ERβ-induced MFN2 inhibits the migration and invasiveness of breast cancer cells by inhibiting the P-AKT signaling pathway

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Abstract

Background Mitofusin 2 (MFN2) is localized on the outer membrane of mitochondria and is closely related to the migration of malignant tumor cells. Estrogen receptor β (ERβ) plays an anticancer role in breast cancer. Our previous experiments showed that ERβ can induce MFN2 expression, which then inhibits breast cancer cell migration. However, the exact mechanism by which ERβ-induced MFN2 inhibits breast cancer cell migration is unknown.

Methods In this study, immunohistochemistry was first used to detect the expression of MFN2 in breast cancer tissues, and its relationship with the clinicopathological characteristics and prognosis of breast cancer patients was analyzed. MCF-7 and MDA-MB-231 cells were transfected with ERβ and MFN2 knockdown or expression plasmids. Western blot was used to detect the effects of ERβ on MFN2 and MFN2 on P-AKT473 and MMP2; the P-AKT pathway inhibitor LY294002 was administered to cells transfected with MFN2 knockdown plasmids, Western blot, immunocytofluorescence, and a wound healing assay revealed the effect of MFN2 on its downstream signaling pathway and the migration of breast cancer cells.

Results This study found that the expression of MFN2 is related to the molecular type and prognosis of breast cancer patients ($P < 0.05$). The positive expression rate of MFN2 in triple-negative breast cancer was significantly lower than that in the HER2 + and luminal types. However, MFN2 expression was unrelated to age, tumor size, lymph node metastasis, TNM stage, histological type and grade ($P > 0.05$); ERβ positively regulated MFN2 expression and reduced the migration of both MCF-7 and MDA-MB-231 cells, while MFN2 knockdown increased the expression of P-AKT473 and MMP2. In contrast, the overexpression of MFN2 inhibited the expression of P-AKT473 and MMP2. These results showed that in MFN2 knockdown cells treated with LY294002, P-AKT473 and MMP2 expression levels were reversed. The reversal of P-AKT473 and MMP2 expression levels inhibits the invasiveness of human breast cancer cells.

Conclusion MFN2 is related to the molecular subtype and prognosis of breast cancer. In human breast cancer MCF-7 and MDA-MB-231 cells, ERβ-induced MFN2 can inhibit the P-AKT pathway, which inhibits the invasiveness and migration of both breast cancer cell lines.

Introduction

Breast cancer is the most common malignant tumor in women, which seriously threatens the health of women. Many studies have confirmed that estrogen is involved in the development of human breast cancer (1, 2). Since estrogen needs to bind to its receptors to function, an in-depth understanding of its two receptors, estrogen receptor α (ER α) and ER β, is essential to explore the pathogenesis of breast cancer (3). Currently, the medical community has a thorough understanding of the role of ER α in the development of breast cancer and uses this knowledge to guide the endocrine treatment of breast cancer. The specific mechanism of action of ER β is unclear and is controversial in many ways (4). In general,

ERα and ERβ differentially contribute to carcinogenesis and tumor progression: ERα acts as an oncogene and ERβ acts as a tumor suppressor (5). Our previous experiments showed that ER β can inhibit the metastasis of breast cancer cells by inducing MFN2 expression (6). However, the exact mechanism by which ERβ-induced MFN2 inhibits the invasiveness and metastasis of the breast cancer cells is unknown.

MFN2, which is located on the outer membrane of mitochondria, plays an important role in the process of mitochondrial fusion and is involved in regulating mitochondrial function and morphological changes. This protein was originally found in vascular smooth muscle cells (7). Recently, MFN2 has been found to exert a tumor suppressive effect in a variety of tumors and tumor-derived cell lines. Several studies have investigated the function of MFN2 in various malignancies, including hepatocellular carcinoma and gastric cancer (8,9). Our previous experiments also showed that MFN2 can inhibit breast cancer cell progression (6). MFN2 may be involved in the pathogenesis and development of tumors, and the difference in the expression of MFN2 in malignant tumor tissues may indicate the vital function of MFN2 in the invasion and metastasis of malignant tumors, and its specific mechanism should be further studied and explored. As a novel tumor suppressor gene, we targeted the signaling pathways related to MFN2 and suppressed them, which provides a new idea for gene therapy of tumors.

Recent studies have shown that ERβ can suppress cancer and that MFN2 may inhibit the P-AKT signaling pathway to reduce the migration and invasiveness of breast cancer (10–13). Moreover, studies have found that MFN2 is associated with cancer prognosis, including that of hepatocellular carcinoma and gastric cancer (14,15). Therefore, in this study, we explored the exact mechanism by which ERβ induced MFN2 and then inhibited the P-AKT signaling pathway to reduce the migration and invasiveness of both MCF-7 and MDA-MB-231 cells; we also analyzed the relationship between MFN2 and the clinicopathological characteristics and prognosis of breast cancer patients.

Materials And Methods

Cell lines and cell culture

The ERβ-positive human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the Scientific Research Center of the Fourth Hospital of Hebei Medical University. Briefly, MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (BI, Israel) and 4.5 g/L glucose, 2 nM L-glutamine, 5000 IU/L penicillin, 5 mg/L streptomycin, 125 U/L Fungizone and 2.2 g/L sodium bicarbonate. All cells were cultured at 37°C in a humidified atmosphere of 5% CO2.

