약학석사학위논문

CYP19A1 발현을 조절하는 유방암 종양미세환경 인자 규명

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

2015년 7월

서울대학교 대학원

약학과 병태생리학전공

박 한 수

CYP19A1 발현을 조절하는 유방암 종양미세환경 인자 규명

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

지도교수 이 미 옥

이 논문을 약학석사 학위논문으로 제출함

2015년 7월

서울대학교 대학원

약학과 병태생리학전공

박 한 수

박한수의 석사 학위논문을 인준함

2015년 7월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

ABSTRACT

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

Hansu Park
College of Pharmacy
The Graduate School
Seoul National University

Estrogen signaling is a critical factor that supports cancer proliferation and metastasis in breast cancer. CYP19A1 is a rate-determining enzyme that catalyzes biosynthesis of estrogen. CYP19A1 is upregulated in adipose tissue adjust to breast cancer, and enhances the local concentration of estradiol and estrogen signaling in breast cancer cells. Therefore, CYP19A1 has been considered as an important target of endocrine therapy for patients with breast cancer. In the present study, we aimed to examine expression of CYP19A1 and estrogen signaling in interaction of cancer cells and adipocytes, and to identify inflammatory, hormonal, and metabolic factors that control CYP19A1 expression in breast cancer microenvironment. First, we found that CYP19A1 expression in mature 3T3-L1 adipocytes was increased by cocultivation with MCF-7 cells. Moreover, pS2, an ERα downstream gene, was increased in cocultivated MCF-7 cells. Next,

among cytokines that are abundant in cancer microenvironment, interferon-ɣ increased CYP19A1 protein level with 100 ng/ml for 24 h treatment in 3T3-L1 mouse preadipocytes. Also, ACTH upregulated CYP19A1 expression when cells were treated with 100 mM for 24 h. Among metabolites, saturated free fatty acids such as palmitic acids and stearic acids increased the protein level of CYP19A1, but unsaturated free fatty acids such as docosahexanoic acids and eicosapentanoic acids decreased expression of CYP19A1. Glucose also upregulated CYP19A1 expression when treated with a concentration of 20 mM for 48 h. Treatment with Interferon-ɣ (IFNɣ), TGF-β, free fatty acids, glucose, IGF-1 was accompanied with an increase in the protein level of p204, a mouse homologue of human IFI16. These findings may help to understand CYP19A1 regulation and estrogen signaling in breast cancer microenvironment.

keywords : CYP19A1, Cancer microenvironment, Estrogen signaling, p204, Breast cancer

Student Number : 2012-21590

CONTENTS

LIST of FIGURES

LIST of TABLES

Ⅰ. INTRODUCTION

Breast cancer is one of the most common cancer in women. In 2013, breast cancer was ranked as the second highest incidence rate and the fifth highest death for Korean female cancer (National cancer information center, Retrieved July, 2015 from http://www.cancer.go.kr). Approximately 75% of female breast cancer patients are estrogen receptor positive (Johnston and Dowsett., 2003). Estrogen receptors are activated by binding of their ligand, expecially estradiol. Activated estrogen receptors promote downstream gene transcription. As a result, breast cancer cell proliferation and metastasis are accelerated. Estrogen receptor α (ERα) -positive breast cancer patients are treated with endocrine therapy targeting estrogen signaling, such as selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs) (Ali et al., 3011). SERMs such as tamoxifen competitively bind to ER α and modulate their activity. AIs, like letrozol, directly inhibit estrogen biosynthesis in breast cancer microenvironment. But when breast cancer acquires resistance against endocrine therapy, cancer cell proliferation and metastasis are increased (Osborne and Schiff., 2011). Therefore, resistance to endocrine therapy is strongly linked to poor prognosis.

One of the critical factors of breast cancer progression and drug resistance is cancer microenvironment (Dittmer and Leyh., 2014).

Cancer microenvironment is the sum of complex interactions played by various types of cells, such as cancer cells, immune cells, adipocytes, fibroblasts, and so on (Figure 1; Ma et al., 2015). These cells release various secretory factors into the cancer microenvironment, including cytokines, hormones, and metabolites (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). First, cytokines regulated molecular signaling of cancer cells. They accelerate cell proliferation, inhibit apoptosis, and cause invasion and metastasis. In addition, they promotes extracellular matrix (ECM) remodeling. Angiogenesis and fibrosis are well-known phenomenon in breast cancer. As a result, modified ECM facilitates cancer cell metabolism, proliferation, and invasion. Next, endocrine hormones are also important factors in breast cancer microenvironment. Estrogen signaling is the most critical factor in breast cancer. Epinephrine is another important factor stimulating the MAPK pathway and STAT3 signaling (Cole et al., 2012). Insulin, IGF-1 and leptin are crucial adipose hormones influence on breast cancer (Calle and Kaaks., 2004; Ando and Catalano., 2012). They activate MAPK, PI3K, and STAT3 signaling in cancer cells and participate in regulation of ERα signaling. Finally, metabolites abundant in breast cancer microenvironment are related with diverse biological process. Nutrients, such as glucose and fatty acid, supply energy and building blocks for cancer metabolism and proliferation. This phenomenon is well-known as “Warburg effect”. Additionally, metabolites are associated with cellular signaling pathway. For

example, oxysterols, the product of cholesterol metabolism, are known as ligands of liver X receptors (LXRs) and estrogen receptors (ERs) (Nelson et al., 2013). Consequently, The microenvironmental factors in breast cancer provoke cancer proliferation, invasion, and drug resistance.

One of the most important player in breast cancer microenvironment is adipose tissue. Adipose tissue is not only just an energy storage structure, but also an important endocrine organ (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). Cancer cells stimulate adipose tissue by secreting inflammatory cytokines. Then stimulated adipose tissue supplies secretory factors including adipokines, hormones, and metabolites to cancer cells. Recently, transformed adipocytes called cancer-associated adipocytes (CAAs) were discovered in adipose tissue adjacent to cancer cells (Dirat et al., 2011; Bochet et al., 2013). CAAs are characterized as delipidated morphology, loss of molecular adipocyte markers and increased secretion of inflammatory cytokines. It was reported that increased cytokine production in CAAs promotes cancer invasion. But involvement of CAAs in ERα signaling has not beed studied, although adipose tissue is the major organ secreting estrogens.

Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (CYP19A1 or aromatase) is a key factor that links estrogen signaling to adipose tissue (Bulun et al., 2011; Simpson et al., 2002). CYP19A1 is the

rate-determining enzyme in estrogen biosynthesis, converts androgens to estrogens. CYP19A1 is upregulated in breast cancer tissue. CYP19A1 induction in tamoxifen-resistant breast cancer has also been reported (Phuong et al., 2014). Moreover, genetic polymorphisms of CYP19A1 are involved with breast cancer. For example, the tetranuleotide [TTTA] repeat polymorphism in intron 4 is significantly associated with breast cancer risk (Haiman et al., 2000; Baxter et al., 2001). Increased local estrogen activates estrogen signaling in breast cancer cells and promotes cancer progression and drug resistance (Figure 2; Yager and Davidson., 2006; Ma et al., 2015). Consequently, CYP19A1 is a crucial target for endocrine therapy in breast cancer (Johnston and Dowsett., 2003; Ali et al., 2011).

The tissue-specific promoters regulate CYP19A1 expression in human (Figure 3; Simpson et al., 2002; Dieudonne et al., 2006; Subbaramaiah et al., 2011; Bulun et al., 2012). PⅠ.1 is the most powerful promoter, but it is only activated in placenta. pⅡ is secondry potent promoter and activated in ovary. pⅡ promoter is strongly activated by FSH through cAMP signaling in female menstrual cycle (Sofi et al., 2003). Other cAMP signaling activators including PGE2 and forskolin also known as CYP19A1 upregulators (Subbaramaiah et al., 2008). In adipose tissue , pⅠ.4 is the major activated promoter region. pⅠ.4 is mainly activated in preadipocytes by signaling pathways including STAT3 and AP-1 (Zhao et al., 1996; Catalano et al., 2003; Zhao et al., 1995). Because placenta and ovary promoter is not activated in

postmenopausal phase, adipose tissue is the major organ expressing CYP19A1 in postmenopausal women. Especially in breast cancer, CYP19A1 expression is upregulated in adipose tissue adjacent to cancer (O'Neill and Miller., 1987; O'Neill et al., 1988; Bulun et el., 1994). Various cytokines and hormones regulate the expression of CYP19A1 through pⅠ.4 by STAT3 and MAPK signaling and pⅡ by cAMP signaling (Zhao et al, 1995; Zhao et al., 1996; Catalano et al., 2003; Sofi et al., 2003; To et al., 2014).

Previous reports showed that human interferon-ɣ inducible protein 16 (IFI16) is involved in estrogen receptor α (ERα) expression in breast cancer (Kang et al., 2014). Moreover, IFI16 increased CYP19A1 expression, estradiol secretion, and ERα target gene transcription in MCF-7 breast cancer cells (Figure 4; Kang, 2014). But involvement of IFI16 in the regulation of CYP19A1 in breast cancer microenvironment has not been studied.

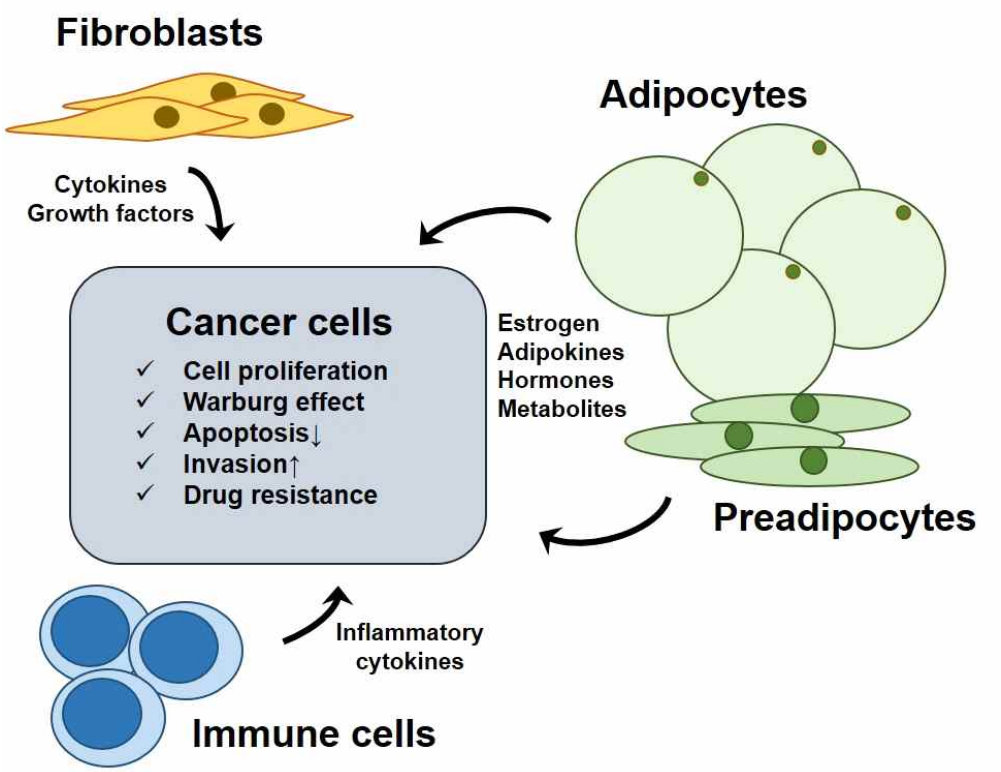

Figure 1. Microenvironmental factors secreted by stromal cells in breast cancer.

Like the 'seeds in soil', breast cancer cells are surrounded with cancer microenvironment composed of various cell types, such as cancer cells, immune cells, fibroblasts, and adipocytes. These stromal cells secrete diverse factors, including growth factors, cytokines, and metabolites. Growth factors and cytokines promote cancer proliferation and invasion. Metabolites supply energy source and building blocks to cancer cells, causing Warburg effect. As a result, the communication between cancer cells and stromal cells promotes cancer malignancy and drug resistance (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014; Ma et al., 2015).

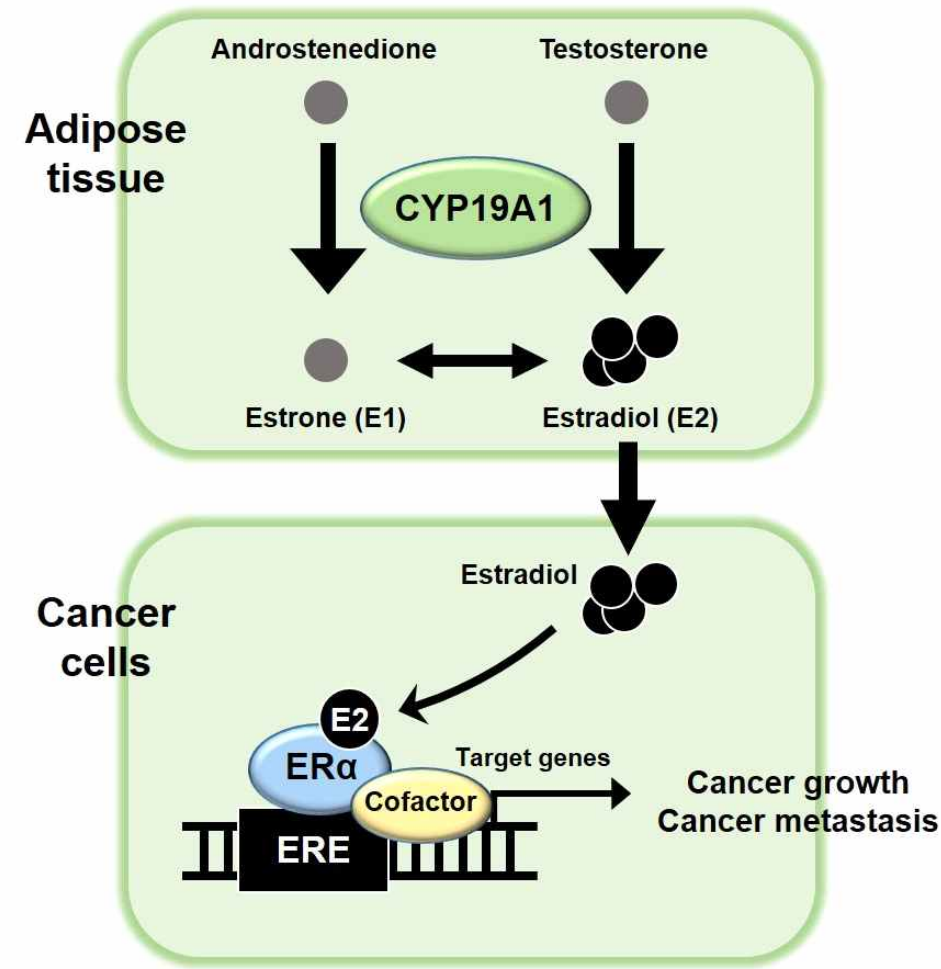

Figure 2. Estrogen biosynthesis and activation of ERα signaling in cancer microenvironment.

CYP19A1 expressed in adipose tissue (especially in preadipocytes) converts androgens to estrogens. Increased local estrogens bind to estrogen receptor α in the cytosol of cancer cells. Activated ERα causes the transcription of downstream genes such as c-Myc and cyclin D1. Consequently, CYP19A1 in adipose tissue promotes breast cancer growth and metastasis (Yager and Davidson., 2006; Ma et al., 2015).

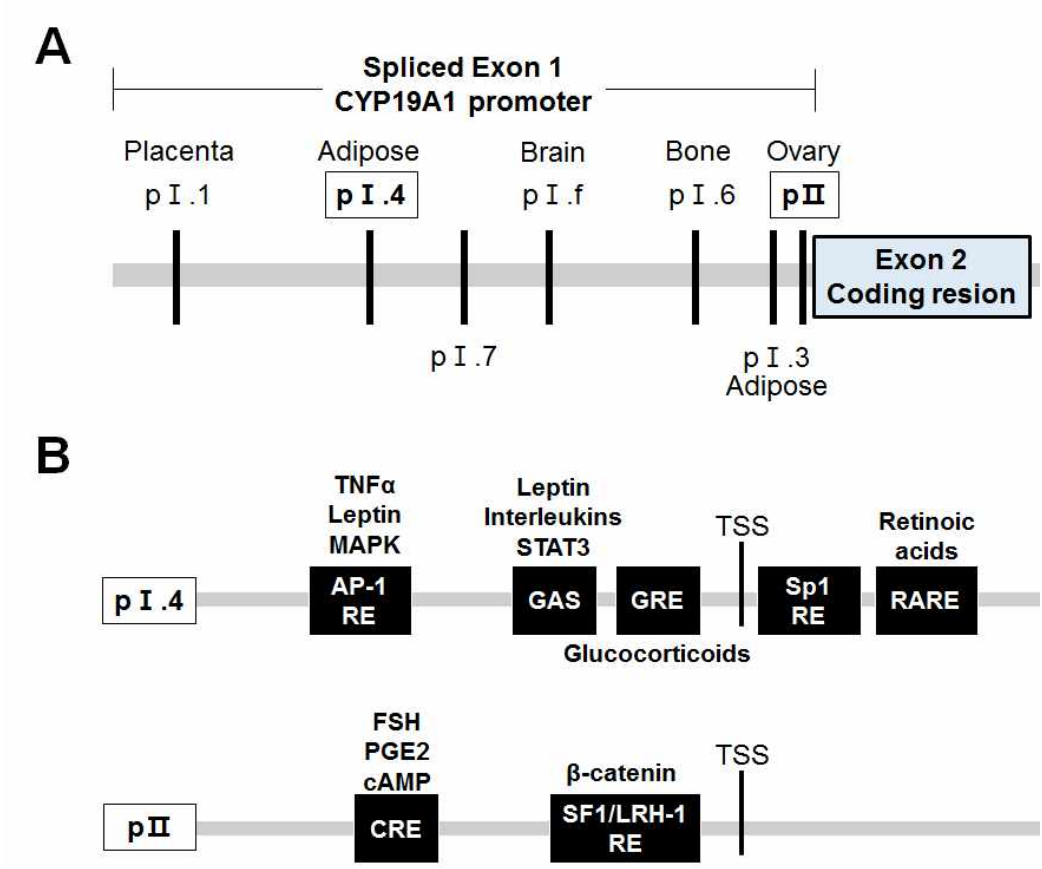

Figure 3. Regulation of CYP19A1 promoter.

