Metabolic characteristics in the breast cancer according to the subtype of cancer-associated fibroblast

Ji Hee Lee

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Ja Seung Koo

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This certifies that the Doctoral Dissertation
of Ji Hee Lee is approved.

Thesis Supervisor : Ja Seung Koo

Thesis Committee Member#1 : Min Jung Kim

Thesis Committee Member#2 : Eunah Shin

Thesis Committee Member#3 : Yoon Jin Cha

Thesis Committee Member#4 : Sangkyu Park

The Graduate School
Yonsei University

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ABSTRACT

Metabolic characteristics in the breast cancer according to the subtype of cancer-associated fibroblast

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In the breast cancer which characteristically produces tumor stroma, reverse Warburg effect is suggested as its tumor metabolism. It is the theory that the metabolic interaction between tumor cells and stromal cells plays an important role in survival and growth of tumor. Cancer-associated fibroblast (CAF), the stromal cell which plays a key role in the theory, is the most important component of tumor microenvironment (TME) and it is recently classified into several subtypes (FAP, S100A4, PDGFRα, and PDGFRβ), each of which has separate functional characteristics. In this study, we aimed to investigate differences in metabolic interaction between tumor cells and stromal cells depending on subtypes of CAF and investigate impacts of tumor stroma-targeted material on tumorigenesis and growth of tumor depending on subtypes of CAF and finally recognize the possibility of tumor stroma-targeted material as a targeted therapy of breast cancer.

We produced four cancer-associated fibroblast (CAF) subtype cell lines, which were stably expressing each CAF marker (FAP, S100A4, PDGFRα, and

PDGFRβ) and analyzed differences in metabolic interaction of each CAF subtype with breast cancer molecular subtypes. We also examined migration assay and invasion assay to investigate the effect of each CAF subtype on metastasis and invasion ability of cancer cells. Then, we produced a tumor xenograft model using BALB/C nude mice to confirm cell line studies.

Among four CAF subtypes, we identified that $FB^{-PDGFR\beta}$ activated glycolysis, mitophagy, and autophagy of MDA-MB-231 and FB^{-FAP} activated glycolysis and autophagy of triple negative breast cancer (TNBC) cell lines. FB^{-FAP} especially played an important role in cancer behavior of TNBC cells; MDA-MB-231 and MDA-MB-468. Also, in mice xenograft model, it showed that FB^{-FAP} activated glycolysis and autophagy metabolism of TNBC cell lines. In inhibition study with knocked down fibroblasts, FB-siPDGFRβ and FB-siFAP cells, the tumor metabolism and its behavior of MDA-MB-231, MDA-MB-468, and mice xenograft models was consistent with the results from studies of TNBC cell lines and mice xenograft models co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP}.

In conclusion, CAF has heterogeneous characteristic in that each breast cancer molecular subtype shows differences in the expression of metabolic related markers and different cancer behavior depending on co-cultured CAF subtype. Among CAF subtypes, FB^{-FAP} seems to promote tumor formation and growth through glycolysis and autophagy of TNBC and we suggest it has a potential role as a therapeutic target for TNBC.

Key words: breast cancer, molecular subtype, triple-negative, tumor cell, cancer-associated fibroblast, tumor metabolism, Warburg effect, reverse Warburg effect, targeted therapy

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I. INTRODUCTION

Most malignant tumor metabolism are explained by Warburg effect theory[1], the metabolic shift from oxidative phosphorylation of mitochondria to glycolysis, focusing tumor cells. However in the breast cancer, one of the tumors with various types of tumor stroma, reverse Warburg effect has been suggested as its tumor metabolism. We can predict that there would be an interaction between breast tumor and various tumor stroma, and the interaction also in the tumor metabolism.

Precedent researches have suggested that breast cancer cells interact with stromal cells in terms of tumor metabolism. Reverse Warburg effect[2-5], or battery-operated tumor growth describes that breast cancer cells produce reactive oxygen species (ROS) such as nitric oxide (NO) to place oxidative stress on stromal cells, so that hypoxia-inducible factor 1-alpha (HIF-1α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) leads to glycolysis, autophagy, and mitochondrial dysfunction in stromal cells. Ketone bodies and lactates produced

by glycolysis in stromal cells enter cancer cells and efficiently produce adenosine triphosphates (ATPs) through oxidative phosphorylation in the mitochondria of cancer cells, which as a whole contributes to their survival and growth of cancer cells. The theory suggests cancer-associated fibroblasts (CAFs) with loss of expression of caveolin-1 as stromal cells which interact with tumor cells in the breast cancer. The loss of expression of caveolin-1 occurs by increased proteolysis of caveolin-1 by enhanced autophagy[2,4,6,7].

CAF[8], which is the most important component of tumor microenvironment (TME), is located around tumor cells and related to tumorigenesis, tumor inflammatory reaction, metabolism, metastasis, drug response, and etc[9]. Though the definition of CAF is controversial[8,9], various markers of CAF such as alpha-smooth muscle actin[10], tenascin-C[11], chondroitin sulfate proteoglycan (NG2)[12], platelet-derived growth factor receptor (PDGFR) α/β[13], fibroblast activation protein (FAP)[14], podoplanin[15], prolyl 4-hydroxylase[16], and fibroblast-specific protein (FSP) 1[12] have been suggested. CAF can be classified into several functional subtypes such as FAP type, FSP1 type, PDGFRα type, PDGFRβ type and each of which has own different characteristics[17].

Although CAF has several functional subtypes according to marker expression, preceding researches about CAF overlooked the heterogeneity of CAF and only referred to the loss of caveolin-1 expression as a marker of CAF. Actually the loss of caveolin-1 expression showed associations with early recurrence of tumor, lymph node metastasis, increase in tumor stage, and low survival rate[18-20] as a marker in several researches using human breast tissue. Those researches also showed that cases with loss of caveolin-1 expression in stromal cells of breast cancer accounted for about 5-40% of total breast cancer cases[18-20]. We need to identify other markers of CAF because only some of breast cancer showed loss of caveolin-1 expression in their stromal cells.

Reverse Warburg effect is an attractive theory which explains that an interaction

between tumor cells and TME is important in survival and growth of tumor. However CAF consists of several functional subtypes with heterogeneous characteristics, we need to identify differences in mechanisms of metabolism and activities depending on CAF subtypes. Moreover, even though a lot of researches about CAF with human breast cancer tissue have done, researches about subtypes of CAF and their characteristics lack.

Nowadays, researchers are trying new breast cancer drugs targeting abundant tumor stroma and drugs targeting various tumor stroma are on study[21]. To make these drugs to function properly, a study about markers of CAF in breast tumor stroma is essential.

In this study, we aimed to investigate differences in the expression of markers related to glycolysis, mitochondrial function, and autophagy in tumor cells and stromal cells depending on subtypes of CAF and investigate differences in metabolic interaction between tumor cells and stromal cells depending on subtypes of CAF and investigate impacts of tumor stroma-targeted material on tumorigenesis and growth of tumor depending on subtypes of CAF and finally investigate the possibility of tumor stroma-targeted material as a targeted therapy of breast cancer.

II. MATERIALS AND METHODS

1. Cell lines and cell culture

Human breast cancer cell lines (MCF-7, MDA-MB-453, MDA-MB-231, MDA-MB-468) and a human normal fibroblast cell line (BJ-5ta) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 was cultured in DMEM/F-12, no phenol (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) (Gibco™, 21041025) supplemented with 10% fetal bovine serum (FBS) and 10μg/ml of insulin. MDA-MB-453 and MDA-MB-468 were cultured in DMEM (HyClone™, SH30243.01) supplemented with 20% FBS and 1% penicillin/streptomycin. MDA-MB-231 was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. BJ-5ta was cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 100μg/ml of hygromycin B. All cell lines were incubated in a humidified CO_2 incubator at 37°C.

Table 1. General information of used breast cancer cell lines

	MCF-7	**MDA-MB-453**	**MDA-MB-231**	**MDA-MB-468**
Organism	Homo sapiens, human	Homo sapiens, human	Homo sapiens, human	Homo sapiens, human
Tissue	Mammary gland, breast; derived from metastatic site: pleural effusion	Mammary gland/breast; derived from metastatic site: pericardial effusion	Mammary gland/breast; derived from metastatic site: pleural effusion	Mammary gland/breast; derived from metastatic site: pleural effusion
Disease	AdenoCA	Metastatic CA	AdenoCA	AdenoCA
Cell type	Epithelial		Epithelial	
Gender	Female	Female	Female	Female
Ethnicity	Caucasian	Caucasian	Caucasian	Black
Molecular subtype	Luminal	HER-2	Claudin- low	Molecular apocrine

Abbreviation: CA, carcinoma

2. Co-culture of normal fibroblasts with breast cancer cell lines

Normal fibroblast, BJ-5ta, was co-cultured with breast cancer cell lines at the ratio of 1:2 for 48 hr using transwell inserts with 0.4μm porous membrane (Costar, 3419). Each breast cancer cell type was grown independently. To reduce the effect of osmotic pressure, all of the cell lines were cultured with 10% FBS in each growth medium.

3. Western blotting

Cells were harvested by trypsinization, washed with PBS, and frozen overnight at -70°C. To thaw the cells, cells were at 4°C for 30 min and lysed in RIPA buffer (Biosesang, R2002) with 1% protease phosphatase inhibitor cocktail (Thermo Scientific, 1861281), and 1% phenylmethylsulfonyl fluoride (Thermo Scientific, 36978). Debris was sedimented by centrifugation for 15 min at 15 000 × g, and the protein concentration of the supernatant was determined by BCA assay (Thermo Scientific 23228 and 23224). Lysates in volumes of fixed protein weights were boiled at 95°C for 5 min, with 5X SDS-PAGE loading buffer (Biosesang, S2002). Equal quantities of protein were separated to SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad 1704158).

Membranes were blocked by incubation in 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween-20 and probed with antibody against PDGFRα (1:1000, GeneTex, GTX107903), PDGFRβ(1:1000, GeneTex, GTX107063), FAP(1:1000, GeneTex, GTX102732), S100A4(1:1000, Abcam, ab197896), Glut1(1:1000, bioworld, BS1149), CAIX(1μg/ml, Abcam, ab15086), BNIP3(1:2000, bioworld, BS70685), p62(1:2000, Abcam, ab56416), LC3B (1:800, Abcam, ab63817), diluted in 1% BSA in TBS, 0.1% Tween 20, and 0.02% NaN_3. The membranes were washed and then incubated with secondary

antibodies (HRP conjugated anti-mouse IgG, or anti-rabbit IgG) (1:20000, Santa Cruz) for 1 hr at room temperature. The bands were visualized using WesternBright ECL (advansata, K-12045-D50) after washing the membrane and exposed to x-ray film

4. Quantitative real time PCR

Total RNA was extracted from cell pellets using the RNeasy Plus Mini kit (Qiagen, 74134). One Step SYBR PrimeScript RT-PCR kit (TaKaRa, RR066A) was used for detecting mRNA expression of CAF related proteins (PDGFRα, PDGFRβ, FAP, and S100A4) from 100ng of total RNA. Quantitative PCR was run on a StepOne Plus Real time system (Applied Biosystems, 4376357). PCR amplification was performed under the following conditions: 95°C for 10 min, followed by 40 cycles at 94°C for 5 sec, 60°C for 33 sec. The primers used are shown below: PDGFRα forward, 5′-GAATCCGCCAGTTACAGGAA-3’; PDGFRα reverse, 5′-CAGCAGTTCCACCTTCATCA-3’; PDGFRβ forward, 5′-GCTCACACTGACCAACCTCA-3’; PDGFRβ reverse, 5′-GTGGGATCTGGCACAAAGAT-3’; FAP forward, 5’-GGCTCACGTGGGTTACTGAT-3’; FAP reverse, 5’-TCCTGGGTCTTTGGACAATC-3’; S100A4 forward, 5’-GAGCAACTTGGACAGCAACA-3’; S100A4 reverse, 5’-CTTCCTGGGCTGCTTATCTG-3’; GAPDH forward, 5′-GGCCTCCAAGGAGTAAGACC-3’; GAPDH reverse, 5′-AAGGGAGATTCAGTGTGGTG-3’. Data were analyzed using StepOne software and are shown as relative fold change. For all PCR reactions, GAPDH was used as an endogenous control and CT values were normalized to levels of GAPDH expression. Results are averaged from three independent experiments.

