

22 : 6n-3 DHA inhibits differentiation of prostate fibroblasts into myofibroblasts and tumorigenesis

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Abstract

Prostate cancer is one of the most common malignancies in men. Epidemiological and experimental studies have revealed that stromal cells of the tumour microenvironment contribute to the development of prostate cancers, while long-chain *n*-3 PUFA-enriched diets reduce the risk of this tumour histotype. These findings prompted us to investigate whether DHA, an *n*-3 PUFA, may abrogate differentiation of prostate fibroblasts into myofibroblasts, the activated form of fibroblasts generally involved in prostate cancer progression. We used the human prostate carcinoma cell line (PC3) as a prostate adenocarcinoma model and found that DHA (1) inhibits α -smooth muscle actin (α -SMA) expression, a typical marker of myofibroblast differentiation, in prostate fibroblasts stimulated *in vitro* with transforming growth factor- β (TGF- β), (2) blocks the matrix metalloproteinase-2-dependent enhanced invasiveness of PC3 prostate adenocarcinoma cells migrated in a medium conditioned by TGF- β -stimulated prostate fibroblasts, (3) prevents epithelial–mesenchymal transition (EMT) and invasiveness of PC3 cells promoted by a medium conditioned by TGF- β -stimulated prostate fibroblasts, and (4) reduces the growth rate of tumours obtained in immunodeficient animals injected with PC3 cells plus TGF- β -stimulated prostate fibroblasts. Moreover, DHA was found to revert α -SMA expression and the invasiveness-promoting activity exerted in PC3 cells by tumoral-activated fibroblasts. Thus, DHA represents a suitable agent to inhibit prostate myofibroblast differentiation, invasiveness and EMT, two most important tumour-promoting activities involved in the progression of prostate cancer cells.

Key words: Prostate cancers: Intratumoral myofibroblast differentiation: 22 : 6n-3 DHA: Epithelial–mesenchymal transition

The contribution of microenvironment host cells to tumour progression is now well established, and fibroblasts are one of the most frequent cell types capable of organising a dialogue with tumour cells^(1–4). Fibroblasts represent an extremely multifunctional cell type that may play an important regulatory role in several steps during tumour progression^(2,5,6). They are capable of producing various paracrine inflammatory mediators such as peptide growth factors, cytokines, chemokines, several proteases and their inhibitors. Fibroblasts undergo important modifications in the tumour stroma, in particular the population of fibroblasts adjacent to transformed epithelial cells as reported in several tumour histotypes. In addition, human studies describing genetic alterations in breast cancer tissues showed that some alterations in fibroblasts precede those in the epithelial component,

suggesting a role of fibroblasts in epithelial cell transformation⁽⁷⁾. Similarly, in prostate cancers, loss of p53 in fibroblasts precedes loss of p53 in the epithelium⁽⁸⁾.

The phenotype of these so-called cancer-associated fibroblasts (CAF) has been intensely investigated in recent years. Dvorak⁽⁹⁾ showed that a certain population of tumour stroma fibroblasts shares similarities with inflammatory fibroblasts present in the granulation tissue, including structural features recalling smooth-muscle cells, such as expression of α -smooth muscle actin (α -SMA). These CAF have been referred to as ‘myofibroblasts’⁽¹⁰⁾. It is interesting to note that α -SMA expression is not mandatory for fibroblast activation and populations of fibroblasts, whether expressing α -SMA or not, have been recognised in tumour tissues. However, the common trait of these heterogeneous fibroblast populations

Abbreviations: α -SMA, α -smooth muscle actin; AA, 20:4n-6 arachidonic acid; BSA, bovine serum albumin; CAF, cancer-associated fibroblasts; CM, conditioned media; DMEM, Dulbecco’s modified Eagle’s medium; EMT, epithelial–mesenchymal transition; MMP, matrix metalloproteinase; MMT, mesenchymal-to-mesenchymal transition; NCAF, non-cancer-associated fibroblasts; PC3, human prostate carcinoma cell line; TGF- β , transforming growth factor- β .

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is their reactivity and secretion of factors affecting tumour aggressiveness^(11,12).

Epithelial–stromal interactions are considered to be particularly important for the development and progression of prostate cancer, one of the most frequent cancers in industrialised nations^(13–15). Epithelial–stromal cross-talk is necessary for normal prostate development, during which the mesenchyme of the urogenital sinus determines the growth and differentiation of the prostate gland, and epithelial–mesenchymal interactions are maintained during life. De Wever & Mareel⁽¹⁶⁾ reviewed the interplay between cancer cells and stromal cells, and proposed that two pathways, called ‘efferent’ and ‘afferent’, determine the final effect on tumour progression. We recently demonstrated that in the efferent pathway, prostate cancer cells stimulate a reactive response in human prostate fibroblasts, which undergo a clear activation⁽¹¹⁾. In turn, activated fibroblasts stimulate through an matrix metalloproteinase (MMP)-dependent pathway an epithelial–mesenchymal transition (EMT) in prostate cancer cells, associated with an enhanced *in vivo* tumour growth and spontaneous metastatic dissemination. In addition, an enhanced expression of stem cell markers was found to characterise prostate cancer cells undergoing the EMT upon contact with CAF. Fibroblasts, expressing α -SMA, from the tumour region of an adenocarcinoma-bearing prostate gland stimulate tumour formation, metastatic spread and stem cell markers in prostate cancer cells with an efficiency similar to that of *in vitro* transforming growth factor- β (TGF- β)-activated prostate fibroblasts⁽¹¹⁾.

