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BET bromodomain proteins control breast cancer aggressiveness promoted by adipocyte-derived exosomes

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BET BROMODOMAIN PROTEINS CONTROL BREAST CANCER AGGRESSIVENESS PROMOTED BY ADIPOCYTE-DERIVED EXOSOMES

by

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ABSTRACT

Cells can release lipid bilayer vesicles of endosomal and plasma membrane origin, which are known as exosomes or extracellular vesicles (EVs). EVs contain diverse shuttling lipids, RNA and transmembrane proteins, and play an important role in communicating between neighboring or distant cells. Breast cancer is the most commonly diagnosed malignancy, with over 2 million new cases in 2018, and is the leading cause of cancer mortality in women all over the world. Some observational studies have suggested that breast cancer is more likely to develop among women who have type 2 diabetes; the association is clear in postmenopausal women. Moreover, women with type 2 diabetes diagnosed before, at the same time, or after breast cancer diagnosis, have decreased overall survival compared to women without diabetes.

The most recent medical studies provide more clues as to why breast cancer is more common and has poorer prognosis in type 2 diabetes patients, by pointing out the role of insulin-resistant adipocytes in the etiopathology. Here, we demonstrate how insulin-resistant adipocytes engage crosstalk with breast cancer cells through EVs in the microenvironment and drive the tumor cells to be more metastatic and aggressive. These progression mechanisms and the effects of insulin-resistant adipocytes on breast cancer cells require Bromodomain and ExtraTerminal (BET) proteins – an important epigenetic

pathway. Targeting this pathway may help reduce morbidity and mortality of women with breast cancer and type 2 diabetes.

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LIST OF ABBREVIATIONS

Alpha-SMA ..... Alpha smooth muscle actin

AMPK ..... Adenosine monophosphate-activated protein kinase

BET ..... Bromodomain Extra Terminal

BRD2 ..... Bromodomain-containing protein 2

BRD3 ..... Bromodomain-containing protein 3

BRD4 ..... Bromodomain-containing protein 4

BSA ..... Bovine serum albumin

BU ..... Boston University

DMEM ..... Dulbecco's Modified Eagle Medium

DNA ..... Deoxyribonucleic acid

ECL ..... Enhanced chemiluminescence

EDTA ..... Ethylenediaminetetraacetic acid

EMT ..... Epithelial-Mesenchymal Transition

EpCAM ..... Epithelial cell adhesion molecule

ER ..... Estrogen receptor

Evs ..... Extracellular vesicles

FBS ..... Fetal bovine serum

HER2 ..... Human epidermal growth factor receptor 2

HR ..... Hazard Ratio

HRP ..... Horseradish peroxidase

IBMX ..... Isobutylmethylxanthine

IF ..... Immunofluorescence

IR..... Insulin-resistant

IS ..... Insulin-sensitive

MVBs..... Multivesicular bodies

NH..... Non-Hispanic

PBS ..... Phosphate-buffered saline

PR..... Progesterone receptor

RIPA buffer..... Radioimmunoprecipitation assay buffer

RNA ..... Ribonucleic acid

RT-PCR..... Reverse transcription polymerase chain reaction

SD ..... Standard deviation

SDS-PAGE ..... sodium dodecyl sulfate–polyacrylamide gel electrophoresis

TBST..... Tris-Buffered Saline, 0.1% Tween 20

TME ..... Tumor microenvironment

TNFα..... Tumor necrosis factor alpha

ZEB1 ..... Zinc finger E-box-binding homeobox 1

INTRODUCTION

Breast cancer is the most commonly diagnosed malignancy among women in the high socioeconomic countries (1). Worldwide, breast cancer impacts 2.1 million women each year and also causes 627,000 deaths, which accounted for 15% of all cancer-related death among women in 2018 (2). According to recent reports, the number of worldwide reported breast cancer cases intensively increased approximately 20% since 2008 (2,3). The global incidence of breast cancer has been increasing by about 3.1% each year, from 641,000 cases diagnosed in 1980 to over 2.1 million in 2018 (4). It is estimated that approximately one new case was diagnosed every 18 seconds in 2018 (4,5). About 250,000 new cases of invasive breast cancer are expected to be diagnosed each year in the U.S. Approximately 13% (1 in 8) of women will be diagnosed with invasive breast cancer in their lifetime and about 42,170 women will die from breast cancer in 2020 (6). Therefore, invasive breast cancer is a major public health problem of deepening seriousness worldwide.

Indeed, the breast cancer burden is increasing in developed countries as well as developing countries, regardless of epidemiological characteristics such as income, high population growth or aging of the population. Although the incidence of female breast cancer death significantly declined from the peak at 33.2 (per 100,000) in 1989 to 19.8 in 2017 because of early detection with mammography and efficient adjuvant therapy (7,8), there are still many problems in treating this disease and decreasing the mortality rate. Menopausal hormone therapy, alcohol consumption and physical inactivity are

potentially associated with higher incidence of breast cancer, while being overweight/obesity is associated with worse prognosis of postmenopausal breast cancer (9). In addition, there are a variety of non-modifiable risk factors that include the mutation of breast cancer susceptibility genes (*BRCA1*, and *BRCA2*) and a personal or family history of breast or ovarian cancer (10,11).

Breast cancer epidemiology and prognosis vary among different races and groups of age. In the U.S., incidence rates are higher among Non-Hispanic (NH) black women before age 40 and survival rates are lower, compared to NH white women (12). At the time of diagnosis, about 64% of patients are diagnosed with local-stage breast cancer, 27% with the regional metastasis, and 6% of breast cancer patients have distant metastasis. Five-year relative survival rates for metastatic breast cancer are approximately 27%. Stage at the time the patients are diagnosed, as well as breast cancer incidence rates and mortality rates, vary by ethnicity (NH Black, NH White, Asian/Pacific Islander, Hispanic/Latina, and American Indian/Alaska Native) (13,14).

