c-Met overexpression of fibroblasts induces angiogenic signal in breast cancer

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Directed by Professor Nam Hoon Cho

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ABSTRACT

c-Met overexpression of fibroblasts induces angiogenic signal in breast cancer.

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The abnormal increase of c-Met expression in cancer cells is associated with tumor progression. c-Met is known to be mainly expressed in epithelial origin cells, but its expression was also observed in non-epithelial origin cells such as fibroblasts. The c-Met expression of cancer-associated stroma may play a role in tumor progression, however, which remains unknown. To confirm our assumption, we isolated normal breast fibroblast (NBF) and then made them CAF-like state using the co-culture with breast cancer cells. In our study, breast cancer cells, regardless of their subtypes, were able to induce the expression of c-Met in NBFs. The exogenous overexpression of c-Met in NBFs led to the alteration of extracellular matrix (ECM) expression. 7 genes were significantly 1.5-fold up regulated, whereas 56 genes were down regulated. In the functional enrichment analysis with the up regulated genes,

ECM organization and blood vessel formation were predicted to be promoted. The conditioned medium (CM) of c-Met overexpressing NBF induced the better-organized tube formation and higher VEGFR2 expression of EA.hy926 cells than that of wild-type. On the other hand, c-Met inhibitor, ARQ197, reduced the effect of c-Met overexpressing NBF CM on the tube formation and VEGFR2 expression of EA.hy926 cells.

In this study, we showed that c-Met overexpression in CAFs results in the alteration of ECM expression, which can be potentially associated with angiogenesis.

Key words : c-Met, CAF, extracellular matrix, VEGFR2, breast cancer, angiogenesis

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I. INTRODUCTION

The abnormal expression of c-Met in cancer cells is associated with tumor progression [1,2]. Although c-Met is known to be mainly expressed in epithelial origin cells [3,4], its expression was also reported in non-epithelial origin cells such as fibroblasts and endothelial cells [5,6]. Therefore, it may be plausible that c-Met expression is up regulated in cancer-associated fibroblasts (CAFs), which is associated with tumor progression.

c-Met plays an important role in tumor progression. c-Met, hepatocyte growth factor receptor, is a tyrosine kinase and known to be essential for embryonic development [7] and wound healing [4]. c-Met is generally expressed in epithelial origin cells, while its ligand, hepatocyte growth factor (HGF) – the only known ligand for c-Met, is normally expressed in mesenchymal origin cells [8]. The deregulation of c-Met expression is found in many types of

human diseases. c-Met expression is often observed to be up regulated in cancers, and the abnormal activation of c-Met is associated with the poor prognosis of cancer patients [9-11]. It was reported that c-Met signal activation triggers tumor growth [12] and angiogenesis [5,13].

The abnormal expression of c-Met in cancer-associated fibroblasts (CAFs) may be important for the formation of tumor microenvironment. CAF is one of the abundant stromal cells within the tumor microenvironment which contributes to tumor progression [14]. They are known to be derived from various types of cells such as normal fibroblasts, mesenchymal stem cells, and pericytes [15]. It was reported that CAFs enable cancer cells to be more resistant to cancer drugs [16]. In addition, CAFs are thought to support tumor growth by transferring high energy metabolite to cancer cells through aerobic glycolysis known as reverse Warburg effect [17]. In general, CAFs promote tumor progression by extracellular matrix remodeling and cytokine secretion [16]. HGF, one of the growth factors contributing to tumor proliferation and progression, is known to be produced by CAFs [18]. As aforementioned above, c-Met, a receptor for HGF, is normally expressed in epithelial origin cells, not mesenchymal origin cells. However, it was reported that c-Met expression can be up regulated in non-epithelial origin cells like fibroblasts [19]. Considering that c-Met signaling pathway play an important role in tumor angiogenesis and CAFs stimulate angiogenesis, HGF-c-Met autocrine signaling axis may be working on CAFs during tumor progression. But, the c-Met expression of

CAFs and its role in tumor progression has not been reported.

In this study, we demonstrated that cancer cells induce the expression of c-Met in CAFs. Using functional enrichment analyses, we also showed the possibility that c-Met-induced alteration of ECM expression may be associated with the establishment of tumor-preferable environment and angiogenesis. With the following experiments, we eventually suggest that c-Met signaling in cancer-associated stroma may play an important role in tumor progression and c-Met signal targeting drugs are still required to be developed.

II. MATERIALS AND METHODS

1. Human fibroblast isolation, cell culture, and co-culture

Based on the zonal concept of our previous study [20], NBFs were isolated from breast cancer patients undergoing surgery at Severance Hospital of the Yonsei University Health System, Korea. The normal region of the patient tissue was grossly examined by an experienced anatomical pathologist not to be contaminated with cancer cells. To isolate primary fibroblasts, as described previously, a fraction of tissue was cut into small pieces, added to an enzyme cocktail solution (ISU ABXIS, Seoul, Korea), and incubated at 37℃ in 5% CO2 overnight. Cells were obtained from digested tissue by being filtered through a 70 μm cell strainer, suspended with medium:Ficoll (3:2), and then separated by centrifugation at 90 g for 2 min. The supernatant containing fibroblasts was further centrifuged at 485 g for 8 min. The resulting pellet was resuspended with Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) medium (Gibco BRL, Grand island, NY) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin with 100 μg/ml streptomycin (Gibco BRL, Grand island, NY) and cultured at 37℃ in a humidified 5% CO2 incubator. The fibroblastic characteristics of the isolated cells was confirmed by both microscopic morphology determination and immunostaining with antibodies against vimentin (Abcam, Cambridge, UK), cytokeratin (Dako, Glostrup, Denmark) and cytokeratin 5 (Novocastra,

Newcastle upon Tyne, UK). Breast cancer cell lines (MDA-MB-231, MCF7, BT-474, SK-BR-3, Korean Cell line Bank, Seoul, Korea) and EA.hy926 (American Type Culture Collection, RockVille, MD) were cultured in the DMEM and RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS, respectively. For co-culture, near-confluent NBF were incubated with the serum-free DMEM/F12 containing 5 μM CellTracker Green CMFDA (5-chloromethylfluroecein diacetate; Invitrogen, Carlsbad, CA) at 37℃ for 30 min, washed with phosphate-buffered saline (PBS), and replaced with fresh, pre-warmed medium. Subsequently, unstrained cancer cells were seeded onto the CMFDA-stained fibroblasts, and then cultured with the reduced serum medium composed of DMED/F12, 0.5% FBS and 100 IU/ml penicillin with 100 μg/ml streptomycin for 4 days.

