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Ph.D THESIS

Studies on New Therapeutic Targets for the Treatment of Tamoxifen-Resistant Breast Cancer

Graduate School of Chosun University

Department of Pharmacy

Nguyen Thi Thuy Phuong

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(타목시펜 저항성 유방암에서
신규 치료 표적 연구**)**

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Graduate School of Chosun University

Department of Pharmacy

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Studies on New Therapeutic Targets for the Treatment of Tamoxifen Resistant Breast Cancer

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Acronyms

ER:	Estrogen receptor
PI3K	Phosphoinositide 3-kinase
CREB	cAMP response element binding protein
ERK	Extracellular signal-regulated kinase
GR	Glucocorticoid receptor
PTEN	Phosphatase and tensin homolog
PGE2	Phostaglandin E2
TAM	Tamoxifen
COX-2	Cyclooxygenase-2
EP1	PGE2 receptor 1
EP2	PGE2 receptor 2
5-Aza	5-aza-2'-deoxycytidine
MAPK	Mitogen-activated protein kinase
EETs	Epoxyeicosatrienoi acids
CYP	Cytochrome P450
TAM	Tamoxifen
4-OH-TAM	4-hydroxy tamoxifent
15-EEZE	14,15-epoxyeicosa-5(Z)-enoic acid
MSPPOH	N-methylsulfonyl-6-(2- propargylozyphenyl) hexanamide

VEGF	Vascular endothelial growth factor
EMT	Epithelial mesenchymal transitions
CAM assay	Chick chorioallantoic membrane assay

요약

타목시펜 저항성 유방암에서 신규 치료 표적 연구

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유방암 치료에 있어서 중요한 난제중 하나는 항암제 저항성 생성이다. 따라서, 항암제 저항성 반전을 위한 새로운 치료표적을 찾는 것은 중요한 의미를 갖는다. 본 학위논문에서는 항암제저항성 유방암의 치료에 기여할 수 있는 신규 치료표적을 제시한다. 본 연구진은 에스트로겐 생합성의 핵심효소인 aromatase 의 기저발현 및 활성이 대조유방암 (MCF-7) 세포에 비하여 타목시펜 저항성 유방암 (TAMR-MCF-7) 세포에서 현저하게 증가된 것을 발견하였다. 또한, 면역조직화학법을 통한 타목시펜 저항성이 있는 사람의 유방암 (TAMR-MCF-7) 세포조직에서 aromatase 면역반응성을 알아본 결과, 타목시펜에 반응성이 있는 사람 유방암 (MCF-7) 세포조직에 비해 높은 면역반응성을 보였다. TAMR-MCF-7 세포에서 phosphoinositide 3-kinase (PI3K)의 억제는 aromatase 유전자 전사활성 및 효소 활성을 억제하였고, PI3K/Akt-의존적 CREB 의 활성화는 aromatase 의 발현 증가에 관여함을 밝혔다. 테스토스테론은 에스트로겐 양성 및 음성유방암에서 세포성장을

억제한다고 알려져있다 (Chottanapund et al., 2013). 그러나 테스토스테론 처치시 MCF-7 세포에 비하여 높은 aromatase 의 발현을 보이는 TAMR-MCF-7 세포에서는 세포성장 억제효과가 유의성있게 감소하였다. 또한, aromatase 억제제인 formestane 이 TAMR-MCF-7 세포에서 4-hydroxytamoxifen 매개에 의한 세포사멸을 증가시켰다. 본 발견은 aromatase 효소와 PI3K/Akt-의존적 CREB 신호전달경로가 타목시펜 저항성 유방암의 치료에 있어서 유망한 타겟이라는 것을 제시한다.

본 연구진은 두번째로 유방암 진행에 있어서 시토크롬 P450 (CYP) epoxygenase 을 통한 아라키돈산(AA)에서 epoxyeicosatrienoic acids (EETs)으로의 전환과정에 관심을 가졌다. 다른 CYP 효소와는 달리 CYP3A4 epoxygenase 가 MCF-7 세포에 비하여 타목시펜 (TAM) 저항성 유방암 세포 (TAMR-MCF-7)에서 현저하게 증가하였다. 또한, EET 산물들 중 11,12-epoxyeicosatrienoic acid (11,12-EET) 농도가 MCF-7 세포에서의 비해 TAMR-MCF-7 세포에서 현저하게 증가하였다. 케토코나졸 (CYP3A4 억제제) 혹은 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE, 합성 EET 길항제) 처치는 TAMR-MCF-7 세포에서 세포증식을 감소시키고 4-hydroxy tamoxifen (4-OH-TAM)에 대한 세포독성을 증가 시켰다. 병아리 융모 막 (CAM) 분석시 대조 MCF-7 세포 이식군에 비하여 증가된 TAMR-MCF-7 세포에서의 암 증식 및 혈관신생이 케토코나졸과 14,15-EEZE 처리에 의해 유의성 있게 억제되었다. 또한, 세포이동능 평가에서도 TAMR-MCF-7 세포의 활발한 이동능은 상기 화합물 처치에 의하여 감소되었다. 이 결과들은 세포증식, 혈관형성 및 이동능을 포함하는 타목시펜 저항성 유방암의 특징들에서 CYP3A4 epoxygense 과발현과 CYP3A4-매개 11,12-EET 합성의 중요한 역할을 제시한다. 더 나아가 케토코나졸 또는 14,15- EEZE 처치는 본 연구진이 타목시펜 저항성 유방암세포의 혈관신생 및 상피-간질세포 형질전환 현상의 원인으로 제시했던 E2F1-의존성 Pin 1 발현을 억제하였다. 반대로 MCF-7 세포에서 EET 처치는 Pin 1 발현을 증가시켰다. 본 연구에서는 aromatase 와

CYP3A4 매개성 EET 생성 과정이 유방암의 내분비 저항성을 해지시킬 수 있는 신규 치료 타겟이 될 수 있음을 제시한다.

I. Introduction

Breast cancer characterized as hormone-dependent cancer type is one of the most common malignances in the world. It accounts for 22.9% of all cancer (excluding non-melanoma skin cancers) in women and caused approximate 13.7 % cancer death in women (World cancer report, 2008). Most breast cancer requires estrogen and a functional estrogen receptor α (ERα) for the proper growth (Mueller *et al.*, 2002). Thus, anti-estrogens such as tamoxifen (TAM), a non-steroidal anti-estrogen has been widely used and elucidated as the most effective therapy in the treatment of ER+ breast cancer patients (Petrangeli *et al.*, 1994). Though TAM treatment prolongs overall survival, reduces mortality and metastatic potential, most of the patients finally acquire the resistance which has been being a serious problem in the treatment of breast cancer patients (Ali *et al.*, 2002; Osborne *et al.*, 1994). So far, there have been diverse molecular mechanisms contributed to the acquired anti-estrogen resistance of breast cancers. For instance, the loss of ERα expression by hypermethylation within *ERα* promoter portends a poor prognosis that these patients may never respond to anti-estrogen therapy (Giacinti *et al.*, 2006). Or the overexpression of HER-2 acts as a molecular mechanism for the development of endocrine resistance in human breast cancers (Pietras *et al.*, 1995). We have also suggested that hypermethylation in *PTEN* gene promoter which regulates activity of phosphatidylinositol 3-kinase(PI3K)/Akt signaling pathway requires for tamoxifen resistance in ER+ breast cancer cells (Phuong *et al.*, 2011). Our collaborators have revealed that overexpression of Pin1 (a peptidyl- prolyl ismerase protein) contributes to

cell proliferation and drug resistance of TAMR-MCF-7 cells (Khanal *et al.*, 2011; Namgoong *et al.*, 2010). Moreover, Pin 1 is a key protein involved in regulating angiogenesis (Kim *et al.*, 2011; Kim *et al.*, 2009) and EMT-like behavior (Kim *et al.*, 2009) in TAM-resistant breast cancer cells, both of which may contribute to metastatic ability of the cell line. Despite the highly informative reports, molecular mechanism for the acquisition of TAM resistance is still unclear. Hence, the aim of present research is to discover the molecular mechanisms for the uncontrolled growth of tamoxifen-resistant human breast cancer and to identify the new potential therapeutic targets.

A. Aromatase and breast cancer

1. Sources of aromatase

Aromatase is a member of cytochrome P450 superfamily and the product of CYP19 gene, whose function is to aromatize androgens, producing estrogens and is an important factor for sexual development. In humans, the gene CYP19 is located on chromosome15q21.1 and circulating C19 steroid precursors are essential substrates for extragonadal estrogen synthesis (Ghosh *et al.*, 2009; Toda *et al.*, 1993). Aromatase is highly expressed in the placenta and in the granulosa cells of ovarian follicles, where its expression depends on cyclical gonadotropin stimulation. Aromatase is also present, at lower levels, in several nonglandular tissues, including subcutaneous fat, liver, muscle, brain, normal breast, and breast-cancer tissue (Miller *et al.*, 1982; Nelson *et al.*, 2001). Residual estrogen production after menopause is solely from nonglandular sources, in

particular from subcutaneous fat. Thus, peripheral aromatase activity and plasma estrogen levels correlate with body-mass index in postmenopausal women (Longcope *et al.*, 1986). Clinical study has revealed that concentration of estrogen in breast tumor tissues is several-fold higher than that in plasma of postmenopausal breast cancer patients (Pasqualini *et al.*, 1996), and most of these patients show a resistance to tamoxifen (TAM), a representative ER antagonist (Ali *et al.*, 2002; Osborne *et al.*, 1994), probably in part because of the presence of intratumoral aromatase.

2. Aromatase inhibitors

Estrogen is the main hormone involved in the development and growth of breast tumors. Tamoxifen inhibits the growth of breast tumors by competitive antagonism of estrogen at its receptor site. In contrast, aromatase inhibitors markedly suppress plasma estrogen levels in postmenopausal women by inhibiting or inactivating aromatase (Johnston *et al.*, 2003). And, unlike tamoxifen, aromatase inhibitors have no partial agonist activity. Aromatase inhibitors such as letrozole and exemestane have been shown to be well tolerated and even more effective than anti-estrogen tamoxifen in postmenopausal breast cancer patients (Brueggemeier *et al.*, 2005; Chumsri *et al.*, 2011; Köberle *et al.*, 2005; Wood *et al.*, 2003). Moreover, aromatase inhibitors are considered as a therapeutic option for tamoxifen-resistant breast cancer. Several clinical trials have revealed a significant benefit of sequential treatment of aromatase inhibitor after tamoxifen administration over tamoxifen alone therapy (Boccardo *et al.*, 2006; Kaufmann *et al.*, 2007).

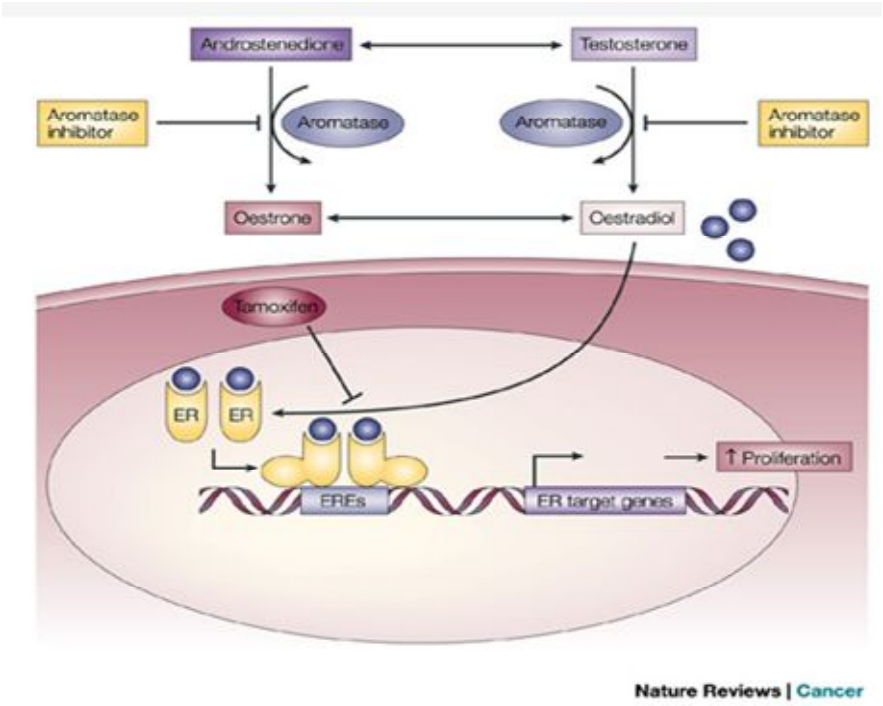


Figure 1. Mechanism of action of aromatase inhibitors and tamoxifen

(Johnston *et al.*, 2003)

3. Regulation of aromatase expression in breast cancer

It has been reported that aromatase is expressed at a higher level in human breast cancer tissue than in normal breast tissue (Chen, 1998). The expression of aromatase (estrogen synthetase) is tissue specifically regulated through the alternative use of multiple exons 1 and promotors (Means *et al.*, 1991; Utsumi *et al.*, 1996). Prostaglandin E2 (PGE2), the product of cyclooxygenase-2 (COX-2), was reported to increase the cAMP level and stimulates aromatase gene expression in breast tumor through binding to EP1 (PGE2

receptor 1) and EP2 receptors (Richards *et al.*, 2003; Zhao *et al.*, 1996). In addition, growth factors secreted by breast cancer cells could also stimulate aromatase expression in both breast cancer and adjacent adipose fibroblasts and stromal cells (Su *et al.*, 2011). The enhanced aromatase activity by growth factor signaling pathways was demonstrated to be via post- transcriptional mechanisms (Su *et al.*, 2011). Previous studies showed that both CREB binding to CRE or glucocorticoids binding to GRE can regulate the transcription of the aromatase gene in breast cancer (Chen *et al.*, 2001b; Simpson and Zhao, 1996; Young and Macphaul, 1998). Besides, in the p53-inactivated mammary epithelial cells, CREB was also shown to play critical role in aromatase overexpression while aromatase independently express and activate on GRE (Choi *et al.*, 2008). In addition, aromatase expression and the nuclear levels of CREB were suppressed by inhibition of ERK but not P38 kinase in this cell line (Choi *et al.*, 2008). Another study has reported about the correlation between PI3K/Akt pathway and aromatase activity, and investigated that PI3K/Akt and MAPK pathway inhibitors suppressed aromatase activity in MCF-7aro aromatase transfected breast carcinoma cells (Su *et al.*, 2011).

Despite the increasing evidence of the regulation and role of aromatase in breast cancer, the knowledge and the studies on the regulation of aromatase expression in TAM-resistant breast cancer have been less understood. Here, we found for the first time that expression and activity of aromatase in TAM-resistant human breast cancer (TAMR-MCF-7) cells were increased compared to control MCF-7 cells. We also revealed that aromatase immunoreactivity in human breast cancer tissues was significantly higher in TAM-

sensitive cases than in TAM-resistant cases. We further tried to clarify cellular signaling pathway(s) for the up-regulation of aromatase and tested its therapeutic implications in TAMR-MCF-7 cells.

B. Cytochrome P450s and Epoxyeicosatrienoic Acids

1. Overview of arachidonic acid metabolism and Cytochrome P450 pathway

Arachidonic acid is converted to eicosanoids including prostaglandins, leucotrienes, lipoxins, and cytocrome P450 (CYP)-derived metabolites by the distinct enzyme systems: cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-LOX, 12-LOX, 15-LOXa, 15-LOXb), and cytochrome P450s (ω-hydroxylases and epoxygenases) (Figure 2). These enzymes are the therapeutic targets of approved drugs for the treatment of pain, inflammation, asthma, and allergies (Imig and Hammock, 2009). The CYP-denpendent metabolism of arachidonic acid occurs in several tissues including the liver, kidney and the cardiovascular system via two distinct pathways the ω-hydroxylases and epoxygenases. The ω-hydroxylases of the 4A and 4F gene families of cytochrome P450 (CYP4A and CYP4F) convert arachidonic acid to autacoids such as HETEs. 20- Hydroxyeicosatrienoic acid (20-HETE) is the principle isoform of this pathway and has shown vasoconstriction acitivity (Miyata and Roman, 2005). CYP epoxygenases are encoded predominantly by the CYP2C and CYP2J genes and generates four regioisomeric epoxyeicosatrienoic acids (EETs; 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET (Capdevila *et al.*, 2000), which have demonstrated vasodilatory and anti-inflammatory activities (Fisslthaler *et al.*, 1999;

Kaspera *et al.*, 2009; Zeldin, 2001). EETs are then further metabolized, mainly by sEH, to dihydroxyeicosatrienoic acid (DHETs) that traditionally been considered to be less active than EETs (Yu *et al.*, 2000; Zeldin *et al.*, 1993).

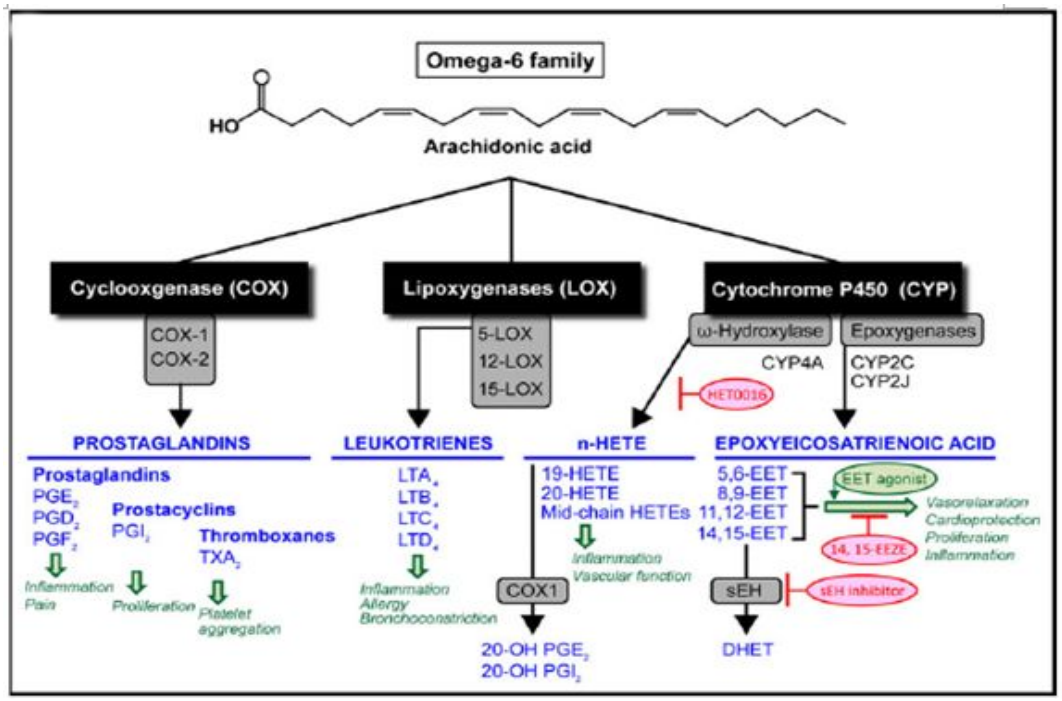


Figure 2. Bioactive eicosanoids derived from the arachidonic acid cascade

(Panigrahy *et al.*, 2011)

2. Role of CYP in pharmacology

Cytochrome P450s are involved in the metabolism of drugs, including anticancer drugs and xenobiotics, and contribute to the metabolism of a variety of procarcinogens (Gonzalez

et al., 1994; Guengerich *et al.*, 1991). The P450s are correlated with both the response to cancer treatment and the initiation and promotion of tumorigenesis (Gonzalez *et al.*, 1994; Guengerich *et al.*, 1991).

3. Functions of EETs

EETs are mainly secreted by endothelial cells and their roles are well known in cardiovascular system with function as vascular relaxation factors independent of nitric oxide (NO) and prostacyclin I2 (PGI2) (Xu *et al.*, 2011) (Figure 3). EETs act as paracrine and autocrine mediators of physiological processes such as proliferation, migration, and anti-inflammation (Fleming, 2007; Spector *et al.*, 2004) (Figure 3).