Breast cancer is a group of diseases in which cells in the breast proliferate in an uncontrollably. Breast cancer can emerge from different parts of the breast such as lobules where milk is produced, ducts which collect the milk and work as a system to bring milk to the nipple, and connective tissue, which includes fibrous and fatty types of connective tissue. On the histological and molecular level, breast cancer is also a heterogeneous disease, defined by diverse features of the malignancy cell of origin and molecular features which include expression of human epidermal growth factor receptor 2 (HER2), hormone receptors such as estrogen receptor (ER) and progesterone receptor

(PR) and mutations in specific genes (15). Hence, treatment decisions differ according to these histological and molecular characteristics. In spite of our remarkable understanding about the molecular features and pathology of breast cancer, the disease remains a massive public health burden where advanced breast cancer is generally considered incurable (16). The mortality rates vary among the subtypes of breast cancer, with the highest rate (among women ≥65 years of age) in non-luminal HER2-positive disease [Hazard Ratio (HR) 2.21 (95% confidence interval (CI) (1.62-3.01)], then luminal B [HR, 1.69; 95% CI, 1.28-2.24], luminal A [HR, 1.51; 95% CI, 1.33-1.71] and triple negative breast cancer [HR, 1.25; 95% CI, 1.03-1.53]. Mortality rates also vary by ethnicity and age (17). These patterns suggest that personalized therapy will be critical and prioritized in breast cancer research for the development of novel strategies to optimize the efficacy and tolerance of recent therapies.

Although a high stress lifestyle and high alcohol consumption play roles in increased incidence risk, obesity is also becoming a crucial risk factor, as well as one of the poor prognostic factors among metastatic breast cancer survivors (19). During the last two decades, the number of overweight and obese individuals has steadily increased (20). Besides older age and being female, being overweight or obese is also well established to be associated with increased risk of developing breast malignancy in *postmenopausal* women (21). Conversely, in *premenopausal* women, obesity is associated with decreased breast cancer incidence (22), but poorer prognosis. Moreover, obesity is independently associated with *overall* poorer prognosis of breast cancer (22,23). Women who are physically inactive, have diabetes or have insulin resistance syndromes are more likely to

have breast cancer (24). Significant evidence demonstrates that obesity increases the risk of recurrence or death by 25% to 50%, which is independent of hormone receptor status (25). Moreover, obesity also causes insulin resistance that promotes hyperinsulinemia and impaired glucose tolerance (26,27). Insulin resistance is related to poor survival in all breast cancer subtypes (28). Not only hyperinsulinemia and impaired glucose tolerance are extensively reported to directly associate with tumor growth and metastasis, but also emerging evidence demonstrates that insulin resistance status clearly drives the epithelial-mesenchymal transition (EMT) of breast cancer through crosstalk between EVs of adipocytes and cancer cells in the tumor microenvironment (29,30). EMT is a process that allows cells to lose their epithelial characteristics by depolarizing and reducing their cell-cell connections. As EMT occurs, cells become more mesenchymal and gain migratory and invasive capacity (31). A recent observation has shown that metformin, a medicine that lowers insulin level and increases insulin sensitivity and is a first line drug for type 2 diabetes (T2D), has a potential anti-cancer effect by reducing proliferation and migration capacity through activation of adenosine monophosphate-activated protein kinase (AMPK) (32). Because of the complexity of insulin resistance caused by inflammation, restricted oxygen intake and/or higher level of insulin, additional research about insulin resistance linked to breast cancer is needed to improve our understanding in order to develop tailored treatment for pre-diabetes and type 2 diabetes patients diagnosed with breast cancer (33).

Tumor microenvironment (TME) has been universally recognized as a substantial contributor to tumor progression. Evasion, invasion and metastasis, evading apoptosis,

inducing angiogenesis and therapy resistance are tightly connected to TME, which includes a variety of cell types, extracellular matrix (ECM), secreted proteins, and small molecules (34). In the last decades, there is a significant growing interest in nano-sized vesicles such as EVs and their roles in TME. Emerging data suggest that EVs shuttle many physiological and pathological biomolecules to play a role in intercellular communication (35). EVs are nanoparticles, phospholipid bilayer membrane organelles that are created by budding of plasma and endosome membranes (35,36). EVs are released from a variety of cells, prokaryotes and eukaryotes, to communicate with targets or distant cells by the fusion with plasma membrane of the recipient cells. EVs can be categorized in two groups, ectosomes and exosomes (37) **(Table 1)**. Because of its distinctive subset of cargo and smaller size vesicle, there has been growing interest in exosome research in the last decade. In the 1990s, the mechanism of exosome release was found in B lymphocytes and dendritic cells in the same way as multivesicular bodies (MVBs) fuse with plasma membranes (38). The early definition of exosomes is considered to be secreted vesicles that are functional in physiology (39). Many more forms of evidence demonstrate that the role of MVBs in releasing the exosomes is crucial in both hematopoietic and non-hematopoietic cells, such as cytotoxic T cells, neurons, and intestinal epithelial cells (40). Furthermore, the physiological function of exosomes and their secretion were reported as general and basic cellular functions in a variety of cells. Exosomes are the lipid bilayer vesicles in the size of ~40 to 160 nm (average ~100 nm) in diameter, containing transmembrane proteins, DNA, RNA, lipids and metabolites (41). Exosomes bring their contents, such as nucleic acids, metabolites, and proteins from

the origin cells to the recipient cells to effectively change the target cells' biological and morphological characteristics (42,43,44,45). The emerging data also suggest that exosomes play an important role in not only intracellular communication in the TME but also in disease diagnosis by characterization of their cargoes.

Table 1. Extracellular vesicles' characteristics.

Characteristic	**Exosomes**	**Ectosomes**
Properties	~40-160nm	~100-1000nm
Origin	Intracellular multivesicular bodies (MVBs)	Plasma membrane
Characteristic	Homogeneous	Heterogenerous
Content	DNA, RNA, lipids, cytosolic and cell-surface proteins and metabolites, amino acids	Proteins, mRNAs, miRNAs
Common markers	CD9, CD81, CD63, ALIX, TSG101, flotillin, ceramide	TyA, C1q

In order to investigate the aggressive behavior of breast cancer cells in breast cancer patients, we profiled EMT characteristics of breast cancer cells. EMT is a biologic and pathological program that facilitates acquisition of the potential of migrating, increased cancer stem-like cell formation and transcriptional plasticity in cancer cells