CYP19A1 is regulated through tissue-specific promoters by alternative splicng (A). Especially promoter pⅠ.4 and pⅡ are activated in breast cancer microenvironment (B). Various cytokines regulate pⅠ.4 region through AP-1 RE and GAS. Endocrine hormones activate GRE in pⅠ.4 and CRE in pⅡ. RE : Response element, GAS : Interferon-ɣ activation sequence, GRE : Glucocorticoid RE, RARE : Retinoic acid receptor RE, CRE : cAMP RE, TSS : Transcription start site (Simpson et al., 1981; Zhao et al., 1995; Zhao et al., 1996; Simpson et al., 2002; Catalano et al., 2003; Sofi et al., 2003; Parakh et a., 2006; Subbaramaiah et al., 2008; Wilde et el., 2013; To et al., 2014.)

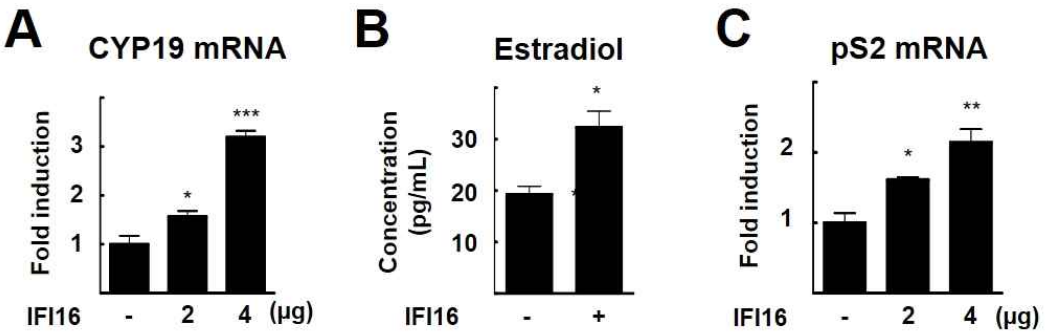

Figure 4. IFI16 upregulates CYP19A1 in breast cancer cells.

In MCF-7 breast cancer cells, transient transfection of IFI16 increased the transcription of CYP19A1. As a result, estradiol biosynthesis and secretion was increased. The transcription of pS2, an ERα downstream gene, was also increased (Kang, 2014).

Ⅱ. PURPOSE of the STUDY

Estrogen biosynthesis and ERα signaling are critical factors in breast cancer progression and drug resistance. Estrogen biosynthesis in microevironment is mediated by CYP19A1 aromatase, where it converts androgens to estrogens. In breast cancer microenvironment, CYP19A1 is mainly expressed in preadipocytes of adipose tissue. In breast cancer, CYP19A1 expression is increased, and elevated local estrogens promote breast cancer proliferation and metastasis. In this study, I aimed to investigate the regulation of CYP19A1 in breast cancer microenvironment. For the purpose, differentiated 3T3-L1 adipocytes were cocultivated with MCF-7 breast cancer cells, and alteration of CYP19A1 expression and ERα signaling was examined. Next, I examined to find regulators of CYP19A1 expression in breast cancer microenvironment. Various microenvironmental factors including cytokines, endocrine hormones, and metabolites were treated to 3T3-L1 mouse preadipocyte cells, and alteration of CYP19A1 expression was evaluated. On the other hand, upregulation of CYP19A1 expression by human IFI16 in breast cancer cells was previously reported. For this reason, I investigated the involvement of p204, a mouse homologue of human IFI16, in the regulation of CYP19A1.

Ⅲ. MATERIALS and METHODS

1. Cell culture and cell treatment

Mouse preadipocyte cell line, 3T3-L1 was a generous gift from professor Minsoo Noh. Human breast adenocarcinoma cell line, MCF-7 was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10% newborn calf serum (NBCS, Gibco 16010-159)) was supplemented for 3T3-L1 cells, and fetal bovine serum (FBS, Hyclone) was added to the medium for MCF-7 cells. Cells were cultured at 37°C in a 5% CO_2 / 95% air incubator.

Mouse Interferon-ɣ (315-05), mouse WNT3A (315-20), mouse IGF-1 (250-19) were purchased from Peprotech. Mouse TGF-β (7666-MB-005) was obtained from R&D systems. Dexamethasone (D4902), 3-isobutyl-1-methylxantine (I5879), human insulin (I9278), ACTH 1-24 fragment (A0298), Palmitic acid (P5585), Stearic acid (S4751), Docosahexanoic acid (D2534), Eicosapentanoic acid (E2011), D-(+)-Glucose (G5400) were purchased from Sigma Aldrich. For the treatment of free fatty acids, 1% fatty acid-free bovine serum albumin (A6003) was dissolved in medium with free fatty acids.

2. Adipocytes differentiation and MCF-7 coculture

For differentiation, 3T3-L1 preadipocytes were seeded at 1 X 10^5

cells/well into 6 well plates. At postconfluent two days, cells were stimulated by MDI (1 μM Dexamethasone, 0.5 mM IBMX, 10 μg/ml human Insulin) in DMEM supplemented with 10% FBS, penicillin and streptomycin. Two days later, MDI was replaced with 10 μg/ml human Insulin. After another two days, medium was changed to DMEM containing 10% FBS, penicillin and streptomycin, which was replaced every two days (Neal and Clipstone., 2002). After 6 days, MCF-7 cells were seeded at 4 X 10^4 cells/well into the upper insert of Corning Transwell system (CLS3450). Media was changed every 3 days.

3. Oil-red O staining

Cells were washed with PBS and fixed by 10% formaldehyde for 15 minutes. Oil-red O (Sigma aldrich, O0625) stock solution (0.5% in isopropanol) was diluted to 3:2 with distilled water and filtered before use. Samples were stained with diluted Oil-red O working solution for one hours and rinsed with distilled water (Wang et al., 2015).

4. Western blotting

Cells were lysed in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 1% NP-40, and protease inhibitors cocktail (Roche) for 30 min on ice, and whole cell lysates were obtained by subsequent centrifugation. The protein concentration was quantified by bicinchoninic acid assay (Pierce). Protein from whole cell lysates were subjected onto sodium dodecylsulfate-polyacrylamide

gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. Blocking was performed in 5% (w/v) non-fat-dried milk in phosphate-buffered saline containing 0.1% Tween-20. The membrane was then incubated with specific antibodies against CYP19A1 (sc-14245), FABP4 (SC-18661), ERα (sc-543), pS2 (SC-22501), p204 (SC-13367) from Santa Cruz Biotechnology, and α-tubulin (calbiochem).

5. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR

Total RNA was prepared using EASY-BLUE^{TM} Total RNA Extraction Kit (iNtRON Biotechnology) according to the manufacturer's instructions. cDNA was synthesized from total RNA in a reaction mixture containing random hexamer (Invitrogen) and MMLV-Reverse transcriptase (Invitrogen). Quantitative real time PCR was performed using SYBR Green PCR mix (Applied Bioscience). The data were normalized to β-actin reference. The sequences of the primers are described in Table 1.

6. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was determined by student's t-test. Differences were considered as statistically significant when p-value was < 0.05.

Table 1. Primer sequences used for real-time PCR analysis

Gene		Primer sequences	Ref.
CYP19A1	Forward	5′-ATG TTC TTG GAA ATG CTG AAC CC-3′	-
	Reverse	5′-AGG ACC TGG TAT TGA AGA CGA G-3′	
p204	Forward	5′-TGG TCC CAA ACA AGT GAT GGT GC-3′	Unterholzner et al., 2010
	Reverse	5′-TCA GTT TCA GTA GCC ACG GTA GCA-3′	
β-actin	Forward	5′-CGT GGG CCG CCC TAG GCA CCA-3′	Kang et al., 2014
	Reverse	5′-TTG GCT TAG GGT TCA GGG GGG-3′	

Ⅳ. RESULTS

1. Alteration of CYP19A1 expression and ERα signaling in cocultivated MCF-7 cells and 3T3-L1 adipocytes.

In order to mimic cancer-associated adipocytes (CAAs) *in vitro*, MCF-7 breast cancer cells and mature 3T3-L1 adipocytes were cocultivated using Transwell coculture system (Figure 5). Lipid accumulation and adipocyte marker FABP4 expression were decreased in cocultivated adipocytes. These alterations demonstrate the transformation of adipocytes, similar to delipidated and de-differentiated phenotype of CAAs (Dirat et al., 2011). In contrast, lipid accumulation and FABP4 expession were increased in cocultivated MCF-7 cells, indicating that lipid was transferred to cancer cells from adipocytes (Nieman et al., 2011). Intriguingly, CYP19A1 expression was upregulated in cocultivated adipocytes (Figure 6). Moreover, expression of pS2, an ERα downstream gene, was induced in cocultivated MCF-7 cells. Furthermore, the protein level of ERα was decreased in cocultivated MCF-7 cells, implying that binding of estradiol triggered the degradation of ERα. These results suggest that upregulated CYP19A1 in cocultivated adipocytes promoted the release of estrogen and activated ERα signaling in cocultivated MCF-7 cells (Figure 13A).

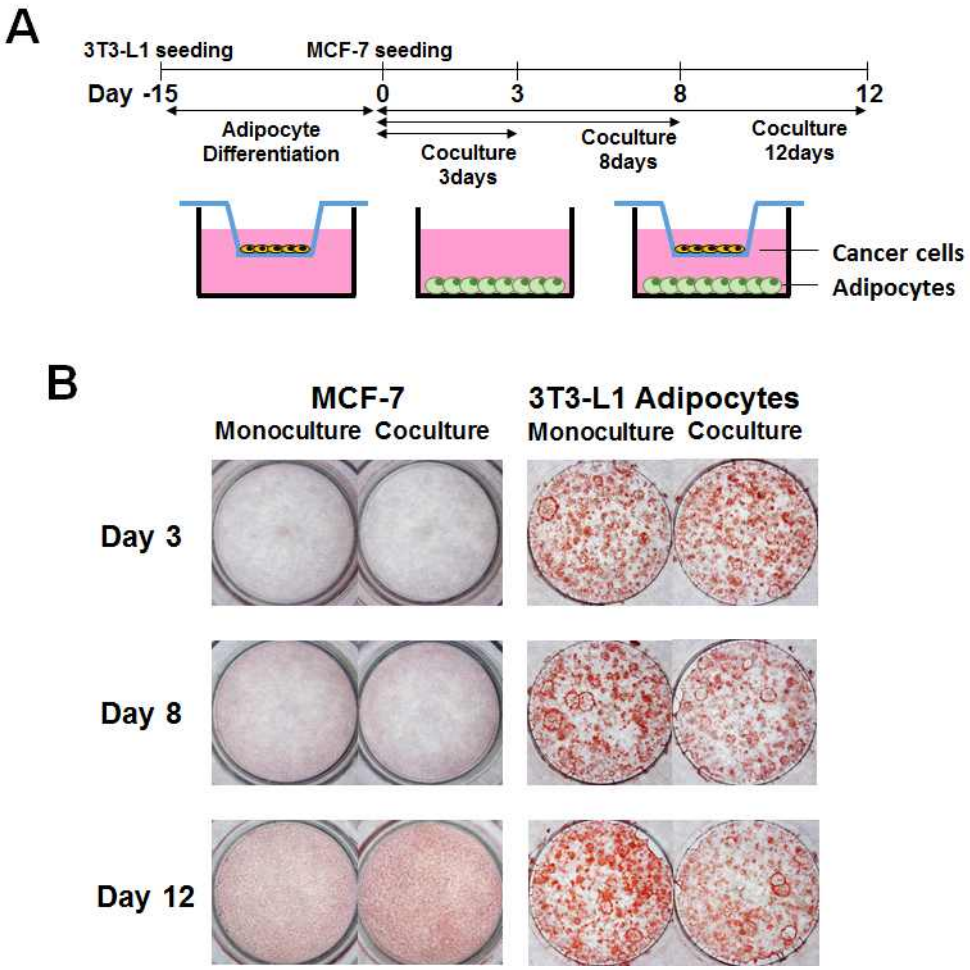

Figure 5. Transwell coculture of MCF-7 cells and 3T3-L1 adipocytes.

MCF-7 cancer cells and 3T3-L1 adipocytes were cocultivated using Transwell system. (A) Schematic illustration of coculture experiment. 3T3-L1 cells were differentiated for 15 days, and cocultivated with MCF-7 cells for 12 days. (B) Lipid accumulation of cocultivated cells was analyzed by Oil-red O staining.

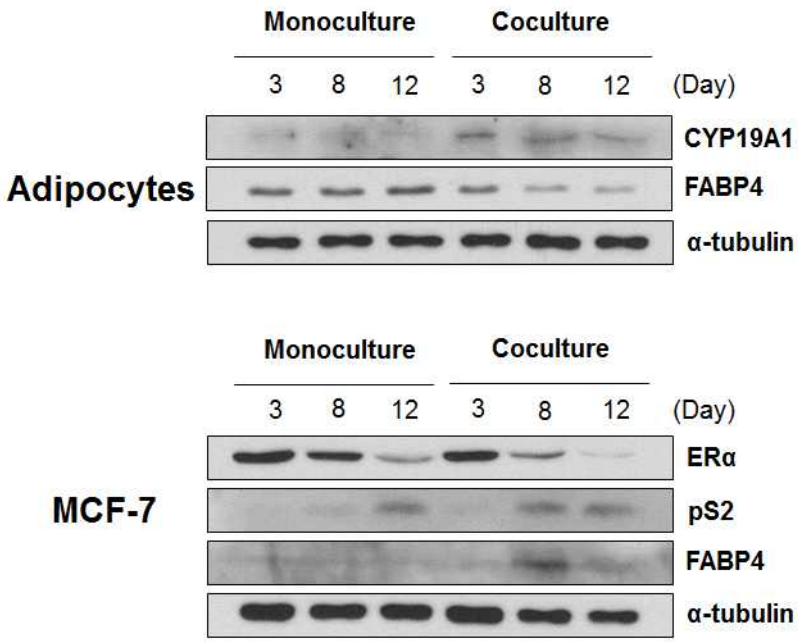

Figure 6. Alteration of CYP19A1 expression and ERα signaling in cocultivated MCF-7 cells and 3T3-L1 adipocytes.

Alteration of the protein expression of cocultivated MCF-7 cancer cells (upper panel) and 3T3-L1 adipocytes (bottom panel). Whole cell lysates were analyzed by western blotting.

2. Breast cancer microenvironmental cytokines increase CYP19A1 expression in 3T3-L1 preadipocytes.

Next, I aimed to investigate factors that cause alteration of CYP19A1 expression in breast cancer microenvironment. Various inflammatory cytokines are known to regulate CYP19A1 expression (To et al., 2014). In order to find cytokines involved in the regulation of CYP19A1, the effects of inflammatory cytokines and adipokines abundant in breast cancer microenvironment were examined. Among inflammatory cytokines, IFNɣ and TGF-β upregulated CYP19A1 protein and mRNA expression in 3T3-L1 preadipocytes, which express a high level of CYP19A1. For adipokines, WNT3A elevated CYP19A1 expression in preadipocytes (Figure 7).

3. Endocrine hormones regulate CYP19A1 expression.

Endocrine hormones, including FSH, leptin, and glucocorticoids, are also known to be crucial CYP19A1 regulators (To et al., 2014). Moreover, insulin signaling is activated in adipose tissue, and has a role in breast cancer development (Calle and Kaaks., 2004). Two important hormones in insulin signaling, insulin and IGF-1, increased CYP19A1 expression in 3T3-L1 preadipocytes. In addition, adrenocorticotropic hormone (ACTH) elevated the protein level of CYP19A1 in preadipocytes (Figure 8).

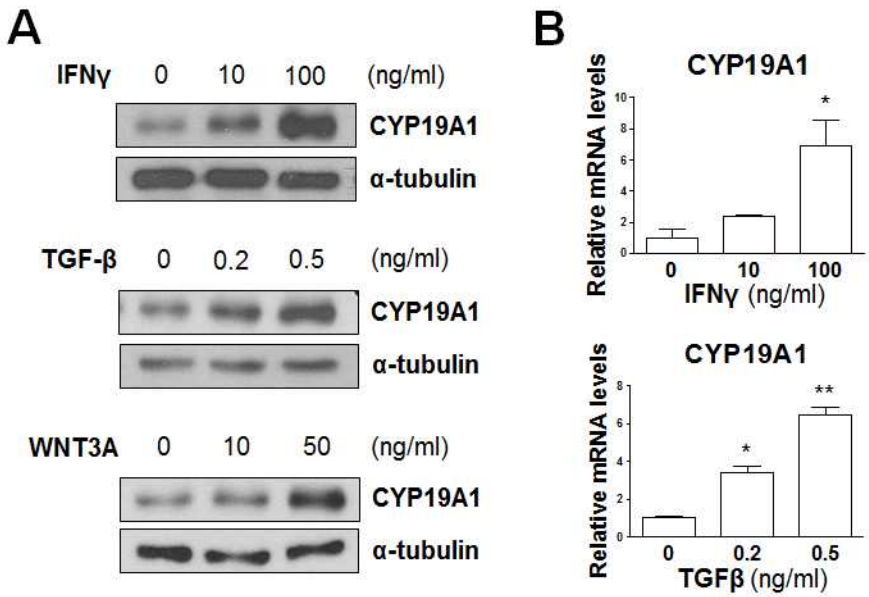

Figure 7. Breast cancer microenvironmental cytokines increase CYP19A1 expression in 3T3-L1 preadipocytes.

