Lipocalin 2 promotes inflammatory breast cancer tumorigenesis and skin invasion

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1. Introduction

Inflamatory breast cancer (IBC) is an aggressive form of primary breast cancer characterized by rapid onset and high risk of metastasis and poor clinical outcomes. The biological basis for the aggressiveness of IBC is still not well understood and no IBC-specific targeted therapies exist. In this study, we report that lipocalin 2 (LCN2), a small secreted glycoprotein belonging to the lipocalin superfamily, is expressed at significantly higher levels in IBC vs non-IBC tumors, independently of molecular subtype. LCN2 levels were also significantly higher in IBC cell lines and in their culture media than in non-IBC cell lines. High expression was associated with poor-prognosis features and shorter overall survival in IBC patients. Depletion of LCN2 in IBC cell lines reduced colony formation, migration, and cancer stem cell populations in vitro and inhibited tumor growth, skin invasion, and brain metastasis in mouse models of IBC. Analysis of our proteomics data showed reduced expression of proteins involved in cell cycle and DNA repair in LCN2-silenced IBC cells. Our findings support that LCN2 promotes IBC tumor aggressiveness and offer a new potential therapeutic target for IBC.

Inflamatory breast cancer (IBC) is a rare form of primary breast cancer characterized by rapid onset and high risk of metastasis and poor clinical outcomes. This aggressive disease is considered rare in the United States (1–4% of all breast cancer cases), and it accounts for a disproportionate 10% of breast cancer-related deaths because of its aggressive proliferation and metastasis and limited

Abbreviations
BrMS, brain metastasis subline; ER, estrogen receptor; ERBB2/HER2, Erb-B2 receptor tyrosine kinase 2; IBC, inflammatory breast cancer; LCN2, Lipocalin 2; LuMS, lung metastasis subline; MAPK, mitogen-activated protein kinase; MMP-9, matrix metallopeptidase 9; MTOR, mammalian target of rapamycin; non-IBC, non-inflammatory breast cancer; PR, progesterone receptor; RPS6KB1, ribosomal protein S6 kinase B1; shCtl, short hairpin RNA control; shLCN2, short hairpin RNA for Lipocalin 2; TNBC, triple-negative breast cancer.
therapeutic options [1–5]. IBC disproportionately affects young and African American women [1,6]. IBC is associated with unique clinical and biological features and a distinctive pattern of recurrence with high incidence in central nervous system, lung, and liver as first site of relapse [4,7,8]. Even with multimodality treatment strategies, survival rates for women with IBC are far lower than for those with other types of breast carcinoma (non-IBC), with estimated 5-year overall survival rates limited to 40% vs 63% for non-IBC [4,6–9]. These features underscore the critical need to better define the mechanisms that drive the aggressive behavior of IBC and to develop novel agents to improve the overall prognosis for women with IBC. Efforts have been undertaken to identify pathways and therapeutic targets distinct to IBC and to better elucidate the mechanisms of IBC aggressiveness [10–15]. However, the molecular and cellular basis for IBC aggressiveness remains unclear. Identification of specific targets and unraveling the mechanisms of growth and metastasis of this aggressive disease could lead to improvements in IBC patient survival.

Lipocalin 2 [LCN2, also known as neutrophil gelatinase-associated Lipocalin (NGAL), siderocalin, or 24p3] is a 25-kDa secreted glycoprotein that belongs to the lipocalin superfamily. LCN2 is known to sequester iron, as it binds siderophore-complexed ferric iron with high affinity, and has significant roles in immune and inflammatory responses, angiogenesis, cell proliferation, survival, and resistance to anticancer therapies [16–21]. LCN2 has been implicated in the progression of several types of human tumors, including breast cancer, through several mechanisms, such as stabilization of matrix metallopeptidase 9 (MMP-9), sequestration of iron, induction of epithelial–mesenchymal transition, apoptosis resistance, lymphangiosis, and cell cycle arrest [16–20,22–26]. Moreover, high LCN2 expression levels have been linked with poorer survival in patients with breast cancer [16,26–28]. Little is known regarding the oncogenic role of LCN2 in IBC tumors.

In the present study, we demonstrate that LCN2 was expressed at significantly higher levels in patients with IBC and that LCN2 promoted tumor growth, skin invasion, and metastasis in xenograft mouse models of IBC.

2. Materials and methods

2.1. Cell lines

The SUM149 cell line was purchased from Asterand (Detroit, MI, USA), and MDA-IBC3 cell line was generated in Dr. Woodward’s laboratory [29,30] and cultured in Ham’s F-12 media supplemented with 10% FBS (GIBCO, Thermo Fisher, Carlsbad, CA, USA), 1 µg·mL⁻¹ hydrocortisone (#H8088, Sigma-Aldrich, St. Louis, MO, USA), 5 µg·mL⁻¹ insulin (#12585014; Thermo Fisher), and 1% antibiotic-antimycotic (#15240062; Thermo Fisher). HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 1% penicillin and streptomycin (#15140122; Invitrogen, Carlsbad, CA, USA). All cell lines were kept at 37 °C in a humidified incubator with 5% CO₂ and were authenticated by short tandem repeat profiling at the Cytogenetics and Cell Authentication Core at UT MD Anderson Cancer Center.

2.2. Lentivirus-mediated knockdown

LCN2 stable knockdown clones were generated in SUM149 or MDA-IBC3 cells by using shRNA (shLCN2-1: TRCN0000060289 from Sigma-Aldrich; shLCN2-2: RHS4430-200252675 or shLCN2-3: RHS4430-200246537 from MD Anderson’s Functional Genomics Core Facility, Houston, TX, USA). The MISSION(R) pLKO.1-puro Empty Vector (SHC001, Sigma) was used as control (shCtl). HEK293T cells were transfected with 4.05 µg of target plasmid, pCMV-VSV-G (0.45 µg; #8584; Addgene, Watertown, MA, USA) and pCMV delta R8.2 (3.5 µg, #12263, Addgene) by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and pCMV delta R8.2 (3.5 µg, #12263, Addgene) by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) for 24 h. SUM149 and MDA-IBC3 cells were incubated with the supernatant-containing virus plus 8 µg·mL⁻¹ of polybrene for 24 h. Stable cell lines were selected with 1 µg·mL⁻¹ of puromycin.

