

miR-21 is targeted by omega-3 polyunsaturated fatty acid to regulate breast tumor CSF-1 expression

Chandi Charan Mandal¹, Triparna Ghosh-Choudhury^{1,5},
Nirmalya Dey⁴, Goutam Ghosh Choudhury^{2,3,4} and
Nandini Ghosh-Choudhury^{1,2,*}

¹Department of Pathology, University of Texas Health Science Center, San Antonio, TX, USA, ²VA Research and ³Geriatric Research, Education and Clinical Center, South Texas Veterans Health Care System, San Antonio, TX, USA, ⁴Department of Medicine, University of Texas Health Science Center, San Antonio, TX, USA ⁵Present address: The Graduate School of Biomedical Sciences, One Baylor Plaza MS: BCM215, Suite N204, Houston, TX 77030, USA

*To whom correspondence should be addressed.

Email: choudhury@uthscsa.edu; 210-567-3108 (phone); 210567-1784 (FAX)
Correspondence may also be addressed to Goutam Ghosh Choudhury.
Email: choudhuryg@uthscsa.edu; 210-567-4830 (phone); 210-567-4712 (FAX)

Increasing evidence shows the beneficial effects of fish oil on breast cancer growth and invasion *in vitro* and in animal models. Expression of CSF-1 (colony stimulating factor-1) by breast cancer cells acts as potent activator of malignancy and metastasis. In this report, we used two human breast cancer cell lines, MDA-MB-231 and MCF-7, to show that the bioactive fish oil component DHA (docosahexaenoic acid) inhibits expression of CSF-1 and its secretion from these cancer cells. We found that the tumor suppressor protein PTEN regulates CSF-1 expression through PI 3 kinase/Akt signaling via a transcriptional mechanism. The enhanced abundance of microRNA-21 (miR-21) in breast cancer cells contributes to the growth and metastasis. Interestingly, DHA significantly inhibited expression of miR-21. miR-21 Sponge, which derepresses the miR-21 targets, markedly decreased expression of CSF-1 and its secretion. Furthermore, miR-21-induced upregulation of CSF-1 mRNA and its transcription were prevented by expression of PTEN mRNA lacking 3'-untranslated region (UTR) and miR-21 recognition sequence. Strikingly, miR-21 reversed DHA-forced reduction of CSF-1 expression and secretion. Finally, we found that expression of miR-21 as well as CSF-1 was significantly attenuated in breast tumors of mice receiving a diet supplemented with fish oil. Our results reveal a novel mechanism for the therapeutic function of fish oil diet that blocks miR-21, thereby increasing PTEN levels to prevent expression of CSF-1 in breast cancer.

Introduction

CSF-1 is expressed as differentially spliced mRNAs, which produce membrane-bound and secreted growth factors. Both soluble and membrane-attached forms of CSF-1 are biologically active and regulate the growth, differentiation and survival of macrophages and their bone marrow precursors (1,2). CSF-1 also acts as a differentiation factor for osteoclast lineage (3). Furthermore, CSF-1 promotes mature osteoclast activity and their adhesion to the bone surface (4). Binding of CSF-1 to its single tyrosine kinase receptor (CSF-1R) induces signal transduction involving various SH-2 domain-containing enzymes and adaptor proteins to elicit its biological activity (5). Earlier studies revealed abnormal expression of both CSF-1 and its receptor in many epithelial tumors originating in endometrium, ovary and breast, indicating the presence of an autocrine mechanism in carcinogenesis (2,6). Normal mouse breast epithelial cells express CSF-1 in the

Abbreviations: CSF-1, colony stimulating factor-1; DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; EPA eicosapentaenoic acid; ER, estrogen receptor; miRNA, microRNA; miR-21, microRNA-21; PIP₃, PI 3,4,5-tris-phosphate.

absence of CSF-1R. Introduction of CSF-1R significantly increases tumorigenicity and invasiveness of these cells (7). In a study, 75% of patients with breast cancer showed increased expression of CSF-1, which correlated with poor prognosis (8). Furthermore, expression of CSF-1 has been shown to be enhanced in invasive rather than in intraductal preinvasive breast carcinomas (9). Thus, the role of CSF-1 in breast malignancy and its metastasis is significant.

MicroRNAs (miRNAs) have emerged as short noncoding RNAs that control the progression and maintenance of many diseases, including cancer (10). Both enhanced and decreased expressions of multiple miRNAs have been reported in breast cancer (11,12). Moreover, the evolutionarily conserved miRNA, miR-21, is markedly increased in breast tumors (13). In fact, high miR-21 levels are linked to hormone receptor negativity, high tumor grade, lymph node involvement and poor disease-free survival (14). miR-21 is present in the intergenic region of TMEM-49 gene in human chromosome 17 (14). The primary transcript of miR-21 (pri-miR-21) is independently synthesized from an intronic promoter by RNA polymerase II (15). Pri-miR-21 is processed by the RNase III activity of Drosha/DGCR8 complex in the nucleus to produce a 72-nucleotide hairpin pre-miR-21, which is exported to the cytosol by exportin 5 (16). In the cytoplasm, the RNase III dicer binds to the stem-loop pre-miR-21 to yield 22-nucleotide-long miR-21 duplex. Unwinding of the duplex RNA generates the guide strand as mature miR-21 (16). Finally, mature miR-21 is associated with RNA-induced silencing complex along with miRNA ribonucleoprotein to target specific mRNAs containing imperfect complementarity in their 3'-UTR. This whole process results in a decrease in expression of protein encoded by the targeted mRNAs.

The beneficial effects of fish oil and its components, ω -3 fatty acids, have been extensively studied in various diseases, including cancer. The incidence of breast cancer is four to seven times higher among women in the USA than those in Japan and China. This shows a strong association of fish oil diet in preventing breast cancer (17). In fact, the recent diminished consumption of fish oil in Japanese population positively correlates with higher incidence of breast tumorigenesis in these women (18). The effect of fish oil components, DHA and eicosapentaenoic acid (EPA), on tumorigenicity of breast cancer cells has been tested both *in vitro* and in animal models to demonstrate beneficial effects (19–21). Recently, we demonstrated that fish oil significantly prevented the metastasis of breast cancer cells to bone (22). We showed that both DHA and EPA, two active constituents of fish oil, blocked *in vitro* invasion of breast cancer cells (22). The mechanism by which these ω -3 fatty acids exert their inhibitory effects on growth and invasion of breast tumor cells is poorly understood. In the present study, we found that fish oil and its bioactive component, DHA, significantly reduced CSF-1 expression in breast tumor *in vivo*, and in MDA-MB-231 and MCF-7 breast tumor cells *in vitro*. We found that dietary fish oil inhibits the expression of miR-21, resulting in increased abundance of the tumor suppressor protein PTEN, which negatively regulates the transcription of CSF-1 via Akt kinase.

Materials and methods

Materials and cell culture

The MCF 10A, MDA-MB-231 and MCF-7 cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained at 37°C in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and penicillin and streptomycin as described previously (22,23). Antibodies against PTEN and the p65 subunit of NF κ B were purchased from Santa Cruz (Delaware, CA). Actin antibody was obtained from Sigma (St Louis, MO). Estrogen receptor (ER)- α antibody was purchased from Calbiochem (San Diego, CA). CSF-1 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN). TRIZol RNA isolation kit was obtained from Sigma. FUGENE-HD was purchased

from Roche Diagnostics (Indianapolis, IN). DHA was obtained from Cayman Chemical Company (Ann Arbor, MI). The fish oil diet was obtained from Harlan Bioproducts (Indianapolis, IN) and prepared as described previously (22,24). The AIN 76 semipurified diet prepared with 10% (w/w) fish oil concentrate containing 34% EPA, 24% DHA and 10% other ω -3 fatty acids was used. SYBR green/ROX PCR master mix was purchased from Super Array Biosciences (Frederick, MD). U6 (for normalization) primers and mirVana qRT-PCR miRNA detection kit were obtained from Ambion (Austin, TX). To detect mature miR-21, primers and probe were obtained from Applied Biosystems (Foster City, CA). Luciferase assay kit was purchased from Promega (Madison, WI). miR-21 Sponge plasmid containing seven copies of bulged miR-21 recognition element, PTEN 3'-UTR-Luc reporter plasmid and pCMV-miR-21 have been described previously (25). miR-21 promoter-driven luciferase (-332 to +1957bp) plasmid (miR-21-Luc) was a kind gift from Dr X.-M. Chen, Creighton University Medical Center, NE, USA (26). Dominant negative PI 3 kinase (Δ p85), dominant negative Akt1 (K179M) and vector expressing PTEN without 3'-UTR were described previously (27,28).

Animal protocol

Immunocompromized (nu/nu) mice were purchased from the NIH. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center, San Antonio, TX, USA, approved the animal protocol. The control mice were fed normal lab chow diet during the period of experiment. The experimental group was fed on AIN 76 diet containing 10% (wt/wt) of a fish oil concentrate containing 34% EPA and 24% DHA for 7 days prior to injection of MDA-MB-231 breast cancer cells. Trypsinized breast cancer cells (10^6 cells in PBS) were injected into the mammary fat pad. The experimental group of mice was fed on fish oil diet throughout the experimental period. Mice were sacrificed 22 days post-injection of breast cancer cells. The tumors were isolated and frozen.

