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ARTICLE

Overexpression of *lnc-ERP44-3:6* Causes Cell Death and Sensitivity to Cisplatin in Breast Cancer Cell Lines

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ABSTRACT

Breast cancer (BC) is one of the leading causes of death in women worldwide. A major challenge in BC is chemoresistance, which is often modulated by epigenetic regulators such as long non-coding RNAs (lncRNAs). Because these regulator lncRNAs may play diverse roles, determining their specific pathways and/or functions is crucial to identify possible biomarkers and/or therapeutic targets for BC. In this study, we used gene expression microarrays in order to identify lncRNAs related to the BC biology. We found, among six differentially expressed (DE) lncRNAs, that the expression of *lnc-ERP44-3:6* was consistently down-regulated in all breast tumor tissues compared to normal adjacent tissues of BC patients from northeastern Mexico. Since the role of *lnc-ERP44-3:6* remains unknown in BC, we induced its transient overexpression in three different BC cell lines (i.e., MCF10A, MCF-7, and HCC1395) by transfection in order to elucidate its potential downstream effects. Remarkably, our results showed a significant increase in cell death and chemosensitivity to cisplatin at 48 h post-transfection ($p < 0.01$). In addition, we observed that *GAPDH* and *FAS* were up-regulated following the overexpression of *lnc-ERP44-3:6*. As *GAPDH* and *FAS* promote apoptosis in cancer, our findings suggest that the *lnc-ERP44-3:6* overexpression induces cell death possibly via up-regulation of one or both genes in BC cell lines. To the best of our knowledge, this is the first study evaluating the overexpression of *lnc-ERP44-3:6* and providing insights about its role in BC cells, which suggests that this lncRNA may be an interesting candidate as a potential therapeutic target for BC in our population. However, further studies would be needed to clarify the mechanisms by which an overexpression of *lnc-ERP44-3:6* causes both cell death and chemosensitivity to cisplatin.

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KEYWORDS

Breast cancer; lncRNA; lnc-ERP44-3:6; biomarker; therapeutic target; chemoresistance; tumor suppressor

1 Introduction

BC is one of the major public health problems worldwide and is the most common cancer, with more than 2 million cases, 600 000 deaths, and an increase of 0.3% per year in the world. It is the second-leading cause of death—after lung cancer—among women younger than 50 years old [1,2]. Since BC is a heterogeneous disease, it has been classified into different subtypes according to overexpression or underexpression of different receptors (e.g., estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)) [3,4]. A further highly aggressive and invasive BC subtype lacking hormone receptors expression is commonly known as triple-negative breast cancer (TNBC) subtype. The current therapies for BC rely on these subtypes [5,6]; yet, notwithstanding the progress in the management of BC patients, reaching a timely diagnostic and/or treatment is often not straightforward (especially for the TNBC subtype) and chemoresistance remains to be the foremost hurdle to overcome as it leads to metastasis and a poor prognosis in BC patients.

The observed heterogeneity in BC may partly be caused by aberrant epigenetic changes that lead to either the silencing of tumor suppressor genes or overexpression of oncogenes. These modifications can include CpG methylation, and hypoacetylation and hypermethylation of histones [7–9]. Recent discoveries have postulated that the lncRNAs may be involved in said changes [10–13]. LncRNAs are essential epigenetic regulators, span more than 200 nucleotides in length, locate mainly in the nucleus, transcribe by the RNA polymerase II, present post-transcriptional modifications, and their expression lower than mRNA is tissue-specific [11,14–18]. LncRNAs participate in multiple molecular processes such as chromatin remodeling, transcription enhancing or interfering, alternative splicing, protein stability, and as microRNAs decoys [19–21]. More recently, lncRNAs have been identified in various cellular and cancer-related processes, including stem cell pluripotency, cell cycle and proliferation, cell migration, lineage differentiation, and tissue invasion [14,15,22], and they may act as tumor suppressors (e.g., *MEG3*) [23], oncogenes (e.g., *BCRT1*) [24], and/or cell sensitizers to chemotherapeutic agents (e.g., *SNORD3*) [22]. Therefore, identifying new lncRNAs and their underlying functions would be essential to propose new biomarkers and/or therapeutic targets that allow for a more precise diagnosis and an improved treatment for BC.

In this study, we focused to find differentially expressed (DE) lncRNAs not yet reported in the literature to be related to BC or with unknown function. Thereby, we found six lncRNAs, among which the expression of *lnc-ERP44-3:6* was consistently down-regulated in all BC tissue samples compared to adjacent normal tissue of patients from northeastern Mexico. *lnc-ERP44-3:6* (also known as *LOC101928438*, *lnc-ERP44-3:6*, *AL359710.1*, *lnc-ALG2-5*, *lnc-ALG2-1*, and *STX17-AS1*) is an intergenic lncRNA that consists of 1917 bp and six exons and is located on chromosome 9 (genomic coordinates 99585324-99773612; hg38) (Fig. S1). Except for a mention in an abandoned patent [25], *lnc-ERP44-3:6* has not yet been reported in other instances related to BC. Hence, we evaluate its effect on cell death and chemoresistance using a BC cellular model. Strikingly, we found that its overexpression in the model decreased cell viability and promoted cell death by apoptosis, as well as enhanced chemosensitivity to cisplatin in tumoral and non-tumoral cell lines.

2 Materials and Methods

2.1 Ethics Committee

The study was approved by the ethics committees of Hospital Universitario "Dr. José Eleuterio González" and Hospital Metropolitano "Dr. Bernardo Sepúlveda" with the protocol numbers of BI15-002 and DEISC-19 01 16 23, respectively. All the procedures performed were carried out according to the ethics committees and the latest version of the 1964 Helsinki Declaration.

2.2 Study Population

Twelve Mexican patients with untreated invasive ductal carcinoma (IDC) were recruited in the period 2015–2017 from the Hospital Universitario "Dr. José Eleuterio González" and Hospital Metropolitano "Dr. Bernardo Sepúlveda" (Monterrey, Nuevo León, México). Primary tumor samples from 12 biopsies (Luminal A, n = 2; Luminal B, n = 2 and HER2+, n = 4; and unidentified samples n = 4) and their corresponding 12 adjacent non-cancerous tissue samples (total = 24 biopsies, 5 × 5 × 5 mm) from these BC patients were dissected and preserved in RNA later solution (QIAGEN, USA). No TNBC subtype samples were found in this cohort. Total RNA was isolated and purified by the All Prep DNA/RNA mini tube kit (QIAGEN, USA) according to the manufacturer's instructions. Purified RNA was later retrotranscribed and used for gene expression analysis by microarrays and real-time quantitative PCR (RT-qPCR). Next, we describe the experiments we conducted in this study to find and investigate the potential role of *lnc-ERP44-3:6* in BC (Fig. 1A).

2.3 Transcriptome Microarrays

To perform a global differential gene expression analysis, the cDNA from each tumor and adjacent non-cancerous tissue sample was labeled with Cy3 dye and hybridized on the SurePrint G3 Human Gene Exp v3, 8x60K microarray (Agilent Technologies, USA), following the manufacturer's instructions for the Agilent Low Input QuickAmp Labeling kit, one color protocol (Agilent Technologies, USA). GeneSpring software v14.5 (Agilent Technologies, USA) was used to process data, and a T-student test with Benjamin-Hochberg correction ($p \leq 0.01$ and fold change ≥2.0) was performed to compare expression changes between tumor and non-cancerous tissues. Through this analysis, we focused on DE lncRNAs.

2.4 Design and Production of pCMV-lnc-ERP44-3:6-GFP-Vector

Once we got the *lnc-ERP44-3:6* sequence from the LNCipedia database (v5.2), it was synthesized by GenScript (Piscataway, USA). This synthesized sequence was cloned into a pCMV-GFP expression vector (ATUM, USA) using the SapI restriction site and a T4 DNA ligase (New England Biolabs, USA). The vector is regulated by a T7 promoter and resistant to ampicillin. We transformed our vectors pCMV-lnc-ERP44-3:6-GFP (Fig. 1B) and pCMV-GFP using chemically competent (Molecular Cloning: A Laboratory Manual Sambrook) *E.coli* (strain TOP10). Then was selected the ampicillin-resistant clones and was confirmed through PCR the positive clones with the *lnc-ERP44-3:6* sequence in the vector. Finally, we grow the bacterial culture and purify the plasmid DNA using the PureYield™ Plasmid Maxiprep system (Promega, USA).

