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Original Article

Inhibition of cervical cancer cells by co-culturing with mesenchymal stem cells

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Abstract: *Background:* The effect of mesenchymal stem cells (MSCs) on tumors remains controversial and requires further exploration. In this study, we isolated MSCs from umbilical cord (UC) and co-cultured with cervical cancer HeLa cells. Flow cytometry was conducted to detect apoptosis and cell cycle. CCK8 and Matrigel-transwell were performed to assay cell proliferation and invasion ability, respectively. *Results:* Our results indicated that UCMSCs inhibited proliferation of HeLa cells 72 hours post co-culture but had no obvious effect on apoptosis and cell cycle. UCMSCs dramatically suppressed invasion of HeLa cells in transwell detection. In mechanism study, we found that UCMSCs could inhibit expression of AKT/PI3K/STAT3/mTOR pathway and influence epithelial-mesenchymal transition markers. *Conclusion:* Our study clearly confirms that UCMSCs could inhibit biological functions of cervical cancer cells.

Keywords: Mesenchymal stem cells, cervical cancer, HeLa cells, anti-tumor effect

Introduction

Cervical cancer is the second leading cause of female malignancy-associated mortality worldwide. There are more than half a million new cases and about 270,000 deaths annually [1]. The major cause of cervical cancer is human papilloma virus (HPV) infection which disrupts normal proliferation and differentiation of cervical squamous epithelial cells [2]. Risk factors for cervical cancer are those associated with HPV exposure such as increased number of sexual partners and immunosuppression [3]. Although vaccines are highly effective in preventing HPV infection, widespread HPV vaccination remains a difficulty. Therapy methods include cisplatin-based chemotherapy, angiogenesis blockade (bevacizumab, sunitinib, pazopanib, etc.), immune checkpoint inhibitors (ipilimumab and pembrolizumab), and target therapy (gefitinib and erlotinib) [4].

Mesenchymal stem cells (MSCs) are multipotent stem cells which can be isolated from multiple tissues such as bone marrow, adipose tissue, periosteum, dermis, deciduous teeth, umbilical blood, and peripheral blood [5]. The most important characteristics of MSCs are their self-renewing capability and multi-lineage differentiation potential. Surface expression markers are positive for CD29, CD59, CD90, CD105, and CD44 but are negative for co-stimulation factors such as CD80, CD86, and CD40. Consequently, MSCs fail to activate allo- or xenogeneic lymphocytes due to a lack of immunogenicity [6]. The third feature of MSCs is that they can inhibit activation, proliferation, and function of immune cells including T cells, B cells, NK cells, and antigen-presenting cells [7]. MSCs are now used to treat many diseases such as liver cirrhosis [8], multiple sclerosis [9], graft versus host disease (GVHD) [10], type I diabetes [11], multiple sclerosis [12], and systemic lupus erythematosus [13]. However, there are contradictory results regarding the effect of MSCs on tumors. Pisati et al. indicated that MSCs could dramatically inhibit proliferation of Kaposi's sarcoma cells [14] while Djouad suggested that the immune-regulatory function of MSCs could benefit development of tumors [15].

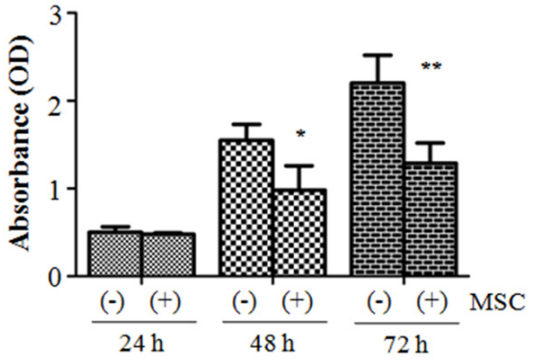


Figure 1. Proliferation rate of HeLa cells co-cultured with UCMSCs. *P < 0.05, **P < 0.01.

In this study, we used MSCs isolated from umbilical cord (UC) and co-cultured with cervical cancer HeLa cells. The aim of this study was to discover whether MSCs could influence biological functions of HeLa.

Materials and methods

Co-culture of UCMSCs and Hela

UCMSCs were kindly provided by Sichuan Umbilical Cord Blood Stem Cell Bank. UCMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 1 × 10^5 cells/well in a 6-well plate (Falcon, Bedford, MA, USA). HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The UCMSCs were co-cultured with HeLa cells at a ratio of 2:1.

