The effect of bone marrow mesenchymal stem cells on highly metastatic MHCC97-H hepatocellular carcinoma cells following OPN and TGFβ1 gene silencing

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Abstract. The metastatic behavior of hepatocellular carcinoma (HCC) is one of the key factors that leads to poor prognosis. The aim of the current study was to determine the changes in metastasis and proliferation potential of bone marrow mesenchymal stem cells (BMSCs) in high metastatic potential hepatocellular carcinoma (MHCC97-H) following gene silencing. The osteopontin (OPN) and transforming growth factor-β (TGFβ1) genes, which are associated with metastasis and tumor proliferation, were silenced in MHCC97-H cells. Transwell assays were used to evaluate the migration of MHCC97-H cells in vitro. Additionally, a murine model of MHCC97-H lung metastasis was established. Following OPN and TGFβ1 silencing, the migration of MHCC97-H cells was significantly reduced following BMSC intervention (P<0.01). Furthermore, there were few MHCC97-H cells in the lung tissues of the OPN- and TGFβ1-silenced animals, and their integrated optical density (IOD) value was significantly lower compared with controls (P<0.05). Immunofluorescence of lung metastasis in the MHCC97-H model revealed that there was no significant difference in the IOD value of integrin αvβ3 expression in the OPN- and TGFβ1-silenced groups compared with controls (P>0.05). The metastasis and proliferation potential of MHCC97-H following BMSC intervention were significantly reduced in vitro and in vivo, especially in the TGFβ1-silenced group. The decrease in the metastatic potential in gene-silenced MHCC97-H cells was not associated with integrin αvβ3 expression. Therefore, OPN and TGFβ1 may be potential targets for HCC treatment, and TGFβ1 may have a higher therapeutic potential for BMSC intervention.

Introduction

In China, hepatocellular carcinoma (HCC) is a common, malignant tumor with poor prognosis (1). The mortality rate of HCC is the 3rd highest among all malignant tumors (1). Metastasis and recurrence are the main causes of poor prognosis (2). The invasion and metastasis of HCC is a complex multi-gene, multi-step and multi-factor process involving interactions between cancer cells, and between cancer cells and the host microenvironment (3). As multiple biological factors are involved in HCC metastasis, HCC models with different metastatic potentials constructed by the Hepatocellular Carcinoma Institute of Fudan University have become an effective means to study the metastatic behavior of HCC (4). For example, the highly metastatic HCC cell line MHCC97-H has a lung metastasis rate of 100% (5). Therefore, this cell line is the most effective model for studying HCC lung metastasis (6).

The development of stem cell technology has provided novel data for the occurrence, progress and treatment of HCC. Stem cell technology can be divided into normal stem cell technology and cancer stem cell (CSC) technology. The CSC concept states that tumor growth, analogous to the renewal of healthy tissues, is fueled by small numbers of dedicated stem cells (7). Normal stem cell technology refers to the use of human normal stem cells for tumor intervention techniques in order to observe their role on tumors or tumor cells (8). Autologous bone marrow mesenchymal stem cells (BMSCs) transplantation is a promising tool for tumor treatment (9). In liver disease, for example liver cirrhosis, autologous BMSCs generate hepatocytes and bile duct cells, which can repair damaged liver tissue and therefore prevent liver transplantation (10). This method has become the point of focus in research for HCC biotherapy due to its advantages, including easy collection, ability to avoid rejection reactions, simple and easy transplantation process, low treatment cost and absence of ethical concerns (11,12).

Current research focuses mainly on the biological intervention of BMSCs for the metastatic potential of HCC, which provides a basis for finding suitable biotherapeutic...
targets (13,14). However, there are still numerous uncertainties regarding the efficacy of BMSCs in tumor intervention, including stem cell tumorigenicity, long term interventional efficacy and the influence of stem cells on the biological behavior of tumors, which all require further research and observation (8).

