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Apoptotic effects of human amniotic fluid mesenchymal stem cells conditioned medium on human MCF-7 breast cancer cell line

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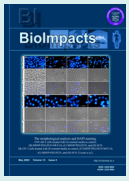

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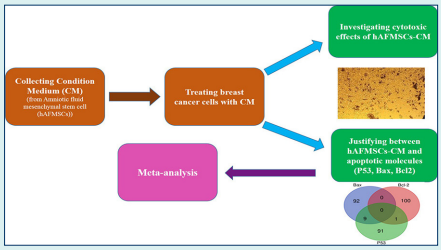
Abstract



Introduction: Breast cancer, as the most common malignancy among women, is shown to have a high mortality rate and resistance to chemotherapy. Research has shown the possible inhibitory role of Mesenchymal stem cells in curing cancer. Thus, the present work used human amniotic fluid mesenchymal stem cell-conditioned medium (hAFMSCs-CM) as an apoptotic reagent on the human MCF-7 breast cancer cell line.

Methods: Conditioned medium (CM) was prepared from hAFMSCs. After treating MCF-7 cells with CM, a number of analytical procedures (MTT, real-time PCR, western blot, and flow cytometry) were recruited to evaluate the cell viability, Bax and Bcl-2 gene expression, P53 protein expression, and apoptosis, respectively. Human fibroblast cells (Hu02) were used as the negative control. In addition, an integrated approach to meta-analysis was performed.

Results: The MCF-7 cells' viability was decreased significantly after 24 hours ($P<0.0001$) and 72 hours ($P<0.05$) of treatment. Compared with the control cells, Bax gene's mRNA expression increased and Bcl-2's mRNA expression decreased considerably after treating for 24 hours with 80% hAFMSCs-CM ($P=0.0012$, $P<0.0001$, respectively); an increasing pattern in P53 protein expression could also be observed. The flow cytometry analysis indicated apoptosis. Results from literature mining and the integrated meta-analysis showed that hAFMSCs-CM is able to activate a molecular network where Bcl2 downregulation stands in harmony with the upregulation of P53, EIF5A, DDB2, and Bax, leading to the activation of apoptosis.

Conclusion: Our finding demonstrated that hAFMSCs-CM presents apoptotic effect on MCF-7 cells; therefore, the application of hAFMSCs-CM, as a therapeutic reagent, can suppress breast cancer cells' viabilities and induce apoptosis.

Introduction

Breast cancer remains as the most common lethal cancer among women around the world.[1] Currently, chemotherapy and surgery are the main approaches in the breast cancer clinical cure. However, the toxicity of chemotherapy agents on normal cells and their resistance to drugs have been considered as the main barrier to proceed with chemotherapy.[2,3] Nowadays, other types of treatments such as hormone replacement therapy and complementary therapies are under clinical consideration, among which targeted therapies, gene therapy, and stem cell therapy have gained considerable attention in the breast cancer research field.[4]

In the last decade, stem cell treatment has been considered as a new method for discovering potential therapeutic approaches in cancer therapy.[5-10] In this regard, a great deal of researches have underscored the mesenchymal stem cells' (MSCs) impact and their related factors on cancer cells.[7,11-13] MSCs are defined as regenerative undifferentiated cells capable of being differentiated into various cell types.[14] Recently, several studies have unveiled MSCs' potential of suppressing tumors by inhibiting tumor cell proliferation and inducing apoptosis in cancer cells.[6,9,10,15,16] It is argued that

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the human amniotic stem cells (hAECs) anticancer effect is associated with the endogenous production of growth inhibitors which target tumor growth and progression. Some studies showed hAECs express a range of cytotoxic cytokines, such as IFN-γ, TGF-β, TNF-α and TNF-β as apoptotic inducer substances.[17] Additionally, hAECs secrete various interleukins, including IL-3, IL-4, and IL-2, to promote cytotoxicity in NK cells, the targeting of cancer cells, and the inhibition of tumor formation.[18,19]

Furthermore, the ability of MSCs to move to primary tumors could be used to deliver anti-cancer factors to the tumor site.[20,21]

Human amniotic fluid mesenchymal stem cell-conditioned medium (hAFMSCs-CM), as effective stem cells in treating a number of human diseases, are achieved from pregnant women at the end or the second trimester of pregnancy using amniocentesis.[22,23] Therefore, not only is the generation of such cell lines considerably easier than human embryonic stem cells (hESCs), they are also not subject to hESCs barriers. Some studies have revealed the inhibitory effects of stem cell conditioned medium (CM) on cancer cells.[24,25] CM has many advantages such as easy production, freezing-thawing competence, and packaging.[26]

There is sufficient evidence about hAMSCs ability to produce IFN-γ and CXCL10 as key inhibitors of angiogenesis in the literature.[27] IFN-γ has the potential to hinder a tumor growth and enhance the apoptosis.[28,29] The hAMSCs-CM targets the ratio of cells in S and G2/M phase of PBMC cells leading to apoptosis induction.[30] In addition, hAFMSCs express a number of miRNAs (miR-195-3p, miR-19b-1-5p, miR-20a-5p, miR-20b-3p, miR-26a-1-3p, 708-3p, miR-16-1-3p, 3p, miR-15b-3p, 5p, miR-93-3p, miR204)[31-35] that negatively interact with anti-apoptotic targets.

hAFMSCs are known to have anti-cancer effects by inducing P53 (tumor suppresser) and P21 expression as well as reducing cyclin B1 and D1 after five days of co-culturing with human ovarian cancer cell lines.[6] P21 acts as a P53 transcriptional target, inhibiting cell cycle activity in G1/G2 phases.[36] P53 inhibits the proliferation of abnormal cells by adjusting cell cycle checkpoints in most tissues.[36] Various breast cancers mutate P53, resulting in more aggressive forms of the disease.[37]

Bcl-2 inhibition by P53, as a transcriptional factor, is crucial for apoptosis induction. As an anti-apoptotic gene with high expression in most breast cancers, Bcl-2 is known as an effective factor in primary breast cancer prognosis.[38,39] Bax is a pro-apoptotic gene within the Bcl-2 family that presents expression in most breast cancers; a low expression of Bax leads to apoptosis resistance in breast cancer.[40]

Based on our literature review and meta-analysis, hardly, we could find any study reporting the effect of the cell-free hAFMSCs conditioned medium on MCF-7 cells viability and the apoptosis. Therefore, the present work aims at assessing the apoptosis and meta-analysis of hAFMSCs-CM on breast cancer cell line.

Materials and Methods

hAFMSCs culture

hAFMSCs were prepared in accordance with previous studies.[22] Cells were plated in 25 cm^2 cell culture flasks and DMEM-F12 (Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12) were supplemented with 15% FBS (Fetal Bovine serum), streptomycin (100 µg/mL), penicillin (100 units/mL), and 10 ng/mL of bFGF (basic fibroblast growth factor). The cells were cultured in an incubator with 5% CO_2 humidified gas environment at 37°C.

Preparing conditioned medium

The hAFMSCs were cultured in 75 cm^2 flasks to prepare the conditioned media**.** When the cells reached 70% to 80% confluency, they were washed with phosphate buffer saline (PBS) for 3 times and were kept in DMEM-L (Dulbecco's Modified Eagle Medium-Low Glucose), penicillin (100 units/mL), and streptomycin (100 µg/mL) for 48-72 hours at 37°C in a 5% CO_2 humidified environment. Afterward, the media were collected from the flasks and centrifuged at 450 g for 10 minutes to acquire the supernatant and discard the pellet. Passing through a 0.22-µm filter, the media were stored at -80˚C (see Fig. 1).

