

## HIC1 Silencing in Triple-Negative Breast Cancer Drives Progression through Misregulation of LCN2

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### Abstract

The tumor suppressor gene HIC1 is frequently deleted or epigenetically silenced in human cancer, where its restoration may improve cancer prognosis. Here, we report results illuminating how HIC1 silencing alters effect or signals in triple-negative breast cancer (TNBC), which are crucial for its pathogenesis. HIC1 expression was silenced only in TNBC compared with other molecular subtypes of breast cancer. Restoring HIC1 expression in TNBC cells reduced cell migration, invasion, and metastasis, whereas RNAi-mediated silencing of HIC1 in untransformed human breast cells increased their invasive capabilities. Mechanistic investigations identified the small-secreted protein lipocalin-2 (LCN2), as a critical downstream target of HIC1 in TNBC cells. Elevating LCN2 expression in cells expressing HIC1 partially rescued its suppression of cell invasion and metastasis. Notably, autocrine secretion of LCN2 induced by loss of HIC1 activated the AKT pathway through the neutrophil gelatinase-associated lipocalin receptor, which is associated with TNBC progression. Taken together, our findings revealed that the HIC1–LCN2 axis may serve as a subtype-specific prognostic biomarker, providing an appealing candidate target for TNBC therapy. *Cancer Res*; 74(3); 862–72. ©2013 AACR.

### Introduction

Triple-negative breast cancer (TNBC) consists of a diverse and heterogeneous group of tumors that by definition lack estrogen and progesterone receptors and amplification of the HER2 gene (1). Although systematic therapeutic approaches have reduced cancer-specific mortality, TNBC remains associated with high rates of cancer recurrence and metastasis, which makes the development of effective treatments difficult (2). Therefore, identifying new biomarkers of TNBC progression may be of value for the prevention of metastasis and the design of effective therapeutic strategies for this disease.

Hypermethylated in cancer 1 (HIC1) is a tumor suppressor gene that is frequently epigenetically silenced, including in prostate, liver, colorectal, lung, and breast cancer (3–5). Recent studies showed that HIC1 promoter hypermethylation is not only present in solid tumors but also in normal breast ductal (6), brain (7), and prostate epithelium tissues (3). These findings indicate that other inhibitory mechanisms besides hypermethylation may exist, such as the modification of HIC1 by

SUMOylation or acetylation or mutation/inactivation of the positive HIC1 regulator p53 (8, 9).

HIC1 encodes a sequence-specific transcriptional repressor with an N-terminal POZ domain and 5 Krüppel like C2H2 zinc fingers at the C-terminal that can bind a specific motif consisting of a 5'-(C/G)NG(C/G)GGGCA(C/A)CC-3' sequence centered on GGCA (reverse 5'-TGCCC/A-3') named HiRE (HIC1 Responsive Element; refs. 10 and 11). The upstream regulation of HIC1 is mediated by p53 through a regulatory feedback loop in which HIC1 directly represses the transcription of SIRT1, which deacetylates and thereby inactivates p53 (11). Recently, downstream target genes of HIC1 responsible for developmental and cell-cycle control have been identified, including the histone deacetylase *SIRT1* (11), the transcription factor *ATOH1* (12), the G-protein-coupled receptor *CXCR7* (13, 14), *Cyclin D1*, *P57KIP2* (*CDKN1C*; ref. 9), and *ephrin-A1*, a cell surface ligand for Eph tyrosine kinase receptors (15). However, given the many potential physiologic roles of HIC1, few HIC1 target genes have been characterized.

In this study, we identified Lipocalin2 (LCN2, also known as NGAL) as a direct target gene of HIC1 using genome expression profiling. LCN2 is a small-secreted glycoprotein involved in multiple processes, such as immunity, renal development, and apoptosis (16, 17). Pathologically, LCN2 is associated with multiple types of human tumors, including breast cancer (16). Orthotopic studies showed that LCN2-expressing breast tumors display a poorly differentiated phenotype (18, 19) and LCN2 expression is a predictor of poor prognosis in human primary breast cancer (20). However, the mechanisms underlying the role of LCN2 in different subtypes of breast cancer remain controversial (18, 21).

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The identification of basal-like tumors by immunohistochemistry remains challenging in clinical practice. Because TNBCs and basal-like breast cancers share many molecular and morphologic features, the TNBC phenotype could reliably be used as a surrogate for basal-like breast cancer (22).

## Materials and Methods

### Cell cultures

The human breast cancer cell lines MCF-7, MDA-231, MDA-468, BT474, and SK-BR-3, and the lung cancer cell line A549 were obtained from the American Type Culture Collection (ATCC) and cultured according to the online instructions of the manufacturer. The immortalized epithelial cell line HBL-100 (Cell Bank of Shanghai Institute for Biological Sciences, Shanghai, China) was maintained in Dulbecco's Modified Eagle Medium (DMEM; Hyclone) and MCF-10A cells (ATCC) were maintained in DMEM/F12 (Invitrogen) supplemented with 5% horse serum, EGF (20 ng/mL), hydrocortisone (0.5 mg/mL), cholera toxin (100 ng/mL), insulin (10 µg/mL), and 1% penicillin/streptomycin. The cell lines were tested and authenticated by DNA typing in the Shanghai Jiao Tong University Analysis Core (last test in April 2013).

### Western blot analysis

Details are provided in the Supplementary Methods.

### Tissue microarray and immunohistochemistry

The immunohistochemical analysis was performed using the avidin–biotin–peroxidase complex method with an anti-HIC1 antibody. Details are provided in the Supplementary Methods.

### Construction of lentiviral vectors

To restore the expression of HIC1 in TNBC cells, human full-length HIC1 cDNA was inserted into the lentivirus vector pHR-SIN-CSIGW. Lenti-x cells were then transfected with the PMD2.G, PSPAX2, and HIC1 expression vectors using Lipofectamine 2000 (Invitrogen). After 48 hours, culture supernatants were collected, passed through 0.45 µm filters, and mixed with fresh media (1:1) and polybrene (8 µg/mL) to infect target cells. Cells with restored expression of HIC1 were designated as MDA-231<sup>HIC1</sup> and MDA-468<sup>HIC1</sup>, and the respective controls were designated as MDA-231<sup>GFP</sup> and MDA-468<sup>GFP</sup>. GV248 lentiviral vectors were used to generate HIC1 knockdown cell lines. Details are provided in the Supplementary Methods.

### Migration and invasion assays

A wound healing assay was used to assess cell migration ability and cell invasion was examined using a reconstituted extracellular matrix membrane (cat. no. 354483; Becton Dickinson). The procedures are described in the Supplementary Methods.

### siRNA transfection

siRNA transfection was performed with 50 nmol/L of each siRNA (GenePharma) using Lipofectamine 2000 according to the manufacturer's instructions. The siRNA sequences are provided in the Supplementary Methods.

### Clinical dataset analysis

The classification included in the dataset (Sorlie classification method) was used to assign patients to the different clinical breast cancer subtypes. For survival analyses, overall survival (OS) and distant metastasis-free survivals (DMFS), stratified by expression of the gene of interest, were presented as Kaplan–Meier plots and tested for significance using log-rank tests. The analysis was performed according to the manufacturer's instructions (<http://kmlplot.com/analysis/index.php?p; ref. 23>).

