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17β-Estradiol treatment inhibits breast cell proliferation, migration and invasion by decreasing MALAT-1 RNA level

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ABSTRACT

Breast cancer cells, which express estrogen receptor α (ERα), respond to estrogen in a concentration dependent fashion, resulting in proliferation or apoptosis. But breast cancer cells without ERα show no effect on low concentration of estrogen treatment. Proliferation, migration and invasion of MCF10a, MCF7 and MB231 cells treated with low (1 nM) or high (100 nM) dose of 17β-Estradiol (E2) was performed. We identified the effects of E2 on these breast cell lines, and looked for the difference in the presence and absence of ERα. Specifically, we looked for the changes of long non-coding RNA metastasis associated lung adenocarcinoma transcript 1 (MALAT-1), which is found extensively and highly expressed in several kinds of tumor cells, including breast carcinoma. It was observed that proliferation, migration and invasion of breast cells were greatly affected by high concentration E2 treatment and were not affected by low concentration E2 treatment in an ERα independent way. We found that the high concentration E2 treatment largely decreased MALAT-1 RNA level. Interestingly, MALAT-1 decreasing by knocking down showed similar effects on proliferation, migration and invasion. E2 treatment affects breast tumor or non-tumor cells proliferation, migration and invasion in an ERα -independent, but a dose-dependent way by decreasing the MALAT-1 RNA level.

1. Introduction

Estrogens are an important class of hormones in the physiology of mammals, regulating cell proliferation, differentiation and apoptosis [1,2]. The effects of estrogens including E2 are depending on their bound to estrogen receptors. After bound by estrogen ligands, the ERαs localized in the cytoplasm dissociate from the heat shock proteins and translocate to the nucleus. In the nucleus, the complex of ligands and ERα functions as a transcription factor (TF), binds to target gene promoter or transcriptional regulating domain, and thereby modulating their expression [3]. Vast amount of evidence indicates that high E2 level in postmenopausal women increases their chance to get breast cancer [4]. E2s act via estrogen receptor (ERα) to affect cell proliferation of breast cell [5], as well as its differentiation and migration ability.

The cellular response to estrogens is concentration-dependent. In MCF7 and MCF10a cells, which express functional ERα, E2 promotes cell proliferation at low concentration (1 nM) [6]. In MCF7, 10 nM or lower concentration of E2 promote cell proliferation, [7], 10 nM or higher concentration of E2 causes apoptosis [8]. Previous studies have been revealed that metabolites of E2 interact with the genome to induce oncogenic mutations [9,10]. Therefore, E2 may affect cells in both ER-dependent and ER-independent ways.

Epithelial–mesenchymal transition (EMT) is a precisely regulated process, through which epithelial cells lose polarity and cell to cell junction and gain a fibroblast like morphology. During EMT, the epithelial protein level, such as E-cadherin and γ-catenin [11], are downregulated; while mesenchymal protein, such as N-cadherin, fibronectin and vimentin are upregulated [12].

MALAT-1 gene that is located in 11q13.1 of human genome, encodes a long non-coding RNA. MALAT-1 is highly conserved in mammals and highly and ubiquity expressed in various tissue [13]. Previous studies have shown that MALAT-1 participate in cell differentiation and development [14,15]. For example, depletion of MALAT-1 in Hela cell causes decrease of cell growth rate by sticking cells in G2/M phase [16]. It was also reported that MALAT-1 has functions in the regulation of translation. Lines of evidence indicates that MALAT-1 play important roles in tumor cells. Interestingly, MALAT-1 induces migration and tumor growth in non-small cell lung cancer [17]. Despite all the evidences above suggesting MALAT-1's involvement in cell proliferation and differentiation, its physiological significance in tissues and organs remains unclear.

