17β-Estradiol treatment drives Sp1 to upregulate MALAT-1 expression and epigenetically affects physiological processes in U2OS cells

QIONGYING HU[1*], SHUIQIN LI[2*], CHANGJIN CHEN[3], MENGLIN ZHU[4], YIMING CHEN[5] and ZIYI ZHAO[3]

Departments of [1]Laboratory Medicine and [2]Hepatobiliary Surgery; [3]Central Laboratory, The Teaching Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan 610072; [4]Chengdu Experimental Foreign Languages School, Chengdu, Sichuan 610000; [5]Chengdu Shude Middle School (Guang Hua), Chengdu, Sichuan 610091, P.R. China

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Abstract. Osteosarcoma is the most common primary bone tumor characterized by high risk of metastasis, thus presents with an overall survival rate of 60%, despite the use of chemotherapy and surgery. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) has been reported to upregulated and epigenetically regulate the metastasis in osteosarcoma; however, the regulatory mechanisms of MALAT-1 expression remain unclear. In the current study, significant upregulation of MALAT-1 was observed subsequent to exposure to low concentrations of 17β-estradiol (E2) in U2OS cells. Using chromatin immunoprecipitation assays, E2-activated estrogen receptor α (ERα) was identified to promote the binding of specificity protein 1 (Sp1) to the MALAT-1 promoter. Electrophoretic mobility shift assay and immunoprecipitation results demonstrate that ERα binds indirectly to the MALAT-1 promoter by binding directly to Sp1 protein. Notably, without E2 stimulation, overexpressed ERα results in no significant promotion of the Sp1/MALAT-1 promoter, indicating that the translocation of ERα to nuclei stimulated by E2 is necessary. The immunofluorescence assay confirmed that E2 stimulation promotes the translocation of Sp1 to the nuclei in an ERα-dependent manner. Subsequently, the effects of E2 on osteosarcoma physiological processes were further analyzed. Consistently, E2 treatment was observed to promote proliferation, colony formation, migration and invasion in U2OS cells. Taken together, the results indicate a role for E2 in regulating the physiological processes of osteosarcoma cells by regulating MALAT-1 expression levels.

Correspondence to: Dr Ziyi Zhao, Central Laboratory, The Teaching Hospital of Chengdu University of Traditional Chinese Medicine, 39 Shierqiao Road, Chengdu, Sichuan 610072, P.R. China
E-mail: zhaoziyi925@163.com

*Contributed equally

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Introduction

As the most frequent primary malignant bone tumor, osteosarcoma occurs most commonly in children and adolescents, and presents with a high risk of metastasis (1). It predominantly occurs around regions involved in the processes of bone growth and repair, including the knee joint, lower femur and upper tibia. Osteosarcoma presents with a low 5-year survival rate, with pulmonary metastases as a common cause of death of patients (2). Therefore, in addition to the traditional surgical cytoreduction of the primary tumor and chemotherapy, the critical strategy for improvement of the prognosis of patients carrying osteosarcoma is to prevent the pulmonary metastases. MALAT-1 has been previously demonstrated to be involved in the novel epigenetic regulatory mechanism (3).

MALAT-1 is a novel large, noncoding RNA, which is highly abundant and is expressed predominantly in healthy organs, and localizes to the nucleus (4). In addition its 3' end can be processed to yield a tRNA-like cytoplasmic RNA (5). In addition to its presence in in healthy organs, MALAT-1 has been demonstrated to be a potential marker for epithelial carcionmas (6-8) and is significantly upregulated in lung adenocarcinoma metastasis (5), endometrial stromal sarcoma of the uterus (8), nonhepatic human carcinomas (4) and placenta previa in trophoblasts (9). Overexpression of MALAT-1 has been observed to predict unfavorable outcomes of drug therapy in patients with osteosarcoma (6). Metastasis has been associated with upregulation of MALAT-1 by functioning as an epigenetic regulator (10), however, the regulatory mechanism of MALAT-1 expression levels remains to be fully elucidated.

Specificity protein 1 (Sp1) binds to GC-rich DNA regions through three C2H2-type zinc fingers in the C-terminal domain (11). When compared with adjacent normal tissues, tumor tissues exhibit significantly increased expression levels of Sp1, including in breast cancer, gastric tumors and lung cancer (12-14), indicating a critical role in tumorigenesis. Increasing evidence indicates that the Sp1 protein promotes the

metastasis of numerous tumor types via an unknown mechanism. In a recent study, it was been demonstrated that Sp1 activates the promoter of MALAT-1 gene by binding to its promoter region. Knockdown of Sp1 markedly reduced the MALAT-1 expression levels and thus caused the inhibition of tumor cell migration and invasion (15). The tight regulation of MALAT-1 transcription by Sp1 suggests a critical role for Sp1 in regulating tumor metastasis.

