

ORIGINAL ARTICLE

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## Human glioma cells-conditioned medium promotes proliferation and migration of human bone marrow mesenchymal stromal cells *in vitro*

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### Summary

**Purpose:** To investigate changes in cell proliferation and migration of human bone marrow-derived mesenchymal stromal cells (hMSCs) in glioma environment, in order to assess the tumorigenic risk of hMSCs in the clinical application to treat human gliomas.

**Methods:** hMSCs were obtained from normal adult persons and identified by their morphological characteristics and test of their stemness. The U251 glioma cell-conditioned medium (U251-CM) was obtained to simulate the human glioma environment *in vitro*. hMSCs were cultured in the U251-CM or the control medium in the same conditions and changes in cell proliferation and migration were detected by MTT assay and wound healing assay.

**Results:** The results of MTT assay showed that, compared with the control group, the proliferation of hMSCs cultured in U251-CM increased significantly, and the results of wound healing assay showed that the migration of hMSCs cultured in U251-CM also increased significantly.

**Conclusions:** Human glioma cell-conditioned medium may promote the proliferation and migration of hMSCs, and we are concerned about the tumorigenic risk of hMSCs in glioma environment before their clinical application.

**Key words:** bone marrow-derived mesenchymal stromal cells, cell migration, cells proliferation, gene therapy, glioma, tumorigenicity

### Introduction

The potential of mesenchymal stromal cells (MSCs) for targeted gene delivery in the context of cancer is an exciting area of research that has gained considerable momentum in recent years, with studies reporting engineered MSCs specifically targeting multiple tumor types [1-5]. Nevertheless, research about the influence of tumor cells on MSCs is still not in the focus. When being applied as delivery vector MSCs would leave the original living place and enter into the targeted tumor environment, it is hardly known what kind of changes would happen to MSCs under the influence of tumor cells.

MSCs possess unique migratory properties within the central nervous system (CNS) and good tropism to glioma cells, and had been demonstrated to be effective for delivering transgenes to treat experimental gliomas [6-10]. More attention has been paid to the potential of hMSCs for clinical application to treat human gliomas, but less to their bio-safety in glioma environment. In our previous study we had found that the co-culture of hMSCs with human glioma cells led to upregulated expression of some important oncogenes in hMSCs, the overexpression of which has been demonstrated to contribute to tumorigenesis [11].

The milieu of growth factors and inflammatory cytokines present within the glioma environment are very different from that in the original living place of hMSCs and we feel concerned about the tumorigenic risk of hMSCs in glioma environment.

Here we investigated the changes in cell proliferation and migration of hMSCs in glioma environment, in order to further assess the tumorigenic risk of hMSCs in the clinical application to treat human gliomas.

## Methods

### *Isolation of hMSCs and cell culture*

To isolate hMSCs, bone marrow aspirates were taken from the iliac crest of normal adult donors after informed consent and under a protocol approved by the Ethics Committee of the Affiliated Hospital of Guangdong Medical University. Nucleated cells were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient, resuspended in Dulbecco's modified Eagle medium: Nutrient mixture F-12 (DMEM-F12) (Gibco BRL, Gaithersburg, MD, USA) plus 10% fetal bovine serum (FBS), and incubated at 37°C with 5% CO<sub>2</sub>. After 24 hrs, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate buffer saline (PBS) and continued to be incubated with the new medium. Following this, the medium was changed every 3 days and cells were passaged once a week.

Human glioma U251 cells were purchased from China Center for Type Culture Collection (Shanghai, People's Republic of China) and maintained in DMEM-F12 plus 10% FBS at 37°C with 5% CO<sub>2</sub>.

### *Determination of hMSCs' stemness*

After 2-5 passages, the conversion of hMSCs into neurosphere like structures was initiated. Specifically, cells were dissociated with 0.25% trypsin/0.01% ethylene diamine tetraacetic acid (EDTA) and plated on uncoated flasks at a concentration of 1x10<sup>5</sup> cells/cm<sup>2</sup> in serum-free DMEM/F12 medium containing supplements B27 and N2 (all purchased from Gibco BRL, Gaithersburg, MD) plus 20 ng/mL of both epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), both purchased from Sigma (St. Louis, MO) and incubated at 37°C with 5% CO<sub>2</sub>. The medium was changed once a week and growth factors were added twice a week. After 10-15 days, neurosphere like structures could be observed. Cells of neurosphere like structures were fixed in 4% paraformaldehyde in PBS. Immunocytochemistry was carried out using standard protocols. The antibodies used and their dilutions were: nestin 1:500 and antirabbit IgG Cy3 conjugate

1:100, both purchased from Chemicon International, Temecula CA, USA.