2.5 Cell Culture and Transfection

MCF10A (normal mammary epithelial) was grown in MEBM medium with 100 ng/ml of cholera toxin; MCF-7 (represents the luminal A subtype) was cultured in EMEM (Gibco, USA) medium with 0.01 mg/ml of human recombinant insulin (Gibco, USA) and 1 μM 4-hydroxytamoxifen (Sigma-Aldrich, USA); BT-474 (represents the luminal B subtype) was grown in DMEM/F-12 (Gibco, USA); MDA-MB-453 (represents the HER2+ subtype) was cultured in Leibovitz's medium; and HCC1395 (represents the triple-negative subtype)

was cultured in RPMI-1640 medium (Gibco, USA). All culture media were supplemented with 10% Fetal bovine serum (FBS; Gibco, USA), Amphotericin B (250 ng ml^{-1}; Gibco, USA), Gentamicin (50 µg ml^{-1}; Gibco, USA), L-Glutamine (20 µM ml^{-1}; Sigma-Aldrich, USA), Penicillin and Streptomycin (10 units ml^{-1} and 10 µg ml^{-1}, respectively; Gibco, USA). The cells were incubated at 37°C with 5% CO_2, under sterile conditions. MCF10A, MCF-7, and HCC1395 were transfected with the pCMV-*Inc ERP44-3:6*-GFP or pCMV-GFP (control vector) plasmids, using Xfect transfection reagent (Takara Bio, USA), following the manufacturer's instructions. Cells were then incubated at 24-, 48-, and 72-h post-transfection by technical triplicate. Statistical analysis was performed in GraphPad Prism 9 (v.9.2.0), using One-way ANOVA with a p-value < 0.05.

2.6 RNA Extraction and RT-qPCR

Trizol was used to extract total RNA according to the manufacturer's instructions (Invitrogen, USA). The RNA pellet was resuspended in nuclease-free water (Corning, USA). Next, we performed RT-PCR using SuperScript III (Invitrogen, USA) to obtain the cDNA, following the manufacturer's instructions. Later, Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent, USA) was used to process the samples on an Applied Biosystem 7,500 fast real-time PCR system (Thermo Fisher, USA). The *lnc-ERP44-3:6* specific primers for RT-qPCR were designed using primer 3 plus [25]; their quality, specificity, and characteristics were analyzed and proved by OligoAnalyzer Tool (IDT) [26] and Amplify4 v1.0 software [27], respectively. *CCSER2* was used as an endogenous gene to calculate relative levels of expression. *GAPDH* (metabolism and apoptosis), *N-RAS* (cell growth, motility, angiogenesis, and immune escape), *EpCAM* (EMT: epithelial-mesenchymal transition), *KI-67* (cell proliferation), *PCNA* (cell proliferation), *SNAIL* (chemotherapeutic resistance, and EMT), and *PIK3CA* (cellular growth, proliferation, survival, angiogenesis, and motility), *FAS* (death receptor) genes were selected to determinate potential pathways affected after overexpressing *lnc-ERP44-3:6* (Tab. S1). The RT-qPCR was performed by technical triplicate and the mean values were calculated to determine the relative expression using the $2^{-\Delta\Delta Ct}$ method. T-student and one and two-way ANOVA tests were performed for statistical analysis with a p-value <0.05 using GraphPad Prism 9 (v.9.2.0).

2.7 Immunoassay with Transfected Cells

MCF10A, MCF-7, and HCC1395 cell lines were used to seed 10,000 cells per well on 96-well plates. The cells were fixed with paraformaldehyde and incubated for 10 min. The plate with the fixed cells was blocked with 200 µl of 5% skimmed milk in PBS (Phosphate Buffered Saline) and incubated for 1 h at room temperature; the next three washes with PBS containing 0.05% Tween-20™ were performed to remove waste. Then we added 100 µl of antibodies anti-GAPDH, anti-PCDNA, anti-KI-67, anti-PI3, anti-N-RAS, anti-EpCAM, and anti-SNAIL with a dilution of 1:2000 (Santa Cruz Biotechnology) and incubated for 1 h at room temperature with three subsequent washes using PBS containing 0.05% Tween-20™. We used an antibody anti-mouse marked with HRP (100 µl, 1:10000 dilution; Pierce, Rockford IL, USA) was then incubated for 1 h at room temperature, followed by three washes with PBS containing 0.05% Tween-20™. The HRP was then detected by adding 100 µl of 1-Step™ Ultra TMB-ELISA (Pierce, Rockford IL, USA) until a blue color was observed. The reaction was stopped by adding 100 µl 1M H_2SO_4, and the absorbance was measured at 450 nm in the Cytation 5 microplate reader (Biotek, USA). The immunoassay was performed by technical triplicate and GraphPad Prism 9 (v.9.2.0) and SPSS (v25) [28] were used for statistical analyses; a two-way ANOVA was performed with a p-value < 0.05.

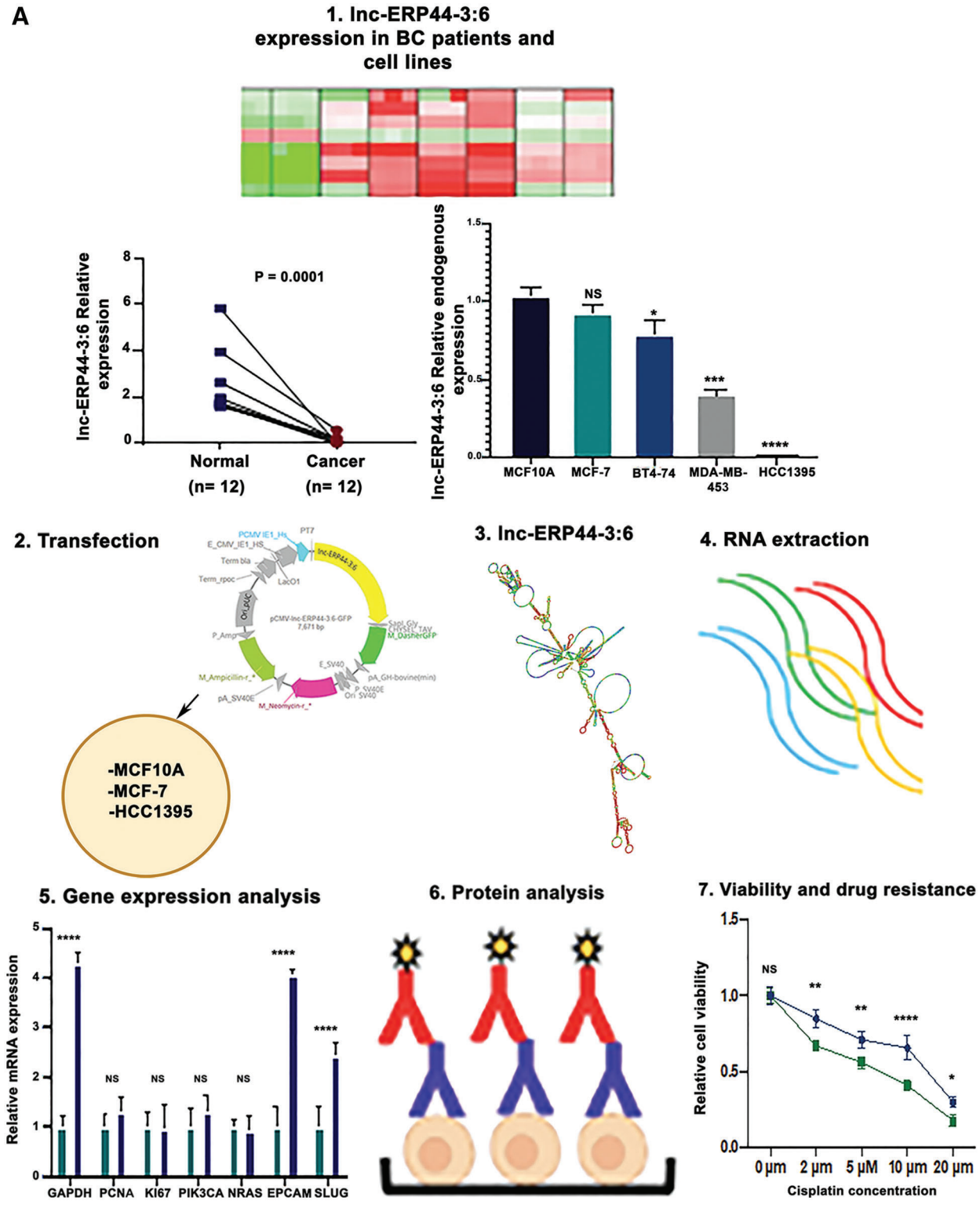

Figure 1: (continued).

