

# Ginkgetin induces cell death in breast cancer cells via downregulation of the estrogen receptor

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**Abstract.** Ginkgetin is a natural biflavonoid isolated from the leaves of *Ginkgo biloba*, and is characterized by its anti-inflammatory and anti-viral activities. Although numerous studies state that it has also antitumor activity, the anti-proliferative effect of ginkgetin and the underlying mechanism in breast cancer cells have not yet been investigated. In the present study, ginkgetin inhibited the cell viability of MCF-7 and T-47D cells dose-dependently, and suppressed the expression of the estrogen receptor (ER) at the mRNA and protein levels. Among the targets of the ER, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), cyclin D1 and survivin were also downregulated by ginkgetin treatment. The anti-proliferative effects of ginkgetin were sufficient to suppress the growth by estradiol stimulation. However, ginkgetin did not significantly affect the viability of MDA-MB-231 cells, which are ER-negative cells. Furthermore, the knockdown of the ER and an inhibitor of PFKFB3 significantly sensitized MCF-7 and T-47D cells to ginkgetin. These findings suggest that ginkgetin induces cell

death in ER-positive breast cancer cells via the inhibition of ER expression and that it is a promising agent for breast cancer treatment.

## Introduction

The estrogen receptor (ER) and its ligand, estrogen, serve a critical role in the development and progression of breast cancer (1). The human ER exists as two subtypes, ER- $\alpha$  and ER- $\beta$ , which regulate the transcription of various target genes upon binding to estrogen response elements present within the regulatory region of the target genes (2). In the majority of ER- $\alpha$ -positive cases of breast cancer, the expression level of ER- $\alpha$  is considerably higher compared with that in normal breast epithelium (3). Accordingly, endocrine therapies, which target ER activity, are standard treatments for patients with ER-positive breast cancer in the early and advanced/metastatic stages. However, despite the substantial benefit from endocrine treatment, resistance is still common, and it significantly influences the overall morbidity and mortality of breast cancer (3).

The fruits and seeds of the *Ginkgo biloba* tree have traditionally been used in Chinese medicine with indications for the treatment of asthma, coughs and enuresis (4). Ginkgetin, which is a biflavonoid isolated from *G. biloba* extract, is known to have anti-inflammatory and anti-viral activities *in vitro* and *in vivo* (5,6). Of note, ginkgetin was reported to exhibit cytotoxic effects in ovarian adenocarcinoma and prostate cancer cells (7-9). However, the anticancer activities and the underlying mechanism of ginkgetin in breast cancer cells have not yet been investigated.

The present study examined the cytotoxicity of ginkgetin against numerous breast cancer cell lines, including ER-positive and negative cells. It was demonstrated that the anticancer activity of ginkgetin in breast cancer cells was associated with the downregulation of the ER and, subsequently, the blockade of the signaling pathway activated by estrogen. The results of the present study suggested that ginkgetin may

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merit further investigation as a chemotherapeutic agent against breast cancer.

## Materials and methods

**Cell culture and reagents.** MCF-7, T-47D, and MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), the T-47D cells were grown in RPMI-1640 growth medium (Invitrogen; Thermo Fisher Scientific, Inc.), and the MDA-MB-231 cells were grown in Leibovitz's L-15 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Corning Life Sciences, Tewksbury, MA, USA) at 37°C in 5% CO<sub>2</sub> incubator. For treatment with 17β-estradiol (E2), the cells were grown in media containing no phenol red and 10% charcoal:dextran-stripped FBS (SciPak Lifesciences, Sacramento, CA, USA). Antibodies against ER-α (cat. no. sc-8005) and β-actin (cat. no. sc-130300) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against cleaved poly (ADP-ribose) polymerase (PARP; cat. no. 5625), caspase 7 (cat. no. 12827), cyclin D1 (cat. no. 2978) and survivin (cat. no. 2808) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against 6-phospho-fructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3; cat. no. 13763-1-AP) was purchased from Proteintech (Chicago, IL, USA). Short interfering (si)RNAs targeting ER-α and negative control (scrambled) siRNAs were purchased from Santa Cruz Biotechnology, Inc. An inhibitor of PFKFB3, 3PO, was purchased from Merck KGaA (Darmstadt, Germany). Ginkgetin and isoginkgetin were isolated from dried *G. biloba* leaves (9).

