### Title

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# Eicosapentaenoic acid induces neovasculogenesis in human endothelial progenitor cells by modulating c-kit protein and PI3-K/Akt/eNOS signaling pathways☆

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**Abstract**

Human endothelial progenitor cells (hEPCs) derived from bone marrow play a crucial in the prevention of ischemic injuries in the course of postnatal neovasculogenesis. Frequent fish oil (FO) consumption is reportedly associated with a significantly lower incidence of cardiovascular disease. However, the molecular mechanisms of eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) are not well elucidated, and the beneficial effect of FO consumption on neovasculogenesis has not been demonstrated yet. In the current study, we investigated the effects of EPA/DHA and FO consumption on neovasculogenesis by using vascular tube formation assay, Western blotting, real-time polymerase chain reaction, immunohistochemical staining and Doppler imaging in both *in vitro* and *in vivo* models. The results demonstrate that EPA and DHA dose-dependently enhance the neovasculogenesis and cell migration of hEPCs *in vitro*. The mechanisms of action included up-regulation of the c-kit protein as well as the phosphorylation of the ERK1/2, Akt and endothelial nitric oxide synthase signaling molecules in hEPCs. Furthermore, EPA significantly suppressed the expression of microRNA 221 *in vitro*. In experimental animal models, FO consumption significantly induced the formation of new blood vessels (neovasculogenesis) and prevented ischemia. Taken together, it is suggested that FO consumption enhances neovasculogenesis mainly through the effects of EPA in hEPCs, thereby exerting a preventive effect against ischemic injury.


*Keywords:* Eicosapentaenoic acid; Neovasculogenesis; c-kit; Akt; eNOS; microRNA 221; Human endothelial progenitor cells

## 1. Introduction

The hemangioblast, an ancestral progenitor of endothelial cells (ECs) during embryogenesis, plays an important role in the *de novo* development of blood vessels [1]. Circulating endothelial progenitors (EPCs) are derived from the hemangioblast and function as a cell source that contributes to neovascularization in tissue regeneration [2–6]. The postnatal formation of new blood vessels is necessary under both pathological and physiological conditions, such as ischemia, cardiovascular diseases (CVDs), stroke and tissue regeneration [7,8]. Adult circulating EPCs are derived from bone marrow and integrate into the sites of blood vessels during an ischemic event [9]. In addition, several studies have reported an inverse association between the circulating EPC level and ischemic conditions [10]. Recent studies have suggested that the number and function of EPCs are inversely correlated with certain risk factors and forms of CVDs, such as coronary artery disease. Previous studies had suggested that low levels of nitric oxide (NO) impaired the migration capacity of ECs

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and contributed to the dysfunction of vasodilatation [11,12]. Therefore, the modulation of EPC activity, cell migration and neovasculogenesis has become the target of several cardiovascular drug research efforts and attracted considerable interest from scientists in a variety of fields [13].

Multiple pathways are involved in the migration of EPCs and postnatal neovascularization following vascular injury [14]. Among them, stem cell factor (SCF) and its c-kit receptor signaling cascades have attracted considerable attention. Previous investigation indicated that the c-kit vasculogenic protein, a receptor tyrosine kinase (RTK) involved in neovasculogenesis, is a biomarker of human endothelial progenitor cell (hEPCs) [15,16]. One study reported that augmentation of c-kit expression was associated with the activation of PI3-K/Akt signaling pathways in hEPCs [17]. Further, SCF (a c-kit ligand) induces neovasculogenesis in hEPCs through the activation of the c-kit RTK protein and multiple downstream signaling pathways, including PI3-K/Akt and the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) signaling cascades [15,18]. The activation of PI3-K/Akt leads to the phosphorylation of the endothelial nitric oxide synthase (eNOS) protein

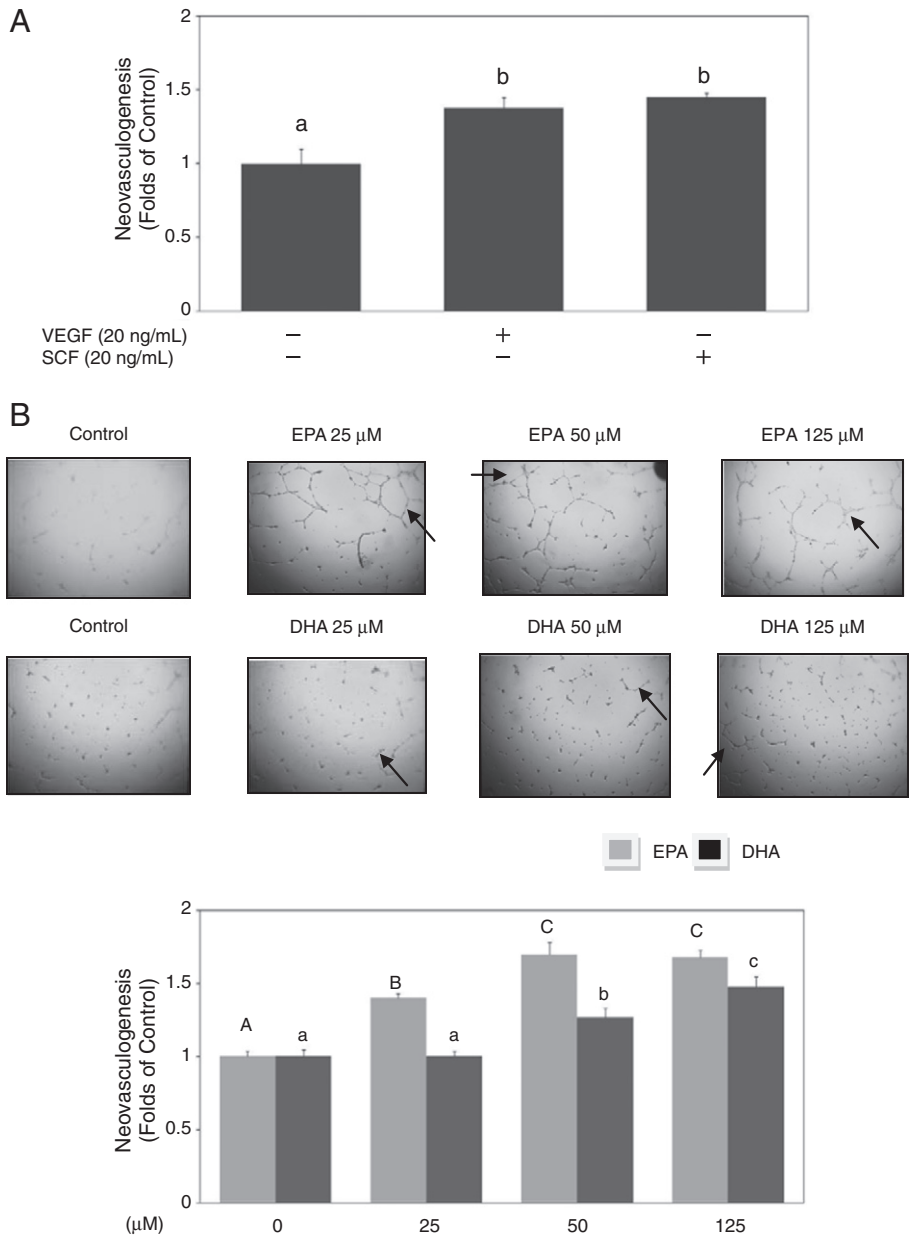


Fig. 1. EPA and DHA induce neovasculogenesis and cell migration of hEPCs *in vitro*. Neovasculogenesis and cell migration of hEPCs in (A) SCF (20 ng/ml), VEGF (20 ng/ml) or (B) EPA or DHA (at concentrations of 25, 50 and 125 μM) for 6 h until the measurement of tubular formation. The values are the mean±S.D. of the total tube length in eight randomly selected fields in each culture dish, each carried out in triplicate and repeated twice. Vascular formation is indicated by the arrow. (C) Cell migration of hEPCs in VEGF or SCF (20 ng/ml) for 4 h until the measurement of migratory capability. (D) Cell migration of hEPCs in the case of EPA or DHA (at concentrations of 25, 50 and 125 μM) for 4 h until the measurement of migratory capability. Each experiment was carried out in triplicate and repeated twice. (A) (C) The different letters represent a statistically significant difference ($P<.05$). (B) (D) The different letters (uppercase for EPA and lowercase for DHA) represent a significant difference in each group ($P<.05$).

