Research Article

IFNα-Expressing Amniotic Fluid-Derived Mesenchymal Stem Cells Migrate to and Suppress HeLa Cell-Derived Tumors in a Mouse Model

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Background. Immunotherapy for cervical cancer with type I interferon (IFN) is limited because of the cytotoxicity that accompanies the high doses that are administered. In this study, we investigated the utilization of amniotic fluid-derived mesenchymal stem cells (AF-MSCs) as a means for delivering IFNα to local tumor sites for the suppression of cervical cancer in a mouse model using HeLa cell xenografts. Methods. The tumor tropism ability of AF-MSCs and AF-MSCs genetically modified to overexpress IFNα (IFNα-AF-MSCs) was examined through Transwell in vitro and through fluorescent images and immunohistochemistry in a mouse model. Tumor size and tumor apoptosis were observed to evaluate the efficacy of the targeting therapy. Mechanistically, tumor cell apoptosis was detected by cytometry and TUNEL, and oncogenic proteins c-Myc, p53, and Bcl-2 as well as microvessel density were detected by immunohistochemistry. Results. In this model, intravenously injected AF-MSCs selectively migrated to the tumor sites, participated in tumor construction, and promoted tumor growth. After being genetically modified to overexpress IFNα, the IFNα-AF-MSCs maintained their tumor tropism but could significantly suppress tumor growth. The restrictive efficacy of IFNα-AF-MSCs was associated with the suppression of angiogenesis, inhibition of tumor cell proliferation, and induction of apoptosis in tumor cells. Neither AF-MSCs nor IFNα-AF-MSCs trigger tumor formation. Conclusions. IFNα-AF-MSC-based therapy is feasible and shows potential for treating cervical cancer, suggesting that AF-MSCs may be promising vehicles for delivering targeted anticancer therapy.

1. Introduction

Cervical cancer, a malignant tumor of the female reproductive system, is a leading cause of mortality and a significant hazard to women’s health worldwide. Although cervical cancer can be effectively treated by cystectomy in the early stages, the treatment of advanced cancer appears to be less efficient resulting in high morbidity rates. New molecular-based therapy has been developing rapidly [1]. Because human papillomavirus (HPV) infection is a known cause of cervical cancer [2], IFNα, an antiviral as well as antitumor cytokine, has been widely applied in clinical therapy of cervical cancer [3, 4]. IFNα is believed to not only be immunomodulatory but to also be able to suppress tumor growth by inducing apoptosis [5]. Nevertheless, due to the high dose of systemically administered IFNα, the side effect of cell toxicity is inevitable, and the short half-life of this protein also hinders it from reaching the desired concentration at tumor sites [6]. Currently, stem cells are being explored as a promising vehicle candidate for targeted therapy.

Mesenchymal stem cells (MSCs), a cell population that can be found in a variety of tissues, are capable of self-renewal and can be easily isolated and cultured because they can adhere to plastic [7, 8]. They are not only of low immunogenicity and oncogenicity, but they can also selectively migrate to tumor sites [2, 3, 9, 10]. There have been several reports on the use of MSCs for effective cytotherapy in diverse tumor models [11–15]. One study demonstrated that

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bone marrow (BM)-MSCs modified to express IFNβ could also be specifically recruited to the tumor site and resulted in tumor suppression [12]. Additionally, Rachakatla et al. showed that in a model of breast carcinoma, MSCs isolated from the umbilical cord matrix (UCMS cells) exhibited similar specific migration to the tumor and that the engineered cells secreting IFNβ significantly reduced the tumor burden [16]. Although studies on the antitumor effects of MSCs from BM and other sources have been widely reported, few have focused on amniotic fluid-derived MSCs (AF-MSCs). AF-MSCs can be acquired through amniocentesis, which is less invasive and safer than bone marrow puncture. AF-MSCs have similar characteristics to human BM-MSCs but are less differentiated [17]. Due to their advantageous properties, including stable characteristics, nontumorigenicity, and low immunogenicity [18], AF-MSCs are emerging as a new candidate in regenerative medicine and anticancer therapy [17, 19]. Most existing studies on AF-MSCs are associated with their application in the field of regenerative medicine, especially in tissue repair in acute injury models [20–23]. Importantly, these studies take advantage of the innate ability of MSCs to migrate to inflammatory signaling sites. Therefore, researchers have deduced that AF-MSCs should be able to engraft to tumor sites, regardless of tissue origin, as they do in injury models, and serve as delivery vehicles for antitumor molecules [3, 13, 24, 25].

Based on the aforementioned studies of MSCs in antitumor applications, in this study, we performed investigations of the ability of AF-MSCs to migrate to cervical cancer cells in vitro and in vivo. In addition, we explored the efficacy of AF-MSCs, especially those engineered to express IFNa in the treatment of cervical cancer.

