CD44 Attenuates Metastatic Invasion during Breast Cancer Progression

Jose I. Lopez, Todd D. Camenisch, Mark V. Stevens, Barbara J. Sands, John McDonald, and Joyce A. Schroeder

Abstract

Metastatic invasion is the primary cause of breast cancer mortality, and adhesion receptors, such as CD44, are believed to be critical in this process. Historically, primary breast tumor epithelium has been investigated in isolation from other tissue components, leading to the common interpretation that CD44 and its primary ligand, hyaluronan, promote invasion. Here, we provide in vivo evidence showing CD44 antagonism to breast cancer metastasis. In a mouse model of spontaneously metastasizing breast cancer (MMTV-PyV mT), we found that loss of CD44 promotes metastasis to the lung. Localization studies, in combination with a novel hyaluronan synthase-GFP transgenic mouse, show a restricted pattern of expression for CD44 and hyaluronan. Whereas CD44 is expressed in tumor epithelium, hyaluronan synthase expression is restricted to stromal-associated cells. This distinct CD44 and hyaluronan pattern of distribution suggests a role for epithelial-stromal interaction in CD44 function. To define the relevance of this spatial regulation, we developed an in vitro invasion assay to emulate invasion into the extracellular matrix. Invasion of CD44-positive tumor cells was inhibited in hyaluronan-containing matrices, whereas blocking CD44-hyaluronan association increased invasion. Collectively, these data show that during breast cancer progression, hyaluronan-CD44 dynamics occurring through epithelial-stromal interactions are protective against metastasis.

Introduction

Metastatic invasion is the primary cause of patient mortality during breast cancer progression. For a transformed cell to metastasize to a distant site in the body, it must first lose adhesion, penetrate and invade the surrounding extracellular matrix (ECM), enter the vascular system, and adhere to distant organs (1). The ability of transformed cells to modulate their adhesion to the ECM is a key mechanism facilitating metastatic invasion to secondary sites.

CD44 (also known as homing cell adhesion molecule, PGP-1, Hermes antigen, and HUTCH-1) is a type I transmembrane glycoprotein receptor that binds primarily to the extracellular glycosaminoglycan, hyaluronan. The engagement of CD44 by hyaluronan results in intracellular signaling that has been linked to such diverse effects as cellular adhesion, migration, and invasion which are important in cancer progression, as well as hematopoietic development and wound healing (2–6). The wide variety of responses resulting from CD44-hyaluronan interactions may be attributed to the alternate expression of CD44 variant isoforms in different cell types and tissues. CD44 is a highly polymorphic protein due to the potential insertion of 10 variant exons into the extracellular portion of the protein (CD44v). Whereas the standard form of CD44 (CD44s) is widely expressed and hyaluronan is ubiquitous to most extracellular spaces, variant isoforms of CD44 have restricted expression to specific conditions such as transformation, wound healing, and lymphocyte activation (4). The insertion of variant regions into CD44 may confer distinct properties to CD44v such as a reduced affinity for hyaluronan and the ability to form homotypic interactions with CD44 on neighboring cells in CD44v8-10, or the binding of growth factors by CD44v3 (7, 8).

The last decade has produced varied observations concerning the expression of CD44 and its variants in cancer progression. Several studies have associated CD44 expression with tumor progression and metastasis, whereas others maintain that CD44-positive cells exhibit a favorable prognosis. Studies have found increased levels of CD44 variant isoform expression correlating with poor prognosis in diseases, such as thyroid cancer, prostate cancer, renal cell carcinoma, and breast cancer; whereas others have shown decreased levels of CD44 correlating with good prognosis in head and neck carcinomas, prostate, colon cancer, breast cancer, and neuroblastoma (9–20). In vitro data showing that the effects of CD44 engagement with hyaluronan during cancer progression and metastatic spread seem cell type specific and dependent upon CD44 isoform expression. Overexpression of CD44s in colon carcinoma cell lines results in decreased tumorigenicity and decreased liver metastasis, whereas transfection of CD44v6 into nonmetastatic pancreatic cancer cells leads to a full metastatic phenotype (21, 22). Similarly, overexpression of CD44s in lymphoma cells does not affect tumorigenesis and progression, whereas overexpression of variant CD44 isoforms leads to accelerated tumor formation and lymph node metastasis (23). These observations suggest a role for CD44v expression in metastatic spread even as CD44s may protect from metastasis in cancers of epithelial or lymphatic origin. Human studies focusing on the role of CD44 in breast cancer have indicated that CD44 expression cannot be confidently used as a reliable prognostic indicator (24). Collectively, these observations emphasize that the differing ability of CD44 to promote migration or adhesion may depend on isoform and ligand expression and highlight the complexity of cancer biology in the intact organism.

