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14. ABSTRACT Recent studies have suggested that a3pl and a6P4 integrins, receptors for extracellular matrix laminin, synergize with oncogenic ErbB receptor kinases to promote mammary tumor malignancy and drug resistance. Given the strong physical link of these receptors with CD15 1, a 30 kDa cell surface protein and member of the tetraspanin family, we proposed to study if CD15 1 is a viable target for human breast cancer treatment. Our project focused on those tumor cells overexpressing EGFR and ErbB2, two oncogenic members of ErbB receptor family. We hypothesized that CD151 acts together with EGFR or ErbB2 to affect tumor cell sensitivity to currently available biological therapeutic agents, including clinically used kinase inhibitors. Using culture breast cancer cell lines and xenograft animal models we examined CDI 5 1 effects on mammary tumor cell motility, , malignancy and proliferation. Our dab demonstrated that ablating CD151 markedly inhibited tumor cell migration and invasion on laminin substrate but had minimal effect on cell proliferation. Also, removal of CD15 1 enhanced mammary tumor sensitivity to treatment with ErbB inhibitor Herceptin and Lapatinib. Furthermore, our results showed that CD15 1 affects ErbB receptor signaling in a laminin-binding integrin-dependent manner. Collectively, our studies support a critical role for CD15 1 in ErBBZpositive breast cancer.					
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Final report for Breast Cancer DOD award

INTRODUCTION

It has been suggested that $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, receptors for extracellular matrix laminin synergize with oncogenic ErbB receptor kinases to drive mammary tumor malignancy and drug resistance (Guo et al., 2006). Given the strong physical link of these receptors with CD151, a 30 kDa cell surface protein and member of the tetraspanin family (Hemler, 2005), we proposed to study if CD151 is a viable target for human breast cancer treatment. We focused particularly on those tumor cells overexpressing EGFR and ErbB2, two oncogenic members of ErbB receptor family. We hypothesized that CD151 acts in concert with EGFR or ErbB2 to affect tumor cell sensitivity to currently available biological therapeutic agents, including clinically used kinase inhibitors. Using cultured breast cancer cell lines and xenograft animal models we performed extensive analyses to examine CD151 effects on mammary tumor cell morphology, malignancy, proliferation and signaling. We also determined the extent to which CD151, together with its laminin-binding integrin partners, affected survival of tumor cells under a range of ErbB receptor inhibitor treatments.

BODY

Our experimental progress is presented according to specific objectives described in our original proposal.

Objective I. Synergy between CD151 and ErbB receptors

We have systematically performed a series of experiments to address if CD151 has a role in EGFR-mediated and laminin-binding integrin-dependent mammary cell behaviors and tumor malignancy. In both normal (MCF-10A) and malignant (MDA-MB-231) mammary cell lines expressing a high level of EGFR, we successfully ablated CD151 by use of a Lentivirus vector containing CD151-specific shRNA. Our subsequent functional analyses demonstrated that removal of CD151 protein markedly affected EGFR-mediated cellular adhesion, migration and invasion on laminin substrates via laminin receptors including $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins, but had minimal impact on tumor cell proliferation in vitro (See Fig 2 of Yang et al., *Cancer Research*, 2008). Also, EGFR activation failed to rescue cell adhesion and invasion defects, due to the absence of CD151 (See Fig 3 of Yang et al., *Cancer Research*, 2008), further emphasizing that the EGFR is difficient in the absence of CD151 on mammary tumor cells.

We also observed that in the absence of CD151 there was diminished activation of small GTPase Rac 1, FAK (*Focal adhesion kinase*) and SFK (*src family kinases*), which are all known to be effectors of integrins and EGFR-mediated pathways (See Fig 4 of Yang et al., *Cancer Research*, 2008). Most importantly, mammary tumor formation on immuno-deficient mice was markedly delayed, when CD151 was removed from breast cancer cells ((See Fig 6 of Yang et al., *Cancer Research*, 2008). Thus, our experimental results strongly suggest that CD151 acts in synergy with EGFR and laminin-binding integrins during breast cancer progression and malignancy.

Objective II. CD151 and tumor cell sensitivity to ErbB inhibitors

i.) We carried out extensive studies to assess how CD151 expression affects breast tumor cell sensitivity to ErbB kinase inhibitors. We used multiple breast cancer cell lines with overexpressed or amplified ErbB2 receptor, including BT474 and SK-BR-3 cells. Again, more than 95% of endogenous

CD151 on these cell lines was successfully removed by using a Lentivirus shRNA approach. Cells with or without CD151 knockdown were then subjected to treatment with two distinct kinase inhibitors at varying doses: *Herceptin*, a humanized monoclonal antibody against the extracellular domain of ErbB2; *Lapatinib*, a small molecule inhibitor of ErbB2 kinase activity.

Our data indicated that ablating CD151 made two ErbB2-positive breast cancer cell lines (BT474 and SKBr3) significantly more sensitive to killing by Herceptin drug treatment, while a non-responsive ovarian cancer line was unaffected by CD151 knockdown. Because CD151 is tightly coupled with $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, which are receptors for laminin-5 substrate, we suspected that laminin substrate might affect Herceptin killing. Our analyses indicated erbB2-positive cells showed a significant increase in resistance to Herceptin killing, when plated on laminin-5. By contrast, cells maintained on non-coated surfaces, or on surfaces coated with other matrices, were more sensitive to Herceptin. We next assessed whether the sensitizing effects of removing CD151 were influenced by plating cells on laminin-5 substrate. Indeed, laminin-engagement dramatically enhanced the sensitizing impact of CD151 removal on mammary tumor cell responses to Herceptin. Sensitivity to another ErbB2 inhibitor, lapatinib, was similarly enhanced when CD151 was knocked down. Similar findings were also obtained with additional ErbB2-positive tumor cell lines, including SK-BR-3. Furthermore, RNAi ablation of CD151-associated $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, together or individually, also caused a marked enhancement of ErbB2-positive cell sensitivity to Herceptin. These results suggest that CD151 is acting together with its associated laminin-binding integrins to contribute to mammary tumor cell resistance to erbB2 inhibitors.

In conclusion, CD151 and associated laminin-binding integrins may be valuable targets for enhancing mammary tumor cell sensitivity to clinically used ErbB2 inhibitors. This may be particularly true in human mammary glands, where laminin-5 is highly enriched.

ii.) We also tried to use CD151-specific antibodies as tools to probe the role of CD151 in human breast cancer cells. However, none of the available CD151-specific antibodies showed blocking activities in our experiments. We are currently in the process of testing additional CD151 antibodies for our functional analyses. We hope to obtain additional tools to further evaluate the potential for targeting CD151 on breast cancer cells.

iii.) We have also begun to test whether CD151 disruption affects tumor cell sensitivity to ErbB inhibitors within xenograft animal models. However, definitive results have not yet been obtained.

KEY RESEARCH ACCOMPLISHMENTS

1. Our most significant achievement is to provide evidence for a strong functional link between CD151 protein and ErbB receptors, during cellular events such as cell adhesion, motility, invasion, and signaling in normal and malignant mammary epithelial cells.
2. Another significant finding is that CD151 markedly affects tumor cell responses to two different clinically used kinase inhibitors, humanized monoclonal antibody (Herceptin) and small molecule (Lapatinib). CD151 had the most pronounced impact on drug sensitivity when tumor cells were engaged with laminin, a physiologically relevant ECM substrate during human breast cancer.

3. We also addressed the molecular mechanisms underlying CD151 effects in ErbB2-positive mammary tumor cells. Our data indicate that CD151 most likely affects mammary tumor cell sensitivity through its partners, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins. Thus, ablating CD151 may also directly disrupt the synergy between ErbB receptor and these laminin receptors in mammary cancer cells. Together, our data suggest that CD151 is an alternative target to its associated integrin partners for conquering human mammary tumor malignancy.

REPORTABLE OUTCOMES

1. A published paper is included in the appendix. Within that paper, figure 2-6 are highly relevant to the aims of this research project
2. Results from this project will be published in the near future.

CONCLUSIONS

- We have presented strong evidence that CD151 acts in synergy with ErbB receptors to promote mammary tumor adhesion, motility and invasion, thereby facilitating breast cancer progression
- Our analyses reveal that CD151, together with its associated integrin complexes, markedly affect mammary tumor cell sensitivities to ErbB2 inhibitors, suggesting that CD151 may be a viable target for enhancing current biological therapy-based treatment of human breast cancer.

