Tumorigenesis and Neoplastic Progression

Evidence for a Role of Tumor-Derived Laminin-511 in the Metastatic Progression of Breast Cancer

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Most studies investigating laminins (LMs) in breast cancer have focused on LM-111 or LM-332. Little is known, however, about the expression and function of $\alpha 5$ chain-containing LM-511/521 during metastatic progression. Expression of LM-511/521 subunits was examined in genetically related breast tumor lines and corresponding primary tumors and metastases in a syngeneic mouse model using real-time quantitative polymerase chain reaction, *in situ* hybridization, and immunohistochemistry. The results from our investigation indicate that LM-511 rather than LM-111, -332, or -521 correlates with metastatic potential in mouse mammary tumors. LM-511 was a potent adhesive substrate for both murine and human breast carcinoma cells and promoted strong haptotactic responses in metastatic lines. Haptotaxis was mediated by $\alpha 3$ integrin in both MCF-7 and MDA-MB-231 cells and was strongly inhibited by blocking antibodies against this integrin subunit. However, whereas nonmetastatic MCF-7 cells migrated toward LM-511 primarily via $\alpha 3\beta 1$ integrin, results from antibody perturbation experiments and flow cytometry analysis suggest that this response is mediated by an as yet unidentified $\alpha 3\beta$ integrin heterodimer (other than $\alpha 3\beta 1$) in MDA-MB-231 cells. These results are consistent with earlier reports implicating $\alpha 3$ integrins in breast cancer progression and support the role of LM-511 as a functional substrate regulating breast cancer metastasis. (*Am J Pathol* 2007, 170:2135–2148; DOI: 10.2353/ajpath.2007.060709)

Laminins (LMs) are heterotrimeric glycoproteins abundant in basement membranes (BMs) and are distinguished by their specific trimeric combination of α , β , and

γ chains.¹ Although there is considerable structural homology between LM isoforms² and overlap in receptors used,^{3,4} they differ in their tissue distribution and functional properties and have different roles under normal and pathological conditions, including cancer.^{1,5–8} Alterations in the composition and level of expression of BM LMs and their receptors in breast cancer have been documented and shown to contribute to disease progression.^{9,10} In particular, the excessive proteolytic activity associated with cancer progression typically results in decreased LM deposition and dissolution of the BM, a hallmark of invasive breast tumors.^{11–13} It is clear, however, that despite decreased BM deposition, some LM remains expressed in invasive breast tumor cells and contributes functionally to their motile and invasive phenotype.^{11,14–16} Consistent with this, increased expression of several LM receptors has been reported in breast tumor cells and shown to be associated with metastatic progression and poor prognosis in breast cancer patients.^{9,17–23} Together, these studies suggest that production of LM by breast cancer cells most likely contributes to their metastatic dissemination through autocrine stimulation.

Although the association between LMs and breast cancer progression is well supported, the critical LM isoform involved in the metastatic progression of breast tumors remains unclear. Most studies investigating the expression and function of LMs and their receptors in breast cancer have been interpreted as evidence for the role of LM-111 ($\alpha 1\beta 1\gamma 1$ trimer, previously known as laminin-1) or LM-332 ($\alpha 3\beta 3\gamma 2$ trimer, previously known as laminin-5) but did not take into account the lack of specificity of some of the LM antibodies^{14,22,24,25} or the overlap in receptors used to bind these isoforms and the more recently identified LM-511/521 ($\alpha 5\beta 1\gamma 1$ and $\alpha 5\beta 2\gamma 1$ trimers, previously known as laminin-10 and -11, respec-

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tively),^{4,26} also present in the breast.²⁷ Current evidence argues against a direct role for LM-111 and LM-332 in the late stage progression of breast tumors because both of these isoforms are down-regulated in most advanced breast tumors.^{28–30} Although these changes can be expected to contribute to the disruption of BM integrity^{12,13} and loss of epithelial polarity in early lesions,²⁹ the absence of LM-332 in the majority of advanced breast tumors has led to the suggestion that LM-332 may have a tumor suppressor role and is unlikely to be used directly by breast tumor cells to promote invasion and metastasis.^{31,32}

Given their more recent identification³³ and the confusion regarding the specificity of the 4C7 antibody,²⁵ few studies have specifically investigated LM-511/521 expression in breast cancer progression. In addition, the function of LM-511 and LM-521 in normal and neoplastic breast epithelial cells has not been investigated directly, in part because of the limited availability of purified proteins and the lack of clinically relevant models of breast cancer metastasis. However, evidence supporting the role of LM-511 in metastatic progression is now emerging. High to moderate expression of LM-511 subunits has been reported in fibroadenoma, ductal carcinoma *in situ* (DCIS), tubular carcinomas, atypical medullary carcinomas, and carcinomas of no specific type.^{10,11} These studies and more recent work by Fujita and colleagues³⁴ specifically investigating vascular expression of LM isoforms in normal and malignant breast reported high levels of LM-511 in the tumor vasculature and increased expression in invasive tumors and brain metastases leading the authors to propose that vascular LM-511 may contribute to the attachment of tumor cells to blood vessel walls during metastasis. Although the relationship between tumor cell expression of LM-511/521 and breast cancer metastasis has not been investigated, the recent findings that tumor cell expression of LM α 5 chain provides the strongest independent prognostic value for identification of non-small cell lung carcinoma patients with high risk of disease recurrence is supportive of an autocrine role for LM-511/521 in cancer progression.³⁵

Here we investigate the expression of α 5-containing LM isoforms (LM-511/521) in a clinically relevant mouse model of spontaneous breast cancer metastasis and in human breast tissue. Using *in vitro* assays, we provide evidence for the functional relevance of LM-511 and its integrin receptors in the metastatic process in mouse and human breast cancer cells. Our results identify the α 3 integrin subunit as an important LM-511 receptor in breast cancer cells and support previous reports implicating this integrin in the regulation of breast cancer metastasis.

Materials and Methods

Antibodies and Reagents

Rabbit polyclonal antisera directed against mouse LM α 1 and LM α 3 chains were kindly provided by Dr. Takako Sasaki (Max-Planck Institute, Leipzig, Germany). Rabbit

