Cancer-associated fibroblasts regulate gene expression of breast cancer cells via exosomal microRNAs

Ji Eun Kim

Department of Medical Science

The Graduate School, Yonsei University

Cancer-associated fibroblasts regulate gene expression of breast cancer cells via exosomal microRNAs

Directed by Professor Nam Hoon Cho

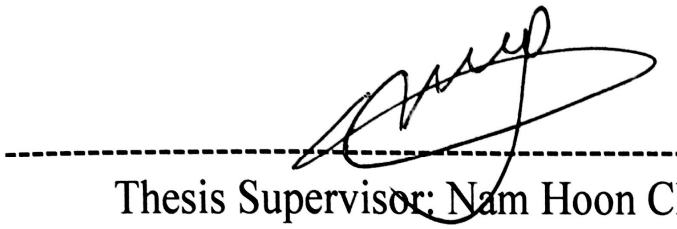
The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Ji Eun Kim

June 2015

This certifies that the Master's Thesis

of Ji Eun Kim is approved.



Thesis Supervisor: Nam Hoon Cho

Thesis Committee Member#1: Joo Hyuk Sohn

Thesis Committee Member#2: Ja Seung Koo

The Graduate School

Yonsei University

June 2015

ACKNOWLEDGEMENTS

I sincerely appreciate all those who gave me emotional support as well as valuable advice during my master's degree program.

Firstly, I deeply appreciate my supervisor Dr. Nam Hoon Cho. His generosity, thoughtful guidance, and enthusiasm motivated me throughout my entire research. It was my honor to study in his laboratory. I truly thank Dr. Joo Hyuk Sohn and Dr. Ja Seung Koo for advising my research as well.

Especially, I would like to express my sincere gratitude to my lab members, Dr. Baek Gil Kim, Dr. Hyun Ho Han, Mrs. Suki Kang, Mr. Joo Hyun Lee and Dr. Sung Hwan Lee. Without their generous help and invaluable advice, this study would not be able to reach the present goal. Deeply appreciate their kindness. Also thank my schoolmates, Jeong Eun, Jee San, Chan Woo, Hae-Ji, Ara, and Ji Hye for their support and warmhearted words.

I want to thank my best friends, Mi Jeong, Hye Rim, Ji Won, Seo Ryung, and Diana for their encouragement.

Lastly, I appreciate my parents, my brother, my entire family, mom Shirley and Baird family for their continuous support. Their patience, belief and love helped me to endure hardships and finish my master's degree at Yonsei University.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Cancer-associated fibroblasts regulate gene expression of breast cancer cells via exosomal microRNAs

Ji Eun Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Nam Hoon Cho)

Cell-derived vesicles known as exosomes facilitate communication between cancer cells and cancer-associated fibroblasts (CAFs). CAF-derived molecules, including microRNAs (miRNAs), were recently found to be transferred to cancer cells via exosomes. However, little is known about the role of CAF-derived exosomal miRNAs in tumor progression. To investigate the relationship between miRNA levels in fibroblast-derived exosomes and breast cancer progression, we analyzed exosomal miRNAs of normal breast fibroblasts (NBFs) and CAFs using microarray, examined the delivery of exosomal miRNAs from fibroblasts to cancer cells by direct exosome treatment, and confirmed the function of exosomal miRNA in gene regulation through overexpression. Several miRNAs were

downregulated more than two-fold in CAF-derived exosomes, compared to NBF-derived exosomes. Endogenous miR-4516, a miRNA downregulated five-fold in CAF-derived exosomes, was also downregulated in CAF cells. In MCF7 cells, NBF-derived exosomes delivered four-fold more miR-4516 than CAF-derived exosomes. Overexpression of miR-4516 in MCF7 and MDA-MB-231 cells induced downregulation of several tumor progression-associated genes (PHF8 and STAT3). Thus, CAFs may regulate gene expression in breast cancer cells via exosomal miR-4516. By modulating the expression level of miR-4516 in CAF and NBF-derived exosomes, breast cancer cells could circumvent the inhibitory effect of exosomal miR-4516, promoting tumor progression.

Key words: exosomes, microRNA, cancer-associated fibroblasts, tumor microenvironment, breast cancer cells

Cancer-associated Fibroblasts regulate gene expression of breast cancer cells via exosomal microRNAs

Ji Eun Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Nam Hoon Cho)

I. INTRODUCTION

Cancer-associated fibroblasts (CAFs) are one type of stromal cells composing the tumor microenvironment (TME) and have been found to build a tumor-preferable microenvironment, such as a dense extracellular matrix, and secrete growth factors or cytokines to communicate with cancer cells.[1,2] At the early stage of tumorigenesis or under non-tumorigenic conditions, cancer is suppressed by surrounding normal fibroblasts and other stromal components, while CAFs promote cancer progression, invasion and migration at the late stages of tumorigenesis.[3-6] Hence, communication mediators between CAFs and

cancer cells have been one of the major issues in cancer research. As one CAF-secreted mediator, the exosome carries several molecules, including microRNAs (miRNAs), to cancer cells exogenously.[7]

The exosome is a small 30–100 nm-sized vesicle and a bi-directional signaling factor between CAFs and cancer cells.[8-10] Secretion of exosomes has been observed in various cell types, such as breast cancer cells,[11] fibroblasts,[8] and mesenchymal stem cells.[12] Exosomal miRNAs are delivered to other cells via exosomes where they are internalized to regulate gene expression in recipient cells.[13] Valadi and his colleagues demonstrated that exosomes enclose miRNAs, as well as proteins and mRNAs, which are transferred to other cells and activate their functions.[10] For example, CD81 positive exosomes, secreted from CAFs, activate the Wnt-planar cell polarity signaling pathway to promote tumor progression in breast cancer cells.[8] Recently, leukemia cell-derived exosomal miR-92a was found to be transferred to endothelial cells, thereby enhancing endothelial cell tube formation.[7] Together, these studies indicate that exosomes may play an important role in cell to cell interaction, but the precise role of exosomal miRNA in this process is not understood; thus, further study of its role in cancer progression is required. MicroRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level.[14,15] It is widely accepted that miRNAs play dual roles and act on multiple targets, allowing them to act as either tumor suppressor genes or oncogenes.[16] In response to various kinds of extracellular stimulation, CAFs endogenously transcribed different types of miRNAs.[17,18]

In this study, we demonstrated that breast cancer cells may affect the composition of miRNAs organized within CAF-derived exosomes, leading to changes in target gene expression in breast cancer cells and in the communication between cancer cells and stromal cells. Thus, we hypothesized that the differential composition of miRNAs in exosomes derived from CAF and normal breast fibroblast (NBF) was responsible for regulation of gene expression in breast cancer cells. Supporting this hypothesis, specific exosomal miRNA was differentially contained between CAF and NBF-derived exosomes, and overexpression of miRNA in cancer cells suppressed the expression of putative target genes required for tumor progression.

II. MATERIALS AND METHODS

1. Cell culture

CAFs and NBFs were isolated from breast cancer patients undergoing surgery at Severance Hospital of the Yonsei University Health System, Korea. A pathologist examined and obtained representative samples of CAFs and NBFs. A fraction of tissue was used to isolate primary fibroblasts as previously described.[19,20] Briefly, isolated tissues were chopped into small pieces, added to a digestion solution of Enzyme Cocktail (ISU ABXIS, Seoul, Korea), and incubated at 37°C in 5% CO_2 overnight. Digested tissue was filtered through a 100μm cell strainer and suspended in medium:Ficoll (3:2), followed by centrifugation at 670rpm for 2 minutes. Then, the supernatant containing fibroblasts was transferred to a tube and centrifuged at 1,400 rpm for 8 min. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) medium (Gibco®, Gaithersburg, MD, USA) supplemented with 1% penicillin/streptomycin and 20% fetal bovine serum (FBS) (Gibco®). Further confirmation of fibroblast characteristics was obtained via morphology portrait and immunofluorescence observation using antibodies against cytokeratin (Dako, Glostrup, Denmark), vimentin (Abcam, Cambridge, UK), and cytokeratin 5 (Novocastra, Newcastle upon Tyne, UK).