The source of ligand production is also important as epithelial versus stromal production of hyaluronan may alter CD44 activity. Whereas native hyaluronan is observed primarily in stromal...
compartments, studies driving exogenous expression of the three hyaluronan synthase enzymes (Has1-3) in fibroblasts and epithelial cells indicate a potential for transformation. Overexpression of any of the mammalian hyaluronan synthases leads to increased cellular growth and migration in vitro, although clones expressing higher levels of hyaluronan displayed inhibited cell growth (25–27). Whereas epithelial cell lines may retain the ability to produce hyaluronan in vitro, the increased levels of hyaluronan found in stromal compartments are most likely the product of fibroblasts because tumor cells can secrete factors to induce hyaluronan production by cells resident in the connective tissue (28–31). These studies exemplify the complex nature of cell adhesion proteins and their ligands in cancer progression and make it clear that the role of CD44 and hyaluronan in cell adhesion and migration can vary greatly when comparing studies on single cell populations done in two-dimensional assays to those done with multiple cell types in the presence of ECM.

In this investigation, we define the in vitro role of CD44 in breast cancer progression through the use of mouse models and complementary in vitro invasion studies. We have crossed CD44 null mice (32) onto the mouse mammary tumor virus–driven polyoma middle T antigen (MMTV-PyV mT) transgenic mice (33) in an effort to understand the epithelial/stromal interactions of CD44/hyaluronan in a physiologically intact and spontaneously arising tumor environment. MMTV-PyV mT mice develop stochastic, metastatic mammary gland tumors with an etiology and molecular phenotype strongly resembling human disease (34). By crossing these MMTV-PyV mT mice onto a CD44 null mouse background, we have determined that CD44 plays a protective role against metastatic invasion to the lung. Further analysis shows that the protective role of the CD44/hyaluronan interaction against metastasis may be based on an epithelial-stromal interaction that inhibits the invasion of transformed epithelium.

Materials and Methods

**Mice.** CD44 null mice were a kind gift from Tak Mak (University of Toronto; ref. 32) and maintained on a C57Bl/6 background. MMTV-PyV mT mice on both the FVB/N and C57Bl/6 background were a gift from William Muller (McGill University) and Sandra Gender (Mayo Clinic Scottsdale). F1 generation–outbred mice (C57Bl/6 X FVB/N) were analyzed for tumor and metastasis formation.

Has2-GFP mouse were generated by microinjection of a GFP-reporter construct under the direction of putative promoter/cis-acting elements of the Has2 allele. Briefly, site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) introduced a NotI restriction enzyme site into exon 2 of Has2 allele. The open reading frame (ORF) and bovine growth hormone polyadenylate tail of green fluorescent protein (GFP, pQBl, Quantum Technologies, Chelmsford, MA) had NotI linkers (New England Biolabs, Beverly, MA) placed onto Nhel and DraIII exposed ends to allow cloning into the site-directed fabricated NotI site within exon 2 of Has2. Identified clones were sequence verified for in-frame placement of the GFP-ORF. The construct was linearized and microinjected into fertilized FVB eggs and transferred into pseudopregnant female mice by standard methodologies. Seventeen of the GFP founder mice were confirmed by Southern blot analysis to carry the transgene. Multiple founder lines demonstrating GFP fluorescence and germ line transmission of the transgene were established and lines 54 and 64 were used in this study. These Has2-GFP mouse lines recapitulate endogenous Has2 gene expression.5 Mice were housed in microisolator cages and fed and watered ad libidum.

**Cell lines.** MDA-MB-231, MDA-MB-468, and BT-20 invasive breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum (Biomedical, Foster City, CA) and 1% penstrep-glutamine (Invitrogen, San Diego, CA) at 5% CO2 in a humidified chamber at 37°C.

