

Estrogen Insensitivity in a Model of Estrogen Receptor–Positive Breast Cancer Lymph Node Metastasis

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Abstract

The lymphatic system is a common avenue for the spread of breast cancer cells and dissemination through it occurs at least as frequently as hematogenous metastasis. Approximately 75% of primary breast cancers are estrogen receptor (ER) positive and the majority of these maintain receptor expression as lymph node (LN) metastases. However, it is unknown if ER function is equivalent in cancer cells growing in the breast and in the LNs. We have developed a model to assess estrogen responsiveness in ER⁺ breast tumors and LN metastases. Fluorescent ER⁺ MCF-7 tumors were grown in ovariectomized nude mice supplemented with estradiol. Once axillary LN metastasis arose, estradiol was withdrawn (EWD), for 1 or 4 weeks, or continued, to assess estradiol responsiveness. On EWD, proliferation rates fell similarly in tumors and LN metastases. However, estradiol-dependent ER down-regulation and progesterone receptor induction were deficient in LN metastases, indicating that ER-dependent transcriptional function was altered in the LN. Cancer cells from estradiol-treated and EWD primary tumors and matched LN metastases were isolated by laser capture microdissection. Global gene expression profiling identified transcripts that were regulated by the tissue microenvironment, by hormones, or by both. Interestingly, numerous genes that were estradiol regulated in tumors lost estradiol sensitivity or were regulated in the opposite direction by estradiol in LN metastases. We propose that the LN microenvironment alters estradiol signaling and may contribute to local antiestrogen resistance. [Cancer Res 2007;67(21):10582–91]

Introduction

Interactions between luminal epithelial cells and the surrounding microenvironment govern the overall physiology of the mammary gland (1), with development of epithelial ducts being dependent on the activity of both stromal and epithelial estrogen receptor α (ER α ; ref. 2). Integrins, growth factors, and steroid hormone signaling pathways all play an important part in maintaining normal glandular architecture (3). Stroma makes up >80% of breast volume and is composed of fat, interstitial/interlobular dense connective tissue, intralobular loose connective tissue, and lymphatic/blood vessels (4). Luminal epithelial cells associate with these stromal

elements as well as with basement membrane and myoepithelial cells. Disruption of this delicate balance results in dramatic changes in both extracellular and intracellular signaling (3).

Seventy percent to 80% of primary breast tumors express ER α and initially respond to estradiol withdrawal (EWD) or antiestrogen therapies (5). Breast cancer ER α expression and function are also influenced by the cellular microenvironment. *In vitro* studies with human ER⁺ breast cancer cells show that extracellular matrix proteins, such as type I collagen and laminin, can modify proliferative responsiveness to estrogen (6). A comparison of *in vitro* and *in vivo* models found that estradiol regulates different genes in human breast tumor xenografts compared with the identical cells in culture (7). These studies suggest that a microenvironment-dependent influence on estradiol-dependent gene expression exists in ER⁺ breast cancers. However, this issue has received little attention, and to the best of our knowledge, *in vivo* experiments that test if metastatic microenvironments alter estradiol-dependent gene expression have not been conducted.

Metastasis to the lymph nodes (LN) is a key prognostic factor that conveys advanced disease status with the possibility that cancer cells have spread to other more distant sites. At diagnosis, 30% to 50% of all breast cancers have spread to the draining or “sentinel” LN (8–10), and if the primary tumor is ER⁺, then ~80% of LN metastases retain ER expression (11–13). As tumor cells disseminate to LNs, they enter a distinct microenvironment. Here, they encounter different supporting elements, such as reticular fibers, flowing lymph fluid, lymphocytes, T and B cells, increased numbers of macrophages, and epithelial reticular cells that are surrounded by adipose tissue (14). It is unknown if the differences in structure, function, and composition of the mammary gland compared with the LN affect ER activities and estradiol-dependent gene expression in breast cancer cells that are located within these two distinct microenvironments.

The studies presented here use a xenograft model of fluorescent, ER⁺, estradiol-dependent human breast cancer LN metastasis to address this question. In the same mouse, LN metastases are found to be transcriptionally estradiol insensitive compared with primary tumors from which they originated, despite retention of ER. We identify genes that may aid in explaining mechanistically why advanced ER⁺ breast cancers become nonresponsive to antiestrogen or EWD therapies and often recur as metastases.

Materials and Methods

Cell lines. MCF-7 human breast cancer cells were originally purchased from the American Type Culture Collection. The generation of fluorescent MCF-7 cells has been previously described (15). In brief, ZsGreen retroviral particles (Clontech) were isolated from PT-67 packaging cells (Clontech), filtered, and overlaid onto MCF-7 cells. Cells were serially transduced twice for 24 h each round and then subjected to G418 selection followed by aseptic fluorescence-associated cell sorting to isolate a homogeneously

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

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bright green subpopulation. Generation of PR-B-expressing T47D human breast cancer cells has been described previously (16).

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 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 abdominal mammary glands with 1 million ZsGreen-expressing MCF-7 cells in 100 μ L of 100% Matrigel (Becton Dickinson). Mice were also implanted with silastic pellets containing 17 β -estradiol (2 mg + 8 mg cellulose) as previously described (17). Fluorescent whole-body imaging (Illumatool 9900, Lighttools Research) was done weekly to determine when axillary LN metastases arose. For EWD mice, once axillary LN metastases were detected, mice were anesthetized with Avertin and estradiol-releasing pellets were removed for 1 week; some were removed for 4 weeks to confirm protein expression and for proliferation studies. Control mice were continued on estradiol. Mice were euthanized by CO₂ asphyxiation and fluorescent intravital optical imaging was done by coupling the Illumatool with an Olympus SZ-61 dissecting microscope and Olympus C-5050 digital camera. Fluorescent tumors in mammary glands and LNs were removed. Lymphatic vessels (LV) draining fluorescent tumor cells from mammary glands were also collected.

Serial passaging of a ZsGreen⁺ T47D-PR-B LN metastasis was done by isolating the LN, pushing the tissue through multiple sieves, expanding and G418 selecting human tumor cells in culture, and reinjecting the cells into the mammary glands of estradiol-supplemented recipient mice.

Laser capture microdissection and expression profiling. Mammary gland tumors (MGT) and LN metastases from estradiol-treated or 1-week EWD mice were harvested, placed in a Tissue-Tek cryomold (EMS), covered with Neg50 frozen section medium (Richard-Allan Scientific), and frozen in isopentane (Sigma) cooled by liquid nitrogen. Sections (8 μ m) were cut through the entire tissue with a cryotome set at -20° C. Each section was collected on an uncharged slide (Fisher), and every 10th slide was stained for H&E or processed for cytokeratin 18 (CK18) immunofluorescence. Slides containing cancer cells were processed through 75% ethanol, water, 75% ethanol, 95% ethanol, and 100% ethanol for 30 s each and finally dehydrated with xylene for 1 min before being immediately subjected to laser capture microdissection using an Arcturus Autopix. Two thousand cells for each sample were extracted within 30 min of fixation and frozen at -70° C until further processing. Suggested protocols from Arcturus were followed for RNA extraction and two rounds of amplification using the PicoPure RNA isolation kit and RiboAmp HS kit (Arcturus), respectively. Samples were *in vitro* transcribed, biotinylated, hybridized to Affymetrix X3P chips that interrogate 47,000 transcripts with 61,000 probe sets, and scanned on an Agilent Bioanalyzer 2100.