5. Cell migration and invasion assay

BJ-5ta cells ($2X10^4$ cells/well) were seeded on the 24 well plate, 24hr before

the assay. And breast cancer cell lines were starved in serum free growth media for 16hr before assay to stop the cell cycles. The invasion and migration assay were performed using transwell inserts with 8μm porous membrane with (CORNING, 354480) or without (Costar, 3422) Matrigel, respectively. Each transwell inserts were installed on top of the 24 well plate where BJ-5ta cells were incubated. In case of the transwell with Matrigel, the inserts were incubated in DMEM with 10% FBS at 37°C for 1hr before installed. Breast cancer cell lines ($5X10^4$ cells/well) were seeded onto the inserts and incubated at 37°C for determined hours. The incubation hours for each breast cancer cell type and assay are shown below: MCF-7 migration 24hr, MCF-7 invasion 48hr; MDA-MB-453 migration 48hr, MDA-MB-453 invasion 48hr; MDA-MB-231 migration 8hr, MDA-MB-231 invasion 24hr; MDA-MB-468 migration 24hr, MDA-MB-468 invasion 24hr. To reduce the effect from concentration differences, all of the cell lines were cultured with 10% FBS in each growth medium. After certain hours of incubation, cancer cells were fixed in 5% glutaraldehyde for 30 min. Then, inserts were stained using crystal violet, and washed with ddH_2O. The non-invading cells were gently removed with a soft cotton swab, and inserts were dried overnight. The invasive cells on the bottom were visualized under a microscope and quantified by counting the number of cells in 5 randomly chosen fields at 200X magnification.

6. Generation of caner-associated fibroblast subtype cell lines

Each CAF-related protein (PDGFRα PDGFRβ, FAP, S100A4), which is stably expressed, was transfected into normal fibroblast cell line (BJ-5ta) to generate each subtype of CAF.

The constructs were cloned and purchased from COSMO GENETECH company (COSMO GENETECH; Seoul, Korea). Every 4 insert was cloned into pEGFP-C1 vector (CLONTECH, 6084-1), which has CMV promoter and

encodes GFP at N-terminal of the insert. The final constructs were confirmed by Sanger sequencing. Each 4 construct was transfected into BJ-5ta cells by jetPRIME (Polyplus, 114-15). Empty vector (pEGFP-C1) was also transfected as a control. Stable cells were generated after being selected with gentamicin (Gibco, 15750-060). Fluorescence microscope and immunoblotting with polyclonal antibodies against GFP validated overexpression of CAF-related proteins in each CAF subtype cell line.

7. siRNA knock down of cancer-associated fibroblast-related proteins

Short interfering RNAs (siRNA) against PDGFRα, PDGFRβ, FAP, and S100A4 were synthesized and purchased from GenePharma Company (GenePharma; Shanghai, China). The siRNA sequences for each gene and their negative control are shown below: PDGFRα, 5'-UAUAAUGGCAGAAUCAUCAUCA-3'; PDGFRβ, 5'-UUGACGGCCACUUUCAUCG-3'; FAP, 5'-UAUUGGCACCUUUCUUUCC-3'; S100A4, 5'-UUUAUUGAACUUGCUCAGC-3'; Negative control, 5'-UUCUCCGAACGUGUCACGUTT-3'. BJ-5ta cells ($4.5X10^5$ cells/dish) were plated 24hr before transfection and each siRNA was transfected into the cells using jetPRIME (Polyplus, 114-15) in 70nM according to manufacturer's instructions. Cells were then collected after 48hr for further experiments. Silencing was confirmed by qRT-PCR as well as western blotting.

To analyze interactions between CAF-related gene knocked down fibroblasts and breast cancer cell lines, co-culture was performed 4hr after siRNA transfection. And cells were then collected after 48hr.

8. Subcutaneous tumor xenograft model and *in vivo* analysis

This study was carried out in strict accordance with the recommendations in

the Guide for the Care and Use of Laboratory Animals under Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine (Seoul, Korea) which obtain certification by AAALAC International.

$3X10^6$ cells of breast cancer cell lines, which were co-cultured with fibroblasts for 48hr, were harvested by trypsinization, resuspended with 250μl of cold PBS, then same amount of Matrigel (Corning, 356231) was added. Each cell was independently injected subcutaneously into the flank of 6-week-old female nude mice. Tumors were harvested after 6 weeks inoculation and weighed. Then a portion of each tumor was fixed with 10% formalin and embedded in paraffin for immunohistochemistry analysis. The rest of the tumor was frozen on liquid nitrogen for western blot analysis.

9. Immunohistochemistry (IHC)

The antibodies used for IHC of metabolism-related proteins in mouse tumor xenograft are shown in Table 2. IHC was performed with formalin-fixed, paraffin-embedded (FFPE) tissue sections. Briefly, 5-μm-thick sections were obtained with a microtome, transferred onto adhesive slides, and dried at 62°C for 30 min. After incubation with primary antibodies, immunodetection was performed with biotinylated antimouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3′-diaminobenzidine chromogen as the substrate. The primary antibody incubation step was omitted in the negative control. Positive control tissue was used according to the manufacturer’s recommendation.

Table 2. Source, clone, and dilution of used antibodies

Antibody	Clone	Dilution	Company
Glut-1	SPM498	1:200	Abcam, Cambridge, UK
CAIX	Polyclonal	1:500	Abcam, Cambridge, UK
MCT-4	Polyclonal	1:100	Santa Cruz, California, USA
BNIP3	Ana40	1:100	Abcam, Cambridge, UK
LC3A	EP1528Y	1:100	Abcam, Cambridge, UK
LC3B	Polyclonal	1:100	Abcam, Cambridge, UK
p62	SQSTM1	1:100	Abcam, Cambridge, UK

10. Interpretation of IHC staining

All IHC markers were assessed by using light microscopy. IHC staining results for Glut-1, CAIX, MCT-4, BNIP3, LC3A, LC3B, and p62 were determined by multiplication of proportion of stained cells and immunostaining intensity. The proportion of stained cells was scored as 0 for negative, 1 for positive with less than 30% of the cells stained, and 2 for positive with greater than or equal to 30% of the cells stained. The immunostaining intensity was scored as 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. The multiplication result of 0 and 1 was regarded as negative and the result between 2 and 6 as positive.

III. RESULTS

1. Differential expression of cancer-associated fibroblast (CAF) markers in fibroblasts co-cultured with breast cancer cell lines

To investigate the effect of breast cancer on fibroblast, BJ-5ta which is normal fibroblast cell line was co-cultured with each breast cancer cell line in the ratio of 1:2 for 48hr. Each subset of breast cancer cell line was MCF-7 (Luminal type), MDA-MB-453 (HER-2 type), MDA-MB-231 (Claudin-low type), and MDA-MB-468 (Molecular apocrine type). After 48hr later, expression of PDGFRα, PDGFRβ, FAP, and S100A4, which are cancer-associated fibroblast (CAF) markers was analyzed by western blotting from the lysate of BJ-5ta. As a result, all of CAF markers were upregulated in co-cultured fibroblasts, compared to the ones in fibroblast which were cultured alone (Figure 1).

To observe these results in more detail, we compared the expressions according to the markers and subsets of breast cancer. In case of PDGFRβ, expression was highly increased in the group which was co-cultured with MDA-MB-231 and MDA-MB-468. This shows that PDGFRβ is significantly increased when fibroblasts are co-cultured with triple-negative breast cancer (TNBC) cell line. In case of FAP, expression was greatly increased in fibroblasts co-cultured with MDA-MB-453 and MDA-MB-231. Taken together, these results suggest that the breast cancer cell affects adjacent fibroblasts and, as a result, normal fibroblasts are transdifferentiated into cancer associated fibroblasts (CAF). Also, the increased markers in fibroblasts were different depending on the subset of breast cancer, which means the fibroblasts are not the one kind, but have different characteristics which could be divided into several molecular subtypes. Even though you can see data of MDA-MB-435S in figures, we won't refer them because it has turned out that MDA-MB-435S is not a female cell line and is not a suitable model for breast carcinoma[22].

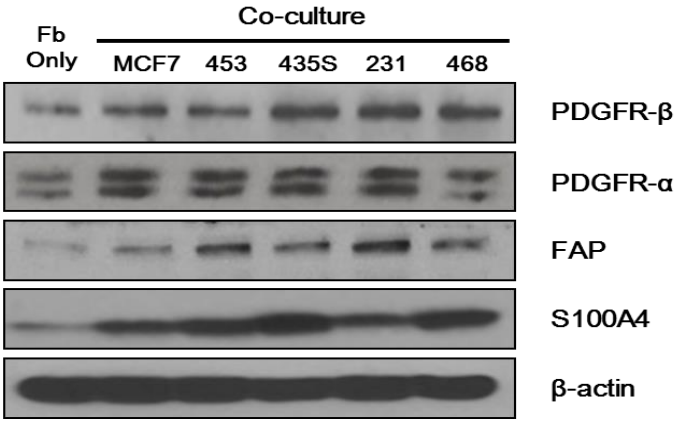

Figure 1. Differential expression of CAF markers in BJ-5ta fibroblasts co-cultured with breast cancer cell lines. Western blotting with the indicated antibodies was carried out using BJ-5ta fibroblasts extracts. All of CAF-related proteins are upregulated in co-cultured fibroblasts, compared to the ones in fibroblast which were cultured alone.

2. Generation of CAF subtype cell lines stably expressing each CAF marker

To analyze differences of metabolism in breast cancer according to adjacent CAF molecular subtypes, each subtype of CAF was generated. The gene of each CAF marker (PDGFRα, PDGFRβ, FAP, and S100A4) was cloned into pEGFP-C1 vector (CLONTECH, 6084-1), and these constructs were transfected into BJ-5ta cell lines so that each CAF marker could be stably expressed in the fibroblast. Fluorescence microscope and immunoblotting with polyclonal antibodies against GFP validated overexpression of CAF-related proteins in each CAF subtype cell line (Figure 2). After confirmation of CAF marker overexpression, we named each cell line as FB^{-GFP} (control), $FB^{-PDGFR\alpha}$, $FB^{-PDGFR\beta}$, FB^{-FAP}, and $FB^{-S100A4}$. And this made our study proceed forward about functional characteristics of each CAF subtype.

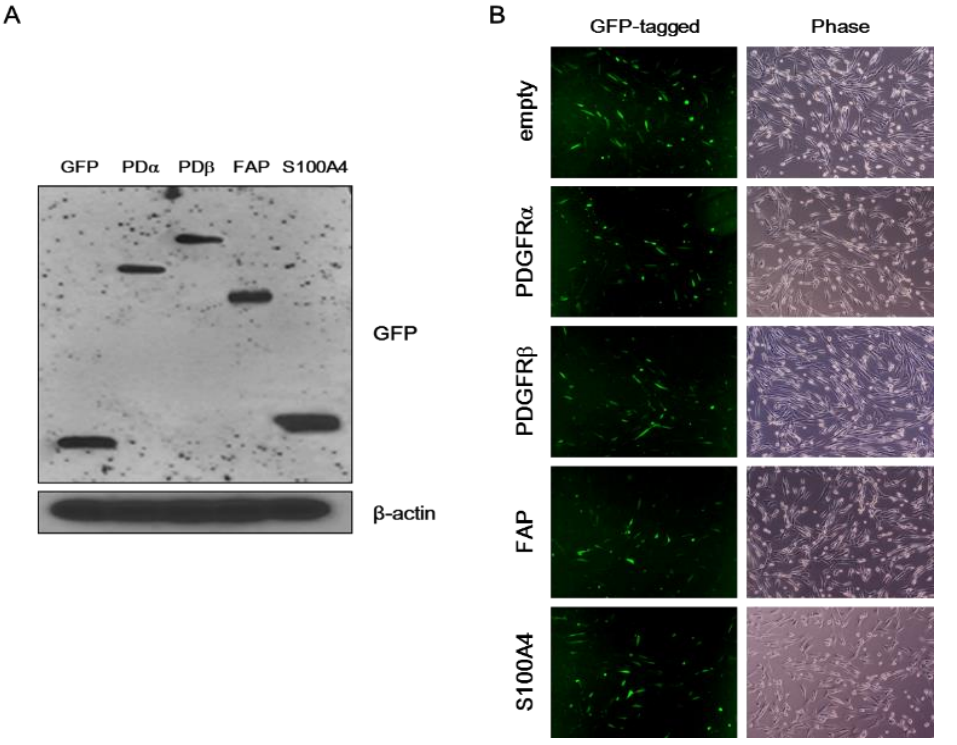

Figure 2. BJ-5ta fibroblasts overexpressing CAF markers. BJ-5ta fibroblasts were transfected with GFP empty vector (control) or GFP-tagged CAF-related protein constructs using Lipofectamine 2000. Stable expression of each construct was examined by immunoblotting (A), and fluorescence imaging (B).