2.3. RNA isolation and real-time PCR

RNA was isolated by using TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. The cDNA was obtained with a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). Real-time PCR was done by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500 Real-Time PCR system (Applied Biosystems). LCN2 forward primer: 3'-CCACCTCAAGACCTGATCCCA-5', reverse primer: 3'-CCCCCTGGAATTGGTTGCTG-5'; GAPDH forward primer: 3'-GAAGGTGGATTGTGCTCCTG-5'; GAPDH reverse primer: 3'-GAAGATGGTGATGGATTTC-5'.
2.4. ELISA

Human LCN2/NGAL Quantikine ELISA Kits (DLCN20; R&D Systems, Minneapolis, MN, USA) were used to measure the levels of LCN2 in the cell lines according to the manufacturer’s instructions. Samples were assayed in duplicate.

2.5. Western blotting

Cells were lysed in RIPA buffer (Sigma) supplemented with 10 µL-mL⁻¹ phosphatase and 10 µL-mL⁻¹ protease inhibitor cocktail. SDS/PAGE and immunoblotting were carried out as described elsewhere[29]. The following primary antibodies were used: LCN2 antibody (1 : 1000, #MAB1757SP; R&D Systems), pMEK1/2 (1 : 1000, #9154; Cell Signaling, Danvers, MA, USA), MEK1/2 (1 : 1000, # 8727; Cell Signaling), pERK1/2 (1 : 1000, # 4370; Cell Signaling), ERK1/2 (1 : 1000, # 9102; Cell Signaling), or GAPDH (1 : 5000, #5174; Cell Signaling), and samples were incubated overnight at 4 °C. Secondary antibodies (1 : 5000), anti-rat IgG (#HAF005; R&D Systems) and anti-rabbit IgG (#7074; Cell Signaling) were incubated with the samples for 2 h at room temperature. Immunoreactivity was visualized with Clarity™ Western ECL Substrate (#1705061; Bio-Rad, Hercules, CA, USA) using ImageQuant LAS4000 (GE Healthcare, Chicago, IL, USA).

2.6. Proliferation

About 2500 cells were seeded in triplicate in a 96-well plate. Cell proliferation was measured every day for up to 72 h with the CellTiter-Blue assay (#G8080; Promega, Madison, WI, USA) according to the manufacturer’s instructions. Absorbance was recorded at OD595 nm with a Multifunctional Reader VICTOR X3 (PerkinElmer, Waltham, MA, USA).

2.7. Colony-formation assay

About 100 SUM149 or 500 MDA-IBC3 shRNA Control or LCN2-silenced cells were plated in triplicate in 6-well plates. After 15 days, cells were fixed with methanol for 2 min and stained with 0.2% (w/v) crystal violet for 30 min. Colonies were counted using GelCount (Oxford OptoroniX, Abingdon, UK).

2.8. Migration and invasion assay

For the migration assay, 50 000 cells per well (triplicate) were seeded in medium without serum onto 8 µm polypropylene filter inserts in Boyden chambers (Fisher). Medium with 10% FBS was added onto the well. After 24 h, cells on the bottom of the filter were fixed and stained with Thermo Scientific Shandon Kwik Diff Stains (Fisher). The invasion assay was done as described above, except that the 8 µm polypropylene filter inserts were coated with Matrigel (#CB-40234; Corning Inc., Corning, NY, USA) and incubated for 24 h. Ten visual fields were randomly chosen under microscopy and cells were quantified by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.9. Mammosphere assay

For primary mammosphere formation, 30 000 SUM149 or MDA-IBC3 control or LCN2-knockdown cells were plated in ULTRALOW attachment six-well plates (Corning Inc.) in mammosphere medium [serum-free MEM supplemented with 20 ng-mL⁻¹ of bFGF (Gibco), 20 ng-mL⁻¹ epidermal growth factor (Gibco), B27 1× (Gibco), and gentamycin/penicillin/streptomycin (Thermo Fisher)]. After 7 days, 5 µg-mL⁻¹ of MTT (Sigma-Aldrich) was added for 30 min and the mammospheres were counted using GelCount (Oxford OptoroniX). For secondary mammosphere formation, primary mammospheres were dissociated and counted, and 10 000 cells were plated in the ULTRALOW attachment six-well plates in mammosphere media and analyzed after 7 days.

2.10. CD44/CD24 flow cytometry

About 2.5 x 10⁵ cells were suspended in CD24-PE mouse anti-human (#555428; BD Biosciences) or CD24-BV421 Mouse Anti-Human (#562789; BD Biosciences, Franklin Lakes, NJ, USA) and CD44-FITC mouse anti-human (#555478; BD Biosciences) or CD44-APC Mouse anti-Human (#559942; BD Biosciences) solutions and incubated for 20 min on ice. Cells only, PE-BV421 only, and FITC/APC only were used as controls to set the gating. Fluorescence was detected by using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) at the Flow Cytometry and Cellular Imaging Core Facility (UT MD Anderson Cancer Center, Houston, TX, USA). FLOWJO software (Treestar, Ashland, OR, USA) was used to analyze the data.

2.11. Kinase enrichment analysis

The RPPA data were also used for the phosphoproteomic analysis using kinase enrichment analysis (KEA —https://maayanlab.cloud/kea3/) [31]. Briefly, the 20 proteins that exhibit the highest phosphorylation fold
change levels in control vs LCN2-silenced cells were analyzed. Two different analyses were performed using KEA: (a) The differentially phosphorylated proteins are queried for enrichment of kinase substrates and (b) the differentially phosphorylated proteins are queried for enrichment of interacting proteins across seven databases. The latter analysis is more general and is not limited to only kinase substrates. Both analyses result in the detection of kinases that are putatively responsible for the observed phosphorylation differences. Identified proteins by both analyses were mapped onto the STRING network (https://string-db.org) to investigate their mutual interactions.

2.12. In vivo experiments

Four- to six-week-old female athymic SCID/Beige mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and allowed to acclimate for 1 week before use. All mice were given free access to food and water in a specific pathogen-free condition. All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center. Mice were euthanized with overdose of isoflurane when they met the institutional criteria for tumor size and overall health condition. For primary tumor growth, cells were injected into the orthotopic cleared mammary fat pad of mice as previously described [32]. Briefly, $5 \times 10^5$ SUM149 shRNA Control / LCN2-knockdown cells were injected (9 mice/Control; 10 mice/LCN2 KD). Tumor volumes were assessed weekly by measuring palpable tumors with calipers. Volume ($V$) was determined as $V = (L \times W \times W) \times 0.5$, with $L$ being length and $W$ width of the tumor. To determine latency, the first day when palpable tumors appeared was used to plot the graph. For brain metastatic colonization studies, we followed our laboratory protocol [33]. Briefly, $1 \times 10^6$ MDA-IBC3 GFP-labeled shRNA Control/LCN2-knockdown cells (10 mice/group) were injected via the tail vein into SCID/Beige mice. At 12 weeks after tail-vein injection, mice were euthanized, and brain tissue collected and imaged with fluorescent stereomicroscopy (SMZ1500; Nikon Instruments, Melville, NY, USA). ImageJ was used to measure GFP-positive areas to quantify the area of brain tumor burden. For mice with more than one brain metastasis, the area of each metastasis was considered and measured.

