Estrogen receptor β inhibits estradiol-induced proliferation and migration of MCF-7 cells through regulation of mitofusin 2

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Abstract. In the present study, we investigated whether estrogen receptor (ER) β affected the proliferation and migration of the human breast cancer cell line MCF-7 through regulation of mitofusin 2 (mfn2). A previous study reported that mfn2 may be regulated by ER through a non-classical pathway; in this pathway, the ER modulates the activities of other transcription factors by stabilizing their binding to DNA and/or recruiting coactivators to the complex. However, the previous study, unlike the study presented here, did not directly explore the interactions between ER and mfn2. Here, RT-PCR and western blot analysis were used to test the expression of mfn2 in MCF-7 cells after exposure to different doses of estradiol (E2). The ability of cells to proliferate and migrate was determined by MTT assay and a monolayer-wounding protocol, respectively. Finally, changes in MCF-7 cell biology after transfection with ERβ or mfn2 expression vectors were investigated, and the role of ERβ in mfn2 expression was also explored. Our results showed that E2 attenuated mfn2 expression in a dose-dependent manner, concomitant with the activation of proliferation and migration of MCF-7 cells. The mfn2 expression vector effectively suppressed E2-induced upregulation of PCNA and migration in MCF-7 cells. ERβ inhibited the E2-induced mfn2 downregulation that accompanied the inhibition of proliferation and migration in MCF-7 cells. Briefly, ERβ may inhibit E2-induced proliferation and migration of MCF-7 cells through regulation of mfn2.

Introduction

Both clinical and epidemiological evidence show that estrogens participate in the initiation and development of human breast cancer (1,2). Understanding the role of both types of estrogen receptor (ER) (ERα and ERβ), in the pathogenesis of breast cancer is important, because effects of estrogen are mediated through both of these ERs (3-6). Although the function of ERα has been established and its remains the most important marker of response to hormonal therapy in breast cancer, the role of ERβ remains elusive with many conflicting studies (7). The two ERs act in distinct ways in several estrogen target cells and tissues (8,9). There are two major conclusions to be drawn from current research situation of ERs. First, ERα and ERβ have different biological functions, which are indicated by their distinct expression patterns and the different phenotypes reported for the two ERs in knockout animals, respectively. Second, ERα and ERβ have overlapping yet unique roles in estrogen signaling, as judged from a number of gene expression profiling studies.

Mitofusin 2 (mfn2), also named as hyperplasia suppressor gene for its antiproliferative effects, localizes to the mitochondrial outer membrane and plays an essential role in mitochondrial fusion, thus regulating mitochondrial morphology and function. Chen *et al* (10) recently demonstrated that mfn2 profoundly suppresses cell growth and proliferation in multiple tumor cell lines and rat vascular smooth muscle cells *in vivo* and in culture systems via inhibition of the Ras-ERK MAPK signaling pathway. Also, there is some evidence suggesting a protective effect of mfn2 in mammalian cells (11-13).

There is a growing body of literature suggesting that estrogen may modulate expression of some genes through a non-classical pathway in which the ER interacts with other transcription factors, a process referred to as transcription factor cross-talk. In this pathway, the ER modulates the activities of other transcription factors, such as activator protein (AP)-1, by stabilizing their binding to DNA and/or recruiting coactivators to the complex. DeNardo *et al* (14) identified sets of estrogen-induced genes, including mfn2, whose promoters contain potential AP-1 sites but no estrogen-responsive element (ERE) sequences, essential for a classical model of estrogen action; these genes thus depend on AP-1 for their expression. Further characterization of the promoters suggested that the ER regulated these genes through the non-classical pathways mentioned above. However, the previous study, unlike the study presented here, did not directly explore the interaction between ERs and mfn2.

In the present study, we showed that ERβ inhibits human breast cancer cell proliferation and migration by inducing

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expression of mfn2. We report for the first time that ERβ acts upstream of mfn2. Moreover, this observation indicated that mfn2 affects the proliferation and migration of human breast cancer cells.

Materials and methods

Cell lines and groups. MCF-7, a human breast cancer cell line, was kindly provided by Professor Mei-xiang Sang, Division of Scientific Research, the Fourth Hospital of Hebei Medical University, Shijiazhuang, China. Cells were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, USA) containing 4.5 g/l glucose, 2 mM L-glutamine, 5,000 IU/l penicillin, 5 mg/l streptomycin, 125 U/l Fungizone, 2.2 g/l sodium bicarbonate and 10% fetal bovine serum (FBS) pretreated by 5% charcoal-dextran, in a 5% CO_2 incubator. For experiments carried out in serum-free conditions, cells were made quiescent by culturing in serum-free medium for 24 h. DMEM with antibiotics and glutamine, was supplemented with 0.5 g/l BSA (1). Cells were randomly divided into six groups and cultured for 48 h with E2 (17β-estradiol, at doses of 0 mol/l group, 10^{-9} mol/l group, 10^{-8} mol/l group, 10^{-7} mol/l group, 10^{-6} mol/l group and 10^{-5} mol/l) to determine the dose-dependent effects of E2 on mfn2 and cell behavior. Cells of each group were cultured for 48 h in DMEM medium containing 10% FBS plus defferent dose of E2 without phenolsulfonphthalein (2). To specially enhance mfn2 expression and explore its effect on proliferation and migration of MCF-7 cells, cells were randomly divided into four groups in gene transfection experiments as follows: normal group (blank control), untransfected E2 group (E2), control vector pEGFP-transfected E2 group (E2 plus control vector) and pEGFP-mfn2-transfected E2 group (E2 plus mfn2 vector). Cells of the three groups treated with E2 were cultured in DMEM with 10% FBS plus 10^{-6} mol/l E2 for 48 h (3). To explore the effect of ERβ on mfn2 expression, cells were randomly divided into four groups as follows: normal group (blank control), untransfected E2 group (E2), control vector pEGFP-N1 E2 group (E2 plus control vector) and pEGFP-N1-ESR2 E2 group (E2 plus ERβ vector). Cells of every group were grown as described in group 2. Each experiment was repeated six times.