Preparation of RNA and real-time RT-PCR

Total RNA was extracted from tumor tissues or MDA-MB-231 cells using TRI reagent as described previously (23,25). To detect CSF-1 mRNA, 500ng of total RNA was reverse transcribed using First Strand synthesis kit. One microliter of cDNA was amplified using SYBR green method in a real-time PCR machine (7300, Applied Biosystems) in the presence of CSF-1-specific primer. CSF-1 mRNA was normalized to the level of GAPDH mRNA. The PCR conditions were: 94°C for 10min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. Primers for CSF-1 were: Forward, CCAGTGTATCCTGGTCTTG; Reverse, CCACCTGTCTGTATCCTGA. Primers for GAPDH were purchased from SuperArray Biosciences. For detection of pre-miR-21, 1 μ g of RNA was employed to synthesize cDNA using the mirVana qRT-PCR miRNA detection kit (25). qRT-PCR was performed in a real-time PCR machine. Samples were processed in triplicate. PCR conditions were: 94°C for 10min, followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30 s. The primers used for detection of premature miR-21 were as follows: Forward primer: 5'-TGTCGGGTAGCTTATCAGAC-3'; Reverse primer: 5'-TTCAGACAGCCATCGACTG-3'. For detection of pri-miR-21, the following primers were used: Forward, 5'-ACAGGCCAGAAATGCCTGGG-3'; Reverse, 5'-GATGGTCAGATGAAAGATAC-3'. mirVana qRT-PCR primer sets for U6 (Ambion) were used for normalization. For mature miR-21, TaqMan miRNA PCR (Applied Biosystems) was used following the manufacturer's protocol. The level of mature miR-21 was normalized to U6 (TaqMan). Data analysis was done by the comparative Ct method as described previously (25).

CSF-1 ELISA

The conditioned media from MDA-MB-231 and MCF-7 human breast cancer cells were used to detect CSF-1 protein levels using the Quantikine ELISA kit as described previously (29).

Immunoblotting

The MDA-MB-231 and MCF-7 human breast cancer cells were lysed in RIPA buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM EDTA, 1mM Na_3VO_4 , 1% NP-40, 1mM PMSF and 0.1% protease inhibitor cocktail) for 30min at 4°C for half an hour. The cell extracts were centrifuged at 10 000g for 20min to collect the supernatant. Protein was estimated using the BioRad reagent. Immunoblotting of equal amounts of protein was carried out with the indicated antibodies as described previously (22–24,30).

Transfection

MDA-MB-231 and MCF-7 human breast cancer cells were transfected with indicated plasmid vectors using Fugene HD as described previously (23,25). The conditioned media from the transfected cells were harvested for CSF-1 ELISA. Transfected cells were used to prepare RNA and protein lysates. The miR-21 Sponge construct encodes green fluorescence protein, which can be used as a surrogate for miR-21 sponge expression. GFP mRNA levels were determined

using the following primers: Forward, 5'-ACGGCAAGCTGACCCTGAAG-3'; Reverse, 5'-GGGTGCTCAGGTAGTGGTTG-3'. PCR cycling conditions were: 94°C for 10min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s.

Luciferase activity

The MDA-MB-231 and MCF-7 human breast tumor cells were transfected with the reporter plasmid along with the indicated expression vectors. The cell lysates were assayed for luciferase activity using a kit as described previously (23,25). The data are presented as luciferase activity per microgram protein as arbitrary units \pm SE of triplicate measurements as described previously (23–25,27,29,30).

Statistical analysis

Statistical significance of the data was calculated by ANOVA followed by paired *t*-test or Student Newman-Keuls analysis. A *P*-value of <0.05 was considered significant.

Results

DHA attenuates CSF-1 expression in breast cancer cells

CSF-1 plays an important role in breast tumorigenesis and metastasis. The invasive human breast cancer cell line, MDA-MB-231 (ER-negative), expresses CSF-1 constitutively and supports osteoclast formation when cultured with stromal cells (31). Furthermore, MDA-MB-231 cells injected into mouse heart metastasize to bone to form osteolytic lesions (32). In this process, CSF-1 has been implicated in increased osteoclastic activity (33). Recently, we reported that fish oil prevented MDA-MB-231 cell metastasis to the bone (22). Therefore, we used these breast tumor cells to study CSF-1 expression. We also used ER-positive MCF-7 breast cancer cell line. We first compared CSF-1 protein secretion between human MCF10A normal breast epithelial cells and these two breast cancer cells. The conditioned medium was analysed for CSF-1 using ELISA. As expected, both tumor cell lines showed increased secretion of CSF-1 compared with the normal breast epithelial cells (Supplementary Figure S1A, available at *Carcinogenesis* Online). Interestingly, metastatic MDA-MB-231 cells secreted significantly higher levels of CSF-1 compared to non-metastatic MCF-7 (Supplementary Figure S1A, available at *Carcinogenesis* Online). Expression of CSF-1 mRNA was also significantly high in these breast tumor cells (Supplementary Figure S1B, available at *Carcinogenesis* Online). Next, we incubated MDA-MB-231 and MCF-7 cells with DHA, an active component of fish oil. The conditioned media were tested for CSF-1 protein by ELISA. DHA significantly reduced the levels of CSF-1 in the conditioned media from both cell lines (Figure 1A and 1D). To confirm this observation, we tested expression of CSF-1 mRNA in these cells. Similar to the effect of DHA on CSF-1 levels, DHA blocked the expression of CSF-1 mRNA (Figure 1B and 1E). Interestingly, DHA did not show any effect on CSF-1 protein and mRNA expression in the MCF10A normal breast epithelial cells (Supplementary Figure S2A and S2B, available at *Carcinogenesis* Online). The results in breast cancer cells indicated that CSF-1 expression might be controlled at the transcriptional level. To directly examine this, we employed a reporter construct in which the CSF-1 promoter drives the luciferase gene (CSF-1-Luc) (29). MDA-MB-231 and MCF-7 cells were transfected with this reporter plasmid and treated with DHA. DHA significantly inhibited the reporter activity (Figure 1C and 1F). These results suggest that the bioactive fish oil component DHA blocks expression of CSF-1 in human breast cancer cells via a transcriptional mechanism.

PTEN regulates CSF-1 expression in breast tumor cells

We have recently demonstrated that fish oil increases the levels of PTEN in a breast cancer xenograft model. We also showed that DHA induced PTEN expression in MDA-MB-231 breast cancer cells (24). Therefore, we tested the role of PTEN in expression of CSF-1. Expression of PTEN in both MDA-MB-231 and MCF-7 cells significantly inhibited the levels of CSF-1 in the conditioned medium (Figure 2A, and Supplementary Figure S3A, available at *Carcinogenesis* Online). The biological function of PTEN is mediated by its lipid phosphatase activity, which dephosphorylates the

