Transition into inflammatory cancer-associated adipocytes in breast cancer microenvironment requires microRNA regulatory mechanism

Jiwoo Lee[1☯], **Bok Sil Hong**[1☯], **Han Suk Ryu**[2], **Han-Byoel Lee**[1,3], **Minju Lee**[1], **In Ae Park**[2], **Jisun Kim**[1,3], **Wonshik Han**[1,3,4], **Dong-Young Noh**[1,3,4], **Hyeong-Gon Moon**[1,3,4]*

1 Center for Medical Innovation, Seoul National University Hospital, Seoul, Korea, **2** Department of Pathology, Seoul National University Hospital, Seoul, Korea, **3** Department of Surgery, Seoul National University College of Medicine, Seoul, Korea, **4** Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

☯ These authors contributed equally to this work.
* moonhg74@snu.ac.kr

Abstract

The role of adipocytes in cancer microenvironment has gained focus during the recent years. However, the characteristics of the cancer-associated adipocytes (CAA) in human breast cancer tissues and the underlying regulatory mechanism are not clearly understood. We reviewed pathology specimens of breast cancer patients to understand the morphologic characteristics of CAA, and profiled the mRNA and miRNA expression of CAA by using indirect co-culture system *in vitro*. The CAAs in human breast cancers showed heterogeneous topographic relationship with breast cancer cells within the breast microenvironment. The CAAs exhibited the characteristics of de-differentiation determined by their microscopic appearance and the expression levels of adipogenic markers. Additionally, the 3T3-L1 adipocytes indirectly co-cultured with breast cancer cells showed up-regulation of inflammation-related genes including *Il6* and *Ptx3*. The up-regulation of IL6 in CAA was further observed in human breast cancer tissues. miRNA array of indirectly co-cultured 3T3-L1 cells showed increased expression of mmu-miR-5112 which may target *Cpeb1*. *Cpeb1* is a negative regulator of *Il6*. The suppressive role of mmu-miR-5112 was confirmed by dual luciferase reporter assay, and mmu-miR-5112-treated adipocytes showed up-regulation of *Il6*. The transition of adipocytes into more inflammatory CAA resulted in proliferation-promoting effect in ER positive breast cancer cells such as MCF7 and ZR-75-1 but not in ER negative cells. In this study, we have determined the de-differentiated and inflammatory natures of CAA in breast cancer microenvironment. Additionally, we propose a miRNA-based regulatory mechanism underlying the process of acquiring inflammatory phenotypes in CAA.

Introduction

The association between the degree of obesity and the risk of breast cancer incidence has long been recognized. The proposed mechanisms relating the adipose tissue and the breast cancer risk are mostly explained by endocrine and systemic effects of the adipokines and sex hormones [1]. However, recent studies have focused on the potential roles of adipocytes in the breast cancer microenvironment that may modulate the growth of the cancer cells [2, 3]. Although controversial, there are clinical studies suggesting that the degree of obesity is related to the risk of loco-regional recurrence in breast cancer patients suggesting a dynamic interactions between the local adipocytes and breast cancer cells in the tumor microenvironments [4, 5].

Studies have shown that the adipocytes in the vicinity of the cancer cells, often named as 'cancer-associated adipocytes (CAA)', show diverse molecular characteristics that can enhance the ability of cancer cell invasion and aid the cancer cell proliferation via conferring metabolic advantages [6–8]. However, studies have conflicting results on the differentiation lineage of the CAA [7–11], and the regulatory mechanisms of the CAA transition is not clearly understood. In this study, we aimed to characterize the CAA in human breast cancer tissues and to explore the regulatory mechanism of the CAA transition.

Materials and methods

Pathologic examination of the adipocytes within the breast cancer specimens

Hematoxylin-eosin stained slides of 55 consecutive breast cancer specimens were reviewed to observe the characteristics of the adipocytes. For each patient, representative images showing adipocytes in the vicinity of the epithelial cancer cells and adipocytes around the normal mammary glandular tissue were obtained and the cell sizes were measured with ImageJ [12]. In human breast cancer specimens, the adipocytes located in the vicinity of breast cancer cells were regarded as cancer-associated adipocytes. The collection of the clinical and pathologic data from the breast cancer patients was approved by the institutional IRB (1208-046-421) and all procedures were done in accordance with the Declaration of Helsinki.

Adipogenic differentiation of 3T3-L1 cells and indirect co-culture with cancer cells

3T3-L1 cell line was purchased from ATCC (Manassas, Virginia) and were cultured in DMEM supplemented with 10% bovine serum at 37˚C in 5% CO2. 3T3-L1 cells were grown to confluency and induced with adipogenesis media (DMEM with 10% FBS, 10μg/ml insulin, 0.5mM IBMX and 1μM dexamethasone). After 2 days, the media was changed with DMEM with 10% FBS and 10μg/ml insulin and maintained for 4 days. After 8 days of differentiation with DMEM with 10% FBS, the cells were used for further experiments. Breast cancer cell lines used for the indirect co-culture study were MCF7, ZR75–1, T47D, MDA-MB231, and Hs578T. MCF7, MDA-MB231, and Hs578T cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100μg/ml streptomycin. ZR75–1 and T47D cells were cultured in RPMI media with same supplements. For the indirect co-culture of the differentiated adipocytes and breast cancer cell lines, we used transwell plate with 0.4μm micron inserts (BD Biosciences, San Jose, CA). Differentiated adipocytes were cultured in the bottom chamber of the transwell co-culture system with or without the breast cancer cells seeded in the top inserts. After the co-culture for certain periods, the adipocytes at the bottom wells were collected and used for further analysis.

mRNA and miRNA expression microarray

For the quality control, RNA purity and integrity were evaluated by OD 260/280 ratio, and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). For gene expression analysis, the Affymetrix Whole transcript Expression array process was executed according to the manufacturer's protocol (GeneChip Whole Transcript PLUS reagent Kit). cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal labeling kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Human 2.0 ST Array at 45˚C for 16hour. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix). Raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip® Command Console® Software (AGCC). After importing CEL files, the data were summarized and normalized with robust multi-average (RMA) method implemented in Affymetrix® Expression Console™ Software (EC). We exported the result with gene level RMA analysis and perfomed the differentially expressed gene (DEG) analysis.