2.13. Statistical analysis

All in vitro experiments were repeated at least three times, and graphs depict mean ± SEM. Statistical significance was determined with Student’s $t$-tests (unpaired, two-tailed) unless otherwise specified. One-way analysis of variance was used for multiple comparisons. Mann–Whitney test was used when normality was not met. LCN2 expression in breast cancer samples was analyzed in the IBC Consortium dataset [34] for IBC and from a meta-dataset previously published [35]. Tumor samples were stratified as LCN2-high when expression in tumor was at least two-fold the mean expression level measured in the normal breast samples; otherwise, the sample was classified as LCN2-low. Kaplan–Meier curves and log-rank tests were used to compare survival distributions. Univariate and multivariate Cox regression models were used to evaluate the significance of LCN2 expression on overall survival. A $P$ value of < 0.05 was considered significant. GraphPad software (GraphPad Prism 8, La Jolla, CA, USA) was used.

3. Results

3.1. LCN2 mRNA is highly expressed in inflammatory breast cancer

Previous studies have shown that high LCN2 expression levels were correlated with poor prognosis in breast cancer patients [17,25–27]. We further validated these findings by analyzing a meta-dataset of 8951 breast cancers, in which 87% of tumor samples were classified as LCN2-low ($n = 7830/8951$) and 13% as LCN2-high ($n = 1121/8951$). Table 1 summarizes the clinico-pathological patient characteristics stratified by LCN2 expression status. High expression of LCN2 was associated with variables commonly associated with poor outcome: younger patients’ age, high grade, advanced stage tumors (pN-positive and pT3), ductal type, estrogen receptor (ER)-negative status, progesterone receptor (PR)-negative status, Erb-B2 receptor tyrosine kinase 2 (ERBB2)-positive status, and aggressive molecular subtypes [ERBB2$^+$ and triple-negative breast cancer (TNBC) subtypes]. In this cohort, we also analyzed the association of LCN2 expression and survival over time using the Kaplan–Meier method. We found that LCN2-high tumors had significantly shorter overall survival ($P < 0.0001$) than LCN2-low tumors (Fig. 1A).

Analysis of microarray data from the IBC World Consortium Dataset [34] consisting of IBC and non-IBC patient samples ($n = 389; $IBC = 137$, non-IBC = 252$) showed that LCN2 expression was significantly higher in tumors from IBC patients compared to non-IBC ($P = 0.0003$; Fig. 1B). We validated this
finding in another independent dataset [36] that compared mRNA expression of microdissected IBC and non-IBC tumors ($P = 0.0379$; Fig. 1C). Here too, $\text{LCN2}$ expression was higher in ER-negative IBC patients ($P = 0.0009$; Fig. 1D) and in more aggressive subtypes, ERBB2-positive and TNBC, compared to hormone receptor (HR)-positive/ERBB2-negative subtype (Fig. 1E). Multivariate analysis showed that $\text{LCN2}$ was expressed significantly higher in IBC tumors relative to non-IBC tumors, independently from the molecular subtype differences (Odds ratio, 1.71, $P = 0.034$; Table 2). Here too, the survival analysis in IBC patients showed that $\text{LCN2}$-high tumors had significantly shorter overall survival ($P = 0.0317$) than $\text{LCN2}$-low tumors (Fig. 1F). Consistent with the patient data, the levels of $\text{LCN2}$ were higher in IBC cell lines (Fig. 1G) and in the supernatants collected from IBC cell lines relative to non-IBC (Fig. 1H). Taken together, our findings show that $\text{LCN2}$ is highly expressed in IBC tumors and is correlated with aggressive features and poor outcome suggesting it may contribute to the aggressive pathobiology of IBC tumors.

### 3.2. $\text{LCN2}$-knockdown reduced aggressiveness features in vitro

We generated stable $\text{LCN2}$-knockdown cell lines [SUM149 (triple-negative IBC); MDA-IBC3 (HER2$^+$ IBC)] to investigate the role of $\text{LCN2}$ in IBC aggressiveness in vitro and in vivo. $\text{LCN2}$-knockdown was confirmed by qRT-PCR and immunoblotting (Fig. 2A, B). Because $\text{LCN2}$ is a secreted protein, we evaluated levels of $\text{LCN2}$ protein in the supernatants from control and $\text{LCN2}$-silenced IBC cell lines by using ELISA. We observed significant reduction of secreted $\text{LCN2}$ in the $\text{LCN2}$-silenced IBC cells (Fig. 2C). Silencing $\text{LCN2}$ slightly reduced proliferation of SUM149 cells but did not affect MDA-IBC3 cells (Fig. 2D). Depletion of $\text{LCN2}$ reduced the capacity of the cells to form colonies (Fig. 2E) and to migrate and invade (Fig. 3A, B). $\text{LCN2}$ silencing also significantly reduced the percentage of cancer stem cell populations in $\text{LCN2}$-silenced IBC cells relative to control, as shown by reductions in primary and secondary mammosphere formation efficiency (Fig. 3C,D) and CD44$^+$CD24$^-$ cell subpopulations (Fig. 3E). These findings indicate

### Table 1. Clinico-pathological characteristics of tumor samples from patients with IBC or non-IBC according to $\text{LCN2}$ expression. The percentage between brackets is relative to the total number of samples informative in each column.