2. Methods

2.1. Isolation and Characterization of AF-MSCs. Human AF-MSCs were isolated from the amniotic fluid in the second trimester of gestation. All clinical investigations were conducted according to the principles expressed in the Helsinki Declaration. The amniotic fluid samples (5–15 ml) were aspirated via amniocentesis under ultrasonographic control with informed consent. Each sample was centrifuged at 1000 rpm for 10 min, and the cell pellet was resuspended in α-Modified Eagle’s Medium (α-MEM) (Hyclone, USA) containing L-glutamine supplemented with 15% fetal bovine serum (FBS, Gibco, USA) and a 1% penicillin-streptomycin mixture in 6-well plates and incubated at 37°C in a 5% humidified CO2 chamber. After 7 days, nonadherent cells were removed and the medium was discarded. The adherent cells were cultured in fresh medium until clones appeared. Subsequently, the medium was changed every 3 days, and the adherent cells were trypsinized and subcultured whenever they reached 80%–90% confluence.

To identify these cells as AF-MSCs, several cell surface markers specific for this type of stem cell (CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR) were assessed by flow cytometry. Furthermore, the capacity of AF-MSCs to differentiate into osteocytes was evaluated. AF-MSCs were seeded at 1 × 10^5/cm^2 in 6-well plates and cultured in an osteogenic medium (α-MEM supplemented with 10% FBS, 0.1 mmol/l dexamethasone, 10 mmol/l glyceral phosphate, and 50 mmol/l ascorbate). The medium was changed every 3 days, and the cells were stained with Alizarin red on day 21 to assess the presence of calcifying nodules.

2.2. HeLa Cells. HeLa cells, originally from a human cervical cancer, were a gift from Dr. Zheng (Department of Gynecology, First Affiliated Hospital of Harbin Medical University). The cells were cultured in 1640 medium (Hyclone, USA) supplemented with 10% FBS and a 1% penicillin-streptomycin mixture.

2.3. Preparation of IFNa-Overexpressing AF-MSCs. IFNa cDNA was reverse-transcribed and amplified from mRNA extracted from the peripheral blood mononuclear cells (PBMCs) obtained from Chinese volunteers. The HIV-1-based lentiviral transfer plasmid, pTY-CMV-eGFP, contains the enhanced green fluorescent protein (eGFP) reporter gene that is driven by the CMV promoter and was provided by Dr. C. Li (Southern Medical University, China). To express IFNa using this lentiviral vector, the eGFP gene was replaced with IFNa cDNA, and the resultant transfer plasmid was named pTY-CMV-IFNa. The transfer plasmid, pTY-CMV-IFNa, and the packaging plasmids, psPAX2 and pMD2.G (Addgene, USA), were used to transfect 293T cells with liposome 2000 (Invitrogen, USA) to produce VSV-G pseudotyped, IFNa-producing lentivirus. The transfected cells were incubated for 48 h to allow the production of recombinant virus. The recombinant viruses were harvested, titrated, and then used to infect AF-MSCs at a multiplicity of infection (moi) of 0.75 pg/ml (defined as the activity of viral reverse transcriptase) for 48 h. The retrovirus-infected, IFNa-producing cells (IFNa-AF-MSCs) were selected due to their resistance to puromycin. For further identification, IFNa-AF-MSCs were also subjected to stem cell surface marker test (CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR) by flow cytometry. The levels of IFNa expressed by IFNa-AF-MSCs and MSCs were detected by ELISA (Pbl Biomedical Labs, USA).

2.4. AF-MSCs Labeled with CM-DIL. To evaluate the tropism of MSCs for cancer cells in vivo, AF-MSCs and IFNa-AF-MSCs were also subjected to stem cell surface marker test (CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR) by flow cytometry. The levels of IFNa expressed by IFNa-AF-MSCs and MSCs were detected by ELISA (Pbl Biomedical Labs, USA).

2.5. In Vitro Transwell Migration Assay. To determine the tropism of MSCs for cancer cells in vivo, AF-MSCs and IFNa-AF-MSCs were labeled with a fluorescent cell surface marker, CM-Dil (Invitrogen), by incubation with a working solution of 5 μg/ml CM-Dil for 5 min at room temperature, followed by a 20-minute incubation at 4°C. After labeling, the cells were washed with PBS and cultivated in fresh medium prior to injection into mice, which occurred within 24 h.

2.6. In Vivo Migration Assay. To further evaluate the potential of MSCs to migrate to neoplastic tissues, the transwell migration assay was performed as previously described [13]. HeLa cells were cultured for 24 h in 1640 medium containing 10% FBS; after being trypsinized, HeLa cells were resuspended and plated at 1 × 10^5/2 ml in the lower wells of Transwell chambers and allowed to adhere for 24 h. After being cultured in α-MEM (0.5% FBS) for 24 h, AF-MSCs and IFNa-AF-MSCs were placed in the upper well of chambers with 8 μm pores (Corning Costar,
USA) at a density of $1 \times 10^5/800 \mu l$. HSF, cultured in DMEM + 10% FBS previously, were used as control. These chambers were incubated at 37°C for 24 h to allow the MSCs to migrate. Then, the cells that had not migrated were removed from the upper chamber with a wet cotton swab, and the cells that had migrated were fixed and stained with trypan blue. Images of the stained cells that had migrated to the bottom of the Transwell chamber through the upper membrane were obtained using a Leica DMI4000B inverted phase-contrast microscope (Leica, Germany). The number of cells in 4 high-power fields ($\times 200$) per membrane was counted manually.

2.6. Analysis of Apoptotic HeLa Cells in the Presence of IFNa- AF-MSCs In Vitro. IFNa produced by IFNa-AF-MSCs was measured by ELISA in CM from P1-P5 IFNa-AF-MSCs. The CM was collected 48 hours post transduction from three random wells.