In a previous study, autologous BMSCs were genetically modified to upregulate osteopontin (OPN) and transforming growth factor β1 (TGFβ1) gene expression in order to observe the effect of stem cells on HCC cells with high (MHCC97-H) and low (MHCC97-L) metastatic potentials. OPN promotes tumor metastasis following interaction with integrin αvβ3 and induces cell movement by altering the cytoskeleton, promoting angiogenesis and cell adhesion, and preventing cell apoptosis (15). Exogenous OPN secretion by BMSCs serves a role in promoting tumor invasion by inhibiting the secretion of endogenous OPN in MHCC97-H cells, mainly by activating matrix metalloproteinase-2 (15). Previous studies have also demonstrated that TGFβ1 is associated with tumor proliferation and has different effects on liver cancer cells with high and low metastatic potentials (16), namely, the ability of BMSCs with TGFβ1 to promote MHCC97-L invasion was higher than that for MHCC97-H (17) and MSCs may be capable of enhancing the angiogenesis of HCC, which may be partly due to the involvement of TGFβ1 (18). In the relationship between TGFβ1, OPN and integrin αvβ3, OPN mediates the adhesion of integrin to the extracellular matrix (ECM) and becomes a critical factor in tumor metastasis, while TGFβ1 activates protein kinase B by regulating the expression of integrin linked kinase. TGFβ1 promotes the phosphorylation of focal adhesion kinase tyrosine and activates downstream signaling molecules to directly or indirectly participate in the adhesion of integrin and ECM, which promotes HCC invasion and metastasis (19,20).

Based on previous findings, the current study was designed to determine the effect of reduced OPN and TGFβ1 levels on MHCC97-H metastasis. Therefore, the metastasis-associated gene OPN and the tumor growth-related gene TGFβ1 were silenced using small interfering RNAs (siRNAs) in MHCC97-H cells and the change in metastatic potential was evaluated. Furthermore, the effect of BMSCs on MHCC97-H following gene silencing was also evaluated.

**Materials and methods**

**Instruments and reagents.** Instruments used included: a frozen microtome (HM525 NX; Thermo Fisher Scientific, Inc.), an upright fluorescence microscope (BX43; Olympus Corporation), a refrigerator (BCD-211KD; TCL Corporation), a rotary microtome (RM2235; Leica Microsystems GmbH), a pathological tissue drift Bakeware (Tec 2500; Changzhou Haosilin Instrument Equipment Co., Ltd.), a rotary table scanning confocal microscope (DSU; Olympus Corporation), an electric thermostatic blast drying oven (101-3, Shanghai Jinping Instrument Co., Ltd.), a water-proof constant temperature incubator (PYX-DHS500BS-II; Shanghai Yuejin Medical Devices Co., Ltd.), an electronic balance (FA1104; Shanghai Tianping Instrument Factory), an ultra-low temperature refrigerator (BS-812; Qingdao Haier Co., Ltd.) and a purification bench (SW-CJ-2D; Shanghai Xinmiao Medical Instrument Manufacturing Co., Ltd.).

Reagents used included: mouse polyclonal integrin αvβ3 antibody (L2206; Santa Cruz Animal Health; 1:50), donkey anti-rabbit fluorescent secondary antibody (15316; Thermo Fisher Scientific, Inc.; 1:800; excitation/emission wavelength: 490/520 nm); citric acid antigen recovery solution (pH 6.0; Fuzhou Maxin Biotechnology Co., Ltd.), xylene (Chengdu Kelong Chemical Reagent Factory), anhydrous ethanol (Chengdu Kelong Chemical Reagent Factory) and DAPI (Sigma-Aldrich; Merck KGaA; absorption wavelength/emission wavelength: 358/461 nm). DMEM medium, trypsin and 0.02% EDTA were purchased from Gibco, Thermo Fisher Scientific, Inc. Phosphate buffered saline (PBS) was purchased from China Pharmaceutical Chemicals Co., Ltd. Fetal bovine serum (FBS) was purchased from Biological Industries, blue streptomyccin (100 x double antibody) from Hangzhou Haotian Biotechnology Co., Ltd., and L-Glutamine medium from Shanghai Ruibosai Biological Technology Co., Ltd.

**Cell experiment and culture methods.** MHCC97-H were provided by the Institute of Liver Cancer at Fudan University. MHCC97-H cells were incubated with 10% FBS with 1X DMEM medium at 37°C in a 5% CO2 incubator. Cells were subcultured by washing with PBS, followed by dissociation with 0.25% trypsin and 0.02% EDTA. BMSCs were provided by Saiye Biotechnology Co., Ltd., (cat. no. HUXMA-90011). BMSCs were derived from the bone marrow of healthy adults (18–45 years), purchased from Saiye Biotechnology Co., Ltd., (cat. no. HUXMA-90011). BMSCs were cultured and passed to the 2nd generation in vitro and then stored frozen. BMSCs activity was tested before each experiment, include BMSCs growth state, differentiation ability and cluster differentiation tests. BMSCs were incubated with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) + 1X MSCM (Beijing Yuhengfeng Technology Co., Ltd.) + L-Glutamine medium at 37°C in a 5% CO2 incubator. When cells confluence reached 90%, BMSCs were passaged by washing with PBS, followed by dissociation with 0.25% Trypsin and 0.02% EDTA.