### *Preparation of U251 glioma cell-conditioned medium (U251-CM)*

U251 cells in exponential phase of growth were dissociated with 0.25% trypsin/0.01% EDTA, planted in 75 cm<sup>2</sup> culture flasks (Corning, Lowell, MA, USA) in DMEM-F12 plus 10% FBS, and incubated at 37°C with 5% CO<sub>2</sub>. Confluent cultures were washed with serum free DMEM/F12, and then 9 ml of fresh DMEM/F12 supplemented with 0.1% bovine serum albumin (BSA) for each flask were added and incubated for 3 days. After conditioning, the medium was aspirated from the cells, centrifuged (1000 x g for 5 min), filtered through 0.22-µm-pore-diameter Millipore filters, and stored frozen until required. The basal medium of the conditioned medium, DMEM/F12 supplemented with 0.1% BSA, was used as the control medium.

### *Measurement of cell proliferation (MTT assay)*

To examine the difference of cell proliferation between MSCs cultured in the conditioned medium derived from U251 (U251-CM) and those cultured in the control medium, MTT assay was performed. An equal number of hMSCs were seeded at a density of 5000/well and cultured in either U251-CM or the control medium in 96-well plate. Cells were incubated at 37°C with 5% CO<sub>2</sub> for 72 hrs, and the mediums were changed at 24<sup>th</sup> and 48<sup>th</sup> hr. To avoid losing cells in the medium in each well, cells were changed by half each time. Then, the cell number was counted with MTT assay. Briefly, 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, from Sigma, St. Louis, MO) was added (100 µg/well) to each well of the 96-well plate and incubated in 37°C for 4 hrs. MTT medium was discarded and 200 µl sterile DMSO (Sigma, St. Louis, MO) were added to lyse cells (10 min, 37 °C, under shaking), and the optical density (OD) was measured at a wavelength of 490nm with microtiter plate reader (Model-550, Bio-Rad, USA). Each experiment was independently performed in triplicate.

### *Measurement of cell migration (wound healing assay)*

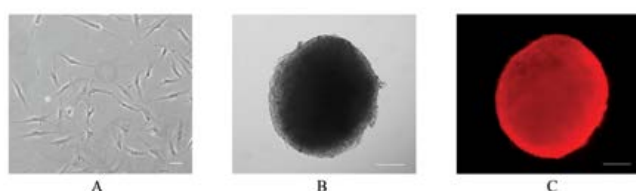
Cell migration was measured by the wound healing assay. hMSCs were cultured in 6-well cell culture plates (Corning, Lowell, MA, USA) until confluence and then serum-starved for 24 hrs. A scratch wound was created in the cell monolayer with a 10-µl pipette tip. The cells were washed twice with PBS to remove the floating cells, and 10 ml of either the U251-CM or the control medium per well were added. The progress of cell movement into the wound area was observed at 0 and 24 hrs, and the representative fields were photographed using a digital camera attached to a microscope (Olympus Corporation, Tokyo, Japan). The percentage of wound closure was measured with OLYSIA

BioReport imaging software (Informer Technologies, Inc., Madrid, Spain). Each experiment was independently performed in triplicate.

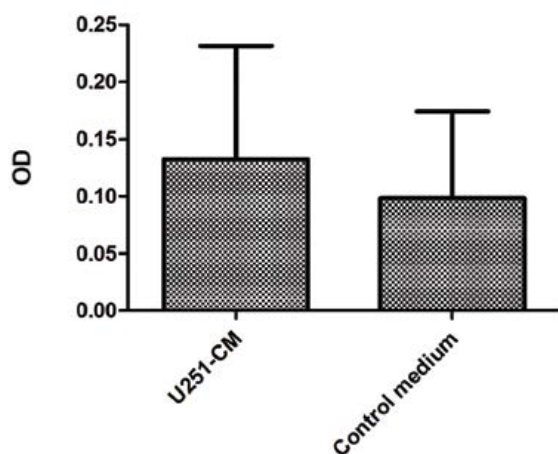
## Results

### Identification of hMSCs

The applied hMSCs were identified by their morphological characteristics and test of their stemness. hMSCs had a fusiform-like shape, adhered to the surface of tissue culture flasks (Figure 1A) and could be converted into cells with characteristics of neural stem cells (NSCs) *in vitro*, which formed cell aggregates that were morphologically similar to NSC-derived neurospheres (Figure 1B) and highly expressed the neural stem cell marker nestin (Figure 1C).



**Figure 1.** Characteristics of human bone marrow-derived mesenchymal stromal cells (hMSCs) and hMSCs-derived neural stemlike cells. hMSCs have a fusiform like shape, adhere to the surface of tissue culture flasks (A) and could be converted into cells with characteristics of neural stem cells (NSCs) *in vitro*, which formed cell aggregates that were morphologically similar to NSC-derived neurospheres (B) and highly expressed the neural stem cell marker nestin (C). Scale bar = 100  $\mu$ m.