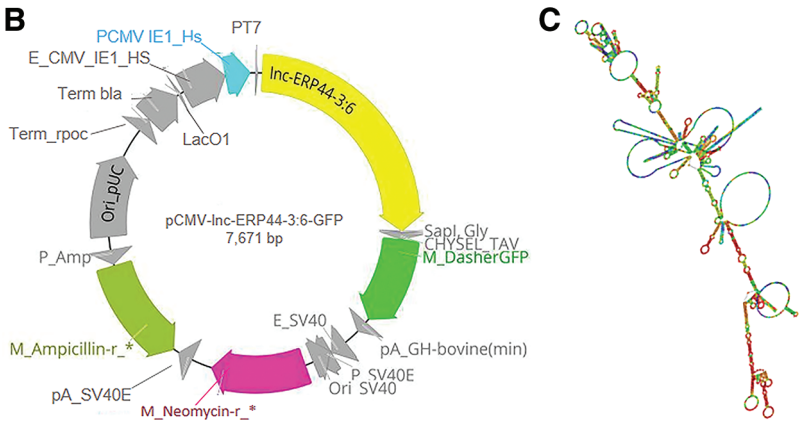

Figure 1: (A) Workflow we follow to determine the role of *lnc-ERPP44-3:6*, steps 1 to 7: 1. Gene expression analysis in BC patients and BC cell lines, 2. Overexpression of *lnc-ERPP44-3:6* transfecting BC cell lines with the pCMV-*lnc-ERP44-3:6*-GFP vector, 3. RNA extraction from the cell lines transfected to perform the RT-qPCR, 4. Analysis of the gene expression, 5. Analysis of the protein profiles through immunoassays, 6. Viability and drug resistance following an overexpression of *lnc-ERPP44-3:6*. (B) Map of the pCMV-*lnc-ERP44-3:6*-GFP vector, which includes a CMV promoter and a GFP protein, and the sequence of *lnc-ERPP44-3:6* inserted using the SapI restriction. (C) Prediction of the secondary structure of *lnc-ERPP44-3:6* using RNAfold WebServer [29]

2.8 Cell Viability, Death, and Cytotoxicity Assays

Sterile 96 well plates were used to seed 10,000 cells of MCF10A, MCF-7, or HCC1395 by technical triplicates and incubated for 24 h. Cells were transfected with pCMV-lncERP44-3:6-GFP and/or pCMV-GFP (empty vector, used as control) vector and incubated for 24, 48, and 72 h post-transfection. The cell viability was determined using 100 µl of CellTiter-Glo® 3D (Promega, USA) to each well, and luminescence was measured after 25 min of incubation at room temperature, following the manufacturer's instructions. To detect cell death, we added 100 µl of Caspase-Glo® 3/7 Reagent to each well of 96-well plates with 10,000 cells of MCF10A, MCF-7, and HCC1395; the cells were incubated for 30 min at room temperature and luminescence was measured according to the manufacturer's instructions. All assays were performed by technical triplicate and statistical analysis was performed in GraphPad Prism 9 (v.9.2.0), using two-way ANOVA with a p-value < 0.05.

Finally, the transfected cells with the *lnc-ERP-443:6* or control vector were incubated with different concentrations of Cisplatin (2.5, 5, 10, and 20 µM) 24 h post-transfection and CellTiter-Glo® and Caspase-Glo® 3/7 reagents were used to determine viability cell and rate of apoptosis, respectively, at 48 h post-transfection. All assays were performed by technical triplicate and statistical analysis was performed in GraphPad Prism 9 (v.9.2.0), using two-way ANOVA with a p-value < 0.05.

3 Results

3.1 lnc-ERP44-3:6 Expression in BC Patients' Tissue and BC Cell Lines

Gene expression microarrays were performed to determine the global differential expression pattern from BC tissue (n = 12) compared to adjacent non-cancerous tissue (n = 12). Our results showed 99 DE entities (p < 0.01, unpublished data), among which, *lnc-C9orf131-1*, *lnc-SLC25A21-2*, and *lnc-GLB1-1* were overexpressed, and *lnc-ERP44-3*, *lnc-BBOX1-1*, and *lnc-SPRYD3-1* were down-regulated (Fig. 2 and data not shown). In this regard, *lnc-ERP44-3:6* (fold change –3.719) was consistently down-regulated in all samples (and probes) of our study population (Figs. 2A and 2B, and data not shown); this result

was validated by RT-qPCR assays (Fig. 2C). Given that the samples collected from patients were positive for either luminal A, B, or HER2+ subtype (Tab. S2), we evaluated the expression pattern of *lnc-ERP44-3:6* in cell lines corresponding to such subtypes as well as in both a non-tumor cell line and a TNBC (Tab. S3). Thus, we determined its endogenous expression by RT-qPCR in all tumoral (i.e., MCF-7 (luminal A subtype), BT-474 (luminal B subtype), MDA-MB-453 (HER2+), and HCC1395 (TNC)) and normal (i.e., MCF10A) cell lines. The results showed no significant expression changes in the MCF-7 cell line compared to MCF10A; conversely, HCC1395 cell line showed the lowest expression of *lnc-ERP44-3:6* compared to MCF10A, MCF-7, BT-474, and MDA-MB-453 (**** $p < 0.0001$) (Fig. 2D).