MCF-7 and Hu02 cells culture and treatment

MCF-7 (human breast cancer cell line) and Hu02 (human skin fibroblast cell line) cell lines were obtained from IBRC (Iranian Biological Resource Center). The cells were grown in 25 cm^2 flasks with ESCs culture medium (DMEM supplemented with 10% FBS, 100 µg/mL of streptomycin, and 100 units/mL of penicillin). Cells were stored in a humid gas environment with 5% CO_2 at 37°C. The media were replaced 3 times per week; 80% (v/v) hAFMSCs-CM was used for the treatment.

Cell viability assay

To determine the effect of hAFMSCs-CM, cell viability was evaluated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, Cas# 298-93-1, USA) assay, as explained elsewhere.[6,41] MCF-7 and Hu02 cells were treated with different percentages of hAFMSCs-CM (20%, 40%, & 80%) for 24, 48, and 72 hours, respectively. In order to determine the cell viability, 0.5 mg/mL of MTT reagent was added to each well and incubated for 4 hours. Then, the MTT solution was removed and 100 µL DMSO (Dimethyl sulfoxide) was added to each well of the 96-well plate to solve formazan crystal. ELISA reader (BioTek, USA) was recruited to measure the absorbance at 570 nm. The untreated cells were considered as the control. To calculate the cell viability, the following formula was used:

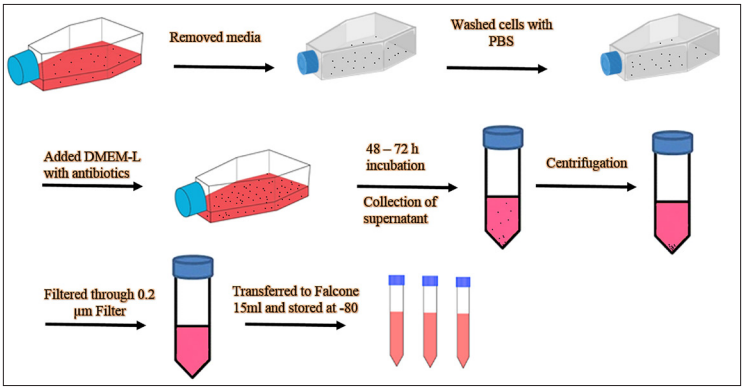


Fig. 1. Schematic diagram showing condition medium preparation from hAFMSCs.

Cell viability (%) = (Mean optical absorbance of the treated cells/ Mean optical absorbance of the control cells) × 100

RNA extraction and cDNA synthesis

While the control cells were maintained using the normal media and incubated, MCF-7 and Hu02 cells were treated with 80% of hAFMSCs-CM for total RNA extraction.

After 24 hours, total RNA of MCF-7 was extracted using the RiboEx kit (Gene All, Cat No.301-001, Korea) and the complementary DNA (cDNA) was synthesized from the total RNA using BioFACT kit (BioFACT, Cat No.BR441-096, Korea) based on the manufacture's protocols.

Real-time PCR

To characterize the hAFMSCs-CM effects on the pro-apoptotic (Bax) and the anti-apoptotic (Bcl-2) mRNA expression, Real-time PCR was carried out using SYBER Green (BioFACT, Cat. No. DQ385-40h, Korea) in ABI (Applied Biosystems Step One Plus) detection system in compliance with the manufacture's instruction. Table 1 illustrates the sequence of the primers used in this study; GAPDH (housekeeping gene) was considered as the internal control.

Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method based on the following formula[42]:

$$\Delta\Delta Ct = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)} = (Ct, Target\ gene - Ct\ GAPDH)_{(treated)} - (Ct\ Target\ gene - Ct\ GAPDH)_{(untreated)}$$

Western blot analysis

After the hAFMSCs-CM treatment, western blot (WB) analysis was performed to evaluate the amount of P53 protein in MCF-7 and Hu02 cells. While MCF-7 cells were treated with 80% of hAFMSCs-CM, untreated cells were considered as the control. The cells were collected and lysed following a 24-hour incubation, then an electrophoresis was performed when equal amounts of crude protein (50 µg) of sample were loaded in each lane for 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel). The extracted proteins were transferred to a polyvinylidene fluoride (PVDF) membrane where they were blocked with 2% no-fat milk for 1 hour. Next, they were incubated with mouse anti-P53 (Santa Cruz Biotechnology, sc-126, 1:300) and anti-β- actin (sc-47778, 1:300) at 4°C overnight. Afterward, the membrane was incubated with a probed secondary antibody conjugated to HRP (horseradish peroxidase) (Anti-rabbit 1:1000) for 1 hour. An Enhanced Chemiluminescence detection system was employed for detection. Beta-actin was used for normalization and internal control, and ImageJ software was utilized to analyze the image.

Table 1. The primers' sequence used for the Real Time PCR

Primer Sequences	Gene
Forward: 5'- CAAGATCATCAGCAATGCCTCC - 3' Reverse: 5'- GCCATCACGCCAGTTTCC - 3'	GAPDH
Forward: 5'- GACTCCCCCCGAGAGGTCTT - 3' Reverse: 5'- ACAGGGCCTTGAGCACCAGTT - 3'	BAX
Forward: 5'- GAGCGTCAACCGGGAGATGTC - 3' Reverse: 5'- TGCCGGTTCAGGTACTCAGTC - 3'	Bcl-2

Flow cytometry

Apoptotic cells were exposed to phosphatidylserin in their outer plasma membrane, which were identified by fluorescein isothiocyanate (FITC) labeled Annexin-V/ PI (propidium iodide) using flow cytometry. Following a 24 hours treatment of cells with 80% hAFMSCs-CM, MCF-7 and Hu02 cells were harvested by trypsin and washed with PBS. After 8 minutes of centrifugation at 1300 rpm, the cells were re-suspended in 100 µL binding buffer (Invitrogen, Lot #4338210) and were mixed with 2 µL Annexin-V (Invitrogen, Lot #1989095); they were then incubated on ice for 20 minutes in a dark place. The cells' solution was centrifuged at 1300 rpm for 8 min, after which the supernatant was removed and 100 µL binding buffer was added. The sample solution was combined with 1 µL of PI (Invitrogen, Lot #1957465) and was incubated for 20 minutes in a dark place. Flow Jo (7.6.1) software was used to run the flow cytometry analysis on samples utilizing BD FACS Calibur Flow Cytometry (BD Biosciences, NJ, USA).

Finding a possible molecular network underlying the hAFMSCs-CM function using integrated approach of meta-analysis and literature mining

We conducted a literature-mining-based network analysis and employed an integrated approach of meta-analysis of expression data to ascertain the possible regulatory network underlying hAFMSCs-CM function in breast cancer cells.

As presented in Fig. 2, the following steps were performed:

1. Recruitment of Mutual Ranking (MR) statistics for Co-expression meta-analysis of Bax, Bcl-2, and P53, consulting public transcriptomic data in Gene Expression Omnibus (GEO).
2. Selection of top 100 co-expressed genes with Bax, Bcl-2, and P53.
3. Finding shared genes between co-expressed profiles of Bax, Bcl-2, and P53.
4. Performing Literature-mining based network analysis: Discovery of common targets and regulators with positive interactions with Bax, P53 and apoptosis, and negative interactions with Bcl-2.

