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# Docosahexaenoic acid increases the expression of oxidative stress-induced growth inhibitor 1 through the PI3K/Akt/Nrf2 signaling pathway in breast cancer cells

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ABSTRACT

Oxidative stress-induced growth inhibitor 1 (OSGIN1), a tumor suppressor, inhibits cell proliferation and induces cell death. N-6 and n-3 PUFAs protect against breast cancer, but the molecular mechanisms of this effect are not clear. We investigated the effect of n-6 and n-3 PUFAs on OSGIN1 expression and whether OSGIN1 is involved in PUFA-induced apoptosis in breast cancer cells. We used 100 μM of n-6 PUFAs including arachidonic acid, linoleic acid, and gamma-linolenic acid and n-3 PUFAs including alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid (DHA). Only DHA significantly induced OSGIN1 protein and mRNA expression. DHA triggered reactive oxygen species (ROS) generation and nuclear translocation of Nrf2. LY294002, a PI3K inhibitor, suppressed DHA-induced OSGIN1 protein expression and nuclear accumulation of Nrf2. Nrf2 knockdown attenuated DHA-induced OSGIN1 expression. *N*-Acetyl-L-cysteine, a ROS scavenger, abrogated the DHA-induced increases in Akt phosphorylation, Nrf2 nuclear accumulation, and OSGIN1 expression. DHA induced the Bax/Bcl-2 ratio, mitochondrial accumulation of OSGIN1 and p53, and cytochrome *c* release; knockdown of OSGIN1 diminished these effects. In conclusion, induction of OSGIN1 by DHA is at least partially associated with increased ROS production, which activates PI3K/Akt/Nrf2 signaling. Induction of OSGIN1 may be involved in DHA-induced apoptosis in breast cancer cells.



## 1. Introduction

Oxidative stress-induced growth inhibitor 1 (OSGIN1), also known as OKL38, is recognized as an oxidative stress response and tumor suppressor gene (Ratliff, 2005; Li et al., 2007). According to the alternative splicing of the 5′-untranslated region of OSGIN1 mRNA, OSGIN1 transcript variants encode proteins including OSGIN1 (34 kDa), OSGIN1-1a/2a (52 kDa), OSGIN1-2b (61 kDa), and OSGIN1-2c (59 kDa) (Ong et al., 2004). Loss at the OSGIN1 genomic locus or a nucleotide variation (G to A substitution at nucleotide 1494) in the OSGIN1 coding region impairs the tumor-suppressive function of OSGIN1, which is frequently found in hepatocellular carcinoma patients compared with adjacent nontumor tissue (Liu et al., 2014). Induction of OSGIN1 expression results in growth

*Abbreviations:* AA, Arachidonic acid; ALA, alpha-linolenic acid; ARE, antioxidant response element; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular signal regulated kinases; GLA, gamma-linolenic acid; $H_2$DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; JNK, c-Jun N-terminal kinase; LA, linoleic acid; MAPK, mitogen-activated protein kinases; NAC, N-acetyl cysteine; Nrf2, nuclear factor erythroid 2-related factor 2; OSGIN1, oxidative stress-induced growth inhibitor 1; PARP, Poly (ADP-ribose) polymerase; PI3K, phosphoinositide 3-kinase; Rac3, receptor-associated coactivator 3; ROS, reactive oxygen species.

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inhibition and cellular apoptosis in a variety of carcinoma cells (Huynh et al., 2001; Hu et al., 2012), whereas knockdown of OSGIN1 expression leads to cell proliferation and survival (Hu et al., 2012). Previous studies showed that OSGIN1 is significantly induced by aqueous extract of Taheebo and that induction correlates positively with the inhibition of breast cancer cell growth and initiation of apoptosis (Mukherjee et al., 2009).

Nuclear factor erythroid 2-related factor-2 (Nrf2), a basic leucine zipper transcription factor, is necessary for the induction of numerous antioxidative genes (Lu et al., 2014). In response to oxidative stress, Nrf2 translocates from the cytosol into the nucleus and forms a heterodimer with small Maf proteins, followed by binding to the antioxidant response elements (AREs) in the promoters of its downstream target genes (McNally et al., 2007; Li et al., 2008). Extracellular signal regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and the p38 mitogen-activated protein kinases (MAPKs) as well as phosphoinositide 3-kinase/Akt (PI3K/Akt) are involved in Nrf2-mediated gene transactivation in carcinoma cells (Kocanova et al., 2007) and human endothelial cells (Yang et al., 2013). A recent study revealed that Nrf2 is essential for OSGIN1 expression in human aortic endothelial cells (Yan et al., 2014). However, the role of Nrf2 and the actual working mechanism of the regulation of OSGIN1 in cancer cells are not fully elucidated.

N-6 and n-3 PUFAs not only play crucial roles in energy metabolism and endogenous hormone synthesis (Spector and Yorek, 1985) but also regulate a number of genes (Li et al., 2006; Liu et al., 2016). Moreover, n-6 PUFAs, such as arachidonic acid (AA), linoleic acid (LA), and gamma-linolenic acid (GLA), and n-3 PUFAs, such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), induce apoptosis in cancer cells (Zhang et al., 2015). Several apoptosis-related proteins such as BCL-2 family proteins, p53, caspases, and cytochrome *c* have been shown to be up-regulated by n-3 and n-6 PUFAs, which contributes to apoptosis in various cancer cells (Kwon et al., 2008; Lee et al., 2009). Although induction of OSGIN1 has been reported to induce apoptotic cell death, whether OSGIN1 plays an essential role in the cancer cell apoptosis induced by n-3 and n-6 PUFAs is still not understood.

Breast cancer is one of the most frequently diagnosed cancers worldwide, and it has maintained a high incidence and mortality rate in women, particularly African American women (DeSantis et al., 2016). The therapeutic choices and prognostic outcomes for patients with breast cancer are usually based on predictive biomarkers and diversified biological phenotypes, including luminal A, luminal B, HER2 positivity, and basal-type tumors (triple-negative breast cancer) (Prat et al., 2013). OSGIN1 is a predictive biomarker in tumorigenesis, and a negative correlation between OSGIN1 and breast cell proliferation was demonstrated in previous studies (Huynh et al., 2001; Wang et al., 2005). In the present study, we used MCF-7 and Hs578T human breast cancer cells to study the individual effects of n-3 and n-6 PUFAs on OSGIN1 expression and the possible mechanisms involved in this gene regulation. Moreover, we further studied whether OSGIN1 is involved in the regulation of apoptosis by DHA in breast cancer cells, and compared with the difference of OSGIN1-relative effects by DHA between human breast carcinoma cells and the non-tumorigenic mammary epithelial cells.

## 2. Materials and methods

### 2.1. Reagents

Albumin, essentially fatty acid–free BSA, sodium bicarbonate, PI3k inhibitor (LY294002), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma–Aldrich (St. Louis, MO); DMEM, FBS, 25% trypsin-EDTA, and penicillin–streptomycin solution were from GIBCO/BRL (Grand Island, NY); SP600125 (JNK inhibitor II), PD98059 (ERK inhibitor), and SB203580 (p38 inhibitor) were from TOCRIS (Ellisville, MO); AA, LA, GLA, ALA, EPA, and DHA were from Cayman Chemical (Ann Arbor, MI); TRIzol reagent was from Invitrogen (Carlsbad, CA); antibodies against OSGIN1 were from GeneTex Inc. (Irvine, CA); antibodies against Akt, *p*-Akt (Thr308), β-actin, and poly (ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (Beverly, MA); antibodies against ERK1/2, *p*-ERK1/2, and *p*-Akt (Ser473) were from MILLIPORE (Billerica, MA); antibodies against Nrf2, Bax, Bcl-2, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against COX IV was from ProteinTech Group, Inc. (Rosemont, IL); antibody against cytochrome *c* was from Abcam (Cambridge, UK). KAPA™ SYBR® FAST qPCR Kit was from KapaBiosystems (Woburn, MA); Lightshift® Chemiluminescent EMSA kit was from Thermo Fisher Scientific Inc. (Rockford, IL).

### 2.2. Cell culture

MCF-10A, a non-tumorigenic epithelial cell line derived from nonneoplastic human mammary tissue, was cultured and maintained in 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5% horse serum, L-glutamine, insulin (10 mg/mL), hydrocortisone (5 mg/mL) and cholera toxin (100 ng/mL), human recombinant epidermal growth factor (20 ng/mL). The human MCF-7 and Hs578T tumorigenic breast cancer cell lines, and H184 A1N4 (H184) non-tumorigenic epithelial cell lines were cultured and maintained in DMEM (pH 7.2) supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% $CO_2$ humidified incubator.

### 2.3. Fatty acid preparation

PUFA samples were prepared and complexed with fatty acid–-free BSA at a 6:1 molar ratio before addition to the culture medium. At the same time, 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate were added to the culture medium to prevent lipid peroxidation.

### 2.4. Cell viability assay

MCF-7 and Hs578T cells were grown to 70%–80% confluence and were then treated with or without indicated concentrations of AA, LA, GLA, ALA, EPA, and DHA for 24 h. MCF-10A and H184 cells were treated with or without 100 μM DHA for 24 h. Afterwards, cell viability assays were performed according to a previous study (Chen et al., 2013).

### 2.5. Cytosolic and nuclear extracts and whole cell protein preparation

After each experiment, cells were washed twice with cold PBS and were then scraped from the dishes with PBS. Cell homogenates were centrifuged at 2000 × *g* for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 350 μL of hypotonic extraction buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF. After centrifugation at 7000 × *g* for 15 min, the resulting supernatant was used as the cytosolic fraction for Western blotting and the pellets containing nuclei were extracted by gentle mixing with 50 μL of hypertonic extraction buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT,

4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol at 4 °C for 30 min. The samples were then centrifuged at 10,000 × *g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at −80 °C until Western blotting was performed. For whole cell protein preparations, cells were washed twice with cold PBS and were harvested in 100 μL of 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9000×*g* for 30 min at 4 °C. The supernatant was used as whole cell proteins for Western blotting.