anti-mouse LM α 5 antiserum was a generous gift from Dr. Jeff Miner (Washington University, St. Louis, MO). Control preimmune rabbit antiserum was purchased from Vector Laboratories (Homebush, Australia). The mouse anti-human LM β 2 antibody (clone C4) and the 4C7 mouse anti-human LM α 5 monoclonal antibody (unpurified ascites) were purchased from Chemicon International (Boronia, Australia) and used for immunostaining of human tissue sections. The mouse monoclonal 3G10 antibody was raised against recombinant human LM-511 and its specificity for the LM β 1 chain verified by immunoblot and immunoprecipitation in our laboratory (L. Zamurs, N. Pouliot, D. Williams, and E. Nice, manuscript in preparation). Control mouse isotype-matched antibodies IgG₁ (1B5) and IgG_{2a} (ID4.5) were kindly provided by Dr. Paul Simmons (Peter MacCallum Cancer Centre, Melbourne, Australia). Function-blocking antibodies against α ₃ (P1B5), α ₆ (GoH3), and β ₄ (ASC-3)³⁶ integrins were purchased from Chemicon. The anti- α 2 antibody (P1H5, hybridoma supernatant) was kindly provided by Dr. Pritinder Kaur (Peter MacCallum Cancer Centre). Anti-integrin β ₁ blocking antibodies were obtained from BD Pharmingen (clone mAb13; San Jose, CA) or purified in our laboratory from hybridoma supernatant (P5D2, a generous gift from Dr. William Carter, Fred Hutchinson Cancer Research Center, Seattle, WA). All anti-human integrin-blocking antibodies were used at 10 or 20 μ g/ml as indicated in the figure legends. Purified function-blocking mouse anti-rat α 3 integrin (Ralph 3.2, 20 μ g/ml) was from Santa Cruz Biotechnology (Santa Cruz, CA). Hamster anti-rat β 1 integrin (Ha2/5) and isotype-matched antibodies were obtained from BD Biosciences (North Ryde, Australia) and used at 20 μ g/ml. Type IV collagen, fibronectin, and vitronectin were from Sigma (St. Louis, MO) and used at optimal coating concentrations of 20, 10, and 10 μ g/ml, respectively, as previously determined.³⁷ Rat tail type I collagen (BD Biosciences) was used at 20 μ g/ml. Recombinant human LM-511 produced by human embryonic kidney 293 cells transfected with full-length cDNAs encoding LM α 5, - β 1, and - γ 1 chains (kindly provided by BioStratum Inc., Durham, NC) was purified from serum-free culture supernatant by affinity chromatography as previously described.³⁸

Cell Lines and Cell Culture

The mouse mammary carcinoma cell lines 67NR, 66cl4, and 4T1 were obtained from Dr. F. Miller (Michigan Cancer Foundation, Detroit, MI).^{39,40} The bone metastatic 4T1.2 and 4T1.13 lines were clonally derived from 4T1 cells in our laboratory.^{41,42} For routine culture, all cell lines were maintained in α -minimal essential medium supplemented with 5% fetal calf serum and 1% penicillin-streptomycin at 37°C with 5% CO₂ and limited to 4 weeks in culture. The human MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in α -minimal essential medium, 10% fetal calf serum, sodium pyruvate (1 mmol/L), glutamine (2 mmol/L)

L), and 1% penicillin-streptomycin. MCF-7 cells were maintained in the same medium supplemented with insulin (0.2 IU/ml).

Tumor Growth

Tumor cells [1×10^5 in 20 μ l of phosphate-buffered saline (PBS)] were injected into the inguinal mammary fat pad of 6- to 8-week-old female BALB/c mice. Tumors developed throughout a period of 4 to 5 weeks and metastasized to secondary organs, the extent to which being dependent on the tumor line as described previously.^{41,42} Animals were euthanized by an overdose of anesthetic inhalation either when the primary tumor reached a size of 1.5 g or when the mouse showed signs of distress. Organs, including primary tumors, femurs, spines, and lungs, were harvested for histological examination and immunohistochemical staining.

Flow Cytometry

Flow cytometric analysis of integrin receptor expression in murine 67NR and 4T1.2 and in human MCF7 and MDA-MB-231 cells was performed essentially as described.³⁷ The following primary antibodies were used for mouse cell lines: $\alpha 2\beta 1$ (clone BMA2.1, 25 μ g/ml; Chemicon), $\alpha 3$ (clone 42, 10 μ g/ml; BD Pharmingen), $\alpha 5$ (clone 5H10-27, 10 μ g/ml; BD Pharmingen), $\alpha 6$ (clone GoH3, 10 μ g/ml; Chemicon), $\beta 1$ (clone MB1.2, 10 μ g/ml; Chemicon), and $\beta 4$ (clone 346-11A, 10 μ g/ml; BD Pharmingen). Anti-human integrin $\beta 1$ (P5D2), $\alpha 3$ (P1B5), and $\alpha 3\beta 1$ dimer (M-KID2) were from Chemicon and used at 10 μ g/ml. All cells were treated for 1 hour on ice, washed twice in PBS with 2% fetal calf serum to remove excess antibodies, and reacted with appropriate fluorescein isothiocyanate-conjugated secondary antibodies for 45 minutes on ice. Specific fluorescence was detected on a Canto flow cytometer (Becton Dickinson, San Jose, CA).

Immunohistochemistry (IHC)

Organs were trimmed of excess connective tissues, fixed for 48 hours at 4°C in zinc-Tris fixation buffer (2.8 mmol/L calcium acetate, 22 mmol/L zinc-acetate, 36.7 mmol/L zinc chloride, and 0.1 mol/L Tris-HCl, pH 7.4), and processed for paraffin embedding. Bones were decalcified in 5% nitric acid for 18 to 24 hours at 4°C before embedding. Serial sections (4 μ m) were stained with hematoxylin and eosin (H&E) or subjected to IHC staining for LM chains as follows. Sections were rehydrated, equilibrated in antigen retrieval buffer (50 mmol/L Tris-HCl, pH 7.8, 0.1% CaCl_2 , and 150 mmol/L NaCl), and subjected to trypsin (0.5 mg/ml; Sigma) digestion for 7 minutes (soft tissues) or 10 minutes (bones) at 37°C. Sections were rinsed extensively in water and blocked for 30 minutes at room temperature in blocking buffer (PBS, 3% normal goat serum, and 0.05% Tween 20) and reacted with specific antisera against LM $\alpha 1$, LM $\alpha 3$, LM $\alpha 5$, or control preimmune rabbit antiserum diluted in blocking buffer

overnight at 4°C under humidified atmosphere. The sections were washed three times with wash buffer (PBS, 0.05% Tween 20) and bound antibodies reacted with a biotin-conjugated goat anti-rabbit secondary antibody (Vector) for 1 hour at room temperature. Unbound antibodies were washed with wash buffer as above and once with PBS. After inactivation of endogenous peroxidases for 30 minutes in methanol with 0.3% hydrogen peroxide, specific primary-secondary antibody complexes were detected using ABC reagent (Vector) and visualized using a diaminobenzidine peroxidase substrate kit (Vector). All slides were developed in parallel and the reaction stopped before detection of nonspecific staining in control preimmune serum-treated sections. Sections were counterstained with hematoxylin, mounted in DPX neutral mounting medium (Chem-Supply, Adelaide, Australia), and photographed on a Zeiss Axioskop 2 microscope (Jena, Germany).

