

## Original Article

# Systemically transplanted human gingiva-derived mesenchymal stem cells contributing to bone tissue regeneration

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**Abstract:** As novel postnatal stem cells, gingiva-derived mesenchymal stem cells (GMSCs) have been considered as an ideal candidate cell resource for tissue engineering and cell-based therapies. GMSCs implanted into sites of injury have been confirmed to promote the injury repair. However, no studies have demonstrated whether systemically transplanted GMSCs can home to the bone injuries and contribute to the new bone formation in vivo. In this study, we transplanted human GMSCs into C57BL/6J mice with defects in mandibular bone via the tail vein to explore the capacity of transplanted GMSCs to promote bone regeneration. Results showed that the transplanted GMSCs were detected in the bone defects and employed in new bone formation. And the newly formed bone area in mice with GMSCs transplantation was significantly higher than that in control mice. Our findings indicate that systemically transplanted GMSCs can not only home to the mandibular defect but also promote bone regeneration.

**Keywords:** Gingiva, mesenchymal stromal cells, bone regeneration, mice

## Introduction

Bone injuries in the oral and maxillofacial area due to trauma, tumor resection and infectious diseases may have a serious impact on masticatory function, facial aesthetics and psychological health of patients. Demand for physiological and functional reconstruction is pressing. The optimal bone reconstruction for healing is the regeneration of the damaged tissues. Similar to embryonic development the process of bone regeneration for repair involves a series of cellular events, the most important of which is mesenchymal stem cells (MSCs) recruitment to the wound site as well as proliferation and differentiation into osteoblasts and chondrocytes [1, 2]. As stem cells especially MSCs present a great hope for repairing almost all type of tissues, the cell-based therapies have been a promising alternative for bone healing [3].

Because of the ability of self-renewal and multiple differentiation potential, bone marrow

stromal cells (BMSCs) have played a significant role in cell-based therapy and tissue engineering in the past years. However, the disadvantages in cell isolation, aging and limited proliferative property restrict the utility of BMSCs for clinical application [4, 5]. In addition, MSCs derived from various dental tissues such as dental pulp, periodontal ligament and dental follicles have also shown the potential to differentiate into many cell types [3, 6]. And yet these cells are not easily obtained or cultured. Gingiva-derived mesenchymal stem cells (GMSCs), obtained from gingival tissue, are a type of pluripotent mesenchymal stem cells with self-renewal, multipotent differentiation potential and immunomodulatory capacities [5, 7, 8]. As novel postnatal stem cells, GMSCs have been paid great attention for ease of isolation, high proliferation capacity, uniformly homogenous property, stable phenotype, and importantly, maintaining normal karyotype and telomerase activity during prolonged culture time [5]. Thus, GMSCs are considered as an ideal candidate cell resource for tissue engi-

neering and cell-based therapies. GMSCs transplanted into the periodontal defects have been confirmed to participate and contribute to the periodontal regeneration in animal models [9]. After implantation into the mandibular defects in animal models, GMSCs showed great promotion to the bone healing [10]. Various studies demonstrated when delivered systemically into humans and animals, MSCs selectively home to the sites of injury and provide therapeutic benefits [11-14]. These reports lead us to hypothesize that systemically transplanted GMSCs can home to the bone defects and be employed in bone regeneration in vivo. In this study, we transplanted human GMSCs into C57BL/6J mice with defects in mandibular bone via the tail vein to explore the capacity of transplanted GMSCs to promote bone regeneration.

### Materials and methods

#### *Animals*

A total of 36 seven-week-old male C57BL/6J mice purchased from Peking University Health Science Center (Beijing, China) were used in this study. The animals were kept under specific pathogen-free conditions with controlled temperature ( $22 \pm 2^\circ\text{C}$ ), humidity (60%) and lighting (12-h light/dark cycle). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Qingdao University, Qingdao, Shandong Province, China.

