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Original Article

TGF-B1-over-expressed adipose stem cells-derived secretome exhibits CD44 suppressor and anti-cancer properties via antagonistic effects against SMAD4 in breast cancer cells

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Abstract: Objectives: This study aimed to investigate the effect of TGF-B1-transfected adipose-derived mesenchymal stem cell (AD-MSC) conditional medium (TGF-B1-CM) on CD44 expression and biological activities in MCF-7 and MDA-MB-231 cells. Methods: In the study, the experimental groups were created as a standard medium, AD-MSC-CM, TGF-B1-CM, and TGF-B1 recombinant protein. The medium and proteins specified in these groups were applied to MCF-7 and MDA-MB-231 cells separately at 24, 48 and 72 hours. Western blot and immunofluorescent staining were performed with antibodies suitable for CD44 and canonical smad signaling pathway analyses between groups. Cellular proliferation in MCF-7 and MDA-MB-231 cells was measured by MTT. Biological activity analyses such as apoptosis, cell cycle, proliferation, DNA damage, and membrane depolarization between groups were tested on the Muse Cell Analyzer using appropriate kits. Cellular migration between groups was determined by showing cells that migrated to the scar area with in vitro scar formation. Statistics were performed with GraphPad Prism 8.02 software. Results: It was determined that TGF-B1-CM activates the smad signaling pathway in MCF-7 and MDA-MB-231 cells. TGF-B1-CM increased pSMAD2/3 expression and decreased SMAD4 expression in breast cancer cells. A decrease in CD44 expression was found at points of increase in pSMAD2/3 expression. Decreased expression of SMAD4 in breast cancer cells with TGF-B1-CM was associated with decreased expression of CD44. In MCF-7 and MDA-MB-231 cells, TGF-B1-CM was found to increase apoptosis, decrease proliferation, disrupt membrane depolarization, and arrest cells at G0/G1 stage. TGF-B1-CM suppressed MCF-7 and MDA-MB-231 migrations. Conclusion: SMAD4-targeted therapeutic strategies may be considered to suppress CD44 expression in breast cancer cells. Both the anti-tumorigenic factors released by AD-MSCs and the secretomes obtained as a result of supporting these factors with the overexpression of TGF-B1, severely suppressed breast cancer cells. With this study, it was planned to obtain a targeted biological product that suppresses breast cancer cells *in vitro*.

Keywords: TGF-B1, CD44, adipose-derived mesenchymal stem cell, conditioned media, breast cancer

Introduction

Breast cancer is a quite common type of cancer in women worldwide and is one of the leading causes of cancer-related death. Many risk factors, especially exposure to ionizing radiation and certain hormones, play a role in the process of breast cancer, but these factors are not limited in terms of tumor formation. It is known that many multifactorial conditions trigger the breast cancer process. Despite significant advances in early diagnosis, diagnosis, and treatment methods, breast cancer plays a leading role in the causes of death in women, along with lung cancer, even in the leading countries of the world. Classical immunohistochemistry markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 receptor (HER2), along with traditional clinicopathological variables such as tumor size, tumor grade, and nodal involvement, have traditionally been used

for patient prognosis and treatment. The molecular classification indicates that breast cancers are luminal A, luminal B, HER2, and triple-negative breast cancer subtypes [1-4].

The mammary gland is located in adipose tissue and adipose-derived mesenchymal stem cells (AD-MSCs) in this niche play a remarkably effective role in the maintenance of tissue homeostasis [5, 6]. Recently, we come across studies showing that MSC paracrine factors may have tumor suppressive effects [7]. Studies conducted in breast cancer cell lines reveal that MSC intrinsic factors have an inhibitory effect on tumor development [6, 8]. It has been reported that the secretion factors released from MSCs exhibit an anti-tumor effect, and conditional media collected in different MSC types have suppressive effects against most solid tumor types [9, 10].

CD44 acts as a cell adhesion molecule. These adhesion molecules are glycoproteins that induce cell signaling, cell-cell interaction, and ECM production [11, 12]. Studies discuss the link between CD44 expression and activation of matrix-metalloproteinases (MMP) in tumor cell types [13, 14]. Reported results show us that CD44 plays a serious role in metastasis and invasion along with MMP activity. In this respect, suppression of CD44 in breast cancer cell lines leads us to think that it may have a tumor suppressor effect.

It has been reported that TGF-B1 plays both tumor-initiating and tumor-suppressing roles in the development of breast cancer. It is thought that this dual role varies according to the tumor stage, and studies also support this situation. It is important to elucidate the mechanisms underlying this condition, known as the TGF-Beta paradox, in cancer cells. Illuminating studies on the cancer-related roles of TGF-beta pathways will also be important for understanding many more cancer-related pathways [15-17]. The TGF-Beta family has three major isoforms, TGF-B1, TGF-B2, and TGF-B3. It has been reported that TGF-B1 plays important role in organizing vital cellular functions such as apoptosis, proliferation, cell growth, and tissue repair [18].

The study aims to suppress CD44 protein expression and biological activities in MCF-7 and MDA-MB-231 cells by using the secretome obtained from TGF-B1-transfected AD-MSCs, both with anti-tumorigenic factors released from AD-MSCs and activating the TGF-B1/SMAD signaling pathway.

Materials and methods

Cell lines and experimental groups

Adipose-derived mesenchymal stem cells (AD-MSCs), MCF-7, and MDA-MB-231 cell lines were used in the study. Cells were provided as ready-made cell lines from Erciyes University Genome and Stem Cell Center (GENKÖK). In the study, the experimental groups were created as a standard medium, AD-MSC-CM, TGF-B1-CM, and TGF-B1 recombinant protein. A control group was formed by applying a standard growth medium on MCF-7 and MDA-MB-231 cells. Study groups were formed as follows: Conditioned medium produced from adipose-derived stem cells (AD-MSC-CM), conditioned medium created from TGF-B1-transfected adipose-derived stem cells (TGF-B1-CM), and TGF-B1 recombinant protein. The medium and proteins specified in these groups were applied separately to MCF-7 and MDA-MB-231 cells at 24, 48, and 72 hours.

The culture and characterization of AD-MSCs

AD-MSCs at the end of the first passage, obtained from Erciyes University Genome and Stem Cell Center as a ready-made cell line, were quickly removed from the liquid nitrogen tank and thawed in a 37°C water bath. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) was removed from the thawed cells by centrifugation. Post-centrifugation pellet low glucose DMEM (Gibco, USA), 10% FBS (Biological Industries, Beit-HaEmek, Israel), 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza, MD, USA), 2 mM L-glutamine (Gibco, USA) and seeded in 75 cm^2 culture dishes by counting 5000 cells/cm^2 on Thoma slide. AD-MSCs seeded in culture dishes were incubated in an incubator containing 37°C, 5% CO_2, and 95% humidity. The culture medium in the incubated cells was replaced with a fresh one every three days. Cells were stained with CD90-FITC, CD44-PE, CD105 PerCP5.5, CD73-APC and CD34-FITC, CD45-PE, CD11b-PerCP5.5, HLA-DR-APC (BD Bioscience, Heidelberg, Germany) antibodies and were measured in flow cytometry (Navios Beckman Coulter, USA). The data were ana-

lyzed with the software KALUZA (Beckman Coulter, USA). Differentiation tests were performed on AD-MSCs with immunophenotypic characterization using appropriate kits (Lonza, USA).

