Original Article

The effects of Stem Cell-Conditioned Media on Malignancy Behavior of Breast Cancer Cells *in Vitro*

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ABSTRACT

Breast cancer represents the most frequently diagnosed form of cancer among women on a global scale. In recent years, there has been a notable increase in interest among researchers in exploring alternative therapeutic methods, including stem cell therapy. The objective of this study was to examine the impact of adipose-derived mesenchymal stem cell-conditioned media (AD-MSCs-CM) on apoptosis induction and migration inhibition of breast cancer cells (MDA-MB-231) in vitro. In this study, malignant breast cancer cells (MDA-MB-231) and adipose-derived mesenchymal stem cells (AD-MSCs) were cultured separately in DMEM/F12/FBS (15%) culture media under standard conditions. Subsequently, the conditioned media derived from AD-MSCs was introduced to the MDA-MB-231 cells. Following a 24- and 48-hour exposure period, the expression levels of CASP3, KRAS, and MMP9 were evaluated using a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. Furthermore, the proliferation and migration abilities of the cancer cells were evaluated using MTT and wound healing assays, respectively. Furthermore, the protein expression of Caspase-3, K-RAS, and MMP-9 was examined using a western blot assay. It is noteworthy that the expression levels of the MMP9 and KRAS genes were significantly reduced following treatment with AD-MSCs-CM in MDA-MB-231 cells. Furthermore, the CASP3 gene expression level was found to have increased significantly in the treated groups. Additionally, the proliferation of MDA-MB-231 cells treated with AD-MSCs-CM was markedly diminished by MTT and wound healing assays. Moreover, the AD-MSCs-CM was observed to induce caspase-3 activation and reduce the protein expression of K-RAS and MMP-9. The results of this study indicate that AD-MSCs-CM may exert an influence on the apoptosis, proliferation, and migration of breast cancer cells. Consequently, it could be proposed as a promising therapeutic strategy for the suppression of breast cancer. However, further testing and research are required to validate these findings and to ascertain the full potential of this approach.

Keywords: Breast Cancer, Stem Cells, Apoptosis, Migration, Gene Expression.

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1. Introduction

Breast cancer (BC) is the most frequently diagnosed malignancy among females and remains one of the primary causes of cancer-related deaths globally (1). The most aggressive form of breast cancer is triple-negative breast cancer (TNBC), which is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (2). In contrast to other clinical subtypes of breast cancer, TNBC does not have a tumor-specific treatment. The range of available treatment options for TNBC is currently limited to surgical intervention, in addition to other therapies such as chemotherapy and radiotherapy. Consequently, researchers are investigating innovative therapeutic strategies with the objective of enhancing the prognosis of TNBC patients (3). Stem cells are fundamental to the processes of tissue development, homeostasis, and regeneration. This is achieved through asymmetrical division, whereby two cells are generated: a new stem cell and an additional progenitor cell (4). Mesenchymal stem cells (MSCs) are a population of highly adaptable, self-renewing stromal cells that have demonstrated notable efficacy in the management of malignancies (5). Mesenchymal stem cells (MSCs) are capable of differentiating into a number of different cellular lineages, including chondrocytes, adipocytes and osteoblasts. This distinctive attribute renders them a promising instrument for therapeutic interventions with the objective of facilitating tissue repair and recuperation in patients afflicted with a spectrum of medical conditions (6). The term "microenvironment of cells" is a widely used term in the scientific community to describe the intercellular matrix, nearby cells, cytokines, and humoral components. Furthermore, it is the site of cell-cell contact. The stability of the microenvironment is of great importance for the maintenance of cell differentiation, metabolism, proliferation, and other functional processes. Abnormal alterations in the constituent parts of the microenvironment can impede cellular functions (7). A substantial body of evidence indicates that mesenchymal stem cells (MSCs) can release exosomes that alter the tumour microenvironment and play a pivotal role in the development of various cancers. Exosomes derived from MSCs play a pivotal role in cancer resistance to chemotherapy, targeted therapies, radiation, and immunotherapy (8). It has recently been observed that MSC-derived conditioned media (CM), including exosomes released during cell culture, is as effective as source cell direct therapy (9). Adipose-derived mesenchymal stem cells (AD-MSC) constitute a subtype of mesenchymal stem cells that offer a number of significant advantages. They can be obtained from minimally invasive procedures and produce a substantial quantity of multipotent stem cells with the capacity to differentiate into multiple lineages, exhibit immunomodulatory properties, and promote tissue regeneration via the secretion of extracellular vesicles containing trophic factors. The secretome is currently being investigated as a potential source for a cell-free based therapy for human cancers. If proven effective, this approach would significantly reduce the associated costs, risks, and legal constraints, allowing for a wider application in actual clinical practice (10). The principal objective of this study was to assess the impact of AD-MSCs-CM on the apoptosis, proliferation, and migration of MDA-MB-231 cells in vitro.