Notably, Sp1 interacts directly with estrogen receptor (ER) (16). The binding activity of Sp1 protein to GC-rich oligonucleotides has been observed to be enhanced by the addition of ER, indicating functional synergy between Sp1 and ER. Although the regulatory mechanism of MALAT-1 transcription by the ER/Sp1 complex remains unclear, one possible mechanism is through 17β-estradiol (E2) stimulation. The regulatory role of E2 on MALAT-1 expression levels has been reported to be dose-dependent (17), however, the detailed mechanisms remain to be fully elucidated.

In the current study, the effects of E2 treatment on the osteosarcoma cell line U2OS were detected. It was identified that treatment with E2 at a concentration of 10-100 pM significantly upregulated MALAT-1 expression. Silencing of either Sp1 or ERα was observed to abolish the regulatory effects of E2 on MALAT-1 expression. Electrophoretic mobility shift assay (EMSA) and the chromatin immunoprecipitation (ChIP) assay confirmed the effect of E2 treatment on DNA binding activity of Sp1 by translocating ER into nuclei.

Materials and methods

Plasmid construction. For Sp1 knockdown, the Sp1 shRNA plasmid (h) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). For ERα knockdown, the short hairpin RNA (shRNA)-expressing lentiviral vector (pLKO.1-shERα) was constructed by targeting to 5'-GTACCAATGACAAGG GAAGT-3'; scrambled control sequence, 5'-AGCCAGGCA GTATAGAGAAT-3'. For constructing the ERα expressing vector, an approximately 1788 base pairs (bp) coding sequence was polymerase chain reaction (PCR)-amplified from cDNA reverse transcribed using 1 μg MCF-7 total RNA (MCF-7 cells from the American Type Culture Collection, Manassas, VA, USA) with the First Strand Synthesis kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) with a poly(dT) oligonucleotide. MCF-7 RNA was used due to high levels of ERα mRNA expression. For the following synthesis of the coding sequence of ERα, 0.5 μl cDNA template was amplified using PrimeSTAR® Max DNA Polymerase (Takara Bio, Inc., Shiga, Japan). For PCR amplification, the following primers were used: ERα, forward 5'-GCCGGCGCTAGCATGCCATGACCCTCCACA CCA-3' and reverse 5'-CCTTAACTTAAGCAGACCGTGGCA GGGAAACCC-3'. Subsequent to 5 min initial denaturation at 98˚C, the mixture was amplified for a total 30 cycles with a two-step cycle process that began with the denaturation at 98˚C for 15 sec and annealing and extension at 65˚C for 2 min. Subsequent to digestion with Nhe I and Hind III, the fragment was inserted into pcDNA3.1. For construction of the different expression vectors, the same procedure was followed described above with the following primer pairs: Hemagglutinin (HA)-ERα, forward 5'-GCCGGCGCTAGCTACCCATAC GACGTCCCAGACTACGCTATGCCATGACCCTCCACA CCA-3' and reverse 5'-CCTTAACTTAAGCAGACCGTGGCA GGGAAACCC-3'; Flag-Sp1 forward 5'-GCCGGCGCTAGC GATTACAAGGATGACGACGATAAGATGGATGAAATG ACAGCTGTGGTGA and reverse 5'-CCTTAACTTAAGTCA GAAGCCATTGCCACTGATA.

Cell line and transfection. The human osteosarcoma cell line U2OS was purchased from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Life technologies; Thermo Fisher Scientific, Inc.) and 100X antibiotic-antimycotic mixed stock solution (Life technologies; Thermo Fisher Scientific, Inc.).

For transfection, Lipofectamine 2000 (Life technologies; Thermo Fisher Scientific, Inc.) was employed following the manufacturer's instructions. These methods were used to establish U2OS-shER, U2OS-shSp1, U2OS-ERα, U2OS-Sp1 and U2OS-shScrambled.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of the target cells were isolated using TRIzol (Life technologies; Thermo Fisher Scientific, Inc.). First-strand cDNA was synthesized from 1 μg total RNA using the First Strand Synthesis kit (Guangzhou RiboBio Co., Ltd.). qPCR was performed with a SoEva Green Super Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the Applied Biosystems 7500 Real-time system (ABI 7500HT instrument; Applied Biosystems; ThermoFisher Scientific, Inc.) was used for measurement. The reaction mixture contained 0.2 μl cDNA, 10 μl SoEva Green Super Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 0.5 μl of each primer and 8.8 μl deionized water. The primers used in the current study were as follows: MALAT-1, forward 5'-GAC TTCAGGTCTGTCTGTTCT-3' and reverse 5'-CAACAATCA CTACTCCAAGC-3'; NEAT1, forward 5'-GCTCTGGGACCT TCGTGACTCT-3' and reverse 5'-CTGCCTTGGCTTGGA AATGTAA-3'; HN1, forward 5'-CACAGCAAGACGAGA AGACCCTATGGAGC-3' and reverse 5'-GTCAAGTTATTG GATCAA-3'; HOTAIR forward 5'-GACAGGGTCTGGGAC AGAAG-3' and reverse 5'-GAGTCAGAGTTCCCCACTGC-3; GAPDH, forward 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'.