### 2.6. Mitochondrial and cytosolic fractionation

Isolation of mitochondrial and cytosolic fractionation was performed by use of the Mitochondria/Cytosol Fractionation kit according to the manufacturer's instructions (Biovision, Inc., Milpitas, CA). The cells were seeded in 10-cm dishes at a density of $1 \times 10^6$ cells/dish and incubated overnight at 37 °C in a 5% $CO_2$ humidified incubator. After incubation, the cells were treated with 100 μM DHA for 24 h. After DHA treatment, MCF-7 cells were washed twice with cold PBS and were then scraped from the dishes with 1 mL PBS. The collected cells were then centrifuged at 600×*g* for 5 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended with 400 μL of 1X cytosol extraction buffer mix containing DTT and protease inhibitors at 4 °C for 10 min. The resuspended cells were then homogenized by using a homogenizer and the homogenates were centrifuged at 700 × *g* for 10 min at 4 °C. The supernatants were collected and centrifuged at 10,000 × *g* for 30 min at 4 °C. The collected supernatants were used as the cytosolic fraction for Western blot analysis and the pellets were resuspended with 80 μL of ice-cold mitochondrial extraction buffer containing DTT and protease inhibitor used as for mitochondrial fraction.

### 2.7. Western blotting

The protein content in each sample was quantified by use of the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Protein aliquots were denatured and separated on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to polyvinylidenedifluoride membranes (New Life Science Product, Inc., Boston, MA). The nonspecific binding sites in the membranes were blocked with 5% nonfat dry milk in 15 mM Tris-150 mM NaCl buffer (pH 7.4) at room temperature overnight. After blocking, the membrane was incubated with anti-OSGIN1, anti-Nrf2, anti-PARP, anti-ERK1/2, and anti-phospho-ERK1/2 or anti-Akt, anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-Bcl-2, anti-Bax, anti-p53, anti-cytochrome *c*, anti-COX IV, and anti-β-actin antibodies at 4 °C overnight. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG at 37 °C for 1 h, and the immune-reactive bands were developed by use of the Western Lightning™ Plus-ECL kit (PerkinElmer, Waltham, MA).

### 2.8. RNA isolation and real-time PCR

Total RNA was isolated from MCF-7 and Hs578T cells by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Amounts of 1 μg of total RNA were used to synthesize complementary DNA by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The conditions of the reverse transcription reactions were as follows: 5 min at 65 °C, followed by 50 min at 50 °C, and 15 min at 70 °C. Real-time RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System using the KAPA™ SYBR® FAST qPCR kit (KAPA™). Oligonucleotide primers for real-time PCR analysis were as follows: OSGIN1 (forward, 5′-AGAAGAAGCGAAGAGGTC -3'; reverse, 5′-CGGACACAAAGTTATGCC-3′), and β-actin (forward, 5′-CGGCATCGTCACCAACTG-3'; reverse, 5′-TCTCAAACATGATCTGGGTCATCT-3′). The conditions of the PCR reaction were as described in our previous study (Li et al., 2015). β-actin was used as an internal standard gene and the threshold cycle (Ct) of a test sample to a control sample (ΔΔCt method) was used for relative quantification of target gene expression.

### 2.9. shRNA

Lentiviral infection was performed according to the method of a previous study (Tang et al., 2011). The sequence targeting human Nrf2 mRNA was chosen and purchased from the National RNAi Core facility platform, Taipei, Taiwan. RNAi clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as follows: TRC0000007555 (responding sequence, GCTCCTACTGT-GATGTGAAAT) was used for shNrf2 targeted to Nrf2. The shLuc was used for vector control targeted to luciferase and was kindly provided by Dr. C.-K. Lii, China Medical University, Taichung, Taiwan. Briefly, MCF-7 cells ($5 \times 10^5$) were plated onto 6-cm plastic culture dishes in DMEM supplemented with 1.5 g/L sodium bicarbonate, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% FBS. After 24 h of attachment, the cells were infected with packaged lentiviruses for another 24 h. On the following day, the medium was removed, and the cells were selected by using 2 mg/mL puromycin for 2 days. The cells were then passaged to 10-cm plastic culture dishes and were ready for assay.

### 2.10. Intracellular reactive oxygen species measurement

Detection of intracellular oxidative states was performed by using the probe $H_2$DCFDA and experimental conditions as in a previous study (Wang et al., 2008). First, cells were plated in 4-well chamber slides ($2 \times 10^5$ cells/well) and then incubated in DMEM supplemented with 10% FBS overnight. Thereafter, cells were stabilized in DMEM with 10% FBS for at least 40 min before exposure to DHA for the indicated time periods. Cells were then incubated with 10 μM $H_2$DCFDA for 30 min at 37 °C. Cells were immediately observed under an Inverted Fluorescent Microscope (ZEISS, AXioskop2) at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm and cellular reactive oxygen species (ROS) levels were assessed as relative fluorescence units (%).

### 2.11. OSGIN1 overexpression plasmid transfection

Mammalian expression plasmid encoding OSGIN1 (BC032476) was obtained from transOMIC Technologies (Huntsville, AL, USA), and the plasmid DNA was transformed into *E. coli* by using the heat shock method as described (Froger and Hall, 2007). The MCF-7 cells were transiently transfected with the pTCN-OSGIN1 plasmid DNA and pTCN control vector by using TransIT®-2020 transfection reagent according to the manufacturer's instructions (Mirus Bio, Inc., Madison, WI).

### 2.12. Annexin V and propidium iodide staining

The apoptosis of MCF-7 cells in response to DHA and OSGIN1-overexpressing vector was analyzed by using the FITC Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ). MCF-7 cells ($1 \times 10^6$ cells) were seeded on 3-cm dishes and incubated overnight in medium with 10% FBS when cells were at 70% confluency. Then cells were treated with DHA in a dose-dependent manner or transfected with OSGIN1-overexpressing vector for 24 h at 37 °C in a $CO_2$ incubator. Supernatants were collected from culture dishes and adherent cells detached with trypsin-EDTA. The

cells were collected by centrifugation at 800 × *g* for 5 min at 4 °C, and the pellets were resuspended by using 1X binding buffer at $1 \times 10^6$ cells/mL. Then, 1 mL of the cell suspension was transferred to a 5-mL culture tube and incubated with 5 μL FITC Annexin V and 5 μL propidium iodide (PI) for 15 min at room temperature in the dark. Finally, 1 mL of 1X binding buffer was added in a 5-mL culture tube. Fluorescence intensity was determined by flow cytometry (FACSCalibur, BD Bioscience).

### 2.13. Statistics

Data were analyzed by using one-way ANOVA (version 10.0; SPSS, Chicago, IL). The significance of the difference among group means was determined by Tukey's test, and the difference between mean values was determined by Student's *t*-test. P values $< 0.05$ were taken to be statistically significant.

## 3. Results

### 3.1. Effect of n-6 and n-3 PUFAs on OSGIN1 expression in MCF-7 and Hs578T cells

As shown by the MTT assay, the cell viabilities of MCF-7 cells treated with 100 μM AA, LA, GLA, ALA, EPA, and DHA were 115.6 ± 5.1%, 110.3 ± 20.6%, 111.8 ± 12.3%, 121.3 ± 16.0%, 107.4 ± 4.7%, and 107.4 ± 4.8%, respectively, compared with the untreated controls (100%). The corresponding cell viabilities of Hs578T cells were 96.2 ± 5.9%, 102.2 ± 6.4%, 100.6 ± 8.7%, 95.5 ± 5.2%, 103.0 ± 14.1%, and 101.1 ± 8.4%, respectively. We chose to use 100 μM PUFAs because concentrations greater than 100 μM caused cell damage (data not shown). Thus, in our study, the observed effects of n-6 and n-3 PUFAs were not due to cell viability.

OSGIN1 is an oxidative stress response gene that is up-regulated by epoxyisoprostane E2 in endothelial cells (Yan et al., 2014). To investigate whether OSGIN1 expression was affected by n-6 and n-3 PUFAs, we assayed the effect of 100 μM AA, LA, GLA, ALA, EPA, and DHA on OSGIN1 expression in breast cancer cells. As shown in Fig. 1A, the expression of OSGIN1 protein, particularly the 52-kDa isoform, was dramatically up-regulated by 100 μM DHA, but this effect was not seen in the other PUFA-treated groups. The OSGIN1 mRNA expression pattern was similar to the protein expression pattern in MCF-7 and Hs578T cells (Fig. 1B). To investigate whether there was a dose-response relation in the effect of DHA on OSGIN1 expression, we assayed the effect of 0, 25, 50, and 100 μM DHA. As noted in Fig. 1C, DHA induced OSGIN1 protein expression in a dose-dependent manner, and the maximal induction of OSGIN1 was achieved by treatment with 100 μM DHA for 24 h. A similar pattern was found for OSGIN1 mRNA expression (Fig. 1D).

### 3.2. Effect of DHA on the nuclear translocation of Nrf2 and Nrf2-mediated OSGIN1 gene expression

Nrf2, an oxidative stress-sensitive transcription factor, has been shown to be involved in the induction of OSGIN1 by oxidized phospholipids in endothelial cells (Yan et al., 2014). In this study, we determined whether Nrf2 played an essential role in DHA-induced OSGIN1 expression in MCF-7 cells. As noted in Fig. 2A, cells treated with 100 μM DHA showed a higher level of Nrf2 accumulation in the nuclear fraction as early as 4 h, and this accumulation was sustained until 24 h. Furthermore, DHA dose-dependently increased Nrf2 translocation from the cytosol to the nucleus (Fig. 2B). To further confirm the role of Nrf2 in DHA-induced OSGIN1 expression, an Nrf2 silencing experiment was performed with a VSV-G pseudotyped lentivirus-shRNA system (Fig. 2C). Compared with shLuc cells, cells transfected with shNrf2 showed a significant reduction in Nrf2 protein expression in the presence of 100 μM DHA. Moreover, induction of the OSGIN1 protein level by DHA was decreased in MCF-7 shNrf2 cells as well (Fig. 2C).

### 3.3. Effect of specific MAPK inhibitors on DHA-induced OSGIN1 expression

Activation of MAPKs and PI3K/Akt signaling pathways is involved in the up-regulation of Nrf2-dependent gene expression by DHA (Yang et al., 2013). To clarify the contributions of the MAPKs and PI3K/Akt signaling pathways in the up-regulation of OSGIN1 by DHA, we used pharmacological inhibitors of MAPKs and PI3K, such as SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), and LY294002 (PI3K inhibitor). As noted in Fig. 3A, DHA-induced OSGIN1 protein expression was suppressed by specific inhibitors, particularly the PI3K inhibitor. Phosphorylation of Akt at both Thr308 and Ser473 was rapidly increased at 30 min after DHA treatment (Fig. 3B). Furthermore, the PI3K inhibitor significantly decreased the DHA-induced nuclear accumulation of Nrf2 (Fig. 3C).