Tissue samples

Breast cancer samples from 107 patients treated from January to December 2010 at the Fourth Hospital of Hebei Medical University were obtained, and the patient follow-up data from January 2010 to

December 2018 were collected. According to the guidelines of relevant institutions, samples were collected after informed consent was obtained from the patients and after approval by the Institute Research Ethics Committee of the Fourth Hospital of Hebei Medical University. None of enrolled patients received neoadjuvant therapy, radiation therapy, or chemotherapy before surgery, and all had complete follow-up data.

Cell transfection

Cells in the logarithmic growth phase were seeded in a 6-well culture plate, and transfection was initiated when the cells grew to approximately 70-80% confluence. The transfection step was performed according to the instructions of the Lipofectamine 2000 reagent (Invitrogen, USA). In all, 4 μg of plasmid and 10 μl of cationic transfection reagent Lipofectamine 2000 were diluted in 250 μl of serum-free DMEM medium and mixed. After 20 minutes, the mixture was added to 1.5 ml of serum-free medium. After 4 h of transfection, the DMEM culture medium containing 10% fetal bovine serum was changed, and then, 48 h after transfection, protein extraction and related detection were performed.

Western blot

In all, $\geq 1 \times 10^6$ cells were added to 500 μl of RIPA lysate, mixed thoroughly, placed at 30°C for 4 minutes, and centrifuged at 12,000 r/min for 20 minutes at 4°C, after which the supernatant was collected to determine the protein concentration by Coomassie blue. An equal amount of total protein was added to each well and was transferred to a PVDF membrane after SDS-PAGE. The membrane was blocked with 5% BSA at 37°C for 1 h. Next, the membrane was incubated with primary antibodies including those against ERβ (1:1000, Affinity, USA), P-AKT473 (1:1000, Cell Signaling Technology, USA), β-actin, MMP2, and MFN2 (1:1000, Proteintech, China) overnight at 4°C. Then, the membrane was incubated with an HRP-labeled secondary antibody (1:5000, Proteintech, China) for 2 h at room temperature, after which the proteins were visualized using enhanced chemiluminescence (TIANGEN, China). Protein quantification was performed using ImageJ software.

Immunochemistry (IHC)

The slides were deparaffinized and incubated for 10 min with 3% H_2O_2 in water. Then, the slides were incubated with 10% goat serum at 37°C for 20 minutes, after which the slides were incubated overnight with an anti-MFN2 antibody (1:100, Proteintech, China). Subsequently, the slides were incubated with the appropriate secondary antibody for 30 min at room temperature. For quantification, 5 random images (400×) per experimental group were captured with a microscope (Leica, Germany). The final weighted expression score (0–2) was obtained by calculating the intensity values of IHC staining and the

percentage of positive cells. Based on previous research, 0-1 points indicated low expression, while 2 points indicated high expression.

Immunofluorescence (IF)

Cells were plated on coverslips in 6-well plates, fixed in 4% paraformaldehyde and washed with PBS. After permeabilization with 0.5% Triton X-100 in PBS and blocking with 10% goat serum, the cells were incubated with a primary antibody against MMP2 (1:100, Proteintech, China) at 4°C overnight. Then, the cells were washed 3 times with PBS and incubated with a secondary fluorescent antibody at room temperature for 2 h. After 3 washes in PBS, cells on the coverslips were analyzed by confocal microscopy.

Wound healing assay

First, 6-well plates were seeded with 1×10^6 cells until the cells were 100% confluent. The cell layer was then carefully scratched with a sterile 200 μL pipette tip to generate a wound, which was then washed and cultured in complete medium without FBS. At 0 h and after 24 h, the wounds were photographed under a light microscope, and the wound closure percentage (%) was evaluated using ImageJ software.

Statistical analysis

All data in this study were obtained independently three times and are presented as the mean±SD. Statistical differences between the results of each group were evaluated using Student's t-test or one-way analysis of variance (ANOVA). A *P*-value <0.05 was considered statistically significant.

Results

The relationship between MFN2 and the clinicopathological features and prognosis of patients with breast cancer

Breast cancer samples from 107 patients treated at the Fourth Hospital of Hebei Medical University were collected from January to December 2010. The analysis of clinicopathological characteristics showed that, among the three breast cancer subtypes, the positive expression rates of MFN2 in the triple-negative, HER2-positive and luminal types were 42.3% (11/26), 81.5% (22/27) and 75.9% (41/54), respectively. Among them, the triple-negative type has the lowest positive expression rate, and the HER2-positive and luminal types have similar higher expression rates than the triple-negative type. This showed that the positive expression rate of MFN2 in breast cancer tissues is significantly related to cancer classification

(P<0.05); the positive expression rate of MFN2 in breast cancer tissues is unrelated to age, tumor size, TNM stage, lymph node metastasis, histological type, and histological grade (P>0.05) (Table 1).