Western blotting. Samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA) pre-blocked in 5% powdered milk + 5% bovine serum albumin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in PBS containing 0.3% Tween 20 for 30 min at room temperature. Subsequently, the blot-transferred membrane was incubated at 4˚C overnight with the following primary antibodies from Abcam (Cambridge, UK): Anti-SP1 (ab13370; 1:1,000), anti-ERα (ab32063; 1:1,000), anti-GAPDH (ab8245; 1:1,000) and anti-TIM antibody (ab66062; 1:1,000). After washing three times with PBS containing 0.3% Tween 20, the membrane was incubated with goat anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibodies (ab6721; 1:5,000; Abcam) for 1 h at room temperature. Then membrane was then developed by using ECL detection systems (Thermo Fisher Scientific, Inc.).

ChIP. For the ChIP assay, $5x10^6$ cells were cross-linked with ice-cold 1% formaldehyde in 4˚C for 15 min and washed three

times with ice-cold PBS. Cells were collected with lysis buffer. DNA was then sheared to an approximate length of 200-500 bp. Sheared DNA (100 μl) was incubated with 10-15 μg primary antibody or rabbit IgG (negative control) followed by IP with 50 μl protein A agarose beads (Life Technologies; Thermo Fisher Scientific, Inc.) during an overnight incubation at 4˚C with rotation. Enriched DNA was extracted from the DNA/antibody/protein A bead complexes by proteinase K digestion, reverse crosslinking process at 65˚C for 4 h and purification via centrifugation at 1,000 x *g* for 10 min at 4˚C. The IP product was amplified using the MALAT-1 promoter region: Forward GGA AGTTGGGCAGCAGCTCCACG and reverse CCACT GGTT CTAACCGGCTCTAG. Dihydrofolate reductase 5'untranslated region was included as a negative control; forward ACCTGG TCGGCTGCACCT and reverse TTGCCCTGCCATGTCTCG.

Co-IP. Protein A agarose bead slurry (50 μl) was incubated with 10 μg anti-Flag or anti-HA antibody overnight at 4˚C with rotation. The beads were then centrifuged at 1,000 x *g* for 10 min at 4˚C and washed three times with PBS. The protein A/antibody complex was then incubated with 500 μg lysate protein overnight with rotation, followed by three washes with PBS. The complex was then heated at 100˚C for 10 min, and 15 μl eluted sample was loaded for the following semiquantitative western blot assay.

Isolation of nuclei. Intact nuclei were isolated from target cells using a nuclei isolaton kit (cat. no. NUC201; Sigma-Aldrich; Merck Millipore) following the manufacturer's instructions.

EMSA. The Sp1-binding fragment of the MALAT-1 promoter region was 5'-biotinylated and annealed. The sequence was as follows: 5'-CAGGCGTTAGGGCGGGGCGCGCGTGC-3'. Either 10 or 30 ng target protein was incubated with 10 pM biotinylated DNA fragment for 30 min at 4˚C. The complexes were fractionated using 4% PAGE gel in 0.5X TBE and transferred onto Hybond-N+ membranes (Life Technologies; Thermo Fisher Scientific, Inc. Subsequently, the assay was conducted and detected using HRP-conjugated streptavidin (LightShift™ Chemiluminescent EMSA kit; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Cell counting kit-8 (CCK-8) assay for proliferation. Target cells were seeded into a 96-well plate at a concentration of 5,000 cells/well. After 24 h, E2 was added if required. At 1, 2, 3 and 4 days after transfection, the cell proliferation assay was conducted by the addition of 10 μl CCK-8 solution [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] to each well, followed by incubation at 37˚C for 2 h. Absorbance was measured at a wavelength of 450-620 nm using a Multiskan spectrum microplate reader (Thermo Fisher Scientific, Inc.).

Invasion and migration analyses. Cell migration was analyzed using a scratch assay. The cell layer that reached confluence was scratched by a 200 μl pipette tip and incubated at 37˚C for 24 h. The average extent of wound closure was imaged. For analyzing invasion, the under surface of the membrane was coated with Matrigel (0.01%) at 37˚C for 2 h. The lower chamber was filled with 1 ml DMEM supplemented with 10% FBS. A total of $1x10^6$ cells in a volume of 0.2 ml were added to the upper chamber. Following incubation at 37˚C for 24 h, the cells on the upper surface of the transwell membrane were removed. The cells attached to the lower surface of membrane were stained with 1% crystal violet and imaged.

Statistical analysis and presentation of data. The results were expressed as the means ± standard deviation. The statistical analysis involving two groups was performed using the means of Student's t-test. All data were processed using SPSS software, version 19. $P<0.05$ was considered to indicate a statistically significant difference.

