Original Article
Mesenchymal stem cells inhibited development of lung cancer induced by chemical carcinogens in a rat model

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Abstract: Mesenchymal stem cells (MSCs) may play a significant role in carcinogenesis; however, data have shown that MSCs can both promote and inhibit tumor growth. We investigated the effect of MSCs on the development of lung cancer in a rat model. Bone marrow-derived MSCs were isolated from male Wistar rats and fluorescently labeled. Genotoxic carcinogens 3-methylcholanthrene (MCA) and diethylnitrosamine (DEN) were instilled into the left lung lobes of female rats to induce tumors. Labeled male MSCs were infused into the female rats via tail vein, and the rats were sacrificed on days 3 and 7. MSC survival and distribution were detected by PCR and fluorescence, respectively. Labeled MSCs aggregated at the injection site in the left lobe (MCA/DEN-treated) on day 3 but not the untreated right lobe. Survival of the MSCs in vivo was confirmed by detection of the male SRY gene in lung tissues by PCR at day 3; however, by day 7, lung tissues were SRY-negative. Next, carcinogen-treated rats were divided into two groups and infused with normal MSCs (experimental group) or PBS (control group) every week for 10 weeks, then sacrificed. Cell proliferation in lung tissues was calculated by Ki67 and PCNA expression. Eighty-percent (8/10) of rats in the control group had tumors, while none of the rats in the experimental group had tumors. There was no difference in cell proliferation in lung tissues between the groups. Therefore, bone marrow-derived MSCs prevented development of carcinogen-induced lung cancer in a rat model. Additional studies are needed to determine mechanism.

Keywords: Lung cancer, mesenchymal stem cells, carcinogenesis, rat model

Introduction

In the last 10 years, an increasing number of studies have shown that mesenchymal stem cells (MSCs) possess pluripotent properties and the ability to differentiate into many lineages of cells. In addition, they also have tropism for tumors, can differentiate into tumor-associated fibroblasts, and develop fibroblast networks within the tumor stroma [1], thus, playing an important role in carcinogenesis [2-6]. However, the role played by MSCs during tumor formation and the mechanisms underlying the tumor-MSC interaction are not completely understood [7-10].

Most investigators agree on the tropism of MSCs for tumors and have proposed that MSCs may serve as target-delivery vehicles for vari-

ous antitumor agents, such as cytokines, receptor apoptosis inductors, interferons, chemical agents, and oncolytic viruses [10, 11]. Presently, there are many conflicting studies showing that MSCs can both promote and inhibit tumor growth. The reasons for such discord among research findings remains unclear as yet, and vary greatly. Still MSCs are most frequently derived from bone marrow (BM) and adipose tissue. Tissue-derived MSCs differ from BM-derived MSCs in a number of characteristics, which may explain the existing discrepancies in the data about the biological properties of these cells [12].

BM-derived MSCs have been thoroughly investigated. The spectrum of their surface markers has been established, and their adhesive properties and capacity to differentiate into other

cells of the mesenchymal lineage (adipose, muscle, cartilage, and bone) under the impact of differentiation factors are known [8, 13]. Moreover, the tropism for tumors and transformation into differentiated fibroblasts have been shown [14, 15]. MSCs in general and from the BM in particular support regeneration and hematopoiesis; however, the function of native BM-MSCs remains unknown. When an organ is injured, MSCs can differentiate into tissue elements, support the formation of new vessels, and synthesize cytokines and growth factors, thereby stimulating regeneration and recovery of the damaged tissues. The recovery potential of MSCs has been established in many diseases, including diabetes, stroke, and Parkinsonism [16]. MSCs play a similar role in malignancies where they perform recovery functions while differentiating into tumor fibroblasts and pericytes and, possibly, into endothelium-like or endothelial cells.

The aim of the present work was to study the effect of BM-derived MSCs on the development of lung cancer induced by chemical carcinogens in a rat model. Our results indicated that BM-derived MSCs inhibited the development of lung cancer induced by MCA and DEN in rats; thus, MSCs depressed the formation of lung cancer induced by chemical carcinogens.

Materials and methods

Cell cultures

Rat bone marrow-derived MSCs were isolated from 6-8 week-old Wistar male rats under the protocol approved by the Tangdu Hospital of the Fourth Military Medical University (Xi'an, China). Mononuclear cells were separated by centrifugation over a Histopaque gradient (Sigma-Aldrich, St. Louis, MO, USA), suspended in regular growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS]; HyClone, GE Healthcare, UK), 0.58 mg/Ml L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and 40 U/mL gentamicin, and cultured in flasks, incubated at 37°C in 5% CO_2 in saturated humidity. After 3 days, non-adherent cells were removed by washing with phosphate buffered saline (PBS), and monolayers of adherent cells were cultured until they reached confluence. The cells were then trypsinized (0.25% trypsin with 0.1% EDTA) and subcultured. When MSCs were 80% to 90% confluent in the culture flasks, the cells were incubated for 3 to 4 days. MSC conditioned medium then was collected, filtered through a 0.22-μm filter, and stored at -80°C.

Immunophenotyping of MSCs

To confirm the identity of the MSCs, the cells were immunophenotyped by flow cytometry (Cell Lab Quanta SC; BeckmanCoulter, Brea, CA, USA) for markers common to MSCs (CD29, CD44H, CD45 (Abcam)). Cell passages 3 to 6 were used for all experiments.