Statistical analyses. Raw expression values for each tumor and LN were downloaded into GeneSpring version 7.3 (Agilent) for exploratory purposes. Four matched pairs of tumors and metastatic LNs (2 estradiol and 2 EWD) as well as 2 unmatched LNs (1 estradiol and 1 EWD) for a total of 10 samples were analyzed. Probe sets that were absent across all 10 samples were filtered from further statistical analysis (18). Remaining probe sets were robust multiarray analysis (RMA) normalized in Partek Genomics Suite 6.2, and a three-way mixed model ANOVA analysis was done to identify differentially expressed genes. Four groups were compared: estradiol-treated MGT, estradiol-treated LN, EWD MGT, and EWD LN. These four groups were divided based on the two levels of hormone treatment (estradiol or EWD) and the two levels of tissue type (MGT or LN). Hormone and tissue were therefore defined as main factors in the model. Interaction effects among hormone and tissue were also included. The random factor animal (which is nested in hormone) was added to account for the fact that multiple tissues (MGT and LN) were taken from the same animal. In total, 1,570 transcripts were identified as having significant differences among the four group means ($P_{\text{model}} < 0.05$) and $P < 0.05$ for main or interaction effects. To further identify differences in hormonal regulation among the 1,570 differentially expressed genes, they were subjected to linear contrasts to make two specific comparisons: EWD versus estradiol-treated MGT and EWD versus estradiol-treated LN. Bonferroni's correction for

multiple tests was then applied to these two comparisons and the level of significance was changed to 0.025.

Gene networking. To define relationships among genes differentially regulated by estradiol in MGTs and LN metastases, pathway analyses were done using Ingenuity software. Probe set identification numbers were used for gene identification.

Immunohistochemistry. Organs were fixed in 4% paraformaldehyde overnight, paraffin embedded, and cut into 4- μ m sections. After high-temperature antigen retrieval in citrate buffer, immunohistochemistries were done on estradiol-treated tissues and 1-week or 4-week EWD mice using primary antibodies directed against ER (1:100, SP1; Lab Vision/NeoMarkers), progesterone receptor (PR; 1:500, 1294; Dako), CK18 (1:200; Calbiochem), CD44 (1:200; Ab4; Lab Vision/NeoMarkers), caveolin 1 (CAV1; 1:125; Epitomics), and cathepsin D (CTSD; 1:125; Epitomics) for 1 h at room temperature. Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse and/or goat anti-rabbit secondary antibodies (Envision HRP; Dako) reacted with 3,3'-diaminobenzidine (Dako). Sections were counterstained with hematoxylin and mounted with Permount (Fisher). For immunofluorescence microscopy, goat anti-rabbit Alexa Fluor 555 (red) secondary antibodies were used (1:200, Alexa Fluor; Invitrogen) and sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in methanol and mounted with Gel/Mount (Biomeda).

Results

EWD model. MCF-7+ZsGreen tumors require estradiol for growth and LN metastasis (15). To generate LN metastases, bilateral tumors were grown in the abdominal mammary glands of ovariectomized immunocompromised mice that were supplemented with an estradiol-releasing pellet (Fig. 1A). Weekly tumor measurements were recorded with a digital caliper. Spread of cancer cells to axillary LNs was determined by weekly fluorescent whole-body imaging (Fig. 1B). To define estradiol-regulated genes (7), once LN metastases were detected, the estradiol-releasing pellet was withdrawn for 1 week in half of the mice (EWD) or left in place in the remaining mice. To assess proliferation rate, EWD was extended for 4 weeks in tumors and LN metastases. Bromodeoxyuridine labeling and mitotic indices indicated a similar decrease of $\sim 50\%$ at both sites. At necropsy, assessment of the uterine mass confirmed the presence or absence of systemic estradiol, with 1- and 4-week EWD uteri exhibiting significant decreases in mass compared with estradiol-treated uteri. To locate cancer cells within LNs, the entire LN was sectioned and every 10th slide was processed for CK18 immunohistochemistry (Fig. 1C). The CK18 map and the unique morphology of MCF-7 cells compared with lymphocytes (Fig. 1D) allowed for identification of the cancer cells in serial sections. We then marked and laser captured pure MCF-7 cell populations from matched tumors and LN metastases.

Estrogen insensitivity in LN metastases: ER and PR expression. Estradiol treatment leads to down-regulation of ER protein levels in tumors (19, 20). To characterize these effects in estradiol-free, estradiol-treated, and EWD conditions, the MCF-7 tumors, LV emboli, and LN metastases were probed for ER and PR by immunohistochemistry. In Fig. 2, the tissues from each treatment group were from the same mouse. Highest ER levels were present in tumors that did not receive supplemental estradiol ($-E$). Seventy-three percent of MCF-7 cells expressed high levels of ER and no cells contained PR (Fig. 2, *top*). In the presence of estradiol ($+E$), ERs were down-regulated by $>40\%$ in the tumor (Fig. 2, *middle*) but decreased by $<20\%$ in LV tumor emboli and LN metastasis of the same mouse. Ligand-dependent down-regulation of ER is required for its transcriptional activity (20), including PR

