On vector (Clontech) using Gene Porter (PeqLab) as a transfection reagent according to the manufacturer's instructions. Selection of stably transfected clones was achieved using 1.2 mg/mL G418. Clones were then transfected with 1 µg pTRE-luc (Clontech) and cultivated in the presence or absence of 1 µg/mL doxycycline. Clones showing the highest inducibility (MTLy mut tet on cells), as assessed using a standard luciferase assay, were used for further experiments.

To create CD24-inducible MTLy cells, MTLy mut tet on cells were cotransfected with the pTRE-CD24 construct and pTK hygromycin resistance plasmid by using Gene Porter II (PeqLab) according to the manufacturer's instructions. Selection of stably transfected clones was achieved using culture medium containing 0.7 mg/mL hygromycin. Isolated resistant clones were tested for inducible CD24 expression by incubating individual clones in the presence or absence of 1 µg/mL doxycycline for 48 hours. Expression of induced CD24 was measured using the HIS50 antibody and flow cytometry.

RNA preparation and Northern blots. Northern blots using 1% agarose-formaldehyde gels and 5 µg polyadenylated RNA were done as described previously (31). Blots were probed sequentially at high stringency using a rat CD24 probe (23) and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (*PstI* fragment of plasmid pGAPDH; ref. 32).

Flow cytometry. Cells were harvested with 5 mmol/L EDTA/PBS and resuspended in PBS/10% FCS. Primary antibodies were applied at 5 µg/mL. After incubation for 30 minutes, the cells were washed with PBS and incubated for a further 30 minutes with fluorescently labeled secondary antibody. The cells were washed with PBS and analyzed by a Becton Dickinson FACStar Plus Flow Cytometer (Mountain View, CA). As negative controls, cells were stained with either isotype-matched control antibodies or with no primary antibody. No difference in staining was observed between these two controls.

Tumor experiments. All experiments were approved by the local review board. IAS clones (1×10^6 cells per animal) were injected s.c. into groups of BDX rats (8 rats per group). MTLy-derived cell lines (5×10^5 cells per animal) were injected s.c. into the no. 4 mammary fat pad of female F344 rats (eight rats per group). Following injection of the tumor cells, tumor volume was measured periodically using a calibrated caliper. The volume was calculated assuming that the tumor approximated to a sphere ($4/3\pi r^3$), where instead of r^3 , half the value of the diameter values obtained in each of three dimensions were used in the calculations. When a tumor reached the German legal limit of 5 cm in one dimension, the animal was sacrificed and autopsied for metastasis formation.

Proliferation assays. Cells were seeded into a six-well plate at a density of 5×10^4 per well. At various time points afterwards, cells were trypsinized and counted. Alternatively, 5×10^4 cells were seeded in 96-well plates and incubated for 24 hours in the presence or absence of an anti- β_1 integrin antibody (25 µg/mL) or 500 µg/mL each of the RDG and CS-1 peptides. Each sample was done in triplicate. The cells were then labeled for 4 hours with 1 µCi [3 H]thymidine. To analyze the amount of incorporated radioactivity, the cells were trypsinized for 30 minutes and harvested onto a glass fiber filter (Wallac, Gaithersburg, MD) with aid of the Harvester 96

cell harvester (Tomtec, Hamden, CT). The filter-immobilized radioactivity was quantified using MicroBeta TriLux Liquid Scintillation together with a luminescence counter (Wallac).

Adhesion assays. Human fibronectin (Roche, Indianapolis, IN), laminin 5 (Chemicon), laminin (Sigma, St. Louis, MO), collagen type IV (Sigma), and collagen type I from rat tails were dissolved at a concentration of 10 µg/mL. P-selectin (RDI, Flanders, NJ) was dissolved at 1 µg/mL and hyaluronan (Rooster Comb, Sigma) at 1 mg/mL, each in PBS. The solutions were used to coat 24-well culture plates (Greiner, Frickenhausen, Germany) by incubation overnight at 4°C, except in the tenascin C experiments, where 96-well plates were coated similarly. The coated wells were washed twice with PBS and then blocked with 1% bovine serum albumin in PBS for an additional hour. Cells (2×10^5 per well) were transferred to the coated wells and incubated at 37°C for 1 hour. Where required, cells were preincubated with peptides (500 µg/mL) or antibodies (10 µg/mL) in PBS for 30 minutes at 4°C before being transferred to the coated wells. Nonadherent cells were removed by gentle washing with PBS. Adherent cells were fixed in 70% ethanol for 30 minutes at 4°C then stained with crystal violet (0.1 mg/mL) at room temperature for 30 minutes. The stained cells were treated with 10% acetic acid, and the absorbance of the resulting solution was measured at 595 nm. Absorbance readings were converted into cell numbers by reference to a standard curve created from defined numbers of stained cells.

Immunoprecipitation. Cells were lysed in lysis buffer (1% lubrol, 25 mmol/L HEPES, 150 mmol/L NaCl, 10 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L NaVO₄) and centrifuged to remove insoluble material. After preclearing with protein A-agarose, the lysates were immunoprecipitated with 2 µg anti-FAK antibody together with protein A-agarose. Precipitates were washed thrice with lysis buffer, boiled in reducing SDS-PAGE sample buffer, subjected to 10% SDS-PAGE, and then Western blotted. The blot was incubated with anti-PY20H antibody diluted 1:2,500 and analyzed with enhanced chemiluminescence detection reagent (Amersham, Buckinghamshire, United Kingdom). The blot was then stripped for 25 minutes at 55°C in stripping buffer [62.5 mmol/L Tris (pH 6.8), 2% SDS, 0.75% β -mercaptoethanol] and subsequently reprobed with the anti-FAK antibody.

Immunofluorescence. Cells were plated on fibronectin-coated (10 µg/mL) chamber slides (Corning, Inc., Corning, NY). After 24 hours, cells were rinsed with PBS and fixed with paraformaldehyde for 10 minutes. The slides were then blocked with 10% FCS in PBS for an additional 1 hour and then incubated for 24 hours at 4°C with primary antibodies. After washing with PBS, the cells were incubated with rhodamine or fluorescein-conjugated secondary antibody for 30 minutes. The cells were then rinsed in PBS, mounted in Immuno-mount mounting medium (Life Sciences International, Philadelphia, PA), and viewed at room temperature with a Zeiss LSM Axiovert 200 M microscope (C-apochrome objective $\times 63/1.2$ W, Zeiss AIM system, Zeiss LSM software, Jena, Germany).

Spreading assays. Glass chamber slides with single wells (Corning) were coated overnight at 4°C with fibronectin (10 µg/mL) in PBS. The coated chamber slides were washed thrice with PBS and blocked with 1% BSA in PBS for an additional hour. Cells were seeded into the chamber slides for various time points (5×10^5 per chamber). After each time point, the slides were washed twice with PBS, then the adherent cells were fixed with paraformaldehyde for 10 minutes and mounted under glass coverslips using Immuno-mount mounting medium (Life Sciences International).

Monolayer wounding (scratch) assay. Cells (1×10^6) were plated on fibronectin-coated (10 µg/mL overnight, 4°C) or noncoated single-well chamber slides (Corning) in complete medium. Upon confluence, the cell monolayer was wounded with the thin edge of a plastic cell scraper (Renner, Dannstadt, Germany). The cells were then cultivated for a further 24 hours in normal medium in the presence or absence of an anti- β_1 integrin antibody (25 µg/mL) or 500 µg/mL each of the RDG and CS-1 peptides. Photographs of the wound were taken using a Zeiss Axiovert 25 microscope at various time points after monolayer wounding. Where necessary, the cells were given a pulse of bromodeoxyuridine (BrdUrd; 10 µmol/L final concentration) for 2 hours before harvesting. The cells were subsequently stained with anti-BrdUrd antibodies (Roche) according to the supplier's instructions.

