Reduction in the Viability of Human Cervical Cancer HeLa Cell Line via Indirect Co-culture With Amniotic Fluid-Derived Mesenchymal Stem Cells

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Abstract
Objectives: This experiment was carried out to evaluate the impacts of unmodified human amniotic fluid-derived mesenchymal stromal/stem cells (hAF-MSCs) on the viability of HeLa cells, as well as the impact of these cells on the expression of common pro-apoptotic and pro-survival genes in tumor cells by establishing an indirect co-culture system.
Materials and Methods: To this end, an indirect co-culture system was established, and hAF-MSCs were co-cultured with HeLa cells at a ratio of 1:2 for five days. The cell viability of co-cultured tumor cells was determined after the incubation period. Then, several parameters were examined, including the gene expression of tumor protein 53 (TP53), BCL2-associated X protein (BAX), B-cell lymphoma 2 (BCL-2), and cyclin-dependent kinase inhibitor 1A (CDKN1A). Finally, gene regulatory networks were analyzed as well.
Results: The results of this study confirmed that the co-culture of hAF-MSCs with HeLa cells could decrease the viability of tumor cells. The reduction of HeLa cell viability was accompanied by an increase in BAX, TP53, and CDKN1A while a decrease in BCL-2 gene expression. Eventually, the analysis of the regulatory network revealed that the co-culture of Hela ¬cells with hAF-MSCs activated several transcriptional factors and microRNAs which regulated the expression of these genes.
Conclusions: In general, hAF-MSCs exerted the inhibitive effects on the growth of HeLa cells, along with alterations in the expression of common pro-apoptotic and pro-survival genes in a timely and concentration-dependent manner.
Keywords: Mesenchymal stem cells, HeLa cells, Amniotic fluid, Co-culture, Cell-based therapy

Introduction

Cervical cancer is a group of cancer that originates from the entrance to the uterus, so-called as the uterine cervix. There are several lines of evidence indicating that the rates of incidence and mortality of cervical cancer cases can be controlled and prevented through comprehensive and integrated approaches. In addition, it is the fourth most frequently diagnosed cancer, and the second major cause of malignancy-related mortality among women globally (1,2). Moreover, deaths which occur due to cervical cancer in developing countries continue to be one of the most important causes of all malignancy-related mortalities. The widespread lack of accessibility to cervical cancer screening tests including Papanicolaou's (Pap) smear and human papillomavirus (HPV) testing for women

susceptible to this malignancy is the main cause of cervical cancer-related mortality in these countries (3).

The incidence rate with an estimation of 570 000 women afflicted with this malignancy was recorded worldwide in 2018 representing 7.5% of all female cancers (4). Although the incidence rate of cervical cancer was strikingly low in Iran in 2012, its mortality rate was high. The obtained data from global cancer statistics-GLOBOCAN 2012 presented the estimates of the incidence rate of cervical cancer as 2.8 in 2012 and a death rate of 1.2 per 100 000 per year (5). The incidence and mortality rates varied across different geographic regions of Iran and relied on different factors. The findings showed that the early age of first pregnancy, smoking, early age at marriage, use of oral contraceptive pills, family history, and multiple sexual partners are

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among the major risk factors that are associated with the incidence and death rates of cervical cancer in Iran (6).

Many types of HPV have been detected in many different groups of species, some of which have been exclusively found in human beings (7). However, HPV infections can lead to cervical cancer in some cases (8). It was estimated that 79.1 million people were infected with HPVs (also known as the most prevalent sexually transmitted infection) in 2008. It is noteworthy that oncogenic HPV types have been detected in more than 99% of cases of invasive cervical cancer worldwide (9). This malignancy usually occurs following persistent infections with oncogenic HPV types, especially high-risk HPV (hrHPV) types 16, 18, 31, and 45 (10).

The latest research findings have indicated that the hrHPVs encode two oncoproteins of E6 and E7 which are responsible for the malignant transformation of human cervical epithelial cells (11). In other words, the main stages of the pathogenic mechanism of this malignancy include the binding of HPV E6 and E7 to p53 and retinoblastoma tumor suppressor protein (pRB) subsequently stimulate the proteasomal degradation of p53 and pRB, respectively (12).

Patients suffering from cervical cancer usually have no noticeable manifestations in the early stages of the disease. However, they can undergo medical check-ups at regular intervals for the early diagnosis of cervical cancer.

