Myocardin-related transcription factor A is up-regulated by 17β-estradiol and promotes migration of MCF-7 breast cancer cells via transactivation of MYL9 and CYR61

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Many lines of evidence have suggested that estrogen plays important roles not only in the initiation and proliferation of breast cancer, but also in cancer metastasis. However, the mechanistic basis of the latter events is poorly understood. In addition, recent studies have suggested that myocardin-related transcription factor A (MRTF-A) might be related to cancer metastasis. However, as reports are contradictory, certain of its roles still remain confusing. In the present study, we showed that excessive 17β-estradiol could promote the migration of MCF-7 breast cancer cells and up-regulate the expression of MRTF-A, myosin regulatory light chain 9 (MYL9), and cysteine-rich angiogenic inducer 61 (CYR61). Overexpression of MRTF-A significantly promoted the migration of MCF-7 cells through its transactivation effects on MYL9 and CYR61 genes, while RNA interference-mediated knockdown of MRTF-A strongly inhibited transcription and expression of the target genes and reduced the migration ability of MCF-7 cells. These results provided novel evidence supporting the metastasis-promoting functions of MRTF-A, and implied that MRTF-A might be a switch for the estrogen pathway to change its proliferation-promoting roles into migration-stimulating roles in breast cancer.

Keywords 17β-estradiol; breast cancer; migration; MRTF-A; MYL9; CYR61

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Introduction

Cancer metastasis is one of the biological characteristics of malignant tumor, and it is the leading cause of death in clinical cancer patients. Of all the types of cancer, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females [1]. Excessive or prolonged exposure to estrogen has been regarded as the most important determinant of the risk of breast cancer. Except for its well-known proliferating effect, increasing evidence has shown that estrogen could also stimulate migration of breast cancer cells, while anti-estrogens or aromatase antagonists could inhibit breast cancer cell motility and invasion [2]. However, the mechanisms underlying the effects of estrogen on cell migration still remain unclear.

It has been shown that the RhoA/Rho kinase pathway participates in the estrogen-stimulated migration of endothelial cells [3]. Rho signaling could control the dynamics of the cytoskeleton by regulating nuclear translocation of myocardin-related transcription factor A (MRTF-A, also named MAL, MKL1), a G-actin-binding coactivator of serum response factor (SRF) [4]. Several reports have suggested that MRTF-A might be involved in cancer metastasis. Depletion of MRTF-A or SRF could reduce cell motility in culture, and knockdown of MRTF-A by RNA interference (RNAi) could inhibit metastasis of tumor xenografts in mice [5]. However, confusedly, the overexpression of MRTF-A in oncogenic ras or src transformed epithelial cells significantly reduced tumor formation and suppressed metastasis, and the enforced activation of MRTF-A also impaired migration of fibroblasts cells, whereas down-regulation of MRTF-A with dominant-negative constructs or RNAi enhanced cell motility [6,7]. These conflicting reports strongly suggested that the certain roles of MRTF-A played in cancer metastasis still need to be further confirmed [8].

With the bioinformatics analysis, we found that CarG boxes [5'-CC(A/T)_{6}GG-3'], the highly conserved cis-regulatory elements of MRTFs/SRF, were present in the promoter region of myosin regulatory light chain 9 (MYL9) and
cysteine-rich angiogenic inducer 61 (CYR61), indicating that these two migration-related genes might be the transcriptional targets of MRTF-A. MYL9 is one of the four light chains of myosin, which is an important component of the cytoskeletal structure involved in cell movement, deformation process, and cell migration [9–11]. Although the direct relationship between estrogen and MYL9 in breast cancer has not been reported yet, the expression of myosin subunits has been found to be regulated by estrogen in smooth and cardiac muscle [12]. In addition, several reports have suggested that CYR61 is an estrogen-inducible tumor-promoting factor which plays important roles in the invasive process, and cell migration [9–11]. However, these hypotheses have not been tested up to now. To address these issues, here, the influence of 17β-estradiol (E2) on cell motility and expression of MRTF-A, MYL9, and CYR61 were detected in MCF-7 cells, an estrogen receptor (ER)-positive low-metastatic breast cancer cell line. In addition, the effects of MRTF-A on the cell migration and the transactivation of MYL9 and CYR61 were also investigated.

Materials and Methods

Cell culture and transfection

Either MCF-7 cells or COS7 cells were conserved in our lab and cultured at 37°C in Dulbecco’s modified Eagle’s medium/F12 (HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO2 incubator. For transfection experiments, the cells were seeded in serum-free medium without antibiotics for 24 h, and then transfected with TurboFect reagents (Thermo, Rockford, USA) according to the manufacturer’s instructions.

