Long noncoding RNA BHLHE40-AS1 promotes early breast cancer progression through modulating IL-6/STAT3 signaling

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Abstract

Ductal carcinoma in situ (DCIS) is a nonobligate precursor to invasive breast cancer. Only a small percentage of DCIS cases are predicted to progress; however, there is no method to determine which DCIS lesions will remain innocuous from those that will become invasive disease. Therefore, DCIS is treated aggressively creating a current state of overdiagnosis and overtreatment. There is a critical need to identify functional determinants of progression of DCIS to invasive ductal carcinoma (IDC). Interrogating biopsies from five patients with contiguous DCIS and IDC lesions, we have shown that expression of the long noncoding RNA BHLHE40-AS1 increases with disease progression. BHLHE40-AS1 expression supports DCIS cell proliferation, motility, and invasive potential. Mechanistically, BHLHE40-AS1 modulates interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3) activity and a proinflammatory cytokine signature, in part through interaction with interleukin enhancer-binding factor 3. These data suggest that BHLHE40-AS1 supports early breast cancer progression by engaging STAT3 signaling, creating an immune-permissive microenvironment.

Keywords

BHLHE40-AS1, breast cancer progression, ductal carcinoma in situ (DCIS), IL-6, ILF3, long noncoding RNA, STAT3
Increased emphasis on early breast cancer detection, accompanied by better imaging technologies, has led to a dramatic increase in the diagnosis of ductal carcinoma in situ (DCIS). Despite this increased diagnosis and treatment of early-stage disease, a complementary decline in late-stage diagnosis has not been observed. This discrepancy underscores the reality that only a small percentage of early-stage lesions progress to invasive disease. In fact, the majority are innocuous. The molecular mechanisms promoting DCIS progression to invasive disease remain largely unknown. Current diagnostic strategies focus on assessing the risk of recurrence after DCIS treatment but do not evaluate or define if a DCIS lesion is predicted to progress. Without a clinical method to identify which DCIS will progress, diagnosed women will undergo surgery and postoperative radiotherapy and/or endocrine therapy. This has created a current state of overdiagnosis, overtreatment, and a significant public health problem where low-risk patients undergo unnecessary intensive treatment without benefit. Therefore, there is an urgent need to define clear molecular determinants of progression.

Long noncoding RNAs (lncRNAs) are noncoding transcripts over 200 nucleotides in length that were previously discarded as spurious transcripts with no function within the cell. Now it is appreciated that lncRNAs can function at every level of gene regulation. The majority of lncRNAs with defined functions are located in the nucleus. Nuclear lncRNAs impact gene regulation through recruiting or blocking transcription factors, supporting chromatin looping around enhancer regions, or through interaction with histone-modifying complexes to impact global chromatin architecture. LncRNAs are also found to impact messenger RNA (mRNA) stability, translation, and splicing can function as micro RNA sponges, and as multiple protein complex scaffolds. It is now appreciated that lncRNAs play critical roles in development, and are often dysregulated in disease progression. In breast cancer, an expression panel of lncRNAs has been shown to perform as well as the Prediction Analysis of Microarray 50 at classifying breast cancer intrinsic subtypes and predicting overall survival. Furthermore, lncRNAs can function as oncogenes as exemplified by HOTAIR which supports tumor cell invasion, cell proliferation, and metastasis in breast cancer. Despite increasing evidence that lncRNAs are functional molecules, the analysis of the majority of lncRNAs is preliminary and has not resulted in a clear pattern of expression in models of progression.

Given the number of potential mechanisms that impact on cell function, and clear regulation in breast cancer subtypes, we sought to identify lncRNAs that play a mechanistic role in breast cancer progression. The molecular study of DCIS progression has been limited by a lack of both DCIS cell lines and early progression models. In this study we utilized a unique cohort of patient-matched biopsies in which a DCIS lesion was identified contiguous with invasive ductal carcinoma (IDC) lesion, allowing for the direct interrogation of transcriptional changes during progression. To validate the findings in vitro, we used the MCF10A progression cell line series. This series is derived from the normal, spontaneously immortalized MCF10A cells, and includes three additional cell lines that mimic progression in the forms of atypia (MCF10A-AT1 cells), DCIS (MCF10A-DCIS), and invasive (MCF10A-CA1) cells. We also validated the results in patient-derived SUM225 cells, HER2+ cells that mimic DCIS in vivo.

This study identifies 132 lncRNAs whose expression can distinguish between early-stage DCIS and patient-matched IDC. From this candidate list, we have identified a previously uncharacterized lncRNA, BHLHE40-AS1, which is increased during breast cancer progression and contributes to invasive phenotypes. BHLHE40-AS1 expression modulates interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling suggesting a potential mechanism by which noninvasive DCIS lesions progress to invasive disease.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The MCF10A progression series was purchased from the Barbara Ann Karmanos Cancer Institute and maintained in a culture of Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin and 1× antibiotic-antimycotic (Gibco, Grand Island, NY). MCF10A-HER2 cells were also maintained in the same medium. SUM225 cells were obtained from Asterand and cultured in DMEM-F12 supplemented with 5 μg/mL insulin, 1 μg/mL hydrocortisone, 10 mM HEPES, 5% fetal bovine serum (FBS) and 1× antibiotic-antimycotic as previously described. 293-Phoenix cells for retrovirus production were
maintained in DMEM supplemented with 10% FBS. All cell lines were verified by STR analysis and routinely screened for mycoplasma contamination.