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In this study, to investigate the effects of E2 on breast cancer and explore the mechanism underlying, we tested the ability of E2 to cause inhibition of EMT, proliferation, migration and invasion of breast cancer cell lines and showed the potential roles of MALAT-1 in these processes.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MB231 and MCF7 were cultured in Minimum Essential Medium Eagle (without phenol red; Life technologies, Santa Ana, CA) supplemented with 1 mM sodium pyruvate, 24 mM $NaHCO_3$, 4 mM L-glutamine, 10% fetal bovine serum (Gibco, Santa Ana, CA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, in 5% CO_2. Human breast cell line MCF10a was cultured in DMEM: F-12 Medium with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, in 5% CO_2. H460 non-small-cell lung carcinoma cell line, the PA-1 ovarian cancer cell line, and the U2OS cell lines were grown in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C, in 5% CO_2.

2.2. Cloning and transfection of an estrogen–receptor expression vector

Coding sequence (1788 bp) of human estrogen receptor was, amplified using long distance polymerase chain reaction (Expand Long Range dNTPack, Roche Diagnostics). Nhe I restriction enzyme site was included in PCR primer 1 (5′-GCCGGCGCTAGCATGCCATGACCCTCCACACCA-3′), and Hind III restriction enzyme site was included in primer 2 (5′-CCTTAACTTAAGCAGACCGTGGCAGGGAAACCC-3′). The reaction followed the steps as below: (1) pre-denaturation in 95 °C for 5 min; (2) 35 cycles: 30 s denaturation in 95 °C, 30 s annealing in 58 °C, 3 min extension in 72 °C; (3) final extension in 72 °C for 10 min. PCR products were subject to gel electrophoresis, and band of the right size was cut from the gel. The PCR product was digested with Nhe I and Hind III and inserted into multiple clone sites of pcDNA3.1 (Life Tech.).

2.3. Design and cloning of short hairpin RNA constructs

Short hairpin RNA (shRNA) sequence targeting human MALAT-1 and the scrambled sequence as negative control were designed on DSIR (Designer of Small Interfering RNA, http://biodev.extra.cea.fr/DSIR/DSIR/html) and subcloned into psilencer4.1-CMV-neo vector. Specificity of the shRNA sequences was verified via BLAST search in www.ncbi.nih.nlm.gov. The nucleotide sequence was as follow: shRNA-F (5′-GGAAGATAGAAACAAGATATATCTTGTTTCTATCTTCC-3′); and shRNA-R (5′-GGAAGATAGAAACAAGATATATCTTGTTTCTATCTTCC-3′); negative control shScramble-F (5′-AGATCCGTATAGTGTACCTTATAAGGTACACTATACGGATCT-3′); shScramble-R (5′-AGATCCGTATAGTGTACCTTATAAGGTACACTATACGGATCT-3′). After transfecting the vector into MB231 cell, neomycin (1 mg/ml, Sigma–Aldrich) was added into the culture medium to obtain cell clones that stably express the shRNA.

2.4. Quantitative mRNA analysis

Total RNA was extracted by using Qiagen RNeasy Micro Kit. 1 μg of the total RNA of each sample was used for reverse transcription using moloney murine leukemia virus reverse transcription system (Life Tech.) with random hexamer primers. The primers for human PSF mRNA were as follows: human (NR_002819) P3: (5′-AAAGCAAGGTCTCCCCACAAG-3′); human P4: (5′-GGTCTGTGCTAGATCAAAAGGCA-3′). The quantitative PCR reaction was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Melting curve was used to analyze specificity of the PCR products. Each sample was duplicated in performing the PCR. GAPDH was used as internal control.

2.5. Northern analysis

Total RNA (5–10 mg) was electrophoresed in a 0.8% agarose gel containing 1 × MOPS, 6% formaldehyde, and then transferred to Hybond Nylon membranes in 10 × SSC containing 1.5 M NaCl, 0.15 M $Na3C_6H_5O_7$). DNA probes were firstly synthesized using Nor-F: (5′-GACTGGAGCTGCCTTTTGTCCTTGGAAG-3′); and Nor-R: (5′-ATCTCCCACCTGCCTAAGGTACTTAG-3′) and then end-labeled with biotin using the Pierce® RNA 3′ End Biotinylation Kit (Thermo Scientific, USA). The nylon membranes were incubated at 68 °C for 2 h in hybridization buffer containing 5 × SSC, 1% Ficoll, 0.5% polyvinylpyrrolidone (PVP), 0.5% bovine serum albumin (BSA), 0.5% sodium dodecyl sulfate (SDS), 100 mg/ml herring sperm DNA) with 50 ng probe. Excess probe was removed from the membrane by serial washes at 65 °C in 2 × SSC containing 0.1% SDS. An β-actin probe was end-labeled as internal control. The hybridized probes were visualized by exposure of the membranes to BioMax MS X-ray film (Kodak). X-ray films were scanned using a densitometer (Molecular Dynamics, Sunnyvale, USA).

