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RESEARCH ARTICLE

CD24 interacts with and promotes the activity of c-src within lipid rafts in breast cancer cells, thereby increasing integrin-dependent adhesion

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Abstract Expression of the glycosylphosphatidylinositol-anchored membrane protein CD24 correlates with a poor prognosis for many human cancers, and in experimental tumors can promote metastasis. However, the mechanism by which CD24 contributes to tumor progression remains unclear. Here we report that in MTLy breast cancer cells CD24 interacts with and augments the kinase activity of c-src, a protein strongly implicated in promoting invasion and metastasis. This occurs within and is dependent upon intact lipid rafts. CD24-augmented c-src kinase activity increased formation of focal adhesion complexes, accelerated phosphorylation of FAK and paxillin and consequently enhanced integrin-mediated adhesion. Loss and gain of function approaches showed that c-src activity is necessary and sufficient to mediate the effects of CD24 on integrin-dependent adhesion and cell spreading, as well as on invasion. Together these results indicate that c-src is a CD24-activated mediator that promotes integrin-mediated adhesion and invasion, and suggest a mechanism by which CD24 might contribute to tumor progression through stimulating the activity of c-src or another member of the Src family.

Keywords CD24 · Heat stable antigen · Integrin · Motility · C-src

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Introduction

CD24, also known as heat stable antigen in the mouse, is a membrane protein bound to the glycosylphosphatidylinositol (GPI) anchor with a variable molecular weight in the range 30–70 kDa (reviewed in reference [1]). The mature protein is only 27–30 amino acids long, and most of the molecular weight of the protein is comprised 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 can 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 L1, 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 of 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]. We have also recently demonstrated a role for CD24 in regulating branching morphogenesis in the developing mammary gland [10].

An expanding body of literature points to a role for CD24 in the tumorigenesis and progression of a number of types of cancer, and it is a prognostic indicator of poor survival in several tumor types [11]. Additionally, CD24 has been repeatedly identified in gene expression profiling screens as a gene whose expression correlates with tumorigenesis and tumor progression [12–16]. Furthermore, CD24 is increasingly used as a marker of both normal and cancer stem cells [17].

How CD24 contributes to tumor progression remains poorly understood. CD24 has been reported to support the rolling of tumor cells on endothelial monolayers [2], and to promote tumor cell invasiveness in vivo [18]. CD24 can also regulate the activity of CXCR4 in breast tumor cells [19]. We have previously shown that CD24 expression promotes lung metastasis formation in rodent tumor models [20]. Using a doxycycline-inducible system to express CD24, we found that CD24 expression also stimulates 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 supports rapid cell spreading and strongly induces cell motility. CD24 also induces cell proliferation, but this is integrin-independent.

The role of the Src family kinase (SFK) c-src in invasion and metastasis has been extensively documented [21]. In non-transformed cells, c-src kinase activity is tightly controlled, but in many types of tumor, enhanced c-src kinase is present and is intimately related to a poor prognosis [22]. Here we report that in the context of lipid rafts CD24 expression stimulates c-src kinase activity in MTLy breast tumor cells. Our results indicate that CD24 can induce c-src signaling that increases the formation of focal adhesion complexes, integrin-dependent adhesion and cell spreading, and tumor cell invasion.

Materials and methods

Cell culture

The rat carcinoma cell line MTLy, the CD24-negative subline (CD24mut) and their doxycycline-inducible derivatives that conditionally express CD24 (MTLyCD24ind) have been described previously [20]. RKO and HCT116 human colorectal cancer cells were obtained from ATCC. The dominant-negative CA10-SrcMF (K295 M, Y527F) and constitutively active CA10-SrcY527F chicken-src constructs have been previously described [23]. MTLy cells were cotransfected with a hygromycin resistance plasmid together with either src or the corresponding empty vector using GenePorter (PeqLab) as a transfection reagent according to the manufacturer's instructions. Transfectants were selected in medium containing 800 μg/ml hygromycin. After selection and isolation of stably transfected clones, the clones were analyzed for src expression using western blot analysis. The cells were treated with 1 mM methyl-β-cyclodextrin (MCD) at room temperature for 5 min. For PP2 treatment, cells were seeded in serum-free medium overnight, followed by the addition of 10 μm PP2 for 3 h at 37°C unless otherwise stated. Genistein was used at a final concentration of 20 mM.

Antibodies and other reagents

The anti-$\beta 1$ integrin antibody (HUTS) was obtained from Chemicon. The other anti-rat integrin subunit antibodies and the anti-rat CD24 antibody HIS50 have been described previously [20]. The anti-human CD24 antibody SWA-11 was a kind gift from Prof. Peter Altevogt. Anti-FAK polyclonal antibodies (C903), the β-actin rabbit polyclonal antibody and the anti-c-src (SRC-2) polyclonal antibody were obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine antibodies conjugated to horseradish peroxidase (HRP) (PY20H) and the anti-paxillin monoclonal antibodies were purchased from BD Transduction Laboratories. The anti-c-src monoclonal antibody (clone 327), PP2, and genistein were from Merck (Darmstadt). Anti-vinculin monoclonal antibody, MCD and rabbit muscle enolase were obtained from Sigma. Anti-chicken-src monoclonal antibody (EC10) was obtained from Upstate Biotechnology. Anti-human c-src rabbit polyclonal antibodies were obtained from Cell Signaling Technology. Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 546 were obtained from Molecular Probes (Leiden, Netherlands). The full-length coding sequence of human CD24 was PCR-amplified and cloned into pcDNA3.1 using standard techniques to create an expression vector for CD24. The sequence was verified by sequencing on both strands. Non-silencing siRNA and c-src siRNA were obtained from Applied Biosystems. The siRNA against c-src had the following sequences: *sense* GCCUCUCAGUGUCUGACUUtt; *antisense* AAGUCA GACACUGAGAGGCag. Cholera toxin B (CTB) subunit conjugated to Alexa Fluor 647 was obtained from Invitrogen/Molecular Probes.

Adhesion assays

Fibronectin-coated culture plates were used for adhesion assays as previously described [20]. Where required, cells

were preincubated with PP2 or MCD before being transferred to the coated wells. Adherent cells were fixed in 70% ethanol for 30 min at 4°C then stained with crystal violet (0.1 mg/ml) for 30 min. 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 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 10 mM $NaVO_4$) and centrifuged to remove insoluble material. After preclearing with protein-A-agarose or with protein-L-agarose the lysates were immunoprecipitated with 2 μg anti-FAK antibody, 1 μg anti-paxillin antibody or 1 μg anti-src antibody together with protein-A-agarose, or with 10 μg anti-CD24 antibody together with protein-L-agarose. Precipitates were washed three times with lysis buffer and western blotted. The blot was incubated with anti-PY20H antibody and analyzed with electrochemiluminescence (ECL) detection reagent (Amersham). Where required, the blot was stripped and reprobed.

Immunofluorescence

Cells were plated on fibronectin-coated chamber slides (Corning). Where required cells were treated with different chemicals as indicated. After 24 h cells were rinsed with phosphate-buffered saline (PBS), fixed with paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% NP-40 for 10 min. The slides were then blocked with 10% fetal calf serum in PBS and incubated with primary antibodies for 24 h at 4°C. After washing with PBS, the cells were incubated with Alexa Fluor 546 or Alexa Fluor 488 secondary antibody for 30 min. The cells were then rinsed in PBS, mounted in Immuno Mount mounting medium (Life Sciences International), then imaged at room temperature with a Zeiss LSM Axiovert 200 M microscope (C-apochrome objective ×63/1.2 W, Zeiss AIM system, Zeiss LSM software). For costaining of CD24 and GM1, cells were incubated with HIS50 and CTB/Alexa Fluor 647 (both at 5 μg/ml in PBS/10% goat serum) for 1 h on ice. Cells were then washed and fixed with 4% PFA on ice for 5 min, and then incubated for 30 min with goat anti-moue antibodies coupled to Alexa Fluor 546. After further washing, the preparation was mounted and viewed with a Leica SPE/Leica DMI 400B confocal microscope using LAS AF software.