2. Real-time PCR

Total RNA was extracted from the samples using TRIzol (Invitrogen, Carlsbad, CA). The integrity of total RNA samples was measured with the NamoDrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA). For cDNA synthesis, 1 μg of total RNA (A260/280 = 1.8 ~ 2.1) was reverse-transcribed with Hyperscript™ First strand synthesis kit (Geneall®, Seoul, Korea) in a final volume of 20 μl by the manufacturer's manual. The resulting

cDNAs were 5 fold-diluted with nuclease-free water. Real-time PCR analysis was performed with the reaction mixture containing 1 μl of the diluted cDNA, 1 μL of forward and reverse primer (0.5 μM), 7 μl of ddH2O, and 10 μl of Labopass™ SYBR Green Master Mix (Cosmogenetech, Seoul, Korea) using the CFX connect™ Real time PCR systems (BIO-RAD, Hercules, CA, USA). The real-time PCR was triplicated with the samples separately prepared. The relative expression of an interest gene was analyzed using ΔΔCt method and the normalization with the geomean values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex flavoprotein subunit A (SDHA), and hypoxanthin phosphoribosyltransferase 1 (HPRT1) [21].

3. ECM array

Total RNAs were extracted from the NBFs transfected with empty or c-Met overexpression plasmid and reversely transcribed as described above. From the resulting cDNAs, c-Met overexpression was first investigated, and then the expression profiling of ECM genes was performed using human RT2 Profiler PCR Arrays (Quiagen, Hilden, Germany). Briefly, 5 μg of total RNA were reverse-transcribed and 6-fold diluted to be the final volume of 120 μl with RNase-free water. For the reaction mixture, 102 μl of the diluted cDNA was mixed with 1350 μl of 2x RT2 SYBR Green Master mix and 1248 μl of RNase-free water. The profiling analysis of ECM gene expression was

performed using the CFX connect™ Real time PCR systems (BIO-RAD, Hercules, CA, USA) as described in the section of real-time PCR.

4. Functional enrichment analysis

Gene ontology (GO) term enrichment was performed with the genes above 1.5-fold up- or down-regulated in c-Met overexpressing NBF compared to the wild-type using STRING [22].

5. Western blot analysis

For protein extraction, cells were harvested, lysed with a PRO-PREP™ kit (iNtRON biotechnology, Seoul, South Korea), and then centrifuged at 10,000 x g for 15 min. The resulting supernatant was transferred to a new 1.5 ml tube and its protein concentration was determined using a Bradford method. For electrophoresis, 20 μg of cell lysate was denatured in NuPAGE sample buffer (Invitrogen, Carlsbad, CA) by being boiled at 95℃ for 10 min, resolved on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) with MOPS SDS running buffer (Invitrogen, Carlsbad, CA), and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked at RT for 1h with Dulbecco's phosphate buffered saline containing 0.05% Tween and 0.5% bovine serum albumin (BSA) (Gibco BRL). Blots were probed with the antibodies against c-Met, phospho-

c-Met (Abcam, Cambridge, UK), MMP1, VEGFR2, VEGFA, GAPDH (Santa Crus Biotechnology, In., CA), followed by incubation with HRP-tagged secondary antibodies (GenDEPOT, Barker, TX), and then visualized using an enhanced chemiluminescence (ECL) detection kit (GenDEPOT, Barker, TX).

6. Immunohistochemical staining analysis

The immunohistochemical staining analysis of c-Met expression was performed on the tissue images from Human Protein Atlas [23]. The URLs of the images as follows. Normal tissue 1 (https://www.proteinatlas.org/images/5282/15802_B_1_4.jpg), Normal tissue 2 (https://www.proteinatlas.org/images/5282/15802_B_2_4.jpg), Ductal carcinoma tissue 1 (https://www.proteinatlas.org/images/18577/42310_A_4_8.jpg), Ductal carcinoma tissue 2 (https://www.proteinatlas.org/images/18577/42310_A_5_3.jpg), Lobular carcinoma tissue (https://www.proteinatlas.org/images/18577/42310_A_5_1.jpg).

7. Transfection of c-Met overexpressing plasmid in CAFs.

NBFs were seeded in 6-well plate and cultured to be near confluent (80 ~ 90 % confluence) at the time of transfection. 2 hour before transfection, growth medium was replaced with pre-warmed Opti-MEM medium (Gibco BRL). Transfection mixtures were prepared as the manufacturer's instruction. Briefly, Lipofectamin solution (3 μl of Lipofectamin® LTX reagent in 150 μl of Opti-MEM medium) and DNA solution (2 μg of c-Met overexpression plasmid and PLUS™ reagent in 150 μl of Opti-MEM medium) were mixed and incubated at RT for 5 min, and then treated onto the NBFs at 37℃ with 5% CO_2 in a humidified incubator overnight. Next day, the Opti-MEM containing transfection mixture was replaced with fresh and pre-warmed growth medium.