2.6. Migration assay

About 5×10^5 MB231 cells (in 100 μL DMEM with 10% FBS) were seeded onto the upper part of a Transwell chamber (Transwell filter inserts in 6.5 mm diameter with a pore size of 5 μm; Corning Incorporated). 600 μL of the same medium was added into the chamber's lower part. The assay was performed for 16 h at 37 °C, in 5% CO_2. Flow cytometry was used in analyzing the migrated cells. Each sample was triplicated, and the assay was performed three times independently.

2.7. Scratch/wound healing assay

The wound healing assay (Cell Biolabs) was used to analyze migration of MB231 WT, E2 treated MB231, and MALAT1 knockout cells. According to the manufacturer's instruction, 2 × 105 cells were used per well. After removing the inserts (0 h), images of wound fields were acquired. Wound closure was documented 24 h after. The phase-contrast microscope (Leica Microsystems) in use was equipped with a digital camera (Leica DFC300FX). Adobe Photoshop CS3 software was employed to analyze the images acquired.

2.8. Transwell assay

Cell migration assays were performed using Transwell migration chambers (8 μm pore size, Costar) according to the vendor's instructions. For all cells, 2×10^4 cells were plated into the insert of the well, and representative photos were taken at 100× magnifications.

2.9. Soft agar colony formation assay

For each 35-mm tissue culture dish, soft agar was made of 3 ml medium (MEM; Invitrogen) with 10% FBS, 0.33% BD Difco Agar (Beckton Dickinson GmbH, Heidelberg, Germany), as well as about 5 × 103 MB231 WT, E2 treated MB231, and MALAT1 knockout cells respectively. Cells were cultured at 37 °C, in 5% CO_2, under high humidity condition. Colony counting was performed 21 days after.

2.10. Cell proliferation assay

For each well of a flat-bottom 6-well dish, 5 × 103 of MB231 WT, E2 treated MB231, and MALAT1 knockout cell were respectively cultured in MEM with 10% FBS at 37 °C, in 5% CO_2. CellTiter 96 (Promega Corporation, Madison, WI) was added to each well in accordance with the manufacturer's instructions. To determine cell viability, the absorbance at 490 nm was measured using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK) every 24 h. Each sample was triplicate in analysis, and was repeated twice independently. 100 μL DMEM was used as blank control.

2.11. Data analysis

All reguls were presented as average ± SEM. Student's *t*-test was used for comparison between two groups. The difference is considered as statistically significant when *p* values are less than 0.05.

3. Results

3.1. E2 treatment inhibits proliferation and tumor formation of breast cells

To test the effects of E2 on proliferation of breast cancer cell line, we treated breast cell lines including MCF10a, MCF7 and MB231 with different concentration of E2. 1 nM E2 significantly increase MCF10a and MCF7 proliferation 7 days after treatment, while showed no effect on MB231 proliferation. When E2 concentration was increased to 100 nM, inhibition of all three cell lines became significant 4 days after treatment. The inhibition rates were elevated with the increase of concentration of E2 (Fig. 1A). We also found E2 (100 nM) inhibit colony formation (Fig. 1B). 1 nM or higher concentration of E2 arrested breast cells at G1 phrase. Moreover, 100 nM E2 also increased the portion of cells in S phrase. (Fig. 1C). Notably, 24 h after the removement of E2, G1-phase cells nearly went back to original level compared with untreated control (Fig. 1D), indicating the inhibition of cell cycle progression induced by E2 treatment is revisable. Several cell lines