TGF-B1 gene transfection into AD-MSCs

The TGF-B1 plasmid (pCMV-TGF-B1, Sino biological, Germany) isolated in our previous study and propagated in *E. coli* strains was used in this study [19]. AD-MSCs were incubated in full growth medium (Low glucose DMEM, 10% FBS, 2 Mm Glutamax) under normal culture conditions. A mixture of 1×10^6 cells and 5 µl of plasmid was formed in the neon buffer R (Invitrogen, USA). The mixture was taken into an electrolytic buffer by a special pipette for transfection (Invitrogen, USA). Transfection was performed by 1100 v, 20 ms, and 1 pulse electroporation parameters (Invitrogen, USA).

Transfection efficiency

Changes in the viability of cells before and after TGF-B1 transfection (0, 24, and 48 h) were measured using the Muse Cell Analyzer (Merck Millipore, Germany) device and cell counting kit (Muse Count & Viability Kit MCH100102, Germany) according to the production procedure. TGF-B1 protein expressions in AD-MSCs were measured by flow cytometry analysis at 24 and 48 hours before hygromycin application to determine the transfection efficiency after transfection. TGF-B1 transfection efficiency was analyzed by western blot method using appropriate primary TGF-B1 antibody (Thermo, USA) and HRP-conjugated secondary antibody (Thermo, USA) in addition to flow cytometric analysis.

The preparation of conditioned medium

To obtain a conditioned medium (AD-MSC-CM and TGF-B1-CM), the cells in the experimental groups were propagated in their growth medium until sufficient numbers were reached. AD-MSC-CM and TGF-B1-CM were harvested from cells in the third passage. When TGF-B1-transfected and non-transfected AD-MSCs reached sufficient numbers, they were conditioned for 24 hours with a serum-free medium. At the end of the 24th hour, the conditioned medium was collected into polypropylene tubes and centrifuged at 3000×g for 25 min at +4°C. Cellular debris was removed after centrifugation and the supernatant was transferred to a new tube. Finally, the collected conditioned medium was passed through 0.22 µm sterile filters (Corning, USA).

Application of conditioned media and recombinant TGF-B1 protein on MCF-7 and MDA-MB-231 cell lines

Low glucose DMEM, 10% FBS, 1% Penicillin-streptomycin, and 2 mM glutamax were used as the standard growth culture medium for MCF-7 and MDA-MB-231 cell lines. Then, conditional media obtained from TGF-B1 transfected AD-MSCs (TGF-B1-CM), conditioned media from non-transfected AD-MSCs (AD-MSC-CM), and a standard culture medium containing 5 ng/ml TGF-B1 recombinant protein were applied on MCF-7 and MDA-MB-231 cell lines for 24, 48 and 72 hours.

Analysis of CD44 protein expression in MCF-7 and MDA-MB-231 cells

The analysis of CD44 protein expressions in MCF-7 and MDA-MB-231 cells was analyzed by two different methods, western blot, and immunofluorescence staining.

TGF-B1/SMAD signaling pathway protein analysis

Total protein isolation was performed from the experimental groups at 24, 48, and 72 hours. Subsequently, western blotting was performed to study the proteins in the TGF-B1/SMAD signaling pathway containing Smad2, Smad3, Smad4, pSmad2, and pSmad3 antibodies (Thermo, USA).

MTT cell proliferation assay

Cell proliferation was measured in breast cancer cells after the treatments by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method. MCF-7 cells were treated with MTT (Sigma-Aldrich, Germany) for 4 hours to a final volume of 500 µg/ml. MTT was then aspirated from the cultures and the resulting formazan crystals were dissolved with DMSO. Absorbances at 560/750 nm wavelength were measured with an ELISA-based microplate

reader (Glomax Multi Detection System microplate reader, Promega, USA) and cellular proliferation was compared statistically between groups.

Annexin V apoptosis assay

After the applications according to the experimental groups, the apoptosis rates in breast cancer cells were measured according to the production procedure in the flow cytometry-based Muse Cell Analyzer (Millipore, Germany) device using Annexin V and Dead cell kit (Muse, Millipore, Germany).

Cell cycle assay

After the applications according to the experimental groups, the cell cycle phases in breast cancer cells were measured according to the production procedure in the flow cytometry-based Muse Cell Analyzer (Millipore, Germany) device using a cell cycle test kit (Muse, Millipore, Germany).

Senescence-associated beta galactosidase assay

X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside, Sigma-Aldrich, Germany) staining was performed to test beta-galactosidase activity in cells at 24, 48, and 72 hours. Cellular senescence rates in X-gal positive stained cells were measured by colorimetric method using a Glomax Multi Detection System microplate reader (Promega, USA) device at 420 nm wavelength.

Determination of mitochondria membrane depolarization

After the applications according to the experimental groups, the mitochondrial membrane potentials in breast cancer cells were measured according to the production procedure in the flow cytometry-based Muse Cell Analyzer (Millipore, Germany) device using a mitochondrial potential test kit (Muse, Millipore, Germany).

H2AX DNA damage assay

After the applications according to the experimental groups, DNA damage in breast cancer cells was measured according to the production procedure in the flow cytometry-based Muse Cell Analyzer (Millipore, Germany) device using an H2A.X activation dual detection kit (Muse, Millipore, Germany).

Wound healing assay

MCF-7 and MDA-MB-231 cells were seeded in 6-well culture dishes at 5000 cells/cm^2 in standard growth media, and cells were cultured in standard growth media until reaching 100% confluency. Using 200 µl sterile micropipette tips, wound areas were created by drawing evenly along the centerline of the wells. Cells stripped from the culture dish at the wound site were removed with PBS. Relevant standard medium, AD-MSC-CM, TGF-B1-CM, and TGF-B1 recombinant protein applications were applied to the wells according to the experimental groups. The first application moment was accepted as the zero hours.

Statistical analysis

All experiments were performed in triplicate. The results were presented as mean ± SD. The distribution of data between groups was determined by one-way ANOVA and two-way ANOVA statistical methods. Tukey and Holm-Sidak tests were used for multiple comparisons. Statistical analysis was performed using the "Graph Pad Prism 8.02" software. The $P<0.05$ value was taken as the basis for statistical significance. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001.

Results

The characterization of AD-MSCs

AD-MSCs were found to strongly express specific MSC markers. AD-MSCs differentiated into adipogenic, osteogenic, and chondrogenic lines *in vitro* (**Figure 1**).