MSCs and IFNa-AF-MSCs from passages 5, 10, 15, and 20 were harvested and resuspended in a-MEM containing 10% (vol/vol) FBS at a concentration of $1 \times 10^4$ cells/ml, plated into 24-well plates at 5000 cells (500 $\mu l$) per well, and incubated 24 hours in a humidified 37°C incubator under 5% CO$_2$ to allow the cells to adhere to the plates. The cells from three random wells were counted for 8 days, and the mean values were used to plot cells.

The AF-MSCs and IFNa-AF-MSCs were cocultured with HeLa cells to investigate the cytotoxic effect of MSCs on the cervical cancer cells. HeLa cells were plated on the lower well of 12-well Transwell plates at $5 \times 10^5$ cells/ml in 1640 (10% FBS), while the MSCs were plated on the upper wells (0.4 $\mu m$ porous inserts) in a-MEM (10% FBS), and then cultured for 24 h. Then, the upper wells with AF-MSCs or IFNa-AF-MSCs were inserted into the bottom well containing HeLa cells, and the two layers of cells were cocultured in Transwell chambers for 72 h. HeLa cells cultured without MSCs were used as the negative control. HeLa cells were then trypsinized and stained with a FITC-labeled Annexin V Apoptosis Detection Kit II (BD, USA) according to the manufacturer's instructions before performing flow cytometry using a BD FACSAria flow cytometer.

2.7. Animal Subjects. Female nude mice (Balb/c nude) were purchased from the Shanghai Slac Laboratory Animal Co. Ltd., and they were housed in the Animal Center of Harbin Veterinary Research Institute under specific pathogen free (SPF) conditions. The mice were held for 5 days after arrival to allow them to acclimatize before the experiments were carried out. All animal and human studies were performed in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the Scientific Research Office of the First Affiliated Hospital of Harbin Medical University.

2.8. In Vivo Migration Assay. The eGFP-HeLa cells were injected subcutaneously at a concentration of $1 \times 10^7$/ml into the backs of 6-week-old female Balb/c nude mice (day 0). Ten days later, when tumors were palpable, mice were given three doses of $5 \times 10^5$ cells/ml CM-Dil-dyed AF-MSCs ($n = 3$) or IFNa-AF-MSCs ($n = 3$) every 5 days and were sacrificed 4 days after the last injection of MSCs. For the tracking of fluorescent signals, tumors and organs (liver, lung, spleen, and kidney) were collected and made into cryosections and paraffin sections. The fluorescent images in cryosections were obtained via laser confocal microscopy (Leica, Germany).

Another set of mice ($n = 12$) was given AF-MSC intravenously (i.v.), and three mice from each group were sacrificed on day 1, day 3, day 7, and day 13. Tumors were collected to analyse the distribution of AF-MSCs in tumors with time. Immunohistochemistry (IHC) with an antihuman CD90 antibody was performed to track the MSCs in paraffin sections.

2.9. Tumor Analysis. For the establishment of tumors, 200 $\mu l$ of HeLa cell suspension (suspended in saline) was administered subcutaneously at a concentration of $1 \times 10^7$/ml into the backs of 6-week-old female Balb/c nude mice. After 10 days, when the tumors were palpable, $1 \times 10^6$ AF-MSCs ($n = 12$) or IFNa-AF-MSCs ($n = 15$) were administered i.v. into the tail vein at a volume of 200 $\mu l$. The MSC injections were performed three times at 7-day intervals. Tumor-bearing mice administered with saline were designated as controls ($n = 10$). The tumors in all living mice were measured by calipers throughout the observation period.

One week after the last injection, randomly selected mice in each group ($n = 3$ for the control group and $n = 5$ for the AF-MSC and IFNa-AF-MSC groups) were sacrificed, and the tumors were collected. Secreted IFNa from IFNa-AF-MSC in tumors sites and other organs was tested by immunohistochemistry. To explore the degree of apoptosis in vivo, TUNEL staining was performed using the in situ Cell Death Detection Kit POD (Roche, USA) according to the manufacturer's instructions. TUNEL-positive cells were counted in 10 randomly selected fields at $\times 400$ magnification in each tumor. Furthermore, IHC studies for the oncogenic proteins c-Myc, p53, and Bcl-2 were used to determine their expression. The expression levels of these apoptotic proteins were presented as the mean density by Image-Pro Plus (IPP 6.0).

In addition, microvessel density (MVD) was also evaluated in these tumors. MVD was assessed by staining with an antibody against the CD34 antigen (Dako, Denmark), and positivity was determined by light microscopy using the counting method introduced by Weidner et al. [26]. Briefly, the areas in the slides containing the maximum number of micro-blood vessels were chosen at low-power fields; individual microvessels were counted at $\times 200$ magnification. Both isolated immunoreactive endothelial cells and luminal microvascular structures were considered as countable vessels.