Expression vectors and transient transfection. The pEGFP-mfn2 and pEGFP-N1-ESR2 vectors and their negative control vectors were purchased from Yingrun Biotechnology Co. Ltd., (Changsha, China). pEGFP-mfn2 and pEGFP-N1-ESR2 plasmids carry full-length mfn2 and ERβ genes, respectively. Transient transfection of MCF-7 cells was carried out using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, MCF-7 cells were cultured in 6-well plates and the medium was changed the following day until 80% confluence was achieved. The cells were transfected with 4.0 μg vector DNA by 10 μl Lipofectamine 2000 in 2 ml serum-free DMEM medium. At 6 h after transfection, the medium was replaced by normal DMEM supplemented with 10% FBS, and cells were cultured for 24 h. Cells were then cultured for 48 h in medium containing 10% FBS and E2 to detect proliferation and migration of MCF-7 cells (group 2) and mfn2 expression (group 3). The efficiency of transfection was approximately 70% for all experimental groups.

Table I. Primers and corresponding products for mfn2 and GAPDH.

Gene		Products (bp)
mfn2		
Sense	5'-ATGCATCCCCACTTAAGCAC-3'	301
Antisense	5'-CCAGAGGGCAGAACTTTGTC-3'	
GAPDH		
Sense	5'-AACGGATTTGGTCGTATTG-3'	214
Antisense	5'-GCTCCTGGAAGATGGTGAT-3'	

Western blot analysis. Protein extracted from MCF-7 cells was separated on a 10% SDS-PAGE gel and then transferred onto PVDF membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked for 1 h at 37˚C with 5% BSA in Tris-buffered saline containing 0.05% Tween-20 (TBST). Next, the membrane was incubated at 4˚C overnight with primary antibodies for mfn2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERβ (1:100, Santa Cruz Biotechnology), and β-actin (1:1,000, Santa Cruz Biotechnology). Subsequently, the membrane was rinsed three times with TBST containing secondary antibody (1:5,000), treated with ECL solution (Pierce, Rockford, IL, USA), and bands detected by exposing the blots to X-ray film. For quantitative analysis (i.e., normalized for β-actin), bands were evaluated with IPP 5.0 software. Integrated optical density (IOD) of each band was measured, and relative IOD calculated as the ratio of the target band IOD compared to the IOD of the β-actin band.

Semi-quantitative RT-PCR. Total RNA was extracted with TRIzol (Invitrogen Co.) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using random primers and M-MLV at 42˚C for 1 h and then heated to 94˚C for 5 min in a total reaction volume of 20 μl. The PCR amplification began with a 5-min denaturation at 95˚C, followed by 40 cycles of denaturation at 95˚C for 45 sec, annealing at 55˚C for 45 sec and extension at 72˚C for 60 sec. The final extension was set for 10 min at 72˚C. The products were electrophoresed on a 1.5% agarose gel, and the levels of mfn2 mRNA were normalized with levels of GAPDH mRNA. All PCR primers are shown in Table I.

Immunofluorescence. MCF-7 cells were planted on cover slides in 6-well plates. After fixing with 10% formalin at room temperature for 15 min, pretreating with 0.3% Triton X-100 for 20 min at 37˚C and blocking with goat serum for 30 min at 37˚C, cells were incubated with anti-mfn2 (1:200) overnight at 4˚C. After washing with PBS for three times, the slides was all incubated with FITC-conjugated secondary antibody (1:200, Santa Cruz Biotechnology) for 2 h at 37˚C. Then slides were viewed after being rinsed with PBS three times.

Cell proliferation. Cell proliferation was measured using methyl thiazolyl tetrazolium (MTT) shade selection experiments. Cells ($5x10^3$ per well) were plated in triplicate in 96-well