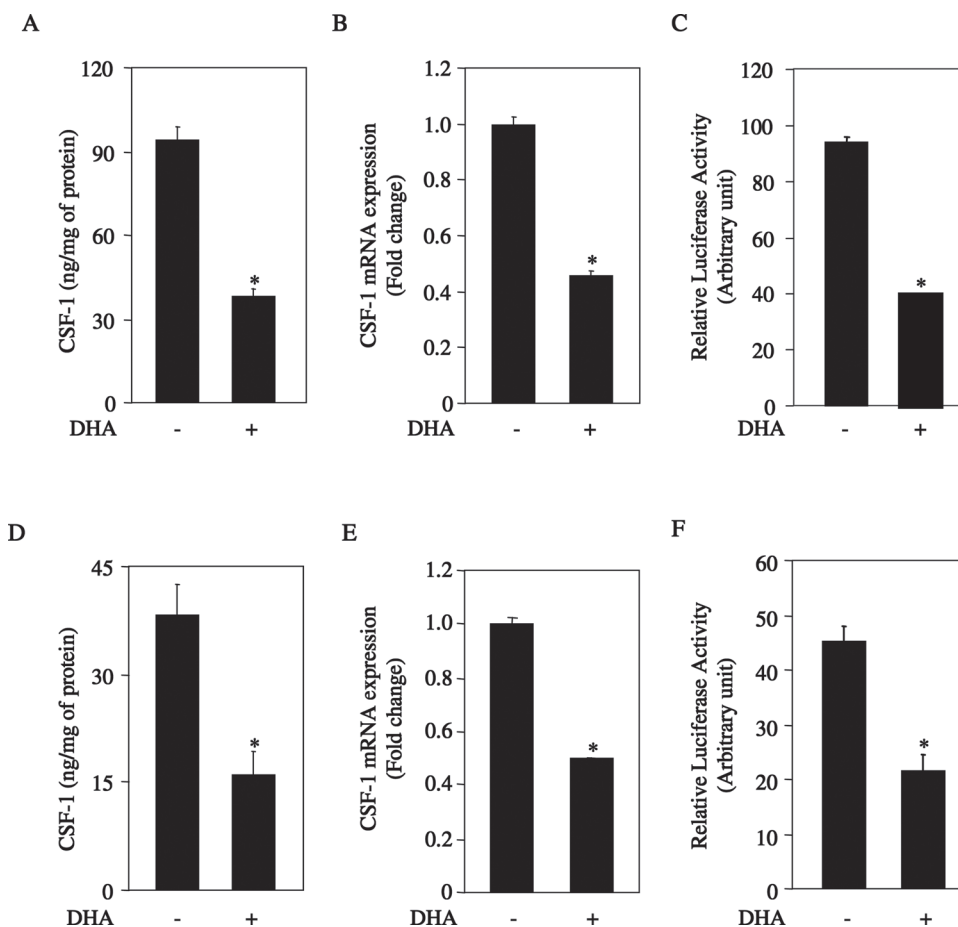


Fig. 1. DHA inhibits CSF-1 expression in MDA-MB-231 and MCF-7 human breast cancer cells. MDA-MB-231 (A and B) and MCF-7 (D and E) cells were incubated with 152 nM DHA for 24 h. (A and D) The conditioned media from untreated and DHA-treated cells were harvested and used in ELISA to detect CSF-1 protein as described previously (29). Mean \pm SE of triplicate measurements is shown. * $P = 0.003$ versus control for panel A; * $P = 0.02$ versus control in panel D. (B and E) Total RNAs were isolated from untreated and DHA-treated MDA-MB-231 and MCF-7 breast cancer cells and used in qRT-PCR to detect CSF-1 mRNA as described in the Materials and methods. Mean \pm SE of triplicate measurements is shown. * $P = 0.0007$ versus control in panel B; * $P = 0.0015$ versus control in panel E. (C and F) MDA-MB-231 (panel C) and MCF-7 (panel F) cells were transiently transfected with CSF-1-Luc reporter construct (29). The transfected cells were incubated with 152 nM DHA for 24 h. The cell lysates were assayed for luciferase activity as described in Materials and methods (23,24,29,30). Mean \pm SE of triplicate measurements is shown. * $P = 0.007$ versus control in panel C; * $P = 0.009$ versus control in panel F.

PI 3 kinase product PI 3,4,5-tris-phosphate (PIP₃). Thus, PTEN may regulate cellular functions by inhibiting PI 3 kinase signaling. We tested the involvement of PI 3 kinase in CSF-1 regulation. Expression of dominant negative PI 3 kinase significantly reduced the levels of CSF-1 in the conditioned medium of both cells (Figure 2B, and Supplementary Figure S3B, available at *Carcinogenesis* Online). Since many physiological and pathological functions of PI 3 kinase are carried out by the downstream kinase Akt and PTEN inhibits the Akt kinase activity (34), we investigated the role of Akt in the regulation of CSF-1. Expression of dominant negative Akt significantly attenuated CSF-1 protein expression (Figure 2C, and Supplementary Figure S3C, available at *Carcinogenesis* Online). Similarly, expression of PTEN, dominant negative PI 3 kinase or dominant negative Akt all blocked expression of CSF-1 mRNA in both cancer cell lines (Figure 2D-F, and Supplementary Figure S3D-F).

We have described above that expression of CSF-1 is modulated by a transcriptional mechanism (Figure 1C and 1F) (29). Transfection of PTEN along with the reporter construct CSF-1-Luc into MDA-MB-231 and MCF-7 breast cancer cells resulted in significant inhibition of the reporter activity (Figure 2G, and Supplementary Figure S4A, available at *Carcinogenesis* Online). Similarly, expression of dominant negative PI 3 kinase or dominant negative Akt kinase abrogated the CSF-1 promoter-driven luciferase activity (Figure 2H and 2I, and Supplementary Figure S4B and C, available

at *Carcinogenesis* Online). Together these data show that PTEN contributes to the transcription-dependent expression of CSF-1 protein in MDA-MB-231 and MCF-7 breast tumor cells by regulating Akt kinase activity.

DHA controls miR-21 expression via NF κ B in breast cancer cells

We have shown recently that fish oil prevented breast tumor growth *in vivo* by increasing PTEN levels (24). Also, DHA blocked MDA-MB-231 breast cancer cell proliferation concomitant with PTEN upregulation (24). Since PTEN regulated the expression of CSF-1 shown above, we investigated the mechanism responsible for PTEN upregulation by the bioactive component present in the fish oil. PTEN mRNA has been validated as a target of miR-21. Furthermore, the expression of miR-21 is increased in many tumors *in vivo*, and in cultured cells *in vitro*, including breast cancer cells (13,14). Cotransfection of miR-21 expression vector with a reporter plasmid containing the 3'-UTR of PTEN mRNA decreased the reporter activity in MDA-MB-231 cells, demonstrating that miR-21 targeted the PTEN 3'-UTR directly in these cells (Supplementary Figure S5A, available at *Carcinogenesis* Online). As expected, expression of miR-21 reduced the levels of PTEN protein in these cells (Supplementary Figure S5B, available at *Carcinogenesis* Online). In order to test the effect of DHA on expression of miR-21, we incubated MDA-MB-231 and MCF-7 breast tumor cells with this ω -3 fatty acid. DHA

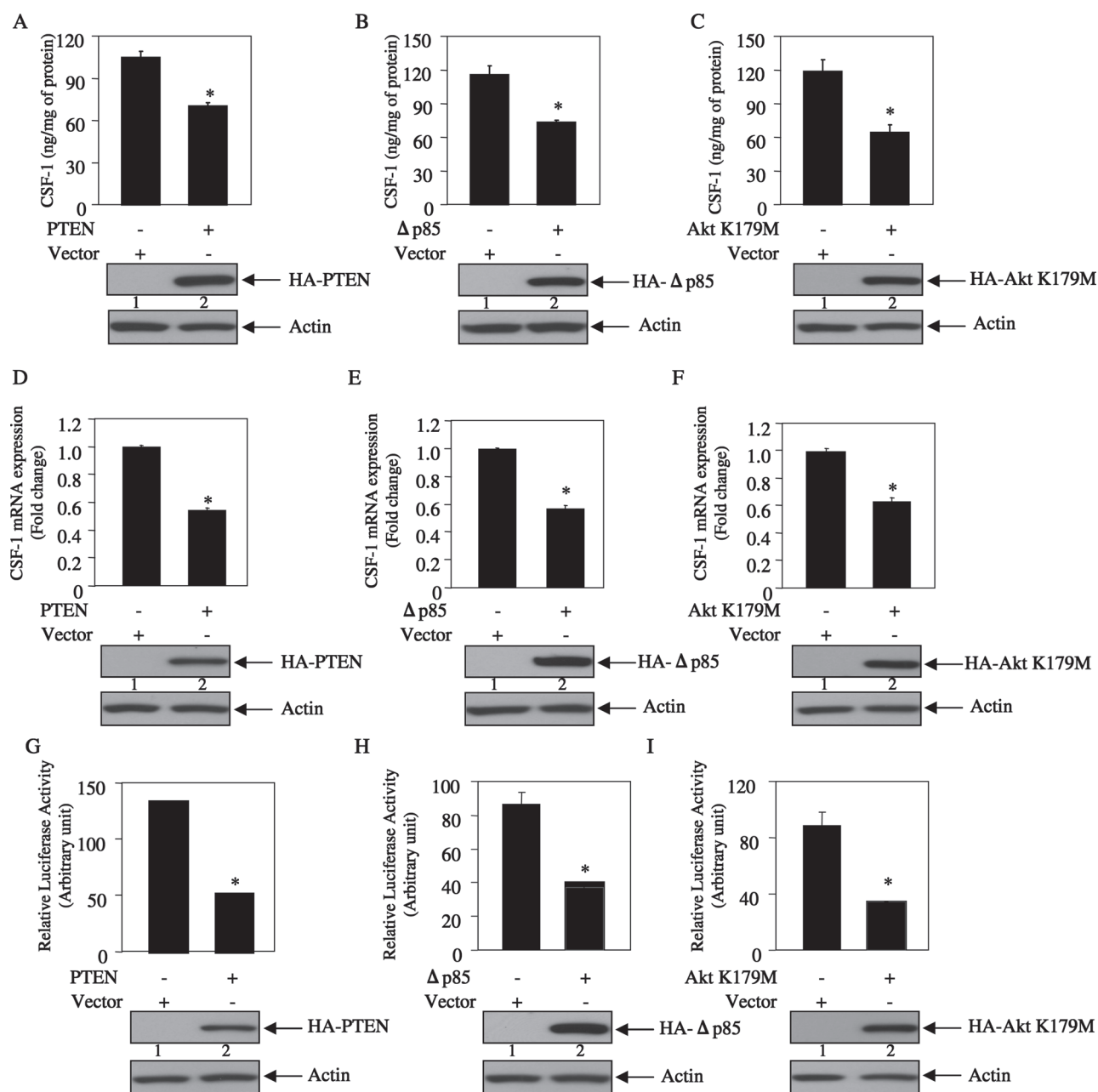


Fig. 2. PTEN regulates CSF-1 expression via PI 3 kinase/Akt signaling. (A and D) MDA-MB-231 breast cancer cells were transfected with vector alone or HA-tagged PTEN plasmids. The conditioned media from vector- and PTEN-transfected cells were used for ELISA to detect CSF-1 protein (panel A). Mean \pm SE of triplicate measurements is shown. * $P = 0.036$ versus control. Total RNAs from these cells were examined for CSF-1 mRNA by qRT-PCR as described in the Materials and methods (panel D). Mean \pm SE of triplicate measurements is shown. * $P = 0.007$ versus control. (B, C, E and F) MDA-MB-231 breast cancer cells were transfected with vector or dominant negative PI 3 kinase (HA-tagged Δ p85; panels B and E) or dominant negative Akt (HA-tagged K179M; panels C and F). The conditioned media from these cells were used for ELISA to detect CSF-1 protein (panels B and C) (29). Mean \pm SE of triplicate measurements is shown. * $P = 0.0042$ and 0.0128 versus control, respectively. Total RNAs from these cells were examined for CSF-1 mRNA by qRT-PCR as described in Materials and methods (panels E and F). Mean \pm SE of triplicate measurements is shown. * $P = 0.0019$ and 0.0263 versus control respectively. Bottom panels show expression of PTEN, Δ p85 and Akt K179M. PI 3 kinase/Akt signal transduction regulates CSF-1 transcription (panels G to I). MDA-MB-231 mammary cancer cells were transfected with CSF-1-Luc reporter and either PTEN (panel G) or Δ p85 (panel H) or Akt K179M (panel I). The cell lysates were assayed for luciferase activity as described in Materials and methods (23,24,29,30). Mean \pm SE of triplicate measurements is shown. * $P = 0.0012$ versus control in panel G; * $P = 0.0268$ versus control in panel H; * $P = 0.0203$ versus control in panel I. Bottom panels show expression of HA-tagged PTEN, Δ p85 and Akt K179M in representative samples.