The choice of standard treatment options for cervical cancer often depends on clinical stages that include traditional surgical excision in the early stages of the disease. Meanwhile, the most common treatment options include radiation therapy and chemotherapy in the advanced stages of cervical cancer. It is noteworthy that 80%-95% of women with early-stage cervical cancer can be cured with chemoradiotherapy and surgery. However, the metastatic and recurrent disease remains the main cause of cancer-related deaths, and patients with metastatic cervical cancer have unfavorable prognosis with the median survival of 8-13 months. In addition, cancer chemotherapeutic drugs are rarely curative and are considered as palliative care options (13,14). The development of new approaches and paradigms in diagnostic, therapeutic, and prognostic strategies should also be taken into consideration which is essential for attaining an upper level of clinical cancer management (15).

Recently, different areas of research have paid particular

attention to the role of two early oncoproteins (i.e., HPV E6 and E7) which are important in transforming the lining cells of the cervix. These oncoproteins are consistently expressed and retained in tumor cells which are necessary for the initial induction and maintenance of the malignant phenotype.

In recent decades, research studies have heavily focused on other molecular targeted drugs, therapeutic vaccines (16), hyperthermia (17), sentinel lymph node biopsy (18), and immunotherapy approaches (19). Although these therapeutic approaches for cervical cancer treatment are not yet approved for clinical use, many studies have focused on these procedures.

Mesenchymal stem cells hold remarkable promise for the application of cancer cell-based therapies. Several recent studies have shown that mesenchymal stromal/stem cells (MSCs) exhibit intrinsic anti-tumor properties against Kaposi's sarcoma (20), glioblastoma (21), Burkitt's lymphoma (22), pancreatic cancer (23), and breast cancer (24). However, the suppressive effects of MSCs on breast cancer still have been incompletely conceptualized (25). The underlying mechanisms responsible for the anti-tumor effects of unmodified MSCs include the induction of apoptosis in tumor cells, inhibition of cell cycle progression, and the inhibition of specific signaling pathways (e.g., PI3K/Akt and Wnt/β-catenin signaling transduction pathways). In addition, other mechanisms such the inhibition of tumor angiogenesis and neovascularization, induction of mesenchymal-to-epithelial transition, suppressive effects on cancer stem cells, a significant impact on immune responses such as the ability to increase inflammatory infiltrations, and the deactivation of the X-chromosome-linked inhibitor of apoptosis protein expression have been observed within tumor cells (20,26,27).

Many findings suggest that important underlying mechanisms responsible for MSC-mediated anti-proliferative effects on Hela cells consist of the inhibition of mitogen-activated protein kinases (MAPKs) and PI3K signaling pathways in tumor cells. The findings have also revealed that the involvement of MAPK-signaling pathways is a turning point for the cellular reaction to p53 activation, apoptosis, and cell cycle arrest (28).

Nowadays, there has been growing attention to human amniotic fluid-derived mesenchymal stromal/stem cells (hAF-MSCs) that can be amenable to cell-based therapies (29,30). The possibility of isolating a large number of these cells, and avoiding moral hazard problems, as well as having multipotent differentiation property, self-renewal ability, and extensive proliferation capacity are the main advantages for clinical use of these cells. In general, these particular characteristics render hAF-MSCs suitable for cell-based therapies (2,31).

Previous research indicated that the direct or indirect co-culture of tumor cells with MSCs or culturing tumor cells with MSC-derived conditioned media can affect

the development of tumor cells (28). Further, evidence suggested that unmodified (intact) MSCs from different sources have the opposite divergent effects on tumor growth and progression. Therefore, the therapeutic effects of hAF-MSCs should be evaluated when faced with different types of cancer.

Accordingly, the main objective of the current investigation was to assess the impacts of hAF-MSCs on the viability of HeLa cells by using an indirect co-culture system with respect to determined cell concentration and incubation period. To the best of our knowledge, no previous study has evaluated this issue. Nonetheless, the effects of this co-culture on common pro-apoptotic and pro-survival genes have been assessed using the quantitative polymerase chain reaction. The regulatory network analysis of miRNAs and transcriptional factors was performed using Pathway Studio software.

Unmodified hAF-MSCs are a promising solution for cell-based cancer therapies and may develop novel anti-cancer strategies against human cervical cancer.

