

H19 lncRNA mediates 17 β -estradiol-induced cell proliferation in MCF-7 breast cancer cells

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Abstract. Estrogen plays a critical role in breast cancer development and progression. However, the mechanism involved in the promotion of breast cancer development and progression by estrogen remains unclear although it has been intensively studied. In the present study, we investigated the estrogen inducibility and functional significance of H19 lncRNA in breast cancer cells and tumor tissues. The screening of 83 disease-related long non-coding RNAs (lncRNAs) revealed that H19 lncRNA was much higher in estrogen receptor (ER)-positive MCF-7 breast cancer cells than in ER-negative MDA-MB-231 cells. 17 β -estradiol produced a dose- and time-dependent induction of H19 expression in MCF-7 cells, which was mediated via ER α as evident by the blockade of this 17 β -estradiol effect with ICI 182780, a specific ER antagonist and knockdown of ER α using specific RNAi. Moreover, knockdown of H19 lncRNA decreased cell survival and blocked estrogen-induced cell growth while overexpression of H19 lncRNA stimulated cell proliferation. Quantitation of H19 lncRNA in human breast cancer tissues showed that the level of H19 lncRNA was >10-fold higher in ER-positive than in ER-negative tumor tissues. These results suggest that H19 is an estrogen-inducible gene and plays a key role in cell survival and in estrogen-induced cell proliferation in MCF-7 cells, indicating that H19 lncRNA may serve as a biomarker for breast cancer diagnosis and progression, and as a valuable target for breast cancer therapy.

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in females worldwide (1). As estrogens acting via estrogen receptors (ERs) play a critical role in breast cancer development, growth and progression, and over 70% of breast cancers are ER-positive (2); ER is therefore a valuable target for breast cancer therapy (3-5). Although it is well documented that estrogens such as 17 β -estradiol (E2), usually stimulate the proliferation of ER-positive breast cancer cells through the regulation of a large number of genes (6,7), it is a great challenge to determine which genes or gene networks underlie the estrogen proliferative response in breast cancer cells.

Recently, long non-coding RNAs (lncRNAs) have been emerged as an important target in cancer development and progression. Studies over the years have shown that lncRNAs, which are non-coding RNAs with a length larger than 200 bp, play a major role in embryogenesis and cell differentiation, and in the development and progression of various diseases including cancers (8-11). lncRNAs may function as either tumor suppressors or tumor promoters in such prevalent cancers as breast and prostate cancer (12). Among lncRNAs, Xist/Tsix, H19, HOTAIR and AIR are a few examples of lncRNAs whose functions have been investigated.

H19 is a well-known imprinted oncofetal gene, which is expressed on maternal alleles without a protein product (13), accumulates in the human placenta and several fetal tissues, and probably plays a pivotal role in embryogenesis and fetal growth and development (14). The H19 gene is localized at human chromosome 11p15.5 (15), in which a variety of disorders including cancer predisposition for both pediatric and adult tumors has been implicated (16). Aberrant expression of H19 is observed in numerous solid tumors, including hepatoma, bladder and breast cancer (16-18), and it has been suggested to be involved in oncogenesis and progression of various types of tumors (19).

Based on the previous observations that H19 expression is associated with the presence of estrogen and progesterone receptors (20), and estrogen regulates H19 expression in breast cancer cells (21), we hypothesized that estrogen-induced cell

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growth in ER-positive breast cancer cells is mediated through the upregulation of H19 gene expression. Our results indicate that H19 lncRNA is a critical factor in estrogen-stimulated cell growth in breast cancer cells, and blockade of H19 function is a potential strategy for the treatment of ER-positive breast cancer.

Materials and methods

Cell culture and hormone treatment. The MCF-7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in RPMI-1640 culture medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA), 20 mM L-glutamine, 100 U/ml of penicillin and 100 μ g/ml of streptomycin. All cells were maintained in a humidified incubator with 5% CO₂ at 37°C. Four days before drug treatment, cells were switched to phenol red-free RPMI-1640 medium supplemented with 5% charcoal-dextran-treated FBS (BI Technologies, Fullerton, CA, USA), 20 mM L-glutamine, 100 U/ml of penicillin and 100 μ g/ml of streptomycin. Medium was changed every 2 days, and cells were treated with vehicle control [0.1% dimethyl sulfoxide (DMSO)], various concentrations of E2 and ICI 182780 alone or in combination for the times as indicated in each experiment. All chemicals and hormones were obtained from Sigma-Aldrich (St. Louis, MO, USA) except those indicated.

Breast cancer tissue samples. Forty-five patients with breast cancer who underwent surgery at Xiangya Hospital were enrolled in the present study. The resected tumor specimens were immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction. The present study was approved by the Ethics Committee of Xiangya Hospital, China (project no. CTXY-140001-5) and a written informed consent was obtained from each subject.

