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ORIGINAL ARTICLE

Docosahexaenoic acid supresses breast cancer cell proliferation and migration by promoting the expression of miR-99a and targeting mTOR signaling

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Abstract Docosahexaenoic acid (DHA) shows different anti-cancer effects on breast cancer (BC) cell proliferation and progression; however, the underlying molecular mechanism yet still is blanketed in mystery. Herein, we aimed to reveal whether the inhibitory effects of DHA on BC proliferation and migration are exerted, at least in part, through promoting the expression of miR-99a and targeting mTOR signaling. DHA lessened the BC cell viability in a time- and concentration-dependent manner. Besides, DHA-treatment significantly suppressed the proliferation and migration, while promoted BC cell apoptosis by regulating Bax and Bcl-2. We also demonstrated that DHA activated caspase-3/7 in MDA-MB-231 BCE cells. Also, we determined that miR-99a was upregulated in DHA-treated cells and mTOR was a direct and functional target of this miRNA, verified by the ability of anti-miR-99a to rescue the suppressive effects of DHA on mTOR expression in BC cells. Furthermore, DHA was shown to inhibit the mTOR-HIF-1α-VEGF signaling via regulating miR-99a in BC cells. DHA treatment caused a significant dose-dependent reduction of VEGF secretion from BC cells. When miR-99a was knocked down, DHA did not inhibit the BC proliferation and migration. We concluded that the anti-cancer effects of DHA can be attributed to the up-regulation of miR-99a that *ipso facto* inhibit the mTOR-HIF-1α-VEGF axis in BC cells. It is thought that DHA treatment might be considered as a promising supplement for BC therapy.

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1. Introduction

As the most common cancer affecting females, breast cancer (BC) is responsible for ~12% of the total of all cancers (Ghaffari-Makhmalbaf et al., 2020; Tahmouresi et al., 2020; Sanchez-Morillo et al., 2018). BC is notorious due to the high

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ability of metastasis to different organs to touch upon lung, brain, and bones (Jin et al., 2018). This cancer is heterogeneously identified according to the status of hormone receptors and human epidermal growth factor receptor 2 (HER-2) expression (Jin et al., 2018). With a more aggressive with de facto poor prognosis nature, triple-negative BC (TNBC) expresses none of these three markers (Tsang and Tse, 2020).

The role of dietary omega-6 and omega −3 polyunsaturated fatty acids (PUFAs), as essential nutrients, in BC pathology remains to be further clarified. However, mounting *in vitro* and *in vivo* studies have shown that high levels of omega-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have repressing effects on the development and progression of BC, compared to omega-6 PUFAs(de Lorgeril and Salen, 2012). Consistently, overconsumption of omega-6 PUFAs and an increased ratio of omega-6 to omega-3 PUFAs, which are common in Western diets, are associated with a variety of malignancies, including BC. Importantly, it was reported that omega-3 fatty acids from fish origins displayed cancer-related preventive activity, with a 14% reduction in the risk of developing BC. In this regard, preclinical studies have demonstrated the protective activity of omega-3 PUFAs in BC development and the correlation between omega-6 PUFAs and BC tumorigenesis. This controversy might be explained by different effects of PUFAs on the regulation of non-coding RNAs and their related signaling pathways (Zanoaga et al., 2018; Freitas and Campos, 2019).

DHA is remarkably beneficial to inhibit the carcinogenesis of mammary glands (Mouradian et al., 2015; Rahman et al., 2013; Liu and Ma, 2014); however, there is a snippet of information considering the underlying molecular mechanisms in BC development. The angiogenesis process in solid tumors is a *sine qua non* of tumor development which is then considered as a mark whereby the tumor steps forward from being benign toward malignancy (Fox et al., 2007); DHA can inhibit different angiogenic mediators such as vascular endothelial growth factor (VEGF) (Matesanz et al., 2010) and exert its anti-angiogenic properties that in turn leads to the anti-cancer characteristic (Yun et al., 2016). The up-regulated VEGF can *per se* pave the way to BC invasiveness and metastasis (Wang et al., 2012).

According to Dong et al., hypoxia-inducible factor-1α (HIF-1α)-VEGF axis plays a crucial pro-angiogenic role under hypoxic conditions (Dong et al., 2011). This axis also functions in BC to induce angiogenesis (Dewangan et al., 2019). Hypoxia and HIF-1α cut both ways because they take a center stage in regulating angiogenesis and also play roles in invasion and metastasis of different types of cancers (Vaupel and Multhoff, 2020; Peng et al., 2018). By binding to the hypoxia response element (HRE) within the promoter of *VEGF*, HIF-1α predominantly induces both normal and pathophysiological angiogenesis (Hoeben et al., 2004; Forsythe et al., 1996). HIF-1α-mediated *VEGF* expression can regulate the mammalian target of rapamycin (mTOR) in BC cells (Del Bufalo et al., 2006). Consistently, it has been shown that tumors with up-regulated mTOR signaling show a high degree of angiogenesis (Land and Tee 2007).

MicroRNAs—also known as miRNAs—are a well-known class of non-coding RNAs (~22 nt in length) that are endogenously expressed in the cell nucleus and control gene expression at the level of post-transcription by suppressing translation and/or clearing the way for mRNA degradation (Razmara et al., 2019). Undeniably, miRNAs control a wealth of biological processes embracing proliferation, differentiation, and apoptosis in different cells (Annese et al., 2020). Aberrant miRNA expression has been contributed to tumorigenesis of different cancers, suggesting they may function as both oncogenes or tumor suppressors (Poursheikhani et al., 2020; Maminezhad et al., 2020). According to Aslan *et al.*, DHA treatment downregulates some oncogenic miRNAs (e.g. miR-21 and miR382), while it upregulates different candidate miRNAs with tumor suppressive activity such as miR-101, miR-199, and miR-342 (Aslan et al., 2020).