#### *Isolation of GMSCs*

Human gingival tissues were obtained from young adult donors (18-25 years old) who had no history of periodontal disease during surgery with informed consent and approval of the clinical research ethics committee of Qingdao University. The gingival tissue was washed several times with phosphate-buffered saline (PBS) containing 400  $\mu\text{g}/\text{ml}$  streptomycin and 400 U/ml penicillin and incubated in a medium containing 2 mg/ml dispase (Sigma) at  $4^\circ\text{C}$  overnight. Then the epithelial layer was easily separated from connective tissue. Afterwards, the connective tissue was minced into small pieces (approximately  $1 \text{ mm}^3$ ) which were subsequently digested with a solution of 2 mg/ml collagenase (Sigma) for 40 min at  $37^\circ\text{C}$ . The tissue explants were then placed into  $25 \text{ cm}^2$  culture vessel containing  $\alpha$ -MEM medium (Hyclone) supplemented with 15% fetal bovine

serum (FBS; Hyclone) at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Cells were subcultured at 80% confluency by 0.25% trypsin/EDTA solution (Gibco).

#### *Flow cytometry*

GMSCs ( $1 \times 10^6$ ) at passage 1 were washed with PBS and incubated with antibodies against human CD73, CD105, CD144, CD31, HLA-DR, Stro-1, CD34, CD44, CD45 (BioLegend), CD29 (eBioscience) at  $4^\circ\text{C}$  for 30 min in the dark. Then the cells were washed and resuspended in 1% paraformaldehyde for flow cytometry analysis with the use of CXP Analysis 2.1 software (Beckman Coulter).

#### *Enhanced green fluorescent protein transfection of GMSCs*

To directly trace the fate of transplanted GMSCs in vivo, the lentiviral vector with enhanced green fluorescent protein (pLV.Ex2d.P/neo-EF1A>eGFP; Cyagen, China) was used to label GMSCs. The first-passage GMSCs ( $5 \times 10^3$  cells/ $\text{cm}^2$ ) were incubated in 6-well plates for approximately 24 h. Then the culture medium was removed and concentrated viral supernatant diluted in serum-free  $\alpha$ -MEM was added. 8 hours later, the viral supernatant was replaced with complete culture medium. Transfected cells were selected with G418 (100  $\mu\text{g}/\text{ml}$ ). The GFP<sup>+</sup> GMSCs were expanded and cells from third to fifth passage were used for in vivo experiments.

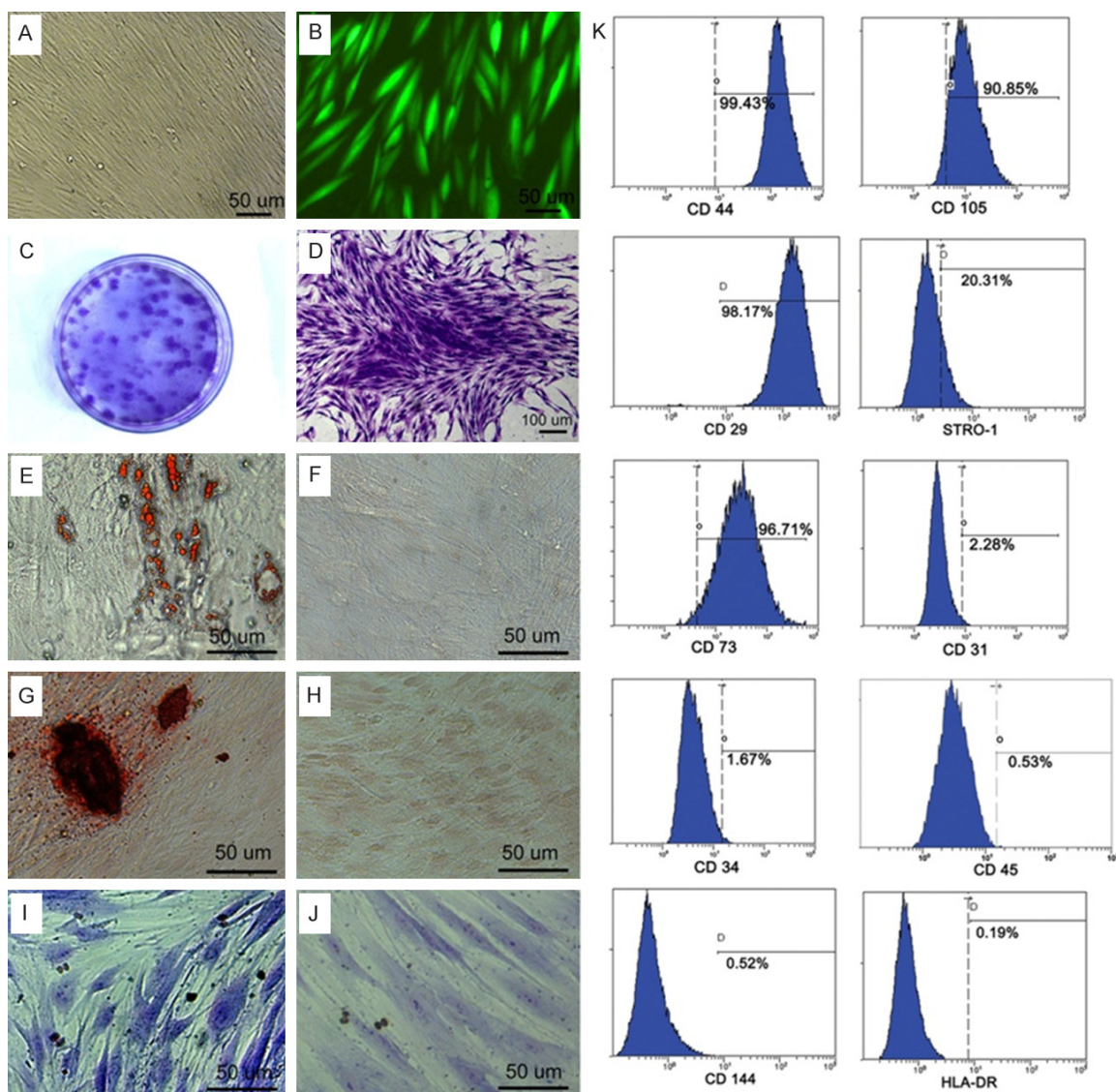
#### *Colony-forming unit-fibroblasts (CFU-F) assay*