### Labeling of MSCs with Hoechst 33342 and DiI

L-DMEM with 10% FBS was added to trypsinized cells at passages 3-6 trypsinized (0.25% trypsin with 0.1% EDTA) to form an MSC suspension. Hoechst 33342 was then added to the suspension to a final concentration of 2 μg/ml and incubated at 37°C for 15 minutes. After two washes with L-DMEM containing 10% FBS (1500 rpm, 3 min), the cells were labeled with 5 μg/ml DiI as done for Hoechst. Double-labeled MSCs were resuspended with PBS at a concentration of 10^6/ml. Labeled MSCs (1 ml per rat) were infused via tail vein into the induced female rats.

Survival and distribution of MSCs in the rat carcinogenesis model

Female Wistar rats (6-8 weeks old, 200±20 g) were purchased from the animal center of Tangdu Hospital, Fourth Military Medical University (Xi'an, China). Rats were housed in plastic cages containing white flake bedding, at a constant temperature of 25°C, with a relative humidity of 40%, and a 12 h dark/light cycle. Food and water were given *adlibitum* throughout the study. All experiments and procedures performed on animals were approved by the laboratory animal production and use committee of the Xi'an City Department of Science and Technology (Institutional Animal Care and Use Committee).

MCA and DEN (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in iodized oil at stock concentrations of 100 mg/ml and 0.1 ml/ml, respectively. After an acclimation period, animals were randomly assigned to two groups. The lefts lungs of 10 animals assigned to Group 1 were instilled with MCA and DEN using a pre-
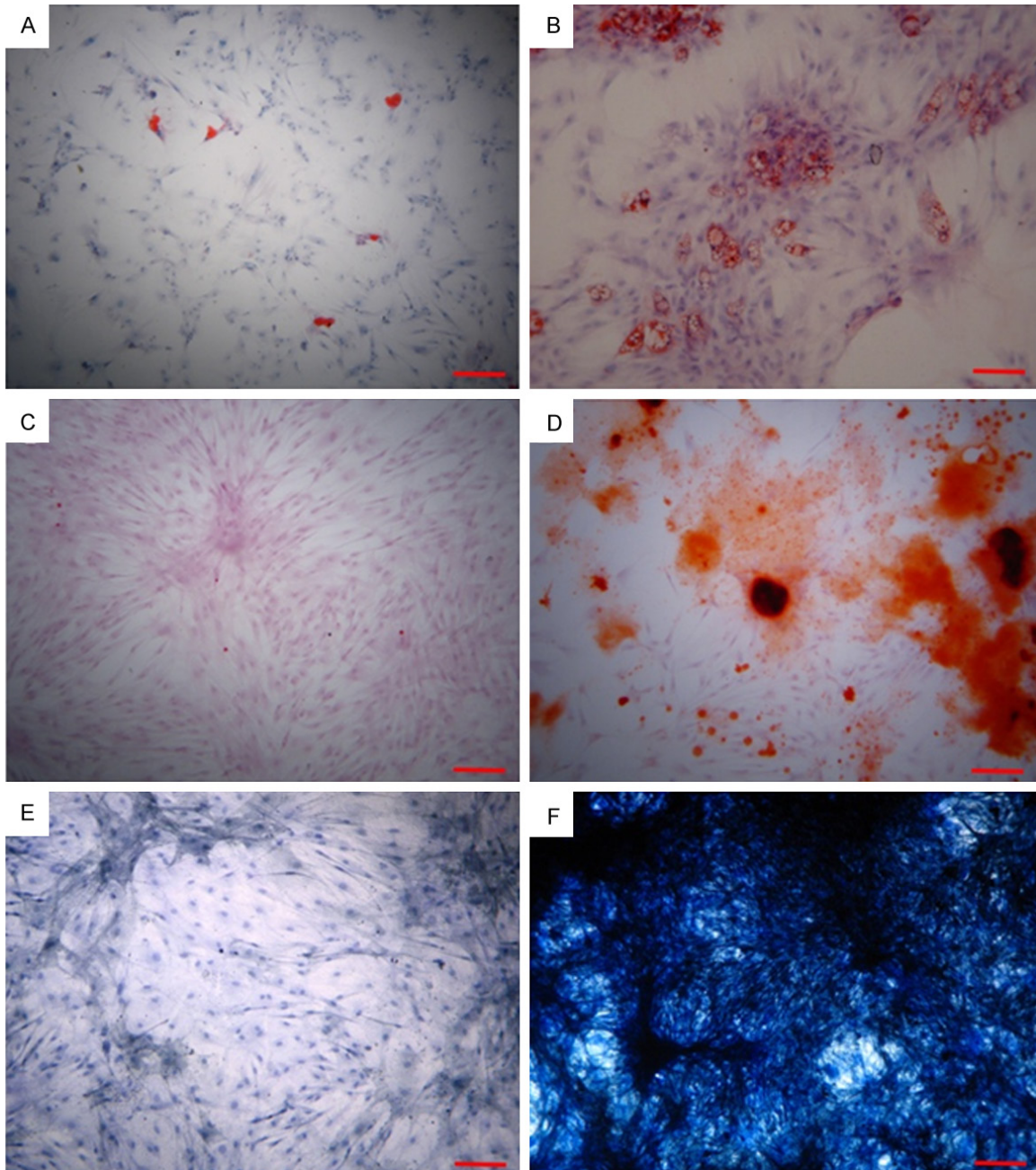
Figure 1. Morphology and differentiation ability of rat BM-MSCs. A. MSCs cultured in adipogenesis medium for 7 days and stained with red oil O. B. MSCs cultured in adipogenesis medium for 14 days and stained with red oil O. C. After 7 days of culture in osteogenic medium, white dots were observed outside of the MSCs. D. MSCs cultured in osteogenic medium for 14 days and stained with alizarin. The white spots outside of the MSCs gradually grew as sheets were confirmed as calcium tubercles by alizarin staining. E. Normal MSCs were stained with alkaline phosphatase (ALP) as a negative control. F. After culture in osteogenic medium for 14 days, MSCs were stimulated to differentiate into osteoblasts. The cells showed strong ALP staining. Scale bar =200 µm.