**Animal experiments and feeding conditions.** Animal experiments were approved by the Ethics Committee of the Fourth Medical Center of the Chinese PLA General Hospital. A total of 32 male BALB/c nude mice, aged 4-5 weeks, were provided by Shanghai SLAC Experimental Animal Co., Ltd. The production license no. was SCXK 2012-0002, SPF grade and the animal quality certification no. was 0205939. At the time of purchase, each animal weighed 20±2 g. Sterilized ultrapure drinking water was provided to the mice and the quality of drinking water met the provisions of the People’s Republic of China National Standard for Drinking Water Hygiene (GB5749-2006). The animal maintenance feed was provided by Shanghai SLAC Experimental Animals Co., Ltd., and the standard GB14924.3-2010 ‘Nutrient Components of Compound Feeds for Experimental Animals’ protocol was implemented (21). The laboratory animal room use permit no. was SYXK 2015-0008. The mice were housed at temperatures of 20-25°C and the relative humidity range was 40-70%. Animal lighting 10-20 Lux, light and dark cycle 10/14 h, work lighting 100-200 Lux. Nude mice were adapted for 6 days in the animal room environment before testing. Following animal experiments, the tumor-bearing animals were euthanized.
MHCC97-H gene silencing. Previous results have demonstrated that MHCC97-H overexpresses the metastasis-associated gene OPN and the proliferation-related gene TGFβ1 (5). Therefore, the current study separately interfered with these genes.

Construction of adenoviral vectors: TGFβ1, siRNA1 sequence (GAGGAGTTGAATGGTGCATAC) and OPN siRNA1 sequence (GGCGATACCTCAGCAACCG) and OPN siRNA2 sequence (GAGGAGTTGAATGGTGCATAC) were used to synthesize oligo sequences purified from a PAGE gel, and gene sequences obtained from GeneBank (http://ncbi.nlm.nih.gov/GeneBank/). Vectors were digested with BamHI and EcoRI (New England Biolabs, Inc.) and recovered by cutting. Following annealing at 50°C for 30 sec, single strands of short hairpin RNA (shRNA) 3′ and 5′ fragments were obtained. The shRNA of the target fragment was ligated with the vector and transformed into TGFβ1. The oligonucleotide fragment was diluted 100 times with water for use, and Annealing Buffer (Beijing Solarbio Science and Technology Co., Ltd.) for Oligos (5X) was added. Annealing was performed at 95°C for 5 min, then naturally cooled to room temperature. The ligation reaction solution was as follows: T4 DNA ligase 5 U, linearized vector carrier 2 µl, diluted oligonucleotide 2 µl and 10X ligase buffer (5X) was added. Annealing was performed at 95°C for 5 min, then naturally cooled to room temperature. The ligation reaction solution was as follows: T4 DNA ligase 5 U, linearized vector carrier 2 µl, diluted oligonucleotide 2 µl and 10X ligase buffer 1 µl, made up to 10 µl with water. Consecutive reactions were performed according to the manufacturer's instructions.