### cDNA microarray analysis

The Agilent SurePrint G3 Human Gene Expression microarray (8 × 60K) was used in this study. Microarray hybridization, data collection, and analysis were performed at Oebiotech Biotechnology Corporation according to Agilent protocols. Detailed procedures are provided in the Supplementary Methods.

### Luciferase reporter assay and chromatin immunoprecipitation

The procedures are described in the Supplementary Methods.

### In vivo metastasis assay

Luciferase tagged MDA-231 cells ( $1 \times 10^6$ ) were injected into the tail vein of *BALB/c* nude mice. Tumor growth and metastasis were examined using the Xenogen IVIS Imaging System. All mouse experiments were performed in an animal center after obtaining approval from the Shanghai Medical Experimental Animal Care Commission. Details are provided in the Supplementary Methods.

### Statistical analyses

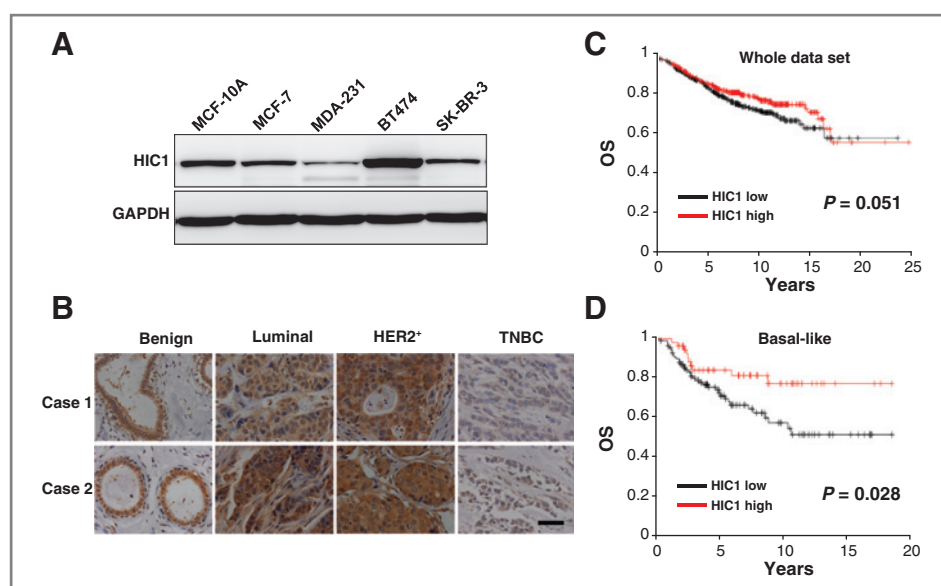
Data are presented as mean ± SD or mean ± SEM. Values of  $P < 0.05$  were considered statistically significant. The results of quantitative real-time PCR were analyzed using the Student *t* test, 2-tailed. HIC1 staining in tissue microarrays was analyzed by Pearson  $\chi^2$  test. All analyses were performed with SPSS 11.5.

## Results

### Expression of HIC1 is silenced in TNBC

Increasing evidence indicates that HIC1 is silenced by epigenetic modifications in many types of prevalent human tumors (11–14, 24). We and other groups recently described the role of HIC1 in tumor progression as partially relying on a variety of downstream targets (11–14, 24). However, the role and regulatory mechanism of HIC1 in different molecular subtypes of breast cancer remain unclear.

We first showed that the expression of HIC1 was weaker in the TNBC cell line MDA-231 than in luminal-type MCF-7 and BT474 cells, HER2-positive SK-BR-3 cells, and in the normal epithelial cell line MCF-10A (Fig. 1A). Next, a high-density tissue microarray was stained with an anti-human HIC1 antibody. Representative images indicated that expression of HIC1 in TNBC tissues was almost absent as compared with benign, luminal-type, and HER2-positive tissues (Fig. 1B). Quantitative analysis confirmed these findings, as shown in Supplementary Table S1.



**Figure 1.** HIC1 is silenced in TNBC and associated with prognosis. A, Western blot analysis of endogenous HIC1 (100 kDa) in breast cancer cell lines. B, HIC1 expression in a human breast cancer tissue microarray. Representative microscopic images of benign and breast cancer tissues stained with an anti-human HIC1 antibody (1:200; Sigma). Scale bar, 50  $\mu$ m. C, Kaplan–Meier plots of OS of patients with breast cancer in whole datasets, stratified by HIC1 expression. Data were obtained from the Kaplan–Meier plotter database (23). The *P* value was calculated by a log-rank test. D, Kaplan–Meier plots of OS of patients with basal-like breast cancer.

However, comparison of benign and malignant breast diseases showed no statistically significant differences ( $P = 0.230$ ). HIC1 expression was significantly correlated with histologic grade, and estrogen receptor (ER), progesterone receptor (PR), and HER2 levels in breast cancer tissues (Supplementary Table S1).

To further explore the relationship between HIC1 and the clinical prognosis, we evaluated the prognostic value of HIC1 in a large public clinical microarray database of breast cancer that includes data from 1,027 patients (23, 25). The results showed that decreased HIC1 expression was associated with poor prognosis only among patients with basal-like breast cancer (Fig. 1D), but not in the overall breast cancer group (Fig. 1C). Overall, these findings indicate that expression of HIC1 is silenced in TNBC and that it may be associated with the subtype-specific prognoses of breast cancer.

#### Effects of restoration of HIC1 expression in TNBC cells

A critical feature of TNBC is its highly aggressive behavior (26, 27). Based on our findings that HIC1 expression is silenced in TNBC cells, its expression was restored in MDA-231 and MDA-468 cells using a lentivirus vector. Figure 2A and Supplementary Fig. 1A show the restored HIC1 expression as confirmed by Western blotting and real-time PCR in both cell lines (noted as MDA-231<sup>HIC1</sup> and MDA-468<sup>HIC1</sup>). The role of HIC1 in the regulation of migration and invasion was assessed by scratch healing assay and by using reconstituted extracellular matrices in porous culture chambers. The results showed that in MDA-231<sup>GFP</sup> cells, wound closure occurred gradually starting at 56 hours after scratch, whereas this effect was significantly reduced in MDA-231<sup>HIC1</sup> cells (Fig. 2B). In line with this finding, MDA-231<sup>HIC1</sup> cells showed reduced invasive capacity, which was also observed in MDA-468<sup>HIC1</sup> cells (Fig. 2C and Supplementary Fig. S1B). By contrast, HIC1 knockdown in HBL-100 cells by shRNAs (Fig. 2D) greatly increased their invasive capacity as compared with the control (Fig. 2E). The combination of apparent shHIC1-2 and -3 was used to decrease HIC1 expression in

MCF-10A cells and similar results were obtained (Supplementary Fig. S8).

To further explore whether HIC1 modulates tumor metastasis *in vivo*, MDA-231<sup>HIC1</sup> and MDA-231<sup>GFP</sup> control cells tagged with luciferase were transplanted into nude *BALB/c* mice by tail vein injection. The results showed a significantly lower rate of lung metastasis in mice injected with MDA-231<sup>HIC1</sup> cells than in those injected with MDA-231<sup>GFP</sup> cells. Bioluminescence imaging confirmed that the incidence of lung metastasis was lower in mice carrying MDA-231<sup>HIC1</sup> than in those carrying MDA-231<sup>GFP</sup> cells at 4 weeks after injection (Fig. 2F). Moreover, examination of the number of micrometastasis and total lung burden also showed that lung metastasis was markedly suppressed in MDA-231<sup>HIC1</sup> mice compared with control mice (Fig. 2G). Representative images using hematoxylin and eosin (H&E) staining further confirmed the macroscopic findings (Fig. 2H). Taken together, these results suggest that restoring HIC1 expression has a significant effect on reducing invasion and metastasis of TNBC cells.