(A) Effect of microenvironmental cytokines on protein expression of CYP19A1 in 3T3-L1 preadipocytes. Recombinant mouse IFNɣ, mouse TGF-β, mouse WNT3A were treated to 3T3-L1 preadipocytes for 24 h, and CYP19A1 protein level was analyzed by western blotting. (B) Alteration of CYP19A1 mRNA level by treatment of IFNɣ and TGF-β. Relative mRNA levels were measured by real-time PCR, and normalized with β-actin. The values represent the means ± SEM of experiments in duplicates. * $P < 0.05$, ** $P < 0.01$ *vs.* vehicle.

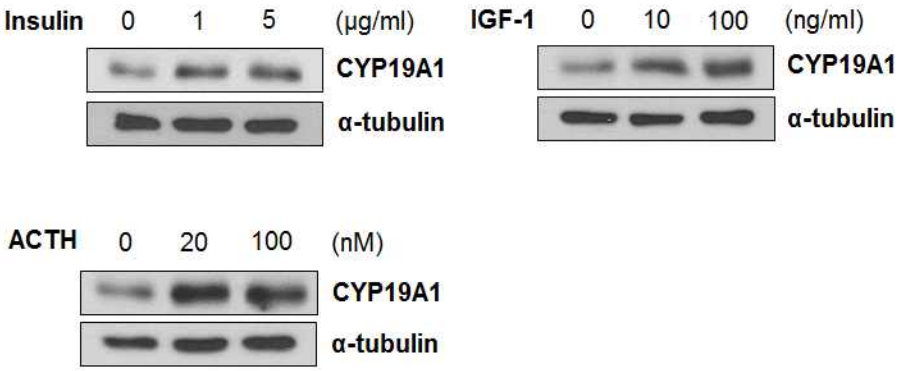

Figure 8. Endocrine hormones regulate CYP19A1 expression.

Human insulin (24 h), mouse IGF-1 (48 h), and ACTH 1-24 fragment (24 h) were treated to 3T3-L1 preadipocytes for indicated doses. The protein expression of CYP19A1 and α-tubulin was analyzed by western blotting.

4. Regulation of CYP19A1 by microenvironmental metabolites.

In adipose tissue, metabolites including free fatty acids and carbohydrates are secreted into the microenvironment. These metabolites are important building block and energy source for breast cancer cells. Interestingly, saturated free fatty acids upregulated CYP19A1 expression, whereas ω-3 polyunsaturated fatty acids were downregulated CYP19A1 (Figure 9A, B). Moreover, treatment of EPA restored IFNɣ- or stearic acid-induced expression of CYP19A1 (Figure 12). On the other hand, glucose increased the protein expression of CYP19A1 in 3T3-L1 preadipocytes (Figure 9C).

5. Microenvironmental CYP19A1 regulators are accompanied by alteration of p204 expression.

According to previous reports, IFI16, the transcriptional factor associated with ERα regulation in breast cancer, increased CYP19A1 expression in MCF-7 breast cancer cells. In the case of mouse, p204 is known as homologue of human IFI16. Intriguingly, CYP19A1 regulation by microenvironmental factors was accompanied with alteration of p204 expression. Among upregulators of CYP19A1, IFNɣ, TGF-β, WNT3A, IGF-1, saturated fatty acids, and glucose increased p204 expression (Figure 10, 11A, 11C, 12). On the contrary, ω-3 polyunsaturated fatty acids, which downregulated CYP19A1, decreased p204 protein level (Figure 11B, 12). These results imply the involvement of p204 in the regulation of CYP19A1 (Figure 13B).

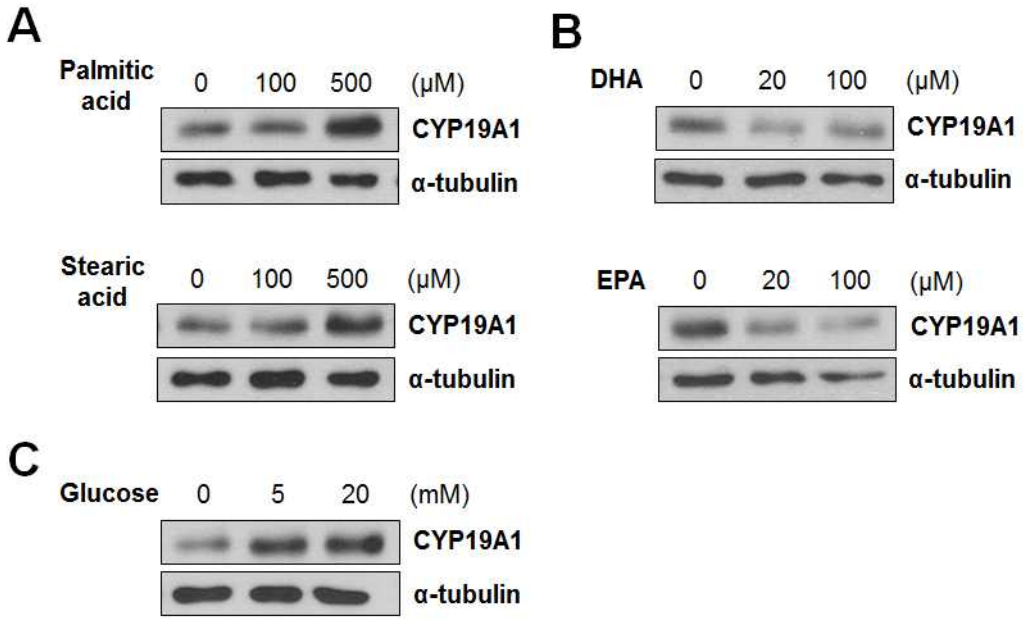

Figure 9. Regulation of CYP19A1 by microenvironmental metabolites.

Effect of Free fatty acids and glucose on CYP19A1 expression in 3T3-L1 preadipocytes. Saturated fatty acids (A) or ω-3 polyunsaturated fatty acids (B) were treated for 24 h. (C) D-(+)-glucose was treated to 3T3-L1 preadipocytes for 48 h. Whole cell lysates were harvested and protein level was measured by western blotting.

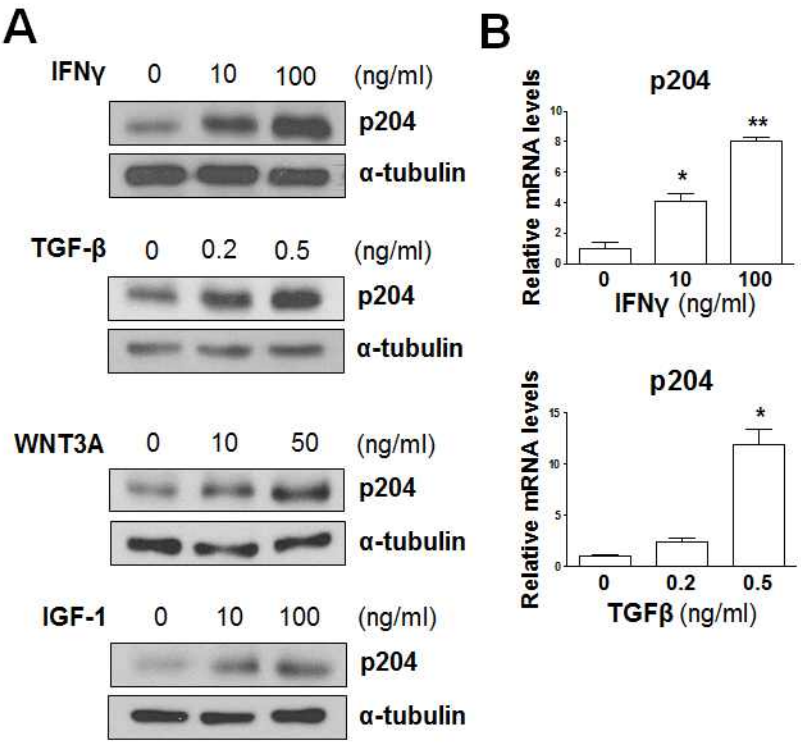

Figure 10. Cytokines and hormones which upregulate CYP19A1 also increase p204 expression.

(A) Effect of microenvironmental cytokines and hormones on protein expression of p204 in preadipocytes. Mouse IFNɣ, mouse TGF-β, mouse WNT3A and mouse IGF-1 were treated to 3T3-L1 preadipocytes, and protein level was analyzed by western blotting. (B) Increase of p204 mRNA by IFNɣ and TGF-β. The mRNA level was measured by real-time PCR and normalized with β-actin. Data represent the means ± SEM of experiments in duplicates. * $P < 0.05$, ** $P < 0.01$ *vs.* vehicle.

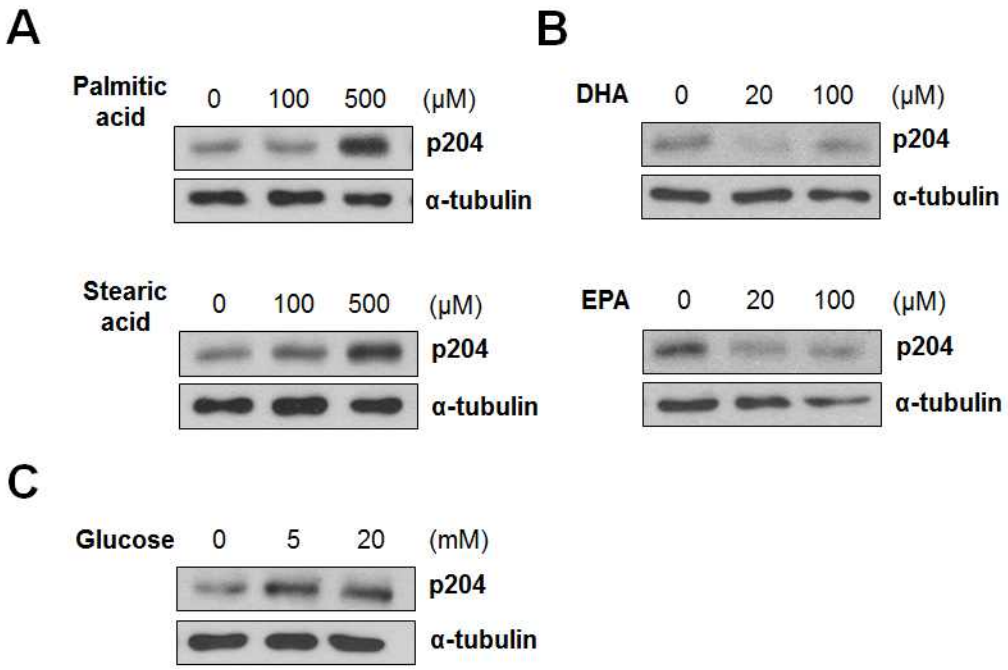

Figure 11. Metabolic CYP19A1 regulators accompany alteration of p204 expression.

Effects of saturated fatty acids (A), ω-3 polyunsaturated fatty acids (B), and glucose (C) on expression of p204 in 3T3-L1 preadipocytes. Whole cell lysates were harvested and protein level was analyzed by western blotting.

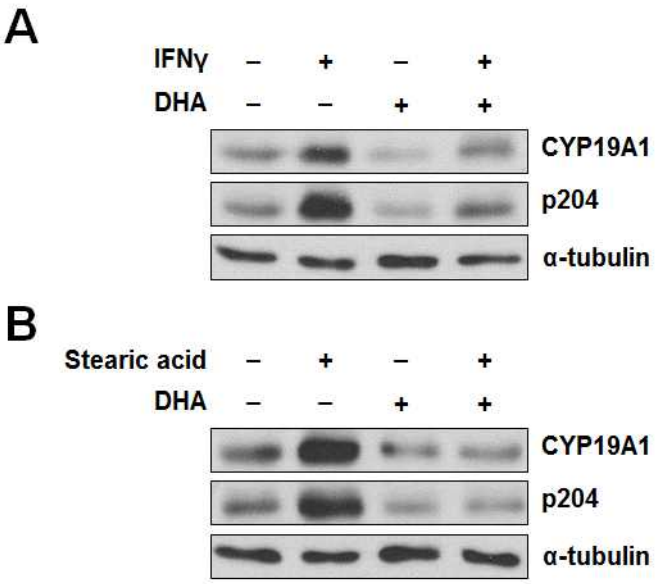

Figure 12. EPA restores IFNɣ- or stearic acid-induced expression of CYP19A1 and p204.

Mouse IFNɣ (A, 100 ng/ml) or stearic acid (B, 500 μM) was co-treated with EPA (100 μM) in 3T3-L1 mouse preadipocytes. After 24 h treatment, whole cell lysates were harvested and protein expression of CYP19A1, p204, and α-tubulin was analyzed by western blotting.

Table 2. Regulation of CYP19A1 expression by breast cancer microenvironmental factors in 3T3-L1 preadipocytes.

Secretory factors	Secreting sites	Previous observations			Expression in this study	
		References	Experimental Models	CYP19A1 Expression	CYP19A1	p204
Cytokines						
IFNɣ	Immune cells	Lai et al., 2014	Granulosa cells	Increase	Increase	Increase
TGF-β	Cancer, Immune cells				Increase	Increase
WNT3A	Cancer cells				Increase	Increase
Adiponectin	Adipose	Ledoux et al., 2006	Follicular cells	Decrease	-	-
CCL2	Adipose, Immune cells				-	-
IL-4	Immune cells				-	-
Resistin	Adipose				-	-
Hormones						
IGF-1	Liver	Sharma et al., 2012	Granulosa cells	Increase	Increase	Increase
Insulin	Pancrea	Chaves et al., 2012	Follicular cells	Increase	Increase	-
ACTH	Hyppocampus				Increase	-
Cortisol	Adrenal cortex	Wang et al., 2012	Placenta cells	Increase	Decrease	-
Epinephrine	Adrenal medula, Nerve				-	-
Metabolites						
Palmitic acid	Adipose				Increase	Increase
Stearic acid	Adipose				Increase	Increase
Oleic acid	Adipose				-	-
DHA	Adipose				Decrease	Decrease
EPA	Adipose				Decrease	Decrease
Glucose	Adipose, Blood				Increase	Increase
Lactic acid	Cancer cells				-	-
27-HC	Liver				-	-
22-S-HC	Liver				-	-
22-R-HC	Liver				-	-
25-HC	Liver				-	-

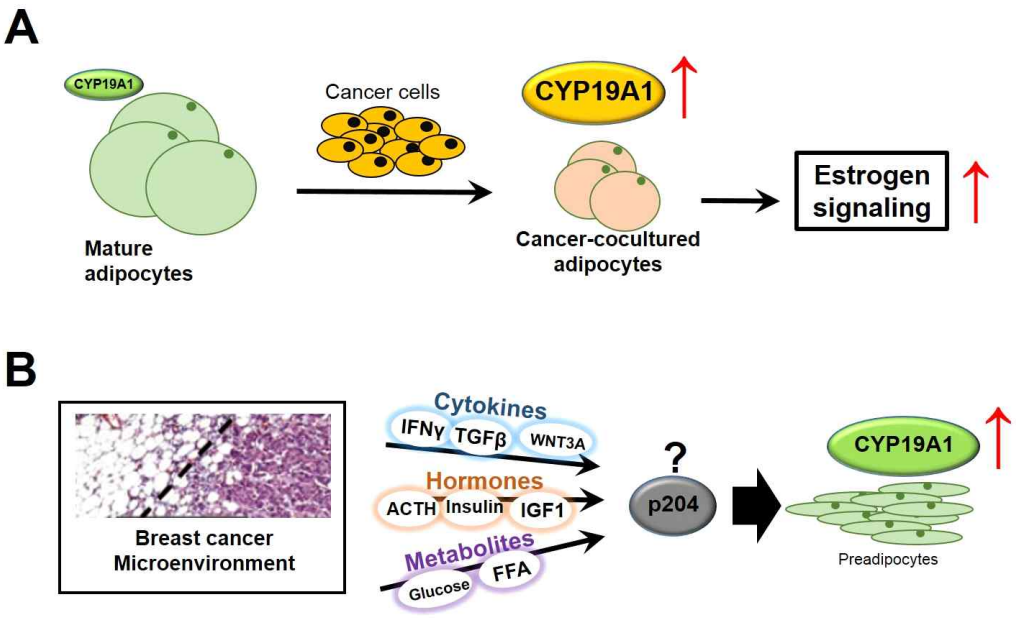

Figure 13. Schematic illustration of CYP19A1 regulation by breast cancer microenvironmental factors.

(A) Alteration of CYP19A1 expression in adipocytes cocultivated with cancer cells. CYP19A1 expression was increased in cocultivated adipocytes, and ERα signaling was activated in cocultivated cancer cells. (B) CYP19A1 regulation by breast cancer microenvironmental factors in preadipocytes. Various cytokines, hormones, metabolites abundant in breast cancer microenvironment regulated CYP19A1 expression in preadipocytes.