### 3.4. DHA increases Nrf2 nuclear accumulation and OSGIN1 expression through ROS-mediated PI3K/Akt signaling pathway

DHA rapidly induces ROS generation in EA. hy926 cells and the increased ROS levels contribute to elevate nuclear translocation of Nrf2 as well as ARE-luciferase reporter activity (Yang et al., 2013). To further demonstrate whether the up-regulation of OSGIN1 expression by DHA was through induction of ROS and the ROS-mediated PI3K/Akt/Nrf2 signaling pathway, we used *N*-acetyl-L-cysteine (NAC), a ROS scavenger. As noted in Fig. 4A, a transient induction of ROS in MCF-7 cells was seen after treatment with 100 μM DHA for 5 min, and with a peak of ROS production at 20 min. Pretreatment with NAC significantly suppressed DHA-induced OSGIN1 expression (Fig. 4B). Moreover, DHA induced the phosphorylation of Akt (308/473), and the nuclear accumulation of Nrf2 was significantly attenuated by NAC (Fig. 4C and D).

### 3.5. OSGIN1 overexpression and DHA treatment increased the Bax/Bcl-2 ratio, mitochondrial accumulation of OSGIN1 and p53, and cytochrome c release in MCF-7 cells

A previous study showed that mitochondrial localization of OSGIN1 is associated with its proapoptotic function in U2OS cells (Yao et al., 2008). We hypothesized that induction of OSGIN1 by DHA would effectively eradicate breast cancer cells through OSGIN1-mediated apoptosis. To clarify the role of OSGIN1 in the regulation of apoptosis in breast cancer cells, we first examined the effect of OSGIN1 on the expression of Bcl-2 and Bax. We used a plasmid vector to overexpress OSGIN1 and analyzed apoptotic cell death by using Annexin V-FITC/PI staining. As noted in Fig. 5A, OSGIN1 overexpression significantly increased OSGIN1 expression and decreased Bcl-2 expression. Compared to the OSGIN1 overexpression group, the related protein levels of OSGIN1 and Bax were slightly increased in the DHA-treated group. Compared to the empty vector control, OSGIN1 overexpression by transient transfection with pTCN-OSGIN1 plasmid DNA dramatically increased early and late apoptosis in 11.4 ± 0.79% and 27.2 ± 2.81% of cells, respectively (Fig. 5B). Moreover, OSGIN1 overexpression significantly increased the mitochondrial accumulation of OSGIN1 and p53 as well as cytochrome *c* release from mitochondria to the cytosol (Fig. 5C). Compared to the OSGIN1 overexpression group, treatment with 100 μM DHA for 24 h slightly increased the mitochondrial accumulation of OSGIN1 and p53 (Fig. 5C). After

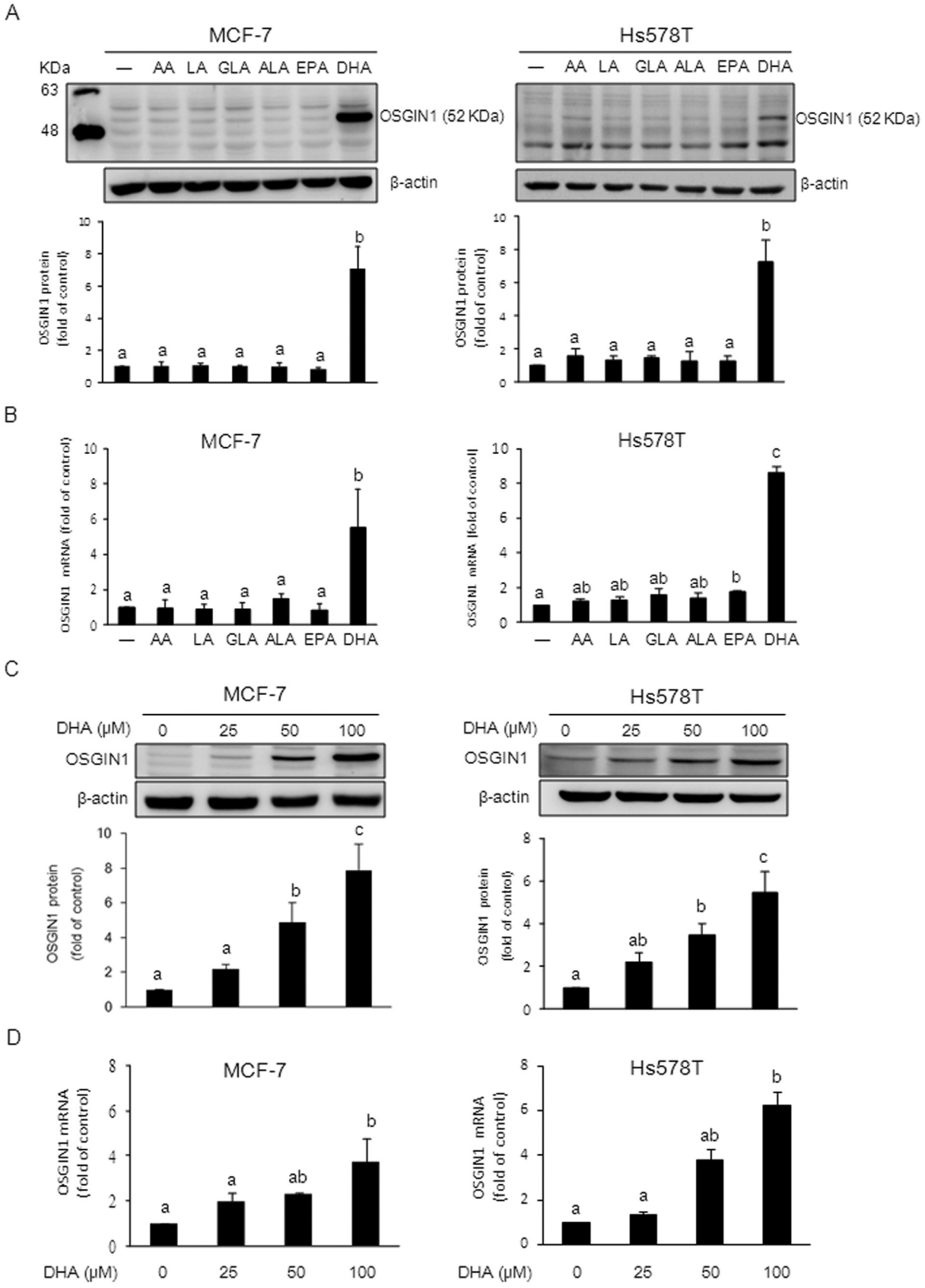


**Fig. 1.** Effect of n-3 and n-6 PUFAs on OSGIN1 expression in MCF-7 and Hs578T cells. MCF-7 and Hs578T cells were treated with 100 μM of the indicated PUFAs for 24 h (A and B) or with various concentrations of DHA for 24 h (C and D). OSGIN1 protein and mRNA expressions were measured by Western blotting and real-time PCR analysis, respectively. Results are mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

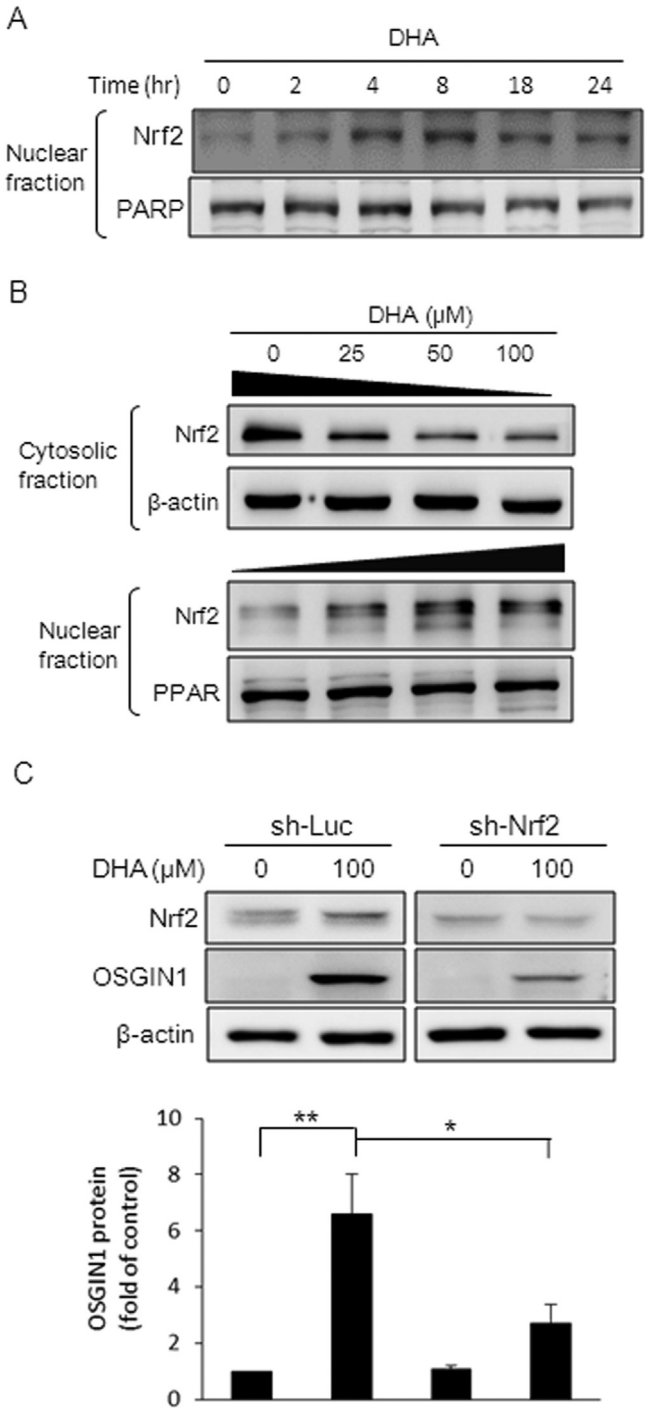


**Fig. 2.** Effect of DHA on nuclear Nrf2 accumulation and Nrf2-mediated OSGIN1 expression. MCF-7 cells were treated with 100 μM DHA for the indicated time periods (A) or various concentrations for 18 h (B), and the nuclear (5 μg) and cytosolic (10 μg) proteins from cells were measured by Western blotting. Cells were transfected with shNrf2 and challenged with 100 μM DHA for 24 h. Aliquots of total protein (10 μg) were used for Western blotting (C). One representative experiment out of three independent experiments is shown. Mean ± SD, n = 3. *P < 0.05 and **P < 0.01.