2. Materials and Methods

2.1. Cell Culture and AD-MSCs-CM Preparation

The human mammary gland adenocarcinoma cells (MDA-MB-231) were sourced from the National Cell Bank at the Pasteur Institute in Tehran, Iran. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, USA), supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA) and 100 units/ml penicillin and 100 µg/mL streptomycin (GIBCO, USA), and incubated at 37°C in a controlled humidified atmosphere of 5% CO2. The human adipose-derived stem cells (AD-MSC) were procured from the Iranian Biological Resource Center, Tehran, Iran. The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM-F12) (GIBCO, US) containing 2 mM L-glutamine and 1% penicillin-streptomycin (GIBCO, USA), supplemented with 15% FBS (GIBCO, USA), and maintained in a controlled humidified atmosphere of 5% CO_2 and 37°C. The fourth passage of cells was employed in the subsequent experiments. MDA-MB-231 cells were subjected to a sequential adaptation process, wherein they were gradually exposed to an increasing ratio of DMEM-F12 to RPMI medium (40:60, 60:40, 80:20, and finally 100:0). The culture media for the mesenchymal stem cells (MSCs) was collected and subjected to centrifugation at 1000 g for a period of five minutes, with the objective of removing the entire cellular content. Subsequently, the supernatant was subjected to filtration through a 22/0 µm pore filter, with the objective of removing cell debris and large vesicles. Following this, the sample was stored at a temperature of -80°C, in preparation for the subsequent experiments.

2.2. MTT Assay

An MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) assay (Bio Basic, Canada) was employed to assess the cytotoxic effects of AD-MSCs-CM on MDA-MB-231 cells. In brief, MDA-MB-231 cells were seeded in 96-well plates at a density of 2×10^3 cells/well and incubated for 24 hours in DMEM-F12. Once the cells had reached 50-70% confluence, they were treated with six different doses of MSC-CM, comprising 60, 80, 100, 150, 200 and 240 µl. Subsequently, the medium of each well was replenished to 200 µl with complete culture medium (DMEM-F12, FBS 15%) and incubated for 48 hours. Subsequently, 50 µl of MTT solution (2 mg/ml) was added to the wells, after the removal of the culture medium. Subsequently, the cells were incubated for a further four hours. Subsequently, the culture medium containing MTT was removed, and 200 µl of DMSO (Applichem, Germany) was added to each well, which were then

incubated at 37°C for an additional 30 minutes. The ocular density of each well was assessed at 570 nm using a Sunrise ELISA reader (Tecan, Switzerland). Furthermore, to substantiate the claim that MDA-MB-231 cells must undergo adaptation to DMEM-F12 prior to treatment in all of the tests under consideration, a series of CM dosages (60, 80, 100, 150, 200, and 240) were administered to non-adapted cells, with each well supplied with 200 µl of RPMI medium. Additionally, a control group of MDA-MB-231 cells that had not been adapted to RPMI medium was included.