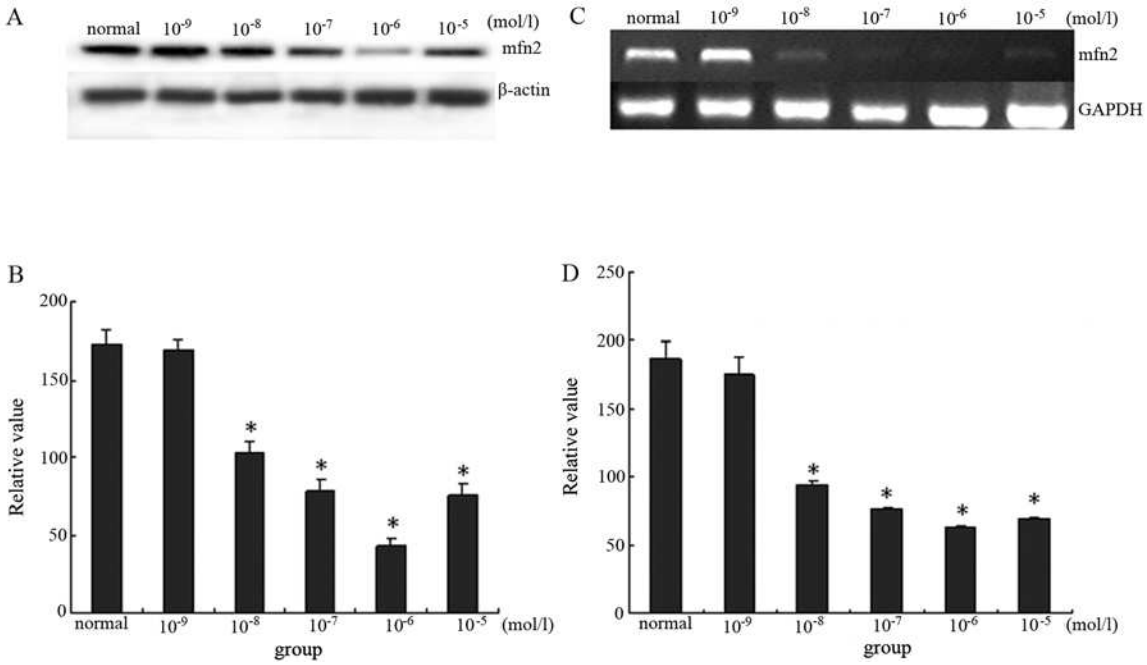

Figure 1. E2 downregulates expression of mfn2 in a dose-dependent manner in MCF-7 cells. (A) Western blot analysis of the dose-dependent effects of E2 on mfn2 expression. (B) Protein levels of mfn2 were quantified by densitometry (mean ± SD, n=6). *P<0.05 vs. normal group. (C) mRNA levels of mfn2 were determined by RT-PCR. (D) mRNA levels of mfn2 were quantified by densitometry (mean ± SD, n=6). *P<0.01 versus normal group.

plates and cultured for 24 h. Then, 3-2,5-dihydro-1-methyl-5h-tetrazole-5-thion sodium salt was added for 4 h before absorbance was determined at 490 nm (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

Measurement of cell migration. Cell migration was measured using a wound-healing protocol developed and described in an earlier publication (15).

Statistical analysis. The figure analysis was carried out by the software of IPP. The quantitative data are presented as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls test. Statistical differences were considered significant at a P-value of <0.05.

Results

E2 downregulates expression of mfn2 in a dose-dependent manner in MCF-7 cells. As described above, the ER might regulate mfn2 expression via a non-classical pathway. To observe the effect of ERα on mfn2, MCF-7 cells, which primarily express ERα, were cultured in medium containing E2. The effect of E2 on mfn2 expression through ERα was measured using immunoblotting for protein levels and semi-quantitative RT-PCR for mRNA levels in MCF-7 cells. E2 inhibited the expression of mfn2 in a dose-dependent manner. Mfn2 was expressed at a higher level in cells cultured with 10% FBS. When cells were pretreated with 10^{-9} mol/l, 10^{-8} mol/l, 10^{-7} mol/l, 10^{-6} mol/l and 10^{-5} mol/l group E2 for 48 h, protein expression of mfn2 decreased by 2.85, 40.00, 55.43, 74.29 and 57.14%, respectively. Thus, the lowest expression of mfn2 was seen in the 10^{-6} mol/l group. Similar changes were seen when cells were analyzed by RT-PCR. These findings demonstrated that E2 decreased mfn2 expression in a dose-dependent manner at both the molecular and protein levels (Fig. 1).

E2 enhances proliferation and migration of MCF-7 cells. MCF-7 cells are the best-characterized ER-positive cell line in terms of known genes regulated by estrogens that promote proliferation. In order to confirm that E2 promotes proliferation, E2-treated MCF-7 cells were examined using an MTT assay. Absorbance of the MTT substrate at 490 nm for each dosage group is showed in Table II. Significant differences were seen among experimental groups and the control group; the maximum effect of E2 on proliferation was seen in the 10^{-6} mol/l group, where mfn2 was expressed at its lowest level. These results demonstrated that E2 treatment resulted in increased proliferation of MCF-7 cells, and that decreased mfn2 might play a positive role in this proliferation.

To determine if E2 influenced cell motility, we examined the ability of treated cells to migrate in a wound-healing assay. In response to wounding the monolayer, the 10^{-6} mol/l group cells were able to almost completely heal the wound. In contrast, the cells of other groups were unable to do so and exhibited an obvious reduction in their rate of migration compared to the 10^{-6} mol/l group. As compared with the 10^{-6} mol/l group cells, the reduction in the migration rate of cells treated with E2 at 10^{-9} mol/l, 10^{-8} mol/l, 10^{-7} mol/l, 10^{-5} mol/l and 0 mol/l were 64.3, 50.0, 31.4, 24.5 and 85.7%, respectively. These results demonstrated that E2 also enhanced cell motility in a dose-dependent manner (Fig. 2).