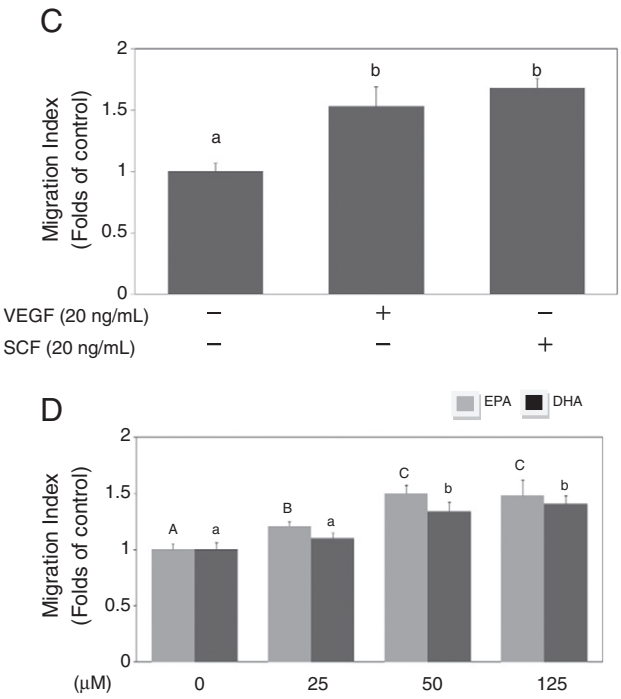


Fig. 1 (*continued*).

downstream. Previous investigation had indicated that the activation of eNOS modulated NO production and enhanced the migration capacity of ECs. In addition to these regulatory pathways, microRNAs (miRs) have emerged as a class of small noncoding RNAs that negatively regulate gene expression through translational inhibition or degradation of the target mRNAs [19–23]. Several studies have indicated that miR down-regulates the translation of targeted mRNA by binding with sites in the 3' untranslated regions [19]. Studies have shown that miR-221 targets the 3' untranslated regions of c-kit mRNA, resulting in an incomplete translation of the c-kit protein [24]. c-kit protein expression was shown to be partially suppressed by miR-221 in ECs [25]. Our previous study also demonstrated that *c-kit* expression and the phosphorylation of the Akt molecule are in part regulated by miR-221 in hEPCs [26]. A clinical study also has reported that the expression of miR-221 was augmented in CVD patients and associated with the degree of ischemic injury [27]. Therefore, the modulation of EPC activity and neovasculogenic regulation has become a target of drug investigation and attracted considerable scientific interest. Although cardiovascular drugs leading to neovasculogenesis constitute the main therapeutic approach to treating CVD, other approaches, such as dietary ones targeted at increasing EPC activity, neovasculogenesis and the preventive effects against ischemic injuries, have yet to be reportedly investigated in any detail at the molecular level.

Epidemiological studies have demonstrated that fish oil (FO) consumption is inversely correlated with the incidence of CVD and stroke [28,29]. For many years, FO consumption has been reported to be a beneficial approach to the prevention of CVD. Studies have reported that FO consumption prevents platelet aggregation, lowered blood pressure and reduced the risk of ischemic attack [30]. The biological functions of FO are partially attributable to certain n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). To our knowledge, to date, no experimental evidence has been presented on the mechanisms underlying the beneficial effects of EPA and DHA on cell migration and neovasculogenesis in hEPCs. Thus, the specific aims of the current study were to examine, both *in vitro* and *in* vivo, the molecular mechanisms of the EPA/DHA effects on neovasculogenesis and the beneficial effects of FO consumption on ischemia prevention.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Anti-phospho-Akt (p-Akt; Ser 473), anti-total-Akt (t-Akt), anti-phospho-ERK 1/2 (p-ERK 1/2; Thr 202/Tyr 204), anti-total-ERK 1/2 (t-ERK 1/2), anti-phospho-GSK-3β (p-GSK-3β;Ser 9), anti-total-GSK-3β (t-GSK-3β), anti-phospho-eNOS (p-eNOS; Ser 1177), anti-total-eNOS (t-eNOS), anti-c-kit and anti-VE-cadherin monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rat tail type I collagen and Matrigels were purchased from BD Bioscience Inc. (San Jose, CA, USA). Anti-β-actin antibody, MEK specific inhibitor (PD098059), eNOS specific inhibitor (L-NAME) and PI3-K specific inhibitor (wortmannin) were purchased from Sigma (St. Louis, MO, USA). SCF and vascular endothelial growth factor (VEGF) recombinant proteins were purchased from R&D Systems Inc. (Minneapolis, MN, USA). The NE-PER nuclear and cytoplasmic extraction reagent kit was purchased from Pierce Biotechnology Inc. (Lackford, IL USA). EPA and DHA were purchased from Cayman Chemical Inc. (Ann Arbor, MI, USA) and dissolved in ethanol at a concentration of 330 mM and stored at −20°C. Fish oil (obtained from Jung-Nan Chemicals, Taiwan) contains approximately 55% of total n-3 PUFA (including 33% EPA and 22% DHA). Corn oil was purchased commercially. Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA). Trizol reagent and Lipofectamine LTX with Plus Reagent were purchased from Invitrogen Inc. (Carlsbad, CA, USA). The two-step real-time polymerase chain reaction (RT-PCR) kit was purchased from Promega Inc. (Madison, WI, USA). The specific Taqman MicroRNA assays, including the primers for hsa-miR-221 and U6 snRNA, were purchased from Applied Biosystems (Carlsbad, CA, USA). The EGM-2 media were purchased from Lonza, Inc. (Allendale, NJ, USA). The control vector and anti-miR-221 plasmids were purchased from System Biosciences Inc. (Mountain View, CA, USA).

### 2.2. Cell culture

hEPCs were a gift kindly provided by Dr. S.C. Chiu (China Medical University, Taichung, Taiwan). Briefly, hEPCs (passage 6–9) cultured in 10% FBS MCDB-131 with an EGM-2 growth kit in a collagen-coated tissue culture dish were lifted off by trypsinization, pelleted by centrifugation and resuspended in heat-inactivated 10% FBS MCDB-131 medium. The working concentration of type I collagen was 50 μg/ml (prepared in 0.02 N acetic acid). For the transfection procedure, 50% confluent hEPCs were transfected with an anti-miR-221 plasmid using Lipofectamine LTX Plus Reagent according to the manufacturer's instructions.

### 2.3. Supplementation with EPA and DHA

The hEPC monolayer was incubated with different concentrations (0, 25, 50 and 125 μM) of EPA or DHA, respectively. For efficient uptake of EPA by hEPCs, EPA was incorporated into FBS for 30 min and mixed with MCDB-131 medium with 10% heat-inactivated FBS, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate in the absence of antibiotics.

### 2.4. Assessment of cell proliferation

3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay was conducted to detect the cell proliferation. hEPCs were seeded in 24-well plates, each well containing $1\times10^5$ cells. The culture medium was replaced by media in which there was present EPA or DHA (at a concentration of 50 μM) in the presence of different inhibitors for 6 h, respectively. There were triplicate tests for each inhibitor. At the end of the experiment, one of the plates was taken out, and fresh 3-[4,5-dimethhylthiaoly]-2,5-diphenyltetrazolium bromide [MTT, final concentration 0.5 mg/ml in phosphate-buffered saline (PBS)] was added to each well. After 2-h incubation, the culture media were discarded, and 200 μl of acidic isopropanol was added to each well and vibrated to dissolve the depositor. The optical density was measured at 570 nm with a microplate reader.

### 2.5. Preparation of protein extraction

Cell nuclear and cytoplasmic fractions were prepared with an NE-PER nuclear and cytoplasmic extraction reagent kit containing protease and phosphatase inhibitors according to the manufacturer's instruction. After centrifugation for 10 min at 12,000×*g* to remove cell debris, the supernatants were retained as a cytoplasmic extract. Cross contamination between the nuclear and cytoplasmic fractions was not observed (data not shown).

### 2.6. Western blotting analysis

Briefly, cytoplasmic proteins (100 μg) were fractioned using 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and blotted with an anti-c-kit or anti-VE-cadherin monoclonal antibody according to the manufacturer's instructions. The blots were stripped and reprobed with β-actin

antibody as the loading control. The levels of p-Akt, p-ERK 1/2, p-GSK-3 β, p-eNOS, t-Akt, t-ERK 1/2, t-GSK3β and t-eNOS in cell lysates were measured using the same procedure described above. The total Akt, total ERK 1/2, total GSK-3β and total eNOS proteins were used as internal controls for phosphorylated Akt, phosphorylated ERK 1/2, phosphorylated GSK-3β and phosphorylated eNOS proteins, respectively.