**Transfections and treatments.** Cells (2x10<sup>5</sup>) in 1 ml of serum-free medium were transfected with control siRNA or ER-α siRNA (100 nM) for 6 h at 37°C in a CO<sub>2</sub> incubator using Lipofectamine (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Upon replacing the culture medium with fresh DMEM with 10% FBS, the cells were treated with 5 μM ginkgetin for 24 h at 37°C to proceed with subsequent experiments.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA, from T-47D cells, was isolated using the TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and was quantified using a spectrophotometer. An aliquot of total RNA (2 μg) was transcribed into complementary (c)DNA primed with oligo dT using an RT2 First Strand kit (Qiagen GmbH, Hilden, Germany). For RT-qPCR, the cDNA was amplified using a KAPA SYBR FASR qPCR kit (Kapa Biosystems, Inc., Wilmington, MA, USA) using specific primer pairs (Origene Technologies, Inc., Rockville, MD, USA). PFKFB3, cyclin D1, and β-actin mRNA expression levels were analyzed using a LightCycler® (Roche Diagnostics, Basel, Switzerland), and thermocycling was performed according to the manufacturer's protocols. The primer sequences were as follows: PFKFB3, 5'-CTGCAGAGGAGATGCCCTAC-3' (forward) and 5'-AGG

TCCCTTCTTTGCATCCT-3' (reverse); cyclin D1, 5'-CCG TCCATGCGGAAGATC-3' (forward) and 5'-CCTCCTCCT CGCACTTCTGT-3' (reverse); β-actin, 5'-GGATTCCCTATGT GGGCGACGA-3' (forward) and 5'-CGCTCGGTGAGGATC TTCATG-3' (reverse). Relative quantification of PFKFB3 and cyclin D1 expression levels was determined by the 2<sup>-ΔΔC<sub>q</sub></sup> method (10).

**Measurement of cell viability.** Cell viability was determined by evaluating the mitochondrial conversion of MTT to a colored product. The cells were treated with drugs as indicated, and the medium was exchanged with serum-free DMEM or RPMI-1640 containing 1 mM MTT. Following 1 h of incubation at 37°C, the cells were solubilized in dimethyl sulfoxide. The amount of formazan, which is the converted form of MTT, was determined by evaluating the absorbance at 590 nm.

**Western blotting.** The cells were harvested and lysed for 20 min at 4°C in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with a protease inhibitor cocktail (GenDEPOT, Barker, TX, USA). Determination of total protein was performed by Bradford method (11). Equal amounts of protein (20-50 μg) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked by incubation with 2.5% skimmed milk for 30 min at RT, followed by incubation overnight at 4°C with the appropriate primary antibodies (diluted 1:1,000; anti-cleaved PARP, anti-caspase 7, anti-ER-α, anti-cyclin D1, anti-survivin, anti-PFKFB3, anti-β-actin) and incubation for 1.5 h at 4°C with the secondary antibody (Santa Cruz Biotechnology, Inc.; diluted 1:10,000; mouse anti-rabbit IgG-horseradish peroxidase; cat. no. sc-2357). Immunoreactive proteins were visualized using enhanced chemiluminescence reagents (GE Healthcare, Chicago, IL, USA).

**Evaluation of apoptosis.** Apoptosis was determined by fluorescence-activated cell sorting analysis using an Annexin V-FITC Apoptosis Detection kit (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's protocol. Briefly, the MCF-7 and T-47D cells were incubated for 24 h at 37°C with ginkgetin and then treated with trypsin (Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min at 37°C. The cells were resuspended with binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) including Annexin V-fluorescein isothiocyanate and propidium iodide (PI). Cell fluorescence was then analyzed by flow cytometry using a FACScaliber and Cell Quest software (version 3.3; BD Biosciences, San Jose, CA, USA).

