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Association of miR-326, SIRT1, and HMGA2 Expression in Breast Cancer Cells Exposed to Mesenchymal Stromal Cell-Derived Extracellular Vesicles

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Abstract

Background: Breast cancer progression is influenced by tumor–microenvironment interactions mediated by Extracellular Vesicles (EVs), which transfer bioactive molecules such as microRNAs. This *in vitro* study investigated the role of miR-326 in modulating SIRT1 (Sirtuin 1) and HMGA2 (High-mobility group AT-hook 2) expression in breast cancer cells after treatment with EVs derived from Mesenchymal Stromal Cells (MSCs).

Methods: EVs were isolated from MSCs using the ExoQuick kit and characterized by Dynamic Light Scattering (DLS). EV internalization was confirmed by PKH26 fluorescent staining. After determining the appropriate dose by MTT assay, EVs were applied to MDA-MB-231 cells. Expression levels of miR-326, SIRT1, and HMGA2 were measured by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR). *GAPDH* and *U6 snRNA* were used as internal reference genes for mRNA and miRNA normalization, respectively. Data were analyzed using the unpaired Student's t-test.

Results: The mean size of EVs was approximately 120 *nm*. The highest viability of MDA-MB-231 cells was observed at an EV dose of 30 *µg/ml*. Treatment with EVs caused a significant decrease in miR-326 expression ($p<0.05$) and significant increases in the expression levels of SIRT1 ($p<0.01$) and HMGA2 ($p<0.05$).

Conclusion: These findings suggest a potential association between miR-326 and its target genes, highlighting a possible therapeutic target within the tumor microenvironment.

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Introduction

Breast cancer is one of the leading cancers in women and still faces significant clinical challenges; its diversity, distribution beyond the original site, and often resistance to therapies add to this problem. Tumor growth is influenced not only by genetic and non-genetic changes in cancer cells but also by signals from the surrounding tumor microenvironment. Extracellular Vesicle (EV)-mediated communication has become an important process in modulating tumor growth, dissemination, and response to therapy [1-3].

EVs are small, membrane-bound particles released by nearly all cell types. They have proteins, lipids, mRNAs, and non-coding RNAs, which can share information between cells. In breast cancer, EVs released by stromal and mesenchymal cells have gained interest. Such EVs can either stimulate or inhibit cancer behavior depending on their contents and cell of origin, adding to the complexity of the tumor environment [4-7].

Among the molecules detected in EVs, microRNAs (miRNAs) are important for gene expression regulation. Changes in miRNA expression are associated with breast cancer development, progression, metastasis, and drug resistance [8-10]. miR-326 has been identified as a specific tumor suppressor in various types of cancer. In breast cancer, lower levels appear to be associated with more aggressive tumors, while miR-326 restoration can slow tumor growth and improve the response to therapy [11-14].

SIRT1 and HMGA2 are two main molecular regulators in the development and progression of breast can-

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cer. SIRT1, an NAD^+-dependent deacetylase belonging to the sirtuin family, participates in multiple cellular processes related to tumorigenesis, such as cell survival, proliferation, and the regulation of signaling pathways [15-18]. HMGA2 is a chromatin-associated architectural transcription factor involved in tumor aggressiveness, epithelial-mesenchymal transition, invasion and metastasis, particularly in aggressive breast cancer subtypes [19-23]. Both genes have been independently linked to the progression of breast cancer, but the mechanisms of regulation of both genes in the setting of EV-mediated communication are not yet fully elucidated.

Earlier research looked into the roles of miR-326, SIRT1, HMGA2, and vesicles derived from mesenchymal cells in cancer biology. Nevertheless, the impact of stromal or mesenchymal EVs on the regulation of the miR-326 pathway, as well as its target genes SIRT1 and HMGA2 in breast cancer cells, is not yet clear. Therefore, this study focused on analyzing the effect of miR-326 on *SIRT1* and *HMGA2* gene expression in MDA-MB-231 breast cancer cells.