(46). When the EMT process is activated, it allows multiple biochemical changes of the polarized epithelial cell that increase aggressiveness, migratory capacity, and chemotherapy resistance (47). Tumor cells start losing their epithelial markers such as E-cadherin, epithelial cell adhesion molecule (EpCAM), as well as their polarized epithelial features (48). The cells begin to detach from the basement membrane and acquire a mesenchymal state by gaining more mesenchymal markers such as vimentin and alpha-smooth muscle actin (alpha-SMA) (49). In the progress of EMT, TME and intercellular communication synergize to direct the cells and enhance their invasive capacity to the metastatic sites. By the end of the EMT program, tumor cells will have lost many of their epithelial markers and will express many mesenchymal markers (50) **(Fig. 1)**. Upon metastasis and arrival at the targeting sites, cancer cells reverse EMT by inducing a MET program to adhere and form metastatic tumors in specific organs (51). Expression of EMT markers associates with poor prognosis and reduced overall survival rate in ovarian cancer, breast cancer and other tumor types (52). EMT will lead to the dissemination of cancer cells and become more invasive in circulating tumor cells (CTCs) (53). Recent studies demonstrate that EMT enhances the expression of important mesenchymal genes such as Vimentin, its transcription factor ZEB1, Snail, Slug, MMP2, and Twist1 to change the skeletal systems to mobilize the cells (54,55). Moreover, after EMT, the tumor cells not only become more plastic but also therapy resistant by expressing cancer stem cells markers (56). The theory “seed and soil” highlighted by Paget (1889) suggested the important role of dynamic interaction between the cancer cells and TME around the tumor to support the proliferation and invasion of cancer cells (57,58). The

emerging data have shown the role of exosomes in the TME, which may induce EMT and transfer the crosstalk signals between the cancer cells and the host microenvironment.

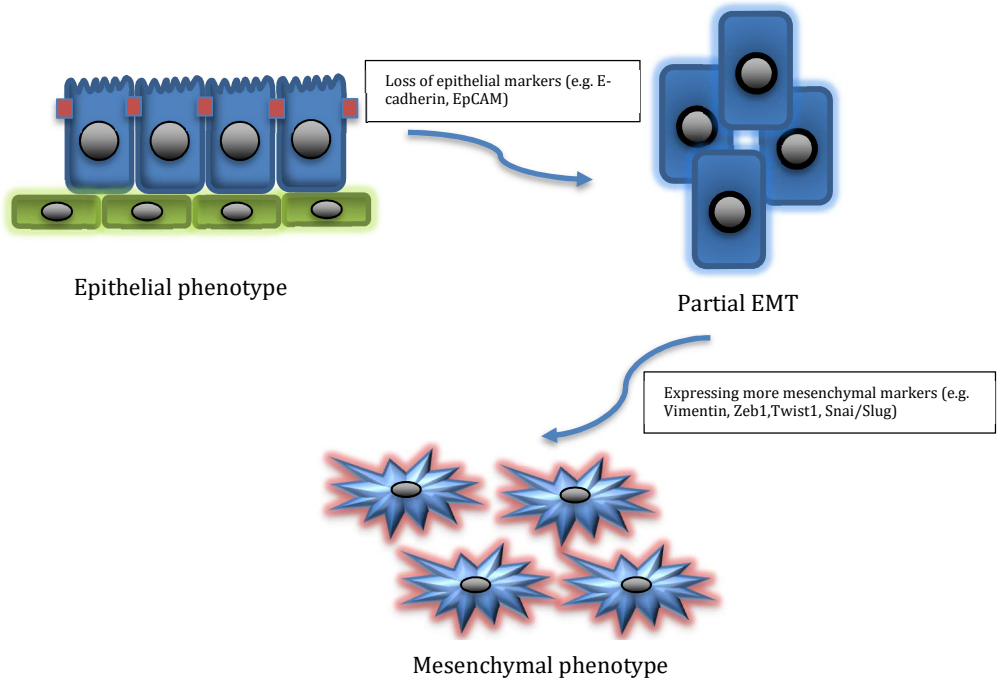


Figure 1. Epithelial-mesenchymal transition

Due to the critical role of Bromodomain and ExtraTerminal domain proteins (BET proteins) in regulating breast cancer metastasis by interacting with metabolic pathways and and the EMT program, much research has focused on the beneficial effects of BET inhibitors on treating a variety of cancer (59) in recent years. Bromodomain and ExtraTerminal domain proteins are a family of proteins that include BRD2, BRD3, BRD4 and germ-cell-specific BRDT (60). BET proteins play an important role in regulating gene transcription by specific binding to acetylated lysine residues in nucleosomal histones as an epigenetic factor (61,62). BET proteins have been established to be

significantly involved in malignancies such as prostate, lung, pancreatic and breast cancer, hence, intense awareness of the possibility of developing multiple BET protein inhibitors is an exciting new way to target particular malignant diseases (63,64,65). Recently, a number of research reports have investigated the ability of JQ1- a pan-BET inhibitor- to reduce the aggressiveness and metastatic capacity of breast cancer (66). However, the wide range of expression and function of each particular BET protein shows that specific BET inhibitors are needed. Recent data also show that a specific target BET inhibitor, MZ1, can modulate the signaling pathway of BRD4 proteins and tumor growth and invasive ability of tumor cells.

Here, we demonstrate how BET proteins regulate breast cancer aggressiveness promoted by insulin-resistant adipocyte-derived exosomes and highlight the effect of targeting BET proteins for breast cancer patients with type 2 diabetes.

MATERIALS AND METHODS

Cell Culture

The 4T1 cell line is a transplantable, highly tumorigenic and invasive breast cancer cell line that works well as an experimental animal model for human breast cancer. When transplanted into the BALB/c strain of mice, the 4T1 cell line spontaneously establishes a highly aggressive tumor that can metastasize to distant organs such as lymph node, lung, liver and brain. 4T1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (penicillin/streptomycin [P/S], Gibco). The cells were grown and maintained in 10 cm^2 tissue culture dishes in a humidified atmosphere in 37ºC and 5% CO_2 incubator.

Swiss 3T3-L1 fibroblasts were cultured in 10 cm^2 dishes until the cells reached confluence, then were kept over-confluent for 48 hours before they were differentiated for another 48 hours with DMEM supplemented with 1 µM dexamethasone, 0.5 mM isobutylmethyl xanthine (IBMX), 1.67 µM insulin, and 10% FBS (67). Subsequently, the cells were washed with phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$, and maintained in DMEM media supplemented with for 1.67 µM insulin and 10% FBS. Next, the cells were divided into two groups to be the insulin-sensitive (IS) and insulin-resistant (IR) adipocytes. We created IR adipocytes by treating 3T3-L1 adipocytes overnight with 1nM TNF-α and IS adipocytes as 3T3-L1 adipocytes without TNF-α treatment.