MFN2 expression levels were divided into the low (n=33) and high (n=74) MFN2 expression groups. A Kaplan-Meier analysis was used to analyze the prognosis of patients during nine years after surgery. The low MFN2 expression group had a worse prognosis than the high MFN2 expression group. The results showed that the expression of MFN2 is related to the overall survival (OS) of breast cancer patients over these nine years (P<0.05) (Figure 1).

ERβ promotes the expression of MFN2 and inhibits the migration and invasiveness of both MCF-7 and MDA-MB-231 breast cancer cells

To our knowledge, compared with normal breast cells, MFN2 is expressed at low levels in breast cancer cells, while in the ERβ-positive breast cancer cells, MCF-7 and MDA-MB-231, MFN2 expression is lower in MDA-MB-231 than in MCF-7 cells. To further explore the specific effect of ERβ on MFN2 in breast cancer cells, we transfected MCF-7 and MDA-MB-231 cells with ERβ expression or knockdown plasmids. Western blot showed that MFN2 was up-regulated in both cell lines after transfection with the ERβ expression plasmid, while MFN2 was down-regulated in both cell lines after transfection with the ERβ knockdown plasmid (Figure 2 A-F). Therefore, we determined that ERβ exerts a positive regulatory effect on MFN2. Then, in the wound healing assay, we found that the overexpression of ERβ inhibited the migration of breast cancer cells. (Figure 2 G-J)

MFN2 inhibits the P-AKT signaling pathway in both MCF-7 and MDA-MB-231 breast cancer cells

To explore the specific mechanism of ERβ-induced MFN2 expression on the migration and invasiveness of breast cancer cells, we transfected the human breast cancer cells MCF-7 and MDA-MB-231 with MFN2 expression or knockdown plasmids. Western blot showed that when MFN2 was overexpressed, P-AKT and MMP2 were inhibited in both cell lines, while MFN2 knockdown led to the upregulation of P-AKT and MMP2 in both cell lines (Figure 3). The above results showed that MFN2 may inhibit the migration and invasiveness of breast cancer cells by inhibiting the P-AKT signaling pathway.

LY294002 reverses the increase in P-AKT and the migration and invasiveness of MCF-7 and MDA-MB-231 cells after MFN2 knockdown

The AKT pathway inhibitor LY294002 was administered to the human breast cancer cell lines MCF-7 and MDA-MB-231, and Western blot demonstrated that LY294002 inhibited P-AKT and MMP2 in both cell lines (Figure 4). For further detection, we divided the cells into the negative control groups, the knockdown-MFN2 group and the LY294002 plus knockdown-MFN2 group. In the group treated with LY294002 in advance, P-AKT and MMP2 levels were reduced compared with the group in which only MFN2 was knocked down. Western blot showed that the expression levels of P-AKT were reduced, and both western blot and immunofluorescence suggested that MMP2 was also reduced(Figure 5 A-D, I-J). Next, the wound healing assay also confirmed that MFN2 inhibited migration and invasion by inhibiting the P-AKT

signaling pathway in both breast cancer cell lines; treatment with the AKT pathway inhibitor LY294002 significantly reversed the increase in P-AKT and MMP2 after MFN2 knockdown and inhibited the migration and invasiveness of breast cancer cells (Figure 5 E-H).

Discussion

Estrogen works by binding to specific receptors, estrogen receptors (ERs), which in turn leads to the activation of transcriptional processes and/or signal transduction events that result in gene expression control (16). It is recognized that ERα and ERβ have different roles in breast cancer cells. Most researchers believe that ERα is crucial to breast cancer development (17), and the function of ERα has been clearly identified (18). However, the exact role of ERβ in breast cancer is unknown. Many studies have shown that ERβ is an anti-oncogene in breast cancer and that the loss of ERβ may promote breast cancer occurrence (19,20). As a novel tumor suppressor gene, MFN2 is related to the antitumor activity of many malignant tumors (21–23). No other studies have provided detailed data or have investigated the effect of ERβ on MFN2 and P-AKT expression in breast cancer. Therefore, in this study, we investigated the exact mechanism by which ERβ induces MFN2 to inhibit the P-AKT signaling pathway and thereby suppresses breast cancer cell migration and invasion; we also analyzed the relationship between MFN2 and the clinicopathological characteristics and prognosis of breast cancer patients.

The human MFN2 gene is located on chromosome 1p36.22. This is a mutation-prone region in many tumors and has been extensively studied. Many tumor patients may have deletions or translocations in this region of this chromosome (24). This region is believed to contain many tumor suppressor genes (25–26), which suggests that the expression of MFN2 is largely related to tumorigenesis and tumor development. MFN2 contains two transmembrane domains across the mitochondrial membrane, a possible protein kinase A or G phosphorylation site and a p21 (Ras) signature motif (amino acids 77–92), this protein plays a vital role in signaling processes (27). In recent years, research on MFN2 and the migration and invasiveness of malignant tumor cells has gradually become a hot spot. Its association with the clinical pathological characteristics and prognosis of patients with malignant tumors has also attracted widespread attention, but the specific mechanism of the relationship between MFN2 and breast cancer requires further investigation (14,15). Consistently, in our study, MFN2 was found to be associated with the patient's nine-year prognosis ($P < 0.05$). However, our study still has some shortcomings. The first is that, due to increasingly effective treatment strategies for breast cancer in recent years, breast cancer patients generally have a good prognosis and extremely low mortality after surgery; the second is the small sample size. Therefore, in order to better study the effect of MFN2 on prognosis, it may be necessary to extend the follow-up time and sample size, the small nature of which are also the primary deficiencies of this study.