RNA extraction and lncRNA screening. Total RNAs from harvested cells or tumor tissues were prepared using TRIzol reagent (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions, and the concentration was determined using a NanoDrop 2000. The quality of the RNA samples was in assurance with an A260/A230 ratio >1.7 and an A260/A280 ratio between 1.8 and 2.0. One microgram of total RNA was subjected to RT-PCR and used for the screening of lncRNAs by the Disease-Related Human lncRNA Profiler kit following the manufacturer's protocol (SBI, Mountain View, CA, USA). A change of log₂⁽ⁿ⁾ >2n was considered a meaningful change.

Quantitative real-time RT-PCR. Total RNA (500 ng) was reversely transcribed in a total volume of 20 μ l with 200 units of reverse transcriptase, 50 pmol random hexamer and 1 mM deoxynucleotide triphosphates. The reaction products were then diluted to a total volume of 100 μ l with distilled water. The real-time PCR reaction consisted of 2 μ l of diluted reverse transcription product, 1X SYBR-Green Master Mix (Applied Biosystems, Foster City, CA, USA) and 50 nM forward and reverse primers. The reaction was carried out in a Roche

LightCycler 480 Sequence Detection System for 40 cycles (95°C for 15 sec, 60°C for 1 min) after an initial 10-min incubation at 95°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were: H19, 5'-GTCCGGCCTTCCTGAACACCTT-3' and 5'-GCTTCACCTTCCAGAGCCGAT-3'; ER α , 5'-CCACCAA CCAGTGCACCATT-3' and 5'-GGTCTTTTCGTATCCCAC CTTTC-3'; and GAPDH, 5'-TTGATTTTGGAGGGATCTCG CTC-3' and 5'-GAGTCAACGGATTTGGTCGTATTG-3'. The level of RNA was expressed as a fold of the control calculated using the $\Delta\Delta$ Ct method.

Knockdown of H19 lncRNA using siRNA and shRNA. Synthetic RNA oligonucleotides targeting ER α and H19 were obtained from RiboBio Co., Ltd. (Guangzhou, China). The siRNA sequences used for ER α were: 5'-CCAGUGCACCAUUGUAU AAdTdT-3'. H19 lncRNA was knocked down using a specific H19 siRNA and a hairpin siRNA vector (shRNAH19) as described by Tsang and Kwok (22). The sequences of H19 siRNA were: 5'-CCTGTAACCAAAAGTGACCG-3'; and the H19 hairpin siRNA sequences were: 5'-CATCAAAGACACCA TCGGA-3' that were subcloned into pSilencer 2.1-U6 neovector (shRNAH19) (RiboBio Co., Ltd.). A negative control siRNA and shRNA were purchased from RiboBio.

H19 expression vector construction. Total cellular RNAs from MCF-7 cells were subjected to reverse-transcription as described above. To construct the H19 expression vector, a 2-step strategy was used. First, the entire H19 RNA was amplified as two fragments by PCR using the following pairs of primers: 5'-AAAAGGATCCAGGGCCCTGCTCTGATTG G-3' and 5'-AAAAAAGCTTCCTCGTCTCCAGCCCCGAA C-3' for the upstream fragment (1,430 bp); and 5'-GGGCGGG GCGGAGTGAATGAGC-3' and 5'-AAAAAAGCTTTTGTCT GTAACAGTGTATTGATGA-3' for the downstream fragment (1,170 bp). These two PCR fragments were inserted into the pGEM-T easy vector and amplified following transformation to *E. coli*. Subsequently, the upstream and downstream H19 fragments in the pGEM-T easy vectors were digested with *Bam*HI plus *Hind*III and *Bbv*CI plus *Hind*III, respectively, ligated and inserted into the pcDNA3.1 vector as a full-length H19 cDNA. The sequences of the H19 expression vector were confirmed by DNA sequencing.

Transfection study. Cells were plated in phenol red-free medium containing 5% stripped FBS in 12-well plates at a density of ~70%. The transfections of various doses of siRNA or shRNA were performed using Lipofectamine RNAiMAX or Lipofectamine 2000 reagent (Invitrogen, Life Technologies, USA) following the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with various hormones for different times and various analyses were performed as indicated in each experiment.