V. DISCUSSION

CYP19A1 is the rate-determining enzyme of estrogen biosynthesis. CYP19A1 converts androgens to estrogens, which bind to estrogen receptors and activate the transcription of ERα downstream (Figue 2; Simpson et al., 2002; Johnston and Dowsett., 2003; Ma et al., 2015). CYP19A1 is upregulated in breast cancer, and increased local estrogen contributes to cancer proliferation and metastasis (Yue et al., 1998). For this reason, CYP19A1 is a crucial target for postmenopausal breast cancer endocrine therapy.

CYP19A1 expression is regulated by tissue-specific promoter regulation (Figure 3; To et al., 2014). In breast cancer microenvironment, CYP19A1 is mainly expressed in adipose tissue, especially in preadipocytes. Recently, adipose tissue is considered as an important endocrine organ, contributing to cancer malignancy by the release of adipokines and endocrine hormones (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). Moreover, transformed adipocytes in breast cancer microenvironment, known as cancer-associated adipocytes (CAAs), have been reported (Dirat et al., 2011; Bochet et al., 2013). CAAs show decreased lipid accumulation, loss of molecular adipocyte markers, and increased secretion of inflammatory cytokines, facilitating the invasion of cancer cells.

Because CYP19A1 expression is decreased during adipocyte differentiation and the phenotype change of CAAs is similar with de-differentiation of adipocytes, I examined the alteration of CYP19A1 expression in CAAs. In order to mimic CAAs *in vitro*, transwell coculture of MCF-7 breast cancer cells with 3T3-L1 mature adipocytes was conducted (Figure 5). As a result, lipid accumulation and FABP4, the lipid carrier protein recognized as representative adipocyte marker, were increased in MCF-7 cells and decreased in adipocytes (Figure 6). This indicates that cocultivated adipocytes were transformed like CAAs (Dirat et al., 2011). On the other hand, lipid transferred from adipocytes to cancer cells provides energy and building blocks for cancer proliferation (Nieman et al., 2011). Interestingly, CYP19A1 expression was increased in cocultivated adipocytes. Moreover, transcription of ERα downstream gene pS2 was increased, and ERα protein level was decreased in cocultivated MCF-7 cells. These results indicate that the increased release of estrogen from cocultivated adipocytes activated ERα signaling, triggering the degradation of ligand-bound ERα and downstream gene transcription.

Next, I aimed to find factors that mediate CYP19A1 regulation in cancer microenvironment. In breast cancer, inflammatory cytokines are typically increased, and contribute to cancer malignancy. In addition, various hormones and metabolites secreted by adipocytes provide fuels for cancer metabolism and building blocks for proliferation. I explored the effects of these breast cancer microenvironmental factors

on CYP19A1 expression in preadipocytes, the main population expressing CYP19A1 in adipose tissue. Consequently, various cytokines, hormones, and metabolites abundant in breast cancer microenvironment regulated CYP19A1 in 3T3-L1 preadipocytes (Figure 7, 8, 9). These results suggest a new type of treatment against CYP19A1, which is different with classical aromatase inhibitor. Modulating microenvironmental cytokines or insulin signaling could be developed as endocrine therapy for breast cancer. In addition, ω-3 polyunsaturated fatty acids have the possibility for mild oral aromatase inhibitor, similar with reports on dietary polyphenols (Subbaramaiah et al., 2013).

Furthermore, I discovered that many CYP19A1 regulators also altered expression of p204, the mouse homologue of IFI16 (Figure 10, 11, 12). Previous reports described that IFI16 is associated with regulation of ERα and CYP19A1 expression in ERα-positive breast cancer(Kang et al., 2014; Kang, 2014). Moreover, IFNɣ, one of the CYP19A1 upregulator, is the most potent upstream of IFI16. IFNɣ has been considered to be a tumor suppressor, because IFNɣ inhibits tumor growth and eliminates cancer cells through immune response. However, pro-tumor functions of IFNɣ were reported recently (Xiao et al., 2009; Zaidi et al., 2011). A downstream of IFNɣ, IFI16, also has a dual role in cancer. IFI16 regulates cell cycle and cell senescence, and it has been recognized as a tumor suppressor (Choubey et al., 2008). But according to previous reports, IFI16 increases ERα and CYP19A1 expression and accelerates cancer

proliferation (Kang et al., 2014; Kang, 2014)). As shown in this study, p204 was involved in CYP19A1 regulation by various cancer microenvironmental factors, implying the oncogenic function of p204 in breast cancer. For the following study, the mechanism of CYP19A1 regulation by microenvironmental factors and the role of p204 in CYP19A1 regulation need to be studied.

REFERENCE

Andò S, Catalano S. The multifactorial role of leptin in driving the breast cancer microenvironment. *Nat Rev Endocrinol*. 2011;**8**(5):263-75.

Ali S, Buluwela L, Coombes RC. Antiestrogens and their therapeutic applications in breast cancer and other diseases. *Annu Rev Med*. 2011;**62**:217-32.

Baxter SW, Choong DY, Eccles DM, Campbell IG. Polymorphic variation in CYP19 and the risk of breast cancer. *Carcinogenesis*. 2001;**22**(2):347-9.

Bochet L, Lehuédé C, Dauvillier S, Wang YY, Dirat B, Laurent V, Dray C, Guiet R, Maridonneau-Parini I, Le Gonidec S, Couderc B, Escourrou G, Valet P, Muller C. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res*. 2013;**73**(18):5657-68.

Bulun SE, Mahendroo MS, Simpson ER. Aromatase gene expression in adipose tissue: relationship to breast cancer. *J Steroid Biochem Mol Biol*. 1994;**49**(4-6):319-26.

Bulun SE, Chen D, Moy I, Brooks DC, Zhao H. Aromatase, breast cancer and obesity: a complex interaction. *Trends Endocrinol*

Metab. 2012;**23**(2):83-9.

Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer*. 2004 **4**(8):579-91.

Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Ando S. Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem*. 2003;**278**(31):28668-76

Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Ando S. Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem*. 2003;**278**(31):28668-76

Chaves RN, Duarte AB, Rodrigues GQ, Celestino JJ, Silva GM, Lopes CA, Almeida AP, Donato MA, Peixoto CA, Moura AA, Lobo CH, Locatelli Y, Mermillod P, Campello CC, Figueiredo JR. The effects of insulin and follicle-stimulating hormone (FSH) during in vitro development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. *Biol Reprod*. 2012;**87**(3):69.

Choubey D, Deka R, Ho SM. Interferon-inducible IFI16 protein in human cancers and autoimmune diseases. *Front Biosci*. 2008;**13**:598-608.

Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. *Clin Cancer Res*. 2012;**18**(5):1201-6

Dieudonné MN, Sammari A, Dos Santos E, Leneveu MC, Giudicelli Y, Pecquery R. Sex steroids and leptin regulate 11beta-hydroxysteroid dehydrogenase I and P450 aromatase expressions in human preadipocytes: Sex specificities. *J Steroid Biochem Mol Biol*. 2006;**99**(4-5):189-96.

Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res*. 2011;**71**(7):2455-65.

Dittmer J, Leyh B. The impact of tumor stroma on drug response in breast cancer. *Semin Cancer Biol*. 2015;**31**:3-15.

Esquivel-Velázquez M, Ostoa-Saloma P, Palacios-Arreola MI, Nava-Castro KE, Castro JI, Morales-Montor J. The role of cytokines in breast cancer development and progression. *J Interferon Cytokine Res*. 2015;**35**(1):1-16.

Gilbert CA, Slingerland JM. Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression. *Annu Rev Med*. 2013;**64**:45-57.

Haiman CA, Hankinson SE, Spiegelman D, De Vivo I, Colditz GA, Willett WC, Speizer FE, Hunter DJ. A tetranucleotide repeat polymorphism in CYP19 and breast cancer risk. *Int J Cancer*. 2000;**87**(2):204-10.

Johnston SR, Dowsett M. Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nat Rev Cancer*. 2003;**3**(11):821-31.

Kang HJ, Lee MH, Kang HL, Kim SH, Ahn JR, Na H, Na TY, Kim YN, Seong JK, Lee MO. Differential regulation of estrogen receptor α expression in breast cancer cells by metastasis-associated protein 1. *Cancer Res*. 2014;**74**(5):1484-94.

Kang HL. The role of interferon, gamma-inducible protein 16 in the estrogen signaling during breast cancer progression [Unpublished Masters Dissertation]. *Seoul-si, South Korea: Seoul National University*, 2014.

Lai WA, Yeh YT, Fang WL, Wu LS, Harada N, Wang PH, Ke FC, Lee WL, Hwang JJ. Calcineurin and CRTC2 mediate FSH and TGFβ1 upregulation of Cyp19a1 and Nr5a in ovary granulosa cells. *J Mol Endocrinol*. 2014;**53**(2):259-70

Ledoux S, Campos DB, Lopes FL, Dobias-Goff M, Palin MF, Murphy BD. Adiponectin induces periovulatory changes in ovarian follicular cells. *Endocrinology*. 2006;**147**(11):5178-86

Ma CX, Reinert T, Chmielewska I, Ellis MJ. Mechanisms of aromatase inhibitor resistance. *Nat Rev Cancer*. 2015;**15**(5):261-75.

Neal JW, Clipstone NA. Calcineurin mediates the calcium-dependent inhibition of adipocyte differentiation in 3T3-L1 cells. *J Biol Chem*. 2002;**277**(51):49776-81.

Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK, Carver NJ, Pillai RV, Sullivan PM, Sondhi V, Umetani M, Geradts J, McDonnell DP. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. *Science*. 2013;**342**(6162):1094-8.

Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med*. 2011;**17**(11):1498-503.

O'Neill JS, Miller WR. Aromatase activity in breast adipose tissue from women with benign and malignant breast diseases. *Br J Cancer*. 1987;**56**(5):601-4.

O'Neill JS, Elton RA, Miller WR. Aromatase activity in adipose tissue from breast quadrants: a link with tumour site. *Br Med J*. 1988;**296**(6624):741-3.

Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med*. 2011;**62**:233-47.

Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, Nilson JH. Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. *Proc Natl Acad Sci U S A*. 2006;**103**(33):12435-40

Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr Rev.* 2011;**32**(4):550-70.

Phuong NT, Lim SC, Kim YM, Kang KW. Aromatase induction in tamoxifen-resistant breast cancer: Role of phosphoinositide 3-kinase-dependent CREB activation. *Cancer Lett.* 2014;**351**(1):91-9.

Sharma I, Singh D. Conjugated linoleic acids attenuate FSH- and IGF1-stimulated cell proliferation; IGF1, GATA4, and aromatase expression; and estradiol-17β production in buffalo granulosa cells involving PPARɣ, PTEN, and PI3K/Akt. *Reproduction.* 2012;**144**(3):373-83

Simpson ER, Ackerman GE, Smith ME, Mendelson CR. Estrogen formation in stromal cells of adipose tissue of women: induction by glucocorticosteroids. *Proc Natl Acad Sci U S A.* 1981;**78**(9):5690-4

Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C, Jones M. Aromatase--a brief overview. *Annu Rev Physiol.* 2002;**64**:93-127.

Sofi M, Young MJ, Papamakarios T, Simpson ER, Clyne CD. Role of CRE-binding protein (CREB) in aromatase expression in breast adipose. *Breast Cancer Res Treat.* 2003;**79**(3):399-407

Subbaramaiah K, Hudis C, Chang SH, Hla T, Dannenberg AJ. EP2 and EP4 receptors regulate aromatase expression in human adipocytes and breast cancer cells. Evidence of a BRCA1 and p300 exchange. *J Biol Chem*. 2008;**283**(6):3433-44

Subbaramaiah K, Hudis CA, Dannenberg AJ. The prostaglandin transporter regulates adipogenesis and aromatase transcription. *Cancer Prev Res*. 2011;**4**(2):194-206.

Subbaramaiah K, Sue E, Bhardwaj P, Du B, Hudis CA, Giri D, Kopelovich L, Zhou XK, Dannenberg AJ. Dietary polyphenols suppress elevated levels of proinflammatory mediators and aromatase in the mammary gland of obese mice. *Cancer Prev Res*. 2013;**6**(9):886-97.

To SQ, Knower KC, Cheung V, Simpson ER, Clyne CD. Transcriptional control of local estrogen formation by aromatase in the breast. *J Steroid Biochem Mol Biol*. 2015;**145**:179-86.

Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol*. 2010;**11**(11):997-1004.

Wang W, Li J, Ge Y, Li W, Shu Q, Guan H, Yang K, Myatt L, Sun K. Cortisol induces aromatase expression in human placental syncytiotrophoblasts through the cAMP/Sp1 pathway. *Endocrinology*. 2012;**153**(4):2012-22

Wang C, Gao C, Meng K, Qiao H, Wang Y. Human adipocytes stimulate invasion of breast cancer MCF-7 cells by secreting IGFBP-2. *PLoS One*. 2015;**10**(3):e0119348

Wilde J, Erdmann M, Mertens M, Eiselt G, Schmidt M. Aromatase activity induction in human adipose fibroblasts by retinoic acids via retinoic acid receptor α. *J Mol Endocrinol*. 2013;**51**(2):247-60

Xiao M, Wang C, Zhang J, Li Z, Zhao X, Qin Z. IFNgamma promotes papilloma development by up-regulating Th17-associated inflammation. *Cancer Res*. 2009;**69**(5):2010-7.

Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N Engl J Med*. 2006;**354**(3):270-82.

Yue W, Wang JP, Hamilton CJ, Demers LM, Santen RJ. In situ aromatization enhances breast tumor estradiol levels and cellular proliferation. *Cancer Res*. 1998;**58**(5):927-32.

Zaidi M, Merlino G. The two faces of interferon-ɣ in cancer. *Clin Cancer Res*. 2011;**17**(19):6118-24.

Zhao Y, Nichols JE, Bulun SE, Mendelson CR, Simpson ER. Aromatase P450 gene expression in human adipose tissue. Role of a Jak/STAT pathway in regulation of the adipose-specific promoter. *J Biol Chem*. 1995;**270**(27):16449-57

Zhao Y, Nichols JE, Valdez R, Mendelson CR, Simpson ER. Tumor necrosis factor-alpha stimulates aromatase gene expression in

human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter 1.4. *Mol Endocrinol*. 1996;**10**(11):1350-7

국 문 초 록

CYP19A1 방향화효소는 에스트로젠 생합성의 율속단계를 촉매하는 중요한 효소이다. CYP19A1은 유방암과 인접한 지방조직에서 발현이 증가하여 종양미세환경의 에스트로젠 농도가 국소적으로 높아지고, 암세포의 에스트로젠 신호전달계가 활성화되어 종양의 성장과 전이를 촉진한다. 그러므로 CYP19A1 방향화효소는 유방암 환자의 내분비 치료에서 중요한 약물 타겟이다. 본 연구에서는 첫째, 유방암 세포주와 공동배양한 지방세포에서의 CYP19A1 발현과 그로 인한 암세포의 에스트로젠 신호전달계 활성화를 규명함과 함께 둘째, 유방암 종양미세환경에 풍부한 사이토카인, 호르몬, 대사물질 중 신규 CYP19A1 조절자를 발굴함을 목표로 하였다. 우선 3T3-L1 지방아세포주를 지방세포로 분화시킨 후 MCF-7 유방암 세포주와 공동배양했을 때, 공동배양한 지방세포에서 Oil-red O 염색을 통해 지방 함량이 줄어들고 지방세포 표지자 FABP4의 발현이 줄어드는 등 형질 변화가 나타남을 확인하였다. 이 때 지방세포에서 CYP19A1의 발현이 증가한 것을 발견하였고, 공동배양한 MCF-7 세포주에서 에스트로젠 신호전달계의 타겟 유전자 pS2의 발현이 증가함을 관찰하였다. 다음으로 3T3-L1 쥐 지방아세포주에 유방암 종양미세환경 인자들을 처리했을 때 사이토카인 중 인터페론-γ와 TGF-β, WNT3A가, 호르몬 중에서는 인슐린과 IGF-1, 그리고 부신피질자극호르몬 (ACTH)이 CYP19A1 발현을 증가시켰다. 또한 대사물질 중 포화지방산인 팔미트산과 스테아르산이 CYP19A1 발현 증가를 일으킨 반면에 오메가-3 불포화지방산인 도코사헥사엔산 (DHA)와 아이코사펜타엔산 (EPA)은 감소시키는 것을 발견하였다.

포도당 또한 CYP19A1 발현을 증가시켰다. 흥미롭게도, 본 연구진은 신규 CYP19A1 발현 조절자 중 인터페론-γ, WNT3A, 팔미트산, 스테아르산, DHA, EPA, 포도당, 그리고 기존에 보고된 CYP19A1 증가 조절자 TGF-β, IGF-1의 처리가 사람 인터페론-γ 유도 단백질 16 (IFI16)의 쥐 상동단백질 p204의 발현 변화를 동반하는 것을 발견하였다. 본 연구는 유방암세포와 지방세포의 상호작용으로 인한 에스트로젠 신호전달계 조절 가능성을 제시하였고, CYP19A1 방향화효소의 신규 조절자를 발굴함으로써 유방암 종양미세환경에서의 에스트로젠 신호전달계의 이해에 기여할 것이다.