viously established method; the doses of carcinogens used to induce lung cancer in these rats were 50 mg/kg MCA and 50 mg/kg DEN (Liu et al., 2008; Tian et al., 1984). Briefly, after the animal was anesthetized completely, it was secured to a slanted surgical board with its mouth open. In this position, 0.1 ml carcinogen-containing iodized oil was delivered through a blunt ZY-type 12-gauge needle into the lower portion of the left lung of each rat at
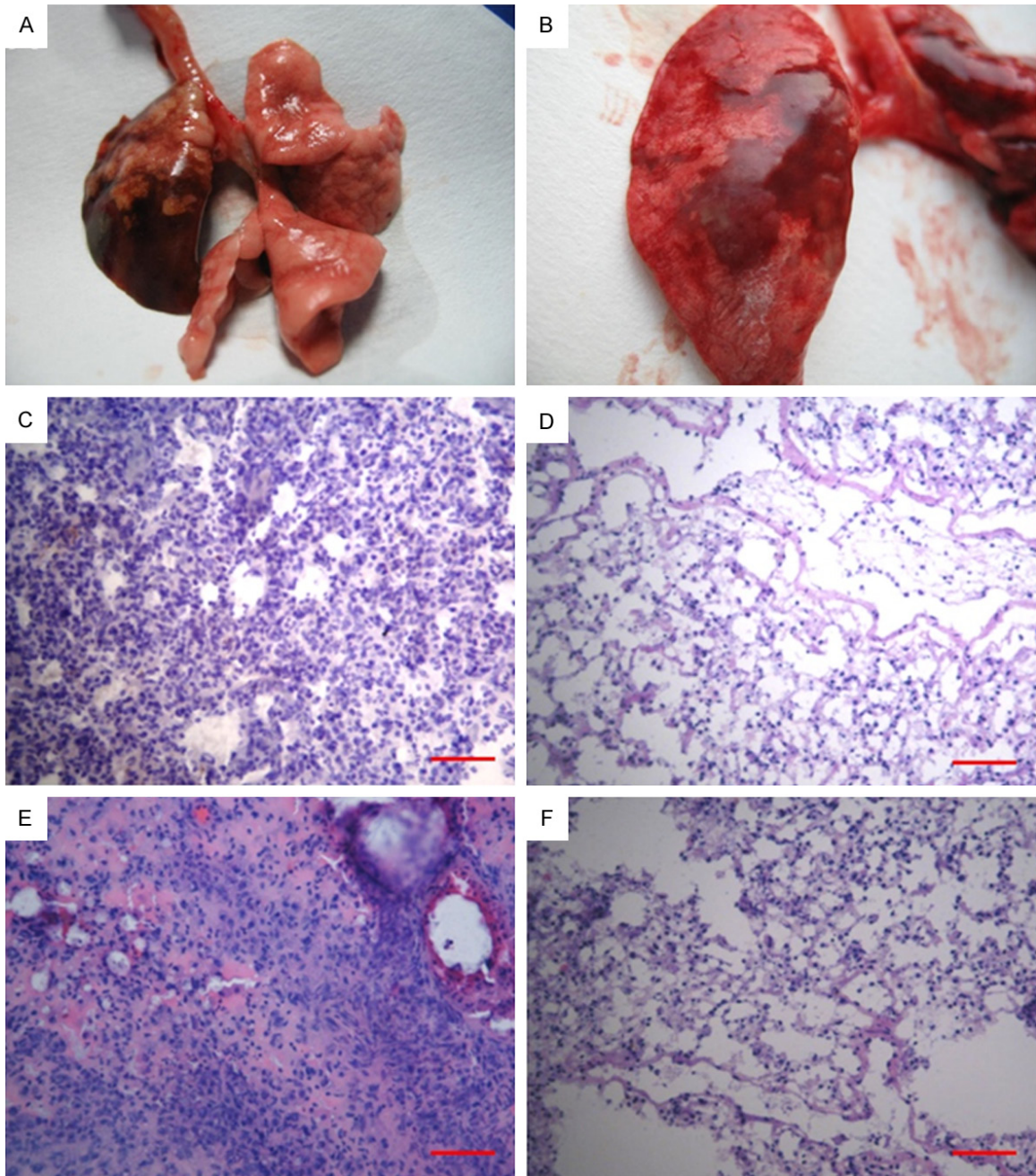
Figure 2. Histological and morphological changes in MCA/DEN-induced rat tumors. A. Morphology of the left lobe 3 days after MCA/DEN injection. B. Morphology of the left lobe 7 days after MCA/DEN injection. C. H&E staining of left lobe 3 days after MCA/DEN injection. D. H&E staining of right lobe 3 days after MCA/DEN injection. E. H&E staining of left lobe 7 days after MCA/DEN injection. F. H&E staining of right lobe 7 days after MCA/DEN injection. Scale bar =100 μm.

the end of an expiration. The three animals assigned to Group 2 were instilled with 0.1 ml iodized oil without any carcinogen into the left lung as a control. All rats were given streptomycin (250 mg/kg bodyweight) and penicillin (100,000 U/kg bodyweight) by intramuscular injection for 1 week after instillation to prevent infections due to the procedure.