Western blot analysis. Western blotting was performed as previously described with minor modifications (23). Briefly, total cellular proteins were extracted from the harvested cells using a lysis buffer [62.5 mM Tris-HCl pH 6.8, 100 mM dithiothreitol (DTT), 2% SDS and 10% glycerol]. The protein concentrations were determined using the Bradford method

Table I. The differentially expressed lncRNAs between the MCF-7 and MDA-MB-231 cells.

lncRNA	Fold-difference (log ² ⁽ⁿ⁾) n-value
Upregulated	
ANRIL	13.20
H19	10.33
NCRMS	9.81
HOTAIR	9.61
HOXA1AS AA489505	9.55
HOTAIRM1	9.52
HAR1B	7.11
LOC285194	5.97
PCGEM1	5.51
HOXA3AS BI823151	3.68
HOXA6AS AK092154	3.32
PCAT-29	3.22
BC017743	3.05
ST7OT3	2.91
BC200	2.80
H19-AS	2.40
HOXA11AS	2.19
BC043430	2.14
Downregulated	
HOTTIP	-2.03
UCA1	-2.05
DGCR5	-2.06
AK023948	-2.15
PCAT-32	-2.20
LincRNA-SFMBT2	-3.24
ZEB2NAT	-4.78

The fold-difference between MCF-7 and MDA-MB-231 cells was calculated by referencing the level of each lncRNA in MDA-MB-231 cells as 1. lncRNA, long non-coding RNA.

with the Bio-Rad protein assay following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Cellular proteins were separated on sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline with 0.5% Tween, TBS-T) at room temperature for 2 h. After washing with TBS-T, the nitrocellulose was incubated with a specific antibody against ER α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β -actin (AC-15; Sigma Chemical Co., St. Louis, MO, USA) overnight at 4°C. Following the incubation with a horseradish peroxidase-conjugated secondary antibody, the signal was detected using an ECL Western Blotting system (Promega, Madison WI, USA) and visualized using the Bio-Rad ChemiDoc MP system.

Cell proliferation assay. For the determination of cell proliferation, MCF-7 cells were plated in 96-well plates at

Table II. The lncRNAs with a change in expression in MCF-7 cells following treatment with 17 β -estradiol.

lncRNA	Fold-difference (log ² ⁽ⁿ⁾) n-value
Upregulated	
H19	2.89
Downregulated	
HOXA1AS AA489505	-6.93
PCAT-29	-3.73
TU_0017629	-4.60

The fold-difference was calculated by comparing cells treated with and without 17 β -estradiol (100 nM). lncRNA, long non-coding RNA.

~10,000 cells/well in phenol red-free RPMI-1640 media containing 5% charcoal-dextran-stripped FBS. Twenty-four hours after plating, the cells were treated with either vehicle control or various hormones as indicated in each experiment. For H19 knockdown and overexpression study, the cells were transfected with siRNA, shRNA or H19 expression vector for 24 h, and then treated with various hormones for 48 h. Cell proliferation was assayed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay as instructed by the manufacturer (Promega).

Statistical analysis. Each experiment was performed at least three times, and the data are presented as mean \pm SEM. For parametric data, the Student's t-test was used to determine the statistical significance between two groups, and one-way ANOVA following *post-hoc* Student-Newman-Keuls test was used to compare the difference among multiple groups using the SPSS software. A p-value <0.05 was considered to indicate a statistically significant result.

Results

Screening lncRNAs associated with estrogen action in breast cancer cells. To identify lncRNAs that are potentially associated with ER-positive breast cancer cells and with estrogen action, we employed the Disease-Related Human lncRNA Profiler kit to screen the expression levels of 83 disease-related lncRNAs in the breast cancer cells. As shown in Table I, out of the 83 lncRNAs screened, 18 had a higher expression and 7 had a lower expression as defined by a log²⁽ⁿ⁾ >2n in the ER-positive MCF-7 cells compared to the ER-negative MDA-MB-231 cells. One of the highest differentially expressed lncRNAs was H19 lncRNA with ~3,300-fold difference between the MCF-7 and MDA-MB-231 cells, which was confirmed using quantitative RT-PCR analysis (1.00 \pm 0.04 in MDA-MB-231 cells vs. 3,335.48 \pm 965.37 in MCF-7 cells). Moreover, out of the 83 lncRNAs screened, only H19 was upregulated while 3 lncRNAs were downregulated when MCF-7 cells were treated with 100 nM E2 for 48 h as shown in Table II.

H19 is upregulated by E2 in ER-positive MCF-7 cells. To further analyze the E2 regulation of H19 expression in breast