*2.7. Cell migration assay*

The migration of hEPCs was analyzed in transwell Boyden chambers with a polyvinyl/pyrrolidone-free polycarbonate filter of 8-μm pore size according the manufacturer's instruction. hEPCs labeled with calcium AM (a green fluorescence

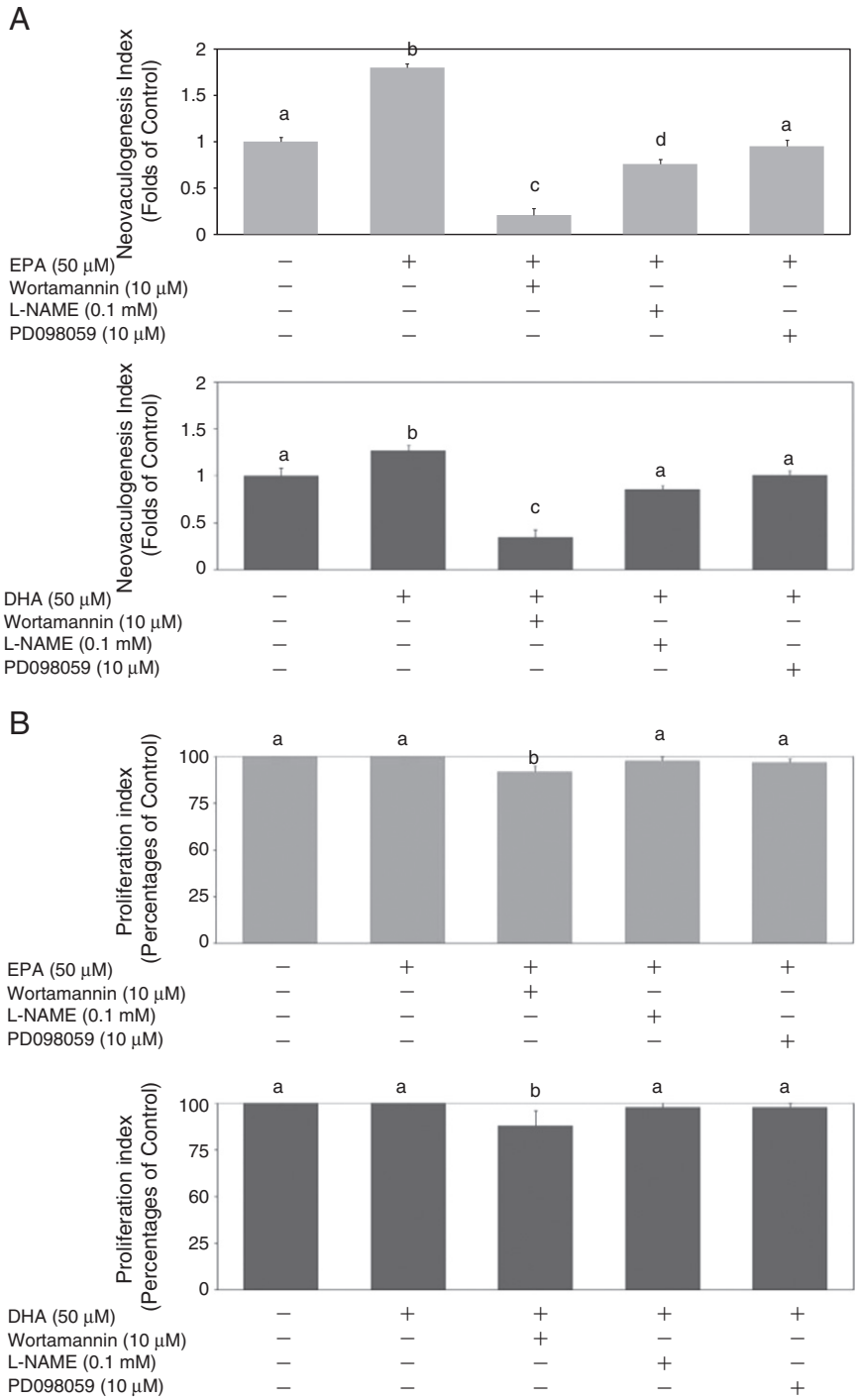


Fig. 2. The PI3-K/Akt/eNOS and MAPK/ERK signaling pathways play an important role in EPA- and DHA-mediated neovasculogenesis in hEPCs. (A) hEPCs were treated with different inhibitors including wortmannin (10 μM), L-NAME (0.1 mM) and PD098059 (10 μM) in the presence of EPA (50 μM) or DHA (50 μM) for 6 h until the measurement of tubule formation. The total length of the vascular tube structure was measured as described in the "Materials and methods" section. The values are the mean±S.D. of the total length of the tubes in eight randomly selected fields in each culture dish, each carried out in triplicate and repeated twice. The different letters represent a significant difference ($P<.05$). (B) hEPCs incubated with wortmannin (10 μM), L-NAME (0.1 mM) and PD098059 (10 μM) in the presence of EPA (50 μM) or DHA (50 μM) for 6 h until the measurement of cell viability. The proliferation index is represented as the mean±S.D. The different letters represent a significant difference ($P<.05$).

dye) stimulated with EPA or DHA (0, 25, 50, and 125 μM) were added to each of upper transwell chamber in serum-free MCDB-131 medium ($1\times10^4$ cells/well). The medium in the lower transwell chamber contained 10% FBS MCDB-131 medium. After incubation for 6 h, hEPCs on the surface of the upper chamber were completely wiped away with a cotton swab. The cells migrating the lower side of the filter were monitored under inverted phase-contrast microscope (200×), and photomicrographs were documented and analyzed by using the Olympus DP-71 digital camera and imaging system (Tokyo, Japan). The number of cells in 10 randomly selected fields in the lower side of the filter was measured as migration activity (migration index).

### 2.8. Neovasculogenesis (vascular tube formation) assay

hEPCs were cultured in MCDB-131-supplemented medium that was changed every 2 days. For the vasculogenesis assay, a confluent monolayer of hEPCs from passages 6–9 was cultured in MCDB-131 medium on 3-D Matrigels. The Matrigel was diluted to a concentration of 4 mg/ml, and the pH was neutralized by adding MCDB-131. Aliquots of 50-μl Matrigel were added to 96-well culture plates and incubated at 37°C until gelatinization. hEPCs were seeded on the Matrigel-coated 96-well plates ($1\times10^4$ cells/well) to form confluent monolayers using MCDB-131 (pH 7.4) containing 10% FBS in the presence of EPA or DHA. After seeding in the 96-well plates for 8 h, hEPCs were fixed with 0.5 ml of a glutaraldehyde/paraformaldehyde mixture (2.5%) and stained with modified May–Gruenwald solution (0.25%). Tubular structure formations on the 3-D Matrigels were visualized under inverted phase-contrast microscopy (40×), and photomicrographs were documented and analyzed with the Olympus DP-71 digital camera and imaging system (Tokyo, Japan).

### 2.9. Quantitative real-time PCR (qPCR)

Total RNA samples from hEPCs were extracted from each group with Trizol reagent and converted into cDNA for further analysis using a two-step RT-PCR kit. Briefly, cDNA samples were used in a PCR mix containing the hsa-miR-221 specific primers, as described above. U6 snRNA was used as the internal control. Quantitative PCR experiments were performed using the real-time PCR detection system (Applied Biosystems, Carlsbad, CA, USA). The expression levels of miR-221 adjusted by the U6 snRNA in hEPCs are presented as a percentage of the corresponding untreated control group.

### 2.10. Xenograft implantation of EPCs

To achieve the specific aims of this study, we used immunodeficient nude mice in which hEPCs were xenografted subcutaneously. Briefly, hEPCs were maintained at 37°C in a 5% $CO_2$ incubator and grown to subconfluency using 10% FBS and EGM-2 kit-supplemented MCDB-131 media. To produce a mouse xenograft model of neovasculogenesis, subconfluent cultures of hEPCs were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed twice and resuspended in serum-free medium. Only single-cell suspensions with a viability greater than 90% were used for the injections.

Female adult (3–4 weeks old) BALB/C AnN-Foxn1 nude mice (18–21 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions in facilities approved by the National Laboratory Animal Center in accordance with current regulations and standards (animal protocol no. 101-134-N). During the entire experimental period, the mice ($n=6$ in each group) were fed according to standard Lab 5010 diet (purchased from LabDiet Inc., St. Louis, MO, USA). The standard diet contains crude fat (13.5% total diet energy), protein (27.5% total diet energy) and carbohydrate (59% total diet energy) and had no detectable amounts of FO as indicated by the supplier. Mice were anesthetized with an inhalation of isoflurane and placed in a supine position. The mice were subcutaneously (s.c.) injected with ~3 million hEPCs ($3\times10^6$/0.1-ml medium) and Matrigel on the right flank of each BALB/C AnN-Foxn1 nude mouse. A well-localized bleb was a sign of a technically satisfactory injection. After the inoculation, mice were divided into three subgroups ($n=6$ in each group). The neovasculogenesis positive subgroup ($n=6$) received injection of EPCs incubated with 20 ng/ml SCF in Matrigel. In the negative control subgroup ($n=6$), mice received only an injection of EPCs and Matrigel only. For the experimental subgroups, mice received FO by gavage feeding (oral tube feeding) at a dosage of 4% w/w of diet per day after the injection of EPCs and Matrigel. At the end of the experiment (second week), the mice were euthanized with $CO_2$ inhalation and sacrificed. The Matrigel implanted for neovasculogenesis was removed from the experimental animals and frozen in liquid nitrogen immediately. For the subsequent investigation, Matrigels were sectioned and probed with a specific c-kit antibody for the identification of this stem cell biomarker.

