中 國 醫 藥 大 學 營 養 學 系

碩 士 論 文

二十二碳六烯酸和亞麻油酸抑制苯二甲酸誘發 MCF-7 人類乳癌細胞中基質金屬蛋白酶-9 表現機制之探討

Effect of Docosahexaenoic Acid and Linoleic Acid on TPA-Mediated MMP-9 Expression in MCF-7 Human Breast Cells

研究生：林俐伶 撰 Li-Ling Lin

指導教授：陳暉雯 博士 Haw-Wen Chen, Ph.D.

中華民國一百年七月

中國醫藥大學營養學系碩士班

論文口試委員審定書

論文題目：

中文：二十二碳六烯酸和亞麻油酸抑制苯二甲酸誘發 MCF-7 人類乳癌細胞中基質金屬蛋白酶-9 表現機制之探討

英文：Effect of Docosahexaenoic Acid and Linoleic Acid on TPA-Mediated MMP-9 Expression in MCF-7 Human Breast Cancer Cells

本論文係 林俐伶 於中國醫藥大學營養學系碩士班完成之碩士論文，經考試委員審查及考試合格，特此證明。

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中華民國 一百 年 七 月 六 日

中國醫藥大學營養學系

論文指導教授推薦書

營養學系碩士班：　林俐伶　君所提之論文

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係由本人指導撰述，同意提付審查。

指導教授　陳暉雲　(簽章)

中華民國　100　年　7　月　6　日

目錄

目錄 i
圖目錄 ii
表目錄 iv
縮寫表 v
中文摘要 vii
Abstract viii
第一部份 1
第一章　前言 2
第二章　文獻探討 3
第一節 乳癌 3
一、腫瘤介紹 3
二、乳癌的發生 4
三、乳癌分期與治療 7
第二節　癌細胞轉移過程 10
一、細胞外基質之組成與功能 10
二、癌細胞轉移 13
第三節 基質金屬蛋白酶和轉移的相關性 14
第四節 Mitogen-Activated protein Kinases (MAPKs)與轉移的相關性 17
第五節 PI3-kinase (phosphatidylinositol 3-kinas)/Akt 與轉移的相關性 19
第六節 轉錄因子 NF-κB 與 AP-1 對 MMP-9 基因表現的影響 21
第七節 脂肪酸的生理色與生化功能 23
第八節 血基質氧化酶(Heme oxygenase, HO) 25
研究目的 29
第二部分 30
Effect of Docosahexaenoic Acid and Linoleic Acid on TPA-Mediated 31
MMP-9 Expression in MCF-7 Human Breast Cells 31
參考文獻 62

圖目錄

第一部分

圖 1-1.乳癌常見的轉移位置 6

圖 1-2.細胞外基質的組成 12

圖 1-3.腫瘤細胞侵犯基底膜步驟 12

圖 1-4.腫瘤細胞轉移步驟 14

圖 1-5 基質金屬蛋白酶之蛋白質結構 15

圖 1-6. 有絲分裂原活化蛋白激酶的級聯反應 18

圖 1-7. Akt 的調節與活化 20

圖 1-8. 人類 MMP-9 基因啟動子區域的調節元素 21

圖 1-9. IκB 影響 NF-κB 調控路徑 22

圖 1-10. DHA 結構圖 24

圖 1-11. n-3、n-6 脂肪酸代謝路徑 24

圖 1-12. LA 結構圖 25

圖 1-13. 血基質氧化酶之作用及其代謝產物 27

第二部分

Figure 1. Effects of TPA with or without DHA or LA on MCF-7 Cell Viability 48

Figure 2. Effect of TPA on MMP-9 gene expression and enzyme activity in MCF-7 cells 49

Figure 3. Effect of DHA or LA on TPA-induced migration and invasion in MCF-7 cells 50

Figure 4. Effect of LA or DHA on TPA-induced MMP-9 expression in MCF-7 cells 51

Figure 5. Effect of protein kinase inhibitors on TPA-induced MMP-9 expression in MCF-7 cells 52

Figure 6. Effect of LA or DHA on TPA-induced MAPKs and Akt activation 53

Figure 7. Effects of LA or DHA on TPA-induced AP-1and NF-κB DNA-binding activity 54

Figure 8. Effect of DHA on HO-1 Expression of MCF-7 Cells in the Presence of TPA..55

Figure 9. Effect of siHO-1 on the inhibition of MMP-9 expression by DHA...................56

Figure 10. Model showing pathways that mediate the inhibition of expression of MMP-9 and metastasis and invasion of MCF-7 cells by DHA or LA..........................61

表目錄

表 1-1. 良性和惡性腫瘤之比較 4
表 1-2. 乳腺癌的危險因素 5
表 1-3. 乳癌的分期判斷 8
表 1-4. 乳癌的分期判斷 9
表 1-5. MMPS 的種類 16

縮寫表

AP-1	Activator protein-1
DHA	Docosahexaenoic acid
DMSO	Dimethylsulfoxide
ERK1/2	Extracellular responsive kinase1/2
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
JNK	c-Jun NH2-terminal kinase/stress-activated protein kinase
LA	Linoleic Acid
MMPs	Matrix metalloproteinases
MAPK	Mitogen activated protein kinases
MTT	thiazolyl blue tetrazolium bromide
NF-κB	nuclear factor kappa B
P38	p38 kinase
PKC	Protein kinase C
PD98059	phosphate-buffered saline
PBS	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran
SDS	sodium dodecyl sulfate
SB203580	4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)-1H-imidazol-4yl] pyridine
SP600125	Anthra[1-9-cd]pyrazol-6(2H)-one

TPA	12-O-tetradecanoylphorbol 13-acetate

中文摘要

基質金屬蛋白酶-9 (Matrix metalloproteinase-9, MMP-9)的表達在癌細胞轉移過程中扮演關鍵角色。不少研究顯示多元不飽和脂肪酸對多種人類癌細胞具有抗癌功效。但對於二十二碳六烯酸 (Docosahexaenoic acid, DHA)和亞麻油酸(Linoleic acid, LA)是否影響乳癌細胞轉移及其相關機制仍尚未清楚。本研究以 12-O-tetradecanoylphorbol-13-acetate (TPA)誘發 MCF-7 人類乳癌細胞轉移為實驗模式，探討 DHA 和 LA 對 TPA 所誘發的乳癌細胞移行(migration)及侵襲(invasion)之影響。實驗結果顯示，TPA 會活化 JNK、ERK1/2、PI3K 和 PKC 路徑並以劑量依賴關係誘發 MMP-9 酵素活性；給予 200 μM DHA 和 LA 會抑制 TPA 所誘發的 ERK1/2 和 AKT 磷酸化作用，並且顯著抑制 TPA 所誘發的 MMP-9 表現和癌細胞的移行及侵襲作用；先前研究結果顯示 NF-κB 及 AP-1 轉錄因子在啟動 MMP-9 基因轉錄活化過程中扮演重要角色，本研究利用 electrophoretic mobility shift assay (EMSA)證實 DHA 和 LA 亦可顯著減少 TPA 所誘發 NF-κB 及 AP-1 與 DNA 的結合能力。此外，本研究發現 DHA 可誘發 MCF-7 乳癌細胞血基質氧化酶(Heme oxygenase-1, HO-1)基因表現且呈現劑量和時間依賴性；並利用 siRNA 將 HO-1 基因 knockdown 後，原本受 DHA 所抑制的 MMP-9 酵素蛋白質表現及活性均可回復。綜合上述結果，DHA 及 LA 均可能透過抑制 ERK1/2 和 PI3K/Akt 訊號傳遞路徑的活化，減少 NF-κB 及 AP-1 對 MMP-9 基因的轉錄活化作用，最終抑制 TPA 誘發 MCF-7 乳癌細胞的移行和侵襲；此外，在 DHA 抑制 TPA 誘發 MMP-9 活化過程中，部分可能是透過活化 HO-1 表現所影響。本研究結果支持了多元不飽和脂肪酸可減少乳癌細胞轉移，因此具有降低乳癌進程之潛力。

關鍵字: 二十二碳六烯酸、亞麻油酸、基質金屬蛋白酶-9、轉移、MCF-7

Abstract

Matrix metalloproteinase-9 (MMP-9) plays a crucial role in the tumor metastasis. Previous studies showed that polyunsaturated fatty acids exhibited anti-cancer effect in various human carcinoma cells. However, the effects of docosahexaenoic acid (Bokor et al.) and linoleic acid (LA) on metastasis of breast cancer cells have not been fully clarified. The model of TPA-induced MCF-7 breast cancer cell metastasis was used in this study. The results showed that TPA-induced MMP-9 gene expression and enzyme activity in a dose-dependent manner, and 200 μM DHA and LA significantly decreased the TPA-induced MMP-9 expression, cell migration and invasion. Treatment with JNK, ERK1/2, PI3K, and PKC inhibitors caused a marked decrease in TPA-induced MMP-9 expression; however, only TPA-induced phosphorylation of ERK1/2 and Akt was attenuated by DHA and LA. The result of EMSA showed that DHA and LA decreased TPA-induced NF-κB and AP-1 DNA binding activity. Moreover, DHA, but LA, dramatically increased HO-1 expression in a dose- and time-dependent manner. HO-1 siRNA alleviated the DHA inhibition of MMP-9 protein and enzyme activities in the presence of TPA in MCF-7 cells. Taken together, these results suggest that DHA and LA inhibites TPA-induced cell migration and invasion by reducing MMP-9 activation, mainly via ERK1/2 and PI3K/Akt pathways and sequentially NF-κB and AP-1 trans-activation. Furthermore, the inhibition of TPA-induced MMP-9 activation by DHA is at least in part through induction of HO-1 expression in MCF-7 cells.

Keywords: Docosahexaenoic acid; Linoleic acid; MMP-9; Migration; MCF-7

第一部份

第一章 前言

惡性腫瘤自民國 71 年起，即列為國人十大死因之首。根據中華民國行政院衛生署於 100 年 6 月 15 日所公布統計資料顯示，99 年十大主要死因死亡人數占總死亡人數的 75.3％，其中仍以惡性腫瘤占 28.4％最多；其次分別為心臟疾病占 10.8％、腦血管疾病占 7.0％。台灣女性乳癌發生率的增加速度為所有癌症之冠，而乳癌的死亡率位居女性癌症第四名，其中更有 30％至 40％的乳癌患者死於癌症轉移後的疾病(Weigelt et al., 2005)。

乳癌的致死率主要來自於乳癌細胞的高度轉移能力，使癌細胞易擴散並侵犯鄰近組織、器官所致。隨著生活型態的改變及飲食習慣的日漸西化，近年來，台灣女性乳癌好發年齡已有逐年下降的趨勢，其發生高峰為四十歲至六十歲之間，相較於歐美國家，台灣女性的乳癌好發年齡，平均小約十歲。研究指出乳癌之發生與飲食攝取的油脂有密切關係，然而，脂肪酸除了作為生物能量所需來源外，也是細胞膜上磷脂質組成之重要成分，飲食中的 n-3 及 n-6 脂肪酸會影響二十碳烯酸（Eicosanoids）的代謝，對於在維持人體正常生長和發展、調控細胞生理、生化及代謝扮演著重要的角色(Simopoulos, 2000)。文獻指出，二十二碳六烯酸(Docosahexaenoic acid, DHA)被證實具有延緩異種移植(xenograft)的生長、減少化學誘導性腫瘤之生長及發展，並可增加人體對化療藥物的敏感性(Kim et al., 2009; Mandal et al., 2010; Shao et al., 1995; Sun et al., 2008)；另有文獻證實，亞麻油酸(Linoleic Acid, LA)或其代謝產物也可抑制結腫瘤細胞生長及發展(Lu et al., 2010b; Yasuda et al., 2009; Zuo et al., 2006)。

因此，本研究將探討 n-3 多元不飽和脂肪酸 DHA 及 n-6 多元不飽和脂肪酸 LA 是否具有降低乳癌死亡率增加相關之癌細胞移行(migration)和侵襲(invasion)之作用為主，並進一步瞭解其可能作用機制，以期望達到預防保健之功效。

第二章 文獻探討

第一節 乳癌

一、腫瘤介紹

癌症的發生會隨著性別、年齡、飲食、生活習慣、遺傳、種族、以及地理環境等因素所影響。自民國71年起，惡性腫瘤即列為國人死因之首位。根據民國100年衛生署所公布統計資料顯示，99年十大主要死因死亡人數占總死亡人數的75.3%，其中仍以惡性腫瘤占28.4%最多，因此，如何有效預防與治療癌症是台灣現今重要的醫療照護與公共衛生課題之一。

Neoplasm 或 Tumor 在中文的意義為贅瘤或腫瘤，是一種組織的異常腫塊(Masson and Mensink)，多屬於自主性的組織新生物(autonomous new growth)，其生長超過正常組織所需，且可不受任何拘束而任意生長(Willis, 1952)。所有的腫瘤不論良性或惡性，都有兩個基本組成：(1)腫瘤的實質(parenchyma)由增殖的贅生細胞構成；(2)支持性的間質(supportive stroma)由結締組織和血管組成。依其分化程度、生長速率、局部侵犯和轉移程度可將腫瘤分為兩種(見表 1-1)：(1)良性腫瘤(benign tumors):多由分化良好細胞組成實質細胞，此細胞不管在形態上或功能上與來源細胞較為相似，也因為生長較為緩慢因此常在周邊出現纖維莢(fibrous capsule)，與外圍宿主組織分隔，不易造成腫瘤侵犯(invasion)及轉移(metastasis)；(2)惡性腫瘤(malignant tumors):多由分化不完全或未分化細胞組成，細胞失去分化能力的回變(anaplasia)癥候為惡性轉變的重要指標。惡性腫瘤細胞與來源細胞的差異頗大，惡性腫瘤細胞與細胞核具有多形性(ploymorphism)，且喪失細胞極性(loss of polarity)進而破壞細胞間原有的排列規則、胞核含大量且濃染(hyperchromatism)的 DNA，使細胞核不對稱增大，與良性及分化較良好的惡性腫瘤相較下，未分化的惡性腫瘤細胞有絲分裂(mitosis)較為旺盛。腫瘤生長速度與分化程度有關，分化程度越不完全腫瘤生長速度越快。惡性腫瘤生長比良性腫瘤快速許多，此與它和周邊正常組織分界不

明顯且缺乏細胞纖維莢膜包圍有關，因此容易隨著腫瘤的生長而侵犯周圍組織(local invasion)，進而造成癌細胞的轉移(Ramzi s. Cotran, 1999/6)。

特徵	良性	惡性
分化/回變	細胞分化良好，為原組織典型結構。	細胞不分化而產生回變，常常不是原組織典型結構。
生長速率	較為緩慢，有絲分裂趨近於正常。	通常較良性快速，有絲分裂異常增加。
局部侵犯	通常為聚成一團且膨大的明顯質塊，但不侵犯或浸潤周圍正常組織。	聚集成團而往外澎大造成，會浸潤到周圍正常組織造成局部侵犯。
轉移	缺乏。	越大或分化程度越不完全的原位癌細胞，越容易轉移。

表 1-1. 良性和惡性腫瘤之比較

二、乳癌的發生

隨著生活型態的改變及飲食習慣的日漸西化，近年來，台灣女性乳癌好發年齡已有逐年下降的趨勢，其發生高峰為四十歲至六十歲之間，相較於歐美國家，台灣女性的乳癌好發年齡，平均要小上約十歲。根據衛生署公佈民國 97 年癌症登記年度報告顯示，乳癌發生率為所有癌症之冠，且死亡率位居女性癌症第四名(行政院衛生署, 2011)。根據流行病學指出，罹患乳癌相關危險因素(見表 1-2)包含年齡、地理位置、社會經濟地位、內分泌與懷孕史(初潮及停經的年齡，懷孕和母乳餵養)，外源激素(服用荷爾蒙藥物和口服避孕藥)、生活方式(酒精，飲食，肥胖和活動型態)、良性乳房疾病史、放射線、骨質密度、IGF-1、化學預防劑，以及遺傳因素(BRCA1，BRCA2 ，P53，ATM，NBS1，LKB1 基因的突變)等(Dumitrescu and Cotarla, 2005)。

Factors that increase breast cancer risk		
	Breast Cancer Risk Factors	**Magnitude of risk**
Well-confirmed factors	Increasing age	++
	Geographical region (USA and western countries)	++
	Family history of breast cancer	++
	Mutations in BRCA1 and BRCA2 genes	++
	Mutations in other high-penetrance genes (p53, ATM, NBS1, LKB1)	++
	Ionizing radiation exposure (in childhood)	++
	History of benign breast disease	++
	Late age of menopause (>54)	++
	Early age of menarche (<12)	++
	Nulliparity and older age at first birth	++
	High mammographic breast density	++
	Hormonal replacement therapy	+
	Oral contraceptives recent use	+
	Obesity in postmenopausal women	+
	Tall stature	+
	Alcohol consumption (~1 drink/day)	+
Probable factors	High insulin-like growth factor I (IGF-I) levels	++
	High prolactin levels	+
	High saturated fat and well-done meat intake	+
	Polymorphisms in low-penetrance genes (see text)	+
	High socioeconomic status	+
	Factors that decrease breast cancer risk	
Well-confirmed factors	Geographical region (Asia and Africa)	--
	Early age of first full-term pregnancy	--
	Higher parity	--
	Breast feeding (longer duration)	--
	Obesity in premenopausal women	-
	Fruit and vegetables consumption	-
	Physical activity	-
	Chemopreventive agents	-
Probable factors	Non-steroidal anti-inflammatory drugs	-
	Polymorphisms in low-penetrance genes (see text)	-

表 1-2. 乳腺癌的危險因素(Dumitrescu and Cotarla, 2005)

腫瘤的散播可經由下列四種途徑: (1)侵襲；(2)直接播種到體腔或體表；(3)淋巴擴散；(4)血液循環散佈。

1. 侵襲(Invasion)：通常是一種局部之蔓延，惡性腫瘤不斷地浸潤、破壞周圍組織器官的生長狀態。

2. 體腔或體表播種(Seeding of body cavities and surface)：當惡性腫瘤穿透而進入開放的空間時，體腔或體表的播種就能發生，最常發生於腹腔之腫瘤，如胃癌破壞胃壁侵及漿膜後，癌細胞可脫落至到大網膜、腹膜、腹腔內器官表面，因而產生與原細胞癌相似的腫瘤細胞，這便是所謂之播種。
3. 淋巴擴散(Lymphatic spread)：癌細胞侵入淋巴管後會形成一種栓子(embolus)的形式，轉移到各處不同之淋巴結(lymph node)或器官，淋巴結侵犯是以淋巴結液自然流向而定，而位於外上方的乳癌最常轉移至同側腋下淋巴結，有時甚至可以進入體循環(systemic circulation)造成與血液散播相同的變化。
4. 血液循環散佈(Hematogenous)：腫瘤細胞以栓子的形式進入血管後，可隨血流到達遠處器官繼續生長，形成轉移瘤，而乳癌細胞最常藉由此一方式轉移到骨、肺和肝(圖 1-1)。