2.3. Wound-Healing Assay

In order to ascertain the influence of AD-MSCs-CM on cellular migration, a wound-healing assay was employed. MDA-MB-231 cells (2.5×10^5 cells/well) were cultivated in 24-well plates. Subsequently, wounds were created in the cell monolayers by introducing a 200 µl sterile pipette tip into the center of each well under standard incubation conditions. A serum-free medium was employed for the removal of debris. Subsequently, the cells were treated with CM in three groups, comprising 25%, 50%, and 100% of the total well volume (2×10^3 microliters), respectively. Additionally, a control group of MDA-MB-231 breast cancer cells was maintained in 100% DMEM-F12 FBS 15% media. Subsequently, the wells were observed under an inverted microscope (Optika, Italy) at 0, 24, and 48 hours post-wounding.

2.4. Gene expression

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was employed to evaluate the expression levels of a panel of genes, including CASP3, MMP9, and KRAS. The primers were designed using the online primer sequence creation tool available on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). In conclusion, total RNA was extracted from cells in accordance with the manufacturer's instructions using TRIzol reagent (GeneAll, South Korea). The NanoDrop (Thermo Scientific, USA) was employed to measure the absorbance at 260 and 280 nm, respectively, to assess the quantity and quality of the isolated RNA. Subsequently, cDNA was synthesized in accordance with the manufacturer's instructions using a commercial kit (Biofact, South Korea). The reactions were conducted using Taq DNA Polymerase Master Mix (Ampliqon, Denmark) and a LightCycler 96 real-time PCR equipment (Roche, Germany). The expression of the target genes was normalized using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as a reference. Table 1 presents the primer sequences utilized in the present study.

2.5. Western Blot

For this purpose, 106 cells were lysed in 500 µl of lysing buffer and subjected to centrifugation at 12,000 x g for 10 minutes at 4°C. The resulting supernatant, which contained the extracted protein, was stored at a temperature of -20°C. The protein concentration was determined by means of the Bradford protein assay. The protein samples were typically separated using 10% SDS-PAGE electrophoresis, followed by transfer onto a PVDF membrane (polyvinylidene difluoride) (GE Healthcare, Amersham, Buckinghamshire, UK). Subsequently, the PVDF membranes were blocked for two hours at room temperature while shaking in a solution of 2% nonfat dried milk in TBST (Tris-buffered saline with Tween 20). Subsequently, the membranes were treated with monoclonal antibodies against MMP-9 (Santa Cruz, USA, 1:300, sc-393859), K-Ras (Santa Cruz, USA, 1:300, sc-30), Caspase-3 (Santa Cruz, USA, 1:300, sc-7272), and ß-catenin (Santa Cruz, USA, 1:300, sc-47778) overnight at 4°C. Subsequently, the membranes were incubated with secondary antibodies, including anti-rabbit IgG-HRP (Santa Cruz, USA, 1:1000, sc-2357) and m-IgGκ BP-HRP (Santa Cruz, USA, 1:1000, sc-516102), for a period of two hours at room temperature. Subsequently, the protein bands were visualized by exposing the membranes to ECL Advance Reagent (Amersham, UK). The signal intensity of each band was quantified using ImageJ 1.62 software (National Institutes of Health, USA), with ß-actin serving as the reference protein. The CM-treated groups and the control group utilized in the Western blotting analysis were comparable to those employed in the wound-healing assay.