To assess colony-forming efficiency of GMSCs, CFU-F assay was performed as described previously [15]. A total of 500 GMSCs at passage 1 were seeded into 6-CM cell culture dish. The culture medium ( $\alpha$ -MEM with 10% FBS) was replaced every 3 days. After 10 days of cultivation, all cultures were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and then washed twice with distilled water. Aggregates of more than 50 cells were counted as a colony under the microscope.

#### *In vitro multipotent differentiation*

**Adipogenic differentiation:** The fourth-passage GMSCs ( $5 \times 10^3$  cells/ $\text{cm}^2$ ) were incubated in 96-well plates in  $\alpha$ -MEM growth media. 24 h later, the culture medium was replaced with adipogenic induction medium supplemented with  $\alpha$ -MEM containing 10% FBS, 1  $\mu\text{M}$  dexamethasone (Sigma), 200  $\mu\text{M}$  indomethacin

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**Figure 1.** Characterization of human GMSCs. A. In vitro culture of GMSCs. B. Expression of GFP in GMSCs cultured in vitro. C. CFU-F assay was carried out. D. Single-colony clusters derived from GMSCs showed typical fibroblastic morphology. E-J. Multipotent differentiation of GMSCs. E. Adipogenic differentiation of GMSCs detected by Oil Red O staining. F. GMSCs cultured under normal conditions detected by Oil Red O staining as control. G. Osteogenic differentiation of GMSCs identified by Alizarin Red S staining. H. GMSCs cultured under normal conditions detected by Alizarin Red S staining as control. I. Chondrogenic differentiation detected by toluidine blue staining. J. GMSCs cultured under normal conditions detected by toluidine blue staining as control. K. Flow cytometry analysis showed that human GMSCs expressed CD44, CD29, CD105, CD73 and STRO-1 but not CD34, CD45, CD31, CD144 and HLA-DR.