Inspecting different functions of miRNAs, we hypothesized whether the anti-cancer effects of DHA can be attributed to miRNA-regulation of oncogenic pathways such as mTOR signaling or not. Since miR-99a has been previously identified to have a substantial role in mTOR regulation (Yin et al., 2018; Tsai et al., 2018; Hu, Zhu, and Tang, 2014), we conducted an investigation into this whether the suppressive roles of DHA on BC cell proliferation and migration are due to the up-regulation of miR-99a. This information can increase our understanding of BC progression and may also pave ways to use DHA in combination with other novel therapeutic strategies against BC.

2. Materials and methods

2.1. Cell culture and chemicals

We used MDA-MB-231 and BT-20 as the human triple-negative BC cell models and MCF10A— a human non-tumorigenic breast epithelial cell line. The cells were maintained in DMEM containing 10% FBS and 1% penicillin–streptomycin (Trace Scientific Ltd., Melbourne, VIC, Australia). To prepare the DHA stock solution (Sigma-Aldrich, USA), DHA with a purity of 99% was dissolved in ethanol (30 mM) and immediately kept at −80 °C for further applications. In order to prepare the optimized solution (5 mM), we diluted DHA by phosphate-buffered saline (PBS) containing 1.5 mM bovine serum albumin (BSA).

2.2. Cell viability and proliferation assay

In order to evaluate the cell viability, a tetrazolium-based colorimetric MTT assay was exploited. To this end, around 4×10^3 cells were incubated with different concentrations of DHA (10, 25, 50, 100, and 150 μM) and the MTT assay was performed at the time point of 24 and 48 h after DHA treatment; subsequently, we measured the absorbance at 450 nm. Besides, the viable cells were stained with trypan blue and counted using a microscope at 12 and 24 h after DHA treatment.

2.3. RNA extraction and quantitative real-time PCR

RNA was isolated from the cells using TRIzol® reagent (Invitrogen, Mulgrave, VIC, Australia). To cut down on the possible DNA contaminations, the RNA samples were incubated with RNase-Free DNase. The RNA purity and concentration were determined by optical density measurements using a Nanodrop™ 2000c (Thermo Fisher Scientific, Rockford, IL,

USA). Approximately, 1 μg of extracted RNA was subjected to turn into complementary DNA (cDNA) using the Prime-Script RT Master mix (TAKARA). The quantitative real-time PCR (RT-qPCR) was exploited to perform the expression analysis on an ABI 7900 System (Applied Biosystems, CA, USA). So as to evaluate the expression level of miR-99a, RNA was polyadenylated and reverse transcription was done using High-specificity miRNA first-strand cDNA synthesis kit (Agilent Technologies, CA, USA); the expression of miR-99a was measured by miR-99a-specific primer and miRNA qPCR master mix kit (Sratagene, LaJolla, CA, USA). The relative expressions of mRNA transcripts and miR-99a were compared to GAPDH and *U48 small nuclear RNA* (*U48 snRNA*), respectively. To calculate the fold change, the $2^{-\Delta\Delta Ct}$ method was applied (Livak and Schmittgen, 2001).

2.4. Luciferase assay

In order to construct a reporter vector, the wild-type (WT) 3′-untranslated region (3′-UTR) sequence of mTOR was amplyfied and cloned into the psi-CHECK2 luciferase reporter vector. We co-transfected MDA-MB-231 cells with mTOR-3′-UTR luciferase reporter vector and 50 nM of hsa-miR-99a precursor molecule (miR-155 mimics) to evaluate whether miR-99a interacts with 3′-UTR of *mTOR* or not. After 48 h, the dual-luciferase assay was done using DualGlo luciferase assay (Promega) which included two specific reporters, 'firefly' and 'renilla' luciferases. The ratio of firefly luciferase to renilla one (as an internal control) was measured and normalized relative to the cells that were transfected only by the mTOR-3′-UTR luciferase reporter vector.

2.5. Short-interference RNA was used to knock down mTOR

MDA-MB-231 BCE cells were maintained in a medium without antibiotics and then were transfected with 50 nmol/L of synthetic short-interference RNAs (siRNAs) against mTOR (si-mTOR) (sc-35409; Santa Cruz Biotechnology, CA, USA) or silencing negative control (si-control) (sc-37007; Santa Cruz Biotechnology, CA, USA) using Lipofectamine RNAiMAX (Invitrogen) as per manufacturer's recommendation.

2.6. Immunoblotting analysis

The Immunoblotting analysis for the candidate proteins was performed. In order to perform cell lysis, we used radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Clayton, VIC, Australia) that in turn contained a mixture of different protease and phosphatase suppressors. We performed protein electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide and transferred the putative proteins to the PVDF membrane (BD Biosciences). The membranes were incubated with primary antibodies (1:1000) at 4 °C overnight; washed by 1 × Tris-buffered saline (TBS) with 0.1% Tween-20; and incubated with horseradish peroxidase-conjugated secondary antibody. The protein bands were detected using an enhanced chemiluminescence kit (ECL, Amersham, Buckinghamshire, UK) and the band intensities were analyzed using ImageJ.

2.7. Enzyme-linked immunosorbent assay

The VEGF concentration was evaluated in the supernatant of BC cells that were previously treated using different DHA concentrations (i.e. 10, 50, and 100 μM) for 48 h. This evaluation was carried out using a human VEGF enzyme-linked immunosorbent assay (ELISA) Kit (ab100662, Abcam, Cambridge, USA). The optical density values were measured at 450 nm and the amount of the putative protein were evaluated. The cells without DHA stimulation were used as the control group.

2.8. Elevation of caspase-3/7 relative activities

The enzyme activity of caspase-3/7 was measured via Caspase-Glo 3/7 assay kit (Promega). To this end, the cells were incubated with the caspase substrate at 23 °C for 1 h, and the luminescence was assessed according to the instructions' manual.