3. Metabolism in breast cancer cell lines when co-cultured with different subtypes of CAF

Breast cancer cell lines were co-cultured with each subtype of CAF ($FB^{-PDGFR\alpha}$, $FB^{-PDGFR\beta}$, FB^{-FAP}, $FB^{-S100A4}$), and the metabolism in breast cancer from the effect of CAF was analyzed. In case of breast cancer cell lines co-cultured with $FB^{-PDGFR\beta}$, MDA-MB-231 showed increase in expressions of glycolysis markers (Glut1, CAIX), mitophagy marker (BNIP3), and autophagy marker (LC3II) (Figure 3). Thus, $FB^{-PDGFR\beta}$ makes MDA-MB-231 activate glycolysis,

mitophagy, and autophagy. Also, LC3II was increased in MDA-MB-468 cell line (Figure 3). This suggests that FB$^{-PDGFR\beta}$ activates autophagy of TNBC. And then, in breast cancers co-cultured with FB^{-FAP}, Glut1 and CAIX were high in MDA-MB-231 and MDA-MB-468, which are TNBC cell lines (Figure 4). The expression of BNIP3, on the other hand, was reduced in those cell lines (Figure 4). Also, LC3II was increased in every breast cancer cell line (Figure 4). The decrease of p62, which indicates autophagy activation, was shown in both MDA-MB-231 and MDA-MB-468 (Figure 4). In short, FB^{-FAP} induces TNBC cell lines to activate glycolysis and autophagy. The rest of 2 subtypes of CAF, FB$^{-PDGFR\alpha}$ and FB$^{-S100A4}$, didn't show any differences in breast cancer cell lines. Consequently, each subtype of CAF induces metabolism in breast cancer cells differently. This confirms that each subtype has distinct characteristics.

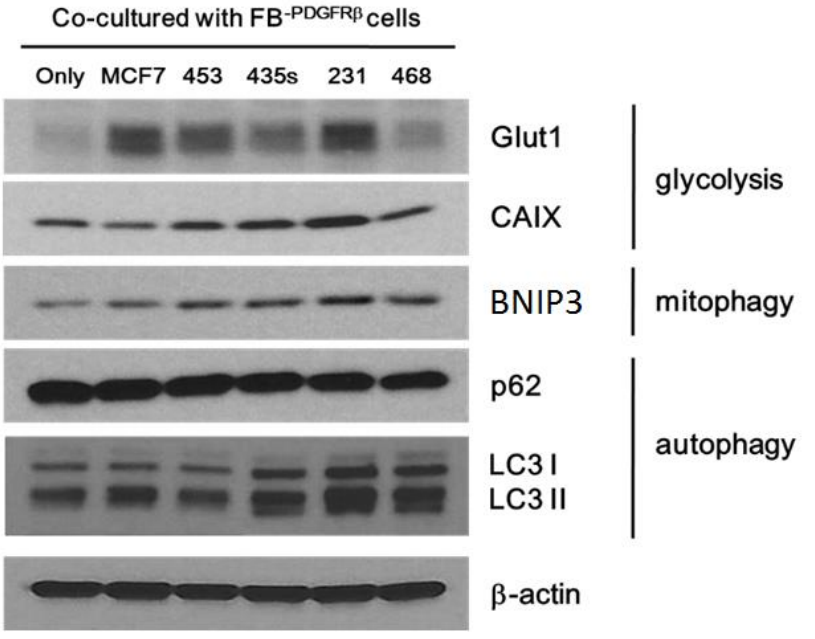

Figure 3. Differential expressions of metabolic related proteins in breast cancer cells co-cultured with FB$^{-PDGFR\beta}$ cells. Breast cancer cells were co-cultivated with FB$^{-PDGFR\beta}$ cells. The lysates were subjected to SDS-PAGE and performed by

western blotting with anti-Glut1, anti-CAIX, anti-BINP3, anti-p62, anti-LC3B, and anti-β-actin antibodies.

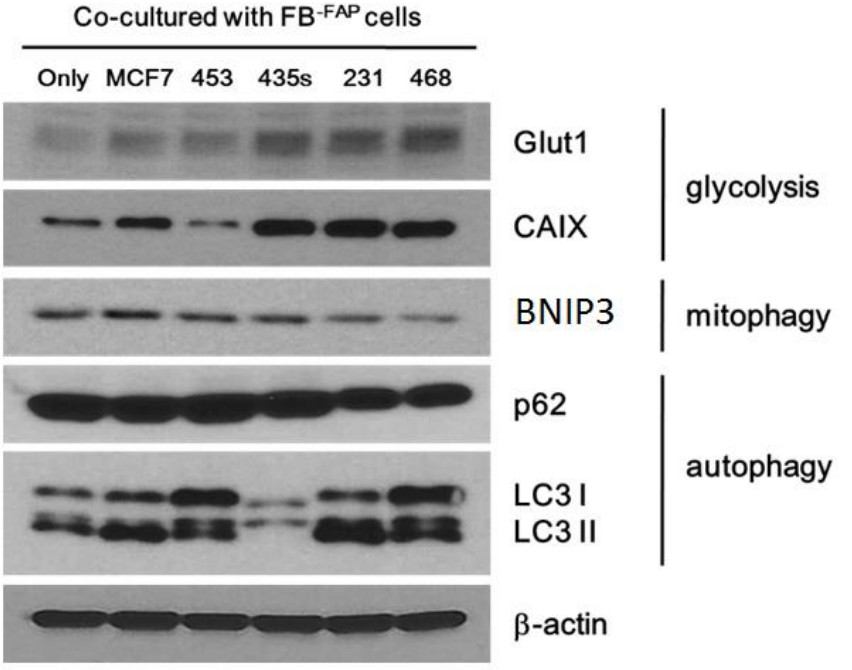

Figure 4. Differential expressions of metabolic related proteins in breast cancer cells co-cultured with FB^{-FAP} cells. Breast cancer cells were co-cultivated with FB^{-FAP} cells. Western blotting with the indicated antibodies was carried out using each breast cancer cell extracts.

4. Differences in characteristics of breast cancer cell lines when co-cultured with different subtypes of CAF

We considered that metabolism interaction between CAF and breast cancer cell would affect cancer behavior, that is, the metastasis and infiltration ability. According to the results above, we carried out later research with $FB^{-PDGFR\beta}$, FB^{-FAP} as CAF subtypes and MDA-MB-231, MDA-MB-468 as breast cancer cell lines.

First, to analyze the effect on metastasis, we conducted migration assay with $FB^{-PDGFR\beta}$, FB^{-FAP}, MDA-MB-231, and MDA-MB-468. In case of MDA-MB-231, all groups of cancer cells which are co-cultured with fibroblasts ($FB^{-PDGFR\beta}$ and FB^{-FAP}) showed increased numbers of migrated cells compared with MDA-MB-231 only group (Figure 5). MDA-MB-468, also, showed increased ability of migration when co-cultured with fibroblasts ($FB^{-PDGFR\beta}$ and FB^{-FAP}). $FB^{-PDGFR\beta}$ group didn't show dramatic change in numbers of migrated cells compared to MDA-MB-468 only group. In contrast, FB^{-FAP} group which showed significant differences in numbers of migrated cells compared to MDA-MB-468 only group (Figure 5).

Secondly, we carried out invasion assay to see the CAF effect on infiltration ability of the breast cancer cell lines. In case of MDA-MB-231, invasion ability was increased regardless of the fibroblast subtypes (Figure 6). But MDA-MB-468 showed significantly increased invasive cells only when co-cultured with FB^{-FAP} (Figure 6).

In summary, the migration and invasion ability of MDA-MB-231 was increased by both CAF cell lines ($FB^{-PDGFR\beta}$, FB^{-FAP}) and the same ability of MDA-MB-468 was increased by FB^{-FAP}. This means, effects on cancer behavior are different by the subsets of CAF. Especially, FB^{-FAP} plays an important role in cancer behavior of TNBC cell lines.

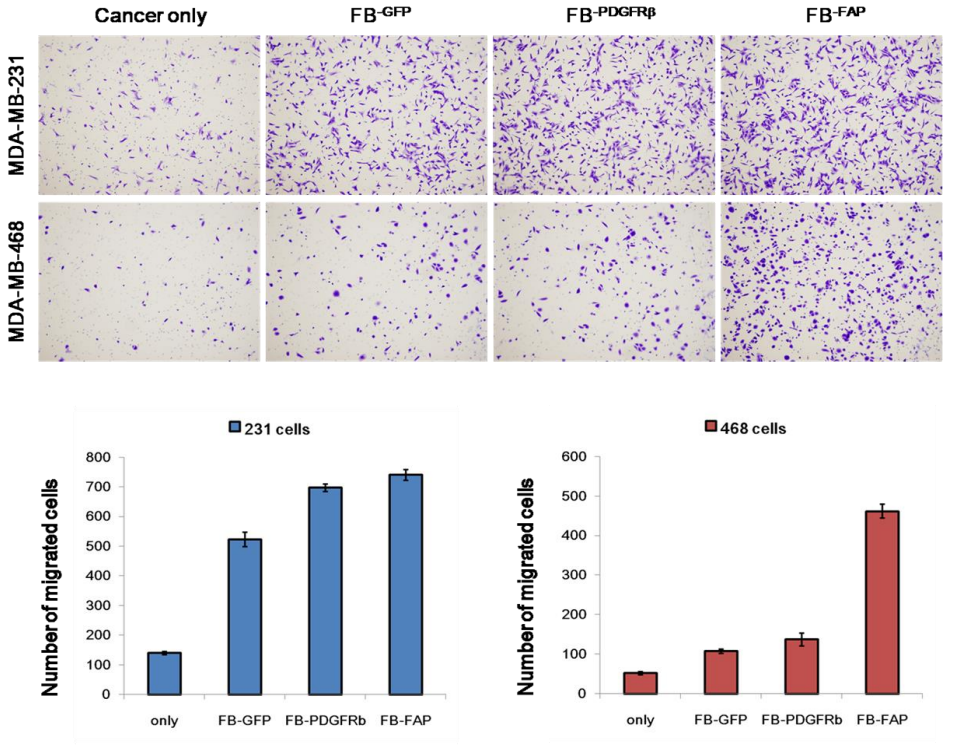

Figure 5. Migration ability of MDA-MB-231 and MDA-MB-468 cells co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP} cells. MDA-MB-231 and MDA-MB-468 cells were cultured with FB^{-GFP}, $FB^{-PDGFR\beta}$, FB^{-FAP}, or medium (control) respectively. Migrated cells attached to the lower surface of the filter were fixed, stained with crystal violet, and counted in five randomly selected fields in each membrane using bright-field microscopy. $FB^{-PDGFR\beta}$ and FB^{-FAP} activated migration of MDA-MB-231 and MDA-MB-468 cells.