OSGIN1 knockdown, DHA-induced mitochondrial accumulation of OSGIN1, p53, and Bax were diminished and the increased cytochrome *c* release was reversed (Fig. 5D). These results suggested that DHA treatment showed a similar effect to OSGIN1 overexpression leading to induction of OSGIN1, elevation of Bax/Bcl-2 ratio, mitochondrial accumulation of OSGIN1 and p53, and cytochrome *c* release.

### 3.6. Effects of DHA on normal cells

Compared with human normal mammary gland tissue, the expression of OSGIN1 mRNA was low in various human breast cancer cell lines, including MCF-7, T47D, Hs578T, and MDA-MB-231 (Huynh et al., 2001). These observations revealed that the existence of OSGIN1 contributes to tumor suppressing properties such as inhibition of proliferation and well differentiation. To demonstrate the difference of OSGIN1 expression and OSGIN1-relative effects by DHA between normal mammary cells and breast carcinoma cells, the non-tumorigenic epithelial cell line MCF-10A and H184 were used. We first demonstrate the effect of DHA on cell death among these cells. Compared with the unstimulated controls, 100 μM and 200 μM DHA increased apoptotic cell death in MCF-7 cells, whereas the extent of apoptosis by DHA was lower in MCF-10A cells (Fig. 6A). The higher concentration of DHA at 300 μM significantly induced MCF-7 and MCF-10A cell death (Fig. 6A). Although the basal level of OSGIN1 expression was similar among these cells. However, treatment with 100 μM DHA significantly induced OSGIN1 expression and Bax/Bcl-2 ratio in MCF-7 than in H184 and MCF-10A (Fig. 6B). Moreover, DHA treatment elevated mitochondrial accumulation of OSGIN1, p53 and Bax as well as cytochrome *c* release in MCF-7, but in H184 and MCF-10A (Fig. 6C). These results indicated that 100 μM of DHA we used does not produce similar effects in normal breast epithelial cell line. DHA has potential properties of inducing OSGIN1 expression and OSGIN1-mediated changes of the proapoptotic proteins in breast cancer cells.

## 4. Discussion

The n-6 and n-3 PUFAs regulate the expression of several genes that modulate cell proliferation, angiogenesis, and metastasis in tumorigenesis (Wen et al., 2013; Galindo-Hernandez et al., 2014; Lii et al., 2016). Although OSGIN1 was recognized as tumor suppressor gene, whether it played an important role in the suppression of breast cancer by n-6 and n-3 PUFAs remained unclear. In this study, we first revealed that DHA, but not other PUFAs, dramatically induced OSGIN1 expression and that this induction was partially through the ROS/PI3K/Akt/Nrf2 pathway in breast cancer cells. Moreover, induction of OSGIN1 was involved in DHA-induced onset of apoptosis in breast cancer cells.

Four OSGIN1 transcript variants encoding protein isoforms with molecular weights of 38, 52, 59, and 61 kDa have been detected (Ong et al., 2004). Among these products, the OSGIN1 1a/2a-encoded 52-kDa protein is recognized for its anti-cancer activity (Wang et al., 2005). As noted in Fig. 1, the finding that DHA, but not other n-6 and n-3 PUFAs, dramatically induced the expression of the 52-kDa OSGIN1 protein isoform suggests that induction of OSGIN1 expression is specific for DHA treatment and may be involved in DHA's anti-cancer properties in breast cancer cells. As shown in Supplementary Fig. S1, the specific induction of OSGIN1 by DHA treatment was also shown in SKOV3 ovarian carcinoma cells, which suggests that induction of OSGIN1 by DHA may be present extensively in different cancer cell types.

ALA, EPA, and DHA belong to the n-3 series of PUFAs and have distinct effects on gene expression. A Venn diagram for cDNA results comparing the effects of EPA and DHA on gene modulation in LT97 human colon adenoma cells showed that a number of differentially expressed genes, such as UDP glucuronosyltransferase 1A3 (UGT1A3), cytochrome P450 3A7 (CYP3A7), and sulfotransferase 1B (SULT 1B), were up-regulated by DHA but not EPA. By contrast, EPA, but not DHA, significantly increased the gene expression of UGT1A1, glutathione S-transferase theta 2 (GSTT2), glutathione

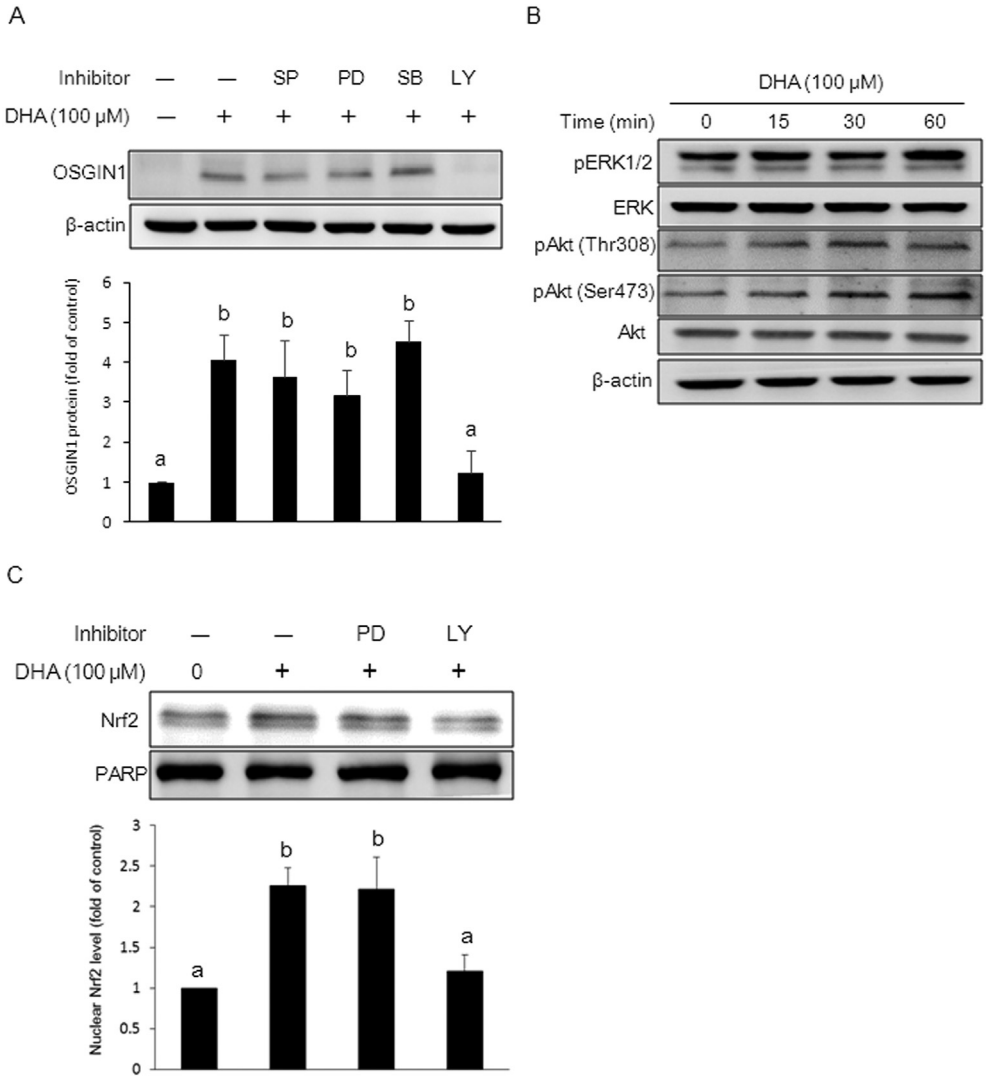


**Fig. 3.** Effect of specific MAPK inhibitors on OSGIN1 protein expression and Nrf2 accumulation in the presence of DHA. (A) MCF-7 cells were pretreated with or without 10 μM of specific MAPK inhibitors for 2 h and then treated with 100 μM DHA for another 24 h. (B) Cells were treated with 100 μM DHA for the indicated time periods, and aliquots of total protein (10 μg) were used for Western blotting. (C) MCF-7 cells were pretreated with or without 10 μM of specific MAPK inhibitors for 2 h and then treated with 100 μM DHA for another 18 h. Nuclear extracts (5 μg) were used for Western blotting. Results are mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

synthetase, and flavin-containing monooxygenase 5 (Habermann et al., 2009). The finding that OSGIN1 was significantly induced by DHA but not EPA suggests that OSGIN1 may be an important index by which to distinguish the action of DHA from EPA and other PUFAs.