Recombinant adenovirus TGFβ1 and OPN shRNA adenoviruses. Recombinant plasmids were prepared and packaged with recombinant adenoviral vectors. The Lentivirus was collected and amplified in ~10^7 cells at an inoculating cell density of 2-5X10^4 293T cells/cm^2 in a 75 cm^2 square bottle containing DMEM +10% FBS. A total of 10 ml of the virus supernatant was added to infect the cells. After 3-4 days, the cells almost became round and half of the cells were in suspension. At this point, all of the cells were collected and centrifuged at 500 x g, 37°C for 60 min and the supernatant was discarded. TGFβ1, and OPN shRNA adenovirus assays were performed and Green Fluorescent protein (GFP, Abcam) was used as a marker gene. PCR was used to identify recombinant viruses. A total of 5 µl virus supernatant was taken and 10 µl protease K was added, incubated at 55°C for 1 h, then boiled for 5 min. After a further centrifugation at 4000 x g, 37°C, 5 min, 1-2 µl was used for PCR. The virus was then used to infect MHCC97-H cells. GFP was used as the marker gene. Expression levels of TGFβ1 and OPN in MHCC97-H cells were measured before and after gene interference using reverse transcription quantitative (RT-q)PCR. RT-qPCR was performed using homo OPN forward, AGGAGGAGGAGGAGGACA and reverse, CTGGTATGGCAGAGGAGTG; and homo TGFβ1 forward, GGCGATACCTCAGCAACCG and reverse, CTAAGGCGAAAGCCCTCAAT. A total of 1X10^6 MHCC97-H cells were collected, lysed and centrifuged (4°C, 12,000 x g, 20 min) using a TRizol®-spin column (Invitrogen; Thermo Fisher Scientific, Inc.). RNA purity was verified by placing 2 µl of RNA solution on the Micro-volume Spectrophotometer SMA4000 (Merinton Instrument, Inc.). RNA was reverse transcribed to cDNA using a High-Capacity RNA-to-cDNA™ kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to operating instructions. β-actin was used as the internal control. The PCR reaction conditions were: 93°C for 2 min, then 93°C for 1 min, and 55°C for 2 min, for a total of 40 cycles. The blank control group of OPN and TGFβ1 were used as the relative quantitative PCR expression levels and were normalized by the internal control (2^ΔΔCq). Relative expression levels were calculated using the 2^ΔΔCq method.

MHCC97-H cell and BMSCs co-culture migration experiment. The Transwell method was used to evaluate the metastatic ability at the cellular level, as it simulates the basement membrane structure of HCC tissue and basement membrane breakdown (23). MHCC97-H blank was set as the control group (Blank control; BC) and MHCC97-H gene interference was the negative control group (Native Contrast; NC), in which GFP gene was transfected as marker gene into the two groups. The experimental groups comprised of the MHCC97H TGFβ1 gene interference and MHCC97-H OPN gene interference groups, with both containing the GFP gene as reporter gene. MHCC97-H cells in log phase were harvested and dissociated with 0.25% Trypsin and 0.02% EDTA. Cells were counted under microscope and 24-well Transwell plates (5.0 µm) were seeded in DMEM medium at densities of 5X10^4 cells/well in the upper chamber and 2X10^5 cells/well in the lower chamber. The plates were incubated at 37°C in a 5% CO2 incubator. After 48 h of incubation, the wells were washed once with PBS and the cells were fixed with 40 g/l paraformaldehyde for 10 min at 37°C. The paraformaldehyde was then removed and the non-migrating cells in the upper chamber were wiped off with a cotton swab. The Transwell inserts were removed, inverted, air dried and rinsed twice with pre-cooled PBS (4°C) and fixed with pre-cooled 100% methanol (4°C) for 10 min. The methanol was removed, and the inserts were incubated for 10 min at room temperature in 200 µl of 0.1% crystal violet (Abcam) that was added to the bottom of each well. Images of three randomly selected fields were taken using an inverted fluorescence microscope (x200; Olympus Corporation; MF53) and cell counts were performed.

MHCC97-H xenograft model. MHCC97-H cells in logarithmic growth phase were collected, centrifuged at 1,000 rpm for 5 min and the cell concentration was adjusted to 2.5X10^7 cells/ml to prepare a single cell suspension in DMEM medium. Nude mice were subcutaneously inoculated with 0.2 ml cell suspension (5X10^6 cells/mouse) of the corresponding group in the right side of the armpit. Light pressure was put on the injection site for 30 sec following injection to prevent leakage of the cell suspension. Anesthesia was performed by intraperitoneal injection of 1% sodium pentobarbital. The dose for nude mice was ~0.3 ml. The method of euthanasia was spinal dislocation.

BMSCs intervention in the MHCC97-H xenograft model and experimental methods. A total of 32 nude mice were randomly divided into four groups: MHCC97-H blank control (BC; n=8), MHCC97-H-NC gene silencing negative control (NC; n=8), MHCC97-H-OPN gene silencing (n=8) and MHCC97-H-TGFβ1 gene silencing (n=8) groups. After the nude mice were inoculated for 14 days and tumor volume was allowed to grow to ~50 mm^3, prior to the initiation of the intratumoral injection of BMSCs (1X10^7 cells/tumor) biweekly for 4 weeks. Pathological observations of tumor tissue were performed using the HE staining method. Sample fixation: 95% ethanol fixation for 20 min and PBS wash twice, 1 min each time. Staining nucleus: Hematoxylin staining for 2-3 min and washing with water.
Staining cytoplasm: Immerse in Eosin staining for 1 min and wash with water. The cells were then air dried and sealed with neutral gum. Observations were performed under a general optical microscope, magnification 200x.

