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EXPRESSION OF TRANSCRIPTION FACTORS INVOLVED IN EPITHELIAL-TO-MESENCHYMAL TRANSITION OF THE BREAST CANCER CELL LINE MCF-7 CO-CULTURED WITH ADIPOSE TISSUE MESENCHYMAL STEM CELLS

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SUMMARY

Breast cancer is the most frequently diagnosed cancer in women globally. The tumor microenvironment plays a vital role in epithelial-to-mesenchymal transition (EMT), leading to the invasion and metastasis of cancer cells. The tumor microenvironment includes all components of the tumor cells, including the extracellular matrix, tumor vasculature, mesenchymal stem cells, immune cells, and fibroblasts. Understanding the interactions between mesenchymal stem cells and cancer cells is essential in determining the role of mesenchymal stem cells in diagnosing and treating breast cancer. In this study, we present the result of co-culture between adipose tissue mesenchymal stem cells (ADMSCs) and breast cancer cells (MCF-7 cell line) and determine the expression levels of transcription factors involved in EMT, including Twist and Snail. The results showed that the proliferation of MCF-7 co-cultured with ADMSCs was not increased compared to MCF-7 mono-cultured. Determination of gene expression levels by qRT-PCR revealed a significant increase in the EMT-related transcription factors (Twist and Snail) in breast cancer cells upon co-culture with ADMSCs. There were also significant differences between the expression levels of IL-6 and AhR in MCF-7 cells co-cultured with ADMSCs and MCF-7 cells mono-cultured. The results suggested that ADMSCs promoted the EMT of MCF-7 cells, potentially via AhR/NF-κB pathways.

Keywords: gene expression, epithelial-to-mesenchymal transformation, co-culture, breast cancer, mesenchymal stem cells

INTRODUCTION

Breast cancer is one of the most common cancers in women and the fifth leading cause of cancer death worldwide, with a 15% female mortality rate from breast cancer (Bray *et al*., 2018). Metastasis of cancer cells is a primary contributor to mortality in most breast cancer patients. The process of metastasis involves cancer cells losing

contact and adhesion, which triggers the epithelial-to-mesenchymal transition (EMT) and promotes cancer cell invasion and migration. Numerous studies have established EMT as the primary mechanism responsible for the metastasis of breast cancer cells (Tang *et al*., 2018).

The tumor microenvironment plays an important role in the maintenance of dormancy as well as the recurrence and metastasis of cancer cells (Shao *et al*., 2015). The tumor microenvironment comprises two main tissue components, namely adipose tissue and cancerous tissue, that are nourished by the vascular system. Within this microenvironment, diverse types of cells, like fat cells, cancer cells, connective cells, etc., exist and interact with each other, influencing one another. Adipocytes are developed from mesenchymal stem cells, while breast cancer cells are developed from cancer stem cells. In the microenvironment of breast cancer, adipocytes act as a source of energy in the form of fatty acids to promote the growth of breast cancer cells (Anderson, Simon, 2020). Breast cancer cells also induced changes in the morphology and function of mesenchymal stem cells from adipose tissue, altering them to act as cancer-associated fibroblasts. These cells facilitate tumor progression by promoting angiogenesis and EMT, two crucial events in tumor metastasis (Ejaz *et al*., 2020). EMT is closely linked to the interaction of various pathways, growth factors, protein molecules, and transcription factors. The transcription factor clusters of twist family bHLH transcription factor 1 (Twist) and snail family zinc finger 1 (Snail) have been implicated in the process of EMT (Grzegrzolka *et al*., 2015; Piotrowska *et al*., 2015). Activated Twist results in the upregulation of N-cadherin and the downregulation of E-cadherin, both of which are characteristic features of EMT. In addition, Twist also plays a significant role in various physiological processes that are associated with metastasis, such as angiogenesis, invadopodia, extravasation, and chromosomal instability (Khan *et al*., 2013). Snail is a significant promoter of EMT and actively suppresses the expression of E-cadherin. The upregulation of Snail expression is strongly associated with tumor grade, recurrence, metastasis, and poor prognosis in breast cancer (Wang *et al*., 2013). Twist and Snail are regulated by many signaling pathways, including interleukin-6/STAT3 (Wang *et al*., 2018). The interleukin-6 is a pivotal inflammatory marker, and it has a predominant role in the promotion of tumor growth. The interaction of IL-6 and its receptor-activated Janus kinases (JAKs) with the following activation of transcription (STAT3) through tyrosine phosphorylation and subsequent transcription of Twist and Snail (Manore *et al*., 2022). In breast cancer cells, the genes that encode IL-6 are under the control of transcriptional nuclear factor-kappa B (NF-κB) (Vyas *et al*., 2017). The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that interacts with a multitude of endogenous and exogenous molecules. AhR participates in several pathways, some of which are associated with inflammation and breast cancer (Guarnieri, 2020). It has been demonstrated that AhR interacts with NF-κB and regulates the metabolism of IL-6 (Vacher *et al*., 2018).