Migration assays. Cell migration assays were done using 6.5-mm transwell chambers (8 μ m pore size, Corning). Cells were resuspended in serum-free medium and added to the top well of the transwell chambers (5×10^5 per chamber) that contained serum-free medium in both compartments. After 24 hours of incubation, the cells that had not migrated were removed from the upper face of the filter using cotton swabs, and cells that had migrated to the lower surface of the filters were fixed in 70% ethanol and stained with a 0.1 mg/mL crystal violet solution. Stained cells were treated with 10% acetic acid, and the absorbance of the resulting solution was measured at 595 nm.

Matrigel invasion assays. Matrigel (100 μ L, 2.5 μ g/mL) was added to the upper side of a transwell chamber and was polymerized for 4 hours at 37°C. Cells were harvested in PBS containing 5 mmol/L EDTA and washed thrice with DMEM without phenol red. The cells were then stained with DiI (15 μ L/mL in DMEM minus phenol red; Molecular Probes, Eugene, OR) for 15 minutes at 37°C. Aliquots of 5×10^5 labeled cells in 200 μ L serum-free DMEM without phenol red were added per transwell chamber. Conditioned medium from cultured rat embryonic fibroblasts was used as a chemoattractant in the lower chamber. After incubation for 24 hours, fluorescence in the lower chamber (520/580) was measured using a Fluorostar fluorescence reader (BMG Labtechnologies, Offenburg, Germany). As a reference, 5×10^5 cells stained with DiI were cultivated in parallel cultures in normal 24-well plates for 24 hours. The fluorescence in these well was used as a 100% reference. Wells containing Matrigel, DMEM minus phenol red in the upper compartment, and conditioned medium from CREF in the lower compartment served as a negative control. All experiments were done in triplicate.

Results

CD24 expression promotes metastasis *in vivo*. We have previously shown that CD24 expression is associated with the metastatic phenotype in rat tumor cells using Northern blotting and in a variety of human carcinomas using immunohistochemistry (23).⁷ Studies from other investigators also correlate CD24 expression with tumorigenesis and progression (see Introduction). To determine whether CD24 expression is sufficient to promote metastasis *in vivo*, we stably transfected the poorly metastatic pancreatic carcinoma cell line 1AS with either a CD24 expression construct or with the corresponding empty vector. 1AS cells do not normally express significant amounts of CD24 endogenously (ref. 23; Fig. 1A). Three CD24-positive 1AS clones were selected, as were three control clones. These were injected s.c. into syngeneic BDX rats; tumors were allowed to grow until they reached the German legal limit; then the animals were sacrificed and autopsied. As can be seen in Fig. 1B, CD24 expression significantly increased the number of lung metastases that developed. These data show that CD24 expression can be sufficient to promote tumor metastasis in experimental tumors.

Next, we sought to substantiate these data using another animal tumor model (i.e., MTLyCD24mut, a CD24-deficient subline of the rat mammary carcinoma cell line MTLy). Northern blot analysis revealed that no CD24 expression could be detected in the MTLyCD24mut cell line, whereas abundant CD24 mRNA was present in the parental MTLy cell line (Fig. 2B). CD24 expression was introduced into the MTLyCD24mut cell line under the control of a tetracycline-inducible promoter using the pTRE vector system (33). This inducible system has the advantage that CD24 can be expressed or not expressed in

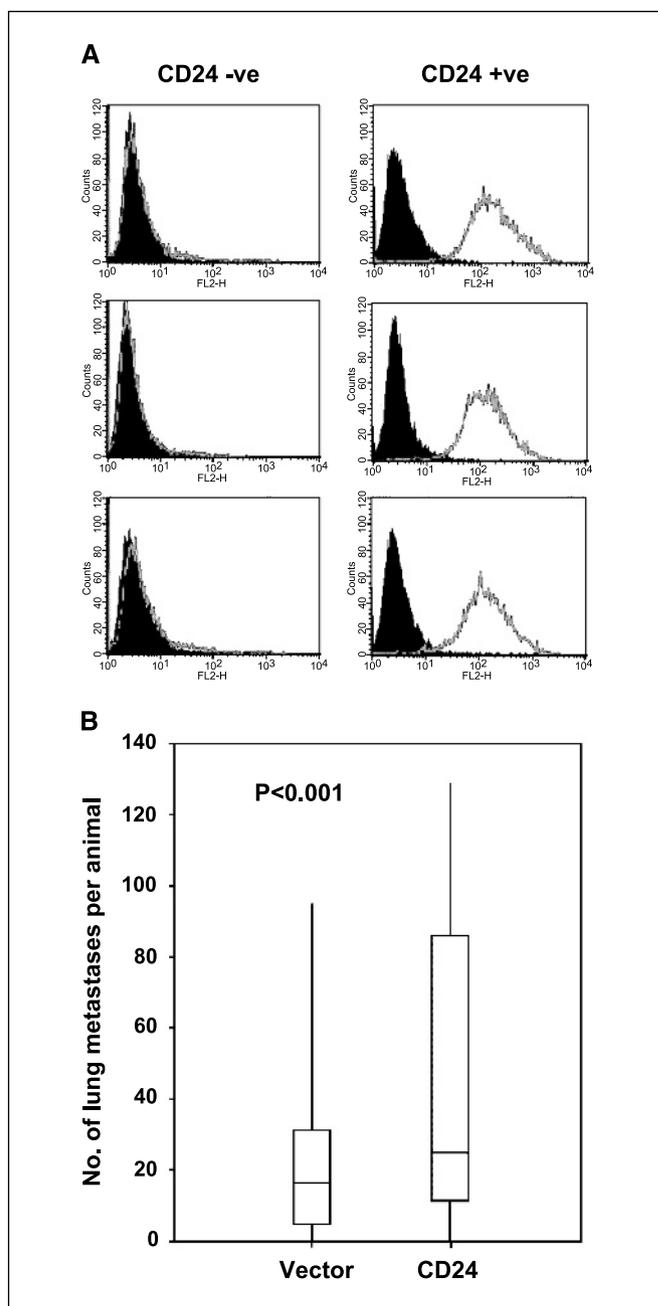


Figure 1. A, FACS analysis of clones of 1AS cells transfected with empty vector (*CD24 -ve*) or with a CD24 expression vector (*CD24 +ve*). The cells were stained with HIS50 anti-CD24 antibodies. *Abscissa*, fluorescence intensity (log scale); *ordinate*, cell number (linear scale). *Filled trace*, background staining; *open trace*, staining with CD24. B, CD24 expression promotes the formation of lung metastases by lung tumors. The CD24-negative 1AS cell clones shown in (A) were injected into syngeneic rats (24 animals), as were the CD24-positive clones. Tumors were allowed to reach 50 mm in one diameter, then the animals were killed and autopsied. The number of lung metastases derived from CD24-negative tumors (*vector*) and CD24-positive tumors (*CD24*) was counted. Box-whisker plots: *boxes*, 75th, 50th, and 25th percentiles; *whiskers*, 95th and 5th percentiles. Statistically compared using the *t* test, which showed that the number of lung metastases in the CD24-positive 1AS cells was significantly higher than in the CD24 negative cells ($P < 0.001$).

exactly the same clonal genetic background, avoiding the possible problems associated with clonal variation.

Several stably transfected MTLyCD24mut cell clones were selected based on their expression of CD24 upon incubation

⁷ Unpublished data.