TGF-B1 transfection efficiency

It was found that the percentage of viability after the transfection was not negatively affected. In non-transfected AD-MSCs, natural TGF-B1 expression was measured as 23.94% at the 24th hour of the culture and 27.01% at the 48th hour of the culture. In transfected AD-MSCs,

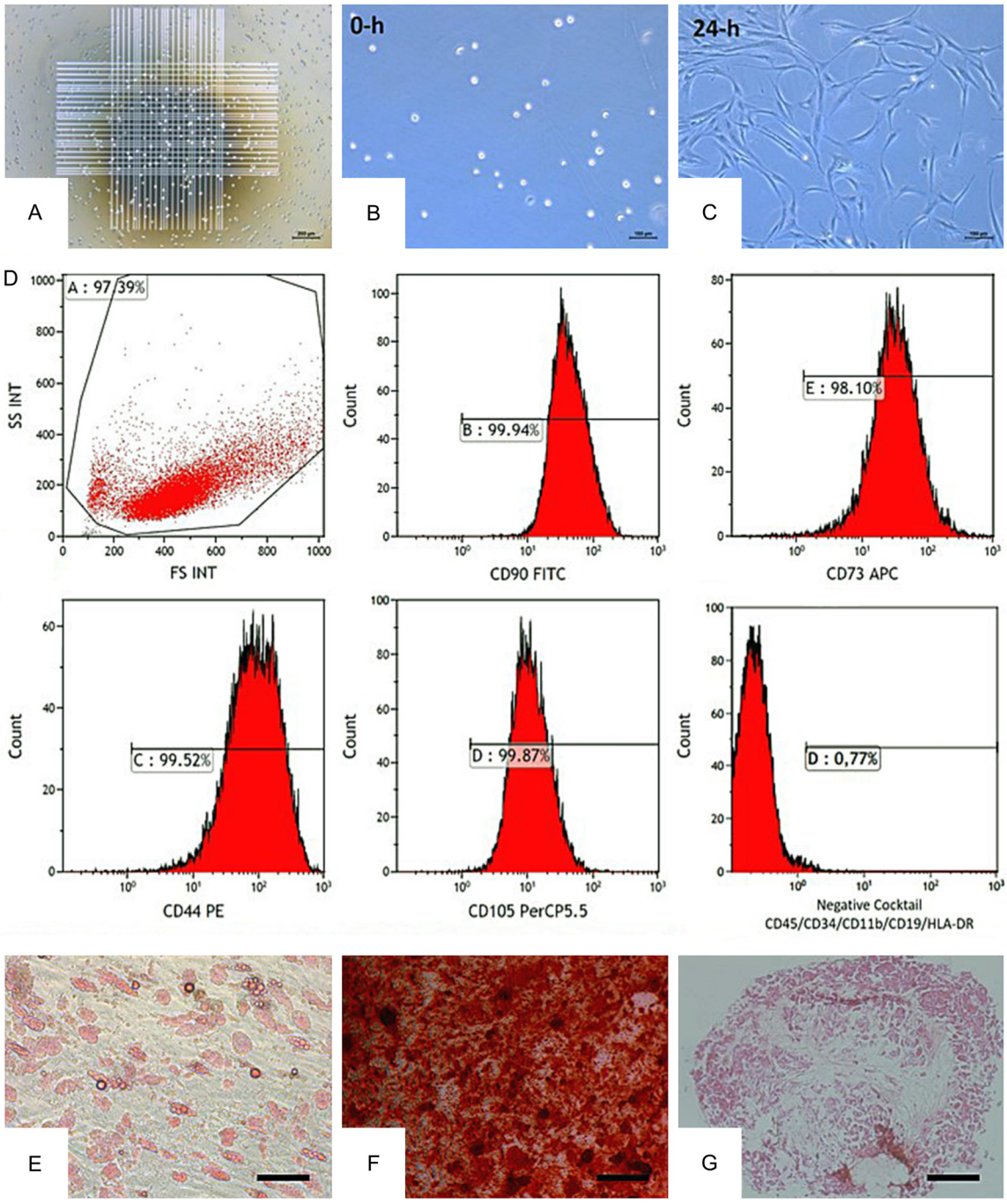

Figure 1. Culture and characterization of AD-MSCs. Count of AD-MSCs in Thoma slide (A) and 0-24 hours in culture morphology (B, C). Microscope objective: 10X, Scale: 100 µm. After flow cytometry analysis of AD-MSCs, 99.94% were CD90 positive, 98.10% CD73 positive, 99.52% CD44 positive and 99.87% CD105 positive. In terms of antibody cocktail containing hematopoietic markers, it was found to be negative with 0.77% expression (D). After adipogenic differentiation, oil droplets are positively stained with adipored (E), mineralization after osteogenic differentiation is positively stained with alizarin red (F), after Safranin-o staining, the ECM and intermediate areas are positively stained, and the cells are differentiated into cubic chondrocyte morphologies (G). Microscope objective: 10X, Scale: 200 µm. All experiments were performed in triplicate.

before being selected with hygromycin, TGF-B1 expression was 45.90% at the 24th hour, and TGF-B1 expression at the 48th hour was analyzed as 45.15%. Western blot analysis showed that TGF-B1 protein expressions supported flow cytometry results (**Figure 2A-G**).