Detergent-insoluble raft preparation

Cells were lysed in ice-cold lysis buffer consisting of 1% Lubrol, 25 mM MES, 150 mM NaCl, pH 6.5, 1 mM PMSF and 10 mM $NaVO_4$ for 30 min on ice and then centrifuged for 10 min at 4°C and 3,500 rpm. The supernatant was mixed with an equal volume of 80% sucrose in MBS (25 mM MES, 150 mM NaCl pH 6.5) and a step gradient was prepared by overlaying with 35% sucrose in MBS, with 25% sucrose in MBS and a final layer of 5% sucrose in MBS. The gradient was centrifuged for 20–22 h at 200,000 *g* using a Beckmann SW50 rotor. Fractions of 0.4 ml were collected from the top of the gradient and either immunoprecipitated with appropriate antibodies as required, or lyophilized overnight for subsequent western blotting analysis. To detect ganglioside GM1, 1 μl of each fraction was spotted onto nitrocellulose, then probed for 1 h at room temperature with CTB coupled to HRP (Invitrogen) diluted 1:5,000, followed by ECL detection.

Evaluation of $pp60^{c\text{-}src}$ kinase activity

Cells were lysed in lysis buffer containing 1% Lubrol, 25 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF and 10 mM $NaVO_4$ for 30 min at 4°C. After centrifugation (10 min, 4°C, 3,500 rpm) supernatants were precleared for 30 min with protein-A-agarose and then incubated for 2 h with 1 μg anti-Src monoclonal antibody (clone 327) preadsorbed on protein-A-agarose beads. Bound material was extensively washed with lysis buffer without Lubrol, then with water before incubation in 45 μl kinase buffer (10 mM Tris, pH7.4, 7 mM $MgCl_2$, 11.4 mM $MnCl_2$, 10 mM ATP, 10 μCi γ-^{32}P-ATP) and 5 μl of acid-denatured enolase (4 μg). Incubations were carried out at 30°C for 10 min. SDS-containing sample buffer was added before SDS-PAGE and autoradiography.

Transwell migration/invasion assays

For MTLy cells, these assays were performed exactly as described previously [20]. RKO and HCT116 human colorectal cancer cells were transfected with either 4 μg of pcDNA3.1 vector control or pcDNA3.1-CD24 expression, together with either non-silencing siRNA or c-src siRNA (both at 50 nm/ml) using Lipofectamine 2000. After 48 h, the cells were harvested, then 2×10^5 cells were plated onto 6.5 mm Transwell chambers (8 mm pore size, Corning) that had been coated with 10 μg Matrigel/well in serum-free medium containing 0.1% bovine serum albumin. Medium containing 10% fetal bovine serum in the lower chamber served as a chemoattractant. After 16 h, non-invaded cells were removed with cotton swabs. Invaded cells were trypsinized and counted using the ATP luminescence-based

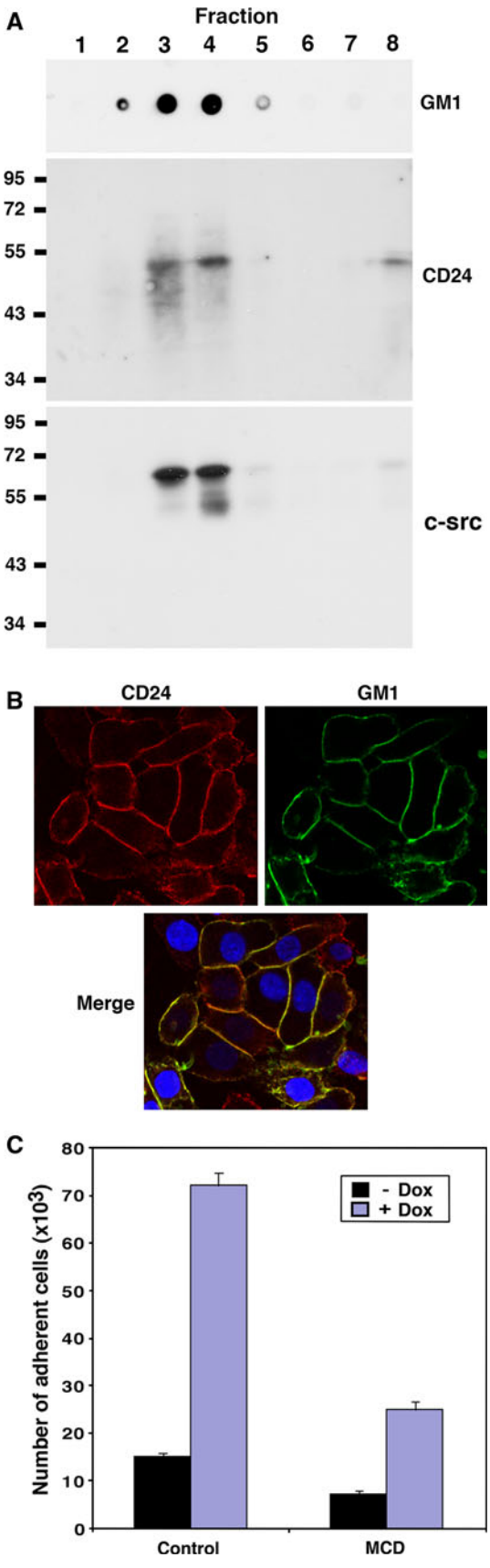


Fig. 1 **a** CD24 is associated with detergent-insoluble lipid rafts. MTLy cells were lysed in lubrol and the cell lysate was separated in a sucrose density gradient. The upper eight fractions containing the lipid rafts and the 35% sucrose buffer between the lipid raft and the non-lipid raft portions of the gradient were analyzed. Portions of the fractions were either spotted onto a nitrocellulose membrane and probed with CTB-HRP to detect GM1 lipid (*GM1*) as a marker of lipid rafts, or western blotted and probed with either anti-CD24 or anti-c-src antibodies. Molecular weight markers are indicated. **b** Coimmunofluorescent staining of MTLy cells for CD24 and GM1 demonstrates localization of CD24 in lipid rafts. Cells were stained with HIS50 (*CD24*), CTB/Alexa Fluor 647 (*GM1*) and DAPI (*blue* nuclear stain). *Bar* 25 µm. **c** Doxycycline-inducible binding of MTLyCD24ind cells to fibronectin is strongly reduced by MCD treatment. Aliquots of cells (2×10^5) treated (*MCD*) with MCD or non-treated (*Control*) were applied to fibronectin-coated plastic and allowed to adhere for 1 h. Non-adherent cells were then washed away and the number of adherent cells was calculated as described in "Materials and methods". The means and standard errors of one representative experiment performed in triplicate are presented

motility invasion assay (Promega). The percentage of invaded relative to control cells transfected with empty pcDNA3.1 vector and non-silencing siRNA was calculated. Statistical significance was calculated using Student's *t* test.

Other methods

Flow cytometry using cells harvested with 5 mM EDTA/PBS was performed exactly as previously described [20], as were the tritiated thymidine incorporation assays to assess cell proliferation, and the cell spreading assays performed on fibronectin-coated surfaces.

Results

CD24-induced binding to fibronectin is dependent on lipid rafts

GPI-linked proteins including CD24 are often associated with cholesterol-rich lipid raft microdomains in the plasma membrane [24, 25]. To confirm that CD24 is associated with lipid rafts, we lysed MTLy breast cancer cells and separated the detergent-insoluble protein lipid fraction from the solubilized proteins using sucrose density gradient centrifugation. As can be seen in Fig. 1a, CD24 was associated with detergent-insoluble lipid rafts. Fractions containing lipid rafts were identified using CTB to detect the lipid raft marker GM1 ganglioside. Consistently, CD24 and GM1 show substantial colocalisation on the surface of MTLy cells (Fig. 1b).