Tissue preparation and histopathologic evaluation

Rats were sacrificed by exsanguination under anesthesia on days 3 and 7 after carcinogen treatment. Upon sacrifice, the heart, liver, spleen, kidneys, and left lung were immediately excised. The left lungs were split into two
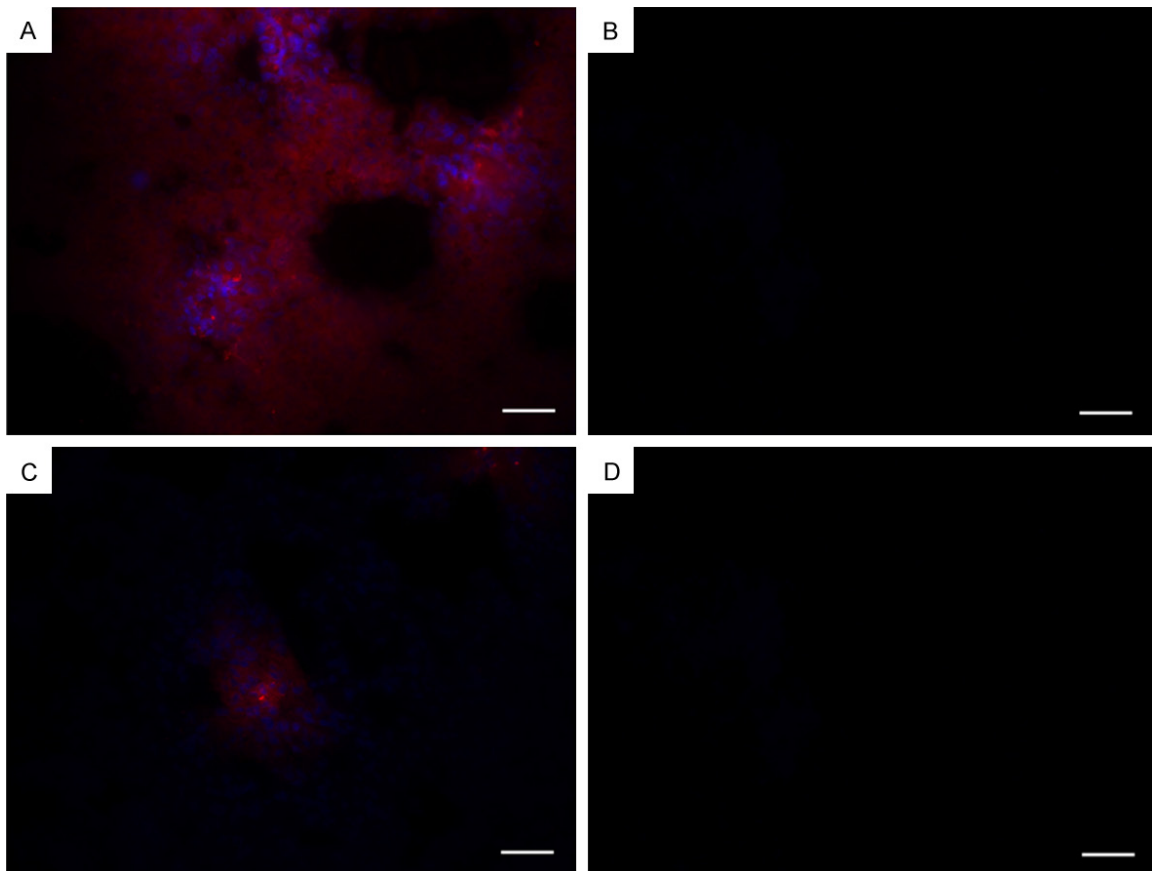
Figure 3. Distribution of MSCs in lung of rats treated with MCA/DEN. A. Many Hoechst 33342- and DiI-stained MSCs were detected on day 3 in the injected left lobe using fluorescent microscopy. B. No fluorescence signal was found in the right lobe on day 3. C. The amount of Hoechst 33342- and DiI-stained MSCs declined dramatically by day 7. D. No fluorescence signal was found in the right lobe on day 7. Scale bar =50 μm.

halves. One was rapidly frozen in liquid nitrogen and stored at -80°C. The other was fixed by immersion in 4% formaldehyde in phosphate buffer for 24 h, embedded in paraffin, sectioned to 4 μm thickness, and routinely processed for hematoxylin and eosin (H&E) staining. H&E-stained slides were microscopically examined and lung tissues were categorized as normal bronchial epithelium, hyperplasia, squamous metaplasia, dysplasia, carcinoma *in situ* (CIS), or infiltrating carcinoma according to the criteria described previously.