For miRNA analysis, the Affymetrix Genechip miRNA array process was executed according to the manufacturer's protocol. 1μg RNA samples were labeled with the Flash-Tag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA). The labeled RNA was quantified, fractionated and hybridized to the miRNA microarray according to the standard procedures provided by the manufacture. The labeled RNA was heated to 99˚C for 5 minutes and then to 45˚C for 5 minutes. RNA-array hybridization was performed with agitation at 60 rotations per minute for 16–18 hours at 48˚C on an Affymetrix® 450 Fluidics Station. The chips were washed and stained using a Genechip Fluidics Station 450 (Affymetrix, Santa Clara, CA). The chips were then scanned with an Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip® Command Console® Software. The CEL files import, miRNA level RMA+DABG-All analysis and result export using Affymetrix® Expression Console™ Software. Array data were filtered by probes annotated species.

Differential expression was analyzed using independent t-test and Benjamini-Hochberg algorithm. Gene-Enrichment and Functional Annotation analysis for significant probe list was performed using DAVID (http://david.abcc.ncifcrf.gov/). All Statistical test and visualization of differentially expressed genes was conducted using R statistical language v. 3.0.2. (www.r-project.org).

Enzyme-linked immunosorbent assay (ELISA)

After 3 days of indirect co-culture with cancer cells, the inserts containing cancer cells were removed and the remaining adipocytes were washed with PBS and were cultured for additional 2 days. The media was collected and the level of IL-6 was determined by enzyme-linked immunosorbent assay. Mouse IL-6 antibody was purchased from eBioscience (CA, USA). After 16 hr of incubation at 4˚C, 100μl amount of media was aspirated from each well. Biotin-conjugated IL-6 was added to the wells and incubated for 1hr at room temperature. After washing off the unbound antibody-enzyme conjugates, the substrate solution was then added to each well and incubated for 30 min before measuring the absorbance at the wavelength of 450nm.

Western blot, RT-PCR, and Taqman PCR

After 3 days of indirect co-culture with cancer cells, adipocytes were lysed with RIPA buffer (Thermo scientific, Korea). After incubation on ice for 30 minutes, cellular debris was removed by centrifugation for 10 minutes at 4˚C. 10 μg of proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membranes were probed with an FABP4 antibody (abcam). Blots were developed with an enhanced chemiluminescence Western blotting detection system (Amersham, Buckinghamshire, UK).

For RT-PCR experiments, total RNA from cultured cells was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) and was quantified by Nanodrop. 1 ug of total RNAs were reverse-transcribed to cDNA using PrimeScript reverse transcriptase (Takara, Japan) The information of the primers used for this study are shown in the S1 Table. All reaction products were analyzed after 28–35 amplification cycles and expression levels were normalized to the values of Gapdh.

For TaqMan PCR, total RNA was extracted from cells using TRIzol reagent (Favorgen Biotech, Taiwan) according to the manufacturer's instructions. RNA was converted to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with TaqMan Universal PCR Master Mix on an Applied Biosystems 7500 System (Applied Biosystems, Foster City, CA). The miRs were normalized to the level of snoRNA412 and the relative expression levels of genes were calculated using the 2−ΔΔ Ct method. All reactions were performed in triplicates.

Luciferase assay

CPEB1 3'-UTR containing the predicted binding site sequences were obtained by annealing sense and antisense strand or amplifying the genomic DNA from 3T3-L1 cells. The sequences were inserted into XhoI and NotI sites in psiCHECK2 dual luciferase reporter plasmid purchased from Promega ((Madison, WI).

HEK 293FT cells were plated in 24-well plates in DMEM supplemented with 10% fetal bovine serum at 37˚C with 5% CO2. When the cells reached 70–80% confluence, 20 ng of psiCHECK-2 recombination vector and 20 nM of miR-5112 mimics or Negative Control were cotransfected with RNAiMAX (Invitrogen, Carlsbad, CA). After 40 h, the cells were washed with PBS and reseeded in 96-well plates at a density of 5×10^3 cells per well with three replicates. Firefly and Renilla luciferase activities were measured with the Dual-Glo luciferase system (Promega, Madison, WI).

Proliferation assay

Proliferation was measured using CellTiter-Glo Luminescent Cell viability Assay (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, after the indirect co-culture with adipocytes, cancer cells in the transwell were washed with PBS, trypsinized and reseeded in 96 well plates. The relative cell viability was measured using microplate luminometer at designated times.

Results

De-differentiated characteristics of CAAs in breast cancer

First, we assessed the microscopic appearance of CAAs in human breast cancer specimens by investigating samples from 55 consecutive breast cancer patients who underwent curative resection. The CAA, located at the invading front of cancer cells, showed smaller cell size (Fig
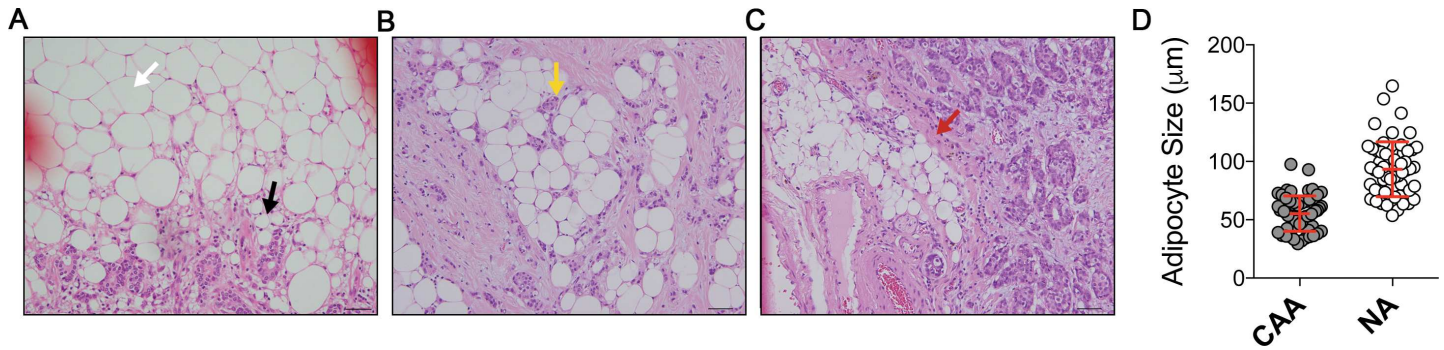
Fig 1. Microscopic appearances of the cancer-associated adipocytes (CAA) in human breast cancer specimens. The CAA (black arrow) showed smaller lipid droplets and hence smaller cell size when compared to normal adipocytes (white arrow) (A). In some cases, the breast cancer cells were clustered in contact with the CAA (B, yellow arrow) while other cases showed stromal tissues separating the nests of cancer cells and CAA (C, red arrow). The cell diameters of CAA and NA (normal adipocyte) are shown in D. Scale bar: 50μm.