### 2.2 Quantitative reverse transcription PCR primers

TaqMan assays were purchased from Thermo Fisher Scientific (Waltham, MA): BHLHE40-AS1 (Hs04274224_m1), 18S (Hs99999901_s1), Actin (Hs99999903_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1). Sybr green assays purchased from Integrated DNA Technologies (Coralville, IA): Actin Primer 1: 5′-chased from Integrated DNA Technologies (GAPDH; Hs02758991_g1). Sybr AAG TG CA GGAGTAC -CC AC 903_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1). Sybr green assays purchased from Integrated DNA Technologies (Coralville, IA): Actin Primer 1: 5′-chased from Integrated DNA Technologies (GAPDH; Hs02758991_g1).

### 2.3 Plasmids

Clonetech SMARTer RACE kit (Takara BIO USA, Mountain View, CA) was used to PCR amplify BHLHE40-AS1 by PCR from MCF10A-DCIS cells. The PCR product was validated by sequencing and ligated into pCR 4Blunt-TOPO for amplification. BHLHE40-AS1 was subcloned into pBabe-puro for retroviral production between restriction sites BamHI and XhoI. Primer 1: 5′-CAATACGATCCGCCCTTTAGTCTT3′ and primer 2: 5′-TTGATCTCGAGTAAACGAATTCGCCCTTACGTCTCTTTGC CG-3′. Primer 1: 5'-GACCTAGGTTAAGCTGC GC-3′. Primer 2: 5'-TGGAATTCGCCCTTACGTCTCTTTGC CG-3′.

### 2.4 RNA sequencing analysis

RNA sequencing of DCIS-IDC patient sample pairs 1 to 5 from NCBI GEO dataset GSE66301 was analyzed. RNA-Seq data were mapped using HISAT2 to the human genome build UCSC hg38. Gene expression quantification was achieved using FEATURCOUTS against the GENCODE20 gene model. Gene expression was further normalized using the R package RUVr. Differential long intergenic noncoding RNA expression was evaluated using the R package edgeR. Significance was achieved for a fold change exceeding 2× and P < .05. Graphical representation as heatmaps were generated using the Python visualization library matplotlib.

### 2.5 Gene expression analysis

Forty-eight hours after seeding, total RNA was collected using Omega Bio-Tek (Norcross, GA) EZNA Total RNA Kit. RNA was reverse-transcribed using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit and analyzed by quantitative reverse transcription PCR (qRT-PCR) on the QuantStudio (Thermo Fisher Scientific) for indicated genes. The ΔΔCt method was used to determine gene expression fold change.

### 2.6 Fluorescence in situ hybridization

A total of 33 DNA probes tiling BHLHE40-AS1 and labeled with Quasar570 were purchased from LGC Biosearch Technologies (Novato, CA). Cells plated on glass cover slides were processed for RNA Fluorescence in situ hybridization following the standard Biosearch protocols. Cells were fixed in 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 and blocked with wash buffer A (LGC Biosearch) with formamide. A total of 250 nM pooled probes in hybridization buffer (LGC Biosearch) were incubated on cells overnight at 37°C followed by DAPI staining. Mounted cells were imaged on the Zeiss LSM780 confocal microscope. Z-stack images were collected with the Plan-Apochromat 63x/1.40 Oil DIC objective and processed in ZEN blue.

### 2.7 Stable cell line generation

A total of 1 μg empty pBabe-puro vector or pBabe-puro-BHLHE40-AS1 vector were transfected into packaging 293-Phoenix cells using lipofectamine X-tremeGENE HP from Millipore Sigma (Burlington, MA). Viral media was collected 36 hours after plasmid transfection and incubated with MCF10A cells overnight. Transduced cells were selected with 1 μg puromycin for 2 weeks with medium changes every 48 to 72 hours.

### 2.8 Wound healing

A total of 2.0 × 10^5 MCF10A-pBabe or MCF10A-BHLHE40-AS1 cells were seeded in six-well plates and grown to confluency. Cells were scratched with a pipette tip, rinsed with phosphate-buffered saline (PBS), and incubated in fresh medium in the EVOS FL Auto OnStage Incubator. Cells were imaged every 30 minutes to monitor wound closing. Images were analyzed by
The MetaViLabs Automated Cellular Analysis System (Austin, TX) to determine the rate of wound closure. The rate of scratch closure during the linear fit portion of the curve is reported.

2.9 | Invasion assay

A total of $2.0 \times 10^5$ MCF10A-pBABE and MCF10A-BHLHE40-AS1 cells were seeded in six-well plates. 48 hours after seeding cells were trypsinized and counted and $2.5 \times 10^5$ cells were resuspended in serum-free medium and loaded into the upper chamber of a growth factor reduced Corning BioCoat Matrigel Invasion Chamber (Corning Life Sciences, Tewksbury, MA) previously rehydrated with serum-free medium. Chambers were placed into 12-well plates loaded with complete media and maintained for 24 hours at 37°C. Cells were then fixed in 100% methanol, stained with 0.1% crystal violet and imaged on the EVOS FL Imaging System (Thermo Fisher Scientific). ImageJ was used to count three fields of view per replicate; three independent biological replicates were performed.