| Characteristics | Level | All (n = 8951) | LCN2-low (n = 7830) | LCN2-high (n = 1121) | $P$ value |
|-----------------|-------|---------------|---------------------|---------------------|----------|
| Age (years)     | $\leq$ 50 | 2587 (36%) | 2218 (36%) | 369 (42%) | 1.10E-04 |
|                 | > 50    | 4520 (64%)  | 4018 (64%) | 502 (58%) | $< 1.00E-06$ |
| Pathological grade | 1       | 717 (11%)  | 680 (13%) | 37 (4%) | 3.89E-02 |
|                 | 2       | 2549 (41%)  | 2359 (43%) | 190 (22%) | $< 1.00E-06$ |
|                 | 3       | 3016 (48%)  | 2389 (44%) | 627 (73%) | $< 1.00E-06$ |
| Pathological node (pN) | Negative | 3666 (57%) | 3253 (57%) | 413 (53%) | $< 1.00E-06$ |
|                 | Positive | 2788 (43%) | 2426 (43%) | 362 (47%) | $< 1.00E-06$ |
| Pathological size (pT) | pT1 | 2116 (37%) | 1912 (38%) | 204 (31%) | $< 1.00E-06$ |
|                 | pT2 | 2931 (52%) | 2588 (52%) | 343 (53%) | $< 1.00E-06$ |
|                 | pT3 | 604 (11%) | 498 (10%) | 106 (16%) | $< 1.00E-06$ |
| Pathological type | Ductal | 4027 (79%) | 3492 (78%) | 555 (86%) | 3.00E-06 |
|                 | Lobular | 500 (10%) | 471 (11%) | 29 (5%) | $< 1.00E-06$ |
|                 | Other | 574 (64%) | 519 (12%) | 55 (9%) | $< 1.00E-06$ |
| ER status$^a$ | Negative | 2753 (31%) | 1955 (25%) | 798 (71%) | 1.97E-215 |
|                 | Positive | 6198 (69%) | 5875 (75%) | 323 (29%) | $< 1.00E-06$ |
| PR status$^a$ | Negative | 4635 (52%) | 3746 (48%) | 889 (80%) | 3.06E-86 |
|                 | Positive | 4284 (48%) | 4055 (52%) | 229 (20%) | $< 1.00E-06$ |
| ERBB2 status$^a$ | Negative | 7862 (88%) | 6975 (89%) | 887 (79%) | 2.37E-21 |
|                 | Positive | 1089 (12%) | 855 (11%) | 234 (21%) | $< 1.00E-06$ |
| HR subtype$^a$ | HR/ERBB2$^+$ | 5914 (66%) | 5598 (72%) | 316 (28%) | $< 1.00E-06$ |
|                 | ERBB2$^+$ | 1089 (12%) | 855 (11%) | 234 (21%) | $< 1.00E-06$ |
|                 | TNBC | 1938 (22%) | 1368 (17%) | 570 (51%) | $< 1.00E-06$ |
| Overall survival$^b$ | 4984 | 1.00 | 1.58 (1.34–1.86)$^c$ | 3.31E-08 | $< 1.00E-06$ |

$^a$mRNA status.

$^b$Univariate analysis.

$^c$Hazard ratio (95% confidence interval)
LCN2 was highly expressed in tumors from patients with IBC. (A) High LCN2 expression was associated with shorter overall survival in a meta-dataset of patients with non-IBC. (B, C) LCN2 mRNA expression was higher in tumors from IBC patients vs non-IBC patients in two independent breast cancer datasets [IBC World Consortium Dataset; GSE45582]. (D) LCN2 mRNA expression was higher in ER-negative compared to ER+ samples IBC samples. (E) LCN2 mRNA expression was higher in more aggressive molecular subtypes, ERBB2+ and TNBC, compared to HR-positive/HERBB2-negative subtype. (F) LCN2-high expression correlates with shorter overall survival in patients with IBC. (G) LCN2 mRNA expression was higher in IBC cell lines compared to non-IBC cell lines. (H, I) LCN2 protein expression was higher in IBC cell lines compared to non-IBC cell lines shown by (H) immunoblotting or (I) ELISA for secreted LCN2 in supernatants. Bar graphs indicate mean ± SEM from three independent experiments. GraphPad Prism software was used to obtain the P values, with Mann–Whitney tests used to compare two categories or one-way analysis of variance to compare three or more categories. Black lines in each group (B–E, and G) indicate mean ± SD.
that suppression of LCN2 in IBC cells reduced in vitro aggressiveness features.

3.3. Silencing of LCN2 inhibited tumor growth and skin invasion

To investigate the effects of LCN2 on tumor growth and skin invasion, key characteristics of IBC tumors [4], we injected SUM149 control or LCN2-silenced cells into the cleared mammary fat pad of SCID/Beige mice. Silencing of LCN2 reduced tumor volumes ($P = 0.0037$; Fig. 4A) and tumor latency, that is, the ability to initiate tumor growth: mice transplanted with SUM149 LCN2-silenced cells took longer to initiate tumors than did those transplanted with SUM149 control cells ($P = 0.0145$; Fig. 4B). Because IBC typically manifests with skin invasion and formation of tumor emboli [4], we assessed skin invasion visually during primary tumor growth, as evidenced by loss of fur at the tumor site and skin redness and thickness, and during tumor excision when tumors were firmly connected with the skin. Analysis of resected tumors showed that significantly fewer mice with SUM149 LCN2-silenced cells had skin invasion/recurrence compared with mice implanted with control cells [shLCN2: two of eight mice (25%) vs shControl: seven of eight mice (87.5%), $P = 0.01$; Fig. 4C,D]. On histologic examination, tumors generated from LCN2-silenced cells were more differentiated than those generated from control SUM149 cells (Fig. 4E); we further

Table 2. Univariate and multivariate Cox regression analysis of IBC patient samples vs non-IBC ($n = 389$).

| IBC vs non-IBC | Univariate | Multivariate |
|----------------|------------|--------------|
|                | Odds ratio | 95% CI | $P$ value | Odds ratio | 95% CI | $P$ value |
| LCN2, high vs low | 2.09 | 1.43–3.06 | 1.43E-03 | 1.71 | 1.13–2.6 | 3.42E-02 |
| Molecular subtype | | | | |
| ERBB2+ vs HR+/ERBB2- | 2.82 | 1.82–4.39 | 1.02E-04 | 2.5 | 1.59–3.93 | 8.16E-04 |
| TNBC vs HR+/ERBB2- | 1.9 | 1.22–2.97 | 1.69E-02 | 1.51 | 0.93–2.44 | 0.162 |

Fig. 2. Silencing LCN2 decreased colony formation efficiency. LCN2 was knocked down (shLCN2) in two IBC cell lines (SUM149 and MDA-IBC3) and confirmed by (A) qRT-PCR and (B) immunoblotting. (C) Secreted LCN2 measured in control and silenced cells by ELISA at the indicated times. Bar graphs indicate mean ± SEM, calculated after three independent experiments; $P$ values from $t$-tests. (D) Proliferation was evaluated in control and LCN2-silenced SUM149 and MDA-IBC3 cells with CellTiter-Blue assay on the indicated days. $P$ values from $t$-tests. (E) Cells were seeded in low numbers to measure the capacity to form colonies in LCN2 knockdown and control. Bar graphs indicate mean ± SEM, calculated after three independent experiments; $P$ values from $t$-tests.
observed tumor emboli, another hallmark of IBC tumors, in SUM149 control-transplanted tumors but not in tumors generated from LCN2-silenced SUM149 cells (Fig. 4E).