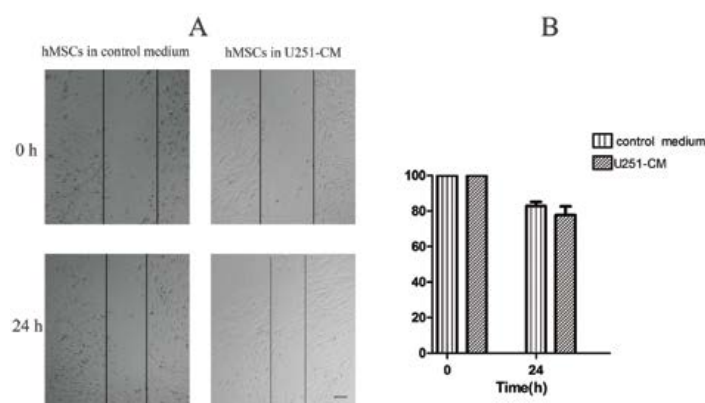
**Figure 2.** Effect of U251 glioma cell-conditioned medium (U251-CM) on hMSCs proliferation. The MTT assay shows that 72 hrs after seeding, the optical density (OD) values of the hMSCs cultured in U251-CM were significantly higher than those of the hMSCs cultured in the control medium (n=6),  $p < 0.05$ .

### Influence of U251-CM on hMSCs proliferation

An equal number of hMSCs were cultured in U251-CM or the control medium. The result of MTT assay (Figure 2) revealed that 72 hrs after seeding, the OD values of the hMSCs cultured in U251-CM were significantly higher than those of the hMSCs cultured in the control medium (n=6,  $p < 0.05$ ).

### Influence of U251-CM on hMSCs migration

The effects of U251-CM on hMSCs motility activity were investigated using the wound healing assay (Figure 3). At 24 hrs after scratching, the distances between the boundaries of the wound region were measured and compared with the initial distances (0 hr after scratching), which were considered as 100%. The results confirmed that the migration capacity of hMSCs cultured in U251-CM was significantly higher at 24 hrs compared with hMSCs cultured in the control medium (n=6,  $p < 0.05$ ).



**Figure 3.** Effect of U251 glioma cell-conditioned medium (U251-CM) on hMSCs migration. The wound healing assay shows that at 24 hrs after scratching, the distances between the boundaries of the wound region were measured and compared with the initial distances (0 hr after scratching) (A). The initial distances were considered as 100% and the distances at 24 hrs after scratching were presented as percentages and shown as mean  $\pm$  standard deviation (n=6),  $p < 0.05$  (B). Scale bar = 100  $\mu$ m.

## Discussion

hMSCs represent an optimal cellular vector for gene therapy to treat human gliomas. What we concern about is the bio-safety of hMSCs in relation with the tumorigenic risk in glioma environment. In our previous study we had found

that some important oncogenes are significantly upregulated (>3-fold) in hMSCs co-cultured with U251 glioma cells, including KIT, CAPNS1, TK1, MMP1, CCND1, CDC20, RELA, and STC1, which are involved in cell growth/proliferation, cell invasion/metastasis, cell cycle, and signal transduction pathways [11]. In the present study we used the conditioned medium from human U251 glioma cells (U251-CM) to simulate the glioma environment *in vitro*, and cultured hMSCs in U251-CM, and then investigated the changes in proliferation and migration of hMSCs.

The results of MTT assay showed that, compared with the control group, the proliferation of hMSCs cultured in U251-CM improved significantly. One of the most important characteristics of malignant cells is their uncontrolled growth. The KIT, CAPNS1 and TK1 genes were found to be upregulated in hMSCs co-cultured with U251 cells [11]. The upregulation of all these genes was demonstrated to be a positive factor in cell proliferation and to contribute to carcinogenesis [12-14].

The results of wound healing assay showed that the migration of hMSCs cultured in U251-CM also increased substantially. Invasive and metastatic behavior is another important characteristic feature of malignant cells. During tracing glioma cells, hMSCs present good migration ability in the CNS [6,7]. This property, however, would contribute to the malignancy of this cell population once they reach uncontrolled growth ability, which should be of concern. Matrix metalloproteinase 1 (MMP1) was found to be upregulated in hMSCs co-cultured with U251 cells [11]. Matrix metalloproteinases (MMPs) have long been associated with cell invasion and metastasis and they

are upregulated in almost every type of human cancer, while their overexpression is often associated with poor survival [15,16].

Taken together, we found upregulation of a number of cancer-related genes in hMSCs co-cultured with U251 cells and the upregulated genes included some important oncogenes the overexpression of which had been demonstrated to promote cell proliferation and migration. In this study we found that human glioma cell-conditioned medium could promote the proliferation and migration of hMSCs. Additional studies would be needed to understand the possible biological significance of these alterations. Though no final conclusion could be made yet that tumorigenic potential in hMSCs would be induced in human glioma environment, our findings still pose concerns about that, and highlight the need for further studies on this problem before their clinical application to treat human gliomas.

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## Conflict of interests

The authors declare no conflict of interests.

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