Analysis of lung metastasis in MHCC97-H xenograft model. The lung metastasis animal model of MHCC97-H was performed according to a previous method (24). Briefly, cell lines in the logarithmic growth phase were collected, centrifuged at 1,000 rpm for 5 min and cell concentrations were adjusted to 2.5x10⁷ cells/ml to prepare a single cell suspension. Nude mice were intravenously inoculated via tail vein with 0.2 ml (5x10⁶ cells/mouse) of prepared cell suspensions. Light pressure was applied to the injection site for 30 sec after injection to prevent leakage of the cell suspension.

Analysis of MHCC97-H metastatic lung tumor pathology. The lungs were excised at the experimental end-point (14 day) and fixed in 4% formaldehyde fixation for 3-5 days. The fixed tissues were processed through serial ethanol gradients (50, 70, 80 and 95%) and xylene, and then embedded in paraffin. The lungs were sectioned (6 µm) and stained with DAPI for 10 min at 37˚C, followed by cover slipping with glycerol (cat. no. 228220; Shanghai Canspec Scientific Instruments Co., Ltd.) and PBS. HCC lung metastasis nodules were observed under a fluorescence microscope (magnification, 400). Differences in fluorescence intensity were analyzed using Image J software (v1.52; National Institutes of Health) and the integrated optical density (IOD) value was used as a semi-quantitative measurement index.

Analysis of expression of integrin αvβ3 in nude mice by immunofluorescence. The excised lung tissues underwent xylene dewaxing at 37˚C, gradient alcohol rehydration with xylene twice for 20 min each at room temperature, 100% ethanol twice for 5 min each, 95% ethanol for 5 min, 80% ethanol for 5 min and then washed three times with PBS for 3 min each at room temperature. Samples were rinsed with 0.001 M PBST (Beijing Solarbio Science and Technology Co., Ltd.) for 5 min, 3 times, and blocked with 10% BSA (Thermo Fisher Scientific, Inc.) in a humidified chamber for 30 min. Slides were placed in 0.01 M citrate buffer (pH 6.0) in the microwave for 10 min and allowed to cool to room temperature, followed by three PBS rinses for 3 min for antigen retrieval. Primary antibodies were added to the slides in a dropwise manner and the slides were incubated at 4˚C overnight. The slides were then washed three times for 3 min each with PBS. Secondary antibodies were added drop by drop and the slides were incubated at 37˚C for 60 min and then rinsed three times with PBS for 5 min each. DAPI staining was performed at room temperature for 10 min. The slides were cover slipped with glycerol and PBS, and observed using a confocal microscope (magnification, x150). Immunofluorescence exhibited a positive green expression. Quantitative analysis of integrin αvβ3 expression by positive pixel levels in the metastatic hepatocellular carcinoma cells of lung tissue was analyzed with Image J software (v1.52; National Institutes of Health).

Analysis of animal model test index. Each group of nude mice was weighed 1 week following cell inoculation and tumor volume was measured. Three times a week, the longest diameter (a) and the shortest diameter (b) of the tumor were measured with Vernier calipers and tumor volume (V) was calculated: \[ V(\text{cm}^3) = \frac{1}{2}ab^2 \times 0.5ab^2, \] and a tumor growth curve was plotted. Furthermore, the animals were weighed three times a week, and the animal tumor weight was calculated as: total animal weight - tumor volume (assuming a tumor density of 1). The following calculations were used for analysis: tumor volume inhibition rate (%) = (1-average tumor volume in experimental group/mean tumor volume in control group)

| Item                  | Proliferation-related gene TGFβ1 | Metastasis-associated gene OPN |
|-----------------------|----------------------------------|-------------------------------|
| MHCC97-H before gene interference | 1.000±0.026                      | 1.000±0.135                   |
| MHCC97-H after gene interference   | 0.557±0.062                      | 0.473±0.095                   |

TGFβ1, transforming growth factor β1; OPN, osteopontin; MHCC97-H, MHCC97-H, high metastatic potential hepatocellular carcinoma.
x100%; tumor weight inhibition rate (%) = (1 - average tumor weight in experimental group/mean tumor weight in control group) x100%.