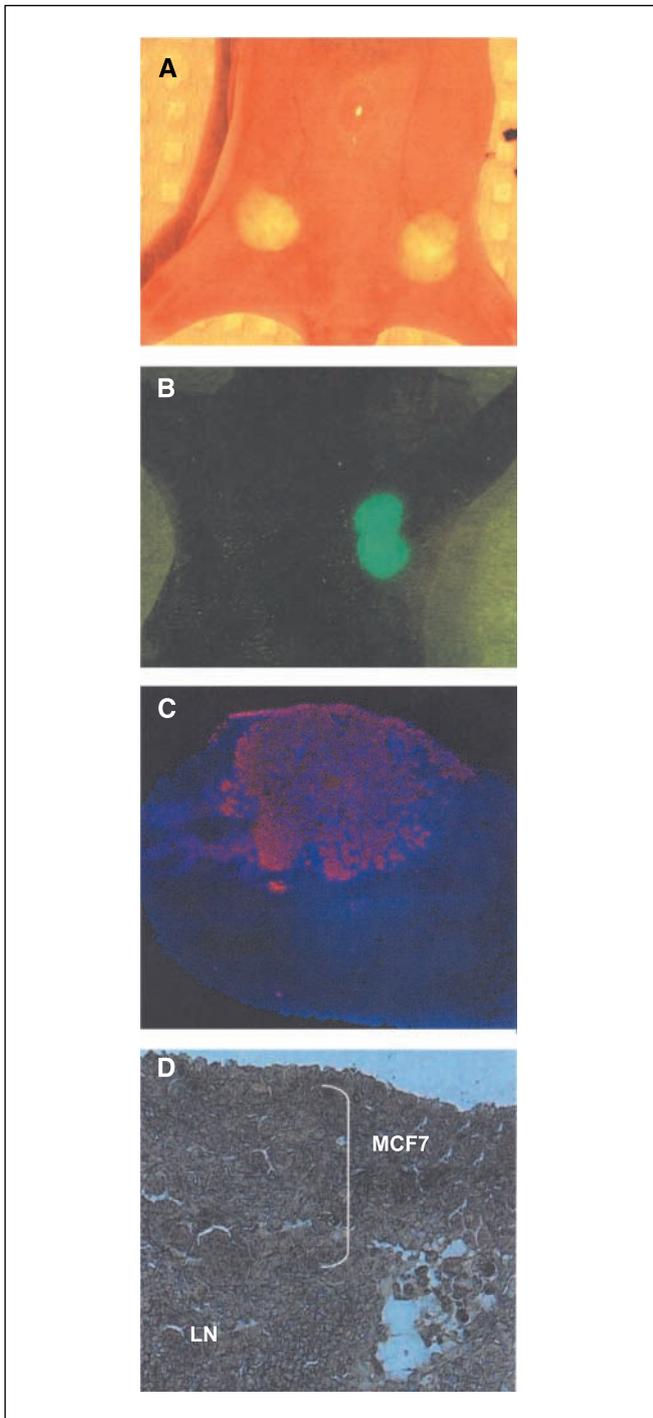


Figure 1. MCF-7+ZsGreen xenograft tumors and LN metastases. MCF-7+ZsGreen tumors were established in mammary glands of ovariectomized *nu/nu* mice supplemented with an estradiol pellet, and LN metastases were allowed to develop. *A*, fluorescent whole-body image of bilateral MCF-7+ZsGreen tumors. *B*, fluorescent whole-body image of MCF-7+ZsGreen metastasis to the left axillary LN. *C*, fluorescence immunohistochemistry for CK18 (red) and DAPI (blue) of an axillary LN metastasis. Magnification, $\times 4$. *D*, white light laser capture microscope image of an axillary LN metastasis showing MCF-7 cells or lymphocytes (LN). Magnification, $\times 40$.

induction. Indeed, PRs were expressed in +E tumors but were only rarely found in +E LV emboli (Fig. 2, arrows) or in LN metastases. On EWD, ERs were restored to control (-E) levels in all tissues, and PRs were absent (Fig. 2, bottom).

Microenvironment versus clonal selection. To determine whether the increased ER expression observed in LN metastases was due to clonal selection of ER⁺ cells from the primary tumor, or to an effect on ER at the metastatic microenvironment, tumor cells were isolated from the axillary LN metastasis of a donor mouse, expanded in culture, and reimplanted in the mammary gland of an

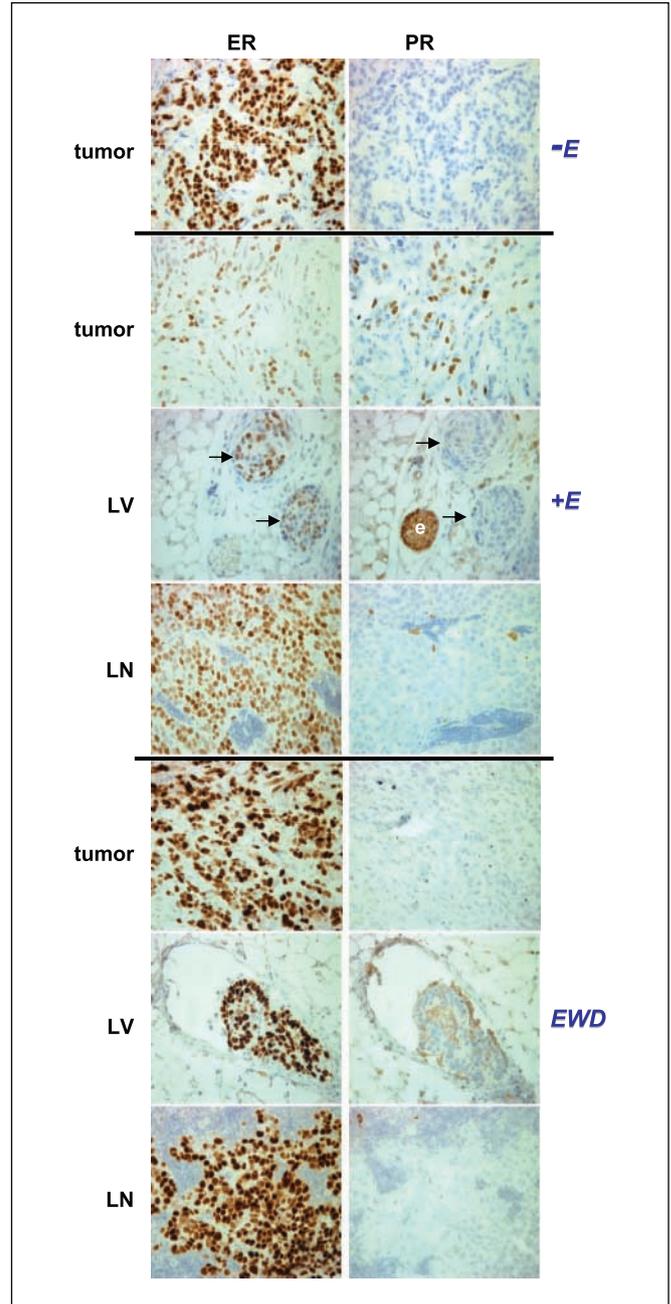
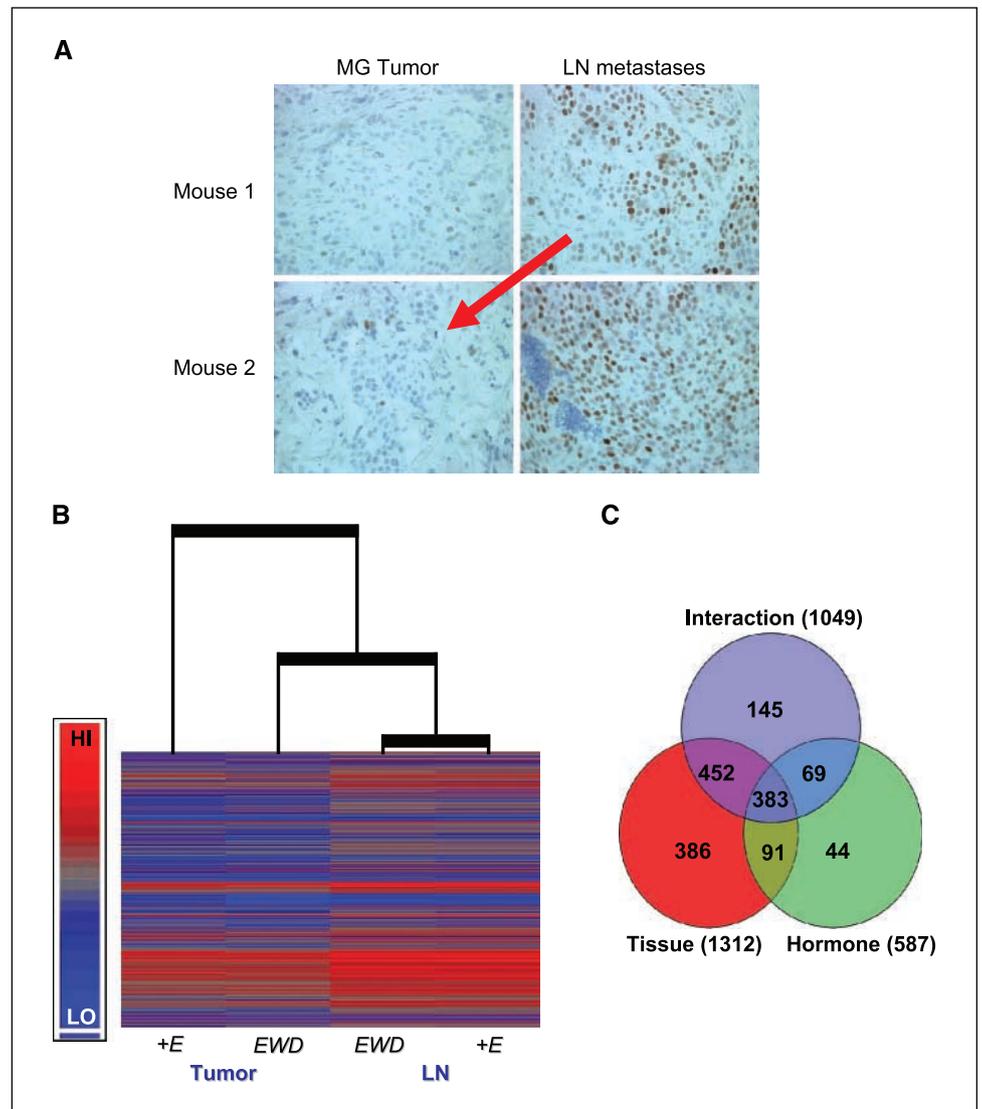


