Lipocalin 2 promotes breast cancer progression

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Here, we report that lipocalin 2 (Lcn2) promotes breast cancer progression, and we identify the mechanisms underlying this function. We first found that Lcn2 levels were consistently associated with invasive breast cancer in human tissue and urine samples. To investigate the function of Lcn2 in breast cancer progression, Lcn2 was overexpressed in human breast cancer cells and was found to up-regulate mesenchymal markers, including vimentin and fibronectin, down-regulate the epithelial marker E-cadherin, and significantly increase cell motility and invasiveness. These changes in marker expression and cell motility are hallmarks of an epithelial to mesenchymal transition (EMT). In contrast, Lcn2 silencing in aggressive breast cancer cells inhibited cell migration and the mesenchymal phenotype. Furthermore, reduced expression of estrogen receptor (ER) α and increased expression of the key EMT transcription factor Slug were observed with Lcn2 expression. Overexpression of ERα in Lcn2-expressing cells reversed the EMT and reduced Slug expression, suggesting that ERα negatively regulates Lcn2-induced EMT. Finally, orthotopic studies demonstrated that Lcn2-expressing breast tumors displayed a poorly differentiated phenotype and showed increased local tumor invasion and lymph node metastasis. Taken together, these in vitro, in vivo, and human studies demonstrate that Lcn2 promotes breast cancer progression by inducing EMT through the ERα/Slug axis and may be a useful biomarker of breast cancer.

epithelial to mesenchymal transition | biomarker | estrogen receptor

Lipocalin 2 (Lcn2, also referred to as neutrophil gelatinase-associated lipocalin) is a member of the lipocalin family. Lipocalins are small extracellular proteins that share the highly conserved structure of an 8-stranded antiparallel β barrel and have been shown to transport and present ligands, to bind to cell surface receptors, and to form macromolecular complexes, thereby playing important roles in cell regulation, proliferation, and differentiation (1).

Lcn2 has been associated with breast cancer. Lcn2 is among the genes most highly associated with estrogen receptor (ER)-negative breast tumors (2). It is also one of the genes that is most increased in the luminal epithelial cells compared with myoepithelial cells (3), a significant finding because the majority of breast carcinomas are thought to arise from the luminal epithelial cells (4). Taken together, these data suggested that Lcn2 may actively participate in breast cancer progression; however, the mechanisms underlying this role remain unknown.

The epithelial to mesenchymal transition (EMT) is one of the key processes involved in tumor progression and metastasis (5). Hallmarks of EMT include the loss of the epithelial marker E-cadherin, an increase in the mesenchymal markers vimentin and fibronectin, and an increase in the migratory and invasive behavior. Several transcription factors, including Snail, Slug, Twist, and SIP1, play key roles during EMT (5, 6). These factors inhibit the epithelial phenotype and induce EMT by repressing E-cadherin transcription.

Here, we report that the overexpression of Lcn2 in human breast cancer cells induces EMT. We have explored the potential mechanisms that may mediate Lcn2-induced EMT and have also assessed the function of Lcn2 in breast cancer progression in vivo by using an orthotopic model and human tissue and urine samples from patients at different stages of disease progression. The results of these studies demonstrate that Lcn2 promotes breast cancer progression and may represent a biomarker of breast cancer.

Results

Elevated Lcn2 Levels Are Detected at Advanced Breast Cancer Stages. Lcn2 levels were examined by immunohistochemistry in human breast cancer tissues representing different stages of disease progression as well as normal nonneoplastic tissues. Nonneoplastic epithelium showed minimal staining (Fig. 1*A*) in contrast to breast carcinomas where the staining was significantly stronger and in more cells (Fig. 1*B*). Staining intensities of Lcn2 in cancer cells were significantly increased in Stages I–III (American Joint Committee on Cancer Staging System) compared with normal epithelium (Fig. 1*C*). Because Lcn2 is a secreted protein, we also examined its levels in the stroma. Staining intensities of Lcn2 in tumor stroma were also significantly higher in Stages II and III samples than normal breast stroma (Fig. 1*D*). Because breast cancers at Stages I–III breach the confinement of the basement membrane and invade into neighboring tissues, local lymph nodes, and distant organs, these results demonstrate that tissue Lcn2 levels correlate with invasive breast cancer.

Given the fact that Lcn2 is secreted from the carcinomas into the normal breast ducts (7), we considered the possibility that Lcn2 might be detected in body fluids and might be associated with disease status. We analyzed Lcn2 levels in urine samples from healthy women and women with metastatic breast cancer. Consistent with the immunohistochemistry results, urinary Lcn2 levels were significantly higher in samples from metastatic breast cancer patients compared with normal controls (Fig. 1*E*). We used logistic regression modeling to determine the probability of metastatic cancer (compared with normal) and found that it was statistically significant (likelihood ratio test = 5.0, $P = 0.025$). This indicates that urinary Lcn2 provides significant prognostic information in differentiating metastatic breast cancer patients from controls, with higher levels of Lcn2 being predictive of a higher probability of metastatic breast cancer.