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Yang XH, Richardson AL, Torres-Arzayus MI, Zhou P, Sharma C, Kazarov AR, Andzelm MM, Strominger JL, Brown M, Hemler ME., 2008. CD151 accelerates breast cancer by regulating alpha 6 integrin function, signaling, and molecular organization. *Cancer Res.* 68: 3204-13

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APPENDICES

A recently published paper on CD151 is also included

CD151 Accelerates Breast Cancer by Regulating α_6 Integrin Function, Signaling, and Molecular Organization

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Abstract

CD151, a master regulator of laminin-binding integrins ($\alpha_6\beta_4$, $\alpha_6\beta_1$, and $\alpha_3\beta_1$), assembles these integrins into complexes called tetraspanin-enriched microdomains. CD151 protein expression is elevated in 31% of human breast cancers and is even more elevated in high-grade (40%) and estrogen receptor-negative (45%) subtypes. The latter includes triple-negative (estrogen receptor, progesterone receptor, and HER2 negative) basal-like tumors. CD151 ablation markedly reduced basal-like mammary cell migration, invasion, spreading, and signaling (through FAK, Rac1, and Ick) while disrupting epidermal growth factor receptor (EGFR)- α_6 integrin collaboration. Underlying these defects, CD151 ablation redistributed $\alpha_6\beta_4$ integrins subcellularly and severed molecular links between integrins and tetraspanin-enriched microdomains. In a prototypical basal-like mammary tumor line, CD151 ablation notably delayed tumor progression in ectopic and orthotopic xenograft models. These results (a) establish that CD151- α_6 integrin complexes play a functional role in basal-like mammary tumor progression; (b) emphasize that α_6 integrins function via CD151 linkage in the context of tetraspanin-enriched microdomains; and (c) point to potential relevance of CD151 as a high-priority therapeutic target, with relative selectivity (compared with laminin-binding integrins) for pathologic rather than normal physiology. [Cancer Res 2008;68(9):3204–13]

Introduction

CD151 (SFA-1, PETA-3), one of 33 proteins in the mammalian tetraspanin protein family (1), is widely expressed on the surface of many cell types, where it associates strongly with laminin-binding integrins ($\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$) and more weakly with a few additional integrins (2). Hence, CD151 is well positioned to modulate integrin-dependent cell spreading, migration, signaling, and adhesion strengthening (3–5). CD151 may function by linking laminin-binding integrins to other tetraspanins (e.g., CD9, CD81, CD82, and CD63), signaling molecules (phosphatidylinositol

4-kinase and protein kinase C), and other proteins within tetraspanin-enriched microdomains (1, 6).

CD151-associated integrins ($\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$) play critical roles in kidney and skin development (7). CD151 itself may support kidney and skin development and other functions in humans (8). Mice lacking CD151 are viable and fertile, with no obvious developmental defects (9) or showing kidney defects (10), depending on genetic background. Under pathologic conditions, CD151-null mice showed *in vivo* defects in wound healing (11) and angiogenesis (12). *Ex vivo* analyses of CD151-null cells and tissues revealed selected alterations in cell outgrowth, migration, aggregation, proliferation, morphology, and signaling (9, 12, 13).

Whereas other tetraspanins suppress tumor cell invasion and metastasis (14), CD151 promotes tumor malignancy (15), and the CD151 gene is up-regulated in human keratinocytes during epithelial-mesenchymal transition (16). In addition, CD151 expression correlated with poor prognosis, enhanced metastasis, or increased motility in several cancer types (e.g., ref. 17). Removal of CD151 by antisense, siRNA knockdown, or knockout may affect the phosphatidylinositol 3'-kinase (PI3K), Akt, and Rac1 pathways (12, 18). In addition, CD151 depletion may either increase (12, 19) or decrease (12, 20) cell motility, whereas effects on cell adhesion vary from minimal to substantial (12, 13, 20, 21), perhaps due to effects on integrin activation (21) and/or internalization (20). Thus, CD151 has diverse and unpredictable functions in different cellular environments.

At present, little has been done about CD151 in breast cancer. The $\alpha_6\beta_4$ integrin (after disconnection from hemidesmosome intermediate filaments) promotes mammary tumor cell motility and invasion by activating the PI3K/Akt pathway or small GTPase Rac1/nuclear factor κ B (22, 23). $\alpha_6\beta_4$ may also promote mammary tumorigenesis by amplifying signaling of ErbB family members (24). In human breast cancers, expression of integrin α_6 and/or β_4 is associated with the estrogen receptor-negative basal-like subtype, high tumor grade, and increased mortality (25–27). Given the CD151 association with laminin-binding integrins, we hypothesized that CD151 influences mammary tumor progression. Indeed, we found elevated CD151 in high-grade and estrogen receptor-negative tumors, including the "triple-negative" (estrogen receptor, progesterone receptor, and HER2 negative) basal-type human breast cancers. CD151 ablation yielded marked alterations in integrin-mediated cell invasion, migration, and/or spreading in mammary cell lines (MCF-10A and MDA-MB-231) with basal-like gene expression patterns (28). We also gained new insights into CD151 effects on integrin signaling, distribution, and collaboration with epidermal growth factor receptor (EGFR). Supporting the relevance of these findings, CD151 ablation delayed human mammary tumor progression in mouse xenograft models.

Note: X.H. Yang and A.L. Richardson contributed equally to this work.

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

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Materials and Methods

Cell culture and reagents. The majority of studies were carried out using immortalized MCF-10A and malignant MDA-MB-231 cells. The former are well suited for analysis of CD151 effects on migration of confluent cell monolayers and integrin organization in cell monolayers. The latter are better suited for studies of cell invasion, epidermal growth factor (EGF)-stimulated responses, signaling, and tumor progression. Both cell types are useful for studies of CD151 contributions to integrin molecular complexes. A few other cell mammary cell lines are also included to illustrate the generality of the findings. Human basal-like mammary epithelial cell lines (MCF-10A, MDA-MB-231, BT549, and Hst578; ref. 28) and J110 (estrogen receptor-positive metastatic mouse mammary line; ref. 29) were cultured in DMEM or RPMI 1640 with 10% FCS (Life Technologies, Inc.), 10 mmol/L HEPES, and antibiotics (penicillin and streptomycin). A Hoechst dye effluxing mammary cell subline (s-MCF-7) was selected for high sensitivity to EGF. *In vivo* passaged p-MDA-MB-231 cells were from nude mouse tumors and were treated with control siRNA (clones C1 and C2) or CD151 siRNA (clones K1, K2, and K3).

Anti-CD151 monoclonal antibodies (mAb) include 5C11, 1A5 (15), and FITC-conjugated IIG5a (GeneTex, Inc.). Anti-CD9 mAb MM2/57 (unconjugated and FITC conjugated) was from Biosource. mAbs to tetraspanins CD81 (M38) and CD82 (M104); mAbs to integrin α_2 (A2-IIE10), integrin α_3 (A3-X8), integrin α_6 (GöH3), integrin β_1 (TS2/16), and integrin β_4 (3E1, ASC-8); and rabbit polyclonal antibodies to the integrin $\alpha_3\alpha_4$ and $\alpha_6\alpha_4$ cytoplasmic domains were referenced elsewhere (5, 12). Anti- β_1 mAb 9EG7 was from PharMingen. Antibodies to FAK, Y397-phosphorylated FAK, Fyn, Src, and p130Cas were from Santa Cruz Biotechnology, Inc. Antibodies to phosphorylated Src, Lck, and FAK (Y925) were from Cell Signaling Technology. PI3K inhibitor (LY294002) was from Calbiochem and mitomycin C was from Sigma.

