Pro-apoptotic and anti-proliferative effects of mitofusin-2 via PI3K/Akt signaling in breast cancer cells

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Abstract. The mitochondrial GTPase mitofusin-2 (Mfn2) gene is a novel gene characterized as a cell proliferation inhibitor. Mfn2 has previously been reported to play a role in regulating cell proliferation, apoptosis and differentiation in a number of cell types. However, there are no studies on the effect of Mfn2 in breast cancer. In this study, we aimed to elucidate the function and mechanism of Mfn2 in breast cancer. A plasmid encoding the complete Mfn2 open reading frame (pEGFP-Mfn2) was used to infect breast cancer cells. The effect of Mfn2 on proliferation was assessed by methyl thiazolyl tetrazolium and the anti-proliferative effects of MCF-7 cells were investigated through western blot analyses. Flow cytometry, immunofluorescence and western blot analyses were used to test the effects of Mfn2 on cell cycle distribution and apoptosis. Additionally, the PI3K/Akt signaling pathway was analyzed after pEGFP-Mfn2 was transfected into MCF-7 cells. The results revealed that Mfn2 suppressed the proliferation of MCF-7 cells by regulating more cells at the G0/G1 phase and decreasing proliferating cell nuclear antigen and cyclin A expression. The results also demonstrated that the PI3K/Akt signaling pathway is involved in Mfn2-regulated proliferation and apoptosis. Taken together, this indicates that Mfn2 mediates MCF-7 cell proliferation and apoptosis via the PI3K/Akt signaling pathway. Mfn2 may thus be a significant therapeutic target in the treatment of breast cancer.

Introduction

The mitochondrial GTPase mitofusin-2 (Mfn2) gene, which is also called the hyperplasia suppressor gene, was originally identified in vascular smooth muscle cells from spontaneously hypertensive rats, which exhibited markedly lower expression than Wistar-Kyoto rats (1). Mfn2 localizes to the mitochondrial outer membrane and plays an essential role in mitochondrial fusion, thus regulating mitochondrial morphology and function. Further research has indicated that Mfn2 has a potential apoptotic effect mediated by the mitochondrial apoptotic pathway (1-3). Chen et al (4) demonstrated that Mfn2 notably suppresses cell growth and proliferation in a number of tumor cell lines through the inhibition of the Ras-ERK MAPK signaling pathway. Recently, Mfn2 has become a focal point in tumor research. Several studies have investigated the function of Mfn2 in various malignancies, including hepatocellular, urinary bladder and gastric cancers, and Mfn2 is considered to perform pro-apoptotic and anti-proliferative functions (5-7).

Clinical and epidemiological evidence reveals that estrogens participate in the initiation and development of human breast cancer (8,9). Understanding the role of estrogen receptor (ER) α and β in the pathogenesis of breast cancer is essential, since the effects of estrogen are mediated through these two ERs (10). Although the function of ERα has been established and this receptor remains the most significant marker of the response to hormonal therapy in breast cancer, the role of ERβ remains elusive as a result of a number of conflicting studies (11). Our previous study demonstrated that ERβ may inhibit the estradiol-induced proliferation and migration of MCF-7 cells through regulation of Mfn2 (12), but the exact mechanism by which Mfn2 exerts its antitumor effects remains unclear. Therefore, exploration of the function of Mfn2 may also help us understand the role of ERβ in the pathogenesis of breast cancer.

A previous study demonstrated that the PI3K/Akt signaling pathway was involved in Mfn2-regulated smooth muscle cell proliferation (13). However, the correlation between them remains unclear in breast cancer. We hypothesized that the outer-membrane protein Mfn2 participates in the apoptotic process in association with the PI3K/Akt signaling pathway. In the present study, we employed a plasmid to deliver Mfn2 to MCF-7 cells, a human breast cancer cell line, in order to evaluate the effect of Mfn2 on apoptosis and proliferation. Furthermore, we investigated the mechanism of Mfn2-regulated pro-apoptosis and the anti-proliferation effects of MCF-7 cells in vitro.

Materials and methods

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Fourth Hospital of Hebei Medical University, Shijiazhuang, China. The cells were cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) containing 4.5 g/l glucose, 2 mM L-glutamine, 5000 IU/l penicillin, 5 mg/l streptomycin, 125 U/l Fungizone, 2.2 g/l sodium bicarbonate and 10% fetal bovine serum (FBS) pretreated with 5% charcoal-dextran in an incubator at 37°C with a humidified atmosphere of 5% CO₂. For the experiments conducted in serum-free conditions, the cells were induced to quiescence by culturing in serum-free medium for 24 h. DMEM with antibiotics and glutamine was supplemented with 0.5 g/l bovine serum albumin (BSA). The cells of each experimental group were cultured for 48 h with 17β-estradiol (E2) at a dose of 10⁻⁶ mol/l, which was confirmed in our previous study to have an optimal effect (12).