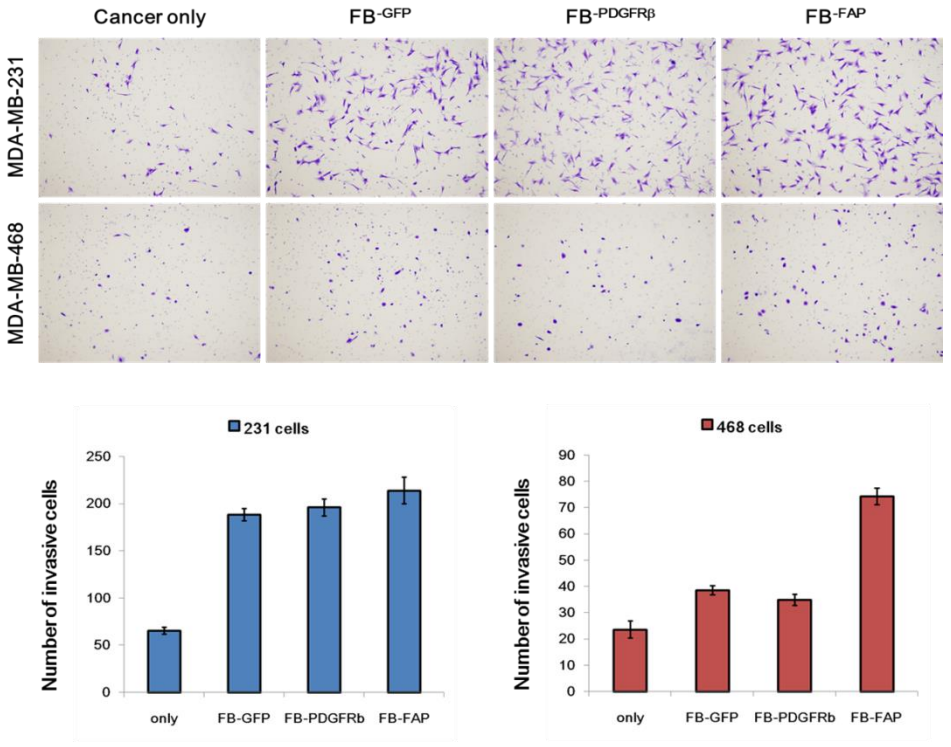

Figure 6. Invasion ability of MDA-MB-231 and MDA-MB-468 cells co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP} cells. MDA-MB-231 and MDA-MB-468 cells were cultured with FB^{-GFP}, $FB^{-PDGFR\beta}$, FB^{-FAP}, or medium (control) respectively. Invasive cells attached to the lower surface of the filter were fixed, stained with crystal violet, and counted in five randomly selected fields in each membrane using bright-field microscopy. $FB^{-PDGFR\beta}$ and FB^{-FAP} activated invasion ability of MDA-MB-231 and MDA-MB-468 cells.

5. Primary tumor growth and expression of metabolic related proteins in mice tumor xenograft models

From the consequences above, $FB^{-PDGFR\beta}$ and FB^{-FAP} have influence on migration and invasion ability of MDA-MB-231 and MDA-MB-468. To confirm this *in vivo*, we carried out tumor xenograft using BALB/C nude mice. Each breast cancer cell line was co-cultured with FB^{-GFP} (control), $FB^{-PDGFR\beta}$, and FB^{-FAP} respectively. $3X10^6$ cancer cells from each group were injected into 6-week-old mice subcutaneously. 6 weeks after injection, the tumor was extracted and weighed. Part of isolated tumor was fixed in 10% formalin for IHC, and the other part was lysed to analyze metabolic related proteins.

In case of MDA-MB-231, the tumor size from the cells co-cultured with $FB^{-PDGFR\beta}$, FB^{-FAP} was much bigger than the one with control (Figure 7A and B). To analyze metabolic differences among those groups, lysates from each tumor were subjected to SDS-PAGE and blotted with the antibodies. The Glut1 level was high in both $FB^{-PDGFR\beta}$ and FB^{-FAP} group. But there were few changes in BNIP3 expression. Decrease in p62 and increase of LC3II, which indicates autophagy activation, was shown in $FB^{-PDGFR\beta}$ and FB^{-FAP} group. The extent of p62 decrease and LC3II increase in FB^{-FAP} was considerable compared to that in $FB^{-PDGFR\beta}$. This tendency was also shown in IHC results (Figure 8). Taken these results together, it might be suggested that FB^{-FAP} promotes tumor formation and growth by metabolism activation like glycolysis and autophagy. And this is similar with the results from cell line study.

As for the MDA-MB-468, the tumor size from the FB^{-FAP} group was larger than the control. But there were little differences between $FB^{-PDGFR\beta}$ group and the control (Figure 9A and B). In the same manner as MDA-MB-231, tumor lysates were subjected to SDS-PAGE and blotted against metabolic markers. In FB^{-FAP} group, Glut1 and LC3II was significantly increased and BNIP3 expression also rose slightly (Figure 9C). Putting these results together, it might imply that FB^{-}

^{FAP} activates glycolysis or autophagy of MDA-MB-468 and contributes to formation and growth of the tumor.

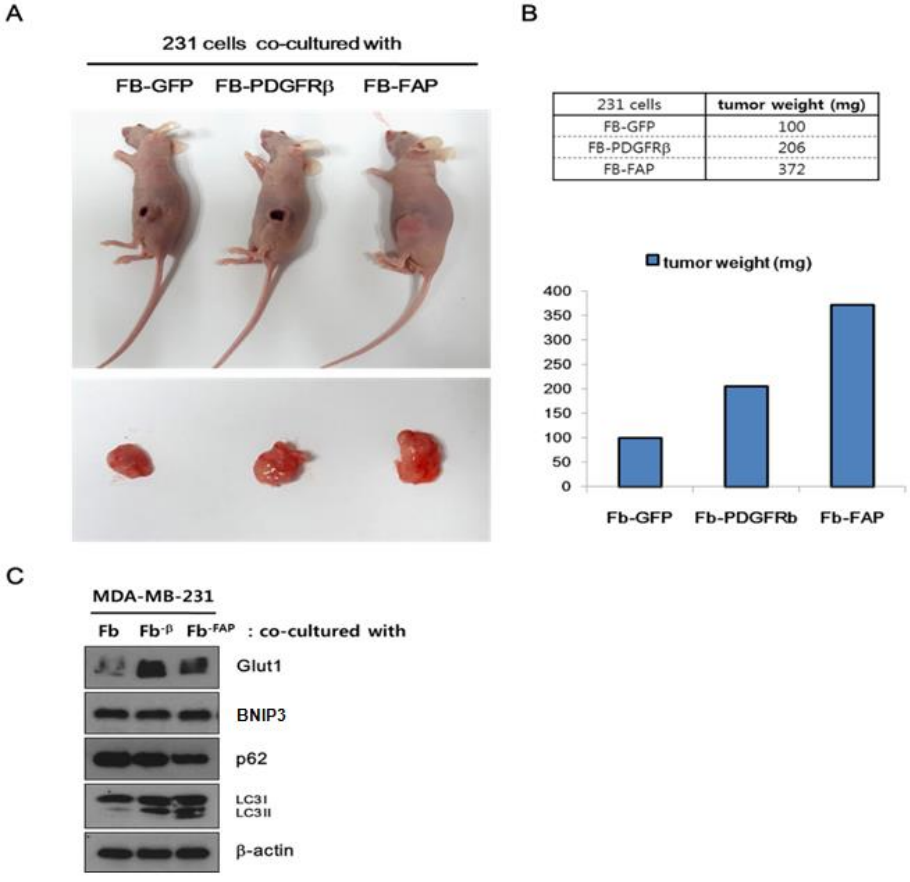

Figure 7. Primary tumor growth and expression of metabolism related proteins in MDA-MB-231 mouse model co-cultured with FB$^{-PDGFRβ}$ and FB^{-FAP} cells. (A) Primary tumor in mice 6 weeks after subcutaneous injection of MDA-MB-231 cells co-cultured with FB^{-GFP} or FB$^{-PDGFRβ}$ or FB^{-FAP} cells. (B) Graphs represent tumor weight measured after isolation of primary tumor. (C) Total lysates from each tissue were subjected to SDS-PAGE and blotted with the indicated antibodies. FB$^{-PDGFRβ}$ and FB^{-FAP} cells promoted primary tumor growth and activated glycolysis, autophagy metabolism in MDA-MB-231 mouse model.

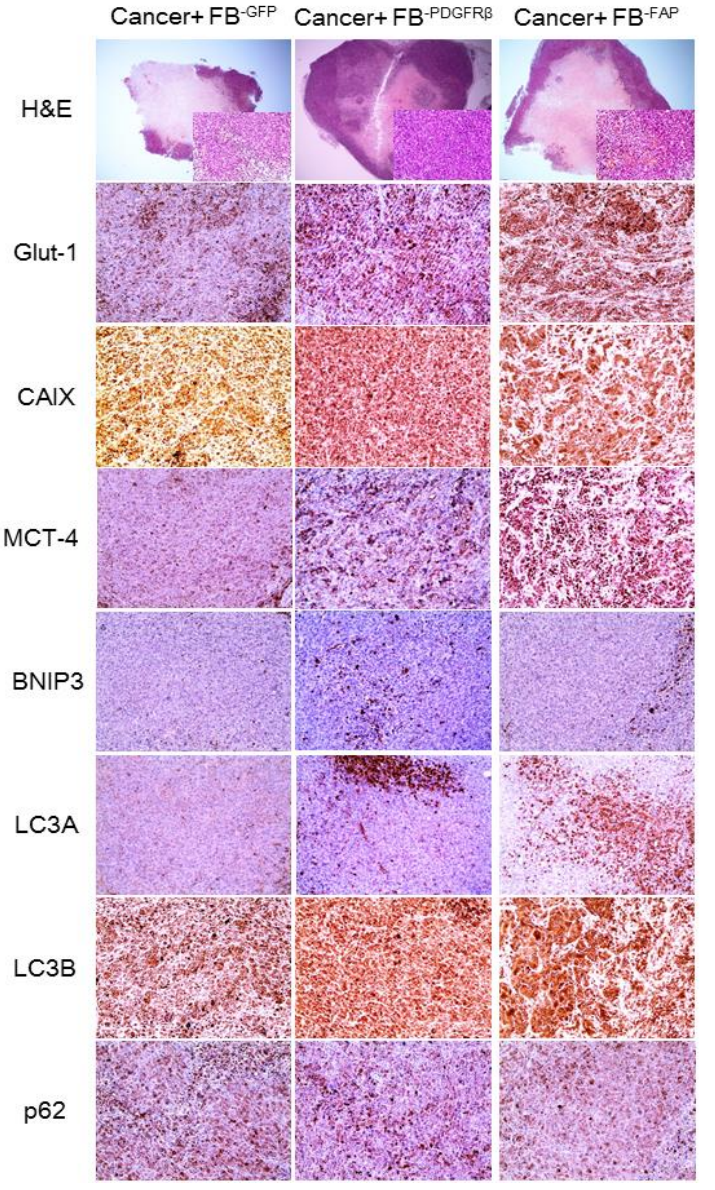

Figure 8. Expression of metabolism related proteins in mouse tumor xenograft. Higher expression of Glut-1, MCT-4, LC3A, and LC3B is noted in mouse tumor xenograft made by injection of MDA-MB-231 cells co-cultured with $FB^{-PDGFR\beta}$ or FB^{-FAP} cells than in the one with $FB^{-GFP.}$