**Statistical analysis.** All data presented are representative of at least three separate experiments. Results are expressed as the mean ± standard deviation. Statistical differences among groups were determined using the Student's t-test (for two groups) or one-way analysis of variance, followed by the post-hoc Tukey's test, for >2 groups using Prism 7 software (GraphPad Software, La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

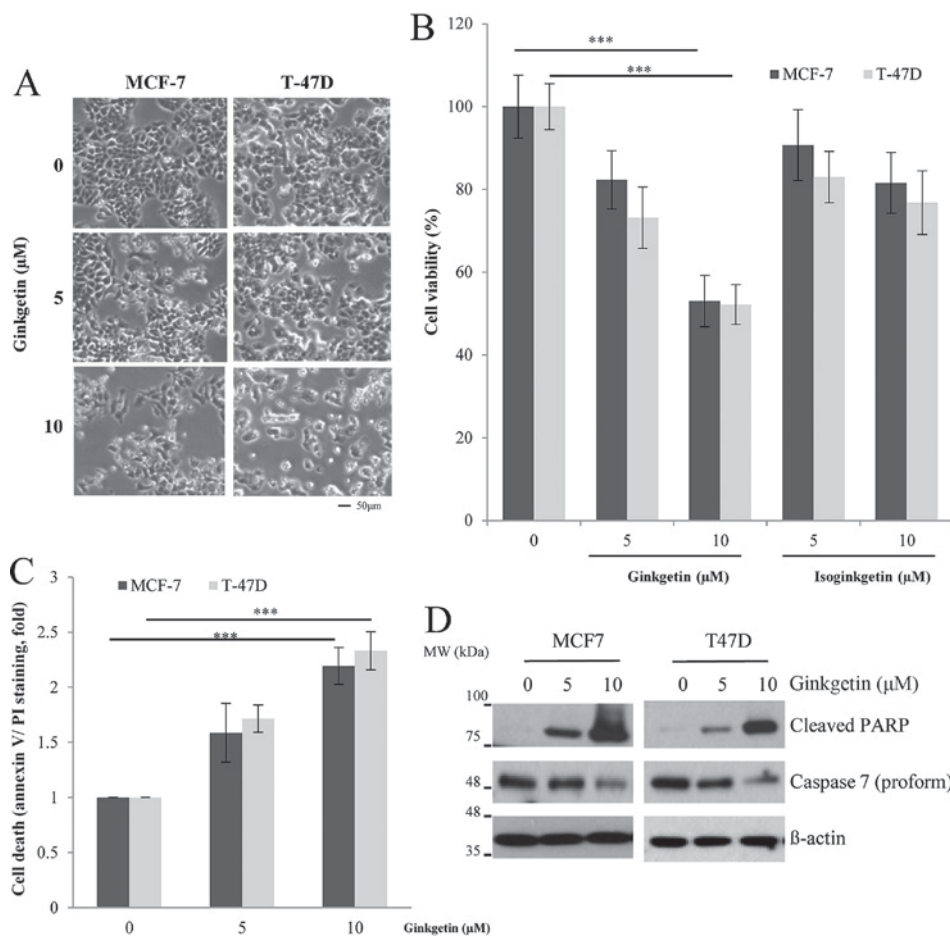


Figure 1. Ginkgetin induces cell death in breast cancer cells. (A) MCF-7 and T-47D cells were treated with the indicated doses of ginkgetin or isoginkgetin for 24 h. The cell morphological changes were observed under an inverted microscope. (B) Cell viability was assessed by an MTT assay, and apoptotic cell death was analyzed by (C) flow cytometry and (D) western blotting. \*\*\* $P < 0.001$ . PI, propidium iodide; PARP, poly (ADP-ribose) polymerase.