Nrf2-mediated OSGIN1 expression was previously noted in human aortic endothelial cells (Yan et al., 2014), but the actual working mechanism by which DHA induced OSGIN1 expression was not fully elucidated. In this study, Nrf2 began to translocate from the cytosol into the nucleus within 4 h after 100 μM DHA treatment, and this accumulation was sustained until 24 h (Fig. 2A and B). Nuclear accumulation of Nrf2 was consistent with DHA-induced OSGIN1 mRNA and protein expression (Fig. 1C). In addition to elevating Nrf2 nuclear translocation, treatment with DHA can induce Nrf2 protein expression as well (Lee et al., 2015). As noted in Fig. 2C, DHA treatment significantly induced Nrf2 protein expression in shLuc-infected control cells. Knockdown of Nrf2 by using shNrf2 in MCF-7 cells abolished DHA-induced Nrf2 and OSGIN1 protein expression (Fig. 2C). These results suggest that up-regulation of Nrf2 expression and nuclear translocation are essential for the induction of OSGIN1 by DHA in breast cancer cells. Although the nuclear translocation and activation of Nrf2 could be induced by various PUFAs, such as AA (Gong and Cederbaum, 2006), EPA (Lee et al., 2015), and DHA (Yang et al., 2013), a differential extent of Nrf2-mediated gene transactivation was observed. For instance, DHA more strongly induced Nrf2/ARE signaling than did EPA at the same concentration of 50 μM in HepG2 cells (Saw et al., 2013). Furthermore, DHA, but not EPA, markedly increased the nuclear accumulation and DNA binding activity of Nrf2 in human umbilical vein endothelial cells (Ishikado et al., 2013). In the present study, significantly elevated nuclear accumulation of Nrf2 was observed in the DHA- and GLA-treated groups (Supplementary data Fig. S2), but the dramatic induction of OSGIN1 was seen only in the DHA-treated group (Fig. 1). We further compared the difference in ARE-luciferase reporter activity between the DHA- and GLA-treated groups. DHA was more effective than GLA at increasing ARE-luciferase reporter activity (Supplementary data Fig. S3). This raises the possibility that DHA acts to up-regulate OSGIN1

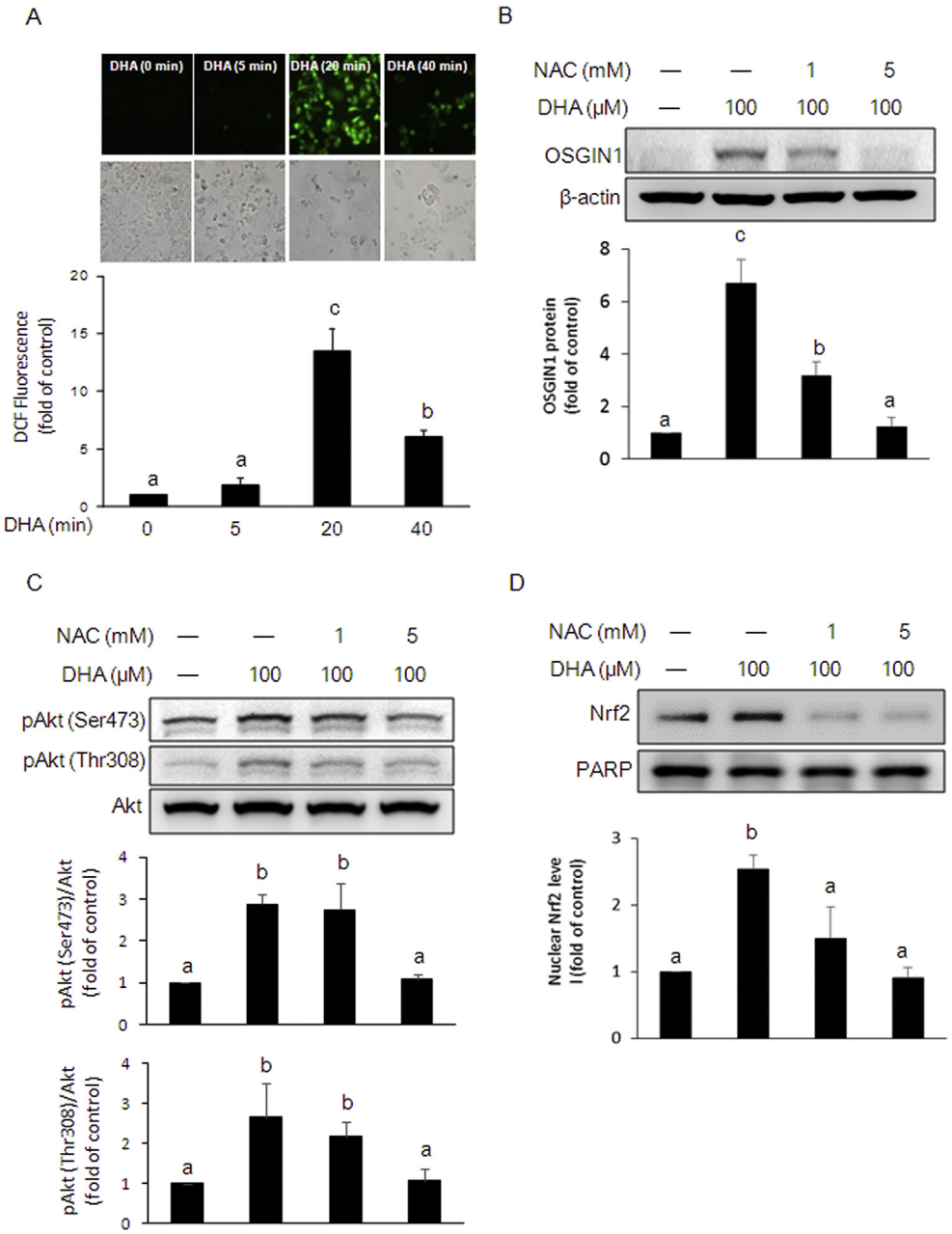


**Fig. 4.** OSGIN1 protein expression is up-regulated by DHA through ROS generation. MCF-7 cells were treated with 100 μM DHA for the indicated time periods and then incubated with $H_2$DCFDA. (A) Quantification of the ROS level as detected by $H_2$DCFDA fluorescence intensities. (B) Cells were pretreated with 1 mM or 5 mM NAC for 2 h followed by treatment with 100 μM DHA for another 24 h. (C) Cells were pretreated with 1 mM or 5 mM NAC for 2 h followed by treatment with 100 μM DHA for another 1 h. (D) Cells were pretreated with 1 mM or 5 mM NAC for 2 h followed by treatment with 100 μM DHA for another 18 h. Aliquots of total protein (10 μg) and nuclear protein (5 μg) were used for Western blotting. Anti β-actin or PARP antibodies were included to confirm equivalent protein loading, respectively. Results are mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

expression by changing the transactivation of transcription factors other than Nrf2. Several coactivators such as Maf, receptor-associated coactivator 3 (Rac3), and SPBP are known to be essential for the Nrf2-mediated activation of ARE-dependent genes (McNally et al., 2007; Kim et al., 2013; Darvekar et al., 2014). We cannot rule out that Maf, Rac3, or SPBP may contribute to enhanced up-regulation of OSGIN1 by DHA in breast cancer cells. The issue of the effect of n-6 and n-3 PUFAs on the interaction between Nrf2 and its coactivators will be considered in a future study.

JNK and p38 MAPK-mediated activation of the Nrf2/Keap1 pathway were essential for $H_2O_2$-induced stress-responsive genes, including heme oxygenase 1, glutathione S-transferase alpha 2, NAD(P)H:quinone oxidoreductase 1, superoxide dismutase 1, and glutathione peroxidase 1 expression in WI38 human embryonic lung fibroblast cells (Roy Chowdhury et al., 2014). PI3K/Akt, but not MAPKs, signaling was involved in ROS-induced stress-sensitive Nrf2/heme oxygenase 1 expression in HepG2 human hepatoma cells and RAW264.7 macrophages (Li et al., 2014; Xu et al., 2015). Our result revealed that LY294002 significantly inhibited the DHA-induced nuclear translocation of Nrf2 and OSGIN1 expression (Fig. 3), which suggests that PI3K/Akt signaling may play a major role in Nrf2-mediated OSGIN1 expression by DHA in

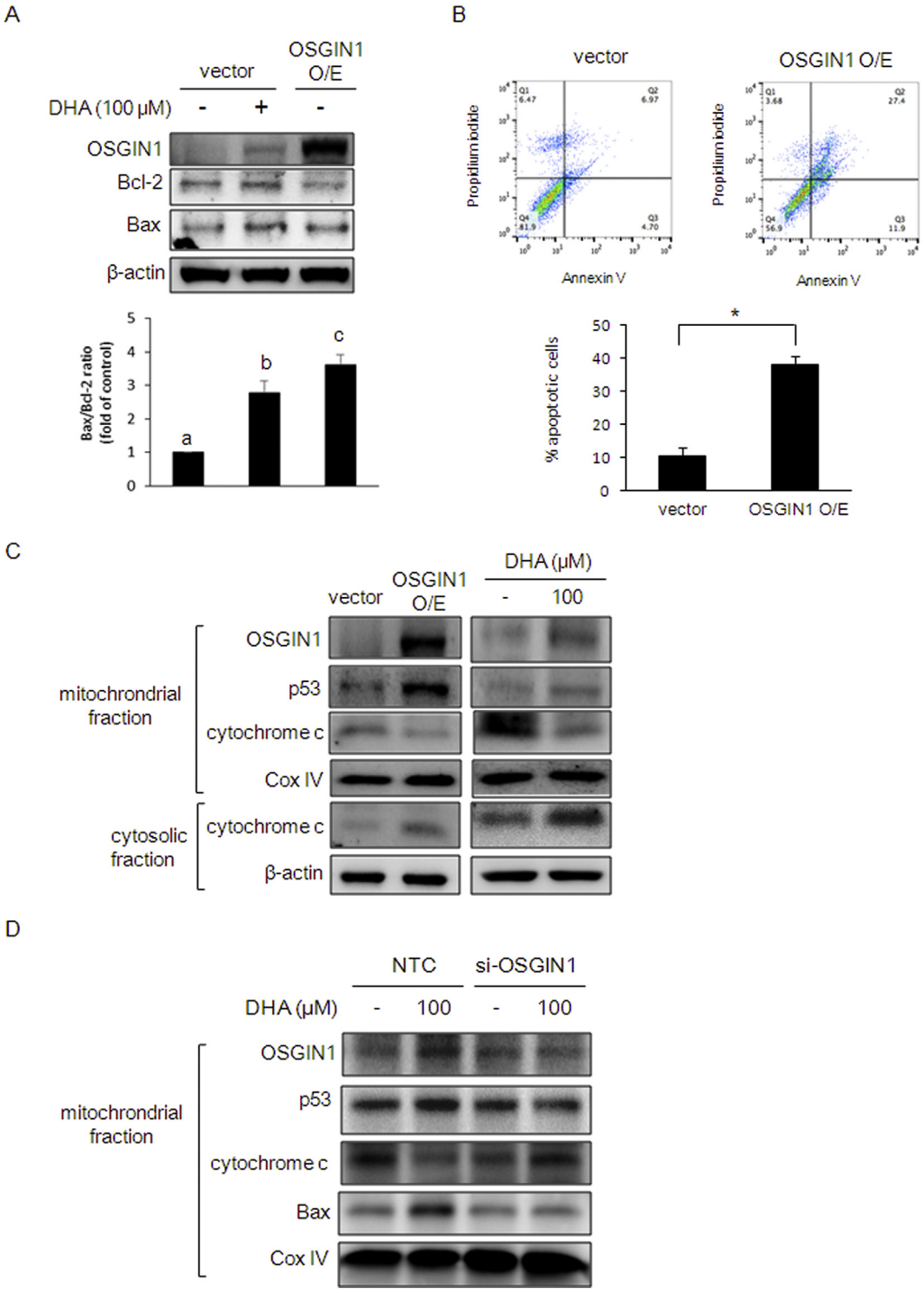


**Fig. 5.** Effect of OSGIN1 overexpression and DHA treatment on apoptotic cell death, Bax/Bcl-2 expression, cytochrome *c* release, and mitochondrial accumulation of OSGIN1, p53, and Bax. MCF-7 cells were transfected with OSGIN1 overexpression vector (OSGIN1 O/E) or pTCN control vector and were then treated with or without 100 μM DHA for an additional 24 h. (A) Aliquots of total protein (10 μg) were prepared and subjected to Western blotting. (B) Cells were incubated with Annexin V-FITC in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. (C) Mitochondrial fractions (2 μg) and (D) cytosolic fractions (2 μg) were prepared and subjected to Western blotting. Anti β-actin or Cox IV antibodies were included to confirm equivalent protein loading, respectively. The data are representative of three independent experiments. Mean ± SD, n = 3. *P < 0.01.

breast cancer cells.

