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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.