Using doxycycline-inducible CD24 expression in MTLy cells, we have previously shown that CD24 promotes the binding of pre-existing $\alpha 3\beta 1$ and $\alpha 4\beta 1$ integrins on the surface of the cells to fibronectin [20]. To investigate whether intact lipid rafts are required for the CD24-promoted

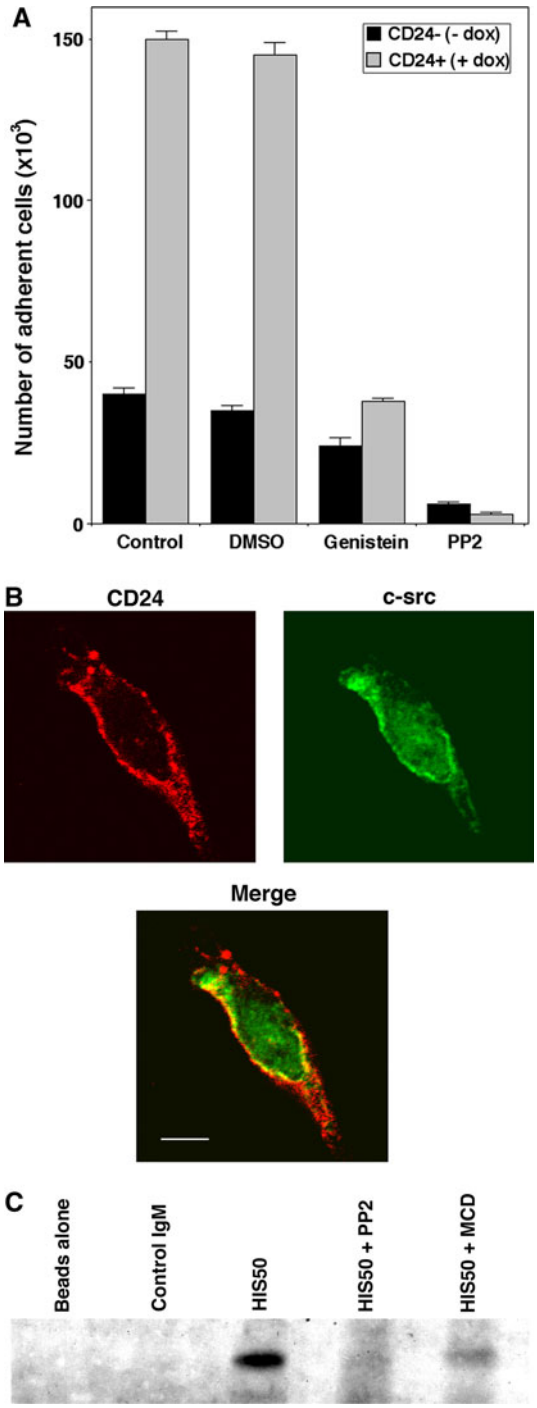


Fig. 2 **a** Blocking of doxycycline-inducible binding of MTLyC-D24ind cells to fibronectin by PP2 (10 μM) and genistein (20 μM). Cells were induced with doxycycline or not induced to express CD24, then treated with the indicated inhibitors for 7 h, transferred to fibronectin-coated plates, and incubated for 60 min at 37°C. The numbers of cells adhering after this time were determined as described in "Materials and methods". The mean and standard error of one representative experiment performed in triplicate is presented. **b** CD24 and c-src partially colocalize in MTLy cells, as assessed by confocal microscopy. Immunostaining with anti-CD24 antibody (*red*) and immunostaining with anti-c-src antibody (*green*) was performed as described in "Materials and methods". Areas of colocalization are indicated by a yellow immunofluorescence. *Bar* 10 μm. **c** Coimmunoprecipitation demonstrates that CD24 and c-src interact. CD24-expressing MTLy were lysed with lubrol lysis buffer and then immunoprecipitated with anti-CD24 antibodies or with IgM as a control. Mock immunoprecipitation with protein-L beads alone served as a further negative control. Samples of cells were also pretreated with either PP2 or MCD as described in "Materials and methods" before immunoprecipitation. The immunoprecipitates were subsequently Western blotted and probed with anti-c-src antibodies. A representative example of three independent experiments is shown

binding to fibronectin, we disrupted lipid rafts on the surface of MTLy cells using MCD, a chemical that withdraws cholesterol from membranes and disrupts lipid raft integrity [26–28]. Treatment of the cells for 5 min with MCD significantly reduced the binding to fibronectin promoted by doxycycline-induced CD24 expression (Fig. 1c). These treatments did not reduce the expression levels of CD24, nor the amount of CD24 on the cell surface (Supplementary Fig. 1). Together these results suggest that disruption of lipid raft integrity strongly reduces the ability of CD24 to activate integrin-mediated binding to fibronectin.

CD24 interacts with c-src and activates its kinase activity

To investigate the molecular pathways by which CD24 promotes integrin binding to fibronectin, we treated MTLy cells with a panel of inhibitors that block various signal transduction components, then examined whether the inhibitors blocked the ability of induced CD24 expression to promote cell binding to fibronectin. Of these, genistein, a non-specific inhibitor of tyrosine kinases, and PP2, an inhibitor of the src family of tyrosine kinases both blocked the CD24-induced binding to fibronectin (Fig. 2a). The findings suggested that src family members might be involved in the CD24-mediated activation of integrins and cell binding to fibronectin. Indeed, CD24 has been reported to interact with Fgr, Lyn, Lck and Hck, members of the src family [29, 30]. In initial experiments, we found evidence for an interaction between c-src and CD24 using confocal immunofluorescence microscopy, which showed that CD24 and c-src partially colocalize (Fig. 2b). Consistently, c-src was also found in lipid rafts (Fig. 1a). We therefore next investigated whether CD24 and c-src associate in a common complex using immunoprecipitation, which showed that CD24 and c-src coprecipitate (Fig. 2b). Significantly, this interaction could be abrogated by pretreatment of the cells with MCD (Fig. 2c), suggesting that intact lipid rafts are required for the interaction.

We next investigated the effect of CD24 expression on c-src expression and activity. While CD24 expression does not substantially change the expression levels of c-src (Fig. 3a), significantly increased c-src activity was observed that could be blocked by PP2 treatment (Fig. 3b).