It is crucial to investigate the interactions between different components present in the tumor microenvironment as well as the molecular associations between ADMSCs and breast cancer cells. The use of transwell

plate cell co-culture to assess cell-to-cell interactions has become a prevalent technique worldwide, including the interactions of human adipose tissue-derived stem cells and different human breast cancer cell lines (Koellensperger *et al*., 2017; Wu *et al*., 2019). In 2017, Koellensperger *et al*. showed that the interaction between ADMSCs and breast cancer cells increases cytokine production and transforms the malignancy of breast cancer cells (Koellensperger *et al*., 2017). By exploring these interactions, it becomes feasible to identify molecular markers or novel molecular targets that could be useful in the diagnosis and treatment of breast cancer.

The aim of this study was to investigate the levels of gene expression of Twist and Snail in MCF-7 cells co-cultured with ADMSCs. The data suggested that ADMSCs enhanced expression levels of Twist and Snail in MCF-7 cells, potentially via the AhR/NF-κB signaling pathway.

MATERIALS AND METHODS

Transwell co-culture of ADMSCs and MCF-7

The mesenchymal stem cells derived from an adipose tissue cell line were purchased from Lonza, USA (code PT-5006, passage 10–30). The breast cancer cell line MCF-7 (passage 10–30) was kindly provided by Dr. La Thi Huyen (Animal Cell Biotechnology Department, Institute of Biotechnology).

The co-culture of ADMSCs and MCF-7 was performed in a transwell system with a 12-mm diameter (Costar, USA). ADMSCs (10^5 cells/mL) were seeded onto a polyester membrane transwell-clear insert (pore size 0.4 μm), while MCF-7 cells were seeded onto the bottom of the cell culture plate. ADMSC and MCF-7 cells were co-cultured for up to 4 days in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) at 37^{o}C and 5% CO_2. MCF-7 cells cultured separately under similar conditions were used as controls. The experiments were repeated three times.

Determination of cell proliferation

The cell proliferation was determined by counting the total number of cells in each culture well every 24 hours. Cell culture supernatants were harvested, and the cell number was determined after trypzinization and trypan blue staining on a Neubauer counter.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from MCF-7 cells using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. A total of 1 μg of RNA was converted into cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). Relative gene expression was measured using PowerUp SYBR Green Master Mix (ThermoFisher Scientific, USA). The primers used for qRT-PCR were synthesized by Phusa Genomics Co., Ltd. (Can Tho, Vietnam), and the primer sequences are listed in Table 1. qRT-PCR reactions and analyses were performed using the QuantStudio™ 6 Pro Real-Time PCR System with Design & Analysis Software v2.6.0. The relative expression levels of the genes were calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen, 2001). β-actin gene was used as an endogenous control to normalize gene expression levels. The graphs and data were processed by Microsoft Excel, with the p-value determined by the t-test method.

Table 1. Primer sequences used in q RT-PCR.

Genes	Forward primers	Reverse primers
Twist	5'-AGCTACGCCTTCTCGGTCT-3'	5'-CCTTCTCTGGAAACAATGACATC-3'
Snail	5'-CTTCCAGCAGCCCTACGAC-3'	5'-CGGTGGGGTTGAGGATCT-3'
IL-6	5'-ACTCACCTCTTCAGAACGAATTG-3'	5'-CCATCTTTGGAAGGTTCAGGTTG-3'
AhR	5'-ACATCACCTACGCCAGTCGC-3'	5'-TCTATGCCGCTTGGAAGGAT-3'
β-actin	5'-TCATGAAGTGTGACGTGGACATC-3'	5'-CAGGAGGAGCAATGATCTTGATCT-3'

RESULTS AND DISCUSSION

The effect of ADMSCs on the morphology and cell proliferation of MCF-7 cells

Co-culturing of cells is widely used to study how they interact with each other in many fields (Vis *et al*., 2020). The transwell co-culture system was established to investigate the effects of ADMSCs on the proliferation and morphology of MCF-7 cells. The cell count after 4 days indicated that transwell culture of ADMSCs did not promote breast cancer cell proliferation (Figure 1; $P>0.05$).