Proliferation, apoptosis, and cell cycle detection

Proliferation was assayed by CCK-8 detection (Beyotime). Apoptosis was stained by Annexin V-FITC and detected by flow cytometry. Cell cycle variation was also completed by flow cytometry.

Western blot

Proteins were extracted using RIPA lysis buffer protein and concentration was measured by BCA method. A total of 20 g was loaded on 12% SDS-polyacrylamide gels, electrophoresed for protein separation, and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA) followed by blocking with nonfat dried milk in TBS containing 0.2% Tween-20 (TBST) for 1 hour at room temperature. Primary antibody was added and incubated 4°C with the primary antibodies. Membranes were then washed in TBST (3 times, 60 min) and incubated with secondary antibody conjugated to horseradish peroxidase (1:5000; Abcam, Cambridge, UK) for 1 hour at room temperature. Antigen-antibody complexes were visualized via x-ray film after exposure to enhanced chemiluminescence reagent (Amersham Biosciences, Fairfield, CT, USA).

Transwell

Twenty four-well (8 um aperture, Millipore) transwell plates were used for co-culture and invasion. HeLa cells were co-cultured with UCMSCs in transwell plates pre-coated with diluted Matrigel (20%) for 12-48 hours. For invasion assay, HeLa cells were plated in the upper chamber and UCMSCs were in the bottom chamber. Invaded cells were stained with toluidine blue 24 and 48 hours post co-culturing.