Human tissue array analyses. Formalin-fixed paraffin-embedded tumor samples annotated with pathologic and prediagnosis clinical data were obtained under an Institutional Review Board (IRB)-approved protocol (Partners IRB #2000-P-001448) from Brigham and Women's Hospital. Immunohistochemistry was done on four paraffin tissue microarrays of 124 primary human breast tumors containing two representative 0.6-mm cores of each tumor and representative cores of normal breast tissues. For CD151 immunohistochemistry, primary antibody (clone RLM30, Novacostra) was used at 1:50 dilution and detected using DAKO EnVision+ System (DAKO). Immunoreactivity was scored semiquantitatively by a breast pathologist (A.R.) using a scale of 0 to 3+, where 1+ staining approximates that in normal breast myoepithelial cells. Each single intensity score is based on two tissue cores, with 0 to 1+ indicating low, and 2 to 3+ indicating high/overexpressed.

siRNA and shRNA targeting. siRNAs were purchased from Dharmacon and used to target human CD151 (#4, GCAGGUCUUUGGCAUGA; #2, CCUCAAGAGUGACUACAUUU), α_6 integrin (CAAGACAGCUCAUUAUUGAUUU), α_3 integrin (UUACAGAGACUUUGACGAUU), CD9 (CCAAGAAGGACGUACUCGAUU), CD81 (CCACCAACCUCUGUAUCUUU), and CD82 (a pool of four siRNAs). Cells were seeded (1.0×10^5 /mL, 12–20 h) before siRNA transfection using Lipofectamine 2000. To enhance knockdown, cells were typically transfected again 2 d later.

For stable knockdown of human CD151, oligo AGTACCTGCTGT-TACCTACA (20) was cloned into lentivirus expression vector plenti-U6BX (CelloGenetics, Inc.) and verified by DNA sequencing. Viral titers were determined by HEK 293T cell infection. Infected cells were sorted by flow cytometry (with mAb 5C11) for CD151 absence.

Matrigel invasion, migration, and spreading assays. To assess invasion, cells were detached using nonenzymatic EDTA-containing dissociation buffer (Life Technologies). Then, cells (3×10^4 – 5.0×10^4) in serum-free DMEM with 0.02% bovine serum albumin (BSA) were added to transwell chambers containing 8- μ m membranes precoated with Matrigel (BD Biocoat). Chamber bottoms contained serum-free medium $\pm$ 10 ng/mL EGF. After invasion through Matrigel (12–18 h, 37°C), membranes were washed, dried, fixed, and stained (Giemsa, Sigma), and then cells were counted.

For monolayer scratch assays, 20% to 30% confluent cells in 24-well plates were transfected with siRNA for 5 d. Confluent cells were starved for ~12 h and gaps were scratched by pipette tip. After removing loose cells, DMEM/F12 was added, which contained MCF-10A-specific supplement at 1% dilution, $\pm$ 10 ng/mL EGF. Cell images were acquired with a monochrome charge-coupled device camera (RT SPOT, Diagnostic Instruments) on an Axiovert 135 inverted microscope (Zeiss Co.) and were controlled by IP Lab software (Scanalytics) running on a G4 Macintosh computer. Cell gaps were quantitated using Scion Image vs.62 (Scion Corp.). For spreading assays, cells (suspended at 37°C, 45 min) were plated onto 48-well plates precoated with extracellular matrix substrates and photographed after 45 min, as indicated above. Cells defined as spread showed a flattened morphology that was not phase bright by light microscopy.

Immunofluorescence microscopy. For confocal analyses, cells cultured on coverslips were treated with siRNAs (5–6 d), stained with various primary antibodies, then incubated with secondary antibody (FITC- or Alexa 594-conjugated goat anti-mouse or anti-rat) alone or combined (Molecular Probes). Cells were visualized under a Zeiss LSM 510 laser-scanning confocal microscope. Using LSM510 Meta software, z-axis images were acquired at 0.5- to 1- μ m increments.

Immunoprecipitation, [3 H]-palmitate labeling, and signaling assays. For metabolic labeling, siRNA-treated cells (80–90% confluent) were washed in PBS, serum starved (3–4 h), pulsed for 1 to 2 h in medium containing 0.2 to 0.3 mCi/mL [3 H]-palmitic acid plus 5% dialyzed fetal bovine serum, and then lysed in 1% Brij-96 for 5 h at 4°C. Immunoprecipitation and detection of [3 H]-palmitate-labeled proteins was as described (12, 30, 31). To assess protein phosphorylation, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS), and then phosphorylated proteins were either immunoprecipitated and blotted with anti-phosphotyrosine antibody (4G10, UBI) or directly blotted in cell lysates using phospho-specific antibodies. Rac1 activation was assessed with a glutathione S-transferase (GST)-PBD pull-down assay kit (UBI).

Nude mouse xenograft assays. For ectopic analysis, five nude mice were each injected s.c. at two sites with MDA-MB-231 cells (1×10^6 per site). For orthotopic analysis, MDA-MB-231 cells were injected into mammary fat pads of 10 nude mice (two sites each, 7.5×10^5 cells per site). Tumor sizes were measured with calipers and volumes were calculated ($L \times W \times H \times 0.52$). Animals were maintained until tumors reached a width of 2 cm or mice became moribund. Apoptotic index was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, using the DeadEnd Fluorometric TUNEL System (Promega Co.), on formalin-fixed, paraffin-embedded tumor sections.

Results

CD151 in normal and malignant human mammary epithelial cells. Samples of normal and malignant human breast tissues were analyzed for CD151 protein expression (Fig. 1A). In normal breast tissue, CD151 was in the basal-myoepithelial cell layer surrounding both ducts and lobular alveolae (Fig. 1A-a, b). Staining was predominantly cytoplasmic with regions of basal or basolateral membrane accentuation. By comparison, a range of CD151 patterns were seen in human breast tumor tissue microarray samples. Some tumors showed absent (score 0, Fig. 1A-c) or only modest CD151 protein (score 1+, Fig. 1A-d). Others showed moderate to high CD151 (score 2+, Fig. 1A-e, f; score 3+, Fig. 1A-g, h). CD151 localization varied from predominantly membranous (Fig. 1A-e, g) to mostly cytoplasmic (Fig. 1A-f, h). CD151 was overexpressed in 31% of invasive breast tumors and significantly correlated with high tumor grade and estrogen receptor negativity (Fig. 1B; Supplementary Table S1). Estrogen receptor-negative/HER2-negative tumors showing the highest