The mfn2 expression vector effectively suppressed E2-induced upregulation of PCNA and migration in MCF-7 cells. A previous study demonstrated that mfn2 profoundly suppresses

Table II. E2 enhances proliferation of MCF-7 cells as quantified by MTT assay.

Group (mol/l)	n	OD value (x ± s)
Control	6	0.45±0.18
10^{-9}	6	0.54±0.10
10^{-8}	6	0.62±0.16[a]
10^{-7}	6	0.71±0.15[a]
10^{-6}	6	0.97±0.06[a]
10^{-5}	6	0.89±0.11[a]

[*]P<0.01 vs. control (0 mol/l) group.

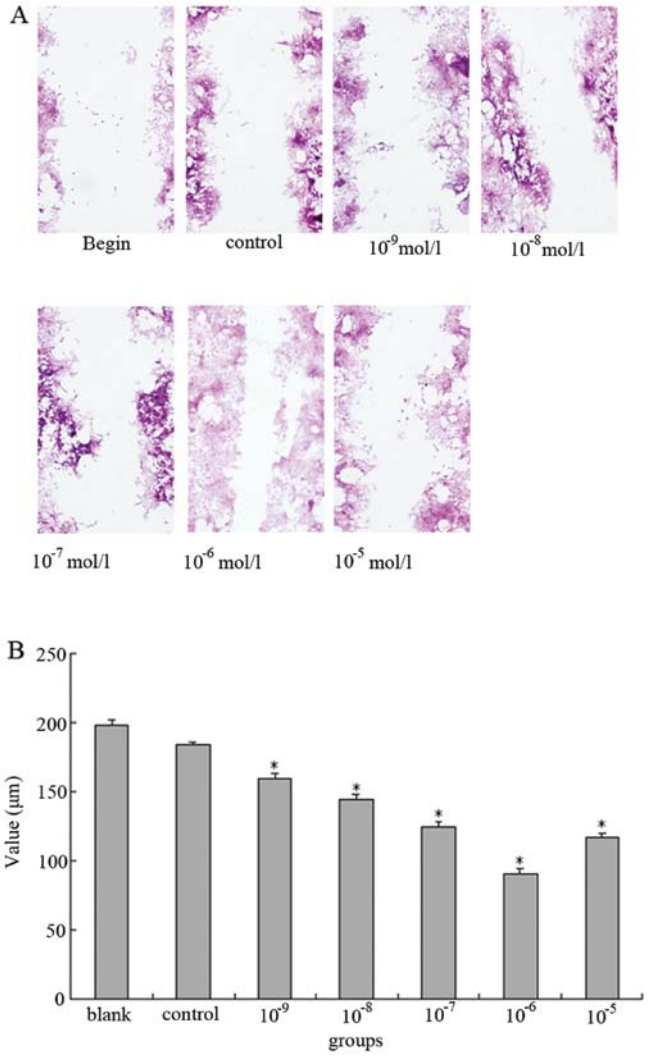

Figure 2. E2 enhances MCF-7 cell migration in a dose-dependent manner. (A) Cell migration was measured by monolayer-wounding protocol. (B) Migration was quantified by distance of monolayer-wounding (mean ± SD, n=6). [*]P<0.01 vs. control group.

cell growth and proliferation in multiple tumor cell lines via inhibition of the Ras-ERK MAPK signaling pathway (10). As described above, the effect of E2 on MCF-7 cells might be partly dependent on inhibition of mfn2. To investigate the involvement of mfn2 in E2-induced cell proliferation and migration, MCF-7 cells were transfected with the expression vector pEGFP-mfn2. As shown in Fig. 3A and B, normal cultured MCF-7 cells (C) had standard expression levels of mfn2 and PCNA. However, both untransfected MCF-7 cells stimulated with 10^{-6} mol/l E2 (E2), and control vector transfected cells stimulated with 10^{-6} mol/l E2 (E2+C) showed notably decreased mfn2 expression and enhanced PCNA expression. In comparison with MCF-7 cells transfected with control vector, mfn2 levels were increased by 2.11-fold and PCNA levels were decreased by about 42.61% in MCF-7 cells transfected with specific mfn2 expression vector (E2+mfn2) (P<0.01). Consistent with the western blot analysis results, immunofluorescence also revealed that the mfn2 vector reversed E2-induced downregulation of PCNA protein (Fig. 3C and D).

Cells transfected with the mfn2 vector showed moderate resistance to E2 stimulation. In comparison with E2-stimulated untransfected or control vector-transfected cells, mfn2 expression vector-transfected cells demonstrated decreased cell migration (Fig. 4). The wound-healing assay indicated that MCF-7 cells and control vector-transfected cells stimulated by E2 almost completely healed the wound, as compared with unstimulated cells. However, this alteration was reversed by transfection with the mfn2 expression vector.