Breast cancer cell lines (MDA-MB-231 and MCF7) were cultured in DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 10% FBS. All cells were cultured at 37°C in 5% CO_2. For the stimulation of CAFs, the media was changed to MDA-MB-231

conditioned medium (231 CM) and incubated for three days. Then, it was replaced with 0.5% FBS reduced serum medium (RSM), and incubated for two days for exosome preparation.

2. Exosome extraction from cell culture supernatants

NBFs were incubated with RSM for two days and CAFs were prepared as described above. The supernatant of NBFs and CAFs was collected and centrifuged at 3,000 ×*g* for 20 min to remove cell debris and filtered through a 0.22 μm filter (Nalgene™, Waltham, MA, USA). Due to the large volume of supernatant needed, 9kD molecular weight cutoff concentrators (Pierce, Rockford, IL USA) were used for concentration,[21] and the volume was concentrated to 10 ml, according to the manufacturer's protocol. The appropriate volume of ExoQuick-TC™ (System Bioscience, Mountain View, CA, USA) was added to the concentrated medium. After overnight incubation at 4°C, the mixture was centrifuged at 3,500 ×*g* for 30 min and the supernatant was removed by aspiration. Exosome pellets were suspended with a large volume of phosphate buffered saline (PBS) and centrifuged again. The supernatant was aspirated, and the exosome pellet was resuspended with serum-free DMEM/F12 (1:1) medium supplemented with 1% penicillin/streptomycin for further experiments.

3. Flow cytometry

For fluorescence-activated cell sorting (FACS) analysis, exosomes were prepared as described with minor modifications.[22,23] Briefly, exosomes were incubated with 4 μm aldehyde/sulfate latex beads (Invitrogen®, Carlsbad, CA, USA) overnight at room temperature in PBS with gentle

agitation. After centrifugation, exosome-coated beads were blocked by 100mM glycine for 1 hr. They were washed twice with PBS and resuspended with incubation buffer (PBS/0.5% (w/v) BSA). They were then incubated with PE mouse anti-human CD63 antibody (BD Pharmingen™, San Diego, CA, USA) or PE mouse IgG1, κ Isotype control antibody (BD Pharmingen™) for 2 hr at room temperature in the dark. Exosome-coated beads were analyzed by flow cytometry, using a FACSVerse (BD Biosciences, San Jose, CA, USA).

4. MiRNA Stem-Loop Real-Time polymerase chain reaction (qRT-PCR)

Total RNAs from CAF and NBF-derived exosomes, fibroblasts, and cancer cells were extracted by TRIzol RNA isolation reagent (Ambion®, Grand Island, NY, USA). RNA concentration was examined by ND-2000/2000c (NanoDrop Technologies Inc., Wilmington, DE, USA). To determine the endogenous level of miR-4516 in NBFs and CAFs, qRT-PCR was performed. A cDNA synthesis kit was used for reverse transcription (Enzinomics, Dae-jun, South Korea). Due to the short length of the miRNA, stem-loop cDNA synthesis was necessary as previously described.[24] Briefly, 1 μg of total RNA and 1 μl miR-4516 RT primer was prepared in a total volume of 20 μl. Reverse transcriptase reactions were performed in Veriti Thermocycler (Applied Biosystems®, Grand Island, NY, USA) for 30 min at 16°C, 30 min at 42°C and for 5 min at 85°C. Power SYBR® Green PCR Master Mix (Applied Biosystems®) was used for StepOnePlus™ qRT-PCR (Applied Biosystems®). Briefly, 1 μl cDNA was mixed with Power SYBR® Green PCR Master Mix, and universal

and miRNA primers in a total volume of 25 μl, according to the manufacturer's recommendations. The reactions were run in triplicate and the relative expression of intracellular miR-4516 was calculated with delta-delta Ct methods after normalization to 18S rRNA. The following PCR primers were used: miR-4516 RT primer: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCCCG-3', universal primer: 5'-CCAGTGCAGGGTCCGAGGTA-3', miR-4516 primer: 5'-CAGAACGGGAGAAGGGTCGG-3', 18S rRNA forward primer: 5′-GTAACCCGTTGAACCCCATT-3′ and reverse primer: 5′-CCATCCAATCGGTAGTAGCG-3′.

5. Transfer of exosomal miRNA

MCF7 cells were seeded in a 6-well plate prior to initiation of the experiment. When MCF7 cells were approximately 70% confluent, previously isolated CAF and NBF-derived exosomes suspended with serum-free DMEM/F12 (1:1) medium supplemented with 1% penicillin/streptomycin were directly added to each well. MCF7 cells were harvested after two days of incubation at 37°C in 5% CO_2. To analyze the amount of exosomal miR-4516 that was absorbed by MDA-MB-231 and MCF7 cells, total RNA from breast cancer cells was isolated as described above and qRT-PCR for miR-4516 and control 18S rRNA was performed. Relative expression of miR-4516 was measured by delta-delta Ct values and cells treated with CAF-derived exosomes were compared to cells treated with NBF-derived exosomes.

6. Transfection of MDA-MB-231 and MCF7 cells with mature miR-4516

MiR-4516 sequence was inserted into pENTR™/H1/TO vector (Invitrogen™). Two hours before transfection, medium in each well was changed to Opti-MEM medium (Gibco®). Lipofectamine® transfection reagent (Life Technologies, Grand Island, NY, USA) was used to transfect miR-4516 into MDA-MB-231 and MCF7 cells. The day after transfection, the medium was changed to fresh serum-free DMEM (Gibco®). After incubation for two days, the cells were harvested, followed by reverse transcription-PCR (RT-PCR) via the AmpONE™ kit (GeneAll, Seoul, South Korea). The following PCR primers were used: PHF8 primers forward: 5'-GGCTGCTGACATTGACCTCT-3' and reverse: 5'-CATGCCCAACCCATCCTTCT-3', STAT3 primers forward: 5'-ACCAGCAGTATAGCCGCTTC-3' and reverse: 5'-AGGCGTGATTCTTCCCACAG-3'.

7. Western blotting

For western blot analysis, proteins in cells were extracted using a PRO-PREP™ kit (iNtRON Biotechnology, Seoul, South Korea) and the concentration of proteins was determined using a Bradford method. Equal amounts of protein from cells were separated on SDS-PAGE gels (Life Technologies) and blotted onto PVDF membranes (Millipore, Billerica, MA, USA). Western blots were incubated with antibodies directed against PHF8 (1:2,000; Santa Cruz Biotechnology, Inc., CA, USA), STAT3 (1:2,000, Santa Cruz), and ß-actin (1:2,000; Santa Cruz), followed by probed with HRP-tagged secondary antibodies (1:10,000;

Invitrogen® and GenDEPOT, Barker, Texas, USA). The Protein-antibody complexes were developed using a luminol reagent (Santa Cruz).

8. MiRNA microarray and profiling

MiRNA microarray was performed by NanoString nCounter® miRNA expression assay (NanoString Technologies, Inc. Seattle, WA, USA). Each CAF- and NBF-derived exosome was collected continuously via ExoQuick-TC™ (System Bioscience) until appropriate amounts of exosome pellets were accumulated. We requested the miRNA microarray with isolated total RNAs from exosomes. The entire process was performed by the PhileKorea Company.

9. Tissue acquisition.

For the isolation of CAFs and NBFs, human breast cancer tissues were extracted from three IDC patients. The tissues were donated by patients who underwent surgery at Severance Hospital of the Yonsei University Health System, Seoul, South Korea. The protocol for the research was approved by the Severance Hospital Ethics Committee (IRB number 4-2008-0383). All patients agreed and signed the consent forms and were informed of the usage of their tissues for comprehensive experiments of breast cancer.