DNA extraction and SRY detection

Cryopreserved left lung tissues were collected into lysis buffer with Proteinase K (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Tween-20, 1 mg/ml ProteinaseK), digested at 55°C overnight, and incubated at 95°C for 10 min to inactivate the Proteinase K. The DNA was precipitated with

ethanol, resuspended in 20 μl ddH$_2$O, checked for concentration and purity using a NanoDrop ND-1000 (Thermo Fisher). Total extracted DNA was incubated with primers against SRY and β-actin. The primer sequences were as follows: SRY Forward 5'-ATGGCCACAGAGGAG-3', SRY Reverse 5'-CTGGTTCTTGGAGGA, β-actin Forward 5'-3'TCAGGTCATCACTATCGGCAAT, and β-actin Reverse 5'-3'AAAGAAAGGGTGTAAAACG-CA.

Effect of MSCs on development of lung cancer in rats treated with MCA/DEN

Twenty female rats treated with MCA/DEN were randomly divided into two groups. MSCs (1 ml at 10^6/ml) were injected into the 'Physiological Saline + MSC' rat group (n=10 rats) via tail vein every week for 10 weeks. As a vehicle control, 1 ml PBS was injected into the 'Physiological Saline' group (n=10 rats). All of the rats were
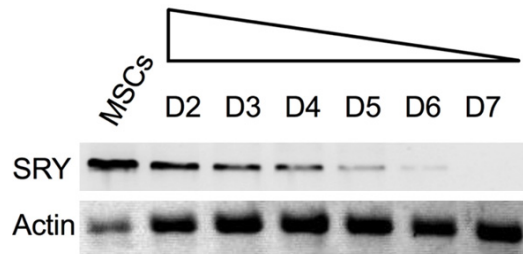
Figure 4. Survival time of injected MSCs was about 7 days. The amount of infused MSCs that localized in the left lung lobe declined over time and had virtually disappeared by 7 days after infusion.

sacrificed by exsanguination under anesthesia at the 10th week and the MCA/DEN-treated left lobes were harvested for histological evaluation.

Immunohistochemistry for dividing cells

Immunohistochemistry was performed as previously described [17]. Briefly, slides were deparaffinized and subjected to antigen retrieval (10 mM citrate buffer, pH 6.0, 95°C for 15 min) and quenching of endogenous peroxidase activity (0.3% H_2O_2 for 15 min). After blocking nonspecific protein binding (10% normal goat serum for 15 min), the samples were incubated at 4°C overnight with a mouse monoclonal antibody against PCNA (1:100; Thermo Fisher, Waltham, MA, USA) or Ki67 (1:100; Thermo Fisher).

Statistical analysis

Statistical analyses were performed with the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Fisher's exact test was used to establish significant differences between groups. Differences were considered statistically significant *P*-value <0.05.

Results

Features of rat BM-MSCs

Isolated rat BM-MSCs showed a spindle-like morphology and were able to differentiate into adipocytes (**Figure 1A**, **1B**) and osteoblasts (**Figure 1C-F**) after 14 days in defined culture media. Flow cytometry revealed that rat BM-MSCs were CD29 (99.4%) and CD44H (99.7%) positive and CD45 (2%) and CD34 (1.9%) negative (Figure S1A-D). All of these results were consistent with the minimal criteria for MSC identification [13].

*Histological and morphological changes in lungs from rats treated with MCA/DEN*

Rats treated with carcinogens had no obviously pathological lesions in any of the main organs, except the treated lungs. Inflammation was found at the injected site in rats sacrificed at day 3 (**Figure 2A**). Greater inflammation was noted in the rats sacrificed at day 7 (**Figure 2B**). No pathological changes were found in any of the control rats. We observed tissues with different morphological features representative of hyperplasia, squamous metaplasia, dysplasia, CIS, and infiltrating carcinoma (**Figure 2C**, **2E**). We captured the multistep process of rat lung tumorigenesis by selecting sacrificing animals at different times following instillation of the carcinogens. All of the lung cancers induced by MCA/DEN were diagnosed as squamous cell carcinoma by two independent pathologists.

Distribution of BM-MSCs during the development of MCA/DEN-induced lung cancer

Rats were sacrificed by exsanguination under anesthesia on days 3 and 7 after instillation. Spherical nodules and inflammation were macroscopic at the injection site in the rats sacrificed at 3 days. Fluorescence imaging *ex vivo* and histological analysis of the left and right lung tissues were performed. Tubercles with fluorescent dye aggregation were found at the injection site in the left lung lobes on day 3 after infusion with labeled MSCs (**Figure 3A**), while neither nodules nor fluorescently labeled cells were seen in the right lung lobes (**Figure 3B**). The same phenomenon was observed on day 7; however, the inflammatory reaction and fluorescence intensity had dramatically declined by this time point (**Figure 3C**, **3D**).