2.9. Wound healing assay

To assess the BC cell motility, the serum-starved cells were grown to 80% confluency. Afterward, we used a 200 ml pipette tip to scratch on the cells as single vertical scratches. To remove debris or detached cells, the monolayer was washed once with PBS. MDA-MB-231 BCE cells were stimulated with 25 μM DHA and incubated at 37 °C for 24 h. We monitored and quantified the wound healing in duplicate wells at 24 h.

2.10. Transwell assay

So as to show the cell migration of BC cells, we carried out the transwell assay based on the previous study (Ghaffari-Makhmalbaf et al., 2020). Briefly, to check the cell migratory capacity after DHA treatment, DHA-incubated cells were suspended in serum-free medium. We added the cell suspension into the upper chamber of the 24-well plates, while for the lower chamber, around 600 μL complete medium was added into. Using crystal violet, we stained the transferred cells and counted them directly after incubation at 37 °C for 24 h. Light microscopy was used to observe and photograph the cells and also to count the cells to reflect cell mobility.

2.11. Statistical analysis

Data were depicted as mean ± standard deviation (SD) of at least three independent assay. We utilized unpaired Student's *t*-test and ANOVA to evaluate the significance of the difference between two or multiple groups, respectively. We set the level of statistical significance to a P-value less than 0.05.

3. Results

3.1. DHA decreases BC cell viability

We investigated whether DHA can induce cytotoxic effects on BC cells or not. To answer, MDA-MB-231 and BT-20 cells were incubated with various DHA concentrations including 10, 25, 50, 100, and 150 μM, followed by performing MTT assay so as to evaluate BC cell viability. We determined that

at 100 μM concentration, DHA decreased 64.22 and 42.32% of MDA-MB-231 cell viability at 24 and 48 h after treatment, respectively. Also, following 24 and 48 h treatment, the viability of BT-20 cells treated with this same concentration of DHA was estimated at 58.03 and 47.48%, respectively. The same concentrations of DHA did not also cause significantly altered viability of the normal human breast epithelial cell line MCF10A. Accordingly, the concentration of DHA at 50 μM was used for further experiments. In a nutshell, the findings underscore that DHA significantly decreases the BC cell viability in a concentration and time-dependent manner (Fig. 1A–D).

3.2. DHA promotes apoptosis in BC cells

To show whether DHA-treatment can promote apoptosis in BC cells, we carried out the caspase-3/7 activity assay by means of a luminescence-based assay. We demonstrated that treating MDA-MB-231 BCE cells with 50 μM of DHA caused a significant augment of caspase-3/7 activity than the control group ($P < 0.0001$; Fig. 2A). To better understand the DHA apoptotic effects, we assessed the protein levels of Bax and Bcl-2 in the DHA-stimulated MDA-MB-231 BCE cells (Fig. 2B). Interestingly, the results revealed that the ratio of Bcl-2 to Bax was considerably lowered in DHA-stimulated MDA-MB-231 cells ($P < 0.001$, Fig. 2C), indicating the apoptotic effects of DHA on BC cells.

3.3. miR-99a functionally targets and suppresses mTOR

To verify whether mTOR can be directly targeted by miR-99a in BC cells or not, we constructed luciferase reporter plasmids that contained the renilla luciferase encoding gene fused to either the 3′-UTR of mTOR that contained miR-99a-5p binding site (WT) or mutated targeting site (Mut). We found that miR-99a specifically suppressed the luciferase activity of a reporter that included the 3′UTR of WT mTOR in BC cells (Fig. 3A, B). This suppression was particular to the predicted miR-99a target sites, as no significant change was detected in the relative luciferase activity of the Mut 3′UTR mTOR reporter. These findings fruitfully highlighted that miR-99a is capable of binding to the specific target sites that were located within 3′-UTR of mTOR mRNA.

To answer this question that how miR-99a affects the mTOR expression level, we transfected MDA-MB-231 BCE cells with either miR-99a mimics or scramble; thence, the mRNA levels of mTOR were analyzed immediately. Compatible with the data of the reporter assay, by the time miR-99 mimics were transfected into BC cells, mTOR expression were considerably reduced ($P < 0.001$; Fig. 3C). Consistently, by using siRNA that was specifically designed for mTOR (si-mTOR), mTOR mRNA levels were decreased ($P < 0.001$, Fig. 3C).

In addition, to better grasp the functional importance of mTOR, we measured the HIF-1α and VEGF transcripts after