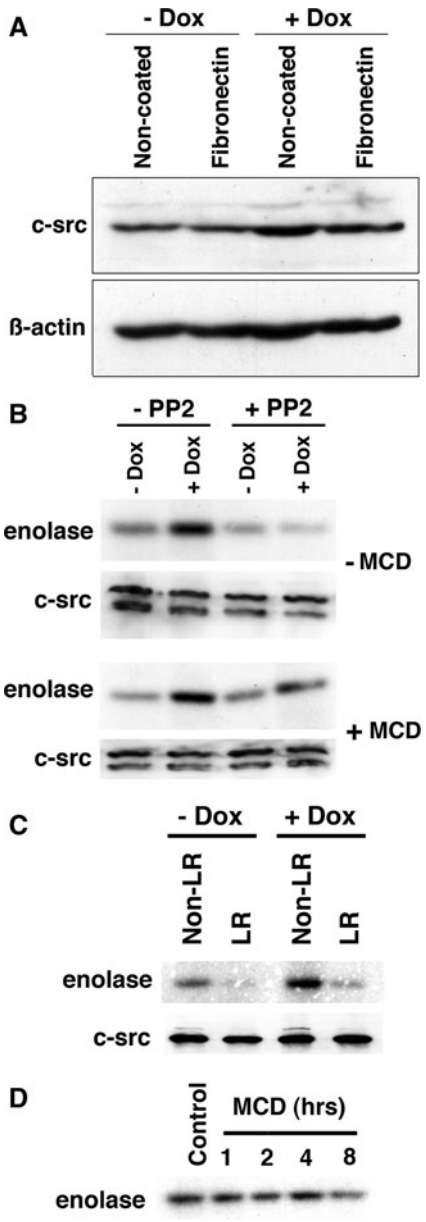


Fig. 3 **a** Expression of CD24 does not influence the expression of c-src. MTLyCD24ind cells were grown on plastic (non-coated) or on plastic coated with fibronectin and either treated or not treated with doxycycline for 24 h as indicated. The cells were then lysed and subjected to SDS-PAGE/western blotting. The blot was probed with anti-c-src antibodies, then stripped and reprobed with anti-β-actin antibodies for a loading control. **b** Expression of CD24 increases c-src kinase activity. MTLyCD24ind cells were not treated (− Dox) or treated with doxycycline to induce CD24 expression (+ Dox). Induced and non-induced cells were then not treated or treated with PP2 as indicated. Induced and non-induced cells were also treated or not treated with MCD as indicated for 5 min immediately before lysis. Cells were lysed and immunoprecipitated with anti-c-src antibody (Mab 327). The immune complexes were then divided. One part was used in an enolase kinase assay to assess c-src kinase activity. Samples were analyzed by 10% SDS-PAGE and autoradiography. The amount of incorporation of [γ-^{32}P]ATP into the enolase (enolase) indicates c-src kinase activity. The other portion of the immunoprecipitate was immunoblotted and probed with anti-c-src antibodies as a loading control (c-src). **c** Expression of c-src and active c-src in non-lipid raft and lipid raft fractions. MTLyCD24ind cells were non-treated (− Dox) or treated with doxycycline to induce CD24 expression (+ Dox). Lysates were separated in a sucrose density gradient. From each gradient, pooled lipid raft fractions (*LR*) and non lipid raft fractions (*Non-LR*) were immunoprecipitated with anti-c-src antibodies. Immune complexes were divided in two parts, one of which subjected to a kinase assay reaction and the other to SDS-PAGE/western blotting. For the kinase reaction the immune complexes were incubated at 30°C for 10 min with [γ-^{32}P]ATP, loaded onto a 10% SDS-PAGE gel and analyzed by autoradiography. Expression of c-src was detected by probing the western blot with an anti-c-src antibody. **d** Effect of MCD on c-src kinase activity. CD24-expressing MTLy cells were not treated (*Control*) or treated for the indicated times with MCD. Cells were then lysed and immunoprecipitated with anti-c-src antibody (Mab 327). Immune complexes were incubated with enolase at 30°C for 10 min together with [γ-^{32}P]ATP, loaded onto a 10% SDS-PAGE gel and analyzed by autoradiography. Incorporation of radioactivity into the enolase as shown in the panel indicates c-src kinase activity

Furthermore, treatment with the src inhibitor PP2 also inhibited the association between CD24 and c-src (Fig. 2c). These data suggest that CD24 may preferentially associate with kinase-active c-src and thereby maintain or increase its kinase activity. We therefore set out to investigate whether enhanced c-src activity in response to CD24 expression is associated with lipid rafts, and whether it requires interaction of CD24 and c-src in lipid rafts. CD24-expressing and non-expressing MTLy cells were fractionated into lipid raft and non-lipid raft fractions, and the amount of c-src kinase activity associated with each fraction assessed. Although we observed that CD24 expression promoted c-src activity (Fig. 3b), the majority of kinase-active c-src was associated with the non-lipid raft fraction, regardless of CD24 expression (Fig. 3c). Furthermore, incubation of doxycycline-induced CD24-expressing cells with MCD to disrupt lipid rafts reduced the CD24-dependent increase in c-src activity only after several hours (Fig. 3d). Together, these observations suggest (a) CD24 and the kinase-active form of c-src colocalize in lipid rafts, (b) that expression of CD24 promotes the kinase activity of c-src, and (c) that disruption of lipid raft integrity disturbs the interaction between CD24 and c-src, but does not immediately affect CD24-mediated enhancement of c-src kinase activity.

CD24-induced binding to fibronectin is dependent on c-src activity, whereas CD24-induced proliferation is not

The src inhibitor PP2 inhibited the CD24-induced binding of MTLy cells to fibronectin (Fig. 2a), suggesting that c-src activity is required for CD24 to promote cell binding to fibronectin. As expression of CD24 results in increased c-src kinase activity (Fig. 3), we next determined whether c-src kinase activity is necessary and sufficient for the

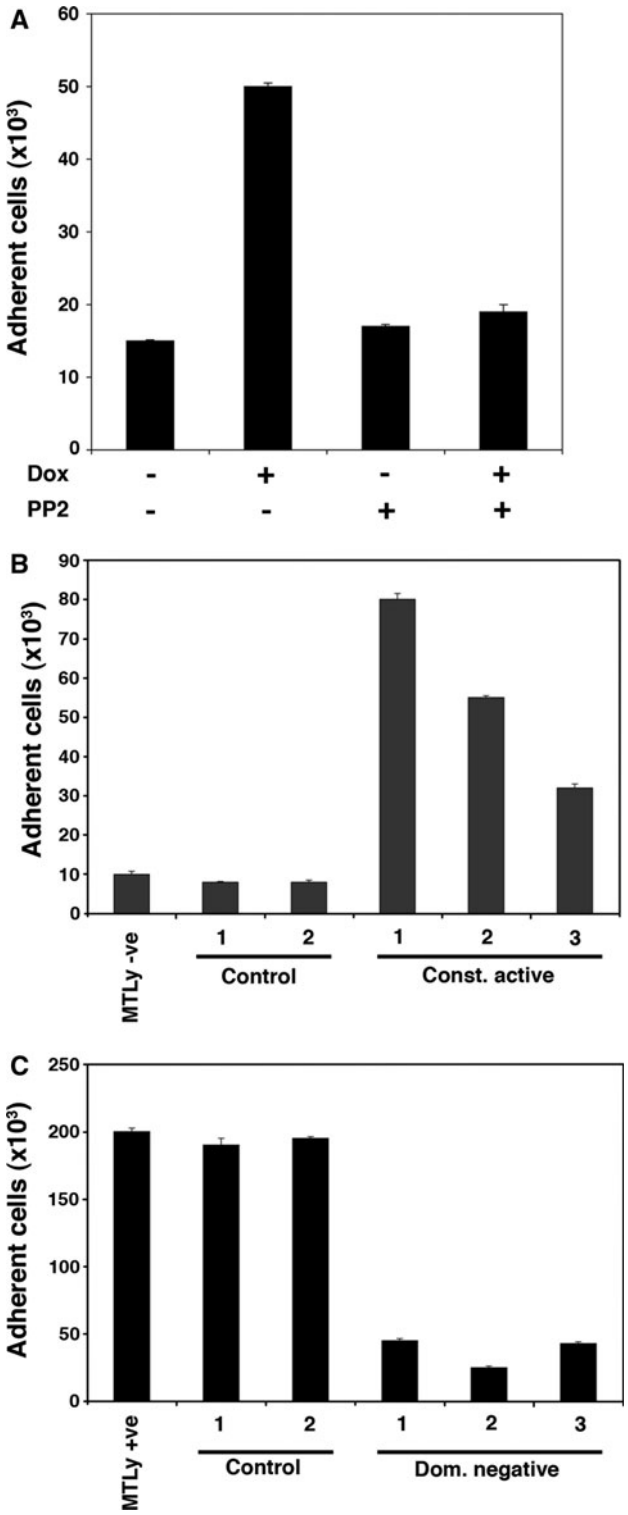


Fig. 4 **a** Treatment with PP2 reduces CD24-mediated cell binding to fibronectin. MTLyCD24ind cells were not treated or treated with doxycycline to induce CD24 expression. Cells were then cultivated in the absence or presence of 10 μM PP2 for 3 h as indicated, and used in adhesion experiments on fibronectin-coated plastic. The numbers of adherent cells were determined as described in "Materials and methods". The means and standard errors of one representative experiment performed in triplicate are presented. **b** Kinase-active c-src induces cell binding to fibronectin in the absence of CD24 expression. MTLy CD24-negative cells were stably transfected with a constitutively active c-src expression vector or with the empty expression vector as a control. Two independent control clones and three independent constitutively active c-src-expressing clones were used in fibronectin adhesion assays, together with the parental CD24-negative MTLy cells (*MTLy -ve*). The numbers of adherent cells were determined as described in "Materials and methods". The means and standard errors of one representative experiment performed in triplicate are presented. **c** Expression of dominant negative c-src blocks the CD24-induced binding of MTLy cells to fibronectin. Parental CD24-positive MTLy cells were stably transfected with a dominant-negative c-src expression vector or with the empty expression vector as a control. Two independent control clones and three independent dominant-negative c-src-expressing clones were used in fibronectin adhesion assays, together with the parental CD24-positive MTLy cells (*MTLy +ve*). The numbers of adherent cells were determined as described in "Materials and methods". The means and standard errors of one representative experiment performed in triplicate are presented

CD24-induced adhesion of cells to fibronectin. Treatment of MTLy cells expressing doxycycline-induced CD24 with PP2 abolished CD24-mediated binding of the cells to fibronectin (Fig. 4a). Furthermore, ectopic expression of constitutively active c-src in CD24-negative MTLy CD24mut cells was able to induce cell binding to fibronectin in the absence of CD24 expression (Fig. 4b). Moreover, ectopic expression of dominant-negative c-src in CD24-expressing MTLy cells substantially reduced the binding of the cells to fibronectin (Fig. 4c). Together, these findings suggest that CD24-mediated enhanced c-src activity is necessary and sufficient for the CD24-induced binding of cells to fibronectin.