주요어: CYP19A1, 종양미세환경, 에스트로젠 신호전달, p204, 유방암

약학석사학위논문

CYP19A1 발현을 조절하는 유방암 종양미세환경 인자 규명

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

2015년 7월

서울대학교 대학원

약학과 병태생리학전공

박 한 수

CYP19A1 발현을 조절하는 유방암 종양미세환경 인자 규명

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

지도교수 이 미 옥

이 논문을 약학석사 학위논문으로 제출함

2015년 7월

서울대학교 대학원

약학과 병태생리학전공

박 한 수

박한수의 석사 학위논문을 인준함

2015년 7월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

ABSTRACT

Breast Cancer Microenvironmental Factors that Regulate Expression of CYP19A1 in 3T3-L1 Cells

Hansu Park
College of Pharmacy
The Graduate School
Seoul National University

Estrogen signaling is a critical factor that supports cancer proliferation and metastasis in breast cancer. CYP19A1 is a rate-determining enzyme that catalyzes biosynthesis of estrogen. CYP19A1 is upregulated in adipose tissue adjust to breast cancer, and enhances the local concentration of estradiol and estrogen signaling in breast cancer cells. Therefore, CYP19A1 has been considered as an important target of endocrine therapy for patients with breast cancer. In the present study, we aimed to examine expression of CYP19A1 and estrogen signaling in interaction of cancer cells and adipocytes, and to identify inflammatory, hormonal, and metabolic factors that control CYP19A1 expression in breast cancer microenvironment. First, we found that CYP19A1 expression in mature 3T3-L1 adipocytes was increased by cocultivation with MCF-7 cells. Moreover, pS2, an ERα downstream gene, was increased in cocultivated MCF-7 cells. Next,

among cytokines that are abundant in cancer microenvironment, interferon-ɣ increased CYP19A1 protein level with 100 ng/ml for 24 h treatment in 3T3-L1 mouse preadipocytes. Also, ACTH upregulated CYP19A1 expression when cells were treated with 100 mM for 24 h. Among metabolites, saturated free fatty acids such as palmitic acids and stearic acids increased the protein level of CYP19A1, but unsaturated free fatty acids such as docosahexanoic acids and eicosapentanoic acids decreased expression of CYP19A1. Glucose also upregulated CYP19A1 expression when treated with a concentration of 20 mM for 48 h. Treatment with Interferon-ɣ (IFNɣ), TGF-β, free fatty acids, glucose, IGF-1 was accompanied with an increase in the protein level of p204, a mouse homologue of human IFI16. These findings may help to understand CYP19A1 regulation and estrogen signaling in breast cancer microenvironment.

keywords : CYP19A1, Cancer microenvironment, Estrogen signaling, p204, Breast cancer

Student Number : 2012-21590

CONTENTS

LIST of FIGURES

LIST of TABLES

Ⅰ. INTRODUCTION

Breast cancer is one of the most common cancer in women. In 2013, breast cancer was ranked as the second highest incidence rate and the fifth highest death for Korean female cancer (National cancer information center, Retrieved July, 2015 from http://www.cancer.go.kr). Approximately 75% of female breast cancer patients are estrogen receptor positive (Johnston and Dowsett., 2003). Estrogen receptors are activated by binding of their ligand, expecially estradiol. Activated estrogen receptors promote downstream gene transcription. As a result, breast cancer cell proliferation and metastasis are accelerated. Estrogen receptor α (ERα) -positive breast cancer patients are treated with endocrine therapy targeting estrogen signaling, such as selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs) (Ali et al., 3011). SERMs such as tamoxifen competitively bind to ER α and modulate their activity. AIs, like letrozol, directly inhibit estrogen biosynthesis in breast cancer microenvironment. But when breast cancer acquires resistance against endocrine therapy, cancer cell proliferation and metastasis are increased (Osborne and Schiff., 2011). Therefore, resistance to endocrine therapy is strongly linked to poor prognosis.

One of the critical factors of breast cancer progression and drug resistance is cancer microenvironment (Dittmer and Leyh., 2014).

Cancer microenvironment is the sum of complex interactions played by various types of cells, such as cancer cells, immune cells, adipocytes, fibroblasts, and so on (Figure 1; Ma et al., 2015). These cells release various secretory factors into the cancer microenvironment, including cytokines, hormones, and metabolites (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). First, cytokines regulated molecular signaling of cancer cells. They accelerate cell proliferation, inhibit apoptosis, and cause invasion and metastasis. In addition, they promotes extracellular matrix (ECM) remodeling. Angiogenesis and fibrosis are well-known phenomenon in breast cancer. As a result, modified ECM facilitates cancer cell metabolism, proliferation, and invasion. Next, endocrine hormones are also important factors in breast cancer microenvironment. Estrogen signaling is the most critical factor in breast cancer. Epinephrine is another important factor stimulating the MAPK pathway and STAT3 signaling (Cole et al., 2012). Insulin, IGF-1 and leptin are crucial adipose hormones influence on breast cancer (Calle and Kaaks., 2004; Ando and Catalano., 2012). They activate MAPK, PI3K, and STAT3 signaling in cancer cells and participate in regulation of ERα signaling. Finally, metabolites abundant in breast cancer microenvironment are related with diverse biological process. Nutrients, such as glucose and fatty acid, supply energy and building blocks for cancer metabolism and proliferation. This phenomenon is well-known as “Warburg effect”. Additionally, metabolites are associated with cellular signaling pathway. For

example, oxysterols, the product of cholesterol metabolism, are known as ligands of liver X receptors (LXRs) and estrogen receptors (ERs) (Nelson et al., 2013). Consequently, The microenvironmental factors in breast cancer provoke cancer proliferation, invasion, and drug resistance.

One of the most important player in breast cancer microenvironment is adipose tissue. Adipose tissue is not only just an energy storage structure, but also an important endocrine organ (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). Cancer cells stimulate adipose tissue by secreting inflammatory cytokines. Then stimulated adipose tissue supplies secretory factors including adipokines, hormones, and metabolites to cancer cells. Recently, transformed adipocytes called cancer-associated adipocytes (CAAs) were discovered in adipose tissue adjacent to cancer cells (Dirat et al., 2011; Bochet et al., 2013). CAAs are characterized as delipidated morphology, loss of molecular adipocyte markers and increased secretion of inflammatory cytokines. It was reported that increased cytokine production in CAAs promotes cancer invasion. But involvement of CAAs in ERα signaling has not beed studied, although adipose tissue is the major organ secreting estrogens.

Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (CYP19A1 or aromatase) is a key factor that links estrogen signaling to adipose tissue (Bulun et al., 2011; Simpson et al., 2002). CYP19A1 is the

rate-determining enzyme in estrogen biosynthesis, converts androgens to estrogens. CYP19A1 is upregulated in breast cancer tissue. CYP19A1 induction in tamoxifen-resistant breast cancer has also been reported (Phuong et al., 2014). Moreover, genetic polymorphisms of CYP19A1 are involved with breast cancer. For example, the tetranuleotide [TTTA] repeat polymorphism in intron 4 is significantly associated with breast cancer risk (Haiman et al., 2000; Baxter et al., 2001). Increased local estrogen activates estrogen signaling in breast cancer cells and promotes cancer progression and drug resistance (Figure 2; Yager and Davidson., 2006; Ma et al., 2015). Consequently, CYP19A1 is a crucial target for endocrine therapy in breast cancer (Johnston and Dowsett., 2003; Ali et al., 2011).

The tissue-specific promoters regulate CYP19A1 expression in human (Figure 3; Simpson et al., 2002; Dieudonne et al., 2006; Subbaramaiah et al., 2011; Bulun et al., 2012). PⅠ.1 is the most powerful promoter, but it is only activated in placenta. pⅡ is secondry potent promoter and activated in ovary. pⅡ promoter is strongly activated by FSH through cAMP signaling in female menstrual cycle (Sofi et al., 2003). Other cAMP signaling activators including PGE2 and forskolin also known as CYP19A1 upregulators (Subbaramaiah et al., 2008). In adipose tissue , pⅠ.4 is the major activated promoter region. pⅠ.4 is mainly activated in preadipocytes by signaling pathways including STAT3 and AP-1 (Zhao et al., 1996; Catalano et al., 2003; Zhao et al., 1995). Because placenta and ovary promoter is not activated in

postmenopausal phase, adipose tissue is the major organ expressing CYP19A1 in postmenopausal women. Especially in breast cancer, CYP19A1 expression is upregulated in adipose tissue adjacent to cancer (O'Neill and Miller., 1987; O'Neill et al., 1988; Bulun et el., 1994). Various cytokines and hormones regulate the expression of CYP19A1 through pⅠ.4 by STAT3 and MAPK signaling and pⅡ by cAMP signaling (Zhao et al, 1995; Zhao et al., 1996; Catalano et al., 2003; Sofi et al., 2003; To et al., 2014).

Previous reports showed that human interferon-ɣ inducible protein 16 (IFI16) is involved in estrogen receptor α (ERα) expression in breast cancer (Kang et al., 2014). Moreover, IFI16 increased CYP19A1 expression, estradiol secretion, and ERα target gene transcription in MCF-7 breast cancer cells (Figure 4; Kang, 2014). But involvement of IFI16 in the regulation of CYP19A1 in breast cancer microenvironment has not been studied.

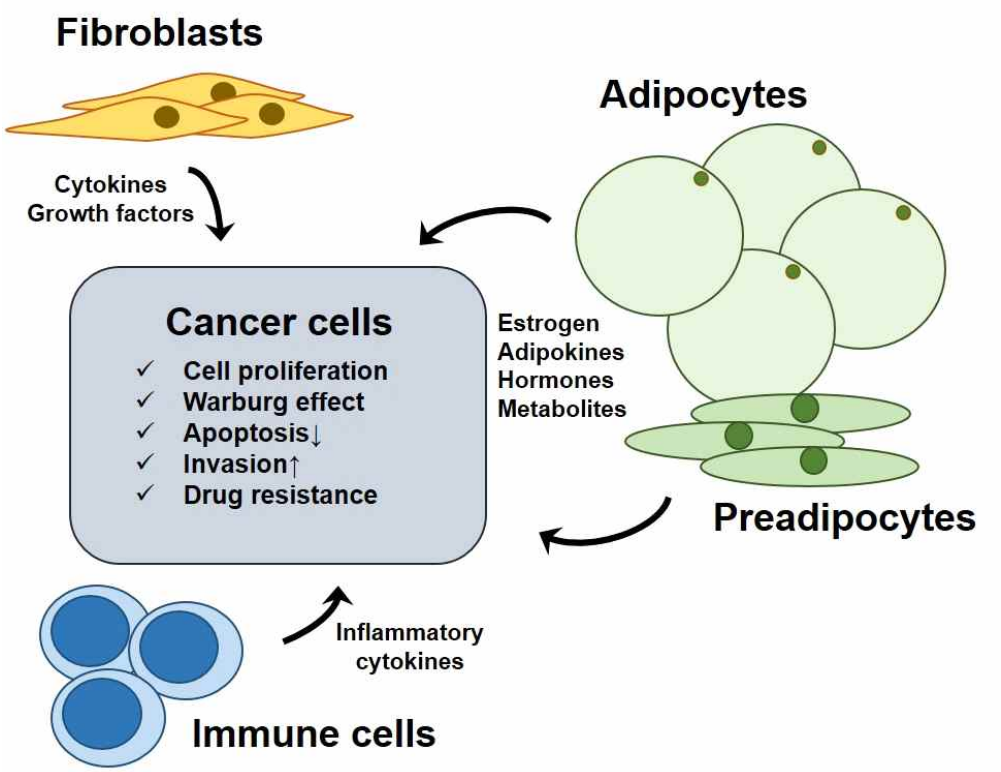

Figure 1. Microenvironmental factors secreted by stromal cells in breast cancer.

Like the 'seeds in soil', breast cancer cells are surrounded with cancer microenvironment composed of various cell types, such as cancer cells, immune cells, fibroblasts, and adipocytes. These stromal cells secrete diverse factors, including growth factors, cytokines, and metabolites. Growth factors and cytokines promote cancer proliferation and invasion. Metabolites supply energy source and building blocks to cancer cells, causing Warburg effect. As a result, the communication between cancer cells and stromal cells promotes cancer malignancy and drug resistance (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014; Ma et al., 2015).

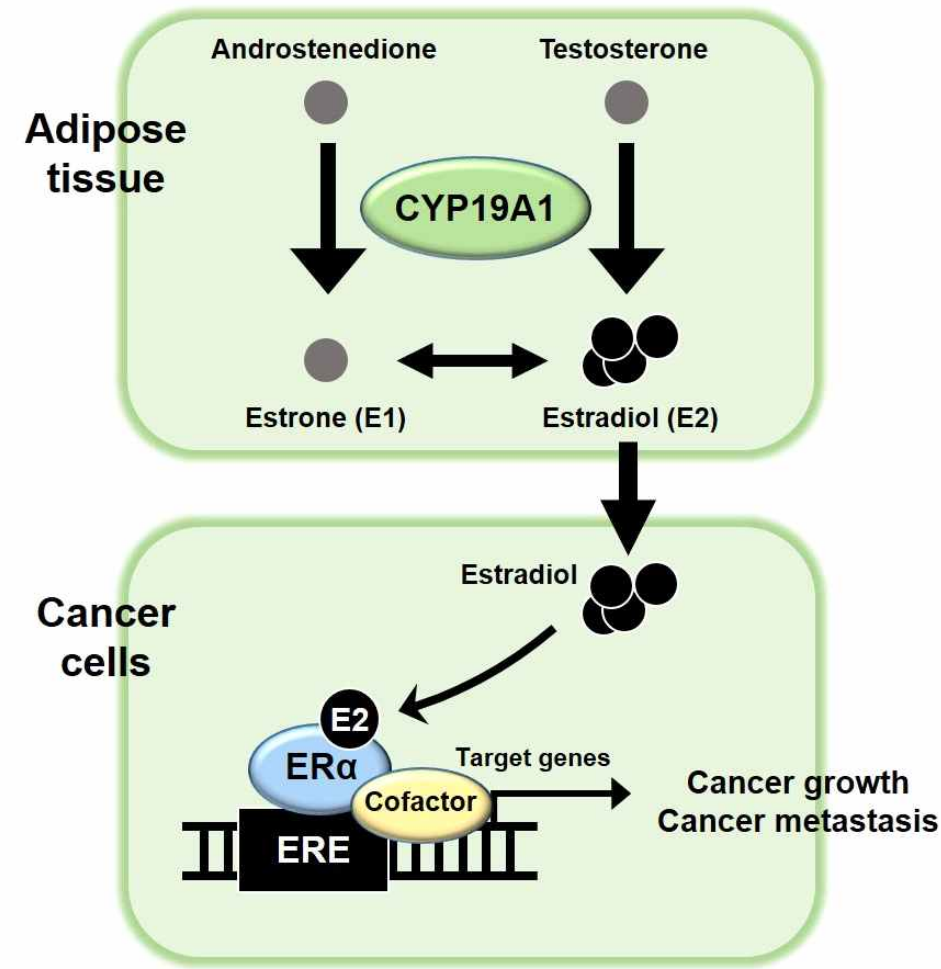

Figure 2. Estrogen biosynthesis and activation of ERα signaling in cancer microenvironment.

CYP19A1 expressed in adipose tissue (especially in preadipocytes) converts androgens to estrogens. Increased local estrogens bind to estrogen receptor α in the cytosol of cancer cells. Activated ERα causes the transcription of downstream genes such as c-Myc and cyclin D1. Consequently, CYP19A1 in adipose tissue promotes breast cancer growth and metastasis (Yager and Davidson., 2006; Ma et al., 2015).

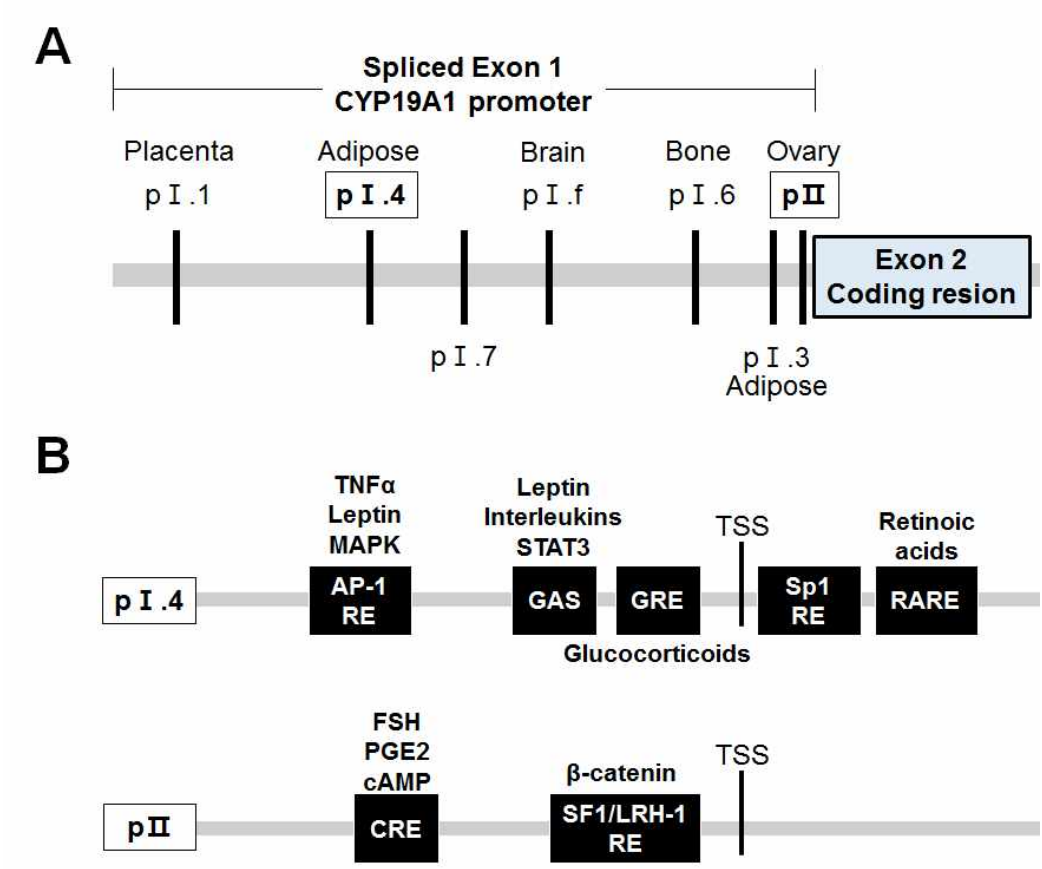

Figure 3. Regulation of CYP19A1 promoter.