1A and 1D), and carried substantial heterogeneity in the patterns of surrounding extracellular matrix and their topographic relationship with cancer cells (Fig 1B and 1C). The smaller cell size is a representative morphologic feature of the less differentiated adipocytes (S1 Fig). These findings led us to hypothesize that the observed microscopic features of the CAA could be resulting from a de-differentiation process of mature adipocytes under the influence of the adjacent cancer cells. The possibility of de-differentiation process in CAA has also been suggested by previous studies [7, 13, 14].

To demonstrate the de-differentiation process of the adipocytes under the influence of breast cancer cells, we used indirect co-culture system for 3T3-L1 preadipocytes and various breast cancer cells. Compared to the adipocyte cultured alone, the adipocytes indirectly co-cultured with breast cancer cells often showed smaller cell size and reduced lipid drops within the cells. (Fig 2A and 2B). The size distribution of adipocytes also showed a shift to smaller cell sizes when they were indirectly co-cultured with breast cancer cells (Fig 2C). Also, the markers of differentiated adipocytes, such as C/EBP-α, PPAR-γ, and FABP4, were down-regulated in co-cultured adipocytes (Fig 2D). The gene C/EBP-α, which is known to be up-regulated in the early phase of adipocyte differentiation, did not show significant changes [15]. The co-cultured adipocytes showed also decreased protein expression levels of FABP4 (Fig 2D). These data suggest that cancer cells influence the adjacent adipocytes by paracrine effect and induce the de-differentiation process.

Effect of CAA on the proliferative capacity of breast cancer cells

To determine the effect of cancer-associated adipocytes on the proliferation of breast cancer cells, we indirectly co-cultured the cancer cells with mature adipocytes for three days. After removal of the cancer cells, a new set of cancer cells were introduced to the co-cultured adipocytes and cultured for three days (Fig 3A). As shown in the Fig 3B, the effect of CAA on the proliferation of breast cancer cells varied between the cell lines. The pro-proliferative effect of CAA was most evident in the ER positive cells such as MCF7 and ZR75–1 while minimal effect was shown for ER negative cells including MDA-MB-231 and Hs578T. Our results suggest that the proliferation promoting effect of CAA, which has been suggested by previous studies [7–9], might be exerted in subtype-specific manners in breast cancer. Indeed, aromatase levels were upregulated in CAA that might favor the growth of ER positive breast cancer cells (S2 Fig).
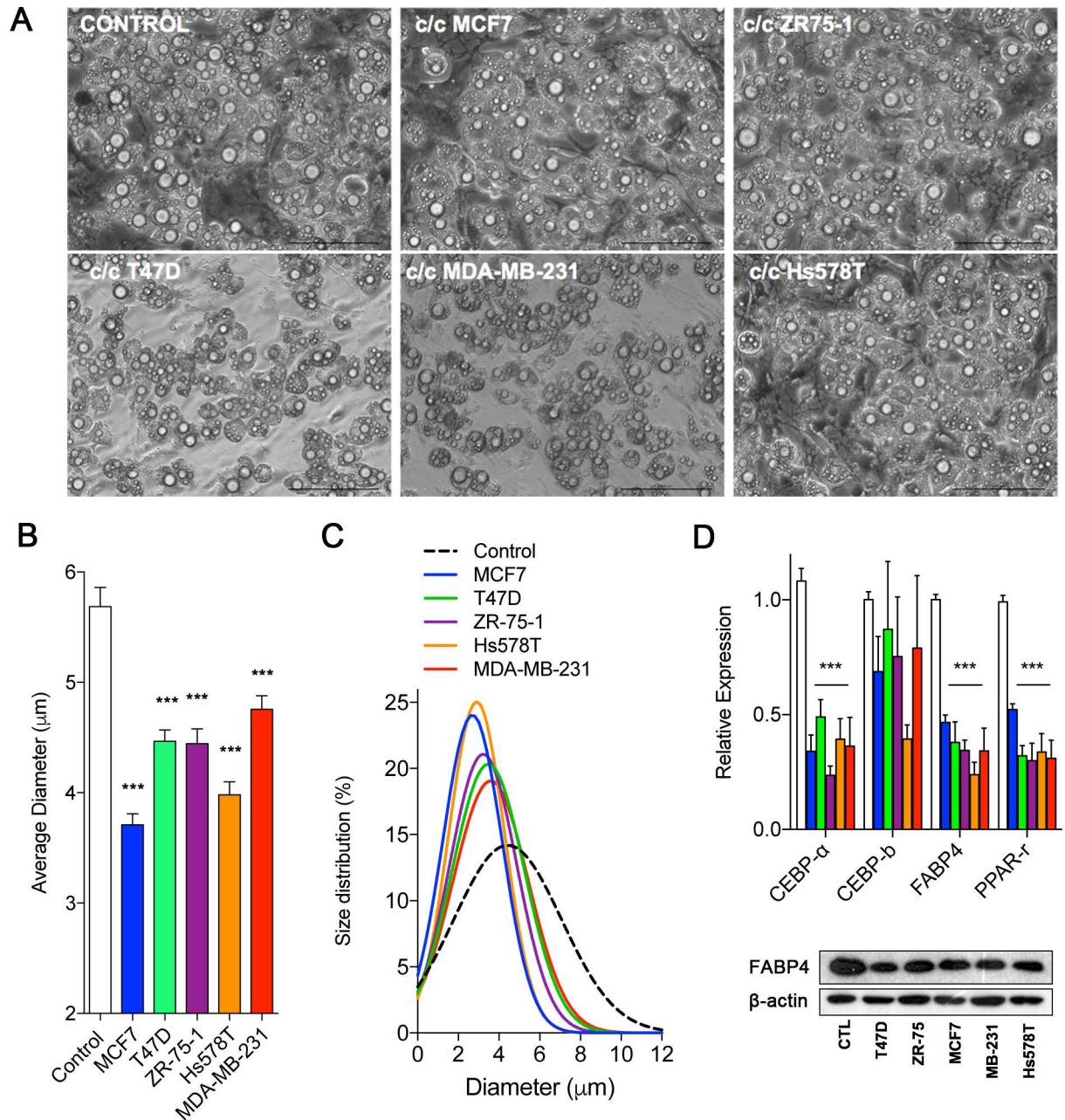
Fig 2. The microscopic appearance and expression characteristics of adipocytes when indirectly co-cultured with various breast cancer cells. Adipocytes indirectly co-cultured with various cancer cells showed reduction in lipid drops and cell size (A). Scale bar: 100μm. The relative size of adipocytes was measured by LAS v3.8 software, and the statistical significance was tested by using Students' t-test (B). The size distributions of co-cultured adipocytes were determined by using nonlinear regression with Gaussian curve fitting models (C). Genes involved in the adipocyte differentiation showed patterns of down-regulation of mRNA levels in adipocytes co-cultured with breast cancer cells. Also, the protein levels of FABP4 in co-cultured adipocytes are shown. The statistical significance was determined by t-testing with Benjamini, Krieger and Yekutieli adjustment (D). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