A recent study on cancer risk indicated that environment and lifestyle play a significant role in the development and progression of certain neoplastic disease, including prostate cancers⁽¹⁷⁾. The most important factor modulating prostate cancer risk appears to be the diet, and also epidemiological and experimental studies have implicated fat and meat as the major risk factors. A diet enriched in SFA and *n*-6 PUFA usually correlates with an increased risk and progression of tumours⁽¹⁸⁾, while *n*-3 PUFA, mainly EPA (20:5*n*-3) and DHA (22:6*n*-3), were found to exert a protective effect against some common cancers, including prostate carcinoma^(19–22). Findings that *n*-3 PUFA-enriched diets inhibited spontaneous and chemically induced carcinogenesis confirm their protective effects⁽²³⁾. In addition, our previous work demonstrated that murine metastatic melanoma cells reduced their capacity to colonise the lung after an endovenous injection into animals fed a fish oil diet⁽²⁴⁾. The lung-colonising potential of murine melanoma cells was also reduced after previous treatment with media enriched with EPA⁽²⁵⁾.

The mechanisms underlying the beneficial effects of *n*-3 PUFA on prostate cancer are still poorly understood, and there are no studies analysing whether the malignant phenotype of prostate cancer cells promoted by fibroblasts might be inhibited by *n*-3 PUFA. We used primary skin fibroblasts as a representative stromal-derived cell model of fibroblast-to-myofibroblast differentiation and subsequently we used prostate-derived stromal cells as a more appropriate model for the human prostate stroma⁽¹¹⁾. In particular, prostate fibroblasts were isolated from both healthy and cancerous

regions of an adenocarcinoma-bearing prostate gland, called non-CAF (NCAF) and CAF, respectively, to test the hypothesis that inhibition of prostate stromal fibroblast differentiation into myofibroblast (a mesenchymal-to-mesenchymal transition, MMT) by *n*-3 PUFA is capable of blocking tumour-promoting activities and prostate cancer progression.

Materials and methods

Cell lines and culture conditions

Human prostate carcinoma cell line (PC3) was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg glucose/l (DMEM 4500; Lonza) supplemented with 10% fetal calf serum (complete medium; Invitrogen), at 37°C in a 10% CO₂ humidified atmosphere. Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test⁽²⁶⁾.

Human dermal fibroblasts were kindly donated by Daniela Monti, Department of Experimental Pathology and Oncology, University of Florence, while human prostate fibroblasts were isolated from surgical explantations⁽¹¹⁾. Briefly, tissue samples from healthy and cancerous regions of prostate carcinoma-bearing patients (Gleason 4 + 5) were obtained aseptically from patients undergoing radical prostatectomy, and digested overnight with 1 mg collagenase I/ml in PBS. NCAF and CAF collected from the healthy and cancerous regions, respectively, were grown in 10% fetal calf serum-DMEM medium.

Ethyl esters of 20:4*n*-6 arachidonic acid (AA), EPA (20:5*n*-3) or DHA (22:6*n*-3) were provided from Cayman Chemical in ethanol solution, shipped in dry ice. Fatty acids were stocked under N₂ and stored at –80°C. For the experiments, fibroblasts (passages 2–4) were grown to subconfluence in complete standard medium, and exposed for the next 24 h to PUFA in a serum-free medium with the addition of 250 μ g/ml bovine serum albumin (BSA; Sigma; DMEM-BSA). AA, EPA or DHA were diluted from a stock solution to the final desired concentration in a DMEM-BSA medium and used immediately; an equal amount of ethanol, not exceeding 0.001%, was used for untreated fibroblasts. Fibroblasts were next exposed for 24 h to a fresh DMEM-BSA medium in the absence or in the presence of recombinant human TGF- β (PeproTech, 10 ng/ml; TGF- β -NCAF) which is known to induce fibroblast differentiation⁽¹¹⁾. Fibroblast and myofibroblast cultures were then grown in fresh DMEM-BSA media for an additional 24 h (conditioned media, CM).

Cell viability assay

NCAF were seeded in 60 mm plates in standard conditions. After 24 h, cells were exposed to a DMEM-BSA medium and divided into control and treatment groups (AA, EPA or DHA (30–90 μ M)). Again, after 24 h, cells were exposed to a fresh DMEM-BSA medium in the absence or in the presence of recombinant TGF- β , and 24 h later, cells were harvested, stained with trypan blue and counted under an



inverted-phase microscope (quadruplicate haemocytometer counts of duplicate cultures were performed).

Invasion assay

PC3 cells (1.5×10^5) were either resuspended in fibroblast CM or in a DMEM-BSA medium, and then placed inside 12 mm, 8.0 μ m pore, Millicell Culture Plate Inserts (Millipore), coated with Matrigel (50 μ g/filter; BD); standard medium was placed in twenty-four-well culture plates. Migration was allowed to occur for 4–6 h at 37°C in 10% CO₂ humidified atmosphere. Inserts were fixed in cold methanol and stained with DiffQuick solution. Cells on the upper surface of the filter were removed with a cotton swab. Migrated cells were counted by a light microscope (10 \times) in ten random fields per each well. In separate experiments, PC3 cells were grown in fibroblast CM for 72 h, and then allowed to migrate in DMEM-BSA medium. Each treatment was performed at least in duplicate. Mean values of the migrated cells for each point were calculated from three independent experiments.

Zymography

Aliquots of different fibroblast cultures CM and human HT1080 fibrosarcoma cell medium, used as markers of molecular weight, were fractionated by 8% SDS–PAGE gels co-polymerised with 0.1% (w/v) type A gelatin. After electrophoresis, the gels were washed in 2.5% (v/v) Triton X-100 for 30 min to remove sodium dodecyl sulfate. Gelatin substrate gels were then incubated in 50 mM-Tris–HCl, pH 7.4, 200 mM-NaCl and 5 mM-CaCl₂ for 24 h at 37°C. After incubation, the gels were stained with 0.1% Coomassie brilliant blue for 60 min at room temperature in a solution of acetic acid, methanol and distilled water (1:2:3, by vol.). After destaining, the gels were immersed in distilled water, scanned immediately and analysed with Image J for Windows (<http://imagej.nih.gov/ij/index.html>). Gelatinolytic activity was demonstrated as clear bands on a blue background.

