Estrogen Receptor Positive Breast Cancer Metastasis: Altered Hormonal Sensitivity and Tumor Aggressiveness in Lymphatic Vessels and Lymph Nodes

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Abstract
Breast cancers commonly spread to lymph nodes (LNs). If the primary tumors are estrogen receptor (ER) and/or progesterone receptor (PR) positive, then the likelihood that LN metastases express receptors exceeds 80%. However, due to lack of ER+ models, little is known about the role of hormones in breast cancer spread or the effects of the LN microenvironment on hormone responsiveness. We have developed metastasis models using ZsGreen labeled MCF-7 and T47D human breast cancer cells. Tumors are tracked in living mice by whole-body imaging, and macrometastases or micrometastases are detected by intravital imaging or fluorescence microscopy. Tumor growth is estrogen dependent and required for intratumoral lymphangiogenesis. Seventy-five percent of all tumors and >95% of larger tumors generate metastases. Occasionally more distant metastases are also observed. “Triads” of primary tumors, tumor-filled draining lymphatic vessels, and tumor-filled LNs from the same mouse are observed. “Triads” of primary tumors, tumor-filled draining lymphatic vessels, and tumor-filled LNs from the same mouse show that (a) proliferation, as measured by 5-bromo-2′-deoxyuridine uptake, is higher in the LN than in the primary tumor. (b) High ER levels are extensively down-regulated by estradiol in primary tumors. However, there is partial failure of ER down-regulation in LNs associated with (c) reduced PR expression. This suggests that ER are dysfunctional in the LN microenvironment and perhaps hormone resistant. (d) CD44 is sparsely expressed in primary tumor cells but homogeneously overexpressed in cells transiting the lymphatics and populating LNs. We hypothesize that CD44 expression targets tumor cells for transport to, and uptake in, LNs. If so, the CD44 pathway could be targeted therapeutically to slow or prevent LN metastases. (Cancer Res 2006; 66(18): 9308-15)

Introduction
Advanced breast cancer kills >40,000 American women each year and 10 times that number worldwide (1, 2). Seventy to 80% of primary breast cancers are estrogen receptor (ER) and/or progesterone receptor (PR) positive and considered to be hormone responsive (3). Importantly, if the primary tumors are ER+, >80% of lymph node (LN) metastases and 65% to 70% of distant metastases retain their receptors (4–6). Despite this, little is known about the role, if any, of estrogen or progesterins in influencing the spread of tumor cells from the primary site, or their deposition and growth at metastatic sites.

This lack of information is due, in part, to a lack of ER+ experimental metastasis models. The three ER+ human breast cancer cell lines most widely used for orthotopic xenograft studies are MCF-7, T47D, and ZR75 cells (7). They develop tumors in mammary glands of nude mice in response to estradiol supplementation (8, 9). However, most reports state that such tumors are poorly invasive and rarely, if ever, metastasize (9). Indeed, most metastasis models do not use these cells, relying instead on ER– cell lines like MDA-231, MDA-435 (10) and similar cells that express putative aggressiveness markers and do metastasize in nude mice. Other studies analyze only late stages of metastasis by injecting ER– tumor cells directly into the circulation (11). Alternative approaches for studying tumor aggressiveness from orthotopic sites with ER+ breast cancer cells involve their modification to overexpress oncogenes like v-Ha-ras (12) or HER-2/neu (13), growth factors like vascular endothelial growth factor (14) or fibroblast growth factor (15), or transcription factors like Id-1 (16) or Fra-1 (17), in an effort to enhance tumor progression while suppressing estrogen-dependent behavior (18). We considered, however, that if the clinical data cited above are a guide, it should not be necessary to suppress hormone responsiveness to achieve a metastatic phenotype.

Observation of metastasis can be difficult, especially if few cells are involved or appropriate target organs are unexplored. The development of fluorescent proteins to genetically tag living cells has greatly increased the likelihood that metastases will be visualized (19). Indeed, jellyfish green fluorescent protein has been widely used for studies of ER+ cells (20). Recently, a set of coral reef fluorescent proteins were identified that require no cofactors or substrates (21). These fluor have been modified to produce variants with even brighter fluorescence and enhanced emission characteristics than green fluorescent protein, and codon usage has been modified to optimize their mammalian expression. Among these fluor, ZsGreen has unique excitation and emission patterns within the visible spectrum.