## Results

*Ginkgetin inhibits cell growth and induces apoptosis in human breast cancer cell lines.* In previous studies, ginkgetin was cytotoxic against tumor cells with a half-maximal inhibitory concentration ( $IC_{50}$ ) of  $\sim 10 \mu M$  (7-9). Thus, the present study exposed MCF-7 and T-47D human breast cancer cells to 5 or  $10 \mu M$  of ginkgetin to evaluate its cytotoxicity against breast cancer cells. As presented in Fig. 1A, the cells treated with ginkgetin demonstrated a decrease in cell number and an increased indication of apoptosis, including apoptotic bodies and cell shrinkage, as observed under an inverted microscope. The results of the MTT assay revealed that ginkgetin reduced cell viability by  $\sim 50\%$  in both cell lines at a concentration of  $10 \mu M$  (Fig. 1B;  $P < 0.001$ ). Subsequently, the present study investigated whether isoginkgetin, a derivative of ginkgetin isolated from *G. biloba* extract, has similar cytotoxic effects on breast cancer cells. However,  $10 \mu M$  isoginkgetin decreased the viability of the MCF-7 and T-47D cells by 17 and 25%, respectively. Thus, the results of the present study are consistent with a previous study demonstrating that ginkgetin was more effective in exerting antitumor activity compared with that of its derivatives (9). To confirm whether ginkgetin induces apoptosis in breast cancer cells, the apoptotic cells were evaluated by flow cytometry. The populations of annexin V and PI-positive cells increased with ginkgetin treatment

in both cell types in a dose-dependent manner (Fig. 1C;  $P < 0.001$ ). In addition, the reduced procaspase 7 and cleaved PARP were detected by western blotting (Fig. 1D), indicating that ginkgetin induced apoptotic cell death in breast cancer cells.

*Ginkgetin impairs the ER signaling pathway via downregulation of ER- $\alpha$  expression.* Certain flavonoids are known to affect the ER signaling pathways in ER-positive breast cancer cells (12,13). To investigate the possibility that the cytotoxicity of ginkgetin in breast cancer cells acts via ER regulation, the present study analyzed the expression level of ER- $\alpha$  in ginkgetin-treated cells. Western blot analysis demonstrated that ginkgetin markedly reduced the ER- $\alpha$  expression level in MCF-7 and T-47D cells in a dose-dependent manner (Fig. 2A). To confirm the inhibition of the ER- $\alpha$  signaling pathways by ginkgetin, the present study determined the expression levels of downstream effectors in ginkgetin-treated cells. Previous studies reported that ER- $\alpha$  directly induced the expression of PFKFB3, cyclin D1 and survivin following estrogen binding to the receptor of breast cancer cells for their survival and growth (14-17). As presented in Fig. 2A, ginkgetin also reduced the expression level of PFKFB3, cyclin D1 and survivin in both cell lines. Isoginkgetin had a small effect on the expression level of ER- $\alpha$  and its effectors, although the expression levels of cyclin D1 and survivin were suppressed