https://doi.org/10.1371/journal.pone.0174126.g002

Acquisition of inflammatory phenotype in CAAs

We analyzed transcriptional changes in adipocytes induced by the paracrine effect of breast cancer cell MCF7 and MDA-MB-231 by using indirect co-culture system. The genes up-
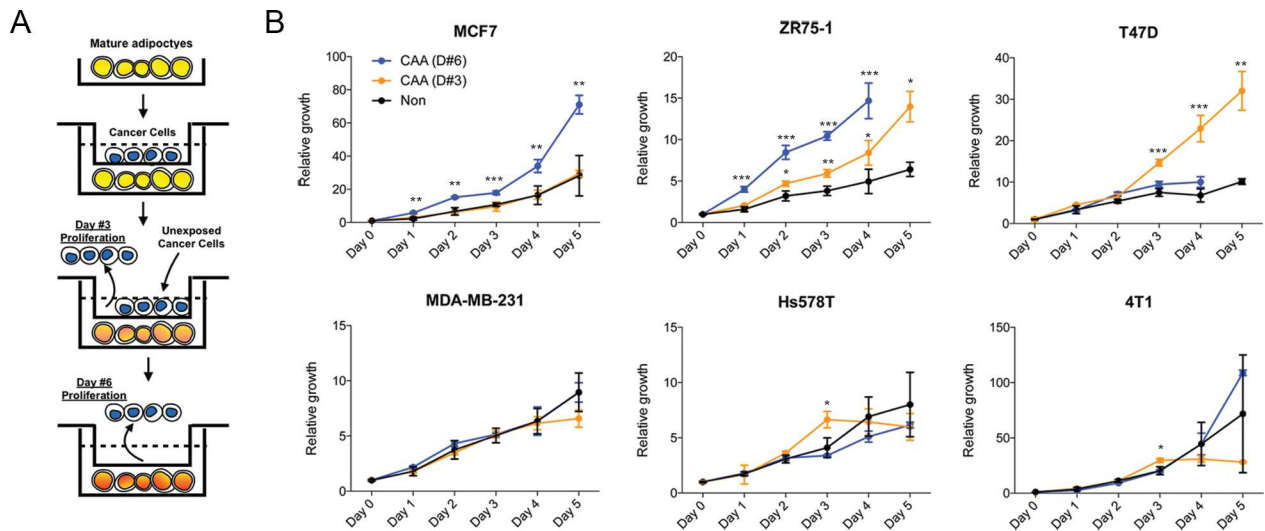
Fig 3. The effect of cancer-associated adipocytes (CAA) on proliferation of various breast cancer cells. The mature adipocytes were indirectly co-cultured with various breast cancer cells for three days, and the resulting CAAs were tested for the pro-proliferative effect on breast cancer cells (A). The degree of cell proliferation was measured by luminescent ATP quantification (B). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

regulated in the indirectly co-cultured adipocytes from both datasets were involved in pathways of inflammatory response, defense response, wound response, and chemotaxis (Fig 4A). Additionally, genes involved in the peptidase inhibitor activity were also significantly changed. *Il6*, *Ptx3*, and *Timp1* were up-regulated genes with highest fold changes (Fig 4B), and the up-regulation of the genes was validated by PCR (Fig 4C). IL6 is suggested to be a major regulator of tumor-stroma interaction in cancer microenvironment [16], and Dirat et al [7] reported similar findings of increased IL6 expression in CAA. TIMP1, another downstream molecule of IL6 [17], has been reported to regulate the cancer cell survival, modulate the proteolytic activity in ECM, and negatively regulate the adipocyte differentiation [18–21].

We then assessed the IL6 protein levels in the media of indirectly co-cultured adipocytes and observed that the up regulation of *Il6* gene resulted in increased secretion of IL6 (Fig 4D). To assess the IL6 expression levels in human breast cancer tissues, we examined the IL6 expression by immunohistochemistry using 39 consecutive breast cancer patients. Normal adipocytes, located far from the cancer cells, showed significantly lower frequencies of IL6 expression when compared to the adipocytes in the vicinity of breast cancer cells (Fig 4E). Our results suggest that breast cancer cells stimulate adjacent adipocytes via paracrine manner to express and secrete IL6 in breast cancer.