significantly inhibited the expression of both pre-miR-21 and mature miR-21 in these cell lines (Figure 3A–D). Together these results demonstrate for the first time that active fish oil constituent, DHA, prevents the expression of miR-21 in breast tumor cells.

Since pre-miR-21 was regulated by DHA, we tested its effect on pri-miR-21 expression. Incubation of both MDA-MB-231 and MCF-7 breast cancer cells with DHA resulted in significant inhibition

of expression of pri-miR-21 (Figure 3E and 3F). These results suggest a transcriptional regulation of miR-21 expression. Recently, miR-21 has been shown to be regulated by the transcription factor NF κ B and the promoter of miR-21 contains NF κ B-binding element (26). To test this in breast cancer cells, we cotransfected the miR-21 promoter reporter plasmid (miR-21-Luc) with the p65 NF κ B subunit expression plasmid. The expression of p65 significantly increased the

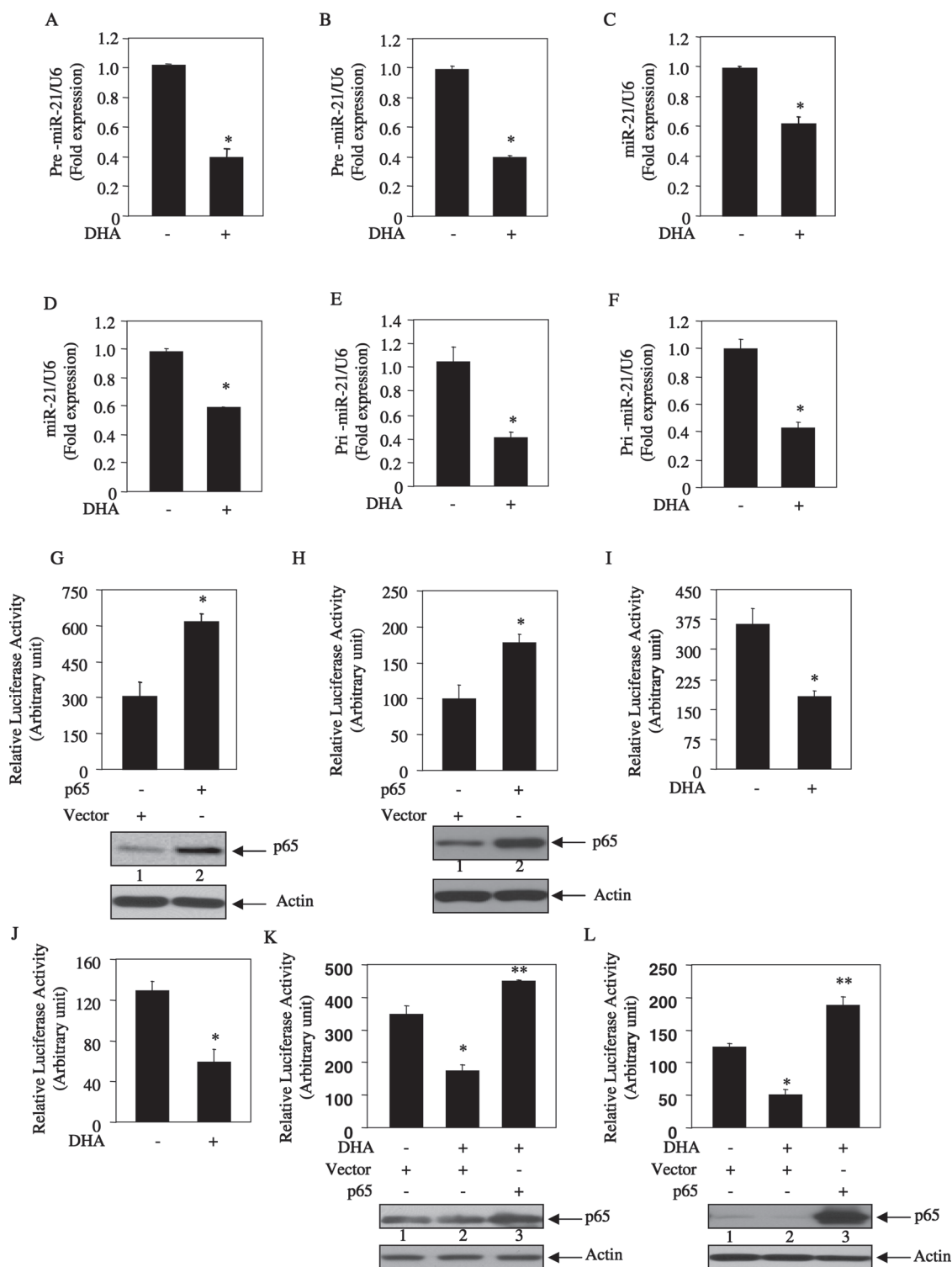


Fig. 3. DHA blocks miR-21 expression. MDA-MB-231 (A, C and E) and MCF-7 (B, D and F) breast cancer cells were incubated with 152nM DHA for 24h. Total RNAs were extracted and used in qRT-PCR to detect pre-miR-21 (panels A and B), mature miR-21 (panels C and D) and pri-miR-21 (panels E and F) as described in Materials and methods. Mean \pm SE of triplicate measurement is shown. * $P = 0.002$ and 0.003 versus control in panels A and B, respectively; * $P = 0.029$ and 0.0057 versus control in panels C and D, respectively; * $P = 0.018$ and 0.0034 versus control in panels E and F, respectively. NF κ B regulates DHA-induced inhibition of miR-21 transcription (panels G to L). (G and H) MDA-MB-231 (panel G) and MCF-7 (panel H) breast tumor cells were transfected with miR-21-Luc reporter plasmids along with p65 expression vector or vector alone. The cell lysates were used for luciferase activity, 48h post-transfection, as described in Materials and methods. Mean \pm SE of triplicate measurements is shown. * $P = 0.016$ and 0.011 versus vector in panels G and H, respectively. (I and J) MDA-MB-231 (panel I) and MCF-7 (panel J) breast tumor cells were transfected with miR-21-Luc reporter plasmid followed by incubation with 152nM DHA 24h post-transfection for additional 24h. Luciferase activity was determined in the cell lysates as described in Materials and methods. Mean \pm SE of triplicate measurements is shown. * $P = 0.049$ and 0.048 versus vector in panels I and J, respectively. (K and L) MDA-MB-231 (panel K) and MCF-7 (panel L) breast tumor cells were transfected with miR-21-Luc reporter plasmids along with p65 expression vector or vector alone. Transfected cells were incubated with 152nM DHA for 24h. The cell lysates were used for luciferase activity as described in Materials and methods. Mean \pm SE of triplicate measurements is shown. * $P < 0.01$ versus control; ** $P < 0.001$ versus DHA in panels K and L. Bottom parts in panels G, H, K and L show expression of p65 and actin by immunoblotting.

reporter activity, indicating that the regulation of miR-21 transcription in these cells is mediated at least partly by NF κ B (Figure 3G and 3H). Since we have demonstrated previously that the DHA inhibits activation of NF κ B in breast cancer cells (24,30), we tested whether DHA regulates transcription of miR-21. As shown in Figure 3I and 3J, DHA significantly inhibited the reporter activity in both MDA-MB-231 and MCF-7 cell lines. Next, we examined the role of NF κ B on DHA-mediated inhibition of miR-21 transcription. Expression of p65 in MDA-MB-231 and MCF-7 cells reversed the DHA-mediated suppression of miR-21-Luc reporter activity (Figure 3K and 3L). Together these results suggest that DHA inhibits miR-21 expression by inhibiting NF κ B activity in the breast cancer cells.