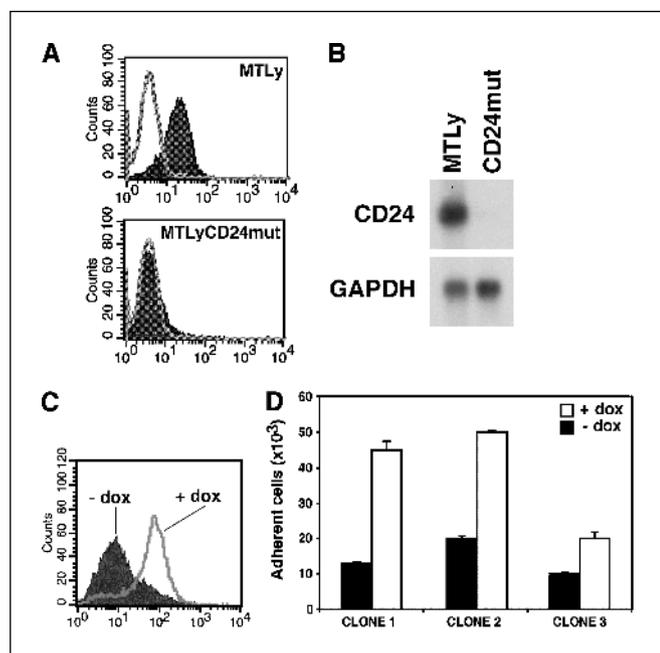


Figure 2. A, FACS analysis showing that CD24 expression is absent from MTLyCD24mut cells. The cells were stained with HIS50 anti-CD24 antibodies. *Abscissa*, fluorescence intensity (log scale); *ordinate*, cell number (linear scale). *Open trace*, background staining; *filled trace*, staining with CD24. B, Northern blot showing that CD24 expression is absent in MTLyCD24mut cells. Polyadenylated RNA was prepared from MTLy cells (MTLy) and MTLyCD24mut cells (CD24mut) and subjected to northern blotting. The blot was hybridized with a CD24 probe. C, induction of CD24 expression in a MTLyCD24ind cell clone. The cells were stained with HIS50 anti-CD24 antibodies. *Abscissa*, fluorescence intensity (log scale); *ordinate*, cell number (linear scale). *Open trace*, CD24 staining after treatment with doxycycline (*dox*); *filled trace*, CD24 staining in the absence of doxycycline induction. D, doxycycline-inducible binding of MTLyCD24ind cells to P-selectin; 24-well plates were coated with P-selectin. Aliquots of cells (2×10^5) were applied to the coated plastic and allowed to adhere for 1 hour. Nonadherent cells were then washed away; and the number of adherent cells was calculated as described in Materials and Methods. *Columns*, means of quadruplicate samples; *bars*, SE.

with doxycycline (e.g., Fig. 2C and Fig. 4B). These cell lines were named MTLyCD24ind. In the absence of CD24 expression, the MTLyCD24mut cells adhered poorly to P-selectin-coated surfaces compared with MTLy cells. However, induction of CD24 expression strongly promoted cell binding to immobilized P-selectin in all inducible MTLyCD24ind cell lines tested (Fig. 2D). These data provide proof of principle that the CD24 protein induced in the MTLyCD24ind cells is biologically active. In this and the following experiments, equivalent results were obtained using the different MTLyCD24ind cell lines; therefore, only a representative experiment is shown with one of the clones.

The MTLy-derived cell lines were tested for their tumorigenicity *in vivo*. When the growth rate of CD24-expressing and nonexpressing tumors growing in syngeneic rats was assessed, the CD24-expressing tumors grew faster than CD24-deficient ones (Fig. 3A). Similarly, when CD24 expression was induced in MTLyCD24ind cells in tissue culture, the cells proliferated at a faster rate than noninduced cells (Fig. 3B). Control experiments using the noninducible MTLyCD24mut cells showed that this was not due to side effects of the doxycycline treatment (data not shown). Together, these data suggest that CD24 expression can promote tumor cell proliferation. Furthermore, we found at

autopsy that four of eight animals with CD24-expressing tumors had developed lung metastases, whereas only one of eight animals with CD24-negative tumors had lung metastases. These data suggest in a further tumor model that CD24 expression can be sufficient to promote metastasis.

Inducible CD24 expression promotes binding to fibronectin and other extracellular matrix components through the activation of integrins. We next set out to characterize further cellular properties influenced by CD24 expression that might be relevant to metastasis. Changed adhesive properties on tumor cells play an important role in tumorigenesis and metastasis. We therefore investigated whether modulation of CD24 expression in MTLy cells changes their ability to bind to a range of extracellular matrix components. Of these extracellular matrix components, binding to fibronectin was dramatically reduced in MTLyCD24mut cells compared with the parental MTLy cells (Fig. 4A). Cell binding to laminin, collagen I, and collagen IV was also reduced. When doxycycline was used to induce CD24 expression in MTLyCD24ind cells, binding of the cells to

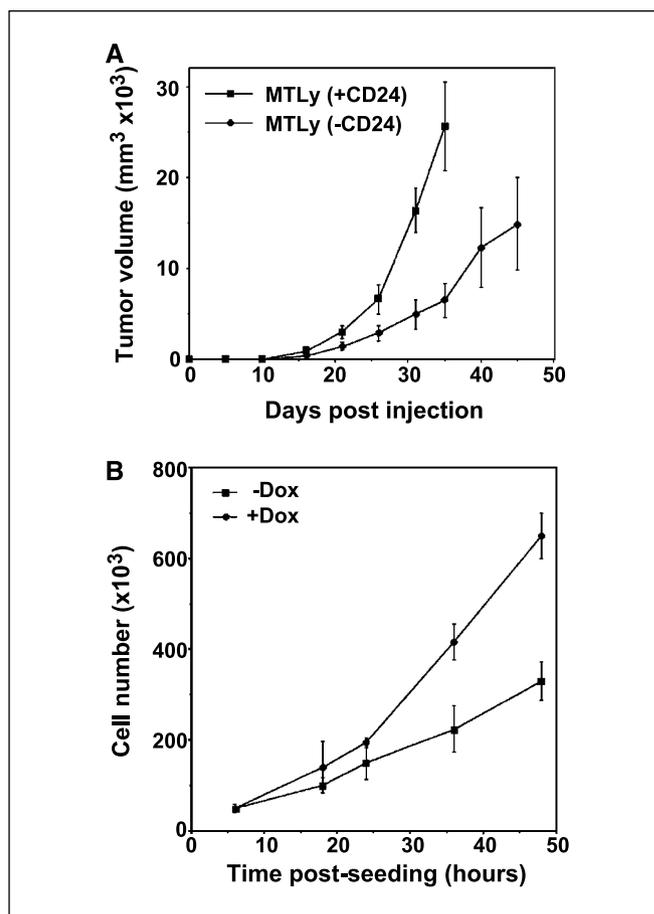


Figure 3. A, growth of CD24-positive (MTLy) and CD24-negative (MTLyCD24mut) tumor cells in experimental animals. Groups of eight animals each were injected with 5×10^5 cells s.c. into the no. 4 mammary fat pads, and tumors were allowed to grow. The size of the tumors in three dimensions was measured at regular intervals using a calibrated caliper. Tumor volume was calculated. *Points*, means of the tumor volume for eight animals; *bars*, SE. B, proliferation in tissue culture of MTLy cells in response to induction of CD24 expression. MTLyCD24ind cells were induced (+Dox) or not induced (-Dox) to express CD24 for 24 hours and plated out in aliquots of 5×10^4 cells in six-well plates. At various time points afterwards, the cells were harvested and counted. *Points*, means of triplicates; *bars*, SE.