Western blot analysis

PC3 cells exposed to fibroblast CM for 24 h and fibroblast cultures were lysed for 10 min on ice in complete radio-immunoprecipitation assay lysis buffer (0.1% SDS, 0.5% deoxycholate, 50 mM-Tris–HCl, pH 7.5, 150 mM-NaCl, 1% Nonidet P-40, 2 mM-ethylene glycol tetraacetic acid, 1 mM-sodium orthovanadate, 1 mM-phenyl-methanesulphonyl-fluoride and 1:1000 Protease Inhibitor Cocktail Set III (Calbiochem)). The lysates were clarified by centrifugation. An equal amount of protein from each sample was run on SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. Immunoblots were probed first with specific primary antibodies diluted 1:1000 (mouse anti-human α -SMA, α -SMA, A2547 from Sigma; rabbit anti-human Met, SC-161, rabbit anti-human E-cadherin SC-7870, mouse anti-human α -tubulin SC-58667 from Santa Cruz) in 1% BSA, 0.05% Tween 20 in Tris-buffered saline buffer (10 mM-Tris, pH 7.5, 100 mM-NaCl), and then with secondary antibodies conjugated with horseradish peroxidase

(AP132P and AP124P, 1:10 000; Millipore) washed, and developed with the ECL Advance Western blotting detection kit (GE Healthcare).

Immunofluorescence

Cells, cultured on 25 mm coverslips, were fixed in 4% paraformaldehyde and membranes were permeabilised in 0.1% Triton X-100 solution. Coverslips were incubated in blocking solution (PBS supplemented with 4% BSA and 1% horse serum) and then incubated at 4°C overnight with anti- α -SMA primary antibodies, washed and incubated for 1 h with sheep Cy3 anti-mouse antibodies (C2181; Sigma).

Xenograft experiments

Male severe combined immunodeficient-bg/bg mice (6–8 weeks old; Charles River Laboratories International) were fed with a regular chow diet (Harlan Laboratories) and water *ad libitum*. The animals were injected subcutaneously with 100 μ l of PBS containing 1×10^6 PC3 cells plus 0.5×10^6 fibroblasts, incubated for 1 h at room temperature before injection. The animals were monitored daily and tumour size was measured every 2–3 d by a caliper; tumour volumes were determined by the formula:

$$LW^2/2,$$

where *L* and *W* are the length and width of the masses.

Mice were killed before the tumour masses exceeded a size to produce evident physical discomfort such as rough hair coat, lack of grooming activity or abnormal posture (huddling, hunching or being stiff).

Statistical analysis

Densitometric data were analysed by a multiple-comparison Student–Newman–Keuls test, after demonstration of significant differences among medians by non-parametric variance analysis, according to the Kruskal–Wallis test. Data are expressed as means and standard errors of the mean, depicted by vertical bars, of the indicated number of experiments.

Ethics approval

All experimental procedures involving animals were performed in accordance with national guidelines, approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and conformed to the legal mandates and Italian guidelines for the care and maintenance of laboratory animals.

Results

DHA prevents differentiation of transforming growth factor- β -stimulated fibroblasts into myofibroblasts

In the present study, before using our experimental model of different populations of prostate fibroblasts, we tested whether the parent *n*-3 compounds DHA and EPA or a

prototype of *n*-6 PUFA, AA, inhibited the TGF- β -stimulated differentiation of skin fibroblasts into myofibroblasts.

To determine the potency of DHA, EPA and AA as inhibitors, we used morphological evidences and Western blot analyses of α -SMA expression levels correlating with the myofibroblast-like phenotype. Among the long-chain fatty acids used in the experiments, DHA inhibited the differentiation of skin fibroblasts into myofibroblasts. In TGF- β -stimulated fibroblasts, 30 μ M-DHA abrogated the typical myofibroblast morphology characterised by a large number of actin stress fibres as well as α -SMA expression (Fig. 1(A) and (B)).

Organ-specific fibroblasts were isolated from the healthy region of a prostate gland resected from prostate adenocarcinoma patients (NCAF) and grown *in vitro* as reported in a previous paper⁽¹¹⁾. NCAF did not show aspects of myofibroblast differentiation, and only after the TGF- β treatment, they expressed α -SMA and the typical myofibroblast morphology. First, we investigated the effect of the AA, EPA or DHA treatment on NCAF viability, and we found that, among the PUFA used in the experiment, 60 and 90 μ M-DHA induced cell death both in NCAF and in TGF- β -stimulated NCAF (20–50% of cells; Fig. 2(A)). We also found that neither EPA nor AA was able to inhibit TGF- β -induced NCAF differentiation as demonstrated by the high levels of α -SMA expression in these fibroblasts (Fig. 2(B)). Immunofluorescence and Western blot analysis of α -SMA demonstrated that

30 μ M-DHA, a dose that did not modify viability of NCAF, inhibited NCAF differentiation into myofibroblasts (Fig. 2(C) and (D)). In addition, both EPA and AA at 60 and 90 μ M increased α -SMA, a finding indicating that the effect of DHA is not mediated through *n*-6 PUFA or AA, but acts through a different mechanism, otherwise EPA would have the same effects as DHA since they both antagonise AA equally (Fig. 2(B)).

In a previous paper⁽¹¹⁾, we demonstrated that TGF- β -stimulated NCAF promote aggressiveness of prostate adenocarcinoma cells; here we demonstrated that media conditioned by DHA-treated TGF- β -stimulated NCAF failed to promote the invasiveness of PC3 cells. Furthermore, we ascribed this effect to the reduced MMP-2 release by DHA-treated TGF- β -stimulated NCAF. Indeed, DHA, but not EPA, was able to inhibit MMP-2 release in TGF- β -stimulated NCAF (Fig. 2(E)). The dependence of PC3-enhanced migration from MMP-2 secreted by TGF- β -stimulated NCAF was strengthened by Ilomostat, a broad range MMP inhibitor, which reduced the increased capacity of PC3 cells to cross Matrigel layers (Fig. 2(F)).