10. Statistical analysis

Two treatment groups were compared using the Mann–Whitney U-test or Student's t-test. Multiple group comparisons were done by one-way

ANOVA. For statistical analysis, GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA) for Windows was used. Results were considered statistically significant when $P < 0.05$.

III. RESULTS

1. Characterization of exosomes derived from CAFs

To confirm that exosomes were correctly collected from the supernatant of fibroblasts (Figure 1), exosome pellets were analyzed by flow cytometry with phycoerythrin (PE)-conjugated CD63 antibody, a widely known exosome marker,[10] or an isotype control antibody (Figure 2A). For further validation, exosomal RNAs were compared with total RNAs from fibroblast cell lysates. Total RNA extracted from the fibroblast cell lysate showed clear peaks representing 28S (5 kb) and 18S (2 kb) rRNAs, and the 28S/18S ratio was 2.3, which indicated intact total RNAs. On the other hand, the RNAs isolated from CAF-derived exosomes contained almost no 28S and 18S rRNAs and relatively small sized RNAs were detected (Figure 2B).

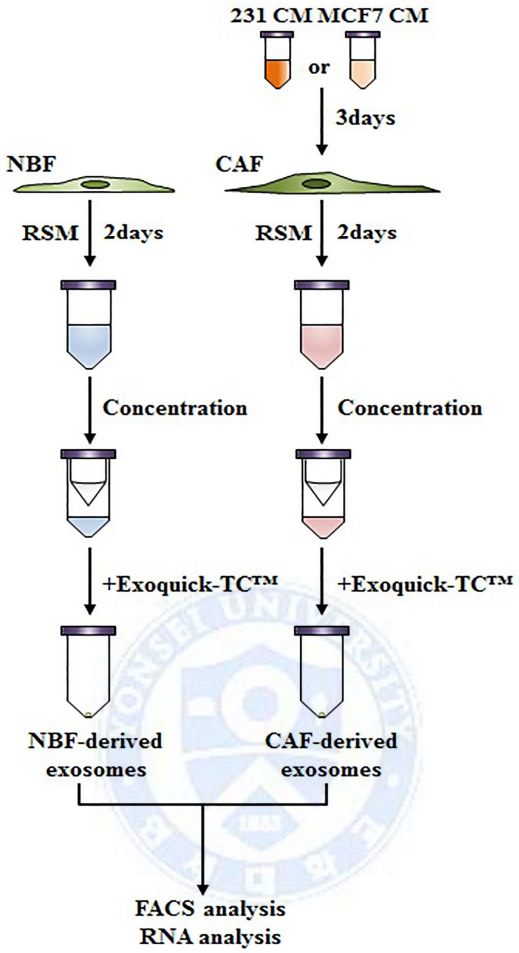


Figure 1. Exosome isolation process. CAFs were treated with MDA-MB-231 conditioned medium (231 CM) or MCF7 CM for three days, followed by replacement with 0.5% FBS reduced serum medium (RSM), and incubation for two days. NBFs were just incubated with RSM for two days. The supernatant from CAFs or NBFs was collected and concentrated using a spin column concentrator. ExoQuick-TC™ was added, and then samples were refrigerated overnight and centrifuged to obtain the exosome pellet, which was used for further analysis.

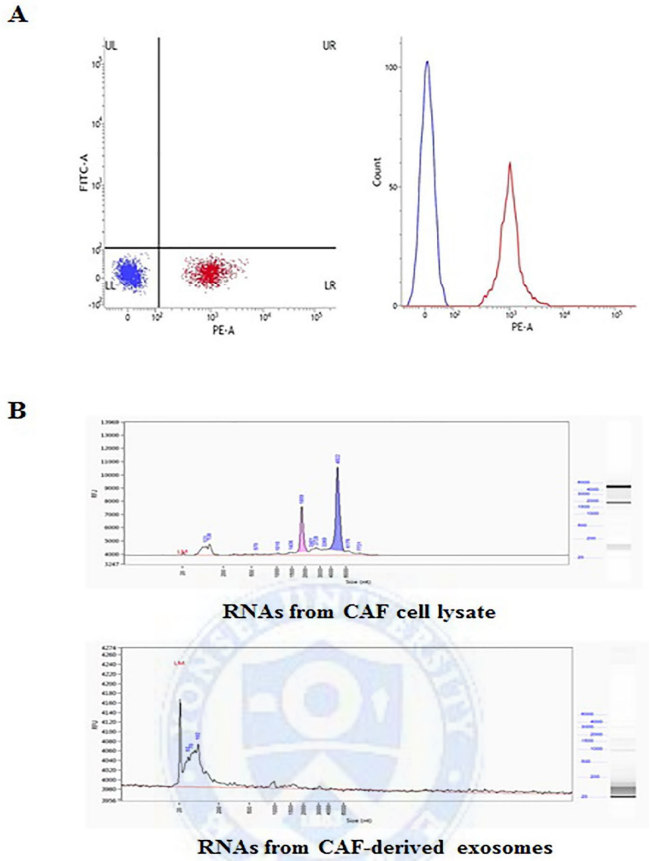


Figure 2. Detection of exosomes isolated from supernatants of fibroblasts grown in cell culture. (A) For FACS analysis of supernatant, the blue area represents the isotype control, while the red area indicates PE-positive CD63 conjugated exosome-coated beads. CD63 is used as a marker for exosomes. (B) Total RNAs were extracted from CAF-derived exosomes and CAF cell lysate and analyzed by a fragment analyzer. RNAs from CAF cell lysate showed clear peaks of 28S and 18S rRNAs, indicating intact RNAs. However, RNAs from CAF-derived exosomes showed no 28S or 18S rRNA, and, instead, relatively small RNAs were detected.

2. Comparative analysis of CAF- and NBF-derived exosomal miRNAs

To demonstrate the different exosomal contents of CAF- and NBF-derived exosomes, exosomal miRNAs were analyzed using microarray. Among 800 miRNAs in the microarray platform, most were expressed at low levels or showed little change between CAF- and NBF-derived exosomal miRNAs (Figure 3A). Ten miRNAs (miR-132-3p, miR-199b-5p, miR-555, miR-99a-5p, miR-720, miR-222-3p, miR-92a-3p, miR-323a-5p, miR-744-5p, and miR-18b-5p) increased by at least 1.7-fold in CAF-derived exosomes compared to exosomes derived from NBFs. Conversely, thirteen miRNAs (miR-451a, miR-630, miR-187-3p, miR-142-3p, miR-371b-3p, miR-136-5p, miR-1284, miR-29b-3p, miR-1253, miR-1915-3p, miR-144-3p, miR-320e, and miR-4516) decrease by at least 1.7-fold in CAF-derived exosomes compared to NBF-derived exosomes (Figure 3B).

As shown in Table 1, miR-29b-3p, miR-1253, and miR-1915-3p showed approximately two-fold expression changes between CAF- and NBF-derived exosomes, while miR-144-3p and miR-320e showed about three-fold changes. MiR-4516 expression in CAF-derived exosomes was highly downregulated compared to NBF-derived exosomes (Table 1). In fact, miR-4516 showed a five-fold change, which was the largest relative change detected in the microarray result. In addition, miR-132-3p and miR-199b-5p were both upregulated two-fold in CAF-derived exosomes compared to NBF-derived exosomes (Table 2).