ERβ ameliorates E2-induced mfn2 downregulation in MCF-7 cells. As stated previously, estrogen's effects are mediated through two ERs, ERα and ERβ (3-6). We hypothesized that ERβ might also act upstream of mfn2, because mfn2 is identically regulated by E2. To test our hypothesis, MCF-7 cells were transfected with an ERβ expression vector. As seen in Fig. 5, MCF-7 cells transfected with the ERβ vector showed high ERβ protein expression after stimulation with E2 for 24 h. However, no changes in ERβ protein levels were found in MCF-7 cells transfected with blank control vector or in untransfected MCF-7 cells. In comparison with MCF-7 cells transfected with blank vector, ERβ protein was increased by about 2.25-fold in MCF-7 cells transfected with the ERβ vector (P<0.01). Cells transfected with the ERβ vector showed antagonistic effects on E2 stimulation; mfn2 protein was upregulated in these cells as compared with blank vector-transfected MCF-7 cells and untransfected cells treated with E2. These results indicated that MCF-7 cells transfected with the ERβ vector showed moderate resistance to E2 stimulation and subsequent decreased downregulation of mfn2 protein (Fig. 5).

An ERβ expression vector effectively suppressed E2-induced enhancement of proliferation and migration in MCF-7 cells. The results above showed that mfn2 negatively regulated E2-induced proliferation and migration of MCF-7 cells and ERβ acted as an upstream signal of mfn2; therefore, we hypothesized that ERβ could also inhibit proliferation and migration of MCF-7 cells. To investigate this hypothesis, MCF-7 cells were transfected with the ERβ expression vector (pEGFP-N1-ESR2). An MTT assay was used to examine the proliferation of MCF-7 cells. As shown in Table III, there were significant differences of absorbance of MTT substrate at 490 nm between experimental groups and the control group. MCF-7 cells transfected with the ERβ expression vector showed moderate resistance to

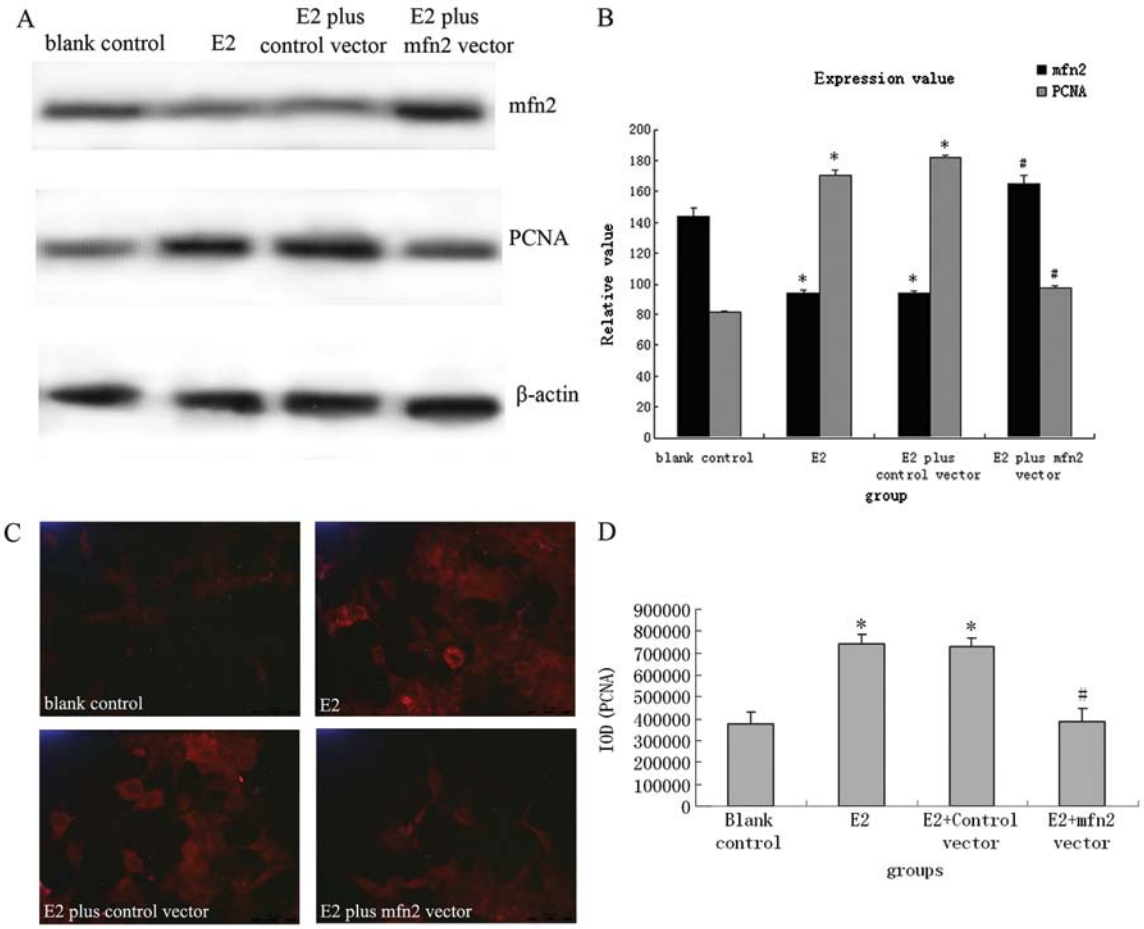

Figure 3. An mfn2 expression vector effectively suppressed E2-induced PCNA expression in MCF-7 cells. (A and B) Expression of mfn2 and PCNA was measured by western blot analysis and quantified by densitometry (mean ± SD, n=6). *P<0.01 vs. control group; #P<0.01 vs. E2 group. (C and D) Expression of PCNA was detected by immunofluorescence and quantified by IOD value (mean ± SD, n=6). *P<0.01 vs. control vector group; #P<0.01 vs. E2 group.