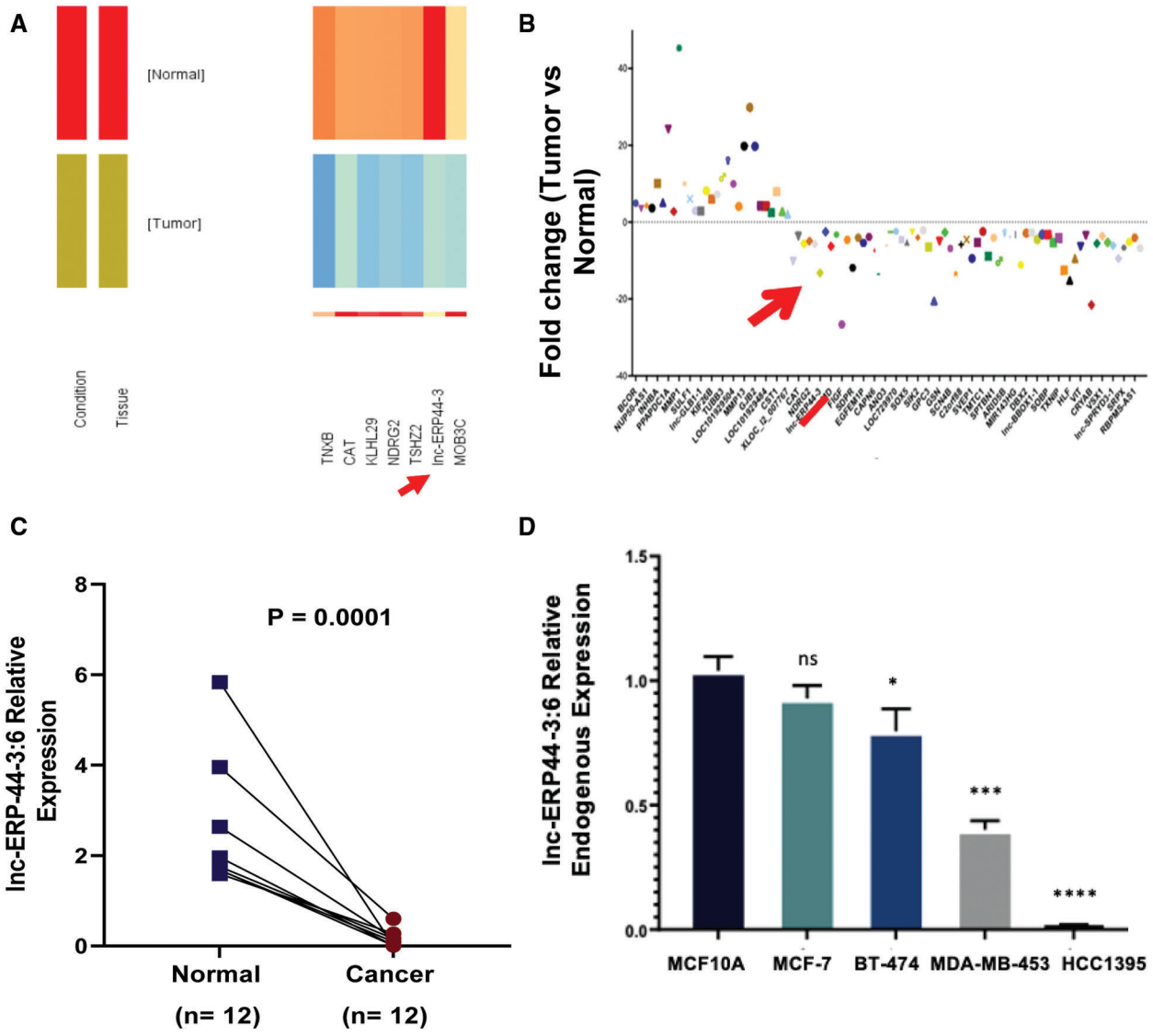

Figure 2: (A) Heat map showing in blue color the down-regulation of *lnc-ERP44-3:6* compared to adjacent normal tissue in red color. (B) Fold change graphic denoting the *lnc-ERP44-3:6* differential expression from microarray results. (C) Validation by RT-qPCR of the expression pattern of *lnc-ERP44-3:6* in BC patients. (D) RT-qPCR results for the endogenous expression of *lnc-ERPP44-3:6* in the BC cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.2 Overexpression of lnc-ERP44-3:6 in BC Cell Lines

Three cell lines were selected to transiently overexpress *lnc-ERP44-3:6* by the pCMV-GFP vector: MCF10A was used as a control cell line, while the MCF-7 and HCC1395 cell lines represented the least aggressive and the most aggressive subtypes of BC, respectively. The results showed the highest expression of *lnc-ERP44-3:6* at 24 h, in all transfected cell lines (Figs. 3 and S2 in Annex B).

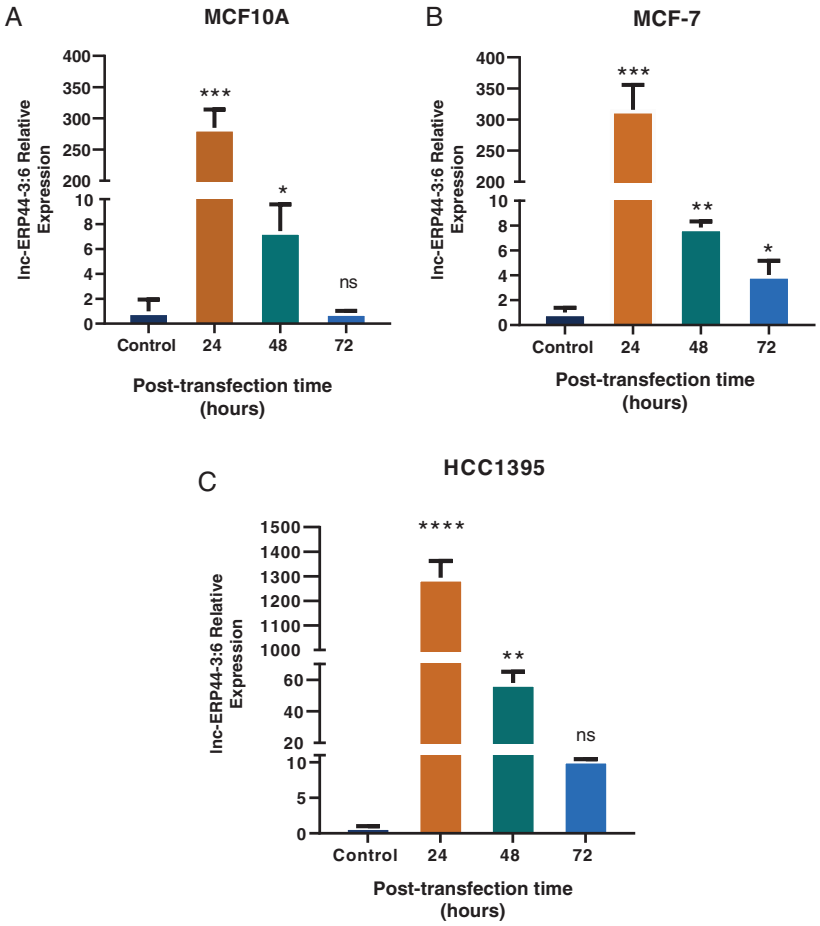

Figure 3: Expression of *lnc-ERPP44-3:6* at 24, 48, and 72 h post-transfection with *lnc-ERPP44-3:6* and control (empty vector) in (A) MCF10A, (B) MCF-7, and (C) HCC1395 cell lines. One-way ANOVA mean ± S.D. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.3 Transcripts and Proteins Affected by lnc-ERP44-3:6 Overexpression

In order to characterize the functional role of this lncRNA, we analyzed the effect of the *lnc-ERP44-3:6* up-regulation on the expression (at both mRNA and protein levels) of pivotal genes involved in cell proliferation (i.e., *PCNA*, *KI-67*, and *PIK3CA*), EMT (i.e., *EpCAM* and *SNAIL*), and oncogenicity (*N-RAS*) in our representative BC cell lines. Because *GAPDH* expression was observed to be altered by overexpressing *lnc-ERP44-3:6*, we used *CCSER2* as an internal control for our RT-qPCR experiments. MCF10A, MCF-7, and HCC1395 cell lines showed the highest expression—at mRNA level—of *GAPDH* (****$p < 0.0001$), *EpCAM* (****$p < 0.0001$), and *SNAIL* (****$p < 0.0001$) at 48 h post-transfection with *lnc-ERP44-3:6*, whereas no significant changes in expression were observed in the remaining

genes (Fig. S2 and 4A). The results were verified by immunoassays at 48 h post-transfection with *lnc-ERP44-3:6* in MCF10, MCF-7, and HCC1395 cell lines, as we detected altered the expression (up-regulated) of GAPDH (**** $p < 0.0001$), EpCAM (*$p \leq 0.05$), and SNAIL (**$p \leq 0.01$) proteins in contrast to control cell lines. We also observed at 48 h post-transfection, that in the MCF-7 cell line both the KI-67 (*$p < 0.05$) and PCNA (*$p < 0.05$) proteins were overexpressed, whereas in the HCC1395 cell line, the expression of KI-67 (*$p < 0.05$), PIK3CA (*$p < 0.05$) and N-RAS (*$p < 0.05$) was up-regulated compared to the control cell line. In contrast, no significant changes in protein expression were observed for the remaining proteins (Fig. 4B).

3.4 lnc-ERP44-3:6 Overexpression Promotes Cell Death and Chemosensitivity to Cisplatin

At 48 h post-transfection, MCF10A, MCF-7, and HCC1395 cell lines showed a significant decrease in cell viability and an increase in apoptosis rate compared to the control cell lines ($p < 0.01$) (Figs. 5A and 5B). After that, MCF10A, MCF-7, and HCC1395 cell lines, at 72 h post-transfection with *lnc-ERP44-3:6*, showed an increase in cell viability compared to 48 h post-transfection, which is consistent with a loss of *lnc-ERP44-3:6* expression at this time point (Fig. 5A).

Moreover, we used cell lines at 48 h post-transfection to analyze if the increase in the apoptosis ratio after overexpression of *lnc-ERP44-3:6* was linked to the activation of the death receptor FAS (cell surface death receptor), which activates the extrinsic apoptosis pathway. Then, we analyze the expression of *FAS* at the mRNA level in MCF10A, MCF-7, and HCC1395 cell lines by real-time PCR at 48 h post-transfection with *lnc-ERP44-3:6* compared to control. We found a significant increase of *FAS*, which suggests us that *FAS* probably participates in the apoptosis after overexpression of *lnc-ERP44-3:6* (Fig. 6).