Paraffin sections or cryosections of three normal human breast and tumor tissues including one DCIS, 11 infiltrating ductal carcinomas (DCs), two infiltrating lobular carcinomas (LCs), two mixed LCs and DCs, and three metastases, were obtained from the Peter MacCallum Cancer Centre Tissue Bank and immunostained using the mouse monoclonal antibodies 4C7, 3G10, and C4 for detection of $\alpha 5$, $\beta 1$, and $\beta 2$ subunits, respectively. Human tissues were either unfixed (cryosections), ethanol fixed, or zinc fixed as indicated in Table 1. Ethanol-fixed and zinc-fixed paraffin sections were treated with trypsin (1 mg/ml, 5 minutes at 37°C) before staining as described above except that a biotin-conjugated goat anti-mouse secondary antibody (Vector) was used. Tumor grades were determined independently by onsite pathologists. LM staining was evaluated semiquantitatively by two independent assessors and given a score (– to +++) based on the intensity (weak to strong) and extent of staining (proportion of positive cells or continuous/discontinuous reactivity in the BM).

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR)

RNA was isolated from monocultures of mouse tumor cells by standard TRIzol extraction (Invitrogen, Mount Waverley, Australia) and cDNA synthesized using 2 μ g of RNA primed with random hexamers and M-MLV reverse transcriptase (Promega, San Luis Obispo, CA) according to the manufacturer's instructions. QRT-PCR was performed on an ABI Prism 7000 thermocycler using SYBR Green I chemistry (Applied Biosystems, Foster City, CA) as previously described.⁴² The following specific primers for LM $\alpha 5$, LM $\beta 1$, LM $\beta 2$, LM $\gamma 1$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Express version 2.0 program (Applied Biosystems): LM $\alpha 5$ (forward: 5'CACCGAAGTGGTCTATTCTGGC; reverse: 5'TAAAACGGAGTGTACGTGGC), LM $\beta 1$ (forward: 5'CCAATCTCTGTGAACCATGTACCT; reverse: 5'GCAATTTGCACCGACACTGA), LM $\beta 2$ (forward: 5'TGGGAGCCAGCGACACTT; reverse: 5'CGAAGCCCTGTGTAGCCTCT), LM $\gamma 1$ (forward: 5'CCTCCAC-

Table 1. Immunohistochemical Staining of LM α 5, β 1, and β 2 Chains in Normal and Malignant Human Breast Tissues

Sample	Type (grade)	Vascular	LM α 5 epithelial BM	Tumor cells*	LM β 1 tumor cells	LM β 2 tumor cells
P04650 [†]	Normal	+++	+++	N/A	N/A	N/A
P04770 [†]	Normal	+++	+++	N/A	N/A	N/A
P06291 [‡]	Normal	+++	+++	N/A	N/A	N/A
P01735 [§]	DCIS (grade II)	+++	++	+	ND	ND
P01544 [§]	Infiltrating DC (grade II)	+++	+	+	ND	ND
P01569 [§]	Infiltrating DC (grade II)	+	+	±	ND	ND
P06635 [‡]	Infiltrating DC (grade II)	+++	++	+	ND	ND
P06291L [‡]	Infiltrating DC (grade II)	+++	++	++	ND	ND
P01843 [§]	Infiltrating DC (grade II)	+++	++	+	ND	ND
P07982 [‡]	Infiltrating DC (grade II)	++	+	±	ND	ND
P06291R [‡]	Infiltrating LC (grade II)	+++	++	++	ND	ND
P01696 [§]	Infiltrating LC (grade II)	+++	±	++	ND	ND
P01780 [§]	Mixed infiltrating LC/DC (grade II)	+++	+	+	ND	ND
P00546 [§]	Infiltrating DC (grade III)	+++	++	+	ND	ND
P07187 [†]	Infiltrating DC (grade III)	+++	++	+++	+	++
P01539 [§]	Infiltrating DC (grade III)	++	+	±	ND	ND
P07970 [‡]	Infiltrating DC (grade III)	+++	+	+++	±	+++
P07916 [‡]	Infiltrating DC (grade III)	+++	+	+++	++	+
P04485 [†]	Mixed infiltrating DC/LC (grade III)	+++	++	+++	++	±
P01019 [§]	Metastasis, bone	++	N/A	++	ND	ND
P01579 [§]	Metastasis, omentum	+	N/A	±	ND	ND
P01289 [§]	Metastasis, omentum	+++	N/A	±	ND	ND

DC, ductal carcinoma; LC, lobular carcinoma; N/A, not applicable; ND, not done.

*Tumor cell expression of LM α 5 was detected in scattered single invasive cells or in cell clusters.

[†]Unfixed cryosections.

[‡]Zinc-Tris-fixed paraffin sections.

[§]Ethanol-fixed paraffin sections. Scores are based on the extent and intensity of staining ($\pm$, fewer than 10 positive cells/section; +, weak reactivity; ++, moderate/discontinuous reactivity; +++, high/continuous reactivity).

CTTTCAGATTGATGAG; reverse: 5'CGCCTCCGAGCC-ATCTC), GAPDH (forward: 5'GGCCTACATGGCCTC-CAA; reverse: 5'GGGATAGGGCCTCTCTTGCT). PCR reactions included 100 nmol/L forward and reverse primers, 1 $\times$ SYBR Green I master mix reagents and 1 μ l of cDNA. LM α 5 transcript levels relative to GAPDH were determined using the formula: relative transcript abundance = $10000/2^{(Ct_{LM\alpha 5} - Ct_{GAPDH})}$.

In Situ Hybridization

A cocktail of 450- to 550-bp riboprobes for LM α 5 was generated by amplification of LM α 5 DNA sequence of interest using primers labeled with the T7 (5' primer) and SP6 (3' primer) promoter sequences for generating sense and anti-sense transcripts. T7 and SP6 polymerases were then used for *in vitro* transcription, with generation of sense and anti-sense riboprobes, respectively. The *in vitro* transcription included labeling of transcripts with digoxigenin using digoxigenin-UTP as per the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). Individual riboprobes were used initially, and those that gave the best signal with minimal background DNA binding were pooled into a cocktail for subsequent hybridizations (including sense and anti-sense cocktails). The protocol for dewaxing and fixation of tissues, pretreatment for access to target nucleic acid sequence, and *in situ* hybridization was as published previously.^{43,44}

Adhesion Assays

Adhesion assays were performed in serum-free medium using a calcein labeling method as described previously³⁷ with minor modifications. In brief, triplicate wells of 96-well plates were coated overnight at 4°C with 100 μ l of extracellular matrix protein, blocked for 1 hour at 37°C with PBS containing 1% bovine serum albumin, and washed twice with PBS before the addition of the cells. Calcein-labeled tumor cells were resuspended in serum-free α -minimal essential medium supplemented with 0.05% bovine serum albumin and added to the wells ($4 \times 10^4/100 \mu$ l/well). The plates were centrifuged for 5 minutes at $400 \times g$ and incubated for 30 minutes at 37°C. Nonadherent cells were removed by gentle washing twice with PBS and once with Tris-buffered saline (pH 7.4) supplemented with 2 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Adherent cells were lysed with 1% sodium dodecyl sulfate, and cell adhesion was determined by measuring specific fluorescence in a Polarstar Optima reader (BMG, Labtech GmbH, Offenburg, Germany). Specific adhesion was expressed as the percentage of total cell input and calculated from a standard curve made up of 0, 12.5, 25, 50, 75, and 100 μ l of calcein-labeled cell lysate derived from the initial cell suspension. For inhibition studies, the cells were pretreated on ice for 15 minutes with function-blocking antibodies directed against specific integrin subunits and added together with the cells to assay wells. Each experiment was repeated

three times, and the results shown represent the mean percentage of total cell input $\pm$ SD of a representative experiment performed in triplicate wells. The statistical differences between treatments were analyzed using a Student's *t*-test; $P < 0.05$ was considered significant.