Materials and Methods
Primary Cells, Cell Culture Media, and Supplements
Human Amniotic Fluid-Derived Mesenchymal Stromal/ Stem Cells
In our previous studies, hAF-MSCs were successfully isolated by using the single-step method of isolation (33). The isolated cells were identified and then moved into a liquid nitrogen storage tank until further use. The cryopreserved hAF-MSCs were then thawed, resuspended, and plated in a 25 cm² cell culture flask at a concentration of 1×10^4 cells/cm². Thereafter, 3-5 mL of pre-warmed (37˚C) low glucose Dulbecco's modified Eagle's medium (LDMEM) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), a 10 ng/mL basic fibroblast growth factor (bFGF, Sigma Aldrich), 1% L-glutamine, and a 1% (v/v) penicillin/streptomycin (Pen/Strep) solution were added to the culture medium. The cultured cells were maintained in an incubator in a humidified atmosphere with 5% CO_2 at 37℃. Moreover, the culture medium was replaced twice per week and the cells were left to grow for four to five days until reaching a confluency of 70%. Finally, hAF-MSCs were sub-cultured, and 0.05% (w/v) Trypsin/0.02% (w/v) EDTA (Sigma Aldrich, USA) was used for the detachment of cells. All experiments were performed using hAF-MSCs from passages 4 to 6.

HeLa Cell Line
The human cervical adenocarcinoma cell line HeLa (HeLa cell line, NCBI_Iran, cat# CD115, RRID: CVCL_0030) was prepared from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (IPI-Tehran). The HeLa cells were plated in a cell culture flask with a surface area of 25 cm², and the DMEM culture medium (Sigma Aldrich) supplemented with 5% FBS (Sigma Aldrich) and 1% Pen/Strep (Sigma Aldrich) solution were then added to the

culture. The cells were then placed in a 5% CO_2 incubator at 37˚C.

Human Fibroblast Cell Line
Human fetal foreskin fibroblasts (HFFF2 cell line, NCBI_ Iran, cat# C163, RRID: CBCL_2489) were obtained from the NCBI, IPI-Tehran and then cultivated in DMEM cell culture media supplemented with 10% FBS (Sigma Aldrich) at 37˚C in a 5% CO_2 incubator.

Experimental Design and Setup
In this study, experimental groups composed of HeLa cells. The viability of HeLa cells and common pro-apoptotic and pro-survival gene expression levels were evaluated on the 3rd and 5th day of the co-culture with hAF-MSCs. HeLa cells in the indirect co-culture system at passage four were plated onto a 24-well cell culture plate in triplicate, and 0.6 mL of culture media per well was added in the lower compartment as we explained earlier (32).

For the indirect co-culture between hAF-MSCs and HeLa cells, Transwell® tissue culture plate inserts with a pore size of 0.4 μm (6.5 mm membrane diameter, Corning) were used to evaluate the impacts of hAF-MSCs on HeLa cells. In this regard, an indirect co-culture system was applied to provide a condition in which a microporous membrane with a pore size of 0.4 μm could be used to separate two cell populations so that each cell population had its own culture-specific condition. Therefore, indirect intercellular communication could take place mostly via soluble molecules.

The hAF-MSCs were seeded onto the upper compartment, then 0.01 mL of DMEM complete media was added to the culture. The ratio number of hAF-MSCs vs. HeLa cells was 1:2 according to literature methods. Previous research findings demonstrated that the anti-tumor effects of MSCs rely on their incubation periods and concentrations. Thus, the selection of sample-size was based on the information that was described in the literature (34-36).

In addition, this experimental study also assessed the impacts of stem cells on HeLa cells, meanwhile the ratio number of hAF-MSCs vs. HeLa cells was 1:2 and the duration of the incubation period was three days. All groups were tested in triplicate under the same experimental conditions. The control groups were composed of untreated HeLa cells which were not co-cultured with hAF-MSCs whereas the comparator control groups included fibroblasts that were co-cultured with HeLa cells under the same experimental conditions.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) Cell Viability Assay
As previously explained, the viability of HeLa cells was measured by the MTT-based assay on the third and fifth days of co-culture with hAF-MSCs (36). Briefly, HeLa cells were cultured and then maintained in an incubator at

37°C under 5% CO_2 in a humidified atmosphere. After the incubation period, the upper media were removed and the MTT solution (5 mg/mL, Sigma-Aldrich) was added to the wells. Next, the cells were maintained for four hours in an incubator at 37°C as well. Thereafter, the MTT solution was discarded and Dimethyl sulfoxide (DMSO, Tokyo, Japan) was poured into each respective well in order to dissolve the purple formazan crystals. The absorbance of each respective well was read at a wavelength of 570 nm using a multi-well plate reader.