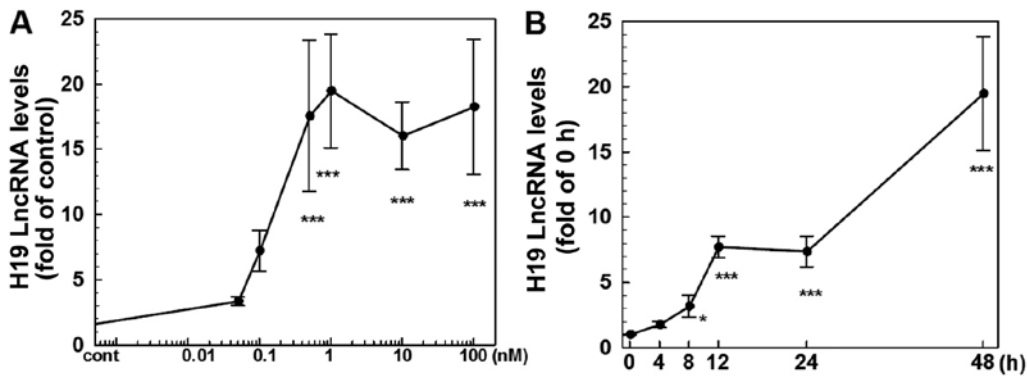


Figure 1. 17 β -estradiol (E2) induces H19 lncRNA expression in a (A) dose- and (B) time-dependent manner. MCF-7 cells were grown in phenol-red-free RPMI-1640 media and treated with various concentrations of E2 for 48 h (A), or with 1 nM E2 for different times (B). Total cellular RNAs were extracted from the cells and H19 lncRNA levels were quantitated by real-time PCR as described in Materials and methods. The data are the mean \pm SEM of three independent experiments. * P <0.05 and *** P <0.001 compared to the corresponding control. lncRNA, long non-coding RNA.

cancer cells, MCF-7 cells were treated with various doses of E2 for 4–48 h. As shown in Fig. 1, E2 produced a dose (Fig. 1A) and time-dependent (Fig. 1B) induction of H19 expression. At 48 h of 1 nM E2 treatment, the level of H19 RNA was increased ~19-fold while there was no significant change observed at 4 h of treatment. At doses ranging from 0.05 to 1 nM, E2 caused a dose-dependent induction of H19 expression with a maximal effect observed at 1 nM (Fig. 1A). On the other hand, treatment of ER-negative MDA-MB-231 cells with E2 at doses up to 100 nM failed to induce H19 expression (data not shown).

E2-induced H19 expression is mediated through ER α . The genomic action of estrogens is mainly mediated through estrogen receptors, ER α and/or ER β . ER α yet not ER β , was expressed in the MCF-7 cells as demonstrated by RT-PCR and western blot analysis (Fig. 2, and data not shown). To determine whether E2 induction of H19 expression is mediated via ER α , a specific ER antagonist, ICI 182780, and a specific ER α siRNA were employed to block ER α action and to knock down ER α expression in the MCF-7 cells, respectively. Transfection of a specific ER α siRNA (100 nM) into MCF-7 cells resulted in a knockdown of ER α of >70% as demonstrated by western blotting (Fig. 2A), leading to a complete elimination of E2-induced H19 expression (Fig. 2A). Furthermore, the E2-induced H19 expression was significantly blocked by ICI 182780, a specific ER antagonist, in a dose-dependent manner (Fig. 2B). The addition of 10 μ M ICI 182780 in the medium completely blocked the E2 induction of H19 expression. These data revealed that the E2 induction of H19 expression in MCF-7 cells was mediated through ER α .

H19 lncRNA promotes cell growth in MCF-7 cells. The functional importance of H19 lncRNA was investigated by analyzing cell growth and cell cycle distribution in the MCF-7 cells. Treatment of MCF-7 cells with E2 produced a dose-dependent increase in viable cell number, and an ~30% induction of viable cells was observed at 1 nM E2 treatment (Fig. 3B). This E2-induced cell growth was completely blocked by transfection of either a specific siRNA (Fig. 3C) or a specific shRNA (Fig. 3D), which knocked down H19 lncRNA by 70 and 55%, respectively (Fig. 3A). In contrast, the transfection of either a non-specific siRNA or a non-specific