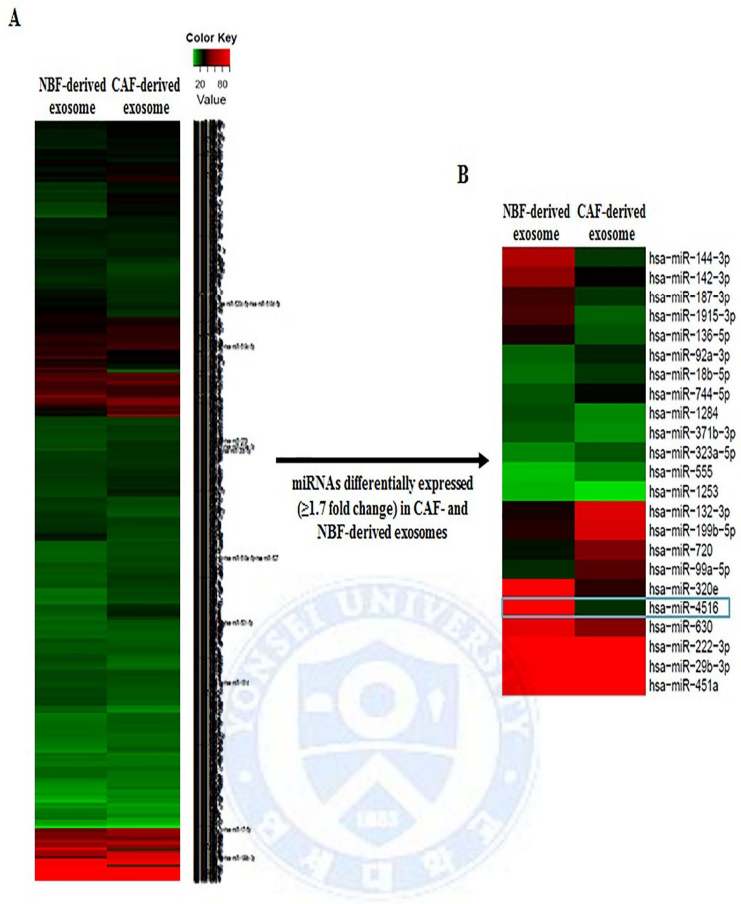


Figure 3. Comparative analysis of miRNAs in CAF- and NBF-derived exosomes. (A) A heat map comparing the expression of eight hundred microRNAs in the microarray platform in CAF-derived exosomes to the expression in NBF-derived exosomes. (B) Twenty-three miRNAs showed at least a 1.7-fold change in expression between CAF-derived and NBF-derived exosomal microRNAs. Thirteen of these miRNAs (miR-451a, miR-630, miR-187-3p, miR-142-3p, miR-371b-3p, miR-136-5p, miR-1284, miR-29b-3p, miR-1253, miR-1915-3p, miR-144-3p, miR-320e, and miR-4516) showed decreased expression

in CAF-derived exosomes compared to NBF-derived exosomes.

Table 1. Downregulated miRNAs in CAF-derived exosomes

microRNA	CAF-derived exosome expression	NBF-derived exosome expression	Fold Change
hsa-miR-4516	22.89	118.46	**-5.18**
hsa-miR-320e	36.5	136.51	**-3.74**
hsa-miR-144-3p	21.68	67.9	**-3.13**
hsa-miR-1915-3p	15.01	41.17	**-2.74**
hsa-miR-1253	2.65	5.95	**-2.25**
hsa-miR-29b-3p	251.34	556.86	**-2.22**
hsa-miR-1284	9.32	18.33	-1.97
hsa-miR-136-5p	17.27	33.44	-1.94
hsa-miR-371b-3p	8.84	16.09	-1.82
hsa-miR-142-3p	30.51	54.65	-1.79
hsa-miR-187-3p	22.18	39.33	-1.77
hsa-miR-630	51.89	91.56	-1.76
hsa-miR-451a	626.42	1068.44	-1.71
hsa-miR-155-5p	7.7	12.8	-1.66
hsa-miR-184	10.64	17.29	-1.63
hsa-miR-203	16.3	26.25	-1.61
hsa-miR-21-5p	215.13	346.01	-1.61
hsa-miR-543	25.69	40.91	-1.59
hsa-miR-765	9.88	15.38	-1.56
hsa-miR-636	8.21	12.46	-1.52

Six microRNAs (with fold changes indicated in bold) showed the most decrease in CAF-derived exosomes. The most negatively changed miRNA was miR-4516 with -5.18-fold change. MiR-144-3p, miR-320e, and miR-4516 were detected at low levels in CAF-derived exosomes.

Table 2. Upregulated miRNAs in CAF-derived exosomes

microRNA	CAF-derived exosome expression	NBF-derived exosome expression	Fold Change
hsa-miR-132-3p	86.14	33.32	2.59
hsa-miR-199b-5p	83.75	35.34	2.37
hsa-miR-555	9.51	5.2	1.83
hsa-miR-99a-5p	43.32	23.61	1.83
hsa-miR-720	49.73	27.26	1.82
hsa-miR-222-3p	197.77	110.8	1.78
hsa-miR-92a-3p	25.18	14.41	1.75
hsa-miR-323a-5p	16.59	9.59	1.73
hsa-miR-744-5p	28.8	16.7	1.72
hsa-miR-18b-5p	21.82	12.75	1.71
hsa-miR-1225-3p	16.5	9.74	1.69
hsa-miR-296-3p	11.46	6.77	1.69
hsa-miR-520g	14.7	8.79	1.67
hsa-miR-490-3p	18.53	11.46	1.62
hsa-miR-582-3p	20.18	12.49	1.62
hsa-miR-30d-5p	25.93	16.11	1.61
hsa-miR-199a-5p	54.53	34.11	1.6
hsa-miR-548ag	24.46	15.3	1.6
hsa-miR-1224-3p	9.43	5.94	1.59
hsa-miR-1267	16.63	10.51	1.58

MicroRNAs upregulated at least two-fold in CAF-derived exosomes compared to NBF-derived exosomes. MiR-132-3p and miR-199b-5p showed the greatest change between CAF- and NBF-derived exosomes.

3. Downregulation of endogenous expression of miR-4516 in CAFs and NBFs stimulated with 231 CM

To confirm whether CAFs expressed less endogenous miR-4516 compared to NBFs, as was the case with exosomes derived from these cells, and to determine whether heterogeneous cancer stimulation would influence miRNA expression changes in CAFs, the expression level of miR-4516 in CAFs was determined upon stimulation with cancer-conditioned medium and compared to NBFs. Both 231 CM and MCF7-conditioned medium (MCF7 CM) were used to stimulate CAFs and observe the difference in miR-4516 expression. These two cancer cells were chosen to represent the influence of different levels of invasiveness on CAFs, as MDA-MB-231 cells are known to be more invasive than MCF7 cells.[25] CAFs treated with 231 CM showed a significant decrease of endogenous miR-4516 compared to control NBFs (Figure 4A). Although CAFs treated with MCF7 CM showed a similar tendency of decreased miR-4516 expression upon treatment, the change was not significant (Figure 4B).

To determine whether cancer stimulation would cause NBFs to exhibit CAF-like behavior such as a decrease of endogenous miR-4516, NBFs were treated with 231 CM for a total of four days. The endogenous level of miR-4516 was measured by qRT-PCR daily (Figure 4C). Like CAFs, NBFs stimulated by 231 CM showed reduced levels of miR-4516. At day 1, approximately a half-fold downregulation of miR-4516 was observed, and after three days, miR-4516 expression decreased continuously in 231 CM treated NBFs compared to the

control non-treated NBFs. NBFs showed the significant decrease of cellular miR-4516 levels over the four days of CM treatment.

4. Transfer of exosomal miR-4516 to breast cancer cells

To investigate the effect of CAF-derived exosomes containing reduced levels of miR-4516 on cancer cells, MCF7 and MDA-MB-231 cells were directly treated with CAF- or NBF-derived exosomes. MCF7 cells were incubated with CAF- or NBF- derived exosomes for two days, and the miR-4516 level was quantified by qRT-PCR. MCF7 cells treated with CAF-derived exosomes showed an insignificance of endogenous miR-4516 levels compared to untreated mock MCF7 cells. On the other hand, compared to MCF7 cells treated with NBF-derived exosomes, containing high levels of miR-4516, miR-4516 expression significantly decreased more than four-fold in cells treated with CAF-derived exosomes (Figure 4D). Like MCF7 cells, low levels of miR-4516 were observed in MDA-MB-231 cells treated with CAF-derived exosomes and untreated mock MDA-MB-231 cells. When MDA-MB-231 cells incubated with CAF-derived exosomes were compared to the cells with NBF-derived exosomes, they also showed a decrease of endogenous miR-4516 (Figure 4E).