Mutual ranking (MR) statistics and Z-transformation of expression data were used for expression data meta-analysis and removal of platform effect, as described elsewhere.[43,44] As compared with the common approach for running the Pearson correlation, MR statistics employs a ranking approach for correlation analysis where it remains unaffected by the experiment. After calculating the rank correlation for each experiment, geometric average of correlation coefficients was ranked in logarithmic manner.[45,46] Correlation rankings were used extensively during the meta-analysis (e.g. Rankprod).[47] The expression data were retrieved from the GEO (NCBI public repository of expression data, https://www.ncbi.nlm.nih.gov/geo/). COXPRESdb v7 tool was performed for analysis.[48] Lower values of MR represent higher level of association where MR value of each gene, including itself, is 0.

Literature mining-based database of Pathway Studio Mammalian (Elsevier)[49,50] was performed, as previously described.[51,52] The database collects data through NLP (Natural Language Processing) algorithm and contains 13 440 356 mined relations from full text published paper and 1 439 833 entities (e.g., proteins/genes, cell process, small molecules, and diseases) (March 2021). The database is enriched with additional inputs from Gene Ontology Consortium for cellular location analysis, MiRbase, and various network construction approaches such as "Common Binding Partner", "Downstream Target Discovery", and "Upstream Regulator Discovery", among others.

Statistical analysis

Each experiment was performed in triplicate. Data were presented as means ± standard error of the mean (SEM). A one-way ANOVA and a *t* test were conducted to compare the three and the two groups, respectively. Any differences were deemed significant when the *P* value was smaller than 0.05 ($P < 0.05$). GraphPad Prism software (La, Jolla, CA) version 8.4.3(686) was utilized to run the statistical analysis.

Results

hAFMSCs-CM effects on MCF-7 cell viability

To investigate the hAFMSCs-CM impact on MCF-7 and Hu02 cell viability, an MTT assay was carried out 3 times (24, 48, and 72 hours) after the treatment. As shown in Fig. 3, hAFMSCs-CM was found to have a cytotoxic effect on MCF-7. Noteworthy, no cytotoxic effects were observed on Hu02 cells. Our data suggest that the cell viability in MCF-7 cells was decreased significantly as a result of CM

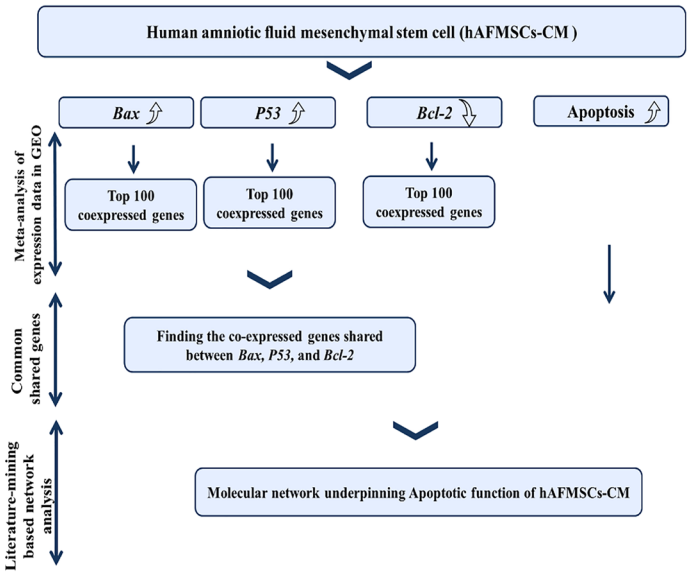


Fig. 2. Bioinformatics pipeline employed in this study.

after the treatments (24, 48, and 72 hours) (see Fig. 3A, 3B, 3C). With 20% of CM no significant effect on cell viability could be observed; however, in 40% and 80% of CMs (after 24 hours), cell growth was inhibited as compared with the control cells. Fig. 3A illustrates the cell viability being declined to 78% ($P<0.0001$) when 80% of CM and to 86.99% ($P=0.0027$) when 40% of CM were used after the phase 1 of the treatment (24 hours). Upon the completion of the 48-hour and 72-hour incubation (40% CM) phases, the hAFMSCs-CM demonstrated an insignificant effect on the cell viability ($P>0.05$). Noteworthy, 80% of the CM was found to have affected the MCF-7 cells considerably ($P<0.05$). Although hAFMSCs-CM failed to affect MCF-7's cell viability, it was found to be capable of promoting the cell viability in Hu02 as normal cells ($P=0.0014$, during the 24 hour-treatment).

hAFMSCs-CM effects on Bax and Bcl-2 genes expression and P53 protein expression

Following the hAFMSCs-CM 24-hour treatment, the Bax and Bcl-2 mRNA level expressions were analyzed. The genes' Ct values were normalized against the GAPDH mRNA level (the housekeeping gene). Notably, as illustrated in Fig. 4A, the pro-apoptotic Bax gene's expression level increased significantly as compared with the control group ($P<0.0001$). On the other hand, the anti-apoptotic Bcl-2 gene's mRNA level decreased considerably when cells were treated with 80% hAFMSCs-CM for 24 hours ($P=0.0012$). Nevertheless, as Fig. 4C shows, in normal cells (Hu02), the level of the Bax gene declined and Bcl2 increased after the hAFMSCs-CM treatment.

Fig. 4B illustrates the WB analysis of P53 protein expression, demonstrating a significant ($P<0.0001$) increase (about 3.7 fold) after the hAFMSCs-CM treatment, as compared with control (untreated) cells. However, we could not observe meaningful differences in P53 expression in Hu02 cells ($P>0.05$) (Fig. 4D).

hAFMSCs-CM effects on apoptosis

Apoptosis was measured using a flow cytometry assay via annexin V and PI staining of the cells. As demonstrated in Fig. 5, apoptosis was induced in the female human breast cancer cells by hAFMSCs-CM. The flow cytometry analysis of MCF-7 cells, treated with 80% hAFMSCs-CM for 24 hours, showed early apoptosis (annexin V+ PI-) of nearly 22.7%, whereas the control cells' apoptotic functions were about 6.2%. Despite insignificant differences among normal cells ($P>0.05$), no considerable apoptosis could be observed in Hu02 cells ($P>0.05$) (Fig. 5F).

Meta-analysis based co-expressed genes with Bax, Bcl-2, and P53

Tables 2, 3, and 4 show the genes that were found to be co-expressed with Bax, Bcl-2, and P53 after a meta-analysis. Fig. 6 also presents the shared genes found within the meta-analysis derived co-expressed profiles of Bax, Bcl-2, and P53. Notably, Bax and P53 were found to be co-expressed. DDB2 (Damage specific DNA Binding protein 2) is among the top 3 co-expressed genes with Bax that co-expresses with P53. In the same vein, EIF5A (Eukaryotic Translation Initiation Factor 5A) is an important protein that co-expresses with Bax and P53.