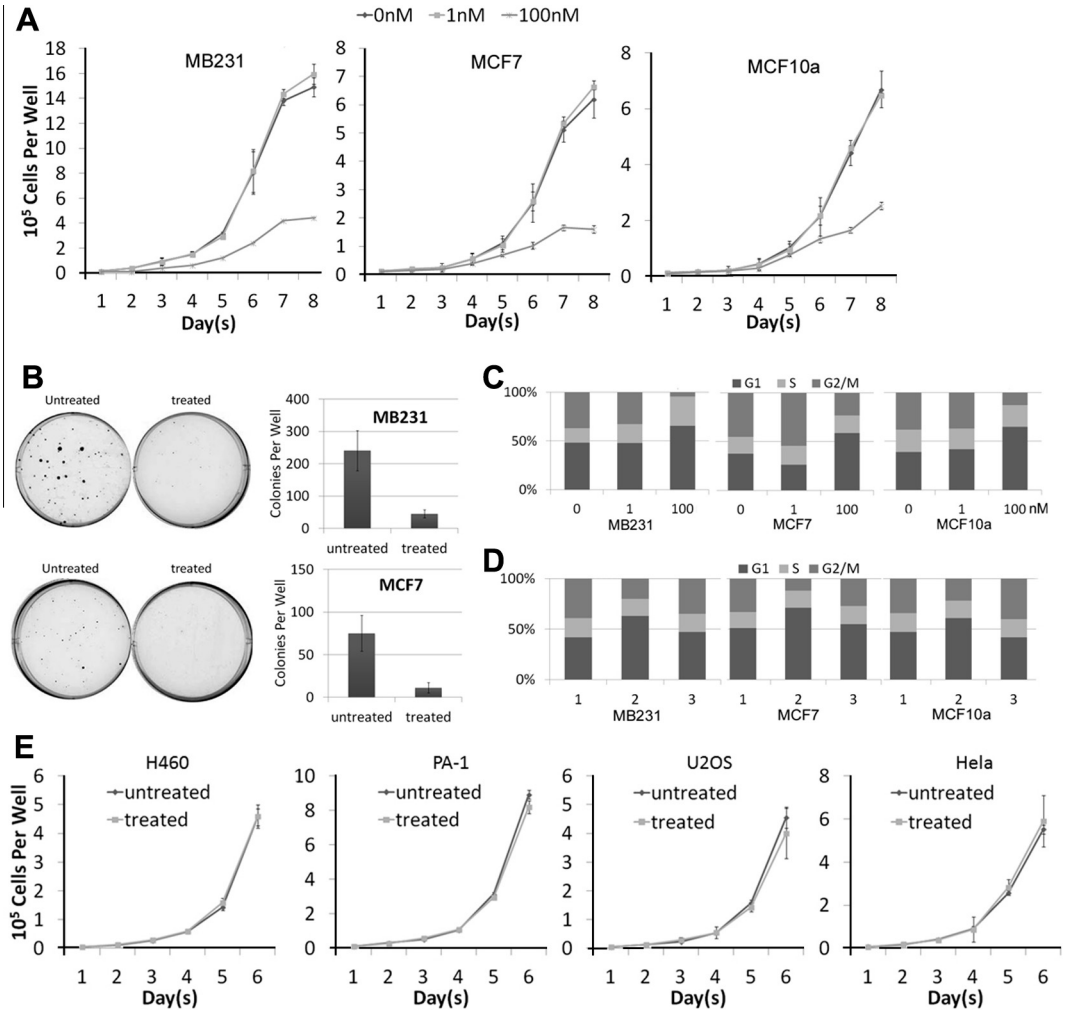

Fig. 1. Effects of E2 treatment on the proliferation, colony formation of breast cells. (A) MB231, MCF7 and MCF10a cells were treated with the indicated concentrations of E2 and the cell numbers were counted every 24 h after treatment, until 8 days. Data are presented as the mean ± SEM of the three separate experiments. (B) MB231 and MCF7a cells were treated with indicated concentration of E2 in semisolid medium ($n = 3$) and grew for 3 weeks before colonies were visualized microscopically. A representative view and counted number of colonies are shown. (C) Cells were treated with indicated concentrations of E2 for 24 h and then were analyzed as described in Section 2. (D) 24 h later after E2 treatment, E2 was removed and the cells were analyzed as the same in C. (E) Effect of E2 treatment on H460, PA-1, U2OS and Hela's proliferation. $^{*}P < 0.01$ *vs.* untreated cells; $^{**}P < 0.05$ *vs.* untreated cells using the Tukey–Kramer multiple comparison test.

derived from different types of tumor were also tested, including H460, U2OS, PA-1 and Hela. No significant effect was observed after 100 nM E2 treatment (Fig. 1E).