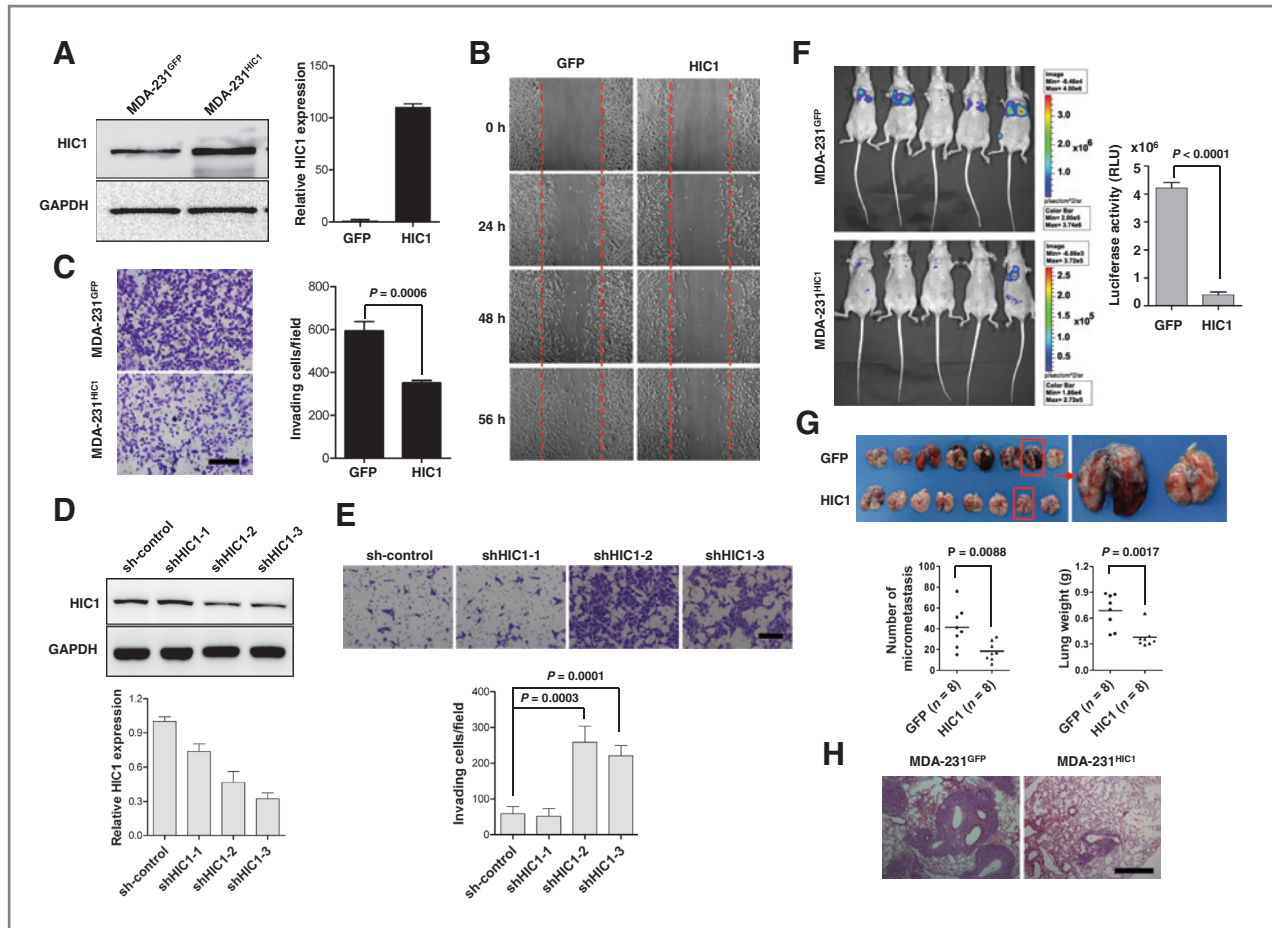
#### Identification of downstream genes of HIC1

To identify potential downstream targets of HIC1, we analyzed the genome-wide transcriptome profile of MDA-231<sup>HIC1</sup> cells and HBL-100<sup>HIC1shRNAs</sup> cells using Agilent Whole Human Genome Microarrays. The microarray dataset has been deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50061>).

Fold-change ( $\times 1.5$ ) screening between cells with positive HIC1 expression and their respective controls identified 1,364 common genes in MDA-231 and HBL-100 cells and 557 down-regulated genes (Fig. 3A). Among the differentially expressed genes, 39 cancer-associated genes were identified that might participate in invasion or metastasis for cluster mapping on the MeV microarray analysis platform ([www.tm4.org/mev.html](http://www.tm4.org/mev.html); Fig. 3C).

Among the genes markedly regulated in MDA-231 and HBL-100 cells, we focused on LCN2 based on our previous microarray





**Figure 2.** Effects of restoration of HIC1 expression in TNBC cells. **A**, restoration of HIC1 expression in MDA-231 cells was confirmed by Western blotting and real-time PCR. **B**, restoring HIC1 expression decreased cell migration in MDA-231 cells. **C**, restoring HIC1 expression impaired cell invasion. Experiments were performed three times; representative images of invaded cells are shown. Mean  $\pm$  SD; scale bar, 200  $\mu$ m. **D**, inactivation of endogenous HIC1 in HBL-100 cells by shRNAs. The knockdown efficiency was confirmed by Western blotting and real-time PCR. shHIC1-2 and shHIC1-3 were more efficient than the sh-control. **E**, cell invasion was evaluated after HIC1 knockdown. Cell invasion increased significantly in shHIC1-2 and shHIC1-3 cells compared with the sh-control. Three independent experiments were performed. Scale bar, 200  $\mu$ m. **F**, HIC1 inhibited lung metastasis in transplantable nude *BALB/c* mouse models. Representative bioluminescence images of animals on week 4 are shown.  $n = 8$ , mean  $\pm$  SEM. **G**, representative gross images of lung nodules (top), quantification of lung metastatic nodules (bottom, left), and total lung burden (bottom, right). **H**, representative images of H&E staining for lung micrometastasis. Scale bar, 500  $\mu$ m.