Plasmids and siRNAs

The plasmid encoding human MRTF-A was purchased from Addgene (Cambridge, USA). Promoters of MYL9 (−1547/+106) and CYR61 (−2365/+63) were amplified by polymerase chain reaction (PCR) using the genomic DNA isolated from MCF-7 cells as templates. For MYL9, sense primer containing MluI (underlined): 5’-GACACGCCTTCTACTTTGGCTCTCTAG-3’, and antisense primer containing XhoI: 5’-CTGCTCGAGTGAGGCCGTCTCTCAAGAC-3’. For CYR61, sense primer containing MluI: 5’-CGACGCCTGTTGGAATGGCGTGTTTG-3’, and antisense primer containing BglII: 5’-AGGAGATCTGGCGTCTTCCGTCAGGTCC-3’. PCR products were double digested with corresponding enzymes and then inserted into the pGL3-basic luciferase reporter vector (Promega, Madison, USA) and confirmed by sequencing. Specific siRNAs were synthesized by Invitrogen Shanghai Co. (Shanghai, China). The sequence of siMRTF-A: forward: 5’-GAAUGUGCUACAGUUGAAAdtdt-3’; reverse: 5’-UUUCAACUGUAGCACAUUCdtdt-3’. Negative control siRNAs: forward: 5’-UUCGCCAACGUGUCGdtdt-3’; reverse: 5’-ACUGACAGAGCUUCGGAGAdtdt-3’.

Proliferation assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-y)-3,5-diphenyltetrazolium bromide (MTT) assay. In brief, MCF-7 cells were transfected with MRTF-A or pcDNA3.1 plasmids in 96-well plates at doses equal to 0.5, 1.0, and 2.0 folds of the dose used in the migration assays. Twenty-four hours later, the medium in each well was replaced by 100 μl of fresh serum-free medium with 1 mg/ml of MTT. After incubation at 37°C for 4 h, the MTT-containing medium was removed and 50 μl of dimethyl sulfoxide was added to each well. After incubation at 37°C for another 10 min, the absorbance value of each well was measured by Synergy4 Hybrid Multi-Mode Reader (BioTek, Winooski, USA) at a wavelength of 490 nm.

Two-dimensional cell migration assay

A two-dimensional cell migration assay was performed using a scratch wound model. In brief, the cells were treated with E2 or transfected with MRTF-A or pcDNA3.1 in 24-well plates, and then scratched with the narrow end of a sterile pipette tip. The remaining cells were washed twice with warm culture medium to remove cell debris. The width of the scratch was photographed at different times using an inverted phase contrast microscope (Olympus, Tokyo, Japan).

Three-dimensional cell migration assay

A three-dimensional cell migration assay was performed using the Transwell cell culture insert for 24-well plates composed of a polycarbonate membrane containing 8 μm pores (Corning Costar, Cambridge, USA). After being transfected for 12 h, the cells were harvested by trypsinization and then seeded into the upper chamber of the Transwell cell culture insert at 1.0 × 10^4 cells in 200 μl of 1% FBS-containing medium. The lower chamber was filled with 600 μl of medium containing 10% FBS. Twenty-four hours later, the cells in the upper chamber were removed using a cotton swab, and the cells that had migrated to the lower side of the membrane were fixed with 4% paraformaldehyde and stained with 4’,6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China). The number of migrated cells was counted and photographed in five fields (the upper, the lower, the left, the right, and the middle) of three independent experiments.
Luciferase reporter assay

Unless otherwise indicated, 200 ng of reporter and 800 ng of activator plasmids were transfected into cells. Twenty-four hours later, the cells were lysed and whole cell extracts were prepared and analyzed by using the Luciferase Assay System (Promega) according to the manufacturer’s instruction. Protein concentration of whole cell extracts was determined by the BCA™ protein assay kit (Thermo). Luminescence was measured and normalized by using a Synergy4 microplate reader (Biotek). Data were expressed as fold(s) of control group. Each sample was examined in duplicate and the experiments were repeated three times.