The expression levels of genes involved in the differentiation process of adipocytes were also explored using the gene expression data. While the indirectly co-cultured adipocyte did not show increased mesenchymal stem cell markers with the exception of *Cd44*, they showed significant down-regulation of the genes involved in the adipocyte differentiation (S3 Fig).

mmu-miR-5112-mediated regulation of IL6 in CAA

MiRNAs can regulate the process of adipocyte differentiation, and are dysregulated in the various pathologic conditions involving adipose tissues.[22] The adipocytes indirectly co-cultured with MCF7 cells were profiled for their miRNA expression patterns to determine the differentially expressed miRNAs in CAA by using miRNA array experiments. Among the differentially expressed miRNAs with statistical significance, mmu-miR-5112 showed highest up-regulation
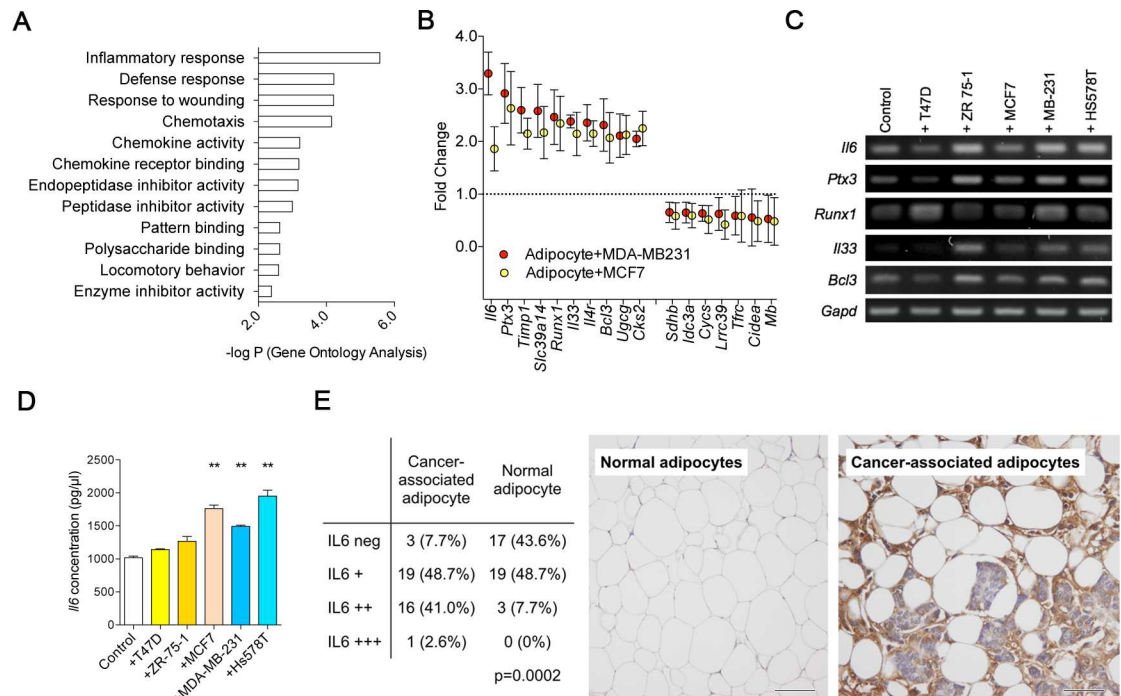
Fig 4. Gene expression profiles of cancer-associated adipocytes. Transcriptome analysis was done by using RNAs obtained from the indirect transwell co-culture system. Gene ontology analysis with DAVID software show that cancer-associated adipocytes are enriched with genes involved in inflammation and ECM remodeling (A). Differentially expression genes with highest fold change are shown in (B) and the differential expression was validated with PCR (C). ELISA against IL6 using secreted proteins from the cancer-associated adipocytes (D) and the IL6 expression levels in adipocytes from human breast cancer specimen (E) are shown. (**$p<0.01$).

in the co-cultured adipocytes (Fig 5A). The induction of miR-5112 expression in adipocytes by cancer cell was validated for MCF7 and MDA-MB-231 breast cancer cells (Fig 5B). The mmu-miR-5112 showed highest expression in the preadipocyte state and the expression was suppressed during the process of adipocyte differentiation (Fig 5C).

Target search using miRDB (http://mirdb.org) and TargetScan (http://www.targetscan.org) suggested *Cpeb1* gene as a potential candidate for mmu-miR-5112 target gene (Fig 5D). *Cpeb1* gene ranked in first and fourth as probable target for mmu-miR-5112 in miRDB and TargetScan, respectively. CPEB1 gene is reported to be a negative regulator of IL6 production and an important regulator of inflammatory response [23]. To test this, we treated differentiated adipocytes with mmu-miR-5112 mimics and observed significant down-regulation of *Cpeb1* and increased level of *Il6* (Fig 5E). To confirm the regulatory role of mmu-miR-5112, the effect of mmu-miR-5112 on the translation activity in 3' UTR of *Cpeb1* was measured by dual reporter luciferase assay. There was a significant decrease in the expression of luciferase reporter in cells transfected with mmu-miR-5112 mimics which demonstrates the suppression of Cpeb1 3'UTR translation by mmu-miR-5112 (Fig 5F). Taken together, our data suggest that the conversion of normal adipocytes into the inflammatory CAA is mediated by a miRNA-mRNA regulatory mechanism involving mmu-miR-5112-*Cpeb1*-*Il6*.