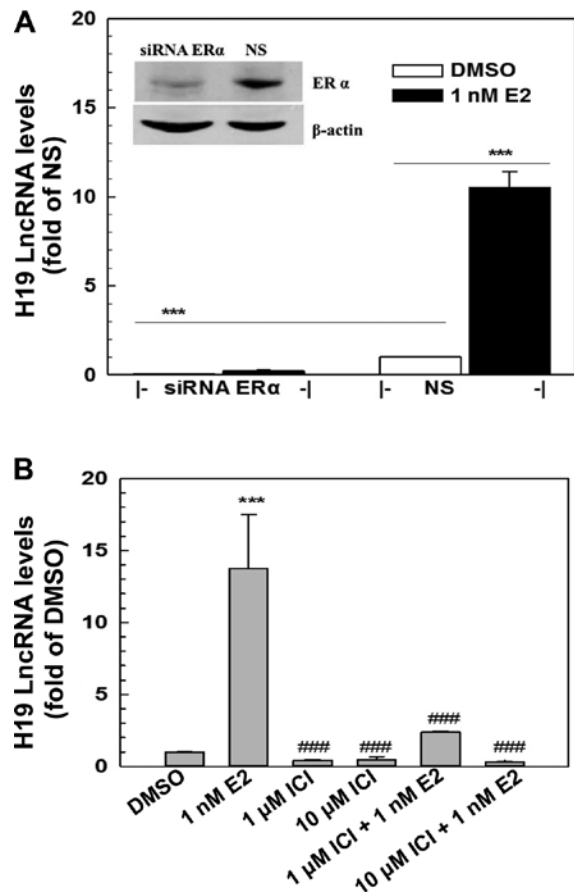


Figure 2. E2-induced H19 expression is mediated via ER α in MCF-7 cells. (A) MCF-7 cells were transfected with 100 nM of a specific ER α siRNA or a non-specific siRNA (NS) for 24 h, and then treated with vehicle control (0.1% DMSO) or 1 nM E2 for 48 h. Western blot analysis of ER α is presented in the insert. (B) MCF-7 cells were treated with 1 nM E2 in the presence or absence of 1 μ M or 10 μ M ICI 182780 (ICI). Total cellular RNAs were extracted from the cells, and H19 lncRNA levels were quantitated by real-time PCR as described in Materials and methods. The data are the mean \pm SEM of three independent experiments. *** P <0.001 compared to the corresponding control and #### P <0.001 compared to 1 nM E2 treatment. lncRNA, long non-coding RNA; DMSO, dimethyl sulfoxide.

shRNA that did not affect H19 expression had no effect on E2-induced cell growth. Notably, knockdown of H19 lncRNA