CYP19A1 is regulated through tissue-specific promoters by alternative splicng (A). Especially promoter pⅠ.4 and pⅡ are activated in breast cancer microenvironment (B). Various cytokines regulate pⅠ.4 region through AP-1 RE and GAS. Endocrine hormones activate GRE in pⅠ.4 and CRE in pⅡ. RE : Response element, GAS : Interferon-ɣ activation sequence, GRE : Glucocorticoid RE, RARE : Retinoic acid receptor RE, CRE : cAMP RE, TSS : Transcription start site (Simpson et al., 1981; Zhao et al., 1995; Zhao et al., 1996; Simpson et al., 2002; Catalano et al., 2003; Sofi et al., 2003; Parakh et a., 2006; Subbaramaiah et al., 2008; Wilde et el., 2013; To et al., 2014.)

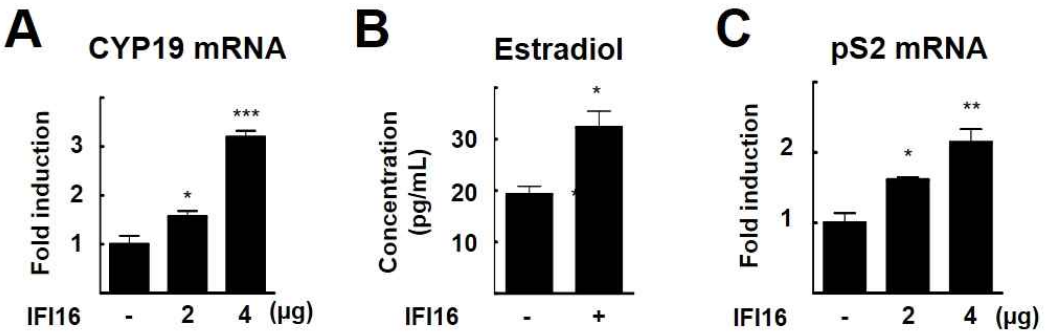

Figure 4. IFI16 upregulates CYP19A1 in breast cancer cells.

In MCF-7 breast cancer cells, transient transfection of IFI16 increased the transcription of CYP19A1. As a result, estradiol biosynthesis and secretion was increased. The transcription of pS2, an ERα downstream gene, was also increased (Kang, 2014).

Ⅱ. PURPOSE of the STUDY

Estrogen biosynthesis and ERα signaling are critical factors in breast cancer progression and drug resistance. Estrogen biosynthesis in microevironment is mediated by CYP19A1 aromatase, where it converts androgens to estrogens. In breast cancer microenvironment, CYP19A1 is mainly expressed in preadipocytes of adipose tissue. In breast cancer, CYP19A1 expression is increased, and elevated local estrogens promote breast cancer proliferation and metastasis. In this study, I aimed to investigate the regulation of CYP19A1 in breast cancer microenvironment. For the purpose, differentiated 3T3-L1 adipocytes were cocultivated with MCF-7 breast cancer cells, and alteration of CYP19A1 expression and ERα signaling was examined. Next, I examined to find regulators of CYP19A1 expression in breast cancer microenvironment. Various microenvironmental factors including cytokines, endocrine hormones, and metabolites were treated to 3T3-L1 mouse preadipocyte cells, and alteration of CYP19A1 expression was evaluated. On the other hand, upregulation of CYP19A1 expression by human IFI16 in breast cancer cells was previously reported. For this reason, I investigated the involvement of p204, a mouse homologue of human IFI16, in the regulation of CYP19A1.

Ⅲ. MATERIALS and METHODS

1. Cell culture and cell treatment

Mouse preadipocyte cell line, 3T3-L1 was a generous gift from professor Minsoo Noh. Human breast adenocarcinoma cell line, MCF-7 was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10% newborn calf serum (NBCS, Gibco 16010-159)) was supplemented for 3T3-L1 cells, and fetal bovine serum (FBS, Hyclone) was added to the medium for MCF-7 cells. Cells were cultured at 37°C in a 5% CO_2 / 95% air incubator.

Mouse Interferon-ɣ (315-05), mouse WNT3A (315-20), mouse IGF-1 (250-19) were purchased from Peprotech. Mouse TGF-β (7666-MB-005) was obtained from R&D systems. Dexamethasone (D4902), 3-isobutyl-1-methylxantine (I5879), human insulin (I9278), ACTH 1-24 fragment (A0298), Palmitic acid (P5585), Stearic acid (S4751), Docosahexanoic acid (D2534), Eicosapentanoic acid (E2011), D-(+)-Glucose (G5400) were purchased from Sigma Aldrich. For the treatment of free fatty acids, 1% fatty acid-free bovine serum albumin (A6003) was dissolved in medium with free fatty acids.

2. Adipocytes differentiation and MCF-7 coculture

For differentiation, 3T3-L1 preadipocytes were seeded at 1 X 10^5

cells/well into 6 well plates. At postconfluent two days, cells were stimulated by MDI (1 μM Dexamethasone, 0.5 mM IBMX, 10 μg/ml human Insulin) in DMEM supplemented with 10% FBS, penicillin and streptomycin. Two days later, MDI was replaced with 10 μg/ml human Insulin. After another two days, medium was changed to DMEM containing 10% FBS, penicillin and streptomycin, which was replaced every two days (Neal and Clipstone., 2002). After 6 days, MCF-7 cells were seeded at 4 X 10^4 cells/well into the upper insert of Corning Transwell system (CLS3450). Media was changed every 3 days.

3. Oil-red O staining

Cells were washed with PBS and fixed by 10% formaldehyde for 15 minutes. Oil-red O (Sigma aldrich, O0625) stock solution (0.5% in isopropanol) was diluted to 3:2 with distilled water and filtered before use. Samples were stained with diluted Oil-red O working solution for one hours and rinsed with distilled water (Wang et al., 2015).

4. Western blotting

Cells were lysed in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 1% NP-40, and protease inhibitors cocktail (Roche) for 30 min on ice, and whole cell lysates were obtained by subsequent centrifugation. The protein concentration was quantified by bicinchoninic acid assay (Pierce). Protein from whole cell lysates were subjected onto sodium dodecylsulfate-polyacrylamide

gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. Blocking was performed in 5% (w/v) non-fat-dried milk in phosphate-buffered saline containing 0.1% Tween-20. The membrane was then incubated with specific antibodies against CYP19A1 (sc-14245), FABP4 (SC-18661), ERα (sc-543), pS2 (SC-22501), p204 (SC-13367) from Santa Cruz Biotechnology, and α-tubulin (calbiochem).

5. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR

Total RNA was prepared using EASY-BLUE^{TM} Total RNA Extraction Kit (iNtRON Biotechnology) according to the manufacturer's instructions. cDNA was synthesized from total RNA in a reaction mixture containing random hexamer (Invitrogen) and MMLV-Reverse transcriptase (Invitrogen). Quantitative real time PCR was performed using SYBR Green PCR mix (Applied Bioscience). The data were normalized to β-actin reference. The sequences of the primers are described in Table 1.

6. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was determined by student's t-test. Differences were considered as statistically significant when p-value was < 0.05.

Table 1. Primer sequences used for real-time PCR analysis

Gene		Primer sequences	Ref.
CYP19A1	Forward	5′-ATG TTC TTG GAA ATG CTG AAC CC-3′	-
	Reverse	5′-AGG ACC TGG TAT TGA AGA CGA G-3′	
p204	Forward	5′-TGG TCC CAA ACA AGT GAT GGT GC-3′	Unterholzner et al., 2010
	Reverse	5′-TCA GTT TCA GTA GCC ACG GTA GCA-3′	
β-actin	Forward	5′-CGT GGG CCG CCC TAG GCA CCA-3′	Kang et al., 2014
	Reverse	5′-TTG GCT TAG GGT TCA GGG GGG-3′	

Ⅳ. RESULTS

1. Alteration of CYP19A1 expression and ERα signaling in cocultivated MCF-7 cells and 3T3-L1 adipocytes.

In order to mimic cancer-associated adipocytes (CAAs) *in vitro*, MCF-7 breast cancer cells and mature 3T3-L1 adipocytes were cocultivated using Transwell coculture system (Figure 5). Lipid accumulation and adipocyte marker FABP4 expression were decreased in cocultivated adipocytes. These alterations demonstrate the transformation of adipocytes, similar to delipidated and de-differentiated phenotype of CAAs (Dirat et al., 2011). In contrast, lipid accumulation and FABP4 expession were increased in cocultivated MCF-7 cells, indicating that lipid was transferred to cancer cells from adipocytes (Nieman et al., 2011). Intriguingly, CYP19A1 expression was upregulated in cocultivated adipocytes (Figure 6). Moreover, expression of pS2, an ERα downstream gene, was induced in cocultivated MCF-7 cells. Furthermore, the protein level of ERα was decreased in cocultivated MCF-7 cells, implying that binding of estradiol triggered the degradation of ERα. These results suggest that upregulated CYP19A1 in cocultivated adipocytes promoted the release of estrogen and activated ERα signaling in cocultivated MCF-7 cells (Figure 13A).

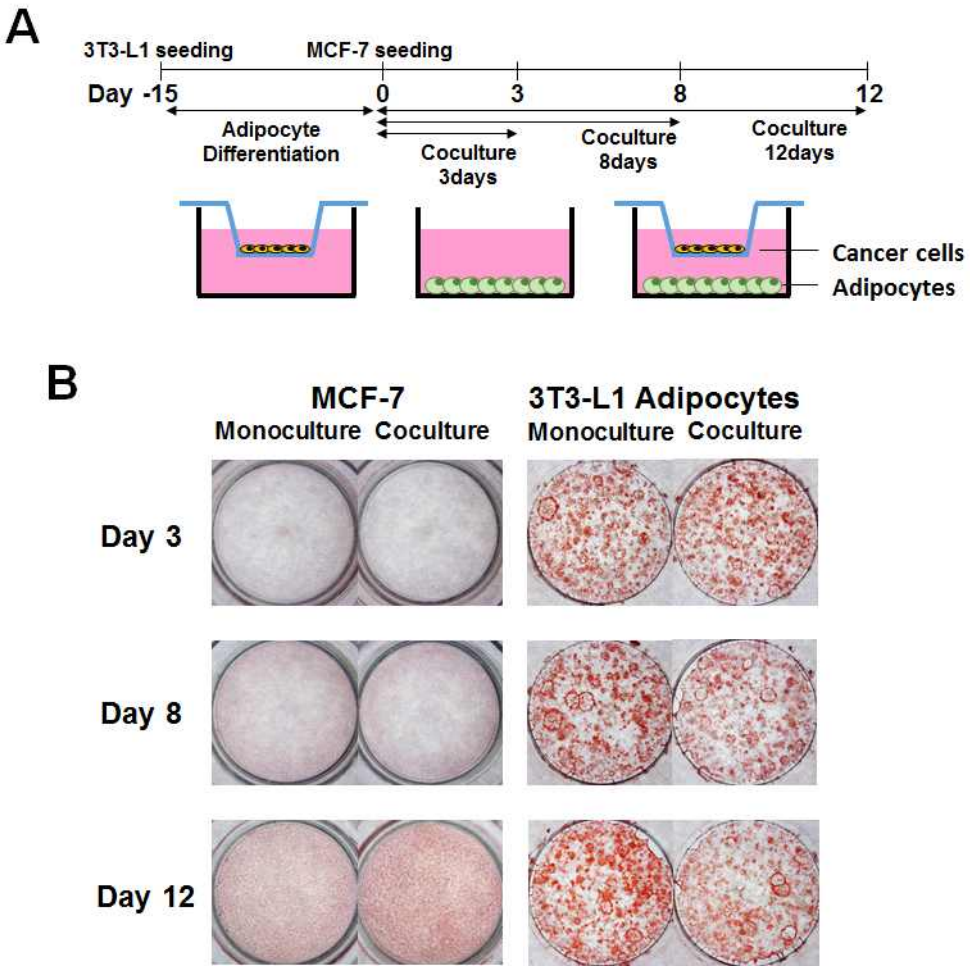

Figure 5. Transwell coculture of MCF-7 cells and 3T3-L1 adipocytes.

MCF-7 cancer cells and 3T3-L1 adipocytes were cocultivated using Transwell system. (A) Schematic illustration of coculture experiment. 3T3-L1 cells were differentiated for 15 days, and cocultivated with MCF-7 cells for 12 days. (B) Lipid accumulation of cocultivated cells was analyzed by Oil-red O staining.

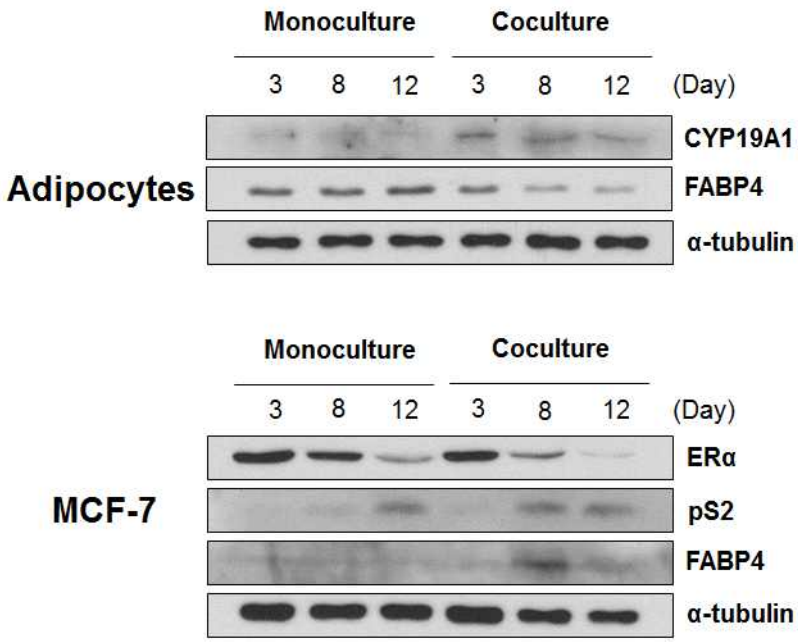

Figure 6. Alteration of CYP19A1 expression and ERα signaling in cocultivated MCF-7 cells and 3T3-L1 adipocytes.

Alteration of the protein expression of cocultivated MCF-7 cancer cells (upper panel) and 3T3-L1 adipocytes (bottom panel). Whole cell lysates were analyzed by western blotting.

2. Breast cancer microenvironmental cytokines increase CYP19A1 expression in 3T3-L1 preadipocytes.

Next, I aimed to investigate factors that cause alteration of CYP19A1 expression in breast cancer microenvironment. Various inflammatory cytokines are known to regulate CYP19A1 expression (To et al., 2014). In order to find cytokines involved in the regulation of CYP19A1, the effects of inflammatory cytokines and adipokines abundant in breast cancer microenvironment were examined. Among inflammatory cytokines, IFNɣ and TGF-β upregulated CYP19A1 protein and mRNA expression in 3T3-L1 preadipocytes, which express a high level of CYP19A1. For adipokines, WNT3A elevated CYP19A1 expression in preadipocytes (Figure 7).

3. Endocrine hormones regulate CYP19A1 expression.

Endocrine hormones, including FSH, leptin, and glucocorticoids, are also known to be crucial CYP19A1 regulators (To et al., 2014). Moreover, insulin signaling is activated in adipose tissue, and has a role in breast cancer development (Calle and Kaaks., 2004). Two important hormones in insulin signaling, insulin and IGF-1, increased CYP19A1 expression in 3T3-L1 preadipocytes. In addition, adrenocorticotropic hormone (ACTH) elevated the protein level of CYP19A1 in preadipocytes (Figure 8).

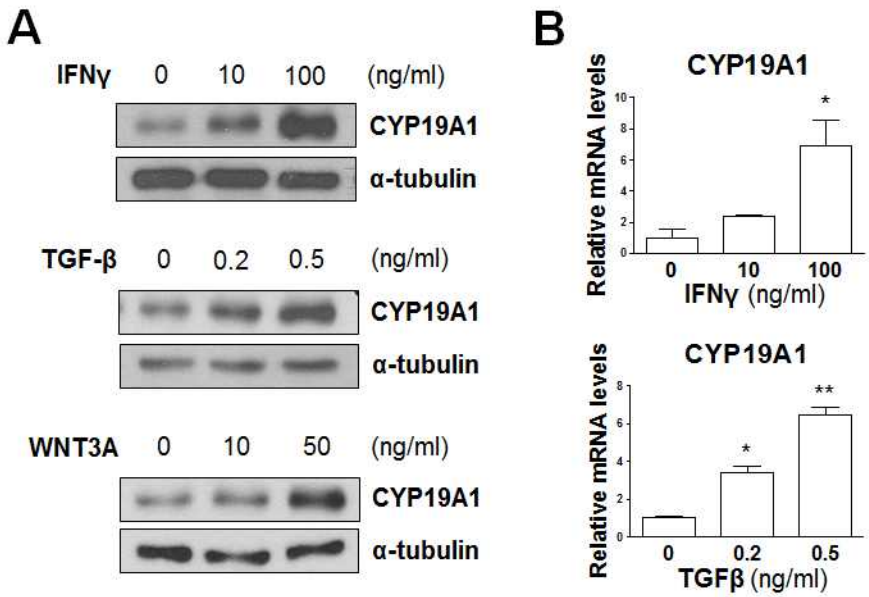

Figure 7. Breast cancer microenvironmental cytokines increase CYP19A1 expression in 3T3-L1 preadipocytes.