Exosome isolation

IS adipocytes and IR adipocytes were washed with PBS and kept in normal DMEM for 72 hours to allow the cells to secrete exosomes into the media (conditioned media). IS and IR adipocyte conditioned media were collected in Falcon 50ml Conical Centrifuge Tubes and centrifuged at 2,000g for 30 minutes at 4^{o}C to remove cells and debris. Total exosome isolation reagent (Invitrogen, US) was added into the cell-free conditioned media and mixed well. The samples were incubated at 4^{o}C overnight and were centrifuged at 10,000g for 1 hour at 4^{o}C. The pellets were resuspended in 500µL of PBS. Exosomes were quantified using NanoSight NS300 system and 4T1 cells were exposed to the same amount of exosomes.

qRT-PCR

Total RNA was extracted from 4T1 cells by using RNAeasy Kit (Qiagen). RNA integrity was assessed by Nano-drop. Reverse transcription reaction to generate cDNA was established with 1µg of total RNA with the QuantiTect® Reverse Transcription Kit (Quiagen). Complementary DNA was amplified using Tag-man PCR Master Mix on an Applied Biosystems (ABI) 7500 Fast Real-Time PCR Systems. The following gene-specific primers from Thermo Fisher were assessed: Vimentin (Mm01333430_m1), TWIST1 (Mm00442036_m1), SNAI1 (Mm00441533_g1), E-CAD (Mm01247357_m1), β-ACTIN (Mm02619580_g1), ZEB1 (Mm00495564_m1), TJP1 (Mm00493699_m1).

Immunoblotting

Cells were harvested from 10 cm^2 dishes and lysed in Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 150 mM

NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100). Sample protein concentrations were calculated by Bradford protein assay (68). Samples containing 20 µg of protein were loaded in 10 sample wells, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% saturated milk protein to prevent non-specific binding of antibodies. Membranes were incubated with specific primary antibodies in 4^{o}C overnight, washed with TBST and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. The membranes were washed with TBST and subsequently exposed in Enhanced chemiluminescent reagent (ECL) for 1 minute. Proteins were visualized with BioRad ChemiDoc^{TM} XRS+ Imaging System. The images were analyzed and quantified by ImageJ software (69).

The specific antibodies used were: anti-β-Actin as a housekeeping protein, anti-Vimentin, anti-E-cadherin, anti-TWIST1 (Cell Signaling), anti-BRD2, BRD3, and BRD4 from Bethyl Laboratories. Goat Anti-Mouse and Anti-Rabbit HRP-conjugated secondary antibodies were purchased from Biorad.

Transwell Migration assay

The migration assay was established with 8-µm-pore size Transwell migration inserts (Costar). 4T1 cells were exposed to insulin-sensitive exosomes, insulin-resistant exosomes, and regular media exosomes as control for 3 days. Next, the cells were serum starved for 3 hours. 4T1 cells were then harvested, counted, and seeded at 7.5 x 10^4 cells/well in the Transwell insert in 300 µL serum free DMEM. For migration assay, the

lower chamber was filled with 500 µL DMEM supplemented 10% FBS and 1% P/S (penicillin and streptomycin). Cell migration was allowed to progress for 10 hours at 37ºC, 5% CO_2 in a standard incubator. At the end of this time, all cells on the upper surface of the insert were wiped away with a cotton swab, and cells on the lower surface were fixed and stained with methanol for 10 minutes and crystal violet for 5 minutes, respectively. To test the effects of BET protein inhibition MZ1, a BRD4 targetted inhibitor was used at concentration ranging from 25-100 nM. Images were taken by EVOS XL Core Imaging System (AMEX-100) and analyzed by ImageJ migration assay macro. All the migration assays were repeated three times. MZ1 was purchased from Tocris.

Immunofluorescence

Cells were fixed in methanol for 5 minutes at -20ºC and permeabilized with PBS supplemented with 0.2% Triton X-100 buffer for 10 minutes. Permeabilized cells were incubated with blocking buffer (PBS supplemented with 0.02% Triton X-100 and 2% bovine serum albumin (BSA)) for 30 minutes to saturate all the non-specific protein-protein binding sites. Sequentially, the cells were incubated with primary antibodies for 30 minutes and then fluorochrome-conjugated secondary antibodies for 1 hour.

List of antibodies are as follows: anti-Vimentin (rabbit mAb, Abcam; 1:1,1000), anti-E-cadherin (mouse mAb, Abcam; 1:1,000), Goat anti-Mouse IgG Secondary antibody Alexa Fluor 647 (Invitrogen, US; 1:1,1000), Goat anti-Rabbit IgG Secondary antibody Alexa Fluor 594 (Invitrogen, US; 1:1,1000), Phalloidin –iFluor 488 (Abcam: ab176753; 1:1,000), DAPI (Thermo Fisher Scientific).

Statistical Analysis

The experiments were statistically analyzed by using Student's t-test and ANOVA in Excel 2010 and GraphPad Prism8. Graphs were displayed by GrahphPad Prism8, and $p<0.05$ was considered statistically significant.

RESULTS

Adipocyte-derived exosomes regulate EMT in breast cancer cells

In type 2 diabetes (T2D), insulin resistance and dysfunction of pancreatic β-cells have been widely demonstrated. Adipose tissue plays central roles in the transition from glucose intolerance to T2D. Recent data suggest that crosstalk between adipocytes and breast cancer by exosomes influences characteristics of breast cancer cells. However, the impact of insulin-resistant adipocytes has not been well established. This study focuses on inter-organ communication by exosomes between adipose tissue and breast tumor to test the hypothesis that metabolic dysfunction of adipocytes will also indirectly impact breast cancer cells.

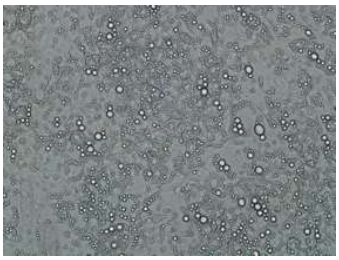
3T3-L1 Swiss pre-adipocytes were differentiated and stained with oil red O to image triglyceride containing lipid droplets (**Fig. 2 A and B**). Differentiated adipocytes were divided into two groups, which were either treated or not with 1 nM TNF-α. Non treated cells were Insulin-sensitive (IS) and TNF treated cells were insulin-resistant (IR). Exosomes from IS and IR 3T3-L1 adipocytes were isolated from media incubated with cells for 72 hours after TNF removal. IS adipocyte exosomes and IR adipocyte exosomes were quantified using a NanoSight NS300 system (**Fig. 2C**).

