

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 ER α in Lcn2-expressing cells reversed the EMT and reduced Slug expression, suggesting that ER α 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 ER α /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. 1A) in contrast to breast carcinomas where the staining was significantly stronger and in more cells (Fig. 1B). 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. 1C). 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. 1D). 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. 1E). 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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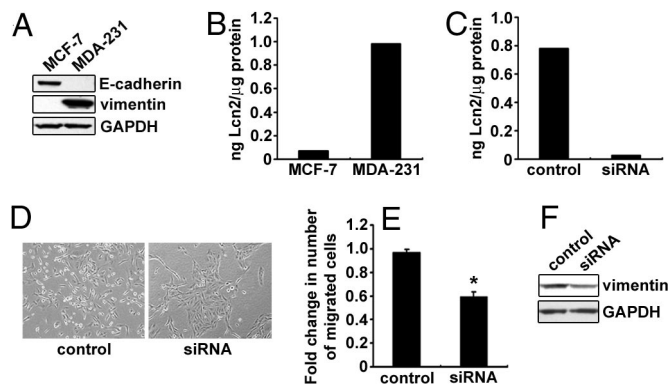


Fig. 5. Reduced Lcn2 expression suppresses the migration and mesenchymal phenotype of MDA-MB-231 cells. (A) Protein levels of the epithelial marker, E-cadherin, and the mesenchymal marker, vimentin, in MCF-7 and MDA-MB-231 cells. (B) Lcn2 levels in the conditioned media of MCF-7 and MDA-MB-231 cells (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.

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. 5A and D). Importantly, these cells produce Lcn2 at a much higher level than MCF-7 cells (Fig. 5B).

Lcn2 was silenced in MDA-MB-231 cells by using a siRNA pool (Fig. 5C). 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. 5D). Moreover, cell migration was significantly reduced compared with controls (Fig. 5E). The mesenchymal marker vimentin was also reduced with Lcn2 silencing (Fig. 5F), 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 ER α 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 ER α /Slug axis alone because Lcn2 silencing reduced vimentin expression but had no effects on ER α 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

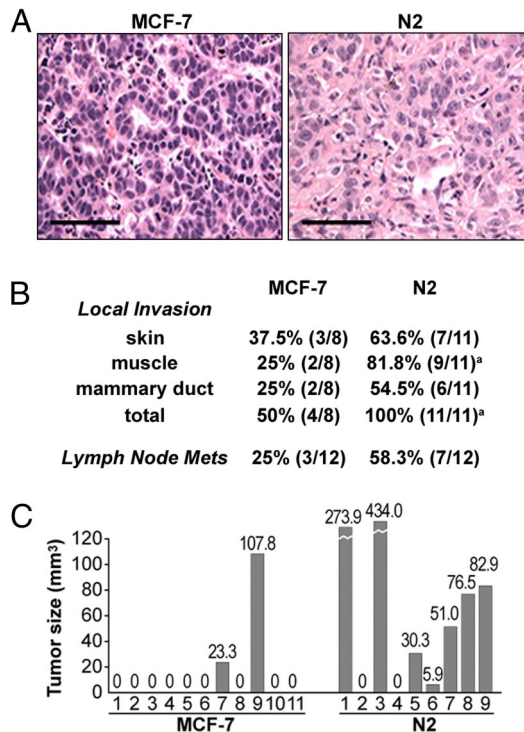


Fig. 6. Lcn2 decreases differentiation and increases breast tumor local invasion, lymph node metastasis, and primary tumor growth in vivo. (A) MCF-7 and N2 tumor sections stained with H&E. (Scale bars, 100 μ m.) (B) Frequencies of local invasion and lymph node metastasis. The value in the parentheses 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.

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. 6A). 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. 6B). Tumor cells were also detected in more lymph nodes in mice injected with N2 cells (Fig. 6B). 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. 6C). 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

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- μ L volume were injected. Slow-release estrogen pellets (0.72 mg of 17 β -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. 1E 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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