This EV-centered modulatory system could provide insights into molecular processes of tumor microenvironment-driven breast cancer progression and highlight potential diagnostic and therapeutic targets.

Materials and Methods

Cell culture

The MDA-MB-231 human breast cancer cell line was purchased from the Pasteur Institute of Iran and was derived from invasive breast carcinoma. Human Mesenchymal Stromal Cells (MSCs) were obtained from primary cultures of breast adenocarcinoma established at Tarbiat Modares University. Isolation of cells was performed from an adult female donor, and characterization was carried out according to the International Society for Cellular Therapy (ISCT) criteria. Immunophenotyping was performed by flow cytometry, confirming positive expression of CD73, CD90, and CD105 (≥95%), and negative expression of CD34, CD45, and HLA-DR (≤2%). The MSCs exhibited an elongated, spindle-shaped morphology with adherence to the culture flask. Cells at passages 3-5 were used for all experiments to ensure phenotypic stability and reproducibility. Culturing of cells was done in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) with 10% fetal bovine serum (Exosome-Depleted FBS, Gibco, USA), 2 *mM* L-glutamine, 100 *U/ml* penicillin, and 100 *µg/ml* streptomycin, maintained at 37°*C* in a humidified space with 5% CO_2. All experiments were performed in biological triplicate.

EVs isolation, characterization and quality control

Conditioned media were collected and subjected to sequential centrifugation at 300×*g* for 10 *min* and 2,000×*g* for 20 *min* to remove cells and debris, followed by filtration through a 0.22 *µm* syringe filter. EVs were then precipitated using the ExoQuick-TC reagent (System Biosciences, USA) according to the manufacturer's instructions with minor modifications. Briefly, ExoQuick-TC reagent was added to the pre-cleared media at a 1:5 ratio and incubated overnight at 4°*C*. The mixture was centrifuged at 1,500×*g* for 30 *min*, and the resulting pellet was washed once with PBS and re-centrifuged at 1,500×*g* for 5 *min* to remove residual polymer and soluble contaminants. The final EV pellet was resuspended in PBS and stored at -80°*C* until use. To control for ExoQuick-related contamination, an ExoQuick-only negative control (reagent processed without media) was included in parallel. In addition, we included an EV-depleted control (conditioned media processed identically but without precipitation) to account for any residual soluble factors.

EV preparations were characterized for size distribution by Dynamic Light Scattering (DLS) analysis using a Zetasizer Nano ZS device (Malvern Instruments, UK).

PKH26 labeling of EVs

To evaluate the ability of breast cancer cells to internalize EVs derived from tumor stromal cells, PKH26-labeled exosomes were directly incubated with MDA-MB-231 cells for 24 *hr* under standard cell culture conditions. PKH26 is a red lipophilic fluorescent membrane dye that readily incorporates into the lipid bilayer of exosomes, generating a stable fluorescent signal that enables visualization and tracking of vesicle uptake by recipient cells. Exosome uptake and intracellular localization were subsequently visualized using a confocal fluorescence microscope.

A PKH26-only control was included, comprising the same dye volume and undergoing the same purification/washing steps as EV-labeled samples, but without EVs. Fluorescence from this control was used to set the baseline threshold for flow cytometry and microscopy analyses.

Determination of optimal concentration of EVs

To evaluate the effect of EVs on the metabolic activity of MDA-MB-231 breast cancer cells depending on concentration, an MTT assay was used. MDA-MB-231 cells were treated with different concentrations of EVs (0, 10, 20, 30, and 40 *µg/ml*) for 24 *hr*. After incubation, the culture medium was replaced with fresh medium containing 20 *µl* MTT solution at a 5 *mg/ml* concentration, and the cells were incubated for an additional 4 *hr* at 37°*C*. The supernatant was then carefully removed, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 490 *nm* using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells, which were considered 100%. All experiments were performed in biological triplicate.