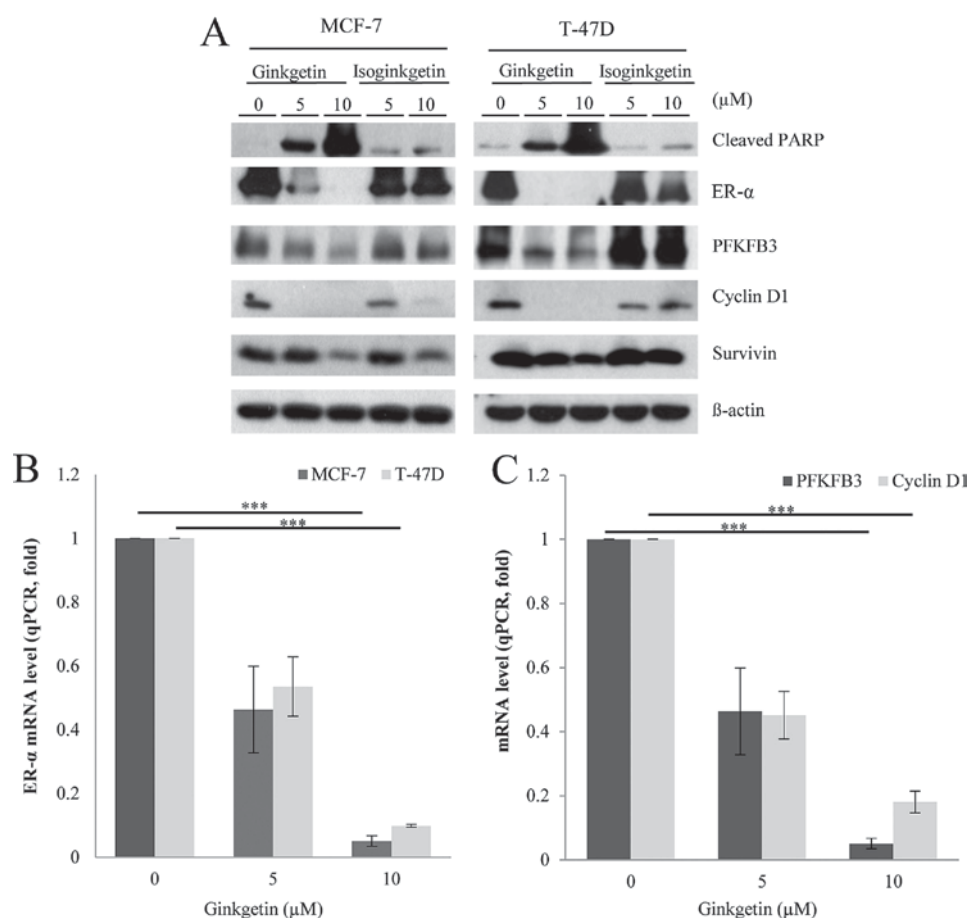


Figure 2. Ginkgetin downregulates the ER signaling pathways in breast cancer cells. (A) Cells were treated with the indicated doses of ginkgetin or isoginkgetin for 24 h, and the cell lysates were subjected to western blotting. (B) ER-α mRNA expression levels were evaluated by RT-qPCR upon exposing the cells to ginkgetin for 24 h. (C) T-47D cells were treated with 5 or 10 μM ginkgetin for 24 h, and the mRNA expression levels of PFKFB3 and cyclin D1 were determined by RT-qPCR. The data were normalized to β-actin, and the relative mRNA expression levels are presented as fold changes. \*\*\*P<0.001. ER, estrogen receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PARP, poly (ADP-ribose) polymerase.

in the isoginkgetin-treated MCF-7 cells (Fig. 2A). Since progesterone reprograms ER-α binding events to novel chromatin loci and transcriptional targets (18), the discrepancy in the effects of isoginkgetin may be due to the differences in the levels of progesterone receptor between these two cell lines. The other possibility is that isoginkgetin may downregulate these molecules by other mechanisms that may be elucidated in the future. To further investigate the decrease in ER-α expression levels in breast cancer cells treated with ginkgetin, the present study performed RT-qPCR to analyze the corresponding mRNA expression levels. Treatment with ginkgetin decreased ER-α mRNA expression levels dose-dependently, which was consistent with the observed reduction in ER-α protein expression levels (Fig. 2B; P<0.001). Conversely, ginkgetin had no effect on ER-β mRNA and protein levels (data not shown), indicating the specificity of ginkgetin in regulating ER-α expression in breast cancer cells. The mRNA expression levels of PFKFB3 and cyclin D1 were also downregulated by ginkgetin in T-47D cells, which was consistent with a decrease in the expression level of these molecules at the protein level (Fig. 2C; P<0.001). Taken together, the results of the present study suggested that ginkgetin may inhibit the ER signaling pathways of breast cancer cells by downregulating ER-α at the transcriptional level.