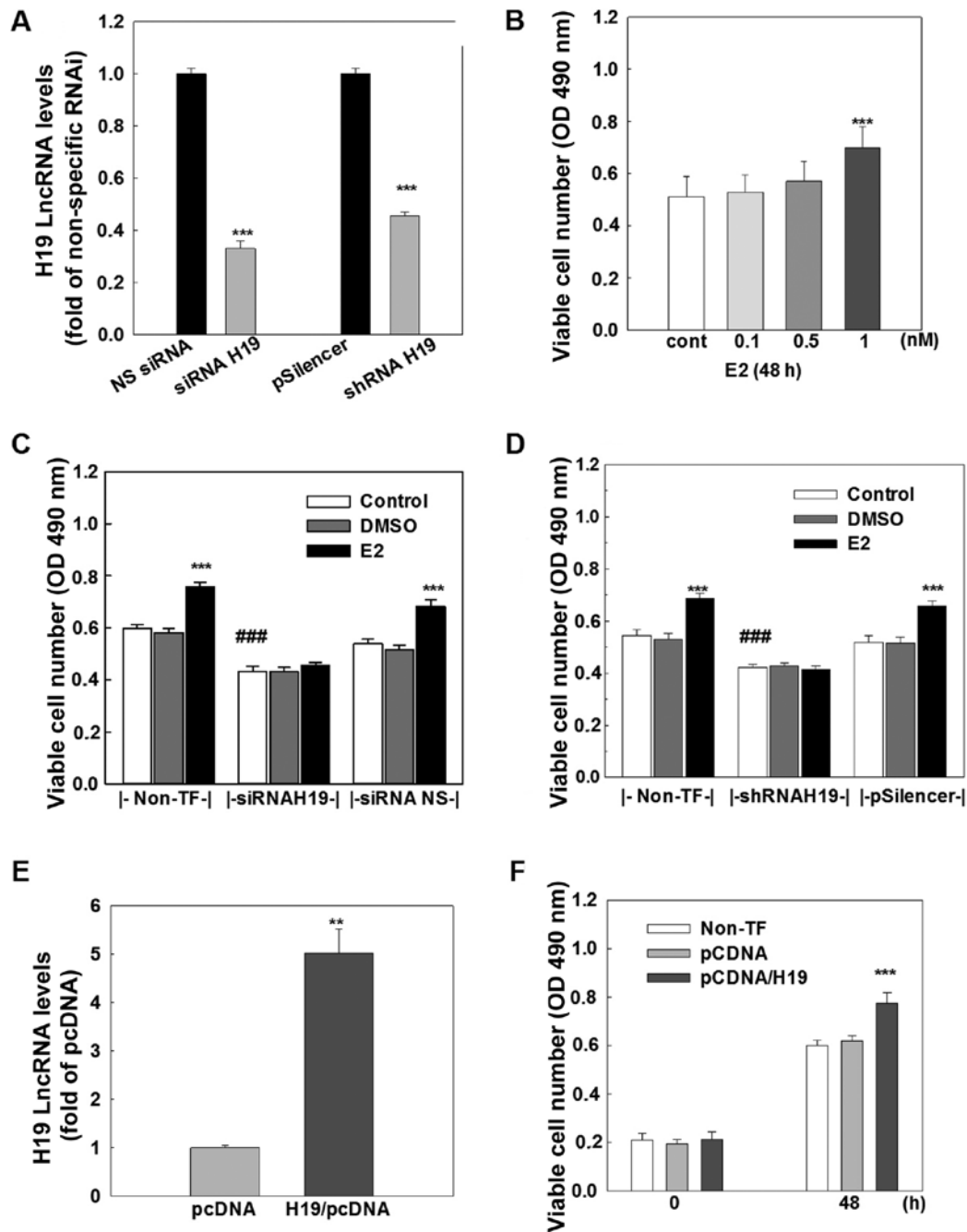


Figure 3. H19 lncRNA mediates cell survival and E2-induced cell growth in MCF-7 cells. MCF-7 cells were transfected with either a specific H19 siRNA (200 nM), or an H19 shRNA vector (1 μ g), or a non-specific (NS) siRNA or a negative control vector (pSilencer) for 24 h. The transfected cells were then harvested for quantification of H19 lncRNA by real-time PCR (A), or treated with or without 1 nM E2 for 48 h for the determination of cell proliferation by MTS assay (C and D). (B) MCF-7 cells were treated with various doses of E2 for 48 h and the viable cell number was determined by MTS assay. (E and F) MCF-7 cells were transfected with an H19 expression vector (H19/pcDNA) or the empty vector control (pcDNA) for 48 h, and the cells were then used for the quantification of H19 lncRNA levels by real-time PCR (E), or for the determination of viable cell numbers by MTS assay (F). All data are presented as mean \pm SEM of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ compared to the corresponding control; ### $P < 0.001$ compared to the corresponding Non-TF group. Non-TF, non-transfection. lncRNA, long non-coding RNA.

caused a significant decrease (~30%) in viable cell number in the MCF-7 cells without E2 treatment (Fig. 3C and D).

Consistent with the cell growth study, cell cycle analysis with flow cytometry demonstrated that E2 treatment increased the fraction of S-phase cells, an indication of an increase in DNA biosynthesis (Fig. 4). This E2-induced elevation in the S-phase cell fraction was eliminated by the knockdown of H19 lncRNA using either a specific siRNA (Fig. 4A) or a specific

shRNA (Fig. 4B). Similarly, knockdown of H19 lncRNA decreased the S-phase fraction of cells in MCF-7 cells without E2 treatment. This analysis demonstrated that expression of H19 is important for the survival and maintenance of MCF-7 breast cancer cells.

To further determine the role of H19 lncRNA in cell growth, H19 lncRNA was overexpressed in the MCF-7 cells. The transfection of an H19 expression vector for 48 h resulted