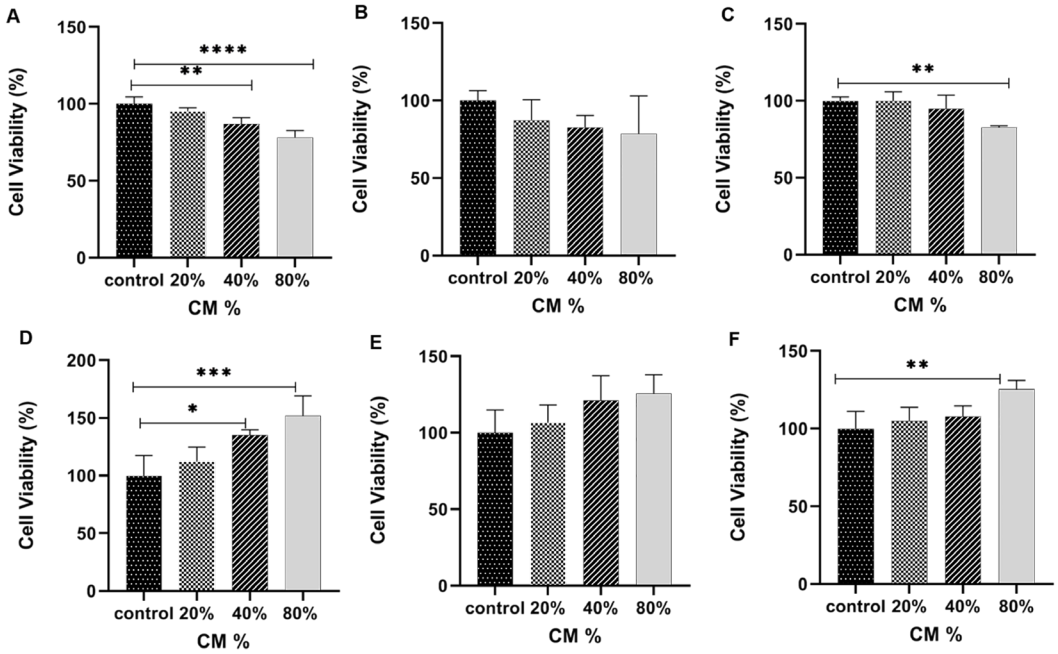


Fig. 3. The MCF-7 and Hu02 cell viability was assessed by MTT assay for MCF-7 cells within 24 (A), 48 (B) and 72 (C) hours and for and Hu02 within 24 h (D), 48 h (E) and 72 h (F) treatment with hAFMSCs-CM. After 24 h, a significant decrease in MCF-7 cells viability ($P<0.0001$) and Hu02 cells viability ($P<0.005$) was observed. The data are presented as mean ± SEM. Significantly different (** $P<0.005$, **** $P<0.0001$).

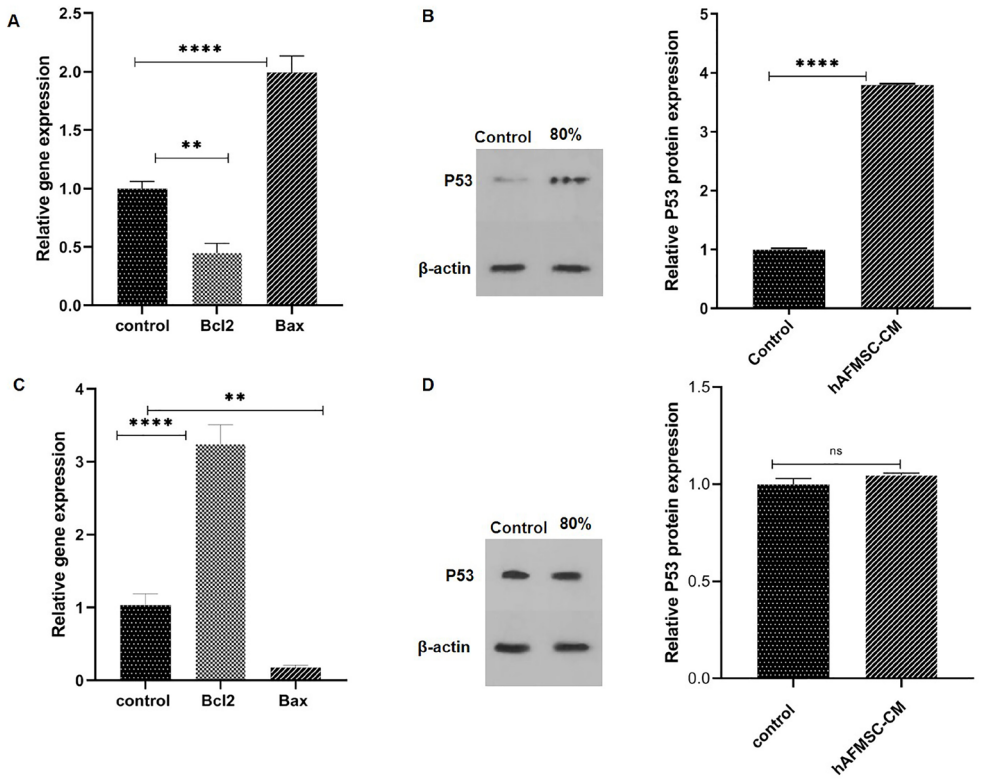


Fig. 4. Real-time PCR and western blot analysis were used to assess hAFMSCs-CM effect on MCF-7 and Hu02 cells. (A) pro-apoptotic Bax and anti-apoptotic Bcl-2 genes of MCF-7 were treated with 80% hAFMSCs-CM in 24 h. (B) WB analysis and P53 protein quantification were used to evaluate 80% hAFMSCs-CM effect on P53 protein expression compared with the control in MCF-7 cells. (C) Hu02's Bax and Bcl-2 genes were treated with 80% hAFMSCs-CM within 24 h. (D) WB analysis and P53 protein quantification to evaluate the 80% hAFMSCs-CM effect on P53 protein expression compared with the control in Hu02 cells. P53 protein level was determined by ImageJ analysis. The data are presented as mean ± SEM. (****P<0.0001, ***P<0.005).

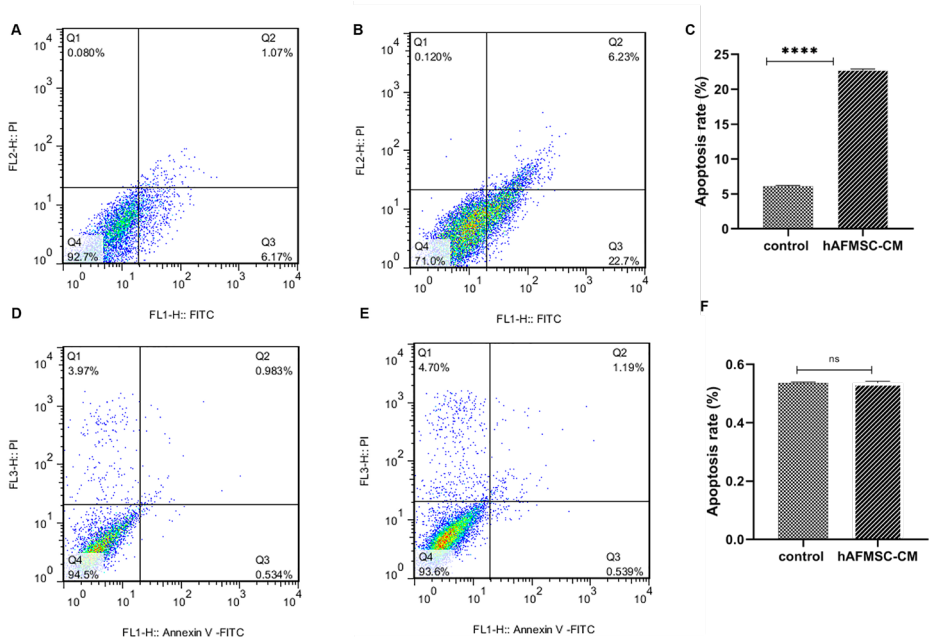


Fig. 5. Apoptotic evaluation using the flow cytometry via annexin V and PI staining. (A) Control MCF-7 cells' (untreated cells) apoptosis. (B) The effect of 80% hAFMSCs-CM on MCF-7 cells after 24 h -- hAFMSCs-CM induces apoptosis in MCF-7 cells. (C) Quantification of apoptosis in MCF-7 cells. (D) Control Hu02 cells (untreated cells) apoptosis. (E) The effect of 80% hAFMSCs-CM on Hu02 cells after 24 h. hAFMSCs-CM do not affect apoptosis in Hu02 cells. (F) Quantification of apoptosis in Hu02 cells. Fig. 5. represents viable cells (annexin V-PI-) population, early apoptosis (annexin V+ PI-), late apoptosis (annexin V+PI+), and necrotic cells (annexin V-PI Flow cytometry analysis was performed for samples using BD FACS Calibur flow cytometry [BD Biosciences, NJ, USA]). Flow Jo. (7.6.1) software was used to analyze the data.