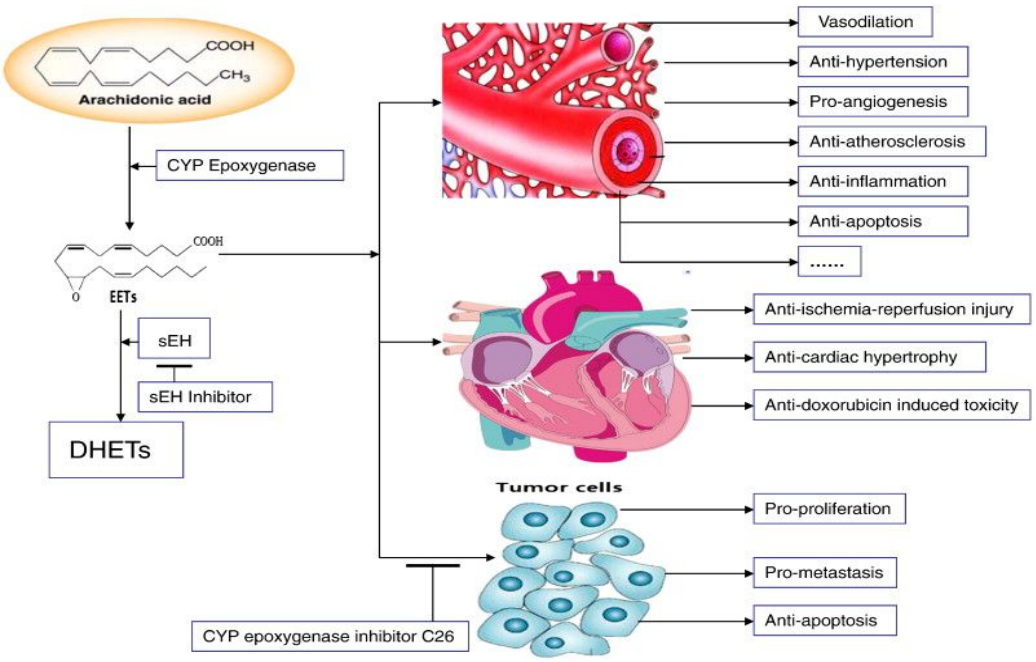


Figure 3. Functions of Epoxyeicosatrienoic Acids (Xu *et al.*, 2011)

3.1. EETs and apoptosis

EETs display an ability of apoptotic inhibition (Chen *et al.*, 2001a; Yang *et al.*, 2007). CYP epoxygenase promotes cell survival by producing 14,15-EET and metabolizing unesterified AA, therefore preventing activation of the neutral sphingomyelinase pathway and pro-apoptotic ceramide formation (Chen *et al.*, 2001). Furthermore, CYP epoxygenases overexpression, which is to increase EET biosynthesis, significantly protects endothelial cells from apoptosis induced by tumor necrosis factor-α (TNF-α). This effect is mediated, at least in part, through the inhibition of ERK dephosphorylation and the activation of PI3K/Akt signaling pathway (Yang *et al.*, 2007). Furthermore, CYP epoxygenases and EETs have also been reported to protect human carcinoma cells from apoptosis induced by TNF- α (Jiang *et al.*, 2005).

3.1. EETs and angiogenesis

Angiogenesis is complex and involves in proliferation, invasion, migration of endothelial cells, and tube formation. Increasing evidence shows epoxygenases and EETs promote endothelial cell proliferation, migration, angiogenesis (Fleming, 2007; Spector *et al.*, 2004). EETs may display a capability to stimulate angiogenesis with both secreted EETs and synthetic EETs (Medhora *et al.*, 2003; Munzenmaier *et al.*, 2000; Zhang *et al.*, 2002). Several pathways have been implicated in EETs- mediated angiogenesis. 11,12-EET may stimulate angiogenesis through the activation of the EGF receptor (Michaelis *et al.*, 2008) or Sphingosine kinase 1 (SK1) activity (Yan *et al.*, 2008). 14,15-EET was shown to induce angiogenesis through several pathways, including Src, PI3K/Akt signaling in

parallel with mTOR-S6K1 activation, and Src-dependent STAT3-mediated VEGF expression (Cheranov *et al.*, 2008). Other studies have shown that the pro-angiogenic activity of EETs is mediated, at least in part, by VEGF activity (Webler *et al.*, 2008); (Yang *et al.*, 2009). The effects of EETs and VEGF regulation are closely related. EETs can enhance the effects of VEGF-induced angiogenesis (Webler *et al.*, 2008). In turn, VEGF can increase the CYP2C promoter activity in endothelial cells and induce the expression of CYP2C8, resulting in increased intracellular EET levels (Webler *et al.*, 2008).

3.2 EETs and tumors

In human, there are four CYP epoxygenases including CYP2C8, 2C9, 2J2 and CYP3A4 which have been well known to convert AA to EETs (Ayajiki *et al.*, 2003; Lundblad *et al.*, 2005; Rifkind *et al.*, 1995). The expression of these CYP epoxygenases has been reported to play critical role in tumorgenesis of diverse cancers, supporting the potential role of EETs in carcinogenesis (Figure 4). CYP2C8, CYP2C9, and CYP2J2 were over-expressed in three prostate carcinoma cell lines (PC3, DU-145, and LNCaP) and contributed to the metastatic capacity (invasion and migration) of these cell types (Nithipatikom *et al.*, 2010). 11,12-EET was the major arachidonic acid metabolite and induced cell invasion and migration in these prostate carcinoma cell lines (Nithipatikom *et al.*, 2010). CYP3A4 overexpression increased the growth of hepatocarcinoma, which was inhibited by the addition of putative EET-receptor antagonist, 14,15-EEZE (Oguro *et al.*, 2011). Moreover, EETs was shown to play a role in cancer metastasis via role of CYP2J2 over-expression

(Jiang *et al.*, 2007). Recently, Mitra et al. have demonstrated that CYP3A4 epoxygenase promotes the growth of estrogen receptor –positive/(ER+) breast cancer cells, in part through biosynthesis of 14,15-EET (Mitra *et al.*, 2011). Both CYP3A4 and CYP2C8 but not CYP2J2 promote breast cancer growth (Mitra *et al.*, 2011). In the comparison to CYP3A4 and CYP2C8, the physiological role of CYP2C9 has not been mentioned in breast cancer biology (Mitra *et al.*, 2011). Despite several studies characterizing the roles of CYP epoxygenase and their derived EETs in breast cancer, their impacts in the development of endocrine resistant acquisition and typical phenomena including the higher angiogenesis and metastasis of TAM resistant breast cancer remains poorly characterized. The aim of the study is to identify the potential role of CYP epxygenases and their derived eicosanoids in the development of endocrine resistant breast cancers.

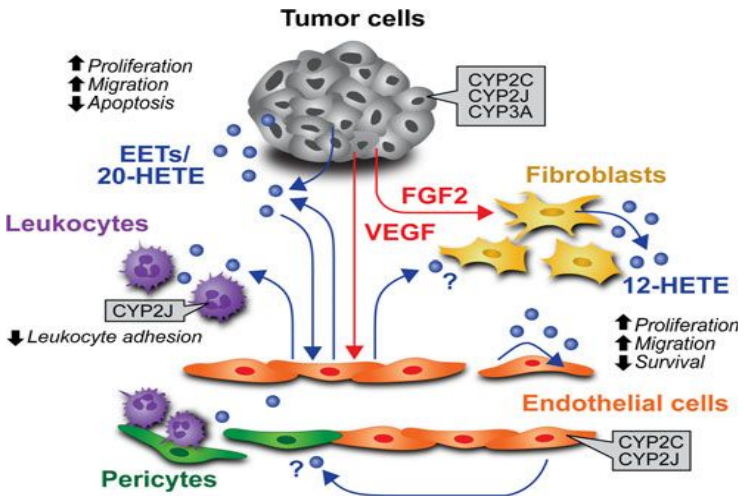


Figure 4. Cytochrome P450 epoxygenases expression in tumor cell compartment and their potential role in cell-cell communication in the tumor stroma. (Panigrahy *et al.*, 2011)

Our studies have revealed that CYP3A4 was overexpressed and played an important role in cell proliferation, angiogenesis and migration ability of TAM resistant breast cancer cells, in part through 11,12-EET biosynthesis. The finding suggest that the inhibition of CYP3A4-mediated EET signaling pathway may be a new therapeutic strategy for the treatment of endocrine resistant breast cancers.

II. Materials and Methods

1. Materials

Antibodies: Antibodies against aromatase were purchased from Abcam (Cambridge, UK, Cat#: ab18995). Antibodies recognizing CREB, P-CREB, P-p38 kinase, p38 kinase, P-extracellular signal-regulated kinase (ERK), ERK, P-AKT and AKT were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against phosphatase and tensin homolog (PTEN) and glucocorticoid receptor (GR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against CYP3A4, CYP2C8, and CYP2C9 were purchased from Abcam (ab3572-100; ab103547; ab80213; Cambridge, UK). Antibodie against p-RB were obtained from Cell Signaling Technology (9307S; Beverly, MA, USA). Antibodies against Pin 1 and E2F1 were purchased from Santa Cruz Biotechnology (sc-15340; sc-22820; Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were acquired from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin and PCNA antibody were obtained from Sigma (St. Louis, MO).

Reagents: Formestane, 17-b-estrodiol, Ketoconazole was obtained from Sigma (St. Louis, MO) and MSPPOH and 14,15-EEZE was obtained from Cayman Chemical Co. (Ann Arbor, MI). Testosterone was purchased from Wako (USA). And most of other reagents used for molecular studies were purchased from Sigma (St. Louis, MO).

Plasmids: pGRE-Luc plasmid was donated from Dr. Lee KY (Chonnam National University, Gwangju, South Korea). pCRE-Luc was purchased from Stratagene (La Jolla, CA, USA). The aromatase-Luc reporter plasmid was kindly provided from Dr. Jeong HG (Chungnam National University, Daejeon, South Korea). VEGF-luc plasmid was kindly donated from Dr. Lee (Chonnam National University, Kwangju, Korea). Full length human MMP-2 and MMP-9 promoter luciferase constructs were kindly provided by Dr. Aree Moon (Duksung women's University, Seoul, Korea). Myc-p85 (a dominant negative form of PI3K) and p110-myc (constitutive active form of PI3K) overexpression vectors were provided by Dr. A. Toker (The Boston Biomedical Research Institute, Boston, MA) and Dr. J. Downward (Imperial Cancer Research Fund, London).

2. Cell culture and establishment of TAMR-MCF-7 cells

MCF-7 cells, MEF cells, and Raw 264.7 cells were cultured at 37^{o}C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. TAMR-MCF-7 cells were established using methods previously reported (Choi *et al.*, 2007; Phuong *et al.*, 2011) and cultured in DMEM containing 10% charcoal-stripped FBS (Hyclone, Logan, UT) and 4-hydroxytamoxifen (3 μM). For the experiment purpose, the chemicals were exposed in serum free medium condition. Hormone-dependent T47D: A18/Neo cells and hormone-independent T47D: A18/PKCα cells were kindly donated from Dr. Miele (Cancer institute, Universtity of Mississippi Medical Center, Jackson, MS). T47D: A18/PKCα cells were cultured at 37^{o}C in 5% CO_2/95% air in RPMI-1640 medium containing 10% fetal bovine

serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1X MEM non- essential amino acids, and 10 ng/ml insulin.

For generation of stable knockdown TAMR-MCF-7 cell lines, the lentiviral transduction particles containing short hairpin RNA (shRNA) for Pin 1 (sc-36230-v), or nontarget control shRNA (sc-108080) were purchased from Santa Cruz. Cells were transduced with virus in the presence of polybrene (5 μg/mL) for 24 hours and then selected by puromycin (3 μg/mL).

3. Immunoblot Analysis.

After washing with sterile PBS, cells were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerolphosphate, 2 mM sodium inorganic pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg/ml leupeptin. Total cell lysates were centrifuged at 10,000*g* for 10 min to remove cell debris, and proteins in the supernatant were fractionated using a suitable separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific antibodies.

Preparation of nuclear extracts. Cells in the dishes were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after adding 100 μl of a hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mMKCl, 0.1mMEDTA, 0.5% Nonidet P-40, 1mMdithiothreitol, and 0.5 mM

phenylmethylsulfonylfluoride. The lysates were incubated for 10 min on ice and centrifuged at 7,200×g for 5 min at 4°C. Pellets containing the crude nuclei were resuspended in 50 µl of an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride and incubated for 30 min on ice. The samples were centrifuged at 15,800×g for 10 min to obtain supernatants containing the nuclear fractions. The nuclear fractions were stored at −80°C until needed.

4. Reporter Gene Analysis.

A dual-luciferase reporter gene assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were plated in 12-well plates and transiently transfected with 1 µg/ml reporter plasmids and phRL-SV plasmid (hRenilla luciferase expression for normalization) using Hillymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in culture medium without serum for 18 h. Firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Bad Wild, Germany). Relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to the hRenilla luciferase.

5. Determination of aromatase activity.

Aromatase activity was measured in both MCF-7 and TAMR-MCF-7 cells with a tritiated water release assay (Kinoshita *et al.*, 2003). 80% confluent MCF-7 and TAMR-

MCF-7 cells were incubated in serum-free medium for 36 h and the cells were treated with [1β-^{3}H] androst-4-ene-3,17-dione (100 nM) for an additional 3 h. The medium was then mixed thoroughly with 5% charcoal/0.5% dextran for 12 h and centrifuged at 10,000 *g* for 30 min at 4°C to remove any residual androst-4-ene-3,17-dione. One ml of the supernatants were added in a scintillation vial containing 10 ml scintillation cocktail, and radioactivity was measured as disintegrations per minute using a liquid scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA).

6. Immunohistochemistry for human cancer tissues.

Blocks for all the samples were consecutively cut in 4 μm sections and mounted on poly-l-lysine coated glass slides. Xylene was used to remove the paraffin from the sections, and the samples were rehydrated. Antigen retrieval was performed by boiling sections for 5 min in 1 M sodium citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 min, followed by three times washing with PBS. Sections were then incubated overnight with anti-aromatase antibody (Abcam, Cambridge, UK) at 4°C. After washing with PBS, sections were incubated with HRP-conjugated anti-rabbit IgG for 30 min and washed with PBS. The color was developed by incubation with DAB solution. Finally, sections were counterstained with hematoxylin, dehydrated, mounted, and observed.

7. Cell proliferation

After exposure of cells to the reagents in 5% FBS or 5% charcoal stripped FBS (depending on each experiment) for the indicated time, viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/mL) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 µl dimethylsulfoxide. Absorbance was assayed at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

8. Flow cytometry

For the apoptosis determination, flow cytometry analysis was performed. Cells were harvested with trypsin treatment, stained with both annexin V–fluorescein isothiocyanate and propidium iodide according to the manufacture's protocol (Invitrogen, Carlsbad, CA) and analysed by flow cytometry (FACStar, BD Biosciences, Mississauga, ON) set for FL1 (annexin V) and FL2 (propidium iodide). A total of 10^4 cells were counted for each sample.

9. VEGF ELISA assay

A commercial ELISA kit (Biosource Diagnostics) was used to determine VEGF concentrations in media according to the manufacturer's protocol. Briefly, cells were plated in six-well culture plates and incubated in serum-free medium for 24 h, and then the culture medium was measured with ELISA. VEGF concentrations were determined by measuring the absorbance at 420 nm and were normalized to total protein concentrations in each well.

10. Migration assay

An in vitro migration assay was performed using a 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA, USA) as previously described (Kim *et al.*, 2011; Kim *et al.*, 2003). The lower side of the filter was coated with type I collagen (Collaborative Research, Lexington, KY, USA). The lower compartment was filled with 10% FBS. Ketoconazole and 14,15-EEZE were added to the Transwell insert in the lower well. Cells were placed in the upper part of the Transwell plate, incubated for 17 hours, fixed with formalin and methanol and stained with Hematoxylin for 10 minutes followed by a brief staining with Eosin. The migration phenotypes were determined by counting the cells that migrated to the lower side of the filter, using microscopy at 400× magnification. Eight fields were counted for each filter.

11. Reverse transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the cells using a total RNA isolation kit (RNAgents, Promega). The total RNA (1.0 μg) was reverse transcribed using an oligo(dT) 18-mer and Moloney murine leukemia virus reverse transcriptase (Bioneer). PCR was done using selective primers for human CYP3A4 (Forward, CATTCCTCATCCCAATTCTTGAAGT; Reverse, CCACTCGGTGCTTTTGTGTATCT), CYP2C8 (Forward, AGATCAGAATTTTCTCACCC; Reverse, AACTTCGTGTAAGAGCAACA), CYP2C9 (Forward, AGGAAAAGCACAACCAACCA; Reverse, TCTCAGGGTTGTGCTTGTC), *VEGF* (sense primer, 5′-GCTACTGCCATCCAATCGAG-3′; antisense primer, 5′-TGCATTCACATTTGTTGTGC-3′), MMP-2 (Forward, AGTCTGAAGAGCGTGAAG; Reverse, CCAGGTAGGAGTGAGAATG), MMP-9 (Forward,

TGACAGCGACAAGAAGTG; Reverse, CAGTGAAGCGGTACATAGG) and *S16 ribosomal protein* (*S16r*) genes (sense, 5′-TCCAAGGGTCCGCTGCAGTC-3′; antisense, 5′-CGTTCACCTTGATGAGCCCATT-3′). PCR was carried out for 40 cycles under the following conditions: denaturation at 95°C for 10 s, annealing at 52°C for 30 s, and elongation at 72°C for 1 min. The band intensities of the amplified DNA were compared after visualization with an FLA-7000 (Fujifilm).

12. Quantitative real-time PCR

Total RNA was isolated by using Trizol (Invitrogen, USA). qPCR was performed with a real time PCR machine and primers specific for Pin1 (Forward, TCGGGAGAGGAGGACTTTG; Reverse, GGAGGATGATGTGGATGCC) and GAPDH (Forward, CATGAGAAGTATGACAACAGCC; Reverse, AGTCCTTCCACGATACCAAAG).

13. Chick chorioallantoic membrane assay

Chick chorioallantoic membrane (CAM) assays were done according to previously published methods (Auerbach *et al.*, 1974; Colman *et al.*, 2003). The surfaces of 10 days-old post-fertilization chick eggs were sterilized and the CAM exposed by cutting a window (1 cm^2) on one side of the egg using the false air sac technique. Both the MCF-7 and TAMR-MCF-7 cells (2x 10^6 cells) were placed on the exposed CAM and the windows were sealed with transparent tape. VEGF (20 ng/mL) was used as a standard proangiogenic agent. The eggs were then incubated in a humidified incubator at 37°C and treated with

ketoconazole and 14,15-EEZE with indicated doses. Eggs were examined every 72 h after inoculation using an SV6 stereomicroscope (Carl Zeiss) at ×50 magnification. Digital images of CAM sections were collected using a three-charge-coupled device color video camera system (Toshiba). Images were analyzed using Image-Pro software (Media Cybernetics). The number of vessel branch points contained in a circular region was counted. The tumors were excised and measured that the size to evaluate the influence of chemicals on the tumorgenesis development.

14. EET extraction from breast cancer cells

Cells grown to full confluence (80%) on 150 x 20 mm plates were washed twice with cold PBS and collected in cold PBS containing 2 μM soluble epoxide hydrolase inhibitor 1471 (a gift from Dr. Bruce Hammock, University of California, Davis). The pellets were extracted with a 1:1 mixture of methanol/chloroform (1.32 ml) and vortexed for 1 minute. Then, 0.33 ml of distilled water was added to the sample, and the mixture was vortexed for another 1 minute. The sample was left at room temperature for 60 minutes, and then separated into two layers by centrifugation for 20 minutes at 15,800 g at 4℃. The lower layer was evaporated with vacuum centrifugation, and the residues were dissolved in 20 μl methanol for mass spectrometric analysis.

15. LC-ESI/MRM/MS Method for Eicosanoid Measurement

Two microlitter of samples were subjected to LC-ESI/MRM/MS analysis with API 2000 Mass Spectrometer (AB/SCIEX) coupled with an Agilent 1100 HPLC (Agilent, CO)

using nitrogen as the collision gas (heater turbo gas temperature: 500 ℃). Negative ion monitoring was performed with the following diagnostic product ions: 319 *m*/*z*3 127*m*/*z* for 8,9-EET; 319 *m*/*z*3 167 *m*/*z* for 11,12-EET; 319 *m*/*z*3 219 *m*/*z* for 14,15-EET. Base-line resolution of EET regioisomers was achieved on a Kinetex C18 analytical column (100 mm x 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA) using the following mobile phase combinations- linear gradient: 10% B (0 min), 90% B (8 min), 95% B (8.1 min), 95% B (18 min), 10% B (20 min), an addition of 10% B (30 min); A: Distilled water, B: Acetonitrile: Methanol (88:12, v/v); 800 μl/min flow rate. A standard curve was obtained by linear regression of the peak area ratio of authentic EET standards. The amount of EETs in samples was calculated according to the standard curve. Only 11,12-EET was detected in the samples.

16. Testosterone ELISA assay

A commercial ELISA kit (Enzo Life Sciences, ADI-900-065) was used to determine testosterone concentrations in media according to the manufacturer's protocol. Briefly, confluent cells in 10 cm^2 plate were incubated in serum-free medium in the absence or presence of testosterone for 3 h, and then cellular lysate was used for ELISA assay. Testosterone concentrations were determined by measuring the absorbance at 405 nm and were normalized to total protein concentrations in each plate.

17. Statistical Analysis.

Scanning densitometry was performed using LAS-3000mini (Fujifilm, Tokyo, Japan). Student's t test was used to examine between group differences. Statistical significance was accepted at either $P < 0.05$ or $P < 0.01$.