Quantitative Real-Time (qRT) Reverse Transcription Polymerase Chain Reaction (PCR)

RT-PCR was performed as described before (33, 36). Briefly, the total cellular RNA was extracted using the RNX-Plus solution (CinnaGen, Iran, Tehran cat# RN7713C) according to the manufacturer's recommendations, and assessed via the Nanodrop ND-2000 spectrophotometer in order to confirm the purity of RNA (Thermo Fisher Scientific). The cDNA Synthesis Kit (Fermentas, cat# K1621) was also used to synthesize cDNA from the extracted RNA. It was prepared at the final volume of 20 μL for each sample in a Thermal Cycler (PEQLAB, Erlangen, Germany). Thereafter, the qRT-PCR amplification was carried out using the SYBR Green 1 dye method for validating candidate gene expression (45,46). In this regard, SYBR® Premix Ex TaqTM II, cat#RR820 (Takara, Bio) was used based on the manufacturer's instructions. The qPCR was conducted with the RT-PCR system (MIC, Switzerland with the following parameters: incubation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 95 $^{\circ}$C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds). The experiments were performed based on primer pair sequences as presented in Table 1. The results were normalized to β-actin mRNA expression patterns which were employed as accurate internal control genes. The relative gene expression of candidate genes including TP53, BAX, BCL-2, and CDKN1A was calculated by the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in triplicate.

Regulatory Network Analysis of Up-Regulated Pro-Apoptotic BAX, CDKN1A, and TP53, and Pro-Survival BCL-2 Genes After Co-Culture

Regulatory network analysis was designed based on literature mining using the Pathway Studio tool (Elsevier) as discussed previously (37-40). To this end, the up-regulated pro-apoptotic BAX, CDKN1A, TP53, and pro-survival BCL-2 genes after the co-culture were used as the input, and relationships were retrieved from the literature based on the relationship interaction of regulation, mRNA-effect, and promoter binding.

Statistical Analysis

All numerical (quantitative) data were expressed as the mean ± standard deviation (SD). Statistically significant differences among experimental and non-experimental groups were calculated by the one-way analysis of variance (one-way ANOVA) and the Tukey's HSD test (post-hoc-test when necessary). Eventually, the statistical data analysis was carried out using GraphPad Prism software, version 7.0 (GraphPad Inc., San Diego, California, USA), and a $P < 0.05$ was considered statistically significant.

Results

The Reduction in the Viability of HeLa Cells

The measurement of HeLa cell viability using the MTT assay indicated that HeLa cell viability was obviously attenuated after indirect co-culture with hAF-MSCs at 37°C in a 5% CO_2 incubator for five days (1:2 ratio of hAF-MSCs to HeLa cells) when the results of experimental co-culture groups were compared to those of the control groups. The obtained results were statistically significant ($P<0.05$) (Figure 1).

However, no statistically significant changes were found in the viability of HeLa cells after three days of incubation with hAF-MSCs at a ratio of 1:2 hAF-MSCs/HeLa cells in an indirect co-culture (the results are not presented).

Pluripotency Gene Expression

The mRNA levels of pluripotency genes (i.e., NANOG, OCT4, and C-MYC) in hAF-MSCs after five days of an indirect co-culture between hAF-MSCs and HeLa cells remained elevated at around the same level before co-culture between HeLa cells and hAF-MSCs ($P>0.05$) (Figure 2).

The mRNA Expression Levels of Pro-Apoptotic and Pro-Survival Genes

The indirect co-culture of hAF-MSCs with HeLa cells at a ratio of 1:2 and an incubation period of five days (in a 5% CO_2 incubator at a temperature of 37°C) led to the up-

Table 1. The Primer Pair Sequences Used for Quantitative Real-time Polymerase Chain Reaction and Their Appropriate Annealing Temperatures