miR-21 regulates CSF-1 expression

Although protumorigenic action of miR-21 is known in breast cancer cells, its role in expression of CSF-1 has not been investigated. We tested the involvement of miR-21 in CSF-1 expression in MDA-MB-231 breast cancer cells. We used a plasmid vector expressing seven copies of bulged miR-21 recognition site placed in the 3'-UTR of GFP mRNA (Supplementary Figure S6A, available at *Carcinogenesis* Online). Expression of this cassette serves as a 'Sponge', which derepresses miR-21 targets (25,35). As expected, miR-21 sponge expression did not alter the expression of miR-21 in MDA-MB 231 cells (Supplementary Figure S6B, available at *Carcinogenesis* Online). The expression of GFP mRNA in the transfected cells serves as a surrogate for miR-21 sponge expression (Supplementary Figure S6B, lower panel, available at *Carcinogenesis* Online). We showed that miR-21 directly targets PTEN 3'-UTR and PTEN protein expression in MDA-MB-231 cells (Supplementary Figure S5, available at *Carcinogenesis* Online). miR-21 sponge expression in MDA-MB-231 cells increased PTEN 3'-UTR-Luc activity (Supplementary Figure S6C, available at *Carcinogenesis* Online) with concomitant increase in expression of miR-21 target protein PTEN (Supplementary Figure S6D, available at *Carcinogenesis* Online). These results indicate that expression of miR-21 sponge dampens endogenous miR-21 activity. MDA-MB-231 and MCF-7 cells were transfected with either miR-21 Sponge or empty vector and the conditioned medium was examined for CSF-1 protein by ELISA. The results show that expression of miR-21 Sponge significantly inhibited CSF-1 protein levels in the conditioned media of both MDA-MB-231 and MCF-7 breast tumor cells (Figure 4A and 4B). Detection of GFP mRNA (Figure 4A and 4B, bottom) served as the surrogate for the expression of miR-21 Sponge sequences (25). Next, we examined the effect of miR-21 Sponge on CSF-1 mRNA expression. Transfection of miR-21 Sponge significantly reduced the expression of CSF-1 mRNA in MDA-MB-231 and MCF-7 cells (Figure 4C and 4D). Since CSF-1 is regulated by transcriptional mechanism, we transfected CSF-1-Luc reporter with miR-21 Sponge or vector into both breast tumor cells. Expression of miR-21 Sponge significantly inhibited the reporter activity (Figure 4E and 4F). Together these results show that miR-21 regulates expression of CSF-1 in breast cancer cells.

miR-21 targets PTEN to regulate CSF-1 expression in breast cancer cells

We have shown above that PTEN and miR-21 independently regulate expression of CSF-1 (Figures 2 and 4). However, these results do not demonstrate whether miR-21-regulated PTEN mediates CSF-1 expression. To test this hypothesis, MDA-MB-231 and MCF-7 breast cancer cells were transfected with CMV-miR-21 alone or along with PTEN lacking its 3'-UTR. miR-21 increased expression of CSF-1 mRNA (Figure 5A and 5B, and Supplementary Figure S7A and B). PTEN without the 3'-UTR prevented the upregulation of CSF-1 mRNA induced by expression of miR-21 in both cells (Figure 5A and 5B, and Supplementary Figure S7A and B). These results indicated that miR-21 regulated expression of CSF-1 via PTEN. Many biological effects of PTEN are mediated by inhibition of Akt activity, as described above (Figure 2). We, therefore, used expression of dominant negative Akt to block endogenous Akt activity. Dominant negative Akt inhibited the increase in CSF-1 mRNA induced by miR-21

(Figure 5C and 5D, and Supplementary Figure S7C and D). We have described earlier that miR-21 regulates CSF-1 expression by transcriptional mechanism. We assessed the effect of 3'-UTR-deficient PTEN on the miR-21-induced transcription of CSF-1. MDA-MB-231 and MCF-7 cells were transfected with CSF-1-Luc and plasmid vector expressing PTEN without 3'-UTR. Analogous to the results obtained with CSF-1 mRNA, expression of PTEN attenuated miR-21-induced reporter activity (Figure 5E and 5F, and Supplementary Figure S7E and F). Similarly, expression of dominant negative Akt blocked the reporter activity in response to miR-21 in MDA-MB-231 and MCF-7 breast cancer cells (Figure 5G and 5H, and Supplementary Figure S7G and H). These results suggest that the action of miR-21 on expression of CSF-1 is mediated by PTEN.

DHA regulates CSF-1 expression through miR-21

We have shown earlier that DHA inhibits expression of miR-21 (Figure 3). This ω -3 fatty acid also attenuated expression of CSF-1 in the MDA-MB-231 and MCF-7 breast cancer cells (Figure 1). We tested the hypothesis that DHA acts through miR-21 to block CSF-1 expression. CMV-miR-21-transfected MDA-MB-231 and MCF-7 breast tumor cells were treated with DHA. As expected, DHA inhibited the secretion of CSF-1 in the conditioned media (Figure 6A and 6B). Expression of miR-21 reversed DHA-induced inhibition of CSF-1 protein secretion (Figure 6A and 6B, and Supplementary Figure S8A and B). Similarly, miR-21 reversed downregulation of CSF-1 mRNA produced by DHA treatment (Figure 6C and 6D, and Supplementary Figure S8C and D). Furthermore, expression of miR-21 abrogated decreased transcription of CSF-1 in response to this ω -3 fatty acid (Figure 6E and 6F, and Supplementary Figure S8E and F, available at *Carcinogenesis* Online). Importantly, the effect of DHA on increased expression of PTEN protein was inhibited by miR-21 in both MDA-MB-231 and MCF-7 breast cancer cells (Figure 6G and 6H, and Supplementary Figure S8G and H, available at *Carcinogenesis* Online). Together, our data demonstrate for the first time that DHA utilizes miR-21 as an intermediate signaling molecule to inhibit the expression of CSF-1 by transcriptional mechanism.

Fish oil attenuates CSF-1 expression in the breast tumors of mice

To investigate the expression of CSF-1 in breast cancer cells *in vivo*, we used a xenograft mouse model of human breast cancer (24). The mice were fed either lab chow or a diet containing 10% fish oil for 1 week prior to inoculation of MDA-MB-231 breast tumor cells. As we showed previously, the fish oil diet significantly prevented the tumor growth in mice (Supplementary Figure S9, available at *Carcinogenesis* Online) (24). We examined the levels of CSF-1 mRNA in tumor tissues. As shown in Figure 7A, tumor tissues isolated from mice maintained on fish oil-supplemented diet showed a significant reduction in CSF-1 mRNA expression compared with those isolated from control animals receiving the regular diet. We have shown above that miR-21 regulates expression of CSF-1 in breast cancer cells *in vitro*. We next tested the expression of miR-21 in the tumor samples. Tumor tissues from fish oil-fed animals showed significantly decreased expression of miR-21, pre-miR-21 and pri-miR-21 (Figure 7B-D). These results demonstrate that fish oil inhibits expression of miR-21, which blocks expression of CSF-1, an essential growth factor for tumor progression.

Discussion

The role of CSF-1 in breast cancer malignancy has been established (2). Due to its osteoclastogenic activity, CSF-1 also contributes significantly to the formation of osteolytic lesions by breast cancer cells metastasizing to bone (33). Our results for the first time show that part of the beneficial effects exerted by the fish oil diet on breast tumor malignancy and metastasis (22,24,30) may include its inhibitory effect on CSF-1 expression. Furthermore, we demonstrate that DHA, the bioactive constituent of fish oil, inhibits expression of miR-21 to increase PTEN protein levels, which in turn attenuates the expression of CSF-1. Our results establish a mechanism for the inhibition of CSF-1 expression by dietary supplementation of fish oil (Figure 7E).