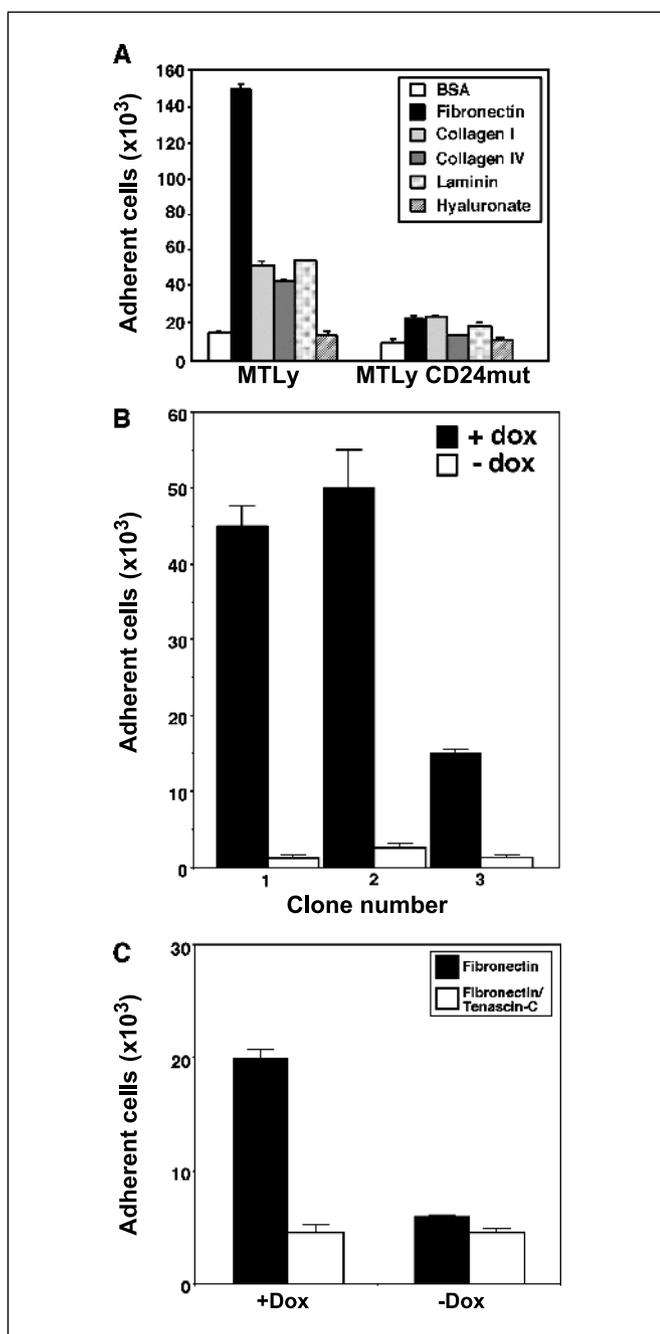


Figure 4. A, binding of MTLy and MTLyCD24mut cells to a panel of extracellular matrix components. Aliquots of cells (2×10^5) were applied to plates coated with the indicated extracellular matrix components and allowed to adhere for 1 hour. Nonadherent cells were washed away, and the number of adherent cells was calculated as described in Materials and Methods. Columns, means of quadruplicate samples; bars, SE. B, doxycycline (*dox*)-inducible binding of MTLyCD24ind cells to fibronectin. Three independent clones of MTLyCD24ind cells were induced (+ *dox*) or not induced (- *dox*) to express CD24. The cells were harvested and aliquots of cells (2×10^5) were applied to fibronectin-coated plastic and allowed to adhere for 1 hour. Nonadherent cells were washed away, and the number of adherent cells was calculated as described in Materials and Methods. Columns, means of quadruplicate samples; bars, SE. C, tenascin C blocks doxycycline-inducible binding of MTLyCD24ind cells to fibronectin. MTLyCD24ind cells were induced (+ *dox*) or not induced (- *dox*) to express CD24. The cells were harvested and aliquots of cells (2×10^5) were applied to either fibronectin-coated or fibronectin/tenascin C-coated 96-well plates and allowed to adhere for 1 hour. Equivalent amounts of fibronectin were used for coating with and without tenascin C. Nonadherent cells were washed away, and the number of adherent cells was calculated as described in Materials and Methods. Columns, means of quadruplicate samples; bars, SE.

fibronectin was strongly promoted (Fig. 4B). Corroborative evidence for a CD24-dependent binding to fibronectin was also obtained in adhesion experiments using tenascin C. Tenascin C binds to fibronectin in an RGD-independent manner and thereby blocks cell attachment to fibronectin (34). Tenascin C was able to block CD24-induced binding to fibronectin (Fig. 4C). Together, these data show that CD24 expression promotes cell attachment to fibronectin.

We next investigated how cell binding to fibronectin is modified by CD24. Antibodies against CD24 were not able to block the CD24-induced binding to fibronectin (Fig. 5). As certain integrins are known to be cell surface receptors for fibronectin, we tested whether CD24 might induce cell binding to fibronectin through integrins. In a first study to identify which integrin α and β subunits might be involved in CD24-mediated binding to fibronectin, we used peptide fragments of fibronectin that are known to block the binding of different integrin subunit combinations to fibronectin (35, 36). These peptides were used in adhesion assays to try and block binding of MTLyCD24ind cells to fibronectin after doxycycline-induced expression of CD24. Two of the peptides used, RGD and CS-1, were able to block CD24-mediated binding to fibronectin (Fig. 5A). The RGD peptide blocks the binding of $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins to fibronectin, whereas the CS-1 peptide blocks the binding of $\alpha_4\beta_1$ integrin to fibronectin (35, 36). However, CD24 expression does not induce cell binding to vitronectin (data not shown), excluding an involvement of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in the binding to fibronectin, as these integrins also bind to vitronectin (36). Together, these data possibly implicated the integrin subunits α_3 , α_4 , α_5 , β_1 , and β_3 in the CD24-dependent binding of MTLyCD24ind cells to fibronectin. To test this possibility, we used blocking antibodies to these integrin subunits and tested whether these antibodies are able to block the CD24-dependent binding of MTLyCD24ind cells to fibronectin. As shown in Fig. 5B and C, the α_3 , α_4 , and β_1 antibodies were able to block binding to fibronectin, whereas the blocking antibodies against the α_5 and β_3 subunits had no effect. These data indicate that $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrins are likely to be involved in the CD24-mediated binding of MTLy cells to fibronectin.

To determine whether CD24 functions by increasing the expression levels of $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrins or rather by activating preexisting integrin subunits, we determined using fluorescence-activated cell sorting (FACS) analysis whether doxycycline-induced CD24 expression alters the levels of the α_3 , α_4 , and β_1 subunits on the cell surface. As can be seen in Fig. 6A, induction of CD24 expression had no effect on the expression level of the α_3 , α_4 , and β_1 subunits, suggesting that CD24 promotes binding to fibronectin by activating preexisting integrin subunits on the cell surface. Furthermore, both FACS analysis (Fig. 6A) and semiquantitative RT-PCR (data not shown) indicated that substantially more α_3 is expressed in these cells compared with α_4 .

Activation of integrin activity leads to phosphorylation of FAK (37). To substantiate the idea that CD24 expression activates preexisting integrin subunits, we analyzed whether CD24 expression has any effect on FAK phosphorylation. As can be seen in Fig. 6B, induction of CD24 expression leads to a substantial phosphorylation of FAK. Furthermore, we also observed that CD24 expression inhibits FAK cleavage (Fig. 6C). Together with the observation that CD24-mediated binding to