3.2. E2 treatment reduces migration and invasion of breast cells

We next investigated the biological effects of E2 on migration and invasion of breast cells. 24 h treatment of 100 nM of E2 or decrease of MALAT-1 by using shRNA inhibit MB231 migration and invasion in both scratch/wound healing assay and transwell assay (Fig. 2A and B). Correspondingly, decrease in E-cadherin and increase in N-cadherin levels were also detected in both two treated ways (Fig. 2C), which is consistent with morphological change of cells. Although the effects of E2 on MCF10a and MCF7 migration were undetectable, the decrease in E-cadherin and increase in N-cadherin levels were significant, indicating the same effects on them.

3.3. E2 treatment to breast cell lines causes MALAT-1 RNA decrease in an ERα independent way

Previous research has been reported that overexpression of MALAT-1 induces, while downregulation of MALAT-1 inhibits cell migration and invasion are induced by MALAT-1 transcript and inhibited by down of RNA [17]. Therefore, we investigated the potential role of MALAT-1 in E2-inhibited migration, invasion or proliferation. Knocking down of MALAT-1 RNA inhibits MB231 migration and invasion, which is similar to effect of E2 treatment on the cell (Fig. 2). We then examined whether MALAT-1 RNA level can be regulated by E2 treatment during EMT inhibition. The results showed that, MALAT-1 levels were decreased in a dose-dependent manner by E2 in breast cells, suggesting E2 plays a role in regulating the MALAT-1 RNA level (Fig. 3A).

To detect whether regulation of E2 treatment on MALAT-1 RNA level is ERα dependent or not, we generated the ERα-positive cell line MB231-ERα by stable expression, ER-knockdown cell line MCF10a-ERα^{KD} and MCF7-ERα^{KD} after E2 treatment, MALAT-1 RNA level is tested by qPCR, HN RNA and hPSF mRNA were employed as internal controls. The results showed that E2 treatment can significantly decrease the MALAT-1 RNA level both in ERα negative and positive MB231 cells, MCF7 and MCF-ERα^{KD}, MCF10a and MCF10a-ERα^{KD} (Fig. 3B). As a control, HN RNA showed no remarkable change after E2 treatment indicating that decrease of MALAT-1 RNA is not caused by general transcriptional inhibition.

3.4. Effects of E2 treatment is caused by MALAT-1 transcriptional regulation of E2

We have showed previously that E2 treatment decreases MALAT-1 RNA level (Fig. 3). To explore the mechanism of MALAT-1 decrease, we confirmed the effect of E2 on MALAT-1 RNA and excluded an effect of E2 on MALAT-1 RNA stability (Fig. 4A). 100 ng/ml actinomycin D and E2 were added together directly into the culture medium, and MALAT-1 RNA levels were detected by qPCR 24 h later. The decreases of MALAT-1 RNA in the E2-treated MB231 cells appears to be due to transcriptional regulation because the half-life of MALAT-1 RNA in these cells wasn't significantly prolonged. We further identified whether the effects of E2 treatment are reversible or not. 24 h later after removal of E2 from medium, MALAT-1 RNA rose up significantly to its original level, and inhibitions of migration, invasion disappeared (Fig. 4B). Because both MALAT-1 RNA level decreases caused by shRNA and E2 treatment show same effects on breast cells, we hypothesized that effects of E2 treatment on breast cells are achieved by regulating MALAT-1 RNA. The MB231 stably expresses shRNA against MALAT-1 was treated with high concentration of E2, but no significant effect and MALAT-1 RNA regulation were observed (Fig. 4C).