Lcn2 Induces EMT in Human Breast Cancer Cells. To understand the mechanism underlying the association between Lcn2 and invasive breast cancer, stable Lcn2 clones were established from the human breast cancer cell line MCF-7, which produces little

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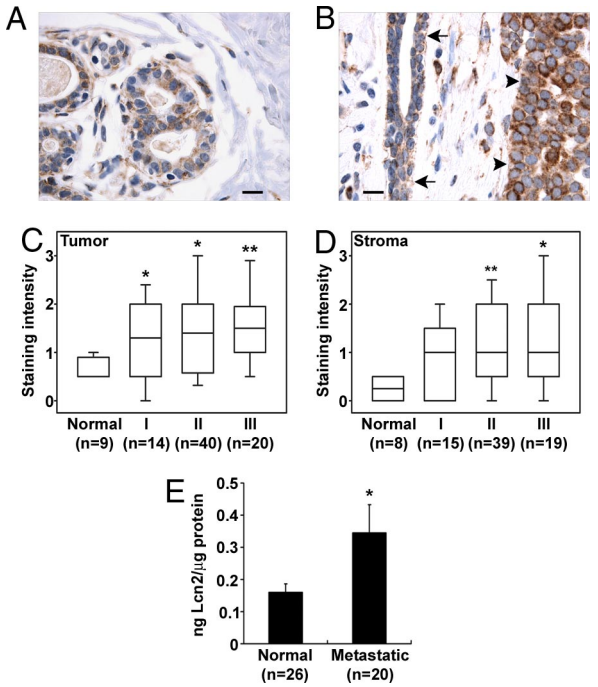

Fig. 1. Lcn2 levels are increased in tissues and urine samples from patients with invasive breast cancer. (*A* and *B*) Representative microscopic images of normal breast epithelium and breast cancer tissue stained with an anti-human Lcn2 antibody, respectively. (*B*) Breast cancer cells with stronger Lcn2 staining (arrowheads) are shown in close proximity to normal mammary epithelium (arrows). (Scale bars, 20 μm.) (*C* and *D*) Quantitation of Lcn2 staining intensity in cancer cells or stroma at different breast cancer stages and in normal breast tissue. Data are presented as box-and-whisker plot (for details see *Materials and Methods*). *, $P < 0.05$; **, $P < 0.01$ compared with normal breast tissue. (*E*) Urinary Lcn2 levels (normalized to total protein concentration) as analyzed by ELISA of normal controls and patients with metastatic breast cancer. *, $P = 0.03$.

endogenous Lcn2 (8). A significant increase in secreted Lcn2 levels was confirmed by using ELISA in Lcn2 clones N1 and N2 (118.8 and 391.2 ng of Lcn2 per mg of protein, respectively) compared with parental MCF-7 cells (16.6 ng of Lcn2 per mg of protein).

The classic phenotypic changes commonly associated with an EMT were observed in Lcn2 clones (Fig. 2*A*). MCF-7 cells exhibited cobblestone-like appearance and strong cell–cell adhesion that is typical of epithelial phenotype, whereas Lcn2-overexpressing N2 cells displayed an elongated morphology and were distributed more evenly across the well surface. N1 clone, which express lower levels of Lcn2 compared with N2 clone, grew in loosely organized cell clusters that looked like a transitional phenotype between MCF-7 and N2 cells.

We next examined the key epithelial marker E-cadherin in all three cell types (5). E-cadherin staining at cell–cell contacts (as seen in MCF-7 cells) was decreased in N1 cells and was nearly lost in most N2 cells, save for that observed in a few of the small cell clusters (Fig. 2*B*). In contrast, the classic mesenchymal markers, vimentin and fibronectin, were markedly induced in N2 cells (Fig. 2*B*). These results were also confirmed by immunoblotting (Fig. 2*C*). The EMT marker expression pattern of N1 cells was similar to parental MCF-7 cells, suggesting that a threshold of Lcn2 expression may be required to induce a complete EMT.

A key feature of cells that have undergone an EMT is their increased migration and invasion. Lcn2 induced markedly higher levels of migration in the clones, especially N2, in contrast to MCF-7 cells in which little to no migration was observed (Fig. 2*D*). High levels of Lcn2 expression in N2 cells also induced significantly more invasion through a layer of Matrigel, whereas no invasion was observed with MCF-7 cells (Fig. 2*E*).