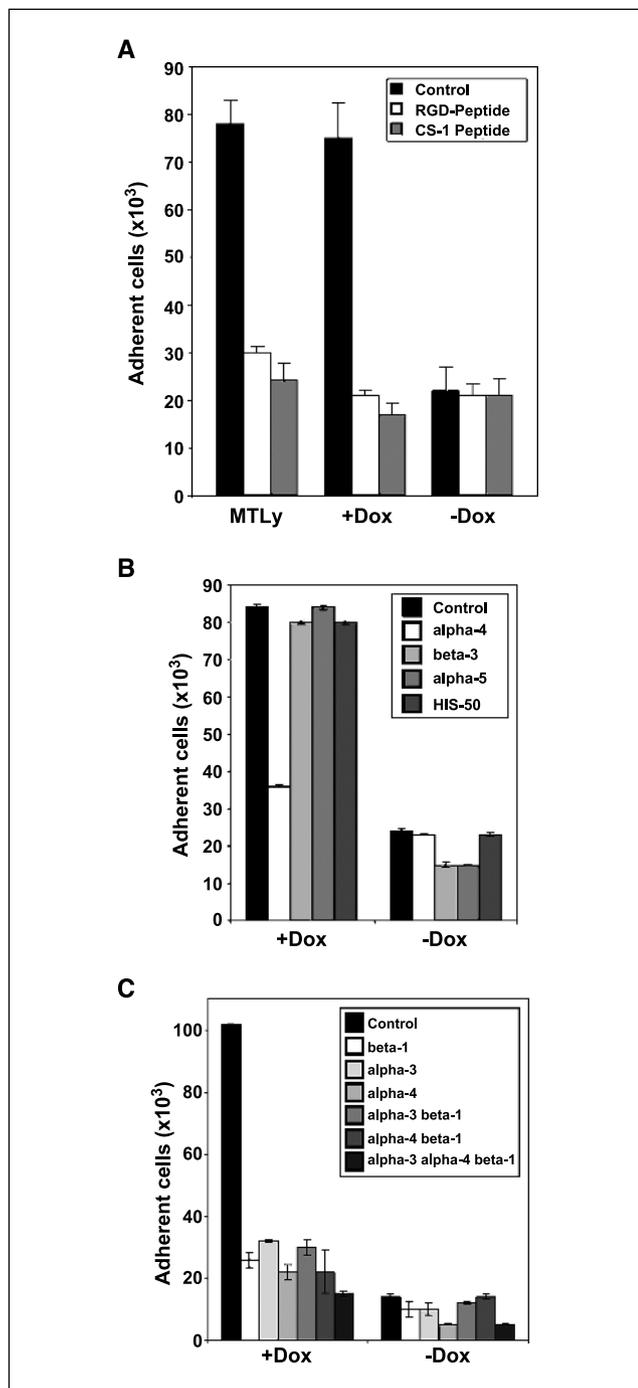


Figure 5. A, blocking of doxycycline (*Dox*)-inducible binding of MTLyCD24ind cells (+ *Dox*, - *Dox*) and parental MTLy cells (MTLy) to fibronectin by RGD and CS-1 peptides. Cells were preincubated with the indicated peptides (500 μ g/mL) in PBS for 30 minutes at 4°C, added to fibronectin-coated plates, and incubated for 60 minutes at 37°C. The number of cells adhering after this time was analyzed as described in Materials and Methods. *Columns*, means of triplicate samples; *bars*, SE. B, blocking of doxycycline-inducible binding of MTLyCD24ind cells to fibronectin by anti-integrin antibodies and anti-CD24 antibody (HIS50). Cells were pretreated with the indicated antibodies (10 μ g/mL) in PBS for 30 minutes at 4°C, added to fibronectin-coated plates, and incubated for 60 minutes at 37°C. The number of cells adhering after this time was analyzed as described in Materials and Methods. *Columns*, means of triplicate samples; *bars*, SE. C, blocking of doxycycline-inducible binding of MTLyCD24ind cells to fibronectin by anti-integrin antibodies. Cells were pretreated with the indicated antibodies (10 μ g/mL) in PBS for 30 minutes at 4°C, added to fibronectin-coated plates, and incubated for 60 minutes at 37°C. The number of cells adhering after this time was analyzed as described in Materials and Methods. *Columns*, means of triplicate samples; *bars*, SE.

fibronectin is magnesium and calcium dependent (data not shown), these data support that notion that CD24 expression activates preexisting integrin subunits.

The data in Fig. 6A suggest that the majority of the CD24-activated fibronectin binding activity might be attributed to the $\alpha_3\beta_1$ integrin. In addition to promoting binding to fibronectin, the $\alpha_3\beta_1$ integrin also binds to other substrates, including collagens I and IV and laminin (38). To provide corroborative evidence that CD24 expression activates $\alpha_3\beta_1$ integrin activity, we therefore did adhesion assays to determine whether doxycycline-induced MTLyCD24ind cells bind better to collagens I and IV and laminin than noninduced cells. This is indeed the case (Fig. 7A), consistent with the results of the adhesion assays in Fig. 4A in which the absence of CD24 reduced MTLy binding to these substrates. Moreover, the enhanced binding to collagens I and IV and laminin in response to the induction of CD24

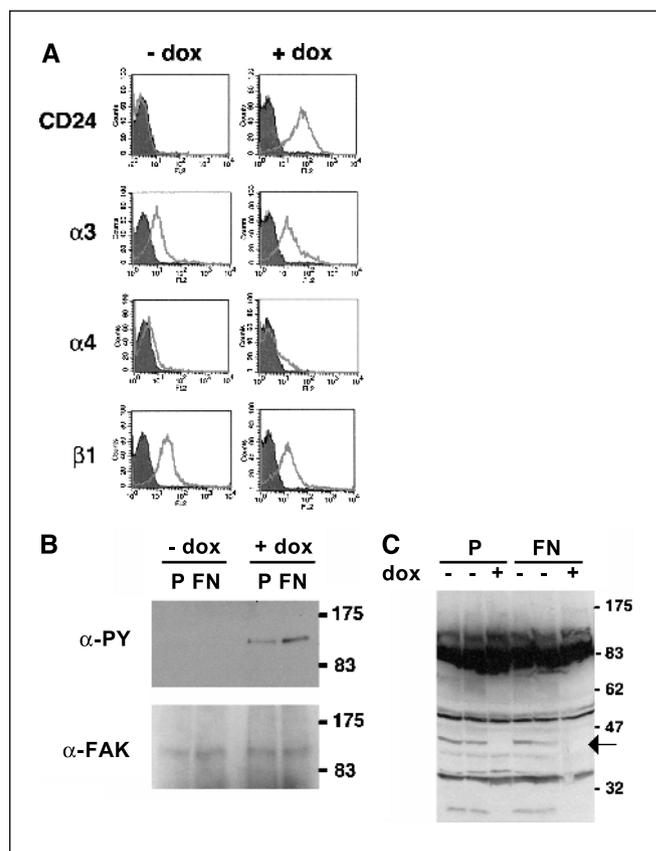


Figure 6. A, FACS analysis showing that doxycycline (*dox*)-inducible expression of CD24 does not influence the expression levels of integrin subunits. MTLyCD24ind cells were induced (+ *dox*) or not induced (- *dox*) to express CD24 and stained with either anti-CD24, anti- α_3 , anti- α_4 , or anti- β_3 antibodies as indicated. *Abscissa*, fluorescence intensity (log scale); *ordinate*, cell number (linear scale). *Open trace*, staining with the antibody (*left*); *filled trace*, staining with CD24. B, MTLyCD24ind cells were grown on plastic (P) or plastic coated with fibronectin (FN) and either treated (+ *dox*) or nontreated with doxycycline (- *dox*) for 24 hours. The cells were then lysed and immunoprecipitated with anti-FAK antibodies. The immunoprecipitates were subsequently Western blotted and probed with anti-phosphotyrosine antibodies (α -PY). The Western blot was then stripped and re-probed with the anti-FAK antibody (α -FAK) for a loading control. Position of the molecular weight markers (kDa). C, MTLyCD24ind cells were grown on plastic (P) or plastic coated with fibronectin (FN) and either treated or nontreated with doxycycline for 24 hours as indicated. The cells were then lysed and subjected to SDS-PAGE/Western blotting. The blot was probed with anti-FAK antibodies. *Arrow*, 42-kDa cleavage product of FAK. Position of the molecular weight markers (kDa).

expression could be partially blocked by anti- α_3 antibodies (Fig. 7A). These data suggest that through activation of integrin, probably in the main $\alpha_3\beta_1$, CD24 expression promotes cellular binding to a range of extracellular matrix components, most prominently to fibronectin, but also to collagens I and IV and laminin.