2.10. Immunohistochemistry. The samples were fixed in 10% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at $4 \mu m$. The sections were deparaffinized in dimethyl benzene for 30 min, 100% alcohol for 2 min, hydrogen peroxide for 10 min, and alcohol for 1 min each and washed. The slides were immersed in the corresponding retrieval solution and heated. After cooling, the sections were
stained with the corresponding primary antibodies for 1 h at room temperature (anti-human CD90 (Biovworld, USA, 1:200), anti-human IFNα (Biosis, China, 1:300), anti-c-Myc (Ebioscience, China, 1:200), anti-P53 (Ebioscience, 1:300), anti-Bcl-2 (Abcam, Hong Kong, 1:200), and anti-CD34 (Dako, Denmark, 1:250)), rinsed in running tap water, stained with secondary antibody-1 (GBI, USA) for 20 min and secondary antibody-2 for 30 min, washed and developed with DAB, and counterstained with hematoxylin, hydrochloric acid, and ammonium hydroxide.

2.11. Evaluation of AF-MSC Tumorigenicity. Female Balb/c nude mice that were 4 weeks old were randomly assigned to experimental groups. AF-MSCs isolated from one single nude mice that were 4 weeks old were randomly assigned

2.12. Statistical Analysis. Differences between groups were evaluated using the parametric or nonparametric Student’s t-test or one-way analysis of variance. All reported P values were considered statistically significant at <0.05.

3. Results

3.1. AF-MSCs Were Isolated and Prepared for IFNα Overexpression. Human AF-MSCs were isolated and cultured as described in Materials and Methods section, and the first clone of adherent cells appeared 7 days after the initial plating. The cells appeared round, spindle shaped, or polygonal in the primary culture and gradually changed to a typical fibroblast-like spindle shaped with increasing passages. The cells were observed in a swirling or radial arrangement after being cultivated for 14 days, which was similar to the morphology of MSCs reported from other sources [7, 27]. To define the MSCs, specific markers of this stem cell were examined by flow cytometry. These cells were positive for CD90, CD105, CD73, and HLA-ABC and negative for CD34, CD14, CD45, and HLA-DR (Figure S1a). In addition, these cells differentiated into chondrogenic lineages, as previously reported [28] (Figure S1b). These results indicated that AF-MSCs were successfully isolated and cultivated. To develop AF-MSCs that consistently overexpressed IFNα, these cells were infected with an HIV-1-based pseudovirus that contained the human IFNα cDNA. Approximately 11,000 pg/ml IFNα was produced by 1×10⁶ IFN-MSCs 24 hours postinfection (hpi), as determined by assaying the culture medium using an IFNα detection ELISA kit. What is more, the stem cell marker (CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR) expression of IFNα-AF-MSCs MSC-alpha is actually consistent with the MSC null ones, which proved the cell stability. These IFNα-producing MSCs (IFNα-AF-MSCs) were used for the subsequent in vitro and mouse experiments.

3.2. AF-MSCs Migrated toward HeLa Cells In Vitro. The tropism of AF-MSCs toward cancer cells was evaluated by examining the migration of AF-MSCs to HeLa cells, a cervical cancer cell line, using an in vitro Transwell system. As demonstrated in Figure 1, these stem cells exhibited significantly higher tropism to HeLa cells compared with HSF cells’ migration toward HeLa (140-fold increase, P < 0.01). Similarly, the MSCs infected with IFNα-expressing lentivirus (IFNα-AF-MSCs) showed a great tropism to the HeLa cells. No significant differences in the migration of AF-MSCs and IFNα-AF-MSCs were observed.

3.3. Transplanted AF-MSCs Migrated toward Tumor Sites In Vivo. Immunohistochemistry result of CD90 tracking (Figure 2(a)) showed that on day 1 after the administration of AF-MSCs, the AF-MSCs were mostly distributed throughout the periphery of the tumors and could hardly be found in the deep tumor regions. On day 3, some CD-90-positive cells began to appear inside the tumor, especially in the stromal regions consisting of nonmalignant tumor cells. On day 7 and day 13, more AF-MSCs were found to have infiltrated the tumor. Furthermore, besides the periphery and stromal region, many AF-MSCs were observed around areas of necrosis. A few AF-MSCs were identified in the spleen and perivessel parts of the liver throughout the study. No CD90-positive cells were found in negative controls. Cryosections showed that there were red fluorescence in edges and inside of tumor mass, AF-MSCs (Figure 2(c)) and IFNα-AF-MSCs (Figure 2(d)) were mostly observed in the connective tissue regions of the tumors, there was also a little red fluorescence in the spleen and liver, but no fluorescent signal can be seen in the kidney or lung tissues (Figure 2(b)).

3.4. IFNα-AF-MSCs Induced Apoptosis of Cocultured HeLa Cells. In CM of IFNα-AF-MSCs from P1-P5, constant IFNα expression was monitored by ELISA test. Levels of expressing IFNα were stable from P1-P5 IFNα-AF-MSCs (11490.5 ± 107.5 to 11,422 ± 68.5 pg/ml, P > 0.05, Figure 3(a)) while hardly any IFNα expressed was tested in CM of AF-MSCs (911.5 pg/ml, P < 0.01, Figure 3(a)).

The growth curves of both AF-MSCs and IFNα-AF-MSCs were roughly shaped “S” (Figure 3(b)), and no significant decrease in MSC proliferation was observed in the presence of IFNα production. The cells stayed in detection period after passing for 2 days, then turned to logarithmic phase, and no longer increased seven days later. The proliferation of cells in the tenth generation was slower than in the fifth generation.