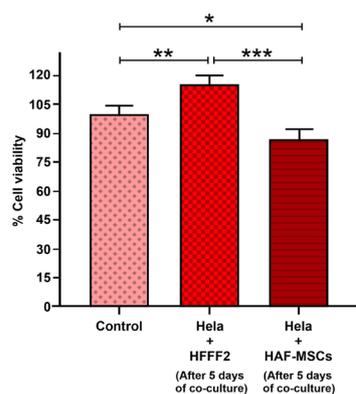| Gene	Primer	Annealing Temperatures (°C)
β-Actin	FWD: 5ꞌ-AAATCTGGCACCACACCTTC REV: 5ꞌ-CCATCTCTTGCTCGAAGTCC	49°C for 45 s
OCT4	FWD: 5ꞌ-AGGTGTTCAGCCAAACGACC REV: 5ꞌ-TGATCGTTTGCCCTTCTGGC	60°C for 30 s
NANOG	FWD: 5ꞌ- ATCCAGCTTGTCCCCAAAG REV: 5ꞌ- ATTTCATTCTGGTTCTGG	60°C for 30 s
BAX	FWD:5ꞌ-TGCTACAAGGTTTCATTTAG REV: 5ꞌ- ATCCACATCAGCAATCATCC	59°C for 30 s
BCL-2	FWD: 5ꞌ- ACTTTGCAGAGATGTCCAGT REV: 5ꞌ- CGGTTCAGGTACTCAGCAT	55°C for 30 s
TP53	FWD: 5ꞌ-TGCTGAGTATCTGGACGACA REV: 5ꞌ- GAGCGTGATGATGGTAAGG	59°C for 30 s
CDKN1A	FWD: 5ꞌ- GCGATGGAACTTCGACTTTGT REV: 5ꞌ- GGTAGAAATCTGTCATGCTGGTC	58°C for 30 s
C-MYC	FWD:5ꞌ-GCTCAAAGCCTAACCTCACAA REV:5ꞌAAAGAAAGAAGATGGGAAGCA	59°C for 30 s

Figure 1. HeLa cell viability in experimental groups were measured by MTT cell viability assay and then compared with the data from the control groups: ˟P- = 0.0163, ˟˟P = 0.0095, ˟˟˟P = 0.0004; hAF-MSCs: Human amniotic fluid-derived mesenchymal stromal/stem cells; HFFF2: Human fetal foreskin fibroblasts.
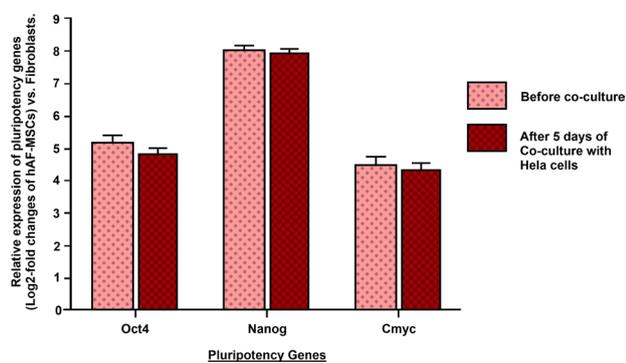

Figure 2. Expression levels of the pluripotency markers of hAF-MSCs before and after indirect co-culture with HeLa cells were compared with the control group (composed of HFFF2). hAF-MSCs: Human amniotic fluid-derived mesenchymal stromal/stem cells; HFFF2: Human fetal foreskin fibroblasts.

regulation of TP53 ($P < 0.0001$), CDKN1A ($P < 0.0001$), and BAX ($P = 0.0001$) in comparison to that of the control groups. In the meantime, the analysis of the basal expression levels of frequent pro-apoptotic genes (i.e., TP53, CDKN1A, and BAX) showed a significant increase in tumor cells while the expression level of the pro-survival BCL-2 gene decreased compared to the control groups ($P = 0.0006$). Figure 3 illustrates the mRNA expression levels of candidate pro-survival BCL-2 and pro-apoptotic CDKN1A, BAX, and TP53 genes in HeLa cells after co-culture with hAF-MSCs.

There were no statistically significant differences among the results of experimental co-culture groups compared to those of the control groups ($P > 0.05$) when hAF-MSCs were indirectly co-cultured with HeLa cells at a ratio of 1:2 after three days of incubation.

An Elevated BAX/BCL-2 Ratio
The indirect co-culture of hAF-MSCs with HeLa cells at 1:2 hAF-MSCs vs. HeLa cells ratio with an incubation period of five days resulted in a significantly higher BAX/

BCL-2 expression ratio ($P < 0.0001$) than that of the control groups (Figure 4).