We recently generated xenograft mouse models of brain and lung metastasis via tail-vein injection of IBC cell lines [29,33]. We also showed that sublines of SUM149 generated from brain metastases (BrMS) and lung metastases (LuMS) have distinct morphologic and molecular features [29]. Microarray profiling of these sublines showed upregulation of LCN2 in the brain metastatic sublines (Fig. S1A), and we confirmed higher levels of secreted LCN2 in the BrMS sublines vs LuMS by ELISA (Fig. S1B). Most recently, Chi et al. [37] elegantly demonstrated that LCN2 promotes brain metastatic growth in mouse models of leptomeningeal metastasis, highlighting a potential brain metastasis-promoting role for LCN2. We investigated the functional role of LCN2 in IBC brain metastasis by using our HER2+ MDA-IBC3 mouse model, which has a high propensity to metastasize to the brain and has been used to identify targets and develop therapeutics against brain metastasis [29,38–40]. We found that the brain metastatic burden was significantly lower in mice that had received tail-vein injection of LCN2-silenced MDA-IBC3 cells than in mice injected with control cells (Fig. 4F, \( P = 0.0059 \)). Also, fewer mice injected with LCN2-silenced cells developed brain metastasis [one of 10 (10%)] than did mice injected with control cells [five of 10 mice (50%)], although this

![Fig. 3. LCN2 knockdown reduced aggressiveness features in vitro](image-url)
trend was not statistically significant ($P = 0.1409$; Fig. 4G). Representative stereofluorescence and hematoxylin and eosin images of brain metastasis are shown in Fig. 4H. Overall, our findings suggest that LCN2 may drive IBC tumor progression, skin invasion/recurrence, and brain metastasis.

3.4. LCN2 silencing impairs cell cycle-associated proteins

To identify potential mechanisms and pathways involved in suppression of tumor growth and skin invasion in LCN2-silenced cells, we used reverse-phase proteomics assay (RPPA) profiling to compare control and LCN2-silenced SUM149 cells. Our analysis showed reduced expression of cell cycle-associated proteins [such as AXL, FOXM1, Chk1, CDK1, Wee1, Aurora-B, and cyclin-B1 and the mammalian target of rapamycin (mTOR)/AKT pathway] in LCN2-silenced IBC cells (Fig. 5A). Gene set enrichment analysis revealed several key signaling pathways that were enriched in the control cells, including those associated with cell cycling, DNA repair, and mTOR signaling (Fig. 5B). Furthermore, we performed KEA [31] on the 20 proteins that exhibited the highest phosphorylation fold changes in LCN2-control vs LCN2-silenced SUM149 cells (Table S1). Based on the set of predicted activated kinases (Tables S1 and S2), an interaction network was generated (Fig. 5C). Based on the node degree distribution (i.e., the distribution of the number of interactions per gene in the network), mitogen-activated protein kinase 1 (MAPK1; $N = 10$), MAPK8 ($N = 7$), ribosomal protein S6 kinase B1 (RPS6KB1; $N = 7$), and MTOR ($N = 11$) appear to be central to LCN2 action in SUM149 cells. Our immunoblotting experiments confirmed that silencing LCN2 reduces the phosphorylated forms of MEK (pMEK) and ERK (pERK) of the MAPK signaling pathway (Fig. 5D). Thus, LCN2 may regulate different pathways, including cell cycle, MAPK, and mTOR proteins to promote tumor growth in IBC.
Inflammatory breast cancer is an aggressive form of breast cancer with poor survival outcomes. Although considerable effort has been undertaken to understand the unique biology of IBC, insights are still limited as to the molecular properties that mediate the development and aggressiveness of IBC. Herein, we report that the secreted glycoprotein LCN2 was highly expressed in tumors from IBC patients and in IBC cell lines. We further demonstrate, with in vitro and in vivo studies, that LCN2 has a tumor promoter function in IBC.

4. Discussion

Inflammatory breast cancer is an aggressive form of breast cancer with poor survival outcomes. Although considerable effort has been undertaken to understand the unique biology of IBC, insights are still limited as to the molecular properties that mediate the development and aggressiveness of IBC. Herein, we report that the secreted glycoprotein LCN2 was highly expressed in tumors from IBC patients and in IBC cell lines. We further demonstrate, with in vitro and in vivo studies, that LCN2 has a tumor promoter function in IBC.

LCN2 has been implicated in the progression of several types of human tumors. LCN2 expression is higher in solid tumors than in corresponding normal tissues [41,25], and it is mainly described as tumor promoter in many cancers, including pancreas, glioblastoma, thyroid, kidney, esophagus, and breast cancer [19,28,42–48].

In breast cancer, increased LCN2 expression was associated with poor outcomes and shown to be an independent prognostic marker of disease-specific-free survival [27,49,48]. LCN2 also correlates with several important unfavorable prognostic factors in breast cancer, such as hormone-negative status, high proliferation levels, high histologic grade, and the presence of lymph node metastases [27,49,48]. Further, serum levels of LCN2 have been shown to correlate with cancer progression and higher likelihood of metastasis in
breast cancer [26,50]. The oncogenic role of LCN2 has been reported in xenograft and LCN2-knockout mouse models. Disruption of the LCN2 gene in MMTV-PyMT mice was found to suppress primary tumor formation without affecting lung metastasis [51]. Using the spontaneous MMTV-ErbB2(V664E) LCN2−/− mouse model, Leng et al. [18] reported delayed tumor growth and reduced lung metastasis burden in these LCN2−/− mice. Another group showed that injection of wild-type PyMT tumor cells into LCN2-deficient mice did not alter primary tumor formation but did significantly reduce lung metastasis [52]. LCN2 has also been shown to promote tumor progression in xenograft mouse models [16,26]. Consistent with these studies, our current work with xenograft mouse models of IBC supports that LCN2 has a tumor promoter function in IBC tumors. We demonstrated that silencing of LCN2 reduced tumor initiation and growth, skin invasion/recurrence, and brain metastasis burden in preclinical mouse models of IBC. We further reported that depletion of LCN2 in IBC cell cultures reduced features associated with aggressiveness in vitro, including migration, invasion, and cancer stem cell populations. Others have also found that reduction of LCN2 levels affected the same features in MDA-MB-231 cells (TNBC cell line) and in SK-BR-3 (HER2+ breast cancer cell line) [18,26]. However, our data demonstrating higher levels of secreted LCN2 in IBC vs non-IBC cell lines and showing significant inhibition of key IBC tumor features such as tumor emboli/skin invasion in LCN2-silenced tumors suggest that LCN2 may exert its influence via an IBC-specific mechanism. The LCN2 protein has many functions, including transport of fatty acids and iron, induction of apoptosis, suppression of bacterial growth, and modulation of inflammatory responses [16–20,26,53]. In malignant cells, LCN2 promotes oncogenesis through several mechanisms, including stabilization of MMP-9, sequestration of iron, induction of EMT, apoptosis resistance, and regulation of cell cycling [16–20,26,53]. Here, we report that LCN2 could regulate cell cycle-associated proteins such as FOXM1, Chk1, CDK1, Aurora-B, Wee1, and cyclin-B1 to promote its oncogenic role in IBC tumors. Others have also found that silencing of LCN2 affected the expression of cell cycle proteins by reducing cyclin-D1 and inducing p21, resulting in G0-G1 cell cycle arrest [22,24,25].