Results

E2 treatment upregulates MALAT-1 RNA levels in the U2OS osteosarcoma cell line. Due to the fact that ERα is strongly expressed in U2OS cells (data not shown), the effects of E2 treatment on the expression of lncRNAs was analyzed in U2OS cells. MALAT-1, NEAT1 and HOX transcript antisense RNA (HOTAIR), which are all associated with malignancy and upregulated in osteosarcoma, were detected quantitatively. As presented in Fig. 1A, 10-100 pM E2 treatment specifically and significantly upregulated the MALAT-1 RNA levels, however did not affect NEAT1 or HOTAIR. To determine whether the E2 treatment is mediated by the stimulation of ERα, ERα was efficiently knockdown by a plasmid that encodes a shERα sequence (Fig. 1B; knockdown efficiency ~65±4.5%). As expected, knockdown of ERα desensitized U2OS-shERα to E2, indicating the necessity of ERα. As mentioned previously, the direct interaction between ERα and Sp1, and the transcriptional regulatory role of Sp1 on MALAT-1 suggests a role of Sp1 in E2-induced MALAT-1 upregulation. Similar to the results of ERα knockdown, Sp1 knockdown also desensitized U2OS-shSp1 to E2. In order to establish whether ERα may regulate MALAT-1 expression without E2 stimulation, ERα was overexpressed in U2OS. It was identified that, compared with the U2OS-vector, MALAT-1 RNA levels were not significantly different without E2 treatment (Fig. 1E, left panel), however were significantly upregulated with E2 treatment (Fig. 1E, right panel).

Following E2 treatment, ERα promotes the nuclear translocation of Sp1 and results in the increase of Sp1/MALAT-1 promoter binding. Due to the fact that Sp1 is reported to bind to MALAT-1 promoter region, its binding activity in ERα-knockdown U2OS cells was analyzed. ChIP was performed with an anti-Sp1 antibody. IgG was employed as a negative control. As presented in Fig. 2A, the binding activity of Sp1 to the MALAT-1 promoter region increased in U2OS-shScrambled, however not in U2OS-shERα. For the quantitative assay, IP products were included in the qPCR reaction. Consistently, the stimulation of MALAT-1 promoter binding activity of Sp1 by E2 treatment was abolished when ERα was knocked down (Fig. 2B). As it has been previously demonstrated, E2 treatment will translocate ERα from the cell membrane into the nucleus, it was hypothesized that the increase of Sp1/DNA binding activity may be associated with the subcellular location. The immunofluorescence assay indicated that, following E2 treatment, Sp1 accumulated in the nuclei instead of the cytoplasm (Fig. 2C). For further confirmation, nuclear fractions from E2-treated