2.10 | Cell cycle analysis

A total of 50 nM control and targeted small interfering RNA (siRNA) were complexed with RNAiMax (Thermo Fisher Scientific) and transfected into indicated cell lines. Seventy-two hours posttransfection cells were trypsinized and fixed in 70% ethanol as a single cell suspension. Cells were rinsed with PBS and stained according to the Muse Cell Cycle Assay kit standardized instructions. A total of 10 000 events were collected for each experimental replicate.

2.11 | CellTiter-Glo analysis

Cells were seeded into 96-well plates. At indicated time points, cells were assayed via CellTiter-Glo (CTG; Promega, Madison, WI). Cells were removed from the incubator and allowed to cool to room temperature for 10 minutes. CTG cell lysis/luminescence reagent was added directly to the well plate and incubated with agitation at room temperature for 10 minutes. Resulting luminescence (generated via the reaction between luciferin and adenosine triphosphate—representing a direct relationship with the number of metabolically active cells) was collected on a VICTOR 3V plate reader (PerkinElmer, Waltham, MA).

2.12 | Microarray

Total RNA was extracted from replicate MCF10A-pBABE and MCF10A-pBABE-BHLHE40-AS1 cells. The RNA quality was assessed by bioanalyzer and 100 ng total RNA with RIN scores of more than 9.8 were arrayed using Applied Biosystems Human Clariom D Assay by the University at Albany Center for Functional Genomics following standard protocols. Raw CEL files were analyzed with the Transcriptome Analysis Console. A total of 365 differentially expressed genes were identified with $P < .05$ and a false discovery rate of less than 0.1. Ingenuity pathway analysis (IPA) (Qiagen, Hilden, Germany) was used to identify predicted upstream regulators with an expression fold change with an absolute value of 2 or greater.

2.13 | Western blot

Cells were lysed in cell lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Whole-cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the following antibodies from Cell Signaling Technology (Danvers, MA): Actin (13E5) 1:1000, GAPDH (14C10) 1:1000, STAT3 (79D7) 1:1000, phospho-Stat3 Tyr705 (D3A7) 1:250, phospho-Stat3 Ser727 (9134) 1:250.

2.14 | Enzyme-linked immunosorbent assay

A total of $2.0 \times 10^5$ cells of each of the MCF10A progression series cell lines were plated in a six-well dish. Forty-eight hours after seeding, conditioned media was collected and centrifuged at 1000g for 10 minutes and assayed for IL-6 concentration using Invitrogen eBioscience Human IL-6 ELISA Ready-SET-Go! Kit (Thermo Fisher Scientific). The remaining cells were trypsinized and counted to determine the cell number for normalization.
2.15 | BHLHE40-AS1 pull-down with biotinylated oligos

RNA purification was performed as previously described. Briefly, MCF10A-DCIS or MCF10A-BHLHE40-AS1 cells were grown to 90% confluence, crosslinked in 3% formaldehyde for 30 minutes followed by quenching with 0.125M glycine for 5 minutes. Cells were lysed in lysis buffer (50 mM Tris pH 7.0, 10 mM EDTA, 1% SDS, protease inhibitors, and 100 U/mL RNase Out) and sonicated until clear. The lysate was diluted in hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris pH 7.0, 1 mM EDTA, 15% formamide, protease inhibitors, and 100 U/mL RNase Out) and incubated with biotinylated probes tiling BHLHE40-AS1 overnight. MyOne Streptavidin C1 Dynabeads were incubated with the lysate for 2 hours, beads were washed in wash buffer (2× NaCl and sodium citrate (SSC), 0.5% SDS, protease inhibitors, and 100 U/mL RNase Out), resuspended in RNA proteinase K buffer (100 mM NaCl, 10 mM Tris pH 7.0, 1 mM EDTA, 0.5% SDS, Proteinase K) at 50°C before boiling for 10 minutes 95°C. Reverse crosslinked samples were then incubated with TRIzol and RNA was purified via chloroform extraction.

2.16 | ILF3 RNA immunoprecipitation

Interleukin enhancer-binding factor (ILF3) immunoprecipitation (RIP) was performed following standard methods. A total of 4.5 x 10^7 MCF10A-DCIS cells were lysed in RIP buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5% NP40, protease and RNase inhibitors), precleared with streptavidin beads, and incubated overnight with 10 μg either control immunoglobulin G (IgG) or anti-ILF3 (cat: #612154; BD Biosciences). Streptavidin beads were added for an additional 2-hour incubation. Beads were washed with RIP buffer, incubated in TRIzol, and RNA was purified via chloroform extraction.