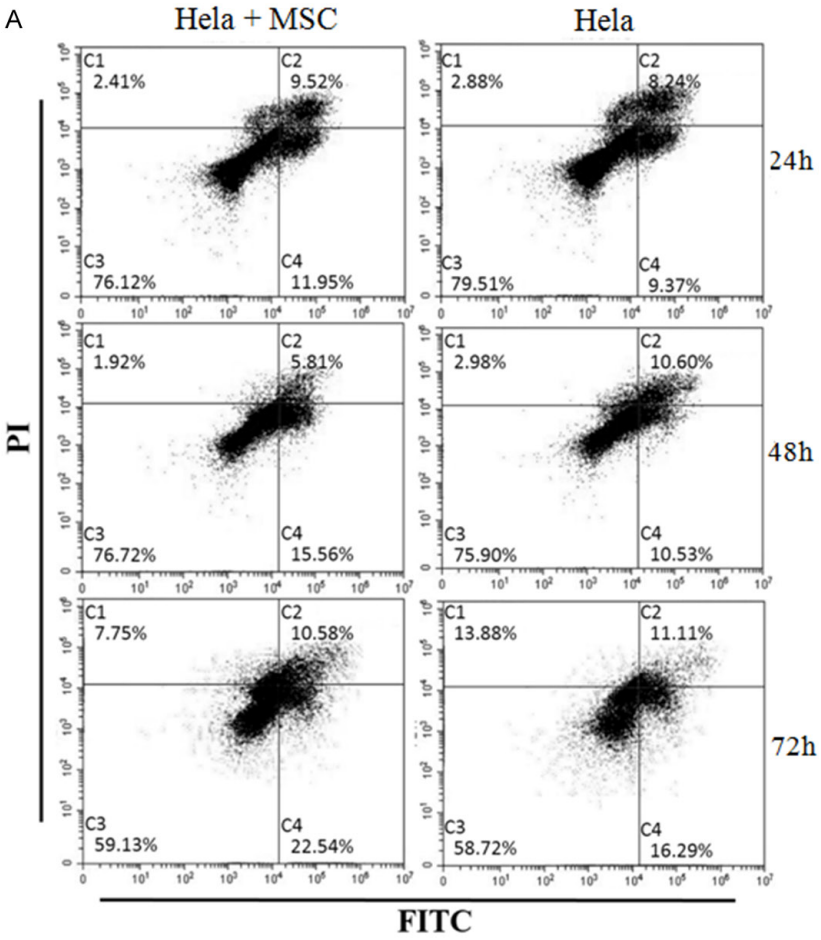
Data analysis

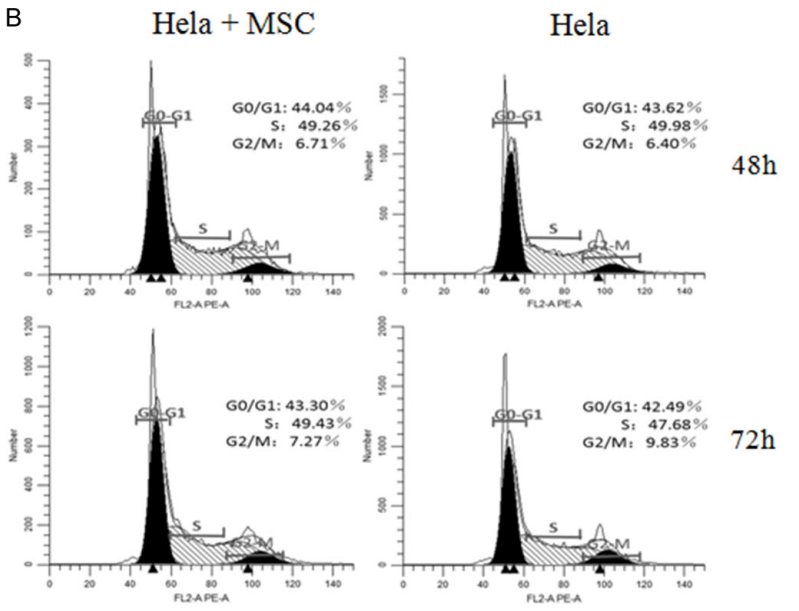
Real-time PCR results were analyzed by Bio-Rad CFX manager software (Bio-Rad, Hercules, CA, USA). Image lab software was used to analyze Western blot data. CytExpert and ModFit were used for cell apoptosis and cell cycle analysis, respectively. All analyzed data are expressed as mean values ± standard error by SPSS18.0 (IBM SPSS Statistics, New York, NY, USA). Values of $P < 0.05$ and $P < 0.001$ were considered significant in comparison to control group. All figures were accomplished by GraphPad Prism5 (GraphPad Software, Inc., LA Jolla, CA, USA).

Results

UCMSC inhibited proliferation of HeLa cells

MSCs isolated from umbilical cords were co-cultured with HeLa in a ratio of 2:1 and CCK8 was introduced to detect proliferation of HeLa 24, 48, and 72 hours post co-culturing. The results indicated that proliferation of HeLa cells was not influenced by UCMSCs at 24 hours post co-culturing (**Figure 1**) but HeLa cells could be significantly suppressed 48 (P < 0.05)





	48 hours		72 hours	
	Hela+MSC	Hela	Hela+MSC	Hela
G0/G1	44.04	43.62	43.30	42.49
S	49.26	49.98	49.43	47.68
G2/M	6.71	6.4	7.27	9.83

Figure 2. Apoptosis and cell cycle assay by flow cytometry. A. Apoptosis; B. Cell cycle.

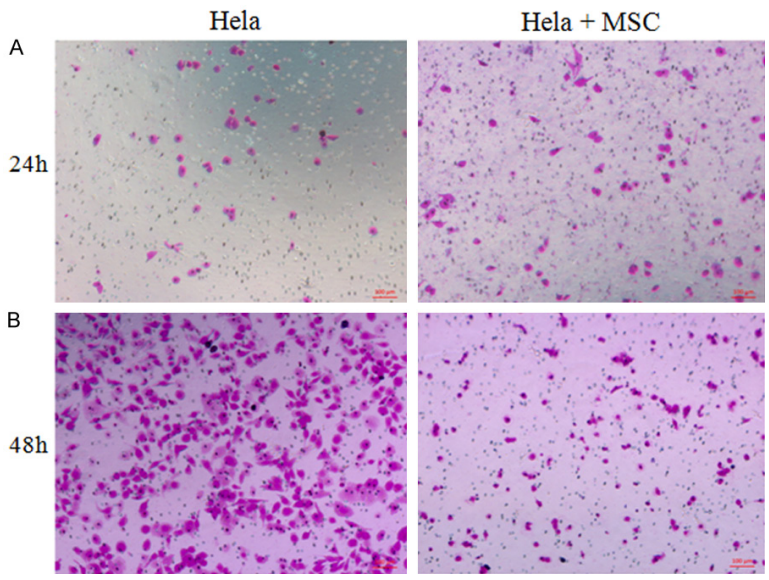


Figure 3. Co-culturing with UCMSCs inhibited invasion of HeLa cells.

and 72 hours ($P < 0.01$) post co-culturing. This indicates that UCMSCs could inhibit proliferation of HeLa cells.