To assess the potential therapeutic effect of IFNα-AF-MSCs on the inhibition of tumor cell proliferation, the IFNα-overexpressing AF-MSCs were cocultured with HeLa cells in a Transwell system, and the apoptotic population of the tumor cells was detected by flow cytometry via annexin/propidium iodide (PI) staining. The results presented in
Figure 3(c) indicated that the coculture with IFNα-AF-MSCs caused a large increase in early (annexin V+/PI−) and late (annexin V+/PI+) apoptoses in HeLa cells. The total percentage of apoptotic (early and late apoptoses) HeLa cells increased from 3.45 ± 1.44% to 84.57 ± 1.67% in the presence of IFNα-AF-MSCs (P < 0.01) (Figure 3(c)). In contrast, the coculture of HeLa cells with AF-MSCs induced much lower apoptosis in the cervical cancer cells (8.12 ± 0.73%), and this apoptotic rate was still significantly higher than that observed in HeLa cells that were cultured alone (P < 0.05).

3.5. Engraftment of IFNα-AF-MSCs Significantly Reduced the Size of HeLa Xenografts in Mice. To explore the efficacy of the cell-based therapy of IFNα-AF-MSCs, AF-MSCs (n = 12) or IFNα-AF-MSCs (n = 15) were administered intravenously (i.v.) into the tail veins of tumor-bearing mice. Tumor-bearing mice that received no MSCs were used as controls. An i.v. injection of 1 × 10⁶ MSCs was administered weekly for 3 rounds, and the mice (n = 3 in the control group, n = 5 in other two groups) were sacrificed 7 days after the last injection of MSCs for tumor collection and tumor apoptosis analysis. Tumor size was measured throughout the experimental period.

As shown in Figure 4, before the administration of MSCs, there was no significant difference in the size of tumors obtained from the mice from the four groups. After 3 injections of MSCs separated by intervals of 7 days, the tumor sizes of the mice that received IFNα-AF-MSCs started to show significant difference compared with those of the control group (P < 0.05) since day 20. The tumor sizes were
Figure 2: Tropism of administered AF-MSCs for tumors in a mouse model. (a) Immunohistochemistry (IHC) showing CD90 expression in AF-MSCs. The tumors were collected and examined at day 1, day 3, and day 7 after the last injection. Untreated tumor stained for CD90 is used as a negative control. Bars, 25 or 50 μm. (b) IHC of AF-MSCs in the liver and spleen. Very few MSCs were observed. Bar, 25 μm. (c, d) Observation of CM-Dil-labeled AF-MSCs and IFNα-AF-MSCs (in red) in the eGFP-HeLa (in green) tumors by fluorescence microscopy. Bars, 10, 25, or 50 μm. HeLa cells that expressed eGFP (eGFP-HeLa) were injected subcutaneously into the backs of nude mice (n = 3) at 1 × 10^7/ml/mouse. AF-MSCs and IFNα-AF-MSCs were labeled with the red fluorescent dye CM-Dil, and 10 days following the initial inoculation of HeLa cells, 5 × 10^6/ml of either MSCs, or IFNα-AF-MSCs were injected into the tail vein of the tumor-bearing mice every 5 days. IHC and fluorescence microscopy were used to observe the infiltration of AF-MSCs.
Figure 3: Induction of apoptosis of HeLa cells by IFNα-AF-MSCs in vitro. (a) Concentration of secreted IFNα in CM of IFNα-AF-MSCs of consecutive passage. IFN-α produced by IFNα-AF-MSCs and AF-MSCs was measured by ELISA in CM from P1-P5. The CM was collected 48 hours post transduction from three random wells. (b) Biological activity of the secreted IFNα on IFNα-AF-MSC proliferation. MSCs and IFNα-AF-MSCs from passages 5, 10, 15, and 20 were plated into 24-well plates at 5000 cells (500 μl) per well, to allow the cells to adhere to the plates. The cells from three random wells were counted for 8 days, and the mean values were used to assess cell proliferation. (c) Induction of apoptosis of HeLa cells for coculture with IFNα-AF-MSCs. AF-MSCs or IFNα-AF-MSCs were cocultured with HeLa cells in 12-well Transwell plates for 72 h. HeLa cells cultured alone were the negative control. After coculture, the apoptotic populations of HeLa cells were evaluated via FITC-labeled annexin V/PI staining flow cytometry. Apoptotic populations, including the early (annexin V+/PI−) and late (annexin V+/PI+) apoptotic cells, were assessed in the HeLa cells cocultured with AF-MSCs and IFNα-AF-MSCs. The data represent three independent experiments. Error bars, standard deviation *P < 0.05, **P < 0.01.
measured on day 32 (7 days after the last injection) as shown in Figure 4(b), and the tumors from the IFNα-AF-MSC group (n = 15) were 434.99 ± 165.79 mm³ significantly smaller than those from the untreated group (653.89 ± 227.84 mm³) (P < 0.05). In addition, the difference in tumor size between the mice treated with IFNα-AF-MSCs and the untreated mice was significant when taken totally through the observation period of 52 days (Figure 4(a)).