Figure 2. Estradiol regulation of ER and PR levels. ER and PR expression in MCF-7 cells from matched tumors, LVs, and LNs. MCF-7+ZsGreen tumors were established in ovariectomized mice supplemented with a placebo pellet (-E), an estradiol-releasing pellet (+E), or an estradiol-releasing pellet until LN metastases arose, after which the estradiol pellet was withdrawn for 1 wk (EWD). The primary tumors, efferent LVs, and draining LNs from the same mouse were dissected and prepared for histology. Paraffin-embedded sections were probed with rabbit anti-ER or mouse anti-PR primary antibodies coupled with goat anti-rabbit (ER) or goat anti-mouse (PR) HRP-conjugated secondary antibodies. Background staining of erythrocytes (e). Arrows point to the LV.

Figure 3. Aberrant ER regulation in tumor cells located in the LN microenvironment and global gene expression profiling of tumors and matched LN metastases. **A**, ER immunohistochemistry of estradiol-treated T47D tumors and their matched LN metastases. An estradiol-treated MGT in mouse 1 developed LN metastases. Cells from the LN metastasis of mouse 1 were expanded in culture and re injected into the mammary gland of a second estradiol-treated recipient mouse 2, which also yielded LN metastases. MGTs and LN metastases from both mice were paraffin embedded, sectioned, and stained for ER by immunohistochemistry. **B**, unsupervised hierarchical clustering from continuously estradiol-treated (+E) or 1-wk EWD tumors and matched LN metastases. *Red*, up-regulated transcripts; *blue*, down-regulated transcripts; *gray*, relatively unchanged transcripts. **C**, ANOVA analysis identified 1,570 genes with tissue effects, hormone effects, and/or interaction effects.



estradiol-treated recipient mouse. This tumor also yielded a LN metastasis. Figure 3A (top) shows ER immunohistochemistry of the estradiol-treated primary MGT and its matched LN metastasis from the donor mouse (mouse 1). ERs in the tumor grown in the mammary gland and LN of the recipient mouse (mouse 2) are shown in Fig. 3A (bottom). There was a 2-fold increase in ER content in the cancer cells populating the LN of mouse 1 and a similar 2-fold increase in the LN ER of mouse 2. These data suggest that the LN microenvironment rather than clonal selection modifies ER expression levels in response to estradiol.

Global gene expression profiling. To determine if the apparent estradiol insensitivity of LN metastases as assessed by ER down-regulation and failure of PR induction extends to other estradiol-dependent genes, global gene expression profiling was done. To ensure that only human tumor cells were evaluated, pure cell populations were isolated by laser capture microdissection. Estradiol-treated and 1-week EWD tumors and their matched LN metastases were analyzed to define the subset of estradiol-regulated genes (7). Unsupervised hierarchical clustering showed that EWD had a lesser effect on genes in LN metastases than on genes in MGTs, with the estradiol-treated and EWD LN metastases clustering closest

together (Fig. 3B). Interestingly, the EWD tumors clustered closer to the LNs than to the estradiol-treated tumors.

To identify genes with differential expression patterns, a three-way mixed model ANOVA was done for genes that were present in at least one condition. Genes with the greatest differences in expression due to the hormone treatment, tissue type, interaction between hormone and tissue, or within and between animals ($P_{\text{model}} < 0.05$) and with significant ($P < 0.05$) main (tissue and/or hormone effects) or interaction effects were sorted to yield a total of 1,570 differentially expressed transcripts. Significant main effects indicate simple differences between the two hormone and/or two tissue groups, an example being that all EWD tissues have higher gene expression levels than all estradiol-treated tissues (regardless of tissue type) or that all LN tissues have higher gene expression levels than all MGT (regardless of hormone treatment). Significant interaction effects, on the other hand, indicate complex differences in which the results of one factor differ depending on the levels of the other factor. For example, a gene that was expressed at higher levels in estradiol-treated LNs than MGTs but in EWD tissues had the opposite pattern with higher levels in MGTs than LNs.