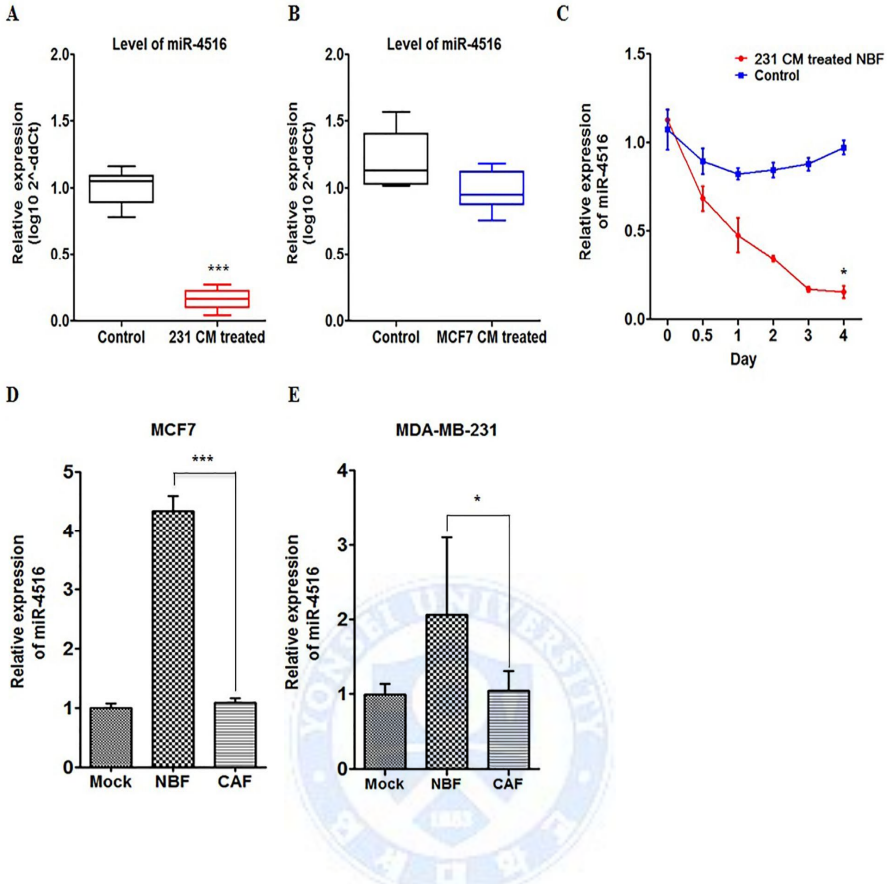


Figure 4. Changed expression of miR-4516 in CAFs and NBFs upon 231 CM stimulation and transfer of miR-4516 to breast cancer cells via exosomes. (A) Box- Whisker plot displays the level of miR-4516 in 231-CM treated CAF and non-treated control NBF. The horizontal line into the boxes indicates the median, and the bars define the 10th and the 90th percentiles. Upon 231 CM stimulations, endogenous miR-4516 expression was significantly downregulated in CAFs (***P < 0.001) compared to control NBF cells without 231 CM treatment. (B) Box- Whisker plot displays the level of miR-4516 in MCF7 CM treated CAF and non-treated control NBF. The horizontal line into the boxes indicates the median, and the bars define the 10th and the 90th

percentiles. MCF7 CM-stimulated CAFs showed no significant change in endogenous miR-4516 expression compared to control NBF cells without MCF7 CM treatment. MiR-4516 expression level was analyzed by using qRT-PCR in triplicate and relative expression was calculated using the delta-delta Ct method. *P*-values were determined using the Mann–Whitney test. (C) During four days, the expression of miR-4516 was analyzed daily by qRT-PCR. NBFs stimulated with 231 CM showed continuous decrease of miR-4516 levels (*P < 0.05) compared with control NBFs without treatment of 231 CM. The values are presented as the mean±SD; n = 3 for each group. *P*-values were determined using an unpaired two-tailed Student's t-test. (D) MCF7 cells were incubated with either CAF- or NBF-derived exosomes, while mock MCF7 cells were treated with RSM. Compared to the cells with NBF-derived exosomes, MCF7 cells incubated with CAF-derived exosomes showed a significant decrease in miR-4516 level (***P < 0.001). Meanwhile, MCF7 cells with CAF-derived exosomes showed insignificance compared to mock MCF7 cells. (E) MDA-MB-231 cells showed a decrease of miR-4516 levels upon treatment with CAF-derived exosomes compared to treatment with NBF-derived cells (*P < 0.05). MDA-MB-231 cells with CAF-derived exosomes, however, showed insignificance compared to mock MDA-MB-231 cells with RSM. MiR-4516 expression level was analyzed, using qRT-PCR in triplicate. The values are presented as the mean±SD; n = 3 for each group. *P*-values were determined using one-way anova.

5. Regulation of tumor progression-related genes in miR-4516 overexpressed breast cancer cells

Putative target genes of miR-4516 was analyzed with the miRNA target gene prediction database TARGETSCAN[26] (http://www.targetscan.org), cancer-associated gene database GeneCards[27] (www.genecards.org), The Biological General Repository for Interaction Datasets[28] (BioGRID: http://thebiogrid.org) and STRING ver. 10 database[29] (http:// http://string-db.org). A total of 9,332 genes were categorized as cancer-associated genes and 2,193 genes were found as oncogenes, while total of 114 genes were predicted for miR-4516 putative target genes. 2,085 genes were showed for cancer-associated and oncogenic genes and 46 genes were predicted as both cancer-associated and miR-4516 putative target genes. After comparing the target genes predicted by two algorithms and overlapped sections, a consensus list of 23 putative target genes was common between them (Figure 5A). The overlapped section of miR-4516 putative genes and oncogenes was also showed exactly the same gene list with 23 putative genes common in all three parts. Among 23 common genes (predicted), Plant homeodomain finger protein 8 (PHF8) and signal transducer and activator of transcription 3 (STAT3) genes were located. PHF8 was ranked at the highest, while STAT3 was listed at 8th. PHF8 was chosen for its favorable context+ score, which is the sum of six features of contribution for specific target sites and STAT3 was focused since the recent study found its direct binding and interaction between STAT3 and miR-4516. These genes indicated as oncogenic cancer-associated miR-4516 target genes.

After sorting a few putative target genes out, several genes were found for their possible interaction with target genes based on the BioGRID database. Four characteristics of cancer progression were categorized: invasion, migration, proliferation and epithelial-mesenchymal transition (EMT). All genes under each category were overlapped in BioGrid and GeneCards database. Additionally, the interactions between all 23 putative target genes were analyzed by STRING database (Figure 5B). It showed that PHF8 and STAT3 could be interactive through HCFC1 and SP1.

To investigate whether decreased exosomal miR-4516 could influence tumor progression-related genes in breast cancer cells, MCF7 and MDA-MB-231 cells were transfected with a miR-4516-containing pENTR™/H1/TO vector. Overexpression of miR-4516 in cancer cells represented transferring NBF-derived exosomes to cancer, while cells transfected with control vector mimicked the condition of transferring CAF-derived exosomes. Overexpression of miR-4516 in breast cancer cells were confirmed by RT-PCR and then, expressions of PHF8 and STAT3 were determined by RT-PCR with 18s rRNA control. When miR-4516 was overexpressed in MCF7 cells, all levels of PHF8 and STAT3 were decreased. In MDA-MB-231 transfected with miR-4516, expressions of both PHF8 and STAT3 showed downregulated compared to cells transfected with control vector (Figure 5C).

Protein expression in MCF7 and MDA-MB-231 cells transfected with miR-4516 was determined. Both cells were confirmed their miR-4516 overexpression beforehand by RT-PCR. Compared to MCF7 cells transfected with control vector, PHF8 and STAT3 protein expression

levels were repressed in MCF7 cells transfected with miR-4516 vector. In the case of MDA-MB-231, PHF8 and STAT3 expressions were also decreased when miR-4516 was overexpressed in the cells (Figure 5D). Thus, as miR-4516 overwhelmed the breast cancer cells, expression of putative target genes was inhibited.