Table 2. Meta-analysis-derived co-expressed genes with Bax

	Gene	Function	Entrez Gene ID
0	BAX	BCL2 associated X: apoptosis regulator	581
1	FDXR	Ferredoxin reductase	2232
2	AP1S1	Adaptor related protein complex 1 sigma 1 subunit	1174
3	DDB2	Damage specific DNA binding protein 2	1643
4	RPS27L	Ribosomal protein S27 like	51065
5	BBC3	BCL2 binding component 3	27113
6	MDM2	MDM2 proto-oncogene	4193
7	ZMAT3	Zinc finger matrin-type 3	64393
8	LINC01759	Long intergenic non-protein coding RNA 1759	1.03E+08
9	PHLDA3	Pleckstrin homology like domain family A member 3	23612
10	PDAP1	PDGFA associated protein 1	11333
11	AEN	Apoptosis enhancing nuclease	64782
12	PFDN6	Prefoldin subunit 6	10471
13	TIGAR	TP53 induced glycolysis regulatory phosphatase	57103
14	PFN1	Profilin 1	5216
15	CDKN1A	Cyclin dependent kinase inhibitor 1A	1026
16	PRMT1	Protein arginine methyltransferase 1	3276
17	APH1A	Aph-1 homolog A: gamma-secretase subunit	51107
18	TRIAP1	TP53 regulated inhibitor of apoptosis 1	51499
19	PHPT1	Phosphohistidine phosphatase 1	29085
20	ZNF428	Zinc finger protein 428	126299
21	HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U like 2	221092
22	PSENEN	Presenilin enhancer gamma-secretase subunit	55851
23	EML2	Echinoderm microtubule associated protein like 2	24139
24	EDA2R	Ectodysplasin A2 receptor	60401
25	ISOC2	Isochorismatase domain containing 2	79763
26	MIR34AHG	MIR34A host gene	1.07E+08
27	AP2S1	Adaptor related protein complex 2 sigma 1 subunit	1175
28	ARPC4	Actin related protein 2/3 complex subunit 4	10093
29	ARPC1B	Actin related protein 2/3 complex subunit 1B	10095
30	RPS19	Ribosomal protein S19	6223
31	RFXANK	Regulatory factor X associated ankyrin containing protein	8625
32	GTF2H4	General transcription factor IIH subunit 4	2968
33	LAMTOR2	Late endosomal/lysosomal adaptor: MAPK and MTOR activator 2	28956
34	THOC6	THO complex 6	79228
35	SYMPK	Symplekin	8189
36	TMEM160	Transmembrane protein 160	54958
37	TP53I3	Tumor protein p53 inducible protein 3	9540
38	PPP4C	Protein phosphatase 4 catalytic subunit	5531
39	PNKP	Polynucleotide kinase 3'-phosphatase	11284
40	GPX1	Glutathione peroxidase 1	2876
41	ITPA	Inosine triphosphatase	3704
42	SPATA18	Spermatogenesis associated 18	132671
43	MPDU1	Mannose-P-dolichol utilization defect 1	9526
44	EIF5A	Eukaryotic translation initiation factor 5A	1984
45	PURPL	p53 upregulated regulator of p53 levels	643401
46	TFPT	TCF3 fusion partner	29844
47	IRF3	Interferon regulatory factor 3	3661
48	CHMP4B	Charged multivesicular body protein 4B	128866
49	GDF15	Growth differentiation factor 15	9518
50	CYBA	Cytochrome b-245 alpha chain	1535
51	PGLS	6-Phosphogluconolactonase	25796

Table 2. Continued

	Gene	Function	Entrez Gene ID
52	DLST	Dihydrolipoamide S-succinyltransferase	1743
53	FBXO22	F-box protein 22	26263
54	PIDD1	p53-induced death domain protein 1	55367
55	GEMIN7	Gem nuclear organelle associated protein 7	79760
56	TNFRSF10B	TNF receptor superfamily member 10b	8795
57	U2AF2	U2 small nuclear RNA auxiliary factor 2	11338
58	UBE2M	Ubiquitin conjugating enzyme E2 M	9040
59	TMED9	Transmembrane p24 trafficking protein 9	54732
60	CTSZ	Cathepsin Z	1522
61	APOBEC3H	Apolipoprotein B mRNA editing enzyme catalytic subunit 3H	164668
62	LAMTOR4	Late endosomal/lysosomal adaptor: MAPK and MTOR activator 4	389541
63	POLH	DNA polymerase eta	5429
64	QTRT1	queuine tRNA-ribosyltransferase catalytic subunit 1	81890
65	PTCHD4	Patched domain containing 4	442213
66	HIRA	histone cell cycle regulator	7290
67	RCC1	Regulator of chromosome condensation 1	1104
68	COMMD4	COMM domain containing 4	54939
69	TRAPPC1	Trafficking protein particle complex 1	58485
70	ARF5	ADP ribosylation factor 5	381
71	BAK1	BCL2 antagonist/killer 1	578
72	RAB35	RAB35: member RAS oncogene family	11021
73	STK16	Serine/threonine kinase 16	8576
74	FTL	Ferritin light chain	2512
75	PIH1D1	PIH1 domain containing 1	55011
76	SHKBP1	SH3KBP1 binding protein 1	92799
77	KDELR1	KDEL endoplasmic reticulum protein retention receptor 1	10945
78	RANGRF	RAN guanine nucleotide release factor	29098
79	TP53	Tumor protein p53	7157
80	PBX2	PBX homeobox 2	5089
81	SDHC	Succinate dehydrogenase complex subunit C	6391
82	MMP14	Matrix metallopeptidase 14	4323
83	DPP3	dipeptidyl peptidase 3	10072
84	TNFRSF10C	TNF receptor superfamily member 10c	8794
85	PPP1CA	Protein phosphatase 1 catalytic subunit alpha	5499
86	PPP2R1A	Protein phosphatase 2 scaffold subunit Aalpha	5518
87	FUS	FUS RNA binding protein	2521
88	CFL1	Cofilin 1	1072
89	TM7SF3	Transmembrane 7 superfamily member 3	51768
90	WDR83	WD repeat domain 83	84292
91	FBXL19	F-box and leucine rich repeat protein 19	54620
92	KPTN	Kaptin: actin binding protein	11133
93	ALYREF	Aly/REF export factor	10189
94	TAX1BP3	Tax1 binding protein 3	30851
95	VASP	Vasodilator stimulated phosphoprotein	7408
96	MRPS12	Mitochondrial ribosomal protein S12	6183
97	LINC02051	Long intergenic non-protein coding RNA 2051	1.08E+08
98	SRSF9	Serine and arginine rich splicing factor 9	8683
99	TWF2	Twinfilin actin binding protein 2	11344
100	DRG2	Developmentally regulated GTP binding protein 2	1819