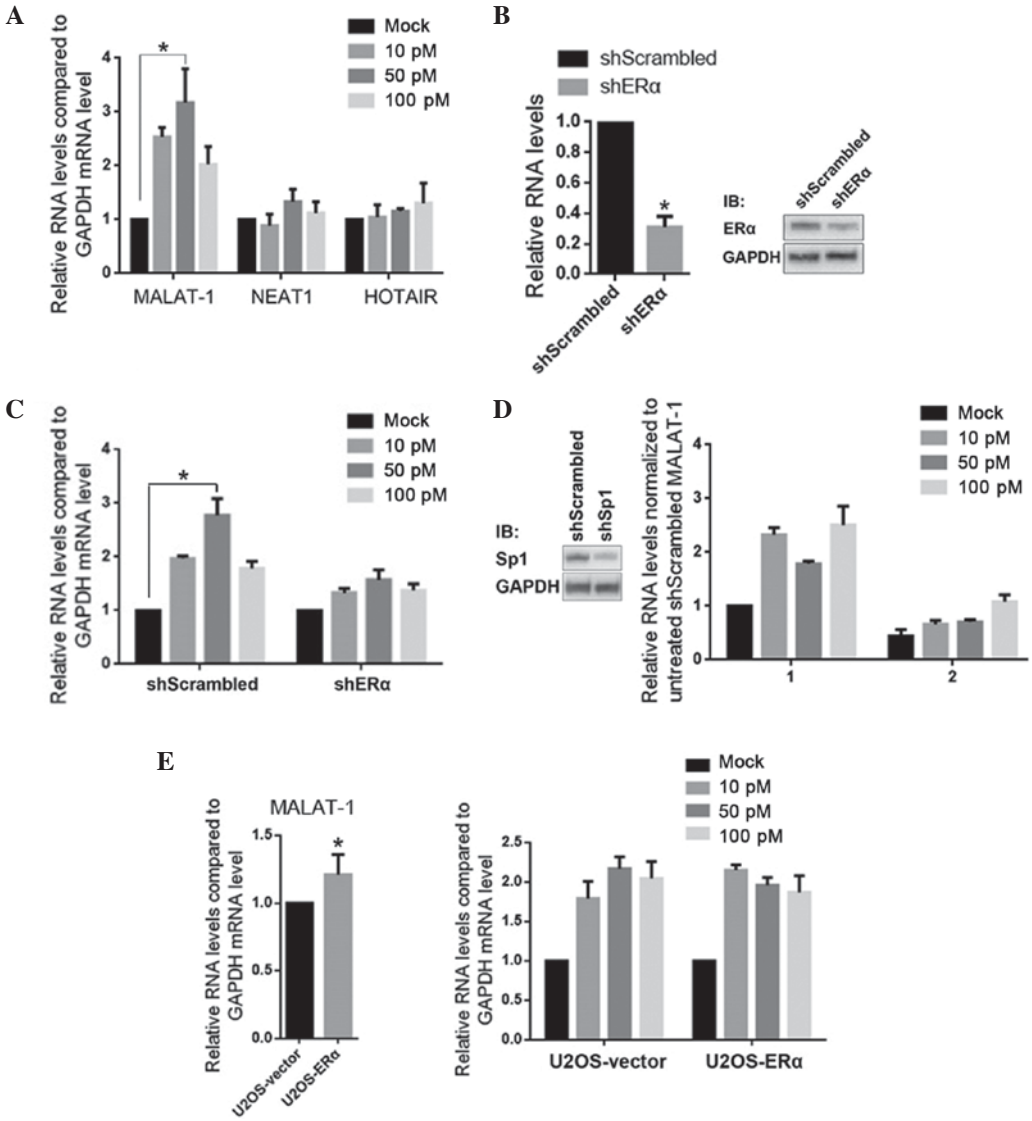

Figure 1. E2 treatment upregulates MALAT-1, however not NEAT1 or HOTAIR, in ERα- and Sp1-dependent manners. (A) U2OS cells were treated with 10, 50 and 100 pM E2. After 24 h, MALAT-1, NEAT1 and HOTAIR were detected by RT-qPCR. (B) ERα expression was knocked down by transfection of shERα, then the ERα mRNA (left panel) and protein (right panel) were detected. (C) Subsequent to ERα knockdown, U2OS-shERα was exposed to 10, 50 and 100 pM E2 respectively, then the MALAT-1 levels were measured by RT-qPCR. (D) Subsequent to the efficient knockdown of Sp1 (left panel), the effects of E2 treatment on MALAT-1 were measured (right panel). (E) The effect of overexpressed ERα on MALAT-1 level was measured with (right panel) or without (left panel) E2 treatment. *P<0.05. E2, 17β-estradiol; MALAT-1, metastasis-associated lung adenocarcinoma transcript 1; NEAT1, nuclear-enriched abundant transcript 1; HOTAIR, HOX transcript antisense RNA; ERα, estrogen receptor α; Sp1, specificity protein 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

U2OS cells were isolated. GAPDH (cytoplasm specific protein) and translocase of the inner membrane (mitochondrial specific protein) were employed as indicators of purity. Western blotting identified nuclear fractions, indicating acceptable cytoplasm and mitochondria contamination (Fig. 2D). Following E2 treatment, ERα and Sp1, particularly Sp1, were significantly accumulated in the nuclear fraction.

ERα binds directly to Sp1, however not to the MALAT-1 promoter region in vitro. Plasmids containing the HA-tagged ERα or Flag-tagged Sp1 coding sequences were established, and they were introduced into HEK293 cells for transient expression. Cell lysates were immunoprecipitated with anti-HA or anti-Flag antibodies individually. Subsequently, the IP products were assayed by immunoblotting using anti-HA or anti-Flag antibodies separately. As presented in Fig. 3A, Flag-tagged and HA-tagged proteins were detectable in the IP products, suggesting the direct binding of Sp1/ERα. It was additionally investigated whether ERα binds to the MALAT-1 promoter by itself. In order to establish this, the EMSA assay was conducted. The MALAT-1 promoter region DNA sequence was labeled with biotin was incubated with purified HA-tagged ERα or Flag-tagged Sp1 separately and fractionated by 4% 0.5X TBE PAGE gel. The binding bands were observed only in Sp1 involved lanes. For further confirmation, E2 treated U2OS cells underwent a ChIP assay. Following E2 treatment, anti-ERα and anti-Sp1 antibodies enriched the MALAT-1 promoter region. However, without E2 treatment, the anti-ERα antibody failed to enrich the MALAT-1 promoter region, while the anti-Sp1 antibody was observed to exhibit no