In our previous study, we reported that ERβ inhibits the migration and invasiveness of MCF-7 cells by up-regulating MFN2 expression (6). In this study, we again confirmed this finding, and with the down-regulation of MFN2, P-AKT expression was elevated. In addition, our immunohistochemistry results showed that the positive expression rate of MFN2 in triple-negative breast cancer was significantly lower

than that of the HER2 + and luminal types. It can be seen that MFN2 expression is related to the molecular type of breast cancer ($P < 0.05$); our survival analysis also confirmed that the prognosis of the MFN2 low-expression group was worse than that of the MFN2 high-expression group ($P < 0.05$). In summary, the above results indicated that MFN2 inhibits the migration of breast cancer cells by inhibiting the P-AKT signaling pathway under the induction of ERβ, and that low expression of MFN2 may indicate a worse prognosis.

AKT is a serine/threonine kinase that is also known as protein kinase B (PKB) due to its high homology with protein kinases. The AKT protein can be involved in regulating cell survival, cycle progression and growth. Pathological findings of highly activated AKT in primary tumors are considered to be negative prognostic markers in different tumor types. Similarly, some studies have shown that AKT activation is also involved in the regulation of cell migration and invasion (28,29). AKT is mainly phosphorylated and activated in a PI3K-dependent manner. Once activated, AKT controls cell function through phosphorylation of downstream substrates, thereby promoting cancer progression (30). These results demonstrated the significance of AKT activity in tumor promotion and growth as well as the potential relevance of AKT as a molecular target. The PI3K/AKT pathway is the most common signaling pathway in human cancers, and this pathway affects multiple processes directly involved in tumorigenesis. The relative levels of negative regulators of the PI3/AKT pathway will also ultimately affect the magnitude of AKT activation. Therefore, the use of AKT inhibitors that inhibit this pathway is an attractive anticancer strategy (31). In recent years, AKT inhibitors have been widely studied and used as anticancer drugs in a variety of cancers (32–35). Recently, promising data have been obtained for the development of PI3K/AKT inhibitors in breast cancer and have provided a theoretical basis for the development of PI3K/AKT-targeted inhibitors (36).

Although many studies have been performed to determine the relationship between the AKT pathway and breast cancer in recent years (21–23), none of these studies has demonstrated that the P-AKT signaling pathway is downstream of ERβ/MFN2. Our data indicated that in MCF-7 and MDA-MB-231 human breast cancer cells, ERβ induced the expression of MFN2, while MFN2 reduced P-AKT, and P-AKT was downstream of ERβ/MFN2. LY294002 was used to determine whether the AKT pathway was involved in the migration and invasiveness of MCF-7 and MDA-MB-231 cells. The results showed that the expression of P-AKT473 and MMP2 was increased in MCF7 and MDA-MB-231 cells when MFN2 was knocked down, while in cells in which MFN2 was knocked down and the AKT pathway was blocked with LY294002, P-AKT473 and MMP2 protein expression was inhibited. The above results showed that the expression of MFN2 induced by ERβ indeed inhibits the migration and invasiveness of breast cancer cells through the P-AKT signaling pathway. Studies on the role of ERβ/MFN2 in inhibiting tumorigenesis and tumor development, especially in the case of P-AKT inhibition, may provide useful information for understanding and treating cancer.

Conclusions

In conclusion, this study provides experimental evidence for the role of ERβ and MFN2 as tumor suppressor genes in breast cancer cells. The MFN2 gene significantly inhibits the migration and invasiveness of breast cancer cells and may inhibit these processes in breast cancer cells through the AKT pathway. We also confirmed that the expression level of MFN2 is closely related to the molecular type and the prognosis of breast cancer patients. These observations highlight the key role of ERβ/MFN2 in breast cancer development via the P-AKT signaling pathway and reveal that P-AKT is a potential therapeutic target for inhibiting tumor migration and invasion.

Abbreviations

MFN2, Mitofusin 2; ER α, Estrogen receptor α; ER β, Estrogen receptor β; MMP2, Matrix Metallopeptidase 2; PBS, Phosphate buffered saline; FBS, Fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium; OS, overall survival; P-AKT, Phosphorylated protein kinase B; AKT, Protein kinase B; PI3K, Phosphatidylinositol 3 kinase; HER 2, Human epidermal growth factor receptor-2; IHC, Immunohistochemistry; IF, Immunofluorescence .

Declarations

Ethics approval and consent to participate

All tissue samples in this study were obtained from the Fourth Hospital of Hebei Medical University with informed consent before sample collection, according to the institutional guidelines; and this study was approved by the Institute Research Ethics Committee.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L Ma and J Hao supervised the project. J Hao, L Ma and M Wei designed and/or performed experiments. Y Liu, R Fu and D Wei provided the study materials or patients; M Wei and X Liao collected and orgnized the data; M Wei wrote the manuscript text and prepared the figures. All authors read and approved the final manuscript.

