

# Comparison and Optimization: Different Medium and a Novel Scheme for Rabbit Bone Marrow Mesenchymal Stem Cells Culture

**Zhu Cong**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

**Lin Jian-biao**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

**Jiang Hui-xiang**

Affiliated Southeast of Xiamen University/909th Hospital of People's Liberation Army

**Lin Wei-bin**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

**Gao Jian-ting**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

**Gao Ming-ming**

Medical College of Xiamen University

**Wu Ben-wen**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

**Feng Bin**

Department of Orthopedics, Number 77th Army Hospital

**Chen Na-na**

Department of Orthopedics, Number 77th Army Hospital

**Guofeng Huang** (✉ [huangguofeng@xmu.edu.cn](mailto:huangguofeng@xmu.edu.cn))

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

<https://orcid.org/0000-0001-6311-680X>

**Ding Zhen-qi**

Affiliated Southeast Hospital of Xiamen University/909th Hospital of People's Liberation Army

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## Research article

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# Abstract

## Background

Rabbit bone marrow mesenchymal stem cells (rBMSCs) are widely used as seed cells for bone tissue engineering, but the effect of different medium on the physiological characteristics of rBMSCs is still not clear and the optimal culture medium for rBMSCs is also uncertain.

## Methods

RBMSCs were divided into 4 groups: DMEM/F12 (F12) group, DMEM/Low glucose (LG) group, DMEM/High glucose (HG) group, and optimizational culture method group which was cultured with DMEM/High glucose for the first 6 days and then DMEM/Low glucose (HG-LG). The morphology, proliferation ability, stem cell surface markers, multi-directional differentiation potential, adhesion ability and vitality in tissue engineering bone (TEB) were detected and compared.

## Results

RBMSCs in the 4 groups shared the same shape, similar surface markers (positive in CD44, CD29, negative in CD45, CD34) and similar osteogenic, adipogenic and chondrogenic directional differentiation capability. For the primary rBMSCs, the proliferation ability of HG group was higher than that of LG group ( $P<0.05$ ), while F12 group was between the former two. The proliferation ability of the 3<sup>rd</sup> passage of rBMSCs in the 4 groups was similar. The adhesion ability of the 3<sup>rd</sup> passage rBMSCs in the 4 groups was listed as follows starting from high to low: LG group and HG-LG group (they had similar ability), F12 group, HG group, and the difference was statistically significant ( $P<0.05$ ). The vitality of TEB in LG group and HG-LG group was higher than that in F12 group and HG group ( $P<0.05$ ).

## Conclusions

The primary rBMSCs cultured with DMEM/High glucose showed good proliferation ability, while the 3<sup>rd</sup> rBMSCs cultured with DMEM/Low glucose showed good adhesion ability and vitality in TEB. RBMSCs cultured with DMEM/High glucose for the first 6 days and then DMEM/Low glucose showed good proliferation ability, good adhesion ability and good vitality in TEB, which might provide a novel optimizational scheme for rBMSCs culture.

## 1. Introduction

Large segmental bone defect is a tough problem for clinical treatment [1, 2]. Tissue engineering bone (TEB) transplantation provides a promising treatment for large segmental bone defect [2, 3], its effect of promoting bone defect repair has been initially confirmed in basic experiments and clinical studies [4, 5]. The biological properties of seed cells are critical to the performance of TEB [6]. Bone marrow mesenchymal stem cells (BMSCs) are a kind of pluripotent stem cells which derived from the early developmental mesoderm and stably present in bone marrow [7-10]. Due to their good self-renewal ability

and multi-directional differentiation potential, coupled with their weak immunogenicity, BMSCs are widely used as biological tissue engineering seed cells for bone and soft tissue repair [11, 12]. New Zealand rabbits are commonly used as experimental animals for bone and soft tissue injury and repair [13, 14]. Although there are many reports on rBMSCs in recent years, there is still no consensus on the choice of medium for rBMSCs culture [15, 16].

The physiological characteristics of BMSCs are strongly dependent on their culture environment [17]. DMEM/F12, DMEM/Low glucose and DMEM/High glucose are the commonly used rBMSCs culture media [15, 16], while the effect of different medium on the physiological characteristics of rBMSCs is still not clear and the optimal culture medium for rBMSCs is also uncertain. The proliferative capacity, multi-directional differentiation potential, adhesion ability and survivability in tissue engineering scaffolds of seed cells play an important role in the repair and reconstruction of damaged tissues [11, 18]. This study focused on the proliferation capacity, multi-directional differentiation potential, adhesion ability and vitality in TEB of rBMSCs cultured in different media. Further more, this study tried to optimize the culture mode of rBMSCs based on these results and find a new effective rBMSCs culture method, which facilitated the culture of rBMSCs in vitro.

## 2. Materials And Methods

### 2.1 Experimental animals

A total of 5 healthy male New Zealand rabbits (aged 4~6 weeks, weight 400~800 g) were obtained from the Animal Experiment Center of Xiamen University. All rabbits were in good condition and had no related diseases based on examination.

### 2.2 Isolation and culture of rBMSCs

The 5-week-old male New Zealand rabbits were sacrificed with an intraperitoneal injection of an excess of 10% chloral hydrate (Sinopharm Chemical Reagent, China) and the hindlimbs of the rabbit were removed and immersed in 75% alcohol for 3 minutes. Then the muscles of femur and tibia were removed and the bone cavity was washed by PBS mixed with an appropriate amount of heparin sodium (0.04 mg/mL, Beijing Solarbio Science & Technology, China) for bone marrow. The suspension was collected and adjusted to the cell concentration of  $2 \times 10^8$  to  $1 \times 10^9$  /mL. A total of 5 mL rabbit mesenchymal cell separation fluid (Tianjin HaoYang HuaKe Biological Technology, China) was added to a 15 mL centrifuge tube, and then the single cell suspension was placed onto the separation liquid level, at 450g centrifugation for 20 minutes. Cells in the second layer were collected in a Petri dish containing MSC complete medium [DMEM/F12 (HyClone, USA, the glucose concentration of 16.67 mmol/L) for F12 group or DMEM/Low Glucose (HyClone, USA, the glucose concentration of 5.56 mmol/L) for LG group or DMEM/High Glucose (HyClone, USA, the glucose concentration of 25 mmol/L) for HG group + 10% FBS (Biological Industries, Israel) + 1% streptomycin/penicillin (HyClone, USA) + 50  $\mu$ mol/L  $\beta$ -mercaptoethanol

(Beijing Solarbio Science & Technology, China)]. The petri dish was placed in a cell culture incubator at 37°C with 5% CO<sub>2</sub> for 3 days, and then the medium was changed every 2 days.

As for the optimizational culture method (HG-LG) group, rBMSCs were isolated and cultured with DMEM/High glucose for the former 6 days, then they were digested with trypsin and cultured with DMEM/Low glucose.

### 2.3 Detection of MSC surface markers

The 3<sup>rd</sup> passage cells of the 4 groups were collected respectively, then CD45 (Acris GmbH, Germany), CD44 (BD Biosciences, USA), CD34 (Thermo Fisher, USA), CD29 (Millipore Corporation, USA), goat anti-mouse secondary antibody (Multi Sciences, China) were used to detect their MSC surface markers according to the instructions [19]. Fluorescence activated cell identify was performed with [flow cytometry](#) (Beckman USA) and data were analyzed with CytExpert (Tree Star, Ashland, OR, USA).

### 2.4 Multi-directional differentiation potential of rBMSCs

**Osteogenic induction:** The 4 groups of rBMSCs were seeded in 6 cm dishes with 3×10<sup>5</sup> in each one and cultured at 37°C with 5% CO<sub>2</sub> for 24 hours. The original medium was then discarded and replaced for osteogenic induction with one containing 10<sup>-2</sup> mol/L of β sodium glycerophosphate (Dalian Meilun Biotech, China), 10<sup>-8</sup> mol/L of dexamethasone (Dalian Meilun Biotech, China), and 3×10<sup>-4</sup> mol/L vitamin C (Dalian Meilun Biotech, China). The medium was changed every 2 days for 2 weeks. Then calcium nodules in different groups were detected by Alizarin Red staining (Beijing Leagene Biotech, China) according to the instructions.