Although CD24 expression promotes cell proliferation [20], PP2 was unable to inhibit the doxycycline-induced CD24-dependent promotion of cell proliferation (Fig. 5a). Furthermore, proliferation of CD24-positive MTLy cells was not affected by ectopic expression of dominant-negative c-src, nor was the proliferation of non-induced CD24-negative MTLy cells affected by the expression of constitutively active c-src (Fig. 5b, c). Taken together, these findings indicate that CD24 promotes cell proliferation independently of its effects on c-src kinase activity.

Activation of c-src by CD24 increases the rate of cell adhesion and spreading and formation of focal adhesions

Our previous studies have shown that CD24 expression promotes the binding of pre-existing integrin subunits on the cell surface to fibronectin [20]. The data in Fig. 4 suggested to us that c-src activation as a consequence of CD24 expression might be sufficient to induce an active integrin conformation, thereby promoting binding of cells to fibronectin. We therefore examined the activation status of β1-containing integrins using an antibody (HUTS) that

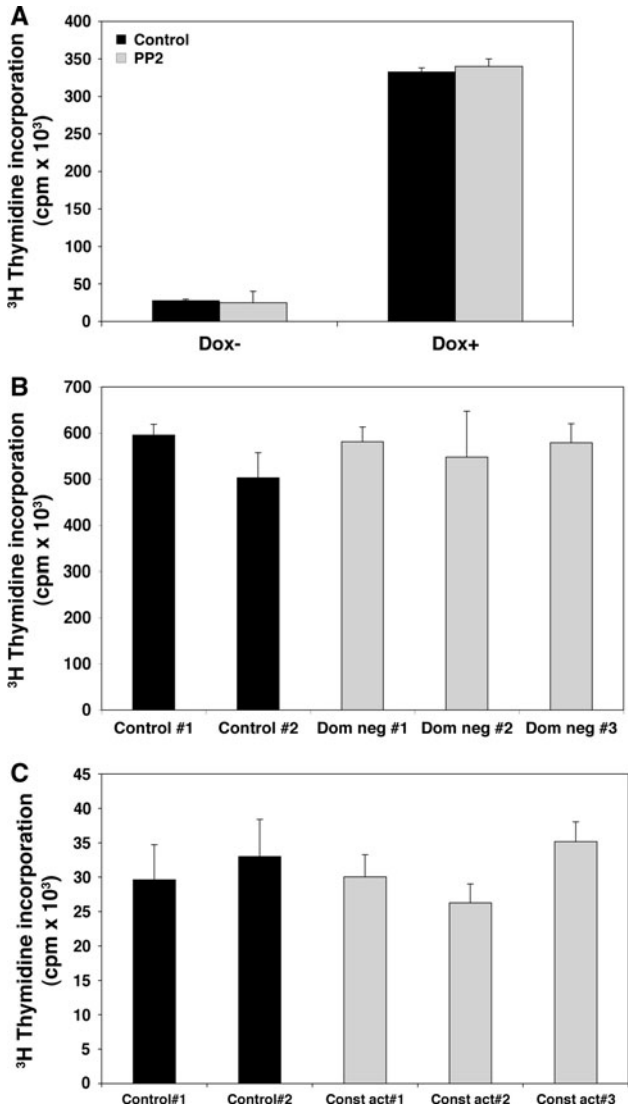


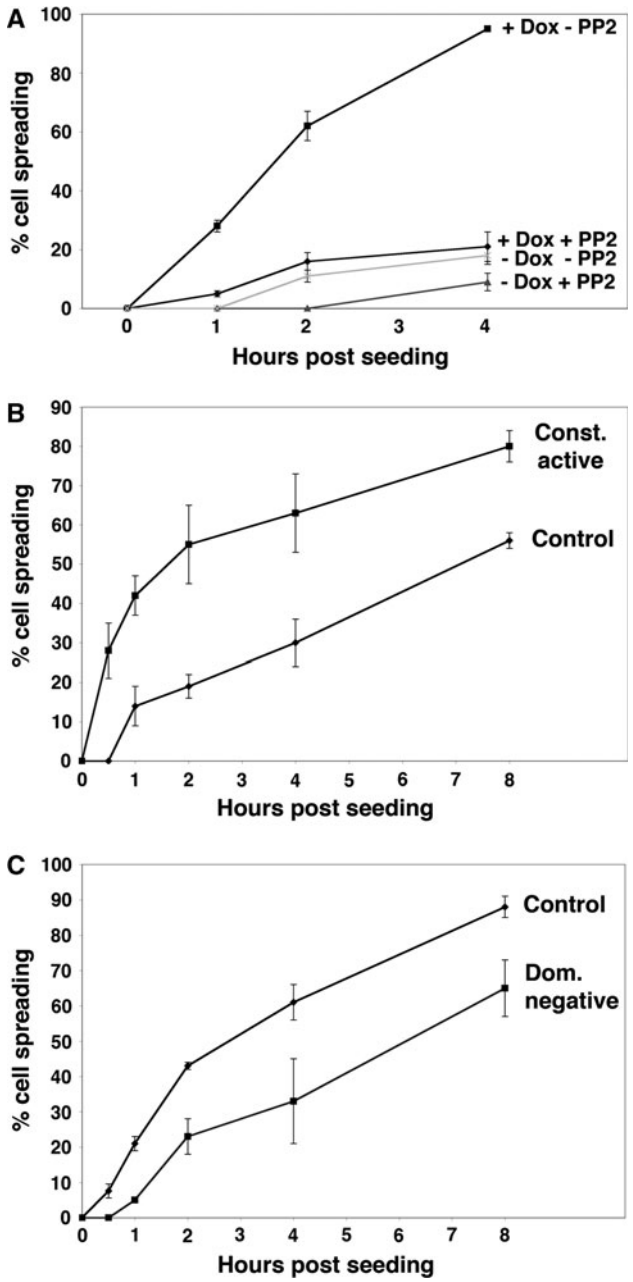
Fig. 5 **a** PP2 does not inhibit CD24-induced proliferation. MTLyC-D24ind cells were induced (*Dox*+) or not induced (*Dox*−) to express CD24 for 24 h. Cells were then treated with 10 μM PP2 for 3 h, labeled with ^{3}H-thymidine, then the incorporated radioactivity was measured. The means and standard errors of one representative experiment performed in triplicate are presented. **b** Expression of dominant-negative c-src does not reduce the proliferation of CD24-expressing MTLy cells. Two independent control clones and three independent clones expressing dominant-negative c-src (see Fig. 4) were used in tritiated thymidine incorporation assays. The means and standard errors of one representative experiment performed in triplicate are presented. **c** Expression of constitutively active c-src in CD24-negative MTLy CD24mut cells has no effect on proliferation. Two independent control clones and three independent clones expressing constitutively active c-src (see Fig. 4) were used in tritiated thymidine incorporation assays. The means and standard errors of one representative experiment performed in triplicate are presented

binds specifically to the activated conformation of β1-containing integrins. Surprisingly we found in FACS analysis that the antibody bound equally well to MTLy cells expressing dominant-negative or constitutively active c-src constructs, and even to doxycycline-inducible MTLy cells with and without doxycycline-induced CD24 expression (Supplementary Fig. 2 and data not shown). This therefore suggested that a mechanism other than induction of an activated integrin conformation might account for increased cell binding to fibronectin as a consequence of CD24 expression.