To ensure that these observed effects were Lcn2-specific and not the result of clonal differences, Lcn2 was silenced in N2 cells, which express the highest level of Lcn2 and exhibit a more pronounced EMT phenotype with two different siRNAs (siRNA5 and siRNA3). siRNA that does not correspond to any known human gene, was used as control. Markedly reduced Lcn2 levels were observed after transfection with the siRNAs (Fig. 3*A*). Lcn2 silencing increased the levels of the epithelial marker E-cadherin and reduced the levels of mesenchymal markers vimentin and fibronectin (Fig. 3*B*). Consistent with the changes in EMT markers, mesenchymal-like N2 cells reverted to a more compact epithelium-like morphology after siRNA transfection (Fig. 3*C*). More importantly, both of the siRNAs significantly inhibited the migration (Fig. 3*D*). Similar results were obtained by using two additional and distinct siRNAs [supporting information (SI) Fig. S1]. Therefore, changes in EMT marker expression, morphology, and migratory behavior of the cells after Lcn2 silencing demonstrate that overexpression of Lcn2 underlies the EMT in MCF-7 cells. Finally, MCF-7 cells were treated with recombinant human Lcn2, and down-regulation of E-cadherin was observed (Fig. 3*E*). We did not observe significant changes in the levels of vimentin or fibronectin. These results suggest that Lcn2 induces EMT, at least in part, in a paracrine fashion.

ERα/Slug Axis Mediates Lcn2-Induced EMT. Because of the importance of estrogen signaling in breast cancer, we examined the estrogen receptor α (ERα) status of our cell lines. This ER isoform mediates most estrogenic responses (9). ERα protein levels were dramatically reduced in N2 cells and slightly reduced in N1 cells compared with parental MCF-7 cells (Fig. 4*A*), consistent with its transcript levels. The inverse correlation between Lcn2 and ERα has also been observed by other groups (2). Moreover, the ERα level in N2 cells was increased after Lcn2 silencing (Fig. 4*B*), suggesting that this change in ERα is an Lcn2-specific event. Decreased ERα in Lcn2 clones correlated with a decreased response to estrogen treatment (Fig. S2).

Down-regulation of ERα results in decreased E-cadherin expression, loss of the epithelial phenotype, and enhanced invasiveness (10). The inverse correlation between ERα and Lcn2 as well as the EMT phenotype in these stable clones suggested that Lcn2 might be disrupting the epithelial phenotype by inhibiting ERα. To test this hypothesis, ERα was induced in N2 cells, which resulted in an increase in E-cadherin levels and a decrease in vimentin and fibronectin levels (Fig. 4*C*), demonstrating that an increase in ERα can inhibit the Lcn2/EMT pathway and reverse the EMT.

A number of EMT pathways converge on the transcription factors Snail, Slug, Twist, and SIP1 to inhibit E-cadherin transcription and the epithelial phenotype (5, 6). We first asked whether the EMT markers were regulated at the transcription level. Consistent with the protein data, the E-cadherin transcript was decreased whereas the vimentin transcript was increased in N2 cells (Fig. 4*D*). Slug expression, at both the mRNA and protein levels, was dramatically increased in N2 cells that expressed the highest Lcn2 level and the lowest E-cadherin level and exhibited a more profound EMT (Fig. 4*E*), suggesting that Slug was part of the pathway underlying Lcn2-induced EMT. Such correlations were not observed with Snail, Twist, or SIP1 (Fig. S3).

Because ERα has been shown to maintain E-cadherin expression by inhibiting Snail, which is a close family member of Slug that shares the ability to repress E-cadherin transcription and induce EMT (11), we asked whether Slug expression in N2 cells is regulated by ERα. We found that Slug levels in N2 cells were