Migration and Invasion Assays

Haptotactic migration of tumor cells toward LM-511 was measured in triplicate Transwell migration chambers (8- μ m pore size) (Corning Inc., Life Sciences, Acton, MA) as described previously.³⁷ Tumor cell migration to the underside of LM-511-coated porous membranes was measured after 3 hours (mouse cell lines) or 5 hours (human cell lines) of incubation at 37°C. Three random fields per membrane were photographed on a Zeiss Axioskop 2 fluorescence microscope and the number of migrated cells counted. Where indicated, function-blocking integrin antibodies were used as pretreatment and added with the cells as described for the adhesion assay. For invasion assays, the cells (7.5×10^4) were embedded in 100 μ l of a 1:1 mixture of serum-free medium and Matrigel (BD Biosciences) in the upper wells and allowed to invade and migrate toward LM-511 coated on the underside of the porous membrane in the absence of serum. The number of cells on the underside of the porous membrane was scored after 20 hours of incubation at 37°C, as described for migration assays. Migration and invasion experiments were repeated three times, and the results show a representative experiment expressed as the mean number of migrated/invaded cells per field $\pm$ SD of nine fields of view per condition. The statistical differences between assay conditions were analyzed using a Student's *t*-test; $P < 0.05$ was considered significant.

Results

mRNA Expression of LM-511/521 Subunits in Mammary Carcinoma Cell Lines of Varying Metastatic Potential

We recently described a syngeneic mouse model of spontaneous breast cancer metastasis that comprises a series of genetically related tumor lines differing in their metastatic potential.⁴¹ cDNA microarray analysis of primary tumors of the model revealed that two subunits of LM-511 ($\alpha 5$ and $\beta 1$) were expressed at higher levels in highly metastatic tumors than in weakly metastatic tumors.⁴² To confirm this finding and to determine whether differential expression of LM-511 occurred specifically in tumor cells rather than in stromal cells of the tumors, QRT-PCR analysis of the LM $\alpha 5$, LM $\beta 1$, and LM $\gamma 1$ chains was performed on RNA isolated from cultures of non-metastatic 67NR, weakly metastatic 66cl4, and highly metastatic 4T1.2 and 4T1.13 cells (Figure 1A). Because both LM-511 ($\alpha 5\beta 1\gamma 1$) and LM-521 ($\alpha 5\beta 2\gamma 1$) trimers are present in the breast and differ only by their β subunit, expression of LM $\beta 2$ was also examined to distinguish between these isoforms.

Figure 1. LM $\alpha 5$ $\beta 1$, $\beta 2$, and $\gamma 1$ mRNA transcript expression in mammary carcinoma cell lines and primary tumors of varying metastatic potential. **A:** QRT-PCR analysis of the LM chains was performed using RNA isolated from monocultures of 67NR, 66cl4, 4T1.2, and 4T1.13 cells and from mammary fat pad. LM mRNA expression was calculated relative to the housekeeping gene GAPDH. Values represent the mean $\pm$ SEM of three replicate cell cultures. **B:** Nonmetastatic (67NR, **a**) and weakly metastatic tumors (66cl4, **c**) expressed LM $\alpha 5$ message primarily in the vasculature (**arrows**) and in epithelial cells of remaining normal mammary glands (**N**, **dotted lines**). **e:** In contrast, high levels of LM $\alpha 5$ transcript were detected in most tumor cells of highly metastatic 4T1.2 tumors using LM $\alpha 5$ anti-sense probes. No nonspecific signal was detected using control sense probes (**b**, **d**, and **f**). Scale bar = 50 μ m.

Whereas transcripts for LM $\beta 1$ and LM $\beta 2$ were amplified efficiently from normal mammary fat pad cDNAs, only weak signals were detected for the LM $\beta 2$ subunit in all tumor lines. In contrast, all cell lines expressed LM $\alpha 5$, LM $\beta 1$, and LM $\gamma 1$ transcripts, and their expression was higher in highly metastatic lines (4T1.2 and 4T1.13). For instance, LM $\alpha 5$ expression in highly metastatic 4T1.2 and 4T1.13 cells was approximately threefold to fourfold higher than that of 66cl4 or 67NR cells. Consistent with this, analysis of primary tumors by *in situ* hybridization detected LM $\alpha 5$ mRNA transcript

Figure 2. IHC detection of LM α chains in normal mammary gland and primary tumors of the model. **A:** Zinc-fixed, paraffin-embedded serial sections of normal mouse mammary fat pad were stained with H&E (**a**) or specific rabbit antisera directed against LM α 1 (**b**), LM α 3 (**c**), and LM α 5 (**d**), as indicated. **Arrows** show strong BM reactivity around mammary glands. **B:** Zinc-fixed, paraffin-embedded serial sections of 67NR (**a–e**), 66cl4 (**f–j**), and 4T1.2 (**k–o**) primary tumors were stained with H&E (**a, f, k**) or specific rabbit antisera directed against LM α 1 (**b, g, and l**), LM α 3 (**c, h, and m**), and LM α 5 (**d, i, and n**), as indicated. Higher magnifications of LM α 5 stains in 67NR, 66cl4, and 4T1.2 tumors (delineated by insets in **d, i, and n**) are shown in **e, j, and o**, respectively. **Arrows** indicate blood vessels. Scale bars: 100 μ m (**A**); 50 μ m (**B**).

in the majority of tumor cells in 4T1.2 tumors (and 4T1.13, data not shown), whereas LM α 5 mRNA localized primarily in the vasculature and remaining normal mammary gland present around 67NR and 66cl4 tumors (Figure 1B). Thus, the abundance of LM α 5, β 1, and γ 1 transcripts and the low level of LM β 2 expression in 4T1.2 and 4T1.13 indicate that LM-511 rather than LM-521 is elevated in highly metastatic breast tumors.