Expression vectors and transient transfection. pEGFP-Mfn2 and its negative control vectors were purchased from Invitrogen Biotechnology Co. Ltd. (Changsha, China). The pEGFP-Mfn2 plasmid carries the full-length Mfn2 gene. The transient transfection of MCF-7 cells was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, MCF-7 cells were cultured in six-well plates, and the medium was changed every 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. Six hours after transfection, the medium was replaced by normal DMEM supplemented with 10% FBS, and the cells were cultured for 24 h. The cells were then cultured for 48 h in medium containing 10% FBS and E2 to detect the proliferation and apoptosis of MCF-7 cells. The efficiency of transfection was ~70% for all the experimental groups.

Cell proliferation. The cell proliferation was measured using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) assay selection experiments. The cells (5x10⁴ per well) were plated in triplicate in 96-well plates and cultured for 24 h. Then, 32-2,5-dihydro-1-methyl-5h-tetrazole-5-thion sodium salt was added for 4 h, and the absorbance was determined at 490 nm (SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. The proteins extracted from MCF-7 cells were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, 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). The membrane was then incubated at 4°C overnight with primary antibodies for Mfn2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt (Cell Signaling Technology, MA, USA), phospho-Akt (Cell Signaling Technology) and β-actin (1:1000; Santa Cruz Biotechnology). Subsequently, the membrane was rinsed three times with TBST containing secondary antibodies (1:5000) and treated with enhanced chemiluminescence solution (Pierce, Rockford, IL, USA), and the bands were detected by exposing the blots to X-ray film. For quantitative analysis (i.e., normalized for β-actin), the bands were evaluated with IPP 5.0 software (?). The integrated optical density (IOD) of each band was measured, and the relative IOD was calculated as the ratio of the target band IOD to the IOD of the β-actin band.

Immuno fluorescence. MCF-7 cells were plated on coverslides on six-well plates. After fixation in 10% formalin at room temperature for 15 min, pretreatment with 0.3% Triton X-100 for 20 min at 37°C and blocking with goat serum for 30 min at 37°C, the cells were incubated with anti-Mfn2 (1:200) overnight at 4°C. After washing three times with phosphate-buffered saline (PBS), the slides were incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:200, Santa Cruz Biotechnology) for 2 h at 37°C. The slides were then viewed after being rinsed three times with PBS.

Bromodeoxyuridine (BrdU) incorporation. MCF-7 cells were plated at 1x10⁴ cells/well in 96-well plates, subjected to growth arrest for 24 h, and exposed to E2 or treated with various agents in serum-free DMEM. BrdU incorporation was measured using BrdU proliferation assay kits (Millipore) according to the manufacturer's instructions. Briefly, the cells were labeled with 10 ng/ml BrdU during the incubation, washed three times with cold wash buffer, fixed, air-dried and incubated for 1 h at room temperature with mouse anti-BrdU monoclonal antibody (diluted 1:200). The antibody was aspirated. The cells were washed three times and then incubated with peroxidase goat anti-mouse IgG (1:2000) at room temperature for 30 min. The cells were washed three times, and 100 µl of the substrates was added to each well. The plate was then incubated for 30 min in the dark. Thereafter, the absorbance was measured at dual wavelengths of 450 to 540 nm.

Flow cytometric analysis. MCF-7 cells were detached using trypsin for 48 h following infection with pEGFP-Mfn2 and the control vector pEGFP. The cells were washed three times with PBS. To detect the cell cycle phases, the cells were treated with 50 µl DNA Prep LPR (Beckman Coulter, Fullerton, CA, USA) for 30 min at room temperature and 500 µl DNA Prep Stain (Beckman Coulter) for 30 min at room temperature. To measure the apoptosis of MCF-7 cells following treatment, an ApoScreen Annexin V apoptosis kit (Southern Biotech, Birmingham, AL, USA) was used according to the manufacturer's instructions. The cell cycle distribution and apoptosis were determined using a flow cytometer (Beckman Coulter).