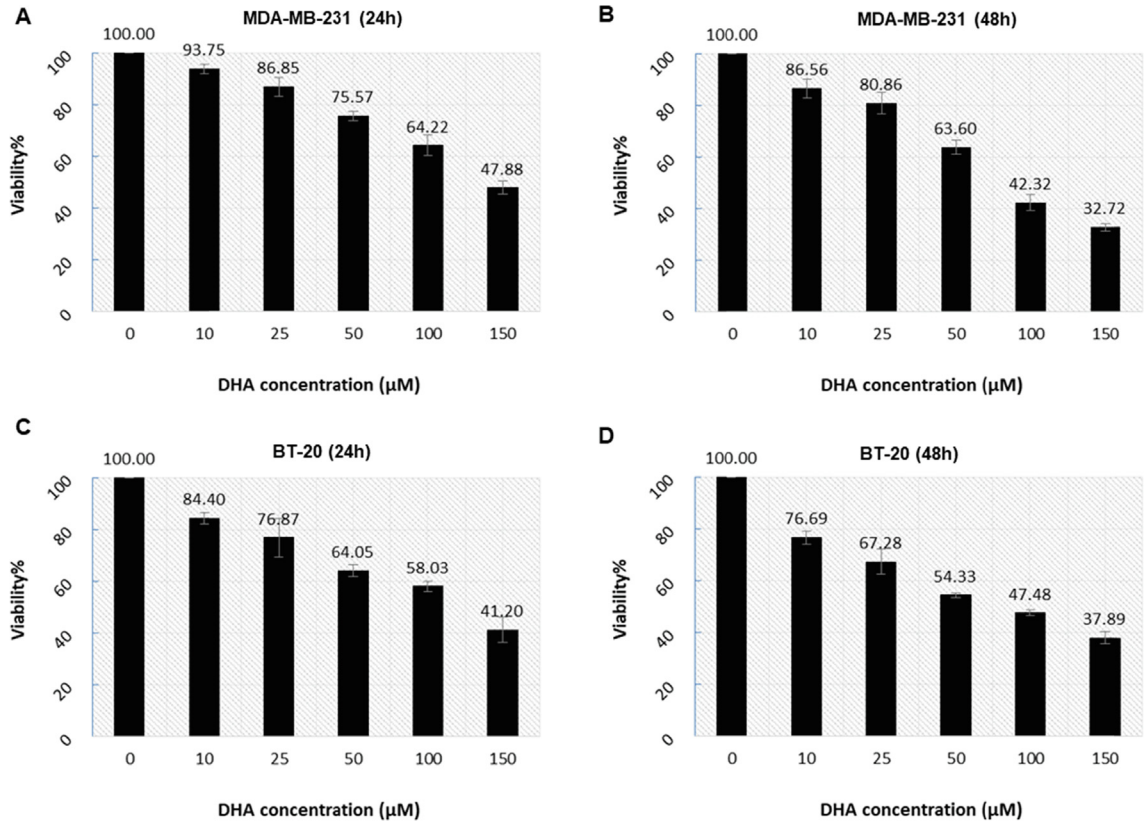

Fig. 1 DHA decreases the BC cell viability in a specific and time-dependent manner. (A–D) Treatment of BC cells (MDA-MB-231 and BT-20) with gradient concentrations of DHA for 24 and 48 h showed that DHA suppresses BC cell survival dose-dependently. The results of the cell viability assay were used to determine the IC50 values for use in further experiments.

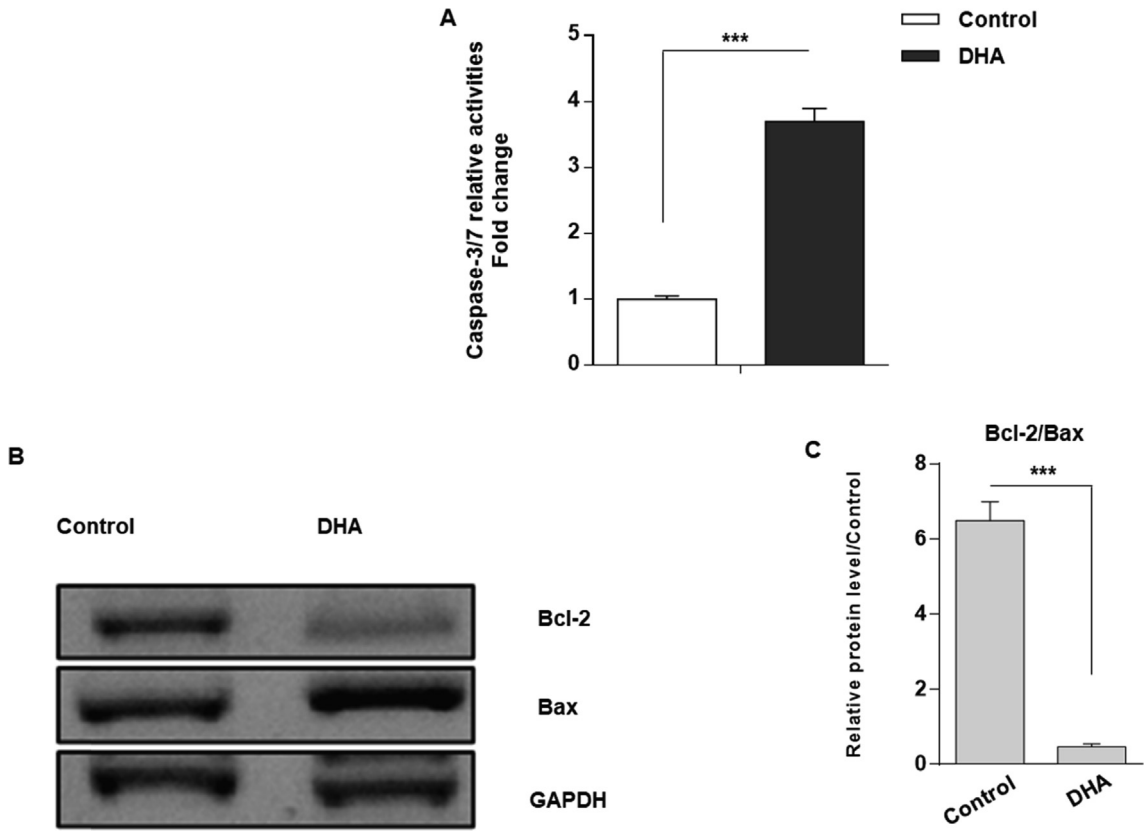

Fig. 2 DHA induces apoptosis in BC cells. (A) The findings show the increased caspase-3/7 relative activity after 48 h in response to 50 μM DHA treatment in MDA-MB-231 cells. (B) Immunoblotting of endogenous Bcl-2 and Bax protein levels in MDA-MB-231 cells 48 h after pretreatment with 50 μM DHA. (C) The ratio of Bcl-2 to Bax was considerably decreased ($P < 0.001$) in MDA-MB-231 BCE cells that were treated with 50 μM DHA, suggesting the pro-apoptotic influences of DHA on MDA-MB-231 BCE cells. ***$P < 0.001$.

transfecting the BC cells using miR-99a. Gene expression analysis that was carried out by RT-qPCR highlighted a considerable reduction of *HIF-1α* ($P < 0.001$) and *VEGF* ($P < 0.01$) transcripts in MDA-MB-231 cells that were transfected with miR-99a mimics than the cells that were transfected with scramble (Fig. 3D). All in all, these data attribute an activator role to mTOR whereby it induces VEGF signaling; We also underscore a supporting model in which miR-99a adjusts VEGF signaling by regulating mTOR.