DHA prevents the epithelial–mesenchymal transition in prostate adenocarcinoma cells

We previously demonstrated that activated fibroblasts through the secretion of MMP may promote the EMT in PC3 cells,

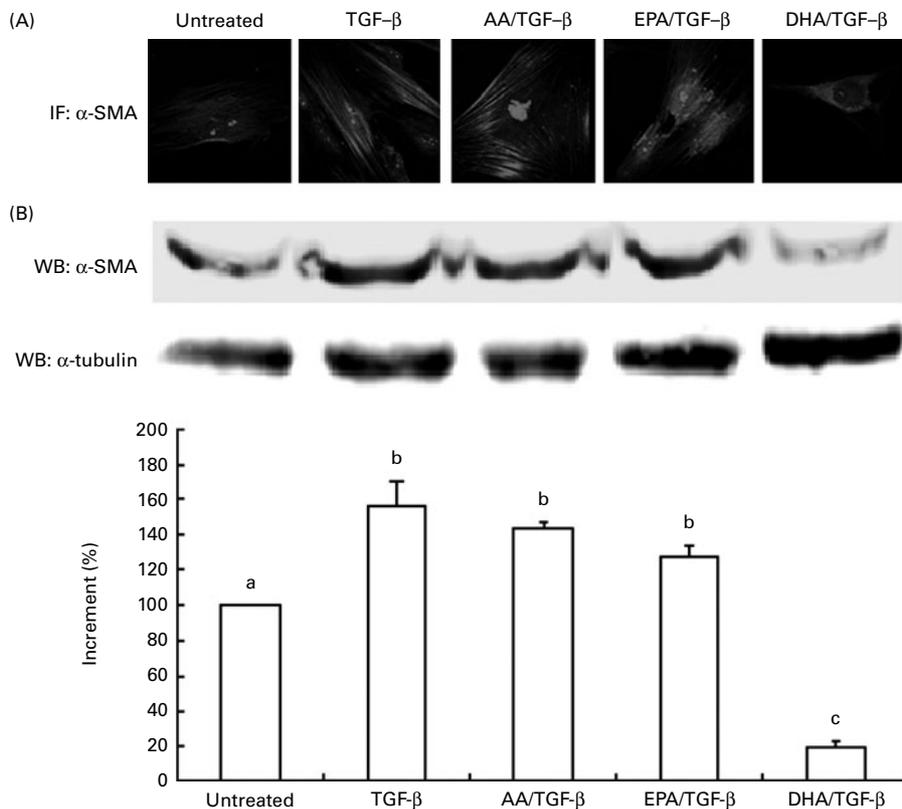


Fig. 1. (A) Immunofluorescence (IF; magnification $\times 100$) and (B) Western blot (WB) analysis of α -smooth muscle actin (α -SMA) expression in human dermal fibroblasts treated with 30 μ M of 20:4 *n*-6 arachidonic acid (AA), EPA or DHA and then stimulated with transforming growth factor- β (TGF- β). Values are means of three independent experiments, with their standard errors of the mean represented by vertical bars. α -SMA expression was quantified by a densitometric analysis of three blots, normalised against α -tubulin and expressed as the percentage of increment. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.005$). A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>