8. Tube formation assay

Matrigel® matrix (Corning, Tewksbury, MA) was first diluted to 10 mg/ml with cold serum free medium, added to the wells of pre-chilled 24-well plate (289 μl / well), and then incubated at 37℃ for 1hr. After a careful removal of the remaining liquid from the wells, 300 ul of the cell suspension containing 1.2 x 10^5 of the EA.hy926 cells stained with Cell Tracker™ Red CMTPX dye (ThermoFisher, Waltham, MA) was added to the wells and incubated 37℃ with 5% CO2 in a humidified incubator for 16 ~ 18 hs. The images were

acquired every 2 hrs for 24 hrs using Eclipse Ti-U (Nickon, Tokyo, Japan)

9. Proliferation assay

$1x10^3$ of the EA.hy926 cells were prepared in 96-well plate. The media was replaced with CM and incubated in a humidified incubator (37℃, 5% CO_2) for 18 hours. The control cells were incubated with a serum-free media in the same condition. 10 ul of CCK-8 solution (Dojindo Molecular Technologies, Inc, Rockville, MD, USA) was added to each well of the plate and incubated for 1-4 hours in the incubator. The absorbance was measured at 450nm and 650nm using a microplate reader. Relative proliferation rate was calculated with the background-subtracted absorbance values (450 – 650 nm).

10. Statistical analysis

Statistical significance was determined by Student t-test. A single, double, and triple asterisk represented $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

III. RESULTS

1. Cancer-associated stroma showed increased expression of c-Met compared to normal stroma in breast cancer.

c-Met is known to be normally expressed in epithelial origin cells. As shown in Figure 1A, a variety of human breast cancer cell lines showed the expression of c-Met. However, c-Met expression was also founded in non-epithelial cells, especially fibroblasts (Figures 1B). This observation made us to hypothesize that c-Met expression in cancer-associated stroma is associated with tumor progression. Therefore, to investigate whether c-Met expression is up regulated in cancer-associated stroma compared to normal stroma, c-Met expression was analyzed using Finak Breast, a dataset of Oncomine database [24]. In Figure 2A, cancer-associated stroma showed a significantly higher expression of c-Met than that of normal stroma (p value = 9.64E-11). In the immunohistochemical images from the Human Protein Atlas, ductal carcinoma and lobular carcinoma tissues showed much higher expression of c-Met in stroma than normal breast tissues (Figure 2B). In addition to up regulated expression, the genetic alteration may be related with the role of c-Met in tumor progression. So, we investigated the genetic alteration of c-Met using the breast cancer databases of The Cancer Genome Atlas (TCGA). The total frequency of c-Met genetic alteration (mutation, deletion, and amplification) in breast cancer tissues was below 4% in most of the databases

(Figure 3). Exceptionally, BCCRC Xenograft database showed around 20% frequency of c-Met amplification.

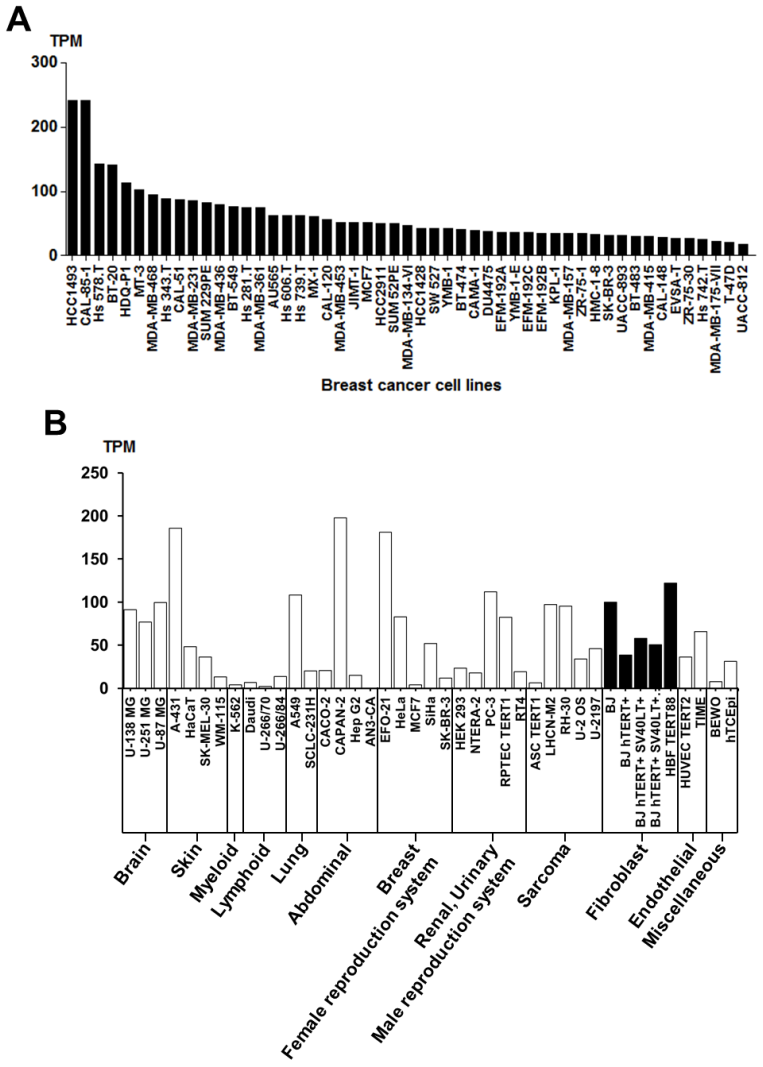

Figure 1. Overview of c-Met RNA expression in human cell lines. A) c-Met RNA expression in human breast cancer cell lines. The TPM values from Expression Atlas were used for comparative analysis. B) c-Met RNA expression in the human cells lines sorted after organ phenotypic resemblance. The TPM values from The Human Protein Atlas were used for comparative analysis.