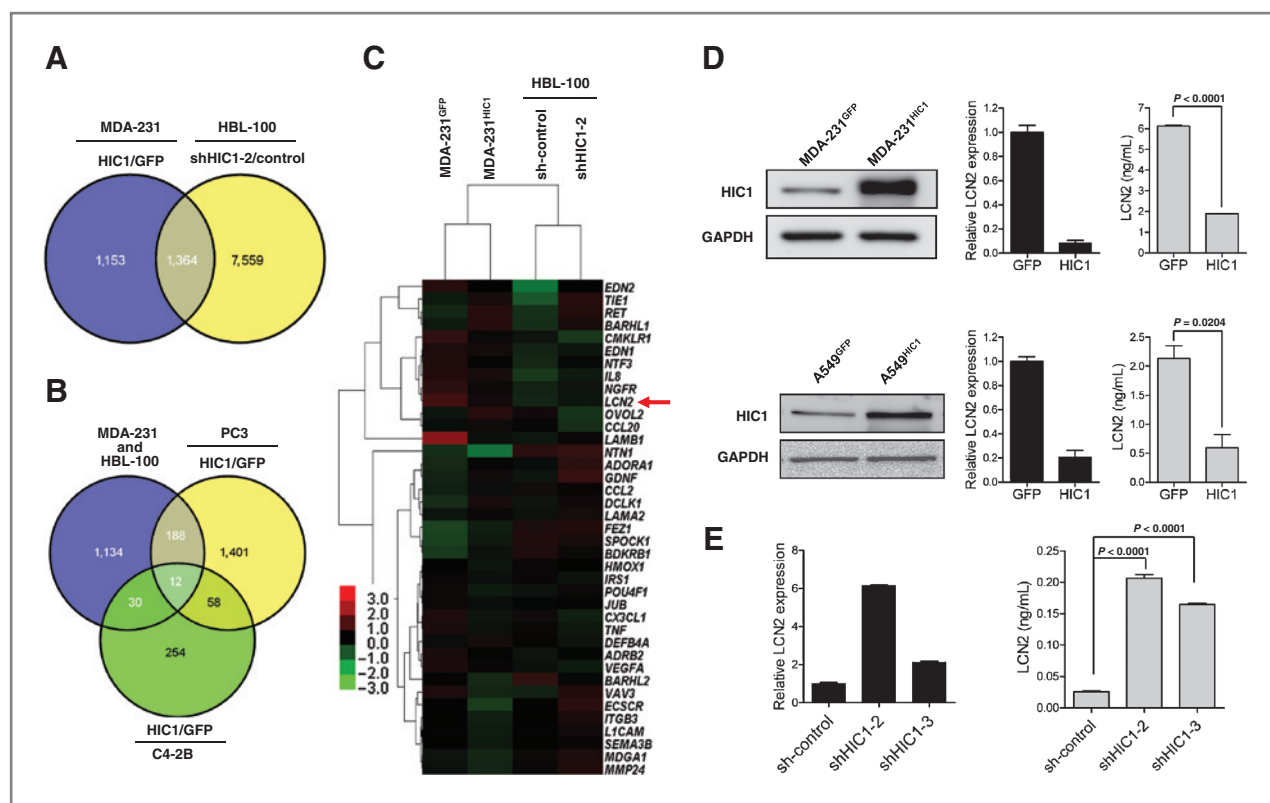
data in PC3 and C4-2B cells (Fig. 3B; ref. 14). Because the regulatory region of the LCN2 promoter might contain several functional HIC1 consensus binding sites (HiRE, HIC1 responsive element; refs. 10 and 11), we next explored the mechanisms by which LCN2 expression is modulated by HIC1 in these cell lines. Real-time PCR and ELISA confirmed that HIC1 down-regulated the expression of LCN2 at the mRNA and protein levels, consistent with the above microarray assays (Fig. 3D). Similar results were obtained in the lung cancer cell line A549 (Fig. 3D). By contrast, shRNAs knockdown of HIC1 in HBL-100 cells significantly enhanced LCN2 expression at the mRNA and protein levels (Fig. 3E). These findings suggest that the expression of LCN2 is modulated by HIC1.

**LCN2 is a direct target gene of HIC1**

To determine whether LCN2 is a direct target gene of HIC1, we firstly constructed a series of LCN2-truncated promoter/

reporter fusion plasmids containing progressive 5' deletions from  $-2955$  to  $+18$ , based on data showing that many putative HIC1 binding elements centered on TGCCC/A are observed on its promoter (10, 11), which allowed us to perform luciferase promoter-reporter assays (Fig. 4A). These constructs were then transfected alone or with the pcDNA3-FLAG-HIC1 expression vector into MDA-231, MCF-7, and SK-BR-3 cells to measure promoter activities in the absence or presence of HIC1. The data showed that the construct containing the LCN2 promoter showed more than 3-fold higher activity than the basic group (Fig. 4B). However, transient transfection of MDA-231 cells with HIC1 markedly inhibited LCN2 promoter activity in a dose-dependent manner (Fig. 4B and C). A similar inhibitory effect was observed in MCF-7 and SK-BR-3 cells (Fig. 4B and C). The effect of the HIC1 expression vector on the inhibition of LCN2 promoter activity in MDA-231 and MCF-7 cells was maintained in the  $-1427/+18$ ,  $-774/+18$ , and

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**Figure 3.** LCN2 expression is regulated by HIC1. **A**, Venn diagram showing overlap of differentially expressed genes in the two cell groups. HIC1/GFP: differentially expressed genes between the HIC1 restored and GFP groups in MDA-231 cells. shHIC1-2/control, differentially expressed genes between shHIC1-2 and control cells in HBL-100 cells. **B**, Venn analysis of differentially expressed genes between the overlap of **A** and our previous data in PC3 and C4-2B. Twelve potential target genes were identified: EDN1, ZBED2, UBD, FGFBP2, LCN2, HKDC1, SAMD9L, PDZK11P1, KIAA1199, MUC17, MAST4, and HS6ST3. **C**, clustering map of differentially expressed genes associated with cell migration and invasion. Rows, genes; columns, experimental cells. **D**, real-time PCR and ELISA confirmed that LCN2 expression was downregulated by restoring HIC1 at the mRNA and protein levels in MDA-231 cells (top), and similar results were obtained in the lung cancer A549 cell line (bottom). Three independent experiments were performed. **E**, inactivation of endogenous HIC1 resulted in LCN2 upregulation at the mRNA (left) and protein levels (right).

–252/+18 truncated constructs, but lost in the smaller construct (–62/+18; Fig. 4D). These results suggest that the regulatory region primarily involved in the HIC1-mediated repression of LCN2 is located in the –252/+18 upstream region of the promoter, which contains 3 putative HIC1 binding sites, namely M1, M2, and M3 (Fig. 4E).