3.4. DHA inhibits mTOR signaling in BC cells through miR-99a regulation

Some scrapes of evidence bear out that DHA exerts its anticancer functions through regulating different miRNAs (Sun et al., 2013; Ghaffari-Makhmalbaf et al., 2020; Aslan et al., 2020). Herein, we investigated how miR-99a could be affected by DHA. For this purpose, the expression status of miR-99a in cells that were pre-incubated with/without DHA was identified. The findings demonstrated that miR-99a was up-regulated in 50 μM DHA-incubated BC cells, in comparison to the control cells (the cells without DHA treatment) (Fig. 4A); this *per se* substantiated that DHA can up-regulate miR-99a expression in BC cells.

We also demonstrated that DHA-induced up-regulation of miR-99a resulted in a significant decrease of VEGF mRNA expression level time-dependently (Fig. 4B). In light of the fact that HIF-1α is a vital regulator of VEGF(Forsythe et al., 1996) and its activation is promoted by mTOR signaling (Land and Tee, 2007), we conjectured that DHA-induced miR-99a up-regulation may affect VEGF expression through modulating the mTOR/HIF-1α. Expectedly, we verified that DHA-induced up-regulation of miR-99a significantly suppressed *mTOR* and *HIF-1α* mRNA expression levels in MDA-MB-231 cells (Fig. 4C). We also investigated the effect of DHA-induced up-regulation of miR-99a on the mTOR-HIF-1α-VEGF pathway using BT-20 cells. We demonstrated that miR-99a up-regulation significantly decreased the mRNA levels of the mTOR-HIF-1α-VEGF axis in DHA-incubated cells (Fig. 4D–F). Data obtained from were in line with these findings and revealed the decreased levels of VEGF secreted from both BC cell models with different concentrations of DHA 48 h after treatment (Fig. 4G).

Moreover, to inspect whether DHA is capable of inhibiting mTOR signaling by promoting the expression of miR-99a or not, BC cells were transfected using the inhibitor of miR-99a. Axiomatically, transfection of MDA-MB-231 cells with miRNA inhibitor reduced the miR-99 expression than the

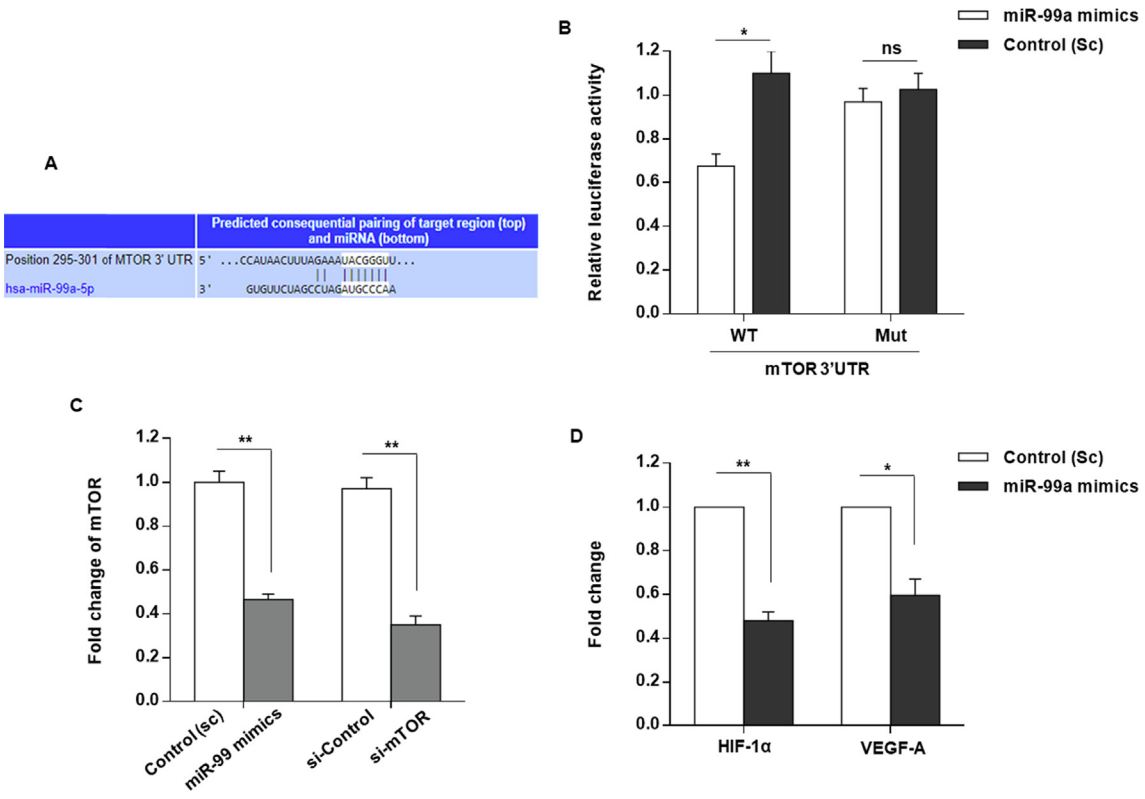

Fig. 3 mTOR is a direct and functional target for miR-99a. (A) Putative complementary sequences between the 3′-UTR of mTOR and miR-99a predicted by TargetScan 5.1. (B) Luciferase assay was done to show direct targeting of mTOR 3′-UTR by miR-99a. Relative luciferase reporter activity of MDA-MB-231 BCE cells at 48 h after co-transfection with wild-type (WT) or mutant (Mut) mTOR 3′-UTR luciferase reporter constructs and miR-155 precursor molecule (miR-155 mimics) are shown. miR-99a was identified to lessen luciferase activity in the cells that were transfected with WT mTOR 3′-UTR ($P < 0.05$), while no significant effect was detected in those cells transfected with Mut mTOR 3′-UTR. (C) mTOR expression at mRNA levels measured in MDA-MB-231 cells that were transfected with either miR-99a mimics or scramble (sc). Transfection of miR-99a mimics led to a significant decrease of mTOR transcript levels in MDA-MB-231 cells. mTOR mRNA levels in MDA-MB-231 cells transfected with either siRNA against mTOR (si-mTOR) or silencing negative control (si-control) is also evaluated; siRNA inhibited mTOR expression. The assays were performed 48 h after transfection. (D) HIF-1α and VEGF-A expressions evaluated by RT-qPCR in MDA-MB-231 cells, 48 h after transfection with either miR-99a mimics or scramble (sc). In this figure: *$P < 0.05$, **$P < 0.01$, and "NS" means "no statistical significance".

control group ($P < 0.01$; Fig. 4H). Interestingly, we elucidated that the miR-99a inhibitor rescued the suppressive effects of DHA on mTOR expression, confirming the functional role of miR-99a in exerting the regulatory effects of DHA on mTOR signaling in BC cells (Fig. 4I).