Statistical analysis. Figure analysis was conducted using IPP software (Media Cybernetics, Inc., Rockville, MD, USA). The quantitative data are presented as the mean ± standard deviation. The statistical analyses were performed using one-way analysis of variance with the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in protein expression levels of Mfn2, Akt and phospho-Akt (p-Akt) in MCF-7 cells exposed to E2. To determine the protein expression levels of Mfn2, Akt and p-Akt, western blot analysis of the total proteins extracted from MCF-7 cells was performed. It was observed that the expression of Mfn2 protein was at a relatively high level in cells


The cells pretreated with 10⁻⁶ mol/l E2 for 48 h demonstrated a 22.54% decrease in the protein expression level of Mfn2 (Fig. 1A and B). These findings demonstrated that E2 decreases the Mfn2 protein expression level. Notably, the protein expression of p-Akt was elevated following culture with E2. However, there was no significant change in the Akt protein expression level in our experiment (Fig. 1A and B).

**Mfn2 mediates E2-induced MCF-7 cell proliferation.** Plasmid transfection technology was used to upregulate the expression of Mfn2, since it is a powerful technology that allows the augmentation of cellular genes with great specificity and potency. To assess the efficiency and specificity of plasmid transfection, the expression of Mfn2 was measured relative to that of β-actin by western blot analysis (Fig. 2A and B). The empty pEGFR-N1 vector, as a green fluorescence-tagged negative control, also demonstrated the efficiency of transfection (data not shown). As shown in Fig. 3A and B, normal cultured MCF-7 cells exhibited standard expression levels of Mfn2. However, untransfected MCF-7 cells stimulated with 10⁻⁶ mol/l E2 and control vector-transfected cells stimulated with 10⁻⁶ mol/l demonstrated a notable decrease in Mfn2 expression. In comparison with MCF-7 cells transfected with control vector, the Mfn2 levels were increased 1.89-fold in cells transfected with the specific Mfn2 expression vector.

To investigate the role of Mfn2 on MCF-7 cell proliferation, the cell viability was examined by measuring the colorimetric conversion of MTT to formazan. The augmentation of Mfn2 with a specific plasmid decreased the cell viability in the presence of E2 compared with cells transfected with the control vector (Fig. 2C). We also examined the population...
Negative role of Mfn2 in the E2-mediated proliferation of MCF-7 cells. To further examine the mechanism underlying the cell biological behavior in the presence of E2, we analyzed whether Mfn2 affects cell cycle progression. The number of cells in the various cell cycle phases was counted by flow cytometry assay. It was observed that E2 induced more cells to enter the S and G2/M phases from the G0/G1 phase; in addition, pEGFP-Mfn2 suppressed cell cycle progression and arrested MCF-7 cells at the G0/G1 phase (Fig. 4). Since cyclin A, a proliferation-related protein, plays a significant role in the S and G2/M phases, we analyzed the expression of cyclin A in MCF-7 cells. The results revealed that cyclin A protein expression was decreased after the cells were treated with pEGFP-Mfn2 (Fig. 3A and B). These results suggested that Mfn2 had a significant effect on cell cycle activity, which inhibited MCF-7 cell proliferation.

Figure 3. Effects of mitofusin-2 (Mfn2) on the expression of proliferating cell nuclear antigen (PCNA) and cyclin A in MCF-7 cells. (A and B) The expression levels of PCNA and cyclin A were measured by western blot analysis and quantified by densitometry. Means ± SD, n=6. *P<0.05 vs. control; †P<0.05 vs. negative control (NC). 17β-estradiol.

Effect of Mfn2 on cell cycle progression. To further examine the mechanism underlying the cell biological behavior in the presence of E2, we analyzed whether Mfn2 affects cell cycle progression. The number of cells in the various cell cycle phases was counted by flow cytometry assay. It was observed that E2 induced more cells to enter the S and G2/M phases from the G0/G1 phase; in addition, pEGFP-Mfn2 suppressed cell cycle progression and arrested MCF-7 cells at the G0/G1 phase (Fig. 4). Since cyclin A, a proliferation-related protein, plays a significant role in the S and G2/M phases, we analyzed the expression of cyclin A in MCF-7 cells. The results revealed that cyclin A protein expression was decreased after the cells were treated with pEGFP-Mfn2 (Fig. 3A and B). These results suggested that Mfn2 had a significant effect on cell cycle activity, which inhibited MCF-7 cell proliferation.