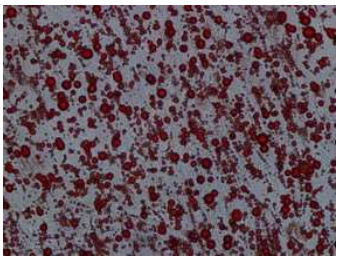
IS and IR adipocyte exosomes were then added to 4T1 breast cancer cell cultures in order to measure the response of these target cells. We found that the IR exosomes significantly increased EMT gene expression in 4T1 breast cancer cells. We assayed mRNA levels of the epithelial marker E-Cadherin (**Fig. 3A**), as well as mesenchymal markers TWIST1 and Vimentin (**Fig. 3B**) in the 4T1 control group, 4T1 treated with IS

exosomes and 4T1 treated with IR exosomes. We observed a significant increase of mesenchymal markers (TWIST1, Vimentin) in the IR exosome-treated group compared with IS exosome-treated group and control. Changes in mRNA levels between the three groups were matched by protein expression using actin as a loading control (Fig. 3C). Our interpretation is that the exosomal cargo will be different depending on the insulin sensitivity of the adipocytes that produced the exosomes.

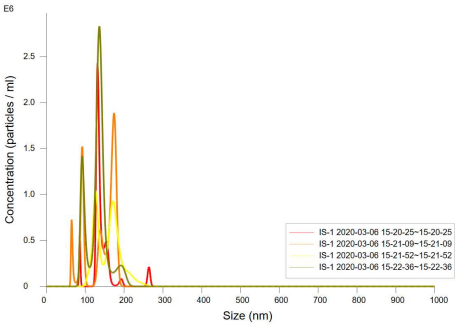
Emerging data by Dr. Naser Jafari suggest that human adipocyte-derived exosomes bring cartilage oligomeric matrix protein (COMP) to MCF7 human breast cancer cells to drive EMT in the targeted breast cancer cells. Proteomic analyses have revealed that insulin-resistant exosomes contain differentially elevated amounts of several proteins, the highest of which was COMP (also called TSP-5) in the payload of IR adipocytes. COMP provided by the exosomes promoted the EMT program in the targeted breast cancer cells. To test the hypothesis that COMP was the critically active factor for EMT function, we overexpressed recombinant COMP/TSP-5 in 4T1 cells using an expression vector and transfection with lentivirus. We observed that the transfected 4T1 cells had EMT gene expression that was significantly increased compared to 4T1 cells transfected with a vector encoding a scrambled sequence (**Fig.4**).

We considered that COMP is just one of several possible factors produced by exosomes that potentially caused the crucial effects on the breast cancer cells. The adipocyte-derived exosomes should be carefully analyzed for all their other contents, which may reveal the general picture of microenvironment around breast cancer cells, including microRNAs.





(A) **(B)**



Results

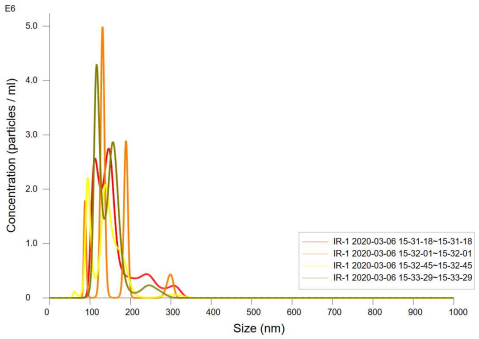
Stats: Merged Data

Mean:	141.9 nm
Mode:	132.6 nm
SD:	33.5 nm
D10:	92.9 nm
D50:	138.6 nm
D90:	180.7 nm

Stats: Mean +/- Standard Error

Mean:	143.6 +/- 5.7 nm
Mode:	142.1 +/- 10.9 nm
SD:	32.2 +/- 2.4 nm
D10:	107.0 +/- 9.9 nm
D50:	147.5 +/- 7.5 nm
D90:	174.7 +/- 9.2 nm
Concentration (Upgrade):	6.24e+07 +/- 1.05e+07 particles/ml
	7.8 +/- 0.9 particles/frame
	8.2 +/- 0.9 centres/frame

(C) Insulin-sensitive exosomes:



Results

Stats: Merged Data

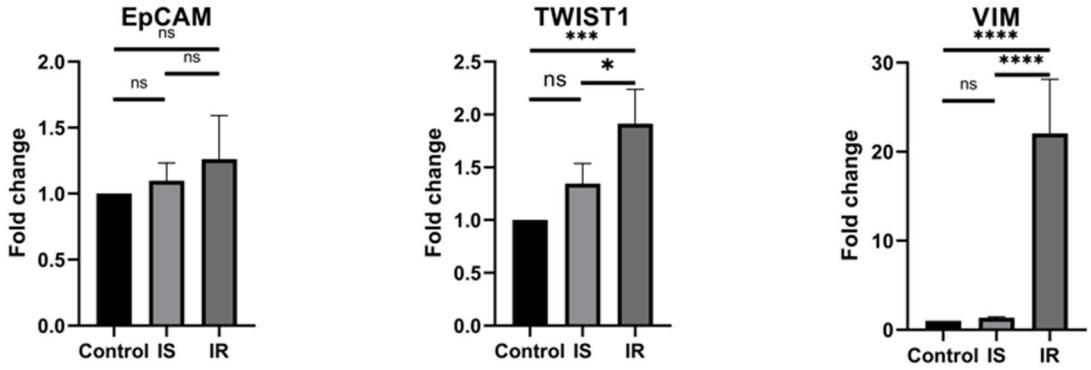
Mean:	151.2 nm
Mode:	131.3 nm
SD:	46.2 nm
D10:	106.3 nm
D50:	140.5 nm
D90:	201.5 nm

Stats: Mean +/- Standard Error

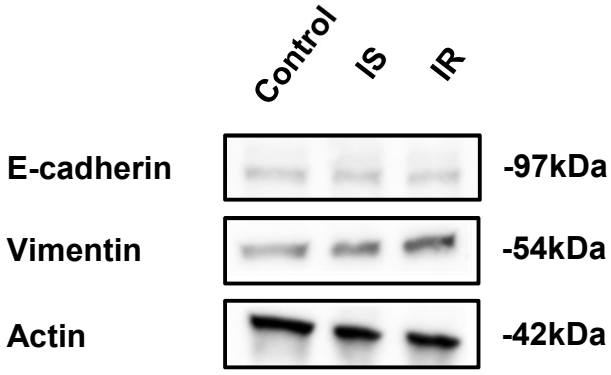
Mean:	150.8 +/- 4.2 nm
Mode:	122.1 +/- 11.0 nm
SD:	45.4 +/- 4.0 nm
D10:	101.8 +/- 5.3 nm
D50:	140.4 +/- 2.2 nm
D90:	201.2 +/- 14.4 nm
Concentration (Upgrade):	1.63e+08 +/- 2.13e+07 particles/ml
	19.6 +/- 2.2 particles/frame
	20.4 +/- 2.2 centres/frame

(D) Insulin-resistant exosomes:

Figure 2. 3T3-L1 adipocytes: light microscopy before staining with Oil Red O, 40X (A), after Oil Red O staining, light microscopy, 40X (B). Adipocyte-derived exosomes tracking analysis using NanoSight NS300 system. Insulin-resistant and Insulin-sensitive exosomes were quantified with manual focus and gain adjustment (C,D).