**Chondrogenic induction:** The 4 groups of rBMSCs were seeded in 6 cm dishes with 3×10<sup>5</sup> in each one and cultured at 37°C with 5% CO<sub>2</sub> for 24 hours. The original medium was then discarded and replaced for chondrogenic induction with one containing 10 μg/L of rabbit TGF-β1 (Dalian Meilun Biotech, China), 10<sup>-7</sup> mol/L of dexamethasone, 5×10<sup>-5</sup> mol/L vitamin C, 6.25×10<sup>-3</sup> g/L of insulin. The medium was changed every 2 days for 2-3 weeks. The induction was terminated and stained with Alison Blue Dye (Beijing Leagene Biotech, China) according to the instructions.

**Adipogenic induction:** The 4 groups of rBMSCs were seeded in 6 cm dishes with 3×10<sup>5</sup> in each one and cultured at 37°C with 5% CO<sub>2</sub> for 24 hours. The original medium was then discarded and replaced for adipogenic induction with adipogenic induction medium A containing 2×10<sup>-4</sup> mol/L of indomethacin, 10<sup>-6</sup> mol/L of dexamethasone, 5×10<sup>-4</sup> mol/L of 3-isobutyl-1-methylxanthine (China National Pharmaceutical Group Chemical Reagent, China) and 10<sup>-2</sup> g/L insulin (Dalian Meilun Biotech, China), and changed to adipogenic induction medium B containing 10<sup>-2</sup> g/L insulin after 2 days, and after 1 day, it was replaced with liquid A, and it was repeated. After 2 weeks, the induction was terminated and stained with Oil Red O (Beijing Leagene Biotech, China) according to the instructions.

The positive rate of dyeing (PRD) was calculated and compared by Image Pro Plus software.

## 2.5 Detection of cell proliferation ability

The cells to be detected were seeded in well plates with  $2 \times 10^3$  cells/well, and then a total of 10  $\mu$ L CCK-8 (Transgen Biotech, Beijing, China) reagents were added into each well and incubated at 37°C with 5% CO<sub>2</sub> for 2 hours after culture for 1, 2, 3 and 4 days, the absorbance at 450 nm (A450) were determined at wave length of 450 nm with Microplate Reader (Bio-Rad, USA) according to the instructions.

## 2.6 Detection of cell adhesion ability

The cells to be detected were seeded in a 9 cm culture dish at a ratio of 80 cells per petri dish. After 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours, the number of adherent cells (number of cell colonies) was counted respectively [20]. Cell adherence rate = (number of adherent cells /80)  $\times$  100%.

## 2.7 Introduction of green fluorescent protein (GFP)

Seed cells were labeled with GFP to monitor the survival of seed cells in TEB scaffold, this was inspired from the methods reported by [Yuriko Kakimoto](#) [21].  $10^5$  primary rBMSCs were put into a 10 cm culture dishes with 8 mL culture medium, the lentivirus with GFP gene were put into each dish. The multiplicity of infection was adjusted to 100 and the density of polybrene was adjusted to 5  $\mu$ g/mL. The medium was changed after 24 hours and then changed every 2nd day. The fluorescence expression of cells were observed by an inverted fluorescence microscope and these GFP-rBMSCs were collected and sorted by a Flow Separation Cell Meter (Beckman USA) and cultivated for amplification.

## 2.8 Preparation of deproteinized defatted cancellous bone scaffold (DDCBS)

Our research team has previously proved that DDCBS obtained from calf femoral heads had good porous structure, and the DDCBS implantation could effectively promote bone defect repair in New Zealand rabbits [22]. In this study, the DDCBS obtained from calf femoral heads was used as scaffold for TEB construction. The cancellous bone of the calf femur was prepared into a cylindrical cancellous bone of 8 mm in diameter, deproteinized with 20% hydrogen peroxide at 37°C for 72 hours, and then degreased with ether at room temperature for 48 hours to prepare for DDCBS, as described before. Scanning electron microscope was used to observe the structure of the DDCBS (Fig 1).

## 2.9 Construction of TEB

The seed cells were adjusted to  $3 \times 10^5$  /mL, 10 mL of cell suspension and a piece of TEB were put into a 15 mL centrifuge tube for mixed culture. The culture was carried out for 7 days with a rotary cell culture apparatus at 20 r/min (Sup. Fig 1). Then the TEB to be detected were placed into another 15 ml centrifuge tube and an appropriate amount of PBS was added to fully infiltrate the stent. The fluorescence intensity of each group was observed and compared by living fluorescence imaging system.

## 2.10 Statistical analysis

Results were expressed as mean  $\pm$  standard deviation. Prism 5.0 (GraphPad Software Inc.) was used to make statistical analysis. Statistical comparisons for different groups were performed using one-way analysis of variance (ANOVA).  $P < 0.05$  were considered statistically significant.

## 3. Results

### 3.1 The morphology of rBMSCs in different culture groups

On the 6th day, the early primary rBMSCs cultured in the 3 media were fusiform, triangular and scattered (Fig 2) while rBMSCs cultured in HG group grew fastest. The 3<sup>rd</sup> passage of rBMSCs cultured in the 4 groups were spindle-shaped or streamlined, closely arranged, while the morphology of rBMSCs in LG group and HG-LG group was more streamlined (Fig 3).

### 3.2 The expression of MSC surface markers in the 3<sup>rd</sup> passage rBMSCs of different culture groups

According to the flow cytometry results, 3<sup>rd</sup> passage rBMSCs of different culture groups were all high expression in CD44 (>95%) and CD29 (>90%), low expression in CD45 (<10%) and CD34 (<5%) (Fig 4A). There was no significant difference in the expression levels of each group ( $P > 0.05$ ) (Fig 4B). This indicated that the 3<sup>rd</sup> passage rBMSCs of the 4 culture groups all had good MSC surface marker expression characteristics.

### 3.3 The multi-directional differentiation potential of the 3<sup>rd</sup> passage rBMSCs in different culture groups

Osteogenic, cartilage and adipogenic induction and staining experiments showed that the 3<sup>rd</sup> passage of rBMSCs in the 4 groups all showed positive staining areas in Alizarin Red staining, Alison Blue and Oil Red O staining after induction (Fig 5A). This indicated that these 4 groups of cells all had certain osteogenesis, cartilage and adipogenic differentiation potential. Further analysis of each group of PRD showed that there was no significant difference in PRD between the 4 groups in the 3 staining experiments ( $P > 0.05$ ) (Fig 5B). The results indicated that the 3<sup>rd</sup> passage rBMSCs cultured in different culture groups had similar multi-directional differentiation potential characteristics.

### 3.4 The proliferative capacity of the primary and the 3<sup>rd</sup> passage rBMSCs in different culture groups

CCK-8 cell proliferation assay showed that there was no significant difference among the A450 of primary rBMSCs in F12, LG, HG and HG-LG group from 0 to 24 hours ( $P > 0.05$ ). At the 48th hour, A450 of F12 group and HG group were higher than that of LG group ( $0.68 \pm 0.07$  and  $0.81 \pm 0.03$  vs  $0.52 \pm 0.04$ ), the differences were statistically significant. At the 72nd hour, A450 of F12 group and HG group were still higher than that of LG group ( $1.05 \pm 0.07$  and  $1.19 \pm 0.10$  vs  $0.78 \pm 0.06$ ), the differences were statistically significant. At the 96th hour, the A450 of HG group was higher than that of LG group ( $1.31 \pm 0.07$  vs  $1.05 \pm 0.06$ ) ( $P < 0.05$ ), while there was no significant difference in A450 between the other groups ( $P > 0.05$ ).

(Fig 6A). There was no significant difference among the A450 of the 3<sup>rd</sup> rBMSCs in F12, LG, HG and HG-LG group from 0 to 96 hours ( $P>0.05$ ) (Fig 6B). This indicated that in the early stage, the proliferation capacity of rBMSCs in HG group was better than those of rBMSCs in LG group and F12 group, while in the latter stage, the proliferative capacity of rBMSCs in the 4 groups was similar.