Hence, we promptly inquired whether an overexpression of *lnc-ERP44-3:6* would stimulate chemosensitivity to cisplatin (Fig. 7). Our results show that the cells treated with cisplatin at different concentrations and 48 h post-transfection with *lnc-ERP44-3:6* exhibit a significant decrease in cell viability (Fig. 7A) and a significant increase in the apoptosis ratio (Fig. 7B) when compared to untransfected control cell lines. Indeed, the overexpression of *lnc-ERP44-3:6* at 48 h post-transfection promoted chemosensitivity to cisplatin.

4 Discussion

LncRNAs play multiple roles in maintaining cell homeostasis and their aberrant expression in cancer cells has been widely associated with promoting tumor development, metastasis, and resistance to anticancer drugs [22,30]. In this study, we found six DE lncRNAs (i.e., *lnc-C9orf131-1*, *lnc-SLC25A21-2*, *lnc-GLB1-1*, *lnc-ERP44-3:6*, *lnc-BBOX1-1*, and *lnc-SPRYD3-1*) whose targets, pathways, and/or functions are largely unknown [31,32]. Among these, *lnc-ERP44-3:6* presented a consistent down-regulated expression in all BC samples compared to their normal adjacent tissue (Figs. 2A–2C). Focusing on this lncRNA, we confirmed a decreased expression pattern ranging from non-significant to significant in BC cell lines here evaluated (i.e., MCF-/luminal, BT4-74/luminal B, MDA-MB-453/HER2+, and HCC1395/TNBC) compared to the non-tumoral one (i.e., MCF10A), which is partially compatible with the observed in our patients' samples. Of note, the TNBC cell line exhibited the lowest expression of *lnc-ERP44-3:6* (Fig. 2D). Moreover, our results agree with those mentioned by Khalil and Markowitz in their patent, in which states (though without providing additional details and/or references) that *linc-ALG2-5* (gene expressing *lnc-ERP44-3:6*) is down-regulated in BC [33]. We also demonstrated that overexpression of *lnc-ERP44-3:6*—induced by CMV-GFP vector—reduces cell viability, promotes cell death, and enhances the chemosensitivity to cisplatin in MCF10A, MCF-7, and HCC1395 cell lines at 48 h post-transfection. Thus, our findings suggest that *lnc-ERP44-3:6* could be a potential universal pro-apoptotic lncRNA, that is, it may act in both pathological and non-pathological states. Partially supporting this scenario, *lnc-ERP44-3:6* presents certain pro-apoptotic characteristics seen in other lncRNAs such as *GAS5* [34] and *SNORD3A* [22], including a low expression in tumor tissue to evade apoptosis, as well as that when overexpressed (by induction) in cancer cell lines, promote chemosensitivity and cell death [35]. These findings and observations underscore the value of *lnc-ERP44-3:6* as a potential therapeutic target in BC, particularly in the TNBC subtype.

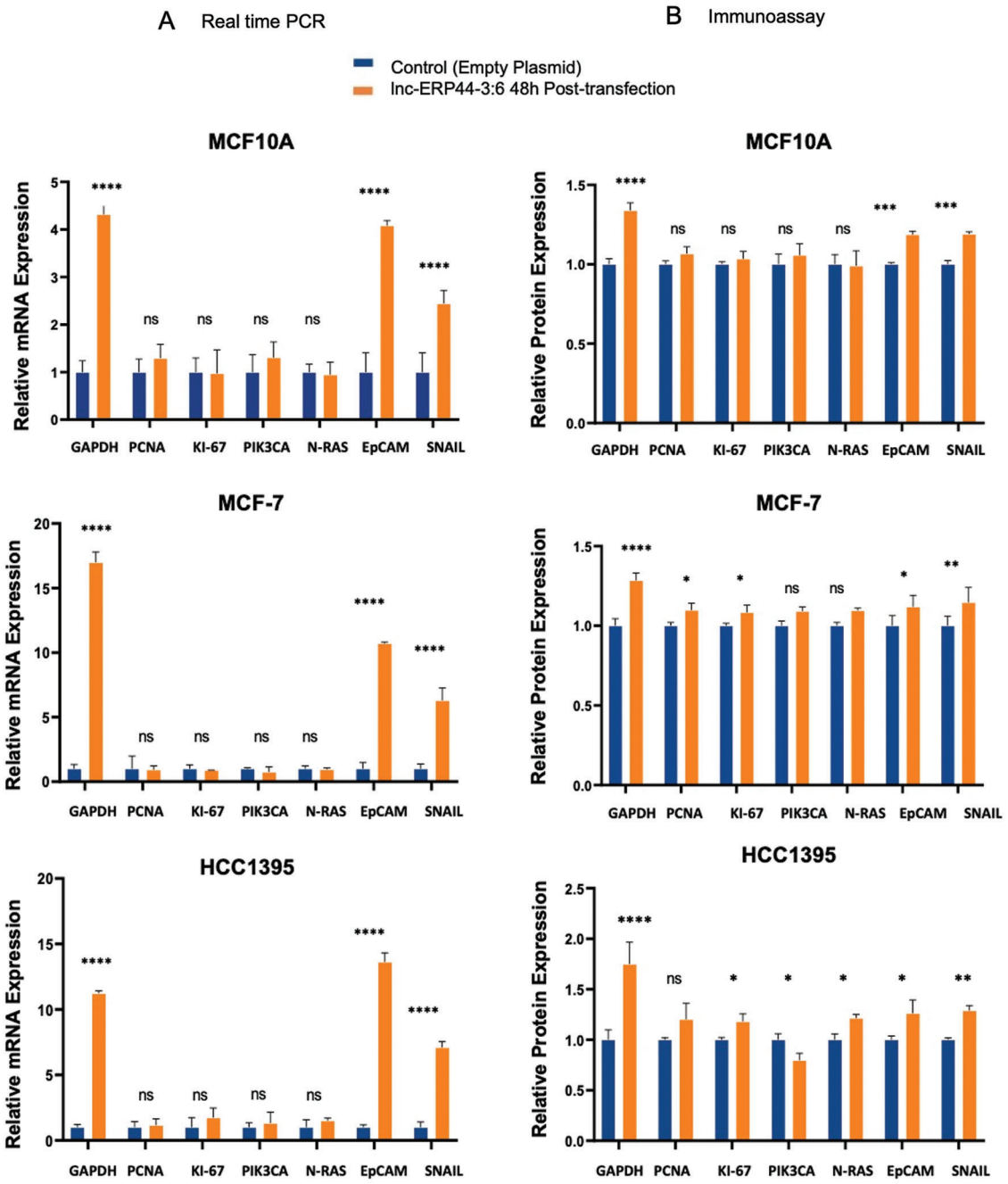

Figure 4: (A) mRNA and (B) proteins expression of GAPDH, PCNA, KI-67, PIK3CA, N-RAS, EpCAM and SNAIL in MCF10A, MCF-7 and HCC1395 cell lines at 48 h post-transfection with control vector and *lnc-ERP44-3:6*. Two-way ANOVA, mean ± S.D. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