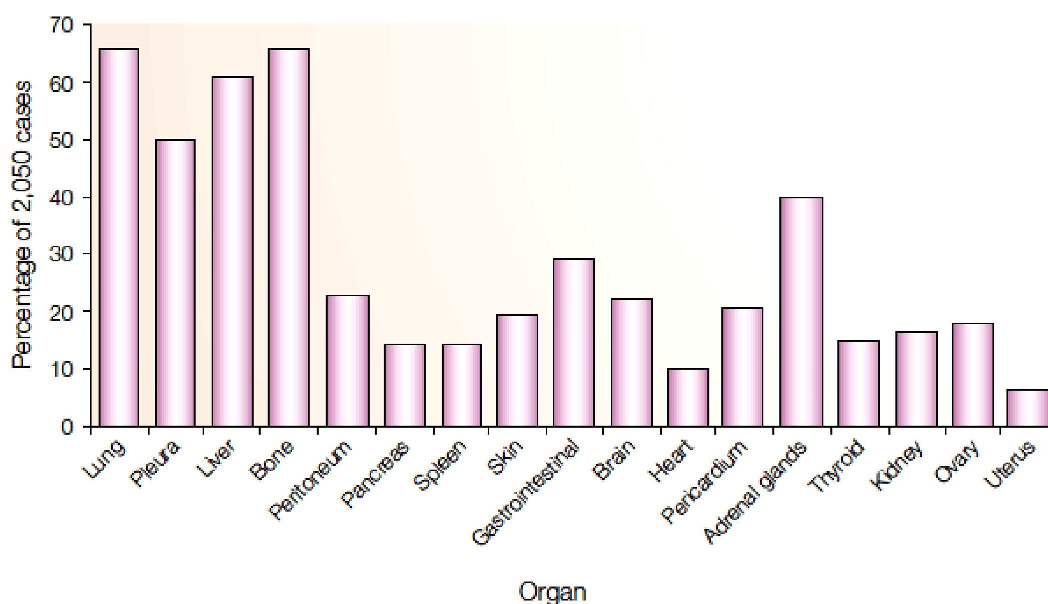


圖 1-1.乳癌常見的轉移位置(Weigelt et al., 2005)。

三、乳癌分期與治療

臨床上，病理性的分期是選擇治療模式及評估預後很重要的依據。美國聯合癌症委員會(American Joint Committee on Cancer , AJCC)最新乳癌的臨床分期標準如下：T為腫瘤大小，Tis為原位癌，N為淋巴轉移，M為遠處轉移，目前將乳癌分為零到第四期如(表1-3)、(表1-4)。

零期：即原位癌，癌細胞仍在乳腺管基底層內，是最早期發現的乳癌。

第一期：乳癌內的腫瘤小於兩公分以下，這種硬塊大部分是無痛性的。且被侷限在乳房組織內，還沒有淋巴腺轉移。

第二期：腫瘤介於兩至五公分之間，或腫瘤小於兩公分但腋下淋巴結有癌細胞轉移。

第三期：局部廣泛性乳癌，腫瘤大於五公分，且腋下淋巴結已有癌細胞轉移，或有胸壁、皮膚的浸潤乳癌。

第四期：轉移性乳癌，已有乳癌細胞轉移至遠處器官，最常見的是骨頭疼痛(骨轉移) ，也可能轉移到肺、肝、胸膜等器官。

T(腫瘤)：	
TX	原發病灶無法評估
T0	無原發病灶
Tis	原位癌(Carcinoma in situ)：管內癌(Ductal Carcinoma in situ, DCIS)、原位小葉癌(Lobular carcinoma is situ, LCIS)或無腫瘤之乳頭柏氏病(Paget's disease)。
T1	腫瘤的最大直徑 2 公分或小於 2 公分
T1a	最大直徑等於或小於 0.5 公分
T1b	最大直徑在 0.5 至 1 公分之間
T1c	最大直徑在 1 至 2 公分之間

T2	腫瘤的最大直徑介於 2 至 5 公分之間
T3	腫瘤的最大直徑大於 5 公分
T4	任何大小的腫瘤直接侵犯胸壁或皮膚
T4a	侵犯至胸壁
T4b	乳房皮膚水腫，潰瘍或附屬的皮膚結節
T4c	T4a+T4b
T4d	發炎性乳癌(Inflammatory carcinoma)
N(局部淋巴結)：	
NX	局部淋巴結無法評估(評估前已被切除)
N0	無局部淋巴結轉移
N1	同側腋下淋巴結轉移
N2	同側腋下轉移之淋巴相互緊縛(fixed)，或固定於附近組織結構。
N3	轉移至同側內乳淋巴結
M(遠處轉移)：	
MX	無法評估的遠處轉移
M0	無遠處轉移
M1	遠處轉移(包括轉移至鎖骨上的淋巴結)

表1-3. 乳癌的分期判斷(AJCC, 2010)。

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

表 1-4. 乳癌的分期判斷(AJCC, 2010)。

而目前治療乳癌方式包括:手術直接切除、放射線治療、化學治療及荷爾蒙治療(台灣癌症臨床研究發展基金會)。

（一）手術治療：

(1)改良式乳房根除術：將乳房及腋下淋巴結切除，僅保留胸大肌，使患者上臂活動能力不致受影響，並可搭配乳房重建手術以維持胸型外觀，適用於局部腫瘤切除，且無遠端器官轉移者。

(2)乳房保留手術：是將小於二公分腫瘤及腋下淋巴結切除，無腋下淋巴結病變者，也可施行此保守療法，切除的範圍僅包括腫瘤本身、直徑二公分內的組織，及下面深部肌膜。術後通常需要配合放射線治療。

(3)單純性全乳房切除手術：常用於乳房腺管原位癌之患者，手術範圍包括了整個乳房、乳頭、乳暈及胸肌的肌膜，但不包含腋下淋巴結清除，術後需加上腋下淋巴腺的放射線治療。

（二）放射線治療：使用高能量的放射線針對生長分裂較迅速的癌細胞進行破壞或停止其生長，一般在手術後會接受放射線治療以減少局部復發的機會。

（三）化學治療：通常合併多種化學藥物，這一類的藥物可對生長較快速之細胞進行直接的毒殺作用。化學治療的給藥方式通分為口服、皮下注射、肌肉或靜脈注射等，其中以靜脈注射較為常見。目前臨床用藥對於乳癌細胞的治療效果相當好，可有效降低局部復發並延長存活時間。

（四）荷爾蒙治療：是藉著荷爾蒙藥物去抑制乳癌的生長，臨床上，乳癌細胞上 estrogen receptor alpha (ERα)的表現與否，常作為乳癌患者荷爾蒙藥物治療之預後的重要指標，乳癌細胞為雌激素陰性者 ERα (－) 會抵抗對荷爾蒙治療的作用，因此預後也較雌激素陽性者 ERα (＋)差(Brinkman and El-Ashry, 2009)。

第二節　癌細胞轉移過程

癌細胞的轉移伴隨著細胞外基質的降解，以下先介紹細胞外基質的組成。

一、細胞外基質之組成與功能

細胞外基質(Extracellular matrix, ECM)位於細胞周圍，除了作為細胞與細胞間支撐與固定的複雜結構體，也在調節細胞的正常生理活動過程扮演著重要角色。ECM 主要由三種生物分子組成(圖 1-2)：(1)蛋白多醣(proteoglycan)：為 ECM 的主要成份，由一個核心蛋白(core protein)加上多個葡萄氨聚醣(glycosaminoglycans, GAGs)所組成；(2)結構蛋白: 包括膠原(collagen)、彈性蛋白(elastin)，這些蛋白可形成堅韌

的三股螺旋結構纖維；(3)特殊蛋白：層黏連蛋白(laminin)和纖維黏連接蛋白(fibronectin)，主要可以增加細胞和其他細胞或基底膜的黏附能力，與細胞的固定、分化、運動、型態有關(Weber, 1992; Yurchenco and Schittny, 1990)。

細胞膜表面可同時表現多種黏著蛋白，黏著蛋白本身不但具有接受器之功能，稱為黏著蛋白接受器(cell-adhesion receptor)，亦可作為其它細胞黏著蛋白接受器之配基(ligand)，與其進行交互作用，其中，細胞與 ECM 則主要透過插入素蛋白(integrins)傳遞細胞與細胞間各項訊息(Weber, 1992)。

在一般胚胎發育、組織重塑(tissue remodeling)、傷口癒合或懷孕等過程中細胞會釋放一些蛋白質分解酶，如(1)基質金屬蛋白酶(matrix metalloproteinases, MMPs)；(2)絲胺酸(Serine)蛋白酶；(3)半胱胺酸(cysteien)蛋白酶；(4) Aspartyl 蛋白酶(Melchiori et al., 1992)，以維持蛋白質正常代謝與細胞生理功能。然而，惡性腫瘤細胞所分泌的蛋白質分解酶雖與正常細胞相似，但其無法正常調控蛋白質分解酶的分泌量，進而促進癌細胞發生轉移。目前已知當蛋白質分解酶分泌異常時會導致各種病理過程，包括類風濕關節炎，骨關節炎，血管生成，侵襲和癌轉移(Chambers and Matrisian, 1997; Egeblad and Werb, 2002)。

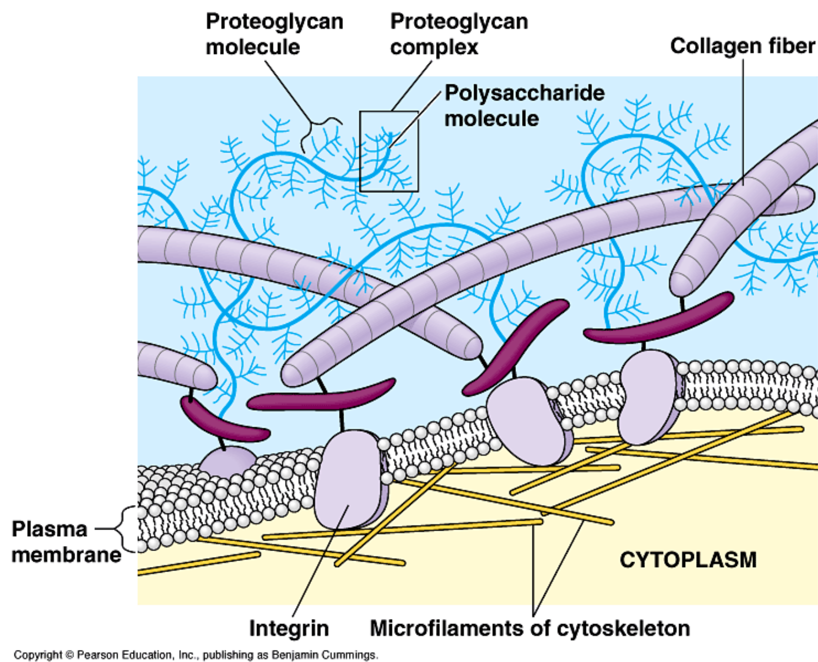


圖 1-2.細胞外基質的組成 資料來源: (kentsimmons.uwinnipeg.ca)

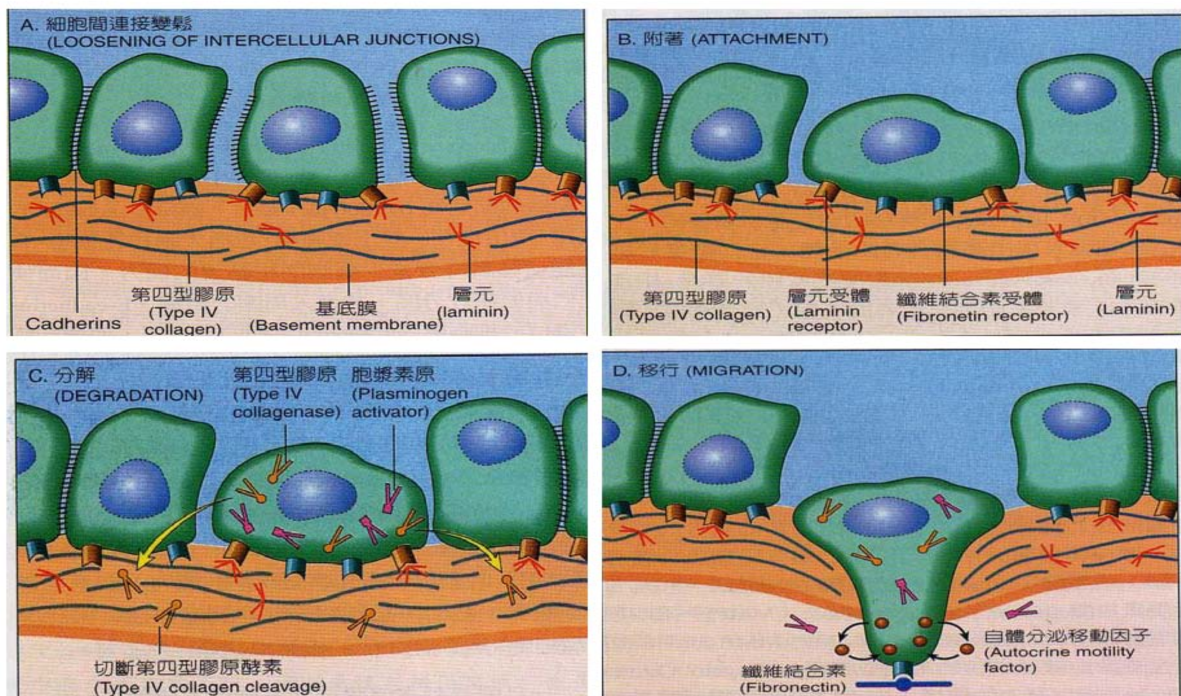


圖 1-3.腫瘤細胞侵犯基底膜步驟 (Ramzi s. Cotran, 1999/6) 。

二、癌細胞轉移

造成乳腺癌患者致死的主要因素大都是癌細胞轉移至其他組織和器官所引起(Woessner, 1991)，完整的癌細胞轉移是經過一連串複雜的步驟，其中包括了黏附(adhesion)、侵入(invasion)、遷移(migration)三個主要階段(Nicolson, 1988)，腫瘤細胞在轉移的過程中需要蛋白質分解酶降解細胞外基質，當癌細胞穿透基底膜後才可經由淋巴或血液循環轉移至其他組織或器官，其轉移過程如下列敘述：(圖 1-4)

1. 轉形(Transformation)：正常細胞經由變成轉型細胞(transformed cell)，當癌細胞不斷增殖後形成原發腫瘤(primary tumor)。
2. 分開(Detachment)：癌細胞彼此間鬆開；癌細胞會降低與其他細胞間吸附力(cell-cell adhiesion)及與細胞質間的附著力(cell-matrix adlhesion)，因而使它們與原病灶分開。
3. 附著(Attachment)：腫瘤細胞產生黏連並侵犯基底膜。
4. 侵襲(Invasion)：癌細胞降解細胞外基質後會侵入血管及淋巴管內和宿主淋巴細胞交互作用，並與血小板形成腫瘤細胞栓子。
5. 移行(Migration)：腫瘤細胞栓子經循環系統黏附到別處的基底膜，存活的癌細胞就能停留在新的組織或器官。
6. 血管新生(Angiogenesis)及生長(Growth)：腫瘤細胞在被轉移器官之組織中移行、增生，並產生轉移瘤(micrometastasis tumor)，此時，轉移瘤會藉由血管新生作用得以在新的組織或器官繼續生長，最後造成腫瘤細胞遠端器官或組織的轉移。

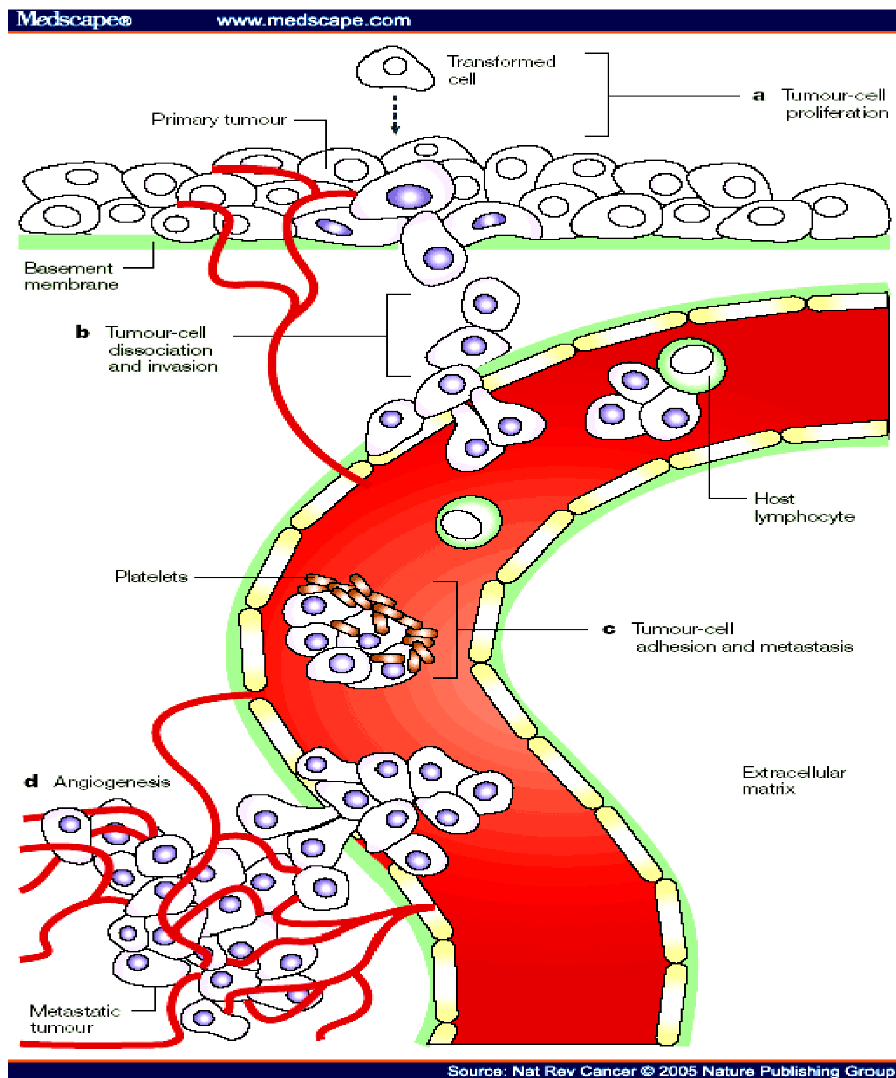


圖 1-4.腫瘤細胞轉移步驟 (資料來源: nature.com) 。

第三節 基質金屬蛋白酶和轉移的相關性

基質金屬蛋白酶(matrix metalloproteinase, MMPs)為一超級蛋白家族，其酵素活性在調節人體正常生理與病理活動過程中扮演重要角色。研究證據證實腫瘤生長、癌細胞轉移、侵襲及血管新生作用皆與基質金屬蛋白酶活化有關，因此，基質金屬蛋白酶常被作為人類癌症臨床診斷及治療的重要指標之一。