**Statistical analysis.** Experimental data are expressed as mean ± SD. The data were analyzed using SPSS statistical software (v16.0; IBM Corp.). The IOD value quantitative comparison between cell counts was performed using one-way ANOVAs with post-hoc LSD tests, and P<0.05 was considered to indicate a statistically significant difference.

**Results**

**TGFβ1 and OPN expression differences before and after gene silencing in MHCC97-H cells.** The relative quantitative results are shown in Table I. OPN and TGFβ1 expression were clearly decreased in the MHCC97-H cells following gene silencing.

**Cell counts from Transwell assay experiments on MHCC97-H cells.** The results demonstrated that there was a significant difference between the control group compared with the other groups in the number of migrated cells (Fig. 1; F=7.461; P<0.001). This indicated that the number of migrating cells following gene interference was significantly reduced, particularly in the MHCC97-H OPN silencing and MHCC97-H TGFβ1 interference groups (P<0.001).

**BMSC intervention in the MHCC97-H animal model.** After the nude mice were inoculated for 14 days and the tumor volume was >50 mm³, the intratumoral injection of human BMSCs was initiated for intervention. The BMSC intervention groups are shown in Table II. At the end of the experiment, each group of mice was sacrificed and tumors were excised. It was found that the boundary between tumors and surrounding tissues was clear, with slight adhesion between the skin and subcutaneous connective tissue for all mice bearing-tumor. Additionally, capsules were intact.

Comparison of the tumor volumes revealed that the MHCC97-H gene-silenced group had statistically significantly smaller compared with the BC group (P<0.05), indicating that BMSCs had a significant inhibitory effect on tumor volume in the gene-silenced group. Comparison of tumor weights demonstrated that BMSCs had no significant inhibitory effect on tumor weight following gene silencing in the MHCC97-H tumor model.

**Pathological results of BMSC intervention in MHCC97-H xenograft model before and after gene modification.** Increased tumor necrosis was observed in the MHCC97-H BC and MHCC97-H NC groups compared to the MHCC97-H group with OPN and TGFβ1 genes silenced groups (Fig. 2). Fibroproliferation was evident and there was an increased number of mitoses in all groups. Furthermore, there was relatively less fibrosis and mitoses in the MHCC97-H OPN-silencing group, while the MHCC97-H TGFβ1-silenced group had no significant inhibitory effect on tumor volume and tumor weight in the MHCC97-H TGFβ1-silenced groups (volume inhibition rate 60.84%, weight inhibition rate 47.23%).

### Table II. Comparison of tumor volume and weight in hepatocellular carcinoma animal models following 28 days of BMSC cell intervention.

| Group      | Tumor volume (mm³) | Tumor weight (g) |
|------------|--------------------|-----------------|
| MHCC97-H BC| 3064.90±821.78     | 20.52±3.32      |
| MHCC97-H NC| 2776.62±704.36     | 18.90±1.87      |
| MHCC97-H OPN| 1200.25±181.59    | 23.72±2.53      |
| MHCC97-H TGFβ1| 595.83±343.83  | 23.34±2.23      |

Data are presented as mean ± SD. *P<0.01 vs. the TGFβ1 gene silence group. **P<0.05 vs. the OPN gene silence group. BMSC, bone marrow mesenchymal stem cell; MHCC97-H blank control group; NC, negative control group; OPN, OPN gene interference group; TGFβ1, TGFβ1 gene interference group.

### Table III. Analysis of the inhibition rate of hepatocellular carcinoma before and after gene modification in animal models.

| Group      | Tumor volume inhibition rate (%) | Tumor weight inhibition rate (%) |
|------------|---------------------------------|---------------------------------|
| MHCC97-H BC| 0.00                            | 0.00                            |
| MHCC97-H NC| 9.41                            | 4.99                            |
| MHCC97-H OPN| 60.84                         | 47.23                           |
| MHCC97-H TGFβ1| 80.56                       | 84.08                           |

BC, blank control group; NC, negative control group; OPN, OPN gene interference group; TGFβ1, TGFβ1 gene interference group.
group exhibited significantly more fibrosis and mitoses, when compared with the control group.

**Lung metastasis animal model fluorescence imaging results of MHCC97-H.** The results demonstrated that there were more tumor cells in the lung tissues of the BC and NC groups (Fig. 3A and B). There were fewer tumor cells in the OPN- and TGFβ1-silenced groups in the lung tissue compared with the BC group (Fig. 3C and D), indicating that the lung metastasis of gene-silenced MHCC97-H tumor potentially declined.