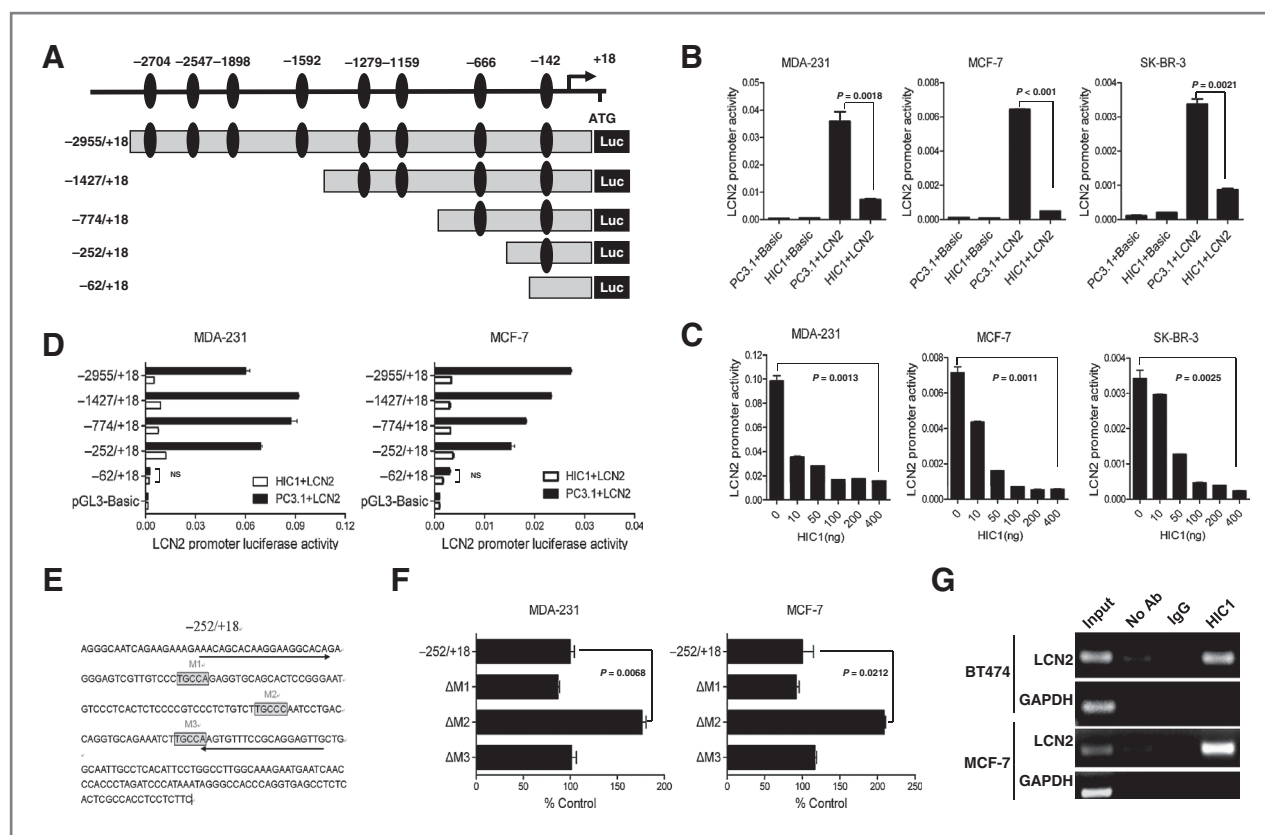
To verify this possibility, these 3 putative binding sites were deleted to abolish HIC1 binding. As shown in Fig. 4F, the  $\Delta$ M2 mutated construct significantly decreased the inhibitory effect of HIC1 as compared with the control, whereas the  $\Delta$ M1 and  $\Delta$ M3 constructs did not affect its function. These results suggest that the putative M2 site in the LCN2 promoter is essential for HIC1-mediated repression, whereas the M1 and M3 sites may have little effect. To confirm that LCN2 is a direct target gene of HIC1, we performed ChIP assays using a polyclonal antibody against HIC1 and then amplified the pull-down DNA by PCR. The primers were designed to amplify the region mediating the repressive effects of HIC1 on the –252/+18 site of the LCN2 promoter. As shown in Fig. 4G, LCN2 was markedly amplified from the HIC1-immunoprecipitated BT474 and MCF-7 chromatin, but absent from chromatin immunoprecipitated by the control rabbit immunoglobulin G.

Taken together, these results demonstrate that endogenous HIC1 proteins can be directly recruited onto the LCN2 promoter to repress its expression, whereas the silenced HIC1 expression in TNBC cells may facilitate the escape of LCN2 from the suppressive modulation.

#### LCN2 partially rescues HIC1-induced phenotypes in TNBC cells

To further demonstrate that LCN2 is a critical target gene involved in the HIC1-induced phenotypes of TNBC cells, we firstly examined the effect of LCN2 on cell invasion *in vitro*. Exogenous LCN2 significantly promoted invasion in MDA-231<sup>GFP</sup> cells compared with the control (Fig. 5A); however, in MDA-231<sup>HIC1</sup> cells, LCN2 partially rescued the reduced invasion caused by HIC1 re-expression.

Next, the invasive ability of MDA-231 cells was examined after restoring LCN2 expression. Effective restoration of HIC1 and LCN2 expression in luciferase-tagged MDA-231 cells was confirmed by Western blotting and ELISA (1#, 2#, 3#, and 4# in Fig. 5B, left). LCN2 overexpression in MDA-231 cells markedly increased invasion compared with the control (Fig. 5C, 2# and 1#). However, coexpression of LCN2 in MDA-231<sup>HIC1</sup> cells



**Figure 4.** LCN2 is a direct target gene of HIC1. **A**, map of the LCN2 promoter region, with the positions of selected consensus binding sites indicated above. The length of different promoter constructs used in reporter assays is shown below. **B**, LCN2 promoter activity after transfection of the full length construct (-2955/+18) alone or together with HIC1 expression vectors. pGL3-Basic, control for promoter constructs; PC3.1, control for the HIC1 expression vector. The results are expressed as a relative ratio of firefly luciferase to *Renilla* luciferase. Three independent experiments were performed in triplicate. **C**, reporter activity after transient cotransfection of MDA-231, MCF-7, and SK-BR-3 cells with the -2955/+18 promoter and increasing amounts of the HIC1 expression vector. The repressive effect increased gradually with the dose of HIC1 expression vector. **D**, LCN2 promoter activity after cotransfection with 100 ng HIC1 expression vector and each of the promoters constructs. The -252/+18 construct had a higher promoter activity and a significant repressive effect, whereas the -62/+18 construct induced loss of function. **E**, analysis of the nucleotide sequence of the -252/+18 construct. Three potential HIC1 binding sites were identified: M1, M2, and M3. Primers used to amplify the LCN2 promoter fragment in the ChIP experiment shown in **G** are indicated by an arrow. **F**, mutations were introduced into the conserved HIC1 binding sites (M1, M2, and M3 deletion) to impede HIC1 binding. The results showed that the  $\Delta$ M2 mutated construct significantly decreased the repression by HIC1 as compared with the control. **G**, ChIP analysis of HIC1 at the LCN2 promoter region in BT474 and MCF-7 cells.

partially rescued the HIC1-induced suppression of invasion (Fig. 5C, 4# and 3#). To further explore whether LCN2 is involved in modulating tumor metastasis *in vivo*, luciferase-tagged MDA-231 cells (1#, 3#, and 4#) were transplanted into nude *BALB/c* mice by tail vein injection. The results showed that lung metastasis was significantly reduced in MDA-231<sup>HIC1</sup> cells (3#) compared with the control group (1#; Fig. 5D), consistent with the *in vivo* results shown in Fig. 2F-H. However, coexpression of LCN2 in MDA-231<sup>HIC1</sup> cells (4#) partially rescued the HIC1-induced reduction of lung metastasis compared with the MDA-231<sup>HIC1</sup> cells alone (3#). Moreover, quantification of micrometastasis in lung tissue further confirmed the above observations (Fig. 5E). As expected, LCN2 overexpression in MDA-231 cells markedly increased lung metastasis (Supplementary Fig. S5A). Assessment of the number of micrometastasis also showed that lung metastasis was markedly increased in MDA-231<sup>LCN2</sup> mice compared with the control (Supplementary Fig. S5B). Representative images using