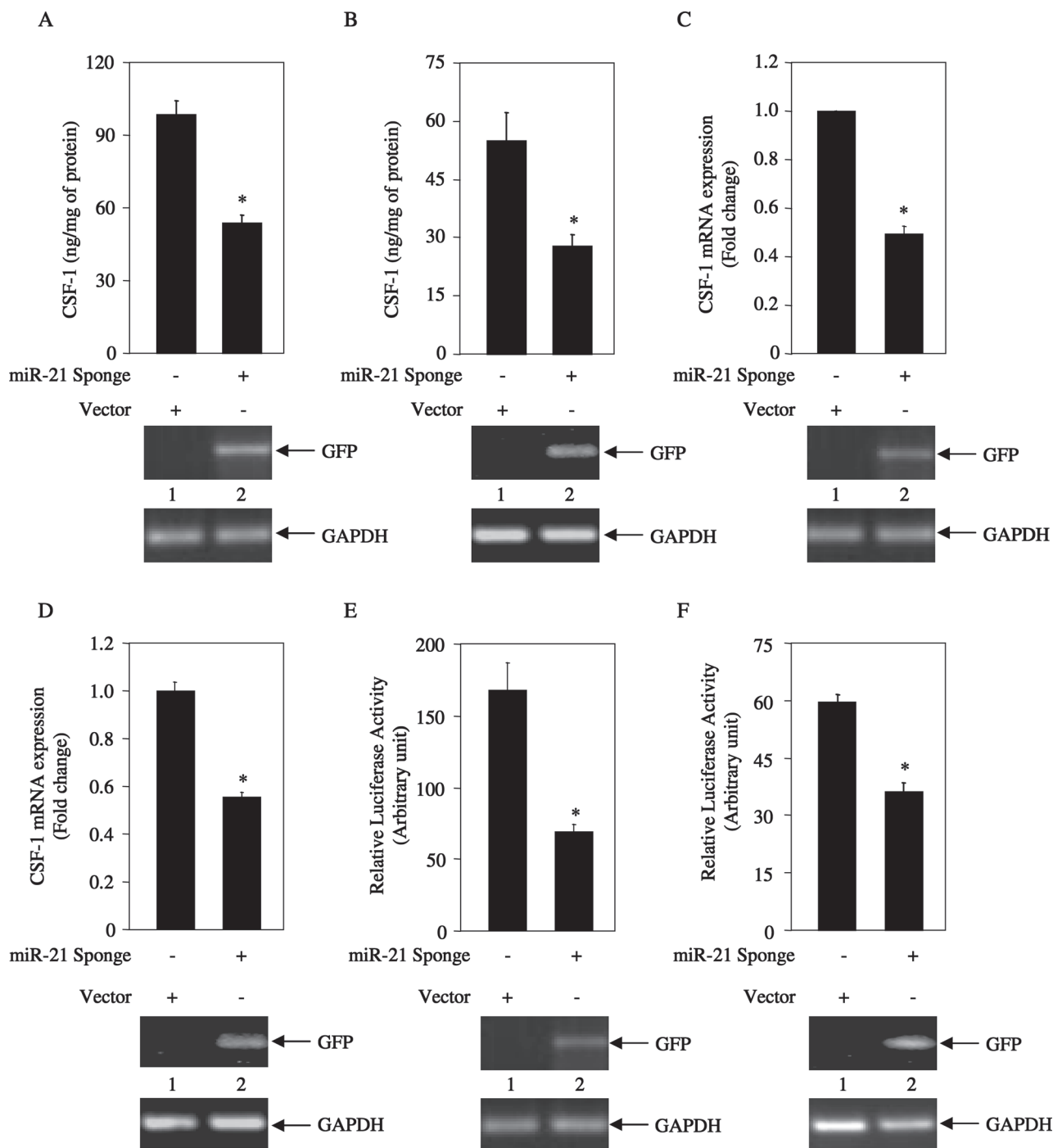


Fig. 4. miR-21 regulates CSF-1 expression. (A to D) MDA-MB-231 (A and C) and MCF-7 (B and D) breast cancer cells were transfected with miR-21 Sponge or vector alone (25). The conditioned media were tested for CSF-1 levels by ELISA as described (panels A and B) (29). Mean \pm SE of triplicate measurements is shown. * $P = 0.0229$ and 0.0146 versus control in panels A and B, respectively. (C and D) Total RNAs from MDA-MB-231 (panel C) and MCF-7 (panel D) cells were used in qRT-PCR for expression of CSF-1 mRNA as described in Materials and methods. Mean \pm SE of triplicate measurements is shown. * $P = 0.0023$ and 0.0417 versus control in panel C and D, respectively. (E and F) MDA-MB-231 (panel E) and MCF-7 (panel F) cells were transfected with CSF-1-Luc and vector or miR-21 Sponge plasmids. The cell lysates were assayed for luciferase activity as described in Materials and methods (23,24,29,30). Mean \pm SE of triplicate measurements is shown. * $P = 0.0165$ and 0.023 versus control in panel E and panel F, respectively. Bottom parts in all panels show the expression of GFP as surrogate for Sponge expression.

Poor prognosis for breast cancer is associated with increased expression of CSF-1 in the breast tumor tissues (36). In addition, high levels of CSF-1 in the breast cancer act as a chemoattractant for the monocytes to the microenvironment of the tumor to produce growth factors and cytokines, which stimulate growth of cells in the stroma and induce angiogenesis (37). Consequently, the breast cancer cells

acquire mutations, lose their positional identity and intravasate into the blood stream and metastasize. In fact, a role of CSF-1 in metastasis was established using a transgenic mammary gland-specific polyoma middle-T antigen mouse model in a background of CSF-1 deficiency (op/op). In this model, the malignancy of breast tumors and their metastasis to lung were significantly reduced (38). Due to lack

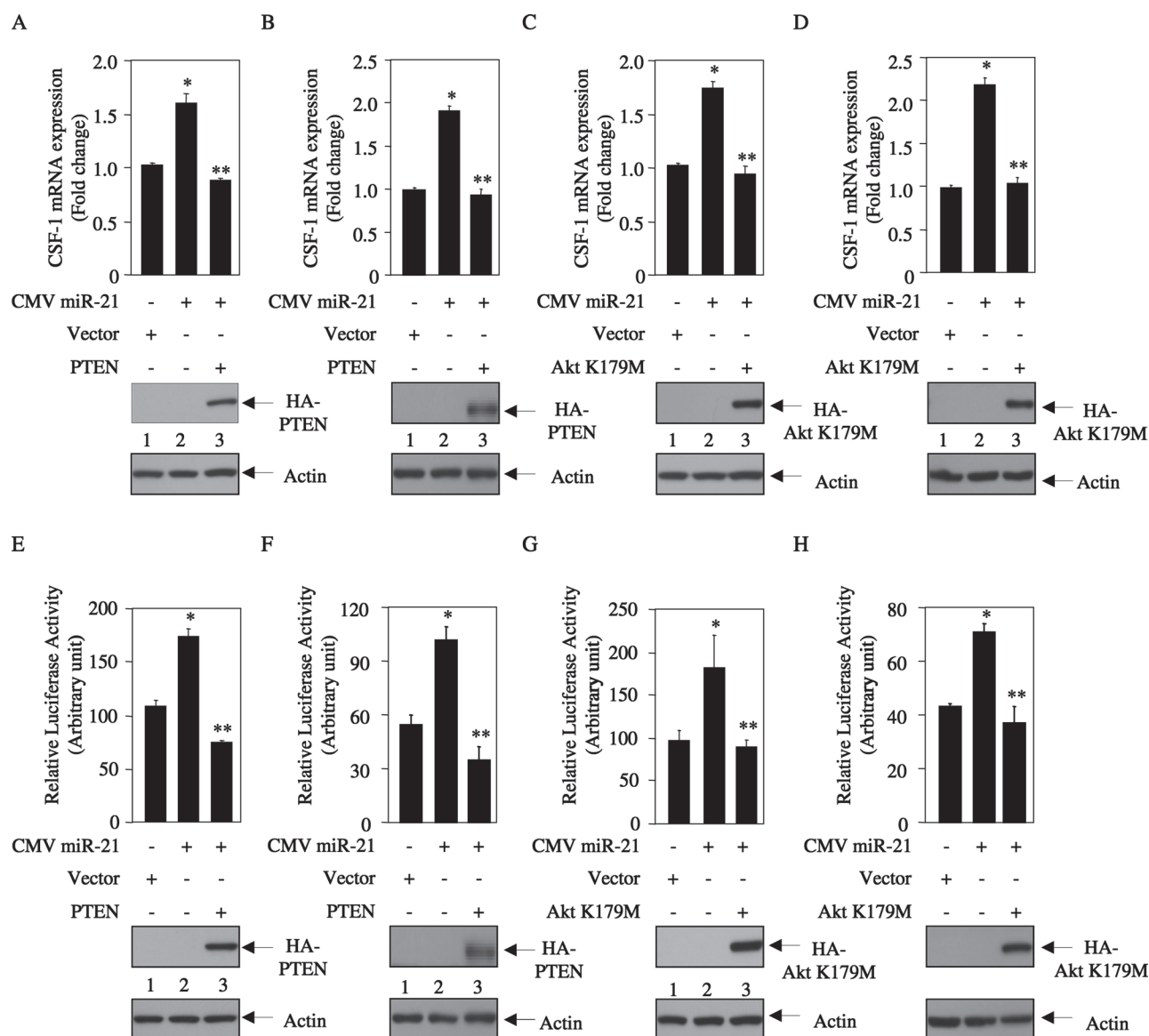


Fig. 5. 3'-UTR-deficient PTEN or dominant negative Akt prevents miR-21-induced CSF-1 expression. MDA-MB-231 (panels A and C) and MCF-7 (panels B and D) breast cancer cells were cotransfected with CMV-miR-21 and 3'-UTR-less PTEN (panels A and B) or Akt K179M (panels C and D). Total RNAs were assayed for the presence of CSF-1 mRNA by qRT-PCR as described in Materials and methods. Mean \pm SE of triplicate measurements is shown.

* $P < 0.001$ versus control in panels A to D; ** $P < 0.001$ versus CMV-miR-21-transfected for panels A to D. Bottom parts show PTEN and Akt K179M expression in cells transfected in parallel. (E to H) MDA-MB-231 (panels E and G) and MCF-7 (panels F and H) cells were transfected with CSF-1-Luc reporter along with 3'-UTR-less PTEN (panels E and F) or Akt K179M (panels G and H). The cell lysates were assayed for luciferase activity as described in Materials and methods (23,24,29,30). Mean \pm SE of triplicate measurements is shown. * $P < 0.01$ versus control; ** $P < 0.01$ versus CMV miR-21-transfected for panel E. * $P < 0.05$ versus control; ** $P < 0.01$ versus CMV miR-21-transfected for panel F. * $P < 0.05$ versus control; ** $P < 0.05$ versus CMV miR-21-transfected for panel G. * $P < 0.01$ versus control; ** $P < 0.01$ versus CMV miR-21-transfected for panel H. Bottom parts show HA-tagged PTEN and Akt K179M expression in cells transfected in parallel.

of CSF-1 in all tissues, this model did not demonstrate a requirement of exclusive expression of this growth and differentiation factor in the breast. To delineate the specific role of CSF-1 in the malignancy of mammary tumorigenesis, the expression of CSF-1 was specifically restored in the Op/Op MMTV-driven middle-T antigen transgenic mouse. Consequently, in this model, breast cancer progressed and metastasized to lung (38). Thus, CSF-1 acts as an organ-autonomous growth factor in promoting breast cancer and metastasis. Therefore, identification of agents that reduce expression of CSF-1 in breast cancer cells is of potentially great therapeutic importance. Our results provide the first evidence that the active ω -3 fatty acid, DHA, present in fish oil significantly blocks the expression and secretion of CSF-1 in the ER-positive and -negative human breast tumor cells (Figure 1).