Efficient adhesion of cells to a substrate requires cell spreading and focal adhesion formation. Previously we have shown that doxycycline-induced CD24 expression potently increases the kinetics of MTLy cell spreading on fibronectin in the hours immediately following seeding of the cells. However, 24 h after the cells were plated out, equivalent spreading was observed when doxycycline-induced and non-induced cells were compared [20]. Together with the FACS analysis with the HUTS antibody, these observations might suggest that the integrins that mediate binding to fibronectin are not in an inactive conformation in the absence of CD24, but rather that the formation of integrin-mediated adhesions are slower in the absence of CD24. We therefore determined whether CD24 expression promotes cell binding to fibronectin by increasing the rate of spreading and focal adhesion formation in a c-src-dependent manner.

As shown previously [20], induction of CD24 expression strongly increased the rate of spreading of CD24-positive cells. This effect was abrogated by treatment with PP2 (Fig. 6a). Consistently, constitutively active c-src accelerated spreading of non-induced CD24-negative MTLy cells (Fig. 6b), while expression of dominant-negative c-src in CD24-positive cells retarded spreading (Fig. 6c).

The kinase activity of c-src is known to play an important role in the formation of focal adhesions, for example through the phosphorylation of focal adhesion components such as FAK and paxillin. We therefore determined whether CD24 expression and the associated activation of c-src kinase activity would increase the rate of focal adhesion complex formation. First we examined the phosphorylation of FAK and paxillin. Doxycycline-induced expression of CD24 promoted a faster phosphorylation of both FAK and paxillin following plating of the cells (Fig. 7a). This effect was abrogated by treatment with PP2 (Fig. 7b). Consistently, expression of dominant-negative c-src in CD24-positive MTLy cells retarded the phosphorylation of FAK and paxillin following plating of the cells, while constitutively active c-src accelerated the phosphorylation of FAK and paxillin following plating of non-induced CD24-negative MTLy cells (Fig. 7c). Correspondingly, these changes in phosphorylation were associated with focal adhesion formation in response to CD24 expression that was abrogated by treatment with PP2



◀ **Fig. 6** **a** Inhibition of c-src kinase activity by PP2 suppresses cell spreading. MTLyCD24ind cells were not treated or treated with doxycycline to induce CD24 expression, then treated with 10 μM PP2 for 3 h as indicated. After seeding onto fibronectin-coated plates, the cells were fixed and photographed after the indicated times. Cells that adhered to the substrate were scored as being spread or remaining rounded up. The percentages of adherent spread cells are indicated. The means and standard errors of one representative experiment performed in triplicate are presented. **b** Expression of constitutively active c-src induces cell spreading in the absence of CD24. Three independent constitutively active c-src-expressing clones (see Fig. 4) and control clones of MTLy CD24-negative cells were seeded onto fibronectin-coated plates for the indicated times. Cells that adhered to the substrate were scored as being spread or remaining rounded up. The percentages of adherent spread cells are indicated. The means and standard errors of one representative experiment performed in triplicate are presented. **c** Expression of dominant-negative c-src in MTLy CD24-positive cells abrogates cell spreading. Three independent dominant-negative c-src expressing clones (see Fig. 4) and control clones were seeded onto fibronectin-coated plates for the indicated times. Cells that adhered to substrate were scored as being spread or remaining rounded up. The percentages of adherent spread cells are indicated. The means and standard errors of one representative experiment performed in triplicate are presented

(Fig. 7d). Furthermore, expression of dominant-negative c-src in CD24-positive MTLy cells, or constitutively active c-src non-induced CD24-negative MTLy cells inhibited and accelerated focal adhesion formation, respectively (Fig. 7e).

Taken together, these findings demonstrate that in the context of MTLy breast cancer cells, CD24 expression promotes c-src kinase activity, which in turn accelerates formation of focal adhesion complexes, as evidenced by enhanced phosphorylation of FAK and paxillin. The accelerated formation of focal adhesion complexes in turn stimulates the spreading and adhesion of the cells. In the absence of the CD24-augmented c-src kinase activity, $\alpha 3\beta 1$ and $\alpha 4\beta 1$ integrins are able to bind to substrates such as fibronectin, but do so only slowly.

CD24 promotes cell motility/invasion in a c-src-dependent manner

We have previously reported that CD24 promotes the motility and invasiveness of MTLy cells [20]. We therefore set out to determine whether this activity is also dependent on c-src. First we examined the ability of CD24-negative MTLy CD24mut cells expressing constitutively active c-src to traverse a matrigel-coated membrane in comparison to control cells. As can be seen in Fig. 8a, constitutively active c-src strongly promoted the ability of MTLy to invade across the matrigel-coated membrane in the absence of CD24. Consistently, expression of dominant-negative c-src in CD24-positive MTLy cells potently reduced the ability of the cells to traverse the matrigel-coated membrane in these assays relative to control cells (Fig. 8b).

To confirm these findings in an independent cellular system, we took RKO and HCT116 human colorectal carcinoma cells that express only very low endogenous levels of CD24. When these cells were transfected with CD24, they showed enhanced invasion across the matrigel-coated Transwell chamber (Fig. 8c). Importantly, knockdown of c-src in these cells abrogated the enhanced invasiveness mediated by CD24 (Fig. 8c). Together, these findings indicate that CD24-mediated invasiveness is also dependent on c-src.

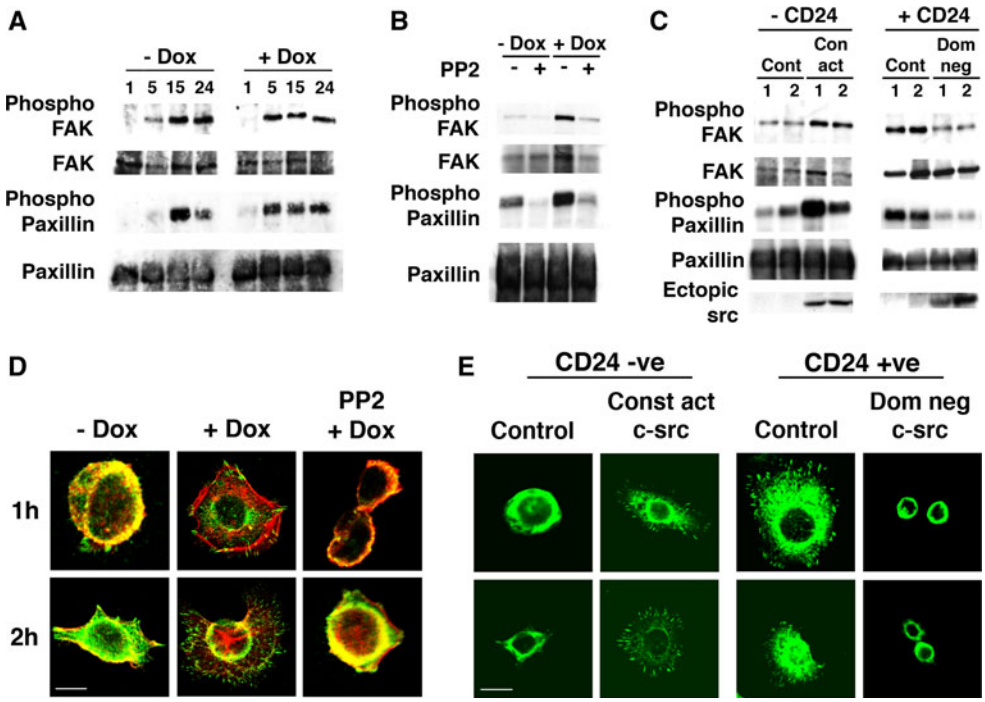
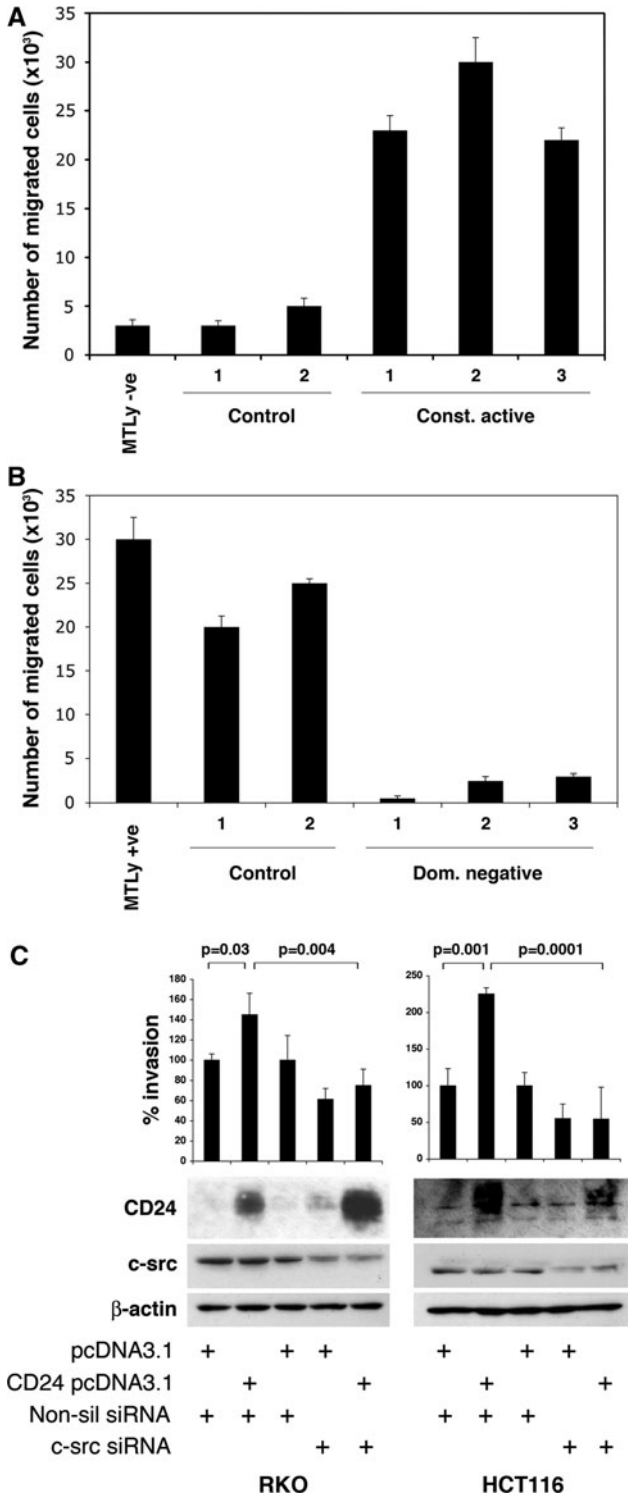