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Table

Table 1 Clinicopathological characteristics of breast cancer patient samples

Group	N	MFN2 protein expression			χ^2	P
		High	Low	Positive rate (%)		
Age (years)						
≤60	86	59	27	68.6	0.063	0.802[a]
>60	21	15	6	71.4		
Tumor size (cm)						
≤2	68	43	25	63.2	3.069	0.080[a]
>2	39	31	8	79.5		
Lymph node metastasis						
Negative	58	38	20	65.5	0.787	0.375[a]
Positive	49	36	13	73.5		
TNM stage						
	39	23	16	59.0	3.519	0.172[a]
	49	38	11	77.6		
	19	13	6	68.4		
Histologic type						
Infiltrative	95	66	29	69.5	0.000	1.000[b]
Non infiltrative	12	8	4	66.7		
Histologic grade						
	1	1	0	100.0	1.902	0.527[c]
	82	54	28	65.9		
	24	19	5	79.2		
Cancer type						
Triple-negative	26	11	15	42.3	11.870	0.003[a]
HER2-positive	27	22	5	81.5		
Luminal	54	41	13	75.9		

Note: a: Chi-square test; b: Fisher exact test; c: Continuity Correction of Chi-square test.

Figures

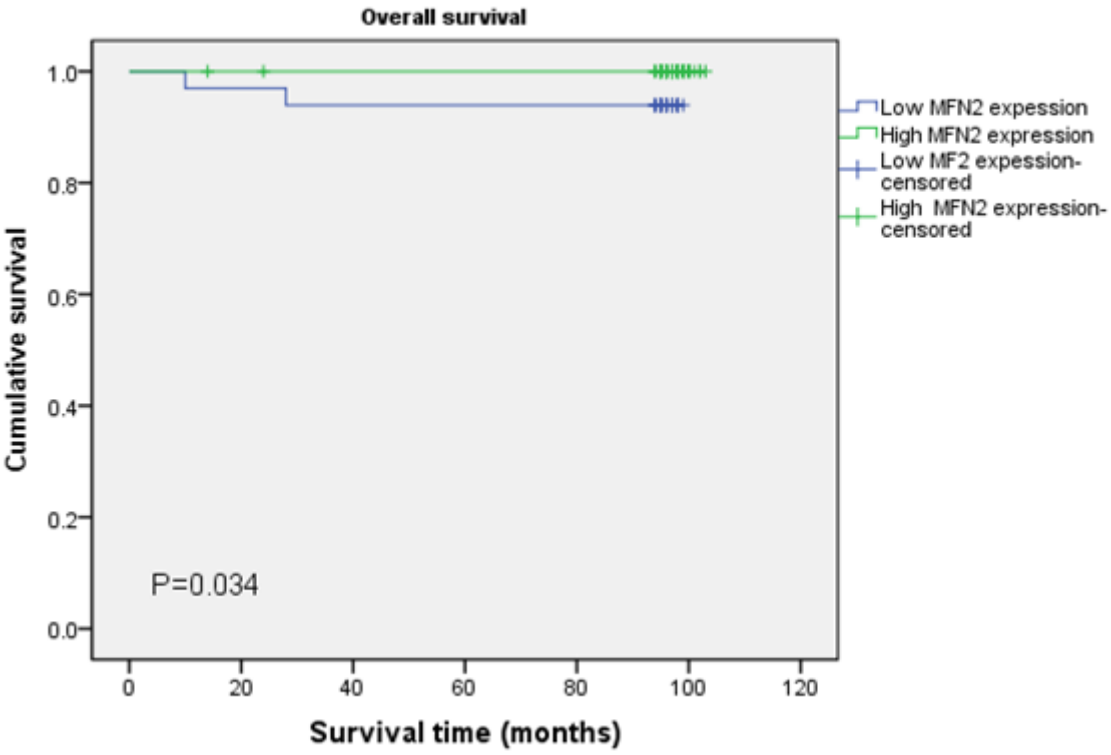

Figure 1

The expression of MFN2 is associated with the prognosis of the breast cancer patients. The MFN2 expression level was identified in the MFN2 low group (n=74) and the MFN2 high group (n=33). Kaplan-Meier analysis was performed and the curve for the nine-year survival of breast cancer patients is shown according to the MFN2 level.