2.6. Statistical Analysis

Each experiment was conducted in triplicate. The non-parametric one-way ANOVA and t-test were employed to evaluate the provided data using the GraphPad Prism 8 program, and the outcomes were presented as the mean standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. AD-MSCs-CM reduced the cell viability of DMEM-F12 FBS 15% adapted MDA-MB-231 cells in a dose-dependent manner

As illustrated in Figure 1, the cell viability rate decreased in MDA-MB-231 cells adapted to DMEM-F12 FBS 15% medium as a result of an increase in the concentration of AD-MSCs-CM. A significant difference was observed between the treated groups in high and low doses of CM ($p<0.05$). As illustrated in Figure 1, the non-adapted MDA-MB-231 cells exhibited no discernible changes in survival rate when compared to the DMEM-F12 FBS 15% adapted cells, irrespective of the dose of CM administered. It should be noted that the medium of MDA-MB-231 cells prior to adaptation was RPMI FBS 10%. Consequently, the addition of increasing doses of AD-MSCs-CM, which includes DMEM-F12 FBS 15% along with secreted factors from MSCs, resulted in an unintended increase in the FBS concentration added to the treated groups. This ultimately led to the observation that the number of cells in the control treatment group remained unaltered. A notable disparity was evident in the cell viability of the adapted groups in comparison to the non-adapted ones ($p<0.05$). Accordingly, all subsequent tests were conducted using adapted cells.

Table 1. Sequences of the primers used in the present study.

Gene Name	Forward/ reverse	Nucleotide sequences
CASP3	F R	5'-TGTCATCTCGCTCTGGTACG-3' 5'-AAATGACCCCTTCATCACCA-3'
MMP9	F R	5'-GGTTCTTCTGCGCTACTGCTG-3' 5'-GTCGTAGGGCTGCTGGAAGG-3'
KRAS	F R	5'-CCTAAACTCTTCATAATGCTTGCTC-3' 5'-CCACAAAATGGATCCAGACA-3'
GAPDH	F R	AACATCATCCCTGCCTCTAC-3''-5 -CTGCTTCACCACCTTCTTG-3''5

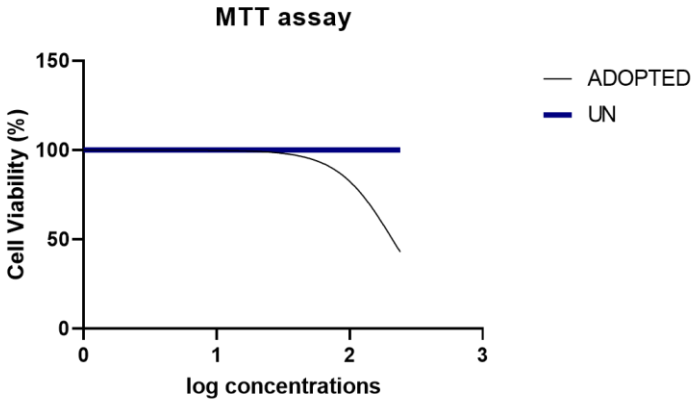

Figure 1. Comparative cell viability rates of CM treated DMEM-F12 FBS 15% adapted and non-adapted MDA-MB-231 cells in 48 hours.

3.2. AD-MSCs-CM reduced the MDA-MB-231 breast cancer cell migration rate

The results of the wound-healing assay indicated that as the dose of AD-MSCs-CM increased, the migratory rate and motility of adapted MDA-MB-231 breast cancer cells exhibited a notable decline. In comparison to the negative controls, the groups treated with conditioned media exhibited a notable inhibitory impact on cell migration after 48 hours (Figure 2).

3.3. AD-MSCs-CM altered the expression levels of migration, proliferation, and apoptosis related genes

The results of the qRT-PCR analysis demonstrated a significant correlation between the 50% AD-MSCs-CM treatment and an increase in CASP3 gene expression ($p<0.01$), as well as a decrease in KRAS and MMP9 gene expression ($p<0.01$ and $p<0.05$, respectively) when compared to the control group (Figures 3A, B, and C).

3.4. AD-MSCs-CM changed the expression levels of migration, proliferation, and apoptosis related proteins

As illustrated in Figure 4, the data indicate a notable rise in Caspase-3 activation (as gauged by the cleaved/pro form) in 50% AD-MSCs-CM. Furthermore, the expression levels of MMP-9 and K-Ras in MBA-MD-231 cells were found to decrease significantly as the concentration of AD-MSCs-CM increased.