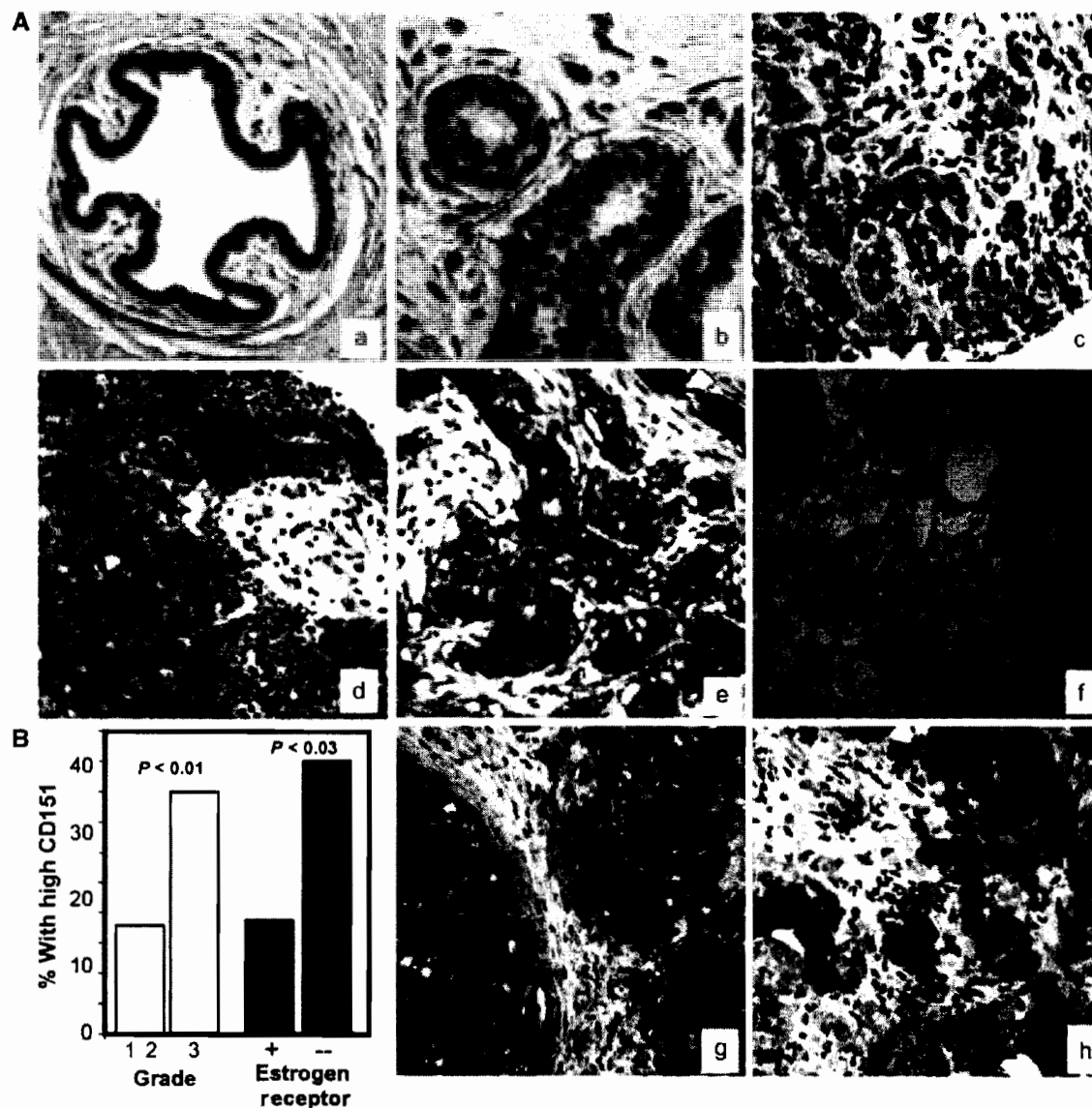


Figure 1. CD151 protein expression in human breast carcinoma. **A**, immunohistochemistry for CD151 was done on paraffin sections of tissue microarrays containing samples of normal breast tissue (*a* and *b*) and invasive breast tumors (*c*–*h*). *a*, normal breast duct ($\times 40$). *b*, normal breast lobule ($\times 100$); arrows, representative CD151-positive cells in the basal/myoepithelial layer. *c* and *d*, human tumors with absent and low degree of CD151 immunostaining, respectively ($\times 40$). *e* and *f*, human tumors with moderate 2+ overexpression of CD151 predominantly located on the membrane or in cytoplasm, respectively ($\times 40$). *g* and *h*, human tumors with marked 3+ overexpression of CD151 on the membrane or in cytoplasm, respectively ($\times 40$). **B**, human breast cancer tissue microarray samples (a total of 124 patients) were subdivided according to modified Bloom-Richardson grade and estrogen receptor protein expression. The percent of each subgroup expressing high CD151 (3+ or 2+) is indicated. For further details, see Supplementary Table S1.

proportion of CD151 overexpression (45% in Supplementary Table S1) were also entirely progesterone receptor negative and contained a basal-like gene expression profile (data not shown). Estrogen receptor-negative/HER2-positive tumors also showed elevated CD151 expression (Supplementary Table S1, 40%). By contrast, luminal tumors (estrogen receptor positive and HER2 negative) had the lowest proportion of CD151 expression (45% versus 17%, respectively; $P < 0.003$). CD151 expression was not associated with patient age, tumor size, ductal or lobular histology, lymph node metastasis, the presence of peritumoral lymphovascular invasion, or HER2 overexpression in this cohort of patients. Long-

term outcome and distant metastatic recurrence data are not yet available.

CD151 effects on mammary cell invasion and motility. For CD151 functional studies, we focused mostly on two distinct, but prototypical, myoepithelial-basal derived mammary cell lines: immortalized MCF-10A and malignant MDA-MB-231. Treatment with siRNA reduced CD151 protein levels by >90% in MCF-10A cells, as seen by blotting (Fig. 2A, right), metabolic labeling (Fig. 5B), or flow cytometry (data not shown). Mobilization of MCF-10A cells into a gap (scratched into a confluent cell monolayer) was reduced by nearly 50% for CD151 siRNA-treated

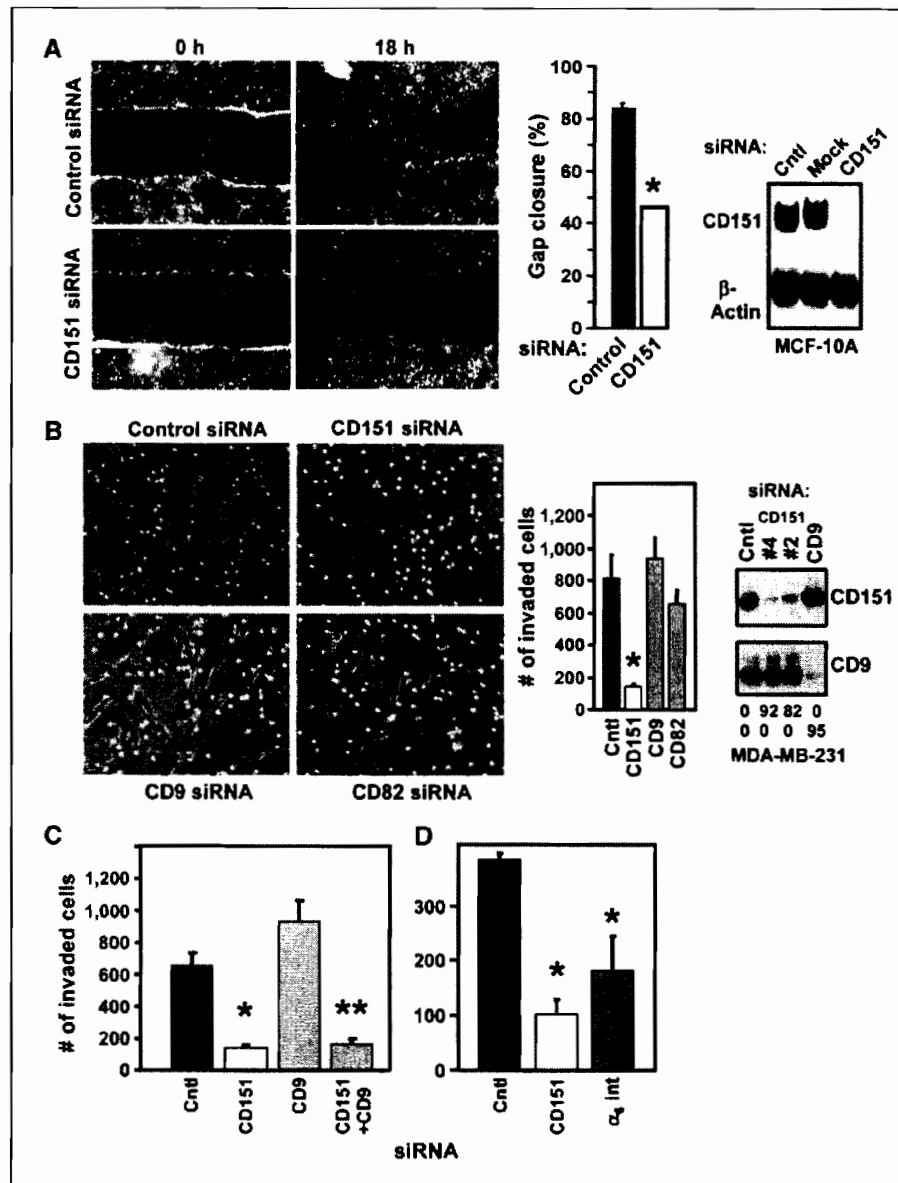
cells compared with control cells (Fig. 2A). Similar results were obtained when CD151 was stably silenced (>95%) by stable shRNA expression in MCF-10A cells (data not shown). Knockdown of integrin α_6 , but not integrin α_3 , essentially eliminated motility (data not shown). Because α_6 mostly associates with β_4 in MCF-10A cells (data not shown), motility in Fig. 2A must depend on integrin $\alpha_6\beta_4$. Neither proliferation nor survival of MCF-10A cells was affected by CD151 silencing (data not shown).

CD151 was also silenced in MDA-MB-231 cells (>80–90%; Fig. 2B, right), resulting in 78% reduction of invasion through Matrigel-coated transwell chambers (Fig. 2B). Stable silencing of MDA-MB-231 CD151 (by shRNA) yielded similar results (data not shown). By contrast, silencing of tetraspanins CD82 and CD9 minimally reduced invasion (Fig. 2B). Although CD9 silencing slightly elevated MDA-MB-231 invasion (Fig. 2B and C), silencing of CD9 and CD151 together mimicked the CD151 effect (Fig. 2C), suggesting that CD151 silencing is dominant. We also silenced CD151 (by ~90%)

in malignant mouse breast cancer J110 cells. Again, invasion through Matrigel was significantly reduced (Fig. 2D). Knockdown of α_6 integrin protein (by 80–90%; data not shown) also caused a >50% reduction in invasion by J110 cells (Fig. 2D), and α_6 integrin silencing caused a >47% decrease in invasion by MDA-MB-231 cells (data not shown). Hence, CD151 contributes considerably to α_6 integrin-dependent motility and invasion in multiple mammary cell lines.