IHC Detection of LM-111, LM-332, and LM-511 in Mammary Tumors and Metastases

LM-111, LM-332, and LM-511 are abundant in the subepithelial BM of the normal mammary gland.²⁷ Although changes in the expression of LM-111 and LM-332 in breast tumors are well documented, little is known about LM-511 protein expression during breast cancer progression, particularly in skeletal metastases. This is attributable in part to the lack of clinically relevant models of spontaneous breast cancer metastasis to bone and the lack of antibodies suitable for IHC detection of LM α 5 chain on standard formalin-fixed, paraffin-embedded archival tissues from human breast cancer patients. To circumvent these problems, we developed an alternative

IHC protocol using zinc fixation and trypsin antigen-retrieval compatible with IHC detection of LM chains in paraffin-embedded tissues, including whole bones (see Materials and Methods). The methodology was used to compare the pattern of expression of LM α 1 (LM-111), LM α 3 (LM-332), and LM α 5 (LM-511) in mammary tumors and metastases of our spontaneous metastasis model. The tissue integrity of normal mammary glands was well maintained using this fixation method as seen by H&E staining (Figure 2Aa). In agreement with previous IHC studies performed on cryosections,²⁷ strong staining for all three LM α chains was detected in the BM of the normal mammary gland using specific polyclonal antibodies previously limited to IHC staining of cryosections (Figure 2A, b–d). No specific BM staining was observed using control preimmune serum (data not shown).

In contrast to the normal mammary gland, analysis of primary tumors revealed significant differences in the expression of LM α 1, α 3, and α 5 subunits (Figure 2B). Although LM α 1 (Figure 2B, b, g, and l) and LM α 3 (Figure 2B, c, h, and m) were absent in all primary tumors irrespective of their metastatic potential, LM α 5 protein expression was detected in all tumors (Figure 2B, d, i, and n). In 67NR and 66cl4 tumors, LM α 5 expression was restricted to vascularized regions near the edges of the

Figure 3. IHC detection of LM α chains in spontaneous lung and bone metastases. Serial sections from spontaneous 66cl4 lung metastases (**a–e**, top), 4T1.2 lung metastases (**f–j**, middle), and 4T1.2 femoral metastases (**k–o**, bottom) were stained with H&E (**a, f, k**) or with specific rabbit antisera against LM α 1 (**b, g, l**), LM α 3 (**c, h, m**), and LM α 5 (**d, i, n**). Higher magnifications of LM α 5 stains delineated by insets in **d, i**, and **n** are shown in **e, j**, and **o**, respectively. **Dotted lines** in H&E-stained sections (**a, f, k**) show the interface between the metastatic nodule (M) and surrounding stroma (S). The tumor-stroma interface in LM α 5-stained sections (**e, j, o**) is also marked with a **dotted line**. Scale bars: 100 μ m (**a**); 50 μ m (**e**).

tumor, and staining was predominantly, although not exclusively, in blood vessels (Figure 2B, e and j; arrows). In contrast, LM α 5 was expressed throughout the 4T1.2 tumor (Figure 2Bo). The highly metastatic 4T1.13 tumors showed a similar pattern of LM α chain expression to that observed in 4T1.2 tumors (data not shown). These observations are consistent with the mRNA data presented in Figure 1 and indicate that tumor cell expression of LM-511, but not LM-111 or LM-332, in mouse mammary tumors directly correlates with their metastatic potential.

Breast tumors most commonly metastasize to lung and bone. Thus, we set out to determine whether the pattern of LM expression observed in primary tumors was maintained in 66cl4 and 4T1.2 spontaneous metastases to these organs. We reasoned that if tumor-derived LM-511 contributes functionally to metastatic spread, the LM α 5 chain should be detected also in distant metastases. For these experiments, we first established that LM α 1 was undetectable in normal lung, whereas LM α 3 and LM α 5 were abundant in epithelial basal lamina (data not shown). In normal femoral sections LM α 1 and LM α 3 were undetectable, consistent with the absence of LM-111 and LM-332 in bone marrow, as reported previously.^{45,46} Also in agreement with these studies, LM α 5 expression was abundant in bone vasculature including the bone marrow sinusoids (data not shown).

LM α 1 was absent in 66cl4 metastatic nodules within the lung (Figure 3b). Although LM α 3 was abundant in the alveolar BM of the lung stroma surrounding metastatic deposits, its expression within 66cl4 tumor nodules was low and varied between tumor samples. In the majority of 66cl4 tumor nodules, LM α 3 was detected predominantly at the tumor-stroma interface and occasionally in small clusters of cells within the metastatic nodule, most likely representing residual lung stroma (Figure 3c). By com-

parison, LM α 5 expression was strong and consistently detected throughout the metastatic nodule (Figure 3, d and e). Likewise, staining of 4T1.2 lung metastases showed strong expression of LM α 5 throughout the tumor nodule (Figure 3, i and j). In contrast, LM α 1 was completely absent (Figure 3g), and LM α 3 expression was restricted to the surrounding lung stroma but undetectable in tumor cells (Figure 3h).

4T1.2 bone metastases within the marrow cavity showed no detectable expression of LM α 1 (Figure 3l) and LM α 3 (Figure 3m), whereas LM α 5 was expressed strongly throughout the metastatic nodule (Figure 3, n and o). A similar pattern of staining to that seen in 4T1.2 lung and bone metastases was observed in 4T1.13 metastases (data not shown). Taken together, these results demonstrate that aggressive and metastatic mammary tumors express high levels of LM-511 protein and that this phenotype is maintained in spontaneous metastases to the lung and bone. High LM α 5 expression in 66cl4 lung metastases and the restricted expression in their corresponding primary tumors suggest up-regulation of tumor LM-511 in the lung microenvironment and/or clonal selection of LM-511-expressing tumor cells.

To address the relevance of these findings in humans, we used the anti-human LM α 5 4C7 antibody to immunostain some normal human breast and breast tumor specimens. In preliminary studies, we found that the 4C7 antibody was unreliable for IHC detection of LM α 5 in formalin-fixed paraffin sections even after citrate/microwave or enzymatic (trypsin, proteinase K, pronase, protease XXV) antigen retrieval (data not shown). However, the antibody was suitable for use on unfixed cryosections as well as ethanol-fixed or zinc-fixed paraffin sections. An example of the pattern of LM α 5 staining in normal breast (P04770) and a tumor sample with high LM α 5 expression

Figure 4. IHC detection of LM α 5 in normal and malignant human breast. Cryosections of normal human breast tissue (specimen P04770) and infiltrating breast tumor (specimen P04485) were treated with the 4C7 antibody specific for the human LM α 5 subunit (**a** and **b**) or control isotype-matched ID4.5 antibody (**c** and **d**) as indicated. **a:** Strong LM α 5 reactivity was detected in the epithelial BM and blood vessels of normal mammary glands. **b:** Diffuse staining and discontinuous BM staining is seen in the tumor tissue. No nonspecific reactivity was detected in normal (**c**) or tumor tissue (**d**) with an isotype-matched control antibody. Scale bar = 50 μ m.

(P04485) is shown in Figure 4. Strong reactivity was detected in the BM of normal mammary glands and surrounding blood vessels (Figure 4a). In contrast, the infiltrating carcinoma showed a diffuse but significant cytoplasmic and pericellular LM α 5 expression despite the absence of a continuous BM (Figure 4b). No nonspecific reactivity was detected using the control ID4.5 isotype-matched antibody (Figure 4, c and d).