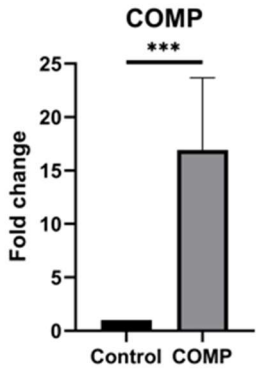
(A) EpCAM expression **(B) TWIST1, Vimentin expression**



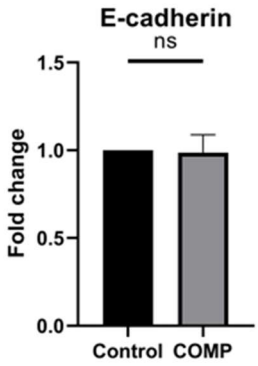
(C) E-cadherin, Vimentin expression

Figure 3. Adipocyte-derived exosomes induce markers of EMT in 4T1 cells. Epithelial markers, EpCAM (A), and EMT markers Vimentin, Twist1 (B) were determined by quantitative reverse transcription polymerase chain reaction with specific primers and beta-actin as house-keeping genes. E-cadherin and Vimentin were confirmed by immunoblot with actin as loading control (C). Mean ± SEM from

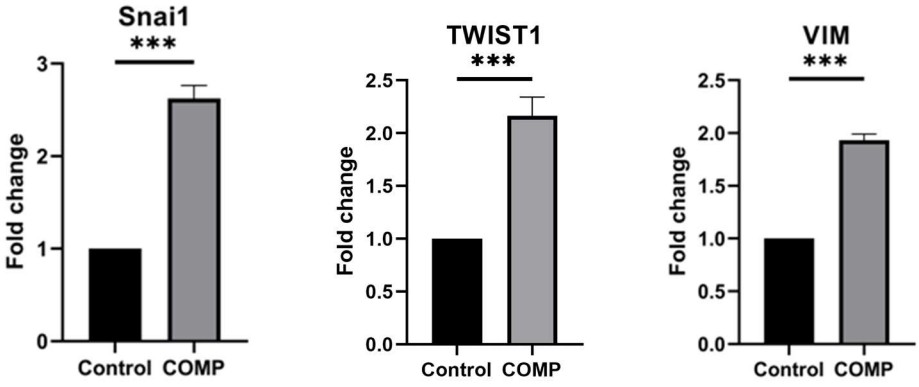
n=3 independent experiments performed in triplicates. *p<0.05, **p<0.01, ***p<0.001.



(A) COMP expression



(B) E-cadherin expression



(C) Snai1, Vimentin, Twist1 expression

Figure 4. Overexpression of COMP induces EMT in 4T1 cells. COMP was overexpressed in 4T1 cells using lentiviral transfection. Epithelial markers, E-cadherin (B), and EMT markers Snai1, Vimentin, Twist1 (C) were determined by quantitative reverse transcription polymerase chain reaction with specific primers and beta-actin as housekeeping genes. Mean ± SEM from n=3 independent experiments performed in triplicates. *p<0.05, **p<0.01, ***p<0.001.

Mesenchymal transition of 4T1 breast cancer cells correlates with increased migration

Exosomes containing RNAs, DNAs, metabolites and proteins regulated EMT in breast cancer cells. The cargoes of the exosomes are transmitted to the recipient cells to regulate the protein transcription and drive the EMT program of the targeted cancer cells. In addition to upregulation of EMT genes as measured by RT-PCR, we confirmed that functional changes also resulted from delivery of the exosome payload.

In order to understand the functional changes of 4T1 cells, which were exposed to IS exosomes and IR exosomes, we performed functional migration assays to demonstrate migration changes consistent with the EMT program and increased invasive/migratory behavior of the treated 4T1 cancer cells. We assayed 4T1 cell migration in a Transwell migration assay. 4T1 cells that were treated with insulin-resistant exosomes displayed the highest migratory capacity, compared to 4T1 cells treated with insulin-sensitive exosomes (which displayed an intermediate capacity) and compared to control (untreated 4T1 cells), which were the reference for the assay (**Fig. 5**). Hence, EMT observed in 4T1 cells induced by crosstalk between exosomes (IS and IR) and the cells themselves, and serves as a model for the TME of insulin resistant compared to insulin sensitive breast adipose tissue.

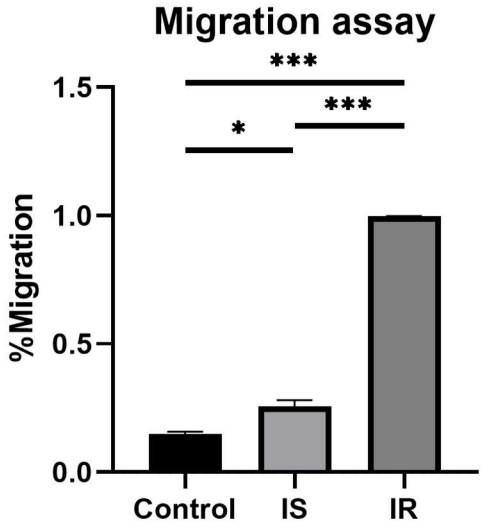


Figure 5. Adipocyte-derived exosomes increase migratory capacity of 4T1 cells. The migratory capacity of 4T1 cells was assessed by Transwell migration assay. Mean ± SD, with n=3 independent experiments established in triplicates.

Adipocyte-derived exosomes alter 4T1 breast cancer cell morphology

The morphology of 4T1 cells that internalized IR exosomes showed more mesenchymal characteristics, hence, they considerably differed from control and 4T1 cells treated with IS exosomes. Immunofluorescence (IF) staining showed that 4T1 cells

treated with insulin-resistant exosomes expressed the highest invasive capacity by expressing lower levels of the epithelial marker E-Cadherin and higher levels of mesenchymal marker Vimentin (**Fig. 6**). These experiments were conducted in collaboration with Dr Naser Jafari, a postdoctoral fellow in the lab. The fluorescence images confirmed immunoblot results of higher Vimentin levels in breast cancer cells treated with IR exosomes (**Fig. 3C**).