### 2.11. Mouse ischemic hind limb model

Nine-week-old female C57BL mice ($n=5$ for each group) were divided into three subgroups: (a) control group, (b) Low_FO group and (c) High_FO group. These experimental animals received either corn oil (control subgroup) or FO (Low_FO: 4% w/w of diet and High_FO: 6% w/w of diet per day, respectively) by gavage. After 2 weeks of corn oil or FO consumption, the hind limb ischemia was performed by removing the right femoral artery as previously described [31]. The hind limb blood perfusion was measured with a laser Doppler perfusion imager system before and after the surgery and then followed every 3–4 days. At the end of the experiment (16 days), the mice were euthanized with $CO_2$ inhalation and sacrificed. The whole limbs were removed from the experimental animals and frozen in liquid nitrogen immediately.

### 2.12. Immunohistochemical and immunofluorescent staining of neovasculogenesis

Frozen Matrigel sections were treated with 0.3% hydrogen peroxide to block the endogenous peroxide activity. Nonspecific protein binding was blocked with 10% normal goat serum for 1 h followed by incubation with an anti-c-kit primary antibody (1:300). Tissue sections were washed with 0.1 M PBS and incubated with biotinyated immunoglobulin G (IgG) (1:300 secondary antibody) at room temperature for 1 h. Tissue sections were stained with avidin–biotin complex, diaminobenzidine and hydrogen peroxide. Imaging was performed at either 200× or 400× magnification. Images of the Matrigel sections were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system.

For immunofluorescent staining, ischemic tissues were frozen, sectioned and subjected to anti-VE-cadherin and anti-c-kit antibodies. The sectioned tissues that were probed with anti-VE-cadherin antibody were further subjected to a secondary antibody with an anti-IgG-conjugated fluorescein isothiocyanate label. The sectioned tissues that were probed with anti-c-kit antibody were further subjected to a secondary antibody with a rhodamine label. Cell nuclei were stained with 4,6-diamidino-2-phenyl indole. Imaging was performed at 200× and 400× magnification. Images of the ischemic tissues were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system.

### 2.13. Statistical analysis

The quantitative methodology was used to determine whether there were differences in vascular formation and cell migration index between the experimental (EPA or DHA groups) and control groups of EPCs. In brief, statistical analyses of the differences in neovasculogenic capacity were performed in triplicate using SYSTAT software. Confirmation of a statistically significant difference in the neovasculogenic index requires rejection of the null hypothesis of no difference between the mean neovasculogenic indices obtained from replicate sets of experimental and control groups at the $P=.05$ level with one-way analysis of variance. The Bonferroni *post hoc* test was used to determine differences among the different groups. A significant difference in protein expression between experimental and control groups was examined using the Student *t* test at the $P=.05$ level.

## 3. Results

### 3.1. EPA and DHA induce neovasculogenesis and cell migration of hEPCs in vitro

The effects of FO components EPA and DHA on neovasculogenesis in hEPCs were investigated *in vitro*. VEGF and SCF were adopted as positive control groups. As shown in Fig. 1A, VEGF and SCF (at concentrations of 20 ng/ml) significantly induced neovasculogenesis, respectively ($P<.05$). VEGF and SCF induced neovasculogenesis up to 1.41- and 1.45-fold that of the unstimulated control group, respectively ($P<.05$) (Fig. 1A). We further investigated the effects of EPA and DHA on cell migration and neovasculogenesis using cell migration assay and vascular tube formation assay, respectively. As shown in Fig. 1 B, EPA and DHA (at concentrations of 0, 25, 50 and 125 μM) significantly induced the neovasculogenesis of hEPCs, respectively ($P<.05$). At concentrations of 25, 50 and 125 μM, EPA significantly induced neovasculogenesis by approximately 1.41-, 1.71- and 1.68-fold ($P<.05$), respectively (Fig. 1B). DHA also significantly induced neovasculogenesis by approximately 1.1-, 1.27- and 1.48-fold ($P<.05$), respectively (Fig. 1B). As shown in Fig. 1C, VEGF and SCF (at concentrations of 20 ng/ml) also significantly induced cell migration up to 1.53- and 1.68-fold that of the unstimulated control group, respectively ($P<.05$). Further analysis revealed that EPA and DHA (at concentrations of 25, 50 and 125 μM) significantly enhanced the cell migration of hEPCs (Fig. 1D). At concentrations of 25, 50 and 125 μM, EPA significantly induced cell migration by approximately 1.25-, 1.51- and 1.48-fold ($P<.05$), respectively (Fig. 1D). Moreover, DHA

significantly induced cell migration by approximately 1.1-, 1.34- and 1.41-fold ($P<.05$), respectively (Fig. 1D). These results indicate that EPA and DHA are able to induce cell migration and neovasculogenesis of hEPCs *in vitro*. Furthermore, EPA was more effective than DHA in terms of both neovasculogenesis and cell migration.

### 3.2. The PI3-K/Akt/eNOS and MAPK/ERK signaling pathways play an important role in EPA- and DHA-mediated neovasculogenesis in hEPCs

To determine the molecular mechanisms underlying these actions, we further investigated the signaling pathways involved in EPA- or DHA-mediated neovasculogenesis. To achieve this goal, different specific inhibitors of the PI3-K, eNOS and the MEK/ERK signaling cascades were utilized. As shown in Fig. 2 A, treatment with wortmannin (a specific inhibitor of PI3-K), L-NAME (a specific inhibitor of eNOS) or PD098059 (a specific inhibitor of MEK) significantly inhibited EPA- and DHA-mediated neovasculogenesis in EPCs ($P<.05$). At the concentration of 0.1 mM, L-NAME significantly inhibited EPA- and DHA-mediated neovasculogenesis up to 58% and 32%, respectively. PD098059 (10 μM) significantly inhibited EPA- and DHA-mediated neovasculogenesis up to 47% and 21%, respectively. Wortmannin (10 μM) significantly inhibited EPA- and DHA-mediated neovasculogenesis to levels as high as 88% and 72%, respectively. Wortmannin reduced cell survival in EPA and DHA conditions by up to 12% (Fig. 2B). Furthermore, PD098059 and L-NAME had no cytotoxic effects on EPA- and DHA-mediated cell survival (Fig. 2B). These results demonstrate that the inhibitory effects of wortmannin, L-NAME and PD098059 on neovasculogenesis were not exerted through their cytotoxic effects. In contrast, wortmannin, L-NAME and PD098059 exerted an effect on neovasculogenesis through an augmentation of vascular signaling pathways. These results clearly suggest that the PI3-K/Akt/eNOS and MEK/ERK signaling pathways are involved in EPA- and DHA-mediated neovasculogenesis.

### 3.3. EPA-mediated neovasculogenesis is associated with up-regulation of the c-kit protein, the activation of the PI3-K/Akt/eNOS pathways and the MAPK/ERK pathways in hEPCs

To verify the neovasculogenic effects, we examined whether EPA and DHA would modulate the expression of the c-kit vasculogenic protein and VE-cadherin adhesion molecule. As shown in Fig. 3A, the expression of the c-kit protein was significantly up-regulated in EPA-stimulated cells, but only moderately up-regulated in DHA-stimulated