III. Results

A. Role of aromatase in chemo-resistance in breast cancer

1. Up-regulation of aromatase expression in TAM-resistant human breast cancer

Aromatase activity plays an important role in breast cancer development through estrogen synthesis (Chen, 1998). We compared the changes in protein expression and activity levels of aromatase in both MCF-7 and TAMR-MCF-7 cells. Western blot analyses revealed that the protein expression of aromatase was significantly increased in TAMR-MCF-7 cells compared to MCF-7 cells (Fig. 5a, upper). To confirm the result and aromatase band size, we determined the basal level of aromatase expression in diverse cell types (Fig. 5a, lower) such as Raw264.7 that have no expression of aromatase (negative control), MEF cells where aromatase expression can be detected by western blot (Wang *et al.*, 2013), and another tamoxifen resistant breast cancer cell line T47D: A18/PKCα that is stably overexpressing PKCα and ER function is down-regulated (Tonetti *et al.*, 2000). The result has showed that the basal level of aromatase was most expressed in TAMR-MCF-7 cells and an induction of this protein was also found in T47D: A18/PKCα cells compared to T47D: A18/Neo cells, suggesting that aromatase up-regulation is related to chemo resistance in breast cancer.

Reporter gene analysis using an aromatase-luc reporter plasmid containing a -294/+20 bp promoter region of aromatase gene and luciferase structural gene showed that

aromatase-luc reporter activity was obviously higher in TAMR-MCF-7 cells than that in MCF-7 cells (Fig. 5b), which implicates that the aromatase is up-regulated in TAMR-MCF-7 cells via transcriptional activation of the aromatase gene (*CYP19* gene). Consistently, the basal aromatase activity was also enhanced in TAM-MCF-7 cells (Fig. 5c). In order to confirm these results by using human cancer cases, tumor tissues were obtained from two groups of patients, depending on the occurrence of relapse after TAM therapy. Four cases showed resistance to tamoxifen and relapsed within 3 years from surgery, and another four tamoxifen-sensitive cases were disease free. Immunohistochemical analyses showed that aromatase staining was significantly higher in TAM-resistant cases than in TAM-sensitive cases (Fig. 5d). The data suggest that aromatase was consistently activated in TAM-resistant breast cancer.

2. Involvement of CREB activation in the up-regulation of aromatase expression in TAMR-MCF-7 cells

To better understand about the regulation of aromatase expression in TAMR-MCF-7 cells, we further examined which transcription factors are required for aromatase induction in TAMR-MCF-7 cells. Several studies have shown that both CREB binding to *cAMP* response element (CRE) or glucocorticoids binding to glucocorticoid response element (GRE) in the promoter region of aromatase gene regulate the transcription of the gene in breast cancer cells (Chen *et al.*, 2001b; Simpson and Zhao, 1996; Young and MacPhaul, 1998). When we assessed the minimal reporter activities of CRE and GRE in both the cell types, the basal CRE-luc reporter activity was distinctly higher in TAMR-MCF-7 cells

than that in MCF-7 cells (Fig. 6a). However, GRE-luc reporter activity was not significantly altered between the two cell types (Fig. 6b). Western blot analysis also confirmed that the levels of Ser133-phosphorylated CREB (active form) and nuclear CREB were increased in TAMR-MCF-7 cells compared to MCF-7 cells, while GR expression was not changed (Fig. 6c). These results suggest that CREB is major transcription factor responsible for the transactivation of aromatase gene in TAMR-MCF-7 cells.

3. Role of PTEN/*phosphoinositide 3-kinase* (PI3K)/Akt pathway in the up-regulation of aromatase in TAMR-MCF-7 cells

It has been shown that PI3K/Akt, ERK and p38 kinase signaling pathways are involved in the regulation of aromatase activity and CREB activation in breast cancer cells (Choi *et al.*, 2008; Su *et al.*, 2011). As shown in our previous studies (Kim *et al.*, 2008; Phuong *et al.*, 2011), the phospho-active forms of these kinases were all enhanced in TAMR-MCF-7 cells (Fig. 7a). However, blocking of either ERK or p38 kinase by specific inhibitors (ERK inhibitor, PD98059 and p38 kinase inhibitor, SB203580) did not affect aromatase expression in TAMR-MCF-7 cells (Fig. 7b). On the contrary, PI3K inhibition by LY 294002 suppressed the enhanced expression of aromatase in TAMR-MCF-7 cells (Fig. 7b). To confirm the role of PI3K in the transactivation of aromatase gene, TAMR-MCF-7 cells was co-transfected with a dominant negative form of PI3K (p85-myc) and aromatase-luc reporter activity was assessed. The increased promoter activity was significantly reversed by myc-p85 overexpression (Fig. 7c, left). Moreover, co-transfection with constitutive

active form of PI3K (p110-myc) significantly enhanced the aromatase-luc reporter activity by about 4 folds in MCF-7 cells (Fig. 7c, right), suggesting that PI3K/Akt signaling is critical for the up-regulation of aromatase gene transcription.

PTEN is an upstream antagonistic phosphatase of PI3K (Fresno Vara *et al.*, 2004; Samuels *et al.*, 2006). Moreover, we have found that the loss of PTEN expression via promoter methylation coincided with constitutive activation of PI3K in TAMR-MCF-7 cells (Phuong *et al.*, 2011). Thus, we hypothesized that a recovery from PTEN loss in TAMR-MCF-7 cells might inhibit the elevated expression of aromastase. When TAMR-MCF-7 cells were transfected with a plasmid expressing PTEN, the basal expression level of PTEN protein was recovered and aromatase expression was concomitantly decreased in TAMR-MCF-7 cells (Fig. 7d, left). In addition, the aromatase promoter reporter activity was also blocked by co-transfection with PTEN overexpression vector (Fig. 7d, right). 5-aza-2'-deoxycytidine (5-Aza, DNA methylation inhibitor) which reverse the PTEN down-regulation and PI3K/Akt activation in TAMR-MCF-7 cells by targeting on the aberrant hypermethylation in PTEN promoter (Phuong *et al.*, 2011) also significantly reduced aromatase expression in TAMR-MCF-7 cells (Fig. 7e). Moreover, LY294002 or 5-Aza treatment inhibited aromatase activity measured by tritiated water release assay (Fig. 7f). The data confirmed that deregulated PTEN/PI3K/Akt pathway plays a crucial role in the up-regulation of expression and activity of aromatase in TAM-resistant breast cancer cells.

4. PI3K/Akt-dependent CREB activation in TAMR-MCF-7 cells

Because both CREB activation and PI3K/Akt signaling pathway are required for the aromatase up-regulation in TAMR-MCF-7 cells, we then assessed whether PI3K/Akt pathway controls CREB activity. Western blot analysis showed that LY294002 completely inhibited the phosphorylation and nuclear accumulation of CREB in TAMR-MCF-7 cells (Fig. 8a). Minimal CRE reporter activity was also blocked by either LY294002 treatment or cotransfection with p85-myc vector in TAMR-MCF-7 cells (Fig. 8b and 8c). The data implicate that PI3K/Akt may be involved in the CREB activation by stimulating the recruitment of CREB from cytoplasm into nucleus, which could be responsible for the transactivation of aromatase gene. We further showed that PTEN overexpression (Fig. 8d) or 5-Aza treatment (Fig. 8e) significantly reduced the CRE reporter activity. In addition, CREB phosphorylation and nuclear accumulation of CREB in TAMR-MCF-7 cells were diminished by 5-Aza treatment (Fig. 8f).

5. Role of aromatase overexpression in TAMR-MCF-7 cells

An aromatase inhibitor, formestane has been used as a chemotherapeutic agent for post-menopausal estrogen-receptor positive breast cancer patients (Pérez *et al.*, 1994). To assess the functional role of aromatase induction in TAM-resistant breast cancer, the combinatorial cytotoxic effect of formestane with 4-hydroxytamoxifen (the active metabolite of TAM) was determined. TAMR-MCF-7 cells were simultaneously exposed to 4-hydroxytamoxifen (10 and 30 μM) and formestane in the indicated doses for 36 h. MTT assay results demonstrated that formestane

additively increased the sensitivity of TAMR-MCF-7 cells to 10 and 30 μM 4-hydroxytamoxifen (Fig. 9a and 9b). Flow cytometry analysis also showed that formestane alone did not induce apoptosis of TAMR-MCF-7 cells, but did increase the number of apoptotic cells seen in combination with 5 μM 4-hydroxytamoxifen (Fig. 9c). However, the effective concentration of formestane was very high (Fig. 9 a,b,c), considering its potency to inhibit aromatase enzyme activity (Veneziani *et al.*, 2007), non-pharmacological dose (100 μM) of formestane was only active in our experimental condition. These results imply that the additive effect of 100 μM formestane may be just due to cytotoxic effects of two drugs with non-specific mechanism. Hence, we further assessed the role of aromatase induction in TAMR-MCF-7 in androgen-containing condition.

We first confirmed the resistance to tamoxifen in both estrogen-free and estrogen-containing conditions. Both MCF-7 cells and TAMR-MCF-7 cells were exposed to 4-hydroxytamoxifen with the indicated doses in DMEM containing 5% charcoal stripped FBS (Fig. 9d) or in DMEM containing 5% charcoal stripped FBS and 17-β-estradiol (10 *p*M) (Fig. 9e). MCF-7 cells showed a marked response to 4-hydroxytamoxifen even at very low concentrations in both mediums, while TAMR-MCF-7 cells showed less or no response in the absence (Fig. 9d) or presence (Fig. 9e) of 17-β-estradiol (10 *p*M), respectively. Interestingly, TAMR-MCF-7 cells did

not response to the presence of 17-β-estradiol (10 *p*M) while MCF-7 cells showed a modest growth response (Fig. 9e). When both cell lines were cultured in androgen-containing condition, testosterone (1-10 nM) caused a significant reduction in cell growth in MCF-7 cells in a concentration-dependent manner (Fig. 9f), which is consistent with a previous report (Chottanapund *et al.*, 2013). While, the inhibitory effect of testosterone on cell growth of TAMR-MCF-7 cells was less than that in MCF-7 cells, and low concentration of testosterone (1 nM) did not inhibit the growth of TAMR-MCF-7 cells (Fig. 9f). Next, we evaluated the effect of formestane on cell proliferation of TAMR-MCF-7 cells in androgen-containing condition (1 nM testosterone). The data showed that low concentration (30 nM) of formestane caused marginally suppressed cell proliferation of TAMR-MCF-7 cells. 30 μM formestane also caused a similar intensity of cell proliferative inhibition effect (Fig. 9g). The cell proliferation was markedly inhibited at 100 μM formestane, however the effect could be due to the combination cytotoxic effects of proliferative inhibition and toxicity of high concentration of formestane. These data demonstrated that aromatase inhibitor partially recovered the physiological function of testosterone. To further confirm the role of aromatase in the conversion of testosterone in TAMR-MCF-7 cells. We determined cellular testosterone level in MCF-7 and TAMR-MCF-7 cells after exposing those cell lines to testosterone (100 nM). The result showed that cellular testosterone concentration in TAMR-MCF-7

cells was at lower level than that in MCF-7 cells (Fig. 9h), implying that testosterone in TAMR-MCF-7 cells may be converted to estrogen by aromatase induction. The data implicate a possible role of aromatase induction in controlling the cellular concentration of exogenous androgens and aromatase up-regulation may be involved in androgen resistance in tamoxifen- resistant breast cancer.

B. Role of CYP3A4-mediated 11,12-EET pathway

1. Increase in CYP3A4- mediated 11,12-Epoxyeicosatrienoic acid synthesis in TAMR-MCF-7 cells

CYP epoxygenases, including CYP2C8, 2J2, 2C9, CYP3A4, were required for the synthesis of EETs and participate in breast cancer progression (Mitra *et al.*, 2011; Murray *et al.*, 2010). We first examined the expression levels of these epoxygenases in both MCF-7 and TAMR-MCF-7 cells. RT-PCR analyses showed that CYP3A4 mRNA level was dramatically increased in TAMR-MCF-7 cells compared to control MCF-7 cells, while CYP2C8 and CYP2C9 mRNA level was marginally enhanced, and CYP2J2 mRNA level trended to decrease (Figure 10a). Immunoblot analyses confirmed that the expression of CYP3A4 protein was up-regulated in TAMR-MCF-7 cells versus MCF-7 cells (Figure 10b).

Because CYP3A4 displays a high ability of AA epoxygenase in breast cancer (Mitra *et al.*, 2011), we then determined the level of EETs in both cell lines using LC-ESI/MRM/MS methodology. The result indicated that 11, 12-EET synthesis was selectively elevated by

about 8 folds in TAMR-MCF-7 cells compared to MCF-7 cells (Figure 10c) whereas the level of 5, 6-EET, 8, 9-EET, and 14, 15-EET were undetectable in both cell lines (data not shown). The data implicate that 11,12-EET is the elevated major epoxy metabolite of the epoxygenase activity resulted from the overexpression of CYP3A4 in TAMR-MCF-7 cells, suggesting a possible role of CYP3A4-derived 11,12-EET pathway in the development of tamoxifen-resistant breast cancer.

2. Effects of CYP3A4 inhibitor and EET antagonist on cell proliferation and apoptosis in TAMR-MCF-7 cells

The expression of CYP3A4 correlates with decreased survival in breast cancer (Mitra *et al.*, 2011). To identify role of the protein in tamoxifen resistant breast cancer cells, we used ketoconazole, a selected strong CYP3A4 inhibitor. We found that treatment with ketoconazole (10 μM) for 72h inhibited cell proliferation of TAMR-MCF7 cells by 43% using MTT assay (Figure 11a), suggesting the CYP3A4 up-regulation could contribute to cell proliferation of TAMR-MCF-7 cells.

EETs were also reported to promote cell proliferation in several cancers including breast cancer (Mitra *et al.*, 2011). To address the role of CYP3A4-synthesized 11,12-EET in TAMR-MCF-7 cells, 14,15-EEZE, an EET antagonist was used. The result have showed that the exposure of 14,15-EEZE (3 μM) for 72h significantly suppressed the cell proliferation of TAMR-MCF-7 cells (Figure 11a). Moreover, flow cytometry analysis showed that co-treatment of ketoconazole (10 μM) or 14, 15-EEZE (3 μM) increased the sensitivity of TAMR-MCF-7 cells to 4-OH-TAM (3 μM) compared to TAMR-MCF-7 cells

alone treated with 4-OH-TAM (3 μM) (Figure 11b). The data implicate that the CYP3A4-mediated 11,12-EET pathway is important for cell proliferation and apoptosis in tamoxifen-resistant breast cancer.

3. Effects of CYP3A4 inhibitor and EET antagonist on angiogenesis in TAMR-MCF-7 cells

Vascular endothelial growth factor-VEGF plays important role in the angiogenic activity in diverse tissues (Webler *et al.*, 2008; Yang *et al.*, 2009). We previously showed that TAMR-MCF-7 cells exhibit an increase in VEGF-mediated angiogenesis compared to MCF-7 cells (Kim *et al.*, 2009). In addition, the pro-angiogenic activity of EETs was mediated at least in part by VEGF (Webler *et al.*, 2008; Yang *et al.*, 2009). We hypothesize that CYP3A4 overexpression and CYP3A4-derived 11,12-EET synthesis are linked to excessive VEGF-mediated- angiogenesis in TAMR-MCF-7 cells. As expectation, after exposure of TAMR-MCF-7 cells to ketoconazole and 14,15-EEZE, the enhanced mRNA level of VEGF in this cell line was dramatically declined (Figure 12a). In addition, ketoconazole and 14,15-EEZE reduced markedly the higher secretion of VEGF level in TAMR-MCF-7 cells (Figure 12b, c). On the other hand, ketoconazole and 14,15-EEZE also blocked the elevated transcription activation of *VEGF* gene in this cell type (Figure 12d, e). The data imply a potential role of CYP3A4 overexpression and CYP3A4-mediated 11,12-EET in regulating the angiogenic activity in TAMR-MCF-7 cells.

Next, we tested the effect of those chemicals to immune deficient embryonic development of chicken embryos in chicken eggs by using chick chorioallantoic membrane

(CAM) assay, which is a convenient model to evaluate the tumor- induced angiogenesis (Ribatti *et al.*, 1999). CAM assay was performed after seeding both MCF-7 cells and TAMR-MCF-7 cells with VEGF (20ng/CAM) treated CAM as positive control. The results showed that CAM bearing TAMR-MCF-7 cells produced higher intensity of blood vessels than that bearing MCF-7 cells or VEGF (20ng/CAM) (Figure 12f, g). Ketoconazole and 14,15-EEZE treatment diminished the number of blood vessels artound tumor produced by TAMR-MCF-7 cells in dose dependently manner (Figure 12f, g). These data suggest that CYP3A4 inhibitor and EETs antagonist (14,15-EEZE) suppress the microvessel formation and angiogenesis in part via down-regulation of VEGF. Moreover, CAM assay also displayed that the tumors formed from TAMR-MCF-7 cell masses in CAM was much bigger than that from MCF-7 cells, implicating that TAMR-MCF-7 cells growth faster than MCF-7 cells. And, ketoconazole and 14,15-EEZE treatment significantly lost the tumor weight formed by bearing TAMR-MCF-7 cell masses in dose dependent manner (Figure 12h). Taken together, our data highlight a critical role of CYP3A4-mediated 11,12-EET signaling pathway in regulation of both angiogenesis and tumorigenesis in tamoxifen-resistant human breast cancer.

4. Effects of CYP3A4 inhibitor and EET antagonist on cell migration in TAMR-MCF-7 cells

CYP epoxygenase and its derived EETs were found to correlate with human cancer metastasis (Jiang *et al.*, 2007). Especially, 11,12-EET was reported to contribute to the migration and invasion capabilities of prostate cancers (Nithipatikom *et al.*, 2010).

However, there have been no studies on the role of CYP3A4 and 11,12-EET in the migration of breast cancers and endocrine resistant breast cancers as well. Previously, Hiscox et al. reported that breast cancer cells exhibited greater metastatic ability *in vitro* characterized by increase in motile and invasion behavior during development of acquisition of tamoxifen resistance (Hiscox *et al.*, 2006; Hiscox *et al.*, 2004). The studies mentioned to the role of EGFR and Scr activity (Hiscox *et al.*, 2006; Hiscox *et al.*, 2004). Consistently, we have confirmed that TAMR-MCF-7 cells displayed a dramatically increase in migration ability compared to MCF-7 cells by approximate 4 folds (Figure 13a). Next, we wanted to identify a role of CYP3A4 and CYP3A4-mediated 11,12-EET in regulating cell migration of TAMR-MCF-7 cells. Treatment with ketoconazole and 14,15-EEZE significantly inhibited the cell migration of TAMR-MCF-7 cells (Figure 13b). The data implicate that up-regulated CYP3A4 and its mediated 11,12-EET formation may contribute to the greater migratory capacity of TAMR-MCF-7 cells.

Cancer metastasis is associated with the enhanced synthesis of metastasis-related genes including matrix metalloproteinases (MMPs) (Stetler-Stevenson, 1999; Sun *et al.*, 2009). Moreover the high levels of MMP-2 and MMP-9 have been related to breast cancer invasion (Sun *et al.*, 2009). We further examined the expression of these proteins in both cell lines. A markedly induction of both MMP-2 and MMP-9 levels was observed in TAMR-MCF-7 cells compared with MCF-7 cells by RT-PCR (Figure 13c), zymography assay (Figure 13d), and immune blot (data not shown). Moreover, reporter gene assay with full-length human MMP2 and MMP9 promoter-luciferase constructs also showed an

increase in the activity of these genes in TAMR-MCF-7 cells compared to MCF-7 cells (Figure 13 e, f). These data suggest that up-regulation of MMP-2 and MMP-9 expression was at transcriptional level and might relate to migratory phenotypic changes in TAMR-MCF-7 cells. To address whether CYP3A4-mediated 11,12-EET pathway is related to the expression of these proteins, TAMR-MCF-7 cells were exposed with ketoconazole and 14,15-EEZE for 24h. The results showed that ketoconazole treatment suppressed the mRNA level (Figure 13g) and the reporter gene activities (Figure 13h, i) of both MMP-2 and MMP-9, but 14,15-EEZE treatment had no effects on these protein expression (data not shown).