OSGIN1 gene expression is induced by distinct stress signals, including damaging reagents or oxidative stress (Li et al., 2007; Romanoski et al., 2011). Early-stage and transient intercellular generation of ROS can be triggered by DHA in PC3 and DU145 cancer cells (Shin et al., 2013). A previous study showed that 10 μM DHA increases cellular ROS early at 1 min, and this induction is constitutively observed for 1 h after DHA treatment in U937 monocytic leukemia cells (Aires et al., 2007). Elevated production of ROS leads to the rapid activation of MAPKs or PI3K/Akt, which can

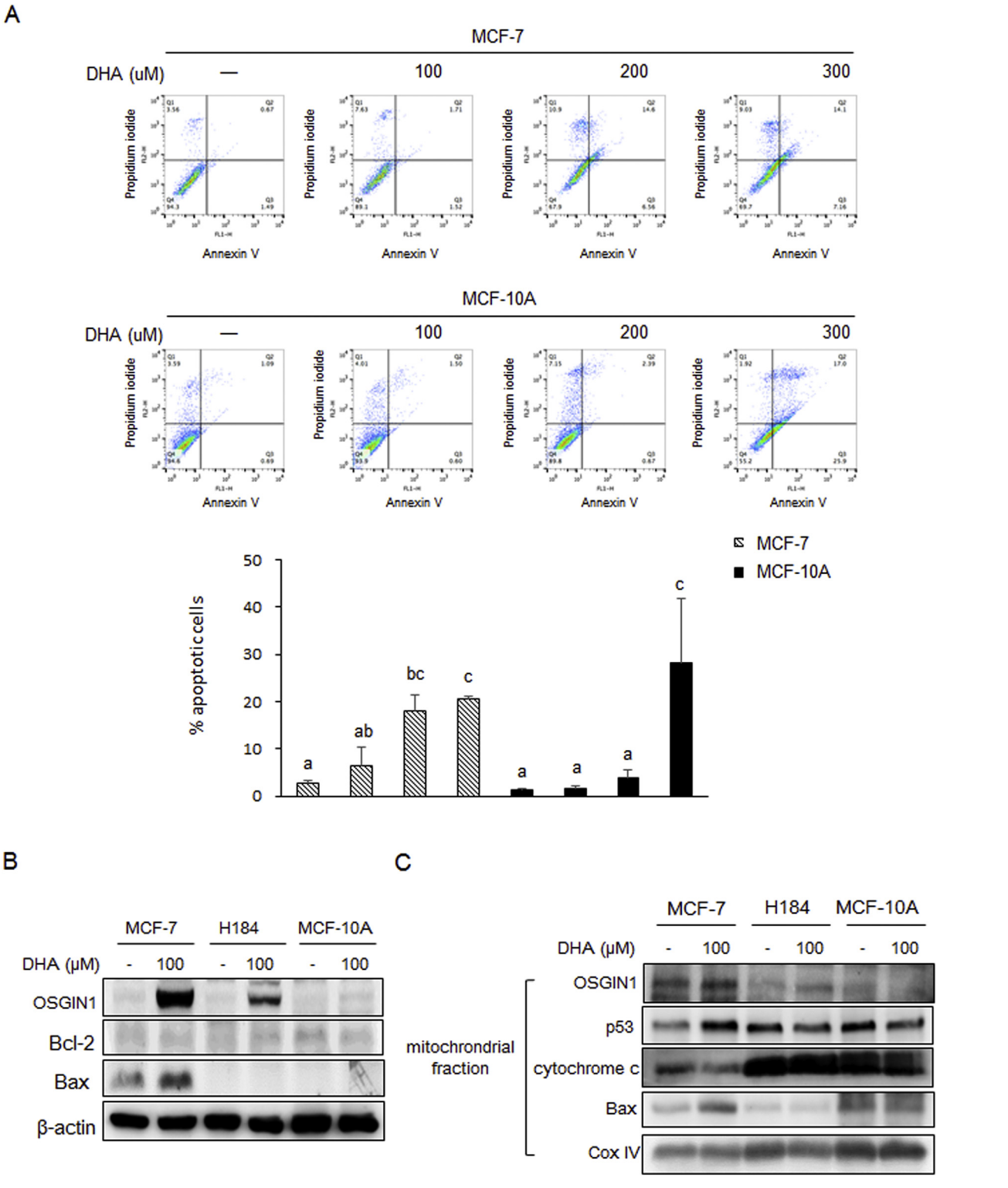


**Fig. 6.** Effect of DHA treatment on apoptotic cell death, OSGIN1, Bax/Bcl-2 expression and mitochondrial accumulation of OSGIN1, p53, cytochrome *c* and Bax in normal mammary cells and breast cancer cells. MCF-10A, H184 cells as well as MCF-7, were treated with or without 100, 200, and 300 μM DHA for 24 h. (A) Cells were incubated with Annexin V-FITC in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. (B) Aliquots of total protein (10 μg) were prepared and subjected to Western blotting. (C) Mitochondrial fractions (2 μg) were prepared and subjected to Western blotting. Anti β-actin or Cox IV antibodies were included to confirm equivalent protein loading, respectively. Results are mean ± SD of three independent experiments. Values not sharing the same letter are significantly different ($P < 0.05$).

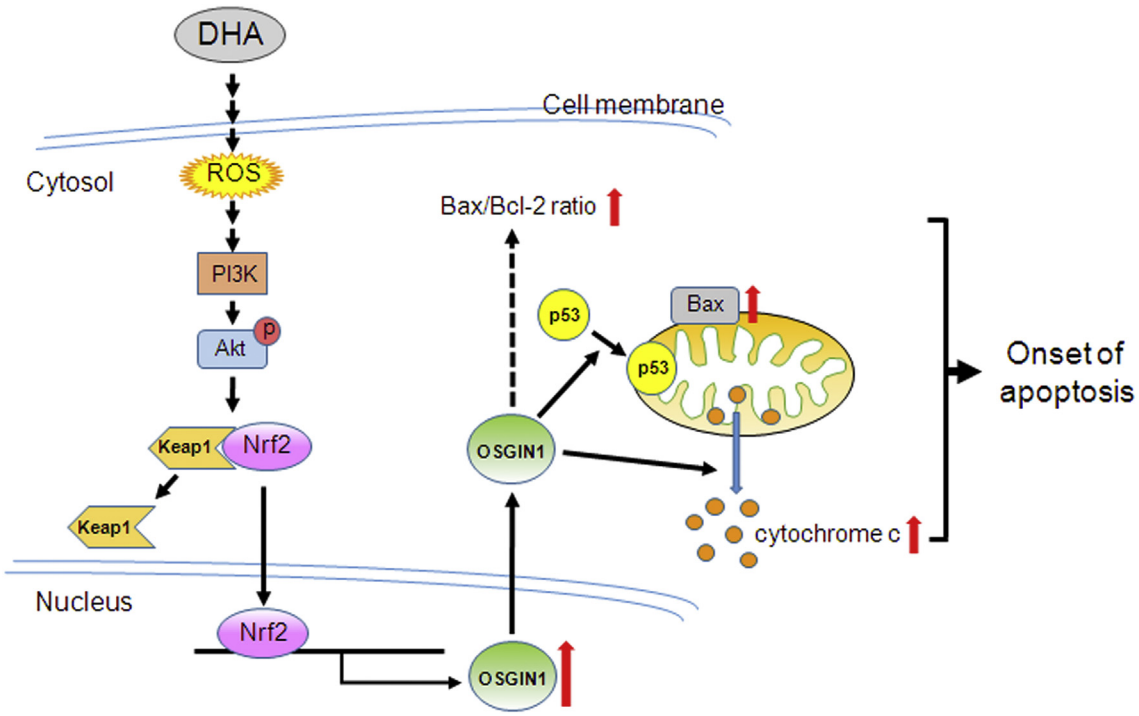


**Fig. 7.** Scheme summarizing the DHA induction of OSGIN1-mediated the onset of apoptosis via the up-regulation of PI3K/Akt/Nrf2 signaling pathway in breast cancer cells.

be inhibited by antioxidants (McCubrey et al., 2006). In this study, the intracellular level of ROS was rapidly increased after 5 min of treatment with 100 μM DHA in MCF-7 cells, and a peak increase was observed at 20 min as measured by the DCF probe (Fig. 4A), which is consistent with the results of a previous study (Yang et al., 2013). Pretreatment with NAC significantly inhibited the DHA-induced phosphorylation of Akt at Ser473/Thr308 as well as the nuclear accumulation of Nrf2 and OSGIN1 (Fig. 4B–D), which suggests that transient elevation of ROS and activation of ROS-mediated PI3K/Akt/Nrf2 signaling were involved in the induction of OSGIN1 by DHA.