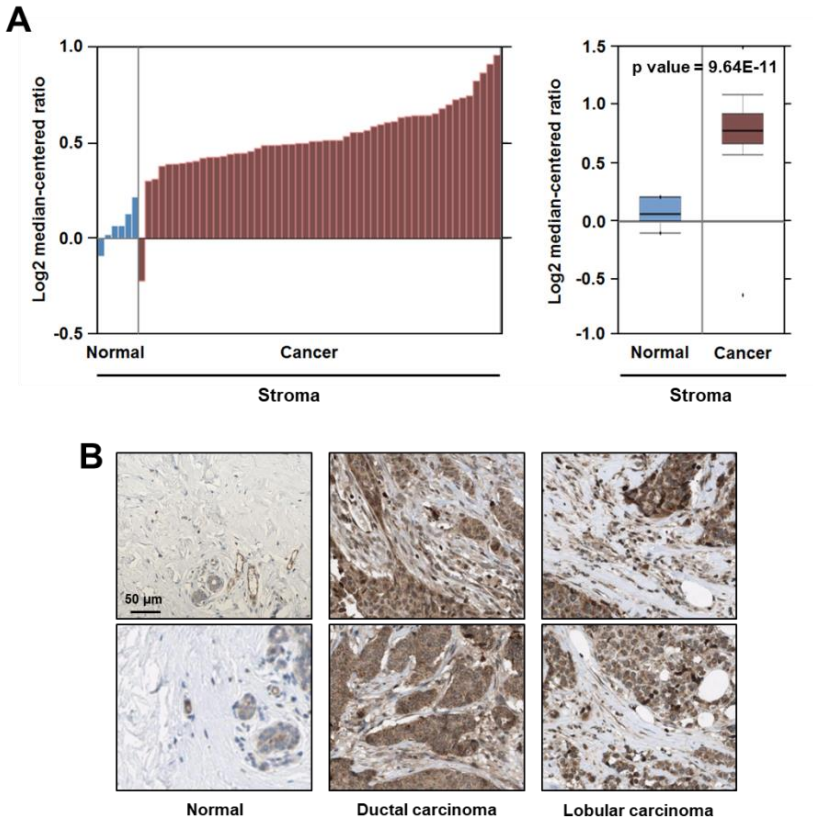

Figure 2. c-Met expression was up regulated in breast cancer-associated stroma. A) Comparative analysis of c-Met expression between breast normal and cancer-associated stroma. The c-Met expression of normal and cancer-associated stroma was analyzed using a dataset of Oncomine database, Finak Breast. B) Immunohistochemical staining of normal and breast cancer tissues. The immunohistochemical images of c-Met staining were obtained from The Human Protein Atlas.

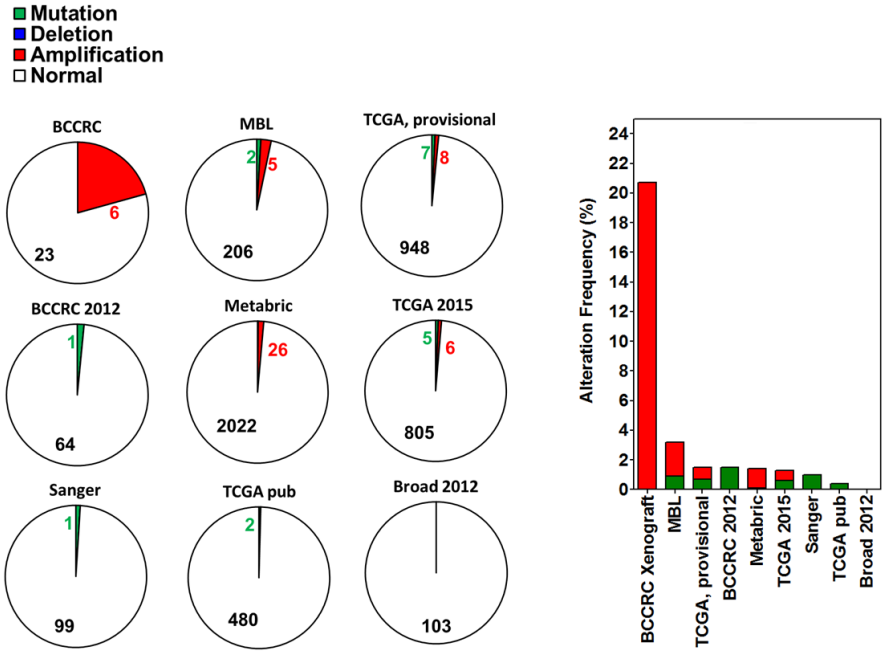

Figure 3. The genetic alteration of c-Met in the breast cancer data sets of TCGA database. The number of the patient with c-Met alteration in each database and the frequency of genetic alteration in breast cancer patient tissues are shown. The mutation, deletion, and amplification of c-Met were analyzed using the breast cancer data sets of TCGA.

2. Breast cancer cells up regulated the c-Met expression in normal fibroblasts.

Cancer-associated fibroblast (CAF) is a major population of breast cancer stromal cells [25], and known to be generated from a various type of cells including normal fibroblast by cancer stimulation [15]. To examine whether c-Met expression is up regulated in NBF by cancer stimulation, NBF was co-cultured with breast cancer cell lines. First, breast cancer cells showed a similar level of c-Met expression before (cultured alone) and after co-culture (Figure 4A). On the other hand, the expression of c-Met mRNA was up regulated in NBF by all 4 intrinsic types of breast cancer cell lines compared to the control NBF cultured alone (Figure 4B). In addition, the expression of HGF, the only known ligand for c-Met, was also found to be up regulated in NBF by co-culture with breast cancer cell lines (Figure 4C). The expression of c-Met and HGF protein was also increased in NBF by cancer stimulation, and the phosphorylation of c-Met (tyrosine 1356) was increased (Figure 4D).