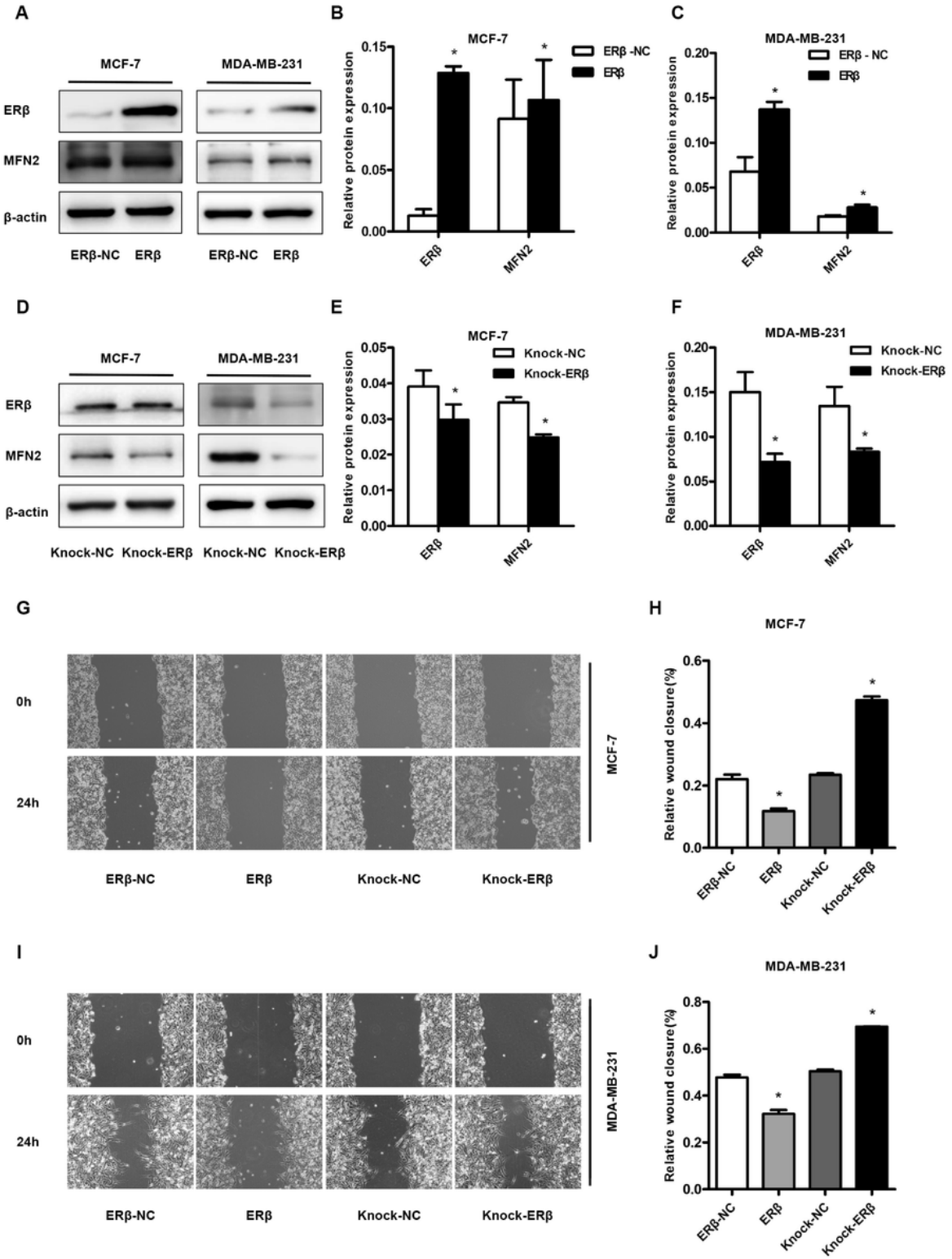

Figure 2

ERβ promotes the expression of MFN2 in both MCF-7 and MDA-MB-231 breast cancer cells. Notes: (A-C) High expression of ERβ up-regulated MFN2 expression in MCF-7 and MDA-MB-231 cells; *P<0.05 compared with ERβ-NC. (D-F) ERβ knockdown reduced MFN2 expression in MCF-7 and MDA-MB-231 cells; *P<0.05 compared with Knock-NC. (G-J) High expression of ERβ promoted the migration and

invasion of both breast cancer cells; *P<0.05 compared with ERβ-NC. ERβ knockdown inhibited the migration and invasiveness of both breast cancer cells; *P<0.05 compared with Knock-NC. Bar=200 μm.