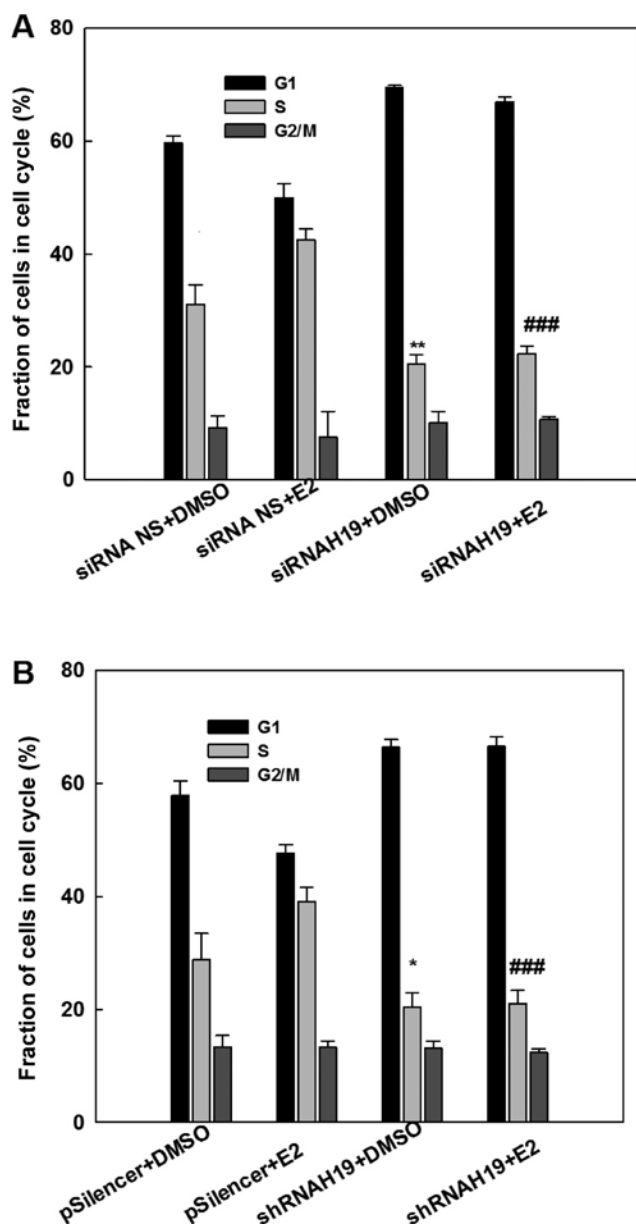


Figure 4. H19 knockdown decreases the fraction of S-phase cells and blocks E2-induced S-phase cells in the MCF-7 cells. MCF-7 cells were transfected with either (A) a specific H19 siRNA (200 nM) or a non-specific (NS) siRNA, or an (B) H19 shRNA vector (1 μ g) or a negative control vector (pSilencer) for 24 h, and then treated with a vehicle control (0.1% DMSO) or 1 nM E2 for 48 h. At the end of the experiments, the cells were harvested, and the fraction of cells in the G1, S and G2/M phases of the cell cycle was quantitated using flow cytometry. The data are the mean \pm SEM of two independent experiments. *P<0.05 and **P<0.01 compared to the corresponding control group; ###P<0.001 compared to the corresponding E2-treated group. lncRNA, long non-coding RNA; DMSO, dimethyl sulfoxide.

in a 4-5-fold increase in the H19 lncRNA level in the MCF-7 cells (Fig. 3E) and a significant increase in cell growth, which was not observed in cells transfected with a negative control vector (Fig. 3F). Taken together, these data suggest that H19 lncRNA possesses cell proliferative activity and the E2-induced cell growth is mediated through upregulation of H19 gene expression in MCF-7 breast cancer cells.

The level of H19 lncRNA is higher in the ER-positive than that in the ER-negative breast cancer tissues. To explore

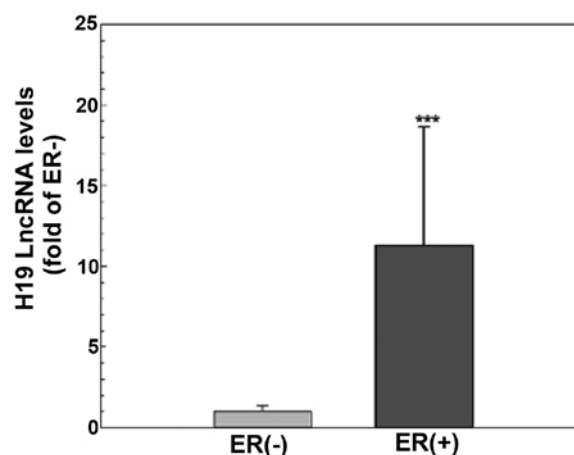


Figure 5. The level of H19 lncRNA is much higher in ER-positive than in ER-negative breast cancer tissues. Breast cancer tissues were collected from patients with ER-positive (n=30) or ER-negative (n=15) breast cancer and the levels of H19 lncRNA were determined using real-time PCR. ***P<0.001 compared to the ER (-) group. lncRNA, long non-coding RNA.

the functional significance of H19 lncRNA and the estrogen inducibility of H19 expression *in vivo*, the levels of H19 lncRNA in primary tumors from breast cancer patients (n=45) were evaluated using quantitative RT-PCR. As shown in Fig. 5, tumor tissues from patients with ER-positive breast cancer (n=30) had a significantly higher H19 gene expression compared to those from ER-negative patients (n=15, p<0.001), suggesting that H19 expression is dependent on and/or induced by ER action in breast cancer cells.

Discussion

In the present study, we demonstrated that 17 β -estradiol mediated through ER α produced a dose- and time-dependent induction of H19 lncRNA expression in the MCF-7 breast cancer cells. Most notably, knockdown of H19 lncRNA by RNAi in the MCF-7 cells resulted in a decrease in viable cell number and a blockade of estrogen-induced cell proliferation (Fig. 3). These results indicate that H19 lncRNA plays a significant role in cell survival and estrogen-induced cell proliferation in MCF-7 cells.

H19 RNA is originally identified as a transcribed, spliced and polyadenylated untranslatable RNA molecule (24), now termed as lncRNA. Functional analysis of H19 lncRNA has linked it to both oncogenic and tumor-suppressive activities (25). Although H19 lncRNA has been shown to cause growth retardation and morphological changes in tumor cells and abrogate the tumorigenicity of G401 tumor cells in nude mice following ectopic overexpression (26), growing evidence suggests that H19 lncRNA possesses oncogenic, proliferative and anti-apoptotic activity in various *in vitro* and *in vivo* systems (26). Baryte-Lovejoy *et al* (27) reported that H19 lncRNA was directly induced by the c-Myc oncogene, resulting in an H19-associated promotion of clonogenicity and anchorage-independent growth in lung and breast cancer cells. Lottin *et al* showed that ectopic overexpression of H19 lncRNA in MDA-MB-231 breast cancer cells promoted tumor progression in a xenograft animal model (19). In the present study, we demonstrated that ectopic overexpression of H19