CD151 effects on integrin-dependent cell spreading and EGF stimulation. MDA-MB-231 cells spread on laminin-1 in an integrin-dependent manner (i.e., spreading was blocked by anti-integrin α_6 antibody; data not shown). This spreading was increased (~31–63%) on stimulation with EGF, which can activate integrin functions (Fig. 3A and B). By contrast, cells lacking CD151 showed lower initial spreading (~8%) that was not stimulated by EGF (Fig. 3A and B). CD151 ablation did not affect cell spreading on fibronectin (Fig. 3C), and MDA-MB-231 cells did not spread on

Figure 2. CD151 supports mammary epithelial cell migration and invasion. **A**, after treatment with siRNAs, MCF-10A cells, grown to confluence, were then incubated in a 24-well plate with serum-free medium containing 10 μ g/mL mitomycin C at room temperature for 1 h. After gaps were scratched into cell monolayers, 0.5 mL of serum-free medium containing 10 ng/mL EGF was added, and gaps were evaluated after 0 and 18 h at 37°C. Percentage of gap closure was determined by measuring the mean change in gap width at three representative sites in three independent experiments ($n = 3$), $P < 0.05$. **Right**, siRNA knockdown efficiency. MCF-10A cells in 24-well plates were treated with siRNAs [mock, control (Cntrl), or CD151#4] for 5 d, and cell lysates (in RIPA buffer) were blotted for CD151 (mAb 1A5) and β -actin. **B**, after treatment with siRNAs, MDA-MB-231 cells (5×10^4) in serum-free medium containing 0.1% BSA were added to the top of Matrigel-coated transwell chambers. Serum-free medium (0.75 mL) containing 10 ng/mL EGF and 0.1% BSA was added to the bottom of transwell chambers. After ~18 h at 37°C, cells that had invaded through the Matrigel were fixed, stained, and photographed, and the mean number of invaded cells was determined from triplicate chambers. Bar, 100 μ m. **Right**, siRNA efficiency. MDA-MB-231 cells were treated with siRNAs and then lysates were blotted for tetraspanins CD151 (mAb 1A5) and CD9 (mAb MM2/57). The two rows of numbers below the figure indicate percent knockdown values for CD151 and CD9, respectively, as determined by densitometry. **C**, after treatment with siRNAs (alone or in combination), MDA-MB-231 cells were again analyzed for invasion through Matrigel, as in **B**. **D**, a metastatic mouse mammary tumor cell line (J110) was treated with siRNA to murine CD151 (70% knockdown) or murine α_6 integrin (>80% knockdown). Invasion was then analyzed as in **B**. *, $P < 0.05$; **, $P < 0.01$.



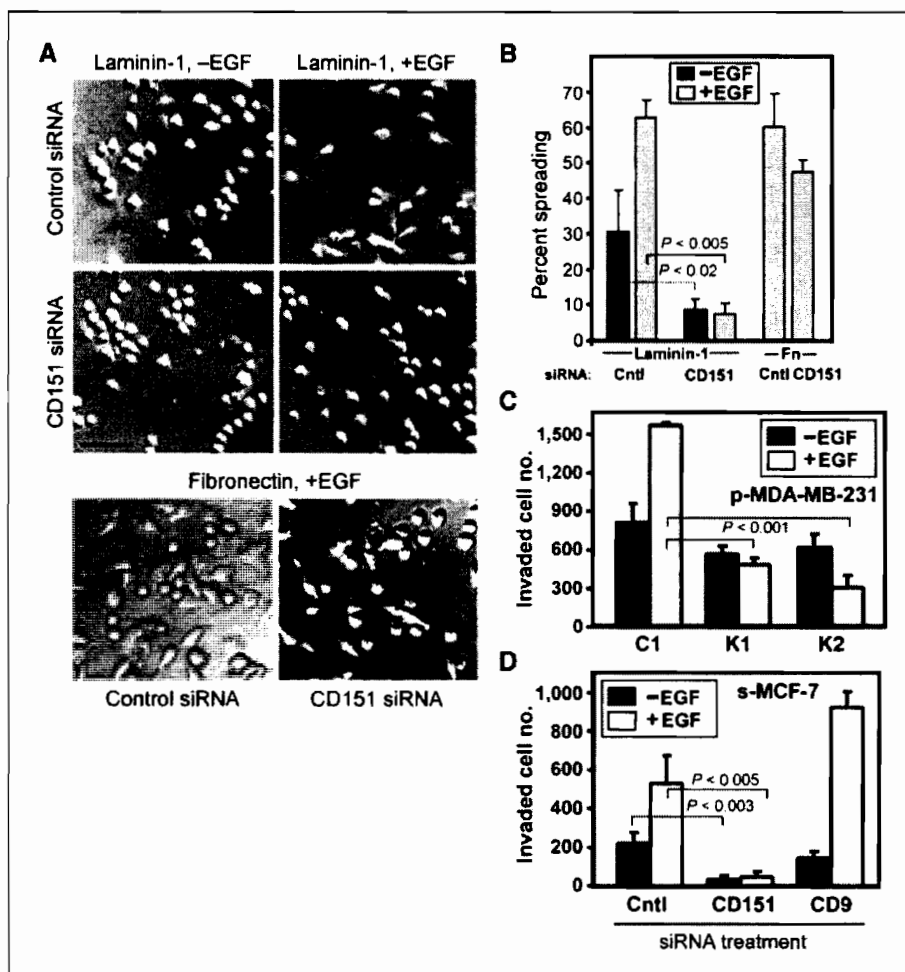


Figure 3. CD151 effects on EGF-stimulated mammary cell spreading and invasion. *A*, after siRNA treatment, MDA-MB-231 cells were suspended in serum-free medium at 37°C for 45 min, and then plated onto laminin-1- or fibronectin (Fn)-coated 24-well plates with or without EGF (10 ng/mL). After 45 min, representative fields were photographed. *B*, percentages of spread cells were determined ($n = 4$). Note: No cell spreading was observed on plastic surfaces coated with BSA (data not shown). *C*, invasion was also analyzed (as in Fig. 2*B*) with or without EGF (10 μ g/mL) added to the bottom of the invasion chamber. After tumor formation in nude mice, MDA-MB-231 cells were reisolated (now called p-MDA-MB-231). These passaged sublines were isolated from tumors originating from MDA-MB-231 cells that had been treated with control shRNA (C1) or CD151 shRNA (K1 and K2; see also Fig. 6*C*). *D*, a subtype of MCF-7 was enriched for Hoechst dye exclusion and elevated EGFR (now called s-MCF-7) and was analyzed for invasion as in *C*.

BSA-coated surfaces (data not shown). EGF stimulation also failed to rescue defective Matrigel invasion caused by CD151 ablation, as seen in p-MDA-MB-231 (Fig. 3*C*), s-MCF-7 (Fig. 3*D*), and BT549 (data not shown) cells. In these multiple mammary cell lines, invasion was stimulated by EGF when CD151 was present, but was not stimulated when CD151 was ablated. Two additional stimulators, phorbol 12-myristate 13-acetate (PMA) and insulin-like growth factor I (which also activate integrins via inside-out signaling), showed similar inability to overcome CD151 depletion effects on MDA-MB-231 cell invasion and spreading (data not shown).

CD151 affects cell signaling. Treatment of MDA-MB-231 cells with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]-pyrimidine, a specific inhibitor of Src family kinases, completely abolished cell spreading on laminin-1 substrate (data not shown), suggesting a role for Src family kinase-mediated tyrosine phosphorylation. Within 30 to 60 minutes after plating on laminin-1, CD151-silenced MDA-MB-231 cells showed reduced tyrosine phosphorylation of FAK (Fig. 4*A*), a kinase crucial for tumor invasion (32). Surprisingly, CD151 ablation did not affect activation of *src* (which typically modulates FAK), as assessed by blotting with anti-pY416 (present in Src, Fyn, and Yes kinases), after cell plating on laminin-1 or fibronectin (data not shown). Phosphorylation of FAK-Y925, which is mediated by Src (33), was also unaffected (data not shown), although overall FAK tyrosine

phosphorylation was diminished (Fig. 4*A*). However, CD151 ablation did diminish activation (at Y505) of *lck*, an Src family kinase member implicated in mammary tumor progression (ref. 34; Fig. 4*A*, bottom).