Table 3. Meta-analysis-derived co-expressed genes with Bcl-2

	Gene	Function	Entrez Gene ID
0	BCL2	BCL2: apoptosis regulator	596
1	BACH2	BTB domain and CNC homolog 2	60468
2	IKZF1	IKAROS family zinc finger 1	10320
3	MDFIC	MyoD family inhibitor domain containing	29969
4	ITPKB	inositol-trisphosphate 3-kinase B	3707
5	KIAA1328	KIAA1328	57536
6	NFATC1	Nuclear factor of activated T cells 1	4772
7	NSD3	Nuclear receptor binding SET domain protein 3	54904
8	KDSR	3-Ketodihydrosphingosine reductase	2531
9	LDLRAD4	Low density lipoprotein receptor class A domain containing 4	753
10	NEMP2	Nuclear envelope integral membrane protein 2	1E+08
11	ANKRD44	Ankyrin repeat domain 44	91526
12	MYB	MYB proto-oncogene: transcription factor	4602
13	RAPGEF6	Rap guanine nucleotide exchange factor 6	51735
14	HNRNPA0	Heterogeneous nuclear ribonucleoprotein A0	10949
15	ZXDA	Zinc finger: X-linked: duplicated A	7789
16	AFF3	AF4/FMR2 family member 3	3899
17	LOC374443	C-type lectin domain family 2 member D pseudogene	374443
18	KIAA1147	KIAA1147	57189
19	TNFRSF13B	TNF receptor superfamily member 13B	23495
20	PTGER4	Prostaglandin E receptor 4	5734
21	BMI1	BMI1 proto-oncogene: polycomb ring finger	648
22	ITPR1	Inositol 1:4:5-trisphosphate receptor type 1	3708
23	LOC100509088	Hypothetical LOC100509088	1.01E+08
24	RHOH	Ras homolog family member H	399
25	KCNQ5	Potassium voltage-gated channel subfamily Q member 5	56479
26	ESR1	Estrogen receptor 1	2099
27	SLC38A1	Solute carrier family 38 member 1	81539
28	ELP2	Elongator acetyltransferase complex subunit 2	55250
29	GPR174	G protein-coupled receptor 174	84636
30	TAF4B	TATA-box binding protein associated factor 4b	6875
31	RCSD1	RCSD domain containing 1	92241
32	SETBP1	SET binding protein 1	26040
33	NOP53	NOP53 ribosome biogenesis factor	29997
34	LINC00341	Long intergenic non-protein coding RNA 341	79686
35	LTA	Lymphotoxin alpha	4049
36	CEP68	Centrosomal protein 68	23177
37	DDHD2	DDHD domain containing 2	23259
38	LRCH1	Leucine rich repeats and calponin homology domain containing 1	23143
39	ZNF24	Zinc finger protein 24	7572
40	TARSL2	Threonyl-tRNA synthetase like 2	123283
41	LRRC8C-DT	LRRC8C divergent transcript	400761
42	PPM1K	Protein phosphatase: Mg2+/Mn2+ dependent 1K	152926
43	SMAD2	SMAD family member 2	4087
44	RGS1	Regulator of G protein signaling 1	5996
45	FMNL3	Formin like 3	91010
46	CD69	CD69 molecule	969
47	C21orf2	Chromosome 21 open reading frame 2	755
48	ZNF407	Zinc finger protein 407	55628
49	GNA13	G protein subunit alpha 13	10672
50	XYLT1	xylosyltransferase 1	64131

Table 3. Continued

	Gene	Function	Entrez Gene ID
51	SP4	Sp4 transcription factor	6671
52	RBBP6	RB binding protein 6: ubiquitin ligase	5930
53	LINC00909	Long intergenic non-protein coding RNA 909	400657
54	IRF4	Interferon regulatory factor 4	3662
55	WDR7	WD repeat domain 7	23335
56	POU6F1	POU class 6 homeobox 1	5463
57	PPP3CC	Protein phosphatase 3 catalytic subunit gamma	5533
58	NTRK2	Neurotrophic receptor tyrosine kinase 2	4915
59	TSHZ1	Teashirt zinc finger homeobox 1	10194
60	PM20D2	Peptidase M20 domain containing 2	135293
61	PRKCE	Protein kinase C epsilon	5581
62	MSI2	Musashi RNA binding protein 2	124540
63	SLC39A6	Solute carrier family 39 member 6	25800
64	RSBN1	Round spermatid basic protein 1	54665
65	ZBTB32	Zinc finger and BTB domain containing 32	27033
66	EPM2A	EPM2A: laforin glucan phosphatase	7957
67	RFTN1	Raftlin: lipid raft linker 1	23180
68	NFATC2	Nuclear factor of activated T cells 2	4773
69	N4BP2L1	NEDD4 binding protein 2 like 1	90634
70	FOXN3	Forkhead box N3	1112
71	LOC107985690	Uncharacterized LOC107985690	1.08E+08
72	ZADH2	Zinc binding alcohol dehydrogenase domain containing 2	284273
73	ARMC5	Armadillo repeat containing 5	79798
74	ANKRD33B	Ankyrin repeat domain 33B	651746
75	LEF1	Lymphoid enhancer binding factor 1	51176
76	PRDM8	PR/SET domain 8	56978
77	STAP1	Signal transducing adaptor family member 1	26228
78	JADE2	Jade family PHD finger 2	23338
79	MIR155HG	MIR155 host gene	114614
80	RABEP1	Rabaptin: RAB GTPase binding effector protein 1	9135
81	P2RY10	P2Y receptor family member 10	27334
82	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	9459
83	RIC8B	RIC8 guanine nucleotide exchange factor B	55188
84	SYNE3	Spectrin repeat containing nuclear envelope family member 3	161176
85	ABCD2	ATP binding cassette subfamily D member 2	225
86	SPNS3	Sphingolipid transporter 3 (putative)	201305
87	FBXL17	F-box and leucine rich repeat protein 17	64839
88	LNPEP	Leucyl and cystinyl aminopeptidase	4012
89	GRASP	General receptor for phosphoinositides 1 associated scaffold protein	160622
90	LINC00938	Long intergenic non-protein coding RNA 938	400027
91	MAST4	Microtubule associated serine/threonine kinase family member 4	375449
92	RNF157	Ring finger protein 157	114804
93	SOCS2	Suppressor of cytokine signaling 2	8835
94	MALT1	MALT1 paracaspase	10892
95	LINC00926	Long intergenic non-protein coding RNA 926	283663
96	CLECL1	C-type lectin like 1	160365
97	CLNK	Cytokine dependent hematopoietic cell linker	116449
98	RASGRP1	RAS guanyl releasing protein 1	10125
99	FCMR	Fc fragment of IgM receptor	9214
100	SDK2	Sidekick cell adhesion molecule 2	54549