3.5. DHA suppresses BC cell proliferation and migration by promoting the expression of miR-99a

We also conducted an investigation into whether DHA-mediated up-regulation of miR-99a suppresses BC cell proliferation and migration. To this end, MDA-MB-231 cells were incubated with DHA (100 μM); remarkably, DHA-treated BC cells showed a somehow low rate of proliferation than the control cells (Fig. 5A). To assess the possible interactions between DHA and miR-99a that in turn can suppress BC cell proliferation and migration, the BC cells were stimulated with DHA and simultaneously co-transfected with either anti-miR-99a or scramble. Consequently, we shed light on the fact that the suppression of miR-99a can negatively affect or reverse the suppressive impressions of DHA on mTOR signaling (Fig. 4I), and consequentially extricated the BC cells from the anti-proliferative impressions of this miRNA (Fig. 5A).

Beyond that, BC cells that were incubated with 25 μM DHA unveiled a lower capacity to migrate than control cells after 48 h. Similarly, BC cell transfection with anti-miR-99a significantly reduced the suppressive outcomes of DHA on the BC cell migration (Fig. 5B, C). To wrap things up, these findings can *ipso facto* show that DHA may impede the BC cell proliferation and migration mainly through inducing the expression of miR-99a.

4. Discussion

Notwithstanding the several attempts that have been made to broaden the horizons toward better BC diagnosis and treatment, the exact molecular mechanisms of BC development and progression yet still remain unclear (Bray et al., 2018; Nigdelis et al., 2020). TNBC embracing around 10–20% of BC cases is the most aggressive BC subtype with little to no beneficial standard therapeutic procedure (Baghi et al.,