Discussion

In this study, we have analyzed the pathologic and molecular characteristics of CAA in human breast cancer specimens and determined the de-differentiated and inflammatory natures of
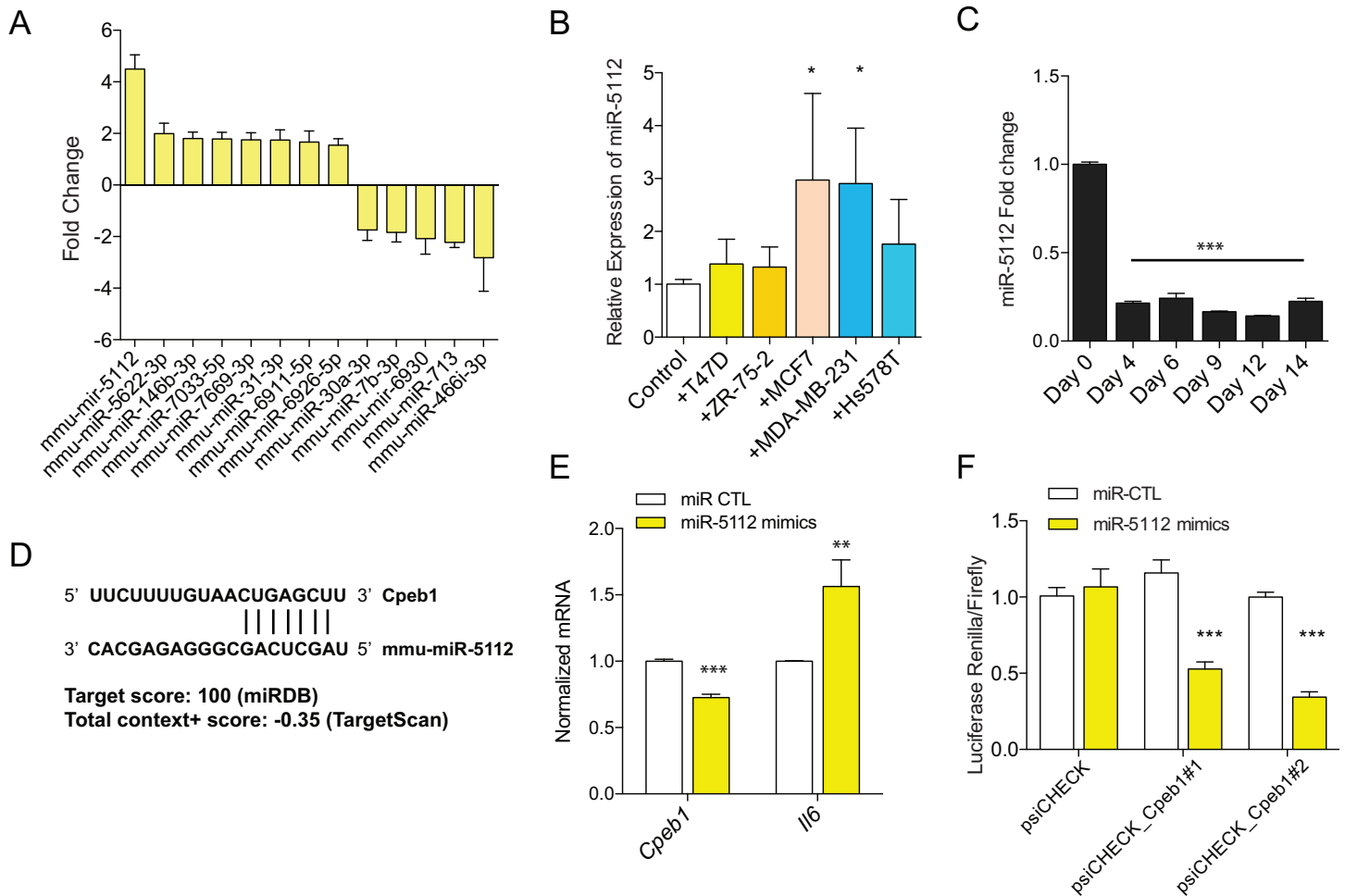
Fig 5. Regulatory role of mmu-miR-5112 in cancer-associated adipocyte (CAA) transition. microRNA profiles of CAA compared to normal adipocytes were determined by microarray (A). Expression of mmu-miR-5112 in adipocytes indirectly co-cultured with various cancer cells (B) and during the process of adipocyte differentiation (C). Predicted target site of Cpeb1 by mmu-miR-5112 (D) and the results of mmu-miR-5112 mimics treatment in Cpeb1 gene expression and translation measured by luciferase assay (E and F). (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

CAA in breast cancer microenvironment. Additionally, by using indirect co-culture system for adipocytes and breast cancer cells, we propose a miRNA-based regulatory mechanism underlying the process of acquiring inflammatory phenotypes in CAA.

Our results show that adipocytes acquire the morphologic and biologic characteristics of cellular de-differentiation process by adjacent breast cancer cells. Several studies have already reported the similar observation showing smaller cell size and down-regulation of adipogenic markers [7–9, 13, 14]. Studies have suggested that the de-differentiation process of CAA can be induced by up-regulation of ECM enzymes such as MMP11 [13] or cytokines such as TNFa or IL11 [14]. Indeed, our gene expression data shows that CAAs show significant upregulation of various cytokines and peptidase genes. The mRNA expression profiles of the CAA showed dysregulation of genes involved in the pathways of inflammation, wound healing, and peptidase activities including *Il6, Ptx3, Il33, and Timp1*. IL6 carries diverse regulatory roles in breast cancer pathogenesis including remodeling the microenvironment, activation of EMT process, malignant transformation, and modulating breast cancer stem cell activities [24–27]. TIMP1, a known transcriptional target of STAT3, carries complex regulatory role in

breast cancer [17]. While the epithelial cell-derived TIMP1 seem to favor cell survival and inhibit apoptosis [19, 28], stromal cell-derived TIMP1 secretion may inhibit breast cancer progression [18]. In consistent with our observation, TIMP1 has been shown to negative regulate the differentiation process of subcutaneous adipocytes [21]. The role of TIMP1 in CAA regulation and ECM remodeling should be investigated further to address its role in breast cancer microenvironment.

The transformed adipocytes showed proliferation-promoting effect on breast cancer cells. However, the pro-proliferative effect of CAA was only seen in ER positive cell lines when tested in a panel of various types of breast cancer cells. Manabe et al [9] have also reported that adipocytes promote tumor growth in ER positive cells but not in ER negative MMT 060562 cells. These findings suggest that the tumor-promoting effect of adipocyte may depend on the molecular subtypes of breast cancer cells. The finding is in contrast to the work of Dirat et al [7] showing the pro-metastatic and invasive effect of CAA in both ER positive and negative cells. It is possible that the local growth promoting effect and the metastasis-promoting effect of adipocytes do not share similar mechanisms. Indeed, recent studies have suggested that growth promoting effect of microenvironmental cells on tumor mainly rely on local cellular interactions such as the exchange of metabolic supports or local production of aromatase enzymes [6, 29].

Based on the miRNA microarray and luciferase reporter assay, we propose a miRNA-based regulatory mechanism for the acquisition of inflammatory nature of CAA in breast cancer. The acquisition of inflammatory characteristics in the neighboring cells is a key regulator of tumor initiation and progression [30], and the process is controlled by numerous miRNAs in the tumor microenvironments [31, 32]. Our data shows that mmu-miR-5112 is markedly upregulated in adipocytes when they are indirectly co-cultured with breast cancer cells. This miRNA has been reported to suppress the *Cpeb1* gene that acts as a negative regulator of IL6 synthesis by inhibiting IL6 translation [23, 33]. In gastric cancer, the stromal fibroblasts can give rise to IL6 expression by epigenetic silencing of miR-149 [34]. Furthermore, Sung et al [35] have shown that let-7 can induce IL6-driven inflammatory stromal reaction in prostate cancer microenvironment. Our study adds another potential regulatory mechanism involving microRNA, CPEB1, and IL6 within the complex microRNA-network of tumor microenvironment.