Isolation of RNA

MDA-MB-231 cells were treated with 30 *µg/ml* EVs for 24 *hr*, and then total RNA was extracted from EV-treated cells and the untreated control cells. RNA was

isolated by Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) at 260 *nm*, and RNA purity was assessed by the A260/A280 ratio to confirm the absence of DNA contamination.

cDNA synthesis and polyadenylation

Total RNA was poly(A)-tailed first for miRNA analysis. Briefly, a reaction with a final volume of 15 *µl* containing less than 5 *µg* total RNA, ATP, poly(A) polymerase buffer, poly(A) polymerase enzyme, and RNase-free water was prepared. The reaction mixture was incubated at 37°*C* for 10 *min*. First-strand cDNA was synthesized using a miRNA cDNA synthesis kit (Takara Bio, Japan), and reverse transcription was performed. The reaction mixture contained oligo(dT), polyadenylated RNA, reaction buffer, DTT, RNase inhibitor, dNTP mix, RTase buffer, and HyperScript reverse transcriptase according to the manufacturer's protocol. Reverse transcription was conducted at 42°*C* for 30 *min*, 25°*C* for 5 *min*, and enzyme inactivation at 70°*C* for 5 *min*. Samples of synthesized cDNA were stored at −20°*C* or −70°*C* until analysis.

Quantitative Real-Time PCR

Expression levels of miR-326, SIRT1, and HMGA2 were assayed by SYBR Green-based real-time quantitative PCR (RT-qPCR) on an ABI StepOne™ Real-Time PCR system with software v2.0.2 (Applied Biosystems, UK). Amplification was carried out using the SYBR Green I Master Mix (Roche Diagnostics, Germany).

PCR cycling conditions were as follows: initial denaturation at 95°*C* for 5 *min*, followed by 40 cycles of denaturation at 95°*C* for 5 *s*, annealing of primers at 62-65°*C* for 30 *s*, and extension at 72°*C* for 20 s. To confirm amplification specificity, melting curve analysis was performed at the end of each PCR run under the following conditions: 95°*C* for 15 *s*, 60°*C* for 30 *s*, and 90°*C* for 15 *s*. Relative expression levels were determined by the comparative threshold cycle method (2^-ΔΔCt). The specificity of the RT-qPCR amplification was verified by melting curve analysis. The endogenous reference gene for miRNA expression analysis was U6 small nuclear RNA (U6snRNA). GAPDH was used as the housekeeping gene for mRNA expression analysis of SIRT1 and HMGA2. Primer sequences used in this study are shown in table 1.

Statistical analysis

Data were analyzed using SPSS version 23 (SPSS Inc., USA). Results were expressed as mean±standard deviation of three independent experiments. Before analysis, data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. Data from the MTT assay were analyzed using one-way ANOVA, followed by Tukey's post hoc test. In qRT-PCR, statistical comparisons between the control and treatment groups were performed using an unpaired Student's t-test. The $p<0.05$ was considered significant.

Results

Characterization of EVs derived from MSCs

The size distribution profile according to the DLS method revealed the shape of a bell curve with a peak at the point of 120 *nm* (Figure 1).

Cellular uptake of EVs by MDA-MB-231 cells

Confocal fluorescence microscopy demonstrated that exosomes derived from tumor stromal cells were efficiently internalized by MDA-MB-231 cells. The red fluorescent signals were predominantly localized within the cytoplasmic region, indicating successful cellular uptake and intracellular accumulation of the exosomes (Figure 2). These findings confirm the active interaction between EVs and breast cancer cells and demonstrate the efficient delivery of vesicular cargo into recipient cells.

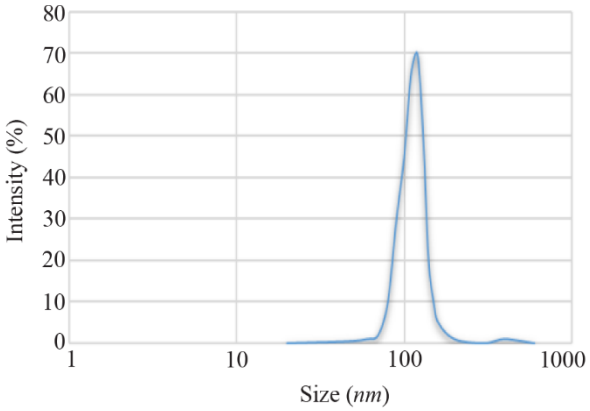

Figure 1. Distribution profile of EVs size according to the data obtained through the DLS method.