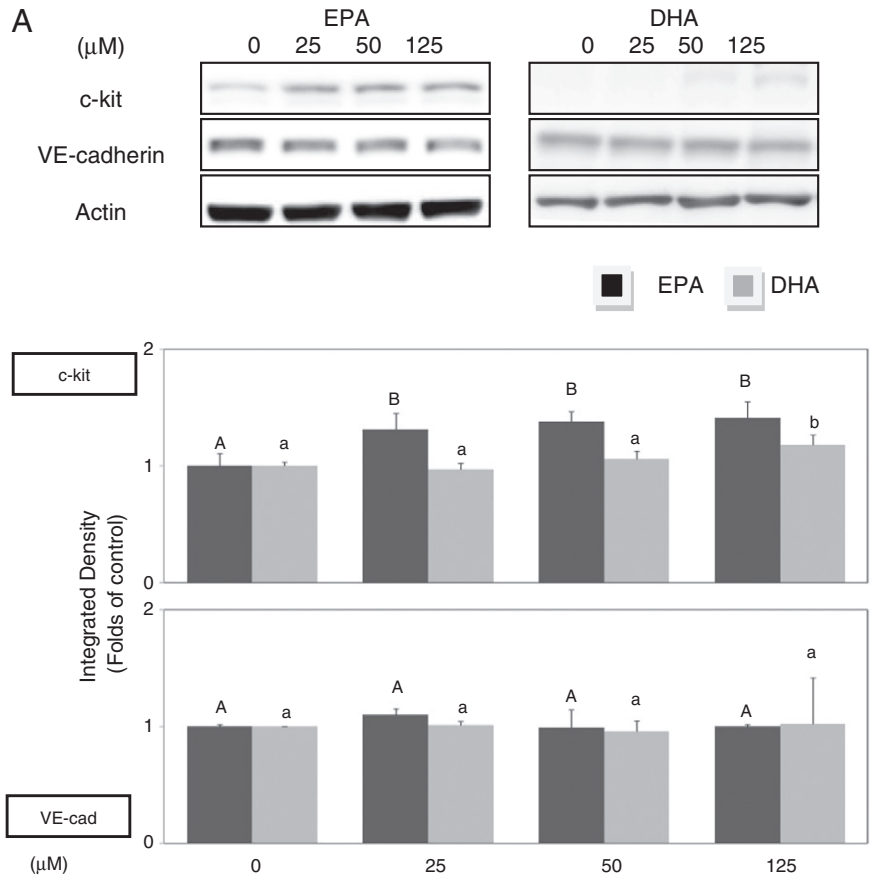


Fig. 3. EPA-mediated neovasculogenesis is associated with up-regulation of the c-kit protein and the activation of the PI3-K/Akt/eNOS pathways and the MAPK/ERK pathways in hEPCs. (A) hEPCs cultured on a 15-cm culture dish were treated with EPA or DHA (at concentrations of 25, 50 and 125 μM) in 10% FBS MCDB-131 for 8 h.Cytoplasmic proteins were prepared for Western blotting analysis using monoclonal antibodies against c-kit, VE-cadherin and actin, as described in "Materials and methods." The levels of detection represent the amount of c-kit and VE-cadherin in the cytoplasm of hEPCs. (B) hEPCs cultured on a 15-cm culture dish were treated with EPA or DHA (at concentrations of 50 μM) in 10% FBS MCDB-131 for different time periods (0, 0.5, 1, 2, 4 h). Cytoplasmic proteins were prepared for Western blotting analysis using monoclonal antibodies against p-Akt, t-Akt, p-GSK3β, t-GSK3β, p-eNOS, t-eNOS, p-ERK 1/2 and t-ERK 1/2 as described in "Materials and methods." The levels of detection represent the phosphorylation levels of Akt, GSK3β, eNOS and ERK 1/2 in the cytoplasm of hEPCs.The results are representative of three different experiments. The integrated densities (mean±S.D.) of these proteins adjusted with the corresponding control proteins (t-Akt, t-GSK3β, t-eNOS or t-ERK 1/2) are shown in the bottom panel. The different letters (uppercase for EPA and lowercase for DHA) represent a significant difference compared to the control in each group, respectively, at $P<.05$.

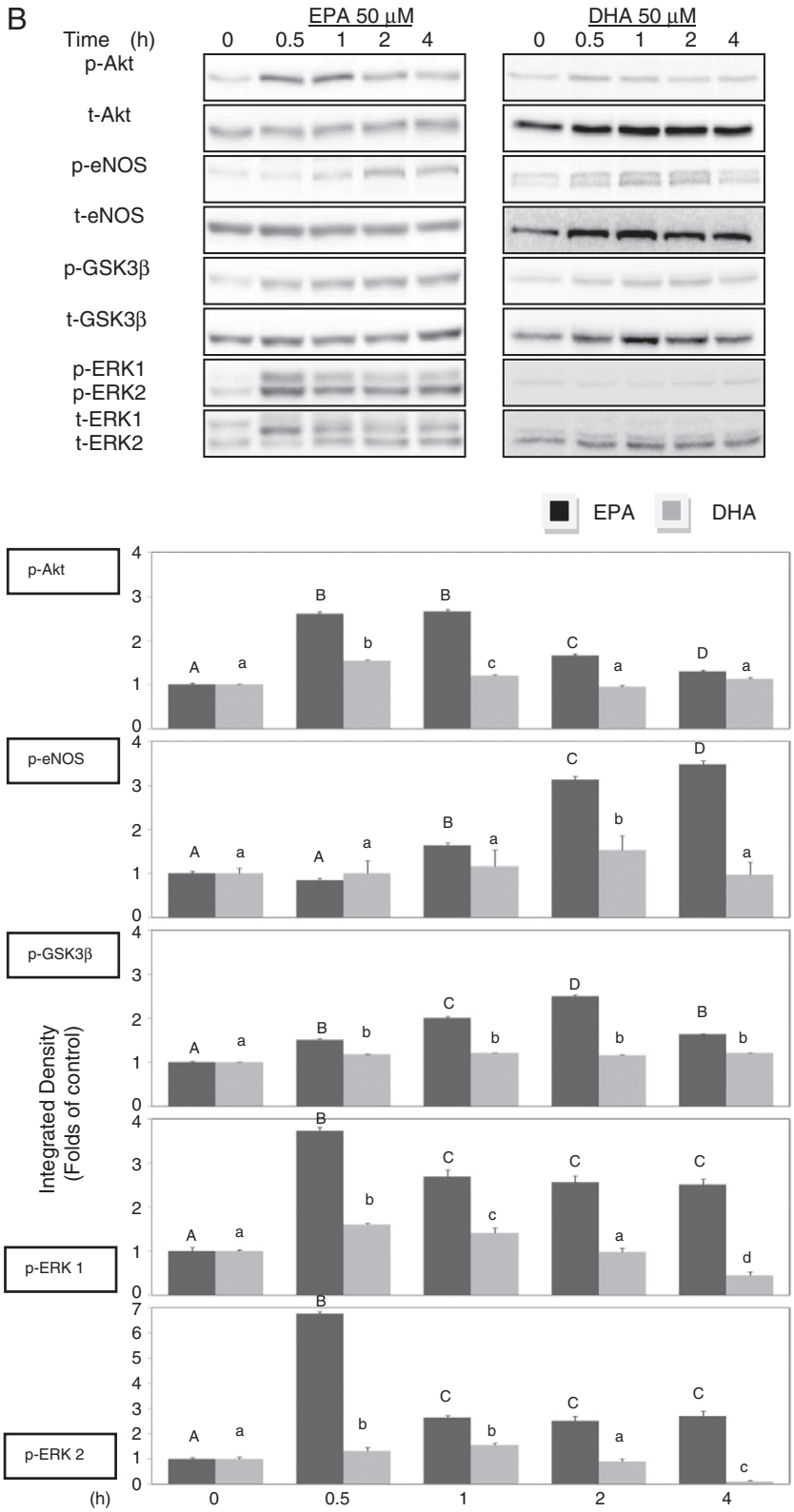


Fig. 3 (*continued*).

EPCs. No significant change in VE-cadherin expression was observed in EPA- or DHA-stimulated cells.

As described above, PI3-K/Akt/eNOS and the MAPK/ERK signaling pathways are involved in EPA- and DHA-mediated neovasculogenesis in hEPCs. To determine the molecular mechanisms underlying these actions, we further examined whether EPA and DHA would modulate the phosphorylation levels of Akt, eNOS and ERK1/2, as well as the downstream target GSK-3β protein. As shown in Fig. 3B, EPA significantly induced the phosphorylation of the Akt, eNOS, ERK 1/2 and GSK-3β proteins in hEPCs ($P<.05$). In contrast, DHA only had a

mild effect on the phosphorylation of these signaling molecules. EPA treatment (at concentration of 50 μM) significantly induced the phosphorylation of the Akt molecule at early time points (at 0.5 and 1 h). The phosphorylation of eNOS and GSK3β was induced at later time points (between 1 and 4 h). Moreover, EPA-mediated phosphorylation of ERK 1/2 was induced and sustained for up to 4 h.