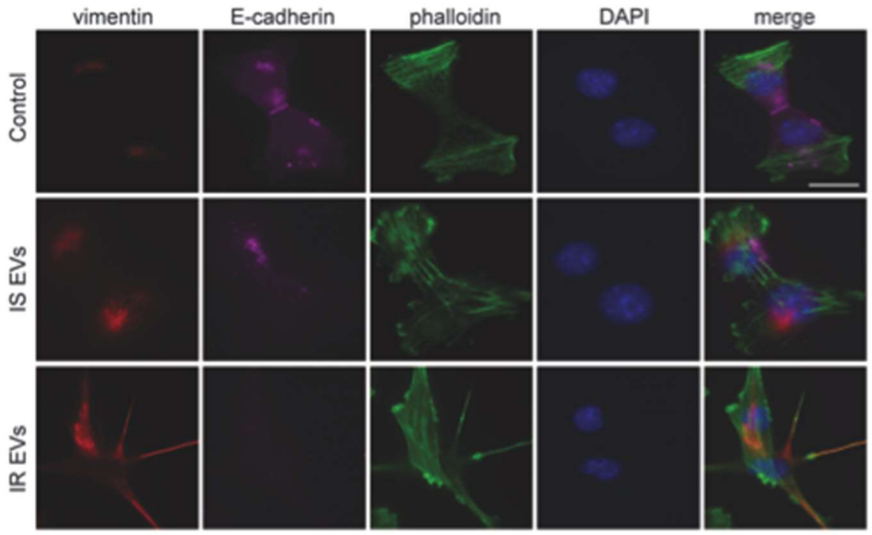
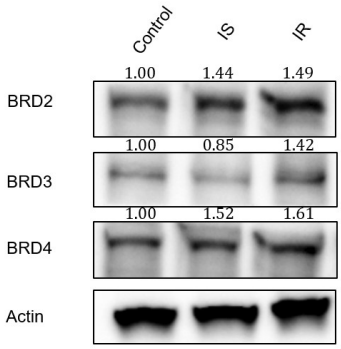


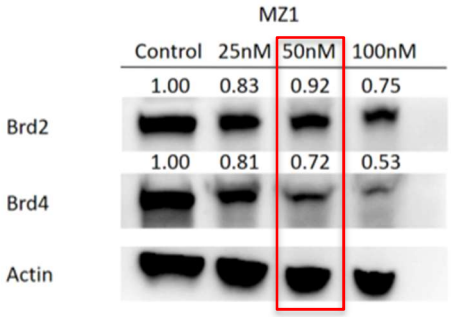
Figure 6. EMT in 4T1 cells after 72 hours on IS, IR exosomes exposure. Immunofluorescence staining of Vimentin, E-cadherin, DAPI and phalloidin was performed on 4T1 cells in control, IS exosomes and IR exosomes treated groups. Multiple immunofluorescence staining of EMT markers for Vimentin (red), E-cadherin (pink); DAPI (blue) and phalloidin (green).

BET protein inhibitor can downregulate aggressiveness and invasiveness of the breast cancer cells triggered by adipocyte-derived exosomes

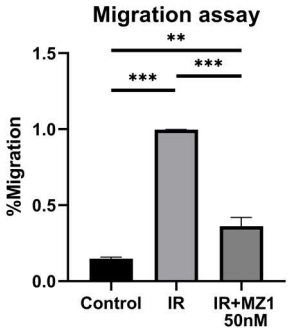
In order to explore how BET proteins regulate aggressiveness of 4T1 breast cancer cells, we assessed BET protein expression in 4T1 cells treated with IS and IR exosomes. Brd2 and Brd4 protein expression increased in 4T1 cells treated with IS and IR exosomes (**Fig. 7A**). The effect of IR exosomes on 4T1 cells is more remarkable compared with IS exosomes. Based on the significant increase of BET protein expression in 4T1 cells treated with exosomes, we tried to decrease the aggressiveness of 4T1 cells by inhibiting BET proteins by MZ1, a BRD4 specific inhibitor. We treated 4T1 cells with different doses of MZ1 (25nM, 50nM, and 100nM) for 72 hours to find out the optimal dose to specifically degrade BRD4 over BRD2. 4T1 cells were treated with IR exosomes, or co-treated with IR exosome and MZ1 50nM for 72 hours to conduct migration assay. The observation suggests that inhibition of BRD4 reduced migration and invasion capacity of 4T1 cells intensively (**Fig.7B**).



A.



B.



C. Migration assay

Figure 7. BET protein inhibition decreases migratory capacity of 4T1 cells stimulated by IR adipocyte-derived exosomes. BET proteins expression in 4T1 cells treated with IS and IR exosomes (A). MZ1 treatment with different doses to test its effect on BET protein expression in specific concentration, MZ1 50nM selectively degrades BRD4 over BRD2 and BRD3 (B). The invasion capacity of 4T1 cells was detected by Transwell migration assay. The area of cells adhered on the bottom of the inserts was quantified and depicted as a chart graph with n=3 independent experiments performed in triplicates (C)

DISCUSSION

Breast cancer has become an increasingly serious burden worldwide in terms of the number of new cases; approximately 2.1 million cases were reported in 2018. A number of complicated factors underlie the origins of the disease, such as lifestyle, environment, and comorbidity, which require more in depth understanding about risk factors, molecular and histological features of disease and their impacts on the progress of disease. Whereas the obesity epidemic has become a heavy burden in the developed country, obesity and its link with breast cancer have been studied with concern, in order to reduce the burden of both diseases and generally improve the outcome of patients who acquire both diseases. Obesity is universally associated with insulin resistance syndrome in type 2 diabetes. Excessive secretion of a variety of factors, such as fatty acids, cytokines, and many hormones, is related to the development of insulin resistance. However, the exact mechanism of this scenario is still not clear. Emerging studies show that exosomes may play an important role in various metabolic complications by the transportation of abnormal cargoes to specific cells. Results suggest that exosomes may be one of the key modulators in the TME.