UCMSCs failed to regulate apoptosis and cell cycle of HeLa

Flow cytometry was introduced to measure apoptosis rate of HeLa, which was stained by Annexin V. It was found that co-culturing with UCMSCs did not significantly influence apoptosis rate of HeLa at 24 hours (Hela + MSC: 21.47% vs Hela: 17.61%), 48 hours (Hela + MSC: 21.37% vs Hela: 21.03%), and 72 hours (Hela + MSC: 33.12% vs Hela: 27.4%) (**Figure 2A**).

We also detected the cell cycle of HeLa cells by flow cytometry. At 48 hours, there were no difference between HeLa (G0/G1: 44.04%, S: 49.26%, G2/M: 6.71%) and HeLa + MSC (G0/G1: 43.62%, S: 49.98%, G2/M: 6.40%). Cell cycle also showed no difference 72 hours post co-culturing, HeLa (G0/G1: 43.3%, S: 49.43%, G2/M: 7.27%) and HeLa + MSC (G0/G1: 42.49%, S: 47.68%, G2/M: 9.83%) (**Figure 2B**).

UCMSCs dramatically inhibited invasion of HeLa

Matrigel was added in transwell chambers to detect invasion of HeLa in the presence of UCMSCs. As shown in **Figure 2A**, the number of invasion HeLa cells was not significantly different between HeLa and HeLa + MSC groups but it was found that invaded HeLa cells were dramatically reduced 48 hours post co-culturing with UCMSCs.

UCMSCs influenced expression of important kinases

To further explore mechanisms of function inhibition of HeLa by co-culturing with UCMSCs, some important kinases formerly reported to play important roles in regulation of tumor biological functions were measured by Western blot. Our study found that AKT was only inhibited at 48 hours ($P < 0.05$) but p-AKT was dramatically suppressed at both 48 ($P < 0.05$) and 72 hours post co-culturing ($P < 0.01$) (**Figure 3A**, **3B**). Expression of PI3K was reduced at 48 ($P < 0.05$) and 72 hours ($P < 0.05$) and p-PI3K was only inhibited at 48 hours ($P < 0.01$). STAT3/p-STAT3 was also inhibited in HeLa cells co-culturing with UCMSCs (STAT3: 48 hours, $P < 0.01$, 72 hours, $P < 0.05$; p-STAT3: 48 hours, $P < 0.01$). In ERK1/2/p-ERK1/2, we also observed downregulation of expression in both phospho and none-phospho ERK (48 hours: ERK1/2 $P < 0.05$, 72 hours: ERK1/2 $P < 0.01$, p-ERK1/2 $P < 0.05$), while in mTOR detection only, p-mTOR was found inhibited in co-culture group (48 hours, $P < 0.01$; 72 hours $P < 0.01$) (**Figure 4A**, **4B**). These results demonstrate the important role of ERK/STAT3/PI3K/mTOR pathways in regulating function of HeLa cells.

UCMSCs regulated EMT biomarkers in HeLa cells

Epithelial-mesenchymal transition is an important characteristic of malignant tumor cells. There are many cytoskeletal molecules playing important roles in EMT. In this study, we found that expression of vimentin was upregulated at 24 hours but was dramatically inhibited at 48 ($P < 0.01$) and 72 hours ($P < 0.01$) post co-culturing. E-cadherin was suppressed at 48 hours ($P < 0.05$) but dramatically increased at 72 hours ($P < 0.01$) (**Figure 5A**, **5B**), suggesting