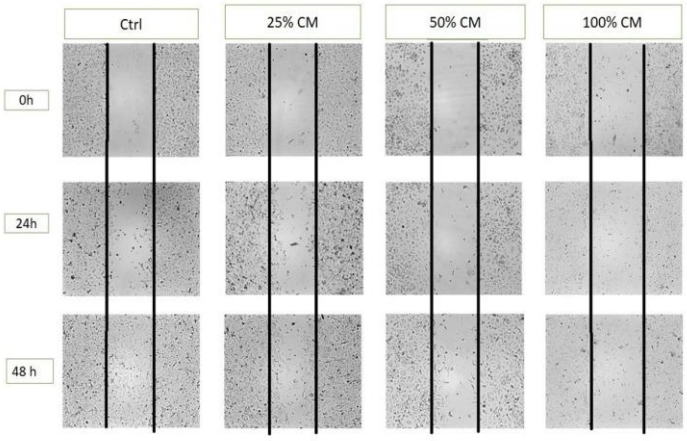

Figure 2. Migration ability of MDA-MB-231 cells were significantly reduced by the increase of CM concentration compared to negative control.

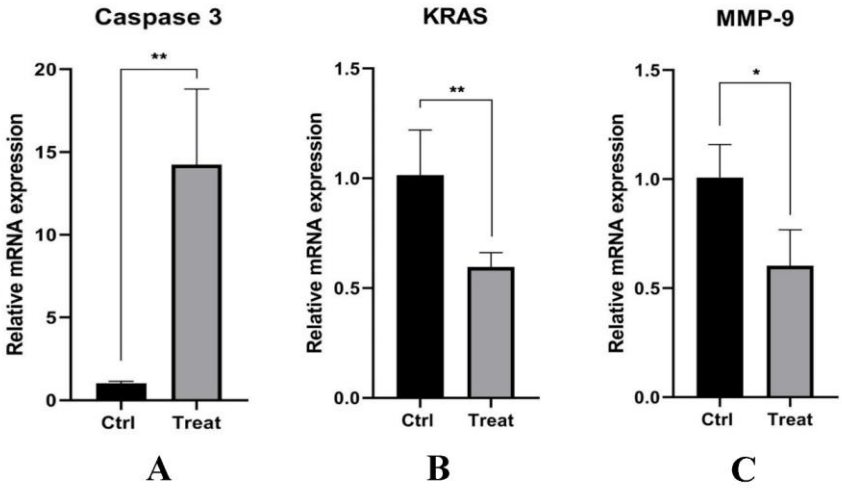

Figure 3. The effects of treatment with AD-MSCs-CM on the expression of apoptosis-related gene, CASP3 (A) compared to the control group. The AD-MSCs-CM treatment down regulates the expression levels of KRAS(B) and MMP9 (C) genes compared to the control group (*p<0.05 **p<0.01, ****p<0.0001).