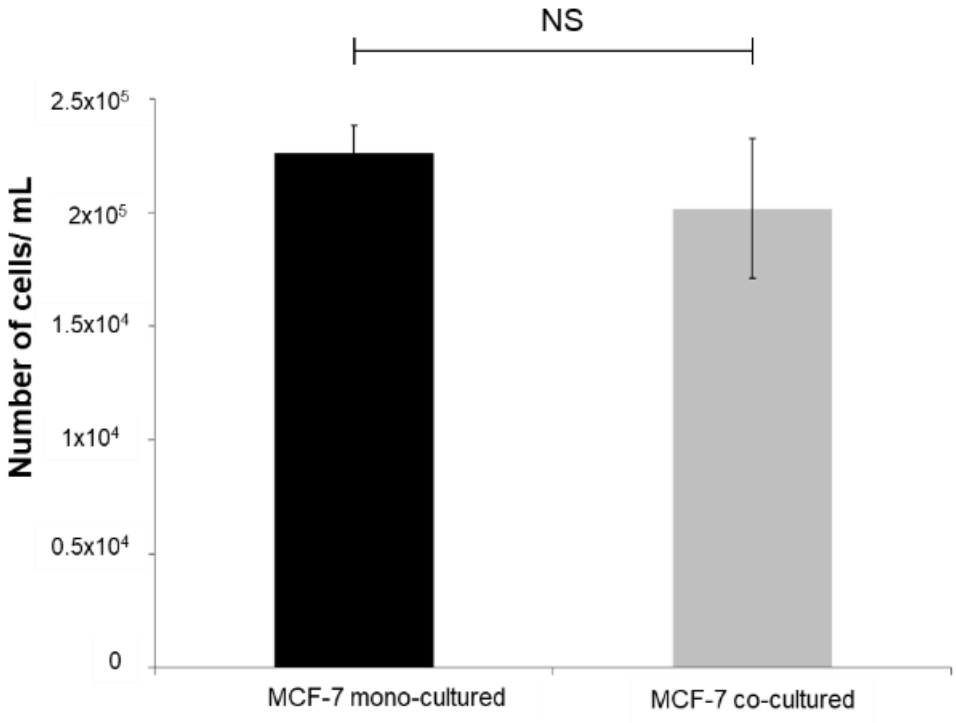

Figure 1. ADMSCs did not promote MCF-7 cell proliferation. There was no significant difference in the number of cells between the co-cultured MCF-7 cells and the mono-cultured MCF-7 cells after 4 days ($P>0.05$). The data are presented as the mean ± standard deviation of three independent experiments.

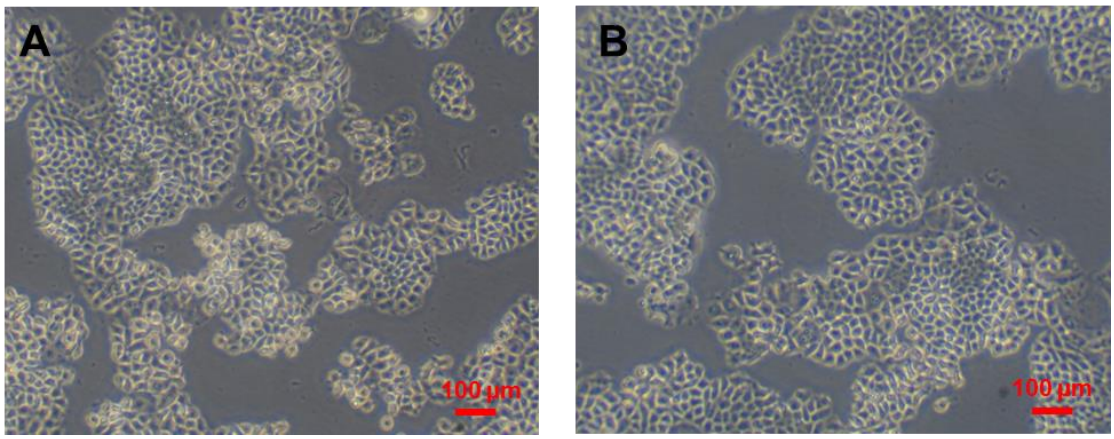

Figure 2. Morphological features of MCF-7 cells mono-cultured (A) and co-cultured with ADMSCs (B). Scale bar, 100 µm.

There were no morphological differences between the breast cancer cell line MCF-7 under mono-culture and co-culture conditions with mesenchymal stem cells from adipose tissue (Figure 2).