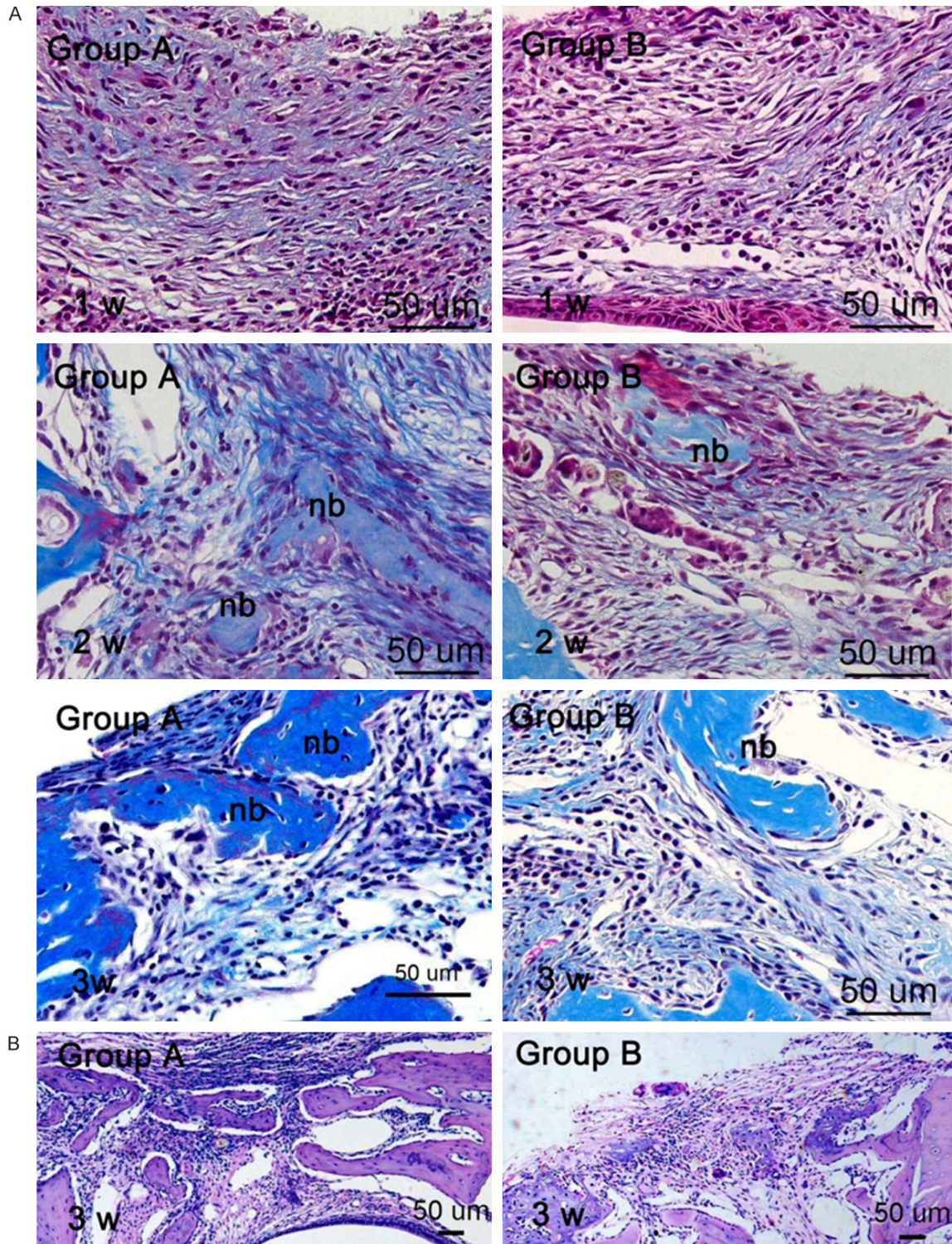
(Sigma), 10  $\mu$ M insulin (Sigma), 0.5 mM isobutyl-methylxanthine (Sigma), and 1% antibiotic/antimycotic. Two weeks later, Oil Red O staining was performed to detect the formation of oil globules.

**Osteogenic differentiation:** As described above, cells plated in 24-well plates were incubated in  $\alpha$ -MEM medium containing 5% FBS, 10 mM

$\beta$ -glycerophosphate (Sigma), 50 mg/ml ascorbate-2-phosphate (Sigma) and 0.1  $\mu$ M dexamethasone. The media was changed twice weekly. Four weeks later, mineral deposition was identified by Alizarin Red S (Sigma-Aldrich) staining.