This report reevaluates the metastatic potential of ER+ MCF-7 and T47D human breast cancer cells in orthotopic mouse tumors using ZsGreen as a sensitive new tracking method. Cells stably expressing this fluor and grown as xenografts in mammary glands of nude mice were absolutely estrogen dependent. Tumor fluorescence was stable and superficial LN metastases were detected by intravital imaging or fluorescence microscopy. Tumor growth is estrogen dependent and required for intratumoral lymphangiogenesis. Seventy-five percent of all tumors and >95% of larger tumors generate metastases. Occasionally more distant metastases are also observed. “Triads” of primary tumors, tumor-filled draining lymphatic vessels, and tumor-filled LNs from the same mouse show that (a) proliferation, as measured by 5-bromo-2′-deoxyuridine uptake, is higher in the LN than in the primary tumor. (b) High ER levels are extensively down-regulated by estradiol in primary tumors. However, there is partial failure of ER down-regulation in LNs associated with (c) reduced PR expression. This suggests that ER are dysfunctional in the LN microenvironment and perhaps hormone resistant. (d) CD44 is sparsely expressed in primary tumor cells but homogeneously overexpressed in cells transiting the lymphatics and populating LNs. We hypothesize that CD44 expression targets tumor cells for transport to, and uptake in, LNs. If so, the CD44 pathway could be targeted therapeutically to slow or prevent LN metastases. (Cancer Res 2006; 66(18): 9308-15)

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5-bromo-2′-deoxyuridine (BrdUrd) were modified by the LN microenvironment. In addition, the hyaluronic receptors, CD44, which control chemotactic behavior of tumor cells (22), were up-regulated in cells transiting the lymphatics and retained in LNs. These data suggest strategies for suppressing LN metastases and indicate that once in the LNs, tumor cells have reduced hormonal sensitivity.

Materials and Methods

Cell lines. MCF-7 cells were originally purchased from American Type Culture Collection (Manassas, VA). Generation of T47D cells that express one PR isoform was previously described (23), and in the present study PR-B expressing T47D cells were used exclusively. The ZsGreen-N1 expression vector, PT-67 packaging cell line, and pLNCX2 retroviral vector were purchased from Clontech/Becton Dickinson (Franklin Lakes, NJ). Fluorescent retroviral vectors were generated by cloning the DNA coding sequence of ZsGreen into the pLNCX2 vector using the restriction enzymes HincII and NotI and T4 ligase. Plasmids were then transfected into PT-67 packaging cells with a standard calcium phosphate protocol. Stable retrovirus producing cells were selected by 2 weeks of treatment with 500 μg/ml G418. MCF-7 or T47D cells were plated at ~20% confluence and incubated in filtered, virus-containing supernatant. Cells were serially transduced two to three times for 24 hours each round, at which point they exhibited heterogeneous green expression. Cells were then subjected to G418 selection, followed by aseptic fluorescence associated cell sorting (FACS), to isolate a homogeneously bright green subpopulation. These cells were returned to culture and remained bright green through multiple passage generations. Expression of ER and PR in the selected cells was confirmed by immunohistochemistry (see below).