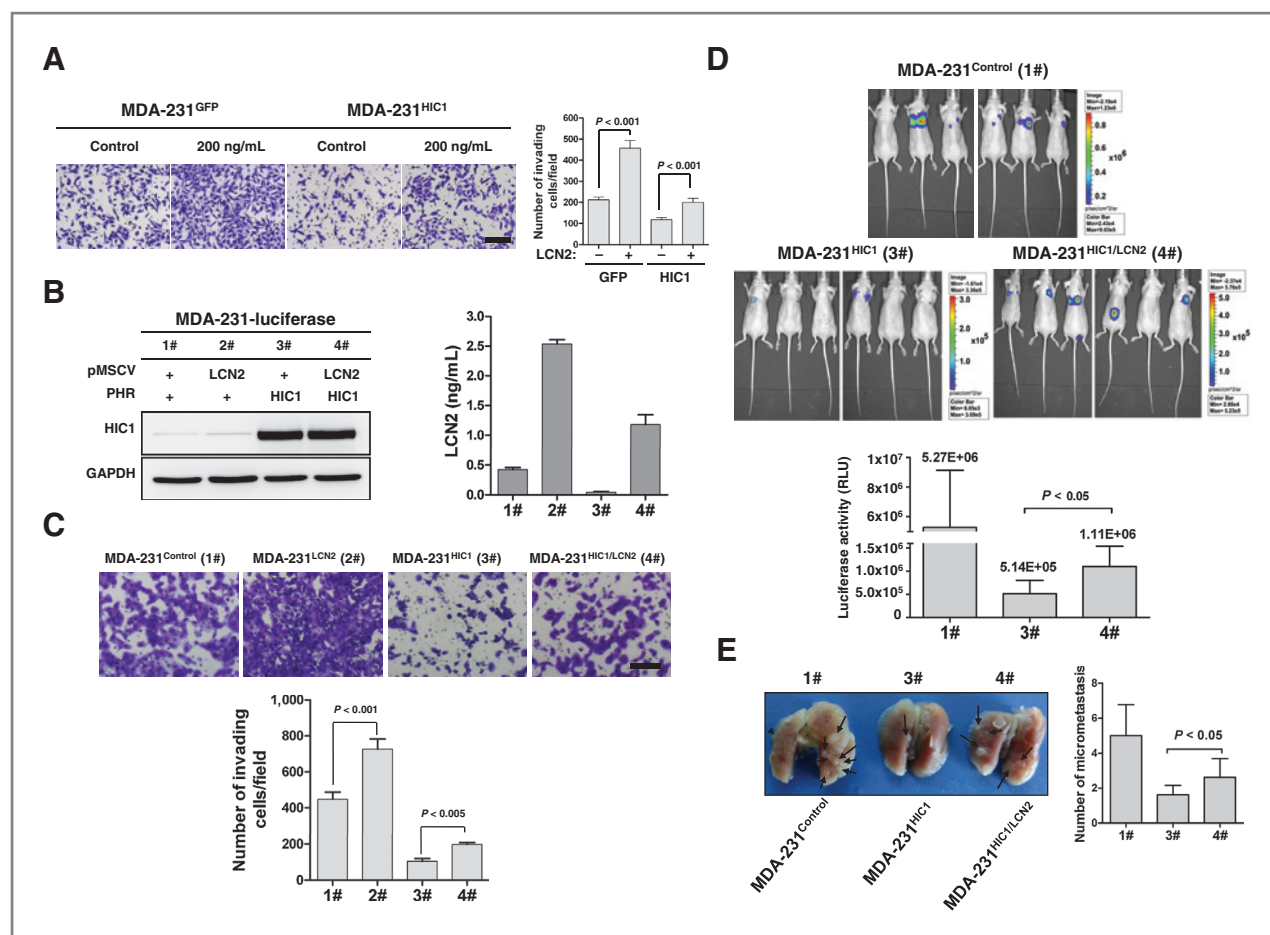
H&E staining further confirmed the macroscopic findings (Supplementary Fig. S5C).

**The AKT pathway is involved in HIC1-induced LCN2 expression in TNBC cells**

Based on our *in vitro* and *in vivo* findings, we next explored the pathways involved in HIC1-induced LCN2 expression associated with TNBC progression. As shown in Fig. 6A, the AKT pathway was inactivated in MDA-231<sup>HIC1</sup> cells, whereas the ERK1/2 and NF- $\kappa$ B pathways were not affected. By contrast, shRNAs knockdown of HIC1 activated the AKT pathway in HBL-100 cells (Fig. 6B). Given that the level of LCN2 is regulated by HIC1, we examined the involvement of LCN2 and found that LCN2 induced AKT activation in MDA-231 and MDA-468 cells (Fig. 6C), whereas it had little effect on the ERK1/2 and NF- $\kappa$ B signaling pathways (Supplementary Fig. S2). These findings suggest that autocrine secretion of LCN2 induced by the loss of HIC1

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**Figure 5.** LCN2 partially rescues HIC1-induced cell invasion and metastasis. **A**, invasion assay in MDA-231 cells with exogenous LCN2 expression (200 ng/mL). Cells and LCN2 were placed at the top and bottom of the chamber, respectively. Three independent experiments were performed. Scale bar, 200  $\mu$ m. **B**, Western blotting assay (left) of MDA-231-luciferase cells infected by a combination of viruses for HIC1 and LCN2 expression, as indicated on the top. Stable infected cell lines were designated as 1#, 2#, 3#, and 4# as described in the main text. LCN2 levels were confirmed by ELISA in these stable cell lines (right). PHR, lentivirus; PMSCV, retrovirus. **C**, invasion assay in stably infected MDA-231-luciferase cells. HIC1 impaired cell invasion significantly (3# and 1#), whereas restoring LCN2 partially rescued HIC1-induced cell invasion (4# and 3#). Three independent experiments were performed. Scale bar, 200  $\mu$ m. **D**, LCN2 partially rescues HIC1-induced lung metastasis. HIC1 inhibited lung metastasis (3# and 1#), whereas restoration of LCN2 partially rescued HIC1-induced lung metastasis (4# and 3#). Representative bioluminescence images of animals at the 6th week.  $n = 6$ , mean  $\pm$  SEM. **E**, representative images of lung nodules (left) and quantification of lung metastatic nodules (right). NS, not significant; mean  $\pm$  SEM.