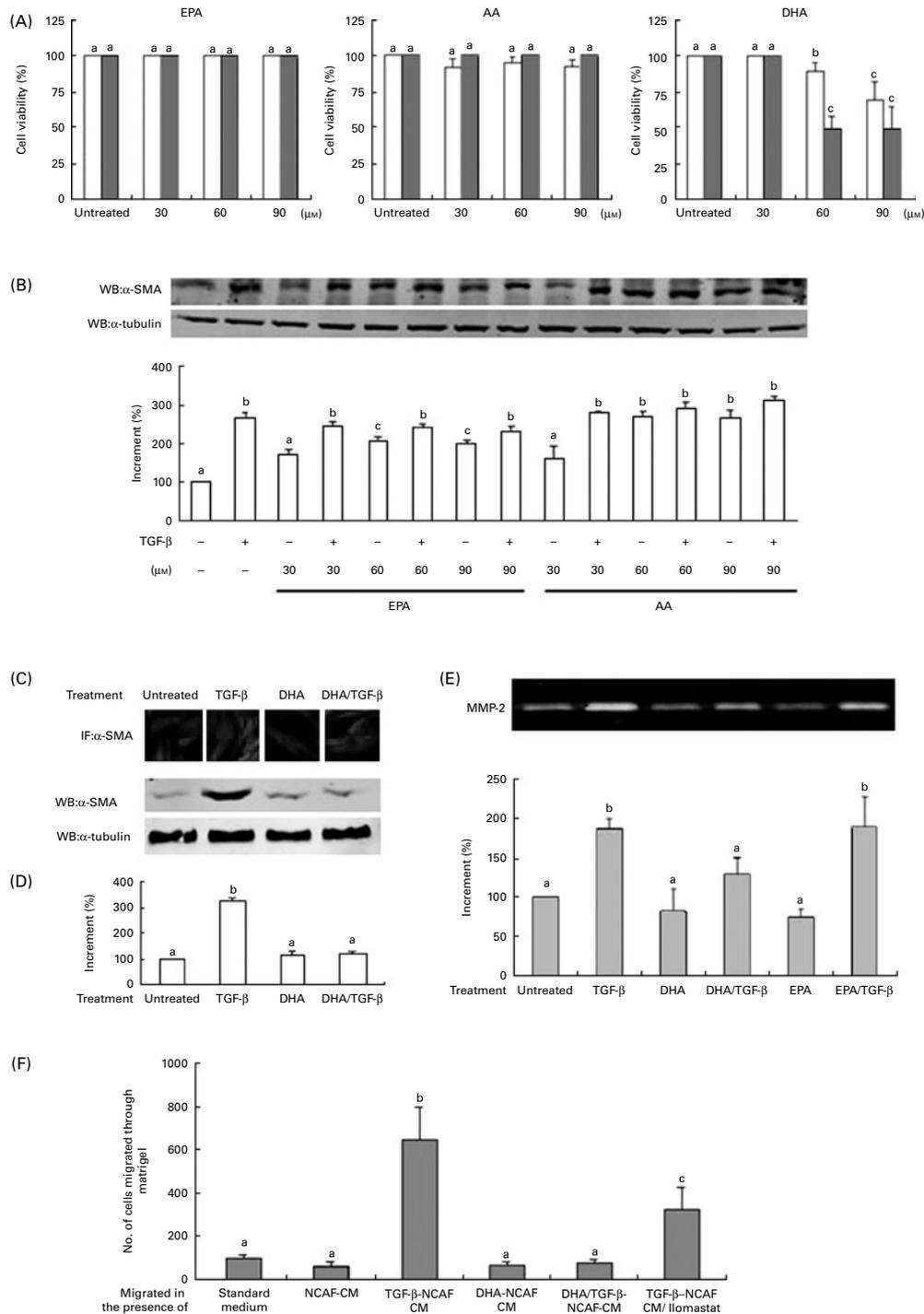


Fig. 2. Effect of PUFA on non-cancer-associated fibroblast (NCAF) differentiation. (A) Cell viability. Cell viability was determined by trypan blue exclusion assay in NCAF treated with 20:4*n*-6 arachidonic acid (AA), EPA or DHA at a dose range from 30 to 90 μ M and then stimulated with transforming growth factor- β (TGF- β) (quadruplicate haemocytometer counts of duplicate cultures). ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.01$). □, Unstimulated; ■, TGF- β stimulated. (B) α -Smooth muscle actin (α -SMA) expression in TGF- β -stimulated NCAF treated with EPA or AA (30–90 μ M) and densitometric analysis of three independent experiments (determined as in Fig. 1). ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.05$). (C) α -SMA expression in (30 μ M) DHA-treated TGF- β -stimulated NCAF determined by immunofluorescence and (D) Western blot (WB) and densitometric analysis of five independent experiments (determined as in Fig. 1). ^{a,b}Mean values with unlike letters were significantly different ($P < 0.005$). (E) Change in matrix metalloproteinase-2 (MMP-2) release in media conditioned by (30 μ M) DHA- or EPA-treated TGF- β -stimulated NCAF, zymogram and densitometric analysis of three independent experiments normalised by the number of NCAF. ^{a,b}Mean values with unlike letters were significantly different ($P < 0.002$). (F) Invasiveness through Matrigel of PC3 prostate adenocarcinoma cells exposed to fibroblast conditioned media (CM). Values are means of three independent experiments, with their standard errors of the mean represented by vertical bars. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.005$). PC3, human prostate carcinoma cell line. A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>