Table 4. Meta-analysis-derived co-expressed genes with P53

	Gene	Function	Entrez Gene ID
0	TP53	Tumor protein p53	7157
1	PFN1	Profilin 1	5216
2	BANF1	Barrier to autointegration factor 1	8815
3	YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon	7531
4	CDK4	Cyclin dependent kinase 4	1019
5	THOC6	THO complex 6	79228
6	RAVER1	Ribonucleoprotein: PTB binding 1	125950
7	ALDH16A1	Aldehyde dehydrogenase 16 family member A1	126133
8	APEX1	Apurinic/apyrimidinic endodeoxyribonuclease 1	328
9	MYBBP1A	MYB binding protein 1a	10514
10	SHMT2	Serine hydroxymethyltransferase 2	6472
11	NONO	Non-POU domain containing octamer binding	4841
12	TRIM28	Tripartite motif containing 28	10155
13	SMARCC1	SWI/SNF related: matrix associated: actin dependent regulator of chromatin subfamily c member 1	6599
14	TRAPPC1	Trafficking protein particle complex 1	58485
15	GEMIN4	Gem nuclear organelle associated protein 4	50628
16	CASP2	Caspase 2	835
17	SF3B3	Splicing factor 3b subunit 3	23450
18	DRG2	Developmentally regulated GTP binding protein 2	1819
19	G3BP1	G3BP stress granule assembly factor 1	10146
20	BTBD2	BTB domain containing 2	55643
21	SF3B4	Splicing factor 3b subunit 4	10262
22	PELP1	Proline: glutamate and leucine rich protein 1	27043
23	EIF5A	Eukaryotic translation initiation factor 5A	1984
24	AAAS	Aladin WD repeat nucleoporin	8086
25	HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U like 1	11100
26	RCC2	Regulator of chromosome condensation 2	55920
27	PFAS	Phosphoribosylformylglycinamidine synthase	5198
28	CHTF8	Chromosome transmission fidelity factor 8	54921
29	DVL2	Dishevelled segment polarity protein 2	1856
30	SCAMP4	Secretory carrier membrane protein 4	113178
31	ASB16-AS1	ASB16 antisense RNA 1	339201
32	WDR6	WD repeat domain 6	11180
33	MTA2	Metastasis associated 1 family member 2	9219
34	CAD	Carbamoyl-phosphate synthetase 2: aspartate transcarbamylase: and dihydroorotase	790
35	CHST14	Carbohydrate sulfotransferase 14	113189
36	HNRNPA0	Heterogeneous nuclear ribonucleoprotein A0	10949
37	IMPDH2	Inosine monophosphate dehydrogenase 2	3615
38	SF3A2	Splicing factor 3a subunit 2	8175
39	G6PC3	Glucose-6-phosphatase catalytic subunit 3	92579
40	APEX2	Apurinic/apyrimidinic endodeoxyribonuclease 2	27301
41	APOBEC3C	Apolipoprotein B mRNA editing enzyme catalytic subunit 3C	27350
42	PRPF8	Pre-mRNA processing factor 8	10594
43	DDB2	Damage specific DNA binding protein 2	1643
44	CTDNEP1	CTD nuclear envelope phosphatase 1	23399
45	UCP2	Uncoupling protein 2	7351
46	VARS	Valyl-tRNA synthetase	7407
47	SET	SET nuclear proto-oncogene	6418
48	PATZ1	POZ/BTB and AT hook containing zinc finger 1	23598
49	NOB1	NIN1/PSMD8 binding protein 1 homolog	28987
50	SNRPA	Small nuclear ribonucleoprotein polypeptide A	6626

Table 4. Continued

	Gene	Function	Entrez Gene ID
51	SLC16A13	Solute carrier family 16 member 13	201232
52	MRPS27	Mitochondrial ribosomal protein S27	23107
53	NCOA5	Nuclear receptor coactivator 5	57727
54	RPA1	Replication protein A1	6117
55	TGIF2	TGFB induced factor homeobox 2	60436
56	C17orf49	Chromosome 17 open reading frame 49	124944
57	MAZ	MYC associated zinc finger protein	4150
58	DNAAF5	Dynein axonemal assembly factor 5	54919
59	GART	Phosphoribosylglycinamide formyltransferase: phosphoribosylglycinamide synthetase: phosphoribosylaminoimidazole synthetase	2618
60	C19orf54	Chromosome 19 open reading frame 54	284325
61	ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	471
62	PHF23	PHD finger protein 23	79142
63	CBX5	Chromobox 5	23468
64	FAM86C1	Family with sequence similarity 86 member C1	55199
65	DAXX	Death domain associated protein	1616
66	ELAVL1	ELAV like RNA binding protein 1	1994
67	MTA1	Metastasis associated 1	9112
68	MEN1	Menin 1	4221
69	TUBB	Tubulin beta class I	203068
70	SIGMAR1	Sigma non-opioid intracellular receptor 1	10280
71	FAM86B1	Family with sequence similarity 86 member B1	85002
72	EIF4A1	Eukaryotic translation initiation factor 4A1	1973
73	ALDH1B1	Aldehyde dehydrogenase 1 family member B1	219
74	ELAC2	elaC ribonuclease Z 2	60528
75	PTBP1	Polypyrimidine tract binding protein 1	5725
76	GLOD4	GLyoxalase domain containing 4	51031
77	EXOSC5	Exosome component 5	56915
78	ALDH18A1	Aldehyde dehydrogenase 18 family member A1	5832
79	RPL22L1	RIbosomal protein L22 like 1	200916
80	RFX5	Regulatory factor X5	5993
81	UNG	Uracil DNA glycosylase	7374
82	C1QBP	Complement C1q binding protein	708
83	BAX	BCL2 associated X: apoptosis regulator	581
84	EEFSEC	Eukaryotic elongation factor: selenocysteine-tRNA specific	60678
85	METTL16	Methyltransferase like 16	79066
86	KDELR1	KDEL endoplasmic reticulum protein retention receptor 1	10945
87	ZNF286A	Zinc finger protein 286A	57335
88	APRT	Adenine phosphoribosyltransferase	353
89	SLC35A4	Solute carrier family 35 member A4	113829
90	ZNF740	Zinc finger protein 740	283337
91	PA2G4	Proliferation-associated 2G4	5036
92	PRR3	Proline rich 3	80742
93	ZNF362	Zinc finger protein 362	149076
94	VPS35L	VPS35 endosomal protein sorting factor like	57020
95	CHAMP1	Chromosome alignment maintaining phosphoprotein 1	283489
96	SENP3	SUMO1/sentrin/SMT3 specific peptidase 3	26168
97	GANAB	Glucosidase II alpha subunit	23193
98	UBTF	Upstream binding transcription factor: RNA polymerase I	7343
99	PRKCSH	Protein kinase C substrate 80K-H	5589
100	TSR1	TSR1: ribosome maturation factor	55720

Molecular network underlying hAFMSCs-CM function in MCF-7 cells

Fig. 7 illustrates the molecular network underlying the apoptotic function of hAFMSCs-CM in MCF-7 cells. Supplementary data represents the underlying relations, mined sentences through literature mining, and the reference publications. P53 (TP53), EIF5A, DDB2, BcL2, and Bax are hubs in the network where BcL2 downregulation stands in harmony with the upregulation of P53, EIF5A, DDB2, and Bax, leading to apoptosis activation.

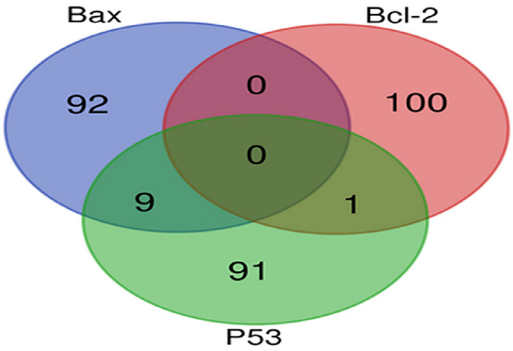
Discussion

Currently, chemotherapy and surgery are the principal approaches in clinical-base breast cancer treatment. However, the side effects of surgery, the toxicity of chemotherapy agents on normal cells, and drug resistance in cancer cells are undeniable post-treatment problems.[2,3] As a result, other types of breast cancer treatments, such as targeted therapies and gene therapy, have become the focus of recent research.[4] Multiple studies have shown that MSCs can fight cancer, which has led researchers to think about using them as a new treatment.[6,7,11,15,53,54] Nonetheless, the MSCs-CM's anti-cancer effects, especially hAFMSCs-CM, on breast cancer apoptosis, have not been clearly understood. TROY, TAIL, and Fas Ligand/TNFSF6 were found in the MSCs-CM made from bone marrow.[55]