Although OSGIN1 was up-regulated by DHA at the concentration of 100 μM, dramatic apoptotic cell death was not seen (data not shown). Treatment with 25 μM DHA for more than 72 h significantly reduces cell proliferation and induces apoptosis in MCF-7 cells (Kang et al., 2010). DHA at a concentration of 30 μM suppresses the viability of DU145 and PC3 cells by about 50% and 70%, respectively (Shin et al., 2013). In this study, treatment with 100 μM n-6 and n-3 PUFAs for 24 h resulted in no damage to cells, which indicates that the cytotoxicity response to PUFAs is dependent on exposure time and dosage as well as cell type. It has been shown that overexpression of the 52-kDa OSGIN1 protein leads to cell death in A498 cells (Ong et al., 2004). We further used the OSGIN1-overexpressing vector to test the hypothesis and looked at differences in expression of proapoptotic proteins, including Bax and Bcl-2, between treatment with 100 μM DHA and OSGIN1 overexpression. As shown, overexpression of OSGIN1 dramatically elevated the total protein level of OSGIN1 more than did DHA treatment (Fig. 5A). Overexpression of OSGIN1 significantly increased the Bax/Bcl-2 ratio more than did DHA treatment (Fig. 5A). Compared with the control group (10.5 ± 2.6%), OSGIN1 overexpression markedly increased apoptotic cell death by 37.9 ± 2.9%, (Fig. 5B), whereas 100 μM DHA treatment resulted in no damage to cells (data not shown). These results suggest that treatment with 100 μM DHA for 24 h can induce endogenous OSGIN1 generation. However, the induction of OSGIN1 by DHA was not better than the results of overexpression of OSGIN1, enough to promote dramatic apoptotic cell death.

OSGIN1 plays a crucial role in the regulation of mitochondrial p53 localization and p53-dependent cytochrome *c* release. OSGIN1 was found to work together with p53 and co-translocate to the mitochondria to induce cytochrome *c* release and apoptosis in U2OS cells (Hu et al., 2012). In response to DNA damage, p53 protein can rapidly translocate to the mitochondria and the increased mitochondrial p53 subsequently induces the oligomerization of Bax and Bak and permeabilization of the outer mitochondrial membrane, resulting in cytochrome *c* release (Mihara et al., 2003; Wolff et al., 2008). Mitochondrial release of cytochrome *c* is involved in DHA-induced apoptosis (Siddiqui et al., 2001). In this study, treatment with DHA mimicked the OSGIN1 overexpression results in accumulation of OSGIN1 and p53 in the mitochondrial fraction as well as the release of cytochrome *c* from mitochondria to the cytosol (Fig. 5C). Upon knockdown of OSGIN1, the DHA-induced increase in mitochondrial accumulation of OSGIN1, p53, and cytochrome *c* release were abolished (Fig. 5D). In addition to cytochrome *c* release, the mitochondrial accumulation of Bax has been recognized as a mitochondrial apoptosis event (Wu et al., 2015). Induction of mitochondrial Bax translocation by cisplatin results in apoptotic cell death in A549 and SPC-A1 cells (Wu et al., 2015). In this study, DHA treatment elevated mitochondrial accumulation of Bax, and knockdown of OSGIN1 diminished DHA-increased Bax accumulation in the mitochondrial fraction (Fig. 5D). Bcl-2 have been reported as transcriptional targets for p53 (Basu and Haldar, 1998). The finding in this study that OSGIN1 overexpression markedly decreased the Bcl-2 protein level (Fig. 5A) suggests that mitochondrial accumulation of p53 by OSGIN1 may interfere with p53-mediated transactivation of its target gene. These results indicate that OSGIN1 may have an essential role in up-regulation of p53 and Bax mitochondrial localization and cytochrome *c* release by DHA, which contribute to the onset of apoptosis in MCF-7 cells.

The findings of the present study are schematically presented in Fig. 7.

To confirm whether the proapoptotic effects of DHA were observed only in breast cancer cells. H184 cells, a chemically transformed human mammary epithelial cells and MCF-10A cells, a non-tumorigenic epithelial cells were used. Treatment with DHA at 100 and 200 μM significantly increased apoptotic cell death in MCF-7 cells, but in MCF-10A (Fig. 6A). However, the higher concentration of DHA up to 300 μM significantly elevated MCF-10A cell death, which was consistent with the previous study (Rescigno et al., 2016). These results indicate that the concentrations of DHA more than 300 μM appears to induce cell death in normal mammary epithelial cells as well as breast cancer cells. The concentrations of 100 μM DHA or less than 100 μM DHA were usually performed in increasing the toxicity of chemotherapy to cancer cells or tumors in several studies (Hajjaji and Bougnoux, 2013), hinted that higher dosages of DHA more than 100 μM may lead to normal mammary epithelial cell death as well. The induction of apoptotic level by 100 μM DHA at was 6.31 ± 4.14%, which was lower than that treatment with OSGIN1 overexpression plasmid (Fig. 5B) suggests that 100 μM DHA may induce early phase of programmed cell death in breast cancer cells. The high levels of p53 expression was found in normal breast tissues compared with breast tumor tissues. Moreover, MCF-10A cells performing higher p53 protein and mRNA levels than MCF-7 cells (Zhang et al., 2016). Upon p53 moves to the mitochondria from cytosol, p53 rapidly induces mitochondrial outer membrane permeabilization, subsequently triggering the release of pro-apoptotic factors from the mitochondrial intermembrane space (Moll et al., 2006). In this study, the basal level of p53 in mitochondria fraction was lower in MCF-7 cells compared with H184 and MCF-10A cells as well (Fig. 6C). Mitochondrial accumulation of p53 and cytochrome *c* release was significantly increased in MCF-7 cells after treatment with 100 μM DHA (Figs. 5C and 6C). In contrast, H184 and MCF-10A cells treated with DHA failed to induce accumulation of p53 and the release of cytochrome *c* from mitochondria (Fig. 6C). Bax translocation to mitochondria is an important event in inducing apoptotic breast cancer cell death (Sarkar et al., 2003). In this study, DHA significantly induced mitochondrial accumulation of Bax in MCF-7 cells compared with H184 and MCF-10A cells (Fig. 6C), providing direct evidence for the differential effects of DHA on pro-apoptotic processes between tumorigenic and nontumorigenic breast epithelial cells.

## 5. Conclusions

In summary, the results of the present study indicate that DHA-induced OSGIN1 expression is mediated at least in part through the PI3K/Akt/Nrf2 signaling pathway. Additionally, induction of OSGIN1 expression caused mitochondrial accumulation of p53 and OSGIN1, which are partially involved in the induction of cytochrome *c* release and onset of apoptosis by DHA in breast cancer cells.

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## Author contributions

The author's responsibilities were as follows: Li C-C (Principal Investigator) and Yao H-T designed the study; Tsai C-H, Chen H-W, Liu K-L, Chang J-W, Chen P-Y, Lin C-Y contributed to sample measurements and data collection; Tsai C-H and Shen Y-C analyzed data and performed statistical analysis; Tsai C-H and Li C-C wrote paper; Li CC had primary responsibility for final content. All authors contributed to the manuscript review and approved the final version.

## Financial disclosure

The authors have nothing to disclose.

## Conflict of interest

Nothing to disclose.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.08.010.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.08.010.

## References

Aires, V., Hichami, A., Filomenko, R., Plé, A., et al., 2007. Docosahexaenoic acid induces increases in [Ca2+]i via inositol 1,4,5-triphosphate production and activates protein kinase C gamma and -delta via phosphatidylserine binding site: implication in apoptosis in U937 cells. Mol. Pharmacol. 72, 1545–1556.

Basu, A., Haldar, S., 1998. The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. Mol. Hum. Reprod. 4, 1099–1109.

Chen, H.W., Chao, C.Y., Lin, L.L., Lu, C.Y., et al., 2013. Inhibition of matrix metalloproteinase-9 expression by docosahexaenoic acid mediated by heme oxygenase 1 in 12-O-tetradecanoylphorbol-13-acetate-induced MCF-7 human breast cancer cells. Arch. Toxicol. 87, 857–869.

Darvekar, S.R., Elvenes, J., Brenne, H.B., Johansen, T., et al., 2014. SPBP is a sulforaphane induced transcriptional coactivator of NRF2 regulating expression of the autophagy receptor p62/SQSTM1. PLoS One. 9, e85262.

DeSantis, C.E., Siegel, R.L., Sauer, A.G., Miller, K.D., et al., 2016. Cancer statistics for African Americans, 2016: Progress and opportunities in reducing racial disparities. CA Cancer J. Clin. 66, 290–308.

Froger, A., Hall, J.-E., 2007. Transformation of plasmid DNA into E. coli using the heat shock method. J. Vis. Exp. 6, 253.

Galindo-Hernandez, O., Serna-Marquez, N., Castillo-Sanchez, R., Salazar, E.P., 2014. Extracellular vesicles from MDA-MB-231 breast cancer cells stimulated with linoleic acid promote an EMT-like process in MCF10A cells. Prostagl. Leukot. Essent. Fat. Acids 91, 299–310.

Gong, P., Cederbaum, A.I., 2006. Transcription factor Nrf2 protects HepG2 cells against CYP2E1 plus arachidonic acid-dependent toxicity. J. Biol. Chem. 281, 14573–14579.

Habermann, N., Lund, E.K., Pool-Zobel, B.L., Glei, M., 2009. Modulation of gene expression in eicosapentaenoic acid and docosahexaenoic acid treated human colon adenoma cells. Genes Nutr. 4, 73–76.

Hajjaji, N., Bougnoux, P., 2013. Selective sensitization of tumors to chemotherapy by marine-derived lipids: a review. Cancer Treat. Rev. 39, 473–488.

Hu, J., Yao, H., Gan, F., Tokarrski, A., et al., 2012. Interaction of OKL38 and p53 in regulating mitochondrial structure and function. PLoS One 7, e43362.

Huynh, H., Ng, C.Y., Ong, C.K., Lim, K.B., et al., 2001. Cloning and characterization of a novel pregnancy-induced growth inhibitor in mammary gland. Endocrinology 142, 3607–3615.

Ishikado, A., Morino, K., Nishio, Y., Nakagawa, F., et al., 2013. 4-Hydroxy hexenal derived from docosahexaenoic acid protects endothelial cells via Nrf2 activation. PLoS One 8, e69415.