**Chondrogenic differentiation:** As described above, GMSCs seeded in 6-well plates were





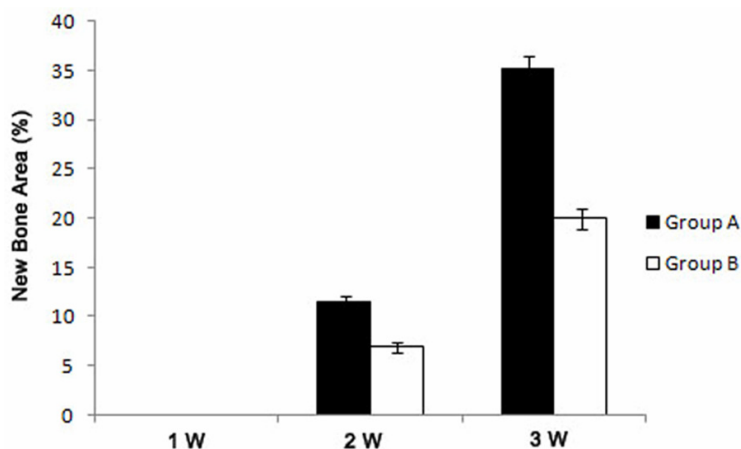
**Figure 2.** Histological observation within the bone defects. A. Observation under  $\times 400$  magnification by MT staining. B. Low power view of the bone defects by HE staining.

cultured in chondrogenic induction medium supplemented with  $\alpha$ -MEM containing 10% FBS, 50 nM ascorbate-2-phosphate, 10 ng/mL

TGF- $\beta$ 1 (Sigma), and 0.1  $\mu$ M dexamethasone. The medium was changed twice weekly. Three weeks later, toluidine blue staining was per-



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**Figure 3.** Analysis of newly formed bone tissue in the bone defects (mean  $\pm$  SD, %). \* $p < 0.05$ .

formed to detect glycosaminoglycan production.

### *Animal surgery and GMSCs transplantation*

As described previously [16], a bone defect with 1.5-mm in diameter in the right mandibular body were created in the 8-week-old C57BL/6J mice. The defect was sited below the mesial root of the lower first molar and drilled with a dental bur under continuous irrigation with phosphate-buffered saline (PBS). Animals were randomly divided into group A and B, with 18 mice in each group. The GFP<sup>+</sup> GMSCs were re-suspended in 100  $\mu$ l of  $\alpha$ -MEM and transplanted into mice in group A by tail vein injection with a very slow speed [17]. Each recipient mouse received approximately  $1 \times 10^6$  cells. Group B animals were injected with 100  $\mu$ l  $\alpha$ -MEM as control. Animals were sacrificed at 1, 2 and 3 weeks after transplantation.

### *Tissue preparation and histomorphometric analysis*

Following perfusion fixation via the intracardiac route with 4% paraformaldehyde (PFA, Sigma) the right mandibular bone was removed. Then the samples were decalcified in 10% EDTA for 3 to 4 weeks, paraffin-embedded and cut into 3  $\mu$ m-thick sections disto-mesially. Sections selected from the most central areas of the bone defect were stained with hematoxylin and eosin (HE) and Masson's Ttichrome (MT). The slides were observed with light microscopy (Olympus, Japan) and photographed using the ProgRes CapturePro software (Jenoptik Optical Systems, Germany). Three HE-stained sections

were analyzed morphometrically for each sample. The new bone formation area as a percentage of the total defect area was calculated with a soft package (Image-Pro Plus 6.0, USA) [18].

### *Homing assay*

Nuclear staining was performed using DAPI. Briefly, the sections were rehydrated and washed with PBS for 5 min and stained with DAPI for 3 min. After washed with PBS, the slices were visualized by a fluorescent microscope (Leica, Germany) under  $\times 400$  magnification and image analysis was performed using Image

J2x (Image J2x Software, USA). GFP<sup>+</sup> cells counterstained with DAPI were examined.

### *Immunohistochemistry for GFP*

Immunohistochemical study was performed using purified rabbit polyclonal antibody (GFP 1:200; Cell signaling, USA) against GFP. De-waxed and hydrated sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min to quench endogenous peroxidase. Then the sections were incubated with the primary antibody at 4°C overnight, washed with PBS and incubated with the anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) at 37°C for 30 min. Counter staining was performed with Mayer's hematoxylin. Control sections were incubated with normal rabbit serum without the primary antibody.