There are several limitations in our study. First, we did not explore the functional effects of CAA in xenograft models. Additionally, we used mouse preadipocyte cells for our in vitro experiments, and therefore, the applicability of the present findings in human breast cancer microenvironment is not known. The in vivo effect of CAA in breast cancer progression and metastasis as well as the regulatory mechanisms in human adipocytes should be determined in further studies. Also, we could not determine the effect of direct co-culture with various cancer cells on mature adipocytes since we only used indirect co-culture methods. Direct co-culture, which mimics the in vivo cell-cell interaction, might have different effects on adipocytes [36]. Finally, the human counterpart microRNAs of mmu-miR-5112 that regulate the CPEB1 expression in cancer microenvironments should be investigated in future studies.

In conclusion, our results describe a potential microRNA-based regulatory mechanism underlying the transition between normal adipocytes and inflammatory/de-differentiated CAA in breast cancer. Furthermore, we show that the pro-survival effect of the CAA can be breast cancer subtype-specific. Additional studies to address the molecular links that explain the subtype-specific effect may provide a window for microenvironment-targeted therapies.

Supporting information

S1 Fig. The changes in the adipocyte differentiation-related genes and microscopic shapes in NIH-3T3-L1 cells during adipogenic differentiation. Along with the adipogenic gene

expression (A), the cells showed increased accumulation of lipid drops and increase in the cell sizes (B).
(TIF)

S2 Fig. The aromatase mRNA levels in adipocytes indirectly co-cultured with breast cancer cell MCF7 and MDA-MB-231. The mRNA levels of adipocytes were measured and shown by their relative levels.
(EPS)

S3 Fig. Expression levels of differentiation markers in the gene expression data. Genes in red and blue represent the genes expressed in mesenchymal stem cells and in differentiated adipocytes, respectively.
(TIF)

S1 Table. Primer sequences used in this study. The primers for PCR experiments for each gene is shown in the Table.
(DOCX)

Author Contributions

Conceptualization: IAP WH DYN HGM.

Data curation: JL BSH HGM.

Formal analysis: JL BSH JK HGM.

Funding acquisition: BSH HGM.

Investigation: JL BSH HSR HBL ML JK.

Methodology: WH DYN HGM.

Project administration: HGM.

Resources: WH DYN HSR.

Validation: HSR HBL ML IAP.

Visualization: JL BSH HSR JK.

Writing – original draft: JL BSH HGM.

Writing – review & editing: WH DYN HGM.

References

1. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nature reviews Cancer. 2004; 4(8):579–91. https://doi.org/10.1038/nrc1408 PMID: 15286738

2. Wang YY, Lehuede C, Laurent V, Dirat B, Dauvillier S, Bochet L, et al. Adipose tissue and breast epithelial cells: a dangerous dynamic duo in breast cancer. Cancer letters. 2012; 324(2):142–51. https://doi.org/10.1016/j.canlet.2012.05.019 PMID: 22643115

3. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and adipocytes support tumorigenesis and metastasis. Biochimica et biophysica acta. 2013; 1831(10):1533–41. PubMed Central PMCID: PMC3742583. https://doi.org/10.1016/j.bbalip.2013.02.010 PMID: 23500888

4. Marret H, Perrotin F, Bougnoux P, Descamps P, Hubert B, Lefranc T, et al. Low body mass index is an independent predictive factor of local recurrence after conservative treatment for breast cancer. Breast cancer research and treatment. 2001; 66(1):17–23. PMID: 11368406

5. Moon HG, Han W, Noh DY. Underweight and breast cancer recurrence and death: a report from the Korean Breast Cancer Society. Journal of clinical oncology official journal of the American Society of

Clinical Oncology. 2009; 27(35):5899–905. https://doi.org/10.1200/JCO.2009.22.4436 PMID: 19805676

6. Nieman KM, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nature medicine. 2011; 17 (11):1498–503. PubMed Central PMCID: PMC4157349. https://doi.org/10.1038/nm.2492 PMID: 22037646

7. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer research. 2011; 71 (7):2455–65. https://doi.org/10.1158/0008-5472.CAN-10-3323 PMID: 21459803

8. Wang C, Gao C, Meng K, Qiao H, Wang Y. Human adipocytes stimulate invasion of breast cancer MCF-7 cells by secreting IGFBP-2. PloS one. 2015; 10(3):e0119348. PubMed Central PMCID: PMC4352027. https://doi.org/10.1371/journal.pone.0119348 PMID: 25747684

9. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. The Journal of pathology. 2003; 201(2):221–8. https://doi.org/10.1002/path.1430 PMID: 14517839

10. Carter JC, Church FC. Mature breast adipocytes promote breast cancer cell motility. Experimental and molecular pathology. 2012; 92(3):312–7. https://doi.org/10.1016/j.yexmp.2012.03.005 PMID: 22445926

11. Bochet L, Lehuede C, Dauvillier S, Wang YY, Dirat B, Laurent V, et al. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. Cancer research. 2013; 73(18):5657–68. https://doi.org/10.1158/0008-5472.CAN-13-0530 PMID: 23903958

12. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012; 9(7):671–5. PMID: 22930834

13. Andarawewa KL, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, et al. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. Cancer research. 2005; 65(23):10862–71. https://doi.org/10.1158/0008-5472.CAN-05-1231 PMID: 16322233

14. Meng L, Zhou J, Sasano H, Suzuki T, Zeitoun KM, Bulun SE. Tumor necrosis factor alpha and interleukin 11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively down-regulating CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma: mechanism of desmoplastic reaction. Cancer research. 2001; 61(5):2250–5. PMID: 11280794

15. Ntambi JM, Young-Cheul K. Adipocyte differentiation and gene expression. J Nutr. 2000; 130 (12):3122S–6S. PMID: 11110885

16. Fisher DT, Appenheimer MM, Evans SS. The two faces of IL-6 in the tumor microenvironment. Seminars in immunology. 2014; 26(1):38–47. PubMed Central PMCID: PMC3970580. https://doi.org/10.1016/j.smim.2014.01.008 PMID: 24602448