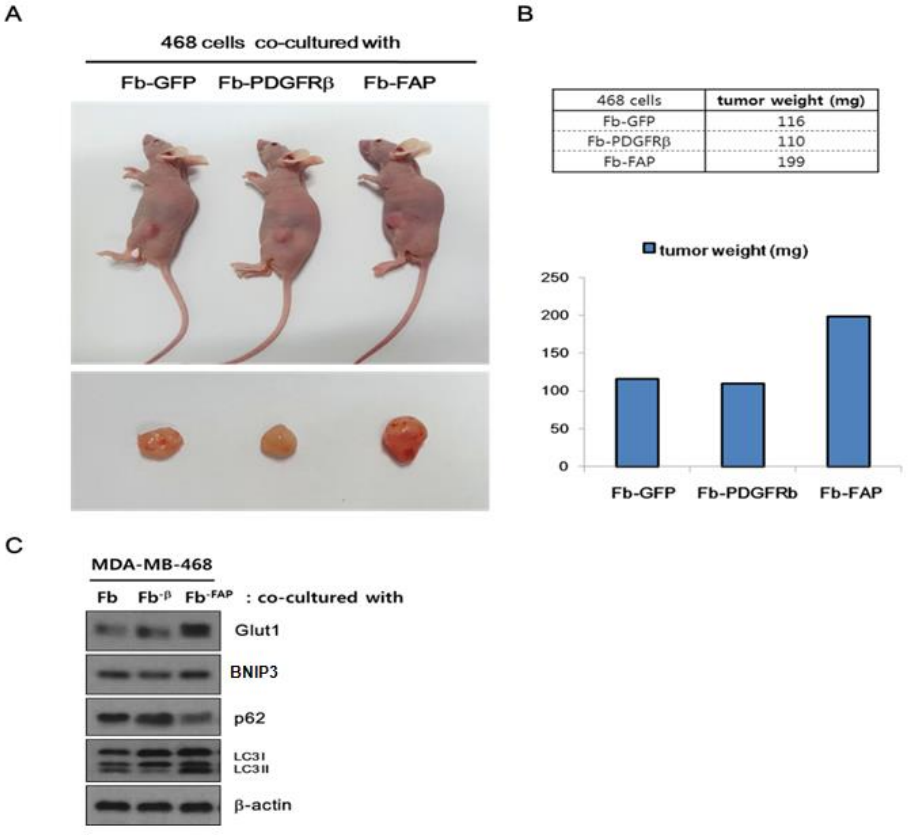

Figure 9. Primary tumor growth and expression of metabolism related proteins in MDA-MB-468 mouse model co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP} cells. (A) Primary tumor in mice 6 weeks after subcutaneous injection of MDA-MB-468 cells co-cultured with FB^{-GFP} or $FB^{-PDGFR\beta}$ or FB^{-FAP} cells. (B) Graphs represent tumor weight measured after isolation of primary tumor. (C) Total lysates from each tissue were subjected to SDS-PAGE and blotted with the indicated antibodies. FB^{-FAP} cells promoted primary tumor growth and activate glycolysis, autophagy metabolism in MDA-MB-468 mouse model.

6. Inhibition of CAF marker genes in BJ-5ta by siRNA knock down

To confirm the effects of CAFs on breast cancer, CAF marker inhibition in normal fibroblasts were also recruited. Each siRNA for PDGFRα, PDGFRβ, FAP, and S100A4 was transfected into BJ-5ta to make it suppress each CAF marker gene. To check whether they were knocked down at mRNA or protein level, qRT-PCR or western blotting was carried out, respectively (Figure 10). After confirmation of CAF marker inhibition, we named each transient cell line as FB-siNC (control), FB-siPDGFRα (or siα), FB-siPDGFRβ (or siβ), FB-siFAP (or siFAP), and FB-siS100A4 (or siS100A4). And our study could be performed with fibroblasts of which CAF markers were inhibited.

7. Metabolism in breast cancer cell lines co-cultured with fibroblasts of which CAF-related genes were inhibited

Breast cancer cell lines (MDA-MB-231, MDA-MB-468) were co-cultured with each subtype of knocked down fibroblasts (FB-siNC (control), FB-siPDGFRβ, and FB-siFAP). Then, the metabolic related proteins of breast cancer cell lines were analyzed.

Firstly, when the cell lines were co-cultured with FB-siPDGFRβ, MDA-MB-231 showed decrease in expressions of Glut1 and CAIX (glycolysis markers), BNIP3 (mitophagy marker), and LC3II (autophagy marker). MDA-MB-468, in addition, showed decrease of LC3II expression (Figure. 11). And then in breast cancers co-cultured with FB-siFAP, both of them showed decrease in glycolysis (Glut1↓, CAIX↓) and autophagy (p62↑, LC3II↓) activity. Expressions of BNIP3, on the other hands, were increased in both cell lines (Figure 12).

Consequently, those results above correspond to the tendency from CAF overexpression studies. Thus, it confirms that adjacent CAF induces metabolism of breast cancer cells and each subtype of CAF ($FB^{-PDGFR\beta}$ and FB^{-FAP}) has distinct characteristics.

8. Differences in cancer behavior of breast cancer cell lines when co-cultured with fibroblasts of which PDGFRβ or FAP is inhibited

To observe the effects of FB-siPDGFRβ and FB-siFAP on cancer behavior, migration assay and invasion assay were conducted. MDA-MB-231 and MDA-MB-468 was cultured with FB-siNC, FB-siPDGFRβ, FB-siFAP, or medium (control). In migration assay, the numbers of migrated cancer cells were counted in each group. Compared to the FB-siNC, FB-siPDGFRβ and FB-siFAP reduced migration ability of MDA-MB-231 greatly. In case of MDA-MB-468, migrated cancer cells were decreased in the FB-siPDGFRβ group compared to the FB-siNC group, but there were little differences between them. FB-siFAP, on the other hands, reduced the migration ability of MDA-MB-468, significantly (Figure 13).

Then, in invasion assay, the numbers of invasive cancer cells were counted in each group. FB-siPDGFRβ reduced invasion ability of MDA-MB-231, while FB-siFAP suppressed the ability of both MDA-MB-231 and MDA-MB-468 (Figure 14).

In summary, the migration and invasion ability of MDA-MB-231 was decreased by both FB-siPDGFRβ and FB-siFAP, and the same ability of MDA-MB-468 was decreased by FB-siFAP. These results confirm again that each subtype of CAF has different effects on cancer behavior, and FB^{-FAP}, especially, plays an important role in cancer behavior of TNBC cell lines.

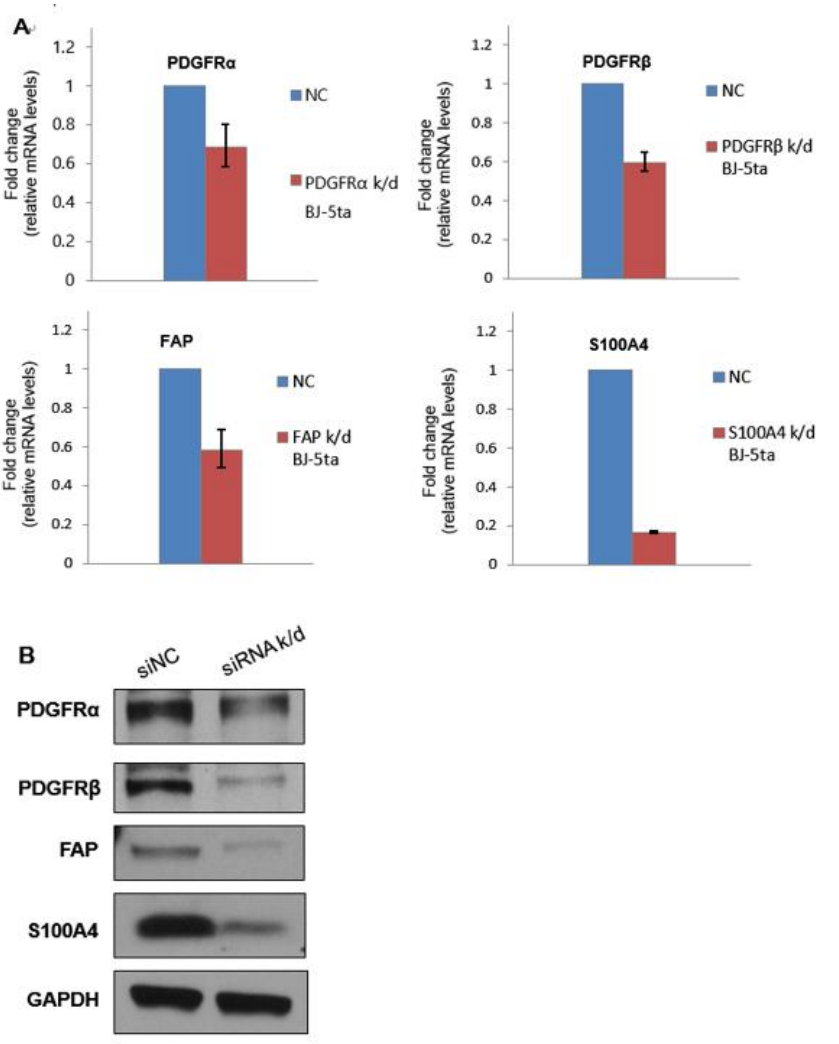

Figure 10. Confirmation of CAF-related gene knock down in BJ-5ta fibroblasts. siRNAs against CAF-related gene were transfected in 70nm for 48hr. (A) Relative mRNA levels of PDGFRα, PDGFRβ, FAP, S100A4 were assessed by quantitative real-time PCR. mRNA expression was normalized to GAPDH and presented as fold change. (B) CAF-related protein levels of PDGFRα, PDGFRβ, FAPα, S100A4 were examined by western blotting.

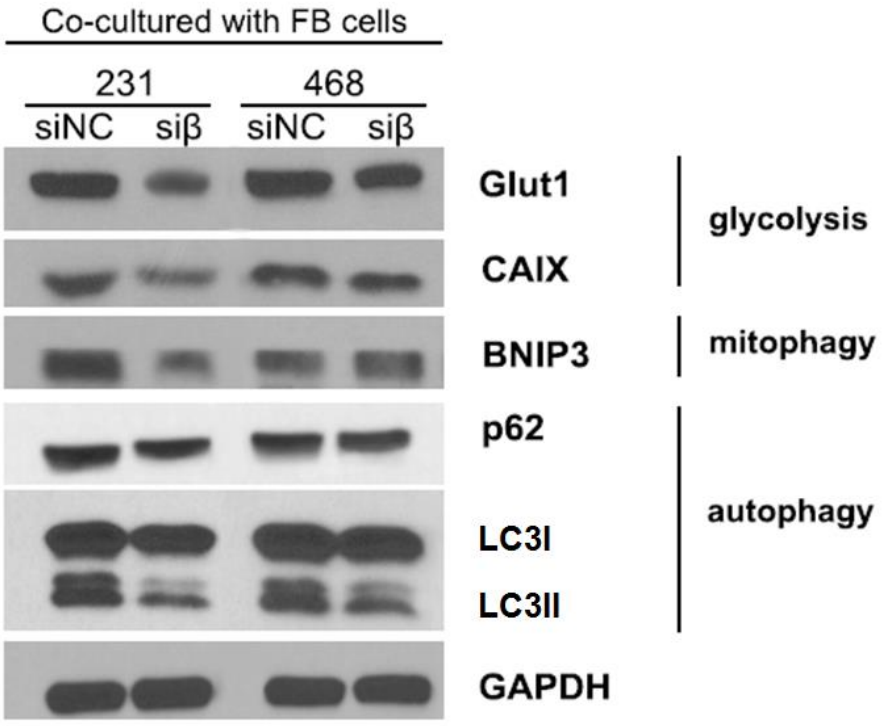

Figure 11. Differential expressions of metabolic related proteins in breast cancer cells co-cultured with FB-siPDGFRβ cells. Breast cancer cells were co-cultivated with FB-siPDGFRβ cells for 48 hr. The cell lysates were subjected to SDS-PAGE and performed by western blotting with anti-Glut1, anti-CAIX, anti-BINP3, anti-p62, anti-LC3B, and anti-GAPDH antibodies.