Previous studies have reported conflicting results on the potential of ADMSCs to promote the proliferation and malignant transformation of breast cancer cells (Koellensperger *et al*., 2017; Goto *et al*., 2019). However, in our study, we found that ADMSCs did not promote the proliferation of breast cancer cells. This is consistent with the results by Ejaz *et al*. (2020).

Expression levels of genes involved in epithelial-to-mesenchymal transition of the breast cancer cell line MCF-7

EMT-associated transcription factors such as Twist and Snail regulate indirectly or directly the gene expression pattern during EMT. The expression of EMT-associated transcription factors, such as Twist and Snail, was examined in the co-cultured MCF-7 cells and the control group. The results showed that in the co-culture system, the expression of Twist (Figure 3A) and Snail (Figure 3B) in MCF-7 cells was significantly upregulated compared to the control, with a fold change of 1.26 and 1.72, respectively ($P<0.05$).

These findings suggest that ADMSCs promote EMT in MCF-7 cells. A previous study by Wu *et al*. (2019) showed that Twist and Snail expressions were upregulated in MCF-7 co-cultured with ADMSCs compared with MCF-7 mono-cultured.

Figure 4 illustrates that MCF-7 cells co-cultured with ADMSCs exhibited significantly higher gene expression levels of IL-6 (fold change of 2.44, $P<0.01$) and AhR (fold change of 3.71, $P<0.01$) than those in the control group. Previous studies illustrated that interaction between ADMSCs and cancer cells prompted the secretion of IL-6 in ADMSCs, and IL-6 was upregulated in breast cancer cells when they were co-cultured with ADMSCs (Wei *et al*., 2015; Koellensperger *et al*., 2017).

It was demonstrated that IL-6 induces EMT in breast cancer cells via activation of the signal transducer and STAT3 (Sullivan *et al*., 2009; Gyamfi *et al*., 2018). In addition, the expression of IL-6 is regulated by NF-

κB. Hennig *et al*. (2002) showed that the activation of AhR by polychlorinated biphenyls (PCBs) promoted inflammatory atherosclerotic phenomena, passing through the transcriptional activation of NF-κB and the increase in IL-6 production in endothelial cells. In breast cancer cells, it has been proven that AhR interacts with NF-κB and modulates the metabolism of IL-6 (Vyas *et al*., 2017; Guarnieri, 2020).

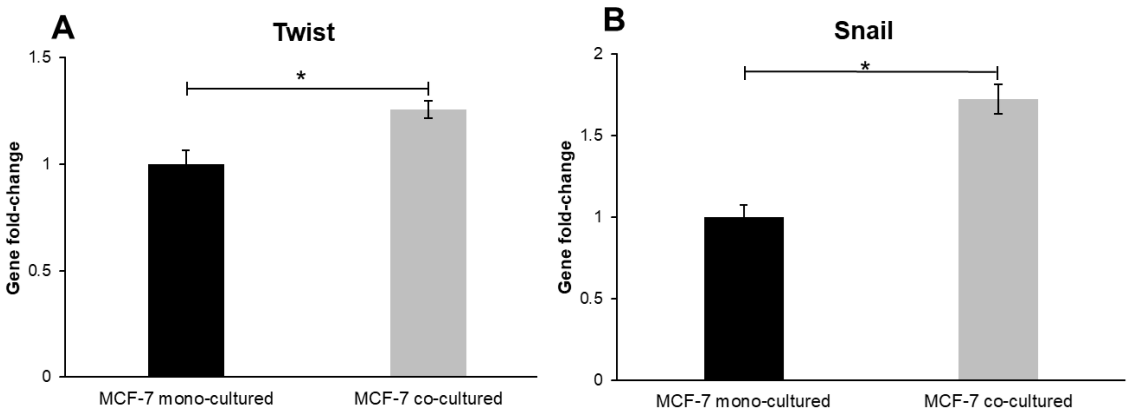

Figure 3. Expressions of Twist (A), and Snail (B) were upregulated in MCF-7 cells co-cultured with ADMSCs. Data are presented as the mean ± standard deviation of three independent experiments. **P*<0.05.