基質金屬蛋白酶屬於鋅依賴性的蛋白家族，幾乎能降解各種細胞外基質的結構(Roy et al., 2009)，圖 1-5 為基質金屬蛋白酶之蛋白質結構。

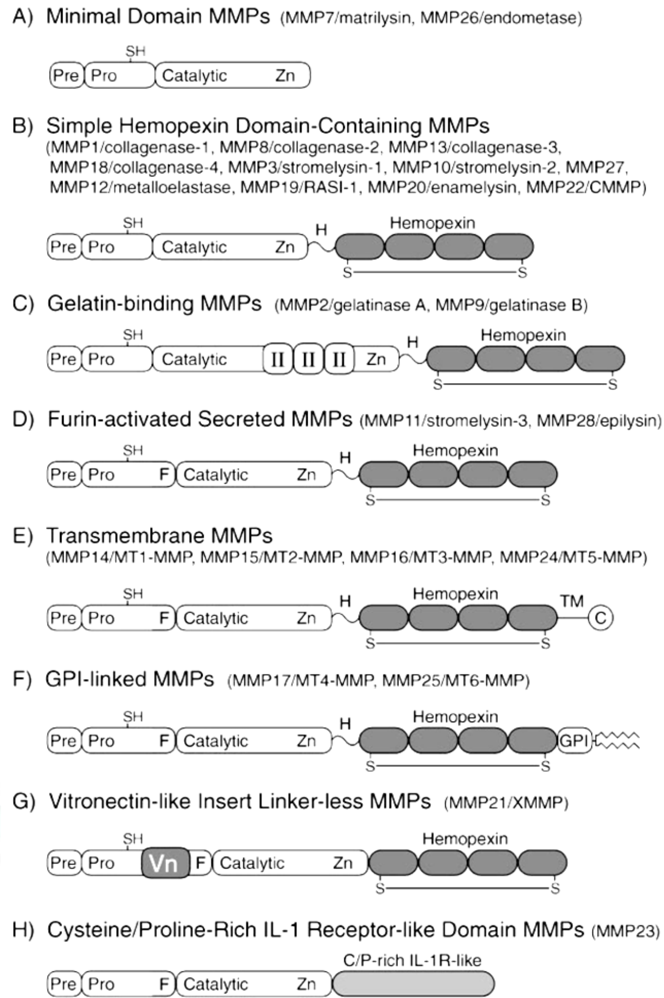


圖 1-5 基質金屬蛋白酶之蛋白質結構(Roy et al., 2009)。

根據基質的不同 MMPs 主要可分成四大類包括：(1)膠原蛋白酶；(2)明膠酶；(3)基質溶解酶；(4)膜型基質金屬蛋白酶，以及其它未被分類的蛋白分解酵素如(表 1-5) (Nelson et al., 2000)。

（1） 膠原蛋白酶 (Collagenases)：主要是降解纖維型的膠原蛋白，包括interstitial collagenase (MMP-1)、neutrophil collagenase (MMP-8)、collagenase-3 (MMP-13)及xenopus collagenase (MMP-18)。

（2） 明膠酶(Gelatinases)：主要降解第四型膠原蛋白，此為基底膜主要成份，包括gelatinase A (MMP-2)及 gelatinase B (MMP-9)。

（3） 基質溶解酶 (Stromelysins)：包括 stromelysin-1 (MMP-3)、stromelysin-2 (MMP-10)、stromelysin-3 (MMP-11)、matrilysin (MMP-7)。

（4） 膜型基質金屬蛋白酶(Membrane-type MMPs)：包括MT1-MMP (MMP-14)、MT2-MMP (MMP-15)、MT3-MMP (MMP-16)、MT4-MMP (MMP-17)和MT5-MMP (MMP-21)。

（5） 其它：包括Enamelysin (MMP-19、MMP-20、MMP-23和MMP-24)。

Table 1. Substrate-Based Classification of MMPs

MMP Family	Enzyme		Principal Substrates
	Descriptive Name	No.	
Collagenases	Interstitial collagenase	MMP-1	Fibrillar collagens, types I, II, III
	Neutrophil collagenase	MMP-8	
	Collagenase-3	MMP-13	
	Xenopus collagenase	MMP-18	
Gelatinases	Gelatinase A	MMP-2	Nonfibrillar collagens, types IV, V
	Gelatinase B	MMP-9	
Stromelysins	Stromelysin-1	MMP-3	Proteoglycans, laminin, fibronectin, nonfibrillar collagens
	Stromelysin-2	MMP-10	
	Matrilysin	MMP-7	
	Stromelysin-3	MMP-11	Serine protease inhibitors
Elastase	Metalloelastase	MMP-12	Elastin, nonfibrillar collagen
Membrane type			Progelatinase A, undefined
MT1-MMP		MMP-14	
MT2-MMP		MMP-15	
MT3-MMP		MMP-16	
MT4-MMP		MMP-17	
MT5-MMP		MMP-21	
Unclassified	Enamelysin	MMP-20	Undefined
		MMP-19	
		MMP-23	
		MMP-24	

表 1-5. MMPs 的種類(Nelson et al., 2000) 。

MMPs 都有相似的結構，從 N 端起分別有 signal peptide、propeptide、catalytic 和 C 端的 hemopexin-like 等四個 domains，MMPs 合成後是以非活化態的型態(Proenzyme)分泌，此時 propeptide domain 高度保留胱胺酸殘基(cysteine residue)，它們以氫硫鍵與 catalytic doamin 上的鋅離子結合，使 MMPs 維持在未活化的狀態，活化 MMPs 需要先要將連接於 cysteine 和鋅離子之間的鍵結打斷，半活化狀態的MMPs 要再將 propeptide domain 切斷，顯露出鋅離子，才可成為具有生理作用活性MMPs(active form)(Nagase and Woessner, 1999; Nelson et al., 2000)。活化的過程中會將 MMPs 結構中 N 端的 propeptide domain 切除，而失去 10kD 的分子量 (Nagase, 1997; Ries et al., 2007)。

MMP-2和MMP-9主要為降解基底膜中的第四型膠原蛋白(type IV collagen)，其與癌症的侵襲及轉移最具相關性(Brinckerhoff and Matrisian, 2002) (圖1-3)。曾有文獻指出MMP-2持續大量表現在高度轉移的腫瘤細胞，而MMP-9可透過生長因子，例如：epidermal growth factor(EGF)、transforming growth factor beta (TGF-β)(Ramirez et al., 2011)；促發炎細胞激素，例如：tumor necrosis factor-α (TNF-α)(Youn et al., 2011)；紫外線輻射(Kimura and Sumiyoshi, 2011)或12-O-Tetradecanoyl-phorbol-acetate (TPA)(Lin et al., 2008a)誘發其表現。研究證實，MMP-9 promoter具有NF-κB和AP-1 binding site(Chung et al., 2004)且誘發NF-κB和AP-1 trans-activation與增加MMP-9表現有關(Garg and Aggarwal, 2002; Lee et al., 2007)。

TPA 為一種 phorbol ester，是腫瘤促進劑(tumor promoter)同時也是蛋白質激酶 C (protein kinase C, PKC)的活化劑，TPA 可直接磷酸化 PKC 是因為在其結構中碳 12 的位置具有一長鏈的肉豆蔻酸(tetradecanoic acid)及碳 13 具有一短鏈的醋酸(acetic acid)，此與二醯甘油酯脂(diacylglycerol, DAG)有相似結構，故能直接活化由鈣離子所調節的磷脂依賴性蛋白激酶(phospholipids-dependent protein kinase)，啟動一連串細胞訊息傳遞(Griner and Kazanietz, 2007)。

根據研究指出 TPA 可透過有絲分裂原活化蛋白激酶(mitogen activated protein kinase, MAPK)、磷酸肌醇 3-激酶(phospho-inositol 3-kinase,PI3K)及 PKC 等傳訊路徑調控轉錄因子 NF-κB 和 AP-1 活化，進而促進 MMP-9 之表現(Blumberg, 1988)。

第四節 Mitogen-Activated protein Kinases (MAPKs)與轉移的相關性

MAPKs屬於serine/threonine kinases蛋白家族，當細胞受到外來刺激時，會藉由MAPK訊息傳遞路徑，將胞外訊號傳遞到細胞核，進而調控與細胞發炎反應(inflammination)、增生、分化、凋亡(apoptosis)及轉移等相關基因表現(Hammaker and Firestein, 2010; Johnson and Lapadat, 2002)。MAPKs級聯反應(cascade reaction)包括3

個順序的活化過程為：MAPK kinase kinase (MAPKKK)、MAPK kinase (MAPKK)及MAP kinase 如(圖1-6)。每一種激酶由不同成分所組成，MAPKKK由C-RAF1、MEKK1、MEKK2、TAK1等組成，MAPKK由MEK1/2、MKK1、MKK4、MKK5和MKK6等組成，MAPK包括ERK 1/2 (extracellar signal-regluatedkinase)，JNK/SAPK (c-Jun NH_2-terminal kinase)以及p38 MAPK (Johnson and Lapadat, 2002)。

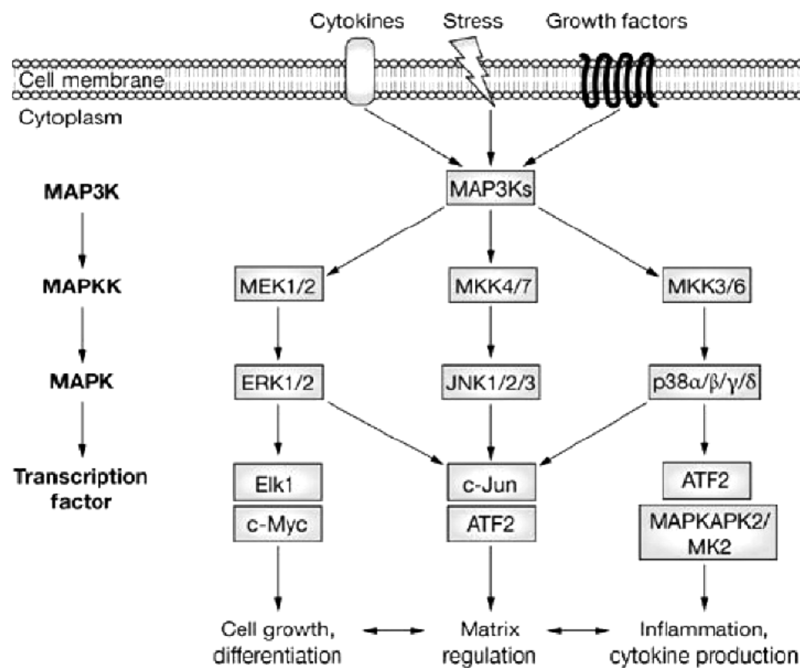


圖 1-6. 有絲分裂原活化蛋白激酶的級聯反應(Hammaker and Firestein, 2010) 。

當 ERK、JNK 和 p38 被活化時，會促進 NF-κB 和 AP-1 等轉錄因子的活化(Force and Bonventre, 1998)，許多研究中已證實可透過活化 MAPKs 訊息傳遞路徑上調 MMP-9 的表現(Hsieh et al., 2010; Hwang et al., 2011b)，因此抑制 MAPKs 路徑可能與抑制癌細胞侵襲及轉移有關，以下將介紹 ERK1/2、P38 與 JNK 路徑:

(1) Extracellular signal-regulated kinase (ERK1/2)：

主要有兩種異構體，分子量分別為 42 kDa 及 44 kDa，ERK 的角色主要是調控細胞的有絲分裂及分化，其可被生長因子、細胞激素、病毒感染和致癌蛋白 Ras 磷酸化而活化，而激活的 Ras 則經由 phosphorylation cascade 依序活化 Ras/Raf/MEK/ERK 進而刺激細胞不斷增生及分化，這亦是造成腫瘤細胞轉移的重要因子之一(Johnson and Lapadat, 2002)。在轉移機制方面，有文獻指出植物化學物質

(quercetin)能透過抑制 PKC/ERK/AP-1 路徑磷酸化而減少 TPA 所誘導的 MMP-9 表現，進而抑制 MCF-7 人類乳癌細胞侵襲和轉移之能力(Lin et al., 2008a)。

(2) c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK)：

主要分為三種異構體：JNK1、JNK2及JNK3；JNK1和JNK2分子量分別為46 kDa及54 kDa，它們普遍分佈於細胞和組織中，而JNK3 (57 kDa)主要表現於腦部、心臟及睪丸。當細胞受到細胞激素、生長因子或環境壓力(UV照射、熱休克、氧化壓力和高滲透壓)等刺激，會透過誘發上游MAPKK4 (MKK4/SEK-1)及MKK7的磷酸化，進一步磷酸化活化下游的JNK訊號傳遞，調控細胞生長、分化、凋亡及轉移(Barr and Bogoyevitch, 2001; Johnson and Lapadat, 2002)。

研究指出洋丁香(Common Lilac)中的acteoside 可以經由抑制JNK磷酸化進而減少NF-κB的轉錄活化作用，降低MMP-9表現，達到抑制人類纖維肉瘤細胞(HT-1080)轉移之能力(Hwang et al., 2011a)。因此，JNK MAPK訊號傳遞路徑在調控癌細胞轉移過程也扮演著重要的角色。

(3) p38 mitogen-activated protein kinase (p38 MAP kinase)：

主要有 α、β、γ 及 δ 四種異構體，細胞在受到發炎的細胞激素、UV 照射、熱休克和高滲透壓等刺激，會透過磷酸化上游的 MKK3 及 MKK6 而活化下游的 p38MAPK。p38 也被證實在調控發炎因子、細胞凋亡與生長上扮演重要角色(Johnson and Lapadat, 2002)。在 p38 調控轉移機制方面，曾有文獻指出紅辣椒中的活性成分辣椒素(capsaicin)可經由下調人類纖維肉瘤細胞中 p38 MAPK 磷酸化及 AP-1 的轉錄活化作用，而減少表皮生長因子所誘導的 MMP-9 表現(Hwang et al., 2011b)。

第五節 PI3-kinase (phosphatidylinositol 3-kinas)/Akt 與轉移的相關性

Phosphatidylinositol 3-kinaes (PI3Ks) 是一種脂質激酶(lipid kinase)，會將 phosphatidylinositol (PI)的3’-OH位置磷酸化(Fruman et al., 1998)，其家族包括Class I、II、III三大類。哺乳動物的Class I 酵素依結構和功能的不同有可分為IA、IB兩類，IA由催化次單位p110與調節次單位p85組成異質二聚體，可受酪胺酸激酶接受器所

活化，而IB次家族由p110γ催化次單位與p101調節單位組成，可受G protein偶合接受器(G protein-coupled receptor)所活化(Katso et al., 2001)。P-Akt也稱為蛋白激酶B (protein kinase B, PKB)，包括Akt1、Akt2和Akt3 (或PKBα/β/γ)三個異構體 (Hers et al., 2011)。在細胞中活化的PI3K將細胞膜Phosphatidylinositol-4,5-bisphosphate (PIP_2)磷酸化成phosphatidyl-inositol-3,4,5-trisphosphate (PIP_3)，PIP_3會與Akt的Pleckstrin homology domain (PH domain)結合，使Akt遷移至細胞膜附近，隨後 3-phosphoinositide-dependent protein kinase 1 (PDK1)及mammalian target of rapamycin complex 2 (mToRC2)與Akt結合並將Akt蛋白的Ser473和Thr308兩個氨基磷酸化(圖1-7)，進而調控細胞生長、增殖和凋亡等作用(Hers et al., 2011)。有許多文獻指出，活化PI3K/Akt訊號路徑與黑色素瘤、乳腺癌與胃癌進展呈現正相關性 (Dai et al., 2005; Nam et al., 2003; Perez-Tenorio and Stal, 2002)。