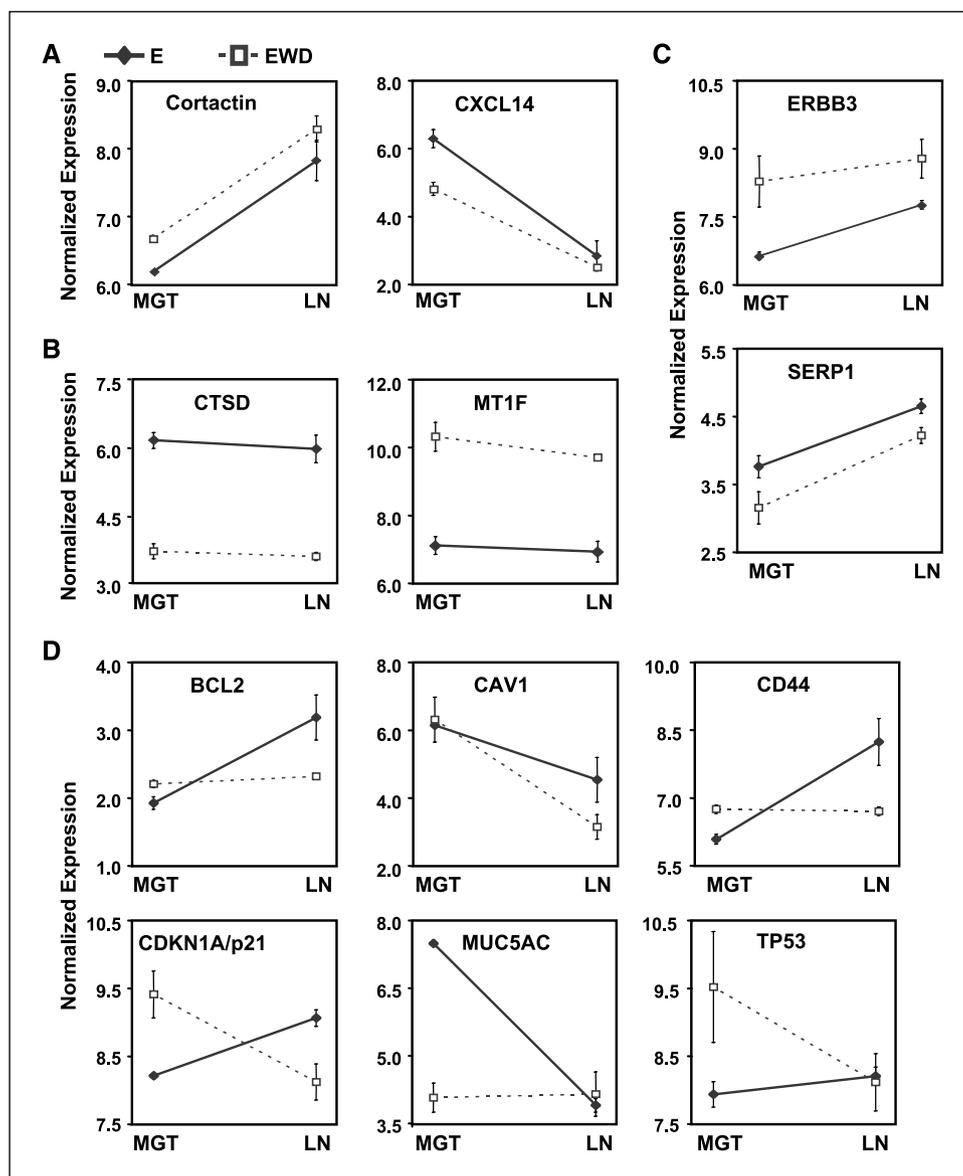


Figure 4. Genes that are tissue and hormone regulated with or without interaction effects. RMA normalized mean RNA expression values with SE. *Solid lines*, data from estradiol-treated tissues; *dashed lines*, data from EWD tissues. *A*, tissue-regulated genes. *B*, hormone-regulated genes. *MT1F*, metallothionein 1F. *C*, genes that are tissue and hormone regulated. *D*, genes with interaction effects that are differentially regulated by estradiol in the two tissues include *CAV1*, *MUC5AC*, *TP53*, *BCL2*, *CDKN1A*, and *CD44*.

Of the 1,570 differentially expressed transcripts, more had increased average expression in LN metastases (70%) than in primary tumors (30%). In addition, on average, genes were more highly expressed in EWD tissues (64%) than in estradiol-treated ones (36%), suggesting that estradiol affects approximately two thirds of genes through suppression of RNA levels. Among the 1,570 transcripts, 386 had only significant ($P < 0.05$; Fig. 3C) tissue effects, 44 had only significant hormone effects, and 91 were affected by both the tissue microenvironment and the presence of hormones (Fig. 3C). However, the majority of genes, 1,049 of 1,570, exhibited a significant interaction effect. The expression values of these genes could not be simply described as due to either of the main effects but rather due to an interrelationship between them. Most of these genes may also have significant main effects as shown by intersection in the Venn diagram, but interaction effects take precedence over these main effects, and the main effects cannot be correctly interpreted without acknowledging the interaction effects (21). For these genes, expression levels in the two tissues (MGT and LN) were influenced differently by the type of

hormone treatment (estradiol and EWD). A subset (145 of 1,049) of these genes exhibited only interaction effects.

Main effects and interaction effects can be visualized by graphical representation with levels of one factor on the *X* axis and separate lines representing levels of the other factor, as in Fig. 4. Lines that are parallel to one another with steep slopes or separation indicate main effects, whereas nonparallel lines indicate interaction effects. Figure 4A shows two examples of genes with significant tissue effects. Cortactin transcripts had significantly higher expression levels in LN metastases, regardless of hormone, as did the majority (290 of 386) of the tissue-regulated genes (other examples include *MAPK* and *cyclin F*). *CXCL14* is one of the 96 of 386 tissue-regulated genes whose levels were higher in the primary tumors compared with LN metastases.

Figure 4B shows two examples of genes with a significant hormone effect. The expression of cathepsin-D (CTSD) transcripts was dependent on estradiol regardless of the microenvironment. Other genes with similar expression patterns were *insulin-like growth factor-I receptor (IGF-IR)*, *cadherin-15*, and *defensin β -1*. Transcript

levels of *metallothionein 1F* were suppressed by estradiol regardless of the microenvironment. Other examples of estradiol-suppressed genes are *cathepsin F* and *BLNK*.

A subset of differentially expressed genes (91 of 1,570) had RNA expression levels that were both hormone and tissue regulated as shown in Fig. 4C. *ERBB3* was significantly suppressed by estradiol regardless of the microenvironment, but its expression was also increased in LN metastases compared with MGTs of both hormone treatments. *SERP1* was also significantly increased in LN metastases compared with MGTs regardless of the presence of estradiol and was also significantly up-regulated by estradiol in both tissues.