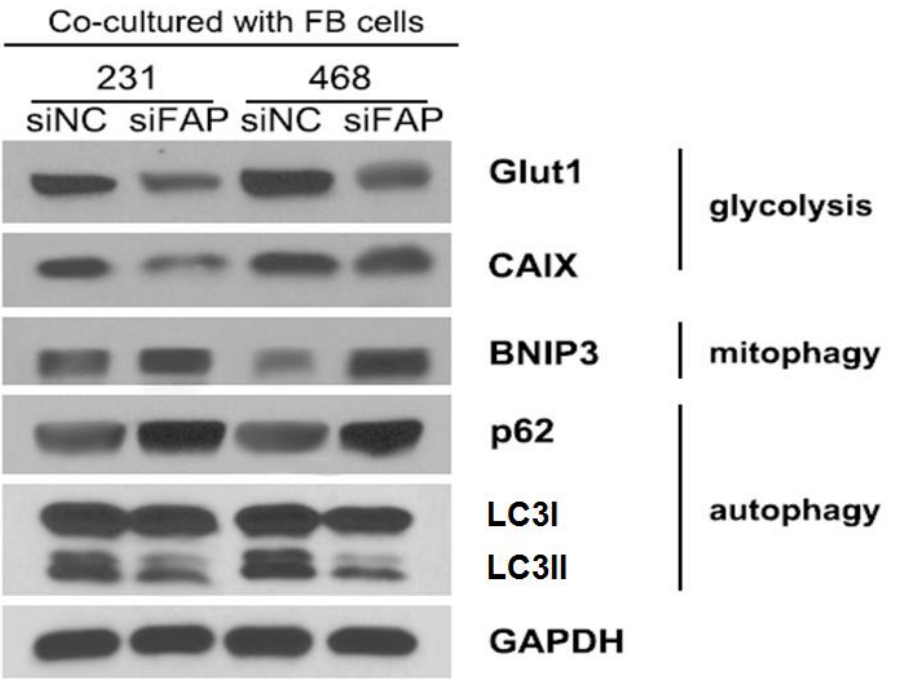

Figure 12. Differential expressions of metabolic related proteins in breast cancer cells co-cultured with FB-siFAP cells. Breast cancer cells were co-cultivated with FB-siFAP cells for 48 hr. Western blotting with the indicated antibodies was carried out using each breast cancer cell extracts.

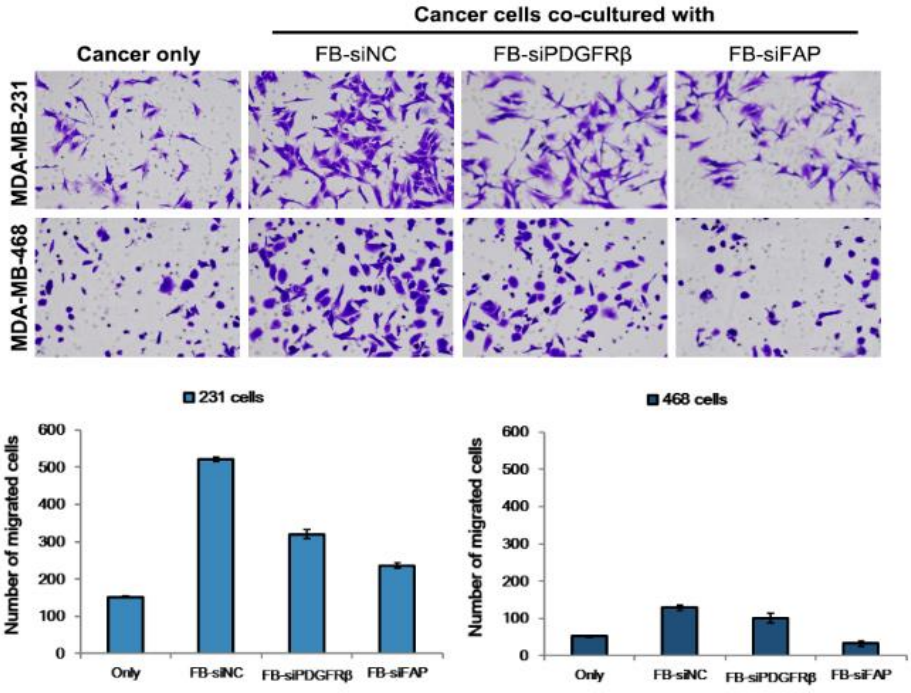

Figure 13. Migration ability of MDA-MB-231 and MDA-MB-468 cells co-cultured with FB-siPDGFRβ and FB-siFAP cells. Cancer cells were cultured with FB-siNC, FB-siPDGFRβ, FB-siFAP, or medium (control) respectively. Cells attached to the lower surface of the filter were fixed, stained with crystal violet, and counted in five randomly selected fields in each membrane using bright-field microscopy. FB-siPDGFRβ and FB-siFAP cells inhibited migration of MDA-MB-231 and MDA-MB-468 cells.

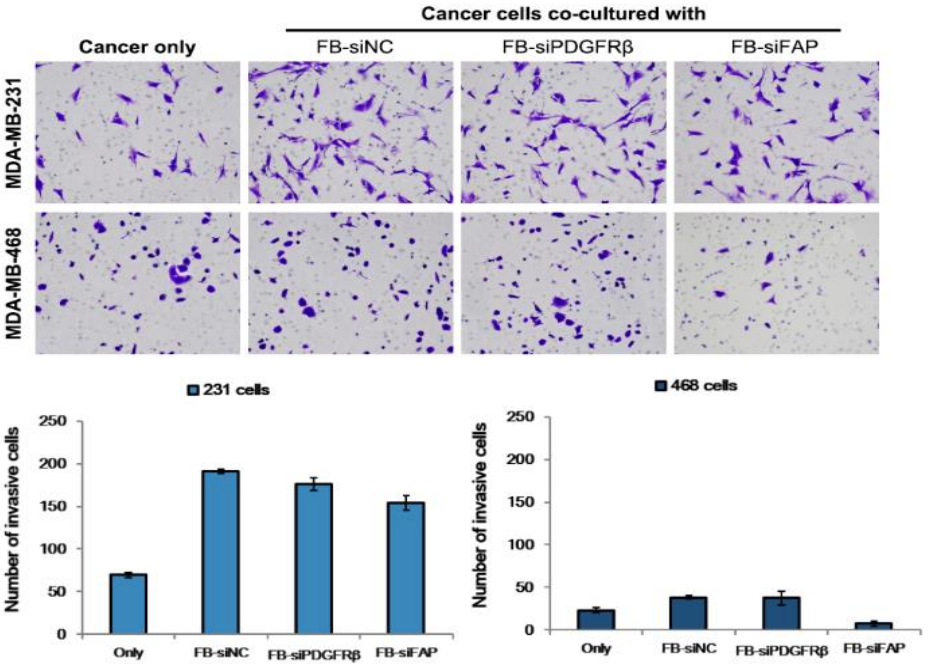

Figure 14. Invasion ability of MDA-MB-231 and MDA-MB-468 cells co-cultured with FB-siPDGFRβ and FB-siFAP cells. Cancer cells were cultured with FB-siNC, FB-siPDGFRβ, FB-siFAP, or medium (control) respectively. Invasive cells attached to the lower surface of the filter were fixed, stained with crystal violet, and counted in five randomly selected fields in each membrane using bright-field microscopy. FB-siFAP cells inhibited invasion ability of MDA-MB-231 and MDA-MB-468 cells.

9. Primary tumor growth inhibition and metabolism suppression in mice tumor xenograft models

To confirm the effects on cancer behavior of FB-siPDGFRβ, FB-siFAP cells *in vivo*, tumor xenograft with BALB/C nude mice was recruited. Each breast cancer cell line was co-cultured with FB-siNC (control), FB-siPDGFRβ, FB-

siFAP, respectively. $3X10^6$ cancer cells from each group were injected into 6-week-old mice subcutaneously. 6 weeks after injection, the tumor was extracted and weighed. Part of isolated tumor was fixed for IHC, and the other part was lysed to analyze metabolic related proteins.

In case of MDA-MB-231, the tumor size from the cells co-cultured with FB-siPDGFRβ and FB-siFAP was smaller than the one with control (Figure 15A and B). To analyze metabolic differences among those groups, lysates from each tumor were subjected to SDS-PAGE and blotted with the antibodies. The Glut1 level was low in both FB-siPDGFRβ and FB-siFAP group. But there were few changes in BNIP3 expression. Increase in p62 and decrease of LC3II, which indicate autophagy suppression, was shown in FB-siPDGFRβ and FB-siFAP group (Figure 15D). Those results support the suggestion from mice xenograft models co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP} that $FB^{-PDGFR\beta}$ and FB^{-FAP} promotes tumor formation and growth by glycolysis and autophagy metabolism activation.

As for MDA-MB-468, the tumor size from the FB-siFAP group was smaller than the control (Figure 16A and B). In the same manner as MDA-MB-231, tumor lysates were subjected to SDS-PAGE and blotted against metabolic markers. The Glut1 level was low in both FB-siPDGFRβ and FB-siFAP group. Also, p62 expression was increased in those groups. Especially in FB-siFAP group, the extent of p62 increase was significantly higher than that in FB-siPDGFRβ group (Figure 16C). In summary, it could be suggested that FB-siFAP reduces glycolysis and autophagy metabolism of MDA-MB-468, and affects tumor formation and growth. And this also corresponds to the results from mice xenograft models co-cultured with $FB^{-PDGFR\beta}$ and FB^{-FAP}.

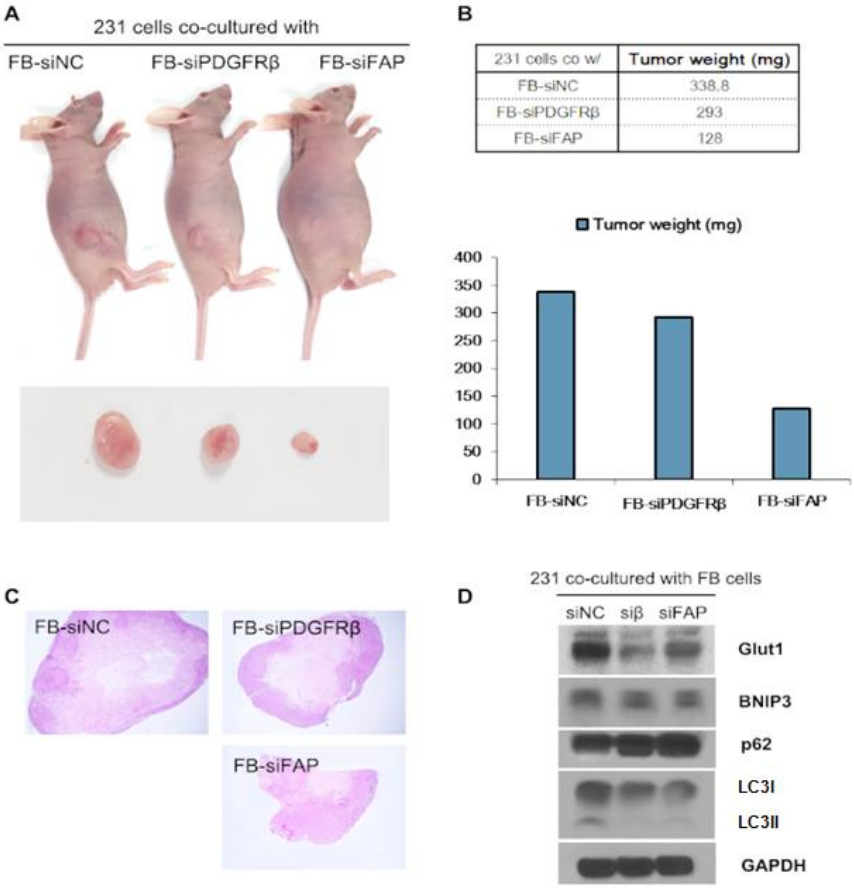

Figure 15. Primary tumor growth and expression of metabolism related proteins in MDA-MB-231 mouse model co-cultured with FB-siPDGFRβ and FB-siFAP cells. (A) Primary tumor in mice 6 weeks after subcutaneous injection of MDA-MB-231 cells co-cultured with FB-siPDGFRβ and FB-siFAP cells. (B) Graphs represent tumor weight measured after isolation of primary tumor. (C) H&E stained mouse tumor images (D) Total lysates from each tissue were subjected to SDS-PAGE and blotted with the indicated antibodies. FB-siPDGFRβ and FB-siFAP cells inhibited primary tumor growth and suppressed glycolysis, autophagy metabolism in MDA-MB-231 mouse model.