5. Influence of CYP3A4-derived 11,12-EET is mediated by RB/E2F1-dependent Pin 1 in TAMR-MCF-7 cells

Pin 1, a peptidyl prolyl iromerase, is an enzyme which has been observed to involve in diverse cancer development processes such as proliferation, differentiation, and apoptosis (Bao *et al.*, 2004). Previously, we have reported that Pin 1 is overexpressed in TAMR-MCF-7 cells and it is critical for the VEGF-mediated angiogenesis and EMT processes (Kim *et al.*, 2009; Kim *et al.*, 2009). Giving important role of Pin 1, we examined a possible relation between Pin 1 and CYP3A4-mediated 11,12-EET signaling pathway in TAMR-MCF-7 cells. Treatment with ketoconazole and 14,15-EEZE significantly diminished the overexpression of Pin 1 in TAMR-MCF-7 cells (Figure 14a). Also, treatment with MSPPOH, an epoxygenase inhibitor, also inhibited Pin 1 overexpression in this cell line (Figure 14a). E2F1 activity is required for Pin 1 overexpression in TAMR-

MCF-7 cells (Lee *et al.*, 2011), moreover E2F activity is dependent on RB phosphorylation (Nevins, 2001). We have also found that the exposure to ketoconazole or 14,15-EEZE blocked the phosphorylation of RB and reduced the expression of E2F1 in TAMR-MCF-7 cells. Furthermore, The treatment of MCF-7 cells with synthetic eicosanoid products including 8,9-EET, 11,12-EET and 14,15-EET significantly up-regulated the Pin 1 expression and the Pin 1 reporter activity in MCF-7 cells (Figure 5c). And, treatment with AUDA, a sEH inhibitor which inhibits EETs biological metabolism also caused a marked increase in the Pin 1 expression and the Pin 1 reporter activity in MCF-7 cells (Figure 14d). Also, exposure to 11,12-EET which has been shown as a major metabolite in TAMR-MCF-7 cells activated Pin 1 expression and RB/E2F1 activity in dose dependent manner in MCF-7 cells (Figure 14e). These data implicate that CYP3A4-derived 11,12-EET signaling pathway is mediated by PR/E2F1- dependent Pin1 overexpression in TAMR-MCF7 cells.

We confirmed the role of Pin 1 in TAMR-MCF-7 cells by establishing a stable Pin1 knockdown/TAMR-MCF-7 cell line using Pin1 shRNA lentivirus. The efficacy of the transfection was confirmed by a decrease in Pin 1 expression (Figure 15a) and mRNA Pin 1 level (Figure 15b). Similar to the influences of CYP3A4 inhibitor and EET antagonist, Pin1 knockdown/TAMR-MCF-7 cell line showed a slower growth than control shRNA/TAMR-MCF-7 cells (Figure 15c). Moreover, Pin 1 knockdown diminished the secretion of VEGF (Figure 15d), implicating an ability of angiogenenic inhibition that was previously demonstrated by Kim et al. (Kim *et al.*, 2009). As well, Pin1 silencing also inhibited cell migration of TAMR-MCF-7 cells (Figure 15e) and reduced the mRNA levels

of MMP-2 and MMP-9 mRNA level in this cell line (Figure 15f). These data strongly interpret that the effect of CYP3A4- derived 11,12-EET signaling pathway is mediated by Pin 1 overexpression in TAMR-MCF-7 cells.

IV. Discussion

A. Role of aromatase induction in tamoxifen resistant breast cancer

Aromatase expression and estrogen levels are frequently elevated in breast cancer tissue compared to normal mammary tissue (Chen, 1998; James *et al.*, 1987; Miller *et al.*, 1997; Zhou *et al.*, 1996; Zhou *et al.*, 1997). Up-regulation of aromatase in tumor tissues is believed to stimulate breast cancer growth in either autocrine or paracrine manner (Sun *et al.*, 1997). It has been also suggested that aromatase can be converted into a more active form in growth factor(s)-exposed breast cancer cells via post-transcriptional modification (Su *et al.*, 2011). Our current study has focused on the regulation of aromatase expression in TAM-resistant breast cancer. Both the protein expression and the gene transcriptional activity of aromatase were obviously enhanced in TAMR-MCF-7 cells compared to MCF-7 cells. Aromatase expression was also up-regulated in a different tamoxifen-resistant breast cancer cell line, T47D:A18/PKCα cells which are stably overexpressing PKCα and ER function are down-regulated (Tonetti *et al.*, 2000). Moreover, immunostaining intensity of aromatase was much higher in TAM-resistant human breast cancer tissues than TAM-sensitive cases. From these results, we can conclude that aromatase expression and its increased activity may correlate with the acquisition of TAM resistance.

Aromatase was reported to be important for the effect of androgens on the proliferation of different breast cancers co-cultured with or without breast adipose fibroblast (Chottanapund *et al.*, 2013). Testosterone displayed inhibitory ability on cell growth in different breast cancers however in co-culture system with breast adipose fibroblasts, where aromatase was highly expressed the inhibitory effect of testosterone was recovered (Chottanapund *et al.*, 2013). Consistently, we have shown that in TAMR-MCF-7 cells where have higher expression of aromatase than MCF-7 cells, the inhibitory effect of testosterone on cell growth was less than that in MCF-7 cells. In combination treatment with an aromatase inhibitor, formestane significantly inhibited cell proliferation of TAMR-MCF-7 cells in testosterone-containing condition. Moreover, the cellular level of testosterone in TAMR-MCF-7 cells after exposing to exogenous testosterone was lower than that in MCF-7 cells. Recent studies have showed that androgens and the androgen receptors are potential targets for hormonal treatment in breast cancer patients, especially in the case of triple negative breast cancer (Gucalp *et al.*, 2010; He *et al.*, 2012; Tang *et al.*, 2012). Our data imply that aromatase induction may be an issue for the therapeutic strategies of breast cancer targeting on androgen and androgen receptors. TAM resistance is typically results from alternation in ER activation, specially the loss of ER-α expression (Giacinti *et al.*, 2006). It may be reason for reducing growth response to estrogen in tamoxifen resistant breast cancer. However, the loss of ER-α expression is not the only mechanism driving acquired tamoxifen resistance because mutation in ERα occurs only in 15% of patients (Gutierrez *et al.*, 2005). Many studies have reported on

changes contributing to endocrine resistance in breast cancer such as HER2 overexpression (Pietras *et al.*, 1995), DNA methylation (Phuong *et al.*, 2011), up-regulation of antioxidant proteins (Kim *et al.*, 2008) and overexpression of cell cycle regulating factor Pin 1 (Kim *et al.*, 2009). Herein, we have found that aromatase induction may be a new mechanism involved in reduced susceptibility to exogenous TAM and androgens in the treatment for breast cancer patients. Considering the clinical reports showing a significant benefit of aromatase inhibitors to prevent the relapse of TAM-resistant breast cancer (Boccardo *et al.*, 2006; Kaufmann *et al.*, 2007); our findings may be meaningful to suggest a mechanistic basis for the treatment regimen.

The regulation of aromatase expression is different in various tissues and is dependent on multiple tissue specific promoters (Means *et al.*, 1991; Utsumi *et al.*, 1996). In breast cancer tissue, the gene transcription is mainly regulated by cAMP-stimulated promoters 1.3 (Zhou and Chen, 1999) and II (Zhou *et al.*, 1996), while in normal mammary tissue it is driven by glucocorticoid-stimulated promoter 1.4 (Chen *et al.*, 2002). cAMP responsive elements (CRE1 and CRE2) are essential for cAMP-induced promoter II activity (Sofi *et al.*, 2003). CREB can be phosphorylation by cAMP-dependent protein kinase at Ser-133 residue (Johannessen *et al.*, 2004). The activated CREB binds to the CRE promoter region, and initiates the gene transcription in response to a variety of extracellular signals including hormones and growth factors (Deutsch *et al.*, 1988). Here, we have shown that CREB but not GR is responsible for the enhanced aromatase transactivation in TAMR-MCF-7 cells. The enhanced CREB phosphorylation at Ser-133 may cause the nuclear

translocation of CREB, and its binding to CRE1 and CRE2 stimulates the transactivation of aromatase gene in TAMR-MCF-7 cells.

In response to diverse stimuli, several kinase family play a key role in the activity regulation of most transcription factors (Treisman, 1996). It has been reported that Ser-133 phosphorylation of CREB is under the control of two mitogen-activated protein (MAP) kinases, ERK and p38 kinase (Cammarota *et al.*, 2001; Gelain *et al.*, 2006; Hokari *et al.*, 2005). In our previous study, ERK, but not p38 kinase was related with CREB-mediated aromatase overexpression in p53-inactivated mammary epithelial cells (Choi *et al.*, 2008). Although both the kinases were consistently activated in TAMR-MCF-7 cells, blocking of ERK or p38 kinase did not change the expression of aromatase, demonstrating that MAPK family is not required for the up-regulation of aromatase in the cell type. Instead, we found out that both the expression and activity of aromatase and CREB were suppressed by PI3K inhibition, suggesting an association of PI3K signaling in CREB-dependent aromatase expression in TAMR-MCF-7 cells. In fact, there have been several previous studies depicting the role of PI3K in regulating aromatase expression in breast cancers. For instance, anti-estrogen ICI-182,780 suppressed aromatase activity in aromatase-overexpressed MCF-7 cells (MCF-7aro) by decreasing AKT phosphorylation (Sabnis *et al.*, 2007). Another study also showed that aromatase activity in breast cancer cells was controlled by growth factor signaling pathways including PI3K/Akt pathway (Su *et al.*, 2011). These reports support our finding that PI3K plays a pivotal role in aromatase up-regulation in TAMR-MCF-7 cells.

The proper PI3K activity is required for the cell growth and the acquired chemo-resistance of breast cancers (Frogne *et al.*, 2005; Jordan *et al.*, 2004), and its enzyme activity is negatively regulated by PTEN expression (Fresno Vara *et al.*, 2004; Samuels *et al.*, 2006; Stambolic *et al.*, 1998). We have previously demonstrated that DNA promoter methylation is a key reason for the loss of PTEN expression and the abnormal characteristics such as rapid proliferation and chemo-resistance in TAMR-MCF-7 cells (Phuong *et al.*, 2011). 5-Aza, a DNA methylation inhibitor restored the loss of PTEN expression and inhibited the exaggerated activity of PI3K/AKT pathway, and consequently recovered endocrine resistance of TAM-resistant breast cancer (Phuong *et al.*, 2011). Thus, down-regulation of aromatase expression by 5-Aza or PTEN overexpression demonstrated in the present study confirming a close correlation between PTEN/PI3K/AKT signaling and aromatase expression during TAM resistance acquisition.

B. Role of CYP3A4-mediated 11,12-EET signaling pathway

The pathological consequences of cancers are mainly correlated to uncontrolled tumor growth and metastasis, causing by abnormal tumor cell proliferation, adhesion, invasion, and migration. Angiogenesis is also implicated in the pathological growth of neoplastic tumors although it is a critical for the processes of wound healing and tissue regeneration (Xu *et al.*, 2011). During acquired chemo-resistant developing progresses, TAM-resistant breast cancer cells have been displaying typical characters of resistance supporting them to become immortal and uncontrolled cancer cells such as rapid growth, microvessels formation for the angiogenesis and tumorigenesis, changing in EMT behavior and high

capacity of metastasis (Hiscox *et al.*, 2004; Kim *et al.*, 2009a; Phuong *et al.*, 2011). The underlying mechanisms implicating for these malignant changes have been poorly understood. Increasing evidences show the effects of CYP epoxygenases and its derived eicosanoids on the pathogenesis of carcinogenesis including cell proliferation, migration, and angiogenesis (Xu *et al.*, 2011; Chen *et al.*, 2011; Jiang *et al.*, 2005; Jiang *et al.*, 2007). Here, our studies have demonstrated a crucial role of CYP3A4-mediated 11,12-EET in regulation of cell proliferation, angiogenesis, and migration in TAM-resistant human breast cancer cells.

CYP3A4 is the most expressed P450 in the human liver and is the major enzyme involved in xenobiotic metabolism (Zhou, 2008). CYP3A4 activates several pro-carcinogens, including polycyclic aromatic hydrocarbons (PAH), and also has critical roles in the metabolism of various anticancer drugs (Shimada *et al.*, 1994; Windmill *et al.*, 1997). CYP3A4 is also expressed in several cancer cell lines such as breast cancer cells, colon cancer cells, and liver cancer cells (Chen *et al.*, 2011; Pfrunder *et al.*, 2003; Yasuda *et al.*, 2008) and can induce cancer growth (Mitra *et al.*, 2011). It is reported that several anticancer drugs such as tamoxifen induce CYP3A4 expression, by which mechanism cancer cells can acquire drug resistance (Dhaini *et al.*, 2003). In our study, we have highly evidenced that CYP3A4 was overexpressed in tamoxifent resistant breast cancer and the knockdown of this protein obstructed significantly cell proliferation and induced synergistic effect to 4-OH-TAM in controlling cell apoptosis. Therefore, the accelerated level of CYP3A4 found is important both to breast cancer development and in influencing

the response of breast cancer to chemotherapy. Moreover, the inhibition of CYP3A4 obviously declined the enhanced angiogenesis and greater migration in TAMR-MCF-7 cells, suggesting a critical role of the overexpression of CYP3A4 as a fundamental mechanism of the malignant changes during the development of drug resistance in breast cancer. The role of CYP2J2 was previously reported in human cancer metastasis including breast cancer (Jiang *et al.*, 2007), however recent studies of both Murray et al. 2010 and Mitra et al. 2011 have showed that CYP2J2 was unassociated with overall survival and its immunoactivity was nearly undetectable in breast cancer (Mitra *et al.*, 2011; Murray *et al.*, 2010). The CYP2J2 mRNA level was decreased in TAMR-MCF-7 cells, suggesting that CYP2J2 was unrelated with malignant changes in this cell line. Both CYP3A4 and CYP2C8 were reported to participate in breast cancer growth (Mitra *et al.*, 2011), however the role of CYP3A4 was specially emphasized by that the knockdown of CYP3A4 induced CYP2C8 and CYP3A5 mRNA level but this induction failed to compensate for the growth inhibition caused by CYP3A4 knockdown (Mitra *et al.*, 2011). In present study, we have showed that CYP3A4 was most highly overexpressed in TAMR-MCF-7 cells while the induction of CYP2C8/2C9 was limited and that of CYP2J2 was even reduced. Taken together, the finding highlights a pivotal role of overexpression of CYP3A4 in multiple regulations of cell proliferation, angiogenesis, tumorgenesis and migration in tamoxifen resistant breast cancer.

EETs play important role in cancers by its ability to promote cell proliferation, angiogenesis and metastasis (migration and invastion) (Jiang *et al.*, 2005; Jiang *et al.*,

2007). Abundant evidences have showed that the influences of CYP3A4 are via producing EETs, or in the other word, EETs participate in the effects of CYP3A4 (Mitra *et al.*, 2011; Oguro *et al.*, 2011). Here, we have found an increase in 11,12-EET biosynthesis in TAMR-MCF-7 cells. As well, blocking the function of EETs by EET antagonist (14,15-EEZE) has showed similar results with the inhibition of CYP3A4 expression in cell proliferation, angiogenesis and migration of TAMR-MCF-7 cells. Our studies suggest that the effects of CYP3A4 in TAMR-MCF-7 cells are at least in part related to 11,12-EET activity. Studies with purified CYP epoxygenases indicate that the catalytic efficiency and region-selectivity of EET formation are P450 isoform- and species-specific (Imaoka *et al.*, 2005; Nishimura *et al.*, 2003; Zeldin, 2001; Chen *et al.*, 2011). Each enzyme is able to convert AA to all four EET regioisomers, the main products in many cases are 11,12- and 14, 15-EET (Capdevila *et al.*, 2000). CYP2C8 and CYP2C9 were reported to produce 14,15-EET at higher concentration than 11,12-EET and 8,9-EET (Zeldin *et al.*, 1995; Thompson *et al.*, 2000; Zeldin, 2001). These reports may in part support for explicating of the selective enhanced CYP3A4-mediated 11,12-EET biosynthesis in TAMR-MCF-7 cells. 11,12-EET was also reported to be a major metabolite in prostate cancer (Nithipatikom *et al.*, 2010). However, the mechanism of the selective biosynthesis and metabolism of eicosanoids in TAMR-MCF-7 cells remains controversy and requires further studies.

EETs promote angiogenesis via up-regulating eNOS, and activating MEK/MAPK and PI3-K pathway (Wang *et al.*, 2005) or the Src-dependent STAT3-mediated VEGF expression (Cheranov *et al.*, 2008). 11,12-EET was reported to stimulate angiogenesis

through activating the EGF receptor (Michaelis *et al.*, 2008) and sphingosine kinase 1 (SK1) (Yan *et al.*, 2008). However, most of studies showed that the pro-angiogenic activity of EETs is mediated at least in part by VEGF (Webler *et al.*, 2008; Yang *et al.*, 2009). The activity of VEGF is required for the angiogenic process in TAMR-MCF-7 cells (Kim *et al.*, 2009b; Kim *et al.*, 2008). Here, we have showed that CYP3A4-synthesized 11,12-EET signaling pathway regulated the angiogenic progression via VEGF activity and it was close associated to the overexpression of RB/E2F1-dependent Pin 1, of which is important for the angiogenesis activity and EMT behavior in TAMR-MCF-7 cells. The effects of EETs and VEGF regulation are closely related. EETs can enhance the effects of VEGF-induced angiogenesis (Webler *et al.*, 2008). In turn, VEGF can increase the CYP2C promoter activity in endothelial cells and induce the expression of CYP2C8, resulting in increased intracellular EET levels (Webler *et al.*, 2008).

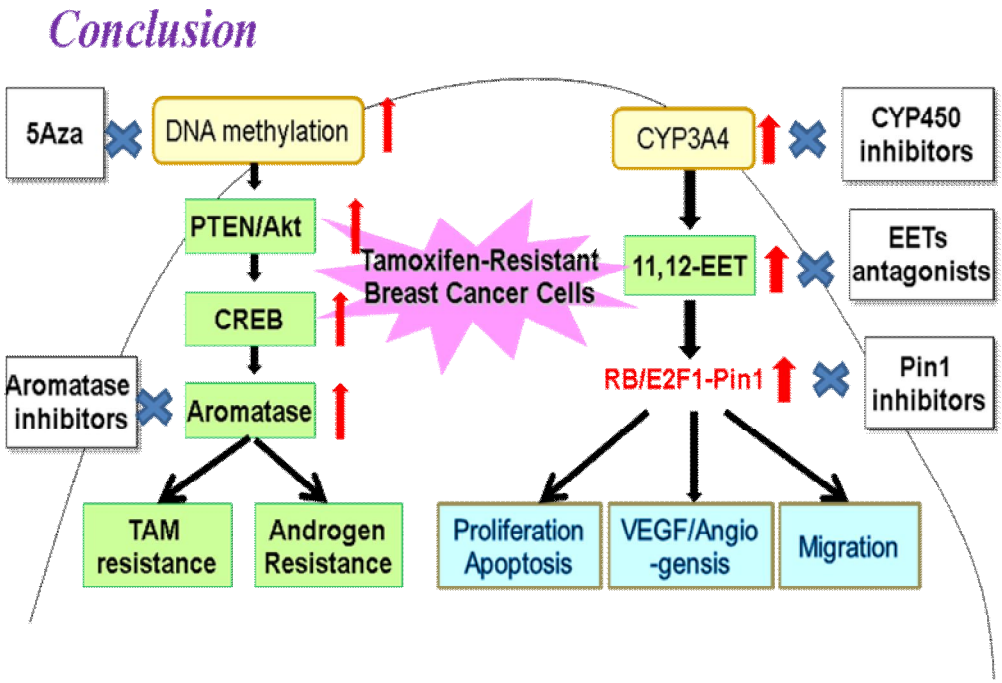
CYP epoxygenases and its derived EETs stimulate the migration and invasion of various cell types (Michaelis *et al.*, 2008), including some cancer cells (Jiang *et al.*, 2007) through the up-regulation of the pro-metastatic MMPs and CD44, and down-regulation of the anti-metastatic genes CD82 and NM23 (Jiang *et al.*, 2007). 11,12-EET was reported to induce cell motility (migration and invasion) in prostate carcinoma cancer via EGFR and Akt (Ser473) activation. The selective CYP epoxygenase inhibitors or synthetic EET antagonists could recover the effect of 11,12-EET on cell invasion and migration in these cell lines (Nithipatikom et al., 2010). In present study, we have showed that CYP3A4 overexpression and CYP3A4-synthesized 11,12-EET contributed to promote the migration

capacity of TAMR-MCF-7 cells. The effects of CYP3A4 on cell migration was likely correlated with the activities of MMP2, MMP9, however the treatment with EET antagonist (14,15-EEZE) had no effect on the activity of these genes (data not shown), implicating an elusive mechanism of CYP3A4-mediated 11,12-EET pathway in regulating the migration in TAMR-MCF-7 cells. Previously, we showed that Pin 1 was overexpressed and closely related with the acquired resistance, the VEGF-mediated angiogenesis, and the EMT process in TAMR-MCF-7 cells (Khanal *et al.*, 2011; Kim *et al.*, 2009a; Kim *et al.*, 2009b). Interestingly, here, we have found a new role of Pin 1 in regulating cell migration. Moreover, CYP3A4-synthesized 11,12-EET signaling pathway is associated to RP/E2F1-dependent Pin 1 overexpression in tamoxifen resistant breast cancer.