LCN2 is also a potential therapeutic target in cancer and other diseases. An antibody against LCN2 was found to decreased lung metastasis in a 4T1-induced aggressive mammary tumor model [18]. In cervical cancer cells, treatment with LCN2-neutralizing antibody reduced the migration and invasion of cells that overexpressed LCN2 [54]. In other diseases, use of an anti-LCN2-neutralizing antibody showed reductions in reperfusion injury after stroke and attenuated skin lesions in a psoriasis mouse model [55,56]. These findings suggest that LCN2 could be an exploitable therapeutic target in IBC and other aggressive tumors. Further studies are needed to explore therapeutic strategies in IBC models by using antibodies against LCN2 or targeting LCN2-associated molecular pathways, including those involved in cell cycling.

5. Conclusion

In summary, our studies provide evidence, for the first time, that LCN2 is highly upregulated in IBC tumors and that it is required for tumor growth and skin invasion in mouse models of IBC; our findings further suggest that LCN2 could be a therapeutic target for IBC and other aggressive cancers.

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Data accessibility

The data that support the findings of this study are available from the corresponding author (bgd-beb@mdanderson.org) upon reasonable request. Additional data are available as supplementary material.

Author contributions

ESV and BGD conceived and designed the project, performed most of the experiments, analyzed the data, and interpreted the results. XH, RL, WB, SRS, and KG performed some experiments. PF, FB, and XS

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helped with data analysis. JS provided statistical analysis support. SK provided pathological expertise and analysis of xenograft tumors. SVL, FB, GS-S, PV-M, NTU, WAW, and DT provided resources and contributed to revision of the manuscript. ESV and BGD wrote and edited the manuscript with input from all other authors.

Conflict of interest
The authors declare no conflict of interest.

References

1 Chang S, Parker SL, Pham T, Buzdar AU & Hursting SD (1998) Inflammatory breast cancer incidence and survival: the surveillance, epidemiology, and end results program of the National Cancer Institute, 1975–1992. Cancer 82, 2366–2372.

2 Dirix LY, Van Dam P, Prove A & Vermeulen PB (2006) Inflammatory breast cancer: current understanding. Curr Opin Oncol 18, 563–571.

3 Hance KW, Anderson WF, Devesa SS, Young HA & Levine PH (2005) Trends in inflammatory breast carcinoma incidence and survival: the surveillance, epidemiology, and end results program at the National Cancer Institute. J Natl Cancer Inst 97, 966–975.

4 Robertson FM, Bondy M, Yang W, Yamauchi H, Wiggins S, Kamrudin S, Krishnamurthy S, Le-Petross H, Bidaut L, Player AN et al. (2010) Inflammatory breast cancer: the disease, the biology, the treatment. CA Cancer J Clin 60, 351–375.

5 Wang Z, Chen M, Pan J, Wang X, Chen XS & Shen KW (2020) Pattern of distant metastases in inflammatory breast cancer - a large-cohort retrospective study. J Cancer 11, 292–300.

6 Abraham HG, Xia Y, Mukherjee B & Merajver SD (2021) Incidence and survival of inflammatory breast cancer between 1973 and 2015 in the SEER database. Breast Cancer Res Treat 185, 229–238.

7 Cristofanilli M, Valero V, Buzdar AU, Kau SW, Brogli KR, Gonzalez-Angulo AM, Sniege N, Islam R, Ueno NT, Buchholz TA et al. (2007) Inflammatory breast cancer (IBC) and patterns of recurrence: understanding the biology of a unique disease. Cancer 110, 1436–1444.

8 Fouad TM, Kogawa T, Liu DD, Shen Y, Masuda H, El-Zein R, Woodward WA, Chavez-MacGregor M, Alvarez RH, Arun B et al. (2015) Overall survival differences between patients with inflammatory and noninflammatory breast cancer presenting with distant metastasis at diagnosis. Breast Cancer Res Treat 152, 407–416.

9 Dawood S, Ueno NT, Valero V, Woodward WA, Buchholz TA, Hortobagyi GN, Gonzalez-Angulo AM & Cristofanilli M (2011) Differences in survival among women with stage III inflammatory and noninflammatory locally advanced breast cancer appear early: a large population-based study. Cancer 117, 1819–1826, doi: 10.1002/cncr.25682.

10 Costa R, Santa-Maria CA, Rossi G, Carneiro BA, Chae YK, Gradishar WJ, Giles FJ & Cristofanilli M (2017) Developmental therapeutics for inflammatory breast cancer: biology and translational directions. Oncotarget 8, 12417–12432.

11 Kleer CG, van Golen KL, Braun T & Merajver SD (2001) Persistent E-cadherin expression in inflammatory breast cancer. Mod Pathol 14, 458–464.

12 van Golen KL, Wu ZF, Qiao XT, Bao LW & Merajver SD (2000) RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res 60, 5832–5838.

13 Villodre ES, Gong Y, Hu X, Luo L, Yoon EC, Ueno NT, Woodward WA, Tripathy D, Song J & Debeb BG (2020) NDRG1 expression is an independent prognostic factor in inflammatory breast cancer. Cancers (Basel) 12, 3711.

14 Wang X, Semba T, Phi LTH, Chaimitikun I, Ihase T, Lim B & Ueno NT (2020) Targeting signaling pathways in inflammatory breast cancer. Cancers 12, 2479.

15 Zhang D, LaFortune TA, Krishnamurthy S, Esteva FJ, Cristofanilli M, Liu P, Lucci A, Singh B, Hung MC, Hortobagyi GN et al. (2009) Epidermal growth factor receptor tyrosine kinase inhibitor reverses mesenchymal to epithelial phenotype and inhibits metastasis in inflammatory breast cancer. Clin Cancer Res 15, 6639–6648.