Reverse transcription-PCR analysis

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, USA), and 2 μg of the sample was reverse-transcribed (RT) by using M-MLV reverse transcriptase (Promega). For PCR amplification, primers specific for GAPDH were: forward 5'-ATTCAACGGCAGCAGTCAA GG-3'; reverse 5'-GCAGAAGGGGCGGAGATGA-3'. For MRTF-A: forward 5'-TGAGCGATGGGAGCATAA-3'; reverse 5'-TTGGGAGCAGTTGG-3'. For MYL9: forward 5'-CA GTGCCAGATCCAGGATTTAAAG-3'; reverse 5'-GGGTG AACTCCACGTAGTTGAAGT-3'. For CYR61: forward 5'-AGCAGCGTTCCTTTCACT-3'; reverse 5'-TGAGTC CCATCACCCACA-3'. Real-time quantitative PCR analysis was performed with StepOne™ Real-Time PCR system (Applied Biosystem, Foster City, USA) using standard procedures. Data were shown as relative expression level after being normalized by GAPDH. Error bars represent the mean (± standard error, SE) of three independent experiments performed in triplicate.

Western blot analysis

The total protein of the cells was prepared using an extraction buffer composed of phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethyl sulfonylfluoride, and protease inhibitors (Roche, Basel, Switzerland). The concentration of protein was determined by the BCA™ concentration of whole cell extracts was determined by the BCA™ protein assay kit (Thermo). Luminescence was measured and normalized by using a Synergy4 microplate reader (Biotek). Data were expressed as fold(s) of control group. Each sample was examined in duplicate and the experiments were repeated three times.

Statistical analysis

Data were expressed as mean ± SE, accompanied by the number of experiments performed independently, and analyzed by t-test. Differences at P < 0.05 were considered statistically significant.

Results

E2 promotes migration of MCF-7 cells and up-regulates MRTF-A, MYL9, and CYR61

To determine the effect of E2 on the motility of MCF-7 cells, the wound healing assay was performed with cells treated with E2 at 10^-10 – 10^-8 M for 72 h. As shown in Fig. 1(A), E2 could dose-dependently promote the migration ability of MCF-7 cells. Furthermore, the results of RT-PCR and western blotting showed that both mRNA levels and protein levels of MRTF-A, MYL9, and CYR61 were significantly increased after being treated with E2 for 24 h, especially when the concentration was over 10^-9 M [Fig. 1(B–E)].

MRTF-A promotes migration of MCF-7 cells

To investigate whether MRTF-A was sufficient to induce migration of MCF-7 cells, the migration ability of cells with overexpression or knockdown of MRTF-A was detected using the wound healing assay and the Transwell chamber assay. The results showed that the scratch of the cells transfected with MRTF-A was markedly narrower than that of the control cells after being transfected for 48 h, while contrary results were observed when the endogenous MRTF-A was suppressed with specific siRNAs [Fig. 2(A,B)]. Similarly, the cells migrating across the membrane of the Transwell chamber in the MRTF-A-overexpressed group was also increased compared with the control group [Fig. 2(C,D)]. In addition, the MTT assay showed that the proliferation of the cells being transfected with MRTF-A remained nearly
unchanging [Fig. 2(E)]. Taken together, these results suggested that the overexpression of MRTF-A could markedly promote the migration of MCF-7 cells under the conditions that it had no obvious effects on cell proliferation.

MRTF-A enhances the transcription and expression of MYL9 and CYR61
According to the result of the bioinformatics analysis, CarG boxes are present in the promoter of *MYL9* and *CYR61*,

E2 up-regulates MRTF-A pathway and promotes breast cancer migration

Figure 1  **E2** promotes migration of MCF-7 cells and up-regulates MRTF-A, MYL9, and CYR61  After being treated with E2 at $10^{-10}$ to $10^{-8}$ M for 72 h, the migration ability was examined by using a scratch wound model (A). The mRNA levels and protein levels of MRTF-A, MYL9, and CYR61 were detected with RT-PCR (B,D) and western blotting (C,E) after being treated with E2 for 24 h.

Figure 2  **MRTF-A** improves the migration ability of MCF-7 cells  After being transfected with MRTF-A for 24 h or siMRTF-A for 48 h, the migration ability was examined by using a scratch wound model (A and B) and Transwell cell culture insert (C). The number of cells migrating to the lower side of the Transwell was counted and photographed in five fields (the upper, the lower, the left, the right, and the middle) of three independent experiments (D). In addition, the effect of overexpression of MRTF-A on the proliferation of MCF-7 cells was measured by MTT assay (E). *P* < 0.05.
indicating that MRTF-A might directly activate the transcription of these two migration-related genes. To further determine this effect, semi-quantitative RT-PCR, real-time RT-PCR, western blotting, and immunocytochemistry assays were performed. As expected, all the results demonstrated that overexpression of MRTF-A could enhance the transcription and expression of both MYL9 and CYR61 (Fig. 3). In contrast, RNAi-mediated knockdown of MRTF-A resulted in significant down-regulation of MYL9 and CYR61 [Fig. 3(E,F)], suggesting that these two migration-related genes are downstream targets of MRTF-A.