Xenograft tumor growth and metastases. All animal procedures were done under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. Ovariectomized female athymic nu/nu mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) at 5 to 6 weeks of age. To establish tumors, animals were anesthetized with Avertin and injected into the opening of the lactiferous duct of the abdominal mammary gland with 1 million ZsGreen-expressing MCF-7 or T47D cells in 100% Matrigel (Becton Dickinson). Mice were also implanted with silastic pellets containing cellulose (10 mg) or 17β-estradiol (2 mg + 8 mg cellulose) as previously described (24). Tumor size was recorded weekly with a digital caliper. Fluorescent whole-body imaging was done weekly (Illumatool 9900, Lightools Research, Encinitas, CA). Two hours before necropsy 100 mg/kg of 5-bromo-2′-deoxyuridine (BrdUrd) in PBS was injected i.p. At the end of the study, mice were euthanized by CO2 asphyxiation and fluorescent intravital optical imaging (www.aacrjournals.org) was used exclusively. The ZsGreen-N1 expression vector, PT-67 packaging cell line, and pLNCX2 retroviral vector were purchased from Clontech/Becton Dickinson (Franklin Lakes, NJ). Fluorescent retroviral vectors were generated by cloning the DNA coding sequence of ZsGreen into the pLNCX2 vector using the restriction enzymes HincII and NotI and T4 ligase. Plasmids were then transfected into PT-67 packaging cells with a standard calcium phosphate protocol. Stable retrovirus producing cells were selected by 2 weeks of treatment with 500 μg/ml G418. MCF-7 or T47D cells were plated at ~20% confluence and incubated in filtered, virus-containing supernatant. Cells were serially transduced two to three times for 24 hours each round, at which point they exhibited heterogeneous green expression. Cells were then subjected to G418 selection, followed by aseptic fluorescence associated cell sorting (FACS), to isolate a homogeneously bright green subpopulation. These cells were returned to culture and remained bright green through multiple passage generations. Expression of ER and PR in the selected cells was confirmed by immunohistochemistry (see below).

Results

An in vivo model of estrogen-dependent human breast cancer LN metastasis. The ER+ human breast cancer cell lines MCF-7 and T47D are commonly used to study the biology of estrogen action in vitro. These cells also grow as estrogen-dependent xenografts in nude mice (8, 24, 26), but most published reports state that orthotopic tumors fail to metastasize. Because, clinically, ER+ tumors metastasize frequently (4–6), we decided to reevaluate the metastatic potential of ER+ human breast cancer cells in xenografts using sensitive new tracking methods. To this end, MCF-7 and T47D cells were retrovirally transduced with ZsGreen, G418 selected, and the brightest green subpopulation was isolated by FACS sorting. Confocal imaging showed that ZsGreen was highly expressed in both cell types, in the cytoplasm and nuclei (not shown). Intense fluorescence is maintained through multiple passages, the fluor is nontoxic, and photobleaching studies show that ZsGreen, a coral reef protein, is much brighter and more photostable than green fluorescent protein, a jellyfish protein (not shown).

Visualizing lymphovascular tumor cell spread. One million fluorescent tumor cells in Matrigel were injected into the lactiferous duct of the abdominal (#4) mammary gland of 5- to 6-week-old, ovariectomized nu/nu mice supplemented with an estradiol-releasing silastic pellet (24). This injection route led to formation of well-circumscribed tumor nodules distant from the subiliac LN in the gland, as shown by a whole mount 3 days after injection (Fig. 1A, top). In contrast, whole mount of a tumor at 12 weeks shows the subiliac LN, still physically distinct from the primary tumor but already highly involved with tumor cells (Fig. 1A, bottom).

This protocol was used to track the spread of ER+ tumors beyond the #4 mammary gland using whole-body imaging of live mice done weekly. Figure 1B shows an image of tumor tracks photographed through the skin of an intact mouse at 12 weeks. Bright green bilateral tumors are present in mammary gland #4 at the groin, and both subiliac mammary gland LNs contain tumor cells. Also clearly visible are tumor cell tracks in the epigastric collecting lymphatic vessels (LV) draining the tumor cranially on the right, as well as tumor cells in the right axillary LN. Similarly, three distinct green fluorescent clusters of tumor cells are faintly seen in the left lymphatics and axillary LN.

At sacrifice, intravital fluorescence microscopy was used to examine tumor spread to visceral tissues as shown in Fig. 1C and D. An axillary LN micrometastasis is seen in one mouse at 12 weeks (Fig. 1C, left) and a fully involved LN metastasis in another mouse at 12 weeks (Fig. 1C, right). Figure 1C also shows the afferent LV full of tumor cells, with the parallel epigastric vein beyond the #4 mammary gland using whole-body imaging of live mice done weekly. Figure 1B shows an image of tumor tracks photographed through the skin of an intact mouse at 12 weeks. Bright green bilateral tumors are present in mammary gland #4 at the groin, and both subiliac mammary gland LNs contain tumor cells. Also clearly visible are tumor cell tracks in the epigastric collecting lymphatic vessels (LV) draining the tumor cranially on the right, as well as tumor cells in the right axillary LN. Similarly, three distinct green fluorescent clusters of tumor cells are faintly seen in the left lymphatics and axillary LN.
(left) and also much more extensive metastases to periaortic and renal LNs (right).