The majority of differentially expressed genes (1,049 of 1,570) had a significant interaction effect, exhibiting an interrelationship between tissue and hormonal factors. Figure 4D shows examples of a few such genes. Transcripts for *BCL2*, *CAV1*, *CD44*, *CDKN1A*, *MUC5AC*, and *tumor promoter 53 (TP53)* all exhibit interaction effects and are affected differently by estradiol in tumors and LN metastases. *MUC5AC* and *TP53* are examples of genes exhibiting main effects (hormone and tissue) but with the interaction term predominating. Main effects of *CDKN1A (p21)* cancel each other out, leaving the dominant role of interaction effects evident.

Immunohistochemistry. Several of the genes that were identified by the expression profiling analysis were investigated further at the protein level by immunohistochemistry (Fig. 5). The estradiol-regulated gene *CTSD* was expressed in 80% to 90% of estradiol-treated MCF-7 primary tumor cells, and EWD reduced this number to 50% to 60%. In the LN metastases in the presence of estradiol, 50% to 60% of cancer cells expressed CTSD; this fell to 10% to 15% on EWD (Fig. 5, *top*). CAV1 protein was unaffected by EWD but was expressed only in MGTs and not in LN metastases (Fig. 5, *middle*). In estradiol-treated tumors, CD44 was heterogeneously expressed; on EWD, nonsignificant increases in the percentage of CD44⁺ cells were recorded. Interestingly, the opposite pattern was observed in LN metastases. The extensive over-expression of CD44 in estradiol-treated cancer cells metastatic to LNs (15) is dramatically reduced by EWD (Fig. 5, *bottom*).

Estrogen-regulated genes. Two linear contrasts were done among the 1,570 differentially expressed transcripts comparing estradiol-treated with EWD tumors and estradiol-treated with EWD LN metastases (Fig. 6A and B). This identified transcripts that were estradiol regulated in MGTs compared with LN metastases. There were 273 significantly hormonally up-regulated or down-regulated genes (fold change >1.3; $P < 0.025$; Fig. 6A). Of these, 256 were estradiol dependent in tumors, 41 were estradiol dependent in LN metastases, and 24 maintained estradiol sensitivity in both MGTs and LN metastases (Fig. 6A). These data confirm the conclusions drawn from Figs. 2 and 3A that tumor cells in the LN microenvironment are relatively estradiol resistant.

To identify genes with any absolute difference in expression due to estradiol in MGTs and LN metastases, the fold change criterion was removed from the two linear contrasts. This increased the total list of significantly ($P < 0.025$) hormone-regulated genes from 273 to 1,176 (Fig. 6B). These additional 903 genes were up-regulated or down-regulated with fold changes >1.0 and <1.3, and in total, there were 707 estradiol-regulated genes in MGTs, 177 estradiol-regulated in LN metastases, and 292 estradiol-regulated in both sites. Seventy-five of these transcripts were regulated by estradiol in opposite directions in MGTs and LN metastases (Fig. 6C). *BCL2*, *CD44*, and *CDKN1A* are examples of such genes (Fig. 4D). *CDKN1A*, also known as *p21/WAF*, encodes a potent cyclin-

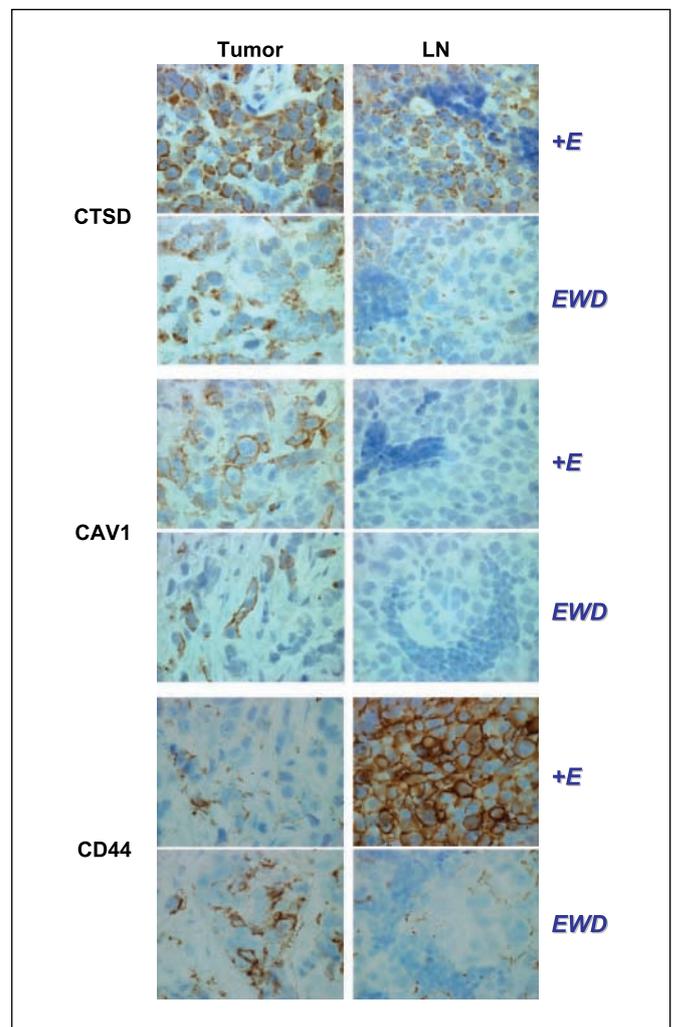


Figure 5. Immunohistochemical analyses of proteins that are hormone and tissue regulated. Estradiol-treated and EWD tumors and matched LN metastases from the same mouse were paraffin embedded, sectioned, and probed with CTSD, CAV1, or CD44 primary antibodies coupled with goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibodies. *Top*, estradiol-regulated CTSD; *middle*, CAV1 protein regulation by the tissue microenvironment; *bottom*, differential estradiol regulation of CD44 expression in tumors and LN metastases.

dependent kinase inhibitor and regulator of cell cycle progression at G₁. Its expression is tightly controlled by *TP53*, one of our set of microenvironment-regulated genes (Fig. 4D). CD44 (Fig. 5) is a putative breast cancer stem cell marker (22) and may target tumor cells to LNs (15).

Network analysis. Ingenuity data analysis was conducted to determine if the genes that had interaction effects and were differentially regulated by estradiol in MGTs and LN metastases are also involved in important functional networks. Twenty-seven unique gene networks that contained at least one differentially regulated gene were identified. Supplementary Fig. S1 shows a network of 33 genes, 100% of which are differentially estradiol regulated in MGTs and LN metastases. This network has *TP53* at its center and functions in cellular responses to “therapeutics, cell death, and connective tissue disorders.” Most of these genes (25 of 33) were estradiol regulated in MGTs but lost estradiol sensitivity in LN metastases (orange outline). Two genes were estradiol regulated in LN metastases but not in tumors (red outline), and the

remaining seven genes were regulated in the opposite direction by estradiol in MGTs and LN metastases (blue outline).