Detection of the SRY gene by PCR to confirm MSC survival in vivo

The SRY gene is a male-specific gene used as a sex determination biomarker [18-20]. After male rat MSCs were infused into female rats via tail vein, PCR was carried out on tissue DNA extracted from the left lung lobes on days 3 and 7, respectively. Gel electrophoresis results showed that lung tissues from MSC-infused rats were SRY-positive from day 2 to day 6, and the copy number decreased over time until day 7 when it was no longer detectable (**Figure 4**). This result indicated that the MSCs injected via
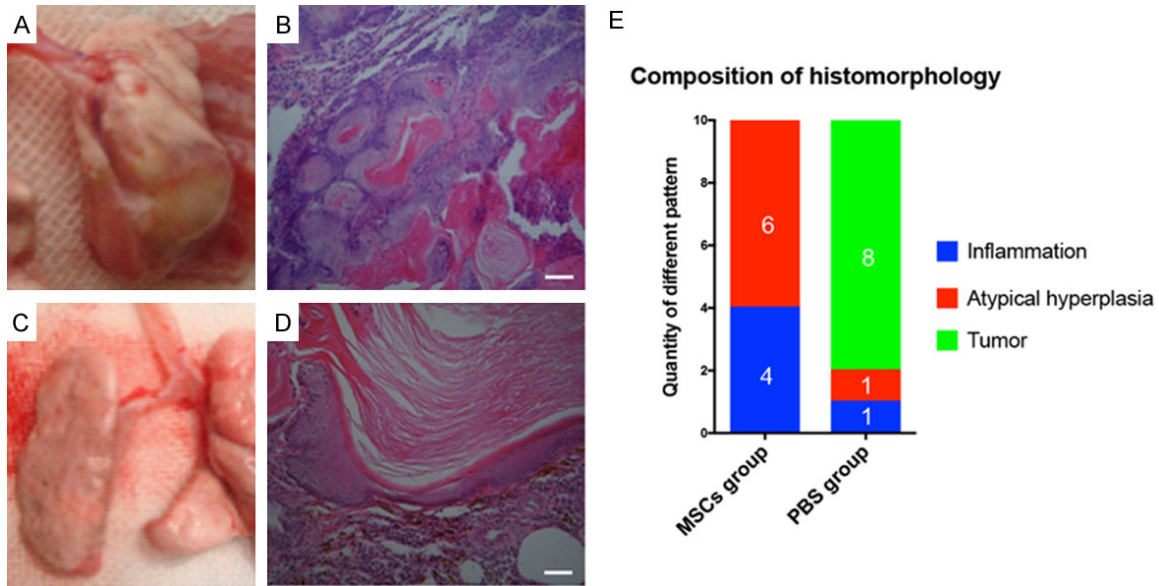
Figure 5. The effect of MSCs on development of tumors induced by injection of MCA/DEN in rats. A. Morphology of representative tumor in the 'Physiological Saline' control group. B. H&E staining shows a typical squamous cell of the 'PBS' control group. C. Morphology of the left lobe of after MSC injection. D. Hyperplasia was the major tissue finding by H&E staining. E. Injection of MSCs prevented tumor formation in rats induced with MCA/DEN. Scale bar =100 μm.

tail vein could directly traffic to the MCA and DEN-injected lung lobes, although they survived less than 7 days.

Effect of MSCs on tumor development

Twenty female rats treated with MCA/DEN were randomly divided into two groups. MSCs (1 ml at 10^6/ml) were infused into the 'Physiological Saline + MSC' rat group (n=10 rats) via tail vein every week for 10 weeks. As a vehicle control, 1 ml PBS was infused into the 'Physiological Saline' group (n=10 rats). All of the rats were sacrificed by exsanguination under anesthesia at the 10th week and the injected left lobes were harvested for histological evaluation. The MCA-DEN treatment resulted in tumors in 80% (8/10) of rats in the control group (**Figure 5A, 5B, 5E**). In contrast, no tumors were observed in the MSC-injected group (0/10) (**Figure 5C, 5D**). Most of MSC-injected rats showed evidence of atypical hyperplasia (6/10) and inflammation (4/10) in the left lobe (**Figure 5D, 5E**).

*Effect of MSCs on tumor cell proliferation in the lungs*

Immunohistochemical analysis was performed on lung tissue sections of animals in the 'Physiological Saline + MSC' group to identify Ki67 and PCNA expression of dividing cells as evidence of tumor cell proliferation *in vivo*. Lung tissues from the 'Physiological Saline' group were used as control. PCNA and Ki67 were highly expressed in tissue samples with squamous cell carcinoma and atypical squamous cell hyperplasia (P>0.05) (**Figure 6A-D**). There was no significant difference between these squamous cell carcinoma and atypical squamous cell hyperplasia with respect to the expression level of Ki67 (25.7±11% vs. 26.9± 6.6%, respectively) and PCNA (91.7±2.7% vs. 87.6±7.3%, respectively) (**Figure 6E**).

Discussion

We investigated the effect of MSCs on lung cancer induced by genotoxic carcinogens. Our findings provided evidence that BM-MSCs inhibited the development of lung cancer induced by MCA and DEN in rats.