In this thesis, we discovered that adipocyte-derived exosomes induce EMT in 4T1 cells, especially 4T1 cells treated with IR exosomes, which show about a 20-fold increase in Vimentin expression **(Fig. 3)**. A potential role in EMT induction is suggested as COMP/TSP5, which is one of the diverse adipocyte-derived cargo was increased **(Fig. 4)**. The induction of EMT changes 4T1 cells morphology to become more spindle-like cells and makes them more migratory and invasive **(Fig. 5 and Fig. 6).** Additionally, 4T1 cells

treated with IS and IR exosomes show an increase in BRD2 and BRD4 protein expression. We show that selective inhibition of BRD4 significantly decreases 4T1 cells migratory and invasive capacity induced by IR exosomes **(Fig. 7). Thus, we show that** BET proteins regulate breast cancer migration and invasion induced by adipocyte-derived exosomes.

In order to investigate the role of exosomes in TME, we utilized the 4T1 cancer cell line to study the association of adipocyte-derived exosomes with the tumor cell invasion capacity and EMT. This syngeneic murine cell line can be used in a future mouse model, to study *in vivo* experiments to mimic the general scenario of metastatic breast cancer.

The molecular and morphological alterations of 4T1 cells exposed to adipocyte-derived exosomes suggested a substantial degree of EMT of cancer cells in this model of TME. Recent studies propose that the aggressiveness of breast cancer is due in part to higher insulin concentration and higher IGF-1 level in patients with obesity. Yet many factors are simultaneously altered in obesity and metabolic disease that could drive breast cancer progression. We considered a role for adipocytes that have not previously been much studied: through crosstalk mediated by Evs. Our studies offer a novel approach to study EMT characteristics and the role of exosomes in the TME. However, exosomes have only relatively recently been identified as biologically important for cellular crosstalk, and our knowledge about exosomes is relatively limited, including the functional interaction of exosomes with recipient cells and the categorization of their

biological structure. Hence, exosomes should be analyzed and carefully quantified to create more in depth knowledge about cell-specific cargo.

Earlier studies demonstrated that tumor-derived exosomes regulate E-cadherin and promote tumor progression. More recently, exosomal miRNAs in mesenchymal cells enhance EMT and associate with metastasis in lung cancer. As emerging evidence shows that IR adipocyte-derived exosomes alter transcriptional profiles of EMT markers, it suggests the association of IR adipocyte-derived exosomes with metastasis in breast cancer. This work focuses on exosomes from IR adipocytes, which play a surprising and critical role in cancer progression. Indeed, isolation of pure exosomes is a major challenge in the field and even today there is no agreement on a standard method. We have used exosome precipitation, which gives a high yield and is good for functional tests. Throughout the study, insulin-resistant exosomes significantly enhanced the migratory ability of cancer cells by regulating the EMT program and changing the BET protein profile of their recipient cells. It is worthwhile to study how to reverse the insulin resistance as well as EMT and BET protein alteration inside cancer cells. These data suggest that weight loss can diminish breast cancer incidence rates, which have been studied and proven by a large, multisite cohort study. It would be interesting to investigate whether patients who have undergone weight loss show different exosomal properties. On the other hand, increasing insulin sensitivity may also provide a solution to reverse the insulin-resistant status of adipocytes. Metformin, a first line diabetes medicine, may improve insulin sensitivity and may reduce the production and release of dangerous exosomes into the microenvironment.

It is noteworthy that adipocyte-derived exosomes changed BET protein expression of 4T1 cells. Several studies suggested that BET proteins also appear to play an important role in metabolic pathways and reprogram cancer cell metabolic activities. A pan-BET protein inhibitor, JQ1, has been shown to increase insulin secretion in pancreatic islet β-cells and also to stimulate fatty acid oxidation, which may enhance cancer cell migration, metastasis and drug resistance. On the other hand, selective knockdown of Brd2 and Brd4 suggested their independent roles as major BET regulators of distinct metabolic pathways in the β cells. Knockdown or inhibition of all BET proteins (Brd2, Brd3, and Brd4) may have undesirable and adverse effects for particular breast cancer patients. Our data show that a Brd4-selective degrader, MZ1, can downregulate migratory capacity of breast cancer cells. It is intriguing that selective BET protein inhibitors may be useful to reprogram insulin resistance status in adipocytes and become an adjuvant therapy in insulin resistance-related cancer.

Our study focuses on the 4T1 cell line, which is derived from murine mammary carcinoma from BALB/c mouse. This cell line is suitable for *in vivo* and *in vitro* study, and is implantable into syngeneic BALB/c mice. The studies about metastatic disease in the exosome field, using mice, are still limited and challenging. Our study based on the 4T1 syngeneic breast cancer cell line suggests the feasability of an *in vivo* study. By being a COMP carrier, insulin-resistant exosomes have shown their ability to enhance the aggressiveness of breast cancer cells even far away from the origin of the exosomes. A next step in the analysis might be to treat BALB/c mice with insulin-resistant exosomes and study its effects on breast cancer metastasis in mice. We previously addressed the

crucial role of IR exosomes in cancer development and progression *in vitro*. Results suggest that if we implant 4T1 breast cancer cells into BALB/c mice, we can reveal whether BALB/c mice treated with IR exosomes show augmented increase of metastasis and tumor aggressiveness in vivo.

Overall, our findings provide important evidence and demonstrate the role of exosomes in crosstalk between cancer cells and adipocytes, especially IR adipocytes. Our results provide preliminary data for an *in vitro* study in a mouse model to be developed in the near future. Upon crosstalk with adipocytes, breast cancer cells can be energized to start metastatic progression and become more invasive and more aggressive in patients who have acquired insulin resistance syndrome. Intriguingly, we pointed out that breast cancer cells overexpressed Brd2 and Brd4 proteins, but Brd3. Specific targeting to knock down individual BET proteins is expected to control the metastatic disease, as our results suggest with MZ1, a Brd4-selective inhibitor. Additional studies are needed to develop a new epigenetic strategy involving specific BET protein transcriptional pathways for breast cancer treatment.

LIST OF JOURNAL ABBREVIATIONS

Anticancer Res……. Anticancer Research

Breast Cancer Res…… Breast Cancer Research

CA Cancer J Clin……. CA: A Cancer Journal for Clinicians

Cancer Metastasis Rev……. Cancer Metastasis Reviews

Cancer Res……... Cancer Research

Curr Diab Rep…….. Current Diabetes Reports

J Biol Chem……. Journal of Biological Chemistry

J Cell Biochem……. Journal of Cellular Biochemistry

J Exp Med…… Journal of Experimental Medicine

Nat Rev Cancer…… Nature Reviews. Cancer

Nat Rev Dis Primers…… Nature Reviews. Disease Primers

Nat Rev Mol Cell Biol……. Nature Reviews. Molecular Cell Biology

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