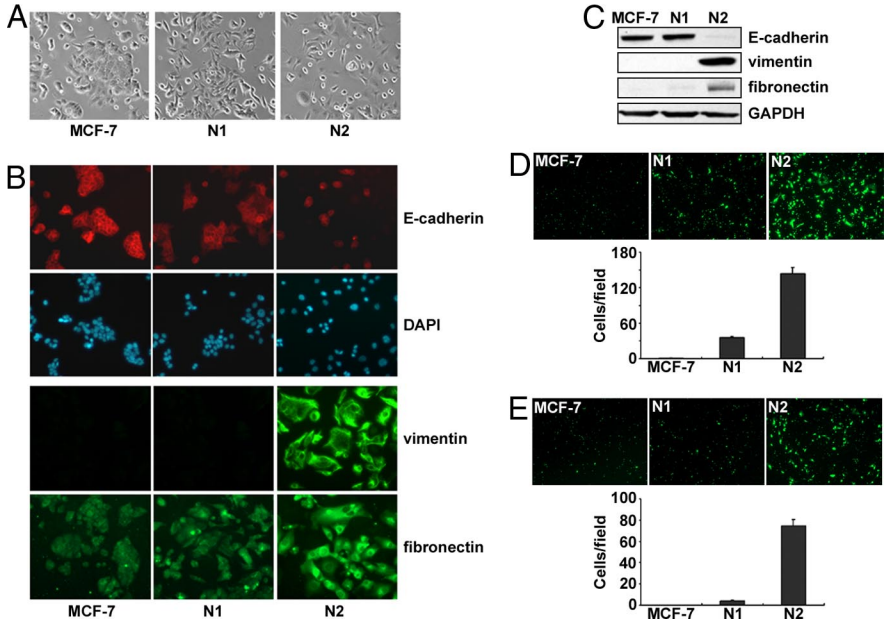

Fig. 2. Lcn2 induces EMT in human breast cancer cells. (*A*) Morphology of parental MCF-7 cells and Lcn2 clones N1 and N2. (*B* and *C*) Expression of the EMT markers as analyzed by immunofluorescent staining and immunoblot. Nuclei are shown with DAPI staining. (*D* and *E*) Migration and invasion of MCF-7 cells and Lcn2 clones. Data are collected from 6 wells. All comparisons between any two of the three groups are significant ($P < 0.0001$). Representative images of migrated or invaded cells are also shown.

reduced with induced ERα (Fig. 4*F*), indicating that Slug is downstream of ERα in the Lcn2/EMT pathway and is negatively regulated by ERα.

In addition to ERα, the MAPK and TGF-β pathways have been implicated in the transcriptional regulation of EMT. Both pathways can activate Slug (5, 12, 13); however, neither of the pathways is the operative upstream regulator of Slug in the Lcn2/EMT pathway. Although phosphorylated Erk was increased in Lcn2 clones, its levels did not change after Lcn2 silencing (Fig. S4) even though a reversal of the EMT occurred at the same time point (Fig. 3), suggesting that activated MAPK pathway is not responsible for Lcn2-induced EMT. As for TGF-β, it has been shown that TGF-β1 does not induce EMT in breast cancer cells, including MCF-7 cells (14).

Taken together, these data demonstrate that Lcn2 induces EMT via the ERα/Slug axis by first down-regulating ERα, which subsequently leads to induced Slug expression, decreased E-cadherin expression, and eventually the transition to the mesenchymal phenotype (see Fig. S5 for the schematic illustration of the pathway).

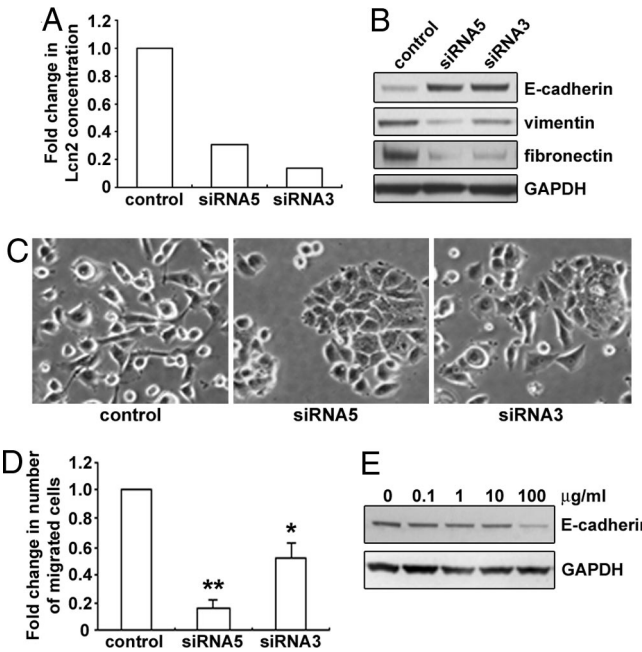

Fig. 3. Lcn2 silencing reverses the EMT phenotype of N2 cells. (*A*) Secreted Lcn2 levels in N2 cells after siRNA silencing. (*B*) Protein levels of EMT markers after siRNA transfection. (*C*) Morphology of N2 cells after siRNA transfection. (*D*) Migration of N2 cells after siRNA transfection. Data are collected from 9 wells. *, $P < 0.05$; **, $P < 0.01$, compared with control. (*E*) E-cadherin and GAPDH protein levels in MCF-7 cells treated with recombinant human Lcn2 at various concentrations.

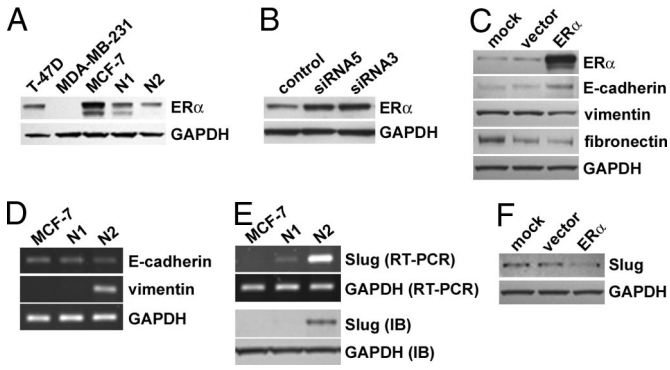

Fig. 4. Lcn2-induced EMT is mediated by the ERα/Slug axis. (*A*) ERα protein levels in MCF-7 cells and Lcn2 clones. T-47D and MDA-MB-231 cell lysates were used as positive and negative controls for ERα, respectively. (*B*) ERα protein levels in N2 cells after Lcn2 silencing. (*C*) Protein levels of ERα and EMT markers in N2 cells after ERα transfection. (*D*) Expression of E-cadherin and vimentin in MCF-7 cells and Lcn2 clones as analyzed by RT-PCR. (*E*) Slug expression in MCF-7 cells and Lcn2 clones as analyzed by both RT-PCR and immunoblot (IB). (*F*) Protein levels of Slug in N2 cells after ERα transfection.