To determine whether there is a direct molecular interaction between CD24 and integrins, we did confocal microscopy (Fig. 7B). CD24 was stained with Texas red, and the β_1 integrin was stained with fluorescein (*green*). Areas of colocalization are indicated by a yellow color. These data show that although CD24 and the β_1 integrin colocalize in part, there are also areas where β_1 is found alone and also areas where CD24 is found alone. We were also unable to detect a direct interaction between CD24 and β_1 integrins in coimmunoprecipitation assays (data not shown). Together, these data suggest that although CD24 and β_1 integrins may interact focally, a significant proportion of the two proteins are not associated.

Integrin activation has been widely reported to promote cell proliferation (39). Is integrin activity responsible for the enhanced cell proliferation observed in response to CD24 expression? In experiments in which CD24 expression was induced and the cells were concomitantly incubated with anti- β_1 integrin antibodies or with RGD and CS-1 peptides, no effect on the CD24-induced increase in proliferation was observed (Fig. 7C). These data therefore suggest that the CD24-mediated increase in cell proliferation is independent of $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrin activity.

CD24 expression promotes cell spreading. The data above show that expression of CD24 indirectly promotes cellular binding to fibronectin by activating integrins already expressed on the cell surface. To determine what effect this might have on cells, we examined the behavior after plating on fibronectin-coated plastic of MTLyCD24ind cells induced or not induced to express CD24. CD24-expressing cells quickly attached to the substratum, whereas CD24-negative cells attached poorly (Fig. 8A), as expected from the previous adhesion experiments. Importantly, when the adherent cells were quantified for spreading, we found that the CD24-expressing cells also rapidly spread, in sharp contrast to CD24-negative cells (Fig. 8). However, 24 hours after plating, the majority of adherent CD24-negative cells had spread (Fig. 8B). These data suggest that although CD24-negative cells are capable of attachment and spreading to a fibronectin substrate, CD24 expression strongly

promotes the kinetics of cell attachment and spreading. As expected, treatment of the cells with blocking anti- β_1 integrin antibodies or a cocktail of the RGD and CS-1 peptides ablated this effect because the initial attachment of the cells was inhibited (data not shown). We could therefore not directly determine the integrin dependency of the CD24-induced spreading. However, we also observed that CD24 expression promotes spreading of cells on noncoated tissue culture plastic (data not shown), suggesting that this effect is not substrate dependent.

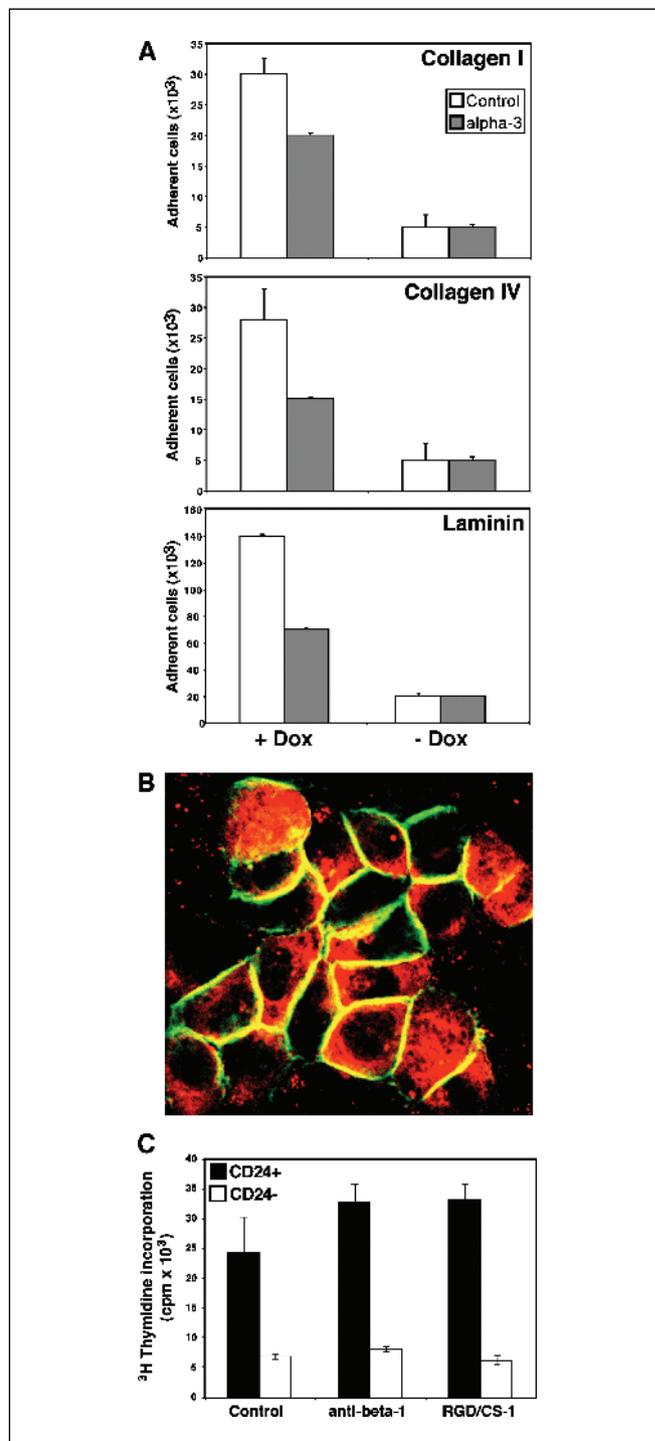


Figure 7. A, binding of MTLyCD24ind cells to collagen I, collagen IV, and laminin is promoted by doxycycline (*Dox*)–induced CD24 expression and blocked by anti- α_3 integrin antibodies. Doxycycline-treated and nontreated cells were preincubated or mock incubated with anti- α_3 integrin antibodies (10 μ g/mL) in PBS for 30 minutes at 4°C. They were then added to 24-well plates coated with collagen I, collagen IV, or laminin and incubated for 60 minutes at 37°C. The number of cells adhering after this time was analyzed as described in Materials and Methods. *Columns*, means of triplicate samples; *bars*, SE. B, colocalization of CD24 and the integrin β_1 subunit assessed by confocal microscopy in MTLy cells. Immunostaining with anti-CD24 antibody (*red*) and immunostaining with the β_1 integrin antibody (*green*). Areas of colocalization (*yellow*). C, enhanced cell proliferation upon CD24 expression is not dependent on integrin activation. MTLyCD24ind cells were induced (*CD24+*) or not induced (*CD24-*) to express CD24 for 24 hours and plated out in the absence (*control*) or presence of 25 μ g/mL blocking anti- β_1 integrin antibody (*anti-beta-1*) or 500 μ g/mL each of RGD and CS-1 peptide (*RGD/CS-1*). After 24 hours, the cells were labelled with [³H]thymidine and harvested, and the incorporated radioactivity counted. *Columns*, means of triplicates; *bars*, SE.