3.6. AF-MSCs Enhanced the Size of HeLa-Derived Tumors in Mice. In contrast to the inhibitory effect of IFNα-AF-MSCs on the growth of HeLa xenografts in mice, the untransfected
AF-MSCs significantly improved tumor growth. For the whole observation, the average tumor size in the AF-MSC group (364.72 ± 272.51) was significantly greater than that in the IFNα-AF-MSC (260.93 ± 189.12, P < 0.05). In particular, when the growth rate of tumors in the control group decreased beginning on day 26, the tumors in the AF-MSC-injected mice continued increasing in size and became significantly larger than those in the control mice.

3.7. Secreted IFNa Was Concentrated in Tumor Sites in IFNα-AF-MSC-Treated Model Mice. To confirm that the therapeutic effect of IFNα-AF-MSCs was the result of IFNα concentration in local tumor, secreted IFNα was detected by immunohistochemistry in tumor tissue, liver, spleen, kidney, and lung of tumor-bearing mice. Tissues were collected on day 7 after the last injection of IFNα-AF-MSCs (n = 5) or AF-MSCs (n = 5). In mice receiving IFNα-AF-MSCs, a large amount of secreted IFNα were found in tumor sites, especially in perivessel and stromal region (Figure 4(d)). A little IFNα was detected in the liver, and no IFNα was found in the spleen, kidney, or lung (Figure 4(d)). What is more, no IFNα was found in mice receiving AF-MSCs, neither in tumors, nor in organs (Figure 4(d)).

3.8. IFNα-AF-MSCs and AF-MSCs Enhanced Cell Apoptosis of Tumors in Model Mice. To explore the mechanism of the inhibitory effect of IFNα-AF-MSCs on the tumor growth of HeLa xenografts, the TUNEL assay for apoptotic cells and IHC for oncogenic proteins (c-Myc, p53, and Bcl-2) were conducted. Tumors from mice receiving IFNα-AF-MSCs (n = 5) or AF-MSCs (n = 5) were removed on day 7 after the last injection of MSCs, and paraffin sections were used for the in situ TUNEL assay and IHC. Tumors from the tumor-bearing mice that received no MSCs (n = 3) were analyzed as controls.

As shown in Figures 5(a) and 5(b), the engraftment of IFNα-AF-MSCs induced a significantly higher proportion of apoptotic cells (32.76 ± 4.67%) in tumors than in that in tumors of untreated mice (7.51 ± 2.67%, P < 0.01) and MSC-treated mice (17.63 ± 3.69%, P < 0.05). Interestingly, although the administration of AF-MSCs largely enhanced tumor growth, they also induce high level of apoptosis (17.63 ± 3.69%) in the tumor cells. Meanwhile, the effect of MSCs or IFNα-overexpressing MSCs on the expression levels of three proteins related to ontogenesis (c-Myc, p53, and Bcl-2) was examined by IHC. The expression levels of these proteins were analyzed by quantifying the density of positively stained cells using the Image-Pro Plus (IPP) 6.0 software. The results demonstrated that the mean density of c-Myc in the tumor sections prepared from the IFNα-AF-MSC-engrafted mouse (0.0223 ± 0.0101, n = 5) was significantly lower than that of the control group (0.0374 ± 0.0064, n = 5, P < 0.05), but no significant differences were observed between either of the other 2 groups. There were no significant differences in the expression levels of p53 and Bcl-2 (Figures 5(d) and 5(e)).

3.9. IFNα-AF-MSCs Significantly Reduced Angiogenesis in the Tumors of Model Mice. To investigate whether the restriction of angiogenesis is associated with the inhibition of tumor growth by IFNα-AF-MSCs, the number of blood vessels in tumor sections from MSC-engrafted and unengrafted mice was examined. Microvessel density (MVD) was assessed for tumor angiogenesis analysis using the CD34 antibody. The paraffin sections used in this experiment were obtained from the same tumors that were used for the apoptosis analysis. The counting method introduced by Weidner et al. [26] was utilized. As shown in Figure 6, at ×200 magnification under light microscopy, there were significantly fewer countable blood vessels in the tumors of IFNα-AF-MSC-engrafted mice (25 ± 6) compared with those in the untreated mice (35 ± 2, P < 0.05). However, no significant difference in MVD was observed in AF-MSC-engrafted group (38 ± 3) compared with that in the untreated controls (P > 0.05).

3.10. Engraftment of AF-MSCs Did Not Result in Tumor Formation in Model Mice. To investigate whether the engrafted MSCs could form tumors or cause significant side effects in the recipients, 4-week-old nude mice were subcutaneously inoculated with 1 × 107 AF-MSCs or IFNα-AF-MSCs (n = 4 for each group). Small, hard swelling resembling non-specific inflammation was observed in the first 3 days after transplantation, but this gradually disappeared. The mice were kept for 50 days before being sacrificed. Neither obvious weight loss nor other symptoms of poor health were observed during the experimental period. No visible solid tumors were found around the injection sites or in any other organs in any of the three groups of mice. The pathological diagnosis via hematoxylin-eosin- (HE-) stained slides confirmed the absence of tumor formation as well (Figure S2). These results suggest that both hAF-MSCs and those engineered to express IFNα are potentially safe for use in transplantation therapy.