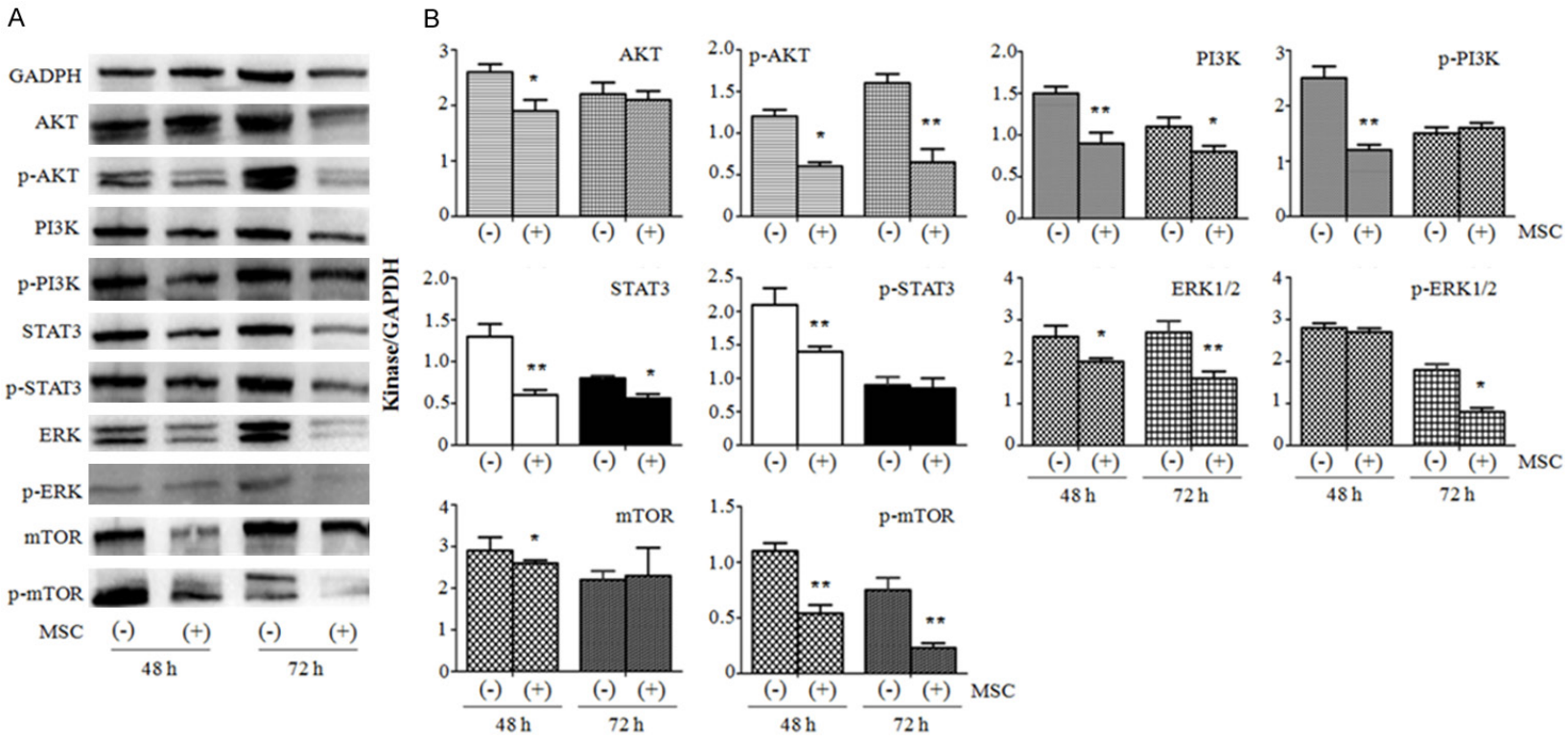


Figure 4. Kinase expression of HeLa cells was dramatically inhibited by UCMSCs. A. Western blot results; B. Data analysis. *P < 0.05, **P < 0.01.