The Rac and Akt signaling pathways exert major influence on cell morphology, motility, and migration (35, 36). Consistent with this, CD151 ablation markedly reduced integrin-dependent Rac1 activation in MDA-MB-231 cells plated on Matrigel for 30 minutes (Fig. 4*B*). However, Akt activation was not notably altered in CD151-silenced cells plated on Matrigel (Fig. 4*B*), although CD151 and associated integrins modulate Akt activation in other cell types (see Discussion) and the PI3K/Akt pathway is critical for MDA-MB-231 cell invasion (37).

CD151 affects integrin subcellular distribution, but not expression levels. We assumed that the CD151 effects seen in Figs. 2–4 arise due to CD151 effects on laminin-binding integrins. However, silencing of CD151 did not affect either the surface expression or activation of $\alpha_6\beta_1$, $\alpha_3\beta_1$, or $\alpha_6\beta_4$ on either MCF-10A or MDA-MB-231 cells (data not shown). Furthermore, amounts of α_3 and α_6 integrins were unchanged, as seen by biosynthetic labeling and immunoblotting (Fig. 5*B*). Hence, although CD151 can closely associate with laminin-binding integrins such as $\alpha_3\beta_1$ and $\alpha_6\beta_4$, it is not required for their expression or activation.

Next, we analyzed CD151 ablation effects on integrin distribution in MCF-10A cells. As seen in ventral sections, integrin α_6 and

CD151 are present in broad patches aligned near cell-cell boundaries (Fig. 5A, a-c). However, CD151 depletion markedly diminished this pattern of staining as bands of α_6 became thinner and more proximal to cell-cell boundaries, whereas CD151 staining itself was greatly diminished (Fig. 5A, d-f). By contrast, CD151 depletion minimally affected integrin α_3 staining (Fig. 5A, compare g-i with j-l) and did not affect integrin α_2 staining (Fig. 5A, m, n). Hence, CD151 markedly affects the subcellular distribution of α_6 integrins (which in this case is mostly $\alpha_6\beta_4$).

CD151 affects integrin associations with other proteins. CD151 may link laminin-binding integrins to other proteins within the plasma membrane (38, 39). Hence, we tested whether CD151 depletion would disconnect α_3 and α_6 integrins from cell-surface partners. Metabolic labeling with [3 H]-palmitate was carried out because tetraspanins and many of their partner proteins are typically palmitoylated, and this method of labeling has proved to be more informative than other types of labeling (12, 30, 31). From [3 H]-palmitate-labeled MCF-10A lysate, recovery of $\alpha_6\beta_4$ integrin was not diminished on ablation of CD151 [Fig. 5B, lanes 5-7; see β_4 (top) and α_6 immunoblot (third row)]. However, recovery was

diminished for CD151 itself, tetraspanins CD9 and CD81, and at least five other proteins (white arrowheads, lane 6). Similarly, immunoprecipitation of α_3 integrin was not diminished (Fig. 5B, second row, lanes 2-4), but levels of CD151 and nearly all other associated proteins were decreased (Fig. 5B, top, lane 3). Diminished recovery of CD9 as an integrin partner, due to CD151 ablation, was confirmed by CD9 immunoblotting (see Figs. 5B, bottom, lanes 2, 3 and 5, 6). In addition, immunoprecipitation of α_6 integrin yielded a small amount of α_3 (Fig. 5B, second row, lanes 5 and 7), which was lost when CD151 was ablated (lane 6), whereas α_2 integrin yielded no prominent proteins, consistent with α_2 not associating with tetraspanins (Fig. 5B, lane 1). As shown here for MCF-10A cells (Fig. 5B), α_3 and α_6 integrin complexes were similarly disrupted on CD151 ablation in MDA-MB-231 cells (data not shown). Together, these results strongly support a critical role for CD151 in linking α_3 and α_6 integrins to multiple components within tetraspanin-enriched microdomains.

CD151 accelerates MDA-MB-231 tumor progression *in vivo*. Soft agar assays were carried out using 5,000 and 10,000 MDA-MB-231 cells per 60-mm dish. No differences were observed in colony numbers, size of colonies, or rate of colony development between control and CD151-knockdown cells. Next, we tested whether CD151 affects tumor progression *in vivo*, using MDA-MB-231 nude mouse xenograft models. In a preliminary ectopic (s.c.) injection experiment, nude mice were injected with MDA-MB-231 cells expressing either control shRNA or CD151 shRNA. Tumors arising from control MDA-MB-231 cells appeared by 8 to 9 weeks, whereas CD151-ablated cells did not yield detectable tumors until 11 to 12 weeks (Fig. 6A). MDA-MB-231 cells were also injected into mammary fat pads, and primary tumor growth was analyzed (Fig. 6B). Again, tumor appearance and growth were markedly delayed (by 4-5 weeks) in mice injected with CD151-ablated cells. However, analysis of H&E-stained slides from several tumors revealed no obvious morphologic differences between tumors formed from CD151-positive and CD151-ablated MDA-MB-231 cells. In addition, from several representative tumors, formalin-fixed paraffin-embedded slides were prepared, and TUNEL staining was carried out to detect apoptotic cells. Although there was a trend toward a higher apoptotic index in CD151-ablated tumors, results did not reach statistical significance (data not shown). In addition, cells were recovered from independent MDA-MB-231 tumors from both control mice (C1 and C2) and CD151-knockdown mice (K1, K2, and K3) and then cultured *in vitro*. Blotting of CD151 confirmed that control MDA-MB-231 cells indeed contained abundant CD151, whereas CD151-ablated cells expressed little or no CD151 (Fig. 6C).

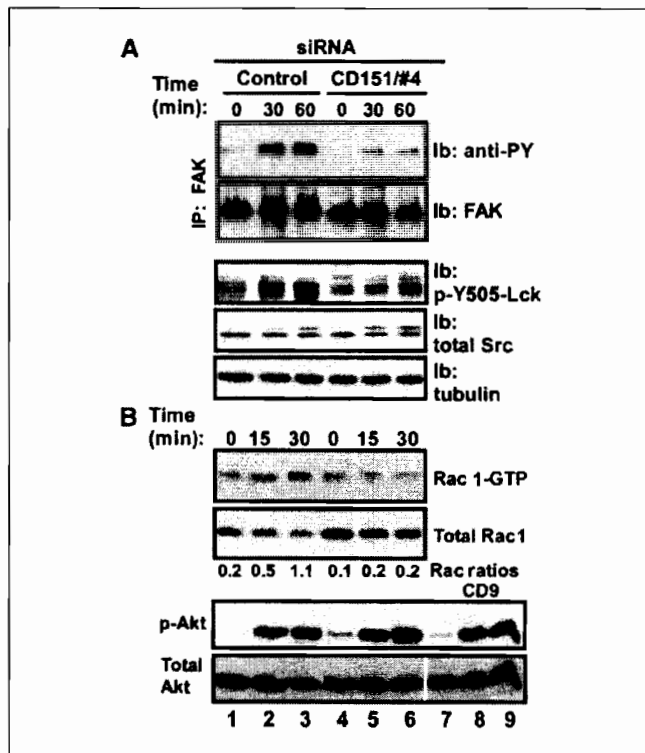


Figure 4. Impact of CD151 ablation on integrin-mediated tyrosine phosphorylation cascade. **A**, after siRNA treatment, MDA-MB-231 cells were detached, washed, and suspended in serum-free DMEM containing 0.1% BSA. Cells were then kept in suspension for 45 min at 37°C to remove residual growth factor effects before being plated on laminin-1. At the indicated times, cells were lysed in RIPA buffer. After immunoprecipitation of FAK from MDA-MB-231 cells, we analyzed FAK tyrosine phosphorylation (mAb 4G10) and total FAK by immunoblotting. Total cell lysates were also probed for activated Lck (p-Y505-Lck), total Src, and tubulin. **B**, to assess activation of small GTPase Rac1, cell lysates (prepared as in A) were incubated with GST-PBD beads (45 min, 4°C) to recover activated Rac1 (Rac1-GTP form). Beads were washed and boiled, and released proteins were blotted with anti-Rac1 antibody (top). Total Rac1 protein in lysates was also blotted to serve as a control (bottom). Note that the observed increase in total Rac1 is due to unequal dilution of starting lysates; the actual amount of Rac1 is unchanged. Activated Akt (p-Akt) and total Akt were also blotted with antibodies to phospho-Akt (p-S473) and total Akt.