(A) Effect of microenvironmental cytokines on protein expression of CYP19A1 in 3T3-L1 preadipocytes. Recombinant mouse IFNɣ, mouse TGF-β, mouse WNT3A were treated to 3T3-L1 preadipocytes for 24 h, and CYP19A1 protein level was analyzed by western blotting. (B) Alteration of CYP19A1 mRNA level by treatment of IFNɣ and TGF-β. Relative mRNA levels were measured by real-time PCR, and normalized with β-actin. The values represent the means ± SEM of experiments in duplicates. * $P < 0.05$, ** $P < 0.01$ *vs.* vehicle.

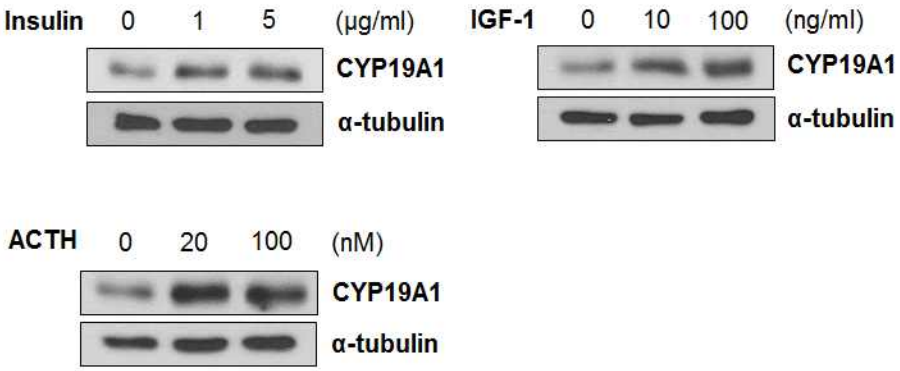

Figure 8. Endocrine hormones regulate CYP19A1 expression.

Human insulin (24 h), mouse IGF-1 (48 h), and ACTH 1-24 fragment (24 h) were treated to 3T3-L1 preadipocytes for indicated doses. The protein expression of CYP19A1 and α-tubulin was analyzed by western blotting.

4. Regulation of CYP19A1 by microenvironmental metabolites.

In adipose tissue, metabolites including free fatty acids and carbohydrates are secreted into the microenvironment. These metabolites are important building block and energy source for breast cancer cells. Interestingly, saturated free fatty acids upregulated CYP19A1 expression, whereas ω-3 polyunsaturated fatty acids were downregulated CYP19A1 (Figure 9A, B). Moreover, treatment of EPA restored IFNɣ- or stearic acid-induced expression of CYP19A1 (Figure 12). On the other hand, glucose increased the protein expression of CYP19A1 in 3T3-L1 preadipocytes (Figure 9C).

5. Microenvironmental CYP19A1 regulators are accompanied by alteration of p204 expression.

According to previous reports, IFI16, the transcriptional factor associated with ERα regulation in breast cancer, increased CYP19A1 expression in MCF-7 breast cancer cells. In the case of mouse, p204 is known as homologue of human IFI16. Intriguingly, CYP19A1 regulation by microenvironmental factors was accompanied with alteration of p204 expression. Among upregulators of CYP19A1, IFNɣ, TGF-β, WNT3A, IGF-1, saturated fatty acids, and glucose increased p204 expression (Figure 10, 11A, 11C, 12). On the contrary, ω-3 polyunsaturated fatty acids, which downregulated CYP19A1, decreased p204 protein level (Figure 11B, 12). These results imply the involvement of p204 in the regulation of CYP19A1 (Figure 13B).

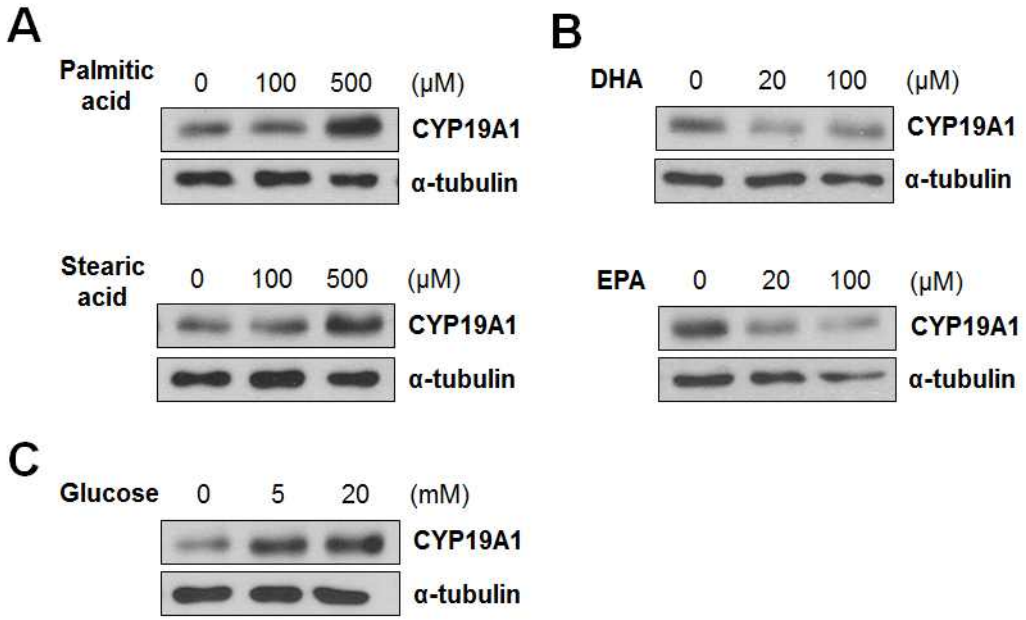

Figure 9. Regulation of CYP19A1 by microenvironmental metabolites.

Effect of Free fatty acids and glucose on CYP19A1 expression in 3T3-L1 preadipocytes. Saturated fatty acids (A) or ω-3 polyunsaturated fatty acids (B) were treated for 24 h. (C) D-(+)-glucose was treated to 3T3-L1 preadipocytes for 48 h. Whole cell lysates were harvested and protein level was measured by western blotting.

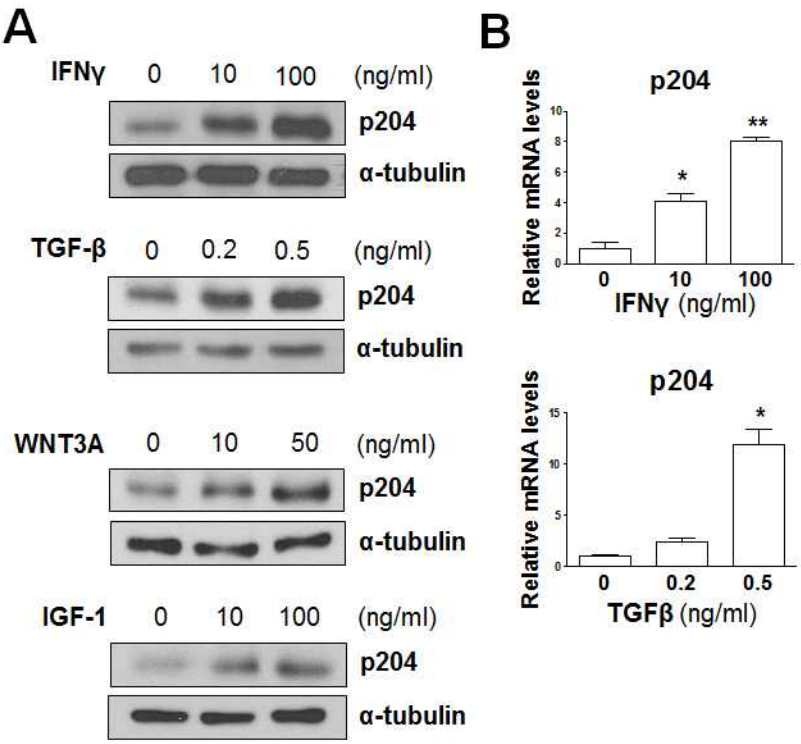

Figure 10. Cytokines and hormones which upregulate CYP19A1 also increase p204 expression.

(A) Effect of microenvironmental cytokines and hormones on protein expression of p204 in preadipocytes. Mouse IFNɣ, mouse TGF-β, mouse WNT3A and mouse IGF-1 were treated to 3T3-L1 preadipocytes, and protein level was analyzed by western blotting. (B) Increase of p204 mRNA by IFNɣ and TGF-β. The mRNA level was measured by real-time PCR and normalized with β-actin. Data represent the means ± SEM of experiments in duplicates. * $P < 0.05$, ** $P < 0.01$ *vs.* vehicle.

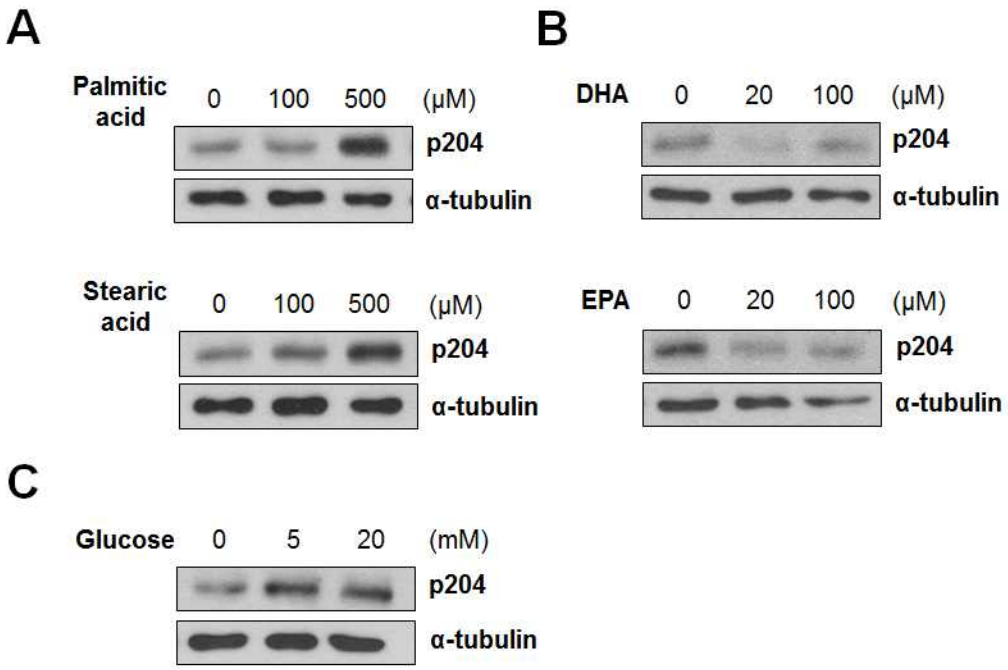

Figure 11. Metabolic CYP19A1 regulators accompany alteration of p204 expression.

Effects of saturated fatty acids (A), ω-3 polyunsaturated fatty acids (B), and glucose (C) on expression of p204 in 3T3-L1 preadipocytes. Whole cell lysates were harvested and protein level was analyzed by western blotting.

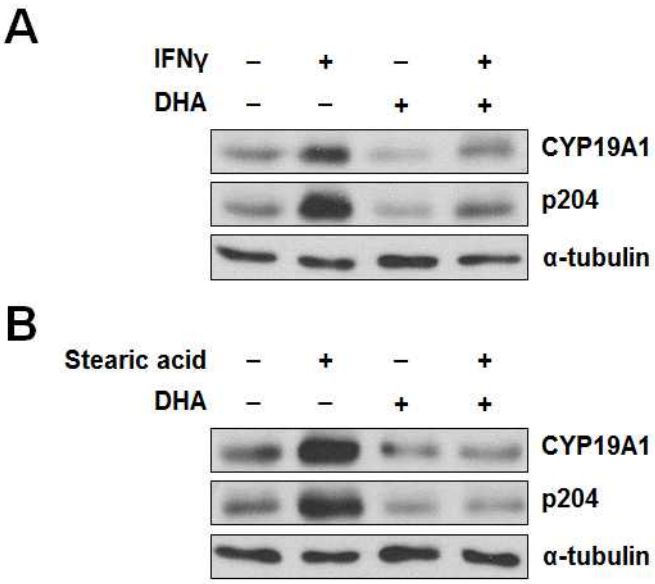

Figure 12. EPA restores IFNɣ- or stearic acid-induced expression of CYP19A1 and p204.

Mouse IFNɣ (A, 100 ng/ml) or stearic acid (B, 500 μM) was co-treated with EPA (100 μM) in 3T3-L1 mouse preadipocytes. After 24 h treatment, whole cell lysates were harvested and protein expression of CYP19A1, p204, and α-tubulin was analyzed by western blotting.

Table 2. Regulation of CYP19A1 expression by breast cancer microenvironmental factors in 3T3-L1 preadipocytes.

Secretory factors	Secreting sites	Previous observations			Expression in this study	
		References	Experimental Models	CYP19A1 Expression	CYP19A1	p204
Cytokines						
IFNɣ	Immune cells	Lai et al., 2014	Granulosa cells	Increase	Increase	Increase
TGF-β	Cancer, Immune cells				Increase	Increase
WNT3A	Cancer cells				Increase	Increase
Adiponectin	Adipose	Ledoux et al., 2006	Follicular cells	Decrease	-	-
CCL2	Adipose, Immune cells				-	-
IL-4	Immune cells				-	-
Resistin	Adipose				-	-
Hormones						
IGF-1	Liver	Sharma et al., 2012	Granulosa cells	Increase	Increase	Increase
Insulin	Pancrea	Chaves et al., 2012	Follicular cells	Increase	Increase	-
ACTH	Hyppocampus				Increase	-
Cortisol	Adrenal cortex	Wang et al., 2012	Placenta cells	Increase	Decrease	-
Epinephrine	Adrenal medula, Nerve				-	-
Metabolites						
Palmitic acid	Adipose				Increase	Increase
Stearic acid	Adipose				Increase	Increase
Oleic acid	Adipose				-	-
DHA	Adipose				Decrease	Decrease
EPA	Adipose				Decrease	Decrease
Glucose	Adipose, Blood				Increase	Increase
Lactic acid	Cancer cells				-	-
27-HC	Liver				-	-
22-S-HC	Liver				-	-
22-R-HC	Liver				-	-
25-HC	Liver				-	-

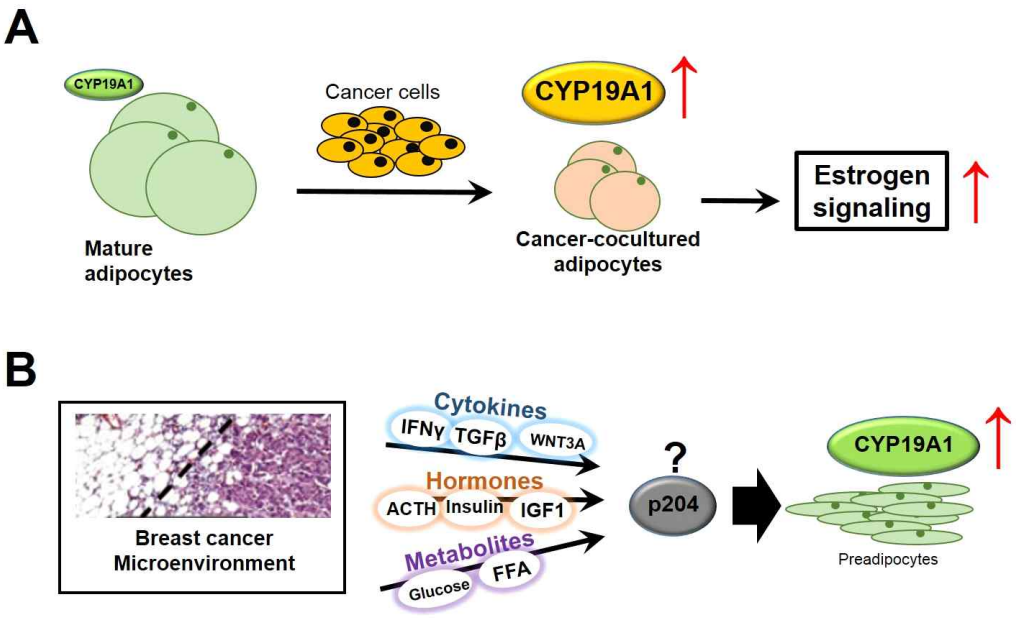

Figure 13. Schematic illustration of CYP19A1 regulation by breast cancer microenvironmental factors.

(A) Alteration of CYP19A1 expression in adipocytes cocultivated with cancer cells. CYP19A1 expression was increased in cocultivated adipocytes, and ERα signaling was activated in cocultivated cancer cells. (B) CYP19A1 regulation by breast cancer microenvironmental factors in preadipocytes. Various cytokines, hormones, metabolites abundant in breast cancer microenvironment regulated CYP19A1 expression in preadipocytes.

V. DISCUSSION

CYP19A1 is the rate-determining enzyme of estrogen biosynthesis. CYP19A1 converts androgens to estrogens, which bind to estrogen receptors and activate the transcription of ERα downstream (Figue 2; Simpson et al., 2002; Johnston and Dowsett., 2003; Ma et al., 2015). CYP19A1 is upregulated in breast cancer, and increased local estrogen contributes to cancer proliferation and metastasis (Yue et al., 1998). For this reason, CYP19A1 is a crucial target for postmenopausal breast cancer endocrine therapy.