C. CONCLUSION

Identification of new phenomena related to the resistance is needed to contribute effective therapies for breast cancer patients. We have demonstrated that aromatase expression is up-regulated through PI3K/Akt-dependent CREB activation and related to exogenous tamoxifen resistance and exogenous androgen sensitivity in TAM-resistant human breast cancer. However, more studies on role of aromatase are necessary to contribute a convincing rationale for using aromatase inhibitors for treatment in tamoxifen-resistant breast cancer. Also, we have revealed another new therapeutic target involved in CYP3A4-mediated 11,12-EET signaling pathway. We have found that CYP3A4-mediated 11,12-EET signaling plays a pivotal role in regulating the malignant changes such as rapid growth, angiogenesis, and migration in tamoxifen resistant breast cancer and is partially

mediated by RB/E2F1-dependent Pin 1 overexpression. In conclusion, our studies provide rationals for using and developing the therapeutic targets involving in aromatase expression and CYP3A4-mediated EET signaling pathway for the treatment of tamoxifen resistant human breast cancer.



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Figures

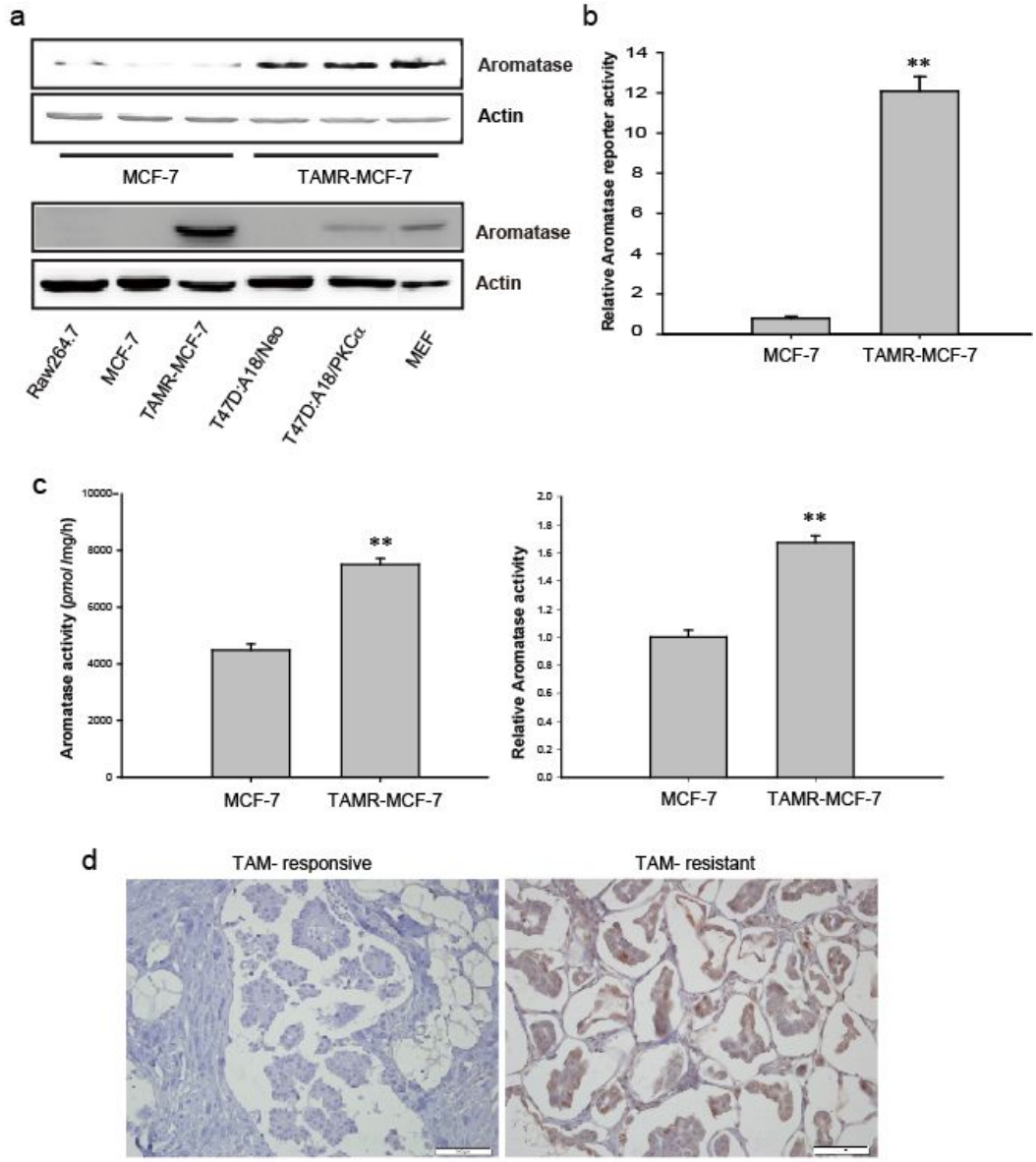


Figure 5. Up-regulation of aromatase expression in TAM-resistant human breast cancer

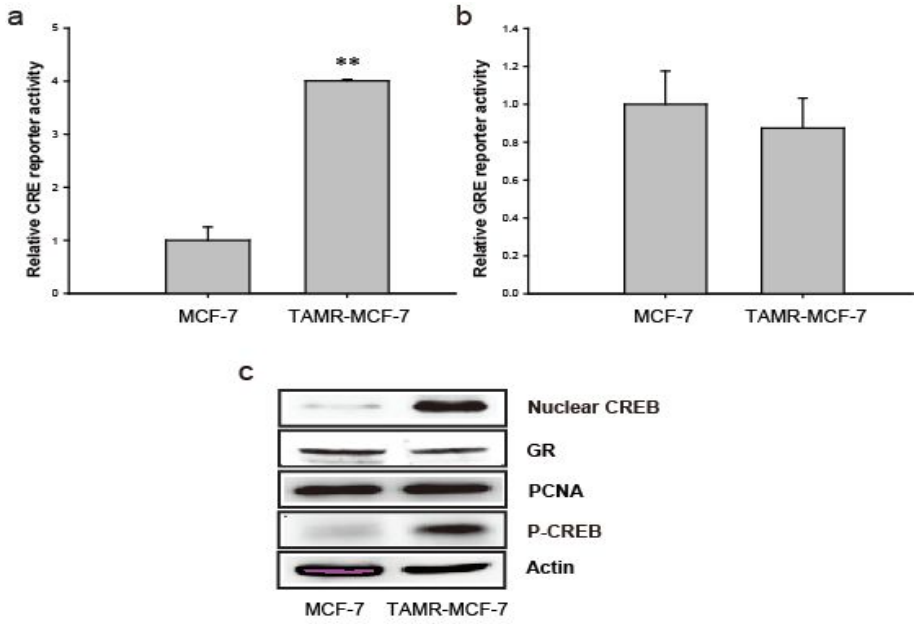


Figure 6. Involvement of CREB activation in the up-regulation of aromatase expression in TAMR-MCF-7 cells

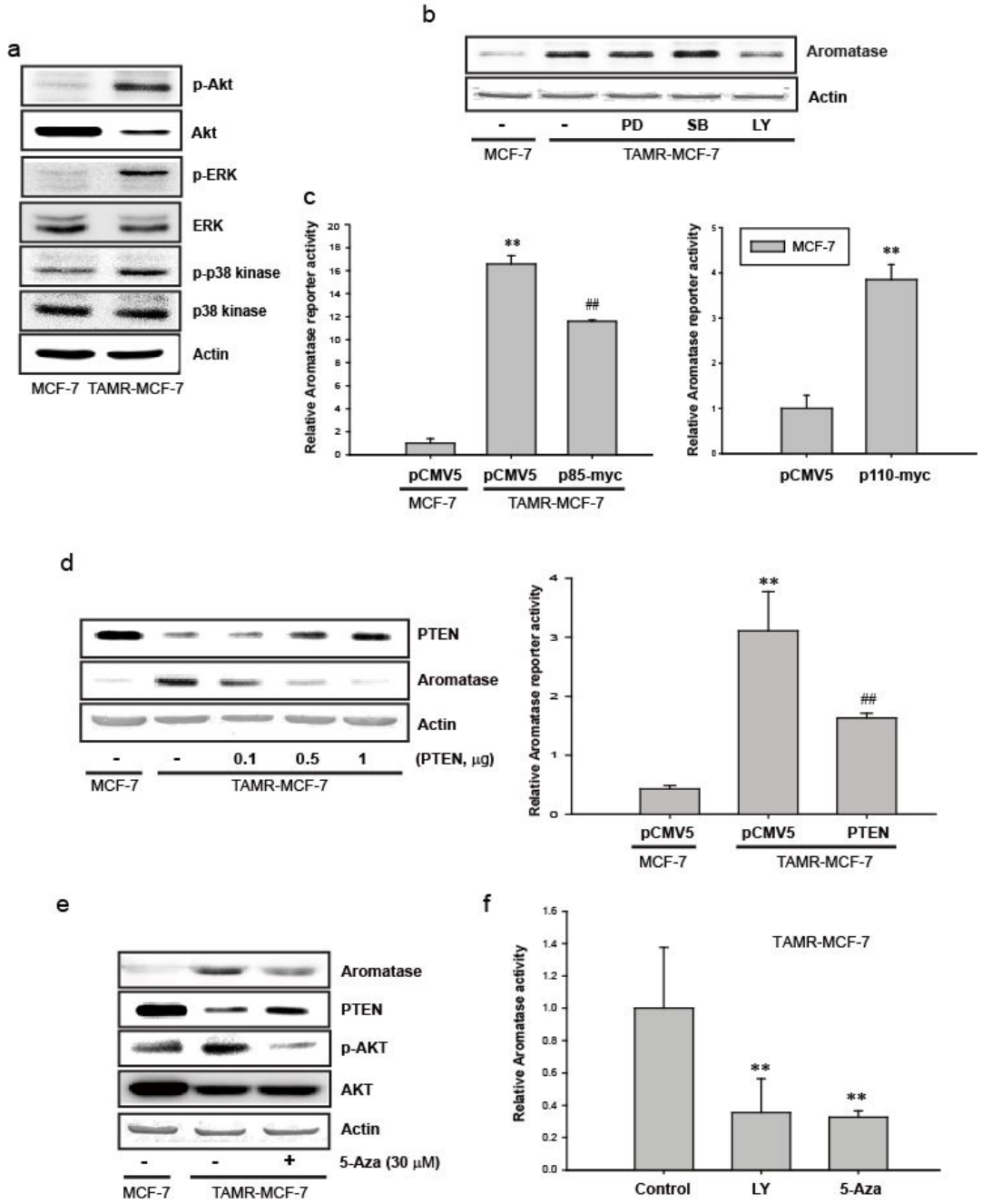


Figure 7. Role of PTEN/phosphoinositide 3-kinase (PI3K)/Akt pathway in the up-regulation of aromatase in TAMR-MCF-7 cells

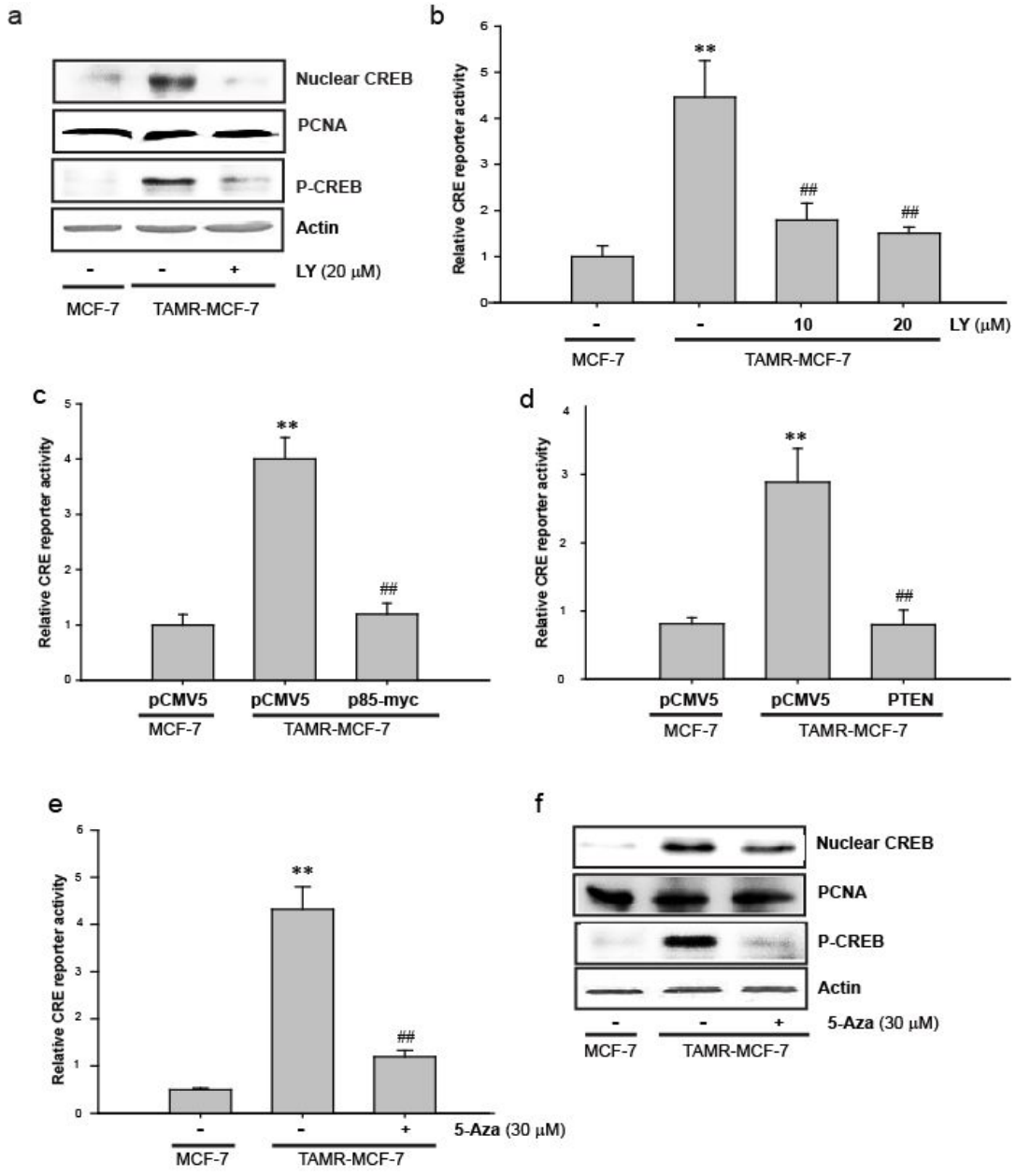


Figure 8. PI3K/Akt-dependent CREB activation in TAMR-MCF-7 cells

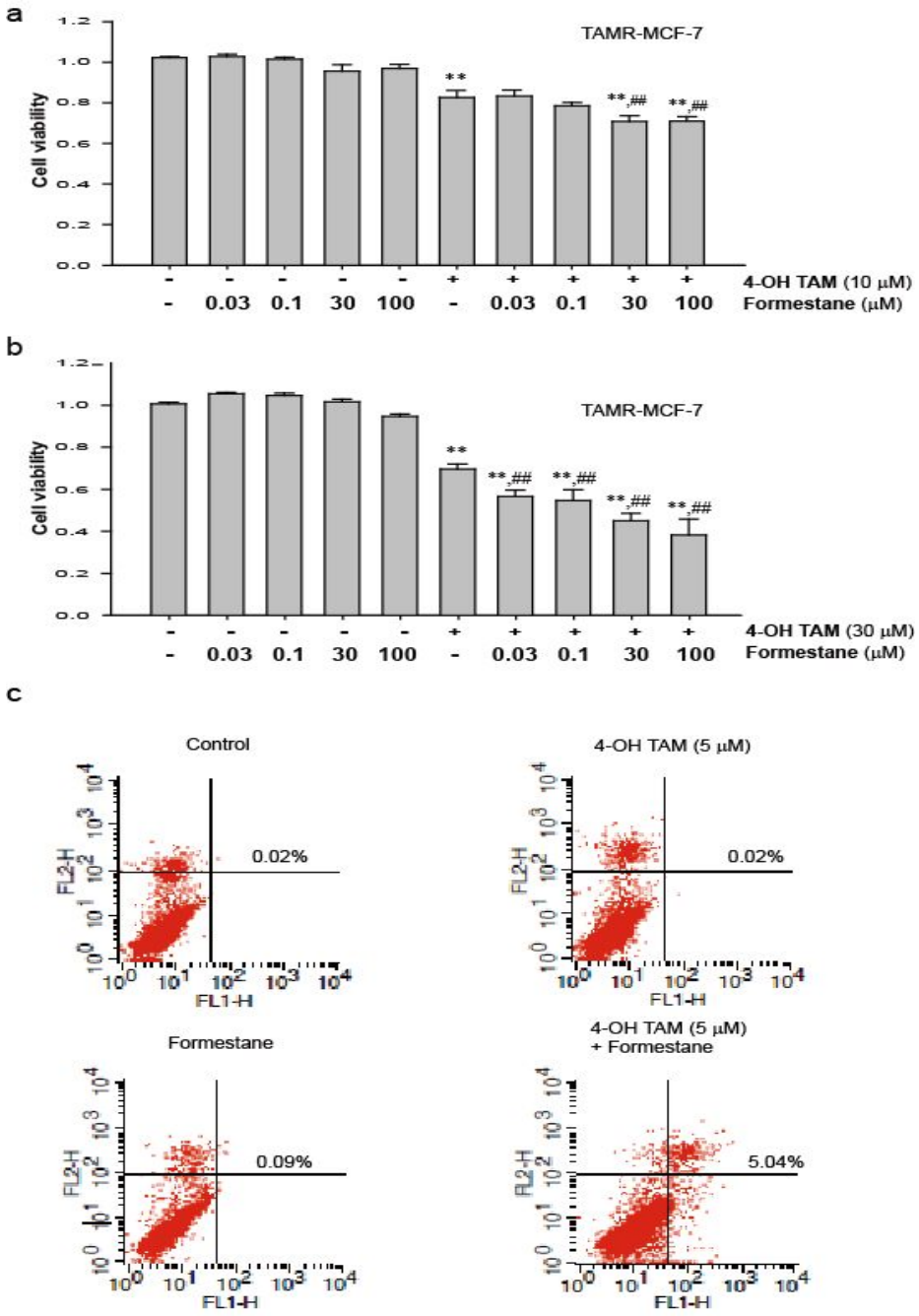
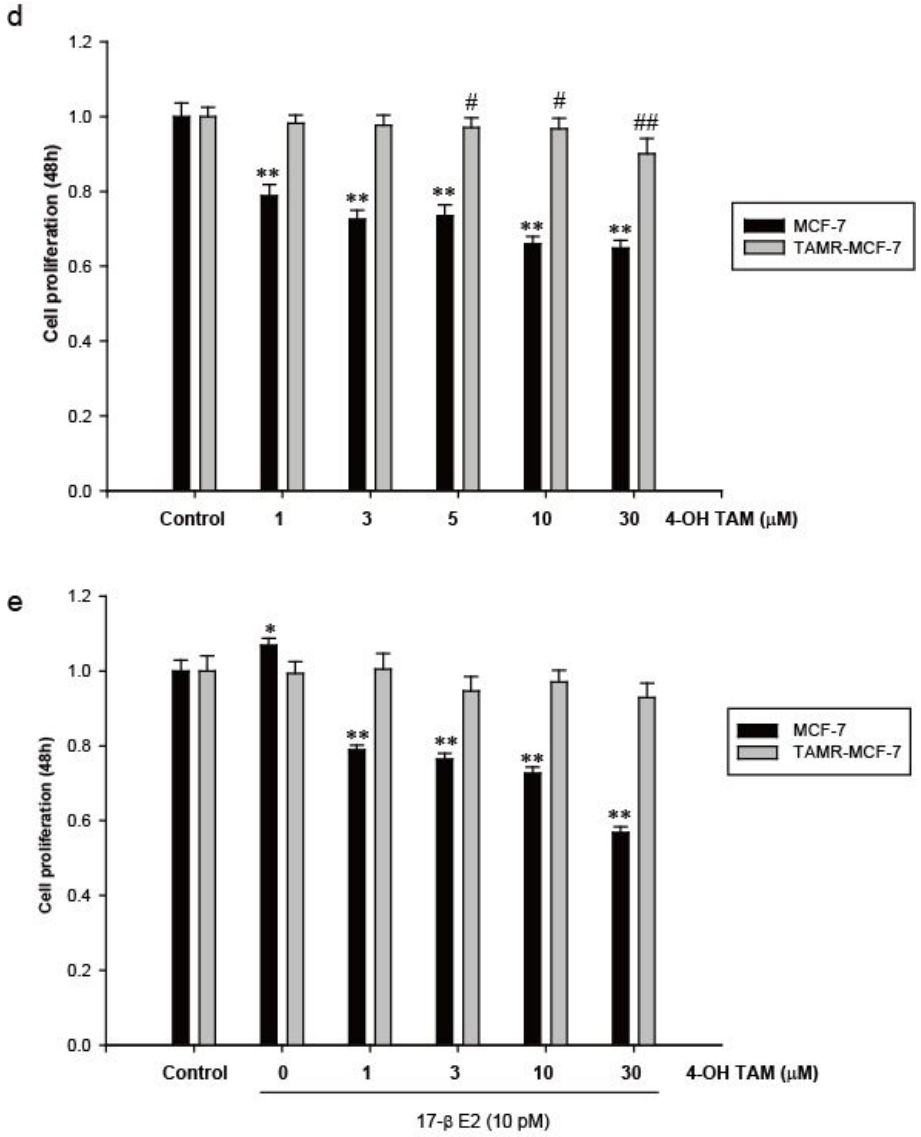