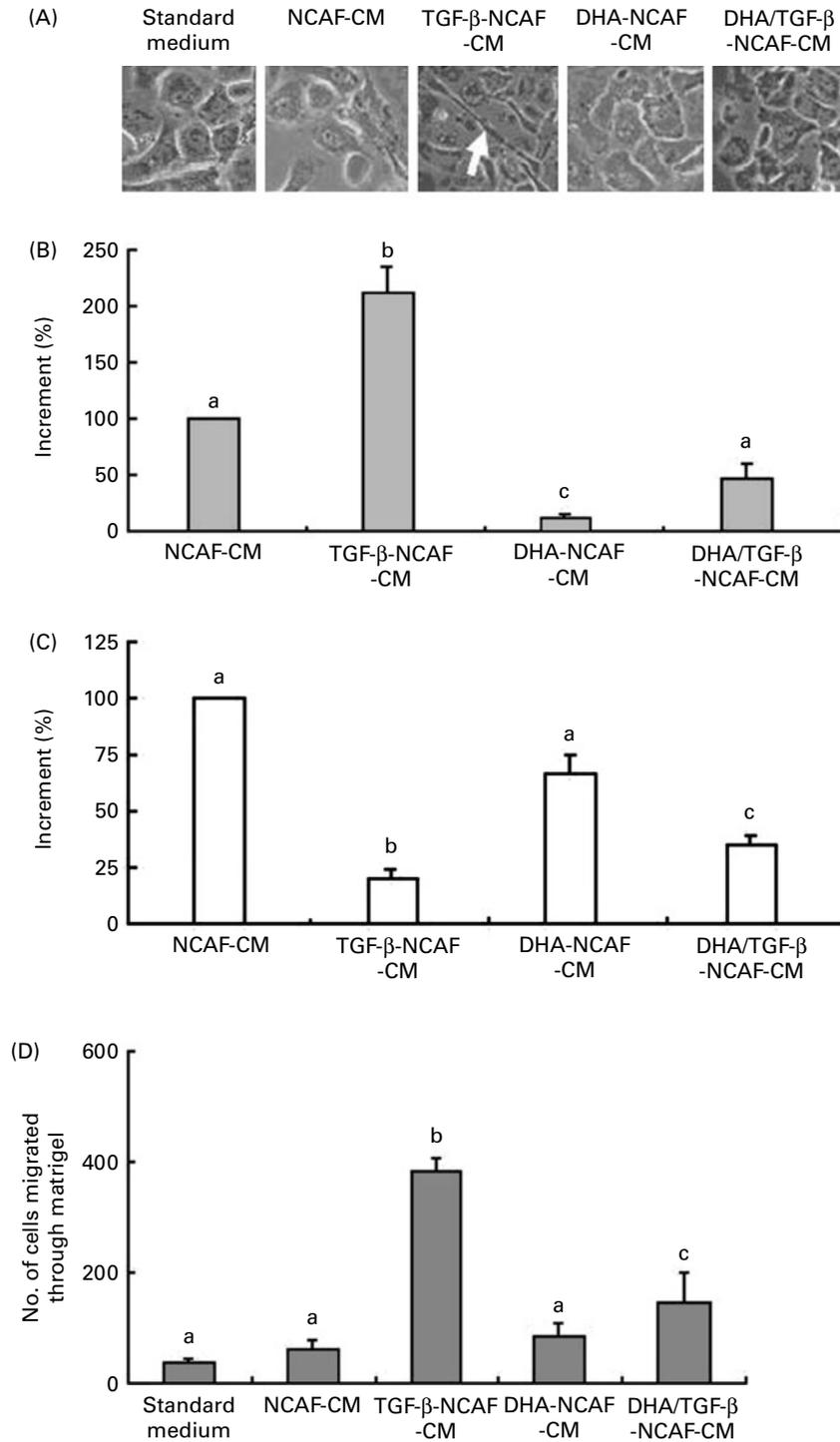


Fig. 3. Change in morphology, epithelial–mesenchymal transition markers and invasiveness of PC3 prostate adenocarcinoma cells grown in a medium conditioned by (30 μ M) DHA-treated transforming growth factor- β (TGF- β)-stimulated non-cancer-associated fibroblasts (NCAF). (A) Contrast microscopy images of PC3 cells exposed for 72 h to different NCAF conditioned media (CM). (B) c-Met (■) and (C) E-cadherin (□) Western blot densitometric analysis of three independent experiments (determined as in Fig. 1). ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). (D) Invasiveness through Matrigel of PC3 cells exposed for 72 h to the different CM. Values are means of three independent experiments, with their standard errors of the mean represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.005$). PC3, human prostate carcinoma cell line.

thereby enhancing cancer cell aggressiveness⁽¹¹⁾. To verify whether the MMT inhibited by DHA was also associated with an abrogation of the capacity of TGF- β -stimulated NCAF treated with DHA to stimulate the EMT in PC3 tumour cells, we analysed cell morphology, some specific markers of EMT and invasiveness of PC3 cells grown under different CM. The PC3 cells, which have undergone the EMT in media conditioned by TGF- β -stimulated NCAF, expressed a mesenchymal morphology, invaded Matrigel-coated filters in association with an enhanced expression of the proto-oncogene c-Met and reduction of E-cadherin (EMT promotes in tumour cells a mesenchymal phenotype, a reduced cell-to-cell contact as well as an increased migration exploiting degradation of three-dimensional matrices). Pre-treatment of TGF- β -stimulated NCAF with 30 μ M-DHA abolished the PC3 expression of mesenchymal morphology, c-Met expression and invasiveness (Fig. 3(A), (B) and (D)), and levels of E-cadherin were restored although not completely (Fig. 3(C)). Thus, the DHA treatment inhibited not only the MMT in NCAF but also the capacity of TGF- β -stimulated NCAF to induce several properties involved in the EMT of prostate cancer cells.

Xenografts of PC3 prostate adenocarcinoma cells co-injected with transforming growth factor- β -stimulated non-cancer-associated fibroblasts treated with DHA

Consequently, we performed an *in vivo* experiment in order to clarify the possibility that DHA exposure influences the capacity of TGF- β -stimulated NCAF to promote xenografts of PC3 prostate adenocarcinoma cells. The animals injected with either PC3 cells alone or PC3 cells together with NCAF (data not shown) did not produce subcutaneous tumours, while PC3 cells injected with TGF- β -stimulated NCAF induced tumours with a short latency (15 d) and high incidence

(100%). On the other hand, animals injected with PC3 cells together with 30 μ M-DHA-treated TGF- β -stimulated NCAF induced tumours with a significantly reduced growth rate, although incidence and latency were unchanged (Fig. 4). This implies that DHA-treated TGF- β -stimulated NCAF were unable to sustain a proper *in vivo* growth of prostate cancer cells.

DHA reverses the myofibroblast phenotype of prostate adenocarcinoma-associated fibroblasts

In order to demonstrate that DHA may also have the capacity to revert myofibroblast phenotype, we used CAF, obtained from a tumour region of an adenocarcinoma-bearing patient, confirmed by Western blot to be strongly positive for α -SMA. Since CAF promote PC3 invasiveness through an MMP-dependent pathway, there was no need for *in vitro* activation by TGF- β to elicit aggressiveness. Treatment of CAF with 30 μ M-DHA reversed α -SMA expression, MMP-2 release and PC3 migration through Matrigel, thereby confirming the capacity of DHA not only to inhibit fibroblast-to-myofibroblast differentiation, but also to revert some crucial aspects of the myofibroblast phenotype involved in the progression of prostate cancer cells (Fig. 5).

Discussion

A growing body of literature describes the reciprocal influence of CAF and tumour cells in prostate cancer progression^(8,11,13-15). These cellular interactions stably induce genetic and behavioural changes that can contribute to cancer progression, and this may provide a molecular basis for a therapeutic approach to change the natural history of prostate cancer. Thus, combining therapeutic strategies that co-target cancer cells as well as CAF will guide new opportunities to delay the progressive phenotype of prostate cancers.

We tested the hypothesis that DHA, a *n*-3 PUFA, directly inhibits the tumour-promoting effect on fibroblasts affecting their differentiation into myofibroblasts, and found that it does by blocking the prostate fibroblast MMT and also by reverting the myofibroblast phenotype.

The observation that DHA, unlike other long-chain fatty acids analysed (EPA and AA), blocks the MMT in skin fibroblasts stimulated by TGF- β prompted us to test DHA in our prostate fibroblast experimental model. In fact, DHA repressed myofibroblast transition in prostate NCAF upon their stimulation with TGF- β , considered one of the most potent, either *in vitro* or *in vivo*, mediators of the MMT. This is in keeping with the capacity of DHA to inhibit osteoclast differentiation, activation and function⁽²⁷⁾ and to modulate pro-inflammatory gene expression associated with colon carcinogenesis⁽²⁸⁾.