### 3.5 The adhesion ability of the 3<sup>rd</sup> passage rBMSCs in different culture groups

Cell adhesion experiment showed that the 75% cell adhesion time of the 4 groups from low to high was: LG group ( $3.75\pm 0.54$ ) h and HG-LG group ( $3.83\pm 0.51$ ) h, F12 group ( $9.40\pm 0.83$ ) h, HG group ( $12.67\pm 1.25$ ) h. There was no significant difference in the 75% cell adhesion time of LG group and HG-LG group, while the difference between the other each two groups were statistically different ( $P<0.01$ ). The cell adherence rates of the 4 groups were: F12 group ( $99.17\pm 0.38$ )%, LG group ( $100.00\pm 0.01$ )%, HG group ( $97.50\pm 1.54$ )%, HG-LG group ( $100.00\pm 0.02$ )%. The cell adherence rate of F12 group was lower than those of LG group and HG-LG group while higher than that of HG group, and the differences were statistically significant ( $P<0.01$ ) (Fig 7). This indicated that the 3<sup>rd</sup> passage rBMSCs' adhesion ability of LG group and HG-LG group had stronger adhesion ability than the other two, followed by F12 group and HG group.

### 3.6 The expression of GFP fluorescence in the 3<sup>rd</sup> passage rBMSCs of different culture groups

Inverted fluorescence microscopy showed that the 3<sup>rd</sup> passage rBMSCs in the 4 groups were highly expressed GFP fluorescence. This suggested that the 3<sup>rd</sup> passage rBMSCs of all 4 culture groups labeled with GFP fluorescence could be used as tracer seed cells for TEB construction (Fig 8).

### 3.7 The vitality of the 3<sup>rd</sup> passage rBMSCs in TEB in different culture groups

After 1 week of TEB construction, the fluorescence intensity of TEB in 4 groups were detected, and they were: F12 group ( $3.42\pm 0.28$ ) $\times 10^8$ , LG group ( $4.58\pm 0.99$ ) $\times 10^8$ , HG group ( $2.68 \pm 0.67$ )  $\times 10^8$  and HG-LG group ( $4.53 \pm 0.45$ )  $\times 10^8$  (Fig 9A). There was no significant difference in the fluorescence intensity of TEB between the LG group and HG-LG group and there was no significant difference in the fluorescence intensity of TEB between F12 group and HG group. However, the fluorescence intensity of the former two groups was stronger than that of the latter two, and the differences were statistically significant ( $P<0.05$ ) (Fig 9B). The results were consistent with the results of cell adhesion experiment. This further indicated that rBMSCs of LG group and HG-LG group had better vitality in TEB than HG group and F12 group.

## 4. Discussion

This study compared the physiological characteristics of rBMSCs cultured with DMEM/F12, DMEM/Low glucose, DMEM/High glucose and DMEM/High glucose for the first 6 days and then DMEM/Low glucose. It was found that the rBMSCs in different culture groups all had good MSC characteristics, while the primary rBMSCs in DMEM/High glucose group had the highest proliferation rate and the 3<sup>rd</sup> passage rBMSCs in DMEM/Low glucose group had the best adhesion ability and the strongest survivability in

TEB. The rBMSCs cultured with DMEM/High glucose for the first 6 days and then DMEM/Low glucose had good MSC characteristics, as well as strong proliferation ability of primary cells, strong adhesion ability and strong survivability in TEB of the 3<sup>rd</sup> passage cells, which might provide a novel optimizational scheme for rBMSCs culture.

Sebastien et al compared physiological characteristics of human BMSCs cultured with DMEM/High glucose [23], DMEM/F12 and DMEM/Low glucose and found that the proliferation capacity of the primary BMSCs cultured with the 3 media from high to low were as follows: DMEM/High glucose group, DMEM/F12 group and DMEM/Low glucose group, while there was no significant difference in cell proliferation ability between the 3<sup>rd</sup> passage of these 3 groups. In this study, the results were consistent with the results of Sebastien's and rBMSCs cultured with DMEM/High glucose with the glucose concentration of 25 mmol/L during the primary cell stage had the best proliferation ability. Gao Hai et al studied physiological characteristics of human BMSCs cultured with media containing different glucose concentration, and found that medium containing 10 mmol/L glucose promoted the proliferation of human BMSCs while high concentration of glucose (>30 mmol/L) inhibited the proliferation of human BMSCs [24]. Although the optimal glucose concentration obtained by Gao Hai is different from that of Sebastien and that in this study, it is indicated that the glucose concentration in the medium had a great effect on the cell proliferation ability.

Studies have shown that medium with high glucose concentration within a certain range could effectively improve the proliferation ability of MSCs [23, 24]. However, some studies also have shown that medium with high glucose might accelerate cell senescence and apoptosis in several pathways [25-27]. Yanan Kong et al cultured MSCs with high glucose (33 mmol/L) medium, and found that the apoptosis rate of cells remained low after culturing for 1 day, while the apoptosis rate of cells increased greatly after culturing for 5 days [25]. And they came to the conclusion that cell apoptosis induced by high glucose was closely related to culture time. Tzu-Ching C et al cultured BMSCs with low glucose (5.5 mmol/L) and high glucose (25 mmol/L) medium respectively, they also found that high glucose could accelerate the senescence of BMSCs by promoting their ability of autophagy after culturing for 2 weeks [26]. Yang Liu et al cultured nucleus pulposus derived MSCs (NPMSCs) with low glucose (5.5 mmol/L) and high glucose (25 mmol/L) medium respectively, and they found that high glucose medium cultured NPMSCs showed significantly decreased expression levels of stemness genes, related mRNA and protein, whereas increased expression levels of cell senescence markers and caspase-3 [27]. Cramer et al applied medium with different concentration glucose (5.56mmol/L, 13.9mmol/L, 27.8mmol/L and 55.6mmol/L) to culture adipose derived stem cells (ADSCs) for 120 hours, and found that the hyperglycemia microenvironment could simultaneously reduce the proliferation ability of ADSCs and increase their apoptosis [28]. In this study, the authors found that BMSCs reached 70-80% confluence on the 7th day and reached the standard of passage, meanwhile, changing the medium at this time point could avoid the adverse effects of long-term high glucose environment leading to apoptosis. Therefore, in this study, rBMSCs were isolated and cultured with DMEM/High glucose for the former 6 days, then they were digested and the medium was replaced with low sugar medium.

The results in this study showed that the 3<sup>rd</sup> passage rBMSCs cultured with DMEM/F12, DMEM/Low glucose and DMEM/High glucose were all high expression in CD44 and CD29, while low expression in CD45 and CD34, and they had similar osteogenic differentiation, adipogenic differentiation and chondrogenic differentiation potential. This indicated that rBMSCs cultured with DMEM/F12, DMEM/Low glucose and DMEM/High glucose all shared good MSC physiological characteristics. The results in this study were consistent with the results of Sebastien [23]. Further more, the study found that in the 3<sup>rd</sup> passage, rBMSCs in LG group had the strongest cell adhesion ability and the strongest survivability in TEB. The adhesion ability of seed cells is important for tissue engineering transplantation, and the stronger the seed cells' adhesion ability is, the easier the successful transplantation of TEB will be [28, 29]. Xue Xingying et al cultured human umbilical cord blood MSCs with DMEM/High glucose and DMEM/Low glucose, and found that the DMEM/Low glucose group had better adherence ability [30]. This also indicated that different media have a effect on the adhesion ability of MSCs. Further, the study transplanted rBMSCs of different groups into DDCBS to observe the viability of these seed cells, and the results were also consistent with the experimental results above.

This study tried to optimize the culture protocol of rBMSCs: culturing rBMSCs with DMEM/High glucose for the first 6 days and then DMEM/Low glucose. And the results showed that rBMSCs in HG-LG group concentrated strong proliferation ability of the primary cells of HG group, strong adhesion ability and strong survivability in TEB of the 3<sup>rd</sup> passage cells of LG group, which might provide a novel optimizational scheme for rBMSCs culture. The physiological characteristics of BMSCs change at different growth stages, and so as to their environmental requirements, providing the corresponding medium according to the different growth stages of BMSCs might be the most beneficial scheme for the culture of rBMSCs.

The physiological characteristics of BMSCs are closely related to their culture environment. The presence or absence of serum [31, 32], glucose content [24], related cytokines such as FGF [33], PH value [34] will all affect. Therefore, further investigations are required to develop a more satisfactory scheme for rBMSCs culture.