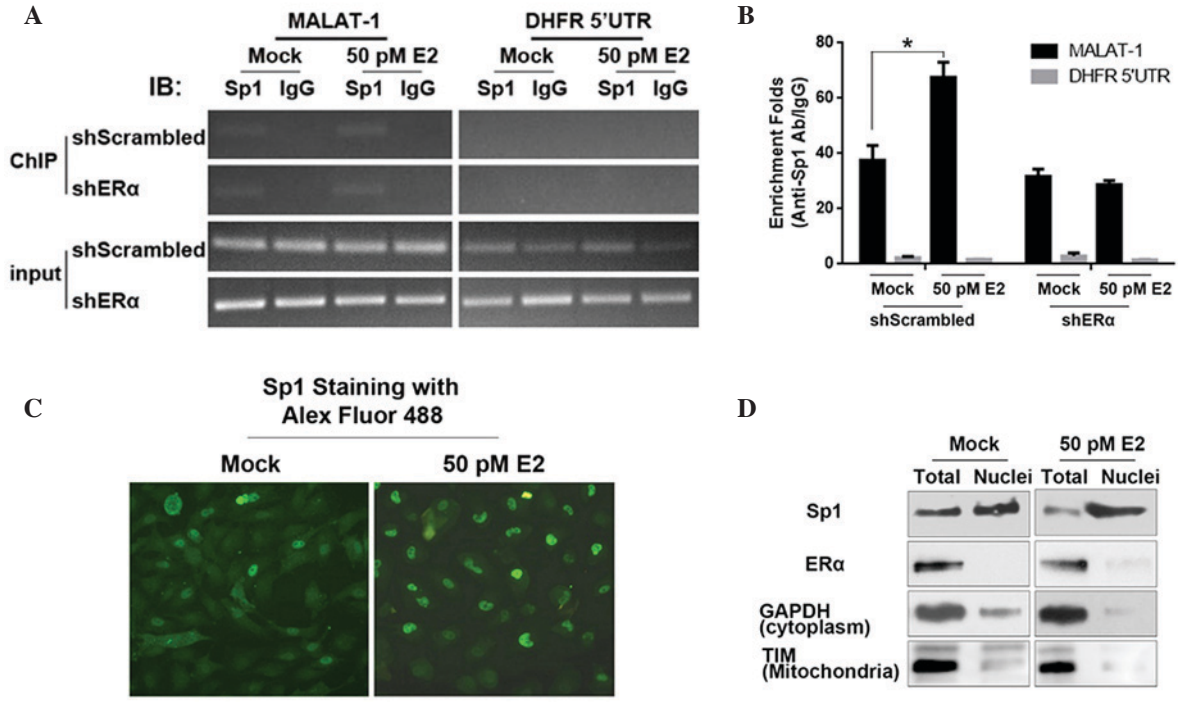

Figure 2. E2 treatment promotes nuclei translocation of Sp1 in the presence of ERα. (A) ChIP assay was performed to detect the binding of Sp1 to MALAT-1 promoter region following E2 stimulation. (B) ChIP products were detected by semiquantitative PCR and quantitative PCR. (C) Following E2 treatment, Sp1 was stained with the Alex Fluor 488-labeled antibody. (D) Following E2 treatment, nuclei of U2OS cells were isolated. The mitochondria-specific TIM, cytoplasm-specific GAPDH, ERα and Sp1 were measured in this fraction compared with total protein. *P<0.05. E2, 17β-estradiol; Sp1, specificity protein 1; ERα, estrogen receptor α; ChIP, chromatin immunoprecipitation; MALAT-1, metastasis-associated lung adenocarcinoma transcript 1; PCR, polymerase chain reaction; TIM, translocase of the inner membrane.

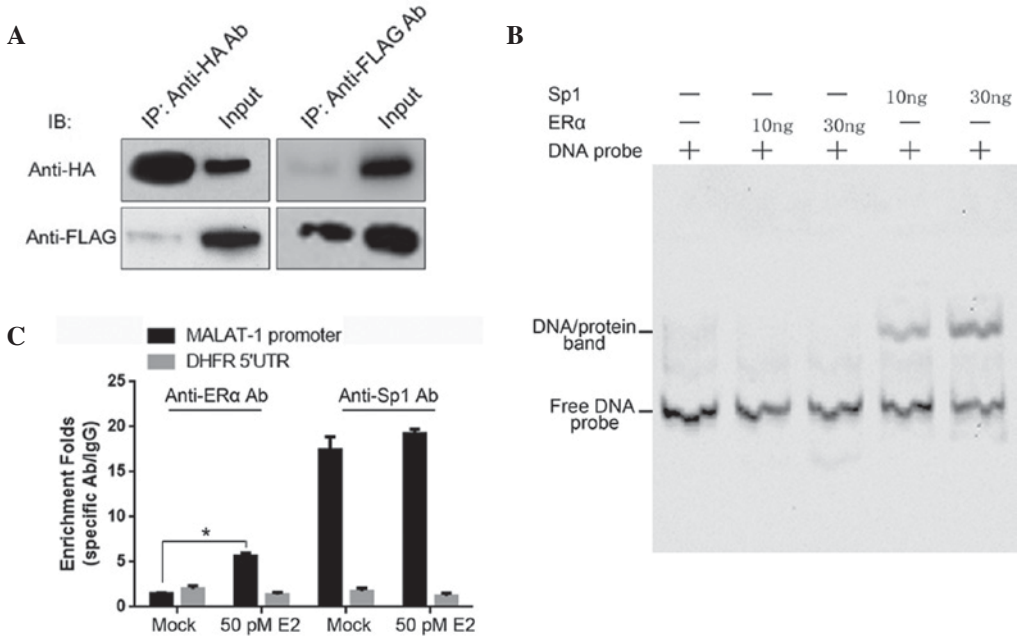

Figure 3. ERα binds to Sp1, however not to the MALAT-1 promoter region. (A) Plasmids expressing Flag-tagged ERα and HA-tagged Sp1 were co-transfected into HEK293 cells. After 48 h, co-IPs were performed using anti-Flag or -HA antibodies, respectively. (B) The binding of Sp1 or ERα to the MALAT-1 promoter region was further confirmed *in vitro* by the electrophoretic mobility shift assay. (C) Plasmids expressing Flag-tagged ERα and HA-tagged Sp1 were co-transfected into U2OS cells, and after 48 h, ChIPs were performed with anti-Flag and -HA antibodies or IgG. *P<0.05. E2, 17β-estradiol; Sp1, specificity protein 1; MALAT-1, metastasis-associated lung adenocarcinoma transcript 1; ERα, estrogen receptor α; HA, hemagglutinin; IP, immunoprecipitation; ChIP, chromatin IP.

difference to the E2-treated sample (Fig. 3C). Taken together, ERα binds to the Sp1 protein, however not to the MALAT-1 promoter.