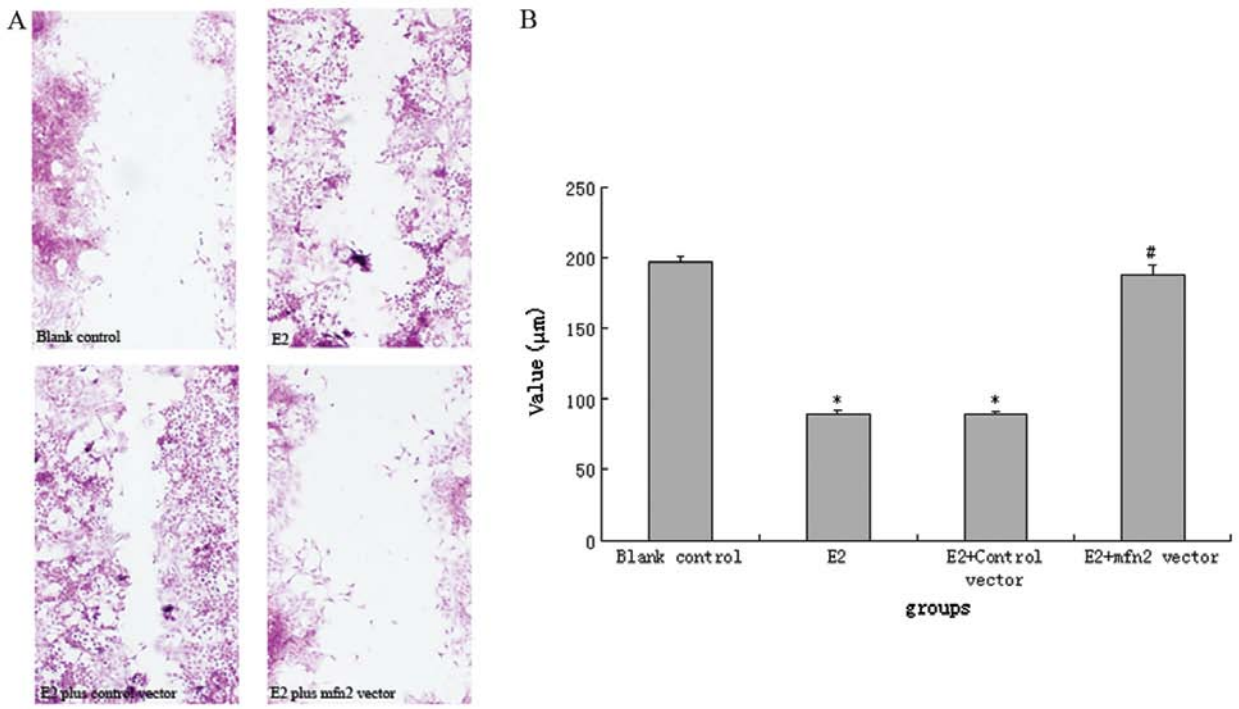

Figure 4. An mfn2 expression vector effectively suppressed E2-induced enhancement of migration in MCF-7 cells. (A and B) Cell migration measured by monolayer-wounding protocol and quantified by distance of monolayer-wounding (mean ± SD, n=6). *P<0.01 vs. control group; #P<0.01 vs. E2 group.

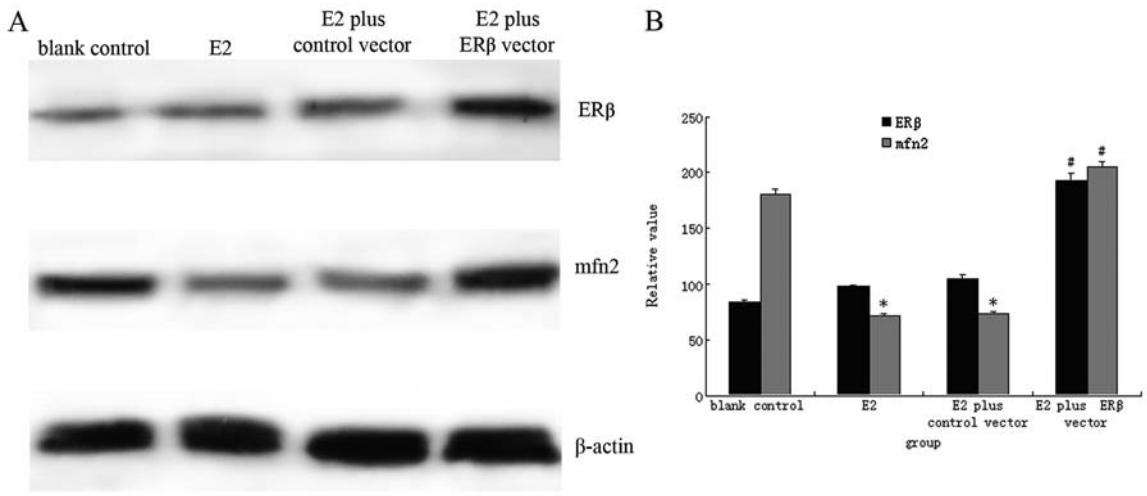

Figure 5. ERβ ameliorates the E2-induced biologic effects in MCF-7 cells. (A and B) ERβ vector reversed E2-induced mfn2 downregulation as measured by western blot analysis and quantified by densitometry (mean ± SD, n=6). *P<0.01 vs. control group; #P<0.01 vs. E2 group.