Discussion

A role for CD151 in breast cancer had not previously been shown. Here we show that CD151 overexpression occurs, at least to some extent, in all subtypes of human breast cancer, with expression most significantly elevated in patient tissue samples that were of high grade and/or of the estrogen receptor-negative type. Among estrogen receptor-negative samples, CD151 was elevated most frequently in triple-negative basal-like tumors. Here we focused mostly on the role of CD151 in basal-like cells. Its role in other types of mammary cells and tumors will be addressed elsewhere.

Not only is CD151 significantly up-regulated in basal-like human breast cancer samples but it also seems to play a functional role.

CD151 depletion, via RNA interference, caused a marked delay in tumor formation by MDA-MB-231 cells, as seen in both ectopic and orthotopic xenograft models. Hence, CD151 seems to accelerate mammary tumor progression in this basal-type cell line. Elevated CD151 expression was previously linked to poor prognosis in human lung (40) and prostate cancers (17). However, CD151 had not previously been shown to promote *in vivo* tumor progression in breast cancer or in any other type of cancer. Depletion of CD151 had no effect *in vitro* on proliferation or survival of MDA-MB-231 cells. Furthermore, *in vivo* studies showed that MDA-MB-231 tumor morphology was not altered, and apoptosis was not significantly increased in tumors formed from CD151-ablated cells. Hence, CD151 most likely affects the early stages of MDA-MB-231 tumor formation, in which cells initially encounter the extracellular matrix and invade into surrounding mammary fat pad tissue.

Consistent with this, CD151 indeed affected the invasion and migration by basal-like mammary cells.

To learn how CD151 functions, we carried out *in vitro* studies using two different human basal-like mammary cell lines (immortalized MCF-10A and malignant MDA-MB-231 cells), with supporting results obtained using a few other cell lines. On ablation of CD151, but not other tetraspanins, MDA-MB-231 cell invasion through Matrigel was decreased by >80%. Support of invasion by CD151 is consistent with *in vitro* results seen in other tumor cell types (17, 40). In MCF-10A cells, removal of CD151 markedly impaired cell migration, consistent with a promigratory role for CD151 in epidermal carcinoma cells (20) but contrasting with antimigratory roles for CD151 in other cells (12, 19). CD151 is known to support adhesion strengthening (5), and cell migration is biphasic with respect to adhesion strength. Hence, we suggest that

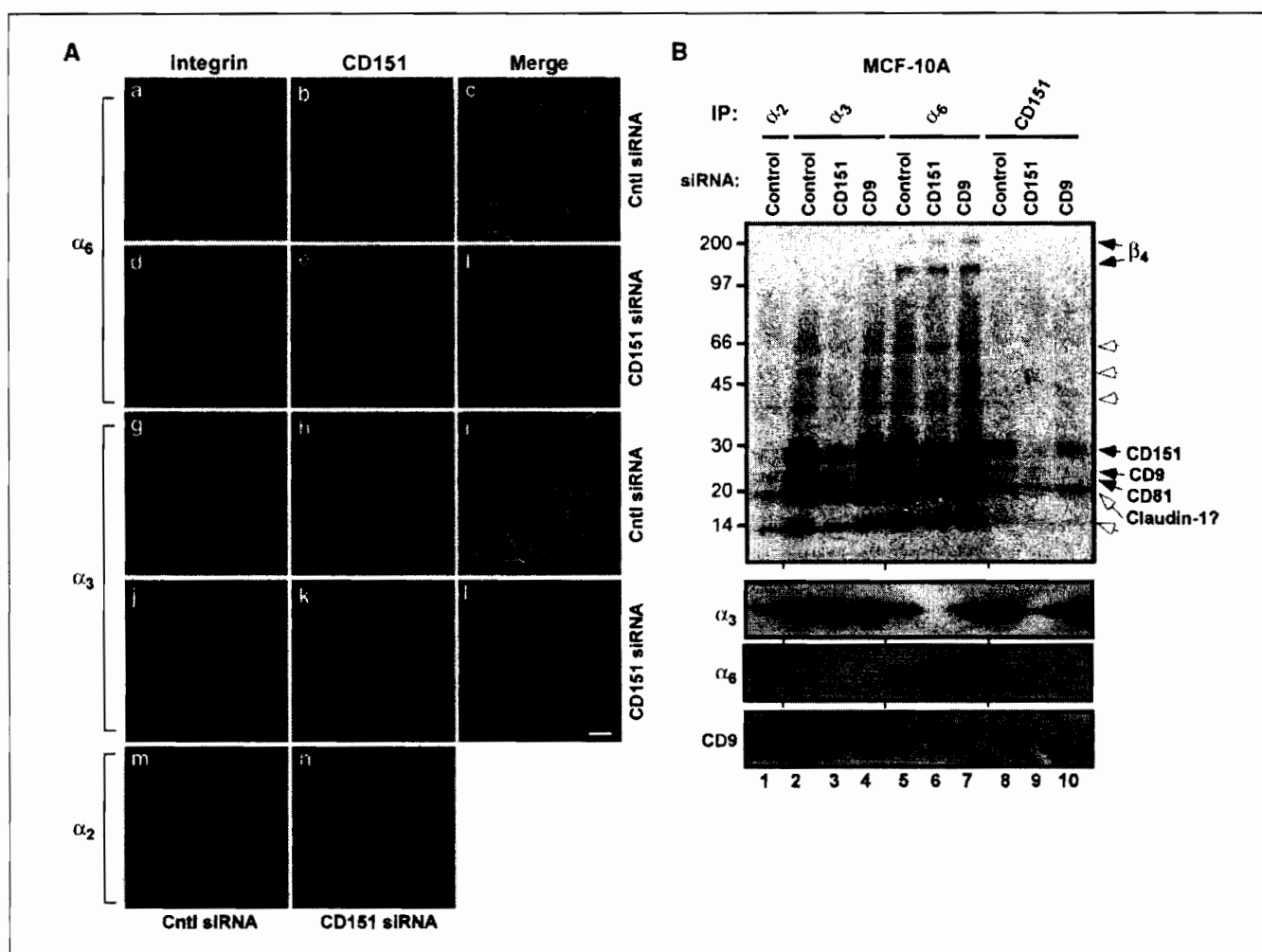
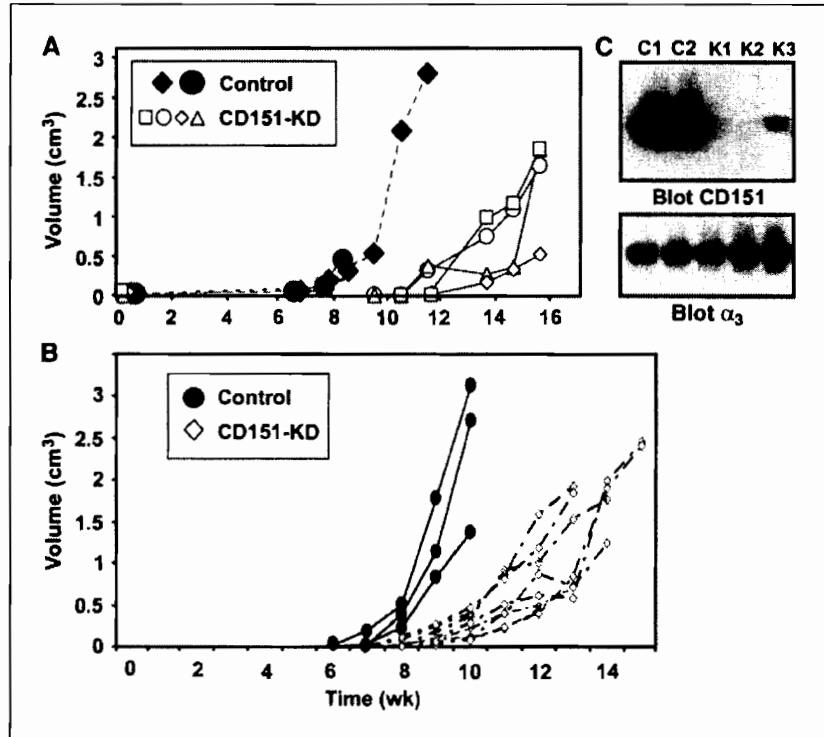