The Analysis of Regulatory Network
Network analysis showed that co-culture activates a combination of transcription factors and miRNAs that regulate BAX, CDKN1A, TP53, and BCL-2. The regulatory transcription factors were SOX2, Sp1, KLF4, POU4F1, EGR1, TP53, TP73, RELA, NR3C1, TBX2, MYC, BCL6, and E2F1. At the miRNA level, many apoptosis-related candidate miRNAs were MiR181B1, MiR20A, MiR19B1, MiR21, MiR22, MiR221, MiR214, MiR181A1, and metastasis-associated candidates were MALAT1, MiR372, MiR23A, MiRLET7A1, MiR222, and MiR296. Two protein kinases ofMAP2K7 and STK4 were involved in network regulation. The extracted relations are depicted in Figure 5.

Discussion
Our findings indicated that the viability of HeLa cells can be decreased in an indirect co-culture system by using an appropriate proportion of hAF-MSCs to HeLa cells, along with optimal incubation time. After 5 days of incubation at a 1:2 ratio of hAF-MSCs/HeLa cells, there was a remarkable decrease in the HeLa cell viability which was accompanied by the up-regulation of the mRNA expression levels of pro-apoptotic CDKN1A, BAX, and TP53, as well as the down-regulation of pro-survival *BCL-2* genes. Furthermore, these data revealed that the co-culture of hAF-MSCs with HeLa cells could lead to a significant augmentation in the BAX/BCL-2 ratio.

In the current study, the effects of hAF-MSCs on HeLa cells were determined after co-culture between hAF-MSCs and HeLa cells. Nowadays, hAF-MSCs hold great promise as a formidable source of stem cells that is amenable to cell-based therapies (41,42). These cells possess a high replicative potential, the capability to differentiate into all three germ-layers or lineages, and the ability to maintain their stemness and multipotency for long periods of time. In addition, these cells can be readily extracted from the amniotic fluid and can be isolated and purified in large amounts.

Despite the controversy surrounding current diversity in MSC-based regenerative therapy and promising perspectives for the future of this field, there has been an emerging area of research which focuses on the development of new MSC-based therapeutic approaches in order to improve clinical results in patients with cancer (43). It is noteworthy that unmodified MSCs exert bidirectional effects on tumor progression via different mechanisms. It means that these cells exhibit both anti- and pro-tumor activities. Moreover, the findings suggest that several conditions and factors alter the pro- or anti-tumor activities of MSCs. For instance, MSCs exert dual effects on tumor growth in a concentration-dependent manner. Thus, MSCs have anti- and pro-tumor activities
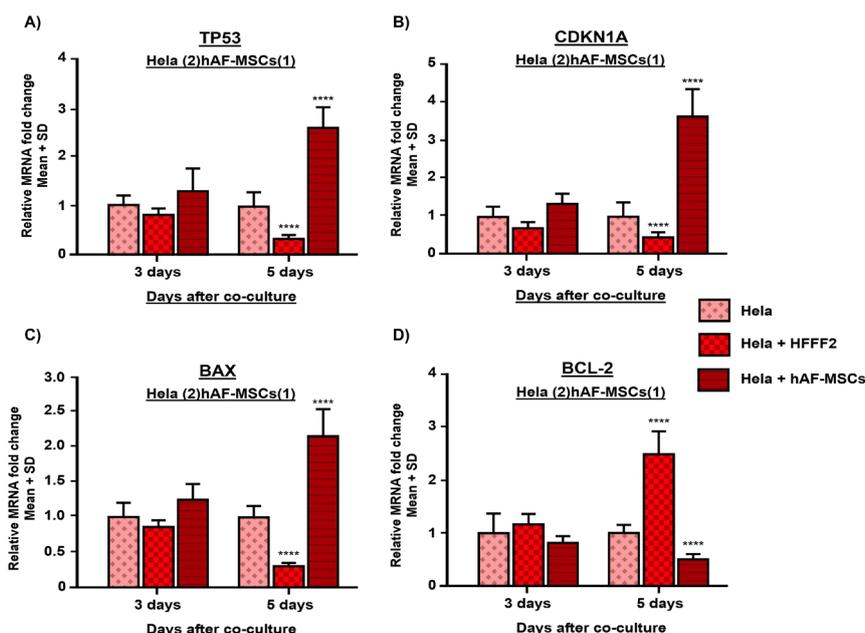
Figure 3. The mRNA expression levels of pro-apoptotic BAX, CDKN1A, and TP53 and pro-survival BCL-2 genes were measured both in experimental and control groups and then were compared against each other. (A) TP53, (B) CDKN1A, (C) BAX, (D) BCL-2. ***P = 0.0001, ****$P < 0.0001$; hAF-MSCs: Human amniotic fluid-derived mesenchymal stromal/stem cells; HFFF2: Human fetal foreskin fibroblasts.

which rely on the ratio of MSCs to tumor cells. Similarly, time-course experiments have demonstrated that MSCs exert time-dependent effects on tumor growth (44). Our experiments were performed in a 1:2 ratio number of hAF-MSCs vs. HeLa cells with an incubation period for five days of co-culture (35). The cells were examined on the third and fifth days of co-culture.