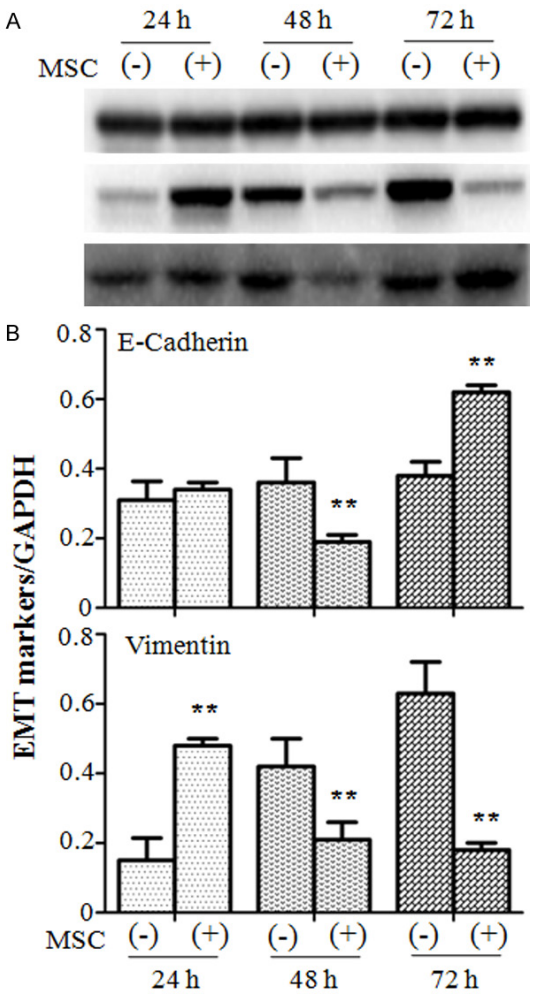


Figure 5. UCMSCs could influence the level of epithelial-mesenchymal transition. A. Western blot results; B. Data analysis. *P < 0.05, **P < 0.01.

the importance of EMT markers in biological functions of HeLa.

Discussion

In order to investigate the effect of mesenchymal stem cells on tumor biological functions, we selected cervical cancer HeLa cells and co-cultured with MSCs isolated from umbilical cord. Different assay methods were performed to detect variation of biological functions 24, 48, and 72 hours post co-culturing. The results indicated that UCMSCs inhibited proliferation and invasion of HeLa cells but had no effect on apoptosis and cell cycle. Further research indicated that UCMSCs inhibited expression of AKT, PI3K, STAT3, ERK1/2, and mTOR. EMT markers (E-Cadherin and vimentin) were also regulated upon UCMSCs co-culturing. Our study clearly suggests that UCMCs could inhibit biological functions of HeLa cells.

There have been contradictory results regarding the effect of MSCs on tumor cells. Kabashima-Niibe et al. reported that α-SMA (smooth muscle actin) positive MSCs promoted occurrence of EMT in pancreatic cancer cells. By regulating expression of stemness-associated genes and enhancing tumor formation, MSCs dramatically benefited development of pancreatic cancer cells. They also found that MSCs induced drug resistance of pancreatic cancer cells in the presence of MSCs [16]. Zhang et al. isolated MSCs from mice bone marrow and co-cultured with mouse breast cancer (4T1) and prostate cancer cells (DU145). Their results indicated that both co-culturing and treatment with BMMSCs conditioned medium increased growth of 4T1 cells and the blood vessel area was also enhanced in tumors co-injection with MSCs in nude mice [17]. However, there is also evidence showing that MSCs could inhibit tumor growth. Wu et al. isolated microvesicels (MVs) from human UCMSCs and indicated that MSC-MVs inhibited bladder cancer cell T24 proliferation by arresting cell cycles and induced apoptosis by increased expression of caspase 3 [18]. Li et al. also confirmed that MSCs from children foreskin suppressed growth of gastric cancer SGC-7901 [19]. The results of these studies suggest that the role of MSCs in tumorigenesis remains controversial.

In our study, we co-cultured HeLa cells with UCMSCs, clearly confirming the anti-tumor effect of MSCs isolated from umbilical cord. Proliferation and invasion of HeLa cells were dramatically inhibited in our co-culture system. The possible mechanism to explain invasion suppression may be the inhibition of PI3K/ERK/STAT3 pathway, since they have been reported to promote metastasis of malignancy tumors. The second explanation could be the role of EMT markers in metastasis regulation. E-cadherin acts as suppressor of EMT and finally inhibits invasion of tumor cells while vimentin activates occurrence of EMT. In our study, E-cadherin was dramatically increased while vimentin was downregulated in co-culturing with UCMSCs. Our results indicate that E-cadherin and vimentin may mediate invasion regulation but more research is necessary to confirm our hypothesis.

Conclusion

Our study reveals that UCMSCs could inhibit biological functions of UCMSCs and our results provide novel ideas regarding cervical cancer therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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