Table 1. Primer sequences

Gene	Forward primers	Reverse primers	Amplicon length (*bp*)	Efficiency (%)
miR-326	GGGGGCAGGGCCTTTGTG	GAACATGTCTGCGTATCTC	80	-
U6 snRNA	CTCGCTTCGGCAGCACAT	TTTGCGTGTCATCCTTGCG	95	110
SIRT1	TAGACACGCTGGAACAGGTTGC	CTCCTCGTACAGCTTCACAGTC	116	99
HMGA2	GAAGCCACTGGAGAAAAACGGC	GGCAGACTCTTGTGAGGATGTC	114	95.51
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA	76	100.59

Figure 2. Evaluation of the cellular uptake of EVs by MDA-MB-231 breast cancer cells. PKH26-labeled exosomes were incubated with MDA-MB-231 cells for 24 *hr*, and cellular internalization was examined using confocal fluorescence microscopy. The presence of red fluorescent signals within the cytoplasm of MDA-MB-231 cells indicates efficient uptake and intracellular localization of tumor stromal cell-derived exosomes.

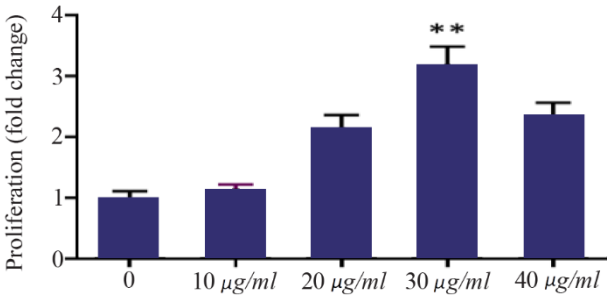

Figure 3. Effect of different concentrations of EVs on the viability of MDA-MB-231 cells. Viability of MDA-MB-231 cells treated with EVs for 24 *hr* was estimated by MTT assay. **p<0.01 *vs.* control.

MTT assay

The MTT results showed that treatment with 30 *µg/ml* EVs for 24 *hr* induced the highest viability response. At this concentration, cell viability increased significantly compared with the control group (p<0.01) (Figure 3).

Effect of EV exposure on miR-326 expression

Treatment of MDA-MB-231 breast cancer cells with EVs (30 *µg/ml*) secreted by mesenchymal/stromal cells resulted in a statistically significant decrease in miR-326 expression (p<0.05) (Figure 4). The treatment group exhibited a relative expression level of approximately 0.5±0.08-fold, corresponding to an approximately 50% reduction compared with the control. The negative control was MDA-MB-231 breast cancer cells without EVs treatment.

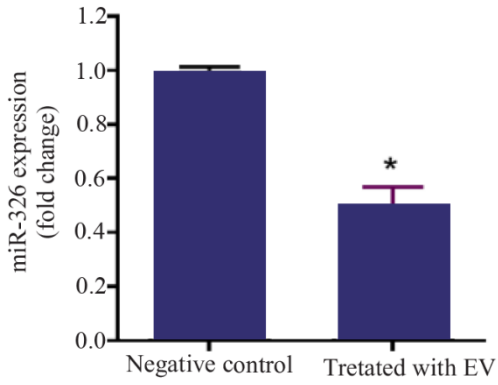

Figure 4. Relative miR-326 expression in MDA-MB-231 cells treated with EVs isolated from stroma/mesenchyme. miR-326 expression in MDA-MB-231 cells in the presence of EVs was significantly decreased (50%) compared to the expression level in control cells, *p<0.05.