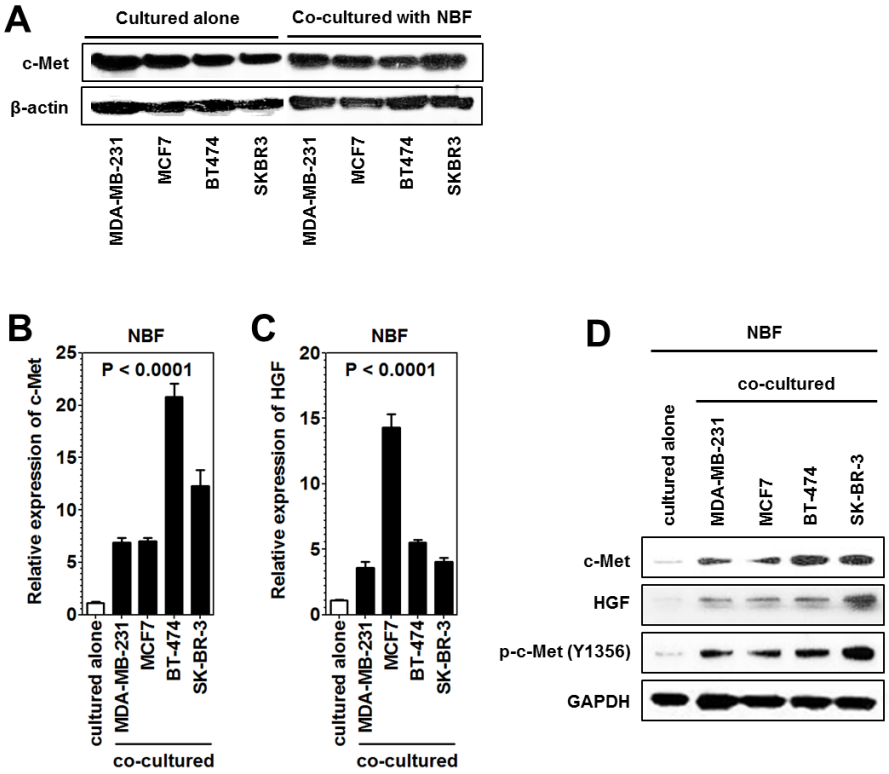

Figure 4. Breast cancer cells up regulated the c-Met expression in normal fibroblasts. A) c-Met expression of the breast cancer cell lines cultured alone or co-cultured with NBF. mRNA expression analysis of B) c-Met and C) HGF in the NBF cultured alone or co-cultured with breast cancer cell lines. D) Western blot analysis of c-Met, HGF and phospho-c-Met in the NBF cultured alone or co-cultured with breast cancer cell lines. Normal breast fibroblasts were isolated from the non-cancerous tissue region of an invasive ductal carcinoma patient, and then immortalized by hTERT overexpression. The breast cancer cell lines represented 4 intrinsic types of breast cancer: MDA-MB-231 for triple negative, MCF7 for luminal B, BT-474 for luminal A, and

SK-BR-3 for Her2. For co-culture, NBF was first cultured to be confluent, stained with green-fluorescent dye, and then cultured with cancer cell lines for 4 days. After co-culture, cancer cells and NBF were separated by FACS sorting.

3. The c-Met overexpression in cancer-associated fibroblasts may be associated with extracellular matrix remodeling and angiogenesis.

CAFs contribute to tumor progression by a distinct pattern of ECM expression [25,26]. To investigate whether c-Met overexpression leads to the alteration of ECM gene expression, total RNA was extracted from the NBF transfected with c-Met overexpression plasmid, reverse-transcribed, and then analyzed using ECM array. The result of ECM array analysis was shown as heatmap of up- or down-regulated ECM genes in NBFs and c-met overexpressing NBFs (Figure 5A). 7 genes (MMP1, ITGB1, TIMP2, COL1A1, VCAN, CTGF, and TGFB1) were significantly up regulated in the NBF transfected with c-Met overexpression plasmid compared to the control, wild-type NBF (Figure 5B). Of them, MMP1 was the most up regulated gene (Table 1). On the other hand, 56 genes were down regulated in the NBF transfected with c-Met overexpression plasmid compared to the control.