E2 treatment tightly regulates physiological processes of U2OS cells in an ERα-dependent manner by upregulating MALAT-1 RNA. MALAT-1 tightly regulates the physiological processes

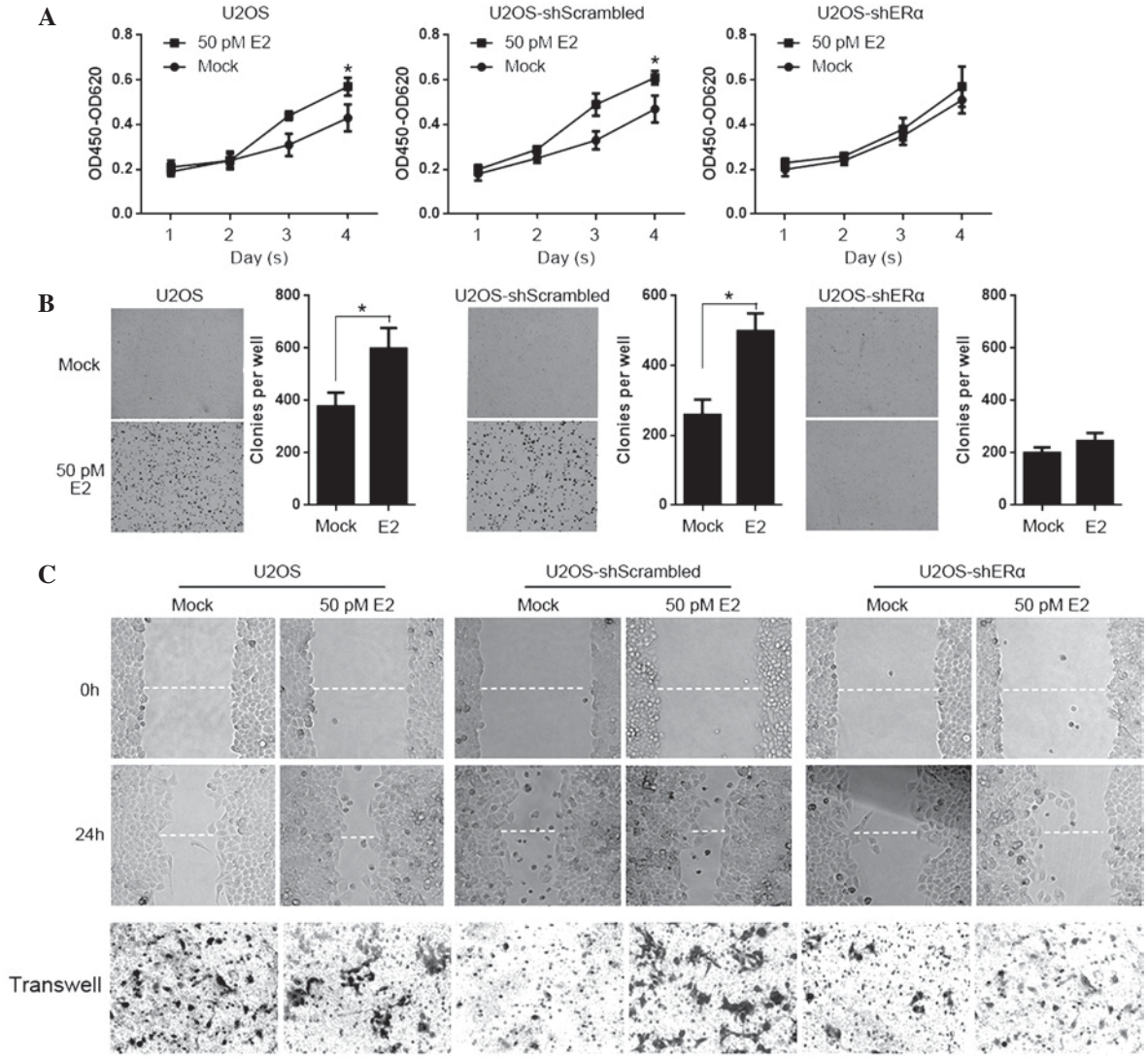

Figure 4. The effects of E2 treatment on physiological processes of U2OS in an ERα-dependent manner. U2OS, U2OS-shScrambled and U2OS-shERα were treated with 50 pM E2, then the proliferation was measured by (A) the cell counting kit-8 assay; (B) colony formation was measured by soft agar; (C) migration and invasion were measured by (upper panel) scratch assay and (lower panel) transwell assay. *P<0.05. E2, 17β-estradiol; ERα, estrogen receptor α; sh, short hairpin; OD, optical density.