Several empirical studies have highlighted that MSCs can have pro- or anti-tumor effects through cell-cell communications, indirect interactions, and when tumor cells are cultured with mesenchymal stem cell-derived conditioned media (45,46). This study aimed to establish an indirect co-culture model to reveal that hAF-MSCs play a pivotal role in decreasing the viability of HeLa cells.

In addition, the current study determined a significant alteration in pro-apoptotic BAX, CDKN1A, TP53, and

pro-survival BCL-2 gene expression. Indeed, the loss of tumor-suppressing functions of p53 in HeLa cells results in the enhancement of tumor cell proliferation.

There is strong evidence for the existence of a direct causal relationship between persistent HPV infection and cervical cancer pathogenesis in more than 99.7% of all cases (9). Persistent HPV infection is the main, but not the only risk factor for women afflicted with cervical cancer (3). In addition to the HPV infection, there are many other risk factors which are related to this malignancy that can increase the risk of acquiring HPV or persistent high-risk HPV infection, including a weakened immune system, exposure to mutagens, and hormonal factors. Meanwhile, genetic predisposition (genetic susceptibility) is found to play a subordinate role in the progression of cervical cancer. The other well-known and important risk factors which can enhance their susceptibility to cervical cancer include the presence of more than one sexual partner, the early commencement of the sexual activity, association with sexually transmitted infections, cigarette smoking, oral contraceptive use, pregnancy, and the family history, as well as women whose mothers took diethylstilbestrol during pregnancy and people with low socioeconomic status (47,48).

Vaccination against HPV can effectively prevent the infections due to oncogenic HPV types which are responsible for nearly 70% of all HPV-associated genital diseases and cervical cancer among eligible candidates (3).

The hrHPV oncoproteins of E6 and E7 have a decisive role in the natural history of cervical HPV infection and the molecular pathogenesis of cervical cancer. After conjugation with ubiquitin-protein ligase E6-AP, the
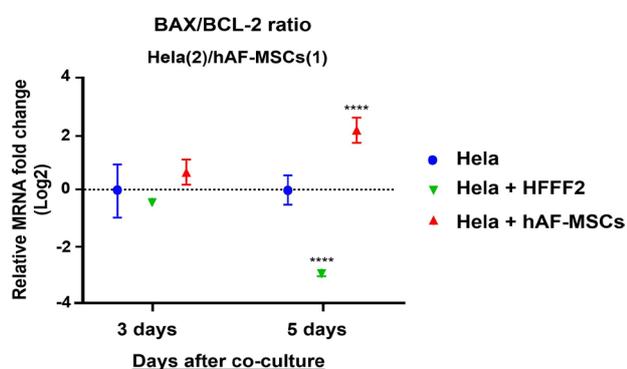

Figure 4. The determination of BAX/BCL-2 ratio in HeLa cells on the third and fifth day in indirect co-culture with hAF-MSCs at a 1:2 ratio of hAF-MSCs/HeLa cells; hAF-MSCs: Human amniotic fluid-derived mesenchymal stromal/stem cells; HFFF2: Human fetal foreskin fibroblasts.
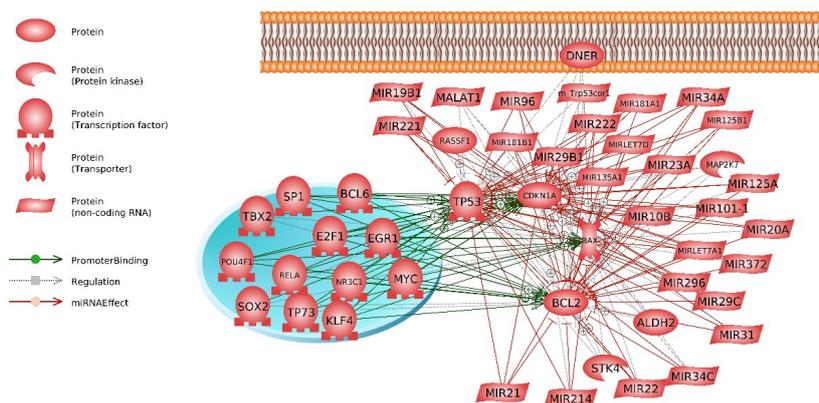
Figure 5. Regulatory network analysis of pro-apoptotic BAX, CDKN1A, TP53, and pro-survival BCL-2 genes after co-culture.