4. Discussion

Estrogen, the main female hormone, is thought to play a role in breast cancer development and tumor growth. Yoshiaki et al. have reported that the cellular sensitivity of mammary carcinoma cell lines to E2 is correlated with the expression of ERα in a dose dependent way. High concentration of E2 treatment (100 nmol/L) induces growth arrest in minimally invasive MCF-7 breast cancer cells, and lower nanomolar concentrations promote proliferation, migration and invasion of breast cancer cells [18]. In women, the physiologic concentrations of estrogen is found ranges from 30 to

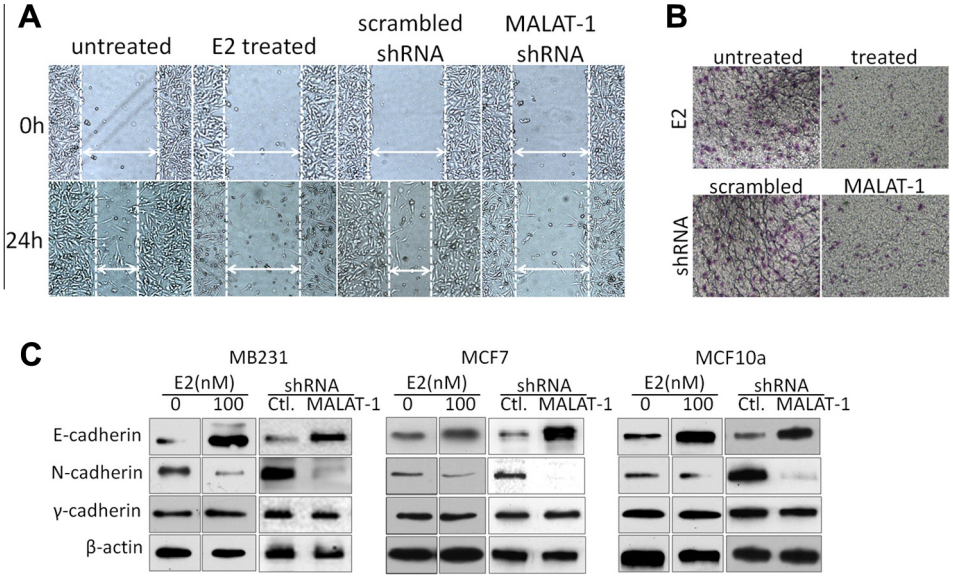

Fig. 2. E2 treatment decreased breast cells' migration by upregulating E-cadherin. (A) The effect of E2 treatment on cell migration was detected by scratch assay at the indicated time point. (B) The effect of E2 treatment on cell migration was detected by transwell assay. (C) MB231, MCF7 and MCF10a cells were treated with E2 (100 nM) for the time indicated, the protein level of E-cadherin, N-cadherin, γ-cadherin and β-actin were detected by Western blot.