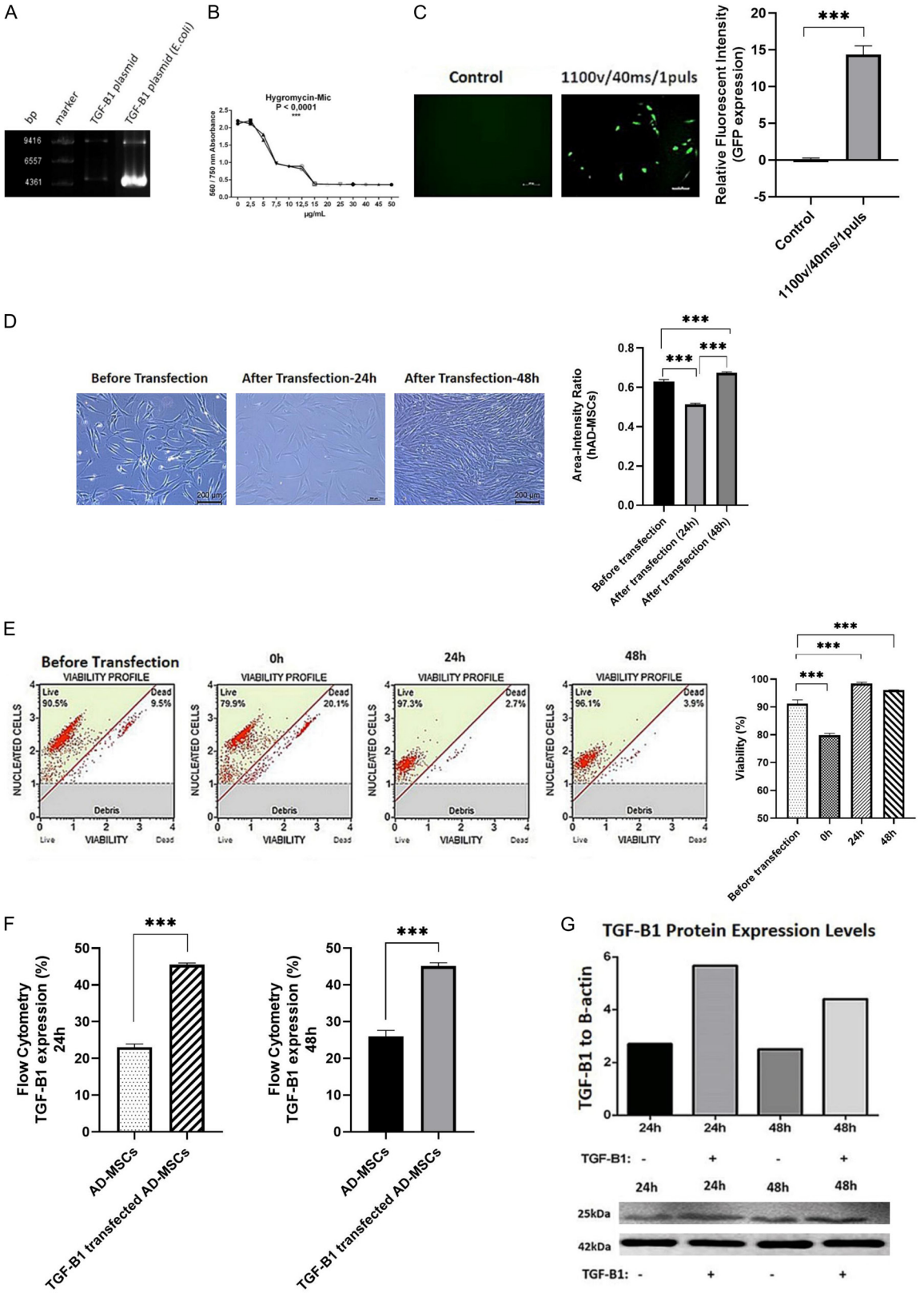

Figure 2. TGF-B1 transfected AD-MSCs and transfection efficiency. As a result of measuring the absorbance values at 560/750 nm wavelength with MTT, the IC50 dose that killed half of the cells on the 7th day, which is half the selection time, was determined as the minimum inhibitory concentration. Considering the absorbance and proliferation curves, it was found that the 6.25 µg/ml hygromycin dose was the appropriate dose for AD-MSCs in terms of

minimum inhibitory concentration (A, B). Transfected cells are seen under an inverted light microscope and fluorescence microscope after GFP plasmid transfection with different parameters for transfection optimization. At 1100 v, 40 ms, and 1 pulse electroporation parameters, it was observed that there was GFP expression in more cells and at higher intensities compared to transfections made with other parameters (C). Microscope objective: 10x, Light microscope images scale bar: 100 µm, fluorescence microscope images scale bar: 200 µm. Culture images of AD-MSCs without hygromycin application just before TGF-B1 gene transfection and 24 hours and 48 hours after transfection. Microscope objective: 10x, scale bar: 200 µm (D). Viability analysis of AD-MSCs before transfection and at 0, 24, and 48 hours after transfection (E). Measurement of TGF-B1 protein expression by flow cytometry and western blot analysis before and 24 and 48 hours after transfection to determine transfection efficiency (F, G). ***: P<0.001. All experiments were performed in triplicate.

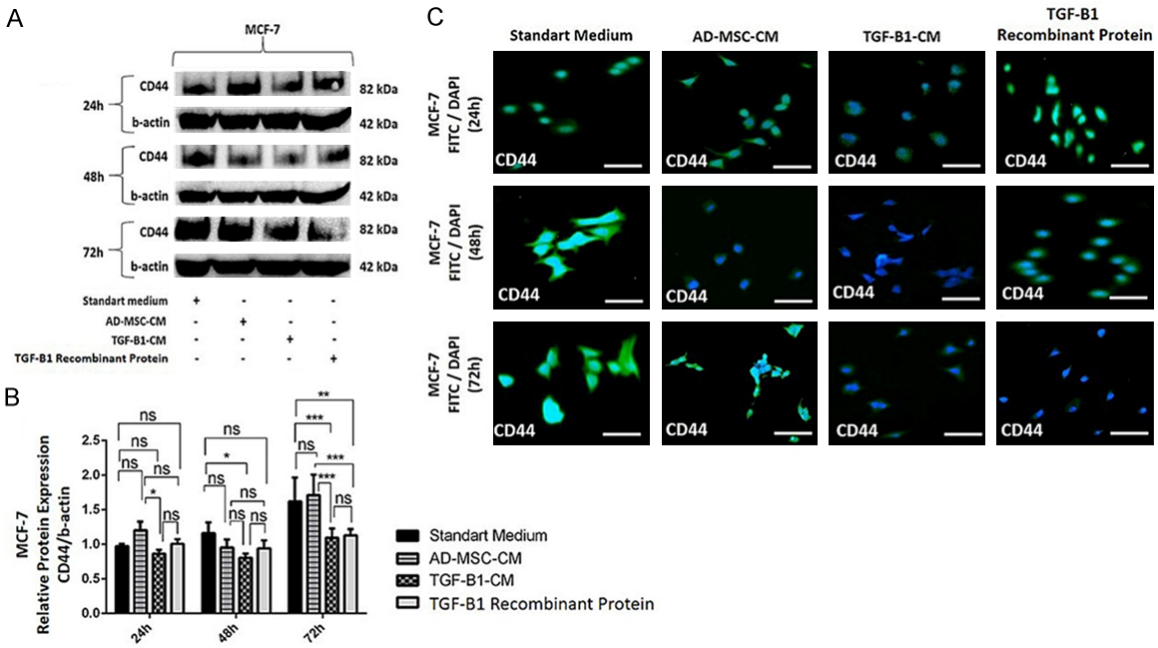

Figure 3. CD44 protein expressions in MCF-7. Analysis of CD44 protein expressions in MCF-7 cells by western blot (A, B) and immunofluorescent staining (C) between groups at 24, 48, and 72 hours. Microscope objective: 10x, scale bar: 100 µm. Immunofluorescent staining results are provided as FITC and DAPI merged images. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001 and ns: not significant. All experiments were performed in triplicate.

Analysis of CD44 and TGF-B1/SMAD signal pathway protein expression in MCF-7 and MDA-MB-231 cells

In MCF-7 and MDA-MB-231 breast cancer cells, it was observed that CD44 protein expression decreased only with the administration of TGF-B1-CM at all periods. Although AD-MSC-CM and TGF-B1 recombinant protein applications decreased CD44 expressions in places, they increased it significantly during some periods (**Figures 3**, **4**). TGF-B1/SMAD canonical signaling pathway has been activated in breast cancer cells treated with TGF-B1-CM. Phospho-SMAD2 and phospho-SMAD3 protein expressions were increased in both MCF-7 and MDA-MB-231 cells and SMAD4 expression was suppressed beyond what was expected. CD44 protein expressions were suppressed according to these changes in the SMAD signaling pathway (**Figures 5**, **6**).

MTT cell proliferation analysis

The application of TGF-B1-CM at the 72nd hour has an inhibitory effect on the proliferation of MCF-7 cells (**Figure 7A**).

Apoptosis results

Our results showed that in MCF-7 cells, especially TGF-B1-CM and TGF-B1 recombinant protein administration significantly increased both early and late and total apoptosis at very early periods such as 24 and 48 hours (**Figure 7B**).