Figure 5. CD151 affects the molecular organization of integrins. **A**, MCF-10A cells were initially seeded onto coverslips and treated with siRNAs for 5 d. Live MCF-10A cells were incubated with primary anti-integrin antibodies for 1 h at 4°C and then stained with Alexa Fluor 594-conjugated secondary antibody. After washing, cells were further stained with FITC-conjugated CD151 antibody. After staining, cells were fixed in 2% paraformaldehyde (20 min, 4°C) and mounted on slides using Prolong antifade solution (Molecular Probes) and then ventral sections were visualized by confocal microscopy. Antibodies used were mAb GoH3 (integrin α₆; a and d), mAb X8 (integrin α₃; g and j), mAb IIE10 (integrin α₂; m and n), and mAb 5C11 (CD151; c, f, i, and l). **Right**, merged green and red staining. Ventral sections of cells were visualized by confocal microscopy. a–c, g, h, and m, treated with control siRNA; d–f, j–l, and n, treated with CD151 siRNA. **Bar**, 50 μm. **B**, MCF-10A cells were treated with siRNAs, labeled with [³H]-palmitate, and then lysed in 1% Brij-96 buffer. Immunoprecipitations of α₂, α₃, and α₆ integrins and CD151 were carried out with mAbs IIE10, X8, GoH3, and 5C11. Note that the integrin β₁ subunit does not appear because it does not undergo palmitoylation. **Bottom**, immunoblots for α₃, α₆, and CD9, present in the immunoprecipitated complexes. The identity of CD81 (top) was confirmed by immunoblotting (data not shown). We assume that the band just below CD81 is claudin-1 because it is known to be palmitoylated and it associates closely with CD9 (50) and thus would be recruited via CD151 into a complex with α₃ and α₆ integrins.

Figure 6. CD151 accelerates tumor formation *in vivo*. **A**, MDA-MB-231 cells expressing either control or CD151 shRNA were then injected s.c. into nude mice, and tumor formation was monitored. **B**, MDA-MB-231 cells expressing shRNA were injected into mammary fat pads of nude mice. Mice were terminated when they became moribund or when tumors reached 2 cm (in any dimension). Statistical significance was analyzed with the log-rank test. **C**, after tumor formation in nude mice, MDA-MB-231 cells were reisolated and cultured *in vitro*. From these sublines (C1 and C2 from control-shRNA expressing cells; K1, K2, and K3 from CD151-knockdown cells), cell lysates were prepared and blotted for CD151 (with mAb 1A5) and integrin α_3 (with rabbit polyclonal antibody).



removal of CD151 may either impair or enhance cell migration depending on whether initial adhesion strength conditions are optimal or excessive, respectively.

CD151 closely associates with laminin-binding integrins ($\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$) and affects their functions (3–5). Using mammary cell lines, we found CD151 association with $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ integrins, and silencing of CD151 affected cell migration, invasion, spreading, and signaling on laminin, but not fibronectin. In our studies, depletion of CD151 from MDA-MB-231 cells mostly modulated α_6 integrin functions. Elsewhere, MDA-MB-231 invasion and migration were shown to be $\alpha_6\beta_4$ dependent (e.g., ref. 41). However, we cannot rule out contributions also from $\alpha_3\beta_1$. Like CD151, laminin-binding integrins play a functional role in mammary tumors (24, 42). Furthermore, like CD151, laminin-binding integrins are elevated in human breast cancer (26), with $\alpha_6\beta_4$ being a major marker of estrogen receptor-negative basal-like mammary tumors (25, 43).

Thus far, little insight has emerged about the mechanisms by which CD151 affects integrins. Although CD151 expression might affect integrin turnover (20), neither we nor others (12, 20, 21) observed any effect on integrin expression levels. Results elsewhere have suggested that the effects of CD151 on $\alpha_3\beta_1$ integrin activation might underlie its effects on cell adhesion (21). However, removal of CD151 did not diminish an integrin β_1 epitope commonly associated with integrin activation (data not shown). Here we show that CD151 affects the distribution and biochemical organization of α_6 integrins on mammary cell lines. On MCF-10A cells, removal of CD151 altered α_6 integrin localization to the cell periphery. Removal of CD151 also diminished the associations of α_6 and α_3 integrins with at least five other proteins, including other tetraspanins (CD9 and CD81). These results are consistent with CD151-integrin complexes functioning in the context of a larger constellation of proteins known as tetraspanin-enriched micro-

domains (1, 6). Major alterations in the integrin microenvironment, due to CD151 depletion, help to explain changes in integrin-dependent cell migration, invasion, spreading, and signaling.

On CD151 silencing, we observed diminished signaling through Rac1 and FAK in MDA-MB-231 cells plated on laminin-1. Because Rac1 and FAK typically play critical roles during invasion and migration, these results are consistent with CD151 silencing affecting mammary cell invasion and migration. Laminin-binding integrins and CD151 itself (12) can also markedly affect signaling through the PI3K/Akt pathway. Indeed, treatment of MDA-MB-231 cells with PI3K inhibitor Ly294002 almost completely abolished spreading and migration on laminin-1 substrate (data not shown). Hence, it was surprising that CD151 depletion did not decrease Akt signaling in MDA-MB-231 cells. One possibility is that the abundance of constitutively activated Ras found in MDA-MB-231 cells (44) maintains Akt in an activated state regardless of CD151. In an unexpected finding, CD151 ablation decreased activation of Lck but not Src (or Fyn or Yes). Although Src typically contributes to FAK signaling, Lck can also contribute (45), suggesting that CD151 depletion in MDA-MB-231 cells may impair Lck-FAK rather than Src-FAK signaling. Lck was recently implicated as playing a role during mammary tumor progression (34). Thus, decreased signaling through Lck may also contribute to the functional effects of CD151 depletion on MDA-MB-231 cells.

Functional and physical collaboration between α_6 integrins and ErbB receptors has been noted (46, 47). For example, EGF stimulation of epithelial cells disrupts hemidesmosomes, releasing $\alpha_6\beta_4$ to participate in cell motility and invasion (46, 48). We have not observed direct physical association of α_6 integrins with ErbB receptors. Nonetheless, three results suggest that CD151 depletion disrupts integrin collaboration with EGFR: (a) Ablation of CD151 diminished EGF-dependent MCF-10A cell migration. (b) CD151 removal caused cell invasion and spreading deficits (in three

different cell types) that could not be overcome by adding EGF, and in fact, (c) EGF no longer stimulated cell spreading and/or migration at all in CD151-silenced cells. These results are particularly relevant for basal-like mammary tumors because they tend to show elevated EGFR (25). Phorbol ester treatment also did not overcome CD151-knockdown effects on cell invasion and spreading (data not shown). Impaired responses to EGF and PMA, arising from CD151 silencing, again may be due to disruption of the α_6 integrin microenvironment. In this regard, EGFR and protein kinase C (a target of PMA) have previously been linked to tetraspanins such as CD82, CD81, and CD9 (1, 49). Hence, ablation of CD151 may lead to diminished integrin proximity for these other tetraspanins and their associated signaling molecules.

In conclusion, we show here that CD151 is significantly elevated in multiple subtypes of breast cancer, including the basal-like subtype. Using prototype basal-like mammary cell lines, we show that CD151 contributes to mammary tumor progression. Whereas we mostly focused on basal-like mammary cells, CD151-depletion also affected invasion and/or EGF responsiveness for two estrogen receptor-positive mammary cell lines [murine J110 cells (29) and an EGF-sensitive subline of MCF-7]. These results suggest that

CD151 can also contribute to other types of breast cancer. In terms of mechanism, CD151 determines the molecular organization of laminin-binding integrins on the cell surface, thereby affecting integrin-dependent mammary cell morphology, migration, invasion, adhesion, signaling, EGFR cross talk, and ultimately, tumor progression *in vivo*. A previous study showed that CD151 could enhance tumor progression by supporting pathologic angiogenesis in host mice (12). Now we show that tumor cell CD151 also plays a key role, thus pointing to multiple levels of CD151 contributions, and emphasizing that CD151 may be a high-priority therapeutic target in certain breast cancers.

Acknowledgments

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