3 | RESULTS

3.1 | BHLHE40-AS1 expression is increased in breast cancer progression

To identify lncRNAs relevant to patient tumor progression, we have taken advantage of a unique model of patient-based DCIS progression wherein patients were identified that exhibited a DCIS lesion directly contiguous with an IDC lesion. As DCIS is a nonobligate precursor lesion, these tandem lesions are a patient-derived model of progression from preinvasive to invasive disease and can be used to interrogate the molecular differences that occur during the transition. RNA sequencing results from five matched DCIS-IDC pairs (GSE66301) were mapped to the human genome build UCSC hg38 and lncRNAs were identified using the GENCODE gene model considering all classes of lncRNAs, and antisense RNAs. 132 lncRNAs showed discrimination potential between DCIS and IDC (Figure 1A). The lncRNA BHLHE40-AS1 is enriched in the patient DCIS-IDC tandem samples and its expression is statistically significant when comparing all DCIS versus all IDC (Figure 1B). In the MCF10A progression model, BHLHE40-AS1 expression increases with disease progression (Figure 1C). Compared to the MCF10A controls, the expression of BHLHE40-AS1 increases threefold in the MCF10A-DCIS cells and fivefold in the MCF10A-CA1 cells. These data suggest that BHLHE40-AS1 is a potential biomarker of disease progression.

3.2 | Characterization of BHLHE40-AS1

BHLHE40-AS1 has not been functionally characterized. Multiple sequence alignments indicate that BHLHE40-AS1 exhibits a high degree of evolutionary conservation in primates, with the exception of a late insertion of a 6000 bp L1HS long interspersed nuclear element into the human intronic region (Figure S1A). The human transcript shares 45% homology with the positionally conserved mouse RIKEN 0610040F04 transcript (Figure S1A). Rapid Amplification of cDNA Ends (RACE) was used to characterize and clone BHLHE40-AS1 from MCF10A-DCIS cells. This study confirms the presence of an 80 kb intronic region. BHLHE40-AS1 is an antisense head-to-head transcript with its 5’ end overlapping the coding gene BHLHE40. RACE results identify a BHLHE40-AS1 transcript with an extended 5’ end increasing the sequence overlap with BHLHE40. GRO-seq data from MCF7 breast cancer cells suggest that the extended transcript is found in additional backgrounds (Figure S1B). Despite this extended overlap, BHLHE40-AS1 does not appear to interact with the BHLHE40 transcript (Figure S1C,D). Although antisense transcripts frequently function to directly regulate the associated overlapping genes, depletion of BHLHE40-AS1 does not consistently impact BHLHE40 transcription (Figure S1E), nor is there a consistent change in BHLHE40 protein accumulation in multiple cell lines (data not shown). While a functional impact of BHLHE40-AS1 on BHLHE40 cannot be ruled out, based on these data, it appears that BHLHE40-AS1 functions independently of BHLHE40.
To determine the molecular context in which BHLHE40-AS1 functions, BHLHE40-AS1 expression was evaluated in a panel of breast cancer cell lines representing all molecular subtypes. BHLHE40-AS1 is expressed in all subtypes and enriched in HER2 positive cell lines (Figure 2A). Further, MCF10A cells stably overexpressing HER2 demonstrate a threefold increase of BHLHE40-AS1 expression that can be attenuated by treatment with the HER2 inhibitor lapatinib (Figure 2B,C). Confocal imaging in MCF10A-CA1 cells and HER2 positive BT474 cells demonstrate the lncRNA is predominantly found in the cytoplasmic compartment (Figure 2D,E). These data demonstrate that BHLHE40-AS1 is a conserved, cytoplasmic lncRNA, expressed downstream of HER2.