## 5. Conclusion

In summary, this study found that rBMSCs cultured with DMEM/F12, DMEM/Low glucose and DMEM/High glucose all had good MSC physiological characteristics, while the primary rBMSCs cultured with DMEM/High glucose showed the best proliferation ability and the 3<sup>rd</sup> passage rBMSCs cultured with DMEM/Low glucose showed the best adhesion ability and the strongest survivability in TEB. RBMSCs cultured with DMEM/High glucose for the first 6 days and then DMEM/Low glucose concentrated strong proliferative ability of the primary cells of HG group, strong adhesion ability and strong survivability in TEB of the 3<sup>rd</sup> passage cells of LG group, which might provide a novel optimizational scheme for rBMSCs culture.

# Abbreviations

A450: the absorbance at 450 nm

ADSCs: adipose derived stem cells

BMSCs: bone marrow mesenchymal stem cells

CCK-8: cell counting kit-8

DDCBS: deproteinized defatted cancellous bone scaffold

DMEM: Dulbecco's modified Eagle's medium

F12: DMEM/F12

FBS: fetal bovine serum

FGF: fibroblast growth factor receptors

GFP: green fluorescent protein

GFP-rBMSCs: green fluorescent protein labeled rabbit bone marrow mesenchymal stem cells

HG: DMEM/High glucose

LG: DMEM/Low glucose

MSC: mesenchymal stem cell

PBS: phosphate-buffered saline

PH: potential of hydrogen

PRD: the positive rate of dyeing

rBMSCs: rabbit bone marrow mesenchymal stem cells

TEB: tissue engineering bone

TGF- $\beta$ 1: transforming growth factor beta 1

# Declarations

**Ethics approval and consent to participate**

This article does not contain any studies involving human participants. This study was carried out in accordance with the ethical standards in the 1964 Declaration of Helsinki. All studies were approved by the Ethics Committee of the Affiliated Southeast Hospital of Xiamen University and had been carried out in accordance with relevant regulations of the US Health Insurance Portability and Accountability Act (HIPAA).

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no conflict of interest.

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### **Authors' contributions**

Zhu Cong carried out cell isolation and differentiation, flow cytometry, CCK-8, analysis of data, drafting of manuscript, and final approval. Lin Jian-biao carried out preparation of DDCBS, construction of TEB, observation of scanning electron microscope and collection of data. Jiang Hui-xiang, Lin Wei-bin and Gao Jian-ting carried out introduction of GFP to rBMSCs and observation of fluorescence microscope. Gao Ming-ming and Wu Ben-wen participated in cell isolation and differentiation. Feng Bin and Chen Nana participated in preparation of DDCBS, construction of TEB and collection of data. Guofeng Huang and Zhenqi Ding participated in the overall design of the study and interpretation of data and helped edit manuscript for intellectual and scientific contents. All authors read, made edits as necessary, and approved the final draft.

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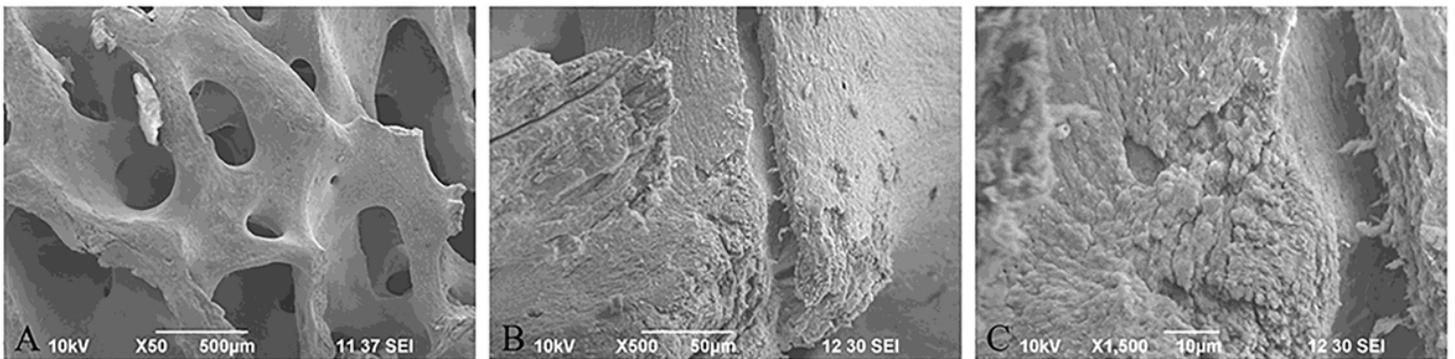
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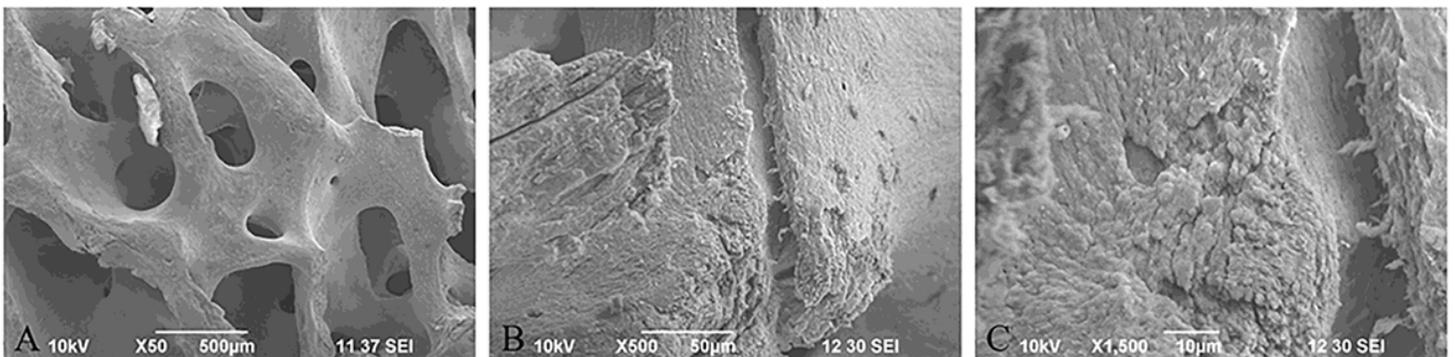
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## Figures