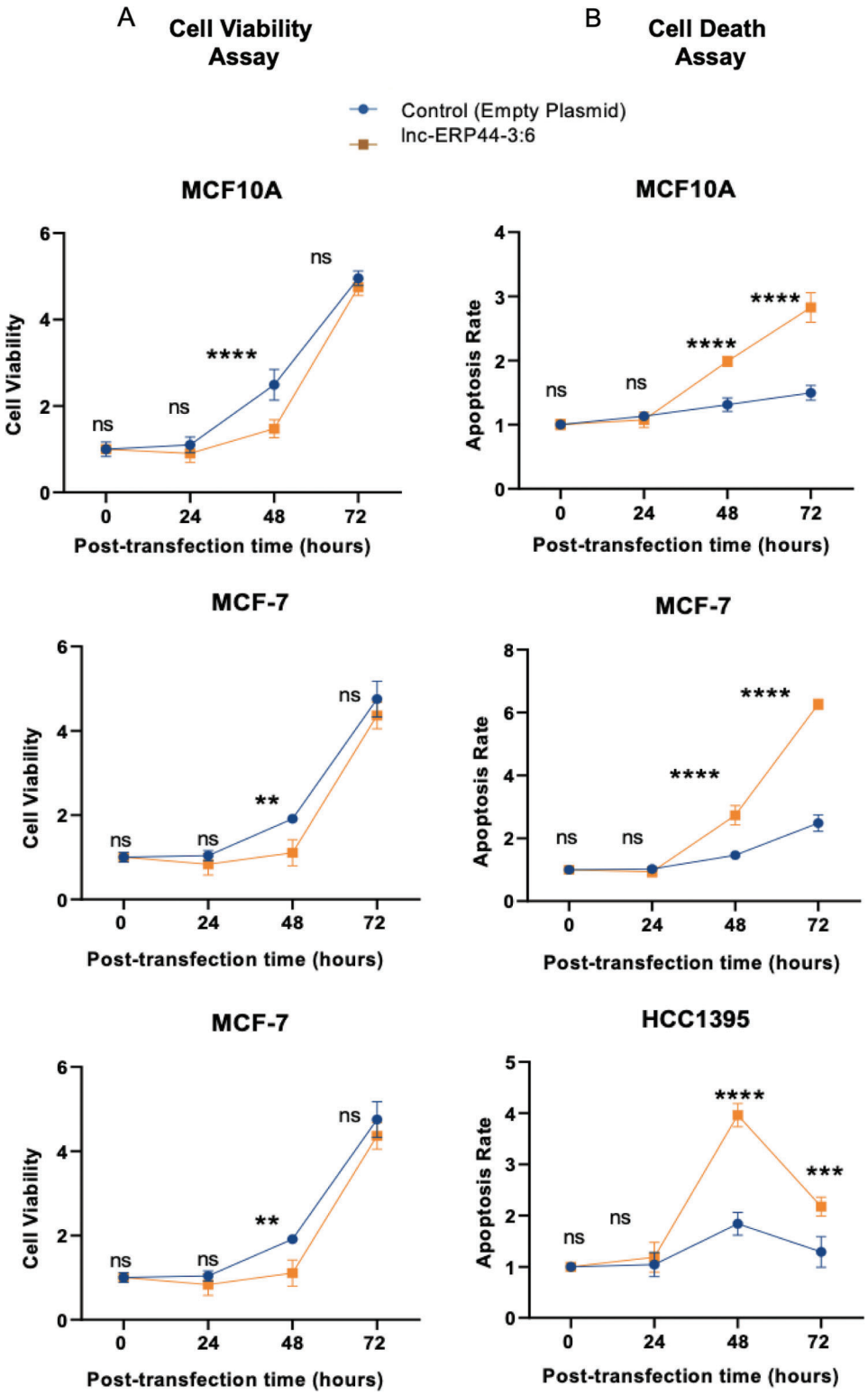

Figure 5: Effect of up-regulation of *lnc-ERP44-3:6* at 0, 24, 48 and 72 h post-transfection on (A) cell viability and (B) apoptosis in MCF10A, MCF-7 and HCC1395 cell lines compared with control cell lines. Two-way ANOVA mean ± S.D. ns: not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

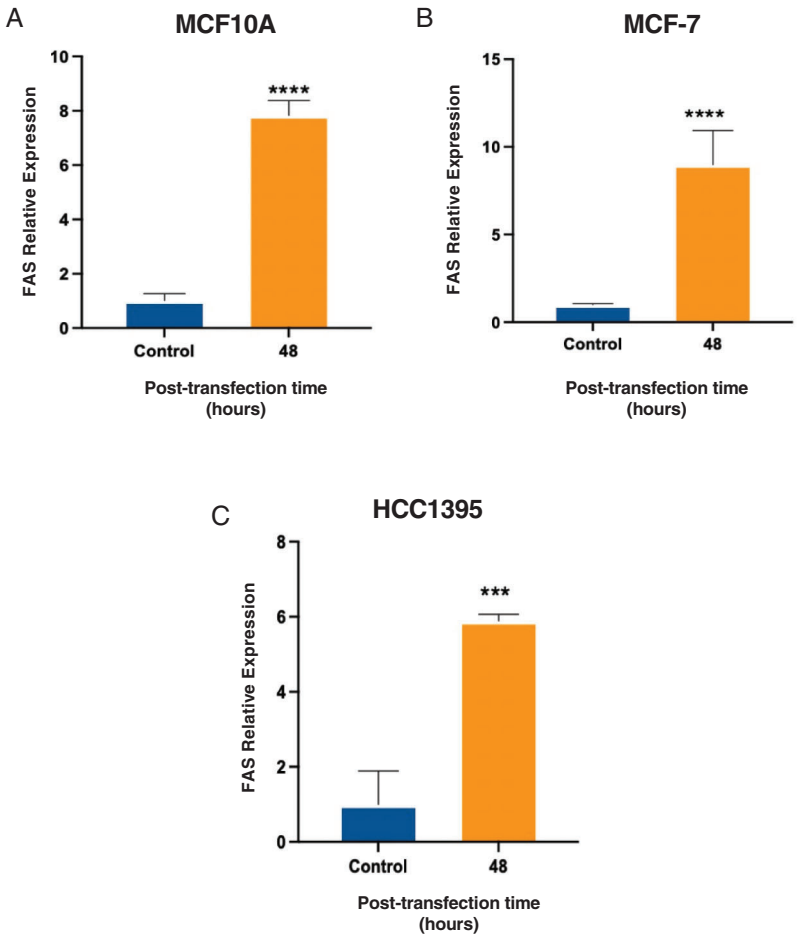

Figure 6: Increase in *FAS* expression at 48 h post-transfection with *lnc-ERP44-3:6* in (A) MCF10A, (B) MCF-7 and (C) HCC1395 cell lines with respect to the control. T-student test mean ± S.D. *** $p < 0.001$, **** $p < 0.0001$

Although we did not evaluate the transcriptome of BC cells overexpressing *lnc-ERP44-3:6*, we obtained interesting expression data from some genes related to cell growth, motility, angiogenesis, and immune escape (i.e., *N-RAS* and/or *PIK3CA*), EMT (i.e., *EpCAM* and/or *SNAIL*), cell proliferation (i.e., *KI-67* and *PCNA*) chemotherapeutic resistance (i.e., *SNAIL*), and apoptosis (i.e., *GAPDH* and *FAS*). In this regard, we identified two key events from our experiments: apoptosis and chemosensitivity. The more plausible explanation about *lnc-ERP44-3:6* overexpression-related apoptosis could be linked to *GAPDH* and/or *FAS*, which were up-regulated in the cells here evaluated. For the former, Tarze et al. [36] observed that the exogenous addition of GAPDH or a high concentration of this protein induced mitochondrial membrane permeabilization, causing activation of the intrinsic apoptosis pathway and releasing of the pro-apoptotic proteins Cytochrome C and AIF (apoptosis-inducing factor). Similarly, FAS is involved in the extrinsic apoptosis pathway activating the initiator caspase 8, which spreads the apoptosis signal by directly processing downstream effector caspases such as caspase-3 [37,38]. Supporting these hypotheses, we indirectly observed a significant increase in the activity of caspase-3 and caspase-7 downstream effectors of both intrinsic and extrinsic apoptosis pathways [37,39] through a high apoptosis rate induced at 48 h post-transfection with *lnc-ERP44-3:6* in the cell death assays (Fig. 5). On the other hand, the increased chemosensitivity to cisplatin observed in the cell lines that overexpressed *lnc-ERP44-3:6* (Fig. 7), may be due to a "double pro-apoptotic hit" as the cytotoxic mechanism of cisplatin also induce the activation of both intrinsic and extrinsic apoptosis pathways [40].