Furthermore, we demonstrate that a diet containing 10% fish oil decreases the levels of CSF-1 in breast tumor tissues (Figure 7).

Activation of class IA PI 3 kinase controls the proliferation and survival of cancer cells, including breast tumor (39). Mutations in the coding sequence of the catalytic subunits of this enzyme, p110 α (*PIK3CA*) and p110 β (*PIK3CB*), have been identified in breast cancer (40–42). The lipid second messenger PIP₃ produced by the activated PI 3 kinase binds to the PH domain of the Akt kinase, resulting in its translocation to the plasma membrane, where it undergoes dual activating phosphorylation. Thus, Akt kinase carries out the proliferative and prosurvival functions of PI 3 kinase (34). In the absence of activated PI 3 kinase, the level of PIP₃ is under the control of another enzyme, the tumor suppressor protein PTEN (34). PTEN

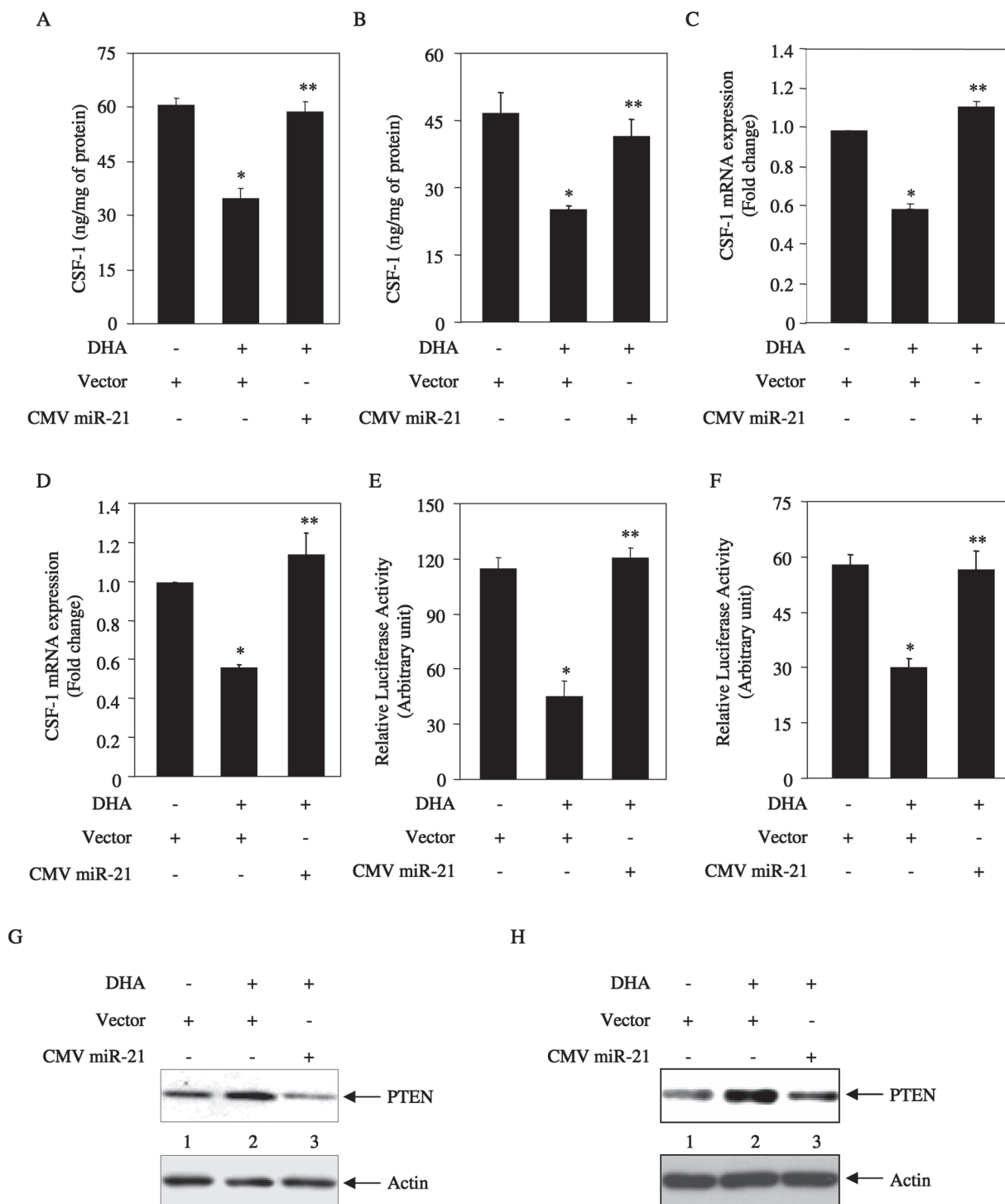


Fig. 6. miR-21 attenuates the downregulation of CSF-1 in response to DHA. MDA-MB-231 (panels A, C, E and G) and MCF-7 (panels B, D, F and H) cells were transfected with CMV-miR-21 or vector. The transfected cells were incubated with 152 nM DHA. The conditioned media (panels A and B) were tested for CSF-1 protein levels by ELISA (29). Mean \pm SE of triplicate measurements is shown. * P < 0.05 versus control; ** P < 0.05 versus DHA-treated only in panel A. * P < 0.01 versus control; ** P < 0.05 versus DHA-treated in panel B. For panels C and D, total RNAs from the cells were examined for CSF-1 mRNA expression by real-time qRT-PCR as described in Materials and methods. Mean \pm SE of triplicate measurements is shown. * P < 0.001 versus control; ** P < 0.001 versus DHA alone in panel C. * P < 0.5 versus control; ** P < 0.01 versus DHA alone in panel D. In panels E and F, MDA-MB-231 (panel E) and MCF-7 (panel F) cells were cotransfected with CSF-1-Luc reporter plasmid and CMV-miR-21. The transfected cells were incubated with 152 nM DHA. The cell lysates were assayed for luciferase activity as described in Materials and methods (23,24,29,30). Mean \pm SE of triplicate measurements is shown. * P < 0.01 versus control; ** P < 0.01 versus DHA alone in panel E. * P < 0.05 versus control; ** P < 0.05 versus DHA alone in panel F. (G and H) Equal amounts of protein from miR-21-transfected and DHA treated MDA-MB-231 (panel G) and MCF-7 (panel H) cells were immunoblotted with PTEN and actin antibodies as indicated.

dephosphorylates the D3 position of PIP₃ to produce PIP₂, thus inhibiting activation of Akt. High frequency of PTEN mutation has been identified in various cancers, including breast tumor (43). Germline

PTEN mutation in the women displays early malignancy in breast (43). Women carrying high risk for breast cancer with BRCA1 mutation commonly lose PTEN to elicit breast tumorigenesis (44). PTEN