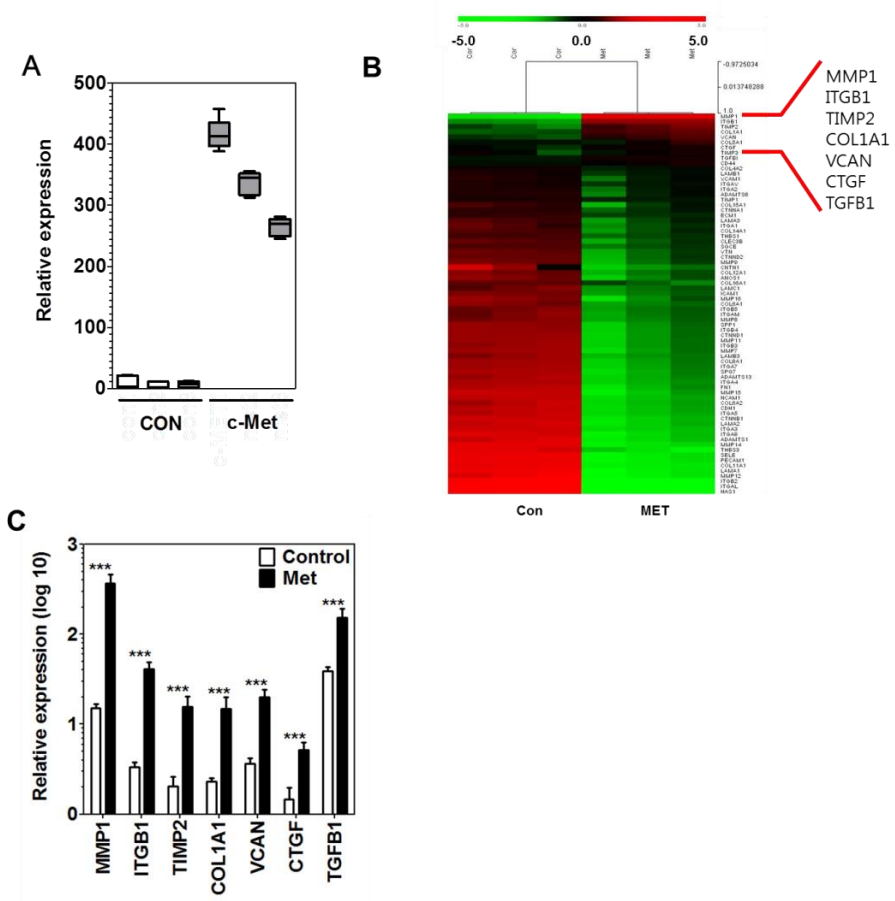

Figure 5. c-Met overexpression induced the alteration of extracellular matrix expression in breast fibroblasts. A) Real-time PCR analysis of c-met expression in wild type NBF and c-met overexpression plasmid transfected NBF. B) The heatmap of ECM expression in wild-type and c-Met overexpressing NBFs. C) Real-time PCR analysis of the up regulated genes in c-Met overexpressing NBFs. Total RNA was extracted from the NBF transfected with c-Met overexpression plasmid, reverse-transcribed, and then analyzed using ECM array. The ECM array was triplicated with separately

prepared cDNA. Wild-type NBF was used as a control.

Table 1. The list of the ECM molecules up regulated in c-Met overexpressing breast fibroblasts.

Gene	Average Relative expression		Fold Up- or Down-Regulation	p-value
	Con	Met		
MMP1	0.464702	4.076488	8.77	0.000000
ITGB1	0.103736	0.438942	4.23	0.000000
TIMP2	0.061741	0.20556	3.33	0.007999
COL1A1	0.071272	0.188955	2.65	0.000339
VCAN	0.107857	0.276331	2.56	0.006058
CTGF	0.043055	0.075125	1.74	0.049617
TGFBI	1.119617	1.747913	1.56	0.000064

4. c-Met overexpressing breast fibroblasts promoted endothelial cell growth and tube formation.

To examine which biological process can be induced by the altered gene expression, functional enrichment analysis was performed using STRING. In Table 2, c-Met-up regulated genes may be associated with extracellular matrix organization and blood vessel development. Angiogenesis, a type of blood vessel development, is a critical event for tumor progression [27]. Based on the functional enrichment analysis, we assumed that c-Met overexpressing NBF promotes endothelial cell growth and/or tube formation. To confirm the assumption, proliferation rate and tube formation capacity were analyzed in the EA.hy926 cells, an immortalized endothelial cells, treated with the conditioned medium (CM) collected from wild-type NBF, c-Met overexpressing NBF, and the c-Met overexpressing NBF treated with ARQ197. The CM from c-Met overexpressing NBF induced faster and better-organized tube formation than the CM from the control, whereas the CM from the c-Met overexpressing NBF treated with ARQ197 induced less organized tube structures than that from c-Met overexpressing NBF (Figure 6A). As shown in Figure 6B, the proliferation rate was also similar with the tube formation pattern. The proliferation of EA.hy926 cells was increased by the CM from c-Met overexpressing NBF compared to the control, whereas it was not increased by the CM from the c-Met overexpressing NBF treated with ARQ197.

Table 2. Functional enrichment analysis of the genes up regulated by c-Met overexpression.

Biological Process (GO)			
pathway ID	pathway description	count in gene set	FDR
GO:0030198	extracellular matrix organization	7	2.37E-09
GO:0072358	cardiovascular system development	5	0.00408
GO:0072359	circulatory system development	5	0.00408
GO:0001568	blood vessel development	4	0.0145
GO:0022617	extracellular matrix disassembly	3	0.0145
GO:0001944	vasculature development	4	0.015
GO:0051216	cartilage development	3	0.0228
GO:0061448	connective tissue development	3	0.0452
GO:0001503	ossification	3	0.0482
GO:0009611	response to wounding	4	0.0482
GO:0010712	regulation of collagen metabolic process	2	0.0482
GO:0016477	cell migration	4	0.0482
GO:0030154	cell differentiation	6	0.0482
GO:0050900	leukocyte migration	3	0.0482
GO:0051674	localization of cell	4	0.0482

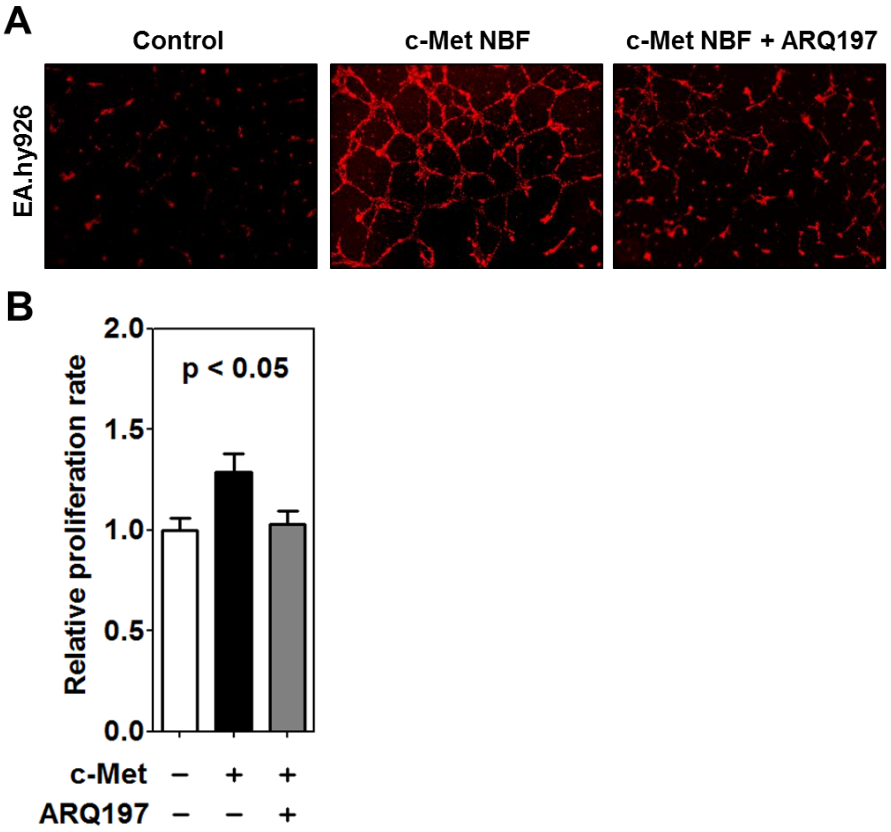

Figure 6. c-Met overexpressing breast fibroblasts promoted endothelial cell growth and tube formation. A) Endothelial cell tube formation assay and B) proliferation assay. EA.hy926 cells were incubated with the CM from wild-type NBF, c-Met overexpressing NBF, and the c-Met overexpressing NBF treated with ARQ197.