To further analyze the metastasis of HCC cells in lung tissue, quantitative analysis was performed on the fluorescence intensity of MHCC97-H in pathological sections using Image J software. IOD was used as a quantitative indicator (Fig. 3E). The IOD values of the MHCC97-H cells were lower in the OPN- and TGFβ1-silenced groups compared with the BC and NC groups. This difference was statistically significant (P<0.05), indicating that the lung metastasis ability of MHCC97-H was reduced following interference by OPN and TGFβ1 genes, which was consistent with the results observed using a microscope.

**Immunofluorescence of metastasis-associated integrin αvβ3 expression.** The positive staining for integrin αvβ3 demonstrated a strong green color (Fig. 4A-C). The density of green fluorescent MHCC97-H cells was significantly reduced in the gene interference groups compared with the control group. The fluorescence intensity of integrin αvβ3 expressed in MHCC97-H cells in the lung was quantitatively analyzed using Image J software (Fig. 4D). The results demonstrated that the IOD value of migrated cells was not statistically different among the three groups (P>0.05), indicating that neither OPN nor TGFβ1 gene interference in MHCC97-H altered the ability of the cells to express integrin αvβ3.

**Discussion**

In the present study, two genes were silenced in MHCC97-H cells. OPN is a type of secreted phosphorylated glycoprotein and its molecular structure contains an RGD (Arg-Gly-Asp) polypeptide sequence. By combining with integrin αvβ3 or CD44, OPN participates in important biological processes, such as cell adhesion and signal transduction. Furthermore, it promotes tumor cell metastasis (25). A previous study demonstrated that OPN is significantly overexpressed in MHCC97-H cells, suggesting that OPN is involved in the metastatic behavior of HCC (26). Furthermore, the metastatic behavior of HCC can be delayed by blocking the expression of OPN (27).

TGFβ1 is an important biological factor involved in tumor growth, invasion and metastasis, and in the occurrence and
progression of HCC in terms of proliferation and metastasis (28). TGFβ1 and integrin αvβ3 receptors are associated with tumor metastasis-related signaling pathways (29). Integrin and TGFβ1 receptor-mediated signal transduction pathways share certain signaling molecules, such as Rac1 and ERK, and interact to exert a variety of biological effects, such as cell collagen hyperplasia and cell fibrogenesis (30). Therefore, based on previous studies, the current study silenced OPN and TGFβ1 genes in MHCC97-H. Changes in metastasis and proliferation ability in MHCC97-H cells were observed following BMSC treatment in vitro and in vivo. The Transwell method was used to evaluate the metastatic ability at the cellular level, as it simulates the basement membrane structure of HCC tissue and basement membrane breakthrough, which is a critical step in metastasis (25). Animal models were used to evaluate metastatic ability in a lung metastasis model of MHCC97-H. MHCC97-H cells were injected before and after gene interference in order to increase the concentrations of BMSCs in tumors and the lung metastasis of HCC was observed after 28 days.

The results of the cytology experiments and the animal model indicated that BMSCs migration ability following gene silencing in MHCC97-H cells was significantly decreased, especially following TGFβ1 silencing. This indicated that biological factors associated with OPN and TGFβ1 were involved in HCC metastasis. Furthermore, the
results suggested that BMSCs are more effective at reducing metastasis in gene-silenced MHCC97-H cells. Therefore, the TGFβ1 gene may be the best target for BMSCs to interfere with metastatic behavior in HCC. Previous studies (31,32) have demonstrated that TGFβ1 overexpression may lead to excessive suppression of immune cells, including T and B lymphocytes, by reducing the immune surveillance function of the host, immune response and clearance to invading pathogens and by increasing tumor cell elimination, leading to the occurrence and metastasis of tumors. This mechanism may explain the reason TGFβ1 gene silencing is more conducive to inhibiting the metastasis of HCC. Further previous studies have demonstrated that low doses of TGFβ1 (≤0.25 ng/ml) increases BMSC proliferation, while higher doses of TGFβ1 (≥1 ng/ml) inhibit their proliferation (33). Additionally, there are data that indicate that low doses of TGFβ1 signaling alongside BMSCs may lower their immunomodulatory potential (34). Therefore, with low TGFβ1 expression in HCC, BMSCs may be better at inhibiting the proliferation and metastasis of HCC.