17. Dien J, Amin HM, Chiu N, Wong W, Frantz C, Chiu B, et al. Signal transducers and activators of transcription-3 up-regulates tissue inhibitor of metalloproteinase-1 expression and decreases invasiveness of breast cancer. Am J Pathol. 2006; 169(2):633–42. PubMed Central PMCID: PMCPMC1698804. https://doi.org/10.2353/ajpath.2006.051109 PMID: 16877361

18. Clarke MR, Imhoff FM, Baird SK. Mesenchymal stem cells inhibit breast cancer cell migration and invasion through secretion of tissue inhibitor of metalloproteinase-1 and -2. Mol Carcinog. 2015; 54 (10):1214–9. https://doi.org/10.1002/mc.22178 PMID: 24819588

19. Liu XW, Taube ME, Jung KK, Dong Z, Lee YJ, Roshy S, et al. Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells from extrinsic cell death: a potential oncogenic activity of tissue inhibitor of metalloproteinase-1. Cancer research. 2005; 65(3):898–906. PMID: 15705888

20. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010; 141(1):52–67. PubMed Central PMCID: PMCPMC2862057. https://doi.org/10.1016/j.cell.2010.03.015 PMID: 20371345

21. Meissburger B, Stachorski L, Roder E, Rudofsky G, Wolfrum C. Tissue inhibitor of matrix metalloproteinase 1 (TIMP1) controls adipogenesis in obesity in mice and in humans. Diabetologia. 2011; 54 (6):1468–79. https://doi.org/10.1007/s00125-011-2093-9 PMID: 21437772

22. Arner P, Kulyte A. MicroRNA regulatory networks in human adipose tissue and obesity. Nature reviews Endocrinology. 2015; 11(5):276–88. https://doi.org/10.1038/nrendo.2015.25 PMID: 25732520

23. Ivshina M, Alexandrov IM, Vertii A, Doxsey S, Richter JD. CPEB regulation of TAK1 synthesis mediates cytokine production and the inflammatory immune response. Molecular and cellular biology. 2015; 35 (3):610–8. PubMed Central PMCID: PMC4285422. https://doi.org/10.1128/MCB.00800-14 PMID: 25452303

24. Dethlefsen C, Hojfeldt G, Hojman P. The role of intratumoral and systemic IL-6 in breast cancer. Breast cancer research and treatment. 2013; 138(3):657–64. https://doi.org/10.1007/s10549-013-2488-z PMID: 23532539

25. Yang L, Han S, Sun Y. An IL6-STAT3 loop mediates resistance to PI3K inhibitors by inducing epithelial-mesenchymal transition and cancer stem cell expansion in human breast cancer cells. Biochemical and biophysical research communications. 2014; 453(3):582–7. https://doi.org/10.1016/j.bbrc.2014.09.129 PMID: 25285632

26. Sansone P, Storci G, Tavolari S, Guarnieri T, Giovannini C, Taffurelli M, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. The Journal of clinical investigation. 2007; 117(12):3988–4002. PubMed Central PMCID: PMC2096439. https://doi.org/10.1172/JCI32533 PMID: 18060036

27. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(4):1397–402. PubMed Central PMCID: PMC3029760. https://doi.org/10.1073/pnas.1018898108 PMID: 21220315

28. Bigelow RL, Williams BJ, Carroll JL, Daves LK, Cardelli JA. TIMP-1 overexpression promotes tumori-genesis of MDA-MB-231 breast cancer cells and alters expression of a subset of cancer promoting genes in vivo distinct from those observed in vitro. Breast cancer research and treatment. 2009; 117 (1):31–44. https://doi.org/10.1007/s10549-008-0170-7 PMID: 18787947

29. Che Q, Liu BY, Liao Y, Zhang HJ, Yang TT, He YY, et al. Activation of a positive feedback loop involving IL-6 and aromatase promotes intratumoral 17beta-estradiol biosynthesis in endometrial carcinoma microenvironment. International journal of cancer. 2014; 135(2):282–94. https://doi.org/10.1002/ijc.28679 PMID: 24347287

30. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646–74. https://doi.org/10.1016/j.cell.2011.02.013 PMID: 21376230

31. Rupaimoole R, Calin GA, Lopez-Berestein G, Sood AK. miRNA Deregulation in Cancer Cells and the Tumor Microenvironment. Cancer Discov. 2016; 6(3):235–46. PubMed Central PMCID: PMCPMC4783232. https://doi.org/10.1158/2159-8290.CD-15-0893 PMID: 26865249

32. Kuninty PR, Schnittert J, Storm G, Prakash J. MicroRNA Targeting to Modulate Tumor Microenvironment. Front Oncol. 2016; 6:3. PubMed Central PMCID: PMCPMC4717414. https://doi.org/10.3389/fonc.2016.00003 PMID: 26835418

33. Groppo R, Richter JD. CPEB control of NF-kappaB nuclear localization and interleukin-6 production mediates cellular senescence. Molecular and cellular biology. 2011; 31(13):2707–14. PubMed Central PMCID: PMCPMC3133380. https://doi.org/10.1128/MCB.05133-11 PMID: 21536657

34. Li P, Shan JX, Chen XH, Zhang D, Su LP, Huang XY, et al. Epigenetic silencing of microRNA-149 in cancer-associated fibroblasts mediates prostaglandin E2/interleukin-6 signaling in the tumor microenvironment. Cell Res. 2015; 25(5):588–603. PubMed Central PMCID: PMCPMC4423088. https://doi.org/10.1038/cr.2015.51 PMID: 25916550

35. Sung SY, Liao CH, Wu HP, Hsiao WC, Wu IH, Jinpu, et al. Loss of let-7 microRNA upregulates IL-6 in bone marrow-derived mesenchymal stem cells triggering a reactive stromal response to prostate cancer. PloS one. 2013; 8(8):e71637. PubMed Central PMCID: PMC3747243. https://doi.org/10.1371/journal.pone.0071637 PMID: 23977098

36. Arrigoni C, De Luca P, Gilardi M, Previdi S, Broggini M, Moretti M. Direct but not indirect co-culture with osteogenically differentiated human bone marrow stromal cells increases RANKL/OPG ratio in human breast cancer cells generating bone metastases. Mol Cancer. 2014; 13:238. https://doi.org/10.1186/1476-4598-13-238 PMID: 25335447