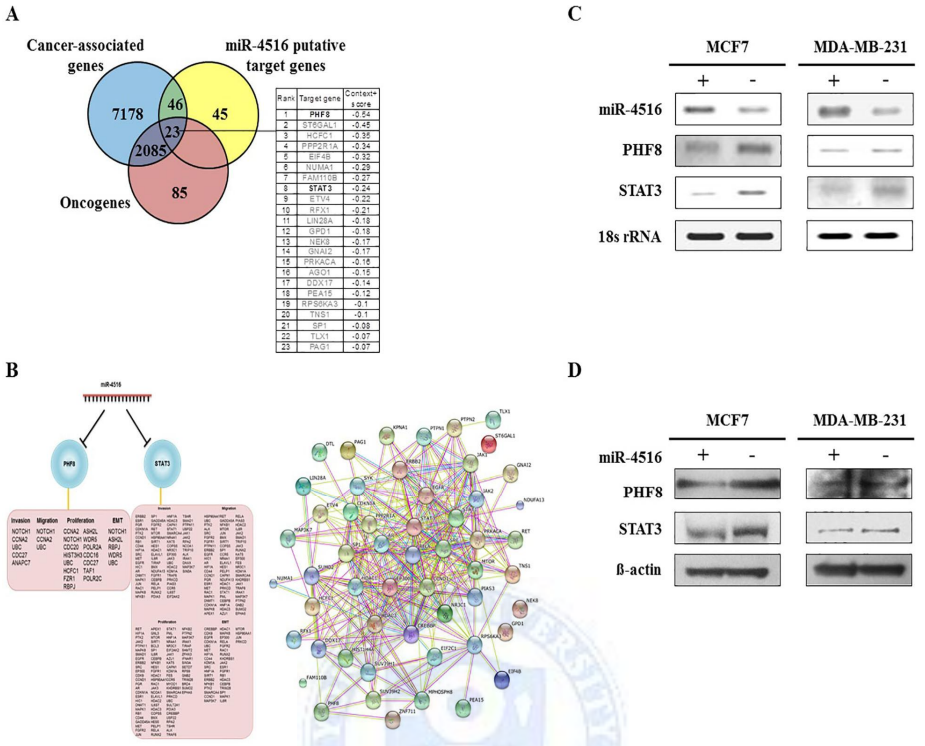


Figure 5. Selection of miR-4516 putative target genes and decreased expression in MCF7 and MDA-MB-231 cells overexpressing miR-4516. (A) Venn diagram illustrating miR-4516 putative target genes predicted by Target Scan and analyzed with GeneCards database. There were 23 genes were predicted as the common genes for oncogenic, cancer-associated as well as miR-4516 putative target genes. The listed genes in the overlapped part of miR-4516 putative target genes and oncogenes were identical to the genes in overlapped in triple overlapped section. PHF8 was ranked 1st place for its favorable prediction score and STAT3 was listed at 8th. (B) By BioGRID and GeneCards database, several genes were found as that they could relate to cancer progression (invasion, migration, proliferation, and EMT) as

well as possibly interact with miR-4516 target genes. The possible interactions of 23 putative target genes and other genes were established by STRING database. (C) Expression of putative target genes of miR-4516 was analyzed by RT-PCR. The plus sign (+) represented overexpression of miR-4516 by transfection and the minus sign (-) represented transfection with the control vector. Both MCF7 cells and MDA-MB-231 cells transfected with miR-4516 vector showed decreased expression of PHF8 and STAT3 compared to the control. (D) Expression of putative target genes of miR-4516 was analyzed by western blotting. The plus sign (+) represented overexpression of miR-4516 and the minus sign (-) represented transfection with the control vector. All overexpression level was previously confirmed by RT-PCR and then, further protein validation was preceded. Compared to MCF7 and MDA-MB-231 cells transfected with miR-4516 vector, cells transfected with control vector showed higher expression in PHF8 and STAT3.

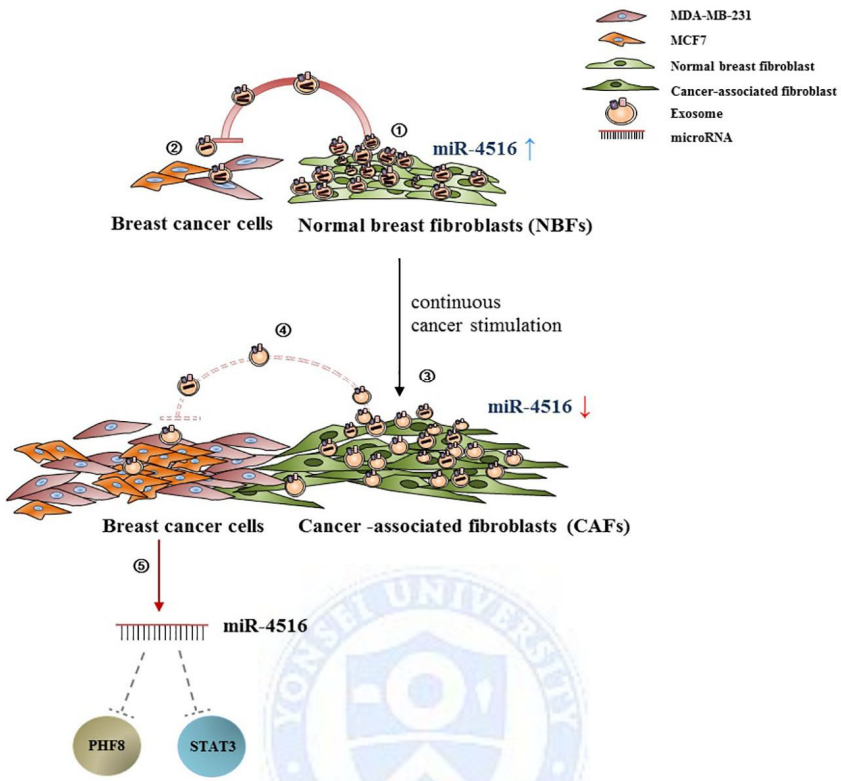


Figure 6. Suggestive model for the communication between CAFs and breast cancer cells via exosomes. (1) At an early stage of tumorigenesis, NBFs could surround the single-cell unit cancer cells, suppressing tumor expansion. (2) They may communicate with cancer cells via secretory exosomes containing high levels of miR-4516. NBF-derived exosomal miR-4516 could inhibit certain genes associated with tumor progression in breast cancer. However, (3) cancers continuously stimulate CAFs during cancer progression; they could start accumulating around the tumor and the environment becomes favorable to cancer cells. CAFs also secrete exosomes, but the miRNA contents could be different that NBF-derived exosomes. CAFs have decreased cellular expression of miR-4516, and CAF-derived exosomes could

contain reduced amounts of miR-4516. (4) The downregulation of miR-4516, due to the increased presence of CAFs and CAF-derived exosomes could lead to decreased inhibition of tumor progression-related genes in cancer cells. (5) Therefore, the suppression of putative target genes, including PHF8 and STAT3 by miR-4516 could be weakened and they could play an important role in breast cancer progression.

IV. DISCUSSION

In TME, CAFs are located within or around the tumor mass and are simultaneously influenced by direct cancer cell contact and cancer-secreting factors.[1] CAFs can communicate with cancer cells via exosomes and their contents, such as miRNAs, could act as endogenous genes in cancer cells.[10] CAFs may compose exosomal miRNA contents in different ratios so that they could influence gene expression associated with tumor progression by transferring upregulated or downregulated exosomal miRNAs. Reduced levels of certain exosomal miRNAs could be advantageous to cancers since the inhibitory effects suppressing tumor progression could be alleviated in cancer cells.