16 Fernandez CA, Yan L, Louis G, Yang J, Kutok JL & Moses MA (2005) The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. Clin Cancer Res 11, 5390–5395.

17 Iannetti A, Pacifico F, Acquaviva R, Lavorgna A, Crescenzi E, Vascotto C, Tell G, Salzano AM, Scalonì A, Vuttariello E et al. (2008) The neutrophil gelatinase-associated lipocalin (NGAL), a NF-kappaB-regulated gene, is a survival factor for thyroid neoplastic cells. Proc Natl Acad Sci USA 105, 14058–14063.

18 Leng X, Ding T, Lin H, Wang Y, Hu L, Hu J, Feig B, Zhang W, Pusztai L, Symmans WF et al. (2009) Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis. Cancer Res 69, 8579–8584.

19 Leung L, Radulovich N, Zhu CQ, Organ S, Bandarchi B, Pintilie M, To C, Panchal D & Tsao MS (2012) Lipocalin2 promotes invasion, tumorigenicity and gemcitabine resistance in pancreatic ductal adenocarcinoma. PLoS One 7, e46677.

20 Shiba M, Saito K, Fushimi K, Ishigami T, Shinozuka K, Nakashima D, Kouzu Y, Koike H, Kasamatsu A,
LCN2 promotes tumorigenesis in IBC

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Sakamoto Y et al. (2013) Lipocalin-2 is associated with radioreistance in oral cancer and lung cancer cells. *Int J Oncol* **42**, 1197–1204.

21 Yang J & Moses MA (2009) Lipocalin 2: a multifaceted modulator of human cancer. *Cell Cycle* **8**, 2347–2352, doi: 10.4161/cc.8.15.9224

22 Chiang KC, Yeh TS, Wu RC, Pang JS, Cheng CT, Wang SY, Juang HH & Yeh CN (2016) Lipocalin 2 (LCN2) is a promising target for cholangiocarcinoma treatment and bile LCN2 level is a potential cholangiocarcinoma diagnostic marker. *Sci Rep* **6**, 36138.

23 Jung M, Oren B, Mora J, Mertens C, Dziumbla S, Popp R, Weigert A, Grossmann N, Fleming I & Brune B (2016) Lipocalin 2 from macrophages stimulated by tumor cell-derived sphingosine 1-phosphate promotes lymphangiogenesis and tumor metastasis. *Sci Signal* **9**, ra64, doi: 10.1126/scisignal.aaf3241

24 Tun MC, Hsieh SC, Yang SF, Cheng CW, Tsai RT, Wang SC, Huang MH & Hsieh YH (2013) Knockdown of lipocalin-2 suppresses the growth and invasion of prostate cancer cells. *Prostate* **73**, 1281–1290, doi: 10.1002/pros.22670

25 Xu J, Lv S, Meng W & Zuo F (2020) LCN2 mediated tumor cell-derived sphingosine 1-phosphate promotes metastasis from breast cancer cells. *Breast Cancer Res Treat* **1720**, 146304.

26 Yang J, Bielenberg DR, Rodig SJ, Doiron R, Clifton MC, Kung AL, Strong RK, Zurakowski D & Moses MA (2009) Lipocalin 2 promotes breast cancer progression. *Proc Natl Acad Sci USA* **106**, 3913–3918.

27 Bauer M, Eickhoff JC, Gould MN, Mundhenke C, Maass N & Friedl A (2008) Neutrophil gelatinase-associated lipocalin (NGAL) is a predictor of poor prognosis in human primary breast cancer. *Breast Cancer Res Treat* **108**, 389–397.

28 Candio S, Abrams SL, Steelman LS, Lertpiriyapong K, Fitzgerald TL, Martelli AM, Cocco L, Montalto G, Cervello M, Polese J et al. (2016) Roles of NGAL and MMP-9 in the tumor microenvironment and sensitivity to targeted therapy. *Biochim Biophys Acta* **1863**, 438–448.

29 Debeb BG, Lacerda L, Anfossi S, Diagaradjane P, Chu K, Bambhroliya A, Huo L, Wei C, Larson RA, Wolfe AR et al. (2016) miR-141-mediated regulation of brain metastasis from breast cancer. *J Natl Cancer Inst* **108**, djw026.

30 Klop AH, Lacerda L, Gupta A, Debeb BG, Solley T, Li L, Spaeth E, Xu W, Zhang X, Lewis MT et al. (2010) Mesenchymal stem cells promote mammosphere formation and decrease E-cadherin in normal and malignant breast cells. *PLoS One* **5**, e12180.

31 Lachmann A & Ma’ayan A (2009) KEA: kinase enrichment analysis. *Bioinformatics* **25**, 684–686.

32 Debeb BG, Lacerda L, Xu W, Larson R, Solley T, Atkinson R, Sulman EP, Ueno NT, Krishnamurthy S, Reuben JM et al. (2012) Histone deacetylase inhibitors stimulate dedifferentiation of human breast cancer cells through WNT/beta-catenin signaling. *Stem Cells* **30**, 2366–2377, doi: 10.1002/stem.1219

33 Hu X, Villodre ES, Woodward WA & Debeb BG (2021) Modeling brain metastasis via tail-vein injection of inflammatory breast cancer cells. *J Vis Exp* **168**, e62249.

34 Van Laere SJ, Ueno NT, Finetti P, Vermeulen P, Lucchi A, Robertson FM, Marsan M, Iwamoto T, Krishnamurthy S, Masuda H et al. (2013) Uncovering the molecular secrets of inflammatory breast cancer biology: an integrated analysis of three distinct affymetrix gene expression datasets. *Clin Cancer Res* **19**, 4685–4696.

35 Bertucci F, Finetti P, Goncalves A & Birnbaum D (2020) The therapeutic response of ER+/HER2- breast cancers differs according to the molecular Basal or Luminal subtype. *NPJ Breast Cancer* **6**, 8.

36 Woodward WA, Krishnamurthy S, Yamauchi H, El-Zein R, Ogura D, Kitadai E, Niwa S, Cristofanilli M, Vermeulen P, Dirix L et al. (2013) Genomic and expression analysis of microdissected inflammatory breast cancer. *Breast Cancer Res Treat* **138**, 761–772.

37 Chi Y, Remsik J, Kiseliovas V, Derderian C, Sener U, Alghamer M, Saadeh F, Nikishina K, Bale T, Iacobuzio-Donahue C et al. (2020) Cancer cells deploy lipocalin-2 to collect limiting iron in leptomeningeal metastasis. *Science* **369**, 276–282.