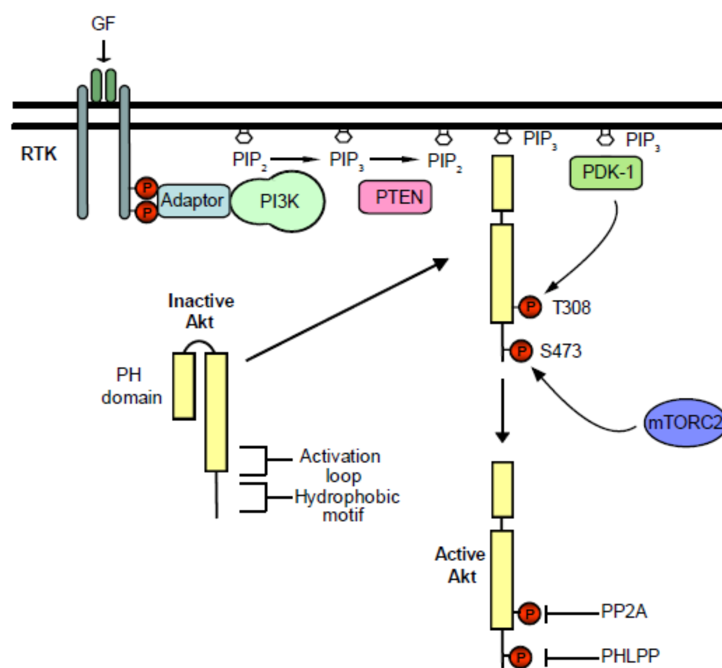


圖1-7. Akt 的調節與活化(Hers et al., 2011) 。

第六節 轉錄因子 NF-κB 與 AP-1 對 MMP-9 基因表現的影響

MMP-9基因的表現主要與NF-κB與AP-1兩種轉錄因子的活化有關(圖1-8)(Hwang et al., 2010)。

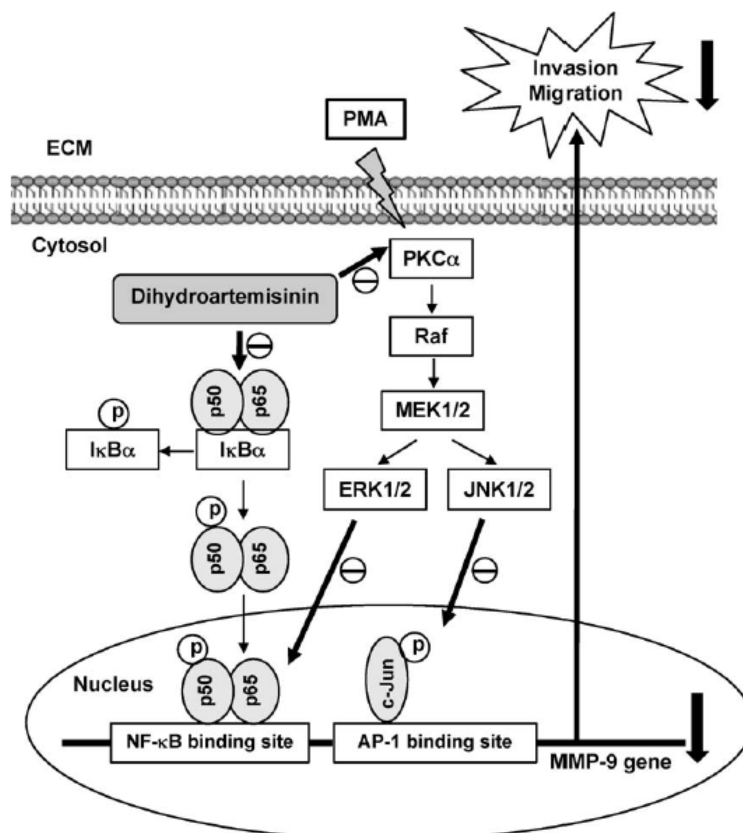


圖1-8. 人類MMP-9基因啟動子區域的調節元素(Hwang et al., 2010) 。

（1） NF-κB：

核轉錄因子NF-κB最初在B淋巴細胞中被發現，其與免疫球蛋白kappa light chain enhancer region上的B位置特定序列GGGACTTTCC結合(Verma et al., 1995)以調控kappa light chain的轉錄，因而稱之細胞核轉錄因子kB。NF-κB家族含有五種次單元包括Rel A (p65)、Rel B (p68)、c-Rel (p75)、NF-κB1 (p50)與NF-κB2 (p52)，能形成不同組合的同質雙聚體(homodimer)或異質雙聚體(heterodimer)，其中最常見的為p65/p50的NF-κB異質雙聚體(De Martin et al., 2000)。細胞未受刺激時NF-κB存在細胞質中並與活性抑制蛋白IκB (包括：IκB-α、IκB-β與IκB-γ)結合而呈現不活化狀態，當

細胞受到刺激，IκB-α會受到IKK (IκB kinase) complex磷酸化修飾，磷酸化的IκB-α隨即被泛素-蛋白酶體系統 (ubiqutin-proteasome system)所降解，IκB-α便脫離NF-κB，使NF-κB釋出具有活性的次單元由細胞質進入細胞核中，透過與目標基因啟動子之NF-κB基因結合位置結合，進行各種基因的轉錄調控作用 (圖1-9)(Hiscott et al., 2001)。研究證據證實，NF-κB在調控細胞發炎、免疫、細胞黏附因子、細胞凋亡與細胞遷移等過程中扮演重要角色。有文獻指出，與腫瘤侵襲與轉移高度相關的MMP-2和MMP-9基因啟動子上皆含有NF-κB轉錄因子的結合位置(Hwang et al., 2010)。

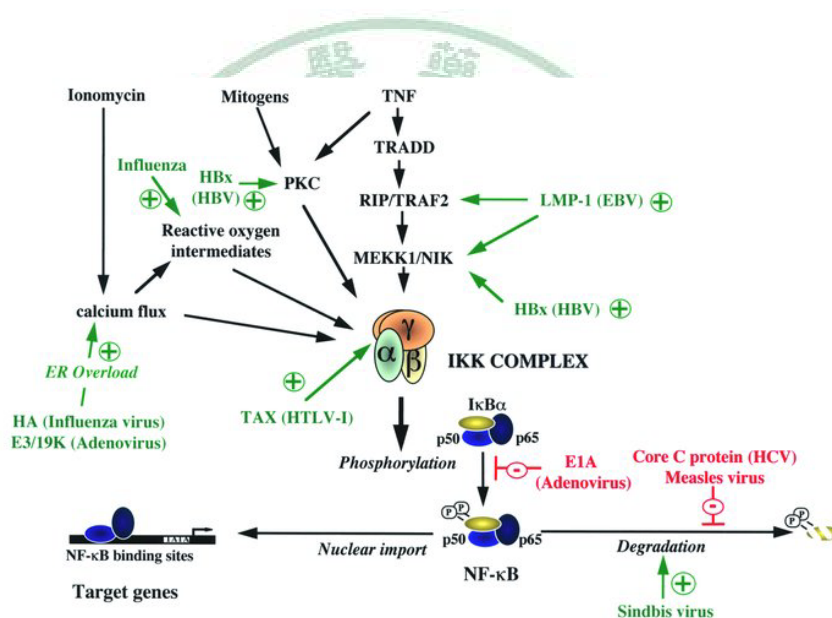


圖 1-9. IκB 影響 NF-κB 調控路徑(Hiscott et al., 2001)。

（2） AP-1：

轉錄因子AP-1調控許多細胞生理反應，包括細胞生長、分化和凋亡，其組成是由Jun family (c-Jun、v-Jun、Jun B 及Jun D)、Fos family (c-Fos, FosB, Fra-1及Fra-2）或其他含有basic region-leucine zipper (bZIP) domain的蛋白質，例如：ATF(包含ATF2, B-ATF, JDP1及JDP2)和Maf (包含Maf A, Maf B, c-Maf及Maf G/F/K)，以同質雙聚體或異質雙聚體形式存在(Mechta-Grigoriou et al., 2001)細胞質中，AP-1的轉錄活性與

其組成的次單位有關，其中以Jun-Fos及Jun-ATF family 結合形成異質雙聚體形式最穩定且與DNA有較高的結合活性。

當細胞受到發炎細胞激素(TNF-α、IL-1、IL-6等)細菌內毒素(LPS)、紫外線、TPA等因子刺激，可經由MAPKs訊息傳遞路徑活化AP-1，進一步啟動目標基因表現(Angel and Karin, 1991)。目前已知與腫瘤侵襲和轉移相關MMPs酵素包括MMP-2及MMP-9基因啟動子上皆含有AP-1轉錄因子的結合位置(Angel and Karin, 1991; Hwang et al., 2010)。

第七節 脂肪酸的生理色與生化功能

研究指出乳癌之發生與飲食攝取的油脂有密切關係。脂肪酸除了作為生物能量所需來源外，也是細胞膜上磷脂質組成之重要成分，飲食中的n-3及n-6脂肪酸參與荷爾蒙的合成(Spector and Yorek, 1985)及二十碳烯酸(eicosanoids)的代謝，對於在維持人體正常生長和發展、調控細胞生理、生化及代謝扮演著重要的角色(Simopoulos, 2000)。

（1）DHA：

Docosahexaenoic acid (DHA, C22:6 n-3)為二十二碳六烯酸(圖1-10)，屬於n-3多元不飽和脂肪酸(polyunsaturated fatty acids, PUFA)。DHA在人體中無法自行合成，需先藉由飲食攝取次亞麻油酸(alpha-linolenic acid, 18:3 n-3)，透過Δ^5和Δ^6去飽和作用(desaturation)及一連串的延長作用(elongation)，最後轉變而成(圖1-11)(Bokor et al., 2010)， DHA也富含於深海魚或魚油及藻類中，臨床研究報告指出，DHA在男性血漿平均濃度為(248.8 ± 107.0 μM)，女性為(279.9 ± 116.6 μM)(Welch et al., 2006)。

許多研究認為DHA可以預防多種疾病的發生。文獻指出DHA能夠促進腦細胞及神經的保護作用(例如提高細胞存活率及抗凋亡)，進而改善腦血管病變所導致的阿滋海默氏病(Alzheimer's Disease, AD)(Lukiw et al., 2005)。另外，動物及細胞實驗中證實DHA具有抗腫瘤之能力(Kato et al., 2002; Schonberg et al., 2006)，例如：DHA可

延緩異種移植(xenograft)的生長、減少化學誘導性腫瘤之生長及發展，並可增加人體對化療藥物的敏感性(Kim et al., 2009; Mandal et al., 2010; Shao et al., 1995; Sun et al., 2008)；以MCF-7人類乳腺癌細胞為研究模式，發現DHA會透過活化過氧化體增殖劑活化受器-γ (peroxisome proliferator-activated receptors-γ; PPARγ)提升syndecan-1表現，進而促使乳癌細胞凋亡(Sun et al., 2008)；另有研究利用人類乳癌細胞株(MDA-MB-231)注射到無胸腺裸鼠(NU/ NU)中的異種移植實驗模式，證實DHA可經由抑制黏附分子CD44的作用進而減少腫瘤細胞侵襲及轉移的發生(Mandal et al., 2010)，由上述文獻可得知，DHA可透過多種訊號傳遞路徑來影響癌細胞發展。

圖1-10. DHA結構圖（資料來源: commons.wikimedia.org） 。

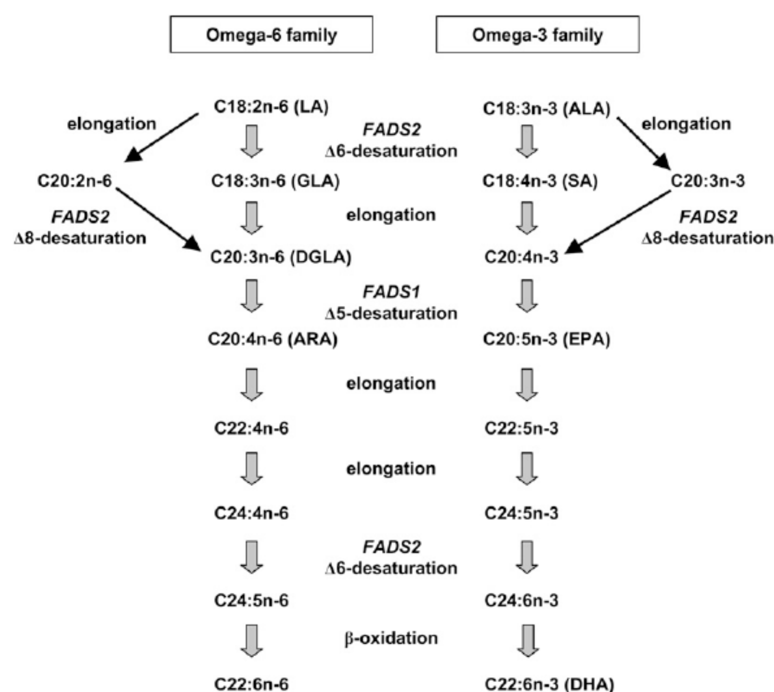


圖1-11. n-3、n-6脂肪酸代謝路徑(Bokor et al., 2010) 。

（2）LA：

Linoleic acid (LA, 18:2 n-6)為亞麻油酸(圖1-12)。人類體內的不飽和脂肪酸可分為四種：n-3、n-6、n-7和n-9型。n-7和n-9型脂肪酸屬於單元不飽和脂肪酸(Mono-Unsatarated Fatty Acid, MUFA)，可自行從飲食中攝取飽和脂肪酸(Satarated Fatty Acid, SFA)進一步合成，但n-3和n-6多元不飽和脂肪酸如次亞麻油酸(ALA, 18:3 n-3)和亞麻油酸是人類無法自行合成的，必須從飲食獲得，因此又稱為必需脂肪酸(Essential Fatty Acids, EFA)。亞麻油酸富含於大豆油、玉米油、葵花籽油、紅花籽油中，其在維持人類正常發育與健康不可或缺的成分。當人體缺乏亞麻油酸時可能會造成生長遲緩、頭髮乾枯，脫髮、皮膚炎、傷口癒合差、不孕等症狀(Cunnane and Anderson, 1997; Simopoulos, 2010) 。

以大腸癌細胞(LOVO and RKO)和人類正常細胞(HUVEC)為研究模式，證實低濃度的亞麻油酸(≤200 µM)下會促進大腸癌細胞增長，而高濃度(≥200 µM)的亞麻油酸劑量下則會誘導大腸癌細胞發生凋亡(Lu et al., 2010b)。動物及細胞實驗證明，亞麻油酸之代謝產物不僅可以減少皮膚炎症反應也可以增加癌細胞凋亡，進而抑制腫瘤之生長(Yasuda et al., 2009; Zuo et al., 2006)。

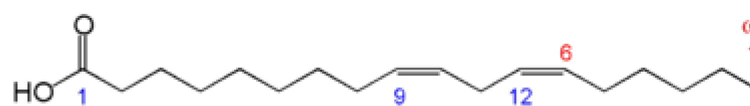


圖1-12. LA結構圖（資料來源: commons.wikimedia.org） 。

第八節 血基質氧化酶(Heme oxygenase, HO)

於 1968 年，血基質氧化酶首度被證實普遍存在大鼠的脾臟、肝、腎和骨髓等組織，且主要分布在細胞內的微粒體(microsomes)中(Tenhunen et al., 1968)。血基質氧化酶是血基質(heme)代謝過程中的速率限制酶，藉由催化血基質的分解，產生等莫耳數的膽綠素(biliverdin)、一氧化碳(carbon monoxide, CO)以及二價鐵離子(Fe^{2+})，

其中膽綠素會進一步經膽綠質還原酶(biliverdin reductase)轉換為膽紅素(bilirubin)，而鐵離子會誘發攜鐵蛋白(ferritin)的生成(圖 1-13)(Farombi and Surh, 2006)。研究發現，血基質的代謝產物，具有許多重要的生理功能，其功能分別敘述如下：

（1） 膽綠素和膽紅素：

已被證實能夠減少脂質過氧化反應、清除過氧化氫避免自由基的產生，有助於降低氧化壓力，減少血管局部缺氧的傷害(Baranano et al., 2002; Stocker and Ames, 1987)。

（2） 一氧化碳：

在體內高濃度的一氧化碳會產生傷害，但有研究指出低濃度一氧化碳卻具有類似一氧化氮(Nitrite oxide, NO)的功能，可作為訊息傳遞分子，活化血管平滑肌胞內的 soluble guanylate cyclase (sGC)，增加環狀鳥嘌呤單磷酸(cyclic guanosine monophosphate, cGMP)的生成，同時具有促使血管舒張、防止血小板凝集及抑制血管平滑肌增生等作用(Morita et al., 1997; Piantadosi, 2008)。

（3） 鐵離子：

游離鐵離子(free iron)對細胞是具有氧化毒性的，而血基質氧化酶分解血基質而產生的二價鐵離子(Fe^{2+})會誘發攜鐵蛋白(ferritin)的產生，攜鐵蛋白除了可捕捉游離鐵以減少氧化傷害之外，並可透過與細胞內鐵離子結合，增加細胞鐵儲存的效率，維持細胞鐵離子濃度的恆定(Balla et al., 2005; Poss and Tonegawa, 1997)。

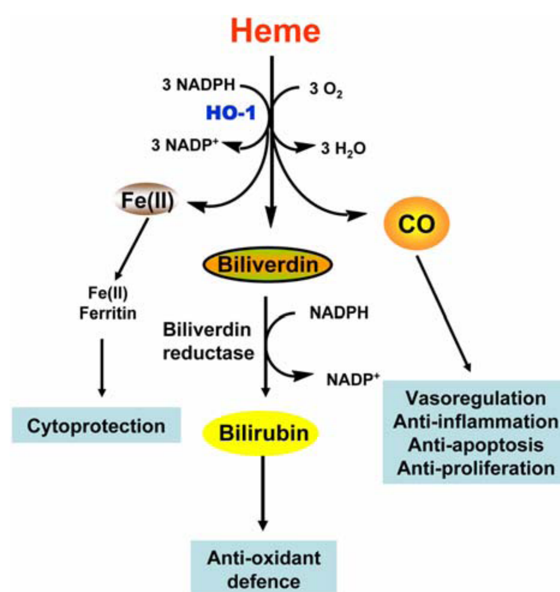


圖 1-13. 血基質氧化酶之作用及其代謝產物(Farombi and Surh, 2006) 。

（一）血基質氧化酶之類型

截至目前，哺乳類生物的HO依不同基因序列和蛋白質分布等特性，區分為HO-1、HO-2和HO-3三種不同亞型(Tenhunen et al., 1968)，分別為第一型血基質氧化酶(HO-1)，分子量約為32 KDa，為熱休克蛋白(Heat shock proteins, HSPs)的一種；第二型血基質氧化酶(HO-2)，分子量約為34 KDa；以及第三型血基質氧化酶(HO-3)分子量為約33 kDa。HO-2為持續表現型(constitutive expression)，存在各組織細胞中，其中以腦、神經系統、心血管以及睪丸等組織具有較高的濃度(Fan et al., 2011; Maines, 1997)；HO-3首度由老鼠腦中發現，存在於脾臟、肝臟、胸腺、前列腺、心臟、腎臟、大腦和睪丸中，HO-3活性為三種亞型中最低且催化能力最弱，其生理功能目前仍未釐清(McCoubrey et al., 1997)。其中HO-1屬於誘發型(inducible form)且為參與血基質代謝中最主要的亞型，HO-1容易被血基質或其他物質如過氧化氫、ultraviolet (UV)、氧化壓力、發炎的細胞激素及重金屬(Lin et al., 2005; Ryter et al., 2006)所誘發。許多研究證據顯示，當組織或細胞受到氧化損傷時會誘導HO-1大量表現，達到保護細胞之目的。HO-1不只能利用其代謝產物達到抗氧化、抗發炎、抗增生及抗細胞凋亡等作用 (Clark et al., 2000; Lee et al., 2009a; Petrache et al., 2000)，最近研究發

現，DHA可透過活化PI-3 kinase/AKT和MEK/ERK訊號傳遞路徑，誘發HO-1基因的轉錄作用，進一步降低BV-2腦微膠細胞(microglia)神經發炎反應(neuroinflammatory responses)，達到抗憂鬱之作用(Lu et al., 2010a)；也有文獻證實sulforaphane及quercetin等植物化學素(phytochemical)可藉由誘導HO-1的增加進而抑制肝癌及乳癌細胞的生長及遷移的能力(Cornblatt et al., 2007; Keum et al., 2006; Lin et al., 2008b)；更有文獻證明骨型態發生蛋白-6 (bone morphogenetic protein, BMP-6)顯著抑制MCF-7乳癌細胞移行及侵襲作用與其誘發HO-1基因表現進而抑制MMP-9活性有關，其中，BMP-6主要透過磷酸化活化Smad1/5蛋白質，並促使其結合至HO-1基因promoter上的Smad-responsive element，進而增加HO-1基因轉錄活性(Wang et al., 2011)。由上述文獻可得知，HO-1可經由多種訊號傳遞路徑所活化，進而達到抗發炎、抗氧化以及抑制腫瘤細胞生長等作用。

研究目的

根據中華民國行政院衛生署於100年所公布統計資料顯示，台灣女性乳癌發生率為所有癌症之冠，且乳癌的死亡率位居女性癌症第四名，乳癌致死原因主要來自於乳癌細胞具有高度轉移能力，使癌細胞易擴散並侵犯鄰近組織及器官所致。不少研究指出乳癌之發生與飲食攝取的油脂有密切關係，而脂肪酸除了作為生物能量的來源外，也是細胞膜上磷脂質組成之重要成分；飲食中的n-3 及n-6 脂肪酸比例會影響類二十碳烯酸(Eicosanoids)的代謝，對於在維持人體正常生長、發展、調控細胞生理、生化及代謝作用上扮演著重要的角色。在目前已有許多研究支持n-3 脂肪酸具有減少腫瘤之進展；然而，對於n-6 脂肪酸調控腫瘤生長及代謝作用仍具有爭議；因此本研究將探討二十二碳六烯酸(Docosahexaenoic acid, DHA)和亞麻油酸(Linoleic acid, LA)是否具有降低乳癌細胞轉移(metastasis)和侵襲(invasion)作用的能力，並進一步瞭解其可能調控的訊息傳遞路徑，以期透過飲食來降低乳癌的發生或惡化的進程。

第二部分

Effect of Docosahexaenoic Acid and Linoleic Acid on TPA-Mediated MMP-9 Expression in MCF-7 Human Breast Cells

Introduction

Breast cancer is the most common female cancer and is the second leading cause of cancer deaths in Western women. About 30% to 40% of women with this form of cancer will develop metastases and eventually die of this disease (Weigelt et al., 2005). According to the statistical data of Department of Health of Taiwan, the incidence of breast cancer has increased 4.5 fold in the past twenty years, and is the fourth leading cause of cancer death in Taiwanese women (Chang, 2006).