We observed that BM-MSCs directly localized to MCA/DEN-injected lobes and not contralateral lung lobes. When an organ is injured or inflamed, MSCs are capable of differentiating into tissue elements, thereby stimulating regeneration and recovery of the damaged tissues. Many investigators have shown the immune-suppressive properties of MSCs when used as
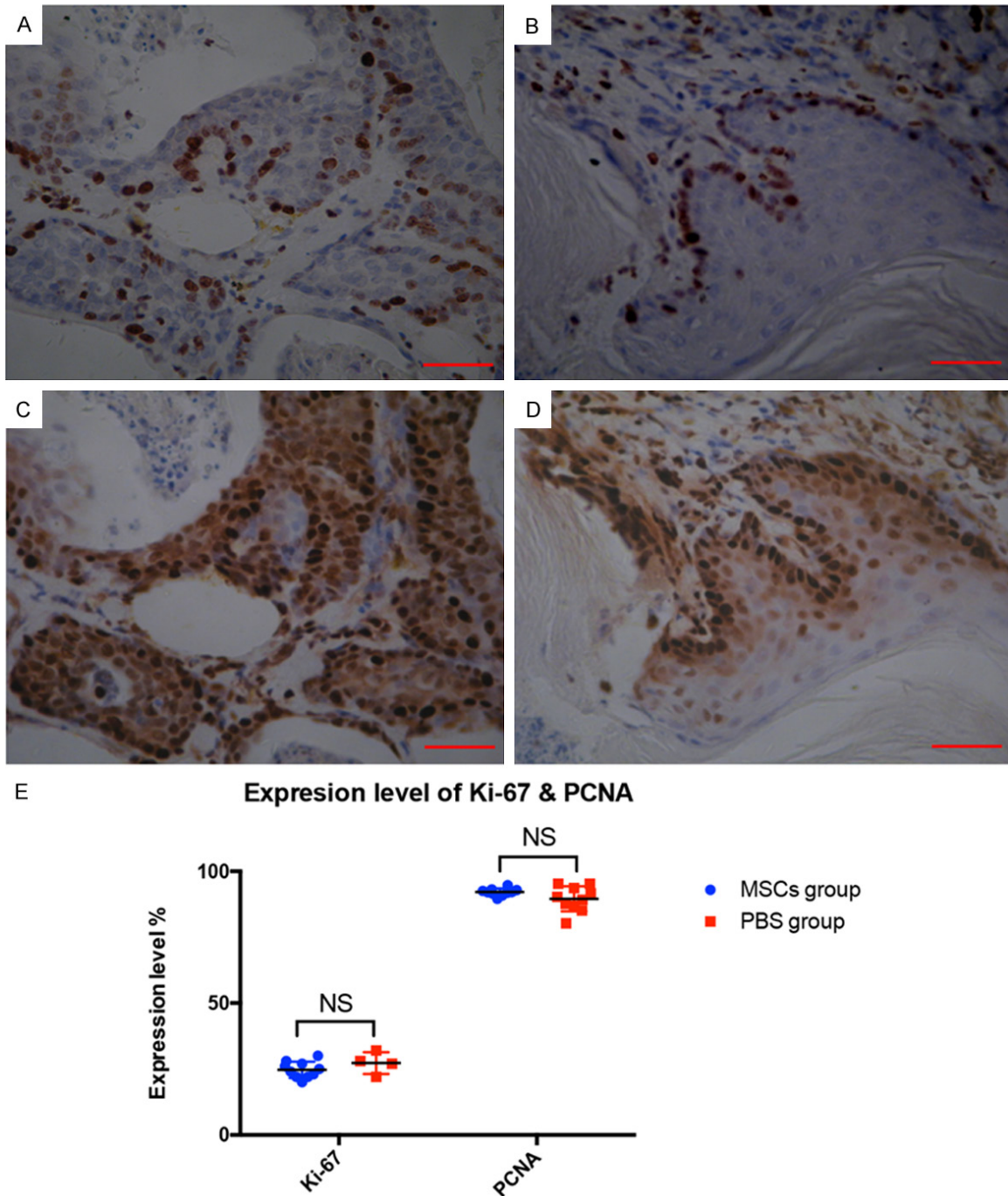
Figure 6. Injection of MSCs did not affect cell proliferation *in vivo*. A. Ki-67 had relatively high expression in hyperplastic epithelium in the 'PBS' group. B. Ki-67 was expressed at a similar level in squamous cells in the MSC-injected group. C. PCNA was highly expressed in hyperplastic epithelium of the 'PBS' group. D. MSCs injected group had the same expression level of PCNA in squamous cells. E. There was no significant difference in Ki-67 and PCNA expression levels between the MSCs and PBS groups. Scale bar =60 μm.

a therapeutic tool for suppression of graft-versus-host disease in BM transplant patients [21, 22]. After injection of MCA/DEN, large amounts of yellow-green purulent exudate were found in the left bronchus. The instillation of MCA/DEN produced an obvious inflammatory reaction, which could have been caused by occlusion due to the iodine oil mixture (vehicle). Therefore, both MCA/DEN and inflammation might have contributed to the localization of MSCs to the injection site.

After infusion of male rat MSCs into female rats via tail vein, SRY-positive cells (MSCs) were

detected on day 3 but not on day 7, which indicated that MSCs could survive between 3 and 7 days after allogeneic transplantation. Others have demonstrated that allogeneic MSCs can survive long-term in the host and differentiate into bronchial and alveolar epithelial cells in animal models of bleomycin-induced lung injury [16, 21, 22]. The mechanisms of lung injury cause by chemical carcinogens used in this study and intravenous injection of bleomycin are entirely different. Furthermore, MCA/DEN had no effect on the inflammatory response, unlike bleomycin. These differences may explain the differences in status of MSCs between these two models.