Bilateral tumor-filled lymphatic tracks exiting a primary tumor are clearly seen in Fig. 2A. LV full of tumor cells extend both cranially and medially from the tumor. It is possible that the tortuously branching network of lymphatics exiting the tumor cranially represents lymphangiogenesis. A higher-power view of the laterally branching iliac LV shows that the tumor cells within it coalesced into emboli rather than moving as discrete cells. In response to mechanical pressure applied either to the tumor or the efferent lymphatic root during dissections, fluorescent emboli in collecting lymphatics can often be seen moving into their draining LN (not shown). This has implications for the order in which breast surgical procedures and sentinel node biopsies are done. In general,
tumor cell spread to the superficial LNs is restricted to the side of the body containing the primary tumor, and spread to deeper LNs becomes bilateral with progressive disease (Fig. 1D). Directional valves (asterisk) within the LV do not inhibit tumor cells moving through LVs, as shown in Fig. 2B. Lastly, fluorescence microscopy can be used to analyze clinicopathologic features of tumors and their metastases. Importantly, ZsGreen fluorescence is maintained throughout the histologic fixation process. Figure 2C shows the H&E stain of a collecting LV filled with tumor cells within the mammary gland fat pad. A milk duct runs alongside it. Fluorescence microscopy of a serial section confirms that tumor cells are restricted to the LV.

**Estrogen-dependent tumor growth and rate of LN metastases.** Figure 3 summarizes the primary tumor growth pattern of MCF-7 and T47D cells during 12 weeks of observation and the extent of LN metastasis as assessed by whole-body and intravitral imaging. Growth from both cell types is estradiol dependent, with tumors nearly tripling in size in ~12 weeks (Fig. 3A). Both MCF-7 and T47D tumors produce LN metastases at the same rate, which increases with time and tumor size. At 2 weeks, no metastases occurred. By 4 weeks, ~14% of tumors generated LN metastases. This increased to ~75% at 12 weeks. Development of LN metastases seems to be size dependent (Fig. 3B). A separate time-course study of tumors grown for 1, 6, or 12 weeks showed that metastases to superficial subiliac and axillary LN, or to deeper visceral lateral iatic and aortic lumbar LN surrounding the aorta and kidneys, tend to occur at the same rate.

**Estradiol and LV development.** Because only estradiol-treated tumors yielded LN metastases, we next studied the effects of estradiol on tumor-associated LV development. For these studies, tumors were grown with or without estradiol supplementation for short term (3-10 days) or long term (6-12 weeks) and LV were quantified with lymphatic-specific LYVE-1 and Prox-1 (not shown) antibodies. Peritumoral LVs were identified in both treatment groups and likely represent lymphatics found adjacent to veins and arteries in the normal mouse mammary gland (Fig. 4). Intratumoral LVs were not seen after short-term estradiol treatment or in the estradiol-deprived dormant tumors. Fifty-eight percent of long-term estradiol-treated tumors contained intratumoral LVs (Fig. 4). ZsGreen fluorescence was quenched during the immunolabeling process, allowing for double immunostaining with anti-LYVE and anti-BrdUrd antibodies (Fig. 4) to assess the proliferation state of the LV. This procedure showed the LVs to be BrdUrd positive, suggesting that lymphangiogenesis was occurring. However, double labeling for LYVE and ER expression showed absence of ER in the LVs (not shown). We conclude that LV proliferation, although restricted to the estradiol-dependent growing tumors, is either not itself estradiol dependent or is occurring in response to paracrine signals from surrounding ER+ tumor cells.