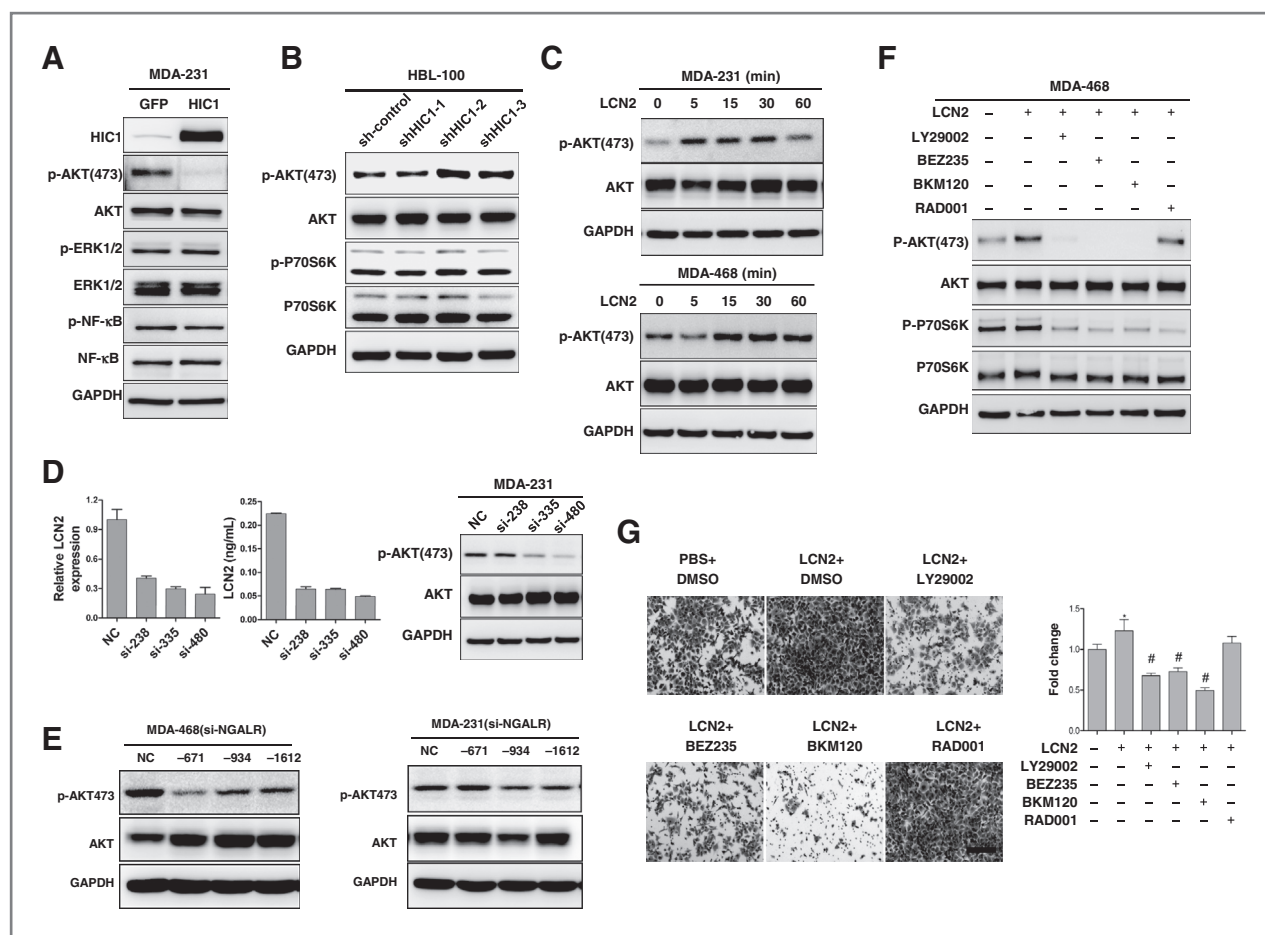
expression may contribute to the activation of the AKT pathway.

Indeed, Fig. 6D shows that knockdown of endogenous LCN2 by siRNAs decreased AKT activation in MDA-231 cells compared with the scrambled control. Emerging evidence has indicated that LCN2, as a secreted inflammatory factor, functions through neutrophil gelatinase-associated lipocalin receptor (NGALR), a multipass membrane protein (28). To test whether NGALR is involved in the AKT signaling pathway, NGALR expression was reduced by siRNAs in MDA-231 and MDA-468 cells. The results showed that the activation of the AKT pathway was inhibited in both cell lines compared with the respective scrambled controls (Fig. 6E and Supplementary Fig. S6). Further experiments are in progress to elucidate the underlying mechanisms and to determine whether other receptors are involved in the process.

To determine whether the LCN2-activated AKT pathway is involved in cell invasion, several small molecule inhibitors of the pathway were used, namely LY29004 [phosphoinositide 3-kinase (PI3K) inhibitor], BEZ235 (PI3K and mTOR inhibitor), BKM120 (selective PI3K inhibitor), and RAD001 (mTOR inhibitor). As shown in Fig. 6F–G, LCN2-induced cell invasion in MDA-468 cells was significantly inhibited by LY29004, BEZ235, and BKM120, whereas RAD001 had no effect, which could be partly attributed to pAkt473 feedback-loop activation. Taken together, these results demonstrate that HIC1-induced LCN2 expression activates the AKT pathway, which is responsible for promoting TNBC cell invasion.

#### LCN2 predicts poor clinical outcome in patients with TNBC

Based on the above findings, we assessed whether LCN2 expression is associated with TNBC progression. Supplementary



**Figure 6.** The AKT pathway is involved in HIC1-induced LCN2 expression in TNBC cells. **A**, the AKT (60 kDa) pathway was inactivated after restoration of HIC1 expression in MDA-231 cells, but no effect on the ERK1/2 (42/44 kDa) and NF-κB (65 kDa) pathways was observed. Three independent experiments were performed. **B**, HIC1 knockdown by shRNA2, 3 can activate the AKT pathway in HBL-100 cells. The experiments were performed three times. **C**, exogenous LCN2 stimulation (200 ng/mL) induced AKT activation in MDA-231 and MDA-468 cells. **D**, knockdown of endogenous LCN2 expression by siRNAs inhibited AKT activation in MDA-231 cells (right). The LCN2 level was confirmed by real-time PCR and ELISA (left and middle). Three independent experiments were performed. **E**, the AKT pathway was inactivated after NGALR siRNA knockdown in MDA-231 and MDA-468 cells, as compared with the scrambled control. **F**, LY29004, BEZ235, and BKM120 inhibited the LCN2-induced activation of the AKT pathway, whereas RAD001 had no effect. Inhibitors were added 30 minutes before initiation of LCN2 stimulation. AKT phosphorylation in response to LCN2 stimulation at 60 minutes was assessed in the presence or absence of inhibitors. **G**, invasion assay in MDA-468 cells stimulated by LCN2 in the presence or absence of inhibitors. LCN2 and inhibitors were added to the bottom and top of chambers, respectively. The results are expressed as fold-change compared with the control. LCN2, 200 ng/mL; \*,  $P < 0.05$ ; #,  $P < 0.01$ . Three independent experiments were performed. Scale bar, 200 μm.

Table S2 shows that serum LCN2 levels were inversely correlated with the ER ( $P < 0.001$ ) and PR status ( $P = 0.012$ ) of patients with breast cancer, consistent with LCN2 expression in breast cancer tissues (19). Compared with the benign group, LCN2 level was markedly higher in patients with TNBC ( $P = 0.007$ ), although the difference did not reach statistical significance ( $P = 0.080$ ) in the HER2-positive subtype (Supplementary Table S2). However, this could be related to the discrete values and sample size limitations. Moreover, the subtype-specific prognostic power of LCN2 was shown in the independent clinical microarray database of breast cancer (23), in which a high level of LCN2 was associated with poor DMFS and OS in patients with basal-like breast cancer (Fig. 7A and B).