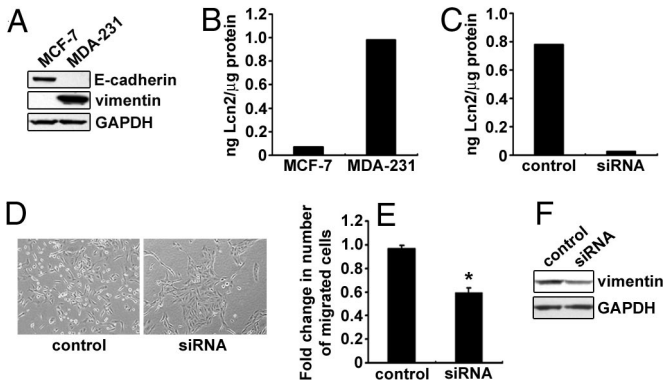

Fig. 5. Reduced Lcn2 expression suppresses the migration and mesenchymal phenotype of MDA-MB-231 cells. (*A*) Protein levels of the epithelial marker, E-cadherin, and the mesenchymal marker, vimentin, in MCF-7 and MDA-MB-231 cells. (*B*) Lcn2 levels in the conditioned media of MCF-7 and MDA-MB-231 cells (normalized to total protein concentration) as analyzed by ELISA. (*C*) Lcn2 levels in the conditioned medium of MDA-MB-231 cells after transfection with a siRNA pool. (*D*) Morphology of MDA-MB-231 cells after Lcn2 silencing. (*E*) Migration of MDA-MB-231 cells after Lcn2 silencing. Data are collected from 6 wells. *, $P < 0.001$. (*F*) Vimentin protein levels in MDA-MB-231 cells after Lcn2 silencing.

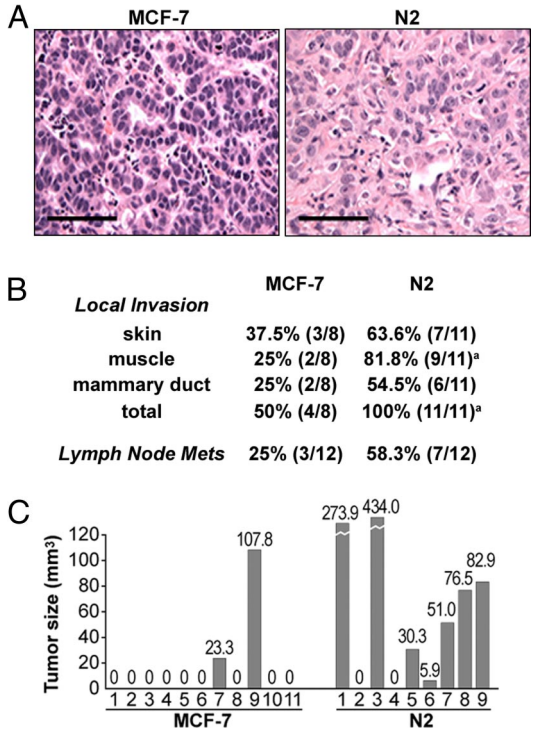

Fig. 6. Lcn2 decreases differentiation and increases breast tumor local invasion, lymph node metastasis, and primary tumor growth in vivo. (*A*) MCF-7 and N2 tumor sections stained with H&E. (Scale bars, 100 μm.) (*B*) Frequencies of local invasion and lymph node metastasis. The value in the parentheses represents the number of tumors with a certain type of local invasion and total number of tumors analyzed (for local invasion) or the number of lymph nodes with tumor metastases and total number of lymph nodes analyzed (for lymph node metastasis). a, $P < 0.05$. (*C*) Individual tumor size at 12 weeks after orthotopic injection.

Inhibition of Lcn2 in Aggressive Breast Cancer Cells Reduces Migration and Suppresses the Mesenchymal Phenotype. We next examined the function of Lcn2 in breast cancer through an opposite approach, reducing its level in MDA-MB-231 cells, which are much more aggressive and invasive than MCF-7 cells (15). These cells display a scattered, more mesenchymal-like morphology, consistent with their EMT marker expression pattern (Fig. 5 *A* and *D*). Importantly, these cells produce Lcn2 at a much higher level than MCF-7 cells (Fig. 5*B*).