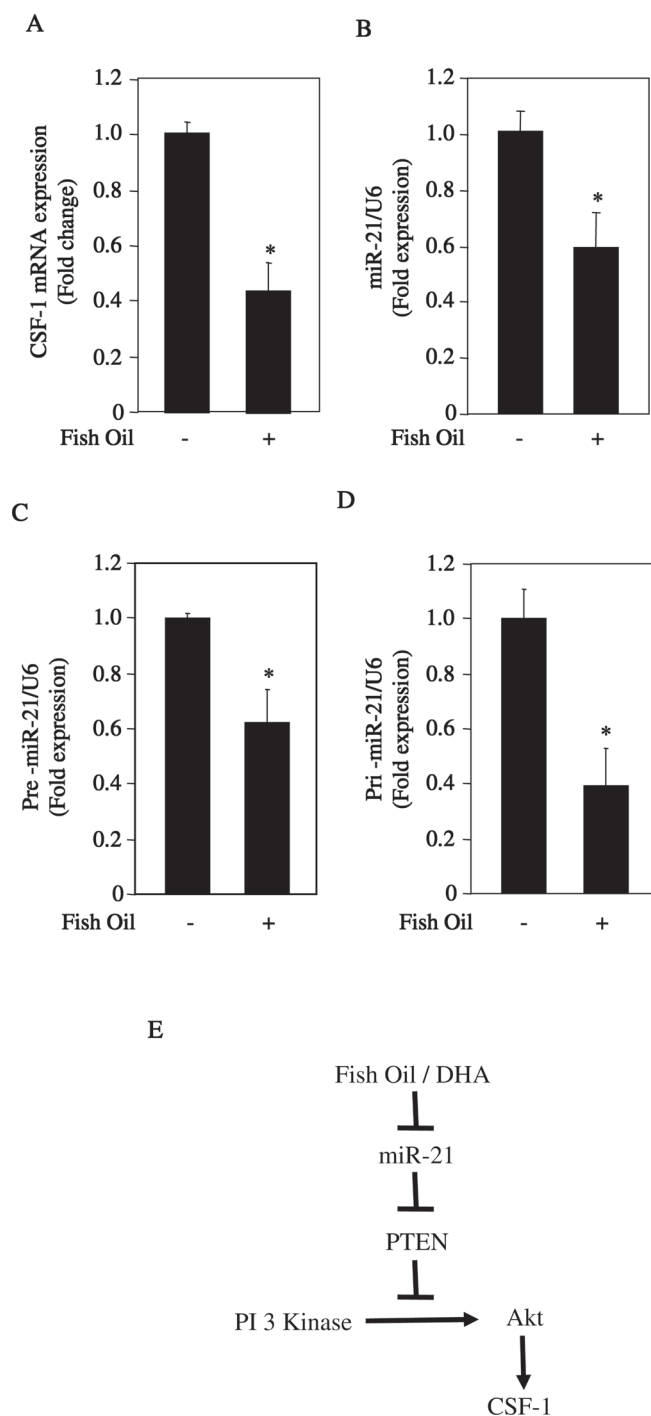


Fig. 7. Fish oil diet prevents expression of CSF-1 and miR-21 in tumors of mice. Mice were fed lab chow or a diet containing 10% fish oil for 1 week prior to injection of 10^6 MDA-MB-231 human breast cancer cells to the mammary fat pad. The mice were maintained in the same diet. The tumors were excised at 3 weeks and tumor lysates were used to prepare total RNAs (24). These RNAs were examined for CSF-1 mRNA expression by real-time qRT-PCR as described in Materials and methods (panel A) (29). The total RNAs were used in real-time qRT-PCR to detect mature miR-21 (panel B), pre-miR-21 (panel C) and pri-miR-21 (panel D). Mean \pm SE of triplicate measurements is shown. * $P = 0.0026, 0.0325, 0.0161$ and 0.0194 versus control in A, B, C and D, respectively. (E) Cartoon summarizing our results.

heterozygous mice develop breast cancer (45). Furthermore, targeted deletion of PTEN in mouse mammary gland resulted in early tumorigenesis, suggesting a significant role of this tumor suppressor protein in breast cancer development (46). Therefore, strategies that would

increase the levels of PTEN in the breast cancer cells may be beneficial. We have shown previously that DHA increases PTEN expression in MDA-MB-231 breast cancer cells and that fish oil diet increases the levels of PTEN in the xenografts, resulting in a reduction in tumor development (24). In the present study, we demonstrate that PTEN reduces the expression and secretion of CSF-1 in both MDA-MB-231 and ER-positive MCF-7 breast cancer cells (Figure 2A, 2D and 2G, and Supplementary Figures S3A, D and S4A, available at *Carcinogenesis* Online). Additionally, we show that this effect of PTEN is mediated by PI 3 kinase/Akt signaling pathway (Figure 2, and Supplementary Figures S3 and S4, available at *Carcinogenesis* Online).

Although only 5% of sporadic breast tumors show mutations in the PTEN gene, nearly 40% of the tumors show reduced levels of PTEN protein and its activity (47). Apart from its downregulation due to transcriptional regulation, PTEN levels and activity are regulated by phosphorylation, oxidation, ubiquitination and protein-protein interaction (48). An additional layer of post-transcriptional regulation of PTEN expression involves specific miRNAs. Thus, multiple PTEN-targeting miRNAs originate from single hairpin structure, such as miR-19a, miR-22, miR-26a, miR-205, miR-22 and miR-21 (49). Similarly, polycistronic miRNAs, such as miR-216a/miR-217, miR-17-92, miR367-302b, miR-221-222 and miR-106b-25, generated from gene clusters also target PTEN to decrease its expression (49). Among these miRNAs, the role of miR-21 has been extensively investigated in many cancers, including breast tumor, where expression of this miRNA has been associated with poor prognosis (50). Doxorubicin-resistant breast cancer cells express elevated levels of miR-21 with concomitant reduction in PTEN protein, indicating a link between chemoresistance and miR-21 (51). Similarly, breast cancer cells resistant to trastuzumab therapy express enhanced levels of miR-21 (52). Consequently, these cells display decreased PTEN protein. An approach to attenuate miR-21 expression may be helpful in achieving antiproliferative and drug sensitivity of breast cancer cells. In fact, anti-miR-21 therapy has been proposed for breast cancer. We demonstrate that DHA, an active component of fish oil, blocks expression of miR-21 in both ER-deficient MDA-MB-231 and ER-positive MCF-7 breast cancer cells while not significantly changing the expression of miR-21 in normal breast epithelial cells MCF10A (Figure 3, and Supplementary Figure S10, available at *Carcinogenesis* Online). Interestingly, activation of ER has been shown to positively as well as negatively regulate expression of miR-21 (53,54). Furthermore, DHA induced degradation of ER α in the presence of estradiol (55). Therefore, our results showing inhibition of miR-21 expression by DHA in MCF-7 cells may have resulted from downregulation of ER. However, no effect of DHA on expression of ER was observed in MCF-7 cells in the absence of estradiol (Supplementary Figure S11, available at *Carcinogenesis* Online). Importantly, tumors from animals maintained on a fish oil diet showed significantly diminished levels of miR-21 (Figure 7B and 7C). These results for the first time demonstrate beneficial targeting of miR-21 in breast cancer by the non-toxic fish oil supplement.

The specificity of breast cancer cells to metastasize in an organ-specific manner is determined by their infiltrative and colonization functions. Expression of genes, which increase invasion of tumor cells into the stroma, attracts and stimulates growth and differentiation of precursor cells from blood to produce mature macrophages. These tumor cells continue to express genes for recruitment of macrophages when they invade distant organs. The expression of CSF-1 in the primary breast tumor represents one such gene, which contributes to initiation of metastasis (2,38). In fact, there are examples of genes that are prominently expressed in the primary tumor that carry out unique functions at the distant organ. The expression of these metastasis virulence genes may increase the metastatic proclivity of the tumor cells to invade specific organs. For example, breast cancer cells that have metastasized to bone express and secrete CSF-1. Although it primarily acts as a growth and differentiation factor for monocyte/macrophage lineage, it contributes to the differentiation, proliferation and maturation of myeloid precursor cells to osteoclasts.

Thus, CSF-1 secreted by the colonizing breast cancer cells in the bone stimulates increased formation of multinucleated osteoclasts that play an important role in the formation of osteolytic lesions. Recently, we have shown in a mouse model that fish oil diet prevents formation of osteolytic lesions induced by the metastasized MDA-MB-231 human breast cancer cells to bone (22). Our current results now provide a new mechanism by which fish oil inhibits the expression of CSF-1 in the breast tumors to attenuate osteoclastogenesis and thus osteolytic lesion formation (Figure 7A). Also, we show that treatment of MDA-MB-231 cells with DHA results in inhibition of endogenous CSF-1 expression (Figure 1A–C). Enhanced expression of miR-21 positively correlates with chemoresistance and antibody therapy resistance in breast cancer cells that often become highly proliferative and metastatic. We demonstrate a positive correlation between expressions of CSF-1 and miR-21 in the human breast cancer cells. Furthermore, we show that miR-21 acts as an upstream regulator of CSF-1 expression in these cells (Figure 4).

PTEN is a haploinsufficient tumor suppressor gene (34). Mouse with PTEN hypomorphic allele with 70–80% decrease in PTEN levels result in prostate cancer with 100% penetrance (56). On the other hand, a PTEN ‘hyper’ mouse was generated to have a hypomorphic allele over a wild-type background, which expressed PTEN mRNA and protein at 80% of the wild-type levels (57). These mice did not show any tumor in the prostate epithelium; however, they predominantly display mammary tumor and showed a gene expression profile of cancer cell proliferation. These results suggest a tissue-specific sensitivity for the levels of PTEN to exhibit tumorigenesis. Only a 20% decrease in PTEN protein sensitizes the animal to develop breast cancer. Similar to these observations in mice, patients with invasive breast cancer showed a significant reduction in PTEN mRNA expression (57). In 20% of these patients, the PTEN levels were found to be slightly lower than the levels present in normal breast tissues with the similar gene signature expression found in the ‘hyper’ mice with only 20% decrease in PTEN. These results reinforce the importance of PTEN levels in sensitizing the mammary tissues to undergo tumorigenesis. Thus in the absence of PTEN mutation, subtle changes in PTEN expression due to post-transcriptional regulation by miRNA may also contribute to the expression of genes, which contribute to breast cancer malignancy. We found that CSF-1, which has been shown to regulate invasion of breast cancer, is regulated by PTEN (Figure 2, and Supplementary Figures S3 and S4, available at *Carcinogenesis* Online). Furthermore, we show that miR-21, which finetunes the expression levels of PTEN protein, also regulates the expression of CSF-1 (Figure 4). Our results provide direct evidence that this effect of miR-21 is indeed mediated by PTEN and its downstream signaling kinase Akt (Figure 5). Importantly, our data demonstrate that miR-21 prevents the DHA-induced downregulation of CSF-1 expression in breast cancer cells (Figure 6). On the basis of these results, we propose a signaling pathway for fish oil action on CSF-1 expression in breast cancer cells involving miR-21 that regulates PTEN/PI 3 kinase/Akt axis (Figure 7E).

Supplementary material

Supplementary Figures S1–S11 can be found at <http://carcin.oxfordjournals.org/>.

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Conflict of Interest Statement: None declared.

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