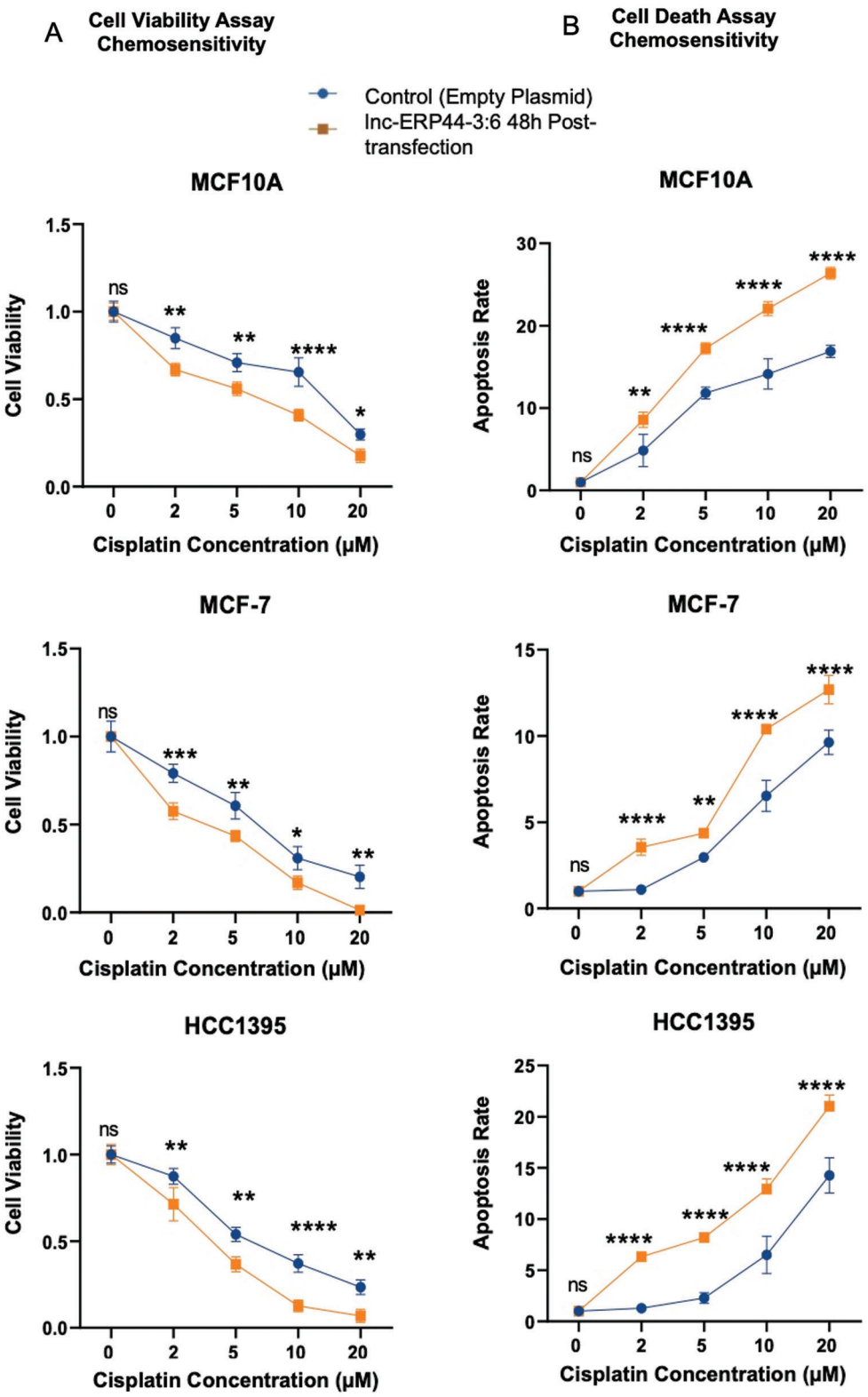

Figure 7: Impact of overexpression of *lnc-ERP44-3:6* at 48 h post-transfection on chemosensitivity at different concentrations of cisplatin in MCF10A, MCF-7 and HCC1395 cell lines was evaluated by (A) cell viability and (B) cell death assays. Two-way ANOVA mean ± S.D. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

As the cancer cells are in a constant internal fight between cell death and survival, they undergo multiple chaotic genomic and transcriptomic changes. For this reason, we often observe unpredictable changes and even opposite or dual roles from multiple genes in cancer cells exposed or not to drugs [41–45]. Here, we observed up-regulation of *SNAIL* and *EpCAM* at both mRNA and protein levels. SNAIL and EpCAM proteins are involved in promoting chemoresistance, aggressiveness, and progression in cancer cells [46,47]. Although we currently unknown the exact mechanisms and meaning of their overexpression, we speculate that they could have increased in response to the pro-apoptotic factors seemingly induced by the *lnc-ERP44-3:6* overexpression (and probably cisplatin exposure). We envisage a similar scenario for *KI-67*, *N-RAS*, and *PCNA*, which were significantly ($p < 0.05$) up-regulated—but only at protein level—at 48 h post-transfection in the MCF-7 and/or HCC1395 cell lines compared to the control (Fig. 4). This apparent inconsistency between mRNA and protein levels of *KI-67*, *N-RAS*, and *PCNA* (Fig. 4) in such cell lines perhaps was due to a strong selective pressure to maintain the protein abundance even though the mRNA abundance diverges [48]. In this regard, protein expression levels depend on protein stability, translation, and post-transcriptional processes, but between 20–60% depend on mRNA levels, which may vary between organisms and conditions [48–51]. However, further studies would be needed to confirm these assumptions and ascertain if the expression patterns observed in our experiments actually depend on the exposure time to the pro-apoptotic factors, since we only evaluated an exposure to *lnc-ERP44-3:6* overexpression of 48 h.

Within the limitations of our study, we are aware that remains to be evaluated the transcriptome of BC cells overexpressing *lnc-ERP44-3:6*, the effects of a stable overexpression of this lncRNA (as opposed to transient overexpression), as well as assess the expression patterns of DE genes in our experiments but at different times and after combining *lnc-ERP44-3:6* overexpression and cisplatin. Nevertheless, our current work represents a preliminary framework to continue studying the pathways and/or functions of this and other lncRNAs found DE in the BC tissues from patients of our population.

5 Conclusions

In this study, we identified the down-regulation of *lnc-ERP44-3:6* in BC tissues compared with their corresponding non-cancerous mammary tissues. According to our results of overexpression, *lnc-ERP44-3:6* is a potential pro-apoptotic lncRNA. Furthermore, we found that combining *lnc-ERP44-3:6* overexpression with cisplatin enhances the chemosensitivity of BC cells to this drug. Strikingly, we observed an exacerbated pro-apoptotic effect induced by this lncRNA on the TNBC cell line. We also found that *GAPDH* and *FAS* were up-regulated by *lnc-ERP44-3:6* transient overexpression. We propose that the *lnc-ERP44-3:6* overexpression-related apoptosis could be linked to one or both of these genes, which can activate both intrinsic and extrinsic apoptotic pathways. Supporting this, we indirectly observed a significant increase in the activity of downstream effectors caspase-3 and caspase-7 of both apoptosis pathways. To the best of our knowledge, this is the first study evaluating the overexpression of *lnc-ERP44-3:6* and providing insights about its role in BC cells, which suggests that this lncRNA may be an interesting candidate as a potential therapeutic target for BC in our population. However, further studies would be needed to better understand the mechanisms by which an overexpression of *lnc-ERP44-3:6* causes both cell death and chemosensitivity to cisplatin, as well as refine the pathways and/or functions of this and other lncRNAs.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix A

Table S1: Genes and sequence of primers used for RT-qPCR

Genes	Sequence of primers
GAPDH	Forward: AGG TCGGAGTCAACGGATTTG Reverse: GTGATGGCATGGACTGTGGT
CCSER2	Forward: GACAGGAGCATTACCACCTCAG Reverse: CTTCTGAGCCTGGAAAAAGGGC
Inc-ERP44-3:6	Forward: GGAAAGTTGCTCTTGCATCA Reverse: AAAAGGCATTGATAGTAGTTCTTGG
N-RAS	Forward: TACATGAGGACAGGCGAAGG Reverse: CTCGCTTAATCTGCTCCCTGT
EpCAM	Forward: GCTGGAATTGTTGTGCTGGTTA Reverse: AGATGTCTTCGTCCCACGC
KI-67	Forward: CCAGAGGAAATTGTGGAGGA Reverse: TGGTTTTCATGTCGTTGCTG
PIK3CA	Forward: TCATGGTGAAAGACGATGGA Reverse: GTCAAAACAAATGGCACACG

(Continued)

Table S1 (continued).