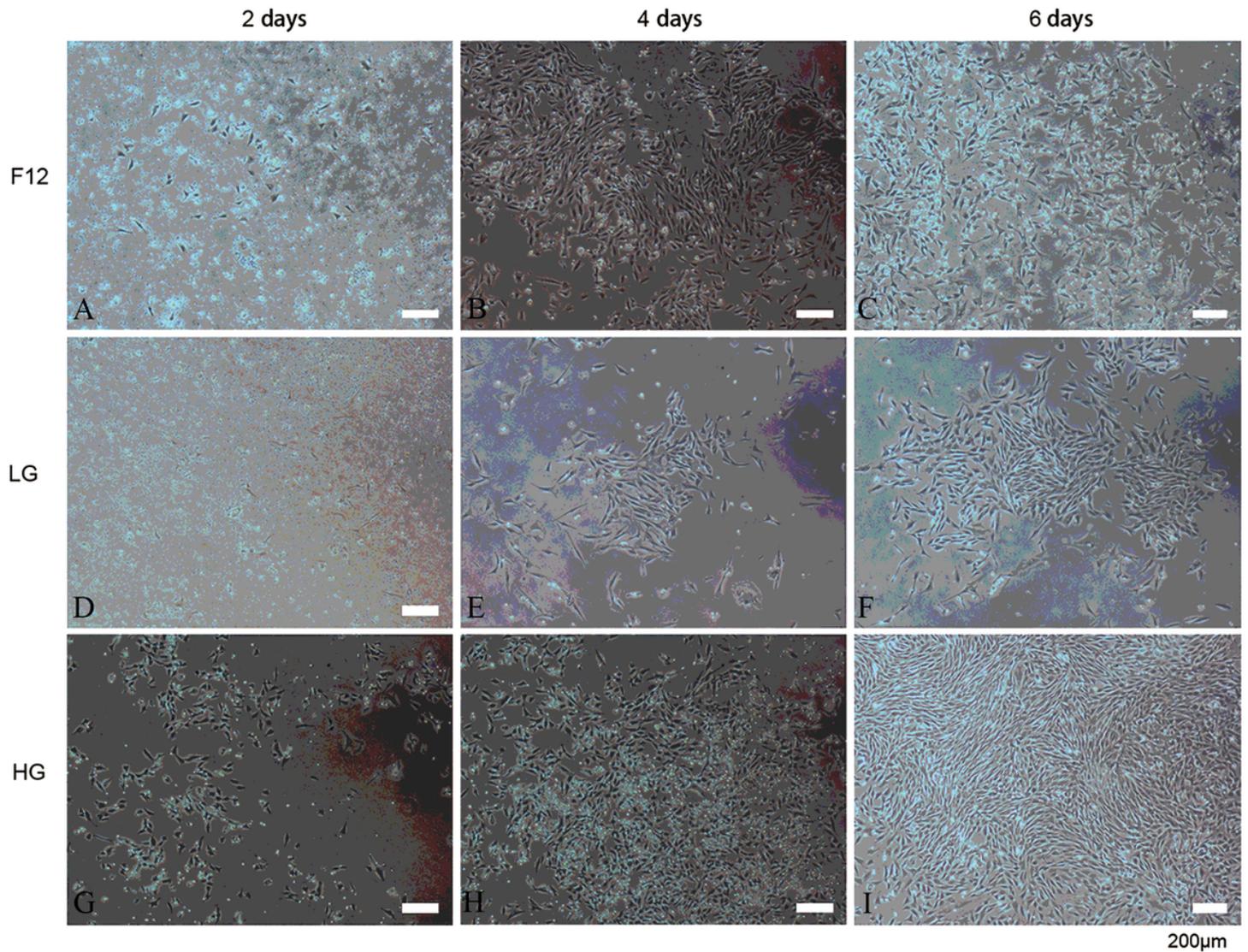
**Figure 1**

Calf deproteinized defatted cancellous bone scaffold Scanning electron microscope observation. A  $\times 50$ ; B  $\times 500$ ; C  $\times 1500$ ; n=5.



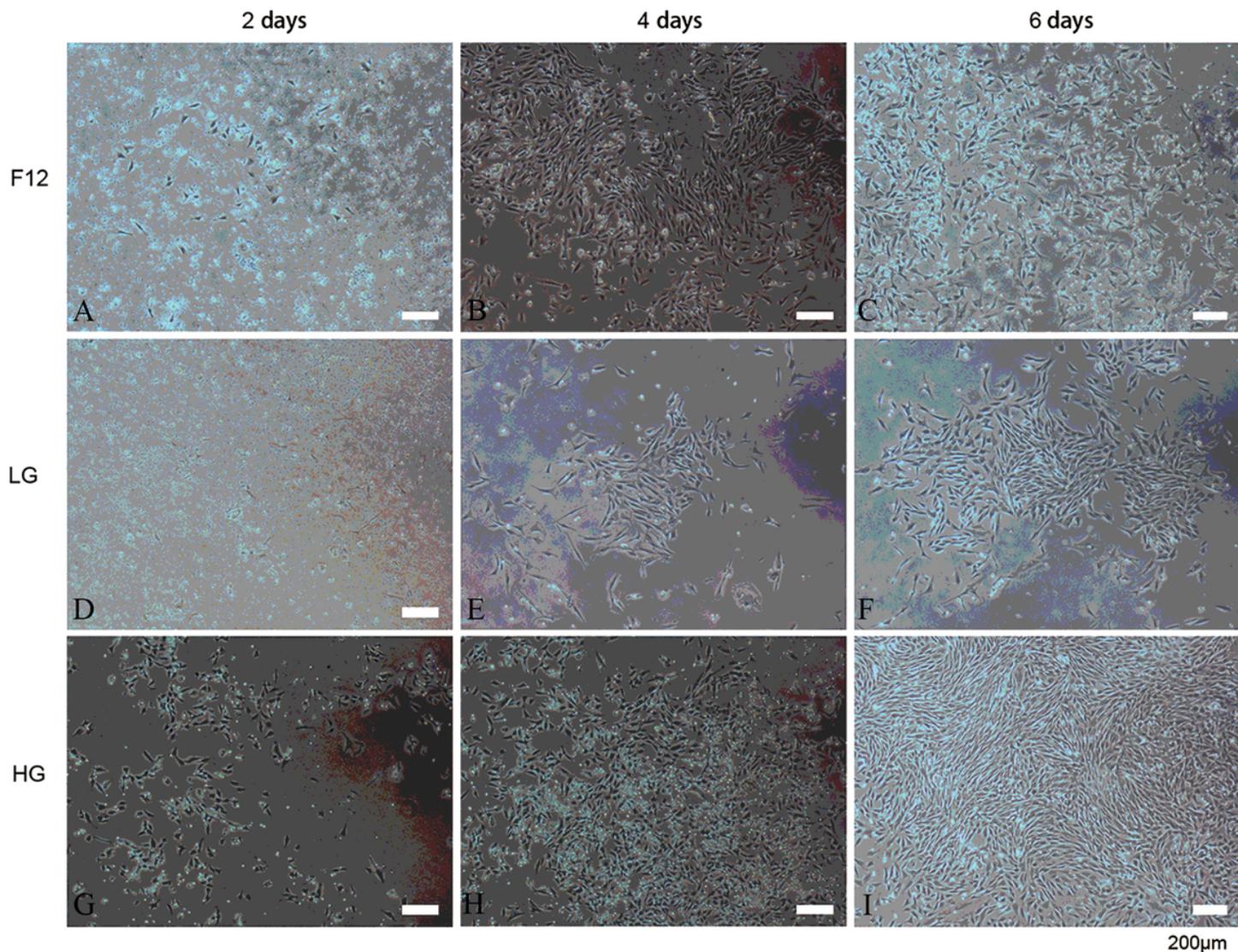
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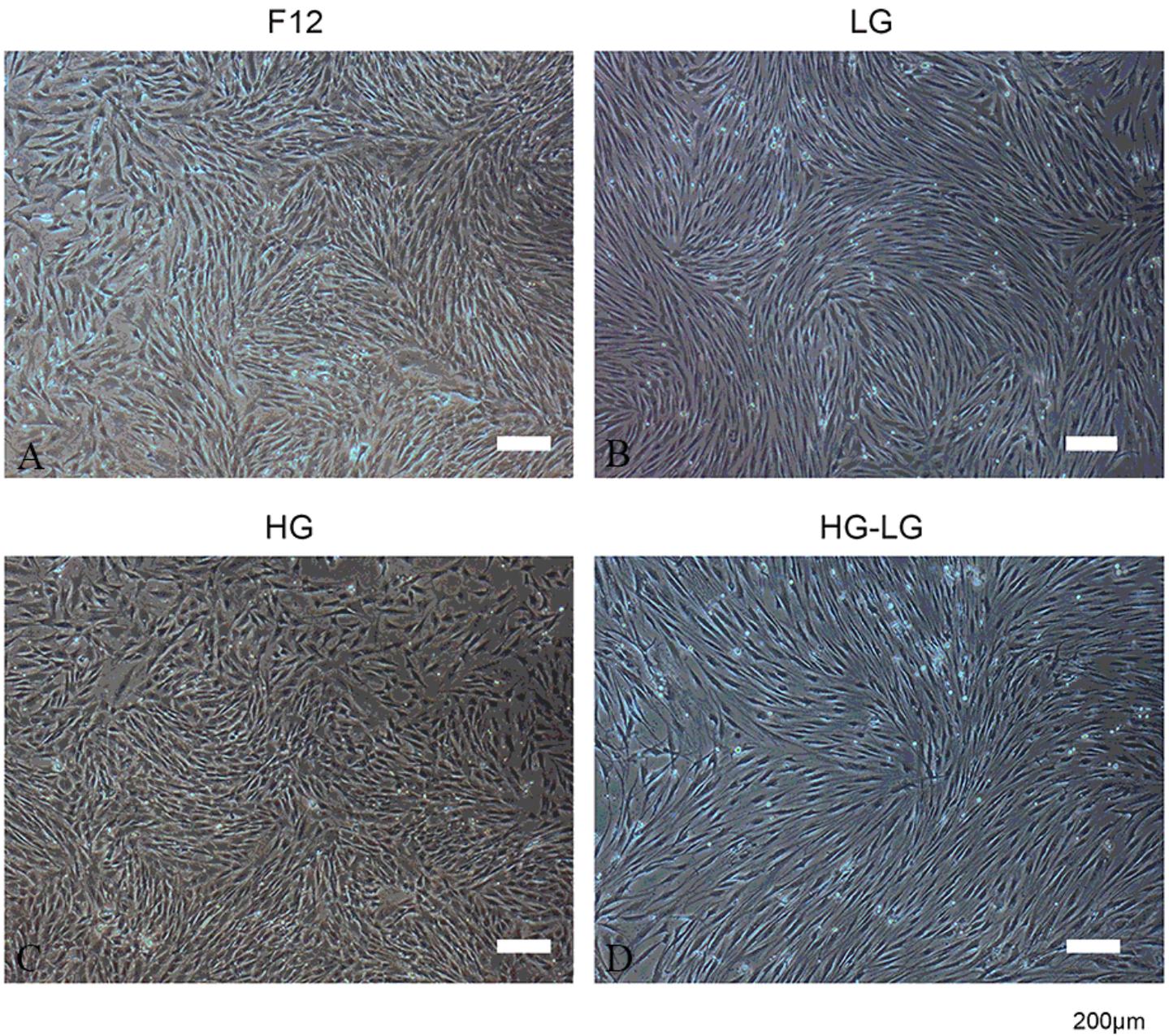
## Figure 2