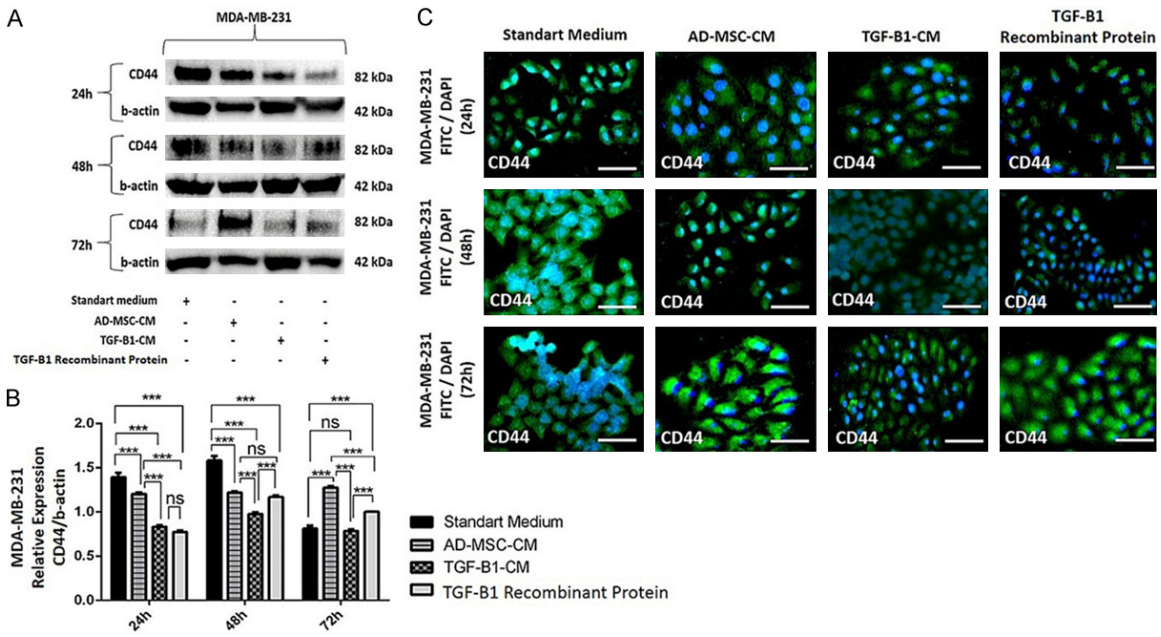

Figure 4. CD44 protein expressions in MDA-MB-231. Analysis of CD44 protein expressions in MDA-MB-231 cells by western blot (A, B) and immunofluorescent staining (C) between groups at 24, 48, and 72 hours. Microscope objective: 10x, scale bar: 100 μm. Immunofluorescent staining results are provided as FITC and DAPI merged images. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001 and ns: not significant. All experiments were performed in triplicate.

Cell cycle assay

It has been determined that TGF-B1-CM application has the function of arresting breast cancer cells in the cell cycle (**Figure 7C**).

Analysis of mitochondria membrane potential

It was found that applications of AD-MSC-CM, TGF-B1-CM and TGF-B1 recombinant protein in MCF-7 cells increased mitochondrial membrane depolarization at 48 and 72 hours (**Figure 7D**).

Senescence-associated beta galactosidase assay

After multiple comparisons between all groups, there was no statistically significant difference in beta-galactosidase activity at 24, 48, and 72 hours in MCF-7 cells (**Figure 7E**).

DNA damage assay

It was determined that significant H2AX phosphorylation took place at 24 and 48 hours in MCF-7 cells treated with AD-MSC-CM, TGF-B1-CM, and TGF-B1 recombinant protein, and DNA damage occurred in response to this. There was no statistically significant difference between the groups at the 72nd hour (**Figure 7F**).

Analysis of wound healing

Wound healing was significantly suppressed by TGF-B1-CM at 44 h in MCF-7 cells compared to other groups (**Figure 8B**). Similarly, the migration of MDA-MB-231 cells was suppressed by the application of TGF-B1-CM (**Figure 8A**, **8B**).

Discussion

In this study we performed, TGF-B1 transfected AD-MSC secretome were used for a new therapeutic approach to breast cancer. Secretomes were applied to the luminal breast cancer cell line MCF-7 and basal MDA-MB-231 breast cancer cell lines. Wu et al. (2019) demonstrated that non-tumor breast epithelial cells and cancerous breast epithelial cells exhibit different responses to the AD-MSC-conditioned medium [20]. Researchers have shown that CM derived from AD-MSCs exerts inhibitory effects on breast cancer cell lines with increased DNA damage and cell apoptosis [20, 21]. The use of TGF-B1 transfected AD-MSC conditioned medi-