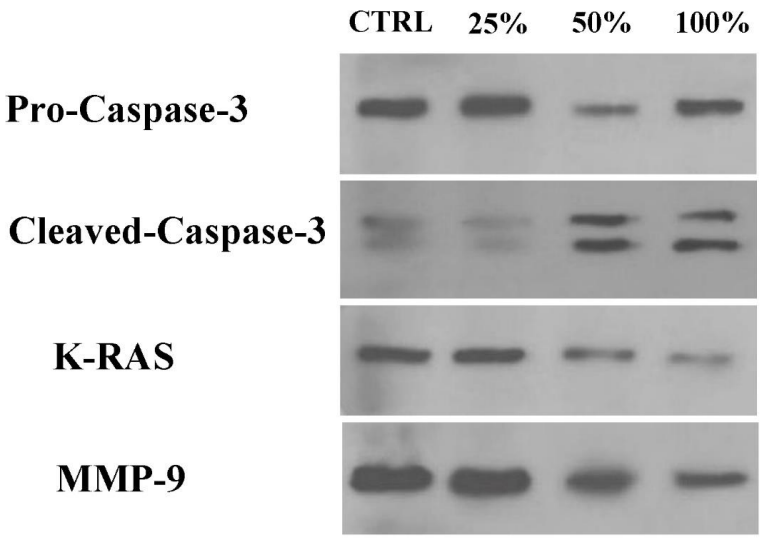

Figure 4. The relative protein expression of Caspase-3, K-RAS and MMP-9 via Western blot. The effects of treatment with different doses of AD-MSCs-CM on the expression levels of proteins compared to the control group. (Groups include: Ctrl 25%CM 50%CM 100%CM).

4. Discussion

The findings of this study demonstrated that the conditioned media derived from adipose mesenchymal stem cells have the potential to influence apoptosis, proliferation, and migration of breast cancer cells via a variety of specific targets, including Caspase-3, K-RAS, and MMP-9. These targets were evaluated using qRT-PCR, MTT assay, Western blot analyses, and scratch test. The current findings indicate that the conditioned media derived from adipose mesenchymal stem cells can mitigate the malignant behavior of breast cancer cells in vitro. The literature indicates that there are multiple challenges in the field of cancer treatment. In this regard, a number of factors contribute to the decreased effectiveness of cancer therapy results, including drug resistance, inadequate understanding of the epigenetic characteristics, the absence of reliable biomarkers for cancer diagnosis, and the presence of cancer stem cells (11). Mesenchymal stem cells have been the subject of extensive investigation for several decades, with encouraging results emerging in the treatment of metastatic malignancies, including breast cancer (12). MSCs offer considerable promise for the targeted delivery of oncolytic viruses, suicide genes, and anticancer medicines to malignant tissues (13-15). Some in vitro and in vivo investigations have demonstrated that MSCs can contribute to tumor development by promoting metastasis, cell