Figure 4. pEGFR-mitofusin-2 (Mfn2) suppressed MCF-7 cell cycle progression and decreased the percentage of cells in the S and G2/M phase. 17β-estradiol (E2) increased the number of cells in the S and G2/M phase compared with the control group, and this change was reversed following treatment with the pEGFR-Mfn2 vector. Cell cycle progression was measured by flow cytometry assay. Means ± SD, n=6. *P<0.05 vs. control; †P<0.05 vs. negative control (NC). E2, 17β-estradiol.

Mfn2 decreases Akt activity in MCF-7 cells. To determine whether the Mfn2-suppressed cell proliferation was mediated by the activation of Akt, the Akt activity and the expression of p-Akt at Ser473 were studied in MCF-7 cells to determine the correlation between Mfn2 and the Akt pathway. We observed a significant decrease in p-Akt 48 h following the transfection of pEGFP-Mfn2 into MCF-7 cells (Fig. 5A and B). However, the change in Akt protein expression was not notable. The results revealed that Mfn2 regulated p-Akt expression and that the Akt pathway was a downstream target of Mfn2.

Mfn2 suppresses proliferation and induces apoptosis in MCF-7 cells via the PI3K/Akt signaling pathway. To examine the effects of Mfn2 and the PI3K/Akt signaling pathway on cell viability, MCF-7 cells were transfected with pEGFP-Mfn2 and stimulated with LY294002 (20 μM), a PI3K inhibitor. The cell viability was determined by measuring MTT. As shown in Fig. 5C, the Akt inhibitor suppressed the cell viability as with MCF-7 cells transfected with pEGFP-Mfn2. However, the cell viability was not further suppressed in MCF-7 cells transfected with pEGFP-Mfn2 and treated with LY294002, compared with the MCF-7 cells transfected with pEGFP-Mfn2 or treated with LY294002. The results also demonstrated that E2 increased the incorporation of BrdU and that this increase was significantly suppressed by LY294002, whereas the cells transfected with pEGFP-Mfn2 and treated with LY294002 exhibited almost no change compared with the cells transfected with pEGFP-Mfn2 or treated with LY294002 alone (Fig. 5D). To elucidate whether the PI3K/Akt pathway participates in the pEGFP-Mfn2-induced downregulation of PCNA expression, we blocked Akt with LY294002 and obtained results similar to those obtained with the BrdU incorporation assay. The PCNA expression in the cells transfected with pEGFP-Mfn2 and treated with LY294002 exhibited almost no change compared with the MCF-7 cells transfected with pEGFP-Mfn2 or treated with LY294002 alone (Fig. 6A and B).

Mfn2 suppresses cell cycle progression via the PI3K/Akt signaling pathway in MCF-7 cells. To further investigate whether the Akt pathway participates in Mfn2-mediated cell cycle activity, we blocked Akt with LY294002. In the presence of E2, the cells entered the S and G2/M phases from the G0/G1 phase. However, the effect was abrogated in the cells transfected with pEGFP-Mfn2 and in the cells in which the Akt pathway...
was blocked by LY294002 alone, even in the presence of E2. Notably, although the number of MCF-7 cells that progressed into the S and G2/M phase of the cell cycle was suppressed following transfection with pEGFP-Mfn2 or treatment with LY294002 alone, no major changes were observed in the cells after blocking the Akt pathway with LY294002 and treatment with pEGFP-Mfn2 (Fig. 7). To further understand the role of Akt in cell cycle progression, the expression of cyclin A was examined in MCF-7 cells. The expression of cyclin A was suppressed in the cells transfected with pEGFP-Mfn2 and in the cells in which the Akt pathway was blocked with LY294002, and the same results were detected in the cells in which the Akt pathway was blocked with LY294002 and treated with pEGFP-Mfn2 (Fig. 6A and B). These findings suggested that Mfn2 suppressed cell cycle progression via the PI3K/Akt signaling pathway in MCF-7 cells.

Figure 5. Mitofusin-2 (Mfn2) decreases the activation of Akt and the proliferation of MCF-7 cells in an Akt-dependent manner. (A and B) pEGFR-Mfn2 downregulated the expression of phospho-Akt (p-Akt) in MCF-7 cells. The expression levels of p-Akt and Akt were measured by western blot analysis and quantified by densitometry. *P<0.05 vs. control; †P<0.05 vs. negative control (NC). (C) Cultured MCF-7 cells were exposed to 17β-estradiol (E2) in the presence of pEGFR-Mfn2, 20 µM LY294002 (LY), or pEGFR-Mfn2 plus LY294002, and the cell viability was determined by methyl thiazolyl tetrazolium assay. *P<0.05 vs. control; †P<0.05 vs. E2 group. (D) Bromodeoxyuridine (BrdU) incorporation studies demonstrated that the E2-induced cell DNA synthesis was inhibited by LY294002. Moreover, pEGFR-Mfn2, LY294002, and pEGFR-Mfn2 plus LY294002 blocked BrdU incorporation to equal extents. *P<0.05 vs. control; †P<0.05 vs. E2 group. Means ± SD, n=6.