Prior to this study, the role of miR-4516 in cancer had not been studied aggressively and also, upregulated miRNAs were not as significant as downregulated miRNAs based on microRNA microarray. qRT-PCR for other two to three-fold upregulated or downregulated microRNAs in CAF-derived exosomes compared to NFB-derived exosomes were performed, however, not significant expression changes were found (data not shown), except for miR-4516. This, along with its five-fold decrease in CAF-derived exosomes compared to NBF-derived exosomes, made it an attractive target for further study. Therefore, we focused on investigating whether exosomal miR-4516 in CAF-derived exosomes could regulate gene expression in breast cancer cells, affecting tumor progression (Figure 3).

231 CM or MCF7 CM was used to compare the influence of

heterogeneous breast cancer cells on CAFs as well as stimulating NBFs to exhibit CAF-like features. 231 CM-treated CAFs showed a significant decrease of miR-4516 compared to control cells, while MCF7 CM treated CAFs did not show significant changes. These results suggest that in a heterogeneous situation, more invasive breast cancer cells could have a larger influence on CAFs compared to less invasive cancer cells. MDA-MB-231 cells induced significant changes in miRNA levels, indicating that exosomes from 231 CM-stimulated CAFs may help regulate gene expression of breast cancer cells more effectively. Moreover, they could indirectly compensate for minor stimulation by less invasive cells (Figure 4A and B).

At the early stages of tumorigenesis, cancer cells could be suppressed by the uptake of excessive exosomal miR-4516 from NBF-derived exosomes. However, as cancers expand, they could constantly stimulate normal fibroblasts, causing them to adopt CAF-like features, such as a decrease in cellular miR-4516 levels. As NBFs underwent 231 CM stimulation, the significant decrease of miR-4516 expression we observed could explain how cancer might influence fibroblasts indirectly to downregulate tumor suppressing miRNA expression. Thus, the decrease in exosomal miRNA in CAF-derived exosomes and cancers can lead to decreased inhibitory effects and regulate gene expression in breast cancer (Figure 4C).

Both MCF7 and MDA-MB-231 breast cancer cells were used as recipient cells since NBF- and CAF-derived exosomes could influence to the heterogeneous breast tumors. Both MCF7 and MDA-MB-231

cells incubated with CAF-derived exosomes showed a significant decrease of cellular miR-4516 levels, while an increase was shown upon treatment with NBF-derived exosomes compared to control (Figure 4D and E). Similar to microarray results, breast cancer cells treated with CAF-derived exosomes showed significant suppression of endogenous miR-4516 compared to cells treated with NBF-derived exosomes. Together, these results indicate that the level of miR-4516 in exosomes was determined by the type of fibroblast from which it was derived: CAFs or NBFs. Like MCF7 cells, MDA-MB-231 cells treated with CAF-derived exosomes showed a downregulation of miR-4516 compared to control cells. Moreover, MCF7 cells treated with CAF-derived exosomes downregulated miR-4516 levels about four-fold, while the level in MDA-MB-231 cells also decreased compared to cells with NBF-derived exosomes. To explain the different level of absorption between MCF7 and MDA-MB-231, we determined that endogenous miR-4516 expression levels were higher in MDA-MB-231 cells than MCF7 cells (data not shown). Thus, it is possible that absorbed exosomal miRNA in MDA-MB-231 cells may be less dramatic than in MCF7 cells.

PHF8 and STAT3 were found by overlapping in three venn diagram. They could be thought as oncogenic cancer associated miR-4516 target genes (Figure 5A). PHF8 presented the most favorable (lowest) context score+ by TargetScan prediction database and was also related to cancer progression, affecting cell proliferation and invasion in prostate cancer.[30] In recent studies, a relationship was established between miR-4516 and STAT3 in human keratinocytes with or without PUVA

treatment, and miR-4516 was found to bind to the STAT3 3'UTR, downregulating its expression.[31] Activation of STAT3 could also promote breast cancer cell migration via mediating upregulation of autotaxin.[32] Taken together, those four genes were selected in this research to show that miR-4516 target genes could be associated with breast tumor progression and overexpression of miR-4516 could suppress their expression.

As Figure 5B showed, when miR-4516 could inhibit the target genes, several genes that could interact with miR-4516 target genes and relate to tumor progression would be suppressed. By BioGrid and GeneCards database analysis, four features of cancer progression were established: invasion, migration, proliferation and EMT. When miR-4516 inhibits target oncogenic genes, possible interaction with other genes associated with possible tumor progression could be influenced like a chain-reaction, thus, cancer progression could be abated. Meanwhile, less miR-4516 exists, less inhibitory effect influences to cancer cells. The interaction between miR-4516 putative target genes was established on a basis of STRING database. PHF8 and STAT3 could communicate through interacting SP1 and HCFC1, which have been known for their association with tumor progression.[33,34]

Overexpression of miR-4516 in breast cancer cells represented cells influenced by NBF-derived exosomes at an early stage of cancer. Since NBF-derived exosomes carried a high level of miR-4516, cancer cells which absorb NBF-derived exosomes would be suppressed by exosomal miR-4516 during early tumorigenesis. Conversely, control

cells represented cells influenced by CAF-derived exosomes, which carried less miR-4516. Comparing these cells explained the roles of miR-4516 and the advantages of low miRNA levels to breast cancer cells (Figure 5C). By RT-PCR, overexpression of miR-4516 was confirmed as well as four putative target mRNA level changes. When miR-4516 overexpressed, expression levels of PHF8 and STAT3 were downregulated compared to control in both MCF7 and MDA-MB-231, which suggested that miR-4516 would influence these genes expression.

Western blot was performed to convince the possible relationship between miR-4516 and putative target genes. As miR-4516 was overexpressed in MCF7 and MDA-MB-231, target protein levels were decreased (Figure 5D) and it could be interpreted as the inhibitory effect of miR-4516 in breast cancer cells. When CAFs secrete exosomes with less amounts of miR-4516 within to recipient cancer cells, cancer could evade influence of miR-4516 and its target genes could function actively, which finally cancer progression proceed.
As miR-4516 overwhelmed the breast cancer cells, expression of putative target genes associated with tumor progression could be inhibited. Thus, the decrease in exosomal miRNA in CAF-derived exosomes and cancers could lead to decreased inhibitory effects and regulate gene expression in breast cancer. Determining the direct interaction of miRNA and each target gene, and the roles of these target genes in breast cancer cells will be the subject of future studies.

In summary, this study demonstrated that miRNA contents differed in

exosomes from CAF and NBFs. Exosomes containing different level of exosomal miR-4516 could be transferred into cells, and they influenced tumor progression-related genes in breast cancer cells. Significant downregulation of endogenous miR-4516 in CAFs could be driven by communication with cancer cells, and NBFs stimulated with cancer CM demonstrated CAF-like features such as decreasing miR-4516. Moreover, direct treatment of cancer cells with CAF- or NBF-derived exosomes demonstrated the influence of exogenous exosomal miRNA in cancer cells. To interact with cancer cells, CAFs secreted exosomes, containing less miR-4516 than NBFs, ensuring that breast cancer cells would not be influenced by exosomal miR-4516 as much as they were at early stages of cancer. This is the first research to suggest that CAFs may contain different exosomal miRNA contents than NBFs and that exosomal miR-4516 derived from CAFs may regulate tumor progression-related genes in breast cancer cells (Figure 6).

V. CONCLUSION

In conclusion, the genes associated with tumor progression could ultimately be regulated via reduced influx of exosomal miR-4516 from CAFs in breast cancer cells. Putative target genes, which have been suppressed by miRNA, could be highly expressed, leading to tumor progression. Our study suggests that exosomal miR-4516 may be positively used as an anti-tumor therapy.