Effect of EV treatment on SIRT1 and HMGA2 expression level

SIRT1 and *HMGA2* expression were significantly increased (3.4- and 2.75-fold, respectively) in MDA-MB-231 cells treated with EVs (30 *µg/ml*) derived from stromal/mesenchymal cells compared to the control group (3.4±0.07-fold, p<0.01 and 2.75±0.2-fold, p<0.05, respectively) (Figure 5). The negative control was MDA-MB-231 breast cancer cells without EVs treatment.

Discussion

In this study, the influence of EVs isolated from MSCs on the regulation of miR-326 expression and its targets, SIRT1 and HMGA2, in MDA-MB-231 breast cancer cells was evaluated. The present study results indicated that treatment with EVs from MSCs leads to increased viability of breast cancer cells and a significant decrease in miR-326 expression and increased expression levels of *SIRT1* and *HMGA2*. These observations can be interpreted as indicating that EVs released by stromal cells may contribute to cancer progression by targeting the miR-326/SIRT1/HMGA2 axis.

The tumor microenvironment has been shown to play an important role in the development, progression, and therapeutic efficacy of cancers. An important feature of MSCs is their capability to influence adjacent cancer cells through the secretion of biologically active EVs. In the present study, DLS analysis confirmed the successful isolation of EVs within the expected size range, and functional experiments were performed to determine their ability to influence breast cancer cell behavior. The enhanced cell viability following EV treatment supports the idea that stroma-derived vesicles contain molecular cargo that can amplify tumor-promoting signaling pathways in recipient cancer cells. One of the key findings from the current study includ-

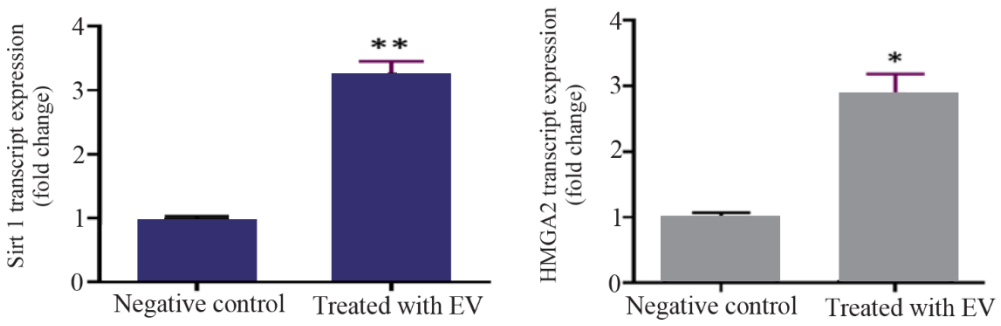

Figure 5. Relative expression of SIRT1 and HMGA2 in MDA-MB-231 cells treated with EVs compared with the control sample, *p<0.05, **p<0.01.

ed downregulation of miR-326 upon treatment with stroma cell-derived EVs. MicroRNA-326 is often regarded as a tumor-suppressive miRNA because it inhibits several oncogenic pathways. Pan *et al* in 2019 reported downregulation of miR-326 in multiple tumors, where low expression is associated with increased proliferation, invasiveness, metastatic potential, and therapy resistance [24]. In this context, the reduced miR-326 observed here may represent one mechanism by which stromal-derived EVs promote tumor-supportive reprogramming in breast cancer cells. More broadly, extracellular vesicles may suppress tumor-suppressive miRNAs and thereby mediate microenvironment-driven molecular changes in cancer cells [4-6].

SIRT1 was significantly upregulated in parallel with decreased miR-326 expression. This is biologically plausible, given the previously reported interaction between miR-326 and *SIRT1*. SIRT1 is an NAD^+-dependent protein deacetylase that regulates cell survival, proliferation, stress responses, and metabolic adaptation. Wei *et al* in 2021 reported that *miR-326* expression was decreased while *SIRT1* expression was increased in non-small cell lung cancer [17]. *SIRT1* was a target of miR-326. In several cancers, elevated *SIRT1* expression has been associated with increased tumor growth, resistance to apoptosis, and reduced sensitivity to therapy. Overall, these findings support the idea that stromal cells can indirectly promote oncogenic signaling by modulating EV-associated miRNA pathways [4-7,11].