Discussion

Gene expression changes in MGTs and LN metastases. Two major theories have been advanced to explain successful cancer metastasis. (a) The “rare tumor cell hypothesis” states that only certain cells within tumors are capable of migrating and/or growing at distant sites. These rare cells may be stem cells, such as those that express CD44 (22). On the other hand, they may represent rare cells within a population of genetically diverse and unstable cells that originated with or acquired the appropriate genetic information needed to seed at specific metastatic sites (23). (b) An alternative hypothesis posits that metastatic capacity is an inherent rather than an acquired feature of breast cancers (24, 25) and is based on data suggesting that metastatic capacity is encoded early in the majority of cells within a tumor. In support of this idea are studies showing that tumors and their LN metastases retain similar genetic signatures, including aggressiveness and proliferation markers (24, 26, 27). One study, for example, found only 27 changed genes between lung tumors and their LN metastases (28). Weigelt et al. (27) identified a 70-gene cluster in primary breast tumors that predicts likelihood of subsequent metastases because the unique expression profiles of these genes were maintained in LNs and more distant metastases. Another study found no “shared genes” between primary tumors that were predictive of LN metastases but, anywhere between 3 and 149 genes, were “antiexpressed” or expressed in opposite directions in tumors versus LN metastases (29).

Our data provide evidence in support of both theories. In these studies, we analyze the changes in gene expression induced by estradiol as revealed by estradiol removal. Fewer than 5% of all genes analyzed were differentially expressed in cancer cells within the mammary gland compared with the LN. Although this might be considered a subtle difference, it nevertheless represents differential regulation of >1,000 genes in these two microenvironments. These genetic differences could be sufficient to define a “metastatic stem cell” (30), expressing the adaptive proteins required for successful colonization and growth of a subpopulation in a metastatic microenvironment, as envisioned by the rare tumor cell hypothesis. Elevated expression levels of the hyaluronan receptor CD44, a putative cancer stem cell marker, in estradiol-treated LN metastases suggest such selectivity in our models. On the other hand, our observations that cancer cell emboli move in bulk through LVs to LNs (15), coupled with our findings here and those of others (24, 27, 29) of modest genetic changes between primary tumors and LN metastases, could be interpreted as supporting the view that the majority of primary tumor cells arise either with or without the predisposition for LN spread. If true, the gene expression changes we observe could be attributed to the plasticity that all tumor cells exhibit when growing in two distinct microenvironments. Evidence supporting these microenvironmental effects on ER expression and function was found when a down-regulation “resistant” ER⁺ LN metastasis regained ER down-regulation capacity when grown in the mammary gland of a recipient mouse (Fig. 3A).

ER function in metastases. Beatson (31) reported >100 years ago that an essential feature of breast cancer is continuous and excessive growth of the epithelium, which invades the surrounding tissues, spreads along the LVs, passes from one set of LNs to

another, and eventually forms deposits in distant organs, steps that occur more rapidly and prove to be more quickly fatal in younger patients. Knowing that the ovaries produced factors required for lactation, and believing “that all pathological changes are merely modified physiological ones,” Beatson was the first to find that removal of the ovaries and the estradiol they elaborate reduced breast tumor size and extended the survival of young women with advanced breast cancer.

Seventy percent to 80% of primary breast cancers are ER⁺, and ~80% of LN metastases retain the receptors (11–13, 32). If such tumors are treated with antiestrogens or EWD therapies, 20% to 50% acquire resistance (33), often recurring as LN metastases. Premenopausal women with LN metastases are less responsive to tamoxifen treatment than LN-negative patients (34), and nodal involvement is a significant predictor of local relapse (35) with a 5.6-fold higher relative risk of local recurrence than women with clear LNs. Preoperative chemotherapy causes significantly less damage to LN metastases in comparison with primary tumors (36). Based on these data, we hypothesized that ER effects on gene expression would be altered by the LN microenvironment. To the best of our knowledge, no previous studies have defined estradiol-regulated genes in tumors compared with their matched LN metastases. Identifying these genes is an important step toward elucidating mechanisms that contribute to breast cancer progression and estradiol resistance in advanced disease.

In the face of continuous estradiol treatment in two separate ER⁺ breast cancer cell lines, higher levels of ER were observed in LN metastases than in matched MGTs (Figs. 2 and 3A). The partial failure of ligand-dependent ER down-regulation in LN metastases suggests that the receptors are relatively estradiol insensitive in the LN microenvironment. Reduced PR expression in estradiol-treated LN metastases supports this conclusion, as does global gene expression profiling, which showed that estradiol had less influence on gene expression in LN metastases than in matched primary MGTs (Figs. 3 and 6). We have now identified numerous genes that are estradiol regulated in primary tumors but not in LN metastases. Examples include *MUC5A* and *TP53*, which were estradiol sensitive in MGTs but lost this regulation in the LNs. Additionally, 75 genes were regulated in the opposite direction by estradiol in MGTs compared with their LN metastases (Fig. 6C), 21 of which, including *BCL2* and *CDKN1A* (Fig. 4), were previously reported to be estradiol regulated (7, 37, 38). *CD44* (Figs. 4 and 5) was also regulated in opposite directions at the two sites. It is one of two cell surface markers that differentiate tumorigenic from nontumorigenic breast cancer cells (22). This putative stem cell and aggressiveness marker (39, 40) is an example of how EWD differentially alters the expression of a potential therapeutic target in primary tumors compared with LN metastases.

Mechanisms of estradiol resistance in LNs. Possible explanations for the differential estradiol sensitivity of MGTs versus LN metastases include increased availability of growth factors in LNs that directly interfere with ER transcription (41), diminished ubiquitination and loss of proteasome-mediated protein down-regulation in LNs (42, 43), loss of CAV1 and subsequent membrane signaling (44), and reduced estradiol availability to LNs. With regard to growth factors, IGFs and epidermal growth factors (EGF) activate the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathways, which alter Jun and Fos activities at the PR promoter and decrease its ER-mediated transcription (45). LN stromal cells produce both IGF and EGF (41), which may explain the reduced PR induction as well as the reduced estradiol