Fig. 7 **a** CD24 expression accelerates paxillin and FAK phosphorylation. MTLyCD24ind cells induced (+ *Dox*) or not induced (− *Dox*) to express CD24 were cultivated on fibronectin-coated dishes for different times (hours) as indicated. Lysates were immunoprecipitated with anti-paxillin antibodies and anti-FAK antibodies and the immune complexes were analyzed by SDS-PAGE/western blotting. The blots were probed with an anti-phosphotyrosine antibody, then stripped and reprobed with anti-FAK and anti-paxillin antibodies as appropriate for loading controls. **b** Treatment with PP2 blocks the CD24-augmented phosphorylation of FAK and paxillin. MTLyCD24ind cells were induced (+ *Dox*) or not induced (− *Dox*) to express CD24, and were then treated or not treated with 10 μm PP2 for 3 h as indicated. Cells were then lysed and immunoprecipitated with anti-paxillin antibodies and anti-FAK antibodies. Immune complexes were analyzed by SDS-PAGE/western blotting. The blots were probed with an anti-phosphotyrosine antibody, then stripped and reprobed with anti-FAK and anti-paxillin antibodies as appropriate for loading controls. **c** Phosphorylation of FAK and paxillin is reduced in CD24-positive MTLy cells expressing dominant-negative c-src, while expression of constitutive active c-src in CD24-negative MTLy CD24mut cells enhances the phosphorylation of paxillin and FAK. Cells were cultivated on fibronectin-coated dishes for the indicated times (hours) then lysed. Lysates were immunoprecipitated with anti-paxillin and anti-FAK antibodies. The immunoprecipitates were subsequently western blotted and probed with anti-phosphotyrosine antibody. Blots were stripped and reprobed with antibodies against FAK and paxillin for a loading control. Expression of exogenous c-src was verified using the chicken src-specific EC10 antibody. **d** Treatment with PP2 reduces the formation of CD24-induced focal adhesions. MTLyCD24ind cells were induced (+ *Dox*) or not induced (− *Dox*) to express CD24, then treated or not treated with 10 μm PP2 for 3 h as indicated. Cells were subsequently immunostained with anti-paxillin antibodies (*green*) and with phalloidin (*red*). *Bar* 10 μm. **e** Expression of constitutive active c-src enhances the formation of focal adhesions in CD24-negative cells, while expression of dominant-negative c-src abrogates the formation of focal adhesions in CD24-positive cells. *Left panel* CD24-negative MTLy CD24mut cells (*CD24 −ve*) stably transfected with either empty vector or with a constitutively active c-src expression vector were seeded for 30 and 60 min on fibronectin-coated plates as indicated. *Right panel* CD24-positive cells (*CD24 +ve*) stably transfected with either empty vector or with a dominant-negative c-src expression vector were seeded for 30 and 60 min on fibronectin-coated plates as indicated. Immunofluorescence staining of paxillin (*green*) was performed to visualize the formation of focal adhesions. *Bar* 10 μm

Discussion

Here we report that in MTLy breast cancer cells, CD24 interacted with c-src in the context of lipid rafts and activated its kinase activity. In turn, the enhanced c-src signaling fostered integrin-mediated adhesion through accelerated focal adhesion formation and hastened phosphorylation of key focal adhesion components. Similarly, CD24 enhanced tumor cell invasion in a manner that is dependent on c-src. CD24-stimulated proliferation in these cells is independent of this mechanism.

Although the concept of lipid rafts has been critically discussed in the past, advances in technology have provided compelling evidence that they represent functional subcompartments of the cell membrane [31]. An important function of lipid rafts is to act as platforms that recruit and organize signaling molecules [32]. Proteins with raft affinity include GPI-linked proteins and the SFKs, and in recent years a number of studies have demonstrated that GPI-linked membrane proteins interact with and activate various SFKs in the lipid raft context. For example the GPI-linked protein Thy-1 physically associates with lyn,



◄ **Fig. 8** CD24 promotes tumor cell invasion in a c-src-dependent manner. **a** Expression of constitutive active c-src promotes MTLy invasion across a matrigel-coated membrane in Transwell assays in the absence of CD24. Three independent constitutively active c-src-expressing clones (see Fig. 4) and control clones of MTLy CD24-negative cells were placed in the upper compartment of a Transwell Boyden chamber coated with matrigel. After 24 h the number of cells that had invaded across the filter was determined. The means and standard errors of one representative experiment performed in triplicate are presented. **b** Expression of dominant-negative c-src in MTLy CD24-positive cells inhibits invasion across a matrigel-coated membrane in Transwell assays. Three independent dominant-negative c-src-expressing clones (see Fig. 4) and control clones were placed in the upper compartment of a Transwell Boyden chamber coated with matrigel. After 24 h the number of cells that had invaded across the filter was determined. The means and standard errors of one representative experiment performed in triplicate are presented. **c** CD24 promotes invasion by RKO and HCT116 human colorectal carcinoma cells in a c-src-dependent manner. RKO and HCT116 cells were transfected with empty pcDNA3.1 control vector, a pcDNA3.1 CD24 expression plasmid, non-silencing siRNA or c-src siRNA as indicated. Some of the cells were then lysed and western blotted to determine expression of CD24 and c-src relative to β-actin as a loading control (*blots*). The remaining cells were used in a Transwell invasion assay. After 16 h the number of cells that had invaded across the filter was determined (*graph*). The means and standard errors of one representative experiment performed in triplicate are presented. Values (percent invasion) are presented relative to invasion by the control cells transfected with empty vector and non-silencing siRNA, which was set to 100%

fyn and c-src in lipid rafts, and has been reported to activate c-src [33, 34]. Similarly, the GPI-anchored protein F3/contactin-1 associates with and activates Fyn [35]. Lyn is activated by the GPI-anchored TAG-1/contactin-2 protein [36]. CD24 itself physically interacts with and activates lyn and lck [30, 37], and the evidence we present here indicates that this is also true for the SFK c-src. Several observations support this notion. First, although CD24 expression does not lead to higher levels of c-src protein expression in MTLy cells, CD24-expressing cells showed higher levels of c-src activity than CD24-deficient cells. Second, CD24 expression led to increased c-src kinase activity as evidenced by enolase assays. Third, CD24 and c-src coimmunoprecipitated, and this interaction was disrupted by MCD. Fourth, the SFK inhibitor PP2 that blocks c-src activation also inhibited the ability of c-src to coimmunoprecipitate with CD24. Fifth, both CD24 and c-src are present in lipid rafts, and immunofluorescence experiments showed that CD24 and c-src partially colocalize in MTLy cells. Although in this study we focused on CD24-stimulated c-src kinase activity, similar mechanisms could conceivably also activate other SFKs.