The morphology of the primary rBMSCs cultured in 3 different media in 2-6 days. The primary rBMSCs in F12 group reached a fusion rate of 60% on the 6th day; the primary rBMSCs in LG group grew slower than the other two groups; the primary rBMSCs in HG group reached a fusion rate of 90% on the 6th day, which grew fastest.  $\times 60$ ; n=5.



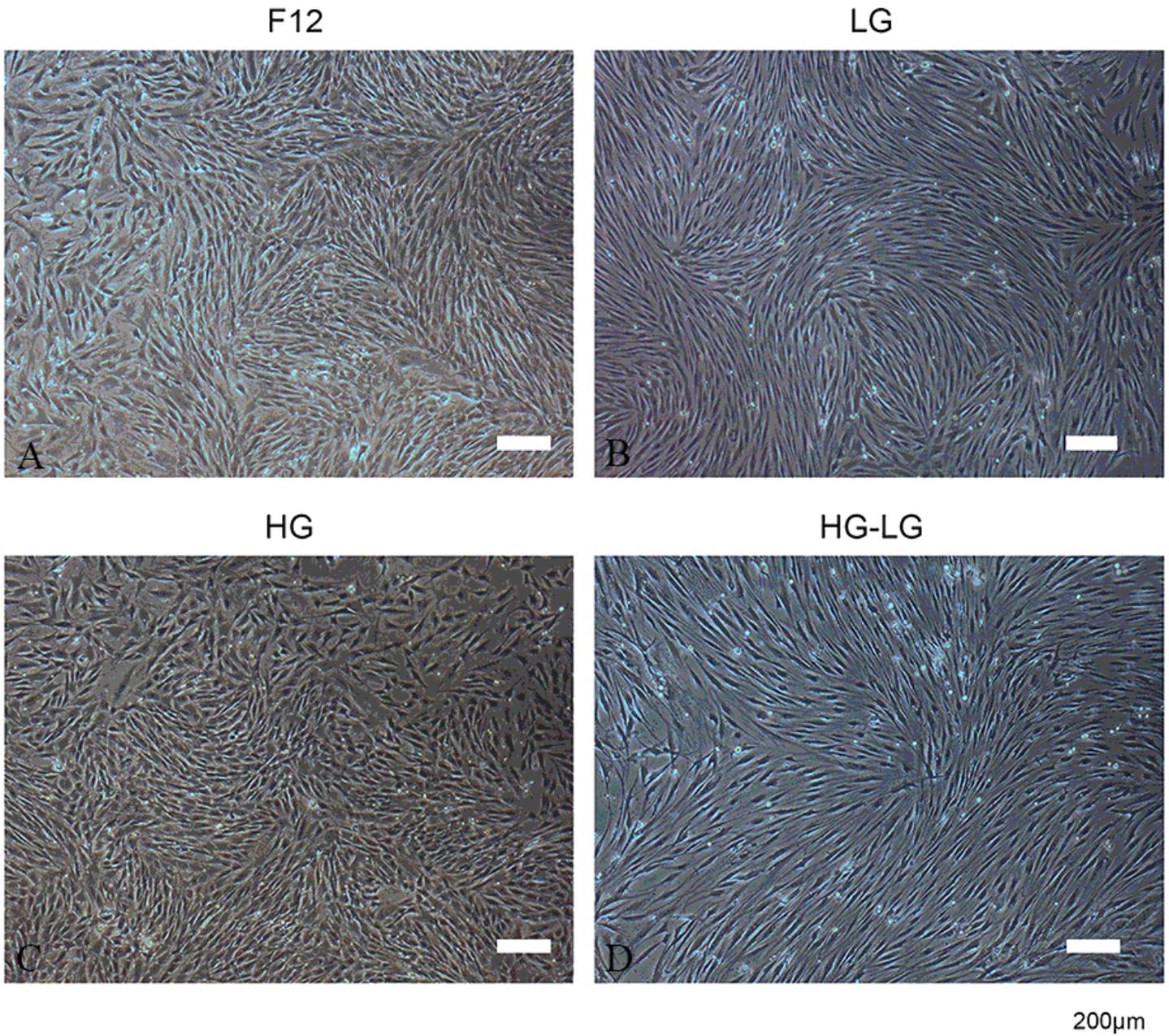
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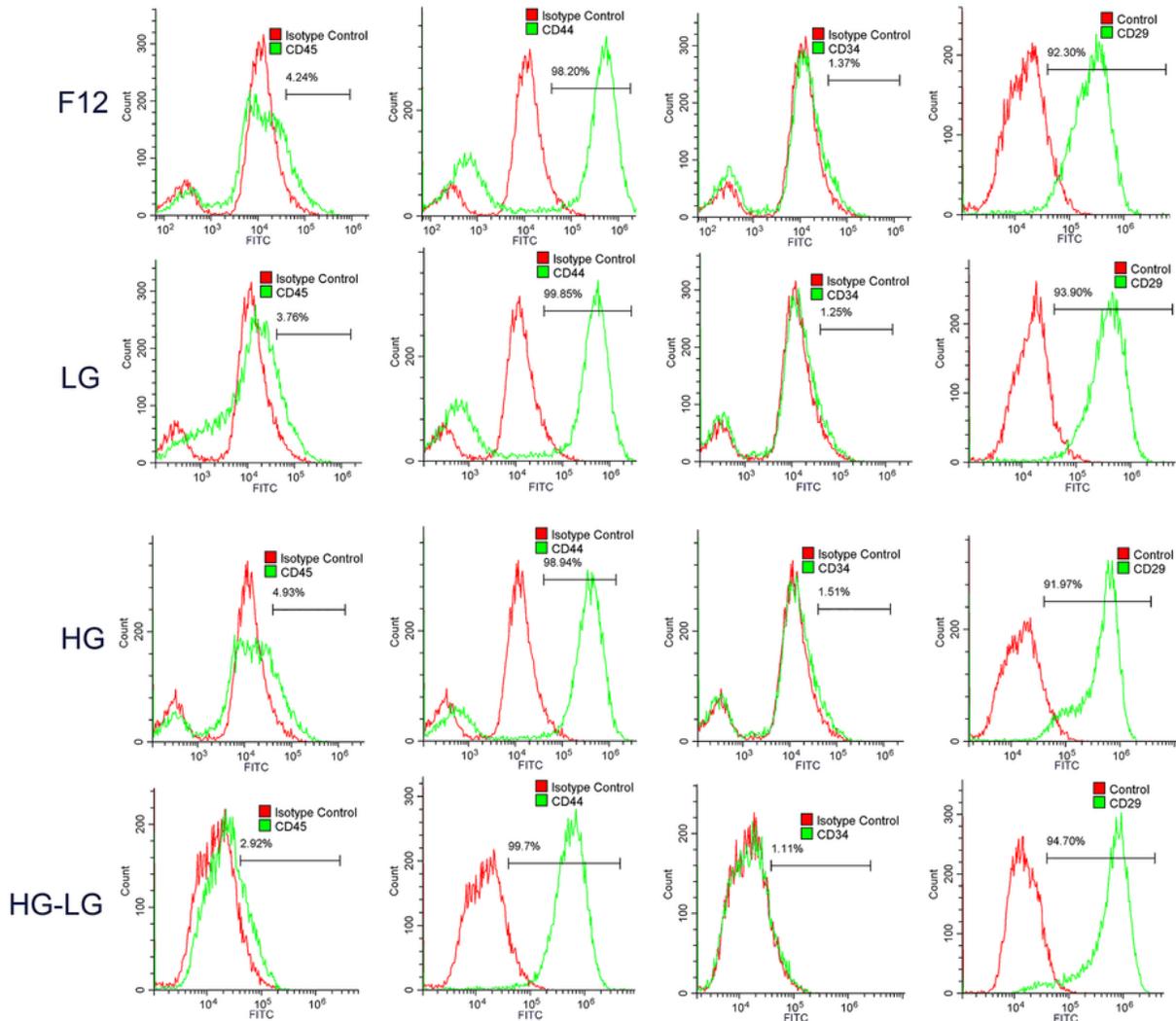
**Figure 3**