Metastatic spread of cancer cells is the main cause of death of breast cancer patients (Weigelt et al., 2005). Breakdown of the extracellular matrix (ECM) by proteinases is an essential step in cancer metastasis (Werb, 1997). Matrix metalloproteinases (MMPs), a family of ECM degrading proteinases, are divided into four subclasses based on the substrate including collagenases, gelatinases, stromelysin, and elastases (Nelson et al., 2000; Yan and Boyd, 2007). Activation of MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) is intensely correlated with the tumor invasion and metastasis in different types of cancer cell, including human breast (Blanckaert et al., 2010; Hanemaaijer et al., 2000), hepatoma (Zhao et al., 2011), prostate (Wegiel et al., 2008) and lung cancer cells (Kamaraj et al., 2010). In general, MMP-2 is constitutively expressed in highly metastatic tumors, whereas MMP-9 can be stimulated by the growth factor, such as epidermal growth factor and transforming growth factor beta (TGF-β) (Ramirez et al., 2011), the inflammatory cytokine such as tumor necrosis factor-α (TNF-α) (Youn et al., 2011), ultraviolet radiation (Kimura and Sumiyoshi, 2011), or phorbol ester (Lin et al., 2008a).

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, stimulates renal tumor cell proliferation through activation of protein kinase C (PKC)(Kolb and Davis, 2004). TPA-induced MMPs activation was mediated by modulating the activation of transcription factors such as NF-κB and AP-1 through PKC, PI3K and mitogen-activated protein kinase (MAPK) signaling pathways (Blumberg, 1988; Jang et al., 2007). Recent studies showed that the dietary factors such as α-lipoic acid,

capsaicin, and conjugated linoleic acid (CLA) are protective against cancer migration, invasion and angiogenesis by suppressing MMP-9 expression or enzyme activity (Hwang et al., 2011b; Kunigal et al., 2007)). In our previous study, phenobarbital-induced JNK1/2 and ERK2 activation was down-regulated by DHA (Lu et al., 2009) which suggests DHA may possess the ability to suppress the MMP-2 or MMP-9 activation. In other words, DHA can be the potential candidate for antitumor.

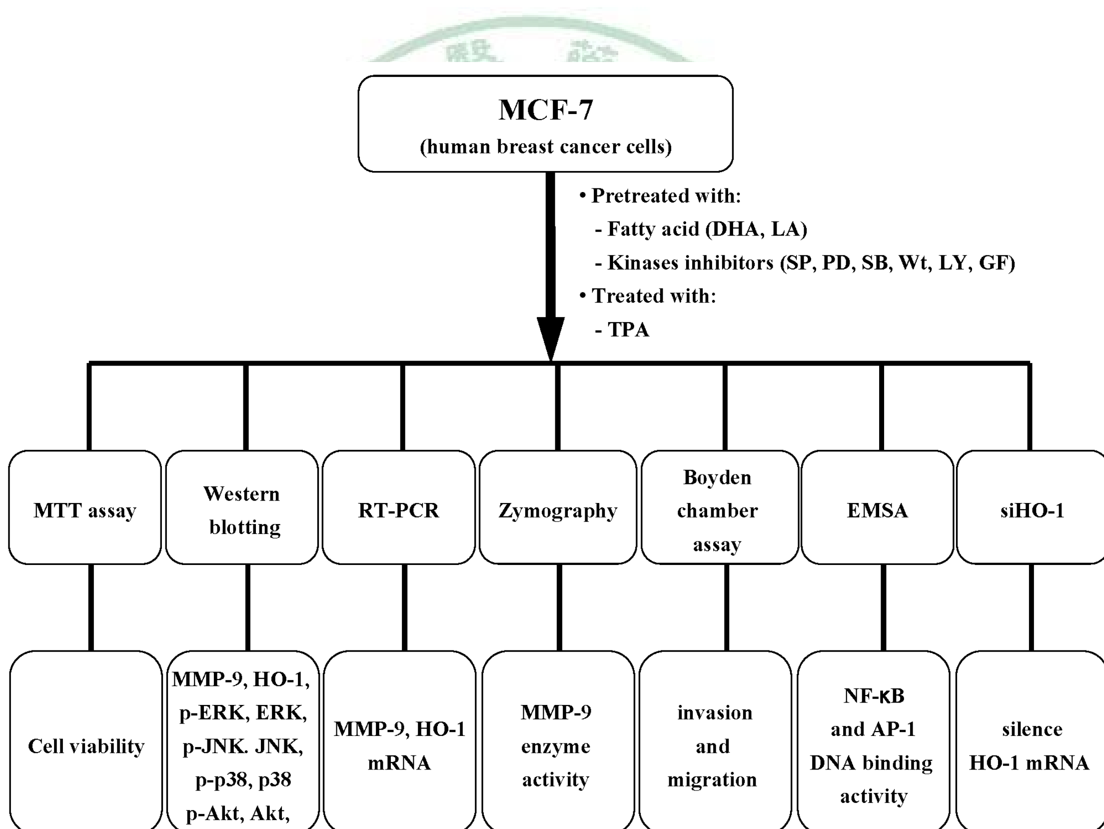
Dietary lipids are important to human beings because of their role in energy and essential fatty acids supplies. Linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) are essential fatty acids that must be obtained from diets. These polyunsaturated fatty acids (PUFAs) and their metabolic products play critical roles in a variety of physiological processes, such as regulation of inflammation (Masson and Mensink, 2011), insulin resistance (Perez-Martinez et al., 2011), blood pressure (Sagara et al., 2011) and lipid metabolism (Neff et al., 2011). Epidemiologic studies showed that high consumption of n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fatty fish is associated with a reduced risk for breast cancer (Kim et al., 2009). Experimental animal and cell culture studies provided evidences that dietary n-3 and n-6 PUFAs inhibit the promotion and progression stages of carcinogenesis (Lee et al., 2009b; Lu et al., 2010a; Sun et al., 2008).

Heme oxygenase 1 (HO-1) is one of the members of HO system. HO-1 is also known as HSP32 (heat shock protein of 32 kDa), and it is an inducible enzyme and expressed relatively low in most tissues under basal conditions. HO-1 is induced by a wide variety of stimuli such as ultraviolet A radiation, endotoxin and cytokines (Chung et al., 2011; Luo et al., 2011; Ronco et al., 2011; Xu et al., 2011; Zhong et al., 2010). In addition to anti-oxidant and anti-inflammatory activities of HO-1 (Seo et al., 2010), HO-1 has also been shown to possess anti-tumorigenic action in breast cancer cells (Li et al., 2011; Pae et al., 2010; Wang et al., 2011). It is also shown that HO-1 is induced by a wide array of phytochemicals through Nrf2 (Velmurugan et al., 2009). In addition to the above mentioned stimuli, induction of HO-1 by DHA in BV-2 microglia (Lu et al., 2010a) and mouse peritoneal macrophages (Wang et al., 2010) was reported. However, the effect of n-3 and n-6 PUFAs on HO-1 induction in human cancer cells lacks.

Because of the HO-1 induction capability of DHA, it is possible that DHA can exert antitumor activity. According to previous studies describing the antitumor activity of n-3

and n-6 PUFAs, we investigated the metastasis and invasion inhibition effects of n-3 and n-6 PUFAs in TPA-induced MCF-7 human breast cancer cell and the possible mechanism involved.

Materials and Methods



Chemicals

Dulbecco's Modified Eagle Medium (DMEM), OPTI-MEM, 25% trypsin-EDTA, and penicillin-streptomycin solution were from GIBCO-BRL (Grand Island, NY); fetal bovine serum (FBS) was from HyClone (Logan, UT); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), albumin, bovine serum essentially fatty acid free (BSA), sodium bicarbonate, calcium chloride, Triton X-100, 12-O-tetradecanoylphorbol 13-acetate (TPA), GF109203X (PKC kinase inhibitor), wortmannin, and LY294002 (PI3K kinase inhibitor) were from Sigma-Aldrich, Inc. (St. Louis, MO); SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 inhibitor) were from TOCRIS (Ellisville, MO); docosahexaenoic acid and linoleic acid were from Cayman Chemical (Ann Arbor, MI); collagen was from Collaborative Biomedical Products (Bedford, MA); TRIzol reagent was from Molecular Research Center, Inc (Cincinnati, OH); antibodies against Akt, phospho-Akt (T308 and S473), ERK1/2, phospho-ERK1/2, p38, and phospho-p38were from Cell Signaling Technology (Danvers, MA); antibodies against JNK1 and phospho-JNK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against HO-1was from Calbiochem (Darmstadt, Germany); and DharmaFECT 1 Transfection Reagent was from Dharmacon (Lafayette, CO).

Cell culture

The human breast cancer cell line MCF-7 was a kindly gift from Dr. Yi-Hsien Hsieh, Chung Shan Medical University, Taichung, Taiwan, and was cultured on collagen-coated cell culture dishes in DMEM (pH 7.2) supplemented with 1.5 g/L $NaHCO_3$, 10% FBS,

100 units/mL penicillin, and 100 μg/mL streptomycin at 37℃ in a 5% CO_2 humidified incubator.

Cell viability assay

Cell viability was assessed by the MTT assay. The MTT assay measures the ability of viable cells to reduce a yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan by mitochondrial succinate dehydrogenase. MCF-7 cells were grown to 70-80% confluence and were then treated with different concentrations of docosahexaenoic acid or linoleic acid (0-200 μM) for 20 h followed by incubation with TPA (100 ng/mL) for another 24 h. Finally, the DMEM was removed, and the cells were washed with PBS. The cells were then incubated with MTT (0.5 mg/mL) in DMEM at 37℃ for an additional 3 h. The medium was removed, and isopropanol was added to dissolve the formazan. After centrifugation at 20,000g for 5 min, the supernatant of each sample was transferred to 96-well plates, and absorbance was read at 570 nm in an ELISA reader. The absorbance in cultures treated with 0.005% ethanol was regarded as 100% cell viability.

Western blot analysis

After each experiment, cells were washed twice with cold PBS and were harvested with 150 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/L leupeptin, 1 mg/L aprotinin, and 2 mM dithiothreitol). Cell homogenates were centrifuged at 14,000g for 20 min at 4℃.The resulting supernatant was used as a cellular protein for Western blot analysis. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology

Inc., Rockford, IL). Equal amounts of proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk in 15 mM Tris/150 mM NaCl buffer (pH 7.4) at 4℃ overnight. After blocking, the membranes were incubated with anti-phospho-Akt (T308 and S473), anti-phospho-JNK1/2, anti-phospho-ERK1/2, anti-phospho-p38, anti-Akt, anti-JNK1, anti-ERK1/2, anti-p38, anti-MMP-9, anti-HO-1, and anti-β-actin antibodies at 4℃ overnight. Thereafter, the membranes were incubated with the secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies at room temperature for 1 h, and the immunoreactive bands were developed by use of the Western Lightning™ Plus-ECL kit (PerkinElmer, Waltham, MA) and were scanned by a luminescent image analyzer (Fujifilm LAS-4000, Japan). The bands were quantified with an ImageGauge (Fujifilm).

RNA isolation and RT-PCR

Total RNA of MCF-7 cells was extracted by using TRIzol reagent. Briefly, after treatment, cells were washed twice with cold PBS and scraped with 500 μL of TRIzol reagent. Samples were mixed with 100 μL of chloroform and centrifuged at 11,000g for 15 min. The supernatant was collected and mixed with 250 μL of isopropyl alcohol. After centrifugation at 12,000g for 20 min, the supernatant was discarded and the cell pellet was stored in 70% ethanol or dissolved in deionized water for quantification.

We used 0.4 μg of total RNA for the synthesis of first-strand cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega Co., Madison, WI) in a 20-μL of final volume containing 250 ng of oligo-dT and 40 U of RNase inhibitor. PCR was

conducted in a thermocycler in a reaction volume of 50 μL which containing 20-μL of cDNA, BioTaq PCR buffer, 50 μM of each deoxyribonucleotide triphosphate, 1.25 mM $MgCl_2$, and 1 U of BioTaq DNA polymerase (BioLine). Oligonucleotide primers of MMP-9 (forward, 5'-CACTGTCCACCCCTCAGAGC-3'; reverse, 5'-GCCACTTGTCGGCGATAAGG-3'), HO-1 (forward, 5'-CTGAGTTCATGAGGAACTTTCAGAAG-3'; reverse, 5'-TGGTACAGGGAGGCCATCAC-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5'-CCATCACCATCTTCCAGGAG- 3'; reverse, 5'-CCTGCTTCACCACCTTCTTG-3') were designed on the basis of published sequences (Lin et al., 2008a; Sun et al., 2009). Amplification of MMP-9 was achieved when samples were heated to 95℃ for 5 min and then immediately cycling 30 times through 30 sec denaturing step at 94℃, 30 sec annealing step at 56℃, and a 1min elongation step at 72℃. Amplification of HO-1 was achieved when samples were heated to 95℃ for 5 min and then immediately cycling 39 times through a 1 min denaturing step at 95℃, a 1 min annealing step at 55℃, and a 2 min elongation step at 72℃, respectively. The GAPDH cDNA level was used as the internal standard. PCR products were resolved in a 1% or 2% agarose gel, scanned by using a Digital Image Analyzer (Alpha Innotech) and quantified with an ImageGauge.

RNA interference by small interfering RNA of HO-1

Predesigned small interfering RNA (siRNA) against human HO-1 and nontargeting control pool siRNA were purchased from Dharmacon (Lafayette, CO). MCF-7 cells were transfected with HO-1 siRNA SMARTpool by using DharmaFECT1 transfection reagent

according to the manufacturer's instructions. Specific silencing was confirmed by at least three independent immunoblotting assays with cellular extracts 24 h after transfection.

Nuclear extract preparation

After each experiment, cells were washed twice with cold PBS and were then scraped from the dishes with 1000 μL of PBS. Cell homogenates were centrifuged at 2,000g for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 200 μL of hypotonic buffer containing 10 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.5 mM DTT, 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM PMSF. After centrifugation at 7,000g for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 0.25 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol at 4℃ for 30 min. The samples were then centrifuged at 20,000g for 15 min. The supernatant containing the nuclear proteins was collected and stored at -80℃ until the Western blot assay and electrophoretic mobility shift assays.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed according to our previous study (Cheng et al., 2004). The LightShift Chemiluminescent EMSA Kit (Pierce Chemical Co., Rockford, IL) and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (forward: 5'-GCCTCAGCTGGTAAATGGATAA-3'; reverse: 5'-AAAGGCCCCAGAGCCAGCC-3') were used to measure AP-1 nuclear protein-DNA binding activity (Tsai et al., 2007). Ten micrograms of nuclear extract, poly (dI-dC), and

biotin-labeled double stranded AP-1 oligonucleotide were mixed with the binding buffer (LightShift EMSA Kit; Pierce Chemical Co., Rockford, IL) to a final volume of 20 μL, and the mixture was incubated at room temperature for 30 min. Unlabeled double-stranded AP-1 oligonucleotide and a mutant double-stranded oligonucleotide were used to confirm the protein-binding specificity. The nuclear protein-DNA complex was separated by electrophoresis on a 6% TBE-polyacrylamide gel and was then transferred to a Hybond-N^+ nylon membrane. The membranes were cross-linked by UV light for 10 min and were then treated with 20 μL of streptavidin-horseradish peroxidase for 20 min, and the nuclear protein-DNA bands were developed with a Chemiluminescent Substrate (Thermo, Rockford, IL). The bands were scanned by a luminescent image analyzer (Fujifilm LAS-4000).

Migration and invasion assays

Transwell (Corning) or BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) in vitro migration or invasion 24-well chambers with 8 μm pore polycarbonate filters were used as directed by the manufacturer's instruction, respectively. Briefly, rehydrated the number of Matrigel inserts before cells setting. $1x10^7$ cells/mL were placed in 500 μL of serum free medium and 750 μL of medium containing 10% FBS in the lower wells. The transwell chambers were incubated with or without 200 μM DHA or LA for 20 h and treated with or without 100 ng/mL TPA for another 48 h. Cells were fixed with 100% methanol for 20 min and then stained with Trypan Blue stain (GIBCO) for 30 min. Non-migrating or non-invading cells on the upper surface of the filter were removed by wiping out with a cotton swab, and the filters were excised and mounted on the

microscope slide. Migration and invasiveness were quantified by counting cells on the lower surface of the filter.