Lcn2 was silenced in MDA-MB-231 cells by using a siRNA pool (Fig. 5*C*). siRNA that does not match any known human genes was used as control. Cells with reduced Lcn2 expression exhibited obvious clustering and a more compact morphology compared with control cells (Fig. 5*D*). Moreover, cell migration was significantly reduced compared with controls (Fig. 5*E*). The mesenchymal marker vimentin was also reduced with Lcn2 silencing (Fig. 5*F*), whereas the epithelial marker E-cadherin did not change. The changes in vimentin can be sufficient to induce the changes in cell migration as reported (16). The level of ERα did not increase with Lcn2 silencing in MDA-MB-231 cells. These results suggest that the regulation of vimentin by Lcn2 may be mediated through mechanisms other than the ERα/Slug axis alone because Lcn2 silencing reduced vimentin expression but had no effects on ERα and E-cadherin.

These data suggest that not only is Lcn2 sufficient to induce EMT in nonaggressive MCF-7 cells, but also contributes to the aggressive behavior and mesenchymal phenotype of MDA-MB-231 cells. These data also demonstrate that Lcn2-induced EMT is not limited to one specific breast cancer cell line but that its effects can be generalized to other breast cancer cell lines as well.

Lcn2 Decreases Cell Differentiation and Promotes Tumor Local Invasion and Tumor Growth in an Orthotopic Breast Cancer Model. To determine whether Lcn2 promotes breast cancer invasion and metastasis in vivo, MCF-7 and N2 cells were injected orthotopically into the inguinal mammary fat pads of female nude mice. This model most reliably recapitulates the process of human breast cancer progression and metastasis.

First, we confirmed that N2 cells continued to express higher Lcn2 levels and maintain the mesenchymal phenotype in vivo (Fig. S6). Robust Lcn2 expression was observed in N2 tumors even 6 months after injection, whereas MCF-7 tumors showed virtually no Lcn2 expression. N2 tumors were robustly positive for vimentin whereas MCF-7 tumors were negative. In contrast, E-cadherin levels were significantly lower in N2 tumors.

MCF-7 and N2 tumors showed distinct differences in the cytology and growth patterns (Fig. 6*A*). In contrast to MCF-7 tumors, N2 tumors consisted of cells with larger, more pleomorphic nuclei, a more open or diffuse chromatin pattern, and very prominent nucleoli. MCF-7 tumor cells exhibited focal tubule and trabecular growth patterns, whereas N2 tumors grew in a disorganized manner with no distinct pattern. Poor differentiation as displayed by N2 tumors corresponds to a higher histologic grade, which is a major indicator of poor prognosis in human cancer (17).

Primary tumors were also analyzed for evidence of invasion into neighboring tissues. N2 tumors showed a significant increase in local invasion with tumor cells invading into skin, muscle, and mouse mammary ducts and even invading into nerves (Fig. S7). Overall, skin, muscle, or mammary duct invasion was observed in all of the N2 tumors, but only in half of the MCF-7 tumors (Fig. 6*B*). Tumor cells were also detected in more lymph nodes in mice injected with N2 cells (Fig. 6*B*). Metastasis to distant sites other than lymph nodes was not observed with either MCF-7 or N2 cells. Taken together, these data indicate that Lcn2 expression increases both local invasion and spontaneous lymph node metastasis in vivo.

Orthotopic tumor growth was also increased in N2 tumors (Fig. 6*C*). At 12 weeks after injection, only 2 of the 11 mice injected with MCF-7 cells grew tumor compared with 7 of the 9 mice injected with N2 cells ($P = 0.022$). In addition, the average

size of the MCF-7 tumors that did grow was smaller than that of the N2 tumors.

Discussion

In this work, we demonstrate that Lcn2 promotes breast cancer progression by inducing the EMT in breast cancer cells. Lcn2 represses the epithelial phenotype, induces the mesenchymal phenotype, and dramatically increases migration and invasion in noninvasive MCF-7 cells. Conversely, Lcn2 down-regulation reduces the migration and the mesenchymal phenotype of the aggressive second breast cancer cell line, MDA-MB-231. We also demonstrate, by using an orthotopic animal model, that Lcn2 induces a poorly differentiated phenotype and increases local invasion and lymph node metastasis. Perhaps most convincing is the fact that tissue and urinary Lcn2 levels are associated with invasive and metastatic human breast cancer, providing in vivo support of our in vitro and animal studies. Our findings are consistent with a recent report describing the strong correlation between Lcn2 levels in primary breast cancer and ER-negative status, poor histologic grade, and lymph node metastasis (18) and provide the mechanism underlying this correlation.