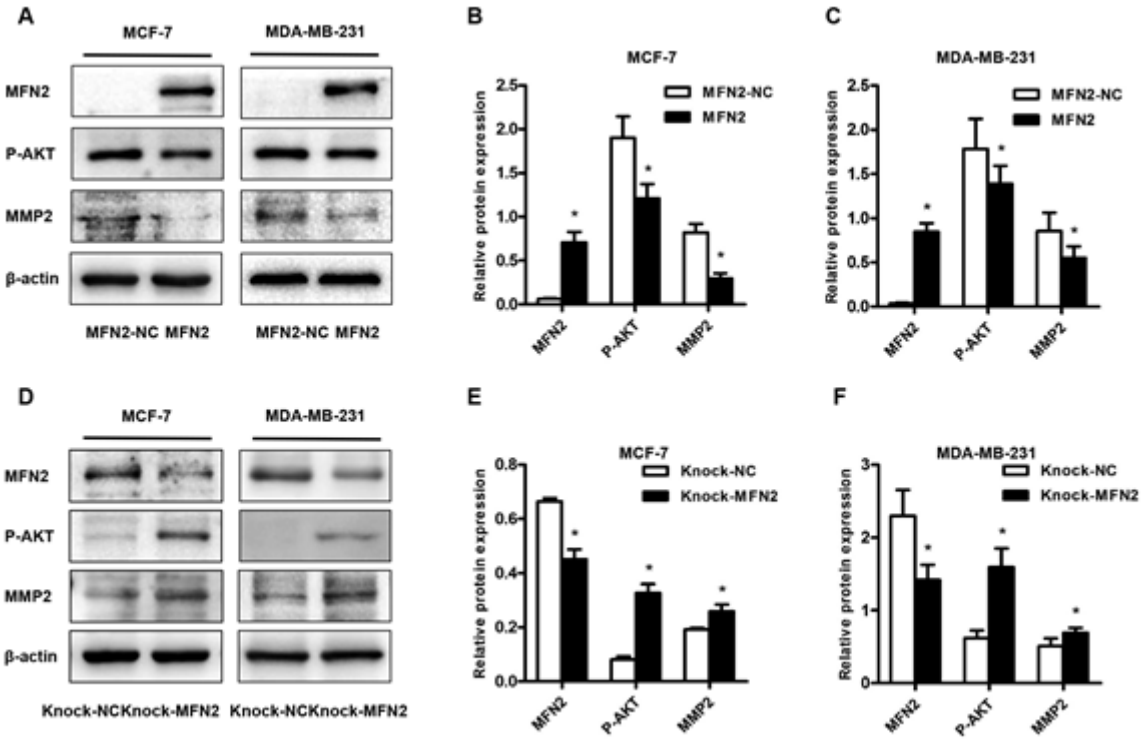

Figure 3

MFN2 inhibits the P-AKT signaling pathway in both MCF-7 and MDA-MB-231 breast cancer cells. Notes: (A-C) In MCF-7 and MDA-MB-231 cells, high MFN2 expression significantly inhibited the expression of P-AKT and MMP2 in MCF-7 and MDA-MB-231 cells; *P<0.05 compared with MFN2-NC. (D-F) MFN2 knockout increased the expression of P-AKT and MMP2; *P<0.05 compared with Knock-NC.

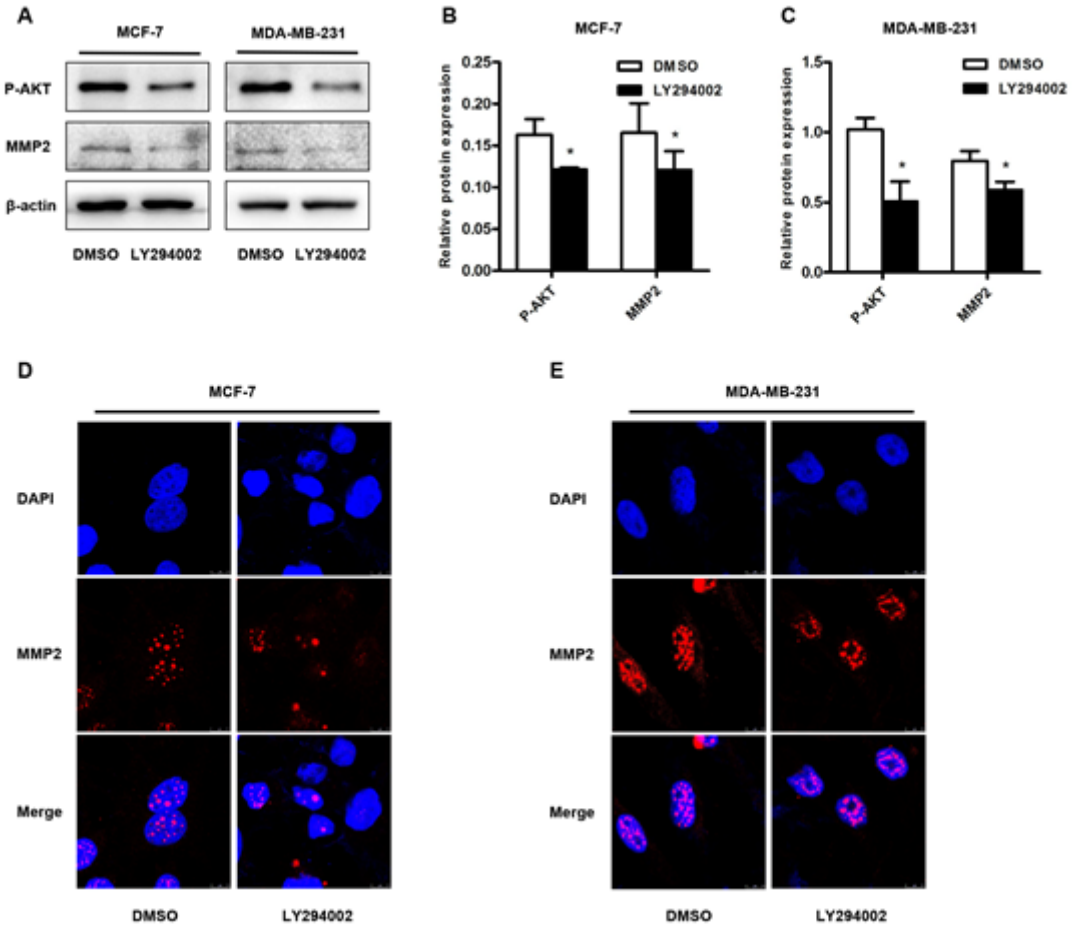

Figure 4

LY294002 inhibits P-AKT and MMP2 in MCF-7 and MDA-MB-231 cells. Notes: (A-C) The AKT pathway inhibitor LY294002 (20 μM) was administered to MCF-7 and MDA-MB-231 cells to significantly inhibit the expression of P-AKT and MMP2; *P<0.05 compared with DMSO. (D-E) Immunofluorescence staining revealed that LY294002 inhibited MMP2 expression. Bar=7.5 μm.