E6 oncoprotein can target p53 for ubiquitination and subsequently its proteasomal degradation whereas the E7 oncoprotein can interact with pRB protein and facilitate its proteasomal degradation (49). Generally, the persistent hrHPV infection of the lining cells of the uterine cervix can alter molecular signature in the affected cells (49, 50).

The increased TP53 expression level in HeLa cells after co-culture with hAF-MSCs led to the restoration of the p53 function. This field of study still requires more in-depth investigations to obtain better results. It was demonstrated that p53 has the ability to transactive downstream target genes by the up-regulation of CDKN1A, MDM2, and cell cycle-regulated gene expression at their transcriptional levels. Several recent studies have shown that many transcriptional targets can be efficiently mediated by p53 activities. Additionally, more than one hundred genes have so far been identified that can be directly targeted by p53 (51,52). Moreover, the expression of the CDKN1A gene is tightly controlled by p53 transactivation activity. Naturally, P21$^{cip1/waf1}$ blocks the G1-to-S phase transition of the cell cycle through the inhibition of D-type cyclin-dependent kinases (i.e., CDK4 and CDK6) and the dephosphorylation of pRB (which is the biologically active form of Rb protein). In addition, p53 tumor-suppressor protein interacts with the pro-survival members of the Bcl-2 family proteins (e.g., Bcl-xL and Bcl-w).

The findings of the present study suggested that the BAX mRNA expression level in HeLa cells was up-regulated after five days of co-culture with hAF-MSCs whereas the BCL-2 gene expression level was down-regulated at the same time point. It is remarkable that the presence of p21 is required for Bax liberation in order to suppress the tumor cell invasion and induction of apoptosis. Indeed, the generation of p53/p21/Bcl-w complex maintains the relationship among all couples such as p21/Bcl-w and p53/p21. In addition, reciprocal interactions between p53 and Bcl-w are required for p53 activity in order to directly bind to BCL-w that finally leads to the liberation of Bax from the complex.

Besides, the Bax/Bcl-2 expression ratio can be used to determine the susceptibility of cells to apoptosis and can actually act as a molecular rheostat. According to this concept, it is more realistic to assume that a high Bax/Bcl-2 ratio is characteristic of the sensitivity of cells to apoptosis. Meanwhile, a decrease in Bax/Bcl-2 ratio is highly positively correlated with the cell resistance to apoptosis. Hence, measuring the expression ratios of pro-apoptotic BAX to anti-apoptotic BCL-2 genes is more crucial for cell fate determination compared to measuring each of them individually (53). Our findings further suggested that the BAX mRNA expression level was markedly up-regulated while simultaneously the BCL-2 gene expression was down-regulated in HeLa cells after five days of indirect co-culture with hAF-MSCs.

Regulatory network analysis demonstrated the involvement of key apoptotic transcription factors, non-coding RNAs, and protein kinases in the regulation of the upregulated genes after co-culture.

The obtained data showed that the clinical use of hAF-MSCs might be a promising choice for improving the treatment of cervical cancer. However, a great effort has been devoted to identify a strategy for controlling the underlying mechanisms which are responsible for the divergent and dual effects of hAF-MSCs on the growth and progression of tumors in order to optimize the outcomes of cell-based therapies.

One suggestion for future research directions in this field of study is the identification of conditions and factors which have an impact on hAF-MSCs to exert dual effects on the growth and development of tumors. In addition, the polarization of hAF-MSCs via toll-like receptors which augment their anti-tumor effects and can generate the anti-tumor phenotypes such as MSC1, and the conflicting role of hAF-MSCs in different molecular pathways which could enhance the tumor-suppressive effects of these cells can be considered as the other suggestions in this regard.

Conclusions

In the present study, we implemented the Guiding Principles underlying previous studies which focused

on the interactions between MSCs and tumor cells. It was experimentally demonstrated that hAF-MSCs can suppress the HeLa cell growth in an indirect co-culture system which was accompanied by alterations in the mRNA expression levels of common pro-apoptotic and pro-survival genes by applying previous experimental outcomes.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This empirical research was approved by the Institutional Review Board and the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, IRAN (Ethical Code: 5/Dal /8713).

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