*The cytotoxicity of ginkgetin is dependent on ER-α expression.* E2, which is the most potent endogenous estrogen, binds to the ER and subsequently stimulates ER proliferation pathways in cultured ER-positive breast cancer cells (19). To further investigate the cytotoxicity of ginkgetin in response to E2 treatment, cells were exposed to ginkgetin with or without E2 treatment. The expression levels of PFKFB3, in MCF-7 and T-47D cells, and cyclin D1, in T-47D cells, were increased by E2 treatment in the absence of ginkgetin, indicating stimulation of the ER signaling pathways (Fig. 3A). However, E2 treatment combined with 5 μM ginkgetin did not induce the expression of PFKFB3, but decreased it in the two cell lines. Additional investigations are required to determine the underlying molecular mechanism. Of note, the ER signaling pathways activated by E2 were also markedly attenuated by ginkgetin treatment in MCF-7 and T-47D cells (Fig. 3A). Ginkgetin effectively induced PARP cleavage and repressed ER-α expression levels, even in E2-treated cells. These results indicated that the cytotoxicity of ginkgetin may be fatal for breast cancer cells and is sufficient to abrogate growth stimulation by E2. To further elucidate the role of ER-α, the present study investigated the cytotoxicity of ginkgetin in MDA-MB-231 cells, which have a relatively low expression of ER-α; thus, these cells are often utilized as a negative control for the investigation of ER-α involvement (20).



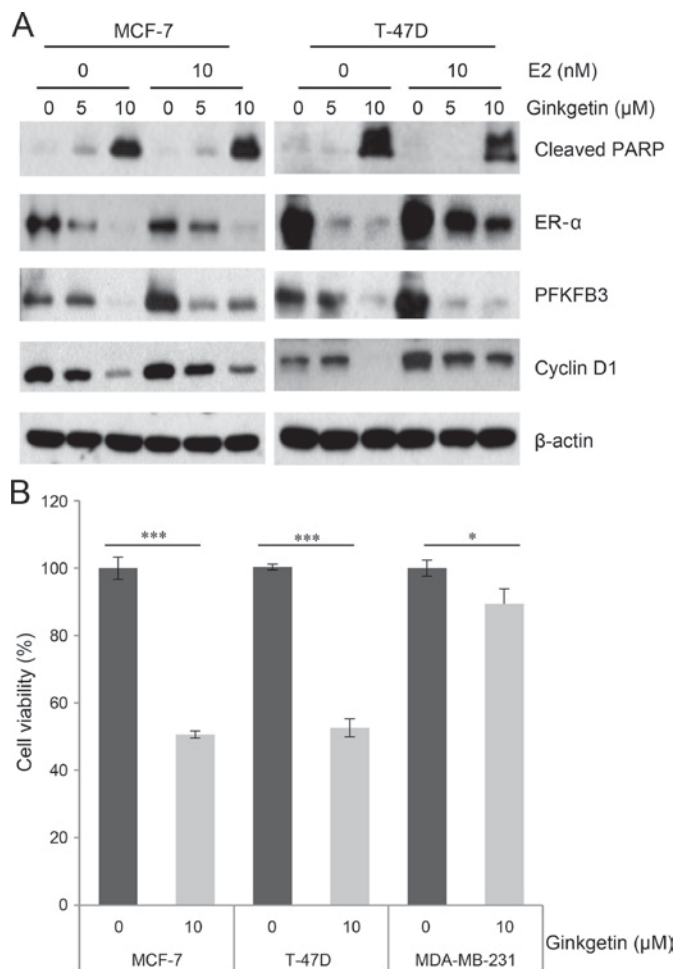


Figure 3. Ginkgetin-induced cell death is dependent on ER expression. (A) The cells were incubated with estrogen-depleted medium in the presence or absence of ginkgetin for 24 h. The cell lysates were subjected to western blotting. (B) The cells were treated with 10 μM ginkgetin for 24 h and then cell viability was evaluated by an MTT assay. \*P<0.05, \*\*\*P<0.001. ER, estrogen receptor; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PARP, poly (ADP-ribose) polymerase; E2, 17β-estradiol.

The MTT assay demonstrated that the growth inhibitory effect of ginkgetin in MDA-MB-231 cells was lower compared with the corresponding effect in MCF-7 or T-47D cells (Fig. 3B), supporting the negative correlation between ER-α expression level and ginkgetin cytotoxicity. Of note, the relatively minor reduction of cell growth in MDA-MB-231 cells resulting from ginkgetin treatment suggests an ER-independent cytotoxic effect of ginkgetin in breast cancer cells (Fig. 3B).