38 Fukumura K, Malguirwar PB, Fischer GM, Hu X, Mao X, Song X, Hernandez SD, Zhang XH, Zhang J, Parra ER et al. (2021) Multi-omic molecular profiling reveals potentially targetable abnormalities shared across multiple histologies of brain metastasis. *Acta Neuropathol* **141**, 303–321.

39 Smith DL, Debeb BG, Thames HD & Woodward WA (2016) Computational modeling of micrometastatic breast cancer radiation dose response. *Int J Radiat Oncol Biol Phys* **96**, 179–187.

40 Villodre ES, Hu X, Larson R, Eckhardt BL, Gong Y, Hu L, Song J, Krishnamurthy S, Ibrahim NK, Ueno NT et al. (2020) Ndr1-l-eegrf axis in inflammatory breast cancer tumorigenesis and brain metastasis [abstract]. In: Proceedings of the 2019 San Antonio Breast Cancer Symposium; 2019 Dec 10-14; San Antonio, TX, Vol. **80**. AACR Cancer Res, Philadelphia, PA.

41 Candido S, Maestro R, Polese J, Catania A, Maira F, Signorelli SS, McCubrey JA & Libra M (2014) Roles of neutrophil gelatinase-associated lipocalin (NGAL) in human cancer. *Oncotarget* **5**, 1576–1594.

42 Du Z, Wu B, Xia Q, Zhao Y, Lin L, Cai Z, Wang S, Li E, Xu L, Li Y et al. (2019) LCN2-interacting proteins and their expression patterns in brain tumors. *Brain Res* **1720**, 146304.

43 Du ZP, Wu BL, Xie YM, Zhang YL, Liao LD, Zhou F, Xie JJ, Zeng FM, Xu XE, Fang WK et al. (2015) Lipocalin 2 promotes the migration and invasion of
esophageal squamous cell carcinoma cells through a novel positive feedback loop. *Biochim Biophys Acta* 1853, 2240–2250.

44 Gomez-Chou SB, Swidnicka-Siergiejko AK, Badi N, Chavez-Tomar M, Lesinski GB, Bekaii-Saab T, Farren MR, Mace TA, Schmidt C, Liu Y et al. (2017) Lipocalin-2 promotes pancreatic ductal adenocarcinoma by regulating inflammation in the tumor microenvironment. *Cancer Res* 77, 2647–2660.

45 Miki M, Oono T, Fujimori N, Takaoka T, Kawabe K, Miyasaka Y, Ohtsuka T, Saito D, Nakamura M, Ohkawa Y et al. (2019) CLEC3A, MMP7, and LCN2 as novel markers for predicting recurrence in resected G1 and G2 pancreatic neuroendocrine tumors. *Cancer Med* 8, 3748–3760.

46 Santiago-Sanchez GS, Pita-Grisanti V, Quinones-Diaz B, Gumpper K, Cruz-Monserrate Z & Vivas-Mejia PE (2020) Biological functions and therapeutic potential of lipocalin 2 in cancer. *Int J Mol Sci* 21, 4365.

47 Viau A, El Karoui K, Laouari D, Burtin M, Nguyen C, Mori K, Pillebout E, Berger T, Mak TW, Knebelmann B et al. (2010) Lipocalin 2 is essential for chronic kidney disease progression in mice and humans. *J Clin Invest* 120, 4065–4076.

48 Wenners AS, Mehta K, Loibl S, Park H, Mueller B, Arnold N, Hamann S, Weimer J, Ataseven B, Darb-Esfahani S et al. (2012) Neutrophil gelatinase-associated lipocalin (NGAL) predicts response to neoadjuvant chemotherapy and clinical outcome in primary human breast cancer. *PLoS One* 7, e45826.

49 Stoesz SP, Friedl A, Haag JD, Lindstrom MJ, Clark GM & Gould MN (1998) Heterogeneous expression of the lipocalin NGAL in primary breast cancers. *Int J Cancer* 79, 565–572.

50 Provatopoulou X, Gounaris A, Kalogera E, Zagouri F, Flessas I, Goussetis E, Nonni A, Papassotiriou I & Zografos G (2009) Circulating levels of matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL) and their complex MMP-9/NGAL in breast cancer disease. *BMC Cancer* 9, 390.

51 Berger T, Cheung CC, Elia AJ & Mak TW (2010) Disruption of the Lcn2 gene in mice suppresses primary mammary tumor formation but does not decrease lung metastasis. *Proc Natl Acad Sci USA* 107, 2995–3000.

52 Oren B, Urosevic J, Mertens C, Mora J, Guiu M, Gomis RR, Weigert A, Schmid T, Grein S, Brune B et al. (2016) Tumour stroma-derived lipocalin-2 promotes breast cancer metastasis. *J Pathol* 239, 274–285.

53 Hu C, Yang K, Li M, Huang W, Zhang F & Wang H (2018) Lipocalin 2: a potential therapeutic target for breast cancer metastasis. *Onco Targets Ther* 11, 8099–8106.

54 Chung IH, Wu TL, Liao CJ, Hu JY, Lin YH, Tai PJ, Lai CH & Lin KH (2016) Overexpression of lipocalin 2 in human cervical cancer enhances tumor invasion. *Oncotarget* 7, 11113–11126.

55 Shao S, Cao T, Lin L, Li B, Fang H, Zhang J, Zhang Y, Hu J & Wang G (2016) Increased lipocalin-2 contributes to the pathogenesis of psoriasis by modulating neutrophil chemotaxis and cytokine secretion. *J Invest Dermatol* 136, 1418–1428.

56 Wang G, Weng YC, Chiang IC, Huang YT, Liao YC, Chen YC, Kao CY, Liu YL, Lee TH & Chou WH (2020) Neutralization of lipocalin-2 diminishes stroke-reperfusion injury. *Int J Mol Sci* 21, 6253.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** LCN2 expression is higher in sublines generated from brain metastasis (BrMS) than those generated from lung metastasis (LuMS). (A) Microarray analysis of sublines generated from BrMS or LuMS of SUM149 cells showed LCN2 to be one of the top upregulated genes in BrMS (red arrow). Samples are described in Debeb 2016 [29]. (B) LCN2 is secreted in higher levels in BrMS vs LuMS.

**Table S1.** Top kinases predicted to be activated based on kinase-substrate interactions of differentially phosphorylated proteins.

**Table S2.** Top kinases predicted to be activated based on kinase-substrate and protein-protein interaction analysis of differentially phosphorylated proteins across 10 different knowledge bases.