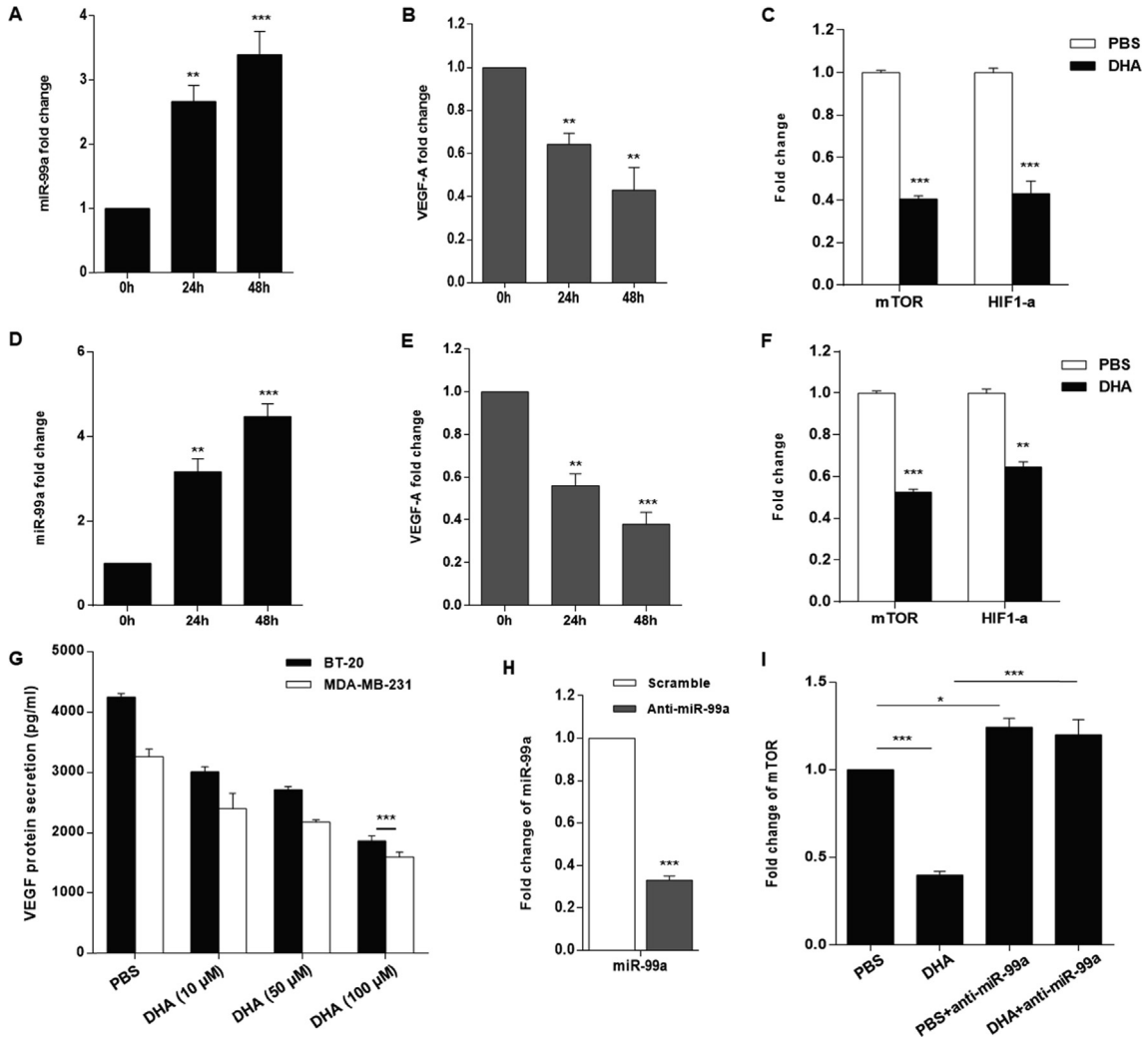

Fig. 4 DHA modulates the mTOR-HIF-1α-VEGF axis through up-regulating miR-99a in BC cells. (A) DHA (50 μM) caused a significant increase of miR-99a expression in MDA-MB-231 BCE cells time-dependently. (B) DHA treatment (50 μM) led to a decrease of VEGF mRNA expression levels. C) mTOR and HIF-1α transcript levels at 48 h after treatment of MDA-MB-231 BCE cells with 50 μM DHA. (D–F) RT-qPCR revealed that DHA-induced up-regulation of miR-99a considerably inhibited the expression of the mTOR-HIF-1α-VEGF signaling in BT-20 BC cells. (G) significant dose-dependent effects of DHA on VEGF protein secretion were measured using MDA-MB-231 and BT-20 BC cells that previously were treated with different concentrations of DHA (10, 50, and 100 μM). (H) Fold change of miR-99a in MDA-MB-231 BCE cells after transfection with anti-miR-99a. miRNA level was measured. I) MDA-MB-231 cells were pre-incubated with 50 μM DHA and transfected with anti-miR-99a (100 nM) for 48 h. The mRNA expression level of mTOR in MDA-MB-231 cells transfected with anti-miR-100 along with either DHA or PBS was considerably much greater than those cells that were only treated with DHA. *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

2018). The fact of the matter is that among inconsistent BC subgroups, TNBC shows divagated biological features that make this type of BC to being more aggressive and justify its increased relapse risk than other subgroups (Rodler et al., 2011). A growing body of evidence has shown that mTOR signaling is often dysregulated in TNBC patients (Miricescu et al., 2021; Ortega et al., 2020; Janku et al., 2012; du Rusquec et al., 2020; Gupta et al., 2020). It has been frequently identified that mTOR signaling can promote tumor onset, metastasis, and proliferation—particularly in TNBC (Massihnia et al., 2016). Tumors that are formed as a result of heightened mTOR signaling have been demonstrated to be highly vascularized, a process that can in turn be imputed to the high rate of angiogenesis in such cells. Indeed, angiogenesis can be regulated through HIF-mediated transcription and also mTOR signaling (Land and Tee, 2007; Pakravan et al., 2017).

DHA functions as an anti-cancer factor in different malignancies, particularly BC; for instance, there are plenty of reports showing the effectiveness of using DHA (alone or combinations with other omega-3 polyunsaturated fatty acids) so as to prevent cancer or even provide a therapeutic strategy (Berquin et al., 2008; Nabavi et al., 2015). Because of the fact that DHA most likely promotes the cytotoxic effects of chemotherapeutic drugs, it is usually combined with such drugs in therapeutic strategies (Nabavi et al., 2015). DHA can successfully suppress tumor angiogenesis in a BC nude