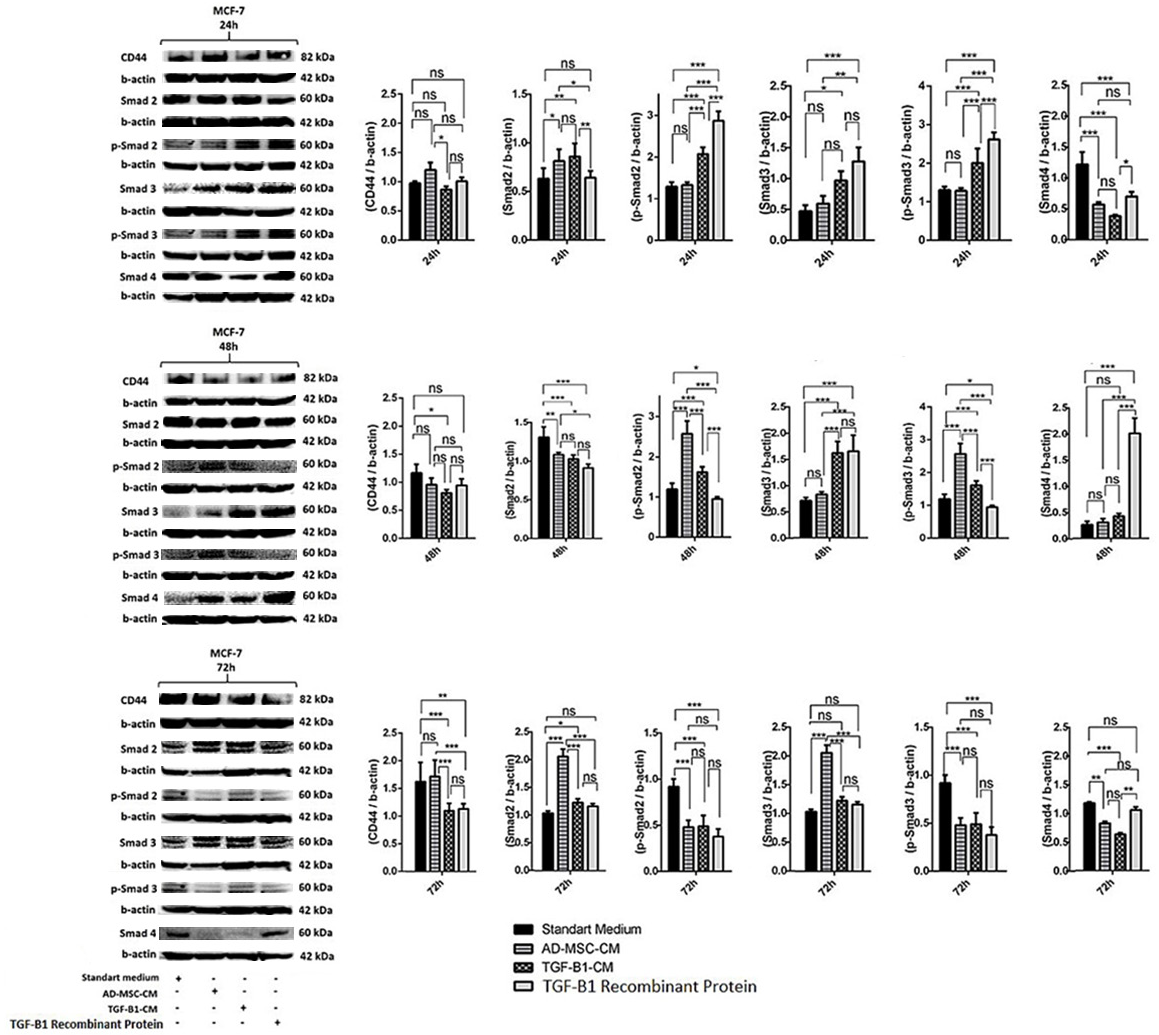

Figure 5. Analysis of CD44, SMAD2, p-SMAD2, SMAD3, p-SMAD3, and SMAD4 protein expressions in MCF-7 cells between groups by western blot at 24, 48, and 72 hours. TGF-B1-CM administration increased p-smad2/3 expression in MCF-7 cells and CD44 protein expression decreased at the times of this increase. SMAD4 expression was decreased by TGF-B1-CM and CD44 expression was decreased in parallel. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001 and ns: not significant. All experiments were performed in triplicate.

um in this study makes our study differs from the literature. He et al. (2018) showed that MSC-CM reduces growth and proliferation in MDA-MB-231 cells, an aggressive breast cancer cell line, and by inhibiting Stat3 activation, it sensitizes cancer cells to radiation therapy [22]. TGF-B superfamily signaling pathways have pleiotropic functions that regulate cell growth, differentiation, apoptosis, motility and invasion, extracellular matrix (ECM) production, angiogenesis, and immune response [23]. The role of TGF-Beta as a tumor promoter or suppressor at the cancer cell level is still under debate due to its different effects in the early and late stages of carcinogenesis. Targeting the TGF-B pathway for cancer therapy can be considered primarily as a microenvironmental targeted strategy [24, 25]. Our previous studies have shown that TGF-B1 plays a healing role in the biological characteristics of mesenchymal stem cells [19]. In MCF-7 breast cancer cells treated with TGF-B1-CM, proliferation was suppressed at the 72nd hour, apoptosis increased at 24 and 48 hours, breast cancer cells were arrested, and DNA damage occurred in the G0/G1 phase of the cell cycle. Cellular senescence was found to be unchanged. The general opinion we have obtained from the literature shows that the effects of TGF-B1 vary according to the early or late stages of cancer cells [26]. In our

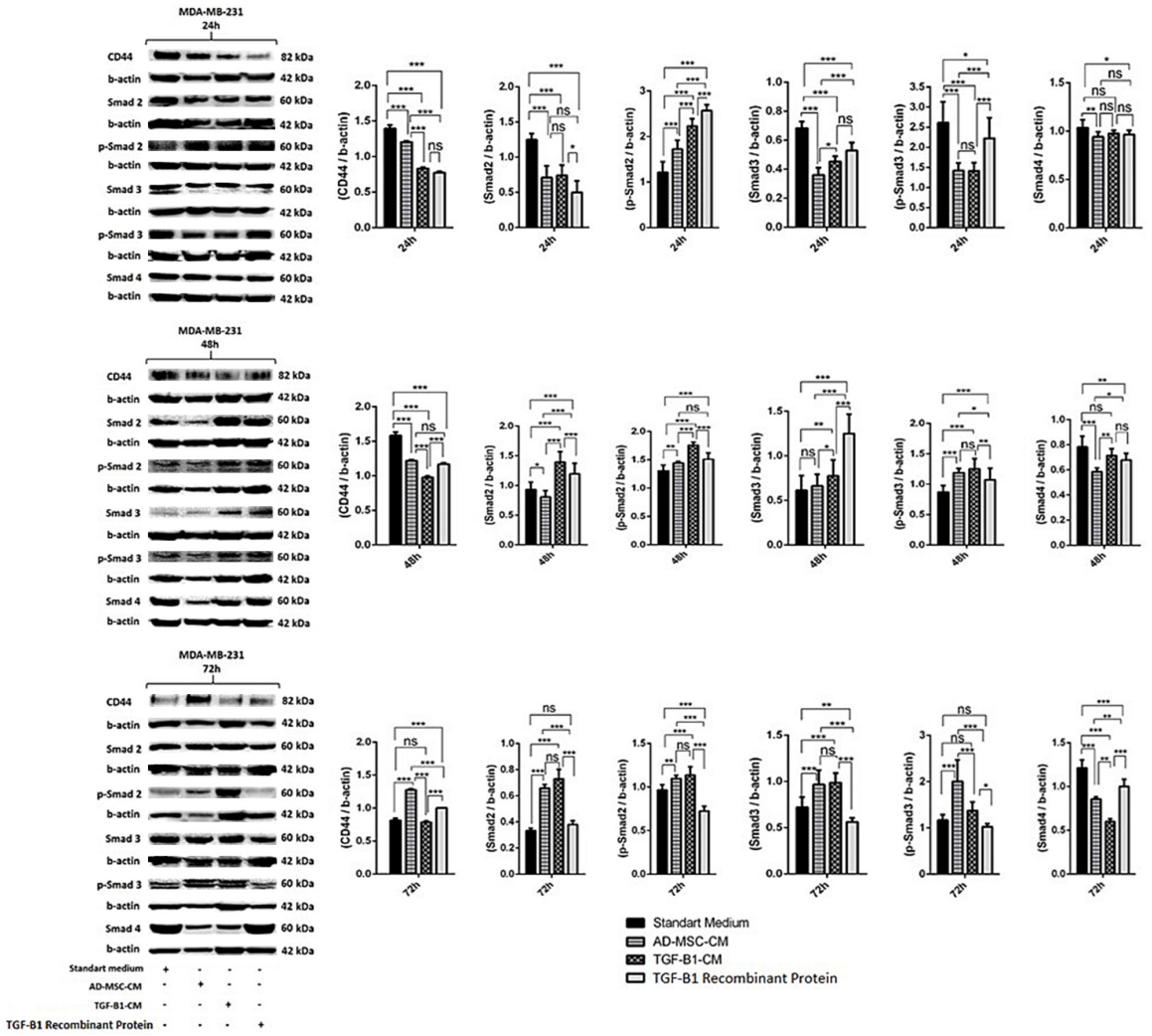

Figure 6. Analysis of CD44, SMAD2, p-SMAD2, SMAD3, p-SMAD3, and SMAD4 protein expressions in MDA-MB-231 cells between groups by western blot at 24, 48, and 72 hours. TGF-B1-CM administration increased p-smad2/3 expression in MDA-MB-231 cells and CD44 protein expression decreased at the times of this increase. SMAD4 expression was decreased by TGF-B1-CM and CD44 expression was decreased in parallel. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001 and ns: not significant. All experiments were performed in triplicate.