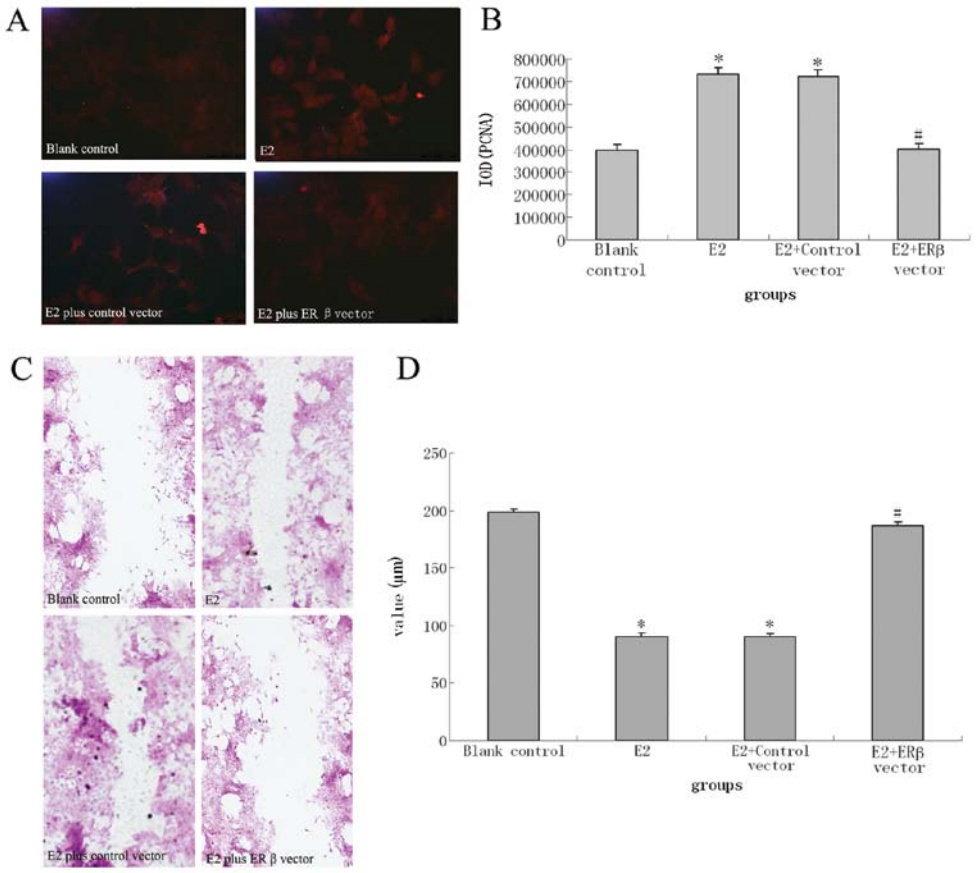

Figure 6. ERβ ameliorates the E2-induced biologic effects in MCF-7 cells. (A and B) ERβ vector reversed E2-induced upregulation of PCNA protein as tested by immunofluorescence and quantified by IOD value (mean ± SD, n=6). *P<0.01 vs. control group; #P<0.01 vs. E2 group. (C and D) Cell migration was measured by monolayer-wounding protocol and quantified by distance of monolayer-wounding (mean ± SD, n=6). *P<0.01 vs. control group; #P<0.01 vs. E2 group.

E2 stimulation and did not exhibit the enhanced proliferation demonstrated by blank vector-transfected and untransfected MCF-7 cells cultured with medium containing E2. The same results can also be seen in the immunofluorescence detection of PCNA expression (Fig. 6A and B).

To determine if ERβ influenced cell motility, we examined the ability of transfected cells to migrate in a wound-healing assay. As showed in Fig. 6C and D, there was decreased cell migration of ERβ expression vector-transfected cells as compared with E2-stimulated untransfected cells and control vector-transfected

Table III. ERβ inhibits the E2-induced proliferation of MCF-7 cells as quantified by MTT assay.

Group	n	OD value (x ± s)
Blank control	6	0.44±0.03
E2	6	0.92±0.06[a]
E2 plus control vector	6	0.91±0.02[a]
E2 plus ERβ vector	6	0.50±0.04[b]

[a]P<0.01 vs. control group, [b]P<0.01 vs. E2 group.

cells. In fact, the wound-healing assay indicated that MCF-7 cells stimulated by E2 and cells transfected with control vector expressed a stronger ability to heal the wound as compared with normal group cells untreated with E2. However, this ability was reversed by transfection with the ERβ vector.