REFERENCES

1. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332-7.
2. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17-32.
3. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999;59:5002-11.
4. Sadlonova A, Bowe DB, Novak Z, Mukherjee S, Duncan VE, Page GP, et al. Identification of molecular distinctions between normal breast-associated fibroblasts and breast cancer-associated fibroblasts. Cancer Microenviron 2009;2:9-21.
5. Sabin AB. Suppression of malignancy in human cancer cells: issues and challenges. Proc Natl Acad Sci U S A 1981;78:7129-33.
6. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 2012;21:309-22.
7. Umezu T, Ohyashiki K, Kuroda M, Ohyashiki JH. Leukemia cell to endothelial cell communication via exosomal miRNAs. Oncogene 2013;32:2747-55.
8. Luga V, Zhang L, Viloria-Petit AM, Ogunjimi AA, Inanlou MR, Chiu E, et al. Exosomes mediate stromal mobilization of

autocrine Wnt-PCP signaling in breast cancer cell migration. Cell 2012;151:1542-56.

9. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. J Biochem 2006;140:13-21.
10. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654-9.
11. Staubach S, Razawi H, Hanisch FG. Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. Proteomics 2009;9:2820-35.
12. Lee JK, Park SR, Jung BK, Jeon YK, Lee YS, Kim MK, et al. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. PLoS One 2013;8:e84256.
13. Yang M, Chen J, Su F, Yu B, Su F, Lin L, et al. Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells. Mol Cancer 2011;10:117.
14. Ambros V. The functions of animal microRNAs. Nature 2004;431:350-5.
15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97.
16. Garofalo M, Croce CM. microRNAs: Master regulators as potential therapeutics in cancer. Annu Rev Pharmacol Toxicol 2011;51:25-43.

17. Musumeci M, Coppola V, Addario A, Patrizii M, Maugeri-Sacca M, Memeo L, et al. Control of tumor and microenvironment cross-talk by miR-15a and miR-16 in prostate cancer. Oncogene 2011;30:4231-42.
18. Zhao L, Sun Y, Hou Y, Peng Q, Wang L, Luo H, et al. MiRNA expression analysis of cancer-associated fibroblasts and normal fibroblasts in breast cancer. Int J Biochem Cell Biol 2012;44:2051-9.
19. Nazareth MR, Broderick L, Simpson-Abelson MR, Kelleher RJ, Jr., Yokota SJ, Bankert RB. Characterization of human lung tumor-associated fibroblasts and their ability to modulate the activation of tumor-associated T cells. J Immunol 2007;178:5552-62.
20. Gao MQ, Kim BG, Kang S, Choi YP, Park H, Kang KS, et al. Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial-mesenchymal transition-like state in breast cancer cells in vitro. J Cell Sci 2010;123:3507-14.
21. Epple LM, Griffiths SG, Dechkovskaia AM, Dusto NL, White J, Ouellette RJ, et al. Medulloblastoma exosome proteomics yield functional roles for extracellular vesicles. PLoS One 2012;7:e42064.
22. Wahlgren J, De LKT, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen P, et al. Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. Nucleic Acids Res 2012;40:e130.
23. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and

characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3 22.

24. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.

25. Nagaraja GM, Othman M, Fox BP, Alsaber R, Pellegrino CM, Zeng Y, et al. Gene expression signatures and biomarkers of noninvasive and invasive breast cancer cells: comprehensive profiles by representational difference analysis, microarrays and proteomics. Oncogene 2006;25:2328-38.

26. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15-20.

27. Rebhan M, Chalifa-Caspi V, Prilusky J, Lancet D. GeneCards: a novel functional genomics compendium with automated data mining and query reformulation support. Bioinformatics 1998;14:656-64.

28. Chatr-Aryamontri A, Breitkreutz BJ, Oughtred R, Boucher L, Heinicke S, Chen D, et al. The BioGRID interaction database: 2015 update. Nucleic Acids Res 2015;43:D470-8.

29. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res 2009;37:D412-6.

30. Bjorkman M, Ostling P, Harma V, Virtanen J, Mpindi JP, Rantala J, et al. Systematic knockdown of epigenetic enzymes

identifies a novel histone demethylase PHF8 overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion. Oncogene 2012;31:3444-56.

31. Chowdhari S, Saini N. hsa-miR-4516 mediated downregulation of STAT3/CDK6/UBE2N plays a role in PUVA induced apoptosis in keratinocytes. J Cell Physiol 2014;229:1630-8.

32. Azare J, Doane A, Leslie K, Chang Q, Berishaj M, Nnoli J, et al. Stat3 mediates expression of autotaxin in breast cancer. PLoS One 2011;6:e27851.

33. Zannetti A, Del Vecchio S, Carriero MV, Fonti R, Franco P, Botti G, et al. Coordinate up-regulation of Sp1 DNA-binding activity and urokinase receptor expression in breast carcinoma. Cancer Res 2000;60:1546-51.

34. Parker JB, Yin H, Vinckevicius A, Chakravarti D. Host cell factor-1 recruitment to E2F-bound and cell-cycle-control genes is mediated by THAP11 and ZNF143. Cell Rep 2014;9:967-82.

ABSTRACT (IN KOREAN)

엑소좀 마이크로RNA를 통한 암-연관 섬유아세포의 유방암 유전자 발현 조절

<지도교수 조 남 훈>

연세대학교 대학원 의과학과

김 지 은

암-연관 섬유아세포는 종양미세환경을 이루는 세포 중 하나로서 엑소좀과 같은 분비 인자들을 통하여 유방암과 소통한다. 암-연관 섬유아세포가 mRNA, 마이크로RNA, 단백질을 담고 있는 엑소좀을 통하여 유방암세포 진행에 영향을 준다는 연구가 활발히 진행되고 있지만 유방암에 미치는 암-연관 섬유아세포가 분비하는 엑소좀 유래 마이크로RNA에 대한 연구가 많지 않아 이에 중점을 두고 연구를 진행하게 되었다. 정상 유방 섬유아세포와 암-연관 섬유아세포가 분비하는 엑소좀 내 마이크로RNA 구성 변화와 특정 엑소좀 마이크로RNA가 유방암세포에 미치는 영향을 밝히기 위해 정상 유방 섬유아세포와 암-연관 섬유아세포에서 각각 추출한 엑소좀 내 마이크로RNA 종류 및 발현을 비교하였다. 그 결과, 정상 유방 섬유아세포와 비교하여

암-연관 섬유아세포 유래 엑소좀에서 miR-4516의 발현량이 약 5배 감소되어 존재함이 분석되었다. 침윤성이 강한 유방암 세포주인 MDA-MB-231의 조건 배지(231 CM)를 처리한 암-연관 섬유아세포와 정상 유방 섬유아세포 내에서도 miR-4516 발현량이 감소되는 것이 보여졌다. 또한, 정상 유방 섬유아세포에 231 CM을 처리하였을 때 miR-4516 발현이 감소하며 암-연관 섬유아세포의 특징을 보였다. 암-연관 섬유아세포와 정상 유방 섬유아세포에서 각각 추출한 엑소좀을 유방암 세포주 MCF7과 MDA-MB-231에 처리 하였을 때, 유방암세포의 miR-4516의 발현이 암-연관 섬유아세포에서 유래된 엑소좀을 처리한 유방암세포에서는 거의 변화가 없었고 정상 유방 섬유아세포에서 유래된 엑소좀을 처리한 세포에서는 약 4배 이상 증가되어 존재함이 보여졌다. 또한, miR-4516을 MDA-MB-231과 MCF7에서 과발현 시켰을 때, 종양진행과 관련된 유전자로 알려진 PHF8와 STAT3의 mRNA 발현과 단백질 레벨이 감소하였다. 따라서 정상 유방 섬유아세포가 많이 옮기던 엑소좀 miR-4516가 종양세포의 지속적인 자극을 받아 암-연관 섬유아세포로 변하면서 발현이 감소되고 엑소좀을 통해 적게 옮겨지면서 유방암세포에 미치던 억제성 영향력이 줄어들어 종양진행에 긍정적 영향을 미칠 수 있다는 것을 보여준다.

핵심되는 말 : 엑소좀, 마이크로RNA, 암-연관 섬유아세포, 종양미세환경, 유방암세포