The triad of primary tumor, lymphatic emboli, and LN metastases: proliferation. Our ability to dissect a primary tumor, its draining lymphatic collecting duct filled with tumor cells, and the downstream tumor-filled LN, from the same mouse, allows assessment of the role of the microenvironment in tumor cell proliferation and steroid receptor expression. BrdUrd uptake, measured with BrdUrd primary antibodies coupled to a HRP or a green fluorescent secondary antibody, was used to assess the proliferation rate of cells in these different compartments. In the absence of estradiol, tumor cells at the injection site remain viable and proliferate slowly with a labeling index <7% (Fig. 5A), but at a rate insufficient to elicit an overall increase in tumor size at 12 (Fig. 3) or 16 to 20 weeks (not shown). These dormant tumors yield no LN metastases. In the presence of estradiol, primary tumors exhibit extensive BrdUrd uptake with a labeling index of ~20%.

To accurately quantify dispersed tumor cells and eliminate the contribution of proliferating normal host cells, tumor cells were immunolabeled with CK18 and a red secondary antibody. In this case, proliferating cells were quantified with anti-BrdUrd and a green fluorescent secondary antibody, and some sections were also counterstained with blue 4’,6-diamidino-2-phenylindole (DAPI). Figure 5B shows two “triads” of tumor, LV and LN. Each triad came from the same mouse; the two sets were taken from two different mice. In triad 1, although the primary tumor cells have a high labeling index, proliferation in the LV embolus is low and mainly restricted to cells at the periphery, and in the LN, tumor cells are present only in the subcapsular sinus. The remainder consists of normal DAPI-stained LN cells (some of which may have contributed to the LN metastases).

**Figure 3. Estrogen-dependent tumor growth is required for LN metastasis.** A. MCF-7 (triangles) and T47D (circles) tumors were grown either with (+E) or without (−E) estradiol for 12 weeks in ovariectomized nude mice. Tumor area was quantified with digital calipers and presence of metastases was assessed by weekly whole-body imaging and by intravitral imaging at necropsy. The percent of mice with LN metastasis is indicated at each time point. B. tumor area at necropsy as assessed by the diameter in two dimensions. Filled symbols, tumors from which metastasis developed; open symbols, tumors that did not generate metastases.
which are also undergoing cell division). Triad 2 exhibits a different pattern, with extensive proliferation in the primary tumor and throughout the LV emboli, and the LN is highly involved with rapidly proliferating tumor cells.

Figure 5C quantifies all proliferation data. It shows, first, that the two methods of assessing BrdUrd uptake yielded similar results (compare the two +E "tumor" sets—one assessed with HRP and the other with dual BrdUrd/CK18 immunohistochemistry). As expected, compared with dormant controls, estradiol-treated primary tumors exhibit a statistically significant increase in proliferation rate from ~7 to ~20%. And, compared with the primary tumors, metastatic cells in the LN microenvironment have a statistically significant (P = 0.003) further increase to ~27%. Proliferation rates of embolic cells in the LV were variable and not statistically different from the other two compartments, although they tended to resemble those in the primary tumor more closely.

The triad of primary tumor, lymphatic emboli, and LN metastases: ER and PR. ER and PR immunohistochemistries for two sets of vehicle versus estradiol-treated triads using MCF-7 derived tumors show the heterogeneity observed (Fig. 6). In the absence of estradiol, levels of ER in tumors are always high. Estradiol treatment leads to extensive tumor ER down-regulation. ER tend to remain low in the LV and LN of the second triad (bottom). No PR are expressed without estradiol induction. Estradiol treatment leads to heterogeneous PR expression in tumors, which tends to decrease in LV and LN of the same mouse. Quantitation of PR data from several mice is shown in Fig. 6. There is a clear trend of decreased PR expression as cells spread into LV and LN (P = 0.052). A second experiment (not shown) using fluorescent immunohistochemistry for ER and PR showed a similar trend of reduced PR between five tumors and their matched LNs (P = 0.09). Each immunohistochemical procedure showed a

Figure 4. Assessment of tumor-associated lymphatics and lymphangiogenesis. Tumors from 3- to 10-day-old or 6- to 12-week-old placebo-treated (–E) and estradiol-treated (+E) ovariectomized mice were paraffin-embedded sections (5 μm) were probed for LVs with an anti-LYVE-1 antibody and an HRP-labeled secondary antibody. Bottom, sections were probed by dual fluorescence immunohistochemistry with mouse anti-BrdUrd and rabbit anti-LYVE primary antibodies, followed by goat anti-mouse (green) and goat anti-rabbit (red) secondary antibodies, to identify lymphatics in S phase. The table quantifies the results.