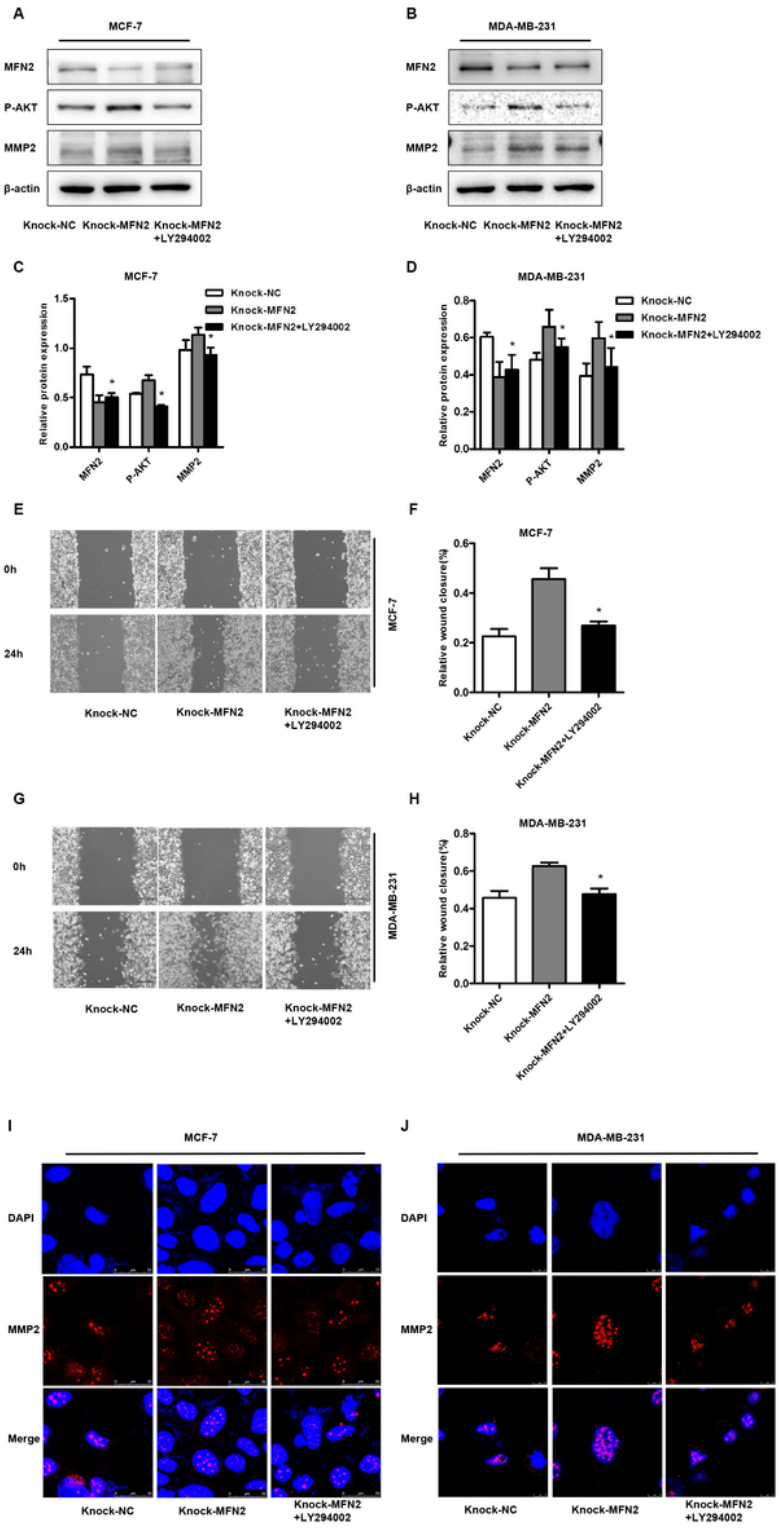

Figure 5

LY294002 reverses the increase in P-AKT after MFN2 knockout. Notes: (A-C) Western blot analysis confirmed that when MFN2 is knocked down, the expression of P-AKT and MMP2 was increased, and LY294002 reversed this increase. *P<0.05 compared with Knock-NC. (D, G) Wound healing assay showed that MFN2 knockdown promoted MCF-7 and MDA-MB-231 cell migration, and LY294002 reversed this

migration. Bar=200 μm; (H, I) Immunofluorescence suggested that MFN2 knockdown promoted the expression of MMP2, and LY294002 then reduced its expression. Bar=25 μm.