**Inhibition of ER-α and PFKFB3 enhances the cytotoxicity of ginkgetin in ER-positive breast cancer cells.** The viability of ER-positive breast cancer cells is dependent on ER-α expression level (3); therefore, the present study determined whether ER-α siRNA enhanced the sensitivity of ER-positive breast cancer cells to ginkgetin. As presented in Fig. 4A, ER-α siRNA markedly enhanced ginkgetin-induced PARP cleavage in MCF-7 cells, along with the downregulation of PFKFB3 and cyclin D1. In the MTT assays, ER-α siRNA had a greater effect on the cytotoxicity of ginkgetin compared with that of the negative control siRNA (Fig. 4B). Finally, the present study employed 3PO, which is a specific inhibitor of PFKFB3, to determine

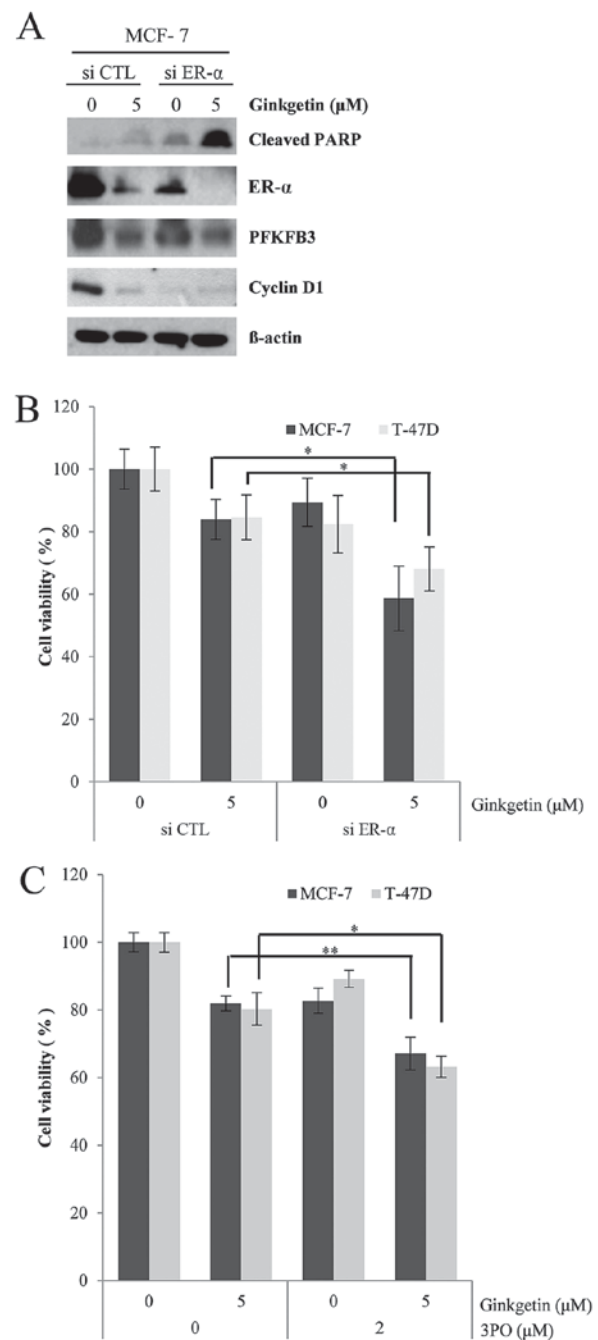


Figure 4. Combination effect of ginkgetin with ER siRNA or 3PO. The cells were transfected with control or ER siRNA and incubated with or without 5 μM ginkgetin for 24 h. (A) The cells were lysed and subjected to western blotting. (B) Cell viability was determined by an MTT assay. (C) The cells were treated with ginkgetin with or without 2 μM 3PO for 24 h, and then cell viability was determined by an MTT assay. \*P<0.05, \*\*P<0.01. ER, estrogen receptor; si, short interfering; CTL, control; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PARP, poly (ADP-ribose) polymerase.