Figure 9. Role of aromatase induction in TAMR-MCF-7 cells



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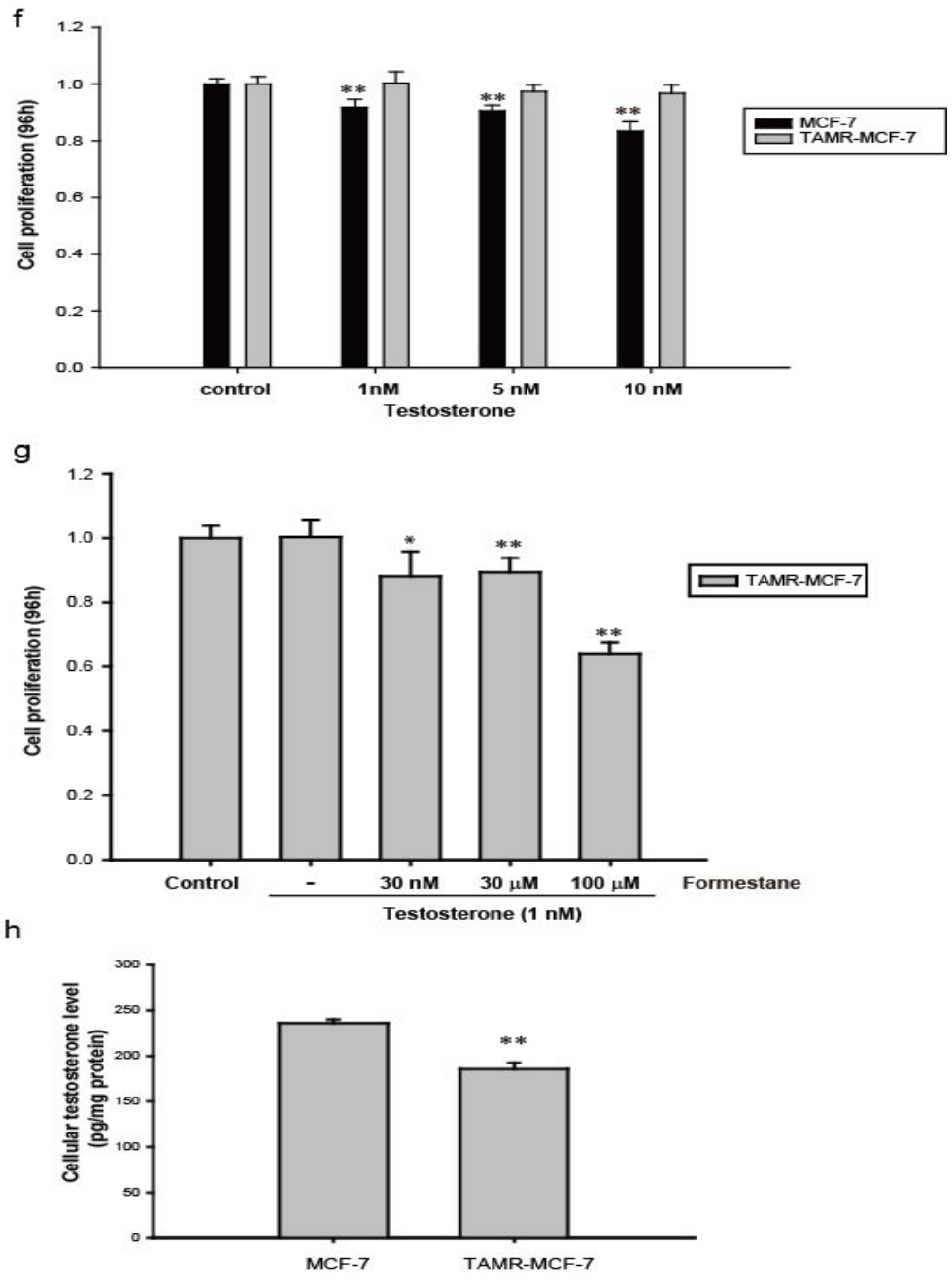

Figure 9. Role of aromatase induction in TAMR-MCF-7 cells

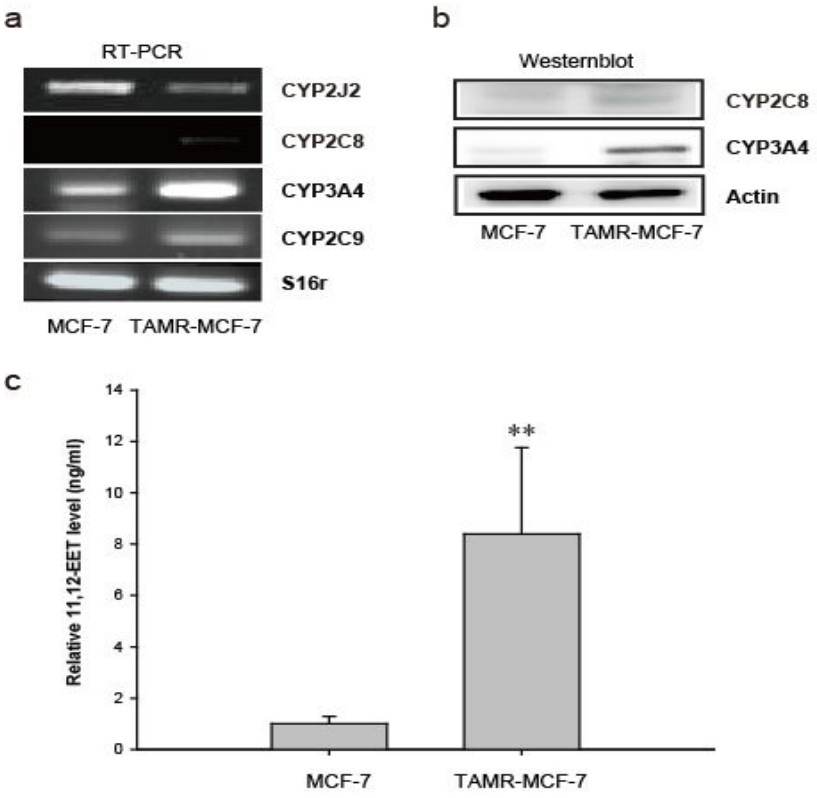

Figure 10. Increase in CYP3A4- mediated 11,12-Epoxyeicosatrienoic acid synthesis in TAMR-MCF-7 cells

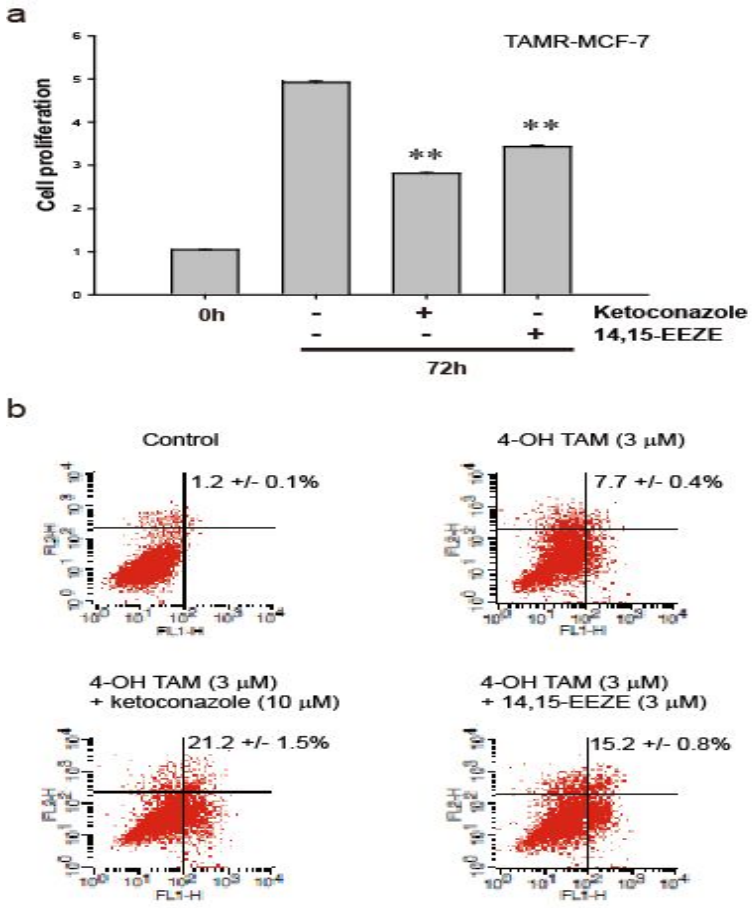

Figure 11. Effects of CYP3A4 inhibitor and EET antagonist on cell proliferation and apoptosis in TAMR-MCF-7 cells

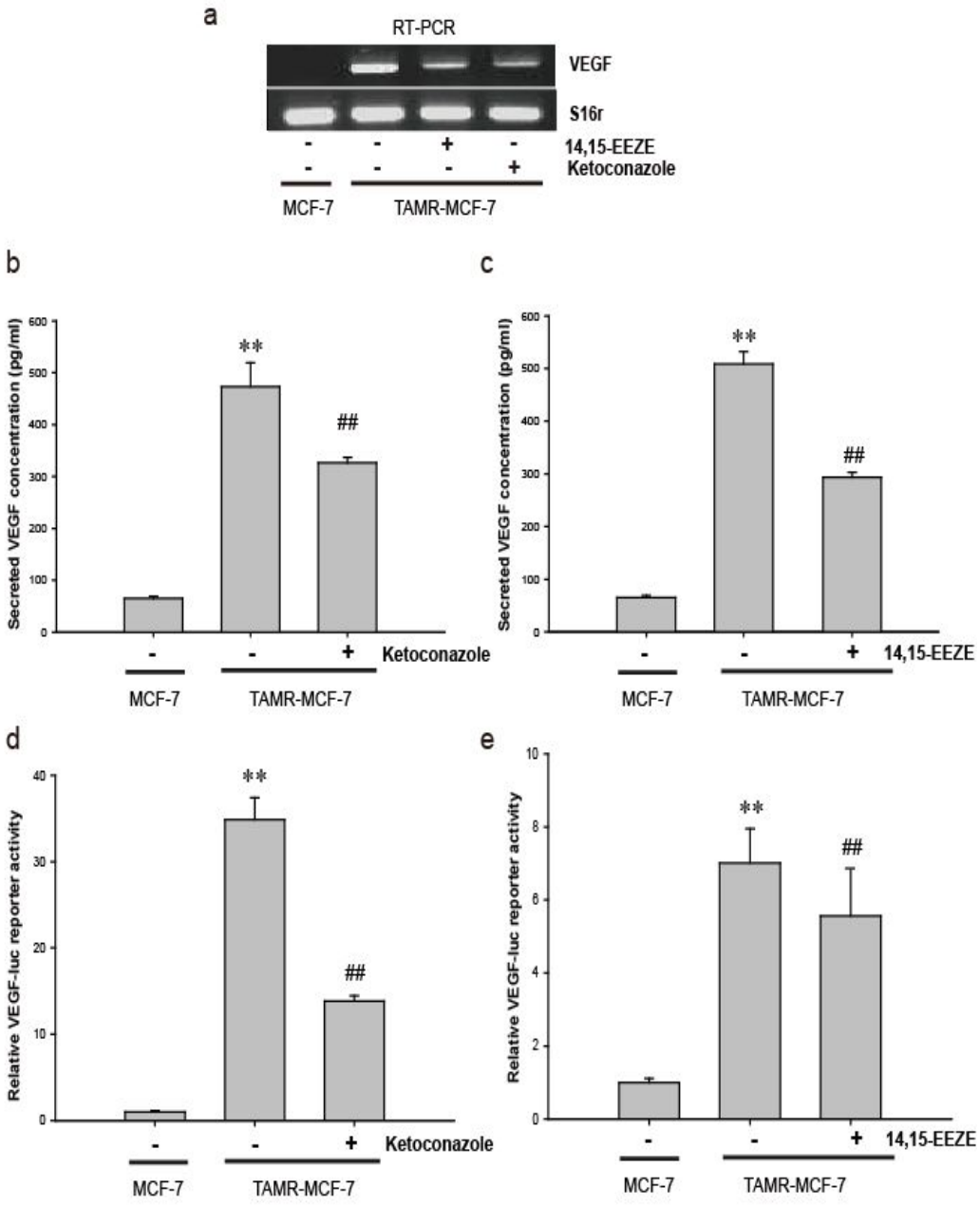

Figure 12. Effects of CYP3A4 inhibitor and EET antagonist on angiogenesis in TAMR-MCF-7 cells

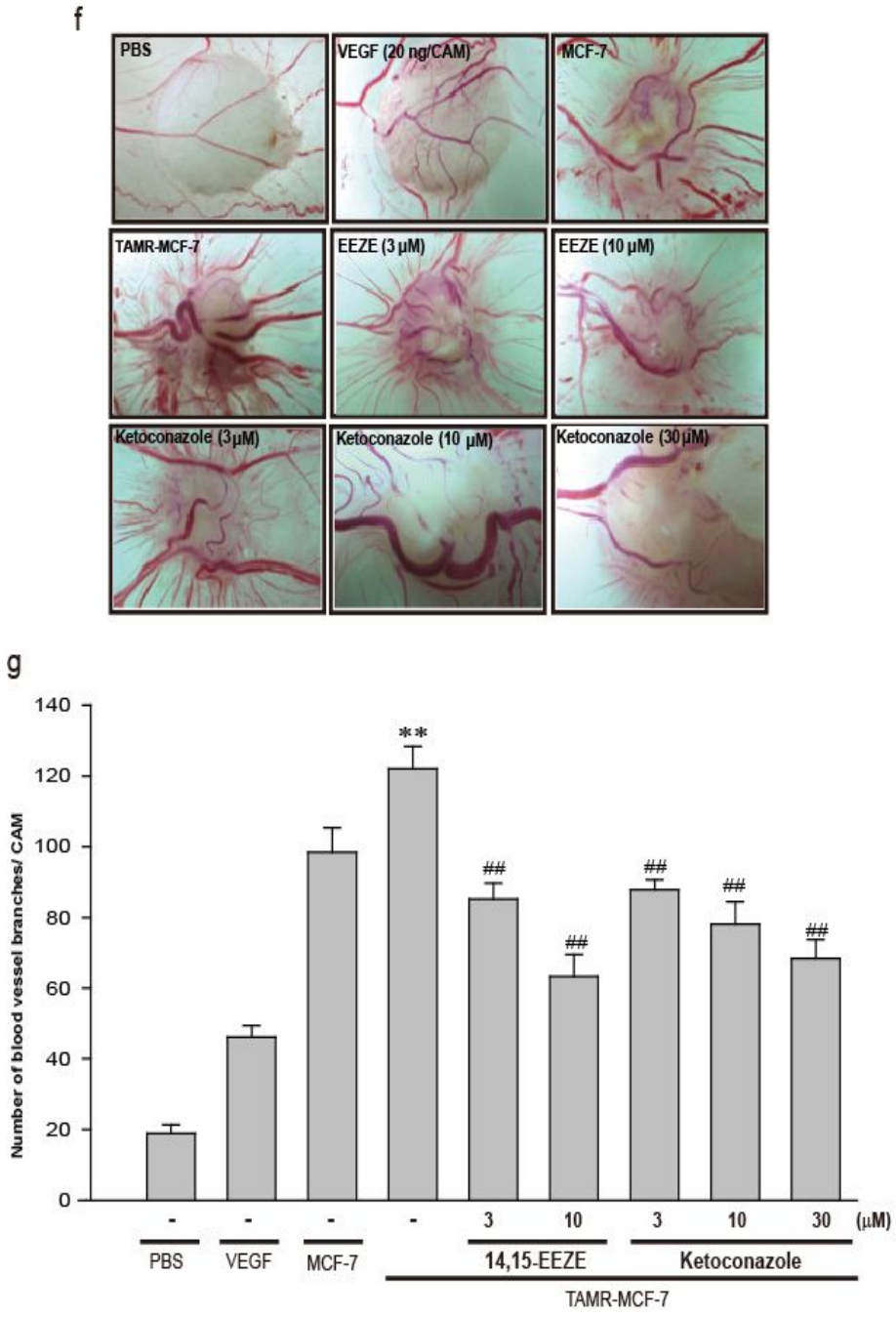

Figure 12. Effects of CYP3A4 inhibitor and EET antagonist on angiogenesis in TAMR-MCF-7 cells

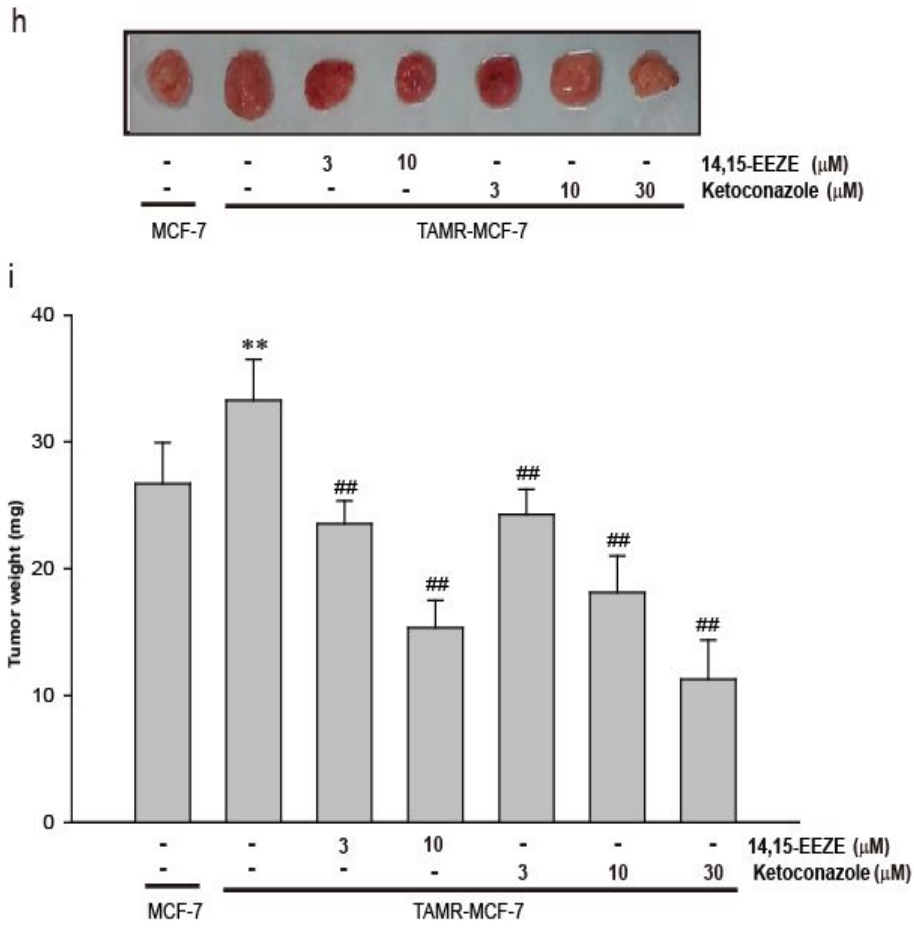

Figure 12. Effects of CYP3A4 inhibitor and EET antagonist on angiogenesis in TAMR-MCF-7 cells