Evidence presented in this work demonstrates that Lcn2 inhibits ERα expression and that this inhibition has two outcomes: reduced response to estrogen treatment and triggering of the EMT pathway. A study by Fujita and coworkers (10) established a mechanistic link between ERα and the invasive phenotype of breast cancer when they showed that ERα signaling maintains the epithelial phenotype through MTA3, a subunit in a histone deacetylase complex (10). MTA3 inhibits Snail and relieves the inhibition on E-cadherin. In the absence of ERα signaling, Snail is induced, E-cadherin is suppressed, and the epithelial phenotype is inhibited. We observed an Lcn2-induced increase in Slug, but not in Snail expression. Slug is a member of the Snail superfamily, and importantly, Slug is both necessary and sufficient for the suppression of E-cadherin in breast cancer cells (19). We demonstrate that Slug is downstream of ERα and that Lcn2 inhibition of ERα leads to up-regulation of Slug and the induction of EMT. However, here, Slug regulation by ERα is not mediated by MTA3, given that no correlation between MTA3 and ERα in Lcn2 clones was detected.

We next asked whether or not Lcn2 might induce EMT in a paracrine manner. To address this question, we treated MCF-7 cells with recombinant human Lcn2 and observed a significant reduction in E-cadherin levels, without cytotoxicity, at a dosage that has also been used by other investigators to induce Lcn2-specific effects (20–22). E-cadherin down-regulation is a prerequisite and hallmark of EMT and is critical for the invasive and malignant phenotype (5, 23, 24). Down-regulation of E-cadherin without a significant change in vimentin and fibronectin as seen with recombinant Lcn2 treatment might represent a transitional stage during the process of the epithelial to mesenchymal transition (25), with the epithelial phenotype being suppressed and the mesenchymal phenotype not yet developed. These results suggest that Lcn2 may induce EMT, at least in part, in a paracrine fashion. The partial effects induced by recombinant Lcn2 might also be because exogenously added protein might not enter the cell and traffic through the same pathways that endogenously expressed protein might. The possibility also exists that endogenous Lcn2 might exert some as yet undefined effect(s) even before it is secreted out of the cell.

To date, Lcn2 has been associated with the mesenchymal to epithelial transition (MET). Lcn2 was identified as an epithelial inducer secreted from the ureteric bud during kidney development (21). As for cancers, Lcn2 inversely correlated with epidermal growth factor-induced EMT in ovarian cancer cell lines (26). When overexpressed in metastatic KM12SM human colon cancer cell line, Lcn2 inhibited tumor cell invasion and metastasis, although the mechanism for the inhibition was not clear (27).

In a study using murine 4T1 breast cancer cells that were engineered to overexpress Ras, it was reported that Lcn2 inhibited the mesenchymal phenotype and induced MET in these Ras-transformed cells (28). This study and the current one differ in a number of important ways. Unlike 4T1-Ras cells, which are murine cells, MCF-7 cells are derived from a pleural effusion of a breast cancer patient and are not experimentally manipulated with activated oncogenes. These cells are widely studied and well documented to be phenotypically and genetically similar to human breast cancers (15). Importantly, the Ras signaling pathway is not genetically altered in MCF-7 cells (29). Second, Lcn2 may perform different functions in mouse and human. This is not without precedence. The mouse ortholog of Lcn2, 24p3, has been reported to induce apoptosis in mouse leukocytes and to be an acute-phase protein (30, 31). However, Lcn2 fails to perform either of these functions in humans (32). Finally, and most importantly, our conclusions are corroborated by analyses of Lcn2 levels in tissue and urine samples from breast cancer patients. Given that Lcn2 levels increase in samples of advanced breast cancer, it is now clear that rather than inhibiting breast cancer progression, Lcn2 appears to promote it.

Our immunohistochemistry studies demonstrate that Lcn2 may be useful as a tissue biomarker of human breast cancer. In addition, given our laboratory's long-standing interest in identifying noninvasive biomarkers for breast and other cancers (33–35), we considered the possibility that Lcn2 levels might be elevated in the urine of women with breast cancer. We found that Lcn2 levels did, in fact, increase in the urine of women with metastatic breast cancer, suggesting that Lcn2 may have potential as a noninvasive biomarker for advanced breast cancer.

Materials and Methods

Cell Lines and Reagents. MCF-7 and MDA-MB-231 cells were purchased from American Type Culture Collection. The pcDNA3.1GS vector containing human *Lcn2* (Invitrogen) was transfected into MCF-7 cells by using the Effectene transfection reagent (Qiagen). Lcn2-expressing clones were selected and established in the presence of zeocin (500 μg/mL; Invitrogen). The ERα plasmid was kindly provided by Paul A. Wade (National Institute of Environmental Health Sciences, National Institutes of Health). Transient transfection with ERα was performed by using the Amaxa Nucleofector system (Amaxa) according to the manufacturer's protocol. Recombinant human Lcn2 was synthesized as described in ref. 20. MCF-7 cells were treated with recombinant Lcn2 at various concentrations (0.1, 1, 10, and 100 μg/mL), and cell lysates were collected 8 days after treatment.