Gelatin zymography assay

The activity of MMP-9 was analyzed by gelatin zymography as described previously (Chu et al., 2004). MCF-7 cells were pretreated with or without 0-200 μM DHA or LA for 20 h, or specific inhibitors of MAPKs (PD98059, SB203580 or SP600125), PI3K (Wortmannin or LY294002) and PKC (GF109203X) for 1 h, followed by incubation in serum-free medium containing 0-200 ng/mL of TPA for an additional 24 h. The conditioned media were collected, mixed with loading buffer and subjected to electrophoresis on 8% SDS-polyacrylamide gel containing 0.1% (wt/vol) gelatin. Electrophoresis was performed at 120 V for 2 h. Gels were then washed twice with washing buffer (2.5% Triton X-100) at room temperature to remove SDS, followed by incubation at 37℃ for 12 to 16 h in reaction buffer (40 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and 0.02% NaN_3), then stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol, and 10% acetic acid) for 1 h and destained with destaining solution (20% methanol and 10% acetic acid) for 30 min. MMP-9 gelatinolytic activity was detected as clear bands in a dark blue background, and the bands were quantified by densitometer measurement using a digital imaging analysis system (Fujifilm).

Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference between mean values was determined by one-way analysis

of variance followed by Tukey's test; p values of <0.05 were taken to be statistically significant.

Results

3.1 Effects of TPA with or without DHA or LA on MCF-7 Cell Viability.

The TPA-induced invasion of human MCF-7 breast cancer cell model has been established by Lin et al. (2008). Our previous study showed that the concentration of DHA and LA greater than100 μM significantly caused rat primary hepatocytes damage (Li et al., 2006). To exclude the cytotoxic effect of TPA, DHA and LA on MCF-7 cell culture system, the MTT assay was performed. As shown in Figure 1A, no significant cytotoxic effect of TPA (0-200 ng/mL) on MCF-7 cells for 24 h treatment. MCF-7 cells treated with 0-200 μM LA or DHA for 20 h showed no significant cytotoxic effects. We chose 200 μM DHA and LA as the highest treatment concentration because cell damage occurred when concentration greater than 200μM (Figure 1B). To confirm the additive effect of cytotoxicity between DHA, LA, and TPA, MCF-7 cells were pretreated with DHA or LA (0-200 μM) for 20 h followed by incubation with TPA (100 ng/mL) for another 24 h. The results showed no adverse effect observed in MCF-7 cells (Figure 1C).

3.2 TPA-Induced MMP-9 Gene Expression and Enzyme Activity in MCF-7 Cells.

To confirm the induction effect of TPA on the expression of MMP-9 in MCF-7 cells, cells were treated with TPA (25, 50, 100, or 200 ng/mL) for 24 h. As shown in Figure 2A, the MMP-9 activity was increased by TPA in a dose-dependent manner. Also, the induction of MMP-9 mRNA, protein expression and enzyme activity by TPA (100 ng/mL) was time-dependent (Figure 2B). In the following experiments, 100 ng/mL TPA and 24 h treatment were used to induce the MMP-9 expression and enzyme activity.

3.3 DHA or LA Suppresses TPA-Induced Migration and Invasion in MCF-7 Cells.

In a mouse study, fish oil was shown to prevent MDA-MB-231 cancer cell metastasis to bone (Mandal et al., 2010). In order to demonstrate whether the metastasis of MCF-7 cells was influenced by DHA or LA, in vitro migration and invasion transwell assays were performed. As shown in Figures 3A and 3B, TPA significantly induced migration and invasion of MCF-7 cells. However, pretreatment with DHA or LA significantly suppressed cell motility of MCF-7. These results suggested that DHA and LA had an inhibitory effect on TPA-induced migration and invasion of MCF-7 cells.

3.4 DHA and LA Inhibited MMP-9 Enzyme Activity via Reducing MMP-9 Gene Expression.

MMP-9 is recognized to play a role in the metastasis of breast carcinoma cells (Lin et al., 2008). Therefore, we study the effects of DHA and LA on TPA-induced MMP-9 enzyme activity and gene expression. The results of RT-PCR and Western blotting analysis showed that MMP-9 mRNA and protein expression were both significantly induced by 100 ng/mL of TPA, and the induction was dose-dependently down-regulated by LA or DHA. The result of gelatin zymography analysis showed that the MMP-9 enzyme activity significantly increased by TPA, and pretreatment with DHA or LA inhibited TPA-induced MMP-9 activity in a dose-dependent manner (Figure 4).

3.5 TPA-Mediated MMP-9 Expression through PKC, PI3K and MAPKs Signaling Pathways.

Several studies have indicated that the induction of protein kinase C (PKC), phosphoinositide 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinases

(MAPKs) signaling pathways is involved in TPA-mediated MMP-9 expression (Cho et al., 2007; Lin et al., 2010). To specify the signaling pathway involved in TPA-mediated MMP-9 expression in MCF-7 cells, we used the pharmacological inhibitors of MAPKs, PI3K and PKC such as SP600125 (JNK inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), wortmannin and LY294002 (PI3K inhibitors), and GF109203X (non-selective PKC inhibitor) to identify the contribution of these signaling pathways. As shown in Figure 5A, there was no cell toxicity caused by the dose of indivisual kinase inhibitor. TPA-induced MMP-9 protein expression and enzyme activity was significantly inhibited by specific inhibitors of SP, PD, Wt, LY and GF, respectively (Figure 5B).

3.6 DHA or LA Down-Regulates TPA-Induced MMP-9 Expression via ERK and PI3K/Akt Signaling Pathways.

Our results showed that TPA-induced MMP-9 enzyme activity was significantly inhibited by specific inhibitors of JNK, ERK, PI3K/Akt and PKC. Furthermore, we investigate whether DHA or LA inhibited TPA-induced MMP-9 expression was through above-mentioned pathways. As shown in Figure 6, the ERK and Akt pathways were activated by TPA at 30 and 60 min treatments and this activation was attenuated by pretreatment with 200 μM DHA or LA. However, DHA or LA had no effect on TPA-activated JNK and p38 pathways. These results suggested that DHA or LA might down-regulate TPA-induced MMP-9 expression via ERK and PI3K/Akt signaling pathways.

3.7 Inhibition of the TPA-Induced DNA Binding Activities of NF-κB and AP-1 by DHA and LA.

Activation of NF-κB and AP-1 is involved in the induction of the MMP-9 gene, which is associated with the invasion and metastasis of tumor cells by TPA (Hwang et al., 2010). EMSA was used to confirm whether the suppression of DHA or LA on TPA-induced MMP-9 gene expression was associated with the attenuation of NF-κB and AP-1 DNA binding. As shown in Figure 7, the DNA-binding complex formation of NF-κB and AP-1was found to increase after 1 h of TPA treatment and peak at 4 h. DHA and LA pretreatment dramatically abolished TPA-induced NF-κB and AP-1 DNA-binding complex formation in MCF-7 cells (Figures 7A and B). These results suggested that attenuation of TPA-induced NF-κB and AP-1 DNA-binding complex formation is involved in the suppression of TPA-induced MMP-9 expression by DHA and LA.

3.8 Effect of DHA and LA on HO-1 Expression of MCF-7 Cells in the Presence of TPA.

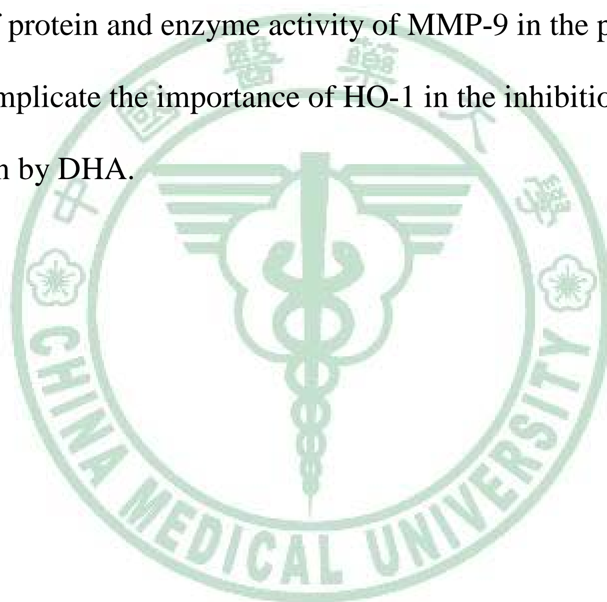
HO-1, a stress response gene, has been shown to suppress MMP-9 gene expression, and subsequently decrease tumor metastasis (Lin et al., 2008). To determine whether the suppression of TPA-induced MMP-9 expression by DHA or LA is via induction of HO-1 expression, we studied the effect of 50, 100, and 200 μM DHA and LA on HO-1 expression in the presence of TPA in MCF-7 cells. In Figure 8A, pretreatment with DHA for 20 h significantly enhanced HO-1 expression in a dose-dependent manner; however, the induction of HO-1 expression was not present by TPA and LA treatments.

We further found the induction of HO-1 expression by DHA is a time-dependent manner. DHA induced HO-1 expression as early as 8 h and effect sustained until 24 h

(Figure 8B). These data suggested that DHA induces HO-1 expression in both dose- and time-dependent manners in MCF-7 cells.

3.9 HO-1 siRNA Alleviates DHA Inhibition of MMP-9 expression in the Presence of TPA.

To clarify whether HO-1 is involved in the suppression of DHA on TPA-induced MMP-9 expression, the HO-1 siRNA SMARTpool system was used. The knockdown efficiency of HO-1 gene was assayed by Western blotting. HO-1 siRNA alleviated the DHA inhibition of protein and enzyme activity of MMP-9 in the presence of TPA (Figure 9). These results implicate the importance of HO-1 in the inhibition of TPA-induced MMP-9 expression by DHA.



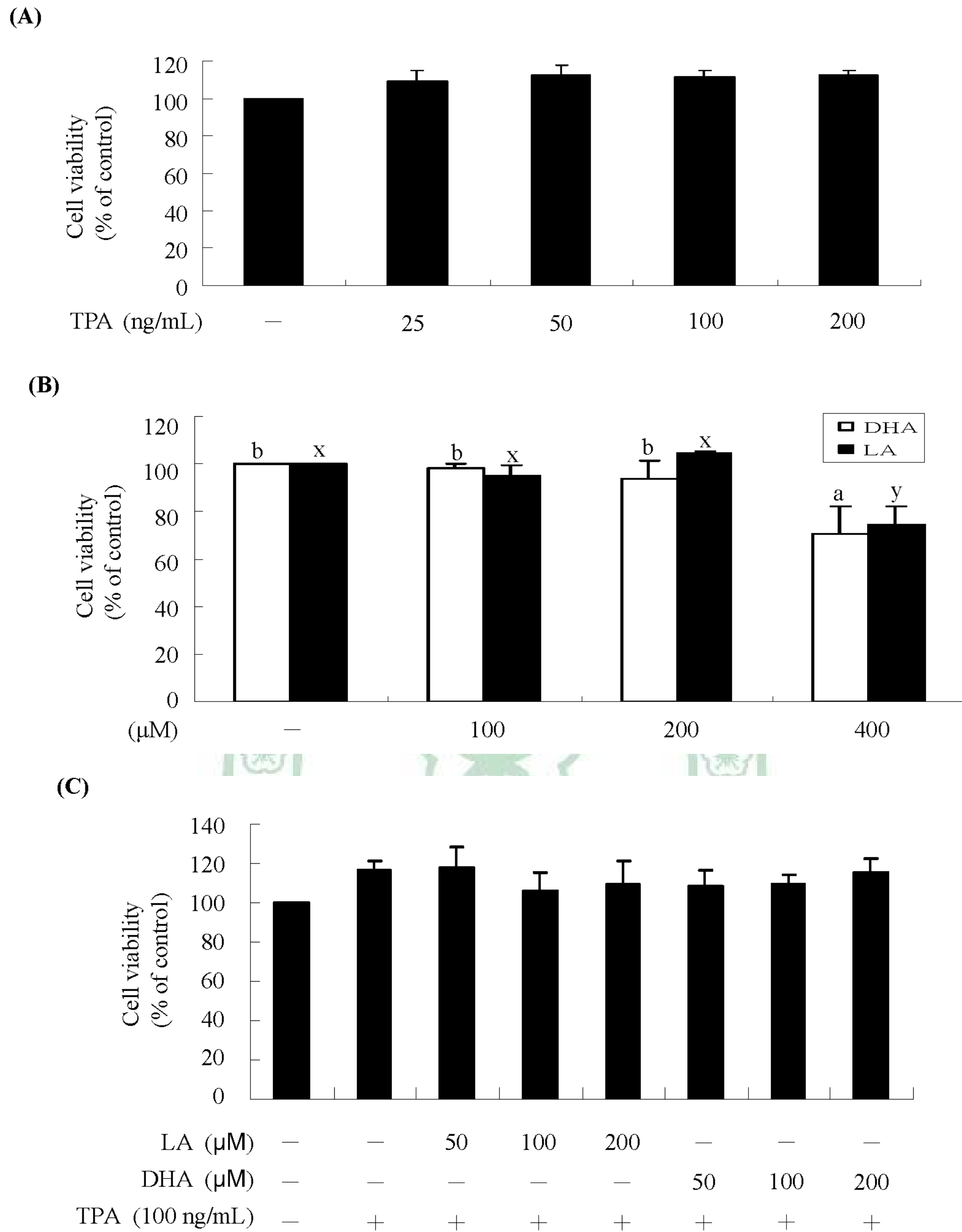


Figure 1. Effects of TPA with or without DHA or LA on MCF-7 Cell Viability. (A) Effect of TPA on cell viability. Cells were treated with 0-200 ng/mL TPA for 24 h. (B) Effects of DHA and LA on cell viability. Cells were treated with 0-200 μM DHA or LA for 24 h. (C) Effects of LA or DHA with TPA on cell viability. Cells were pretreated with 0-200 μM LA or DHA for 24 h followed by incubation with 100 ng/mL of TPA for another 24 h. Cell viability was measured by using the MTT assay. Values are means ± SD of three independent experiments. Bars not sharing the same letters or symbols are significantly different (p<0.05).

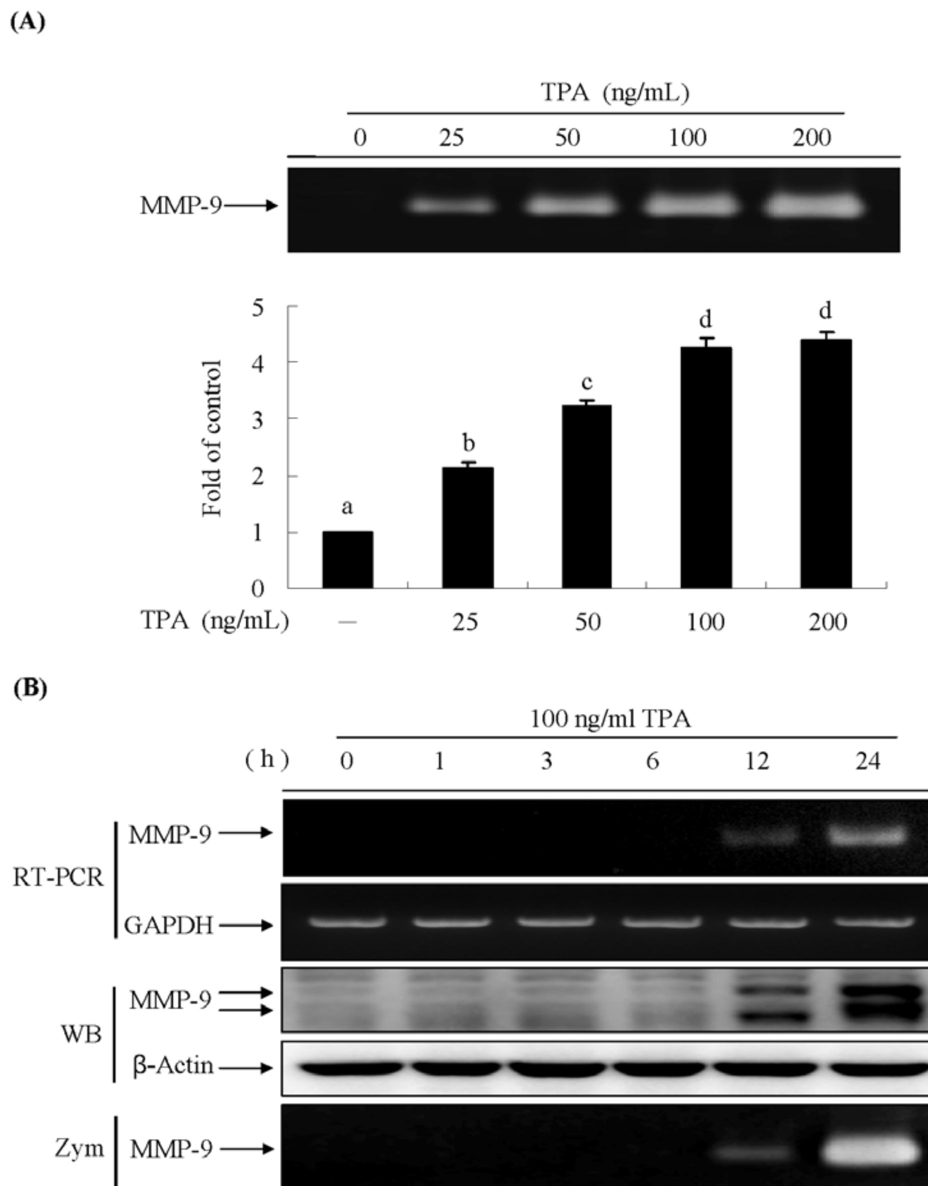


Figure 2. Effect of TPA on MMP-9 gene expression and enzyme activity in MCF-7 cells. (A) TPA induces MMP-9 mRNA expression in a dose-dependent manner. MCF-7 cells were treated with various doses of TPA for 24 h. Total RNA (0.1 μg/μL) were used to detect the MMP-9 mRNA expression, which was measured by RT-PCR. (B) TPA induces MMP-9 mRNA and protein expression and enzyme activity in a time-dependent manner. MCF-7 cells were treated with 100 ng/mL of TPA for 0-24 h. MMP-9 protein expression was measured by Western blotting (WB) and MMP-9 enzyme activity was measured by gelatin zymography assay (Zym). Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p<0.05).