### *Statistical analysis*

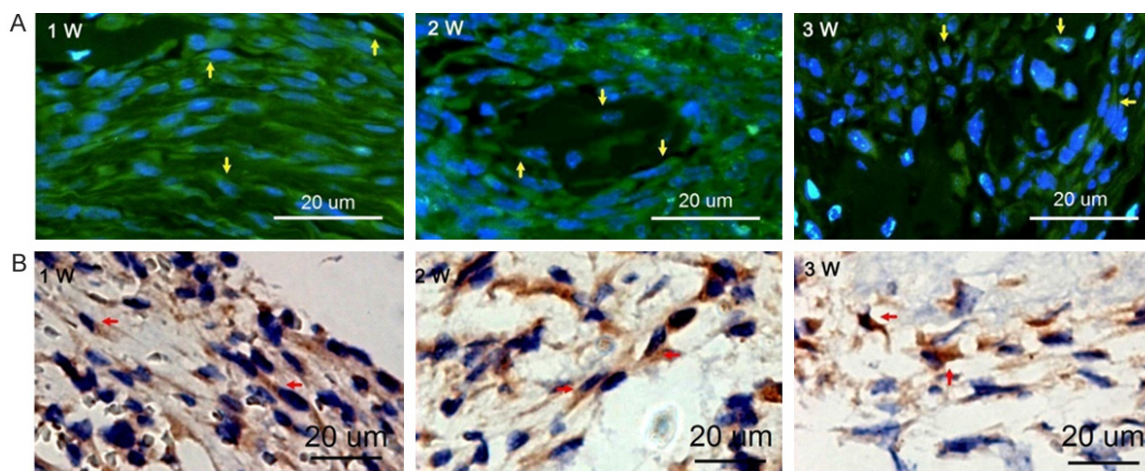
Statistical analysis was performed using a statistical package (SPSS 19.0, SPSS Inc, USA). Data were expressed as mean  $\pm$  SD. Comparison of histomorphometry data for bone formation were analyzed by Independent-Samples T test. The statistical significance chosen was  $p < 0.05$ .

## **Results**

### *Characterization of human GMSCs*

Human GMSCs isolated from healthy gingival appeared fibroblast-like spindle shape (**Figure 1A**) and were able to form clonogenic cell clusters confirmed by the CFU-F assay (**Figure 1C, 1D**). Flow cytometry analysis showed that the

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**Figure 4.** Detection of GFP<sup>+</sup> GMSCs within the bone defect. A. Observation under fluorescence microscopy. Green cells counterstained with DAPI-stained nuclei in blue were detected (as yellow arrowheads indicated). B. Immunohistochemical staining for GFP. Expression of GFP was intense (as red arrowheads indicated).

1st generation GMSCs expressed CD73, CD105, CD29, CD44, and Stro-1 but not CD144, CD31, HLA-DR, CD34 and CD45 (**Figure 1K**). Strong expression of GFP could be detected 72 h post-transfection in GMSCs and GFP expressed stably accompanied the subculture of GMSCs (**Figure 1B**).

### *Multiple differentiation potential of human GMSCs*

Cultured in adipogenic induction medium for 2 weeks, lipid-rich cells were detected by Oil Red O staining, which indicated GMSCs could differentiate into adipocytes (**Figure 1E**). Under osteogenic induction conditions for 4 weeks, GMSCs showed formation of mineralized nodules which were identified by Alizarin Red S staining (**Figure 1G**). After 3 weeks of culture in chondrogenic induction medium, GMSCs were observed an increase in size and blue stained in their cytoplasm using toluidine blue staining (**Figure 1I**). Whereas, no cells positive for Oil Red O staining, Alizarin Red S staining and toluidine blue staining were detected in the control group under normal noninduction conditions (**Figure 1F, 1H, 1J**). These findings suggest that human GMSCs have the potential to differentiate into bone, fat and cartilage.

### *Histology observation and morphometric assessment of the bone defect*

Histology changes were observed in the bone defect by HE and MT staining. In the 1st week

post transplantation, no bone formation was detected in mandibular defect area of both groups. Abundant connective tissue was observed with plenty of fibroblast-like cells and a little newly formed collagen matrix distributed throughout the whole defect area. In the 2nd week post transplantation, the bone defect was rich in blue color by MT staining implying new collagen and bone formation. Some islands of osteoid were observed in the center of the bone defect and newly formed woven bone was present on the defect margins. At 3 weeks post transplantation, active bone formation was observed in both groups and mice in group A showed osteogenesis ability more strongly than those in group B (**Figure 2**).