The morphology of the 3rd rBMSCs in 4 different cultured groups The 3rd passage rBMSCs of the 4 groups were all spindle-shaped or stream-lined, closely arranged, while the morphology of rBMSCs in LG group and HG-LG group was more regular.  $\times 60$ ;  $n=5$ .

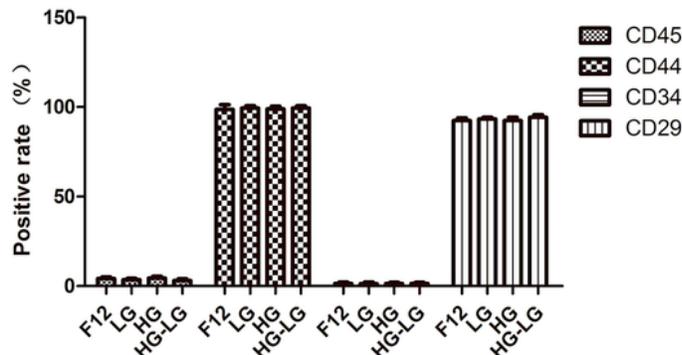


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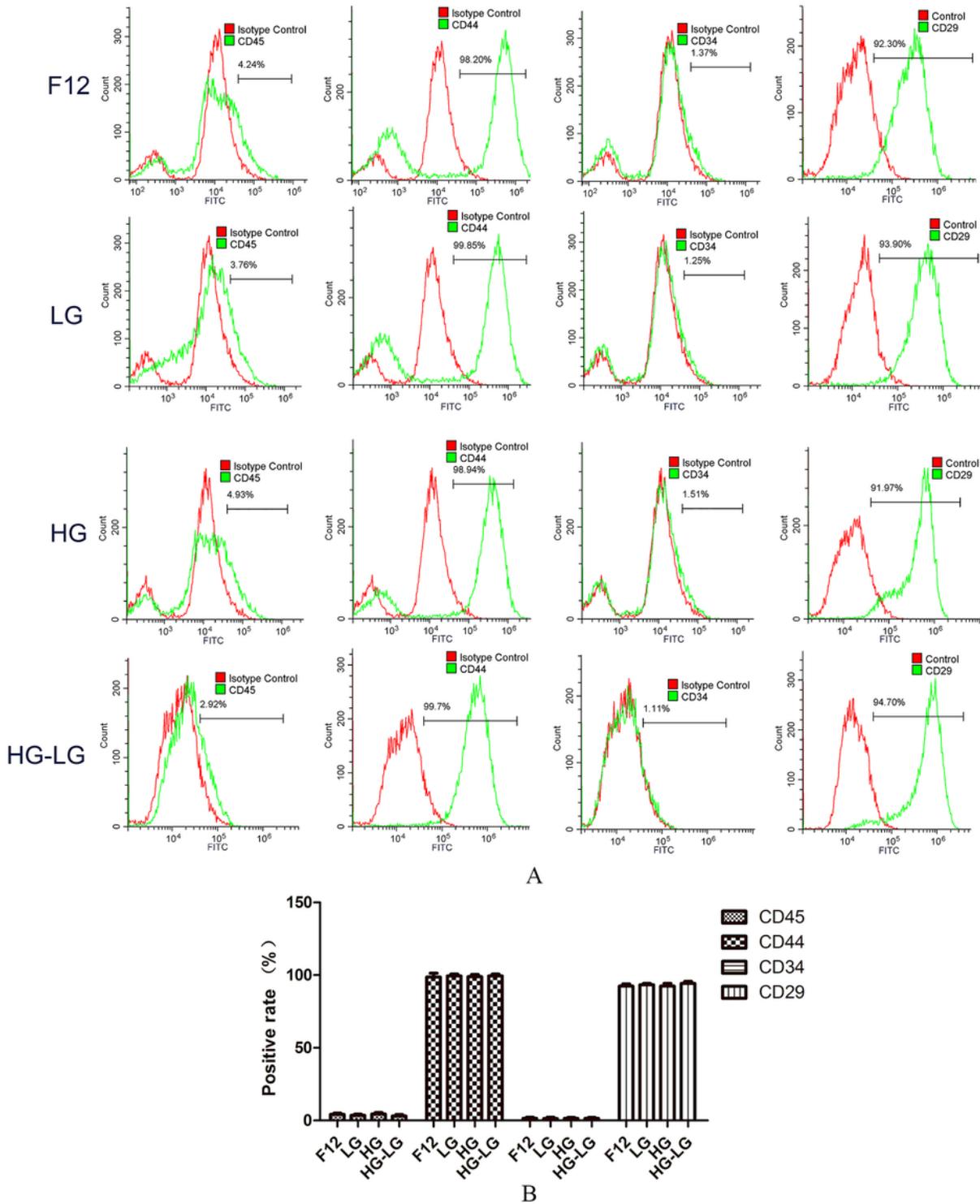
A