We also showed that abrogation of the MMT in NCAF by DHA was associated with a repression in the MMP-mediated enhanced invasiveness of PC3 cells. In parallel experiments, we demonstrated that abrogation of the MMT in prostate NCAF by DHA led to the inhibition of TGF- β -stimulated NCAF, induction of typical properties of the EMT in PC3 cells, such as a mesenchymal morphology, a reduced

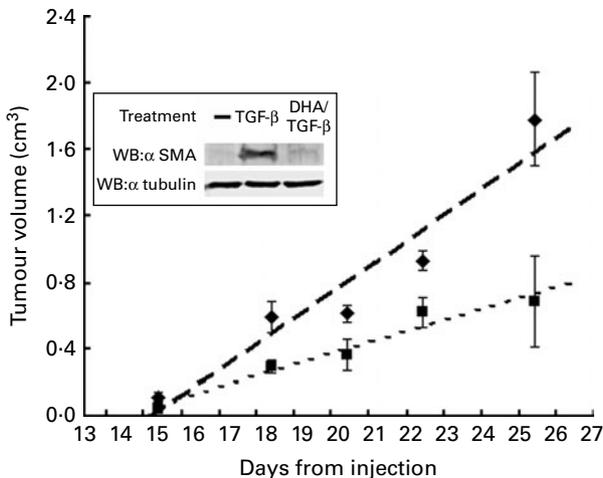


Fig. 4. Tumour growth of PC3 prostate adenocarcinoma cells co-injected with transforming growth factor- β (TGF- β)-stimulated non-cancer-associated fibroblasts (NCAF) or (30 μ M) DHA-treated TGF- β -stimulated NCAF. In the inset, α -smooth muscle actin (α -SMA) expression in TGF- β -stimulated NCAF and in DHA-treated TGF- β -stimulated NCAF analysed before co-injection with tumour cells. Comparison between regression coefficients (slopes) was performed by two-tailed Student's *t* test (*n* 4; *P*<0.02). ◆, PC3 co-injected with TGF- β -NCAF; ■, PC3 co-injected with DHA/TGF- β -NCAF. PC3, human prostate carcinoma cell line. WB, Western blot.

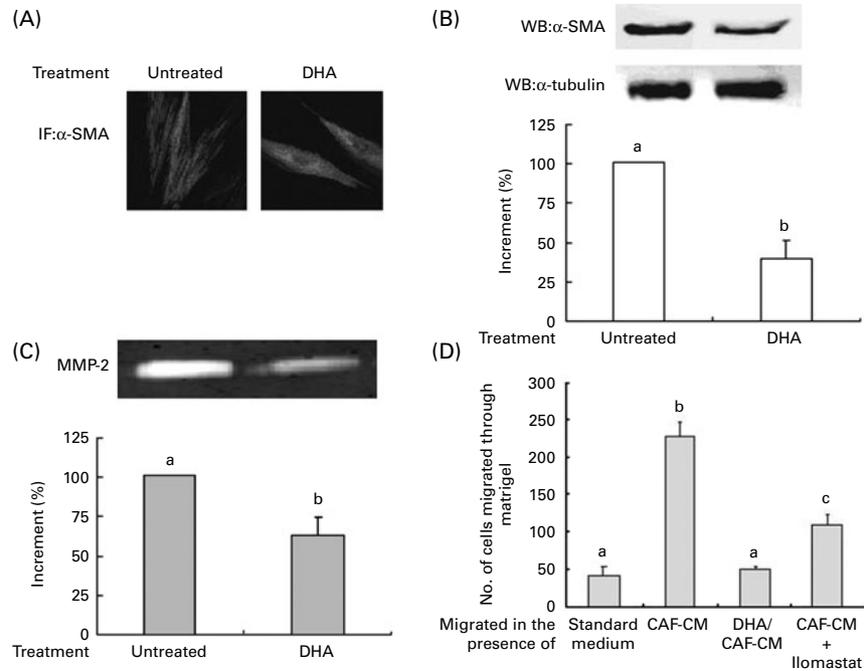


Fig. 5. Effect of (30 μ M) DHA on prostate cancer-associated fibroblasts (CAF). (A) α -Smooth muscle actin (α -SMA) expression in DHA-treated CAF determined by immunofluorescence (IF) and (B) Western blot (WB) and densitometric analysis of three independent experiments (determined as in Fig. 1). ^{a,b}Mean values with unlike letters were significantly different ($P < 0.005$). (C) Zymogram and densitometry analysis of matrix metalloproteinase-2 (MMP-2) released in media conditioned by CAF and DHA-treated CAF of three independent experiments normalised by the number of CAF. ^{a,b}Mean values with unlike letters were significantly different ($P < 0.002$). (D) Invasiveness of PC3 tumour cells exposed for 72 h to media conditioned by CAF or DHA-treated CAF. Values are means of two independent experiments, with their standard errors of the mean represented by vertical bars. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.005$). PC3, human prostate carcinoma cell line. CM, conditioned media. A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>

E-cadherin and an increased c-Met and invasiveness through Matrigel. It is well known that many types of cancer cells secrete molecules that enhance the production of hepatocyte growth factor, a ligand for c-Met receptor tyrosine kinase, by fibroblasts, and fibroblast-derived hepatocyte growth factor, in turn, stimulate in c-Met-expressing tumour cells an increased invasiveness towards the host's tissues. Overexpression of c-Met and increased production of hepatocyte growth factor in tumour tissues have been related to aggressive tumour growth and to a poor therapeutic outcome⁽²⁹⁾.

Finally, the DHA-treated TGF- β -stimulated NCAF co-injected with PC3 cells produced tumours in immunodeficient mice with a lower growth rate with respect to that of PC3 cells with TGF- β -stimulated NCAF. It is worth noting that this result is not applicable to a DHA dietary intervention in tumour prevention; however, together with *in vitro* results, it strengthens the concept of the inhibition of prostate myofibroblast differentiation by DHA.