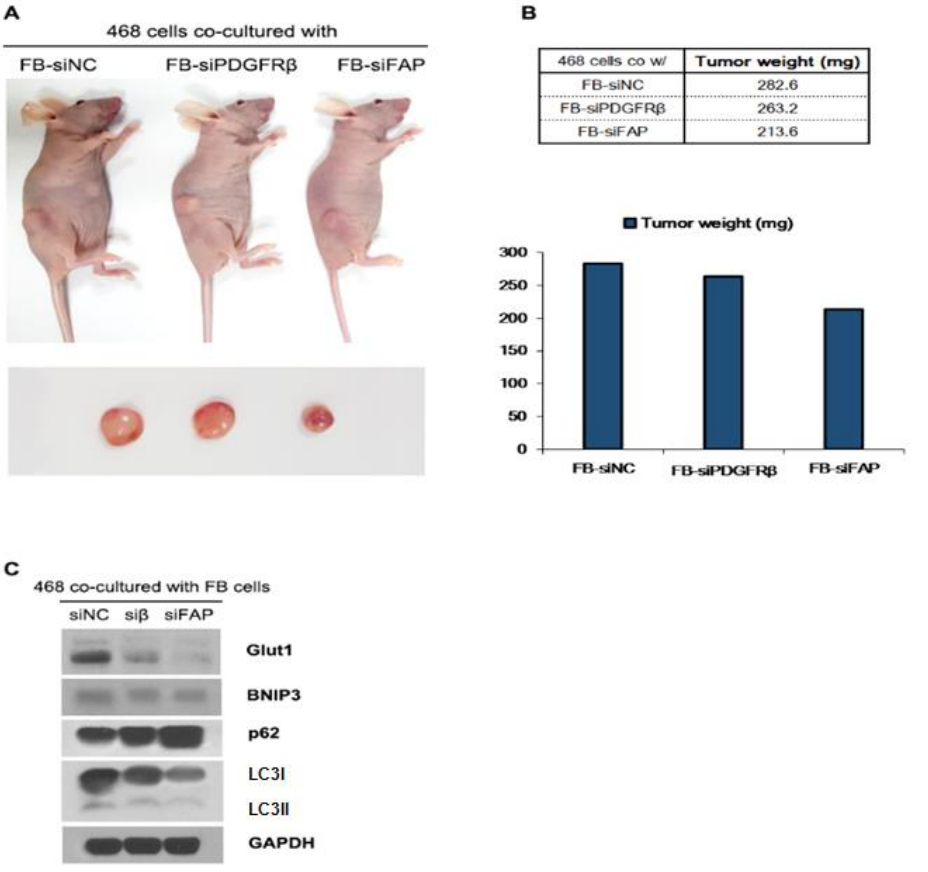

Figure 16. Primary tumor growth and expression of metabolism related proteins in MDA-MB-468 mouse model co-cultured with FB-siPDGFRβ and FB-siFAP cells. (A) Primary tumor in mice 6 weeks after subcutaneous injection of MDA-MB-468 cells co-cultured with FB-siNC, FB-siPDGFRβ, or FB-siFAP cells, respectively. (B) Graphs represent tumor weight measured after isolation of primary tumor. (C) Total lysates from each tissue were subjected to SDS-PAGE and blotted with the indicated antibodies. FB-siFAP cells inhibited primary tumor growth and glycolysis metabolism in MDA-MB-468 mouse model

IV. DISCUSSION

Breast cancer is one of the tumors with various types of stroma. Therefore, we could reach an idea that cancer cells and stromal cells interact in the respect of metabolism. Before dealing with an interaction between cancer cells and stromal cells in the breast cancer, we should refer to breast cancer-associated fibroblasts (CAFs), the most prevalent cellular component of breast cancer tumor microenvironment (TME). Breast CAFs have the potential to function in tumor initiation, tumor progression, invasion, metastasis, metabolic reprogramming of TME, and even therapy resistance[23,24]. It is the reason why therapies against CAFs are continuously considered and studied as a way to control cancer. Nonetheless, fundamental understanding of characteristics of breast CAFs, including their origin, definition, and biologic heterogeneity still remains to be elucidated. CAF can be classified into several functional subtypes expressing widely used markers such as FAP type, FSP1 type, PDGFRα type, PDGFRβ type and each of which has own different characteristics. Above markers, in other words CAF-related proteins, have an intrinsic function as follows: FAP; extracellular matrix modulation and cell invasion, FSP1/S100A4; metastatic colonization, PDGFRα; tumor cell growth and angiogenesis, PDGFRβ; metastatic spread[17]. Due to lack of researches which considered these heterogeneity of CAFs, it is challenging but worthwhile to study CAF-specific markers.

According to reverse Warburg effect, one of the well-known theories explaining the metabolic interaction between cancer cells and stromal cells in the breast cancer, cancer cells induce oxidative stress in neighboring fibroblasts, triggering aerobic glycolysis and producing high energy metabolites in stromal cells, which are in turn transported and used for anabolic need of cancer cells. And we already know that we need to study a biomarker concerning breast cancer metabolism other than loss of stromal caveolin-1.

In this study, we produced four cancer-associated fibroblast (CAF) subtypes,

which were stably expressing each CAF marker (PDGFRα, PDGFRβ, FAP, and S100A4) and analyzed differences in metabolic interaction of each CAF subtype with breast cancer cells by their molecular subtypes. Among four CAF subtypes, we identified that $FB^{-PDGFR\beta}$ activated glycolysis, mitophagy, and autophagy of MDA-MB-231 and FB^{-FAP} activated glycolysis and autophagy of MDA-MB-231 and MDA-MB-468. In mouse xenograft model, it also showed that FB^{-FAP} activated glycolysis and autophagy of MDA-MB-231 and MDA-MB-468. Moreover, we identified that breast cancer cells behaved differently depending on co-cultured CAF subtypes through *in vitro* and *in vivo* experiments. It especially showed that FB^{-FAP} played an important role in migration and invasion of cancer behavior of TNBC cells; MDA-MB-231 and MDA-MB-468. Through these, we suggested improved results which could reinforce precedent researches which overlooked heterogeneous characteristics of CAF by its subtypes. And we judged that FB^{-FAP} promoted tumor formation and growth by metabolic activation such as glycolysis and autophagy.

Researches dealing with the relationship and the mechanism between CAFs and cancer metabolism or cancer behavior in the breast cancer have been ongoing. Wang et al.[25,26] reported that autophagy of CAFs whose identification was checked by high expression of α-smooth muscle actin (α-SMA) and vimentin enhanced the migration, invasion, proliferation, and epithelial-mesenchymal transition (EMT) process of TNBC cell lines. The EMT process could be induced through the Wnt/β-catenin pathway by overexpression of Beclin 1, an autophagic regulator gene. A recent study identified correlation between autophagy and YAP expression in TNBC cells. Autophagy in TNBC cells promoted YAP nuclear localization and the expression of YAP target gene ankyrin repeat domain 1 (ANKRD1) remarkably[27]. One study demonstrated that CAFs, with high expression levels of α-SMA and stromal cell-derived factor 1/C-X-C motif chemokine 12 (SDF1/CXCL12), enhanced metastatic potential of breast cancer

cells through EMT process induced by paracrine transforming growth factor-β (TGF-β) signaling[28]. However, those researches did not consider the heterogeneity of CAFs enough in suggesting a possible mechanism of tumor metabolism or its behavior.

In our study, even though both MDA-MB-231 and MDA-MB-468 belonged to TNBC cell lines, metabolism of each cell line showed a difference in CAF marker which had an effect on its tumor metabolism and behavior. Tumor metabolism of claudin-low subtype (MDA-MB-231) was activated by both $FB^{-PDGFR\beta}$ and FB^{-FAP}, and that of molecular apocrine subtype (MDA-MB-468) was mainly activated by FB^{-FAP}. This difference seems to be attributed to differences in characteristics of intrinsic molecular subtype. As one of the molecular subtypes of TNBC, claudin-low subtype, discovered and named by Herschkowitz et al.[29] in 2007, is characterized by the low expression of genes involved in tight junctions and cell-cell adhesion, including claudin 3, 4, 7, occludin, and E-cadherin. It is differentiated from basal-like tumors by higher expression of genes involved in immune response, cell communication, extracellular matrix, cell migration and angiogenesis[30]. And this subtype is enriched for EMT markers, immune system responses, and cancer stem cell-associated biological processes[30]. Another subtype of TNBC, molecular apocrine subtype, identified by Farmer et al.[31] in 2005 is characterized by apocrine histology, androgen receptor (AR) positivity, estrogen receptor (ER) negativity outside the basal-like subtype, and frequent HER-2 amplification. Similar to the genomic heterogeneity observed in TNBC, analysis of metabolic phenotype of TNBC revealed metabolic heterogeneity among TNBC subtypes and demonstrated that understanding metabolic profiles and drug responses is valuable in targeting TNBC subtypes[32-34]. Compared to ER-positive breast cancer cell lines, MDA-MB-231 and MDA-MB-468 TNBC cell models are reported to have metabolic characteristics of high glucose uptake, increased lactate production, and low mitochondrial respiration

(OXPHOS activity) which is correlated with attenuation of mammalian target of rapamycin (mTOR) pathway and decreased expression of p70S6K[35]. Metabolic profiling showed that MDA-MB231 cells are more likely to rely on glycolytic metabolism, while MDA-MB-468 cells are more likely to rely on oxidative metabolism in the basal state. MDA-MB-231 cells exhibited the least metabolic flexibility and MDA-MB-468 cells exhibited the greatest metabolic flexibility, meaning the greatest glycolytic reserve and the greatest spare respiratory capacity in maximal capability among multiple TNBC subtypes[32].

We demonstrated that CAF markers which had an impact on tumor metabolism and behavior of MDA-MB-231 and MDA-MB-468, in other words, TNBC, turned out to be PDGFRβ and FAP. Stalker et al.[36] showed that both luminal-like and claudin-low breast cancer cells relied on PDGFR signaling for migration and that PDGFR inhibitors such as Sunitinib, Regorafenib and Masitinib can effectively suppress migration of breast cancer cells and metastasis. And it was consistent with their previous observations that knock down of PDGFRs using PDGFR RNAi significantly reduced migration but not proliferation of the claudin-low mammary tumor cells[37]. Primac et al.[38] identified an integrin α11/PDGFRβ-positive CAF subset displaying tumor-promoting features in breast cancer. Mechanistically, integrin α11 proinvasive activity relies on its ability to interact with PDGFRβ in a ligand-dependent manner and to promote its downstream c-Jun N-terminal kinase (JNK) activation, leading to the production of tenascin C, a proinvasive matricellular protein, and finally increased tumor invasion. They identified the integrin α11/PDGFRβ/JNK axis as an important mediator of CAF-promoted tumor invasiveness. Triple colocalization of integrin α11, PDGFRβ, and tenascin C was particularly evident in more aggressive breast cancer molecular subtypes (HER-2 and TNBC) than ductal carcinoma in situ or luminal type. Jia et al.[39] showed FAP-α promoted proliferation and inhibited migration of breast cancer cells, potentially by regulating the focal adhesion

kinase (FAK) pathway. Overexpression of FAP-α resulted in a reduction of phosphorylated FAK level in both MCF-7 cells and MDA-MB-231 cells. Huang et al.[40] generated FAP-expressing MDA-MB-231 cells and identified that they formed tumors with increased growth rates and higher microvessel density compared with control transfectants when implanted into mammary fat pads of female severe combined immunodeficient mice. Their results indicated FAP-α could act as a tumor promoter. Breast cancer cells expressing a catalytically inactive mutant of FAP-α (FAPS624A) also produced tumors that grew rapidly[41]. It referred that FAP-α promoted tumor growth and invasion of breast cancer cells probably through non-enzymatic functions. In contrast, there was a conflicting study that expression of FAP had an association with longer survival in patients with invasive ductal carcinoma of the breast[42]. It suggests that the mechanism underlying physiologic response to FAP is complex and needs to be considered on the exact context of the expression within different microenvironments.

Though previous literature also revealed that PDGFRβ or FAP promoted migration, invasion, and proliferation of the breast cancer, especially TNBC, similar results to ours on the surface, our study was meaningful in that we revealed PDGFRβ and FAP promoted migration, invasion, and proliferation of TNBC through metabolic activation such as glycolysis, mitophagy (autophagy of mitochondria), and autophagy. Although the precise mechanism which explains the correlation between PDGFRβ and FAP of CAF markers and glycolysis and autophagy has not yet been elucidated, it is obvious that those markers have a potential as a therapy target of TNBC. Treatments targeting breast tumor stroma have been attempted. For TNBC tumors with enhanced collagen expression, Pirfenidone (PFD), an FDA-approved drug for idiopathic pulmonary fibrosis, showed a potential as a therapy targeting tumor-stromal interaction[43]. Huang et al.[44] generated a FAPα-targeting prodrug of Doxorubicin (FTPD) by conjugating the drug to the FAP-selective N-terminal benzyloxycarbonyl (z)-blocked

dipeptide and showed antitumor efficacy in 4T1 metastatic breast cancer xenografts with a minimal effect on body weight. For CAF-targeted therapy to perform properly, a study about markers of CAF in breast tumor stroma is necessary. Our study recognized differences of metabolic interaction between tumor cells and stromal cells depending on CAF subtypes, and impacts of each CAF subtype on tumor formation and growth. Therefore, this study provided a clue to develop promising breast cancer drug according to CAF subtypes especially in TNBC which has no satisfiable therapy target yet.