Figure 6. pEGFR-mitofusin-2 (Mfn2) downregulates proliferating cell nuclear antigen (PCNA) and cyclin A expression through the PI3K/Akt pathway. (A and B) The expression levels of PCNA and cyclin A were measured by western blot analysis and quantified by densitometry. Means ± SD, n=6. *P<0.05 vs. control; †P<0.05 vs. E2 group. E2, 17β-estradiol; LY, LY294002.

Figure 7. pEGFR-mitofusin-2 (Mfn2) suppresses MCF-7 cell cycle progression and decreases the percentage of cells in the S and G2/M phase through the PI3K/Akt pathway. The cell cycle progression was measured by flow cytometry assay. Means ± SD, n=6. *P<0.05 vs. control; †P<0.05 vs. E2 group. E2, 17β-estradiol; LY, LY294002.
Discussion

The specific mechanisms of breast carcinogenesis are unclear, although estrogen and its receptors (ERα and ERβ) have been considered essential factors for a long time. ERα was established due to its function in the development and progression of breast cancer, whereas the function of ERβ is unclear and requires further studies. ERβ may inhibit estradiol-induced proliferation and migration of MCF-7 cells through the regulation of Mfn2, as demonstrated in our previous study (12). Thus, the penetration study of Mfn2 may help us understand the exact role of ERβ in breast carcinogenesis. In addition, dysregulation of the balance between proliferation and apoptosis is essential in human carcinogenesis (14). The two main pathways involved in apoptosis are the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (15). Mitochondria are the core organelles of apoptosis, even in the extrinsic pathway. Thus, studies of the exact functional mechanism of Mfn2, which has been confirmed to exert a potential apoptotic effect via the mitochondrial apoptotic pathway (5), are essential. In the present study, we investigated Mfn2, which has been widely studied in Charcot-Marie-Tooth disease (16). Mfn2 is known to be correlated with antitumor activity in a number of malignancies (5-7); however, its effect in breast cancers has not been previously reported. The present study confirmed this association in breast cancer cell lines, and we identified that the pro-apoptotic and anti-proliferative effects of Mfn2 in breast cancer cells occur via PI3K/Akt signaling.

The Mfn2 gene is located on the 1p36.22 chromosome in humans. This chromosome region has been extensively studied, and is considered to contain a number of tumor suppressor genes (17). Mfn2, which controls mitochondrial fusion, is a highly conserved GTPase (18). Similar proteins have been identified in fruit flies and mammals (19). Mfn2 possesses two trans-membrane domains which span the outer mitochondrial membrane, a possible protein kinase A or G phosphorylation site and a p21 (Ras) signature motif (amino acids 77-92), which plays an essential role in signaling (4,18).

In our previous study, we suggested that E2 induces the proliferation of MCF-7 cells by downregulating the expression of Mfn2 (12). In the present study, we confirmed this finding. Notably, accompanied with the downregulation of Mfn2, the protein expression of p-Akt was elevated in the presence of E2. However, no significant change in Akt protein expression was previously reported. The present study confirmed this association in breast cancer cell lines, and we identified that the pro-apoptotic and anti-proliferative effects of Mfn2 in breast cancer cells occur via PI3K/Akt signaling.

Mfn2 possesses two trans-membrane domains spanning the outer mitochondrial membrane, and one of these domains is a p21 (Ras) signature motif (amino acids 77-92) (4,18). A number of studies have suggested that Ras may act as an upstream signaling pathway of PI3K/Akt in cancer carcinogenesis and development (24-26). These studies partly explain our findings. Further experimental studies, including in-depth promoter analysis and chromatin immunoprecipitation, are required.

In conclusion, this study provides experimental evidence confirming the role of Mfn2 as a tumor suppressor gene in breast cancer. The Mfn2 gene significantly promotes apoptosis and inhibits the proliferation of breast cancer cells, and Mfn2 may also induce apoptosis in breast cancer cells via the PI3K/Akt pathway. These observations highlight a previously unexplored role of Mfn2 in cancer development, revealing Mfn2 as a potential therapeutic target for the treatment of tumors and hyper-proliferative diseases.

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References