5. c-Met expression in cancer-associated fibroblasts contributes to angiogenic process.

MMP1 was the most up regulated gene in c-Met overexpressing NF. Therefore, it may be plausible that MMP1 mainly affects the proliferation and tube formation of EA.hy926 cells. According to a previous study, the expression of vascular endothelial growth factor receptor 2 (VEGFR2) can be up regulated by MMP1 [28]. The proliferation and vascular structure formation of endothelial cells are associated with VEGFR2 expression [29,30]. To examine whether the VEGFR2 expression of EA.hy926 cells is related to the ECM alteration caused by c-Met overexpressing NBF, the EA.hy926 cells were treated with the CM from c-Met overexpressing NBF. The CM from c-Met overexpressing NBF increased the expression of VEGFR2 in EA.hy926 cells, whereas, it was suppressed by the inhibition of c-Met activation (Figure 7A). Also, to make in-vivo like situation, the CM from co-cultured CAF and breast cancer cells were treated. VEGFR2 expression is induced by the CM from co-cultured cells, while it was inhibited by the treatment of ARQ197 (Figure 7B). VEGFA is a ligand of VEGFR2 and the activation of VEGFR2 is dependent on VEGFA. So, we analyzed the expression of the VEGFA from the co-culture of breast cancer cells and CAFs. In Figure 7C, VEGFA was up regulated in the co-cultures of CAFs and breast cancer cells compared to the control, the breast cancer cells and NBF cultured alone.

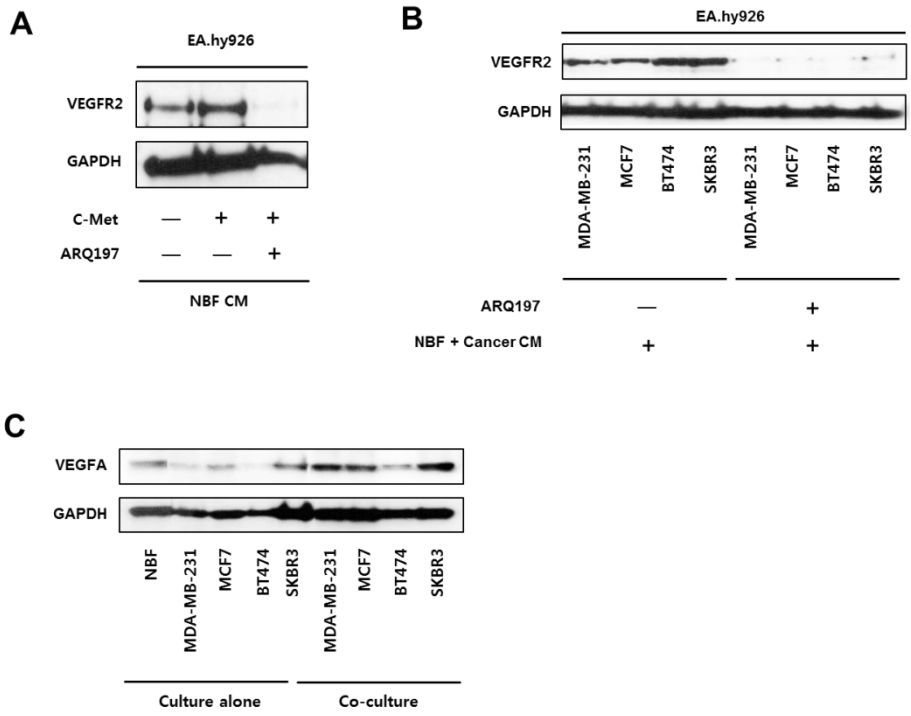

Figure7. The c-Met expression in cancer-associated fibroblasts contributes to angiogenic process. Western blot analysis of VEGFR2 in A) the EA.hy926 cells incubated with the CM from wild-type NF, c-Met overexpressing NF, and the c-Met overexpressing NF treated with ARQ197, B) the EA.hy926 cells incubated with the CM from co-cultured CAF and breast cancer cells and the CM including ARQ197, and C) VEGFA expression in the breast cancer cells and NBF cultured alone and co-cultured NBF and breast cancer cells.

IV. DISCUSSION

For recent years, CAFs have been emerging as an appealing therapeutic target for cancer treatment. The drug resistance of cancer cells is caused by genetic and/or epigenetic instability [31], which is thought to be the biggest limitation of the cancer therapeutic strategy targeting cancer cells. Compared to cancer cells, CAFs are very genetically stable, and therefore less likely to have drug resistance [32]. In this regard, CAFs have recently been considered as an alternative target for cancer therapy [33,34]. Interestingly, the dense fibrosis composed by CAFs acts as a barrier to block antitumor agents into tumor tissues [16]. This may mean that targeting CAFs is one of the ways to compromise the dense fibrosis, thereby enable antitumor agents to be delivered into tumor tissues. In addition, targeting CAFs may result in a prohibitory effect on tumor progression. It is acknowledged that CAFs provide tumor-preferable microenvironment by ECM remodeling, which contributes to the survival, proliferation, and metastasis of cancer cells [16,34].