**Immunofluorescence.** Tissues were dissected and fixed in 10% buffered formalin, transferred to 70% ethanol, embedded in paraffin, and sectioned by the Tissue Acquisition and Cellular/Molecular Analysis Shared Service at the Arizona Cancer Center. Sections (5 μm) were deparaffinized in xylenes and stained with the following: anti-CD44 PE-IM7.8.1 (1:100; Pharmingen, San Diego, CA), hyaluronic acid–binding protein (HARP)-biotinylated (1:200; U.S. Biological, Swampscott, MA), and Living Colors GFP monoclonal antibody (BD Biosciences, San Jose, CA).

**Green fluorescent protein analysis.** Tissues were dissected and placed in 1× PBS for visualizing and documenting GFP fluorescence using a Leica MZFLIII stereodissecting microscope and Magmáfrere Imaging Software.

**Protein analysis.** Cells were lysed in Triton X-100 buffer [20 mmol/L HEPES (pH 8.0), 150 mmol/L NaCl, 1.0% Triton X-100, 2 mmol/L EDTA, 2 mmol/L sodium orthovanadate, 50 μmol/L ammonium molybdate, 10 mmol/L sodium fluoride, and Complete protease inhibitor (Sigma, St. Louis, MO)]. Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL) and proteins were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA) and immunoblotted with anti-CD44 antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, CA).

**Invasion assays.** A collagen gel matrix (0.9 mg/mL; Type I rat tail collagen (BD Biosciences), 83.0% (v/v) M-199 medium (Life Technologies), 0.18% NaHCO3 (Fisher, Hampton, NH), ±0.75 μg/mL hyaluronan reconstituted in medium and boiled to inactivate contaminants (mean MW of 757,000 kDa, Calbiochem-Novabiochem Co., San Diego, CA) was poured into 24-well plates. Costar Transwell inserts (Corning, Cambridge, MA, 8-μm pore) were placed into the wells and gels were allowed to polymerize. Gels were then hydrated with DMEM (Life Technologies) supplemented with 20% fetal bovine serum (Biomedical) overnight. Cells were labeled with a 5.0 μL/mL Vybrant DiO solution (Molecular Probes, Eugene, OR) in serum-free DMEM for 30 minutes. Cells were washed twice in serum-free medium, trypsinized, counted, and placed in the upper chamber of the transwell insert and allowed to invade for the indicated time points. After invasion, the transwell inserts were removed from the plate and the quantity of invading cells into the gel matrix was determined by reading the fluorescence of DiO in a fluorescence plate reader at 485/538 nm (Spectramax Gomini, Molecular Devices, Sunnyvale, CA). CD44 blocking antibody (KM201, Southern Biotechnology) and IgG control (Rat IgG1, Southern Biotechnology) were used at 1.0 μg/100,000 cells.

**Metastasis evaluation.** Lungs were excised and fixed in 10% buffered formalin and transferred to 70% ethanol. Pulmonary lobes were dissected and metastatic foci were enumerated under a dissection microscope. To verify that these foci were lung metastases, lungs from eight independent mice were sectioned and stained with H&E. Metastatic foci were enumerated by multiple observers naive to the genotype of the tissue, under 100× magnification and 100% concordance was obtained with counting foci under the dissection microscope.

Results

**Absence of CD44 does not affect tumor onset or size in transgenic mice.** The metastatic MMTV-PyV mT transgenic mouse line (33) was crossed onto a CD44-deficient background (32) to understand how CD44 affects breast cancer progression in vivo. MMTV-PyV mT is a transgenic mouse line that results in the spontaneous development of multifocal, metastatic breast cancer due to the hyperactivation of a number of oncogenes, including Src, phosphatidylinositol-3 kinase, and Shc (33, 35). The MMTV-PyV mT mouse serves as an excellent model for human breast cancer progression because the MMTV-PyV mT

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5. T.D. Camenisch, data not shown.
transgenic mammary gland undergoes a multistep progression towards malignancy similar to that observed in human disease (34).

MMTV-PyV mT/CD44-/- mice (n = 23) and MMTV-PyV mT/CD44+/+ (n = 26) littermate controls were palpated once weekly for 16 weeks to assess mammary tumor onset. Mice were considered to have developed tumors when a tumor of ≥0.5 cm formed and did not regress upon subsequent palpation. Tumor growth rates between the MMTV-PyV mT/CD44-/- and MMTV-PyV mT/CD44+/+ mice were statistically similar (Fig. 1A), as were tumor sizes upon sacrifice (data not shown). Fifty percent of both CD44-/- and CD44+/+ mice developed mammary gland tumors at 13.5 weeks (Fig. 1A), with no considerable differences at other time points. The comparable onset of tumor growth rates between these two populations suggest that CD44 does not play a crucial role in either the initiation of transformation or the growth of established tumors in this model.