3.3 | BHLHE40-AS1 supports cell migration, invasion, and proliferation

To determine if expression functionally impacts invasive phenotypes, BHLHE40-AS1 was overexpressed in the normal, non-transformed, MCF10A cells. Cells overexpressing BHLHE40-AS1 exhibit increased cell spreading relative to pBABE vector control cells (Figure S2). To determine if BHLHE40-AS1 impacts cell migration, pBABE control cells, and BHLHE40-AS1 overexpressing cells were grown to confluence, scratched, and monitored for migration. Cells overexpressing BHLHE40-AS1 migrate at an average rate of 310 µm²/minute compared to the vector control cells at 184 µm²/minute (Figure 3A,B and supporting videos). BHLHE40-AS1 overexpressing cells also exhibit increased invasive potential in the Boyden chamber assay (Figure 3C). BHLHE40-AS1 overexpression did not significantly impact cell growth as evaluated by CTG suggesting migration and invasion phenotypes were not affected by changes in cell proliferation (Figure 3D). To complement these gain of function studies, MCF10A-DCIS cells were transfected with siRNA targeting BHLHE40-AS1 (Figure 4A). Depletion of the lncRNA for 72 hours attenuates cell cycle progression leading to significant accumulation of cells in G0/G1 from 51.8% ± 8.8% with control siRNA to 71.0% ± 9.9% and 61.5% ± 6.6% with siRNAs #1 and #2, respectively (Figure 4B,C and Table 1). CTG analysis 96 hours after the depletion of BHLHE40-AS1 shows a significant loss in overall cell number (Figure 4D).
To elucidate the molecular impact of BHLHE40-AS1 expression, RNA from pBABE vector control and BHLHE40-AS1 overexpression cells were assessed by the transcriptome profiling Clariom D array. Filtering the results for expression fold change ±2, \( P < .5 \) and false discovery rate less than 0.1, 345 genes were identified as differentially regulated (Figure 5A and Table S1). IPA was used to integrate expression data to gain biological insight into the BHLHE40-AS1 signaling network. 27 Upstream regulator analysis predicts the activation state of master regulators by integrating gene expression changes with known biological networks. This generates activation of \( Z \)-score which assesses the match of observed and predicted regulation patterns and an associated pathway overlap \( P \) value that measures the enrichment of network-regulated genes in the dataset. 27 This analysis, rank-ordered by \( P \) value, identifies a strong proinflammatory cytokine signature (Figure 5B). Two IL-1 family members (IL-1\( \alpha \) and IL-1\( \beta \)), IL-6, as well as STAT3, a transcription factor downstream of all three cytokines, 28-30 are found in the top seven predicted upstream regulators with activation \( Z \)-scores of 4.286, 3.881, 3.667, and 2.367, respectively (Figure 5B). These data strongly suggest that BHLHE40-AS1 plays an important role in inflammation. Validation of the microarray results using qRT-PCR confirmed a significant increase in the expression of IL-1\( \alpha \), IL-1\( \beta \), and IL-6 in the MCF10A-BHLHE40-AS1 cells (Figure 5C). Given that STAT3 is functionally downstream of the indicated cytokines, we focused on validating the impact of BHLHE40-AS1 on STAT3 signaling.
To determine if BHLHE40-AS1 overexpression leads to the predicted increase in STAT3 pathway activity, vector control, and BHLHE40-AS1 overexpressing cells were immunoblotted for phospho-STAT3 (Tyr705) and phospho-STAT3 (Ser727) (Figure 5D). The data demonstrate a clear increase in the level of p-STAT3 (Tyr705) and p-STAT3 (Ser727) compared to pBABE vector control cells indicating that stable overexpression of BHLHE40-AS1 leads to increased phosphorylation of pSTAT3 and downstream proinflammatory cytokine response. However, these signaling changes may also result from long-term overexpression and may not be indicative of the immediate signaling network of BHLHE40-AS1. Therefore, IL-6 and STAT3 expression and phosphorylation were assessed 48 hours after the depletion of BHLHE40-AS1 to determine if they rapidly respond to BHLHE40-AS1 signaling. siRNA mediated depletion of BHLHE40-AS1 reduces IL-6 transcripts in the MCF10A-DCIS cells and SUM225 cells by 60% and 50%, respectively (Figures 6A and 6C). Further, at 48 hours there is a loss of total STAT3 protein and pSTAT3 in both cell lines, suggesting that STAT3 activity responds rapidly to BHLHE40-AS1 attenuation (Figures 6B and 6D). BHLHE40-AS1 was identified as a primary gene of interest due to its increased expression both in patient-matched DCIS/IDC samples as well as in the MCF10A progression series. Investigating IL-6 in the MCF10A progression series, IL-6 protein expression increases in the progression model (Figure S3). Taken together, these data suggest that BHLHE40-AS1 supports early breast cancer progression through modulation of IL-6/STAT3 signaling.

### 3.5 BHLHE40-AS1 interacts with ILF3 to mediate IL-6 signaling

To determine the mechanism by which BHLHE40-AS1 impacts IL-6 signaling, we identified interacting proteins.
BHLHE40-AS1 and control antisense transcripts were in vitro transcribed and biotin-labeled. These RNAs were incubated with whole cell lysate from MCF10A-DCIS cells and purified with streptavidin magnetic beads. IncRNA associated proteins were separated by SDS-PAGE and stained with colloidal coomassie for visualization (Figure S4). Mass spectrometry analysis identified ILF3 as a top candidate interacting protein. To validate ILF3 as an interacting protein, BHLHE40-AS1 pull-down assays were performed in MCF10A-BHLHE40-AS1 cells using biotinylated DNA oligos tiling BHLHE40-AS1, or nontargeting antisense oligos as control. qRT-PCR confirms the successful isolation of BHLHE40-AS1 and immunoblotting of associated coprecipitating proteins confirms an association with ILF3 (Figure 7A). Furthermore, RNA RIP using an ILF3 antibody was performed to monitor the levels of BHLHE40-AS1 associating with ILF3 relative to control IgG. Copurifying RNA was isolated and assessed by qRT-PCR for 18S RNA, H19, a cytoplasmic IncRNA, and BHLHE40-AS1. BHLHE40-AS1 is specifically enriched in ILF3 relative to control IgG and control RNAs (Figure 7B). Taken together these data demonstrate an interaction between BHLHE40-AS1 and ILF3.

ILF3 is a known RNA binding protein, that, is alternately spliced producing the NF90 and NF110 proteins. These RNA binding proteins interact with several cellular and viral RNAs and participate in diverse cellular functions including microRNAs biogenesis, translational regulation, mRNA stabilization, although the physiological importance of some interactions is still to be determined. ILF3 has previously been identified to interact with IL-6 mRNA in lung fibroblast (WI38 and IDH4) cells.