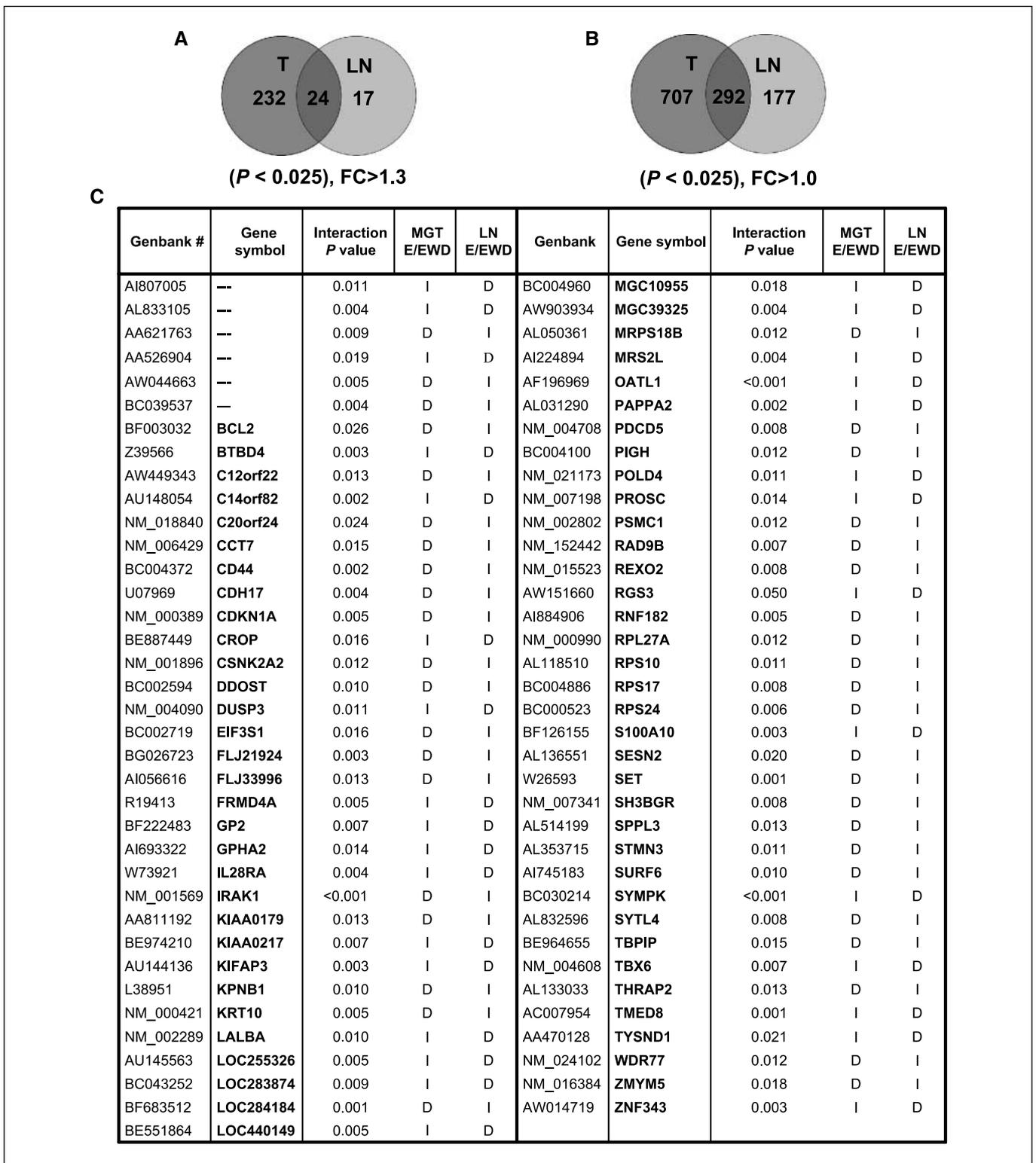


Figure 6. Pairwise comparisons to identify estradiol-regulated transcripts in tumors (T), LN metastases, or both. A, transcripts regulated in a significant manner ($P < 0.025$) by estradiol >1.3-fold. B, transcripts regulated in a significant manner ($P < 0.025$) by estradiol regardless of fold levels. C, list of 75 transcripts that are regulated in a significant manner in opposite directions by estradiol in MGTs compared with LN metastases. I, increases; D, decreases with estrogen.

regulation of other target genes at this site. Increased cortactin levels (Fig. 4) inhibit ubiquitin-mediated degradation of EGF receptors following their internalization, resulting in sustained ligand-induced EGF signaling (42, 43). It is possible that similar

ubiquitin-dependent mechanisms explain the ER down-regulation defects we observe in LNs (Fig. 2; ref. 15). Supporting this hypothesis, nine transcripts known to be involved in the ubiquitination process were differentially expressed in MGTs and LN metastases.

This includes two known enzymes that function in the ubiquitination process, UBE2D3 and USP7, which were increased in LN metastases and also lost estradiol sensitivity in LN metastases (Supplementary Fig. S1). CAV1, a scaffold protein that transduces ER signaling at the plasma membrane (44), was decreased in LNs, possibly explaining loss of ER responsiveness. Finally, although it is possible that the diminished estradiol sensitivity of LN metastases is due to an overall decrease of estradiol levels within LNs, this is highly unlikely given the number of estradiol-regulated genes whose levels are maintained or increased in LN metastases (Fig. 4).

Networks. We speculated that genes differentially regulated in MGTs versus LN metastases may contribute to tumor aggressiveness, recurrence, and/or growth in LNs. We therefore analyzed the functional networks characterizing these genes. Of interest was a 33-gene network composed entirely of transcripts regulated differently by estradiol in MGTs and LNs (Supplementary Fig. S1), which included CDKN1A, TP53, and BCL2-linked signaling pathways. In this network, the majority of the genes were significantly regulated by estradiol in tumors while losing estradiol sensitivity in LN metastases or suppressed by estradiol in tumors but increased by estradiol in LN metastases. These findings are interesting given the fact that 70% of the 1,570 global genes differentially expressed in the two sites were increased in LN metastases compared with MGTs, whereas 64% of the differentially estradiol-regulated genes were suppressed by estradiol. This corroborates previous data with MCF-7 cells showing that 70% of genes are down-regulated by estradiol treatment (38) and is consistent with an overall lifting of the suppressive effects of estradiol in MCF-7 tumor cells growing in LNs. It is possible that these results do not completely mimic effects in a patient with an intact immune system. The LNs of nude mice lack or have reduced numbers of T cells (46). However, they have B cells, natural killer cells, reticular cells, and macrophages as well as a nodal stromal network and architecture. Because different

substrata are known to alter growth and function of ER⁺ human breast cancer cells *in vitro* (6), it is likely that the LN microenvironment, including its unique architecture, cytokines, and growth factors, modifies ER function. Given the experimental nature of our models, these findings stress the need for further studies to show whether estradiol insensitivity occurs in clinical ER⁺ LN metastases and whether other metastatic microenvironments differentially modulate estradiol sensitivity and to define estradiol-regulated genes uniquely influenced by the microenvironment of tumor cells. This approach may identify key genes that can be targeted therapeutically at specific metastatic sites.

In summary, we describe a xenograft model of EWD after LN metastasis and show that estradiol regulation of genes is different in ER⁺ primary MGTs compared with their matched ER⁺ LN metastases. Through global gene expression profiling and immunohistochemical analyses, we identify genes that maintain, lose, or are regulated in the opposite direction by estradiol in tumors and LN metastases. These results suggest that current ER-targeted therapies may differentially affect tumor cells depending on the microenvironment in which the cells reside. If so, our studies provide possible new therapeutic targets, which, in combination with standard hormonal therapies, might improve responses of advanced breast cancers.

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Estrogen Insensitivity in a Model of Estrogen Receptor–Positive Breast Cancer Lymph Node Metastasis

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