**Absence of CD44 expression promotes metastasis in MMTV-PyV mT transgenic mice.** Female MMTV-PyV mT transgenic mice develop lung metastases with complete penetrance when allowed to progress to advanced tumor development. Pulmonary metastases were compared and contrasted between MMTV-PyV mT mice on a CD44-/- or CD44+/+ background. We sacrificed both MMTV-PyV mT/CD44-/- (n = 27) and MMTV-PyV mT/CD44+/+ mice (n = 28) at 14 weeks of age and extracted the lungs. At this time point, only 60% of animals had formed tumors of ≥0.5 cm, whereas all animals exhibited tumors of ≤0.5 cm accompanied by glandular hyperplasia. This time point was chosen to amplify our power to detect an increase in metastases in the absence of CD44 because typically, 10% to 15% of wild-type MMTV-PyV mT mice have visible lung metastasis at this stage of tumor progression. We found that a striking 66% of MMTV-PyV mT/CD44-/- mice had developed lung metastasis at 14 weeks of age (Fig. 1B), contrasted to only 11% of MMTV-PyV mT/CD44+/+ littermates having visible lung metastasis at this age. This 5.7-fold difference is highly significant (P = 0.0005) as determined by a two-tailed Student's t test. In addition to an increase in the percentage of mice developing metastases at 14 weeks, MMTV-PyV mT/CD44-/- mice also developed a greater number of metastases per lung. MMTV-PyV mT/CD44-/- mice developed an average of 29 metastatic tumors per lung, whereas MMTV-PyV mT/CD44+/+ mice developed an average of 4.7 metastatic tumors per lung (Fig. 1C). The number of metastasis in MMTV-PyV mT/CD44-/- mice was highly variable with mice possessing as many as 99 pulmonary metastatic tumors, whereas MMTV-PyV mT/CD44+/+ mice developed a maximum of only 12 pulmonary metastatic tumors.

As this initial study was done on a mixed genetic background (FVB/N X C57BL/6), we next repeated the study on a fully inbred C57BL/6 background. We observed a similar increase in metastasis formation in the lungs of MMTV-PyV mT/CD44-/- (n = 9, 67% metastases) compared with MMTV-PyV mT/CD44+/+ (n = 19, 16% metastases). This second study indicates that the high metastatic potential of CD44 null mammary tumors is due to CD44 expression itself, instead of genetic modifiers as a result of differences in background strain.

Taken together, these results show a protective role for CD44 against breast cancer metastasis. Not only do fewer CD44-positive mice succumb to metastatic disease, but these mice also display a lower number of metastatic tumors in the lung compared with CD44-/- mice.
CD44 and hyaluronan have restricted and alternative localization in tumor tissues. Our in vivo data show a protective role for CD44 against the ability of primary breast cancer to form pulmonary metastases. To examine how CD44 inhibits metastatic progression, we examined the interaction between CD44 and its primary ligand, hyaluronan.

We first examined the expression patterns of both CD44 and hyaluronan in mammary tissue to determine if interactions between them could occur in vivo. Whereas CD44 is detected at low levels throughout the normal mammary gland (Fig. 2A, see ductal epithelium, arrow, and adipocytes; arrowhead), it is highly expressed throughout the primary mammary gland tumor epithelium (Fig. 2B, arrow). Alternatively, hyaluronan is localized mainly to the stromal area surrounding ductal epithelial cells in the normal mammary gland (Fig. 2C, arrow). Similarly, hyaluronan is found primarily in the stromal area surrounding tumors (Fig. 2D, arrowhead) and in the tumor capsule (Fig. 2E-F, arrowhead). Hyaluronan localization within the tumor epithelium itself was virtually undetectable (Fig. 2D, arrow). Therefore, whereas CD44 is expressed throughout the mammary gland tumor epithelium, hyaluronan is found almost exclusively in the ECM, indicating that CD44 may facilitate interactions between epithelial and stromal components, thereby antagonizing the ability of tumor cells to spread beyond the primary site.