A number of mechanisms might be involved in the DHA inhibition of prostate fibroblast MMT, including the influence on transcription factor activity and gene expression. It is possible that DHA might abrogate the reactive oxygen species-dependent expression of α -SMA and the pro-invasive signals in TGF- β -stimulated prostate fibroblasts. It has been shown that TGF- β increases the reactive oxygen species level in stromal fibroblasts, which initiate the MMT⁽³⁰⁾, and DHA enhances the antioxidant response of human fibroblasts by up-regulating γ -glutamyl-cysteinyl ligase and glutathione reductase⁽³¹⁾.

In conclusion, the present findings suggest a new activity of DHA in preventing the pro-tumorigenic effect of prostate adenocarcinoma-associated fibroblasts that may delay the progressive phenotype of prostate cancer cells.

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References

- Witz IP (2008) Tumour–microenvironment interactions: dangerous liaisons. *Adv Cancer Res* **100**, 203–229.
- Kalluri R & Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* **6**, 392–401.

3. Coussens LM & Werb Z (2002) Inflammation and cancer. *Nature* **420**, 860–867.
4. Calorini L & Bianchini F (2010) Environmental control of invasiveness and metastatic dissemination of tumor cells: the role of tumor cell-host cell interactions. *Cell Commun Signal* **7**, 24–34.
5. Orimo A, Gupta PB, Sgroi DC, *et al.* (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**, 335–348.
6. Erez N, Truitt M, Olson P, *et al.* (2010) Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* **17**, 135–147.
7. Allinen M, Beroukhim R, Cai L, *et al.* (2004) Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* **6**, 17–32.
8. Hill R, Song Y, Cardiff RD, *et al.* (2005) Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* **16**, 1001–1011.
9. Dvorak HF (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **25**, 1650–1659.
10. Hinz B, Phan SH, Thannickal VJ, *et al.* (2007) The myofibroblast: one function, multiple origins. *Am J Pathol* **170**, 1807–1816.
11. Giannoni E, Bianchini F, Masieri L, *et al.* (2010) Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. *Cancer Res* **70**, 6945–6956.
12. Sugimoto H, Mundel TM, Kieran MW, *et al.* (2006) Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther* **5**, 1640–1646.
13. Chung LW, Baseman A, Assikis V, *et al.* (2005) Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *J Urol* **173**, 10–20.
14. Olumi AF, Grossfeld GD, Hayward SW, *et al.* (1999) Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* **59**, 5002–5011.
15. Verona EV, Elkahlon AG, Yang J, *et al.* (2007) Transforming growth factor-beta signaling in prostate stromal cells supports prostate carcinoma growth by up-regulating stromal genes related to tissue remodeling. *Cancer Res* **15**, 5737–5746.
16. De Wever O & Mareel M (2003) Role of tissue stroma in cancer cell invasion. *J Pathol* **200**, 429–447.
17. Chavarro JE, Stampfer MJ, Li H, *et al.* (2007) A prospective study of polyunsaturated fatty acid levels in blood and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* **16**, 1364–1370.
18. Neuhouser ML, Barnett MJ, Kristal AR, *et al.* (2007) (n-6) PUFA increase and dairy foods decrease prostate cancer risk in heavy smokers. *J Nutr* **137**, 1821–1827.
19. Reddy BD (2004) Omega-3 fatty acids in colorectal cancer prevention. *Int J Cancer* **112**, 1–7.
20. Xia S, Lu Y, Wang J, *et al.* (2006) Melanoma growth is reduced in fat-1 transgenic mice: impact of omega-6/omega-3 essential fatty acids. *Proc Natl Acad Sci U S A* **103**, 12499–12504.
21. Berquin IM, Edwards IJ & Chen YQ (2008) Multi-targeted therapy of cancer by omega-3 fatty acids. *Cancer Lett* **269**, 363–377.
22. McEntee MF, Ziegler C, Reel D, *et al.* (2008) Dietary n-3 polyunsaturated fatty acids enhance hormone ablation therapy in androgen-dependent prostate cancer. *Am J Pathol* **173**, 229–241.
23. Rose DP & Connolly JM (1999) Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* **83**, 217–244.
24. Mannini A, Kerstin N, Calorini L, *et al.* (2009) An enhanced apoptosis and a reduced angiogenesis are associated with the inhibition of lung colonisation in animals fed an n-3 polyunsaturated fatty acid-rich diet injected with a highly metastatic murine melanoma line. *Br J Nutr* **101**, 688–693.
25. Reich R, Royce L & Martin GR (1989) Eicosapentaenoic acid reduces the invasive and metastatic activities of malignant tumor cells. *Biochem Biophys Res Commun* **160**, 559–564.
26. Chen TR (1977) *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp Cell Res* **104**, 255–262.
27. Rahman MM, Bhattacharya A & Fernandes G (2008) Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. *J Cell Physiol* **214**, 201–209.
28. Narayanan BA, Narayanan NK, Simi B, *et al.* (2003) Modulation of inducible nitric oxide synthase and related proinflammatory genes by the omega-3 fatty acid docosahexaenoic acid in human colon cancer cells. *Cancer Res* **63**, 972–979.
29. Matsumoto K & Nakamura T (2006) Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. *Int J Cancer* **119**, 477–483.
30. Cat B, Stuhlmann D, Steinbrenner H, *et al.* (2006) Enhancement of tumor invasion depends on transdifferentiation of skin fibroblasts mediated by reactive oxygen species. *J Cell Sci* **119**, 2727–2738.
31. Arab K, Rossary A, Flourié F, *et al.* (2006) Docosahexaenoic acid enhances the antioxidant response of human fibroblasts by upregulating gamma-glutamyl-cysteinyl ligase and glutathione reductase. *Br J Nutr* **95**, 18–26.