in osteosarcoma, and aids in the determination of the effects of E2 treatment on these processes in an ERα-dependent manner. For testing proliferation of E2 treated cells, U2OS, U2OS-shScrambled and U2OS-shERα were seeded into 12-wells and were stained with CCK-8 on days 1, 2, 3 and 4. As presented in Fig. 4A, E2 treatment significantly promoted proliferation, however not in U2OS-ERα. Subsequently, cells were seeded in 0.3% soft agar for testing colony formation for 14 days. Consistently, E2 promoted the colony formation, however not in ERα-knockdown U2OS cells (Fig. 4B). In order to further determine the effects of E2 treatment on migration and invasion, the scratch assay and transwell assay were performed with the above mentioned cell lines. It is observed that E2 treatment affected the migration and invasion independent of the presence of ERα. (Fig. 4C).

Discussion

Large number of lncRNAs have now been identified and characterized in mammals (18). Although the function of these novel lncRNAs remains unclear, growing evidence has indicated their crucial roles in various biological processes through various mechanisms, including epigenetic regulation, transcriptional regulation and post-transcriptional regulation. Their functional activities are identified not only in normal developmental processes, however additionally in disease, particularly in cancer (19,20). Originally, MALAT-1 was identified to be tightly associated with tumorigenesis, and it is also termed nuclear-enriched abundant transcript 2 (21). Further research on the molecular mechanisms of MALAT-1 identified its various regulatory roles, including regulation of cell cycle arrest, and epigenetic regulation of collagen triple helix repeat containing 1, chaperonin containing TCP1 subunit 4, nuclear receptor subfamily 4 group A member 1 and polypyrimidine tract binding protein 3 (6,22).

Previous studies have indicated that MALAT-1 is upregulated during tumorigenesis, in metastatic tumor tissues and additionally in patients who have poor prognosis (10,23). However, the mechanism of MALAT-1 regulation remains unclear. In a breast cancer cell line, it was reported that high doses of E2 treatment

(100 nM) markedly downregulate MALAT-1 RNA levels in an ERα-independent manner (24). A previous study additionally identified this mechanism in the MG63 osteosarcoma cell (17). The effects of physiological doses (≤100 pM) of E2 on MALAT-1 expression remains unclear.

Sp1 contains highly conserved C2H2 zinc finger motifs in its C-terminus and binds to GC-rich sites (11). For example, it transcriptionally activates the fibroblast growth factor receptor 1 promoter by binding to its GC-rich region in proliferating myoblasts (25). It has additionally been observed to activate the promoter of the human MALAT-1 gene. *In vitro*, EMSA assay have indicated the binding of Sp1 protein to the MALAT-1 promoter region. The ChIP assay further confirmed this tight binding and thus upregulation of MALAT-1 expression levels (15). Notably, Sp1 binds directly to ERα and serves a synergistic role in transcriptional regulation of their target genes (26). Considering this, the molecular mechanisms by which E2-ER positively regulates the gene expression of MALAT-1 were investigated in the current study.

U2OS cells were treated with with 10-100 pM E2, and it was identified that E2 treatment significantly downregulated MALAT-1 expression. In ERα-knockdown U2OS cells, the effects of E2 treatment were abolished, suggesting that the necessity of ERα on the effects caused by E2 treatment. Consequently, ChIP and immunofluorescence assays observed that, following E2 treatment, Sp1 translocated into the nuclei dependent on the presence of ERα. This result indicates the direct binding of ERα to Sp1 after E2 forms the E2-ER complex.

E2-ER signaling stimulates target gene expression in two different ways. Firstly, the E2-ER complex binds directly to a palindromic ERE or half-ERE in the promoter region of a target gene (17); secondly, instead of binding directly to the target gene, the E2-ER complex interacts with other transcription factors, such as Sp1 (27). In the current study, direct binding of ERα to Sp1 was observed with Co-IP. The presence of a supershifted band of the ERα/Sp1/DNA complex was predicted following addition of ERα, however no band of ERα/Sp1/DNA was observed in EMSA (data not shown); however, the ChIP assay indicated that ERα bound to the MALAT-1 promoter region in an E2-dependent manner. To explain this, two theories were proposed; firstly that the ERα/Sp1/DNA complex is too fragile to be detected as a supershifted band in EMSAs; secondly that the unstable dynamic balance of ERα/Sp1/DNA leads to the dissociation of this complex. As a result of E2-treatment-induced MALAT-1 upregulation, consistent with the results of previous studies, the proliferation, colony formation, migration and invasion of U2OS cells were observed to be significantly promoted.

Taken together, the current study demonstrated that E2 treatment of U2OS cells significantly promotes the nuclear translocation of Sp1 potentially through the formation of the ERα/Sp1 complex. The nuclei-accumulated Sp1 activates the transcriptional activity of MALAT-1, then the upregulated MALAT-1 RNA causes promotion of proliferation, colony formation, migration and invasion.

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