whether inhibition of the ER downstream signaling pathways also promoted the cytotoxicity of ginkgetin. As expected, ginkgetin-induced inhibition was further augmented by 3PO treatment (Fig. 4C). Therefore, the present study suggested that the cytotoxic effects of ginkgetin on breast cancer cells may be mediated by the downregulation of ER-α, which may suppress the survival of ER-positive breast cancer cells.

## Discussion

Breast cancer is one of the most common types of cancer and is the leading cause of cancer-associated mortalities among females worldwide (19). For developing breast cancer therapies, ER- $\alpha$  has been utilized as a target molecule due to its high expression in ~70% of all breast tumors (20). The activation of the ER by estrogens serves a critical role in cancer initiation and progression, and ER antagonists have demonstrated efficacy in the treatment of breast cancer. Endocrine therapy modalities are based on three main strategies: i) Depriving the tumor of its ligand by systemically depleting estrogen production using aromatase inhibitors or ovarian suppression; ii) inhibiting estrogen binding to the ER by using selective ER modulators, including tamoxifen; or iii) degrading the ER using selective ER down-regulators (SERD), including fulvestrant, which results in a more complete inhibition of the ER signaling pathway (21). Fulvestrant, the only SERD approved by the USA Food and Drug Administration to treat patients with breast cancer, has the 100-fold affinity of tamoxifen for ER with no adverse effect on endometrial ERs (21). Recently, an orally bioavailable SERD, TAS-108, has been through phase II clinical studies, and phase III studies are currently being planned (22).

Ginkgetin has a wide spectrum of biological functions, including anti-inflammatory, antifungal, anti-influenza, neuroprotective and antitumor activities (4,6); however, these antitumor activities have been reported in limited cancer types (7-9), and the investigation of the toxicity of ginkgetin in other types of cancer is required to fully understand the underlying mechanism of its effects. The present study reported evidence of a mechanism of ginkgetin-induced cell death in ER-positive breast cancer cells via the downregulation of ER- $\alpha$  expression level. Ginkgetin selectively inhibited the growth of ER-positive breast cancer cells and did not affect ER-negative (MDA-MB-231) or normal cells (MCF-10A; data not shown). Therefore, the present study hypothesized that ginkgetin, at an effective dose against tumor cells, may not have severe toxicity toward breast tissues with low ER expression level. Of note, human epidermal growth factor receptor 2 (HER2) -positive (BT-474) breast cancer cells were less sensitive to ginkgetin compared with HER2-negative (MCF-7 and T-47D) cells, suggesting that HER2 expression level may be associated with the antitumor activity of ginkgetin (data not shown). Further studies are required to determine the mechanism underlying the resistance to ginkgetin in HER2-positive breast cancer cells.

The present study indicated that ginkgetin suppressed ER- $\alpha$  expression at the mRNA and protein levels. Evidence from previous studies indicated that the therapeutic effect of ginkgetin may involve the modification of gene expression, including genes implicated in antioxidant and stress responses (4). In addition, the apoptosis of human ovarian adenocarcinoma cells induced by ginkgetin was mediated mainly by hydrogen peroxide generated most likely via the autooxidation of ginkgetin (7). Thus, the present study speculated that oxidative stress may regulate the activity of the regulator(s) responsible for the gene expression of ER- $\alpha$ . Further experiments are required to determine whether an antioxidant affects the cytotoxicity of ginkgetin in breast cancer cells.

In conclusion, the present study demonstrated that the downregulation of ER- $\alpha$  expression in breast cancer cells by treatment with ginkgetin serves a key role in inducing apoptotic cell death. The results provided novel insight into the action of ginkgetin, which may inhibit the ER signaling pathway in breast cancer. Further studies will provide further evidence for ginkgetin as a promising candidate with minimal adverse effects for drug development against breast cancer.

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