The results for tumor weight and volume indicated that BMSCs have a positive effect on the inhibition of HCC tissues growth after gene silencing. The lack of significant change in tumor weight may be explained by the pathological results, as tumor necrosis and fibrosis increased, and number of mitoses decreased in the experimental groups compared with the control group. Therefore, the reason that the tumor weight did not change significantly was associated with the increase in fibrous tissue in tumors with necrotic components. Furthermore, solid tumor weight inhibition ratios were different from tumor weight measurements. When the tumor inhibition rate was calculated, the weight of the animal itself was taken into account. Because of excessive nutrient consumption of tumor-bearing nude mice, the total weight of the animals reduced (35). However, changes in tumor volume inhibition were consistent with the trend of simply measuring volume changes. Moreover, OPN silencing promoted cell apoptosis, delayed tumor cell proliferation and promoted fibrous tissue proliferation, a result that is consistent with that of Zhu et al (36). Following TGFβ1 silencing, the promotion of
tumor cell proliferation was weakened and replaced by fibrous tissue proliferation, which was consistent with the function of TGFβ1, reported in the literature (37). The ability of metastasis and proliferation of gene-silenced MHCC97-H cells and animal models following BMSC intervention was markedly reduced. These data were confirmed by the lung metastasis fluorescence imaging in the MHCC97-H animal model.

In order to further explore the metastatic potential changes in gene-silenced MHCC97-H, the expression of the key biological molecule integrin αβ3 in HCC tissue with lung metastasis was analyzed. However, the experimental results revealed no significant changes in the expression of integrin αβ3 in MHCC97-H cells in the lungs of the OPN- and TGFβ1-gene-silenced groups compared with the control group. This may be related to the expression of integrin αβ3. The vascular endothelium, as well as the cancer tissue, is also involved in the expression of integrin αβ3 (38). Thus, the decrease in metastatic potential following gene silencing in MHCC97-H was not associated with integrin αβ3 expression, but was related to the decrease in OPN and TGFβ1 expression. At lower levels, OPN and TGFβ1 do not bind to integrin αβ3 (39). Therefore, it is possible that low OPN and TGFβ1 expression in MHCC97-H may reduce metastasis by other mechanisms. Previous findings have suggested that OPN promotes tumor proliferation and metastasis by mediating TGFβ1-dependent mesenchymal stem cell-to-fibrocyte transformation mechanisms (40). Another previous study investigated the relationship between TGFβ1 and MHCC97-H metastasis and the results demonstrated that high TGFβRII expression may inhibit the proliferation, migration and invasion of MHCC97-H (41).

BMSCs have different efficacies for HCC interventions, such as influencing tumor progression, and these differences may be related to the tumor microenvironment (13). Numerous factors may be involved in BMSC intervention in tumors. MCP-3 can increase the migration of liver cancer cells with different metastatic potential and STRAIL can promote apoptosis of liver cancer cells (42,43). In the present study, TGFβ1 and OPN were involved in the proliferation and metastasis of tumors. The results of the current study indicated that the efficacy of BMSCs intervention in MHCC97-H following gene silencing was significantly increased compared with the experimental control group.

In conclusion, the current study employed gene silencing methods and the results revealed that the reduction of OPN and TGFβ1 expression led to reduced metastatic potential in MHCC97-H. The metastatic potential and proliferative potential of MHCC97-H following BMSC intervention were significantly reduced both in vitro and in vivo, particularly in the TGFβ1-silenced group. The reduced metastatic potential following gene silencing in MHCC97-H was not related to integrin αβ3 expression. Therefore, OPN and TGFβ1 factors may be considered to be targets for BMSC intervention, of which TGFβ1 may be the best targets. Since the regulation mechanism of MHCC97-H metastatic potential was not further studied, research into stem cells in metastasis mechanisms in HCC is necessary. The current study provides a basis for treatment of HCC using BMSCs. Future research should involve searching for a suitable gene that inhibits the metastasis and proliferation of HCC.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
TL designed the experimental trial, acquired and analyzed the data, and drafted the manuscript. BZ designed the clinical trial, collected experimental data and analyzed the data. LS and YZ acquired data and drafted the manuscript. YF contributed to the conception and design, administrative support. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal experiments were approved by the Ethics Committee of the Fourth Medical Center of the Chinese PLA General Hospital.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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