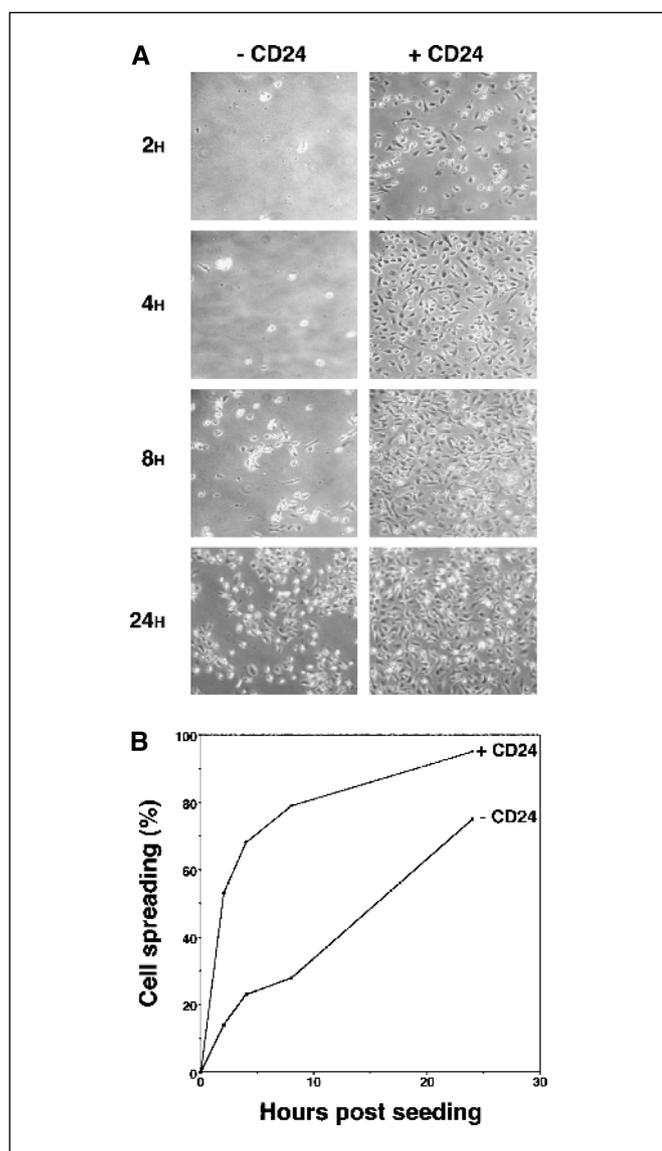


Figure 8. A, attachment and spreading of MTLyCD24ind cells on a fibronectin substrate in response to induction of CD24 expression. CD24-expressing and nonexpressing cells were seeded on fibronectin-coated dishes, fixed, and photographed after 2, 4, 8, and 24 hours as indicated. B, quantitative analysis of spreading of MTLyCD24ind cells on a fibronectin substrate in response to induction of CD24 expression. Cells that adhered to the substrate were scored for being spread or remaining rounded up. The percentage of adherent spread cells is plotted against time after seeding of the cells.

CD24 expression promotes tumor cell migration and invasion. A large body of literature correlates enhanced cell spreading with increased motility (e.g., refs. 40–42), suggesting that CD24 expression might also enhance motility of MTLy breast cancer cells. To determine whether this is the case, we made wounds in monolayers of MTLy cells induced or not induced to express CD24 and monitored the healing of the wounded monolayers. As can be observed in Fig. 9A, CD24-expressing cells closed the wound in the monolayer dramatically faster than non-CD24-expressing cells. This closure cannot be accounted for by the enhanced proliferation of CD24-expressing cells, which based on the growth curves in Fig. 3A could only account for a 2- to 3-fold increase at most in cell numbers at the edge of the

wounded area. Furthermore, when wounded monolayers were pulsed with BrdUrd and stained for BrdUrd incorporation, no difference in incorporation (and therefore proliferation) could be observed between the cells within the wounded area and those in the nonwounded areas (data not shown). These data therefore suggest that CD24 expression enhances breast cancer cell motility. This notion was substantiated by Boyden chamber assays done in the absence of chemotactic or chemokinetic stimuli, which showed that MTLy cells induced to express CD24 are more randomly motile than non-CD24-expressing cells (Fig. 9B). In monolayer wounding assays, neither blocking anti- β_1 integrin antibodies nor a cocktail of the RGD and CS-1 peptides influenced the CD24-dependent increase in cell motility (Fig. 9C). Furthermore, CD24-mediated promotion of monolayer wound closure occurred on both fibronectin-coated and non-coated plastic (Fig. 9A). Together, these data suggest that CD24 expression promotes cell motility and that this motility is not dependent on $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrin activation.

Enhanced cell motility is a prerequisite for tumor invasion, a highly important component of the process of metastasis. We therefore determined whether CD24 expression has any influence on the invasive properties of MTLy tumor cells. In Boyden chamber assays, we found that CD24 expression significantly promoted the ability of the tumor cells to invade through a layer of Matrigel (Fig. 9D). These data therefore suggest that CD24 expression can also promote the invasiveness of tumor cells, which could contribute to the metastasis-promoting activity of CD24.

Discussion

Numerous studies have linked CD24 expression with tumorigenesis and tumor progression. Here, we show that ectopic CD24 expression can be sufficient to promote metastasis *in vivo* and directly influences a number of cellular properties of direct relevance to tumor cell dissemination. CD24 promotes tumor cell proliferation and also changes the adhesive properties of tumor cells (e.g., by promoting their adhesion to P-selectin, fibronectin, collagens I and IV, and laminin). Additionally, cell spreading, motility, and invasiveness are strongly increased upon CD24 expression.

In agreement with previous studies, we show using the CD24-inducible MTLy cells that CD24 expression allows cells to bind to P-selectin. A number of observations suggest that CD24 interacts directly with P-selectin (43, 44). Binding to P-selectin is likely to be highly relevant to the process of tumor metastasis, as tumor cells in the circulatory system that are able to bind to P-selectin have the potential to bind to activated platelets and endothelium. Binding to platelets may potentiate clumping of tumor cells, in turn facilitating enlodgement of the clustered tumor cells in capillary beds. The binding of circulating tumor cells to endothelium is also an important first step in the formation of metastases, as it is a prerequisite for the extravasation of the tumor cells from the circulatory system and subsequent penetration into the underlying tissue. Indeed, others have shown that CD24 expression promotes rolling of breast cancer cells on endothelial monolayers (27) and initiates arrest in the lung of lung adenocarcinoma cells (45). We too found that CD24 expression promoted binding of MTLy cells to P-selectin-expressing endothelial cells (Supplementary Fig. S1). However, we also found that IAS cells bind to P-selectin in the

absence of CD24 expression, and that ectopic CD24 expression does not increase the ability of the cells to bind to P-selectin (Supplementary Fig. S2), suggesting that CD24 expression can promote metastasis by mechanisms other than through binding to P-selectin.

We show here that CD24 can also indirectly alter the adhesive properties of tumor cells by activating integrins. Our data are consistent with the notion that $\alpha_3\beta_1$ is the main integrin that is activated upon CD24 expression in MTLy cells. This integrin can interact with a number of extracellular matrix components, including fibronectin, laminin, and collagens (46). Accordingly, CD24 expression regulates cell binding to these components (Fig. 4 and Fig. 7A). The $\alpha_3\beta_1$ integrin is known to have several functions in tumor cell invasion (38), including the promotion of cell attachment, spreading, and motility through its interaction with extracellular matrix components.