CYP19A1 expression is regulated by tissue-specific promoter regulation (Figure 3; To et al., 2014). In breast cancer microenvironment, CYP19A1 is mainly expressed in adipose tissue, especially in preadipocytes. Recently, adipose tissue is considered as an important endocrine organ, contributing to cancer malignancy by the release of adipokines and endocrine hormones (Park et al., 2011; Gilbert and Slingerland., 2013; Esquivel-Velazquez et al., 2014). Moreover, transformed adipocytes in breast cancer microenvironment, known as cancer-associated adipocytes (CAAs), have been reported (Dirat et al., 2011; Bochet et al., 2013). CAAs show decreased lipid accumulation, loss of molecular adipocyte markers, and increased secretion of inflammatory cytokines, facilitating the invasion of cancer cells.

Because CYP19A1 expression is decreased during adipocyte differentiation and the phenotype change of CAAs is similar with de-differentiation of adipocytes, I examined the alteration of CYP19A1 expression in CAAs. In order to mimic CAAs *in vitro*, transwell coculture of MCF-7 breast cancer cells with 3T3-L1 mature adipocytes was conducted (Figure 5). As a result, lipid accumulation and FABP4, the lipid carrier protein recognized as representative adipocyte marker, were increased in MCF-7 cells and decreased in adipocytes (Figure 6). This indicates that cocultivated adipocytes were transformed like CAAs (Dirat et al., 2011). On the other hand, lipid transferred from adipocytes to cancer cells provides energy and building blocks for cancer proliferation (Nieman et al., 2011). Interestingly, CYP19A1 expression was increased in cocultivated adipocytes. Moreover, transcription of ERα downstream gene pS2 was increased, and ERα protein level was decreased in cocultivated MCF-7 cells. These results indicate that the increased release of estrogen from cocultivated adipocytes activated ERα signaling, triggering the degradation of ligand-bound ERα and downstream gene transcription.

Next, I aimed to find factors that mediate CYP19A1 regulation in cancer microenvironment. In breast cancer, inflammatory cytokines are typically increased, and contribute to cancer malignancy. In addition, various hormones and metabolites secreted by adipocytes provide fuels for cancer metabolism and building blocks for proliferation. I explored the effects of these breast cancer microenvironmental factors

on CYP19A1 expression in preadipocytes, the main population expressing CYP19A1 in adipose tissue. Consequently, various cytokines, hormones, and metabolites abundant in breast cancer microenvironment regulated CYP19A1 in 3T3-L1 preadipocytes (Figure 7, 8, 9). These results suggest a new type of treatment against CYP19A1, which is different with classical aromatase inhibitor. Modulating microenvironmental cytokines or insulin signaling could be developed as endocrine therapy for breast cancer. In addition, ω-3 polyunsaturated fatty acids have the possibility for mild oral aromatase inhibitor, similar with reports on dietary polyphenols (Subbaramaiah et al., 2013).

Furthermore, I discovered that many CYP19A1 regulators also altered expression of p204, the mouse homologue of IFI16 (Figure 10, 11, 12). Previous reports described that IFI16 is associated with regulation of ERα and CYP19A1 expression in ERα-positive breast cancer(Kang et al., 2014; Kang, 2014). Moreover, IFNɣ, one of the CYP19A1 upregulator, is the most potent upstream of IFI16. IFNɣ has been considered to be a tumor suppressor, because IFNɣ inhibits tumor growth and eliminates cancer cells through immune response. However, pro-tumor functions of IFNɣ were reported recently (Xiao et al., 2009; Zaidi et al., 2011). A downstream of IFNɣ, IFI16, also has a dual role in cancer. IFI16 regulates cell cycle and cell senescence, and it has been recognized as a tumor suppressor (Choubey et al., 2008). But according to previous reports, IFI16 increases ERα and CYP19A1 expression and accelerates cancer

proliferation (Kang et al., 2014; Kang, 2014)). As shown in this study, p204 was involved in CYP19A1 regulation by various cancer microenvironmental factors, implying the oncogenic function of p204 in breast cancer. For the following study, the mechanism of CYP19A1 regulation by microenvironmental factors and the role of p204 in CYP19A1 regulation need to be studied.

REFERENCE

Andò S, Catalano S. The multifactorial role of leptin in driving the breast cancer microenvironment. *Nat Rev Endocrinol*. 2011;**8**(5):263-75.

Ali S, Buluwela L, Coombes RC. Antiestrogens and their therapeutic applications in breast cancer and other diseases. *Annu Rev Med*. 2011;**62**:217-32.

Baxter SW, Choong DY, Eccles DM, Campbell IG. Polymorphic variation in CYP19 and the risk of breast cancer. *Carcinogenesis*. 2001;**22**(2):347-9.

Bochet L, Lehuédé C, Dauvillier S, Wang YY, Dirat B, Laurent V, Dray C, Guiet R, Maridonneau-Parini I, Le Gonidec S, Couderc B, Escourrou G, Valet P, Muller C. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res*. 2013;**73**(18):5657-68.

Bulun SE, Mahendroo MS, Simpson ER. Aromatase gene expression in adipose tissue: relationship to breast cancer. *J Steroid Biochem Mol Biol*. 1994;**49**(4-6):319-26.

Bulun SE, Chen D, Moy I, Brooks DC, Zhao H. Aromatase, breast cancer and obesity: a complex interaction. *Trends Endocrinol*

Metab. 2012;**23**(2):83-9.

Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer*. 2004 **4**(8):579-91.

Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Ando S. Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem*. 2003;**278**(31):28668-76

Catalano S, Marsico S, Giordano C, Mauro L, Rizza P, Panno ML, Ando S. Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem*. 2003;**278**(31):28668-76

Chaves RN, Duarte AB, Rodrigues GQ, Celestino JJ, Silva GM, Lopes CA, Almeida AP, Donato MA, Peixoto CA, Moura AA, Lobo CH, Locatelli Y, Mermillod P, Campello CC, Figueiredo JR. The effects of insulin and follicle-stimulating hormone (FSH) during in vitro development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. *Biol Reprod*. 2012;**87**(3):69.

Choubey D, Deka R, Ho SM. Interferon-inducible IFI16 protein in human cancers and autoimmune diseases. *Front Biosci*. 2008;**13**:598-608.

Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. *Clin Cancer Res*. 2012;**18**(5):1201-6

Dieudonné MN, Sammari A, Dos Santos E, Leneveu MC, Giudicelli Y, Pecquery R. Sex steroids and leptin regulate 11beta-hydroxysteroid dehydrogenase I and P450 aromatase expressions in human preadipocytes: Sex specificities. *J Steroid Biochem Mol Biol*. 2006;**99**(4-5):189-96.

Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res*. 2011;**71**(7):2455-65.

Dittmer J, Leyh B. The impact of tumor stroma on drug response in breast cancer. *Semin Cancer Biol*. 2015;**31**:3-15.

Esquivel-Velázquez M, Ostoa-Saloma P, Palacios-Arreola MI, Nava-Castro KE, Castro JI, Morales-Montor J. The role of cytokines in breast cancer development and progression. *J Interferon Cytokine Res*. 2015;**35**(1):1-16.

Gilbert CA, Slingerland JM. Cytokines, obesity, and cancer: new insights on mechanisms linking obesity to cancer risk and progression. *Annu Rev Med*. 2013;**64**:45-57.

Haiman CA, Hankinson SE, Spiegelman D, De Vivo I, Colditz GA, Willett WC, Speizer FE, Hunter DJ. A tetranucleotide repeat polymorphism in CYP19 and breast cancer risk. *Int J Cancer*. 2000;**87**(2):204-10.

Johnston SR, Dowsett M. Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nat Rev Cancer*. 2003;**3**(11):821-31.

Kang HJ, Lee MH, Kang HL, Kim SH, Ahn JR, Na H, Na TY, Kim YN, Seong JK, Lee MO. Differential regulation of estrogen receptor α expression in breast cancer cells by metastasis-associated protein 1. *Cancer Res*. 2014;**74**(5):1484-94.

Kang HL. The role of interferon, gamma-inducible protein 16 in the estrogen signaling during breast cancer progression [Unpublished Masters Dissertation]. *Seoul-si, South Korea: Seoul National University*, 2014.

Lai WA, Yeh YT, Fang WL, Wu LS, Harada N, Wang PH, Ke FC, Lee WL, Hwang JJ. Calcineurin and CRTC2 mediate FSH and TGFβ1 upregulation of Cyp19a1 and Nr5a in ovary granulosa cells. *J Mol Endocrinol*. 2014;**53**(2):259-70

Ledoux S, Campos DB, Lopes FL, Dobias-Goff M, Palin MF, Murphy BD. Adiponectin induces periovulatory changes in ovarian follicular cells. *Endocrinology*. 2006;**147**(11):5178-86

Ma CX, Reinert T, Chmielewska I, Ellis MJ. Mechanisms of aromatase inhibitor resistance. *Nat Rev Cancer*. 2015;**15**(5):261-75.

Neal JW, Clipstone NA. Calcineurin mediates the calcium-dependent inhibition of adipocyte differentiation in 3T3-L1 cells. *J Biol Chem*. 2002;**277**(51):49776-81.

Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK, Carver NJ, Pillai RV, Sullivan PM, Sondhi V, Umetani M, Geradts J, McDonnell DP. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. *Science*. 2013;**342**(6162):1094-8.

Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med*. 2011;**17**(11):1498-503.

O'Neill JS, Miller WR. Aromatase activity in breast adipose tissue from women with benign and malignant breast diseases. *Br J Cancer*. 1987;**56**(5):601-4.

O'Neill JS, Elton RA, Miller WR. Aromatase activity in adipose tissue from breast quadrants: a link with tumour site. *Br Med J*. 1988;**296**(6624):741-3.

Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med*. 2011;**62**:233-47.

Parakh TN, Hernandez JA, Grammer JC, Weck J, Hunzicker-Dunn M, Zeleznik AJ, Nilson JH. Follicle-stimulating hormone/cAMP regulation of aromatase gene expression requires beta-catenin. *Proc Natl Acad Sci U S A*. 2006;**103**(33):12435-40

Park J, Euhus DM, Scherer PE. Paracrine and endocrine effects of adipose tissue on cancer development and progression. *Endocr Rev.* 2011;**32**(4):550-70.

Phuong NT, Lim SC, Kim YM, Kang KW. Aromatase induction in tamoxifen-resistant breast cancer: Role of phosphoinositide 3-kinase-dependent CREB activation. *Cancer Lett.* 2014;**351**(1):91-9.

Sharma I, Singh D. Conjugated linoleic acids attenuate FSH- and IGF1-stimulated cell proliferation; IGF1, GATA4, and aromatase expression; and estradiol-17β production in buffalo granulosa cells involving PPARɣ, PTEN, and PI3K/Akt. *Reproduction.* 2012;**144**(3):373-83

Simpson ER, Ackerman GE, Smith ME, Mendelson CR. Estrogen formation in stromal cells of adipose tissue of women: induction by glucocorticosteroids. *Proc Natl Acad Sci U S A.* 1981;**78**(9):5690-4

Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, Speed C, Jones M. Aromatase--a brief overview. *Annu Rev Physiol.* 2002;**64**:93-127.

Sofi M, Young MJ, Papamakarios T, Simpson ER, Clyne CD. Role of CRE-binding protein (CREB) in aromatase expression in breast adipose. *Breast Cancer Res Treat.* 2003;**79**(3):399-407

Subbaramaiah K, Hudis C, Chang SH, Hla T, Dannenberg AJ. EP2 and EP4 receptors regulate aromatase expression in human adipocytes and breast cancer cells. Evidence of a BRCA1 and p300 exchange. *J Biol Chem*. 2008;**283**(6):3433-44

Subbaramaiah K, Hudis CA, Dannenberg AJ. The prostaglandin transporter regulates adipogenesis and aromatase transcription. *Cancer Prev Res*. 2011;**4**(2):194-206.

Subbaramaiah K, Sue E, Bhardwaj P, Du B, Hudis CA, Giri D, Kopelovich L, Zhou XK, Dannenberg AJ. Dietary polyphenols suppress elevated levels of proinflammatory mediators and aromatase in the mammary gland of obese mice. *Cancer Prev Res*. 2013;**6**(9):886-97.

To SQ, Knower KC, Cheung V, Simpson ER, Clyne CD. Transcriptional control of local estrogen formation by aromatase in the breast. *J Steroid Biochem Mol Biol*. 2015;**145**:179-86.

Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol*. 2010;**11**(11):997-1004.

Wang W, Li J, Ge Y, Li W, Shu Q, Guan H, Yang K, Myatt L, Sun K. Cortisol induces aromatase expression in human placental syncytiotrophoblasts through the cAMP/Sp1 pathway. *Endocrinology*. 2012;**153**(4):2012-22

Wang C, Gao C, Meng K, Qiao H, Wang Y. Human adipocytes stimulate invasion of breast cancer MCF-7 cells by secreting IGFBP-2. *PLoS One*. 2015;**10**(3):e0119348

Wilde J, Erdmann M, Mertens M, Eiselt G, Schmidt M. Aromatase activity induction in human adipose fibroblasts by retinoic acids via retinoic acid receptor α. *J Mol Endocrinol*. 2013;**51**(2):247-60

Xiao M, Wang C, Zhang J, Li Z, Zhao X, Qin Z. IFNgamma promotes papilloma development by up-regulating Th17-associated inflammation. *Cancer Res*. 2009;**69**(5):2010-7.

Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. *N Engl J Med*. 2006;**354**(3):270-82.

Yue W, Wang JP, Hamilton CJ, Demers LM, Santen RJ. In situ aromatization enhances breast tumor estradiol levels and cellular proliferation. *Cancer Res*. 1998;**58**(5):927-32.

Zaidi M, Merlino G. The two faces of interferon-ɣ in cancer. *Clin Cancer Res*. 2011;**17**(19):6118-24.

Zhao Y, Nichols JE, Bulun SE, Mendelson CR, Simpson ER. Aromatase P450 gene expression in human adipose tissue. Role of a Jak/STAT pathway in regulation of the adipose-specific promoter. *J Biol Chem*. 1995;**270**(27):16449-57

Zhao Y, Nichols JE, Valdez R, Mendelson CR, Simpson ER. Tumor necrosis factor-alpha stimulates aromatase gene expression in

human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter 1.4. *Mol Endocrinol*. 1996;**10**(11):1350-7

국 문 초 록

CYP19A1 방향화효소는 에스트로젠 생합성의 율속단계를 촉매하는 중요한 효소이다. CYP19A1은 유방암과 인접한 지방조직에서 발현이 증가하여 종양미세환경의 에스트로젠 농도가 국소적으로 높아지고, 암세포의 에스트로젠 신호전달계가 활성화되어 종양의 성장과 전이를 촉진한다. 그러므로 CYP19A1 방향화효소는 유방암 환자의 내분비 치료에서 중요한 약물 타겟이다. 본 연구에서는 첫째, 유방암 세포주와 공동배양한 지방세포에서의 CYP19A1 발현과 그로 인한 암세포의 에스트로젠 신호전달계 활성화를 규명함과 함께 둘째, 유방암 종양미세환경에 풍부한 사이토카인, 호르몬, 대사물질 중 신규 CYP19A1 조절자를 발굴함을 목표로 하였다. 우선 3T3-L1 지방아세포주를 지방세포로 분화시킨 후 MCF-7 유방암 세포주와 공동배양했을 때, 공동배양한 지방세포에서 Oil-red O 염색을 통해 지방 함량이 줄어들고 지방세포 표지자 FABP4의 발현이 줄어드는 등 형질 변화가 나타남을 확인하였다. 이 때 지방세포에서 CYP19A1의 발현이 증가한 것을 발견하였고, 공동배양한 MCF-7 세포주에서 에스트로젠 신호전달계의 타겟 유전자 pS2의 발현이 증가함을 관찰하였다. 다음으로 3T3-L1 쥐 지방아세포주에 유방암 종양미세환경 인자들을 처리했을 때 사이토카인 중 인터페론-γ와 TGF-β, WNT3A가, 호르몬 중에서는 인슐린과 IGF-1, 그리고 부신피질자극호르몬 (ACTH)이 CYP19A1 발현을 증가시켰다. 또한 대사물질 중 포화지방산인 팔미트산과 스테아르산이 CYP19A1 발현 증가를 일으킨 반면에 오메가-3 불포화지방산인 도코사헥사엔산 (DHA)와 아이코사펜타엔산 (EPA)은 감소시키는 것을 발견하였다.

포도당 또한 CYP19A1 발현을 증가시켰다. 흥미롭게도, 본 연구진은 신규 CYP19A1 발현 조절자 중 인터페론-γ, WNT3A, 팔미트산, 스테아르산, DHA, EPA, 포도당, 그리고 기존에 보고된 CYP19A1 증가 조절자 TGF-β, IGF-1의 처리가 사람 인터페론-γ 유도 단백질 16 (IFI16)의 쥐 상동단백질 p204의 발현 변화를 동반하는 것을 발견하였다. 본 연구는 유방암세포와 지방세포의 상호작용으로 인한 에스트로젠 신호전달계 조절 가능성을 제시하였고, CYP19A1 방향화효소의 신규 조절자를 발굴함으로써 유방암 종양미세환경에서의 에스트로젠 신호전달계의 이해에 기여할 것이다.

주요어: CYP19A1, 종양미세환경, 에스트로젠 신호전달, p204, 유방암