Kang, K.S., Wang, P., Yamabe, N., Fukui, M., et al., 2010. Docosahexaenoic acid

induces apoptosis in MCF-7 cells in vitro and in vivo via reactive oxygen species formation and caspase 8 activation. PLoS One. 5, e10296.
Kim, J.H., Yu, S., Chen, J.D., Kong, A.N., 2013. The nuclear cofactor RAC3/AIB1/SRC-3 enhances Nrf2 signaling by interacting with transactivation domains. Oncogene 32, 514–527.
Kocanova, S., Buytaert, E., Matroule, J.Y., Piette, J., et al., 2007. Induction of heme-oxygenase 1 requires the p38MAPK and PI3K pathways and suppresses apoptotic cell death following hypericin-mediated photodynamic therapy. Apoptosis 12, 731–741.
Kwon, J.I., Kim, G.Y., Park, K.Y., Ryu, C.H., et al., 2008. Induction of apoptosis by linoleic acid is associated with the modulation of Bcl-2 family and Fas/FasL system and activation of caspases in AGS human gastric adenocarcinoma cells. J. Med. Food. 11, 1–8.
Lee, H.J., Han, J., Jang, Y., Kim, S.J., et al., 2015. Docosahexaenoic acid prevents paraquat-induced reactive oxygen species production in dopaminergic neurons via enhancement of glutathione homeostasis. Biochem. Biophys. Res. Commun. 457, 95–100.
Lee, S.E., Kim, G.D., Yang, H., Son, G.W., et al., 2015. Effects of eicosapentaenoic acid on the cytoprotection through Nrf2-mediated heme oxygenase-1 in human endothelial cells. J. Cardiovasc. Pharmacol. 66, 108–117.
Lee, S.E., Lim, J.W., Kim, H., 2009. Activator protein-1 mediates docosahexaenoic acid-induced apoptosis of human gastric cancer cells. Ann. N. Y. Acad. Sci. 1171, 163–169.
Li, C.C., Lii, C.K., Liu, K.L., Yang, J.J., et al., 2006. n-6 and n-3 polyunsaturated fatty acids down-regulate cytochrome P-450 2B1 gene expression induced by phenobarbital in primary rat hepatocytes. J. Nutr. Biochem. 17, 707–715.
Li, C.C., Yao, H.T., Cheng, F.J., Hsieh, Y.H., et al., 2015. Docosahexaenoic acid down-regulates EGF-induced urokinase plasminogen activator and matrix metalloproteinase 9 expression by inactivating EGFR/ErbB2 signaling in SK-BR3 breast cancer cells. Nutr. Cancer. 67, 771–782.
Li, L., Dong, H., Song, E., Xu, X., 2014. Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. Chem. Biol. Interact. 209, 56–67.
Li, R., Chen, W., Yanes, R., Lee, S., et al., 2007. OKL38 is an oxidative stress response gene stimulated by oxidized phospholipids. J. Lipid Res. 48, 709–715.
Li, W., Yu, S., Liu, T., Kim, J.H., et al., 2008. Heterodimerization with small Maf proteins enhances nuclear retention of Nrf2 via masking the NESzip motif. Biochim. Biophys. Acta. 1783, 1847–1856.
Lii, C.K., Chang, J.W., Chen, J.J., Chen, H.W., et al., 2016. Docosahexaenoic acid inhibits 12-O-tetradecanoylphorbol-13- acetate-induced fascin-1-dependent breast cancer cell migration by suppressing the PKCδ- and Wnt-1/β-catenin-mediated pathways. Oncotarget 7, 25162–25179.
Liu, K.L., Yang, Y.C., Yao, H.T., Chia, T.W., et al., 2016. Docosahexaenoic acid inhibits inflammation via free fatty acid receptor FFA4, disruption of TAB2 interaction with TAK1/TAB1 and downregulation of ERK-dependent Egr-1 expression in EA.hy926 cells. Mol. Nutr. Food Res. 60, 430–443.
Liu, M., Li, Y., Chen, L., Chan, T.H., et al., 2014. Allele-specific imbalance of oxidative stress-induced growth inhibitor 1 associates with progression of hepatocellular carcinoma. Gastroenterology 146, 1084–1096.
Lu, C.Y., Yang, Y.C., Li, C.C., Liu, K.L., et al., 2014. Andrographolide inhibits TNFα-induced ICAM-1 expression via suppression of NADPH oxidase activation and induction of HO-1 and GCLM expression through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways in human endothelial cells. Biochem. Pharmacol. 91, 40–50.
McCubrey, J.A., Lahair, M.M., Franklin, R.A., 2006. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxidants Redox Signal. 8, 1775–1789.
McNally, S.J., Harrison, E.M., Ross, J.A., Garden, O.J., et al., 2007. Curcumin induces heme oxygenase 1 through generation of reactive oxygen species, p38 activation and phosphatase inhibition. Int. J. Mol. Med. 19, 165–172.
Mihara, M., Erster, S., Zaika, A., Petrenko, O., et al., 2003. p53 has a direct apoptogenic role at the mitochondria. Mol. Cell. 11, 577–590.
Moll, U.M., Marchenko, N., Zhang, X.K., 2006. p53 and Nur77/TR3-transcription factors that directly target mitochondria for cell death induction. Oncogene 25, 4725–4743.
Mukherjee, B., Telang, N., Wong, G.-Y., 2009. Growth inhibition of estrogen receptor positive human breast cancer cells by Taheebo from the inner bark of Tabebuia avellandae tree. Int. J. Mol. Med. 24, 253–260.
Ong, C.K., Ng, C.Y., Leong, C., Ng, C.P., et al., 2004. Genomic structure of human OKL38 gene and its differential expression in kidney carcinogenesis. J. Biol. Chem. 279, 743–754.
Prat, A., Adamo, B., Cheang, M.C., Anders, C.K., et al., 2013. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist 18, 123–133.
Ratliff, T.L., 2005. Genomic structure of human OKL38 gene and its differential expression in kidney carcinogenesis. J. Urol. 173, 1429–1430.
Rescigno, T., Capasso, A., Tecce, M.F., 2016. Effect of docosahexaenoic acid on cell cycle pathways in breast cell lines with different transformation degree. J. Cell Physiol. 231, 1226–1236.
Romanoski, C.E., Che, N., Yin, F., Mai, N., et al., 2011. Network for activation of human endothelial cells by oxidized phospholipids: a critical role of heme oxygenase 1. Circ. Res. 109, e27–e41.
Roy Chowdhury, S., Sengupta, S., Biswas, S., Sinha, T.K., et al., 2014. Bacterial fucose-rich polysaccharide stabilizes MAPK-mediated Nrf2/Keap1 signaling by directly scavenging reactive oxygen species during hydrogen peroxide-induced apoptosis of human lung fibroblast cells. PLoS One. 9, e113663.
Sarkar, F.H., Rahman, K.M., Li, Y., 2003. Bax translocation to mitochondria is an important event in inducing apoptotic cell death by indole-3-carbinol (I3C) treatment of breast cancer cells. J. Nutr. 133, 2434S–2439S.
Saw, C.L., Yang, A.Y., Guo, Y., Kong, A.N., 2013. Astaxanthin and omega-3 fatty acids individually and in combination protect against oxidative stress via the Nrf2-ARE pathway. Food Chem. Toxicol. 62, 869–875.
Shin, S., Jing, K., Jeong, S., Kim, N., et al., 2013. The omega-3 polyunsaturated fatty acid DHA induces simultaneous apoptosis and autophagy via mitochondrial ROS-mediated Akt-mTOR signaling in prostate cancer cells expressing mutant p53. Biomed. Res. Int. 2013, 568671.
Siddiqui, R.A., Jenski, L.J., Neff, K., Harvey, K., et al., 2001. Docosahexaenoic acid induces apoptosis in Jurkat cells by a protein phosphatase-mediated process. Biochim. Biophys. Acta 1499, 265–275.
Spector, A.A., Yorek, M.A., 1985. Membrane lipid composition and cellular function. J. Lipid Res. 26, 1015–1035.
Tang, S.C., Wu, M.F., Wong, R.H., Liu, Y.F., et al., 2011. Epigenetic mechanisms for silencing glutathione S-transferase m2 expression by hypermethylated specificity protein 1 binding in lung cancer. Cancer 117, 3209–3221.
Wang, G., Gong, Y., Burczynski, F.J., Hasinoff, B.B., 2008. Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay. Free Radic. Res. 42, 435–441.
Wang, T., Xia, D., Li, N., Wang, C., et al., 2005. Bone marrow stromal cell-derived growth inhibitor inhibits growth and migration of breast cancer cells via induction of cell cycle arrest and apoptosis. J. Biol. Chem. 280, 4374–4382.
Wen, Z.H., Su, Y.C., Lai, P.L., Zhang, Y., et al., 2013. Critical role of arachidonic acid-activated mTOR signaling in breast carcinogenesis and angiogenesis. Oncogene 10 (32), 160–170.
Wolff, S., Erster, S., Palacios, G., Moll, U.M., 2008. p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severely disrupts mitochondrial membrane integrity. Cell Res. 18, 733–744.
Wu, H.M., Jiang, Z.F., Ding, P.S., Shao, L.J., et al., 2015. Hypoxia-induced autophagy mediates cisplatin resistance in lung cancer cells. Sci. Rep. 5, 12291.
Xu, X., Li, H., Hou, X., Li, D., et al., 2015. Punicalagin induces Nrf2/HO-1 expression via upregulation of PI3K/AKT pathway and inhibits LPS-induced oxidative stress in RAW264.7 macrophages. Mediat. Inflamm. 2015, 380218.
Yan, X., Lee, S., Gugiu, B.G., Koroniak, L., et al., 2014. Fatty acid epoxyisoprostane E2 stimulates an oxidative stress response in endothelial cells. Biochem. Biophys. Res. Commun. 444, 69–74.
Yang, Y.C., Lii, C.K., Wei, Y.L., Li, C.C., et al., 2013. Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-κB pathways. J. Nutr. Biochem. 24, 204–212.
Yao, H., Li, P., Venters, B.J., Zheng, S., et al., 2008. Histone Arg modifications and p53 regulate the expression of OKL38, a mediator of apoptosis. J. Biol. Chem. 283, 20060–20068.
Zhang, C., Yu, H., Shen, Y., Ni, X., et al., 2015. Polyunsaturated fatty acids trigger apoptosis of colon cancer cells through a mitochondrial pathway. Arch. Med. Sci. 11, 1081–1094.
Zhang, L., Yang, W., Zhu, X., Wei, C., 2016. p53 inhibits the expression of p125 and the methylation of POLD1 gene promoter by downregulating the Sp1-induced DNMT1 activities in breast cancer. OncoTargets Ther. 9, 1351–1360.