Figure 5. Assessment of tumor proliferation in MCF-7 tumors, LV emboli, and LN metastases. A, ovariectomized mice bearing MCF-7 tumors were treated for 12 weeks with placebo (–E) or estradiol (+E). Tumors were paraffin embedded, sectioned, and stained with H&E or analyzed by BrdUrd-HRP immunohistochemistry. B, dual fluorescence immunohistochemistry for cytokeratin 18 (red) and BrdUrd (green) of estradiol-treated MCF-7 tumors, LV emboli, and LN metastases. Each triad was taken from the same mouse. Anti-rabbit CK18 and anti-mouse BrdUrd primary antibodies and goat anti-rabbit (red) and goat anti-mouse (green) antibodies were used. A DAPI counterstain (blue) was occasionally included. The centrally necrotic regions of tumors were excluded from analyses. C, quantification of A and B (n = 5 for tumors and LNs; n = 3 for LVs), showing the data obtained from the HRP based protocol in A (white columns) and the dual CK18/BrdUrd protocol in B (black columns).
nonsignificant slight increase in ER expression in the LN metastases compared with the down-regulated ER in the estradiol-treated primary tumor.

**Selection of cells for lymphatic transit: CD44.** Hyaluronan regulates various aspects of cell behavior, particularly cell migration and invasiveness. It does so by binding to specific receptors on the cell surface, the best characterized of which is CD44 (22). Human breast cancer cells migrate directionally in response to a hyaluronan gradient depending on CD44 expression levels (22). Breast cancers secrete factors that up-regulate hyaluronan expression in osteoblasts (27), which may promote bone metastasis. Figure 7A shows CD44 expression levels in two primary tumors and in their LV and/or LN metastases. Set A (top) clearly shows extensive overexpression of CD44 in tumor cells occupying the LN microenvironment, compared with the same tumor cells in the mammary gland, where CD44 expression is sparse and heterogeneous. Set B includes a matched LV embolus and again shows sparse and heterogeneous CD44 in the tumor, but homogenous and elevated CD44 in both the LV embolic tumor cells, and the LN metastases.

**Distant metastases.** Metastases to distant organs were occasionally observed with these models. Figure 7B (left) shows H&E staining of tumor cells metastatic to the lung. Their identity is confirmed by intense ZsGreen fluorescence in a serial section (Fig. 7B, right). These lung metastases could have arisen by direct peritumoral venous invasion and spread (the most direct route to the lungs from distant body sites) or they may reflect indirect spread from an overwhelmed lymphatic system via lymphatic-venous anastomoses. Both mechanisms may contribute to distant organ metastasis in humans (28). It should be stressed that these metastases were rare and they occurred only in animals that also had extensive LN spread.

**Perineural invasion.** Perineural invasion was also noted in tumors arising from both cell lines. Perineural invasion is observed in a variety of human carcinomas and may contribute to local-regional tumor spread, although it has not been shown to be an independent prognostic indicator in human mammary carcinomas (29). Although not as frequent as lymphatic invasion, perineural invasion was occasionally found at tumor margins and along the peripheral nerves that parallel the iliac veins. Figure 7C shows an H&E stain (left) and CK18 immunohistochemistry (right) of T47D tumor cells (arrow) tracking along a peripheral nerve (asterik).