Because of the strong ability of CD44 to attenuate metastatic invasion to the lung, we sought to determine whether hyaluronan expression was altered in lung metastasis of the MMTV-PyV mT/CD44−/+ mice compared with MMTV-PyV mT/CD44+/+ controls. We assessed metastatic tumors from both groups of mice with biotinylated HABP to determine if hyaluronan accumulation was altered due to loss of CD44. Both MMTV-PyV mT/CD44−/+ and MMTV-PyV mT/CD44+/+ pulmonary metastatic tumors showed similar patterns of hyaluronan deposition (Fig. 2E-F, arrowheads). Hyaluronan deposition was strongest in the stromal tissue surrounding tumor cells in both the MMTV-PyV mT/CD44−/+ and MMTV-PyV mT/CD44+/+ mice (Fig. 2E-F, arrowheads), a staining pattern similar to that seen in primary gland tumors. This indicates that hyaluronan deposition in the pulmonary metastases is not altered in absence of CD44 receptors.

Has2-GFP × MMTV-PyV mT bitransgenic mice express stromally derived green fluorescent protein expression. Detection of high levels of hyaluronan in the stromal area surrounding the tumor along with the minimal presence of hyaluronan within the tumor epithelium led us to hypothesize that tumor-associated hyaluronan is produced by stromal cells and not tumor epithelial cells. To test this, we employed the use of a transgenic mouse that expresses GFP under the control of the putative Has2 (a membrane bound hyaluronan synthase) promoter and cis-acting elements (Fig. 3A). The Has2-GFP transgenic reporter mice express GFP in areas previously determined to express Has2, such as in the embryonic epithelium and invading mesenchymal cells.

Has2-GFP mice were crossed with MMTV-PyV mT mice to generate bitransgenic animals. When viewed under a fluorescent dissection microscope, Has2 expression is visible in a subset of primary mammary tumors (Fig. 3B, arrow) as well as pulmonary metastatic tumors (Fig. 3C, arrow). Note that tumors (Fig. 3B, arrow) and metastases (Fig. 3C, arrow) displayed substantially higher GFP expression than the surrounding mammary gland (Fig. 3B, arrowhead) or lung parenchyma (Fig. 3C, arrowhead). This activation of the Has2 promoter would indicate that Has2 expression is specifically up-regulated in a tumor-specific manner. To determine what cell type in the tumor was expressing the GFP bitransgenic tumors were sectioned and examined for GFP expression patterns histologically using an anti-GFP antibody. Although intense GFP expression can be seen in the intact tumor, closer inspection shows that the expression is restricted to cells residing in the stromal compartment (Fig. 3D, arrow). These data indicate that it is the contribution of the stromal compartment that should be addressed if hyaluronan production is important in tumor development and metastatic invasion.

CD44 and hyaluronan interactions cause inhibition of invasion and migration in a three-dimensional in vitro invasion assay. Invasive disease requires epithelial cell penetration of the basement membrane followed by movement through the interstitial ECM. Based on our findings that hyaluronan production and expression is largely confined to the stromal area surrounding tumors, we sought to do an in vitro invasion assay that could simulate cellular movement through such layers. We developed a three-dimensional invasion assay based on a modified Boyden chamber assay. Briefly, a type I collagen gel is cast in the presence or absence of hyaluronan under an 8-μm pore filter. The chamber above the filter is seeded with fluorescently labeled breast cancer cells suspended in serum-free medium, whereas the gel under the filter is hydrated in medium containing 20% fetal bovine serum to induce chemotaxis. In this system, cells must both migrate through the filter pores as well as invade into the gel matrix, a process similar to invasive disease. After the indicated times, the filters are removed and cells that have invaded into the matrix are quantified based on fluorescence.

Invasion assays were done on a variety of breast cancer cell lines, including MDA-MB-231, BT-20, and MDA-MB-468 cells. CD44 expression in these breast cancer cell lines was analyzed via Western blot, and all three cell lines were found to express CD44 of 85 kDa, the expected size for CD44s (Fig. 4A). Alternatively, only BT-20 and MDA-MB-468 cells expressed significant levels of variant CD44 (~200 kDa; Fig. 4A). The ability of each cell line to invade into collagen matrix gel was then evaluated in the presence or absence of hyaluronan. In each case, invasion into gels containing hyaluronan occurred at rates much lower than invasion into gels without hyaluronan. MDA-MB-231, BT-20, and MDA-MB-468 cells, respectively, had an initial 10-, 3-, and 15-fold inhibition of invasion into collagen gels embedded with hyaluronan compared with collagen alone (Fig. 4B-D). Invasion of each cell line continued to be significantly inhibited throughout a 24-hour time course in the presence of hyaluronan. By 48 hours, invasion into gels cast with or without hyaluronan had reached similar levels (data not shown). Therefore, hyaluronan can initially attenuate but not prevent invasion into collagen gels.