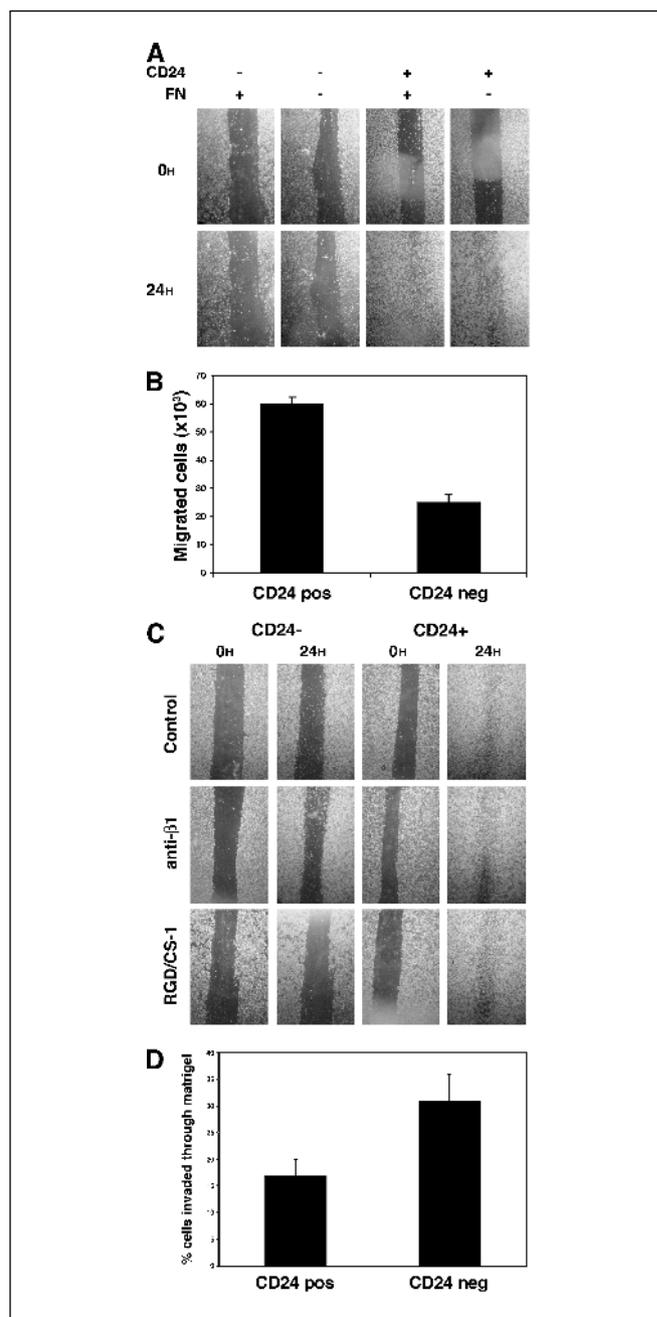
Our data show that when breast cancer cells are induced to express CD24, they also acquire enhanced spreading, motility, and invasive properties. However, enhanced motility is not dependent on the activity of $\alpha_3\beta_1$ or $\alpha_4\beta_1$ integrins (Fig. 9C). These data suggest that CD24 influences motility in two ways, first by promoting adhesion to substrate in an integrin-dependent manner, then by promoting cell movement in an integrin-independent manner. We also note that the promotion of cell proliferation upon CD24 expression is $\alpha_3\beta_1$ and $\alpha_4\beta_1$ integrin independent (Fig. 7C). Together, these data suggest that CD24 expression allows tumor cells to acquire a variety of properties associated with invasion and metastasis, in part through activation of integrin activity and in part by other mechanisms.

Interestingly, CD24 expression in pre-B cells has previously been reported to activate binding of VLA-4, the $\alpha_4\beta_1$ integrin, to fibronectin, and also to alter the binding properties of this integrin to its counter ligand VCAM-1 (47). Here, we show that CD24 has a similar integrin-activating function in breast tumor cells. During tumor progression, cancer cells acquire many properties associated with normal lymphocyte function, which they use for invasion and metastasis (48). The activation of

integrin activity by CD24 is therefore a further example of such a property acquired by cancer cells during tumor progression.

How might CD24 activate integrin activity? Our confocal data indicate that β_1 integrins colocalize focally with CD24 (Fig. 7B), suggesting that there may be a direct interaction between CD24 and β_1 -containing integrins. However, it should be noted that not all available β_1 and CD24 proteins are colocalized, and that we were not able to coimmunoprecipitate CD24 and β_1 . In common with other glycosylphosphatidylinositol-linked proteins, CD24 can also interact with signal transduction machinery and has been shown to interact with the src family members c-fgr, lck, hck, and lyn in different cell lines (49, 50). We have also analyzed the signaling pathways through which CD24 regulates integrin

Figure 9. A, CD24 expression promotes the healing of wounds in monolayers of MTLyCD24ind cells. Monolayers of cells either expressing or not expressing CD24 in response to doxycycline treatment were grown on tissue culture plastic either coated or noncoated with fibronectin (FN). Confluent monolayers were then wounded and photographed immediately (0H) or after 24 hours as indicated. B, CD24 expression promotes motility of MTLyCD24ind cells in Boyden chamber assays. Cells either expressing (CD24 pos) or not expressing CD24 (CD24 neg) in response to doxycycline treatment were introduced into transwell chambers and cultivated in the absence of chemotactic or chemokinetic stimuli. After 24 hours, the number of cells that had migrated across the filter was quantified. Columns, means of triplicate samples; bars, SE. C, enhanced healing of monolayer wounds of MTLyCD24ind cells in response to CD24 expression is not dependent on integrin activation. Monolayers of cells either expressing (CD24+) or not expressing CD24 (CD24-) in response to doxycycline treatment were grown on tissue culture plastic coated with fibronectin. Confluent monolayers were then wounded and photographed immediately (0H) or after 24 hours either in the absence (control) or presence of 25 $\mu\text{g}/\text{mL}$ blocking anti- β_1 integrin antibody (anti- β_1) or 500 $\mu\text{g}/\text{mL}$ each of RGD and CS-1 peptide (RGD/CS-1) as indicated. D, CD24 expression promotes invasion of MTLy cells. CD24 expressing (CD24 pos) and nonexpressing cells (CD24 neg) were labeled with Dil and placed in the upper compartment of a transwell Boyden chamber coated with Matrigel. Invasion of the cells through the Matrigel was analyzed after 24 hours by measuring the fluorescence in the lower chamber. The percentage of input cells that had invaded through the Matrigel was calculated by reference to the fluorescence of defined numbers of Dil-labeled cells. Columns, means of triplicate samples; bars, SE.



activation in MTLy cells.⁸ Our data show that CD24 physically interacts with c-src in the context of lipid rafts. This interaction requires myristylation of c-src and is dependent on intact lipid rafts. The interaction between c-src and CD24 stabilizes the kinase-active form of c-src. The activity of c-src is necessary and sufficient to activate integrin adhesion to extracellular matrix components, such as fibronectin. Interaction of CD24 with signal transduction machinery may also be responsible for the increased proliferation and motility upon CD24 expression we observed in breast cancer cells in tissue culture and *in vivo*. In this regard, it is interesting to note that upon CD24 expression, we observed stabilization and phosphorylation of FAK (Fig. 6), a protein intimately involved with cell proliferation, migration, and adhesion (51). An increasing body of evidence implicates FAK activation in tumor growth and metastasis (39). FAK may therefore be an important mediator of CD24-dependent cellular properties. The further investigation of such mechanisms will be the subject of future studies.

To what extent does CD24 expression always correlate with tumor growth and progression? A wide body of literature implicates CD24 expression in tumorigenesis and progression, in several cases in a prognostically relevant manner (10–26). Thus, although two gene expression profiling studies failed to observe such a correlation (52, 53), the vast majority of literature points towards a critical role for CD24 in tumor growth and progression.

⁸ P. Baumann and J.P. Sleeman, in preparation.

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Al-Hajj et al. (54) have reported that although the bulk of breast cancer cells in a tumor can be CD24 positive, tumor stem cells capable of efficiently initiating tumor formation are CD44 positive and CD24 negative. The study also suggests that CD44-positive, CD24-negative cells can be present in one and the same tumor, and that CD24-negative tumor cells can give rise to CD24-positive cells. Our data show that upon CD24 expression, tumor cells can acquire a number of properties that have the potential to contribute to tumor growth and metastasis. Furthermore, CD24 expression in tumors has been established as an indicator of tumor progression and poor prognosis in several tumor types (e.g., refs. 10–13). Together, these data suggest that although CD44-positive, CD24-negative tumor cells may be highly efficient in initiating tumors in experimental settings, they are unlikely to be the tumor cell population responsible for tumor invasion and metastasis. Rather, the tumor growth and metastasis-promoting properties endowed on cells through CD24 expression mean that CD24-positive tumor cells are likely to be those involved in tumor dissemination.

Acknowledgments

Received 2/22/2005; revised 7/31/2005; accepted 9/21/2005.

Grant support: Deutsche Forschungsgemeinschaft grant SL 37/2-1 and European Union grant FP6 STREP project BRECOSM (contract no. LSHC-CT-2004-503224) (J.P. Sleeman).

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We thank Anja Steffen for excellent technical assistance and Margaret Frame for helpful discussions.

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CD24 Expression Causes the Acquisition of Multiple Cellular Properties Associated with Tumor Growth and Metastasis

Petra Baumann, Natascha Cremers, Frans Kroese, et al.

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