### Table 1: Distribution of cells in the cell cycle (%)

|        | G0/G1 | S    | G2/M    |
|--------|-------|------|---------|
| siCtrl | 51.8 ± 8.8 | 14.3 ± 3.7 | 31.2 ± 6.5 |
| si#1   | 71.0 ± 9.9 | 9.1 ± 3.3 | 17.7 ± 5.2 |
| si#2   | 61.5 ± 6.6 | 13.4 ± 3.2 | 22.9 ± 4.1 |
depletion in the MCF10A-BHLHE40-AS1 cells was sufficient to rescue the increased IL-6 expression (Figure 7C). These data demonstrate that BHLHE40-AS1 modulates IL-6 expression through direct interaction with ILF3.

4 | DISCUSSION

There are currently no known functional determinants of DCIS progression to an invasive lesion; in fact, DCIS and IDC lesions are very similar transcriptionally and epigenetically.33-36 This study profiles global IncRNA expression in a unique patient-based model of breast cancer progression wherein early DCIS lesions are directly contiguous with an IDC lesion. From this unbiased patient-based model, we have identified 132 IncRNAs that are differentially expressed with early breast cancer progression. Further, we identify the IncRNA BHLHE40-AS1 as a novel IncRNA that increases with disease progression both in the patient biopsies and in cell culture models of early breast cancer progression.

Phenotypically, BHLHE40-AS1 expression contributes to cell migration, invasion, and potentially cell proliferation. Cell cycle progression is a complex mechanism regulated by many controls and checkpoints. Given that overexpression of BHLHE40-AS1 did not result in a significant change in cell cycle progression, we did not anticipate a function in proliferation. However, BHLHE40-AS1 depletion attenuates normal cell cycle progression and results in accumulation in G0/G1. Taken together this suggests that with overexpression of the IncRNA, the G0/G1 checkpoint remains intact; however, loss of BHLHE40-AS1 attenuates normal cell proliferation. It is possible that the IncRNA may be playing an indirect role in cell cycle progression as opposed to directly regulating a checkpoint. Future studies will be needed to elucidate the IncRNAs role in cell cycle progression.

The data presented here demonstrate that BHLHE40-AS1 modulates a proinflammatory cytokine signature and is an important mediator of IL-6/STAT3 signaling. IL-1α and IL-1β are pleiotropic activator cytokines that function as critical signaling mediators of the inflammatory tumor microenvironment.37 IL-1β in particular is found to be upregulated in many cancers including breast cancer.
cancer, and to support angiogenesis, proliferation, metastasis, and inflammasome activation at both primary and metastatic sites. In breast cancer, IL-1β expression induces IL-6 and additional cytokines and growth factors. Further, while less studied in cancer than IL-1β, IL-1α has been found to support inflammation and cancer stem cell expansion downstream of HER2 expression. IL-1α induces IL-6 and STAT3 signaling creating a feed-forward proinflammation signaling loop supporting tumor progression.

STAT3 is a core signaling integrator of proinflammatory cytokines in addition to many oncogenes and growth factors. The canonical IL-6/STAT3 signaling pathway is well known to play a key role in the development of many cancers including breast where it is found constitutively activated in greater than 50% of patient tumors. In breast cancer cells, STAT3 activation by IL-6 enhances tumor cell migration, invasion, and metastasis and is tightly linked to cellular transformation, proliferation, and tumor initiation. BHLHE40-AS1 mediates STAT3 activation in DCIS cell lines. In an inducible PyVmT mammary tumor model, in STAT3 deficient mice, early-stage lesions were cleared by immune cell infiltration resulting in delayed tumor development. In contrast, intact STAT3 signaling resulted in protumorigenic inflammation, immune evasion, and tumor formation. The induction of cytokine expression and STAT3 activation by BHLHE40-AS1 implicates the IncRNA as a key mediator of protumorigenic inflammatory response.

Mechanistically, BHLHE40-AS1 may directly target IL-6 transcripts through an interaction with the known RNA binding protein ILF3. ILF3 holds many diverse roles within the cell and has been implicated in promoting invasive potential, proliferation, and migration in breast cancer cells. Future studies will be needed to fully understand the consequences of the interaction between BHLHE40-AS1 and ILF3.

The data presented here demonstrate that BHLHE40-AS1 is a novel IncRNA whose expression is important to the progression of DCIS lesions into invasive IDC. Integrating BHLHE40-AS1 as a marker of increased disease progression and as an activator of STAT3 signaling, we propose BHLHE40-AS1 expression serves as a mediator between primary DCIS lesions and the surrounding cells serving to create a permissive immune microenvironment. Future studies will expand our patient analysis and confirm BHLHE40-AS1 as a biomarker of progression. Incorporating BHLHE40-AS1 and IL-6 expression with STAT3 activation in biopsied DCIS lesions may serve to distinguish those DCIS lesions more likely to progress from those that will remain innocuous.
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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study can be found under GSE136579.