MRTF-A activates the promoter of MYL9 and CYR61

To further investigate the transactivation effect of MRTF-A on the promoter of MYL9 and CYR61, the luciferase plasmid driven by the promoter of MYL9 or CYR61 was constructed and the reporter assay was conducted. As shown in Fig. 4(A), the overexpressed MRTF-A could increase the promoter activity of either MYL9 or CYR61. As there are two CarG boxes in the MYL9 core promoter region [Fig. 4(B)], to examine which one was the key binding site of MRTF-A in the transactivation of MYL9, distal and/or proximal CarG box mutated luciferase plasmids were further constructed. The results of the reporter assay showed that the luciferase activity was significantly reduced when the proximal CarG box was mutated, while the distal CarG box mutant exhibited little changes compared with the wild-type promoter, indicating that the proximal CarG box might be the major element for the transactivation effects of MRTF-A on MYL9 in MCF-7 cells [Fig. 4(C)].

Discussion

Cumulative exposure to estrogen has been regarded as a major risk factor associated with breast cancer. So far, a number of established pathways have been extensively studied in estrogen-induced proliferation of breast cancer cells. However, the effects of estrogen on breast tumor cell motility or invasion are poorly understood. In the present study, we found that E2 could dose-dependently promote the migration of MCF-7 breast cancer cells via up-regulation of MRTF-A and its target genes, MYL9 and CYR61. In addition, these effects were dramatically increased when the concentration of E2 was higher than $10^{-9}$ M. It was reported that the plasma concentration of serum estradiol typically fluctuates in a wide range of $1.7-7.5 \times 10^{-10}$ M in the normal menstrual cycle of adult female, and it always decreases to about $1.5-2.5 \times 10^{-11}$ M in postmenopausal women [16,17]. Therefore, our results suggested that the MRTF-A signal pathway could be markedly activated when the breast cancer cells were exposed to estrogen at higher physiological levels, and this event might contribute to the excessive estrogen-stimulated breast cancer metastasis.

MRTF-A belongs to the MRTFs family that also includes MRTF-B and myocardin. They all have similar structural features and transcriptional regulation functions, especially in the development of the cardiovascular system [18]. However, their roles in cancer were a bit confusing. Myocardin was proposed to be a tumor suppressor and its overexpression could induce differentiation and inhibit growth of sarcoma cells, while MRTF-A, as well as MRTF-B, were shown to be more closely related to cancer metastasis [19,20]. Furthermore, published reports are contradictory, some studies indicated that MRTF-A might promote migration and invasion of cancer cells, but some researches demonstrated that MRTF-A should be a suppressor of cancer metastasis. Besides, MRTF-A may inhibit tumor necrosis factor-induced cell death by suppressing activation of caspases, and it is found to be

Figure 3 MRTF-A enhances the expression of MYL9 and CYR61 After being transfected with MRTF-A for 24 h, the transcription levels and the expression levels of MYL9 and CYR61 in MCF-7 cells were determined by either semi-quantitative or real-time RT-PCR (A and B) and western blotting (C), respectively. The expression and subcellular localization of MYL9 were also examined by immunocytochemistry assay (D). The mRNA levels and protein levels of MRTF-A, MYL9, and CYR61 were detected with RT-PCR (E) and western blotting (F) after being treated with siMRTF-A for 24 h. *P < 0.05 vs. Control.
fused to the RNA-binding motif protein 15 (RBM15) via (1;22)(p13;q13) translocation, and this fusion generates a deregulated protein which induces acute megakaryoblastic leukemia in infants and children, indicating that MRTF-A might be more like an oncogene [21–23]. Here, our results provided novel evidence supporting the metastasis-promoting functions of MRTF-A.

Furthermore, MRTF-A-knockout female mice failed to feed pups due to the defect in mammary gland differentiation, indicating that it should be a key regulator of mammary development and function [24,25]. Guillaume et al. [26] also found that activation of the Rho/actin/MRTF-A signaling pathway impaired the transactivation efficiency of ER α. By combining these reports with our results, we proposed that MRTF-A might be a switch for the estrogen pathway to change its proliferation-promoting roles to migration-stimulating roles, and then promote breast cancer transforming from proliferative type to invasive type.

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References