Whereas these data show that breast cancer cells have reduced invasion into an hyaluronan-embedded collagen matrix, these experiments do not show a direct role for CD44. To elucidate whether inhibition of invasion by hyaluronan is a CD44-dependent event, we used the CD44 blocking antibody KM201 in the three-dimensional invasion assay to functionally block CD44 binding to hyaluronan (36, 37). MDA-MB-231 cells were incubated with the blocking antibody immediately before
their addition to the upper chamber of the assay. When invading into gels containing hyaluronan, the addition of the blocking antibody results in increased invasiveness. By 24 hours, KM201-treated cells invaded into gels containing hyaluronan more readily than into gels without hyaluronan (Fig. 4E), whereas the addition of an IgG isotype control antibody does not affect invasion (Fig. 4F). Note that invasion into gels lacking hyaluronan does not differ in the presence or absence of KM201 (Fig. 4E). This shows that hyaluronan is not simply providing steric hindrance to prevent cellular invasion but that the effect is due to a specific ligand-receptor interaction. This ties the role of hyaluronan during invasion

Figure 2. CD44 and hyaluronan have distinct but separate localization within MMTV-PyV mT breast tumors. MMTV-PyV mT nontransformed mammary glands (A and C) and tumors (B and D) were fixed, sectioned, and probed with anti-CD44 antibodies (A and B) or HABP (C and D). A, ductal epithelia are shown (arrow) surrounded by mammary gland adipocytes (arrowhead) and ECM. B, tumor epithelium (arrow) and ECM (arrowhead) are designated. C, stromal area surrounding ductal epithelial cells (arrow) and the surrounding parenchyma (arrowhead). D, stromal tissue (arrowhead) surrounding the tumor epithelium (arrow). MMTV-PyV mT/CD44−/− (E) and MMTV-PyV mT/CD44+/− (F) pulmonary metastatic tumors were stained with HABP. Sections were counterstained with either DAPI (A-D) or hematoxylin (E-F) to show nuclei. Arrow, tumor epithelium; arrowhead, ECM.
directly to the binding of CD44. Importantly, whereas hyaluronan may inhibit cellular invasion in the presence of CD44, the absence of CD44 may promote hyaluronan-mediated invasion.

Discussion

To determine the precise role of CD44 in tumor growth and metastasis, we have crossed the MMTV-PyV mT onto a CD44−/− background. The absence of CD44 in this model results in a striking 5.7-fold increase in pulmonary metastases when compared with control mice, whereas not affecting either primary tumor formation or growth rate. Additionally, there is a 5-fold increase in the number of metastases per lung of the CD44 null mice compared with CD44-expressing mice. Based on epithelial localization of CD44 and stromal localization of hyaluronan, we developed an in vitro approach to analyze these interactions. Three-dimensional in vitro invasion assays show that CD44 in breast tumor epithelial cells inhibits the ability of cells to invade hyaluronan-containing collagen matrices. These data strongly suggest that epithelial-stromal interactions in vivo need to be taken into account when identifying roles for adhesion proteins in cancer progression.

Hyaluronan can interact with several receptors in addition to CD44, including the receptor for hyaluronic acid–mediated motility (RHAMM), LYVE-1, HARE, Layilin, and Toll-4 (2). Of these receptors, only CD44 and RHAMM are reported to be expressed in invasive breast cancer (2, 38). RHAMM responds to hyaluronan binding by inducing cellular motility and transformation in vitro and in injectable tumor models (39). Hence, hyaluronan activity in vivo may vary depending upon the receptor it interacts with in the transformed state. The action of hyaluronan may be that of homotypic adhesion when binding to the CD44 receptor (as occurs in tumor growth and encapsulation), while binding to an alternate receptor, such as RHAMM or LYVE-1 on lymphatic endothelium, may induce an entirely different phenotype, that of invasion. These studies may be pertinent to our findings with the CD44 null model. It is possible that in the absence of CD44, hyaluronan interactions with an alternate receptor such as RHAMM are not balanced by the anti-invasive effects of CD44 binding.