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REFERENCES

1. Gorringe KL, Fox SB. Ductal carcinoma in situ biology, biomarkers, and diagnosis. *Front Oncol*. 2017;7:248. https://doi.org/10.3389/fonc.2017.00248

2. Groen EJ, Elshof LE, Visser LL, et al. Finding the balance between over- and under-treatment of ductal carcinoma in situ (DCIS). *Breast*. 2017;31:274-283. https://doi.org/10.1016/j.breast.2016.09.001

3. Behbod F, Gomes AM, Machado HL. Modeling human ductal carcinoma in situ in the mouse. *J Mammary Gland Biol Neoplasia*. 2018;23(4):269-278. https://doi.org/10.1007/s10911-018-9408-0

4. Solin LJ, Gray R, Baehner FL, et al. A multigene expression assay to predict local recurrence risk for ductal carcinoma in situ of the breast. *J Natl Cancer Inst*. 2013;105(10):701-710. https://doi.org/10.1093/jnci/djt067

5. Bremer T, Whitworth PW, Patel R, et al. A biological signature for breast ductal carcinoma in situ to predict radiotherapy benefit and assess recurrence risk. *Clin Cancer Res*. 2018;24(23):5895-5901. https://doi.org/10.1158/1078-0432.CCR-18-0842

6. Morlando M, Ballarino F, Matica A. Long noncoding RNAs: new players in hematopoiesis and leukemia. *Front Med (Lausanne)*. 2015;2:23. https://doi.org/10.3389/fmed.2015.00023

7. Gutschner T, Diederichs S. The hallmarks of cancer: a long noncoding RNA point of view. *RNA Biol*. 2012;9(6):703-719. https://doi.org/10.4161/rna.20481

8. Kumar M, DeVaux RS, Herschkowitz JI. Molecular and cellular changes in breast cancer and new roles of IncRNAs in breast cancer initiation and progression. *Prog Mol Biol Transl Sci*. 2016;144:563-586. https://doi.org/10.1016/bs.pmbts.2016.09.011

9. Su X, Malouf GG, Chen Y, et al. Comprehensive analysis of long noncoding RNAs in human breast cancer clinical subtypes. *Oncotarget*. 2014;5(20):9864-9876. https://doi.org/10.18632/oncotarget.2454

10. Avazpour N, Hajjari M, Tahmasebi Birgani M. HOTAIR: a promising long noncoding RNA with potential role in breast invasive carcinoma. *Front Genet*. 2017;8:170. https://doi.org/10.3389/fgene.2017.00170

11. Hu M, Yao J, Carroll DK, et al. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*. 2008;13(5):394-406. https://doi.org/10.1016/j.ccr.2008.03.007

12. Imbalzano KM, Tatarkova I, Imbalzano AN, Nickerson JA. Increasingly transformed MCF10A cells have a progressively tumor-like phenotype in three-dimensional basement membrane culture. *Cancer Cell Int*. 2009;9:7. https://doi.org/10.1186/1475-2867-9-7

13. Miller FR, Santner SI, Tait L, Dawson PJ. MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. *J Natl Cancer Inst*. 2000;92(14):1185-1186.

14. Soule HD, Maloney TM, Wolman SR, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res*. 1990;50(18):6075-6086.

15. Barnabas N, Cohen D. Phenotypic and molecular characterization of MCF10DCIS and SUM breast cancer cell lines. *Int J Breast Cancer*. 2013;2013:872743. 10.1155/2013/872743

16. Behbod F, Kittrell FS, LaMarca H, et al. An intraductal human-in-mouse transplantation model mimics the subtypes of ductal carcinoma in situ. *Breast Cancer Res*. 2009;11(5):R66. https://doi.org/10.1186/bcr2358

17. Elsarraj HS, Hong Y, Valdez KE, et al. Expression profiling of in vivo ductal carcinoma in situ progression models identified B cell lymphoma-9 as a molecular driver of breast cancer invasion. *Breast Cancer Res*. 2015;17:128. https://doi.org/10.1186/s13058-015-0630-z

18. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie, and Ballgown. *Nat Protoc*. 2016;11(9):1650-1667. https://doi.org/10.1038/nprot.2016.095

19. Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper, and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res*. 2019;47:e47. https://doi.org/10.1093/nar/gkz114

20. Frankish A, Uszczynska B, Ritchie GR, et al. Comparison of GENCODE and RefSeq gene annotation and the impact of reference geneset on variant effect prediction. *BMC Genomics*. 2015;16(Suppl 8):S2. https://doi.org/10.1186/1471-2164-16-S8-S2

21. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol*. 2014;32(9):896-902. https://doi.org/10.1038/nbt.2931

22. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140. https://doi.org/10.1093/bioinformatics/btp616

23. Chu C, Zhang QC, da Rocha ST, et al. Systematic discovery of Xist RNA binding proteins. *Cell*. 2015;161(2):404-416. https://doi.org/10.1016/j.cell.2015.03.025

24. Chu C, Quinn J, Chang HY. Chromatin isolation by RNA purification (ChIRP). *J Vis Exp*. 2012;(61):e3912. https://doi.org/10.3791/3912

25. Feng Y, Hu X, Zhang Y, Zhang D, Li C, Zhang L. Methods for the study of long noncoding RNA in cancer cell signaling. *Methods Mol Biol*. 2014;1165:115-143. https://doi.org/10.1007/978-1-4939-0856-1_10

26. Latge G, Poulet C, Bours V, Josse C, Jerusalem G. Natural antisense transcripts: molecular mechanisms and implications in breast cancers. *Int J Mol Sci*. 2018;19(12):123. https://doi.org/10.3390/ijms19010123