Genes	Sequence of primers
SNAIL	Forward: TGCATATTCGGACCCACACATTA Reverse: AAAAGGCTTCTCCCCCGTGTG
PCNA	Forward: AGGCACTCAAGGACCTCATC Reverse: TAGGTGTCGAAGCCCTCAGA
FAS	Forward: GAAGGACATGGCTTAGAAGTGG Reverse: ACATTTGGTGCAAGGGTCAC

Table S2: Histopathological classification of the patients participating in the study

	Patient	Stage	Immunohistochemistry	The histological type of BC
1	LCM34	N/A	ER+	IDC
2	LCM49	IIIB	ER-, PR-, HER2+	IDC
3	LCM51	IIA	ER+, PR+, HER2++	IDC
4	LCM55	N/A	N/A	N/A
5	LCM57	IIIB	ER-, PR+, HER2+++	IDC
6	LCM61	IIA	N/A	IDC
7	LCM64	N/A	ER+, PR+, HER2-	IDC
8	LCM65	IIB	ER+	IDC
9	LCM 67	N/A	N/A	IDC
10	LCM76	N/A	ER+, PR+, HER2-	IDC
11	LCM77	N/A	ER-, PR+, HER2+++	IDC
12	LCM78	N/A	N/A	N/A

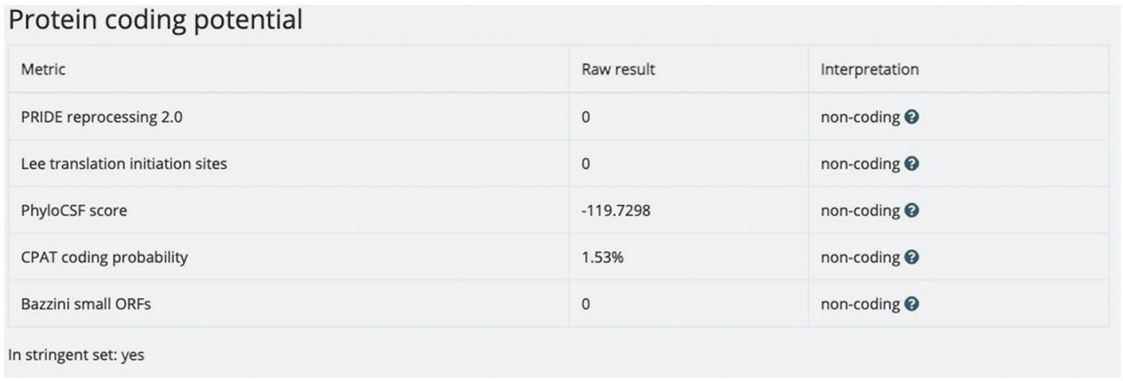
Note: N/A = Not Available, ER = Estrogen Receptor, PR = Progesterone receptor, HER2 = Human Epidermal Growth Factor Receptor 2, IDC = Invasive Ductal Carcinoma.

Table S3: Cell line characteristics

Cell line	Subtype	The histological type of BC	Tissue
MCF10A	**Not cancerous**	**—**	**Mammary gland**
MCF-7	**Luminal A**	**N/A**	**Mammary gland**
BT-474	**Luminal B**	**DC**	**Mammary gland**
MDA-MB-453	**HER2+**	**N/A**	**Mammary gland**
HCC1395	**TN-BC**	**DC**	**Mammary gland**

Note: —— = Not apply, N/A = Not Available, HER2 = Human Epidermal Growth Factor Receptor 2, DC = Ductal Carcinoma, TN-BC = Triple-Negative BC.

Appendix B



Protein coding potential

Metric	Raw result	Interpretation
PRIDE reprocessing 2.0	0	non-coding
Lee translation initiation sites	0	non-coding
PhyloCSF score	-119.7298	non-coding
CPAT coding probability	1.53%	non-coding
Bazzini small ORFs	0	non-coding

In stringent set: yes

Figure S1: *lnc-ERP44-3:6* has no encoding potential. The image represents the bioinformatic analysis by the LNCipedia version 5.2 database to determine the non-coding potential of *lnc-ERP44-3:6* [1]

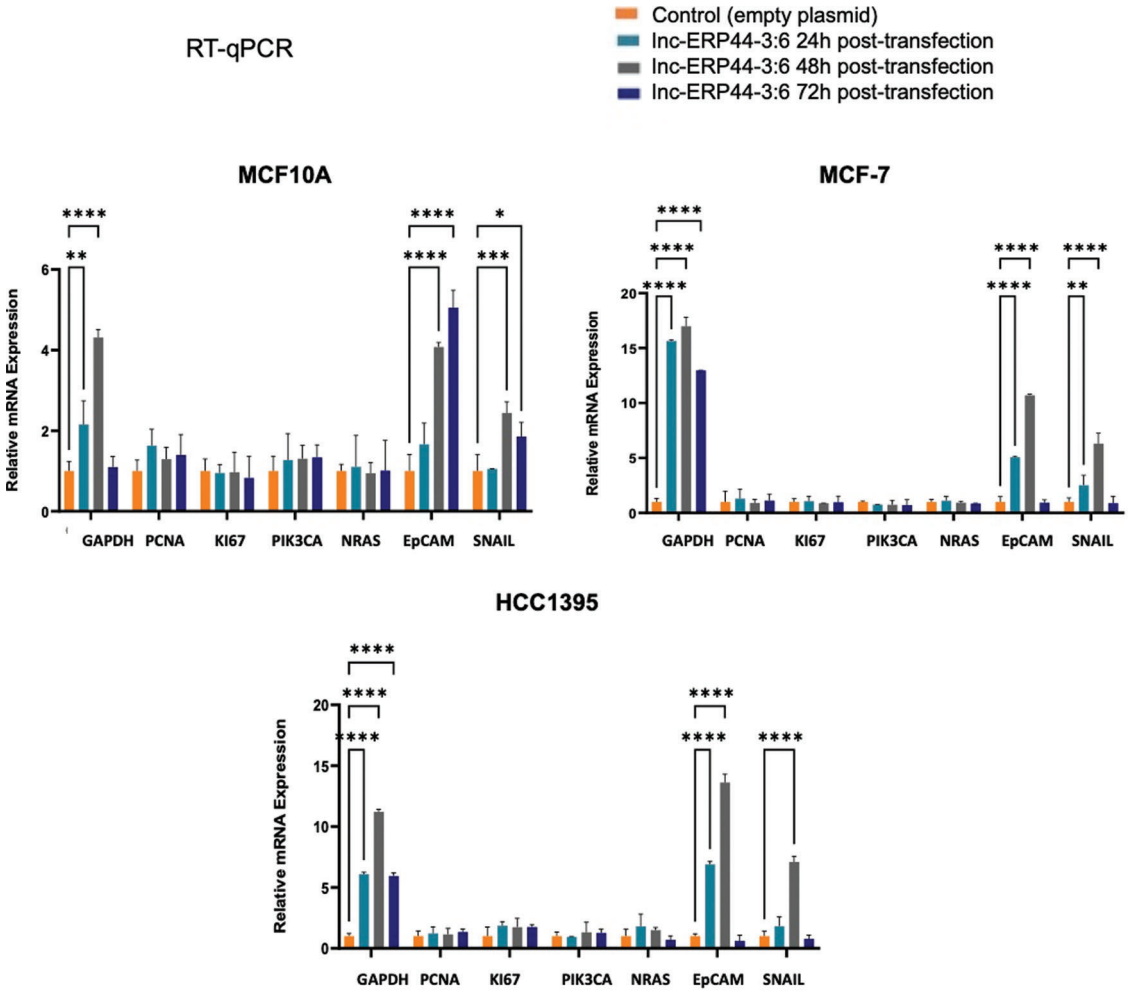


Figure S2: Expression pattern at 24, 48 and 72 h post-transfection of genes involved in BC. The relative mRNA expression of *GAPDH*, *PCNA*, *KI-67*, *PIK3CA*, *N-RAS*, *EpCAM* and *SNAIL* was measured in MCF10A, MCF-7 and HCC1395 cell lines at 24, 48 and 72 h post-transfection with control vector and *lnc-ERP44-3:6*. Two-way ANOVA, mean ± S.D. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

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