Discussion

It is acknowledged that ERα and ERβ have distinct roles in breast cancer cells. Although the majority considered that ERα promotes proliferation and migration in breast cancer cells and the function of ERα had been clearly elucidated, the exact roles of ERβ in the pathogenesis of breast cancer are unclear. In fact, the function of ERβ in the pathogenesis and development of breast cancer is contradictory. Some studies indicate that ERβ may function as a tumor suppressor and that the loss of ERβ may promote breast carcinogenesis (16-18); however, there are other studies suggesting that ERβ may promote cell proliferation and breast tumor formation (19,20). Regardless of these contradictions, the majority of studies focus on the classical model of estrogen signaling through ERs, ERα and ERβ, in which ERs act at ERE-containing promoters. In the classical model, ligand-activated ER binds specifically to DNA at EREs through its DNA binding domain and brings coactivators and corepressors to the transcription site via its activator function (AF)-1 and AF-2 domains. However, an increasing number of studies show that estrogen also modulates gene expression by a second mechanism in which the ER interacts with other transcription factors through a process referred to as transcription factor cross-talk. In this case, the ER modulates the activities of other transcription factors such as activator protein (AP)-1, or SP-1 by stabilizing their binding to DNA and/or recruiting coactivators to the complex (21,23). DeNardo *et al* (14) reported a model of estrogen-ER activation of AP-1 through interaction with existing coactivator complexes that in turn stabilize the entire complex and/or induce this complex into a higher state of activity. They also identified 6 estrogen-induced/AP-1 dependent genes, including mfn2, which might fit this model. However, their conclusions were only speculative, as they did not provide detailed data or investigated the interaction of ERs and mfn2 *in vitro*. In this study, we investigated the role of ERβ in estradiol-induced proliferation and migration of human breast cancer cells and studied whether mfn2 participated in this behavior.

First, we explored whether E2 (17β-estradiol) affected proliferation and migration of MCF-7 cells, a human breast cancer cell line primarily expressing ERα and thus mimicking the majority of ER-positive breast tumors. Similar to some previous studies that revealed that E2 affected biological behavior of human breast cells (24-26), our results showed that both the proliferation and migration abilities of MCF-7 were significantly increased when cultured with increasing doses of E2. Furthermore, regulation was in a dose-dependent manner, with the maximum effect seen in the 10^{-6} mol/l group. These data suggest that E2 and ERα are positive regulators of MCF-7 cells.

Whether mfn2 was involved in the initiation and progression of human breast cancer has not been previously reported. To investigate the role of mfn2, we observed the expression of mfn2 in MCF-7 cells cultured within defferent doses of E2. Interestingly, we found that E2 could decrease mfn2 expression in a dose-dependent manner, and that the changes in mfn2 levels were correlated with the proliferation and migration of MCF-7 cells. These results indicated that mfn2 might negatively regulate estradiol-induced proliferation and migration of MCF-7 cells. Furthermore, we found that introduction of mfn2 blocked the response of MCF-7 cells to E2. Thus, mfn2 plays an important regulatory role in E2-induced proliferation and migration of MCF-7 cells. Considering the reports that mfn2 is one of the estrogen-induced/AP-1 dependent genes (14), the above results suggested that mfn2 might negatively regulate E2-induced MCF-7 cell proliferation and migration by a non-classical pathway. Mfn2, a proliferation-inhibiting gene, targets to the outer membrane of mitochondria. The mfn2 gene was found to play roles in the inhibition of cellular proliferation and the promotion of apoptosis (10) and exhibits antitumor activity in a wide range of cancer cell lines (27-29), suggesting that mfn2 may be important in the development of human cancers. Again, the present study also provided a potential target for prevention or treatment for breast cancer patients with ERα positive expression.

Approximately 70% of breast tumors express ERβ, and most tumors coexpress both ERα and ERβ (30,31). However, whether ERβ is involved in E2-induced downregulation of mfn2 is still unknown. Clearly, additional studies are needed to clarify the role of ERβ in breast cancer. In the present study, we introduced ERβ into MCF-7 cells and investigated the effects of ERβ on proliferation and migration of MCF-7 cells as well as its effects on mfn2 expression. Our studies demonstrate that ERβ changes the phenotype of MCF-7 cells in response to E2. In ERα-expressing MCF-7 cells, E2 causes proliferation and migration, as well as suppression of mfn2. In contrast, when ERβ is expressed along with ERα, MCF-7 cells are directed to antitumor pathways and high levels of mfn2 even in the presence of estrogens. These results suggest that ERβ can alter the response of MCF-7 to estrogens and demonstrate that ERβ may function as a tumor suppressor through the mfn2 pathway. Many cell-based studies suggest that ERβ acts as a negative modulator of ERα action. When ERα and ERβ are co-transfected into ER negative (ER-) cells, ERβ inhibits ERα transcriptional activity and decreases the sensitivity of the cells to E2 (6,32). ERβ also lowers both ERα mRNA and protein levels in MCF-7 cells, thus indirectly influencing function of ERα (33,34). ERβ overexpression in MCF-7 breast cancer cells can not only inhibit ERα regulation of a subset of genes involved in DNA replication, cell-cycle regulation, and proliferation (35,36), but also inhibit cell proliferation in response to E2 (34,36-38), in part by increasing expression of antiproliferative genes ($p21^{Cip1}$

and $p27^{Kip1}$). Our results were quite similar to these reports, and minor deference lie in downstream factors. There may be diverse mechanisms for the effect of ERβ on the response of ERα to E2. Recently, some studies revealed in series that the responses of breast cancer cell lines to 17β-estradiol are dependent on the ERα/ERβ ratio (39,40). Most importantly, ERβ might regulate mfn2 expression directly in a non-classical pathway similar to ERα. Therefore, further studies are needed to determine the exact mechanisms of interaction between ERs, ERα and ERβ, and mfn2, especially to delineate the mechanism of action through experiments such as in-depth promoter analysis and CHIP.

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