The newly formed bone area within mandibular defects at the 2nd and 3rd week was assessed in both groups. At 2 weeks after transplantation, the newly formed bone area in group A and B was 11.4% and 6.9% respectively. And at 3 weeks that was 35.2% and 20% respectively. Both at 2 weeks and 3 weeks after transplantation, the differences in bone formation between group A and B were all statistically significant, which indicated a significant effect of GMSCs transplantation on bone regeneration (**Figure 3**).

### *Homing properties of GMSCs*

Both fluorescent microscope observation and immunohistochemical staining showed the existence of GFP<sup>+</sup> cells in the bone defect,

which indicated the transplanted GMSCs exhibited homing specificity and could survive in the bone defect (**Figure 4**). 1 week and 2 weeks after transplantation, a large number of GFP<sup>+</sup> fibroblast-like cells were observed in the bone defect and at 3 weeks the number of donor cells decreased. GFP<sup>+</sup> osteoblasts were identified in the surface and inside of newly formed bone at 2 and 3 weeks post transplantation.

### Discussion

Stem-cell-based therapies aiming at reconstruction of bone injuries have been a promising alternative for clinical trial [19]. As novel postnatal stem cells, GMSCs have been paid extensive attention for their therapeutic potential in regenerative medicine [9, 10]. GMSCs can be easily isolated from human gingival tissue which is usually discarded as biological waste in the clinic and proliferate rapidly in vitro to meet the transplantation requirement for cell amount. In this study, GMSCs were positive for MSC-associated surface markers such as CD73, CD105, CD29, CD44 and Stro-1 and negative for hematopoietic stem markers (CD34 and CD45) and endothelial cells markers (CD144 and CD31). Moreover, GMSCs were negative for HLA-DR indicating the applicability for allogeneic stem cell transplantation. We also found GMSCs had self-renewal capacity and showed multiple differentiation potential, which were consistent with previous studies showing that GMSCs possessed the basic characteristics of mesenchymal stem cells (MSCs) [5, 10].

In our previous study we demonstrated systemically delivered BMSCs can home to the mandibular bone defect in mice and provide therapeutic benefits [14]. However, up to date no study has been reported to evaluate the contribution of GMSCs migrating from peripheral circulation to bone regeneration though local application of GMSCs showed great promotion to the bone healing [10]. Therefore, we designed a study to explore the capacity of systemically transplanted GMSCs to promote bone regeneration. We labeled GMSCs with GFP to directly trace the fate of transplanted GMSCs in vivo. The results of fluorescent microscope observation and immunohistochemical staining showed GFP<sup>+</sup> fibroblast-like cells were detected within bone defects at 1, 2, 3 weeks post transplanta-

tion, which were consistent with previous demonstrations that MSCs have a tendency to home to the sites of injury [11-13]. We also detected GFP<sup>+</sup> osteoblasts in the newly formed bone area in the 2nd and 3rd week post transplantation. These findings strongly supported our hypothesis that systemically transplanted GMSCs can be employed in bone regeneration in vivo.

The contribution of GMSCs to bone regeneration was confirmed by histology observation and morphometric assessment of the bone defect in the present study. As we found, at 2 and 3 weeks post transplantation, the newly formed bone area in Group A mice was significantly higher than that in Group B mice. In addition to the osteogenic potential of GMSCs to promote the new bone forming, another possible explanation responsible for the therapeutic effects of GMSCs on bone injuries was the transplanted GMSCs triggered the endogenous MSCs recruitment which is known to be crucial for successful bone repair [2, 16], though the mechanisms of MSCs recruitment to the injury sites were unclear.

Stem-cell-based therapies have been a promising alternative for bone regeneration. Selection of appropriate donor cell types plays an important role in successful cell transplantation. The present study provides evidence that systemically transplanted GMSCs can not only home to the mandibular defect but also promote bone regeneration. Given the basic characteristics of MSCs and advantages such as ease of isolation, high proliferation capacity, uniformly homogenous property, and so on, GMSCs are considered as an ideal candidate cell resource for cell-based therapies. Future studies using large animal models are needed to assess the long-term safety and efficacy of GMSCs for bone regeneration.

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### Disclosure of conflict of interest

The authors declare that they have no competing interests.

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