**Discussion**

LN metastasis in association with tumor size is the single most powerful indicator of poor prognosis in mammary carcinoma (30, 31). Pathologic data sets of patients with breast cancer indicate that, at diagnosis, presence of tumor-infiltrated LNs is common, with estimates ranging from 30% to 50% of cases (32–34), depending on tumor size. The dominant feature of the present model is rapid and reliable LN metastasis of ER+ cells...
from orthotopic tumor sites. Two different cell lines, MCF-7 and T47D, generated estrogen-dependent solid tumors and reliably produced metastases to local and distant LNs. Under conditions of the studies, which were terminated at 12 weeks, tumor cells in LV and LN metastases were observed in 75% of all mice and in essentially 100% of mice, the primary tumors of which exceeded \( \sim 75 \text{ mm}^2 \) in area (Fig. 3). Clinically, the association between tumor size and LN involvement is well known (35). Metastases to other sites, like the lungs (Fig. 7B), were occasionally observed, as were cells migrating along peripheral nerve tracks (Fig. 7C). The ability of the latter routes to reliably colonize distant organs undoubtedly will require longer observation times. This is suggested by the studies of others. Half a million ER– tumors are more likely to spread to bones whereas ER+ tumors more commonly metastasize to the viscera (40, 41). ER+ metastasis models, reflecting the clinical situation, could help define molecular mechanisms involved in such tissue specificity.

Expression profiling of matched primary and metastatic LN tumor pairs in clinical samples has failed to identify genes that distinguish one from the other (42) because metastases are genetically more closely related to the primary tumor from which they arose than to any other metastasis. However, within matched pairs, select genes are differentially expressed between the tumor and LN metastasis. Common among these are extracellular matrix and cell-matrix interacting genes, like the hyaluronan receptor CD44, which mediates rolling, attachment, and migration of cells on a hyaluronan substratum (43, 44). We show here that CD44 is sparsely and heterogeneously expressed in primary tumors but homogeneously overexpressed in LV tumor cell emboli and LN metastases (Fig. 7). Because directional migration of breast cancer cells towards hyaluronan is dependent on CD44 (22), we propose that the CD44+ subpopulation of primary tumor cells is preferentially propelled into tumoral lymphatics, and from there to LNs. If so, therapeutic targeting of the CD44+ subpopulation in a primary tumor could prevent LN metastases.

There is a high concordance rate (\( \sim 80-90\% \)) in ER expression between primary tumors and matched LN metastases (5). However, in the same patient, considerable disparities can exist in the concentrations of ER at the two sites, with ER levels often (6, 45) but not always (45) higher in the LN than in the local primary tumor. We observe a pattern (Fig. 6) in which ER levels are higher in the LN than in the primary tumor of the same mouse, an indicator of ER down-regulation failure in the LN microenvironment. It has been reported that there is an association between estrogen-dependent ER down-regulation and improved transcriptional efficacy of the receptors (46). Although it might be counterintuitive to think that in the presence of estrogen, lower ER levels signal a stronger hormone response, this seems to be the case. Thus, the inefficient ER down-regulation in the LN could be associated with poorer hormone responsiveness at that site. In the mouse tumors, PR expression levels (Fig. 6), which are markers of estrogen action and ER function, support this hypothesis. Assessment of PR levels in LNs and matched primary tumors shows a trend towards a decrease in LN PR (Fig. 6). This would be predicted by inefficient ER down-regulation. Clinically, both up-regulation and down-regulation of PR have been reported in the LN compared with primary tumors (45), but this has not been correlated to ER in the same samples.

We also observe a statistically significant increase in the proliferation rate of LN metastases compared with matched primary tumors (Fig. 5B and C). Clinically, increased mitoses in nodal metastases compared with primary tumors have been reported (47), possibly due to release of cytokines and growth factors, such as insulin-like growth factor-I and epidermal growth factor, by the LN (48). The implications of this growth promotion within LNs for failure of hormone treatments in metastatic disease could be important. If, in the LN microenvironment, cytokines and growth factors are the dominant mitogens, then LN metastases would be less sensitive than primary tumors to estradiol-suppressive therapies. Indeed, in metastatic disease,
tamoxifen, which targets ER signaling pathways, is rarely associated with long-term remission, exhibiting decreased mean time to disease progression and decreased duration of response from local disease (41, 49, 50).

In summary, we have developed models of ER+ human breast cancers that reliably spread to LNs from solid tumors. Through the use of a new generation fluorescent protein, ZsGreen, we are able to identify cancer cells in tumors, LV emboli, LNs, and, occasionally, more distant sites for further molecular analysis. These studies show that the tumor cells found in LNs express marker-associated with tumor aggressiveness. At the same time, the LN microenvironment may suppress estrogen sensitivity of tumor cells, which would make them more resistant than primary tumors to ER-targeted treatments.

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