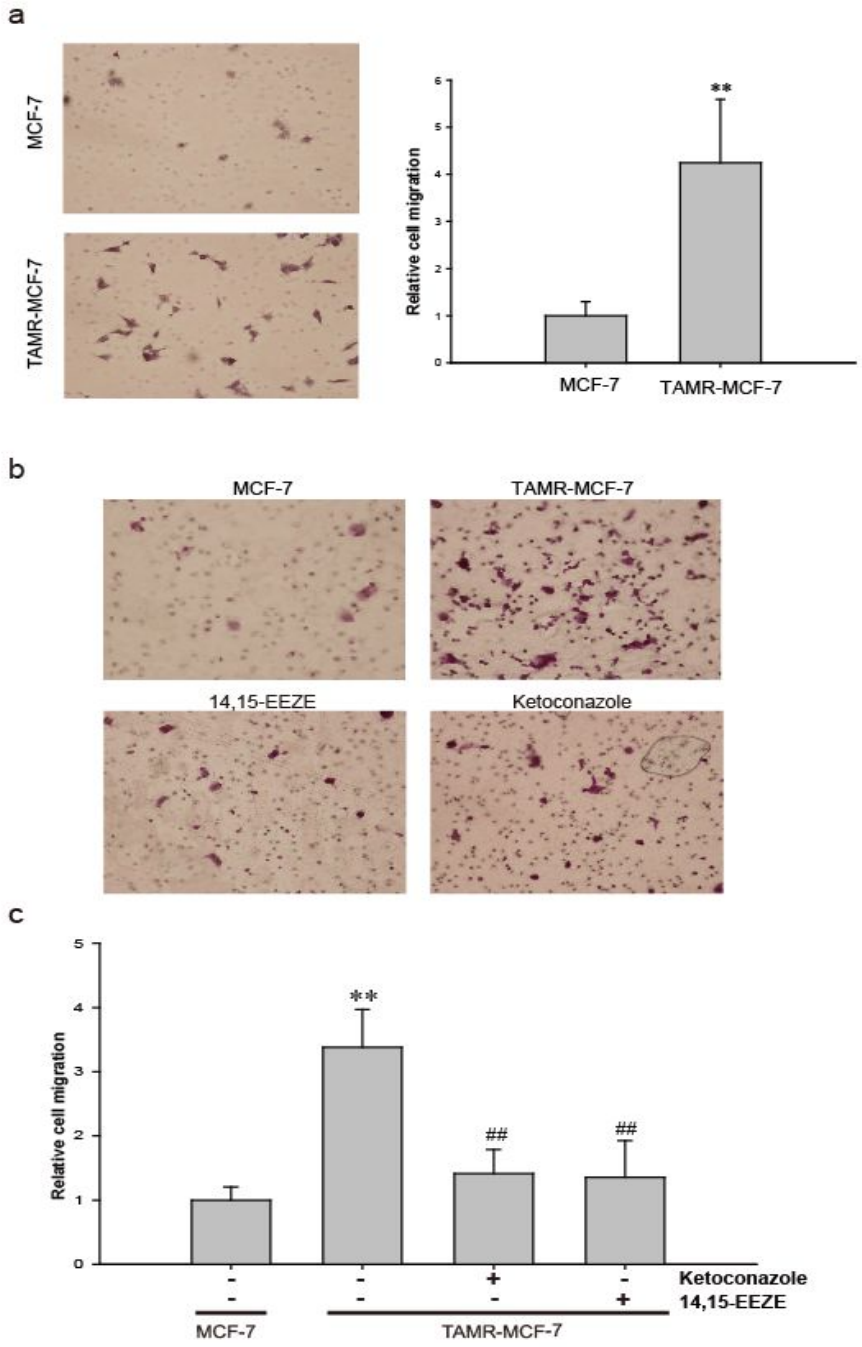

Figure 13. Effects of CYP3A4 inhibitor and EET antagonist on cell migration in TAMR-MCF-7 cells

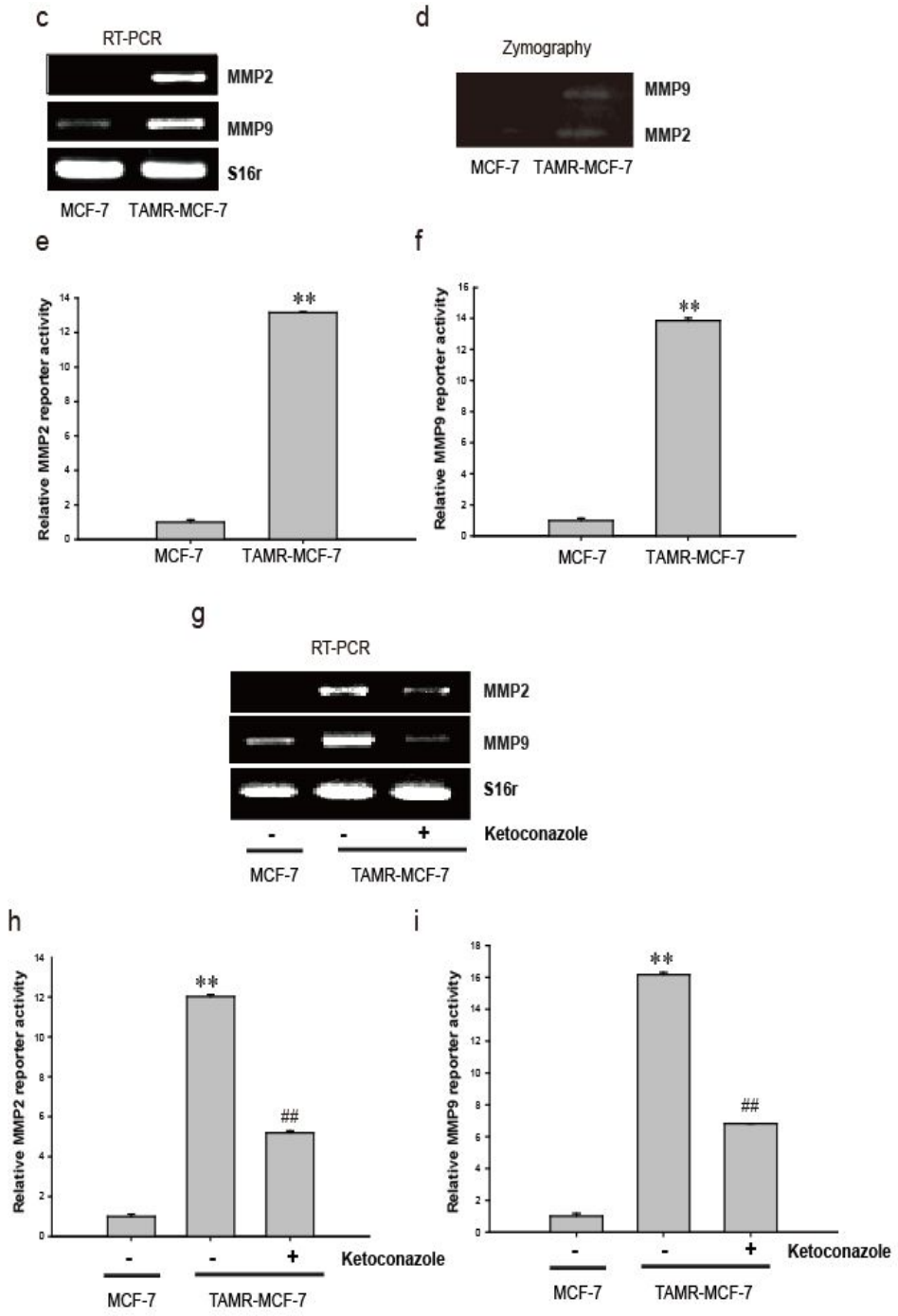

Figure 13. Effects of CYP3A4 inhibitor and EET antagonist on cell migration in TAMR-MCF-7 cells

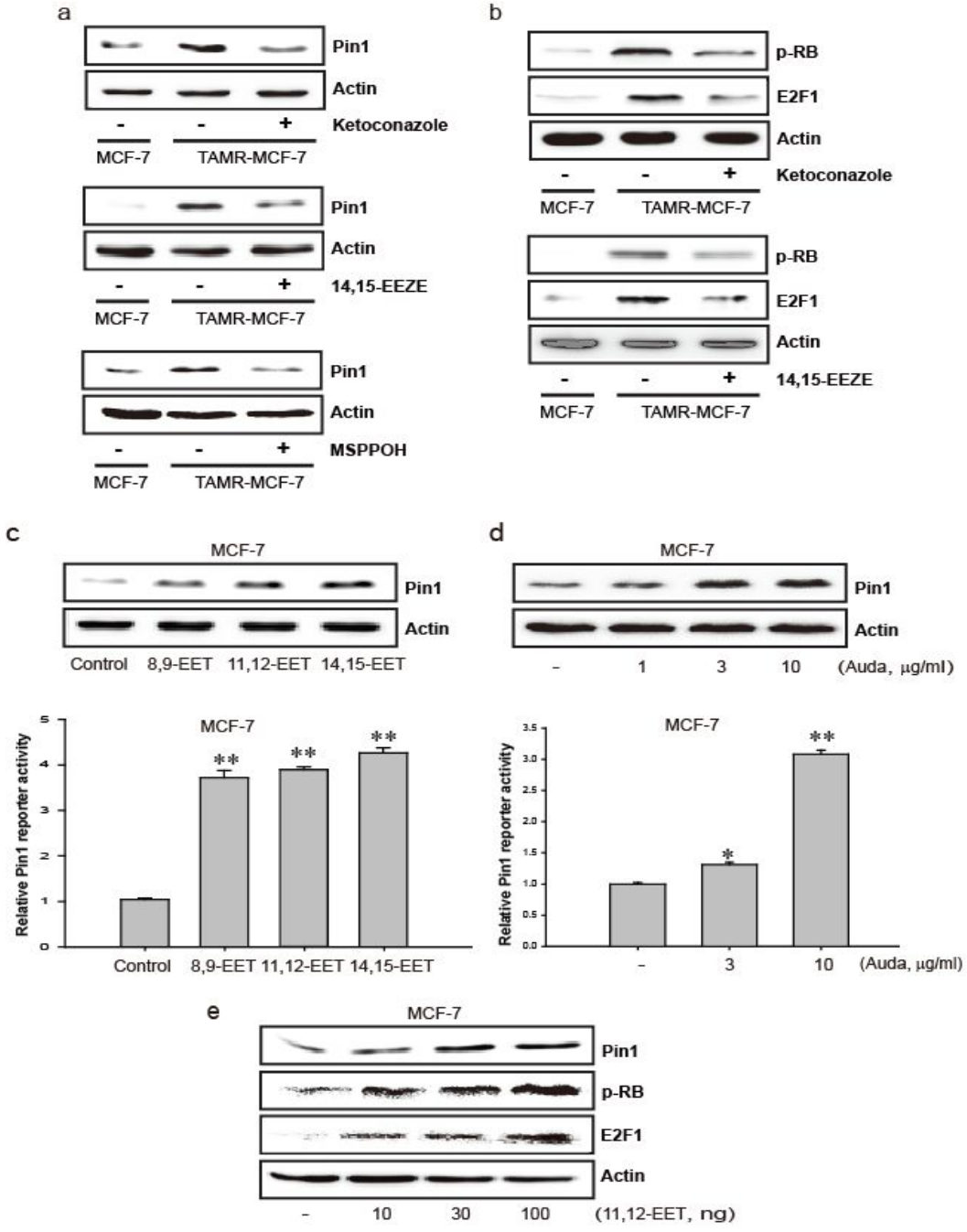

Figure 14. Influence of CYP3A4- derived 11,12-EET is mediated by RB/E2F1-dependent Pin 1 in TAMR-MCF-7 cells

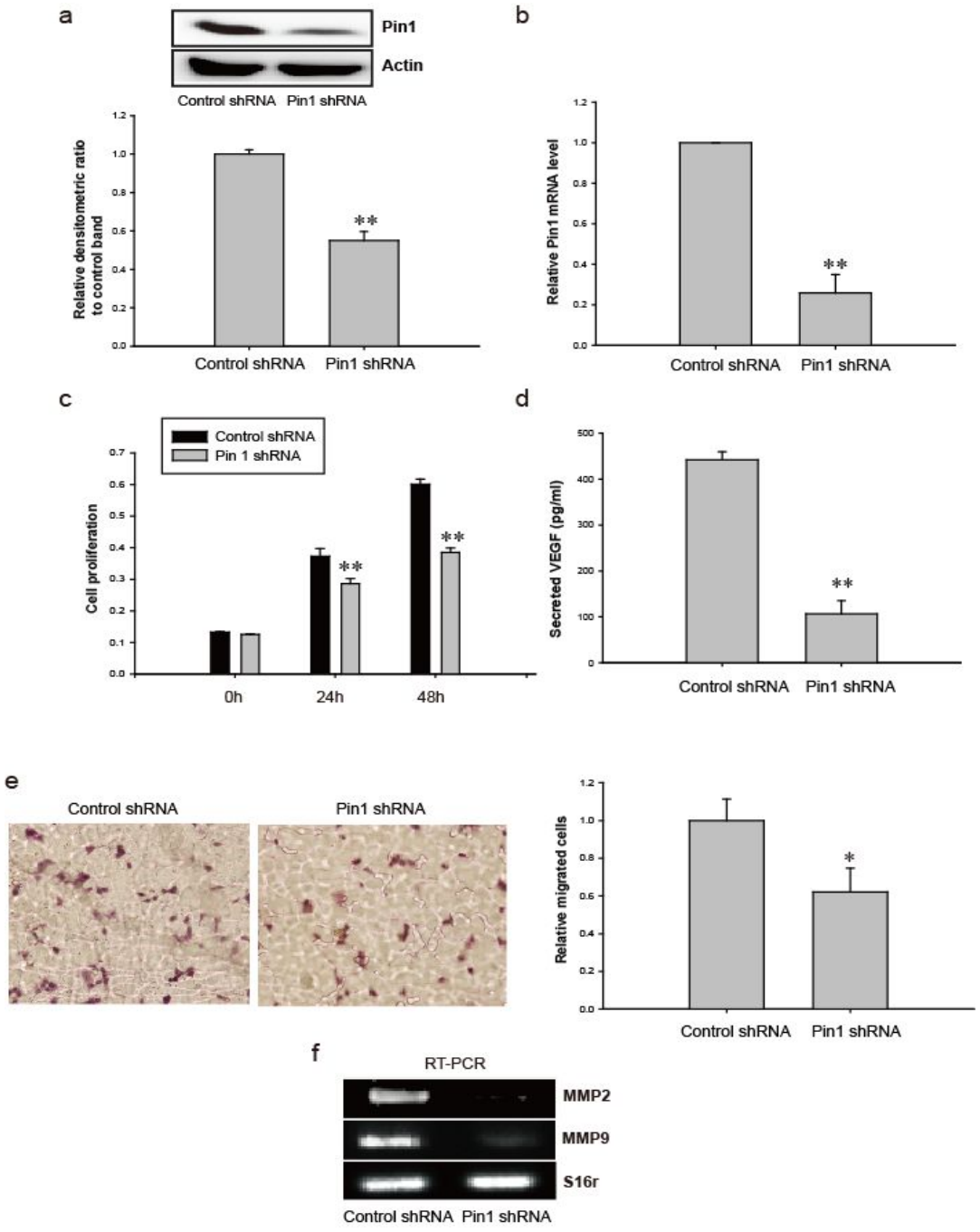

Figure 15. Influence Pin 1 knockdown in TAMR-MCF-7 cells

Figure legends

Figure 5. Up-regulation of aromatase expression in TAMR-MCF-7 cells. (**a**) upper: Immunoblot analysis of aromatase in MCF-7 and TAMR-MCF-7 cells. Each lane represents different sample. (**a)**, lower: expression of aromatase in diverse cell types: Raw264.7 (negative control); hormone-dependent breast cancer T47D: A18/Neo; hormone-independent breast cancer T47D: A18/PKCα ; and MEF cells (positive control) (**b**) Basal aromatase-luc activities in MCF-7 and TAMR-MCF-7 cells. MCF-7 and TAMR-MCF-7 cells were transiently transfected with aromatase-luc reporter (1 μg/ml) and phRL-SV (hRenilla) (1 ng/ml) plasmids. Dual luciferase reporter assays were performed on the lysed cells 18h after transfection. Reporter gene activity was calculated as a relative ratio of firefly luciferase to hRenilla luciferase activity. Data represent mean±SD with 6 different samples (significant versus MCF-7 cells, **P<0.01). (**c**) Increase in aromatase activity in TAMR-MCF-7 cells compared to MCF-7 cells. Data represent mean±SD with 3 different samples (significant versus MCF-7 cells, **P<0.01). (**d**) Representative figures for aromatase immunohistochemistry in human breast cancer tissues of 2 groups of patients: TAM-responsive cases (left) and TAM-resistant cases (right), respectively. Data represent significant change with 4 different samples.

Figure 6. Role of CREB in the up-regulation of aromatase expression in TAMR-MCF-7 cells. Basal activities of minimal reporter of (**a**) CRE and (**b**) GRE in MCF-7 and TAMR-MCF-7 cells. Cells were transiently transfected with CRE reporter plasmid or GRE-

reporter plasmid (1 μg/ml) and phRL-SV (hRenilla) (1 ng/ml). Reporter activity was determined as described in figure legend of Figure 1b. Data represent mean±SD with 6 different samples (significant versus MCF-7 cells, **P<0.01). (**c**) Immunoblot analyses of nuclear CREB and GR from nuclear fractions, and phosphorylated-CREB (p-CREB) from total cell lysates in MCF-7 and TAMR-MCF-7 cells.

Figure 7. Role of PI3K/Akt signaling pathway in the up-regulation of aromatase expression in TAMR-MCF-7 cells. (**a)** Immunoblot analyses of p-Akt, Akt, p-ERK, ERK, p-p38 kinase and p38 kinase in MCF-7 and TAMR-MCF-7 cells. **b** Effect of kinase inhibitors on aromatase expression in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with PD98059 (PD, 20 μM), SB203580 (SB, 20 μM) and LY294002 (LY, 20 μM) for 24 h and total cell lysates were subjected to immunoblotting. (**c**), left: effect of p85-myc (dominant negative mutant form of PI3K) transfection on aromatase-luc reporter activity in TAMR-MCF-7 cells. TAMR-MCF-7 cells were transiently co-transfected with aromatase-luc (1 μg/ml), and pCMV5 or p85-myc (0.5 μg) plasmids. Dual luciferase reporter assays were performed as described in the figure legend of figure 1b. Data represent mean±SD with 4 different samples (significant versus MCF-7 cells, **P<0.01; and significant versus control pCMV5-transfected TAMR-MCF-7 cells $^{\#\#}$P<0.01). (**c**), right: effect of p110-myc (active form of PI3K) transfection on aromatase-luc reporter activity in MCF-7 cells. MCF-7 cells were transiently co-transfected with aromatase-luc (0.5 μg/ml), and pCMV5 or p110-myc (0.2 μg) plasmids. Dual luciferase reporter assays were performed as described in the figure legend of figure 1b. Data represent mean±SD

with 3 different samples (significant versus control pCMV5-transfected MCF-7 cells, **P<0.01). **(d)** Effects of PTEN overexpression on protein expression and gene transcription of aromatase in TAMR-MCF-7 cells. TAMR-MCF-7 cells were transfected with PTEN overexpression vector (0.5 μg/ml) and protein expression of aromatase (left) and aromatase promoter activity (right) were determined. (**e)** Effects of 5-Aza (DNA methylation inhibitor) on PTEN expression, AKT phosphorylation and aromatase expression in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with 30 μM 5-Aza for 24 h. (**f)** Effects of LY294002 and 5-Aza on the aromatase activity in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with 20 μM LY294002 or 30 μM 5-Aza for 24 h. Data represent mean±SD with 3 different samples (significant versus vehicle-treated TAMR-MCF-7 cells, **P<0.01).

Figure 8. PI3K/Akt-dependent CREB activation in TAMR-MCF-7 cells. (**a)** Effect of PI3K inhibition on CREB activity. TAMR-MCF-7 cells were incubated with 20 μM LY294002 for 24 h and nuclear CREB and phosphorylated-CREB (p-CREB) were determined. (**b), (c)** Effect of PI3K inhibition on CRE minimal reporter activity in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with (**b**) LY294002 (10, 20 μM) or transfected with (**c**) p85-myc (0.5 μg) and minimal CRE reporter activity was determined as described in the figure legend of figure 1b. Data represent mean±SD with 3 different samples (significant versus to MCF-7 cells, **P<0.01; significant versus vehicle-treated or PCMV5-transfected TAMR-MCF-7 cells, $^{\#\#}$P<0.01. (d) Inhibition of CRE reporter activity by PTEN overexpression. TAMR-MCF-7 cells were transfected with PTEN

overexpression vector (0.5 μg) and minimal CRE reporter activity was determined as described in the figure legend of figure 1b. Data represent mean±SD with 3 different samples (significant versus PCMV5-transfected TAMR-MCF-7 cells, $^{\#\#}P<0.01$). Dual luciferase reporter assays were performed as described in figure 1b. Data represent mean±SD with 3 different samples (significant versus to MCF-7 cells, $^{**}P<0.01$; significant versus vehicle- treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$). (**e**), (**f**) Effects of 5-Aza on CREB activation in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with 5-Aza (30 M) for 24 h and minimal CRE reporter activity (**e**), and nuclear CREB and phosphorylated- CREB (p-CREB) (**f**) were determined in TAMR-MCF-7 cells. Dual luciferase reporter assays were performed as described in figure 1b. Data represent mean±SD with 3 different samples (significant versus to MCF-7 cells, $^{**}P<0.01$; significant versus vehicle- treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$).