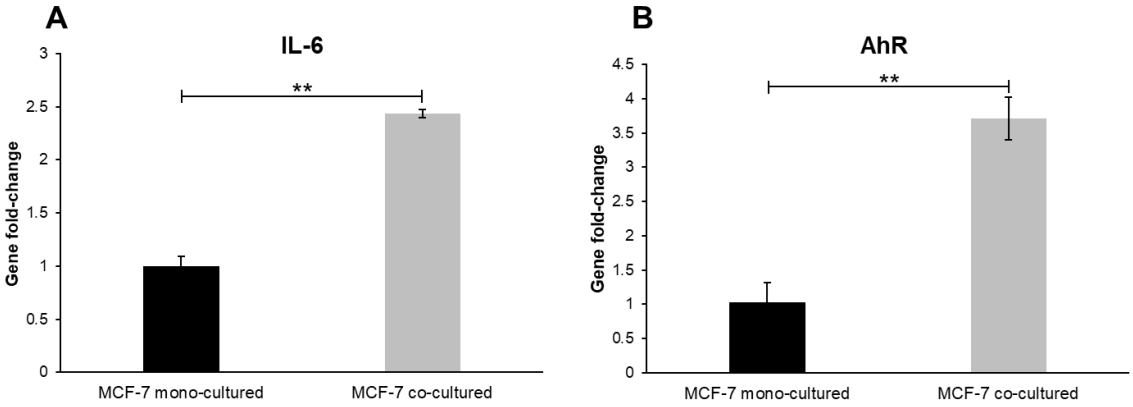

Figure 4. Expressions of IL-6 (A) and AhR (B) were upregulated in MCF-7 cells co-cultured with ADMSCs. Data are presented as the mean ± standard deviation of three independent experiments. ***P*<0.01.

In this study, based on the gene expression data of Twist, Snail, AhR, and IL-6, we hypothesized that ADMSCs have the potential to induce EMT in MCF-7 cells through the AhR/NF-κB signaling pathway (Figure 5). However, further studies are needed to completely clarify the mechanism by which ADMSCs promote the EMT of MCF-7 cells.

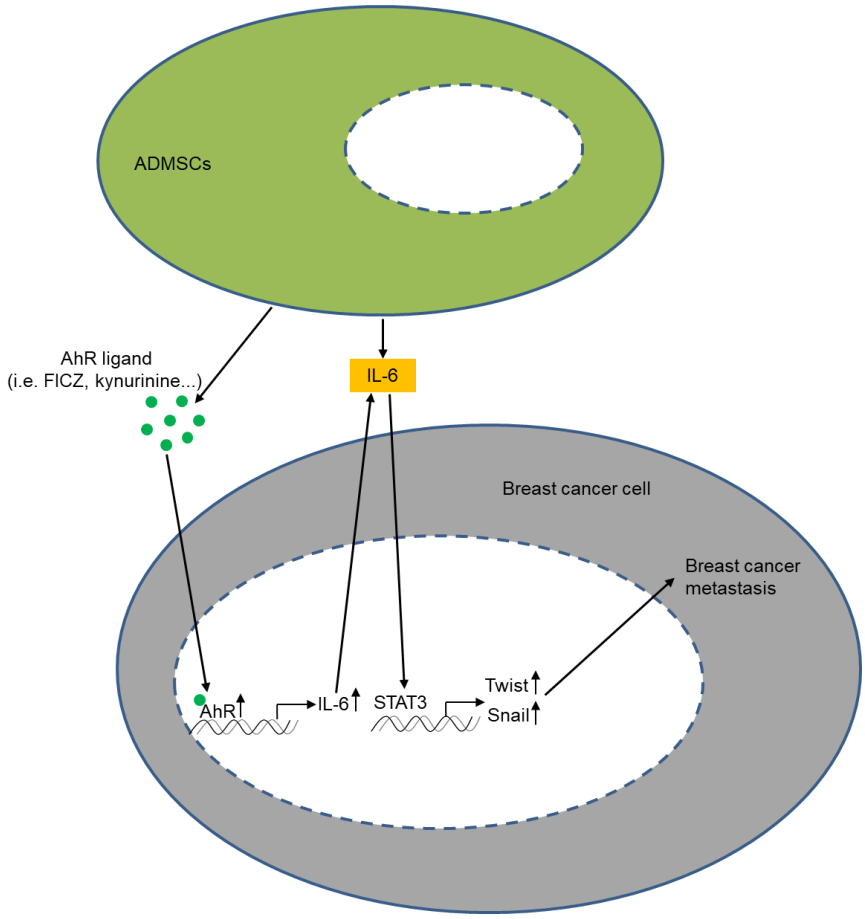

Figure 5. ADMSCs induce EMT in breast cancer cells, potentially via the AhR/NF-κB signaling pathway.

CONCLUSION

In conclusion, this study utilized a transwell co-culture system to investigate the paracrine effects of ADMSCs on tumor development by studying their interaction with MCF-7 cells. This provided evidence that ADMSCs increased expressions of EMT-related transcription factors, including Twist and Snail, which were at least partially mediated by the AhR/NF-κB signaling pathway.

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