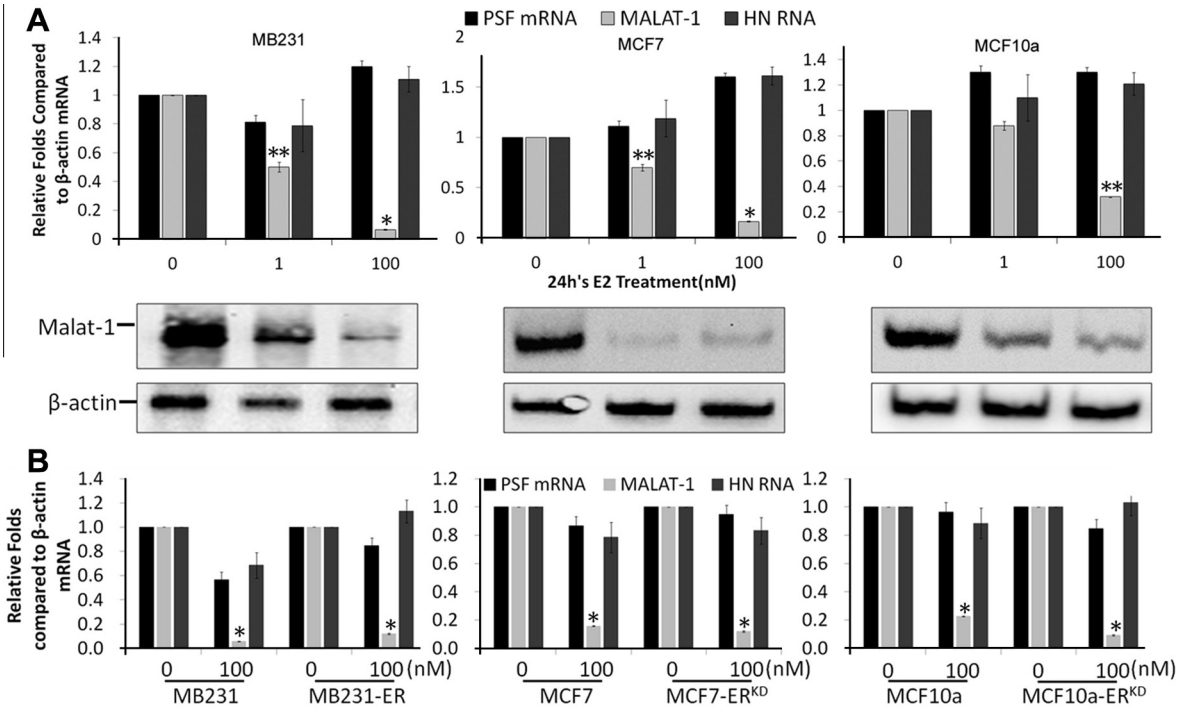

Fig. 3. E2 treatment decreases breast cells' MALAT-1 RNA level in an ERα independent way. After treated with 100 nM E2, MB231, MCF7 and MCF10a total RNA were extracted and been assayed by RT-PCR and Northern blot. (A) RT-PCR result shows 100 nM E2 treatment decreased the MALAT-1 RNA amount. Lower panel is Northern blot which were performed to show that certain concentrations of E2 treatment will decrease the full length of MALAT-1 RNA. (B) MB231 cells stably expressing ERα were tested after E2 treatment showed no difference with original MB231 cells; MCF7-ER^{KD} and MCF10a-ER^{KD} were tested after E2 treatment also showed no difference with original breast cells. Each data point is the mean ± SEM of three independent plates of cells. $^{*}P < 0.01$ *vs.* untreated cells; $^{**}P < 0.05$ *vs.* untreated cells.