V. CONCLUSION

In conclusion, CAF has heterogeneous characteristic in that each breast cancer molecular subtype shows differences in the expression of metabolic related markers when co-cultured with each CAF subtype. Also, each breast cancer molecular subtype shows different cancer behavior depending on co-cultured CAF subtype. Among CAF subtypes, FB^{-FAP} seems to promote tumor formation and growth through glycolysis and autophagy of TNBC and we suggest it has a potential role as a therapeutic target for TNBC.

REFERENCES

1. Warburg O. On the origin of cancer cells. Science 1956;123:309-14.
2. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, et al. The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 2009;8:3984-4001.
3. Pavlides S, Tsirigos A, Vera I, Flomenberg N, Frank PG, Casimiro MC, et al. Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "reverse Warburg effect": a transcriptional informatics analysis with validation. Cell Cycle 2010;9:2201-19.
4. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle 2010;9:3256-76.
5. Bonuccelli G, Tsirigos A, Whitaker-Menezes D, Pavlides S, Pestell RG, Chiavarina B, et al. Ketones and lactate "fuel" tumor growth and metastasis: Evidence that epithelial cancer cells use oxidative mitochondrial metabolism. Cell Cycle 2010;9:3506-14.
6. Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, Zhou J, et al. Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFκB activation in the tumor stromal microenvironment. Cell Cycle 2010;9:3515-33.
7. Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, et al. Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: implications for breast cancer and DCIS therapy with autophagy inhibitors. Cell Cycle 2010;9:2423-33.
8. Franco OE, Shaw AK, Strand DW, Hayward SW. Cancer associated fibroblasts in cancer pathogenesis. Semin Cell Dev Biol 2010;21:33-9.
9. Östman A. Cancer-associated fibroblasts: recent developments and emerging challenges. Semin Cancer Biol 2014;25:1-2.
10. Desmoulière A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol 2004;48:509-17.
11. De Wever O, Nguyen QD, Van Hoorde L, Bracke M, Bruyneel E, Gespach C, et al. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. Faseb j 2004;18:1016-8.
12. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. Cancer Biol Ther 2006;5:1640-6.
13. Pietras K, Sjöblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. Cancer Cell 2003;3:439-43.
14. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science 2010;330:827-30.
15. Kawase A, Ishii G, Nagai K, Ito T, Nagano T, Murata Y, et al. Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung

adenocarcinoma. Int J Cancer 2008;123:1053-9.
16. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. J Biol Chem 2013;288:10819-29.
17. Cortez E, Roswall P, Pietras K. Functional subsets of mesenchymal cell types in the tumor microenvironment. Semin Cancer Biol 2014;25:3-9.
18. Koo JS, Park S, Kim SI, Lee S, Park BW. The impact of caveolin protein expression in tumor stroma on prognosis of breast cancer. Tumour Biol 2011;32:787-99.
19. Sloan EK, Ciocca DR, Pouliot N, Natoli A, Restall C, Henderson MA, et al. Stromal cell expression of caveolin-1 predicts outcome in breast cancer. Am J Pathol 2009;174:2035-43.
20. Witkiewicz AK, Dasgupta A, Nguyen KH, Liu C, Kovatich AJ, Schwartz GF, et al. Stromal caveolin-1 levels predict early DCIS progression to invasive breast cancer. Cancer Biol Ther 2009;8:1071-9.
21. Gonda TA, Varro A, Wang TC, Tycko B. Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy? Semin Cell Dev Biol 2010;21:2-10.
22. Korch C, Hall EM, Dirks WG, Ewing M, Faries M, Varella-Garcia M, et al. Authentication of M14 melanoma cell line proves misidentification of MDA-MB-435 breast cancer cell line. Int J Cancer 2018;142:561-72.
23. Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. Semin Cell Dev Biol 2010;21:19-25.
24. Buchsbaum RJ, Oh SY. Breast Cancer-Associated Fibroblasts: Where We Are and Where We Need to Go. Cancers (Basel) 2016;8.
25. Wang M, Zhang J, Huang Y, Ji S, Shao G, Feng S, et al. Cancer-Associated Fibroblasts Autophagy Enhances Progression of Triple-Negative Breast Cancer Cells. Med Sci Monit 2017;23:3904-12.
26. Wang MC, Wu AG, Huang YZ, Shao GL, Ji SF, Wang RW, et al. Autophagic regulation of cell growth by altered expression of Beclin 1 in triple-negative breast cancer. Int J Clin Exp Med 2015;8:7049-58.
27. Chen W, Bai Y, Patel C, Geng F. Autophagy promotes triple negative breast cancer metastasis via YAP nuclear localization. Biochem Biophys Res Commun 2019;520:263-8.
28. Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ, Feng YM. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-β signalling. Br J Cancer 2014;110:724-32.
29. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007;8:R76.
30. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12:R68.
31. Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D,

et al. Identification of molecular apocrine breast tumours by microarray analysis. Oncogene 2005;24:4660-71.

32. Lanning NJ, Castle JP, Singh SJ, Leon AN, Tovar EA, Sanghera A, et al. Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities. Cancer Metab 2017;5:6.
33. Kim S, Kim DH, Jung WH, Koo JS. Metabolic phenotypes in triple-negative breast cancer. Tumour Biol 2013;34:1699-712.
34. Choi J, Kim DH, Jung WH, Koo JS. Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype. Breast Cancer Res 2013;15:R78.
35. Pelicano H, Zhang W, Liu J, Hammoudi N, Dai J, Xu RH, et al. Mitochondrial dysfunction in some triple-negative breast cancer cell lines: role of mTOR pathway and therapeutic potential. Breast Cancer Res 2014;16:434.
36. Stalker L, Pemberton J, Moorehead RA. Inhibition of proliferation and migration of luminal and claudin-low breast cancer cells by PDGFR inhibitors. Cancer Cell Int 2014;14:89.
37. Campbell CI, Moorehead RA. Mammary tumors that become independent of the type I insulin-like growth factor receptor express elevated levels of platelet-derived growth factor receptors. BMC Cancer 2011;11:480.
38. Primac I, Maquoi E, Blacher S, Heljasvaara R, Van Deun J, Smeland HY, et al. Stromal integrin α11 regulates PDGFR-β signaling and promotes breast cancer progression. J Clin Invest 2019;129:4609-28.
39. Jia J, Martin TA, Ye L, Jiang WG. FAP-α (Fibroblast activation protein-α) is involved in the control of human breast cancer cell line growth and motility via the FAK pathway. BMC Cell Biol 2014;15:16.
40. Huang Y, Wang S, Kelly T. Seprase promotes rapid tumor growth and increased microvessel density in a mouse model of human breast cancer. Cancer Res 2004;64:2712-6.
41. Huang Y, Simms AE, Mazur A, Wang S, León NR, Jones B, et al. Fibroblast activation protein-α promotes tumor growth and invasion of breast cancer cells through non-enzymatic functions. Clin Exp Metastasis 2011;28:567-79.
42. Ariga N, Sato E, Ohuchi N, Nagura H, Ohtani H. Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast. Int J Cancer 2001;95:67-72.
43. Takai K, Le A, Weaver VM, Werb Z. Targeting the cancer-associated fibroblasts as a treatment in triple-negative breast cancer. Oncotarget 2016;7:82889-901.
44. Huang S, Fang R, Xu J, Qiu S, Zhang H, Du J, et al. Evaluation of the tumor targeting of a FAPα-based doxorubicin prodrug. J Drug Target 2011;19:487-96.

ABSTRACT (IN KOREAN)

종양 연관 섬유아세포 아형에 따른 유방암 대사 특성

<지도교수: 구 자 승>

연세대학교 대학원 의학과

이 지 희

종양 기질을 특징적으로 형성하는 유방암에서는 종양 대사로 리버스 와버그 효과가 제시되고 있다. 이는 종양 세포와 기질 세포 간 대사 상호작용이 있으며, 종양의 생존 및 성장에 중요한 역할을 한다는 이론이다. 리버스 와버그 효과 이론에서 핵심 역할을 하는 기질 세포인 종양 연관 섬유아세포는 종양 미세 환경을 구성하는 가장 중요한 구성 요소로, 최근 이것이 여러 개의 아형 (FAP, S100A4, PDGFRα, and PDGFRβ) 으로 분류되며 그것들 각각은 서로 다른 기능성 특성이 있는 것으로 알려졌다. 본 연구에서, 우리는 유방암에서 종양 연관 섬유아세포의 아형에 따른 종양 세포와 기질 세포 간 대사 상호 작용의 차이를 규명하고, 종양 기질 표적 물질이 종양 연관 섬유아세포의 아형에 따라 종양의 발생 및 성장에 미치는 영향을 조사하여, 최종적으로는 종양 기질 표적 물질의 유방암 표적 치료제로의 가능성을 조사하는 것을 목표로 하였다.

각각 안정적으로 종양 연관 섬유아세포 표지자 (FAP, S100A4, PDGFRα, and PDGFRβ) 를 발현하는 네 종류의 종양 연관 섬유아세포 아형들을 제작하여, 각 종양 연관 섬유아세포 아형과 각 유방암 분자 아형별 세포 간 대사 상호작용의 차이를 알아보았다. 또한 이동 분석과 침윤 분석을 통해 각 종양 연관 섬유아세포 아형이 암세포의 전이 및 침윤 능력에 끼치는 영향을 알아보았다. 그리고 나서는 BALB/C 누드 마우스를 이용하여 종양 이종 이식 모델을 제작하여 세포주 실험들을 확인해 보았다.

제작한 네 가지 종양 연관 섬유 아세포 아형 중에서, $FB^{-PDGFR\beta}$가 MDA-MB-231세포의 해당작용, 미토파지, 자가포식 활성을 증가시키고 FB^{-FAP}는 삼중 음성 유방암 세포주의 해당작용과 자가포식 활성을 증가시킴을 확인하였다. FB^{-FAP}는 특히 삼중 음성 유방암 세포인 MDA-MB-231과 MDA-MB-468의 종양 세포 특성에 중요한 역할을 하는 것을 확인하였다. 또한, 마우스 이종 이식 모델에서도 FB^{-FAP}가 MDA-MB-231과 MDA-MB-468 세포의 해당작용과 자가포식 대사 기전을 활성화 시킴을 확인하였다. Knocked down 섬유아세포인 FB-siPDGFRβ와 FB-siFAP 세포를 이용한 억제 실험에서, MDA-MB-231, MDA-MB-468 및 마우스 이종 이식 모델의 종양 대사 및 종양 세포 특성은 $FB^{-PDGFR\beta}$ 와 FB^{-FAP}를 함께 배양한 삼중 음성 유방암 세포주 및 마우스 이종 이식 모델 연구에서 보여준 결과와 일관되게 나타났다.

종합하면, 각 유방암 분자 아형이 함께 배양한 종양 연관 섬유아세포 아형에 따라 대사 관련 표지자의 발현 및 종양 세포 특성에 차이를 보인다는 있다는 점에서 종양 연관 섬유아세포는 이질적인 특성을 가진다. 종양 연관 섬유아세포 아형들 중, FB^{-FAP} 는 삼중 음성 유방암 세포주에서 해당작용과 자가포식을 통해 종양의 형성 및 성장을

촉진하는 것으로 판단하였고, 이것이 삼중 음성 유방암의 치료 표적으로서 잠재적인 역할이 있음을 제안한다.

핵심되는 말: 유방암, 분자 아형, 삼중 음성, 종양 세포, 종양 연관 섬유아세포, 종양 대사, 와버그 효과, 리버스 와버그 효과, 표적치료