Another result observed in the current study was the increased expression level of *HMGA2* in EV-stimulated cells. The HMGA2 protein is an architectural transcription factor that is crucial for cell plasticity, self-renewal, epithelial-to-mesenchymal transition, and malignant transformation. Ma *et al* in 2024 showed that the increased expression level of *HMGA2* was associated with a more aggressive phenotype in multiple cancers, including breast cancer [25]. In this study, it was found that miR-326 was down-regulated and *HMGA2* was up-regulated simultaneously, indicating a functional modulatory relationship between these molecules. Given that miR-326 has been reported to negatively modulate pathways associated with tumor progression, miR-326 knockdown may release the repressive control on *HMGA2* expression, thus allowing the acquisition of more aggressive cell features [19-23].

Specifically, the co-modifications of miR-326, *SIRT1*, and *HMGA2* suggest the possibility that these molecules may function as components of an interconnected modulatory network under the control of stromal-derived EVs. Changes in miR-326 expression may not be independent but may trigger subsequent molecular events that together promote tumor-supporting phenotypes [11-14,15-18,19-23].

This is further evidenced by the viability response seen in MDA-MB-231 cells upon treatment with EVs. The optimal biologically active concentration of 30 *μg/ml* indicates dose-dependency of the effects of stromal-derived EVs on cancer cell behavior. The increase in metabolic activity with the activation of oncogenic molecular markers suggests that the EV-mediated signaling could be involved in the establishment of a microenvironment favorable to tumor progression. Such effects are consistent with the suggested role of stromal cells as active players in cancer development, rather than passive components of the tumor microenvironment [3-7].

From a translational perspective, the findings of this study could be of significant interest for the development of new therapeutic strategies aimed at tumor microenvironment-mediated signaling. EVs are important mediators of intercellular communication, and, therefore, targeting EV biogenesis, secretion, internalization, or cargo transfer may be a useful approach to disrupt tumor-promoting interactions between stromal and cancer cells. In addition, the miR-326/*SIRT1/HMGA2* axis identified in this study may be a potential molecular target for future research to rescue the tumor-suppressive signaling pathways in breast cancer [5,8-10,26].

Several limitations of the present study should be acknowledged. First, the characterization of the EVs was preliminary; therefore, further studies incorporating orthogonal characterization approaches in accordance with current MISEV recommendations are war-

ranted to confirm their identity and characteristics. Second, although our findings demonstrate associations between EV treatment and the observed molecular and cellular responses, they do not establish direct causal or mechanistic relationships. Further functional studies, including pathway-specific inhibition or activation and other mechanistic approaches, are needed to elucidate the underlying mechanisms. Third, the findings require further validation in additional relevant cell lines and, ultimately, in appropriate *in vivo* models to determine their reproducibility, biological relevance, and potential translational applicability.

Conclusion

In this work, it was shown that EVs derived from MSCs exert a major influence on the expression profile of MDA-MB-231 breast cancer cells. Indeed, the presence of EVs induced miR-326 downregulation and SIRT1 and HMGA2 upregulation. This was associated with cell viability, implying that EVs derived from stroma may contribute to the formation of a supportive environment for tumor development.

The expression profile observed in this work supports the hypothesis that there might exist a potential molecular association network involving miR-326 and its target genes in breast cancer cells under EVs stimulation. Although the underlying mechanisms still need to be uncovered, the data suggest that EVs signaling is implicated in the regulation of gene expression programs linked to tumor progression.

Overall, the current research generates novel insights into the role played by EVs from MSCs during breast cancer progression caused by disruptions of the miR-326/*SIRT1/HMGA2* signaling pathway. Such results expand the scope of knowledge concerning the molecular regulation exerted by the tumor microenvironment and indicate that elements of this pathway may be prospective biomarkers for breast cancer applications in diagnostics, prognosis, and therapy in the future.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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