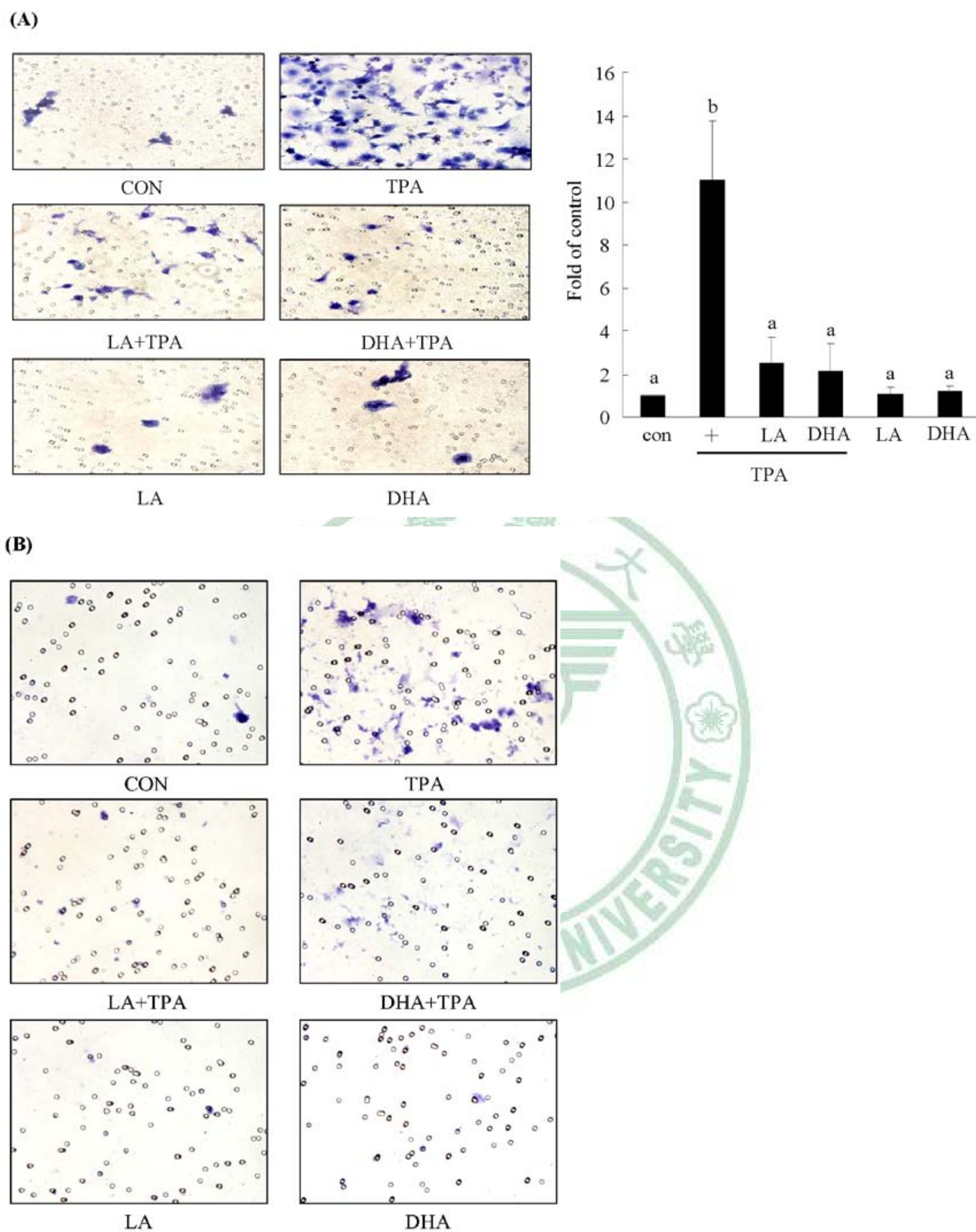


Figure 3. Effect of DHA or LA on TPA-induced migration and invasion in MCF-7 cells. (A) LA or DHA inhibits TPA-induced cell migration in MCF-7 cells. (B) LA or DHA inhibits TPA-induced cell invasion in MCF-7 cells. MCF-7 cells were pretreated with or without 200 μM LA or DHA for 20 h followed by incubation with or without 100 ng/mL of TPA for an addition 24 h. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p<0.05).

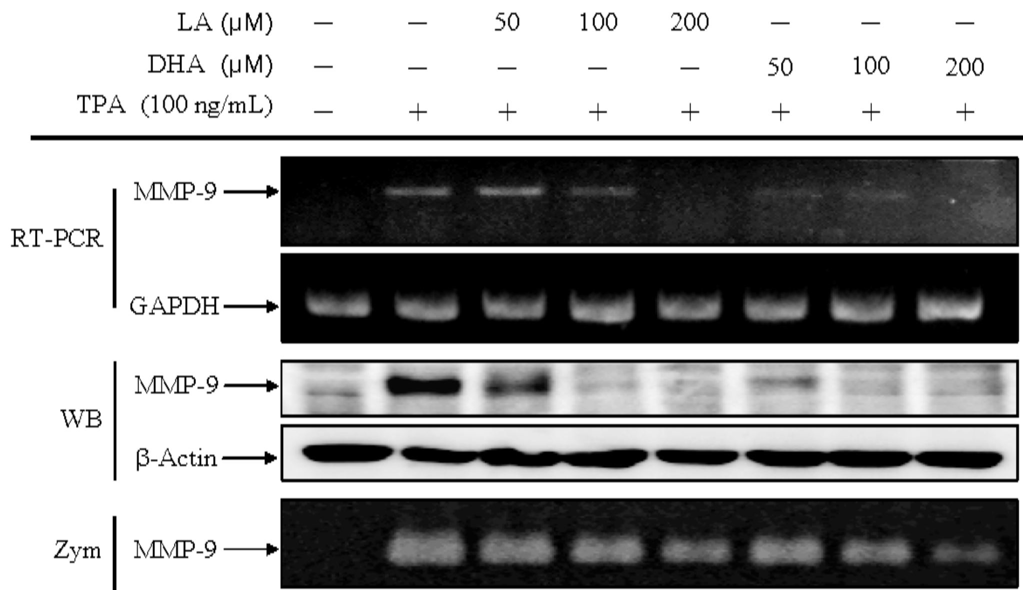


Figure 4. Effect of LA or DHA on TPA-induced MMP-9 expression in MCF-7 cells. Effect of LA or DHA on TPA-induced MMP-9 mRNA and protein expression, and MMP-9 enzyme activity. MCF-7 cells were treated with 100 ng/mL of TPA for 0-24 h. MMP-9 mRNA expression was measured by RT-PCR. MMP-9 protein expression was measured by Western blot (WB) and MMP-9 enzyme activity was measured by gelatin zymography assay (Zym). Total RNA (0.1 μg/μL) were used for RT-PCR. Aliquot of cell lysates (20 μg) were used for Western blot assay. One representative experiment out of three independent experiments is shown. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (*p*<0.05).

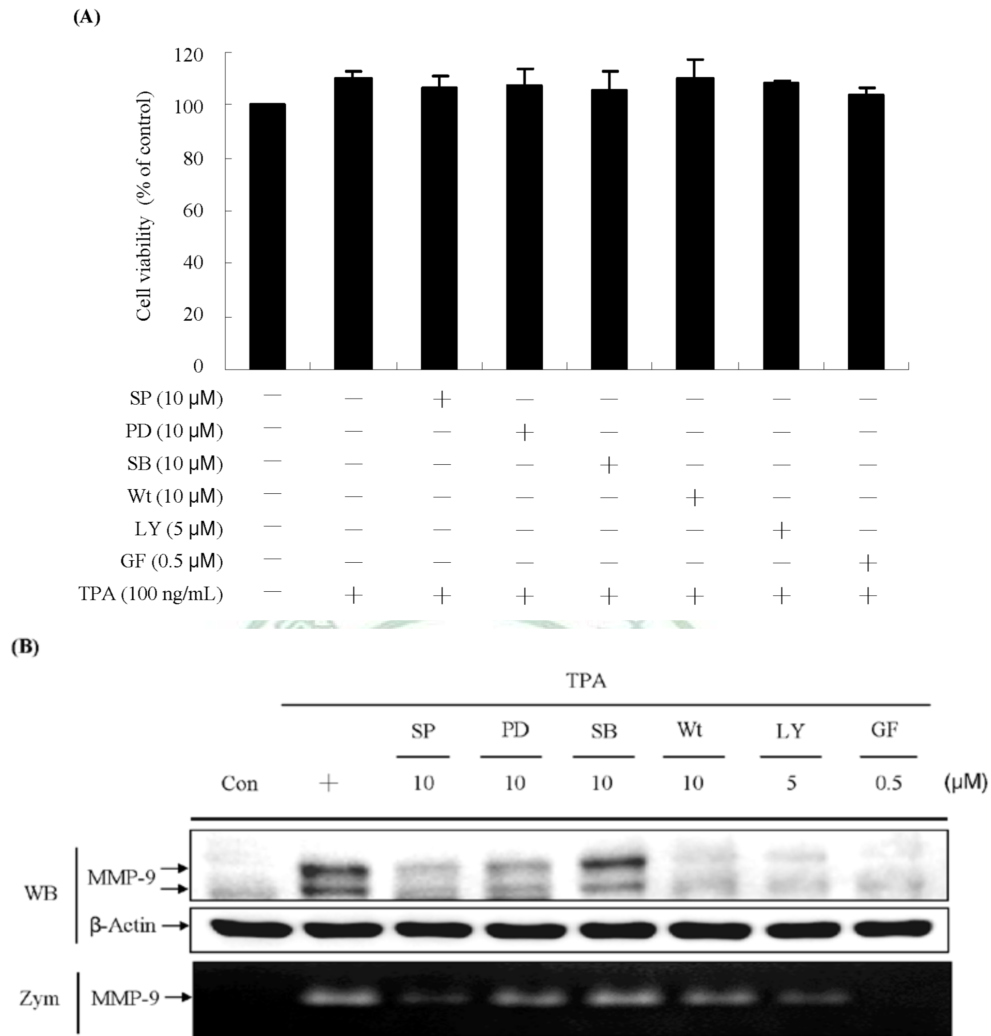


Figure 5. Effect of protein kinase inhibitors on TPA-induced MMP-9 expression in MCF-7 cells. (A) Effects of protein kinase inhibitors on MCF-7 cell viability. Cells were pretreated with pharmacological inhibitors of MAPKs, PI3K and PKC including SP600125 (JNK inhibitor, SP), PD98059 (ERK inhibitor, PD), SB203580 (p38 inhibitor, SB), wortmannin and LY294002 (PI3K inhibitors, Wt/LY), and GF109203X (non-selective PKC inhibitor, GF) for 24 h followed by incubation with 100 ng/mL of TPA for another 24 h. Cell viability was measured by using the MTT assay. (B) Effects of protein kinase inhibitors on TPA-induced MMP-9 protein expression and enzyme activity. MCF-7 cells were treated with 100 ng/mL of TPA for 0-24 h. MMP-9 protein expression was measured by Western blot (WB) and MMP-9 enzyme activity was measured by gelatin zymography assay (Zym). Aliquot of cell lysates (20 μg) were used for Western blot assay. One representative experiment out of three independent experiments is shown. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p<0.05).

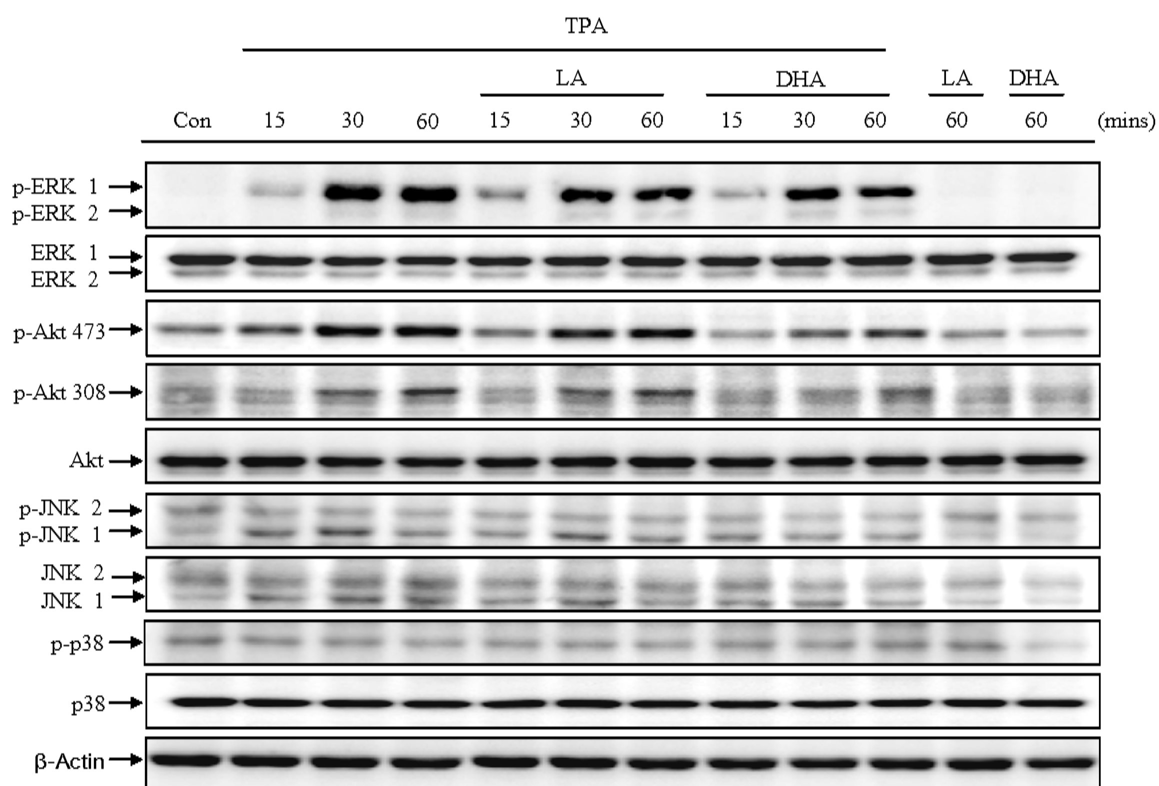


Figure 6. Effect of LA or DHA on TPA-induced MAPKs and Akt activation. Cells were treated with or without 200 μM LA or DHA for 24 h followed by incubation with or without 100 ng/mL of TPA for indicated time periods. The phosphorylation of protein kinases was measured by Western blot. Aliquots of cell lysates (20 μg) were used. One representative experiment out of three independent experiments is shown.

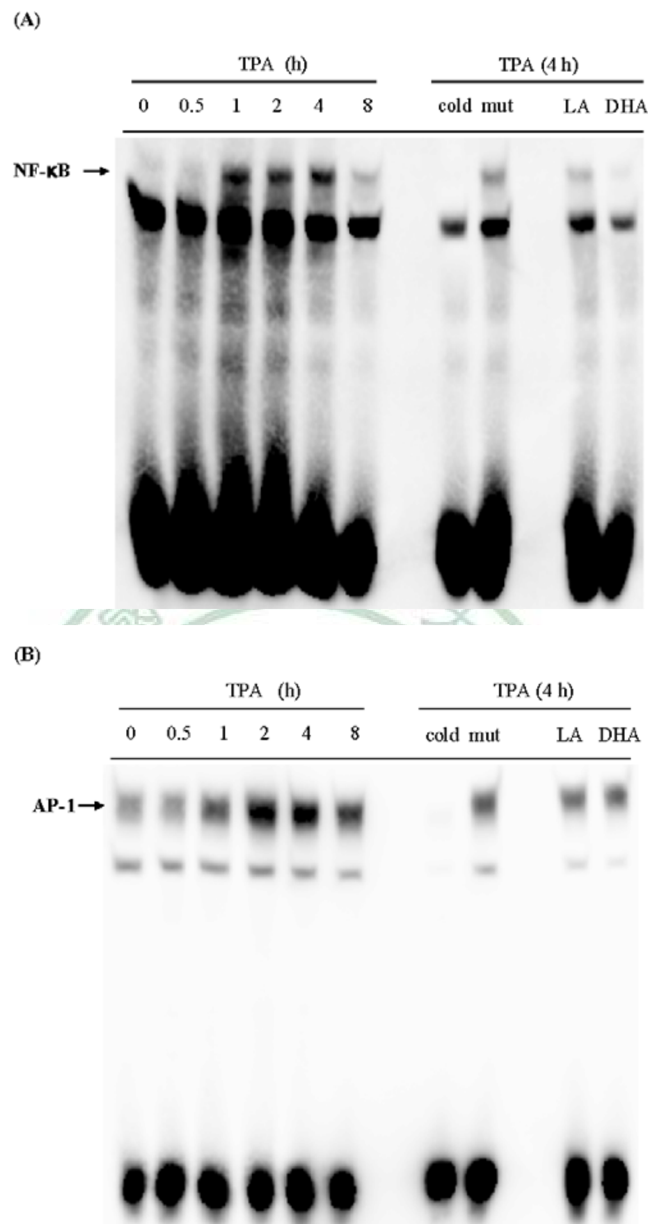


Figure 7. Effects of LA or DHA on TPA-induced AP-1and NF-κB DNA-binding activity. (A) Effects of LA or DHA on TPA-induced NF-κB DNA-binding activity. (B) Effects of LA or DHA on TPA-induced AP-1 DNA-binding activity. MCF-7 cells were treated with 100 ng/mL of TPA for indicated time periods, and cells pretreated with 200 μM LA or DHA for 24 h followed by incubation with 100 ng/mL of TPA for 4 h. Aliquots of nuclear extracts (10 μg) were used for EMSA. To confirm the specificity of the nucleotide, 25-fold of cold probe (biotin-unlabeled AP-1 or NF-κB binding site, cold) and biotin-labeled double-stranded mutant AP-1 or NF-κB oligonucleotide (mut, 4 μg) were included in the EMSA. One representative experiment out of three independent experiments is shown.