The results of immunostaining for LM α 5, β 1, and β 2 in 22 samples, including three normal breast samples, one DCIS, 15 infiltrating carcinomas, and three metastases are summarized in Table 1. Staining was scored semi-quantitatively based on the extent and intensity of the staining in the vasculature, in the subepithelial BM, and in tumor cells. As previously reported, all normal breast specimens showed abundant LM α 5 in the blood vessels, and with the exception of two specimens (P01569, P01579), high vascular expression was maintained in tumor samples. Strong to moderate staining of the epithelial BM was observed in normal breast and DCIS specimens, whereas lower intensity and/or discontinuity of the BM became apparent with increasing grade of the lesion. Although the association between metastatic disease and high tumor cell expression of LM α 5 would be expected to identify only a small proportion of tumors, the results obtained in the mouse model prompted us to examine whether higher tumor cell expression could be detected also in some human tumors. As expected, tumor cell reactivity in infiltrating tumors was variable. LM α 5 expression in invasive human breast tumors showed a focal distribution possibly attributable to the lower cellularity of the human lesions compared with mouse tumors. LM α 5 was detected either in single cells or in clusters of invasive tumor cells. Interestingly, the highest tumor cell score (+++) was observed in four of

six grade III lesions (P07187, P07916, P07970, and P04485) consistent with a potential role of LM α 5 isoforms in more advanced tumors. Among the three metastases examined, only the bone metastasis showed moderate LM α 5 expression in tumor cell clusters.

To clarify which α 5-containing LM isoform was present in human tumors, grade III tumors expressing elevated levels of LM α 5 were further examined for the presence of LM β 1 and LM β 2 subunits. Both subunits were detectable in each tumor, albeit at different levels. Tumors P07916 and P04485 contained predominantly the LM β 1 subunit whereas LM β 2 was more abundant in P07187 and P07970 tumors. Taken together, these data indicate that LM α 5 remains expressed in some invasive human breast tumors, consistent with the results obtained in the mouse model. However, the α 5 subunit seems to be associated with either the β 1 or the β 2 subunit, suggesting the presence of either LM-511 or LM-521 in advanced human breast tumors.

Functional Role of LM-511 in Breast Cancer Cells

The results above are strongly suggestive of a functional role of LM-511 in the metastasis of breast tumors. However, to our knowledge, the biological responses of mammary tumor cells to LM-511 and its receptors have not been characterized, in part because of the limited availability of purified LM-511 protein. To establish the functional significance of LM-511 expression in metastatic mammary carcinomas, we compared the adhesive properties of purified recombinant LM-511 to that of other adhesion proteins previously characterized in the tumor lines of the model.³⁷ LM-511 promoted rapid adhesion (30 minutes) of all lines of the model irrespective of their metastatic potential and was more potent than all other substrates tested (Figure 5A). Adhesion of 4T1.2 cells to vitronectin was enhanced compared with the other lines because of expression of α v β 3 integrin in these cells, as previously reported.³⁷ LM-511 was also a potent haptotactic factor for metastatic 4T1.2 cells in standard Transwell migration and Matrigel invasion assays (Figure 5, B and C, respectively). To determine which receptor may be mediating these responses, we examined the pattern of integrin receptor expression in 67NR and 4T1.2 cells by flow cytometry (see supplementary Figure 1 at <http://ajp.amjpathol.org>). From this analysis, we established that both tumor lines express high levels of α 5, α 6, and β 1 and moderate levels of β 4 integrin subunits. The α 3 subunit and α 2 β 1 integrin dimer are detected in 4T1.2 cells but absent in 67NR. In addition, we showed previously that all tumor lines of the model express low levels of α v integrin subunit whereas only highly metastatic lines (4T1.2 and 4T1.13) express the β 3 subunit.³⁷ As shown in Figure 5B, control isotype antibodies or neutralizing antibodies against the α 6 subunit failed to inhibit 4T1.2 cell migration toward LM-511. In contrast, both migration (Figure 5B) and invasion (Figure 5C) were inhibited by treatment of the cells with function-blocking antibodies directed against β 1 integrin.

Figure 5. LM-511 promotes integrin-dependent adhesion, migration, and invasion of murine mammary carcinoma lines. **A:** Adhesion of mammary carcinoma cells to extracellular matrix proteins. Short-term adhesion (30 minutes) of murine tumor cell lines was measured in triplicate wells coated or not with the indicated concentrations of extracellular matrix proteins. **B:** Haptotactic migration of 4T1.2 cells toward LM-511. The underside of Transwell chambers was coated with control bovine serum albumin (1%) or LM-511 (5 µg/ml). The haptotactic response of 4T1.2 cells in the absence of serum was measured after 3 hours of incubation at 37°C in the absence or presence of control (Ig) or blocking antibodies against $\alpha 6$ or $\beta 1$ integrin. **C:** Matrigel invasion of 4T1.2 cells toward LM-511. Invasion assays were performed in Transwell chambers in the absence of serum using LM-511 coated on the underside of the porous membrane as described in **B**. The cells were embedded in Matrigel in the upper well, and the number of cells invading the Matrigel and migrating to the underside of the membrane was measured after 20 hours. All experiments were repeated three times; and the data represent the mean $\pm$ SD of a representative experiment performed in triplicate wells. * $P < 0.01$.

Attempts to characterize further the specific integrin receptor for LM-511 in the mouse tumor lines were inconclusive because of the lack of commercial function-blocking antibodies for the mouse $\alpha 3$ and $\beta 4$ integrin subunits and the lack of inhibition in this assay using a mouse anti-rat $\alpha 3$ antibody (clone Ralph 3.2, Santa Cruz; data not shown). Therefore, the human MCF-7 and MDA-MB-231 tumor lines were selected for further receptor characterization studies as they differ in their metastatic potential and the repertoire in integrin receptors in these cells has been characterized previously.^{23,47,48} Both tumor lines express the $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, and $\beta 1$ subunits. The $\beta 4$ subunit is expressed exclusively in MDA-MB-231, whereas integrin $\alpha v \beta 3$ is expressed at low and moderate levels in MCF7 and MDA-MB-231 cells, respectively. Adhesion of nonmetastatic MCF-7 cells to LM-511 was inhibited partially by neutralizing antibodies targeting the $\alpha 3$ (38%) or $\beta 1$ subunit (clone P5D2) alone (38%) and more strongly by anti- $\alpha 2$ plus $\alpha 3$ plus $\alpha 6$ antibodies (57%) but not by other α or β integrin antibody combinations (Figure 6A). MCF-7 migration toward LM-511 was strongly inhibited by blocking of the $\alpha 3$ (59%) or $\beta 1$ (76%) subunit alone (Figure 6B). Combination of anti- α or anti- β antibodies did not provide greater inhibition of migration than $\alpha 3$ or $\beta 1$ integrin antibodies used singly, indicating that migration toward LM-511 is mediated primarily via $\alpha 3 \beta 1$ integrin receptors.