Tumor formation occurred in 80% of control rats and 0% of MSC-infused rats, which indicated that MSCs inhibited rat lung tumor development induced by MCA/DEN. Based on the observation that MSCs aggregated at the site of MCA/DEN infusion, and the >3 days survival time of MSCs in lung, we infused MSCs via caudal vein at weekly intervals for 10 weeks to ensure the persistence of MSCs. As mentioned above, there is no general opinion among investigators regarding the mechanism of action of the MSCs on the malignant process [8]. In our study, multiple infusions of MSCs likely inhibited the local inflammatory response and thus reduced tissue damage and carcinogenesis. The infusion of exogenous MSCs probably enhanced repair of injured tissue and inhibited tumor formation; however, we still do not know exactly how MSCs specifically are involved in regulating tissue damage repair and tumor formation. More studies are needed to answer this question.

Ki67 is a nucleoprotein antigen expressed during cell proliferation that also is involved in ribosomal RNA transcription [23, 24]. PCNA is another nucleoprotein antigen that localizes in eukaryotic cell nuclei and participates in DNA extension [25]. Hence, Ki67 and PCNA are used as an index of cell proliferation. We observed that PCNA and Ki67 were highly expressed in squamous cell carcinoma and atypical squamous cell hyperplasia (P>0.05), which indicated a high amount of cell proliferation in both the experimental and control groups due to the carcinogen treatment of the lungs. Although H&E staining of lungs from the experimental group showed atypical hyperplasia, dysplasia still turned into cancer under the continuous

induction of carcinogens. As a consequence, there was no histological difference between the experimental and control groups. However, the development of tumors in 80% of control rats compared to 0% in the experimental group illustrated the effect of MSCs on the development of lung cancer induced by MCA and DEN in rats.

Acknowledgements

Disclosure of conflict of interest

None.

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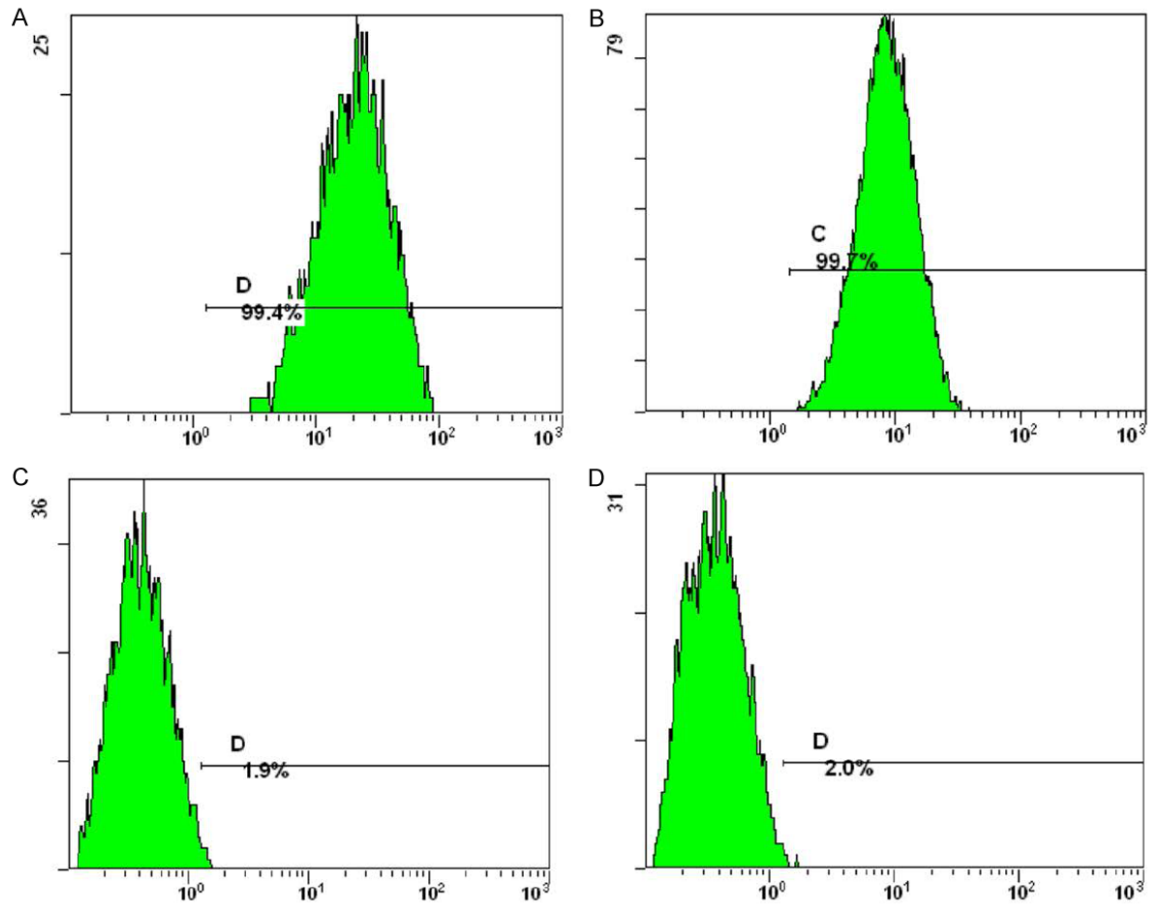
Figure S1. Flow cytometry results of molecular biomarkers of BM-MSCs. A. CD29 on BM-MSCs. B. CD44H on BM-MSCs. C. CD34 on BM-MSCs. D. CD45 on BM-MSCs.