study, it was found that the application of TGF-B1-CM completely stopped the migration of the luminal breast cancer type MCF-7 and basal and more aggressive MDA-MB-231 cell lines and prevented the migration of these breast cancer cells to the wound areas *in vitro* in culture. In our study, it was observed that CD44 expression decreased in MCF-7 cells whose migration was suppressed after TGF-B1-CM application, Among other biological features such as proliferation, apoptosis, cell cycle, mitochondrial membrane depolarization, and DNA damage, TGF-B1-CM administration was found to be more critical for the 48th hour.

The results we obtained from our study showed that TGF-B1 transfected AD-MSC conditioned medium suppresses breast cancer cells. Zhao et al. (2017) reported that TGF-Beta increased the migration and invasion of breast cancer cells by inducing an increase in epidermal growth factor expression and SMAD3 and MAPK signaling pathways [27]. The researchers found that SMAD3 expression was associated with EMT. Given this situation, targeting the SMAD3 signal may be critical to suppress breast cancer cells. In our study, the data of the SMAD signaling pathway showed that active SMAD3 expression decreased in the 24th hour

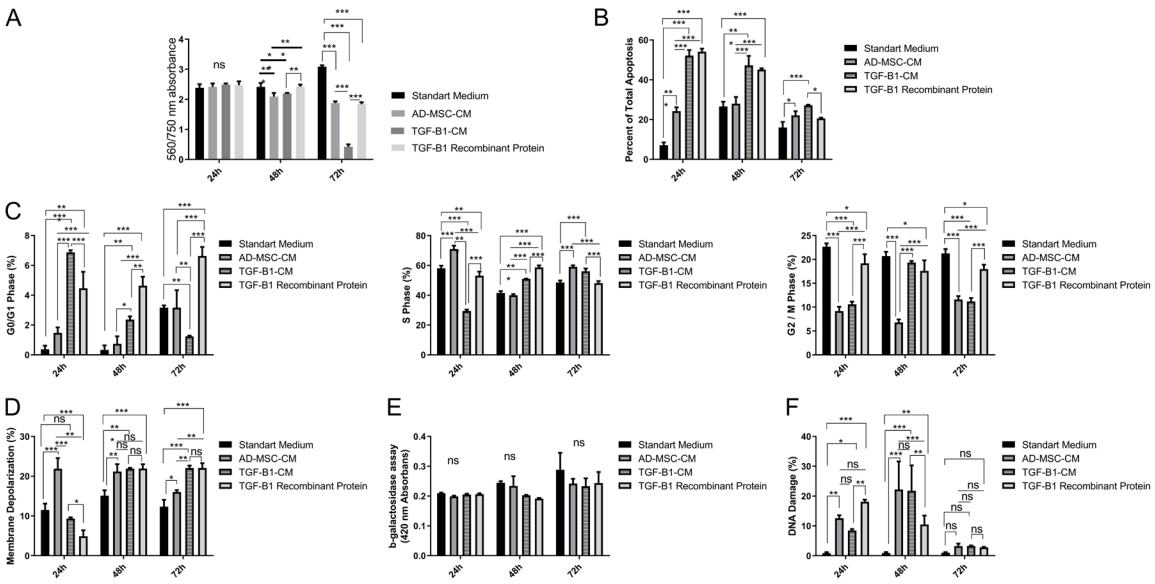

Figure 7. Biological activity assays in MCF-7 cells. Statistical comparisons of findings of proliferation (A), apoptosis (B), cell cycle (C), mitochondrial membrane depolarization (D), senescence-associated beta-galactosidase assay (E), and DNA damage due to H2AX activation (F) between groups. Administration of TGF-B1-CM in breast cancer cells significantly increased apoptosis. TGF-B1-CM suppressed the proliferation of breast cancer cells and arrested breast cancer cells in the G0/G1 phase of the cell cycle. TGF-B1-CM administration induced apoptosis by inducing mitochondrial membrane depolarization in breast cancer cells. Cellular senescence did not change with the administration of TGF-B1-CM. DNA damage was induced in breast cancer cells by the administration of TGF-B1-CM. *: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001 and ns: not significant. All experiments were performed in triplicate.

in the aggressive breast cancer cell line MDA-MB-231, which was applied to TGF-B1-CM. Migration of MDA-MB-231 cells was suppressed by the decrease of phosphorylated SMAD3 expression. Although this is accepted as a clear indicator of the TGF-beta paradox, it shows that not only does the event occur through SMAD3, but it may also be more critical to elucidate the relationship between the suppression of CD44 expressions and the SMAD signaling pathway. To clarify this, our study examined how TGF-B1 transfected AD-MSC secretomes alter TGF-B1/SMAD signaling pathway and CD44 expressions in breast cancer cells. The proven effective role of CD44 in the progression of tumor cells, especially breast cancer, has led to the idea that anti-CD44 agents can be used therapeutically in cancer [28, 29]. Therefore, in our study, it was aimed to suppress CD44, which is intensely expressed in breast cancer cells and is a typical breast cancer stem cell marker, through the TGF-B1/SMAD signaling pathway. Our results showed that in MCF-7 and MDA-MB-231 breast cancer cells, CD44 protein expression decreased statistically significantly with only TGF-B1-CM administration at all periods. Although AD-MSC-CM and TGF-B1 recombinant protein applications decreased CD44 expressions in some places, they increased significantly in some periods. These results show that TGF-B1-CM administration is more stable and effective in suppressing CD44 expression in breast cancer cells. In our study, instead of supplementing the TGF-B1 recombinant protein to the conditioned medium, we transferred the gene transfer to AD-MSCs and applied the anti-tumorigenic factors released from AD-MSCs together with excessive TGF-B1 protein expression and achieved more successful results we did. Sarkar et al. (2019) reported that after transfection of the recombinant human proteoglycan 4 (rhPRG4) gene into MDA-MB-231 cells, the TGF-Beta-hyaluronan-CD44 signaling pathway was inhibited in these cells and breast cancer cell invasion was suppressed. The increase in proteoglycan 4 protein expression inhibited TGF-B-induced EMT, but it is not clear that rhPRG4 regulates the TGF-B1/SMAD signaling pathway [30].