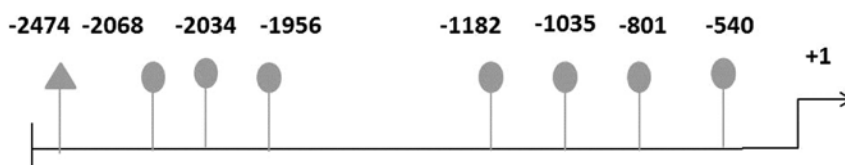


Figure 6. Multiple potential estrogen-response elements (EREs) are present in the promoter region of the H19 gene. The 5'-flanking promoter region of the human H19 gene (GenBank accession no. AC000143) is illustrated with a straight line. The numbers on the top row indicate the nucleotide position upstream from the transcriptional start site which is considered as +1. Solid grey circles indicate potential EREs which match five bases out of the six bases of the consensus half ERE sequence (AGGTCA or TGACCT); and the solid grey triangle indicates a potential ERE which matches the consensus half ERE. lncRNA, long non-coding RNA.

lncRNA in MCF-7 cells significantly increased cell growth while H19 lncRNA knockdown resulted in a decrease in cell survival (Figs. 3 and 4), further indicating the significance of H19 lncRNA in tumor cell proliferation and survival. Moreover, we observed that knockdown of estrogen-induced H19 lncRNA expression completely blocked estrogen-induced cell growth in MCF-7 cells (Figs. 3 and 4), suggesting that H19 lncRNA is a key factor mediating estrogen-induced cell growth in ER-positive breast cancer cells. Although the molecular mechanism of how H19 lncRNA stimulates cell proliferation remains to be investigated, a previous study demonstrated that H19 promoted the cell entry into S-phase in breast cancer cells through the E2F1 factor (20). Taken together, these data support the concept that H19 lncRNA plays a critical role in breast cancer development and progression, and it is therefore a valuable target for breast cancer therapy.

Previous studies have shown that H19 lncRNA is overexpressed in breast cancer cells (18,28,29). To determine whether H19 expression is associated with estrogen action, we compared the expression levels of 83 lncRNAs including H19 between an ER-positive and an ER-negative breast cancer cell line, and evaluated the inducibility of the 83 lncRNAs upon 17 β -estradiol treatment in ER-positive MCF-7 cells. We revealed that the H19 lncRNA level was much higher in the ER-positive MCF-7 cells compared to the ER-negative MDA-MB-231 cells, and 17 β -estradiol produced a dose- and time-dependent induction of H19 gene expression in the ER-positive MCF-7 yet not in the ER-negative MDA-MB-231 cells (Figs. 1 and 2, Tables I and II). The demonstration of estrogen induction of H19 expression is consistent with a previous report by Adriaenssens *et al*, in which an estrogen-associated induction of H19 expression was observed in mouse mammary gland and uterus as well as in MCF-7 cells (21). Using both chemical and genetic approaches, we further elucidated that the estrogen induction of H19 gene expression was mediated through ER α (Fig. 2). ICI 182780, a specific ER antagonist, inhibited 17 β -estradiol-induced H19 expression in a dose-dependent manner; and knockdown of ER α by either a specific siRNA or an shRNA blocked this estrogen action. Furthermore, bioinformatic analysis of the H19 promoter region showed that multiple potential estrogen-response elements (EREs) are present in the promoter region of the H19 gene (Fig. 6) although the functional significance of these elements remains to be elucidated experimentally. Based on current and previous studies, we propose that similar to the estrogen regulation of protein-encoding genes, estrogen upregulation of lncRNA H19 expression is mediated through the traditional genomic pathway via the estrogen-ER α complex interacting with EREs in the H19 promoter region.

The biological significance of H19 lncRNA is further illustrated by quantification of H19 lncRNA levels in breast cancer tissues. Consistent with a previous observation of a steroid receptor dependency in H19 expression (18) and the screening result of a higher level of H19 lncRNA in ER-positive MCF-7 cells (Table I), we demonstrated that H19 lncRNA expression was >10-fold higher in the ER-positive tumor tissues compared to the ER-negative tissues (Fig. 5). These results not only provide evidence to support that H19 expression is dependent on estrogen-ER action, yet also indicate that H19 lncRNA is a valuable target for the diagnosis and treatment of ER-positive breast cancers.

In summary, lncRNAs have emerged as a critical field in cancer development and progression, and H19 lncRNA has been shown to be an oncogenic factor in breast cancer. The present study provides additional valuable evidence that H19 lncRNA plays a crucial role in cell survival and proliferation in ER-positive breast cancer cells. Our results indicate that H19 expression is ER-dependent and induced by estrogen through the traditional genomic pathway of estrogen-ER interaction with the gene. Since H19 is an ER-dependent gene, and mediates estrogen-induced cell proliferation, it may serve as a potential biomarker for breast cancer diagnosis and progression and as a valuable target for breast cancer therapy.

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