Metastatic MDA-MB-231 cells attached more avidly to LM-511 than MCF-7 cells, with greater than 80% of the cells adhering after 30 minutes (Figure 6C). Interestingly, the adhesion of MDA-MB-231 cells was inhibited only marginally by the presence of anti- $\alpha 2$ plus $\alpha 3$ (7%, $P < 0.05$) or anti- $\beta 1$ (clone P5D2, 8%; $P < 0.05$) integrin antibodies even when used at 20 µg/ml. Slightly greater inhibition (20%, $P < 0.05$) was observed when using another anti- $\beta 1$ blocking antibody (clone mAb13) but adhesion was unaffected by $\alpha 2$ -, $\alpha 3$ -, $\alpha 6$ -, or $\beta 4$ -blocking antibodies alone (Figure 6C). Increasing the concentration of $\beta 1$ antibodies to 40 µg/ml (data not shown) did not result in further inhibition nor did other combinations of α or β antibodies. The lack of substantial inhibition by anti- $\beta 1$ antibodies was not attributable to poor blocking ability of these antibodies or availability of the $\beta 1$ epitope because both antibodies dramatically inhibited adhesion of MDA-MB-231 cells to an LM-111-rich matrix (Matrigel).

MDA-MB-231 migrated much more efficiently than MCF-7 cells toward LM-511, and this response was almost completely inhibited (88%) by anti- $\alpha 3$ integrin antibodies (Figure 6D). Combination of $\alpha 3$ with $\alpha 2$ and/or $\alpha 6$ antibodies did not inhibit MDA-MB-231 migration further. Surprisingly, migration was only weakly inhibited by blocking of the $\beta 1$ integrin subunit with P5D2 antibodies (20 µg/ml). Unlike anti- $\alpha 3$ antibodies, inhibition by P5D2 antibodies was weak and variable between assays (5 to 35%). Similar results were obtained with the mAb13 antibody (data not shown). In contrast, both antibodies completely abolished Matrigel-mediated migration (Figure 6D) or LM-511-mediated migration of MDA-MB-435 cells (data not shown), which have now been shown to be melanomas.⁴⁹

Figure 6. LM-511-mediated integrin-dependent adhesion and migration of human breast cancer cell lines. **A:** Adhesion of MCF-7 cells to LM-511. **B:** Haptotactic migration of MCF-7 cells toward LM-511. **C:** Adhesion of MDA-MB-231 cells to LM-511. **D:** Haptotactic migration of MDA-MB-231 cells toward LM-511. Rapid adhesion (30 minutes) of nonmetastatic MCF-7 (**A**) and metastatic MDA-MB-231 cells (**C**) was measured as described for Figure 5 and expressed as percentage of total cell input. Haptotactic migration of MCF-7 (**B**) and MDA-MB-231 cells (**D**) was determined by counting fully migrated cells on the underside of the Transwell porous membrane after 5 hours as described in Figure 5. For MCF-7 cell adhesion and migration and for MDA-MB-231 migration, all antibodies directed against α chains were used at 10 μ g/ml and those against β chains used at 20 μ g/ml. For MDA-MB-231 adhesion, all antibodies were used at 20 μ g/ml. All experiments were repeated three times, and the data represent the mean $\pm$ SD of a representative experiment performed in triplicate wells. * P < 0.05, ** P < 0.001. Cell surface expression of α 3 and β 1 integrin subunits and α 3 β 1 dimers on MCF-7 (**E**) and MDA-MB-231 cells (**F**) was determined by standard flow cytometry as described in Materials and Methods.

Although the results above implicate the $\alpha 3$ integrin subunit in LM-511-induced migration, the higher rate of migration of MDA-MB-231 and the lack of substantial inhibition by anti- $\beta 1$ antibodies despite almost complete inhibition by blocking of the $\alpha 3$ subunit suggest that migration may be mediated by an $\alpha 3$ receptor other than $\alpha 3\beta 1$ in these cells. Flow cytometric analysis of individual $\alpha 3$ and $\beta 1$ subunits and $\alpha 3\beta 1$ dimer in MCF-7 and MDA-MB-231 cells using specific antibodies revealed that MDA-MB-231 (Figure 6F) expressed considerably higher levels of the $\alpha 3$ and $\beta 1$ subunits than MCF7 cells (Figure 6E). Interestingly, the ratio of total $\alpha 3$ subunit to $\alpha 3\beta 1$ dimer was markedly higher in MDA-MB-231 than in MCF-7, consistent with the possibility that a proportion of $\alpha 3$ subunits is either expressed as monomers or as an alternative $\alpha 3\beta$ dimer in MDA-MB-231 cells. Taken together, these results demonstrate the functional relevance of LM-511 in regulating the adhesive and migratory phenotype of human breast tumor cells and support previous reports implicating $\alpha 3$ integrin in the metastatic progression of breast tumors.²³

Discussion

Strong evidence supports the role of the LMs and their receptors in cancer progression.^{1,5,15} However, because of the lack of suitable antibodies and availability of metastasis samples from breast cancer patients, few reports have specifically documented the changes in LM-511 expression during the metastatic progression of human breast tumors.^{10,11,34} In animal studies, progress has been hampered until recently by the lack of clinically relevant models of breast cancer metastasis that recapitulate the whole metastatic cascade from the mammary gland to bone as seen in the majority of patients with metastatic breast tumors.

To investigate the expression and function of LM-511 in breast cancer metastasis, we used a well-characterized syngeneic model of spontaneous breast cancer metastasis.^{41,42} QRT-PCR analysis of the cell lines of the model as well as *in situ* hybridization and IHC performed on their corresponding tumors and spontaneous metastases clearly show that higher expression of LM-511 rather than LM-111 or LM-332 observed in 4T1.2 and 4T1.13 lines correlates with their greater metastatic potential *in vivo*. Although some expression of LM-521 protein in these tumors cannot be completely excluded, the weak signal obtained for the LM $\beta 2$ subunit by QRT-PCR further suggests that LM-511 rather than LM-521 is the predominant $\alpha 5$ -containing isoform expressed in these lines. Detection of tumor cell expression of LM $\beta 2$ protein by IHC in mouse primary tumors and metastases will be possible when suitable antibodies become available. Enhanced LM $\alpha 5$ expression in 66cl4 lung metastases compared with that seen in primary tumors (compare Figure 2B, i and j, and Figure 3, d and e) suggests either clonal selection of LM-511-expressing tumor cells or up-regulation of this LM isoform in metastatic tumor cells induced by the lung stroma. Further work will be required to distinguish between these possibilities.