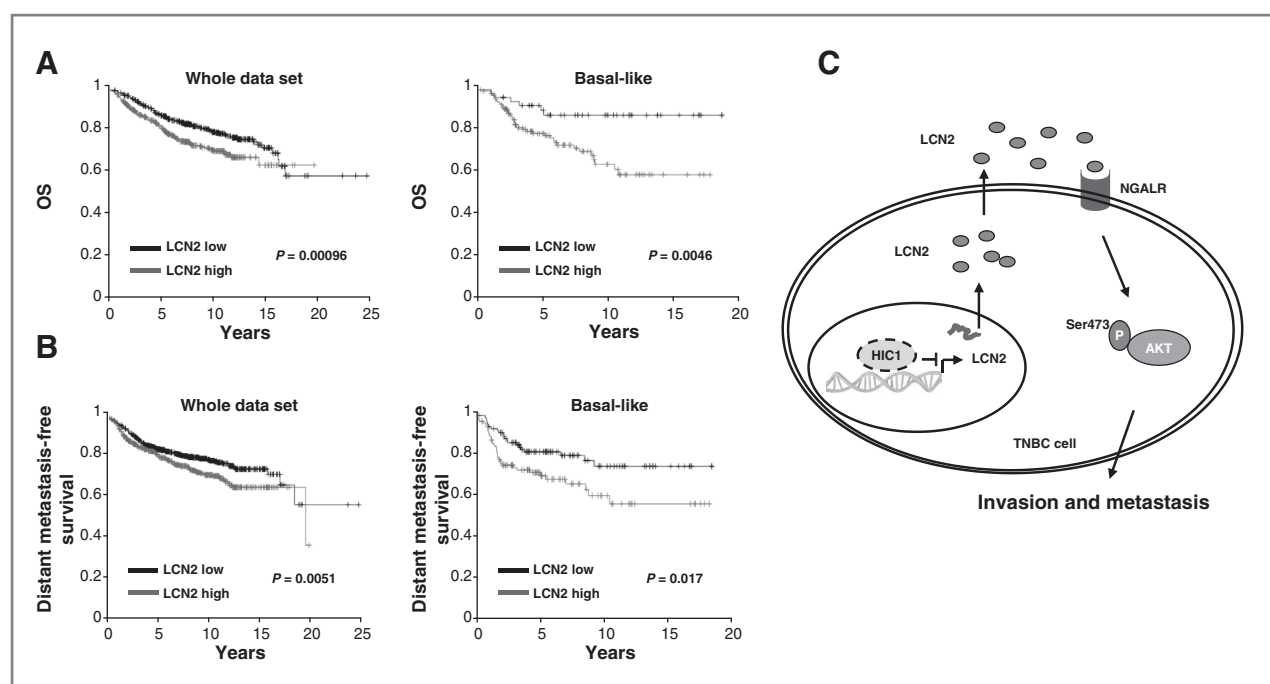
Figure 7C shows a schematic model of the function of the HIC1–LCN2 axis in TNBC progression. HIC1 expression is

silenced in TNBC by epigenetic modifications, which increases the secretion of LCN2 because of the loss of the direct inhibitory effect of HIC1. LCN2 can activate the AKT pathway via an autocrine loop potentially mediated by the NGALR receptor, therefore promoting TNBC invasion and metastasis.

### Discussion

HIC1 is a suppressor gene that is frequently hypermethylated in human cancers, including breast carcinomas. Emerging evidence suggests that re-expression of HIC1 is responsible for an improved prognosis in human cancers (29, 30). Breast cancer is a heterogeneous disease and several distinct subtypes exist, of which the triple-negative subtype has the worst clinical prognosis (29, 30). However, the role





**Figure 7.** LCN2 predicts poor clinical outcome in TNBC. A, Kaplan–Meier plots of OS in whole datasets and patients with basal-like breast cancer, stratified by LCN2 expression. Data were obtained from the Kaplan–Meier plotter database (23). The *P* value was calculated by a log-rank test. B, Kaplan–Meier plots of DMFS in patients with breast cancer and patients with basal-like breast cancer, stratified by LCN2 expression. High levels of LCN2 were associated with significantly poorer OS and DMFS in patients with basal-like breast cancer. C, schematic model of the role of the HIC1–LCN2 axis in TNBC progression.

and regulatory mechanism of HIC1 in each subtype remain unclear.

Here, we showed that HIC1 expression was not significantly different between benign and malignant breast cancer tissues. This result could be related to the finding that HIC1 hypermethylation is a late event in some neoplasms, which suggests that other inhibitory mechanism may exist that modulate HIC1 expression (31–33). Our data showed that HIC1 was only silenced in the triple-negative subtype as compared with the benign, luminal, and HER2 subtypes. These findings indicate that HIC1 expression may be linked to a more aggressive type of TNBC and raise the question of whether lower HIC1 expression may serve as a marker of poor prognosis. Indeed, analysis of a public clinical microarray database (23) showed that HIC1 expression in patients with basal-like breast cancer was significantly associated with OS.

Our *in vitro* and *in vivo* experiments showed that restoring HIC1 expression in TNBC cells had a marked effect on reducing cell migration, invasion, and metastasis. However, reduction of HIC1 expression by shRNAs in MCF-10A and HBL-100 cells greatly increased their invasive ability. Microarray analysis and *in vitro* experiments identified LCN2 as an immediately downstream target of HIC1. LCN2 is a small-secreted glycoprotein involved in multiple processes, such as immunity, renal development, and apoptosis (16, 17). It binds iron by associating with 2,5-dihydroxybenzoic acid, a siderophore that shares structural similarities with bacterial enterobactin, and delivers or removes iron from the cell, dependent on the context (34). Indeed, LCN2 is expressed in a variety of tumors, including

breast cancer (18). Leng and colleagues reported that inhibition of LCN2 impairs breast tumorigenesis and metastasis in MMTV-ErbB2 (V664E) mice (35). Several recent studies have suggested that LCN2 expression plays a role in breast cancer initiation and progression by promoting epithelial–mesenchymal transition (EMT), modulating MMP9 activity and regulating iron homeostasis in cancer cells (18, 36, 37). However, in this study, LCN2 had no effect on the regulation of EMT or MMP9 activity (Supplementary Figs. S3 and S4), which suggests that the function and potential mechanisms of LCN2 in breast cancer need to be further elucidated (21, 38).

Here, we showed that LCN2 activated the AKT pathway and promoted cell invasion in TNBC cells. The PI3K inhibitors LY29004, BEZ235, and BKM120 partly inhibited LCN2-induced breast cancer cell invasion, whereas the mTOR inhibitor RAD001 had no effect, possibly because of pAkt473 feedback-loop activation. These results suggest that in addition to the involvement of the AKT pathway in LCN2-induced cell invasion, other pathways may be activated via crosstalk. A physiologic receptor for LCN2 has not been defined (39). One of its potential receptors, NGALR, has been reported to internalize iron-laden LCN2. LCN2 is also known to undergo endocytosis through the megalin receptor (40, 41), a single-pass type I membrane protein that acts together with cubilin to mediate high-density lipoprotein endocytosis and may participate in the regulation of parathyroid hormone (42). Few studies have explored the mechanism underlying the role of LCN2 and LCN2 receptors in breast cancer progression. Here, we found for the first time that NGALR knockdown could inhibit the activation

of the AKT pathway. Moreover, our data showed that PTEN activity is not greatly induced in the presence of LCN2 (Supplementary Fig. S7). These results suggest that the LCN2-induced AKT activation may be associated with the NGALR receptor, and PTEN may not be involved. However, the mechanisms underlying the role of LCN2 in TNBC progression require further investigation.

We showed that the LCN2 level in serum was higher in patients with TNBC than in those with benign and other subtypes of breast cancer, and it was negatively correlated with the ER and PR status. A similar finding was reported in patients with ovarian cancer, where a correlation between LCN2 expression and tumor differentiation was identified (43). Analysis of the clinical microarray database of breast cancer (23) showed that high levels of LCN2 are associated with poor DMFS and OS in patients with basal-like breast cancer, suggesting that serum LCN2 level may serve as a subtype-specific prognostic factor in breast cancer.

In summary, the findings suggest that the HIC1–LCN2 axis may serve as a subtype-specific prognostic biomarker and provide an attractive therapeutic target for the treatment of patients with TNBC.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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