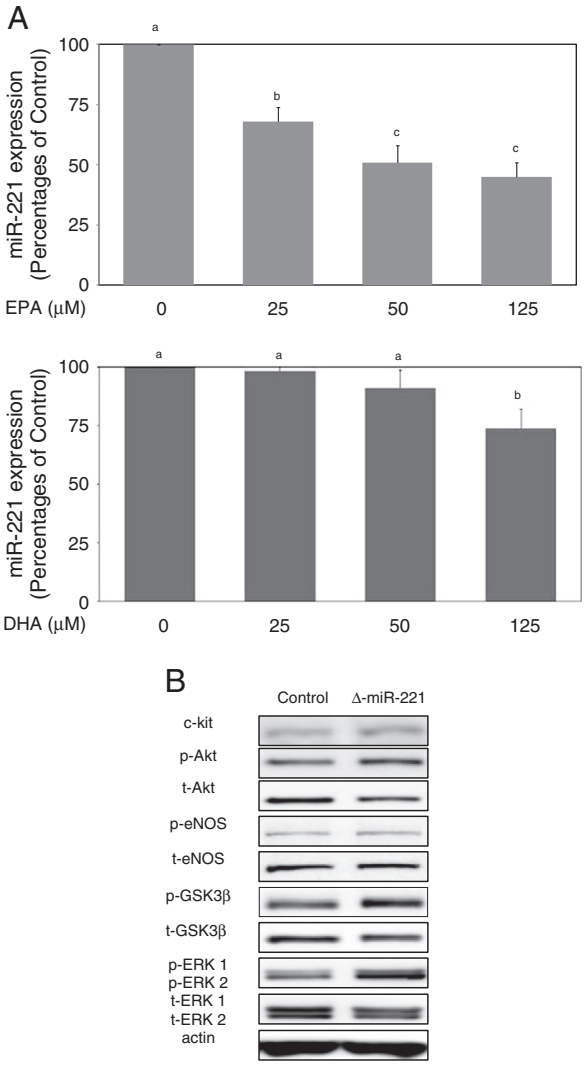


Fig. 4. EPA induces the up-regulation of c-kit and the phosphorylation of Akt/eNOS molecules through suppression of antivasculogenic miR-221. (A) hEPCs were treated with EPA or DHA (at concentrations of 25, 50 and 125 μM) in 10% FBS MCDB-131 for 4 h. Total RNA was extracted from each group and reverse-converted into cDNA for further analysis. Briefly, cDNA samples were used in a PCR mix containing hsa-miR-221 specific primers (Applied Biosystems) as described in "Materials and methods." U6 snRNA was used as internal control. The expression level (mean±S.D.) of miR-221 adjusted by the U6 snRNA (internal control) is shown in the panel. Different letters represent a statistically significant difference at $P<.05$. (B) hEPCs cultured in 10% FBS MCDB-131were transected with a control vector or anti-miR 221 plasmid (△-miR-221) for 8 h. Cytoplasmic proteins were prepared for Western blotting analysis using monoclonal antibodies against c-kit, p-Akt, t-Akt, p-eNOS, t-eNOS, p-GSK3β, t-GSK3β, p-ERK 1/2, t-ERK1/2 and actin as described in "Materials and methods." The levels of detection indicate the amount of c-kit, p-Akt, p-eNOS, p-GSK3β and p-ERK 1/2 in hEPCs. The results are representative of three different experiments. The integrated densities (mean±S.D.) of these proteins adjusted by the corresponding internal control proteins (actin, t-Akt, t-eNOS, t-GSK3β, t-ERK 1/2) are shown in the bottom panel. The asterisk indicates a significant difference compared to the control group ($P<.05$). (C) hEPCs were transfected with a control vector or anti-miR 221 plasmid (△-miR-221) for 4 h. Total RNA was extracted from each group and reverse-converted into cDNA for further analysis. U6 snRNA was used as internal control. The expression level (mean±S.D.) of miR-221 adjusted by the U6 snRNA (internal control) is shown in the panel. The asterisk represents a statistically significant difference at $P<.05$.

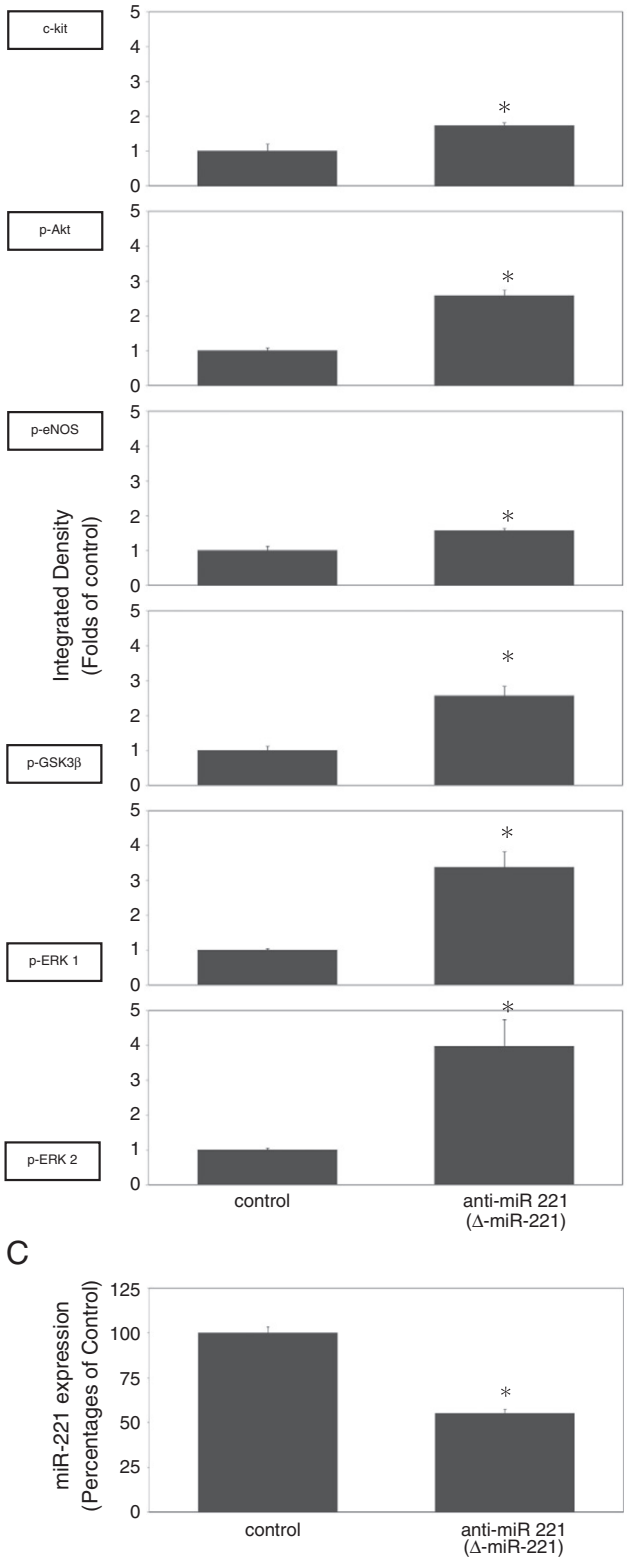


Fig. 4 (*continued*).

Further, these results indicate that EPA was more effective than DHA in terms of the transactivation of the Akt/eNOS and ERK 1/2 signaling pathways as well as the up-regulation of c-kit protein in hEPCs. These results suggested that the activation of PI3-K/Akt and MAPK/ERK signaling cascades and the consequent phosphorylation of the downstream target eNOS protein are involved in neovasculogenesis.

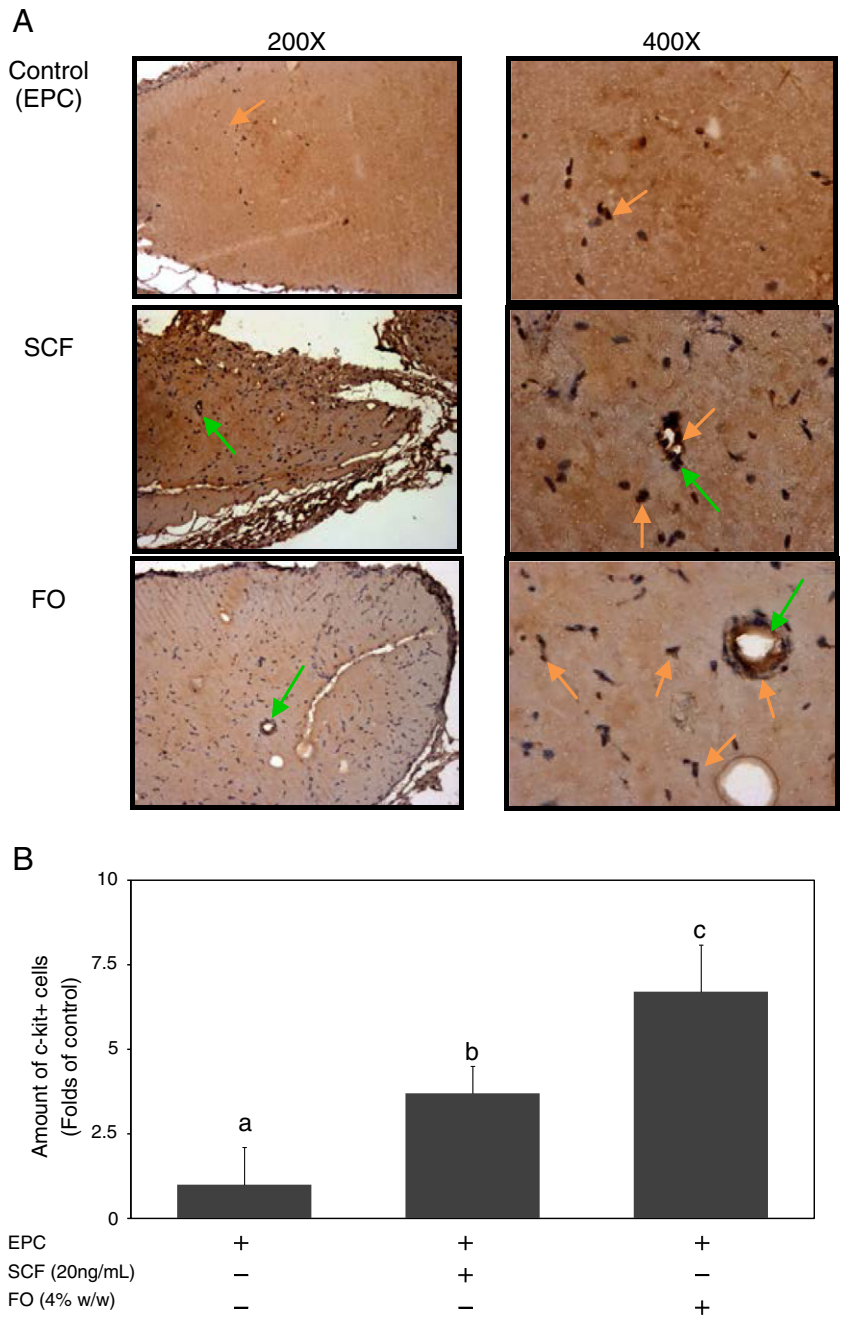


Fig. 5. FO consumption induces blood vessel formation and c-kit protein expression in a mouse xenograft model of neovasculogenesis. For the control group, hEPCs alone were transplanted into the experimental animals. For the SCF group, hEPCs were mixed with SCF (20 ng/ml) and s.c. transplanted into experimental animals. For the FO group, experimental animals were inoculated with hEPCs and received FO (4% w/w of the diet per day) by gavage (oral tube feeding) for 2 weeks. (A) The Matrigel samples from the experimental animals were frozen, sectioned and documented at 200× and 400× magnification. Vascular vessel formation is indicated with a green arrow. The c-kit protein expressed in EPCs is indicated with an orange arrow. (B) The quantitative results (mean±S.D.) of the c-kit+ cells are shown in the bottom panel. The letters indicate a significant difference at $P<.05$.