In regard to CD44 isoform expression, the CD44−/− mouse model represents cells lacking all forms of CD44, whereas breast cancer cell lines typically express both standard and variant isoforms. In vitro studies have provided strong evidence to suggest that variant CD44 expression on cancer cells may facilitate disease progression and metastasis. Isoforms such as CD44v6, CD44v3,8-10, and CD44v10 have enhanced cellular metastasis when transfected into colon and breast cancer cell lines, whereas transfection of CD44s into colorectal cells decreases metastasis (21, 22, 40, 41). Additional studies have also identified a number of partners that CD44 may interact with to promote cell signaling that may lead to tumor progression such as c-Src, ErbB2, ankyrin, or the ERM proteins (42–44). Taking this into account, two of the cell lines

Figure 3. Has2-GFP transgenic mice reveal restricted activation of hyaluronan synthase enzymes in MMTV-PyV mT mammary gland tumors. A, Has2-GFP construct showing insertion of the Has2 promoter region before GFP. GFP detection is shown for a (B) MMTV-PyV mT/Has2-GFP bitransgenic mouse mammary tumor and (C) pulmonary metastasis (arrows). Surrounding tissue parenchyma (arrowheads). D, MMTV-PyV mT/Has2-GFP bitransgenic mammary gland tumors were sectioned and treated with anti-GFP antibody. Arrows, GFP-positive cells in the stromal capsule surrounding the tumor; arrowheads, GFP-negative tumor epithelium. Tissue sections were counterstained with hematoxylin.
used in our studies (BT-20 and MDA-MB-468) express variant isoforms; yet invasion in both of these cell lines are inhibited by hyaluronan-embedded collagen. Further studies examining the role of CD44v in hyaluronan binding may clarify this issue.

In the CD44-deficient mouse model used in this study, neither CD44s nor CD44v are expressed, and the only effect of complete CD44 absence can be evaluated. Transformed fibroblasts from the CD44<sup>−/−</sup> mice were previously shown to form significantly...
larger tumors which develop at a much faster rate when injected into nude mice (32). Although we found no effect on primary tumor growth in our model, this disparity is probably due to the differences between injectable and transgenic models. Furthermore, studies done by Weber et al. (45) using this model of CD44 ablation (32) indicate that CD44 can promote metastatic invasion in a p53-dependent model of osteosarcoma, while not affecting primary tumor growth. This may be due to the alternative ECM components available in bone or perhaps alternative isoforms of CD44 expressed in osteosarcoma compared with breast cancer.

Studies in our MMTV-PyV tM mouse model, crossed onto both the CD44+/− and the Has2-GFP transgenic, provide compelling evidence for a protective role for CD44 against breast cancer metastasis. Whereas hyaluronan is highly expressed and even induced in the ECM of these tumors, loss of the primary receptor for hyaluronan results in an increase in tumor metastasis. This strongly implicates an adhesive role for the CD44/hyaluronan interaction. Strikingly, our in vitro model precisely recapitulated this CD44/hyaluronan mechanism. Addition of the CD44 blocking antibody, essentially removing CD44/hyaluronan interactions, causes an increase in invasion over IgG treatment controls. Importantly, this only happens in the presence of hyaluronan not in collagen gels without hyaluronan. This indicates that in the absence of CD44 expression, hyaluronan is prometastatic. Previous studies show that high levels of hyaluronan in breast tumor stroma are correlated with poor patient survival (46). Our data suggest that this correlation may be due to a loss of CD44s expression in this patient group. Alternatively, hyaluronan deposition in vivo has been shown to inhibit tumor growth, as s.c. injection of hyaluronan into breast cancer xenografts induced tumor regression (47). These findings suggest that stromally localized hyaluronan can also serve a protective role in breast cancer progression. These previous studies, in addition to our current study, indicate that the tumor microenvironment can significantly alter the effect of CD44 and hyaluronan expression in tumor progression. In sum, these data indicate that epithelial-stromal interactions as well as ligand and isoform expression profiles should be considered in their entirety when evaluating a role for CD44 in breast cancer progression.

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