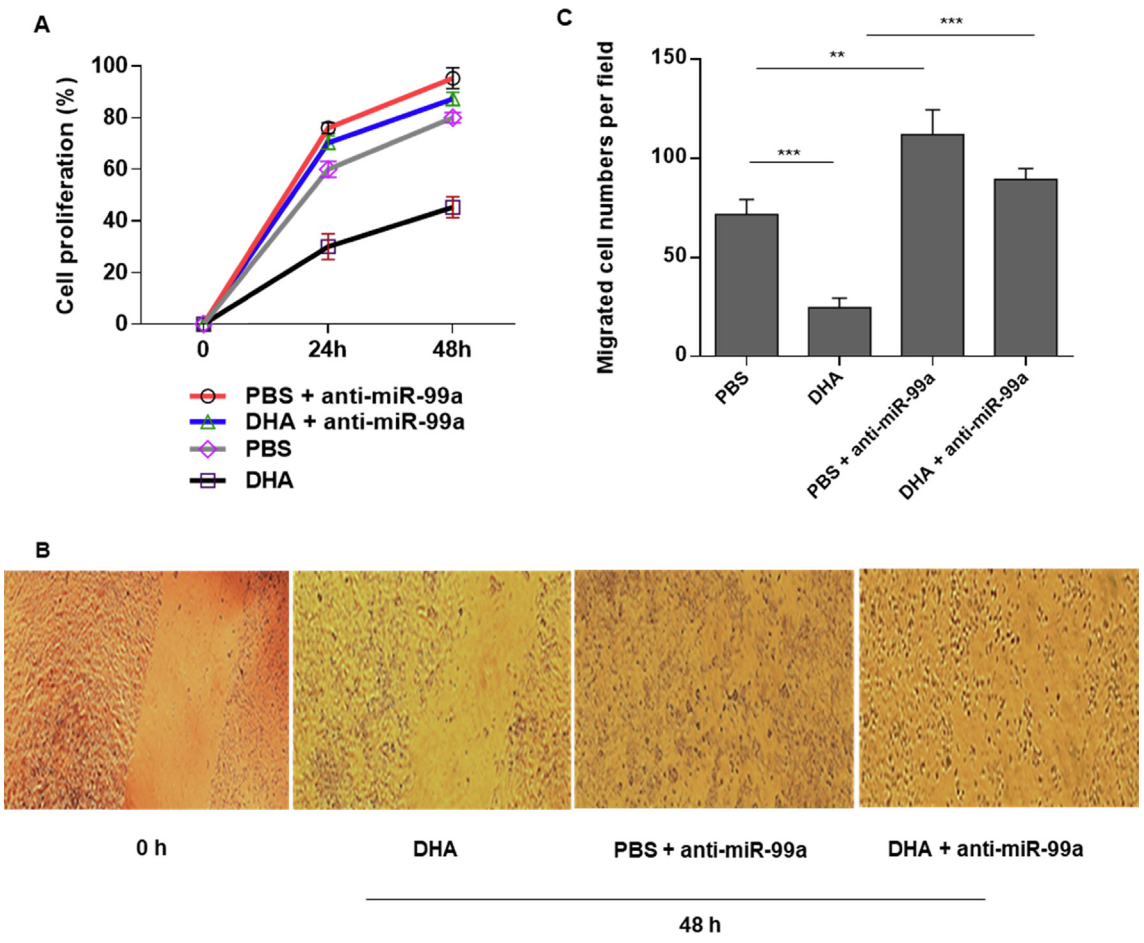

Fig. 5 Inhibitory effects of DHA on BC cell proliferation and migration are exerted by miR-99a up-regulation. (A) DHA (100 μM) has inhibitory effects on the proliferation of MDA-MB-231 BCE cells at time points of 24 and 48 h. Transfection of anti-miR-99a abolished the anti-proliferative effects of DHA in MDA-MB-231 cells. (B) anti-miR-99a could diminish the anti-migratory effects of DHA on MDA-MB-231 BCE cells at 48 h after treatment. (C) The migration of MDA-MB-231 cells decreased after DHA treatment with a concentration of 25 μM than those cells were treated with PBS after 48 h. Transwell migration assay revealed that the anti-migratory effects of DHA on MDA-MB-231 cells were decreased following transfection with anti-miR-99a. $^{**}P < 0.01$ and $^{***}P < 0.001$.

mouse model (Rose and Connolly, 1999) and further confirmed in murine mammary tumor models that were fed with a fish oil diet (Mukutmoni-Norris et al., 2000). From molecular points of view, it was demonstrated that DHA can suppress angiogenesis maybe through VEGF (Hannafon et al., 2015). On the other hand, the exact molecular processes by which DHA prevents breast tumor angiogenesis yet are shrouded in mystery. The concentration of DHA ranging from 50 to 100 μM is commonly used in cancer cell studies and is physiologically attainable *in vivo* (Magalhaes et al., 2020; Collett et al., 2001; Wang et al., 2014b; Thiebaut et al., 2009). Here, the DHA concentration was selected to be achievable through diet. In this regard, the tumor growth suppression of an empirical tumor model that was fed with a fish oil-supplemented diet suggests that omega-3 fatty acids are non-toxic *in vivo* when used at an effective anti-cancer dose (Kang et al., 2010).

Caspase-3 is an apoptotic enzyme that can be induced using different intrinsic and extrinsic pathways (Kiechle and Zhang, 2002). Herein, we demonstrated that DHA activates caspase-3/7 (Fig. 2A). Caspase-3/7 contributes to the majority of cleavage that takes place during apoptosis (Slee et al., 2001). In the intrinsic apoptosis pathway, Bax binds to Bcl-2 which in turn results in releasing cytochrome *C* and ultimately kindling the processes that will result in apoptosis (Gao and Dou, 2001). Remarkably, our data showed that DHA down-regulates Bcl-2, while up-regulates Bax protein (Fig. 2B, C). In other words, DHA triggers apoptosis by regulating Bax and Bcl-2 in MDA-MB-231 BCE cells which is in line with other studies (Chiu et al., 2004; Narayanan et al., 2001).

DHA may exert its anti-tumor and anti-angiogenic activities via modulation of various miRNAs (Ghaffari-Makhmalbaf et al., 2020; Aslan et al., 2020; Javadian et al., 2020). In fact, according to the previous studies, miRNAs are considering an important target in cancer therapy since this fact that they fundamentally play in tumorigenesis. Herein, we demonstrated that DHA treatment can increase the expression of miR-99a in BC cells (Fig. 4A, D). As a miRNA that inhibits cell growth, miR-99a has been verified to modulate mTOR signaling (Wang et al., 2014a; Li et al., 2016). Using luciferase assay, we confirmed that miR-99a directly targets mTOR (Fig. 3A, B). Additionally, RT-qPCR analysis of BC cells transfected with miR-99a mimics confirmed miRNA-mediated repression

of mTOR expression (Fig. 3C). Furthermore, due to the fact that mTOR takes part importantly in HIF-1α-mediated expression of VEGF in BC cells (Del Bufalo et al., 2006), we drew a conclusion that miR-99a might influence BC cells through modulating the mTOR-HIF-1α-VEGF axis. Importantly, we elucidated that after BC cell transfection with miR-99a mimics, mRNA levels of HIF-1α and VEGF were reduced (Fig. 3E). Using BC cell models, we also demonstrated that DHA regulates the mTOR/HIF-1α/VEEGF signaling axis mainly through up-regulating miR-99a (Fig. 4A-G). Besides, we shed light on the inhibitory effects of DHA on the mTOR transcript levels that were partially rescued by the time the BC cells were transfected by anti-miR-99a (Fig. 4I). Besides, in the current study, DHA was identified to reduce the BC cell proliferation and migration capabilities. By the time anti-miR-99a was transfected into BC cells, the suppressive effects of DHA on the proliferation and migration of such cells were partially rescued (Fig. 5), suggesting that DHA suppresses BC cell proliferation and migration mainly through inducing the expression of miR-99a as well as targeting mTOR signaling. Considering the tumor-suppressive role of miR-99a in various tumors (Mei et al., 2017; Wang et al., 2017; Shi et al., 2017; Liu et al., 2019), we demonstrated that DHA-mediated anti-tumor activity in BC cells possibly might be attributed to regulating miR-99a expression.

In sum, this study elucidates that DHA suppressed proliferation and migration, and triggered apoptosis in BC cells. The anti-cancer activity of DHA on BC cells can be meaningfully attributed to the up-regulation of miR-99a that *ipso facto* inhibit mTOR-HIF-1α-VEGF axis. Regarding the significant effects of DHA on the expression levels of miR-99a, it seems that DHA treatment might be used as a useful supplement for BC therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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