The results indicate that EPA augmented the phosphorylation of the Akt, eNOS, GSK-3β and ERK 1/2 proteins in hEPCs ($P<.05$). It seems likely that EPA effectively acted as a neovasculogenic agent via an induction of c-kit expression as well as an activation of the PI3-K/Akt/eNOS and MAPK/ERK pathways.

### *3.4. EPA induces the up-regulation of c-kit and the phosphorylation of the Akt/eNOS molecules through suppression of antivasculogenic miR-221*

Previous studies indicated that miR-221 functions as an anti-vasculogenic miR that impacts neovasculogenic c-kit expression by mRNA degradation and translational inhibition [26]. To investigate whether EPA-mediated up-regulation of c-kit expression was in part carried out through miR regulation, we examined the effects of EPA on the expression of miR-221 in hEPCs using RT-PCR analysis. As shown in Fig. 4A, EPA dose-dependently and significantly inhibited the expression of antivasculogenic miR-221 in hEPCs. However, DHA only had mild effects on the suppression of miR-221 expression at the highest concentration (125 μM). In a previous study, our findings showed that c-kit expression and the phosphorylation of the Akt protein were induced by treatment with anti-miR-221 (△-miR 221) plasmid transfection in hEPCs [26]. In this study, the results further

showed that the transfection with anti-miR-221 plasmid (△-miR 221) increased the phosphorylation level of the Akt, GSK3β, eNOS and ERK1/2 molecules in hEPCs (Fig. 4B). Fig. 4C plots the transfection of anti-miR-221 effectively suppressing the expression of miR-221 in hEPCs. These results indicate that a suppression of miR-221 leads to an up-regulation of c-kit expression and the phosphorylation of the Akt/GSK3β/eNOS and ERK 1/2 signaling molecules in hEPCs. Taken together, EPA augments the expression of c-kit protein and neovasculogenesis in part through the suppression of miR-221 in hEPCs.

### 3.5. FO consumption induces blood vessel formation and c-kit protein expression in a mouse xenograft model of neovasculogenesis

To verify these *in vitro* findings, we examined whether FO consumption would induce neovasculogenesis in experimental animals. Each mouse was given FO (at dosage of 4% w/w of the diet per day) for 2 weeks. SCF was adopted as a positive control group. As shown in Fig. 5A, FO consumption significantly induced the formation of new blood vessels in an EPC-transplanted mouse model of neovasculogenesis. It was shown that the neovasculogenic effect in the FO group was significantly more than the SCF group. Moreover, FO consumption significantly induced the expression of the c-kit protein in these animals (Fig. 5B). These results show that FO consumption enhanced the expression of vasculogenic c-kit protein and the formation of new blood vessels in a mouse xenograft model. It is clear that FO has potential as an agent for augmenting the expression of the neovasculogenic c-kit protein and thereby the formation of new blood vessels (neovasculogenesis) *in vivo* as well as *in vitro*.

### 3.6. FO consumption enhances neovasculogenesis and collateral blood flow recovery and exerts a preventive effect against ischemic injury in experimental animals

To further investigate these findings and the benefits of FO, we examined the beneficial application of FO-mediated neovasculogenesis and its preventive effect against ischemic injuries in experimental animals.

An animal model of ischemia was carried out by unilateral hind limb ischemia surgery in C57/BL mice ($n$=5 for each group). As shown in Fig. 6A, the control mice showed delayed blood flow recovery after ischemic surgery as determined by laser Doppler imaging. However, consumption of FO (at dosages of 4% and 6% w/w of the total diet per day) significantly improved blood flow recovery in the surgically treated mice. Moreover, the immunofluorescence staining results showed that FO consumption significantly increased the expression of c-kit and VE-cadherin proteins in the ischemic tissues (Fig. 6B).

These results show that FO consumption enhanced the expression of the vasculogenic c-kit protein and hence the formation of new blood vessels in ischemic tissues. It is thus evident that FO is a potential agent for augmenting the expression of the neovasculogenic

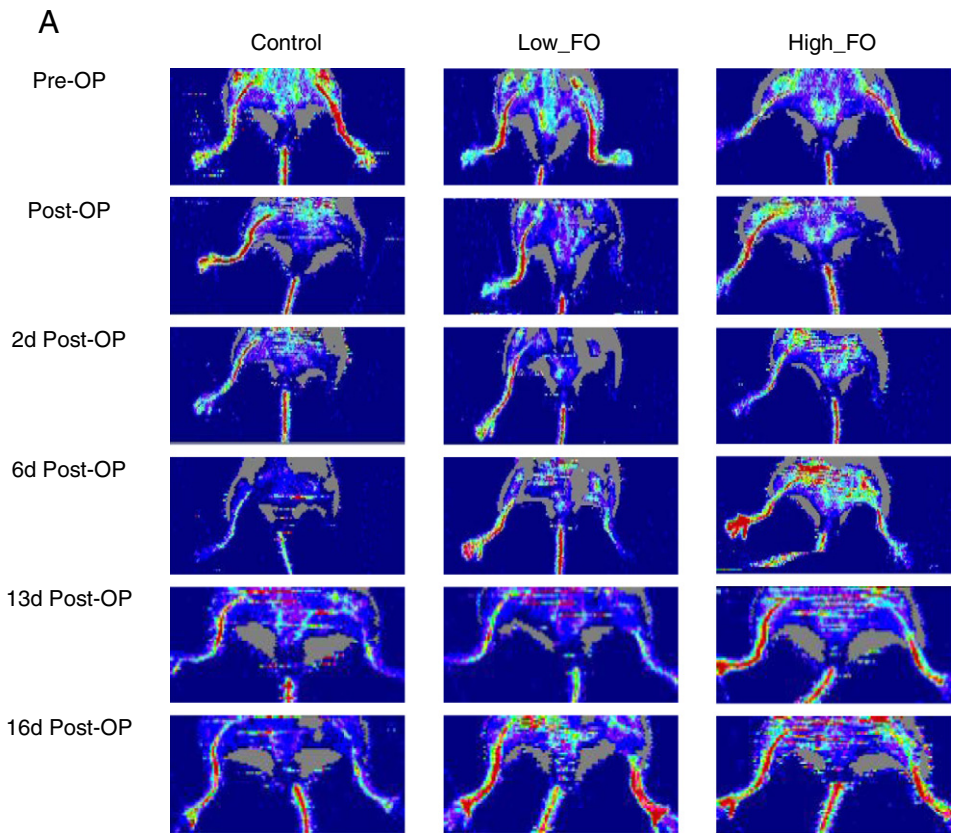


Fig. 6. FO consumption enhances neovasculogenesis and collateral blood flow recovery and exerts a preventive effect against ischemic injury in experimental animals. For the control group, experimental mice received the corn oil by gavage (oral tube feeding) for 2 weeks ahead of the start of the surgical experiment (OP). For both the Low_FO and High_FO groups, experimental mice received the FO by gavage at different dosages (4% w/w and 6% w/w of the diet per day, respectively) for 2 weeks ahead of the start of the surgery (OP). (A) On the day of the experiment, mice underwent Doppler imaging scanning right before and after the vessel-removal surgery (OP). After the surgery, the experimental animals received the scheduled scanning by Doppler imaging on day 2, 6, 13 and 16, respectively.At the end of the experiment, the ischemic tissue samples from the experimental animals were frozen, sectioned and subjected to anti-c-kit and anti-VE-cadherin antibodies using immunofluorescence staining as described in "Materials and methods." Imaging was documented at 200× (B) and 400× (C) magnification, respectively. Green fluorescence indicates the distribution and VE-cadherin protein level during neovasculogenesis stained with the monoclonal antibody. Red fluorescence indicates the distribution and c-kit protein level during neovasculogenesis stained with the monoclonal antibody. Yellow fluorescence indicates the codistribution of c-kit and VE-cadherin protein in the neovasculogenesis sites. Vascular vessel formation is indicated with a yellow arrow. The blue area represents the location of cell nuclei.