The present work has investigated the apoptotic potential effects of hAFMSCs-CM through cellular and molecular approaches. Our data indicated that MCF-7 cell viability declined as a result of hAFMSCs-CM treatment as compared with control cells. Our findings are consistent with studies that have highlighted the promising aspects of human amniotic-derived MSCs' effects on cancer inhibition.[6,56]

Moreover, we have shown that hAFMSCs-CM induces apoptosis in MCF-7 breast cancer cells due to the increase in Bax gene expression and the decrease in Bcl-2 gene expression. Furthermore, based on the protein analysis and compared with the untreated cells, our data revealed that the level of tumor suppressor protein expression, P53, was enhanced in MCF-7 due to the hAFMSCs-CM treatment ($P<0.0001$). There is ample evidence confirming that P53 overexpression in breast cancer downregulates Bcl2 expression, promotes Bax expression, and stimulates Bax function as a result of P53-induced apoptosis.[57-59]

Gholizadeh et al stated that hAFMSCs medium could significantly promote p53 expression in the ovarian cancer cell line ($P<0.05$).[6] Apoptosis can be caused in breast cancer cells by giving them hAFMSCs-CM, and this could lead to more P53 protein in the cells. Consistently, Kalamegam et al. found that CM from Wharton's jelly stem cell had inhibitory effects on an ovarian cancer cell line.[12] Similarly, Serhal et al isolated CM from adipose-derived MSCs and assessed its effect on hepatocellular carcinoma cells. They posited that, after the CM treatment, the apoptosis rate increased due to P53 upregulation and retinoblastoma gene expression. They also highlighted the significant decrease in cell proliferation by dint of hTERE downregulation and c-Myc expression. Likewise, the present study found a noticeable decrease in Bcl-2 mRNA level expression and an increase in Bax mRNA level within the treated cells with hAFMSCs-CM in comparison with the untreated cells ($P<0.005$). Consistent with our findings, in 2020, Rahmatizadeh et al showed that indirect hAFMSCs co-culturing with human cervical



Genes that co-express with *Bax* and *P53*	
TP53 (P53)	tumor protein p53 inducible protein 3
EIF5A	eukaryotic translation initiation factor 5A
DDB2	damage specific DNA binding protein 2
PFN1	profilin 1
KDELR1	KDEL endoplasmic reticulum protein retention receptor 1
TRAPPC1	trafficking protein particle complex 1
BAX	BCL2 associated X: apoptosis regulator
DRG2	developmentally regulated GTP binding protein 2
THOC6	THO complex 6

Genes that co-express with *Bcl-2* and *P53*	
HNRNPA0	heterogeneous nuclear ribonucleoprotein A0

Fig. 6. Shared genes between meta-analysis derived co-expressed profiles of Bax, Bcl-2, and P53.

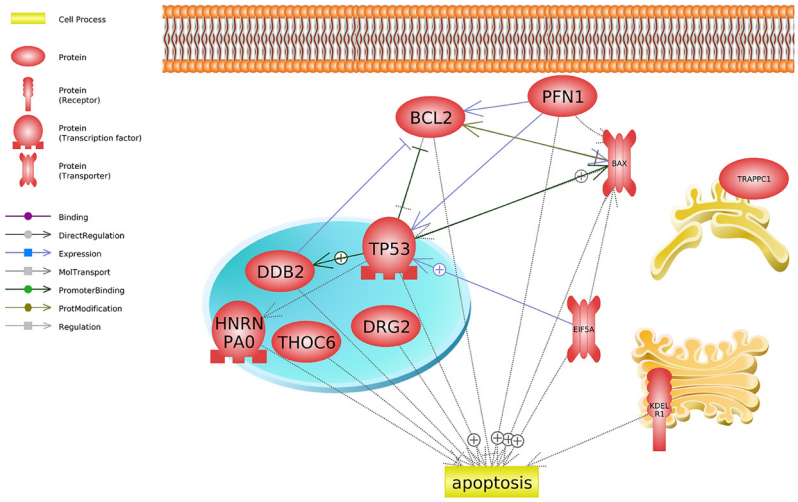


Fig. 7. Molecular network underlying apoptotic function of hAFMSCs function in MCF-7 cells. The positive sign represents the positive/upregulation and the negative sign represents the negative/downregulation interaction.

cancer (HeLa) resulted in an increase in the Bax/Bcl-2 ratio and cells' sensitivity to apoptosis. They also said that the level of p53 mRNA in Hela cells rose a lot after day 5 of co-culture with indirect hAFMSCs, which is when they were mixed with the cells.[60] In 2018, Rodrigues et al reported that P53 is active in human amniotic fluid stem cells.[61] More importantly, we found that P53's protein level increased after the hAFMSCs-CM treatment. According to our findings, it seems that hAFMSCs-CM could interfere with the apoptosis signal pathway associated with P53, inhibiting Bcl-2 expression. Consistent with this study, Jiao et al. demonstrated that hAMCs decreased tumor size significantly ($P < 0.05$) in gliomas by increasing Bax expression and reducing Bcl-2 levels.[62] In addition, Qiao and her colleagues reported that MSCs inhibited hepatoma cancer cell lines by downregulating the levels of Bcl-2, c-Myc, Survivin, PCNA, and β-catenin.[9] Conversely, Farahmand et al showed that bone marrow derived stem cell CM has tumorigenic effects on human breast cancer.[63] In this study, we developed a systems biology analysis approach by integrating the meta-analysis of expression data, using rank correlation and Z standardization, and performing literature mining analysis. The employed systems biology approach led us to an apoptotic-promoting gene interaction network, including P53, EIF5A, DDB2, and Bax, activated by hAFMSCs-CM treatment. More research should be conducted to validate this type of treatment.

Conclusion

The present work revealed that hAFMSCs-CM could promote apoptosis in MCF-7 cells. Our data shown a high level of P53 in MCF-7 cells, but not in normal cells (Hu02). After the treatment, P53 was found competent to downregulate Bcl2 expression and upregulate Bax to induce apoptosis in MCF-7 cells. On the other hand, our data suggest that hAFMSCs-CM has proliferation effects on normal cells but not on p53 expression; thus, we observed a decrease in Bax and an increase in Bcl2 mRNA levels. As per our findings, amniotic fluid-derived stem cells could seemingly target the tumor cells, inhibiting their growth rate by expressing various apoptotic factors. In the end, we suggest that more research be conducted on hAFMSCs' effects on cancer therapy for stem cell CM.

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Research Highlights

What is the current knowledge?

√ The role of MSCs in clinical application is well researched.

√ Stem cells such as hAFMSCs have anticancer effects in some tumors.

√ hAFMSCs-CM ability to downsize tumors should be investigated.

√ Given that the MSCs is the best among different sources, hAFMSCs-CM could target tumor cells and inhibit their growth rate through expressing apoptotic factors.

What is new here?

√ The current study focused on apoptotic effect of the cell-free hAFMSCs-CM on the cancer cells, especially the breast cancer.

√ This study explained the relationship between hAFMSCs-CM and the apoptotic molecules (antitumor).

√ The meta-analysis study illustrated that an apoptotic-promoting gene interaction network, including P53, EIF5A, DDB2, and Bax, can be activated by hAFMSCs-CM treatment.

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Ethical Statement
This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (ID number IR.TUMS.MEDICINE.REC.1398.690).

Competing Interests
The authors declared no conflict of interest.

Authors' Contribution
MP and MPA: Conceptualization & experiments design; RPA & EE: Analyzed the data & Meta-analysis; RPA & MLA: Performed the experiments; RPA: data presentation & draft preparation; RPA & MP & MPA: Writing and reviewing; MPA & MP: Project administration.

Supplementary Materials
Supplementary file 1 contains molecular network relations underlying hAFMSCs function in MCF-7 cells.

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