4. Discussion

Although IFNα is effective in treating cervical intraepithelial neoplasia (CIN) and cervical cancer in clinical trials [1, 29], its cytotoxic side effects, which result from high doses of administration, are inevitable. Thus, cell-based-targeted treatment was explored. Due to their innate capacity for tumor tropism, MSCs derived from various sources, such as the BM and umbilical cord, have been widely studied as delivery vehicles for therapeutic agents in the treatment of various types of cancers [11, 12, 16, 30]. Because AF-MSCs are easily obtained and have stable self-renewal and proliferative properties, they have great potential in treatment application. Our study showed that modified AF-MSCs expressing IFNα were effective in suppressing cervical tumor growth.

Since cervical cancer is mostly caused by HPV infection, we chose HeLa cell line, which was originated from human papillomavirus 18-affected cervical cells, as our research subject. Our study demonstrated that AF-MSCs selectively migrated to the tumor site after engraftment and that this tropism capacity was retained even after the MSCs were genetically engineered to express IFNα. In addition, we found that AF-MSCs and IFNα-AF-MSCs were specifically detected in the tumor periphery, inside stromal regions, and around
Control AF-MSCs IFNα-AF-MSCs

(a)

Percent of apoptotic tumor cells

⁎ ⁎ ⁎

(b)

Figure 5: Continued.
areas of necrosis. This finding is in agreement with many previous studies on the homing capacity of MSCs [11–13]. The MSCs that engrafed at the tumor site were believed to contribute to the population of stromal fibroblasts and participate in tumor formation [3]. The solid tumor environment is composed of malignant cells and matrix components, and MSCs have the potential to transform into activated myofibroblasts or differentiate into fibrocytes, which produce ECM components, and perivascular or vascular structures. The tumor microenvironment is a site where chemokines and cytokines, such as VEGF, urokinase plasminogen activator (uPA), IL-8, transforming growth factor beta-1 (TGF-b1), and monocyte chemotactic protein-1, are abundantly secreted, and these molecules may induce the homing of MSCs [13, 31].

Because MSCs are capable of selectively engrafing and participating in tumor stroma development, this recruitment was employed in a "Trojan horse" approach in cell-based-targeted therapy. Our study demonstrated that AF-MSCs engineered to express IFNα were effective in tumor suppression in a mouse model of cervical cancer. When HeLa cells were cocultured with IFNα-AF-MSCs, the large amount of IFNα produced by IFNα-AF-MSCs induced high levels of tumor cell apoptosis. In IFNα-AF-MSC-treated mice, secreted IFNα was only found assembled in tumor sites and even presented similar distribution as AF-MSCs showed in the previous tropism investigation. Meanwhile, significant decrease in tumor size was also observed in the IFNα-AF-MSC group. Overall, IFNα-AF-MSCs’ significant therapeutic effect on tumor suppression confirmed that the targeted delivery of IFNα by IFNα-AF-MSCs was efficient, even in the complex in vivo environment consisting of metabolic and cytokine interactions. What is more, we also found larger areas of necrosis inside the tumor mass in IFN-MSC-treated mice than in other groups which implies that the efficacy of IFN-MSC treatment was not simply displayed through or judged on the visual appearance of the tumor mass.

The tumor suppressive effects of IFNα-overexpressing MSCs could be due to an induction of damage to the tumor cells (such as higher apoptosis, as demonstrated in both in vitro and in vivo experiments) and/or inhibition of the proliferation of the tumor cells (such as a reduction in the supply of nutrition and hypoxia environment). Tumor suppressor gene p53 and apoptosis control gene bcl-2 are two important genes for cell survival. They were reported to be involved in cervical cancer carcinogenesis [32]. P53 expression was detected in early stages in cervical cancer carcinogenesis and has a role in progression to cervical cancer [32]. Protooncogenes such as c-Myc direct changes in metabolism and protein synthesis supporting enhanced proliferation rates. The gene copy number gain of c-Myc was significantly higher in the cervical lesion of advanced histologic grade [33, 34]. In our research, c-Myc expression dropped in cervical cancer cells as a result of IFNα-overexpressing MSC treatment, while neither p53 nor bcl-2 is related. It is also known that enhanced angiogenesis is essential for tumor growth. Previous study evaluating the potential of bone marrow-derived mesenchymal stem cells (MSC), genetically modified to express interferon (IFN)-alpha, for the treatment of lung metastasis in an immunocompetent mouse model of metastatic melanoma, indicated that the antitumor effect might be related to a potential decrease of angiogenesis and/or induction of tumor cell apoptosis [35]. Therefore, we evaluated the effect of IFNα-AF-MSCs on angiogenesis in our xenograft model by examining the MVD in the tumor and found a significant reduction in blood vessels compared with...
that in the control groups. This reduced angiogenesis could, in turn, promote the apoptosis of tumor cells induced by the IFNα-AF-MSCs. Our finding of angiogenesis inhibition was in line with a previous study [13] that used a bladder tumor model, which demonstrated that IFNβ-AF-MSC treatment resulted in a reduction in vascularization.