In our study, we focused on c-Met expression in cancer-associated stroma, especially CAF, and its role in tumor progression. According to previous studies, c-Met is generally expressed in epithelial origin cells, whereas its ligand, HGF, is expressed in mesenchymal origin cells such

as fibroblast [8]. However, the c-Met expression of non-epithelial origin cells was also reported in The Human Protein Atlas, a public database (Figure 1B). The report made us to investigate whether c-Met is expressed in CAFs and if so what role they play in tumor progression. c-Met signaling is known to be associated with tumor growth and angiogenesis [5,12,13]. Therefore, many drugs targeting c-Met or HGF, the only known ligand for c-Met, have been developed and gone through clinical trials. Unfortunately, however, few drugs show a significant inhibitory effect on tumor progression. Despite the development difficulty of effective c-Met targeting drugs, the role of c-Met signaling in tumor progression still remains valuable to be studied.

To understand whether the c-Met expression is a characteristic of CAF-state, NBFs were isolated and then stimulated by breast cancer cells through co-culture. As shown in Figure 4C, c-Met expression was dramatically increased in the CAFs co-cultured with breast cancer cells. Next, to investigate if the c-Met expression of NBFs contributes to tumor progression, we analyzed the alteration of ECM gene expression in the NBF transfected with c-Met overexpression plasmid since a major role of CAF in tumor progression is to establish tumor-preferable ECM environment [16]. In the functional enrichment analysis with up

regulated genes, ECM organization and blood vessel formation were predicted to be promoted by c-Met overexpression in CAFs (Table 2). On the other hand, in the functional enrichment analysis with down regulated genes, ECM organization and cell adhesion were predicted to be suppressed by c-Met overexpression. Based on the functional enrichment analyses, we cannot make a conclusion that c-Met overexpression in CAFs contributes to tumor-preferable ECM remodeling. But, this result certainly implicates that c-Met expression in CAFs can be associated with tumor progression and therefore needed to be investigated more. As an example, we examined the effect of c-Met overexpressing NBFs and CAFs on the proliferation and tube formation of endothelial cells because blood vessel formation is a process of angiogenesis, a critical event for tumor metastasis. Interestingly, c-Met overexpressing NBFs showed the increased level of MMP1 expression, an ECM to increase VEGFR2 expression in ECs [28]. The proliferation and vascular structure formation of endothelial cells are associated with VEGFR2 expression [29,30]. Moreover, the CM from c-Met overexpressing NBFs were able to induce faster and better-organized tumor formation of EA.hy926 cells than the wild-type CM (Figure 5B).

In this study, we showed that cancer cells induce the expression of c-Met in CAFs, which leads to the alteration of ECM gene expression. According to our functional enrichment analyses, c-Met-induced ECM alteration may contribute to the establishment of tumor-preferable environment and be associated with angiogenesis. Therefore, our results suggest that c-Met signaling in cancer-associated stroma may play an important role in tumor progression and c-Met signal targeting drugs are still required to be developed.

V. CONCLUSION

In conclusion, these findings suggest that c-met signaling in cancer-associated stroma influence the alteration of ECM molecule expression and may play an important role in tumor progression. Therefore, c-Met signal targeting drugs are still required to be developed.

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ABSTRACT (IN KOREAN)

유방암 섬유아세포에서의 c-Met 과발현에 의한 신생혈관 신호 유도

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문 성 경

종양에서 c-Met의 과발현은 암의 진행과정과 관련이 있다. C-Met은 주로 상피세포 유래의 세포에서 발현되는 것으로 알려져 있지만, 섬유아세포와 내피세포와 같은 비상피세포 유래의 세포에서도 발현되는 것으로 보고되었다. 따라서 암 관련 기질에서의 c-met 발현이 종양의 진행에 중요한 역할을 할 수 있다고 생각되지만 그에 관련해서 아직 밝혀진 바가 없다. 이 연구에서는 종양미세환경에서 가장 풍부하게 존재하는 암 관련 섬유아세포에서 c-Met의 발현이 종양의 진행에 미치는 영향을 알아보고자 한다. 먼저, 정상 유방에서 섬유아세포를 얻은 뒤, 유방암세포와 함께 배양함으로써 유사 암 관련 섬유아세포 상태로 만들었다. 그 결과 유방암세포의 고유한 아형에 상관없이 정상 유방 섬유아세포에서 c-Met의 발현이 증가하

는 것을 발견하였다. 이를 통해 c-Met의 과발현이 CAF의 주요한 기능인 세포외기질 재구성에 영향을 미칠 것이라고 가정하였고, cDNA 마이크로어레이를 이용하여 c-Met 과발현 플라스미드를 주입한 유방 섬유아세포에서 세포외기질 분자들의 c-Met 의존적인 발현 변화를 분석하였다. 마이크로어레이 분석 결과 7개의 유전자 발현이 1.5배 이상 증가하였고, 56개의 유전자 발현이 감소하였다. 발현이 증가한 7개의 유전자로 실행한 기능 강화 분석에서 세포외기질 구조변화와 혈관 생성 과정이 촉진됨을 확인하였다. c-Met 과발현 유방 섬유아세포에서 발현되는 세포외기질 분자들이 신생혈관과정에 미치는 영향을 알아보기 위해 내피세포의 튜브 형성 실험을 진행하였다. 그 결과 유방 섬유아세포에서의 c-Met 활성에 의존적으로 튜브 형성이 조절되는 것을 관찰하였다. 또한, c-Met 과발현 섬유아세포에 의한 혈관내피증식인자 수용체의 발현이 조절되는 것을 확인하였다. 따라서 이번 연구는 암 관련 섬유아세포에서의 c-Met 과발현이 세포외기질의 발현 변화를 통해 신생혈관과정을 촉진할 수 있다는 것을 시사한다.

핵심 되는 말: c-Met, 암 관련 섬유아세포, 혈관내피성장인자수용체, 유방암, 신생혈관