In our study, TGF-B1/SMAD signaling pathway was activated by the TGF-B1 transfected

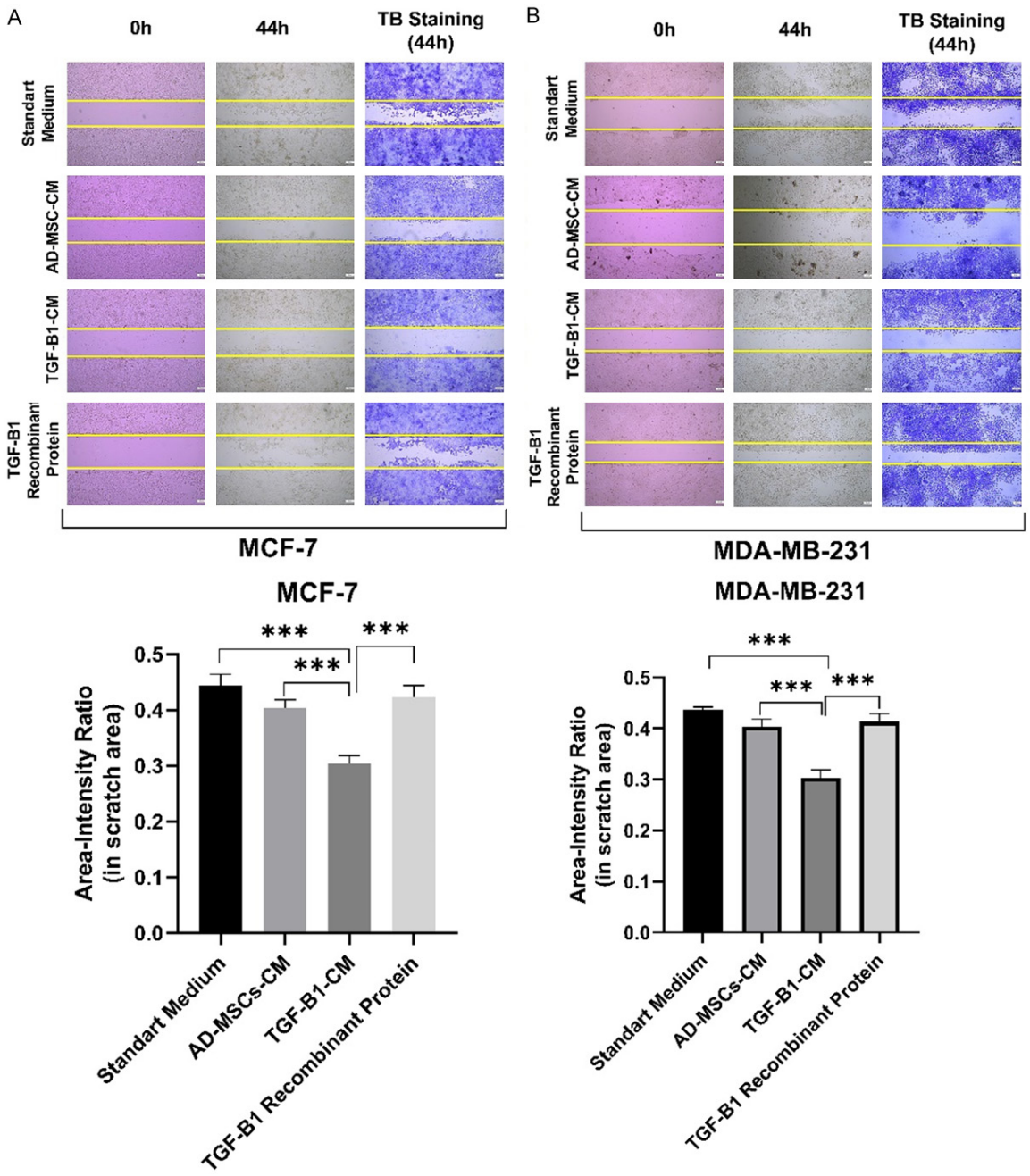

Figure 8. *In vitro* wound healing assay in MCF-7 and MDA-MB-231 cells. Images of the cells showing migration to the wound areas formed after the applications made to the MCF-7 and MDA-MB-231 cell lines according to the experimental groups under the inverted microscope (A and B). The yellow lines represent the edges of the wound sites created at zero hours. Microscope objective: 4x, Scale bar: 50 μM. The migrations of both MCF-7 and MDA-MB-231 breast cancer cell lines were suppressed by TGF-B1-CM administration. ***: P<0.001. All experiments were performed in triplicate.

AD-MSC secretome. When the relationship of this pathway with CD44 expression in breast cancer cells was examined, it was determined that secretomes collected from TGF-B1 transfected AD-MSCs provided SMAD2 and SMAD3 phosphorylation. p-SMAD2 and p-SMAD3 protein expressions were increased in both MCF-7 and MDA-MB-231 cells. CD44 expression decreased in breast cancer cells, inversely proportional to the increase in p-SMAD2/3 expres-

sion. This situation proves our hypothesis. While TGF-B1 recombinant protein administration alone increased the migration abilities of both MCF-7 and MDA-MB-231 breast cancer cells, administration of overexpressed TGF-B1 protein together with adipose-derived mesenchymal stem cell secretome suppressed the in vitro migration of breast cancer cells. TGF-B1-CM administration initiated the SMAD canonical pathway in breast cancer cells, but interestingly, SMAD4 expression was decreased when SMAD2 and SMAD3 were phosphorylated, and CD44 inhibition occurred simultaneously. SMAD4 binds to SMAD2 and SMAD3 is activated in the TGF-B1 signaling pathway, allowing this complex to translocate to the nucleus and form a transcription factor complex for the expression of relevant target genes [31]. Our finding that SMAD4 expression decreased after SMAD signaling pathway analysis suggests that there may be suppression in the transition of p-SMAD2 and p-SMAD3 to the nucleus. The active SMAD complex, which cannot be transported to the nucleus in sufficient quantity as a result of suppression of SMAD4 by TGF-B1-CM, may have decreased the production of hyaluronic acid synthase (HAS). CD44 expression may be suppressed in cancer cells due to the decrease of hyaluronic acid, the main ligand of CD44. If this is the way we think it is, SMAD4-targeted therapeutic strategies can be considered to suppress CD44 expression in breast cancer cells and, in parallel, induce apoptosis and arrest it in the cell cycle. Our discovery of its relationship with CD44 in the canonical pathway, especially in terms of SMAD4, after TGF-B1/SMAD signaling pathway analysis may provide a new perspective on the development of breast cancer therapeutic agents.

Conclusions

SMAD4-targeted therapeutic strategies may be considered to suppress CD44 expression in breast cancer cells. Both the anti-tumorigenic factors released by AD-MSCs and the secretomes obtained as a result of supporting these factors with the overexpression of TGF-B1, severely suppressed breast cancer cells. With this study, it was planned to obtain a targeted biological product that suppresses breast cancer cells *in vitro*. The study we have conducted to fully elucidate the TGF-B1-SMAD-CD44 pathway will make serious contributions to the literature. However, our study provides *in vitro* findings. The successful results we obtained *in vitro* should be supported by *in vivo* studies. Gene and protein interactions between the TGF-B1-CD44-SMAD signaling pathways in breast cancer cells can make our knowledge in this field more enlightening with transcriptomics and proteomics studies. The work presented here is capable of paving the way for further molecular and *in vivo*-based studies in this area.

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Disclosure of conflict of interest

None.

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