The experimental results regarding whether MSCs alone promote or inhibit tumor growth have been controversial. MSCs were shown to suppress tumor growth in some models of tumors, such as glioma, Kaposi’s sarcoma, malignant melanoma, Lewis lung carcinoma, and colon carcinoma [15, 30, 36]. These studies revealed that the mechanisms for suppression of tumor growth varied and included inhibition of certain enzymatic proteins and concentration-dependent inhibition of angiogenesis. However, a few studies have reported that MSCs can contribute to tumor growth [37–39]. Lis et al. demonstrated that the properties of ovarian cancer cells, namely, metastatic abilities (adhesion, migration, and invasion), proliferation, and chemoresistance, can be promoted by MSCs [37]. This promoting effect was considered to be due to the induction of cytokines, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which induce the division and blood vessel formation of endothelial and stroma cells under abnormal conditions, such as wound healing or tumorigenesis [3]. In fact, the tumor type, model, route of MSC transplantation, cell dose, and time course all tended to influence the experimental outcomes [12]. Our study showed that the AF-MSCs significantly increased tumor size. However, interestingly, in situ TUNEL assay showed that AF-MSCs alone also showed induction of apoptosis. According to our previous MVD detection results, tumors of the AF-MSC group did not enjoy a higher standard of angiogenesis inside, even if they showed in the greatest size among all the groups. Thus, relative lack of blood and nutrition supply might occur and induce an increase in the percentage of apoptotic cells in solid tumors. Anyway, tumor-promoting effect of AF-MSCs was reversed after they were modified to express IFNα, and these stem cells demonstrated an inhibitory effect on tumor cell growth.

In addition, considering the potential tumorigenesis risk of AF-MSCs and gene-modified IFNα-AF-MSCs, we performed further experiments to evaluate the possibility that AF-MSCs may form tumors. Up to $1 \times 10^7$ of AF-MSCs and IFNα-AF-MSCs were subcutaneously administered to nude mice, and no visible signs of tumorigenesis were

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**Figure 6:** Reduction of tumor angiogenesis by IFNα-AF-MSCs in a mouse model. (a) Representative images of areas with the highest density of microvessels in each group. Bar, 25 μm. (b) Comparison of MVD between the different experimental groups. Error bars, standard deviation. Solid tumors in nude mice formed by $1 \times 10^7$/ml of subcutaneously administered HeLa cells were removed and processed into paraffin sections to measure microvessel density (MVD). The tumors were collected one week following the three weekly injections of $1 \times 10^6$ AF-MSCs and IFNα-AF-MSCs ($n = 5$ per group), and tumors from three untreated mice were analyzed as controls. Micro-blood vessels were indicated by IHC staining for CD34, and individual microvessels in at least five fields in each tumor were counted at $\times 200$ magnification. Both isolated endothelial cells and luminal microvascular structures were considered countable vessels. *$p < 0.05$.
identified at the injection sites during the 50-day observation period; pathological diagnosis further verified that no tumorigenesis existed, either in the injected subcutaneous tissue or in the collected organs. Therefore, we deduced that even if AF-MSCs promoted tumor growth in vivo, they do not contribute to tumorigenesis in regions of nonmalignant cells. Collectively, we assume that IFNα-AF-MSCs are safe and effective in restricting cervical cancer cell growth in short terms.

In conclusion, our study demonstrated that IFNα-AF-MSCs are capable of homing to the tumor site in a HeLa cell xenograft model and exhibit therapeutic efficacy in tumor suppression. We also demonstrated that although AF-MSCs alone can selectively engraft into the tumor site and participate in tumor construction, they are safe and effective after being genetically engineered to express IFNα, with the aim of treating cervical cancer. Our data suggest that the tumor suppression efficacy of IFNα-AF-MSCs is attributed to angiogenesis reduction, as well as the induction of apoptosis and inhibition of cell proliferation in tumor cells mediated by these IFNα-overexpressing stem cells. Further investigation is needed to determine the optimal cell dose and administration routes, as well as how to prolong the duration of therapeutic efficacy. Additionally, the long-term risk of further immunosuppression triggered by AF-MSCs should also be taken into consideration when AF-MSCs are administered to patients with immunodeficiency [18]. Ultimately, further studies utilizing other therapeutic agents in different tumor models are necessary to validate the efficacy of AF-MSC-based-targeted therapy.

Conflicts of Interest
The authors declare no conflicts of interest.

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Supplementary Materials
Figure S1: identification of AF-MSCs. (a) Identification of specific cell surface markers for AF-MSCs by flow cytometry. AF-MSCs of the 1st, 3rd, 7th, and 15th generations were stained with CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR and were analyzed by flow cytometry. AF-MSCs were stained with CD90, CD105, CD73, HLA-ABC, CD34, CD14, CD45, and HLA-DR. IFNα-AF-MSCs are consistent with the MSC null ones in terms of stem cell markers, which proved the cell stability. Representative results from one of three comparable experiments are shown.

(b) Osteogenic differentiation of AF-MSCs. AF-MSCs were stained with Alizarin red S after being cultured in osteogenic medium for 21 days. Osteogenic induction was shown by positive staining (indicated by arrows), which occurs as a result of mineralized differentiation. The photograph is a representative image of two independent experiments. Figure S2: absence of tumor formation from inoculated AF-MSCs. H&E staining of tissues from mice that received subcutaneous injection of 1 × 107 AF-MSCs or IFNα-AF-MSCs. Samples were obtained from four mice 50 days after injection. The images show representative fields of the tissues from the injected sites, specifically the liver, kidney, spleen, and lung. Bar: 50 μm. (Supplementary Materials)

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