The following antibodies were used: anti-E-cadherin (Chemicon), anti-vimentin (Chemicon), anti-fibronectin (BD Biosciences), anti-ERα (Santa Cruz Biotechnology), anti-Slug (Santa Cruz Biotechnology), anti-phospho-Erk and anti-Erk (Cell Signaling), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon), and anti-human Lcn2 (R&D Systems).

Lcn2 protein levels in the conditioned medium from cell culture or human urine samples were determined by using the human lipocalin-2 quantikine ELISA kit (R&D Systems).

In Vitro Migration and Invasion Assays. Cells were seeded at 50,000 cells per well in Dulbecco's modified Eagle's medium (DMEM) into the BD Falcon HTS FluoroBlok 24-multiwell insert system for the migration assays and BD BioCoat tumor invasion systems for invasion assays (BD Biosciences). DMEM with 10% fetal bovine serum was added to the lower wells to stimulate migration or invasion. Cells were incubated for 20 h before they were stained with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, Invitrogen). Three fields were counted for each well.

RNA Interference Studies. Lcn2 siRNA constructs and DharmaFECT1 transfection reagent were purchased from Dharmacon and the transfection performed according to the manufacturer's instructions. The hLcn2 siRNA pool used for MDA-MB-231 is a mixture of four siRNA constructs (siRNA1, 2, 3, and 5). The sequences of siRNA constructs are available in *SI Materials and Methods*.

Reverse Transcription–PCR (RT-PCR). RNA was collected with the RNeasy kit (Qiagen). RNA was treated with DNase I (Invitrogen) before the cDNA was synthesized by using random primers and SuperScript III reverse transcriptase (Invitrogen). The PCR was performed by using platinum PCR SuperMix (Invitrogen). The sequences of PCR primers are available in *SI Materials and Methods*.

Orthotopic Breast Tumor Model in Nude Mice. All animal studies were conducted in compliance with the Children's Hospital Boston IACUC guidelines. Mice used in these studies were 8- to 10-week-old female BALB/c nude mice (Massachusetts General Hospital). Cell lines were engineered to express firefly luciferase fused to neomycin phosphotransferase as described in ref. 36. The inoculation of cells was performed as described by Price et al. (37). Briefly, the right inguinal mammary fat pad was exposed by incision, and 2×10^6 tumor cells in a 40-μL volume were injected. Slow-release estrogen pellets (0.72 mg of 17β-estradiol per pellet) (Innovative Research of America) were implanted s.c. on the dorsum of the mice.

Tumor growth was monitored weekly with calipers, and the tumor volume was calculated based on the formula (length $\times$ width $\times$ width)/2. Mice were killed when the tumor size reached 1 cm in diameter or when mice were moribund. Mice were imaged with the Xenogen IVIS 200 imaging system for metastasis after i.p. injection of D-luciferin (Xenogen) at 65 mg/kg body weight. Tumors were fixed in 10% formalin and embedded in paraffin. Routine H&E staining was performed on the tumor slides.

Immunohistochemistry. Immunohistochemistry was performed by using paraffin-embedded tumor xenografts or human breast cancer tissue microarrays (AccuMax Array; ISU ABXIS Co.) as described in ref. 38. The individual tissue cores in the microarrays were scored by a surgical pathologist, with no knowledge of sample identity, for no staining (0), weak staining (1+), moderate staining (2+), or strong staining (3+). Photomicrographs were taken on an Olympus BX41 microscope by using an Olympus Q-color5 digital camera and analyzed with the software Adobe Photoshop Elements 2.0.

Human Urine Sample Collection. Urine samples were collected as reported according to the institutional bioethical guidelines pertaining to discarded clinical material (33, 35). Some of the samples studied in Fig. 1*E* were the kind gift of Predictive Biosciences. All 20 breast cancer urine samples were from patients with distant metastases (Stage IV). Thirteen of these 20 samples were from patients with lymph node metastasis as well. Information about the lymph node status for 5 of the samples was not available.

Statistical Analyses. Lcn2 staining intensities in tissue microarrays were compared by the nonparametric Mann–Whitney *U* test because these values did not follow a normal distribution, as evaluated by the Kolmogorov–Smirnov test (39). Box-and-whisker plots were used to present the staining intensities of Lcn2 in normal and breast cancer specimens. Boxes in these plots represent upper and lower quartiles (75% and 25%), and the central lines denote the median (50%). The points at the ends of the whiskers are the 97.5% and 2.5% values. Lcn2 concentrations in urine samples from metastatic breast cancer patients and healthy controls were analyzed by using Student's *t* test. The detailed information for the logistic regression model is available in *SI Materials and Methods*. Statistical analysis was performed with the SPSS software package (version 15.0; SPSS). Two-tailed P values < 0.05 were considered statistically significant. Data are presented as mean $\pm$ SEM.

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