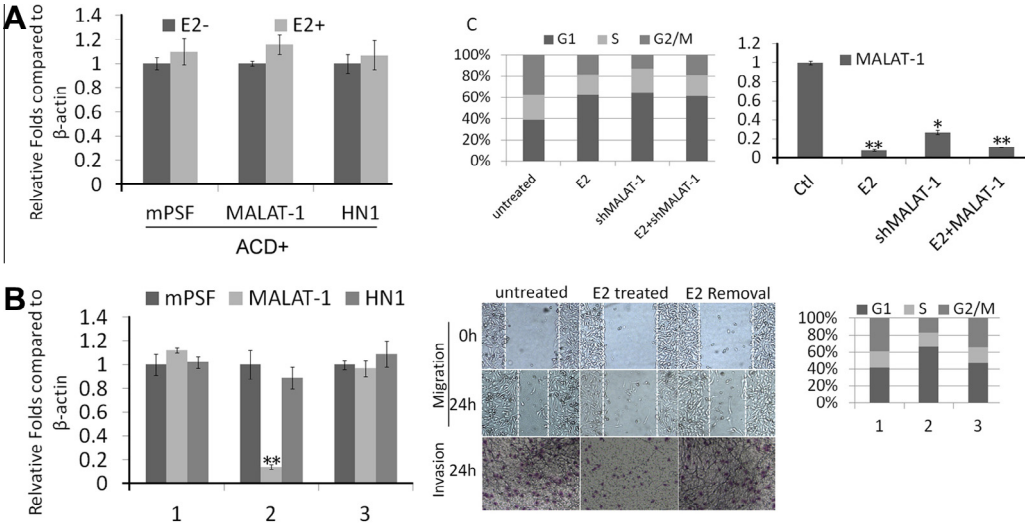

Fig. 4. E2 reversably regulates MALAT-1 transcription. (A) qPCR analysis of target RNAs in MB231 treated with 100 ng/ml ACD (E2−) or ACD and E2 (E2+). (B) Effects of E2 treatment on MALAT-1 level (left panel), proliferation (middle panel), migration and invasion (right panel). 1: untreated MB231 cells, 2: 100 nM E2 treated MB231 cells, 3: MB231 cells treated with 100 nM for 24 h, after E2 was removed, cells were grown for 24 h without E2. (C) The combined effect of shMALAT-1 and E2 treatment. $^{*}P < 0.01$ *vs.* untreated cells; $^{**}P < 0.05$ *vs.* untreated cells using the Tukey–Kramer multiple comparison test.

300 pg/mL midcycle (0.1–1 nmol/L), and in pregnant women it will be much higher to 100 nmol/L. Nuliparous women are more risky to breast cancer, indicating that exposure to high level of E2 may reduce the risk to mammary carcinogenesis [19]. In contrary, longer-term E2 exposure to lower concentrations may result in carcinogenesis. With the accumulated evidence in recent years, it is widely believed that estrogen treatment provide prevention of programmed cell death (PCD) in human breast cancer.

In this study, we have demonstrated that E2 treatment strongly affects mammary breast cells proliferation, migration and invasion in a dose-dependent way. Importantly, this effect is ERα-independent. Lower E2 treatment on ERα negative cell line MB231 showed no effect (Fig. 1A), meanwhile on ERα positive cell line MCF7 and MCF10a promoted proliferation (Fig. 3D). This is consistent with previous report, showing E2 treatment effects on MCF7 growth promotion and arrest depending on E2 concentration. MB231 is

not affected by lower E2 concentration but strongly affected by higher one, indicating that it is achieved by other pathway, and this uncertain pathway is activated under higher E2 level, but not lower one. It's also unclear whether the effect of high E2 concentration on ERα positive cell lines, such as MCF7, is depending on the ERα or the uncertain pathway.

MALAT-1 localizes in specific subcellular position, indicating that this RNA could work with some nuclear proteins to regulate gene expression and/or pre-mRNA splicing. In this regards, one study has revealed some phenotypes upon MALAT-1 knockdown in human cells. It is reported that knockdown of MALAT-1 resulted in a G2/M phase arrest and significant levels of cell death in both human Hela cells and mouse EpH4 cells [16]. In contrary, other researchers found that depletion of MALAT-1 gene leaded to a G1/S phase arrest [11]. Meanwhile, MALTA-1 also strongly regulates migration and invasion. Schmidt et al. reported that MALAT-1 was identified as a strong regulator for non-small cell lung cancer (NSCLC) migration and invasion in vitro, and proliferation in vivo [20]. Considering this, phenotype of MALAT-1 knocking down is employed as a positive control of E2 treatment. As expected, phenotypes of MALAT-1 knocking down showed highly similarity to E2 treatment (Fig. 2). After testing the MALAT-1 RNA level in E2 treated cells, surprisingly, we found that it is significantly decreased in an E2 when the E2 concentration increased. This indicates that phenotypes of E2 treatment are caused by decreasing MALAT-1 RNA level directly. Further experiments also support our hypothesis (Fig. 4).

Taken together, our in vitro studies demonstrate that E2 treatment of breast cells causes transcriptional inhibition of MALAT-1 RNA. Then, decreasing of MALAT-1 RNA causes inhibition of proliferation, migration and invasion. The effects of E2 treatment is reversible after removal of E2, including regain of MALAT-1 RNA. We have shown that prolonged exposure of breast cells with E2 (after at least 24 h) induces a dose-dependent decrease of MALAT-1 with growth arrest despite the fact that E2 is a well-established growth-stimulatory hormone in mammary cells. In addition, we have also shown that effects of E2 treatment are ERα-independent.

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