Figure 9. Aromatase induction partly contributes to tamoxifen resistance in breast cancer. (**a**), (**b**), (**c**) synergistic cytotoxic effect of formestane with 4-hydroxytamoxifen in TAMR-MCF-7 cells. (**a**) TAMR-MCF-7 cells were incubated with formestane (30 nM, 100 nM, 30 μM and 100 μM) and 4-hydroxytamoxifen (**a** 10 μM; **b** 30 μM, respectively) for 36 h and cell viability was determined by MTT assay. Data represent mean±SD with 8 different samples (significant versus vehicle-treated TAMR-MCF-7 cells, **P<0.01; significant versus 4-OH-TAM-treated TAMR-MCF-7 cells, $^{\#\#}P< 0.01$). (**c**) Representative dot plots of TAMR-MCF-7 stained with annexin V-FITC (FL1-H) and propidium iodide (FL2-H). TAMR-MCF-7 cells were treated with 5 μM 4-hydroxytamoxifen in the presence or

absence of formestane (100 μM) for 24 h. Lower right panel represents early apoptotic cells. Upper left and upper right panels represent late necrotic and apoptotic cells, respectively. Lower left panel represents survival cells. The percentage counts of each quadrant are indicated. (**d**) Effect of 4-OH-TAM alone treatment on cell proliferation in MCF-7 cells and TAMR-MCF-7 cells. Both cell types were incubated with 4-OH-TAM in the indicated dose in DMEM containing 5% charcoal stripped FBS for 48 h and cell viability was determined by MTT assay**.** Data represent mean±SD with 8 different samples (significant versus vehicle-treated MCF-7 cells, **P<0.01; significant versus vehicle-treated TAMR-MCF-7 cells, $^{\#\#}$P<0.01; $^{\#}$P<0.05). (**e**) Effect of 4-OH-TAM treatment in combination with 17ß-Estrodiol (10 pM) on cell proliferation in MCF-7 cells and TAMR-MCF-7 cells. Both cell types were incubated with 4-OH-TAM in the indicated dose in DMEM containing 5% charcoal stripped FBS and 10 pM of 17ß-Estrodiol for 48 h and cell viability was determined by MTT assay**.** Data represent mean±SD with 8 different samples (significant versus vehicle-treated MCF-7 cells, **P<0.01). (**f**) Effect of testosterone alone treatment on cell proliferation in MCF-7 cells and TAMR-MCF-7 cells. Both cell types were incubated with testosterone in the indicated dose in DMEM containing 5% charcoal stripped FBS for 96 h and cell viability was determined by MTT assay**.** Data represent mean±SD with 8 different samples (significant versus vehicle-treated MCF-7 cells, **P<0.01; significant versus vehicle-treated TAMR-MCF-7 cells, $^{\#}$P<0.05). (**g**) Effect of formestane in combination with testosterone on cell proliferation in TAMR-MCF-7 cells. TAMR-MCF-7 cells were incubated with formestane in the indicated dose and testosterone

1 nM for 96 h and cell viability was determined by MTT assay (significant versus testosterone-treated TAMR-MCF-7 cells, **P<0.01). (**h**) Cellular level of testosterone in MCF-7 and TAMR-MCF-7. Both cell lines were exposed to testosterone (100 nM) for 3 hours in serum free condition, then were harvested and cellular testosterone level was measured using testosterone ELISA kit. Data represent mean±SD with 4 different samples (significant versus testosterone-treated MCF-7 cells, **P<0.01).

Figure 10. CYP epoxygenases expression and 11,12-EET level in MCF-7 and TAMR-MCF-7 cells. (**a**) mRNA levels of CYP2J2, 2C8, 2C9 and 3A4 in MCF-7 and TAMR-MCF-7 cells. (**b**) Western blot analysis of CYP2C8 and CYP3A4 protein expression in MCF-7 and TAMR-MCF-7 cells. (**c**) 11,12-EET level in MCF-7 and TAMR-MCF-7 cells. Extracted samples of both MCF-7 and TAMR-MCF-7 cells were submitted to LC-ESI/MRM/MS analysis in a mass chromatography coupled with HPLC assay and 11,12-EET product was determined. Data represent mean±SD with 4 different samples (significant versus MCF-7 cells, **P<0.01).

Figure 11. Effect of CYP3A4 inhibitor and 11,12-EET antagonist on cell proliferation and apoptosis in TAMR-MCF-7 cells. (**a**) Effect of ketoconazole (10 μM) and 14,15-EEZE (3 μM) on proliferation of TAMR-MCF-7 cells. TAMR-MCF-7 cells were plated in 96 well plate and cell proliferation rate was determined by MTT assays after 72h treatment with ketoconazole (10 μM) and 14,15-EEZE (3 μM). Data represent mean±SD with 8 different samples (significant versus vehicle-treated TAMR-MCF-7 cells, **P<0.01). (**b**)

Representative dot plots of TAMR-MCF-7 stained with annexin V-FITC (FL1-H) and propidium iodide (FL2-H) using flow cytometry assay. The TAMR-MCF-7 cells were treated with 3 μM 4-hydroxytamoxifen (4-hydroxy TAM) in the presence or absence of ketoconazole (10 μM) or 14,15-EEZE (3 μM) for 24 h. Lower right panel represents early apoptotic cells. Upper-left panel and upper-right panel represent late necrotic and apoptotic cells, respectively. Lower-left panel represents survival cells. The indicated percentage value is sum of apoptotic cells from lower right panel and upper-right panel (n=3).

Figure 12. Effects of CYP3A4 inhibitor and EET antagonist on angiogenesis in TAMR-MCF-7 cells (**a**) Effect of CYP3A4 inhibitor and EET antagonist on VEGF mRNA level in TAMR-MCF-7 cells. TAMR-MCF-7 cells were exposed to ketoconazole (10 μM) and 14,15-EEZE (3 μM) for 24h in serum free condition. VEGF mRNA level was determined by RT-PCR. (**b**), (**c**) Effect of CYP3A4 inhibitor and EET antagonist on secreted VEGF level. TAMR-MCF-7 cells were treated with ketoconazole (10 μM) (**b**) and 14,15-EEZE (3 μM) (**c**) for 24h in serum deprived condition. Secreted VEGF concentration was measured by human VEGF ELISA kit. Data represent mean±SD with 3 different samples (significant versus MCF-7 cells, $^{**}P<0.01$; significant versus vehicle treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$). (**d**), (**e**) Effect of ketoconazole (10 μM) (**d**) and 14,15-EEZE (3 μM) (**e**) on the reporter gene activity of VEGF-luc in TAMR-MCF-7 cells. Each cell type was transiently co-transfected with VEGF-luc reporter plasmid (1 μg/ml) and phRL-SV (hRenilla) (1 ng/ml). Dual luciferase reporter assays were performed on the lysed cells 18 h after reagent treatment. Reporter gene activity was calculated as a relative ratio of firefly luciferase to

hRenilla luciferase activity. Data represent mean±SD with 4 different samples (significant versus MCF-7 cells, $^{**}P<0.01$; significant versus vehicle treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$). (**f**) Representative pictures of angiogenesis originated from both MCF-7 and TAMR-MCF-7 cells using CAM assay. The control CAMs of a 10 day old chick embryo were exposed to PBS or VEGF (20 ng/ml). The additional embryos were implanted with MCF-7 or TAMR-MCF-7 ($2x10^6$ cells/CAM). The MCF-7 cells and TAMR-MCF-7 cells were not pre-treated with VEGF. (**g**) The quantification of new small branches formed from existing blood vessels was done 3 days after cancer cell implantation. Data represent mean ±SD with (n= 5-7) different samples (significant versus MCF-7 cell- implanted group, $^{**}P<0.01$; compared to vehicle- treated TAMR-MCF-7 cells- implanted group $^{\#\#}P<0.01$). (**h**) Representative pictures of tumor growth formed by CAMs bearing MCF-7 and TAMR-MCF-7 cells. (**i**) Ketoconazole and 14,15-EEZE treatment reduced tumor weight formed by CAMs bearing TAMR-MCF-7 cells in dose dependent manner. Data represent mean ±SD with (n= 5-7) different samples (significant versus MCF-7 cell-implanted group, $^{**}P<0.01$; compared to vehicle- treated TAMR-MCF-7 cells- implanted group $^{\#\#}P<0.01$).

Figure 13. Effects of CYP3A4 inhibitor and EET antagonist on cell migration of TAMR-MCF-7 cells. (**a**) Transwell migration assays were performed with MCF-7 cells and TAMR-MCF-7 cells for 17 hours. Represestative figures of migrated cells (left). The relative cell numbers of migrated cells between MCF-7 cells and TAMR-MCF-7 cells (right). Values are means ±SD with (n= 8-10) different areas (significant versus MCF-7

cells, $^{**}P<0.01$). (**b**) Inhibitory effect of ketoconazole (10 μM) and 14,15-EEZE (3 μM) on cell migration of TAMR-MCF-7 cells. Represestative figures of migrated cells (upper). The relative migrated cells (lower). Values are means ±SD with (n= 8-10) different areas (significant versus MCF-7 cells, $^{**}P<0.01$; significant versus vehicle treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$). (**c**) Basal level of MMP2 and MMP9 mRNA level in MCF-7 and TAMR-MCF-7 cells. (**d**) The activity of MMP2, MMP9 was measured in free serum medium by Gelatin zymogram assay in MCF-7 and TAMR-MCF-7 cells. (**e**), (**f**) Luciferase assays were performed to detect promoter activities of MMP2 (**e**) and MMP9 (**f**). Each cell type was transiently co-transfected with MMP2-luc reporter plasmid or MMP9-luc reporter plasmid (1 μg/ml) and phRL-SV (hRenilla) (1 ng/ml). Dual luciferase reporter assays were performed as described in Figure 12d or 12e. Data represent mean±SD with 4 different samples (significant versus MCF-7 cells, $^{**}P<0.01$). (**g**) Effect of ketoconazole (10 μM) on mRNA level of MMP2 and MMP9 in TAMR-MCF-7 cells. (**h**), (**i**) Effect of ketoconazole (10 μM) on the basal reporter activity of MMP2 (**h**) and MMP9 (**i**) in TAMR-MCF-7 cells. Each cell type was transiently co-transfected with MMP2-luc reporter plasmid or MMP9-luc reporter plasmid (1 μg/ml) and phRL-SV (hRenilla) (1 ng/ml). Dual luciferase reporter assays were performed as described in Figure 12d or 12e. Data represent mean±SD with 3 different samples (significant versus MCF-7 cells, $^{**}P<0.01$; significant versus vehicle treated TAMR-MCF-7 cells, $^{\#\#}P<0.01$).

Figure 14. Effects of CYP3A4 inhibitor and EET antagonist on RB/E2F1-dependent Pin 1 expression in TAMR-MCF-7 cells. (**a**) Effect of ketoconazole and 14,15-EEZE on the

expression of Pin1 protein in TAMR-MCF-7 cells. Pin 1 overexpression in TAMR-MCF-7 cells was suppressed by treatment of ketoconazole (10 μM; upper), 14,15-EEZE (10 μM; middle), and MSPPOH (10 μg/ml; lower) for 24h. (**b**) Effect of ketoconazole and 14,15-EEZE on the phosphorylation of RB and the expression of E2F-1 in TAMR-MCF-7 cells. Ketoconazole (10 μM; upper) and 14,15-EEZE (10 μM; lower) treatment inhibited the elevated phosphorylation of RB and the enhanced expression of E2F-1 protein in TAMR-MCF-7 cells. (**c**) Effect of epoxyeicosanoid acids on Pin 1 expression and Pin 1 reporter activity in MCF-7 cells. MCF-7 cells were exposed to 8,9-EET; 11,12-EET; and 14,15-EET (100ng/ml) for 24h. The expression of Pin 1 protein (upper) and basal activity of Pin 1 promoter (lower) in MCF-7 cells were determined (**d**) Effect of Auda, a a sEH inhibitor on Pin 1 expression and Pin 1 reporter activity in MCF-7 cells. Treatment with Auda for 24h accelerated the expression of Pin 1 (upper) and the basal activity of Pin 1 promoter (lower) in MCF-7 cells in dose dependent manner. (**e**) Effect of 11,12-EET on RB/E2F1-dependent Pin 1 pathway in MCF-7 cells. 11,12-EET treatment with the indicated concentration for 24h increased Pin 1 expression, the phosphorylation of RB and the activity of E2F-1 in MCF-7 cells in dose dependent manner.

Figure 15. Effects of Pin 1 knockdown in TAMR-MCF-7 cells. (**a**) Western blot analyses (upper) and relative densitometric ratio (lower) of Pin 1 expression in control shRNA and Pin 1 shRNA- transfected TAMR-MCF-7 cells. (**b**) mRNA level of Pin 1 in control shRNA and Pin 1 shRNA- transfected TAMR-MCF-7 cells. (**c**) Cell proliferation of Pin 1 shRNA/TAMR-MCF-7 cells and control shRNA/TAMR-MCF-7 cells. Values are means

±SD with 8 different samples (significant versus control shRNA/TAMR-MCF-7 cells, $^{**}P<0.01$). (**d**) Secreted VEGF level in control shRNA/TAMR-MCF-7 cells and Pin 1 shRNA/TAMR-MCF-7 cells. Values are means ±SD with 3 different samples (significant versus shControl/TAMR-MCF-7 cells, $^{**}P<0.01$). (**e**) Migratory ability of control shRNA/TAMR-MCF-7 cells and Pin 1 shRNA/TAMR-MCF-7 cells. Transwell migration assays were performed with both cell lines for 17 hours. Represestative figures of migrated cells (left). The relative migrated cell numbers between control shRNA/TAMR-MCF-7 cells and Pin 1 shRNA/TAMR-MCF-7 cells (right). Values are means ±SD with (n= 8-10) different areas (significant versus control shRNA/TAMR-MCF-7 cells, $^{*}P<0.05$). (**f**) mRNA level of Pin 1, MMP2, and MMP9 in control shRNA/TAMR-MCF-7 cells and Pin 1 shRNA/TAMR-MCF-7 cells.

List of Publications

1. Choi HK, Cho KB, **Phuong NT**, Han CY, Han HK, Hien TT, Choi HS, Kang KW. SIRT1-Mediated FoxO1 Deacetylation is Essential for Multidrug Resistance-Associated Protein 2 Expression in Tamoxifen-Resistant Breast Cancer Cells. Mol Pharm. 2013 Jun 13. [Epub ahead of print]

2. Ryu CS, Kwak HC, Lee JY, Oh SJ, **Phuong NT**, Kang KW, Kim SK. Elevation of cysteine consumption in tamoxifen-resistant MCF-7 cells. Biochem Pharmacol. 2013 Jan 15; 85(2): 197-206.

3. Kim JA, Kim MR, Kim O, **Phuong NT**, Yun J, Oh WK, Bae K, Kang KW. Amurensin G inhibits angiogenesis and tumor growth of tamoxifen-resistant breast cancer via Pin1 inhibition. Food Chem Toxicol. 2012 Oct; 50(10): 3625-34.

4. **Phuong NT**, Kim SK, Lim SC, Kim HS, Kim TH, Lee KY, Ahn SG, Yoon JH, Kang KW. Role of PTEN promoter methylation in tamoxifen-resistant breast cancer cells. Breast Cancer Res Treat. 2011 Nov; 130(1): 73-83.

Manuscripts in Preparation for Publication:

1. **Phuong NT**, Kim SK, Lim SC, Lee KY, Yoon JH, Kang KW. Induction of methionine adenosyltransferase 2A in tamoxifen-resistant breast cancer cells: Role of miR-146b down-regulation. Oncogene (in preparation for submission)

2. **Phuong NT,** Lim SC, Kang KW. Aromatase induction in tamoxifen-resistant breast cancer: Role of phosphoinositide 3-kinase-dependent CREB activation. Breast Cancer Res Treat (submitted).

3. **Phuong NT**, Kang KW, et al. CYP3A4-mediated 11,12-Epoxyeicosatrienoic Acid in multiple regulations of endocrine resistance, angiogenesis, and migration in tamoxifen-resistant breast cancer. Oncogene (in preparation for submission).

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1. **Nguyen T.T. Phuong** and Keon Wook Kang. Aromatase induction in Tamoxifen Resistant Human Breast Cancer: Role of Phosphoinositide 3-kinase-dependent CREB activation. *The XIII International Congression of Toxicology. July 1st- 3rd, **2013, Seoul, Korea**.*

2. **Nguyen T.T. Phuong** and Keon Wook Kang. Aromatase induction in Tamoxifen Resistant Human Breast Cancer: Role of Phosphoinositide 3-kinase-dependent CREB activation. *The Pharmaceutical Society Conference. April, **2013, Suncheon, Korea.***

3. **Nguyen T.T. Phuong** and Keon Wook Kang. Methionine adenosyltransferase 2A expression in tamoxifen-resistant breast cancer cells and its molecular mechanism. *Social Toxicology Conference. December, **2012, Kwangju, Korea.***

4. **Phuong Nguyen T.T** and Keon Wook Kang. Crucial role of PI3K in MAT2A expression via regulating Nrf-2 and NF-kB in tamoxifen-resistant breast cancer cells. *Proceedings of the Spring international Convention of the Pharmaceutical Society of Korea. April 21-22, **2011, Busan, Korea.***

5. **Nguyen T.T. Phuong** and Keon Wook Kang. PTEN promoter methylation by MAT2A- dependent SAM increase in TAMR-MCF-7 cells. *The Sixth International Symposium on Hormonal Oncogenesis, September 12-16th, **2010, Tokyo, Japan.***

6. Identification of new therapeutic target for tamoxifen-resistant breast cancer. Innovative Drug Research Center (IDRC) for Metabolic and Inflammatory Disease, Korea, 12/2011 and 02/2013.

7. Role of CYP3A4- mediated 11,12-epoxyeicosatrienoic acid in tamoxifen- resistant breast cancer. Pharmacological symposium, Kangwon, Korea, 07/2013.

ABSTRACT

Studies on New Therapeutic Targets for the Treatment of Tamoxifen-Resistant Breast Cancer

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The frequent development of resistance has been being a serious problem in the treatment of breast cancer. Hence, finding new therapeutic targets for reversal of chemo-resistance is of great interest. In the present studies, we have revealed several potential therapeutic targets which could contribute to the treatment of tamoxifen-resistant breast cancer. Immunohistochemistry also showed that aromatase immunoreactivity in TAM-resistant human breast cancer tissues was higher than that in TAM-responsive human breast cancer tissues. Inhibition of phosphoinositide 3-kinase (PI3K) suppressed the transactivation of aromatase gene and its enzyme activity, and PI3K/Akt-dependent CREB activation was required for the enhanced expression of aromatase in TAMR-MCF-7 cells. Testosterone displays an inhibitory activity on cell growth of several breast cancer cell lines, however in TAMR-MCF-7 cells where express higher level of aromatase than MCF-7 cells, the inhibitory effect of testosterone on cell growth was less than

that in MCF-7 cells. In addition, formestane, an aromatase inhibitor significantly potentiated 4-hydroxytamoxifen-mediated apoptosis in TAMR-MCF-7 cells. The finding suggest that aromatase enzyme and the PI3K/Akt-dependent CREB signaling pathway are promising targets of the treatment of endocrine resistant breast cancer.

We were also interested in cytochrome P450 (CYP) epoxygenases converting arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs) which closely correlate with breast cancer progression. CYP3A4 epoxygenase was dramatically up-regulated in TAMR-MCF-7 compared to control MCF-7 cells. Moreover, we also found that cellular 11,12-epoxyeicosatrienoic acid (11,12-EET) concentration in TAMR-MCF-7 cells was about 8 fold higher than MCF-7 cells. Either CYP3A4 inhibition by ketoconazole (a CYP3A inhibitor) or antaginism of EET activity by 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE, a synthetic EET antagonist) decreased cell proliferation and increased the sensitivity of TAMR-MCF-7 cells to 4-hydroxytamoxifen (4-OH-TAM). Chick chorioallantoic membrane (CAM) assay demonstrated that the increased angiogenic intensity and tumor formation of TAMR-MCF-7 cells was significantly suppressed by ketoconazole or 14,15-EEZE treatment. Moreover, the greater migration of TAMR-MCF-7 cells was also attenuated by these chemicals. These data implicate a critical role of CYP3A4-mediated 11,12-EETs production during the development of TAM-resistant breast cancer. Interestingly, ketoconazole or 14,15-EEZE treatment diminished the overexpression of PB/E2F1-dependent Pin 1 which is crucial for endocrine resistance and higher angiogenesis and epithelial-mesenchymal transition in TAM-resistant breast cancer. We further found that exposure of MCF-7 cells to EETs enhanced Pin1 expression in MCF-7 cells. Pin 1 silencing also blocked cell proliferation, angiogenesis, and migration in TAMR-MCF-7 cells. The data suggest that the influences of CYP3A4-mediated EETs pathway may be partly mediated by Rb/E2F1-dependent Pin1 overexpression in TAMR-MCF-7 cells. Taken together, our studies have shown that aromatase overexpression and CYP3A4-mediated EET production are new potential therapeutic targets for the reversal of endocrine resistance in breast cancer.

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