To our knowledge, this is the first demonstration that spontaneous breast cancer metastasis, including metastasis to lung and bone, is specifically associated with high tumor cell expression of LM-511. Our results complement earlier findings that vascular LM-511 is increased in breast tumors and further support the view that LM-511 expression may be more relevant than LM-111 or LM-332 in the metastatic progression of breast tumors. The IHC methodology used in our study may prove useful to investigate the expression and function of LM isoforms in other tumor types such as prostate cancer in which metastasis to bone is common.⁵⁰ Indeed, a similar shift toward LM-511-dependent interactions has been documented during prostate cancer progression.⁵¹

IHC results in human samples are consistent with those obtained in our animal model and in agreement with previous studies showing high levels of LM-511 in the vasculature of normal human mammary glands and breast tumors.^{10,11,34} Although tumor cell expression of LM $\alpha 5$ subunit was variable, the highest tumor scores (+++) were observed in four of six grade III lesions, and as such, the results are supportive of a role for $\alpha 5$ -containing LM isoforms in tumor progression. Interestingly, high tumor cell expression of LM $\alpha 5$ was associated with either the LM $\beta 1$ or the LM $\beta 2$ subunit (and thus LM-511 or LM-521 isoform) in advanced tumors. Functional characterization of purified LM-521 in breast tumor cells will be useful to determine whether these two $\alpha 5$ -containing LM isoforms have distinct or overlapping activity in advanced breast tumors. Tumor cell expression of LM $\alpha 5$ has been linked to poor prognosis and is a predictor of disease recurrence in patients with non-small cell lung carcinoma.³⁵ Whether tumor cell expression of LM $\alpha 5$ has a similar predictive/prognostic value in breast cancer patients will require the analysis of a large number of tumor samples from breast cancer patients with known clinical outcome. This will be difficult before the development of anti-LM-511 antibodies compatible with standard formalin fixation and paraffin embedding typically used for archival tissues. Isolation of such antibodies is currently underway.

LM-511 is a potent adhesive substrate secreted by many epithelial tumor lines *in vitro* and promotes their proliferation and migration.⁵²⁻⁵⁴ Our results from *in vitro* experiments, together with the high expression of LM-511 in metastatic breast tumors compared with LM-111 or LM-332, are strongly supportive of the functional relevance of LM-511 expression in metastatic mammary tumors and indicate that LM-511 regulates breast tumor cell adhesion, migration, and invasion. These observations and the high protein expression detected in metastatic tumors suggest that targeting LM-511 expression and/or function may provide benefits for the treatment of metastatic breast cancer. This is currently being tested in our mouse metastasis model.

Several integrin receptors have been implicated in breast cancer progression and shown to mediate cell attachment to LM-511 or a commercial preparation of LM-511/521⁵⁵ in other tumor types.^{52,56} In particular, engagement of $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ integrin receptors by LM-511 is known to promote migration of tumor cells *in*

vitro.^{53,57} Consistent with these studies, the results from receptor inhibition experiments indicate that the migratory and invasive responses of 4T1.2 cells to LM-511 are dependent in part on β 1-type integrin receptors. The results obtained with the human tumor lines indicate that multiple integrin receptors are involved in breast tumor cell adhesion and migration on LM-511 and reveal a major role for α 3 integrins in regulating migration. Importantly, metastatic MDA-MB-231 cells were significantly more migratory toward LM-511 than nonmetastatic MCF-7 cells. This can be attributed in part to higher expression of α 3/ α 3 β 1 in MDA-MB-231 cells (Figure 6). However, a number of observations raise the intriguing possibility that metastatic cells may also use an α 3 receptor other than α 3 β 1 dimer to adhere and migrate on LM-511. For instance, although MCF-7 migration was strongly inhibited by blocking of either subunit of the α 3 β 1 receptor, MDA-MB-231 migration was inhibited strongly by anti- α 3 but only weakly by anti- β 1 antibodies. The weak inhibition by β 1-blocking antibodies is unlikely to be attributable to the poor inhibitory activity of the antibody used or the high level of β 1 subunit in MDA-MB-231 cells because the same results were obtained using two different antibodies (P5D2 and mAb13), and migration was not further inhibited by increasing the antibody concentration to 40 μ g/ml. Moreover, β 1-blocking antibodies efficiently inhibited MCF-7 (Figure 6B) or highly migratory MDA-MB-435 cells despite high levels of β 1 expression in these cells (data not shown). It is also unlikely that the weak inhibition by these antibodies was attributable to the expression of a variant or alternative splicing of the β 1 integrin receptor in MDA-MB-231 cells because P5D2 or mAb13 antibodies used at 10 to 20 μ g/ml were sufficient to completely block their migration on Matrigel.

Comparison of the results from antibody perturbation experiments and those obtained in similar studies investigating the receptors used by MCF-7 and MDA-MB-231 to adhere and migrate on LM-111 and LM-332^{23,48} suggests a high degree of receptor specificity for LM isoforms in metastatic breast tumor cells. For instance, in contrast to the almost complete inhibition of MDA-MB-231 migration toward LM-511 by anti- α 3 neutralizing antibodies, Plopper and colleagues⁴⁸ reported that MDA-MB-231 attachment and migration on LM-332 was dependent on β 1-type integrins but unaffected by the presence of α 3- or α 6-blocking antibodies. The same study showed that MCF-7 adhesion and migration on LM-111 was mediated by α 3 β 1 integrin as shown here for LM-511-mediated migration (Figure 6). Interestingly, others²³ have reported that MDA-MB-231 cells migration toward LM-111 could be inhibited by neutralizing antibodies against the α 3 or α 6 integrin subunits. The authors concluded that MDA-MB-231 uses α 3 β 1 or α 6 β 1 integrin to adhere and migrate on LM-111. It should be noted, however, that the effect of anti- β 1 antibodies was not tested in this study, and the role of α 3 β 1 in LM-111-mediated migration was assumed based on the fact that the β 1 subunit is the only known binding partner for the α 3 integrin. Our observation that migration of MDA-MB-231 toward Matrigel, a LM-111-rich matrix, was com-

pletely abrogated by the presence of β 1 antibodies is consistent with their conclusion.

From those studies and ours, it seems that metastatic breast tumor cells may use integrins other than the traditional integrin dimers typically associated with LM-332 and LM-511 responses. Although a novel form of the α 3 integrin subunit comprising the heavy chain of integrin α 3 covalently bound to a monomer of the transferrin receptor has been reported in prostate carcinoma lines, this receptor still associates with the β 1 integrin subunit.⁵⁸ To our knowledge, the α 3 integrin subunit has never been reported to pair with a β integrin subunit other than the β 1 chain. Thus, our results suggest that α 3 integrin may combine with an as yet unidentified β integrin to form a novel LM receptor in MDA-MB-231 cells. The precise nature of this receptor is currently being investigated.

Taken together, the data presented here are highly supportive of the role of LM-511 in breast cancer progression and are consistent with the contention that LM-511 contributes to breast cancer metastasis through autocrine mechanisms. The development and characterization of reliable antibodies for IHC detection of LM α 5 in archival tissues will be most valuable in assessing the predictive/prognostic value of this LM subunit in breast cancer. It will be important also in future studies to clarify the relative contribution of tumor and endothelial-derived LM-511 to the metastatic spread of breast tumors. The mouse model developed in our laboratory will be ideally suited to target LM-511 *in vivo* and address these questions.

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