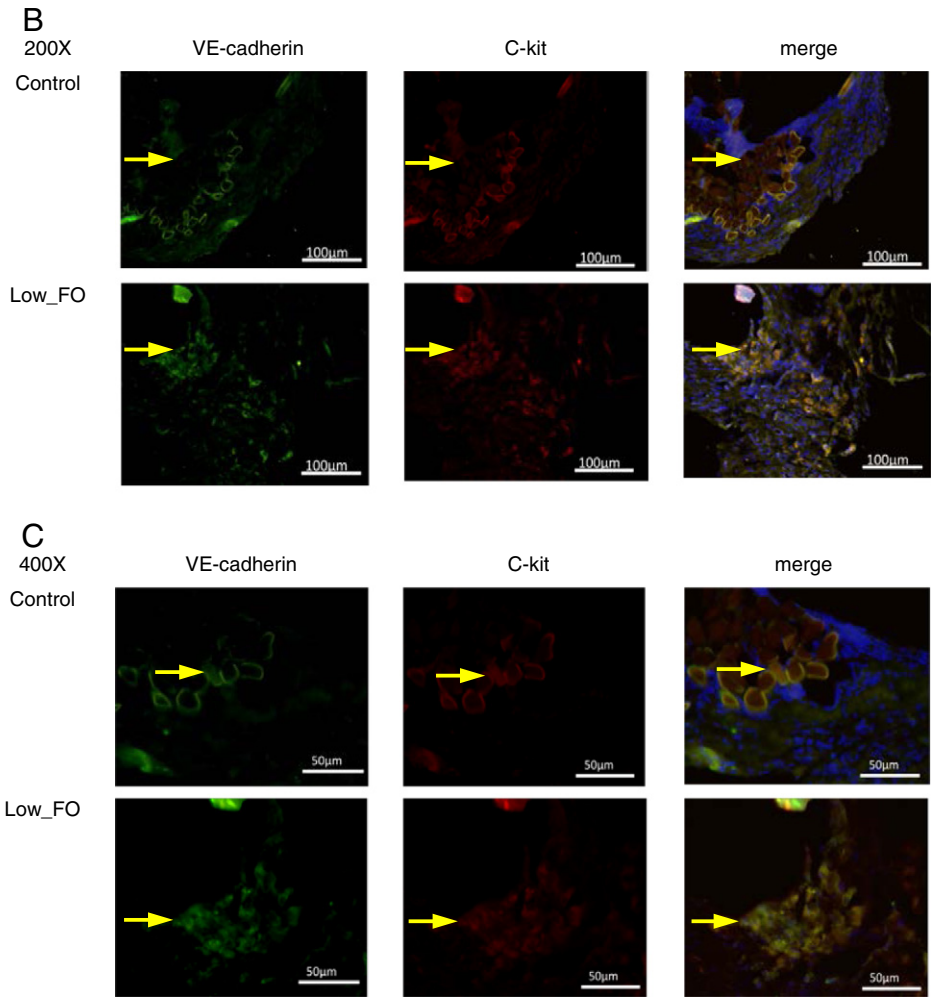


Fig. 6 (*continued*).

c-kit protein and helps EPCs become integrated into the sites of blood vessels (neovasculogenesis) during an ischemic event *in vivo*.

## 4. Discussion

Cardiovascular disease, including stroke as well as heart disease, is one of the leading causes of death worldwide. The formation of new blood vessels plays an essential role in a variety of conditions, such as CVD and tissue regeneration. Therefore, revascularization in ischemic tissue is a key step in the repair of and prevention against injury from stroke and heart disease. Adult angiogenesis occurs through the migration and proliferation of mature ECs derived from preexisting vessels [32–34]. However, these mature ECs are terminally differentiated and have a low proliferative potential. Therefore, the capability of these mature ECs for the repair and replacement of damaged cells is strictly limited. hEPCs from adult bone marrow (BM) are less strictly limited and have excellent repair capability. These human BM-derived EPCs have neovasculogenic characteristics similar to angioblasts. These EPCs have the potential to differentiate into ECs and integrate naturally into the sites of blood vessels [5,35].

A variety of factors, such as an unhealthy lifestyle and/or inadequate nutrient intake, have been shown to contribute to an increased risk for CVD. It is well known that consumption of FO proportionally lowers the risk for CVD. In this study, we demonstrated that n-3 PUFA, especially EPA, is an effective agent in the induction of neovasculogenesis in an *in vitro* study. The normal blood range of EPA is approximately 30 μM. The results showed that EPA dose-dependently enhanced neovasculogenesis in hEPCs *in vitro*, in part through the up-regulation of c-kit protein. The results indicate that EPA was more effective than DHA in terms of up-regulation of the c-kit protein as well as the phosphorylation of the Akt/eNOS and ERK 1/2 signaling cascades. EPA further induced the phosphorylation of the downstream target GSK-3 β proteins. Moreover, EPA mediated an up-regulation of c-kit by reducing miR-221 expression in hEPCs.

There are several possible scenarios in which an FO such as EPA would have the capacity to induce neovasculogenesis. One explanation might be that the augmented activation of Akt/eNOS and ERK 1/2 results in enhanced cell migration. The activation of Akt and ERK1/2 pathways would be expected to lead to an enhanced migration of hEPCs. It seems likely that the PI3-K/Akt pathway may cross talk with the MAPK/ERK 1/2 pathway and affect the migratory capability and neovasculogenesis of hEPCs. Akt activation may further induce the phosphorylation of eNOS protein. A previous study demonstrated that eNOS (nitric oxide synthase)/NO may play important roles in protecting against CVD. The activation of eNOS is regulated by the activation of the PI3-K/Akt signaling pathway. Inhibition of the PI3-K/Akt/eNOS pathway is reported to reduce EC migration. These studies suggested that EPA protects the ischemic myocardium by the induction of NO bioavailability in vascular tissues. Here, we identify a novel role of EPA in EPC-mediated neovasculogenesis. Our results suggest that EPA up-regulates the expression of the neovasculogenic c-kit protein and activates the PI3-K/Akt/eNOS signaling pathways.

Suppression of the respective Akt, eNOS and ERK 1/2 activities by specific inhibitors resulted in reduced levels of neovasculogenesis in hEPCs. Therefore, maintaining the expression of c-kit and the activation of crucial signaling pathways, such as the Akt, eNOS and ERK 1/2 molecules, is apparently the underlying mechanism in EPA-mediated neovasculogenesis. Previous studies indicated that up-regulation of miR-221 was shown to suppress the expression of vasculogenic c-kit protein and thus impact angiogenesis in human ECs [24]. Our previous results also demonstrated that hEPCs transfected with an anti-miR-221 plasmid exhibited an augmentation of the c-kit protein. The results showed that EPA modulated the expression of c-kit protein via a suppression of antineovasculogenic miR 221 expression. The level of antivasculogenic miR-221 is significantly higher in EPCs from CVD patients than in those from healthy subjects [27]. Studies have suggested that the expression of miR-221 is associated with the proliferation of human ECs [25]. Here, our results also demonstrated that transfection with an anti-miR-221 plasmid significantly increases the expression of c-kit protein as well as the phosphorylation of ERK 1/2 and eNOS. These results suggest that EPA induces neovasculogenesis by modulating crucial signaling pathways and suppressing the miR-221 level.

To validate these *in vitro* findings, we utilized a murine model of neovasculogenesis. As shown in Fig. 5, the results showed that consumption of FO enhanced the formation of new blood vessel derived from hEPCs in a mouse xenograft model of neovasculogenesis. Further, FO consumption significantly augmented the expression of the neovasculogenic c-kit protein in these experimental animals (Fig. 5). At dosage of 4% w/w of the total diet per day, FO significantly induced the formation of new blood vessels compared with the control group. No hepatoxicity at dosage of 4% w/w of the total diet per day was observed in this study (data not shown). This dosage in mice is equivalent to physiological levels of EPA for humans. The current results show that EPA effectively enhances the functional activities and neovasculogenic potential of hEPCs *in vitro*. Furthermore, in experimental animals, the consumption of FO induced neovasculogenesis and exerted a preventive effect against ischemic injury (Fig. 6). The ability of EPA to induce neovasculogenesis appears to be dependent on the modulation of the c-kit protein, as shown *in vitro*. The EPA effect on neovasculogenic function is probably exerted through an enhancement of cell migration as well as a modulation of eNOS. Although these effects of FO have not been demonstrated in clinical trials, it is considered that a consumption of EPA-enriched FO would enhance neovasculogenesis and thus help in the prevention against ischemic injury. More detailed studies will be undertaken in the near future.

In conclusion, FO with abundant EPA has potential in clinical applications in the prevention against ischemic injury via an induction of neovasculogenesis by hEPCs.

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