The mechanism by which GPI-anchored proteins in the context of lipid rafts promote SFK activity remains poorly understood. Recent diffusion and single molecule tracking studies suggest that SFKs frequently but only transiently associate with GPI-anchored receptor clusters, and that their mobility is lower in lipid rafts than elsewhere in the membrane or in the cytoplasm [38, 39]. In principle enhanced SFK activity could arise either through activation of SFKs within lipid rafts, or through stabilization of pre-existing SFK activity within the lipid raft environment. Evidence for both possibilities exists. Lyn, for example, is transiently recruited to GPI-anchored clusters, and in this

lipid raft context is activated by Gαi2 [38]. In other contexts it has been proposed that lipid rafts exclude phosphatases, leading to maintenance of the activated phosphorylated state of SFKs in the lipid rafts [40]. Moreover, the lipid raft-anchored protein Cbp sequesters c-src in lipid rafts and inactivates it [41]. Exclusion of negative regulators like Cbp from lipid rafts by particular GPI-linked proteins may conceivably also be a means by which SFK activity can be augmented, although this remains speculation.

The SFK inhibitor PP2, which binds at the ATP binding site [42], abrogates the interaction between CD24 and c-src. This indicates that c-src kinase activity is required for its association with lipid raft-associated CD24, seemingly ruling out a scenario in which inactive c-src is recruited to CD24-containing lipid rafts for activation. Our findings therefore suggest a role for CD24-containing lipid rafts in the maintenance of the pool of kinase-active c-src, thereby leading to enhanced cellular c-src activity, rather than in de novo activation of c-src. As the majority of activated c-src and its associated kinase activity was found in the non-detergent-resistant lipid raft compartment (Fig. 3c), our results indicate a transient association between CD24 and c-src that leads to augmented cellular c-src kinase activity. Specifically, disruption of lipid rafts using MCD abrogated the association between c-src and CD24, yet the increased cellular c-src kinase activity induced by CD24 expression only diminished after several hours (Fig. 3d). These findings are consistent with the reported half-life of active c-src kinase [43], particularly as we measured steady-state c-src kinase activity in our experiments rather than pulse-chase of protein levels. Taken together with the published observations regarding the mechanisms by which GPI-anchored proteins in the context of lipid rafts promote SFK activity, our findings are consistent with a model in which lipid raft-associated CD24 can act as a transient refuge for kinase-active c-src, reducing its rate of inactivation and thereby increasing overall cellular levels of c-src kinase activity. Assessment of c-src kinase activity immediately following 5 min of MCD treatment therefore showed no significant difference between treated and non-treated cells (Fig. 3b), and reduced c-src activity was only observed following turnover of c-src kinase activity (Fig. 3d) which takes several hours [43].

It is formally possible that c-src activation is an indirect consequence of enhanced integrin-mediated focal adhesion formation that is promoted by CD24. However, this scenario seems unlikely. In published work we have previously shown that MTLy cells induced or not induced with doxycycline to express CD24 show only transient differences in their integrin-mediated binding to fibronectin. By 24 h after plating, CD24− and CD24+ cells exhibit essentially equivalent binding to and spreading on fibronectin-coated surfaces [20]. In the present study, differences in c-src activity between CD24− and CD24+ cells were measured after cells had been plated for 24–48 h. During this period, integrin-mediated adhesion would be equivalent between the two conditions, thus ruling out the possibility that c-src activation results indirectly from an increase in integrin-mediated adhesion.

The PP2 inhibitor suppresses the activity of several SFKs, raising the possibility that other SFKs in addition to c-src may have been inhibited in our experiments. In addition, the SRC2 anti-src antibody used for some of the experiments in this paper is also able to cross-react with yes, fyn and fgr. However, it should be noted that the antibody only recognizes bands on western blots of MTLy lysates of a size consistent with that of c-src. Furthermore, siRNA specific for c-src reduced the intensity of bands detected by the SRC-2 antibody in western blots of MTLy lysates (data not shown). In addition, CD24 promoted invasion across matrigel-coated Transwells in a manner that was abrogated by knockdown of c-src (Fig. 8c). Thus, although we cannot rule out the possibility that CD24 acted in the experiments presented here by enhancing the activity of SFKs in addition to c-src, these observations support the notion that the activity of c-src is modulated by CD24 expression.

CD24 recruits β1-containing integrins into lipid rafts [25]. Together with our finding using MTLy cells that CD24 has the propensity to promote c-src kinase activity in a lipid raft-dependent manner and thereby to stimulate integrin-mediated adhesion, these observations suggest that CD24 may act to bring together and stimulate key components of the focal adhesion complex. This notion is supported by the finding that lipid raft-specific knockdown of c-src activity inhibits cell adhesion of breast cancer cells [44].

Our finding that CD24 can interact with and promote c-src activity at least in MTLy cells may help to explain why CD24 expression correlates with poor prognosis for many tumors, as there is a broad body of literature that implicates c-src in promoting invasion and metastasis (reviewed in reference [21]). Furthermore, c-src signaling is required for the survival of breast cancer cells in the bone marrow environment and the formation of latent bone metastasis [45]. Although we have focused here on c-src-mediated integrin activation, c-src kinase activity regulates many other pathways of relevance to tumor progression [46, 47], and therefore CD24-mediated activation of c-src would be expected to have pleiotropic effects that might contribute to the CD24-induced metastasis we have previously reported [20]. Indeed, we found that CD24 increased tumor cell invasiveness in a manner that was dependent on c-src activity. In contrast to the case in

fibroblasts, c-src does not appear to promote proliferation of epithelial and carcinoma cells [21]. Consistently, we found that CD24-regulated c-src activity was not responsible for CD24-induced proliferation of MTLy cells.

Although enhanced c-src kinase activity has been reported for many types of tumor [22], very few activating mutations have been found in the c-src gene in tumors [21]. While c-src is often overexpressed in tumors, high protein levels do not necessarily correlate with activity, and a number of mechanisms have been uncovered that activate c-src in the tumor context [22, 46]. Our finding that CD24 expression in tumor cells can activate c-src kinase activity reveals a further mechanism that could account for increased c-src kinase activity in tumors, particularly as CD24 expression itself correlates with a poor prognosis. Moreover, it also suggests that attempts to therapeutically target CD24 may act at least partially by inhibiting the activity of c-src (and possibly other SFKs). Consistently, targeting of CD24 results in downregulation of the Ras/ERK/MAPK pathway [47] that is activated by c-src [48].

In conclusion, we showed that in MTLy breast cancer cells, CD24 and c-src interact in the context of lipid rafts, which enhances cellular c-src kinase activity and fosters integrin-mediated adhesion, as well as invasion. The degree to which our findings are generally applicable to other CD24-expressing cells remains to be demonstrated. Nevertheless, our findings provide a putative mechanism that might contribute to CD24-mediated metastasis formation, and suggest an additional means by which c-src kinase activity might be enhanced in the context of tumors. Future work will focus on improving our understanding of how CD24 promote cell proliferation, which we showed to be independent of the CD24-mediated increase in c-src kinase activity.

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