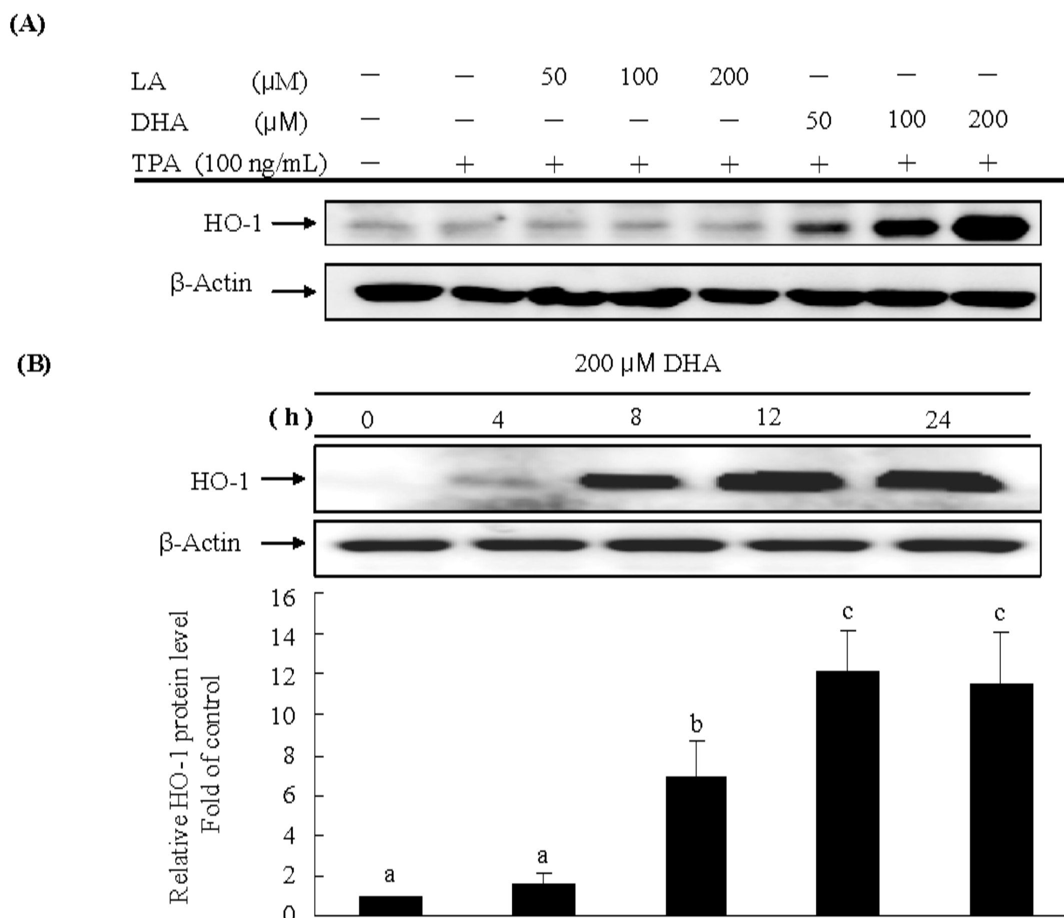


Figure 8. Effect of DHA on HO-1 Expression of MCF-7 Cells in the Presence of TPA. (A) DHA induced HO-1 protein expression in a dose-dependent manner. MCF-7 cells were pretreated with various doses of LA or DHA for 20 h, followed by treatment with 100 ng/mL of TPA for another 24 h. (B) DHA induced HO-1 protein expression in a time-dependent manner. MCF-7 cells were treated with 200 μM DHA for indicated time periods. Aliquots of cell lysates (20 μg) were used for Western blot assay. One representative experiment out of three independent experiments is shown. Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p<0.05).

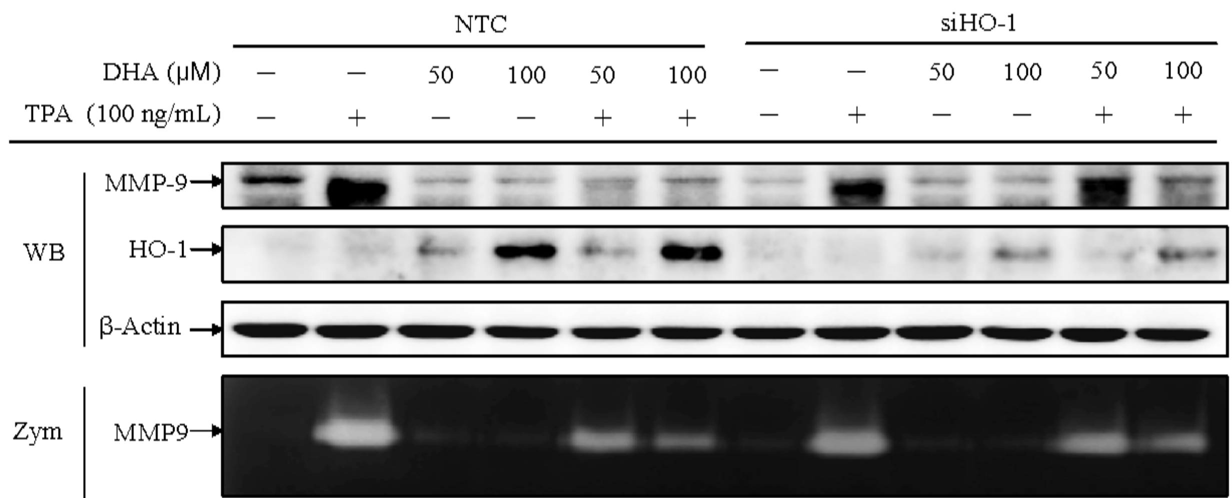
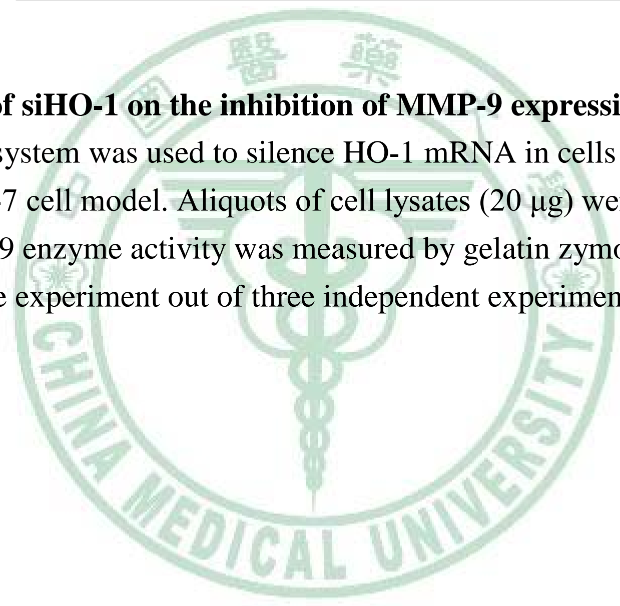


Figure 9. Effect of siHO-1 on the inhibition of MMP-9 expression by DHA.
An HO-1 siRNA system was used to silence HO-1 mRNA in cells and to create a siRNA knockdown MCF-7 cell model. Aliquots of cell lysates (20 μg) were used for Western blot (WB). MMP-9 enzyme activity was measured by gelatin zymography assay (Zym). One representative experiment out of three independent experiments is shown.



Discussion

Breast cancer is the second leading cause of cancer deaths in Western women, and the majority of breast cancer deaths result from metastases (Culhane and Quackenbush, 2009). T*he* dietary high fat consumption has long been recognized to play a role in the development of breast cancer (Tannenbaum and Silverstone, 1953), but the relation between intakes of dietary fat and *mortality rates* of breast cancer is still in contention. Previous study suggests that differences in breast cancer growth and metastasis are partly related to dietary fatty acid intake and especially to the quality and quantity of fatty acid consumed (Fay et al., 1997). Several experimental evidences indicate that n-3 PUFAs may have an anti-tumor activity (Bordeleau et al., 2011; Schley et al., 2005), whereas total fat, saturated, and n-6 PUFAs may stimulate various mammary tumor growth and metastasis, including breast cancer cells (Funahashi et al., 2008). This study was designed to examine the effect of n-3 and n-6 PUFAs, such as DHA and LA, on MCF-7 breast cancer cell migration and invasion and to clarify the possible molecular mechanism involved.

MCF-7 breast cancer cell are usually recognized as weakly invasiveness, however, the invasive potential of MCF-7 cells could be dramatically increased by TPA (Johnson et al., 1999). The tumor promoter TPA has been shown to induce tumor migration and invasion by stimulating MMP-2 or MMP-9 expression in human astroglioma cells (Jung et al., 2006) and hepatoma (Hah and Lee, 2003). Current studies found that medicinal herb such as kalopanaxsaponin A and flavonoid quercetin inhibits TPA-induced cell invasion by reducing MMP-9 expression in MCF-7 cells (Lin et al., 2008b; Park et al., 2009). In the present study, the MMP-9 activity was induced by TPA in a dose-dependent manner (Figure 2A) and both mRNA and protein levels were dramatically increased at 24 h after treatment with 100 ng/mL of TPA in MCF-7 breast cancer cells (Figure 2B), which is in line with the results of a previous study (Lin et al., 2008b).

Previous studies showed that the low concentrations of DHA or EPA (25 μM) alone had minimal inhibitory effect on cell migration against serum (Siddiqui et al., 2005), but the relatively high concentration of 100 μM or 152 μM (about 50 ng/ml) DHA significantly decreased cell migration and invasion in MDA-MB-231 breast cancer cells (Blanckaert et al., 2010; Mandal et al., 2010). In our previous study, results showed that

the concentration of PUFAs, including arachidonic acid (AA), LA, EPA, and DHA, granter than 100 μM significantly caused rat primary hepatocyte damage (Li et al., 2006). In the present study, the relative high concentration of 200 μM of DHA and LA used had no cytotoxic effect on MCF-7 breast cancer cells (Figure 1B), which indicated the difference in cytotoxic potency between normal cells and breast tumor cells (Crawford et al., 2003). Both 200 μM of DHA and LA have significant suppressive effect on TPA-induced cell migration and invasion (Figures 3A and B). Pharmacokinetic study showed that the human plasma concentration of DHA is able to achieve 120 mg/L (about 315 μM) after two-week administration of daily dose of 3 g fish oil supplement, of which contains 1.365g DHA (Rusca et al., 2009). The mean concentration of plasma phospholipid-esterified LA of about 824 μM was reported (Hwang et al., 2010). These results supported the dosage of DHA and LA used in our present study is within the reasonable physiologically relevant levels in human beings.

It is well established that tumor cell migration and invasion depend on MMP-2 and MMP-9 expression and enzyme activities. Previous studies indicated that DHA and dietary CLA are capable of reducing MMP-2 and MMP-9 production in reproductive tissues of pregnant rats (Harris et al., 2001). In vitro cell experiments, migration of vascular smooth muscle cells was decreased by DHA via suppressing MMP-2 and MMP-9 activity (Delbosc et al., 2008). In the present study, we provide the first evidence that TPA-induced MMP-9 expression and activity were down-regulated by DHA in MCF-7 cells (Figure 4). The result indicated the benefit of DHA in the process of breast tumor metastasis (Mandal et al., 2010). Generally, high intake of n-6 PUFA and saturated fat are more likely to increased risk of breast cancer (Do et al., 2003). However, treatment with higher dose of LA (300 μM) was found to suppress colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction (Lu et al., 2010b). In our present study, MMP-9 expression and migration and invasion of MCF-7 cells were suppressed by LA.

TPA increases the migration and invasion of various types of cancer cells by activating MMP-9 via PKC, MAPKs, and PI3K/Akt signaling pathways and transcription factors (Hwang et al., 2010; Park et al., 2009). TPA-induced MMP-9 expression and activity were significantly inhibited by treatment with JNK inhibitor (SP600125, SP), ERK inhibitor (PD98059, PD), PI3K inhibitors such as wortmannin (Wt) and LY294002

(LY), and non-selective PKC inhibitor (GF109203X, GF) (Figure 5) in our culture system. Our previous study reported that the activation of JNK1/2 and ERK2 could be down-regulated by DHA (Lu et al., 2009), suggesting DHA may possess the ability to suppress the MMP-9 expression and it can be developed for anti-breast cancer migration and invasion agent. However, only TPA-induced activation of ERK1/2 and Akt473/308 was attenuated by DHA and LA and the attenuation was observed after 30-60 min DHA and LA treatments (Figure 6). The results of figure 5 were different from that incubation of MCF-7 cells with GF and PD, but not Wt and LY, inhibit TPA-induced MMP-9 expression and activity (Lin et al., 2008b). The other study supported our data that the PI3K/Akt signaling pathway should be involved in the induction of MMP-9 expression by TPA in MCF-7 cells (Park et al., 2009). These results suggested that DHA or LA down-regulates TPA-induced MMP-9 expression and activity is through inhibition of ERK1/2 or PI3K/Akt signaling pathways, and subsequent suppression of MCF-7 breast cancer cell metastasis. TPA-induced MMP-9 expression was mediated by modulating the activation of transcription factors such as NF-κB and AP-1 through PKC, PI3K and MAPK signaling pathways (Blumberg, 1988; Jang et al., 2007). A recent study reported that dihydroartemisnin inhibits TPA-induced MMP-9 activation through suppression of PCKα/Raf/ERK and JNK phosphorylation and subsequent NF-κB and AP-1 trans-activation in HT-1080 cells (Hwang et al., 2010). In the same study, dihydroartemisnin directly suppressed degradation of IκBα and then decreased p65 nuclear translocation. These results supported the notion that one of the possible inhibitory mechanisms of DHA or LA on TPA-induced MMP-9 activation may be associated with suppression of IκBα degradation and p65 or c-jun nuclear translocation in MCF-7 cells. Kalopanaxsaponin A (KPS-A) inhibits TPA-induced invasion by reducing MMP-9 activation, mainly via PI3K/Akt/NF-κB and PKCδ/ERK/AP-1 pathways in MCF-7 cells (Park et al., 2009). EMSA results of the present study indicated that NF-κB and AP-1 play an important role in the suppression of MMP-9 expression by DHA and LA (Figure 7). The activation of ERK1/2 and PI3K/Akt signaling pathways were also involved in TPA-induced MMP-9 activation in our culture system, suggesting DHA or LA inhibits TPA-induced migration and invasion by reducing MMP-9 expression and activity may be via PI3K/Akt/NF-κB and PKCδ/ERK/AP-1 pathways.

HO-1 is induced by a wide variety of stimuli such as hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin and cytokines (Chung et al., 2011; Luo et al., 2011; Ronco et al., 2011; Xu et al., 2011; Zhong et al., 2010). Except for anti-oxidant and anti-inflammatory activities of HO-1, it has also been shown to suppress breast cancer migration and invasion in recent studies (Li et al., 2011; Pae et al., 2010; Wang et al., 2011). A previous study indicated that 25 μM of DHA alone does not have a significant effect on MDA-MB-231 breast cancer cell migration, but when the cells treated with DHA and propofol, significant inhibition of cell migration by about 50% was obtained (Siddiqui et al., 2005). Interestingly, propofol, a widely used sedative and anesthetic agent, was found to up-regulate HO-1 expression in human umbilical vein endothelial cells in a recent study (Liang et al., 2011). These results suggest that the greater inhibitory effect of DHA and propofol combination on cell migration may be via the induction of propofol-mediated HO-1 expression in MDA-MB-231 cells. In the present study, we are the first to provide the evidence that HO-1 gene expression was significantly induced by DHA, but not LA, in a dose- and time-dependent manner in MCF-7 human breast cancer cells (Figure 8). Knockdown HO-1 gene by siRNA reversed the effect of DHA inhibition of the TPA-induced MMP-9 gene expression and activity (Figure 9). These results indicate the importance of HO-1 in the inhibition of TPA-induced MMP-9 expression by DHA.

Taken together, these results suggest that DHA and LA down-regulate TPA-induced MMP-9 gene expression and MCF-7 breast cancer cell metastasis is at least in part through inhibition of ERK1/2 and PI3K/Akt signaling pathways and reduction of NF-κB and AP-1 transcriptional activation. Moreover, we suggest that DHA and LA exhibit a novel function to prevent TPA-induced cell migration and invasion by reducing MMP-9 activation through inhibition of the ERK1/2 and PI3K/Akt pathways in MCF-7 breast cancer cells. Furthermore, the inhibition of TPA-induced MMP-9 activation by DHA is at least in part through induction of HO-1 expression in MCF-7 cells.

Conclusion

In the present study, we demonstrate that DHA and LA inhibits TPA-induced cell migration and invasion by reducing MMP-9 activation, mainly via ERK1/2 and PI3K/Akt pathways and sequentially NF-κB and AP-1 trans-activation. Furthermore, the inhibition of TPA-induced MMP-9 activation by DHA is at least in part through induction of HO-1 expression in MCF-7 breast cancer cells. The findings of this study are schematically presented in Figure 10.

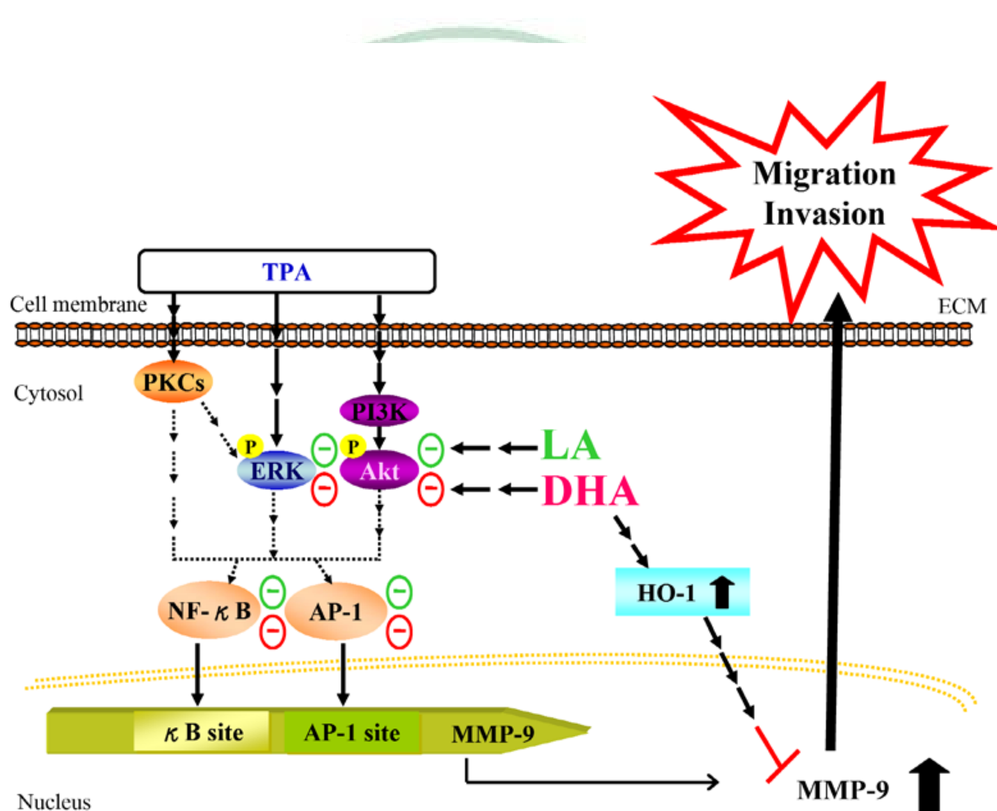


Figure 10. Model showing pathways that mediate the inhibition of expression of MMP-9 and metastasis and invasion of MCF-7 cells by DHA or LA. DHA or LA down-regulates TPA-induced MMP-9 gene expression, cell migration and invasion might involve inhibition of either ERK1/2 or PI3K/Akt signaling pathway, and reduction of NF-κB and AP-1 transcriptional activation. Moreover, HO-1 may play an important role in DHA down-regulation of TPA-induced MMP-9 expression.

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台灣癌症臨床研究發展基金會 認識乳癌.

行政院衛生署 (2011). 中華民國 97 年癌症登記年度報告.