growth, proliferation, and treatment resistance (16). Conversely, MSCs have been demonstrated to possess anticancer properties, including the ability to slow the cell cycle, prevent cell growth, trigger apoptosis, and encourage immune cell infiltration (17). A substantial body of evidence indicates that MSCs can inhibit cell proliferation, induce apoptosis, and impede invasion through the modulation of specific targets, including Caspase-3, K-Ras, and MMP-9 (18-21). The safety of adipose-derived mesenchymal stem cells (ADSCs) has been extensively demonstrated in the context of breast reconstruction, with extensive characterization of their properties (22). Therefore, the objective of this study was to examine the anti-cancer impact of adipose-derived mesenchymal stem cell-conditioned media on the MDA-MB-231 breast cancer cell line in vitro. The MTT assay demonstrated a dose-dependent reduction in viability of MDA-MB-231 cells following treatment with AD-MSCs-CM. A previous study indicated that the proliferation of MCF-7 breast cancer cells was inhibited by AD-MSCs-CM in a dose-dependent manner (23). Another study demonstrated that mesenchymal stem cells (MSCs) and their conditioned media (CM) inhibited the proliferation of four distinct ovarian cancer cell lines (20). This finding aligns with other studies that have reported the anti-proliferative effect of AD-MSCs-CM on various cancer cells (21, 24, 25). In this study, the expression levels of CASP3, as determined by qRT-PCR, exhibited a notable increase following treatment with AD-MSCs-CM. Furthermore, western blot analyses indicated that AD-MSCs-CM facilitated caspase-3 activation in MDA-MB-231 cells. These findings indicate that apoptosis was markedly enhanced following AD-MSCs-CM treatment. Caspase-3 is a member of the cysteine-aspartate specific protease family of enzymes that initiates the extrinsic apoptosis pathway in response to external stimulation of death receptors. It functions as both an executioner and an initiator caspase. Our findings are consistent with previous reports that have demonstrated the anti-apoptotic effects of MSC-CM in a range of malignant diseases. Prior research indicates that MSC-CM from diverse tissue sources can induce apoptosis by enhancing caspase-3 activity (26-28). The results of the current study, which employed a wound healing assay, qRT-PCR (gene expression levels), and western blot analyses of MMP-9 and K-RAS, indicated that treatment with AD-MSCs-CM had a significant inhibitory effect on the proliferation and migration of MDA-MB-231 breast cancer cells. The results of the wound healing assay demonstrated that AD-MSCs-CM exhibited a dose-dependent inhibitory effect on cell migration after 48 hours. This finding is in accordance with previously published literature regarding the inhibitory effect of AD-MSCs-CM on glioma, liver, lung, and breast cancer cell migration (27, 29, 30). MMP-9, a member of the zinc-dependent endopeptidase family, has been linked to the development of breast cancer, particularly in patients with triple-negative and HER2-positive subtypes (31). These subtypes are more aggressive and are associated with a higher risk of metastasis. The expression level of MMP-9 has been demonstrated to correlate with the metastatic potential and staging of breast cancer (31). The current findings indicate a reduction in MMP-9 expression following treatment with AD-MSCs-CM. This downregulation indicates that the AD-MSCs-CM treatment may have a suppressive effect on the expression of MMP-9, which could contribute to the inhibition of human breast cancer cell metastasis. The RAS superfamily of GTPases, which includes K-RAS, H-RAS, and N-RAS, is the most commonly mutated oncogene in numerous human cancers. Although RAS mutations are relatively uncommon in breast cancers, there is substantial experimental evidence indicating that hyperactive Ras can facilitate the growth and development of breast cancer (32). It is noteworthy that K-RAS is the isoform within the RAS family that is most frequently mutated in high-grade cancer (33, 34). The expression level of K-RAS was markedly diminished following treatment with AD-MSCs-CM, as determined by qRT-PCR and western blot analysis. Previously, Huo et al. demonstrated that K-Ras overexpression increased PI3K signaling in MSCs, and K-Ras/PI3K-activated MSC-CM inhibited tumor cell proliferation and migration in pancreatic ductal adenocarcinoma (18). Despite the extensive documentation of the involvement of the oncogenic K-RAS pathway in a multitude of aggressive cancer types, the mechanism of activation in chemo-resistant, relapsed, and metastatic TNBC remains unclear (35). Moreover, there is a paucity of research examining the impact of MSC-CM on K-RAS expression in diverse cancers, particularly breast cancer. The findings of this study indicate that adipose-derived mesenchymal stem cell-conditioned media treatment has the potential to be an effective therapeutic strategy for reducing cell viability, inhibiting proliferation, and impeding migration of breast cancer cells. Further investigation is required to elucidate the precise mechanisms underlying the efficacy of MSC-CM treatment in breast cancer. Further research should be conducted to identify the specific cellular and molecular pathways affected by MSC-CM treatment and to elucidate the underlying molecular mechanisms by which it exerts its anti-cancer effects. Furthermore, a comprehensive investigation into the potential adverse effects and long-term safety of MSC-CM treatment is essential for evaluating its translational potential.

Abbreviations

BC: Breast cancer; TNBC: Triple-negative breast cancer; MSCs: Mesenchymal stem cells; AD-MSCs: Adipose-derived mesenchymal stem cells; CM: Conditioned media; KRAS: Kirsten rat sarcoma viral oncogene homolog; MMP9: Matrix metallopeptidase 9.

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Authors' Contribution
MKh, BB, and RA conceived and planned the experiments; MN, SN and MA carried out the experiments; all authors discussed the results and contributed to the final manuscript.

Ethics
Not applicable.

Conflict of Interest
The authors certify that they have no competing interests.

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Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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