27. Kramer A, Green J, Pollard J Jr., Tugendreich S. Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics*. 2014; 30(4):523-530. https://doi.org/10.1093/bioinformatics/btt703

28. Johnson DE, O’Keeffe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat Rev Clin Oncol*. 2018;15(4):234-248. https://doi.org/10.1038/nrclinonc.2018.8

29. Liu S, Lee JS, Jie C, et al. HER2 overexpression triggers an IL1alpha proinflammatory circuit to drive tumorigenesis and promote chemotherapy resistance. *Cancer Res*. 2018;78(8):2040-2051. https://doi.org/10.1158/0008-5472.CAN-17-2761

30. Mori T, Miyamoto T, Yoshida H, et al. IL-1beta and TNFalpha-initiated IL-6/STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis. *Int Immunol*. 2011;23(11):701-712. https://doi.org/10.1093/intimm/dxr077

31. Castella S, Bernard R, Corno M, Fradin A, Larcher JC. IL5 and NF90 functions in RNA biology. *Wiley Interdiscip Rev: RNA*. 2015;6(2):243-256. https://doi.org/10.1002/wrna.1270
32. Tominaga-Yamanaka K, Abdelmohsen K, Martindale JL, Yang X, Taub DD, Gorospe M. NF90 coordinately represses the senescence-associated secretory phenotype. Aging (Albany NY). 2012;4(10):695-708. https://doi.org/10.18632/aging.100497
33. Abba MC, Gong T, Lu Y, et al. A molecular portrait of high-grade ductal carcinoma in situ. Cancer Res. 2013;73(19):5537-5547. https://doi.org/10.1158/0008-5472.CAN-12-3060
34. DeVaux RS, Herschkowitz JI. Beyond DNA: the role of epigenetics in the premalignant progression of breast cancer. J Mammary Gland Biol Neoplasia. 2018;23(4):223-235. https://doi.org/10.1007/s10911-018-9414-2
35. Nelson AC, Machado HL, Schwertfeger KL. Breaking through to the other side: microenvironment contributions to DCIS initiation and progression. J Mammary Gland Biol Neoplasia. 2018;23(4):207-221. https://doi.org/10.1007/s10911-018-9409-z
36. Pang JB, Savas P, Fellowes AP, et al. Breast ductal carcinoma in situ carry mutational driver events representative of invasive breast cancer. Mod Pathol. 2017;30(7):952-963. https://doi.org/10.1038/modpathol.2017.21
37. Baker KJ, Houston A, Brint E. IL-1 family members in cancer; two sides to every story. Front Immunol. 2019;10:1197. https://doi.org/10.3389/fimmu.2019.01197
38. Holen I, LeFley DV, Francis SE, et al. IL-1 drives breast cancer growth and bone metastasis in vivo. Oncotarget. 2016;7(46):75571-75584. https://doi.org/10.18632/oncotarget.12289
39. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med. 2006;4:48. https://doi.org/10.1186/1479-5876-4-48
40. Oh K, Lee OY, Park Y, Seo MW, Lee DS. IL-1beta induces IL-6 production and increases invasiveness and estrogen-independent growth in a TG2-dependent manner in human breast cancer cells. BMC Cancer. 2016;16(1):724. https://doi.org/10.1186/s12885-016-2746-7
41. Dethlefsen C, Hoffeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. Breast Cancer Res Treat. 2013;138(3):657-664. https://doi.org/10.1007/s10549-013-2488-z
42. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumour Biol. 2016;37(9):11553-11572. https://doi.org/10.1007/s13277-016-5098-7
43. Ling X, Arlinghaus RB. Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. Cancer Res. 2005;65(7):2532-2536. https://doi.org/10.1158/0008-5472.CAN-04-2425
44. Segatto I, Baldassarre G, Belletti B. STAT3 in breast cancer onset and progression: a matter of time and context. Int J Mol Sci. 2018;19(9):2818. https://doi.org/10.3390/ijms19092818
45. Snyder M, Huang XY, Zhang JJ. Signal transducers and activators of transcription 3 (STAT3) directly regulates cytokine-induced fascin expression and is required for breast cancer cell migration. J Biol Chem. 2011;286(45):38886-38893. https://doi.org/10.1074/jbc.M111.286245
46. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. Nat Rev Cancer. 2014;14(11):736-746. https://doi.org/10.1038/nrc3818
47. Jones LM, Broz ML, Ranger JJ, et al. STAT3 establishes an immunosuppressive microenvironment during the early stages of breast carcinogenesis to promote tumor growth and metastasis. Cancer Res. 2016;76(6):1416-1428. https://doi.org/10.1158/0008-5472.CAN-15-2770
48. Hu Q, Lu YY, Noh H, et al. Interleukin enhancer-binding factor 3 promotes breast tumor progression by regulating sustained urokinase-type plasminogen activator expression. Oncogene. 2013;32(34):3933-3943. https://doi.org/10.1038/onc.2012.414
49. Zhang Y, Yang C, Zhang M, et al. Interleukin enhancer-binding factor 3 and HOXC8 co-activate cadherin 11 transcription to promote breast cancer cells proliferation and migration. Oncotarget. 2017;8(64):107477-107491. https://doi.org/10.18632/oncotarget.22491

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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