B

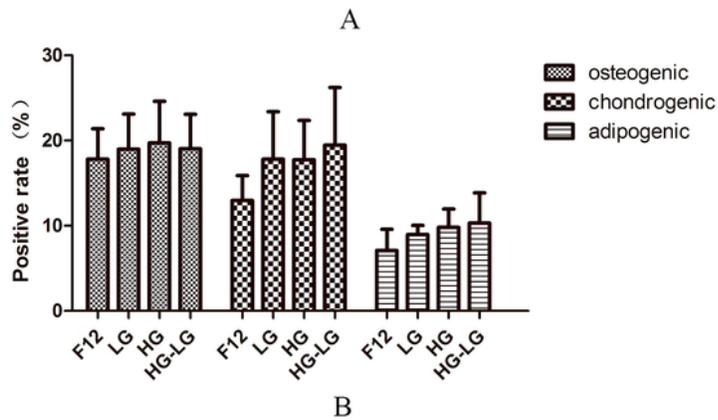
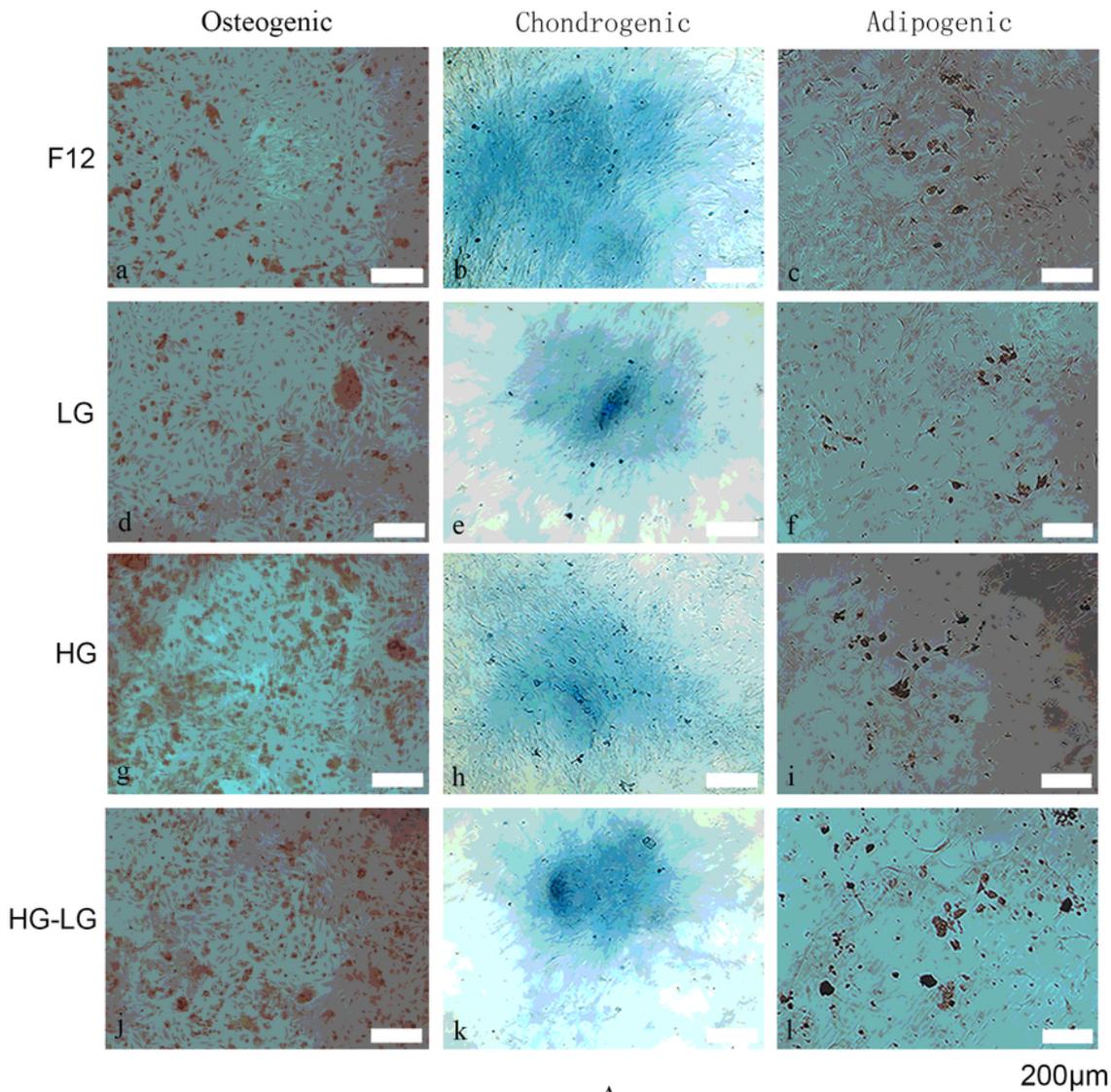
**Figure 4**

The expression of stem cell surface markers in the 3rd passage rBMSCs of 4 different culture groups A: According to the flow cytometry results, CD44 (>95%) and CD29 (>90%) were prominently expressed, whereas CD45 (<10%) and CD34 (<5%) were barely expressed in the 3rd passage rBMSCs of different culture groups. Stained cells were represented in green, whereas unstained cells were in red. B: Statistical analysis of flow cytometry results. n=3.



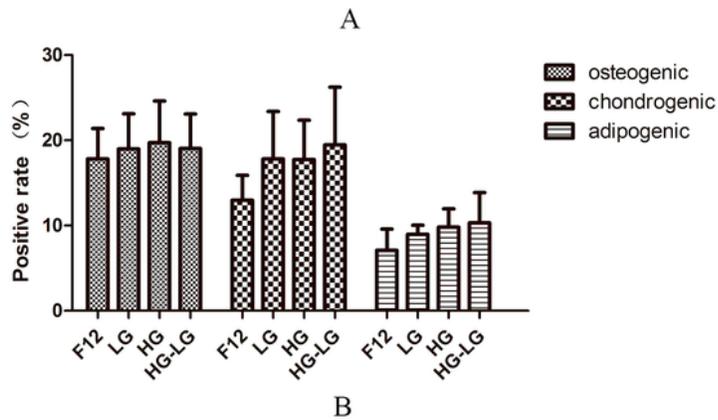
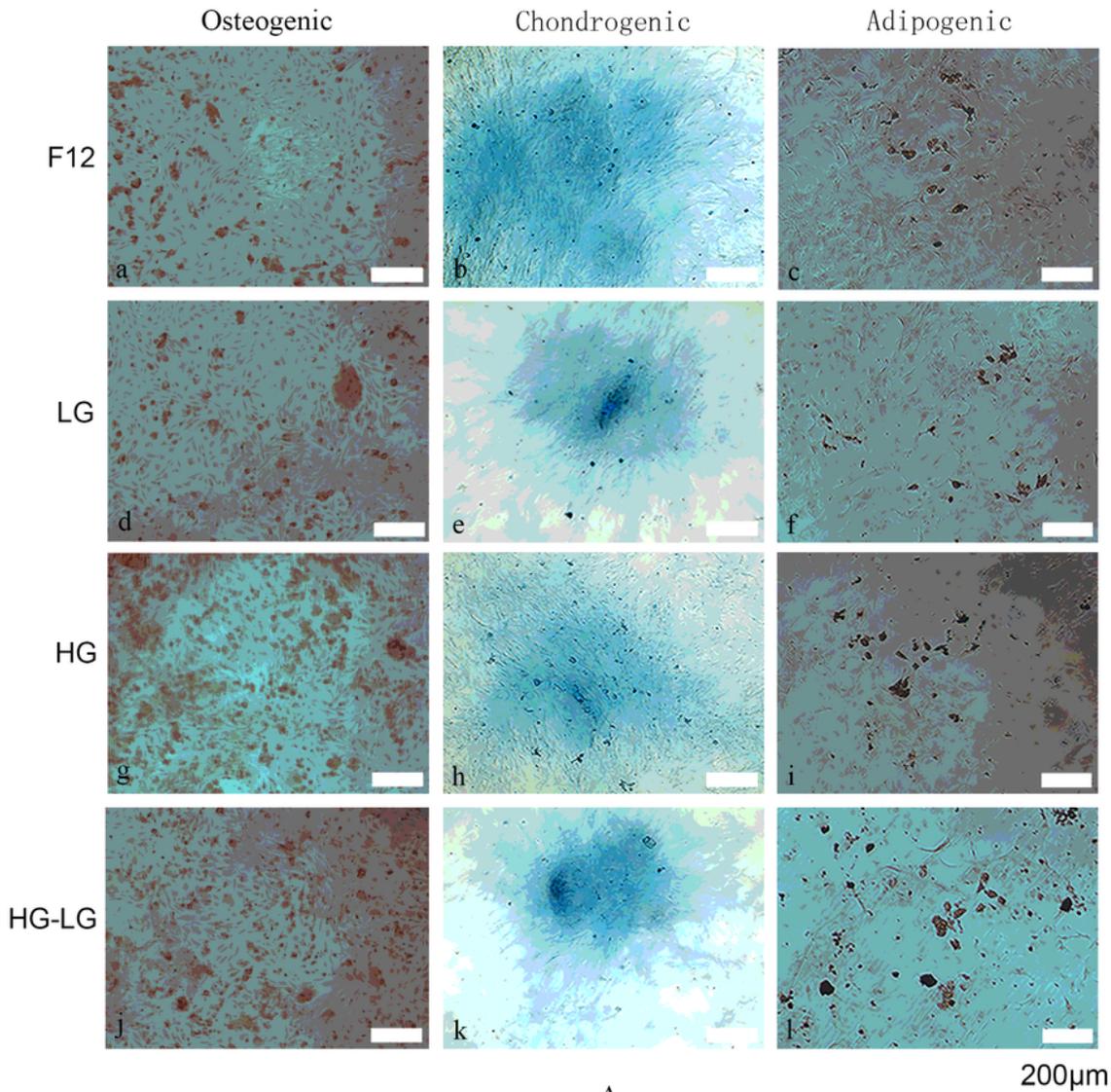
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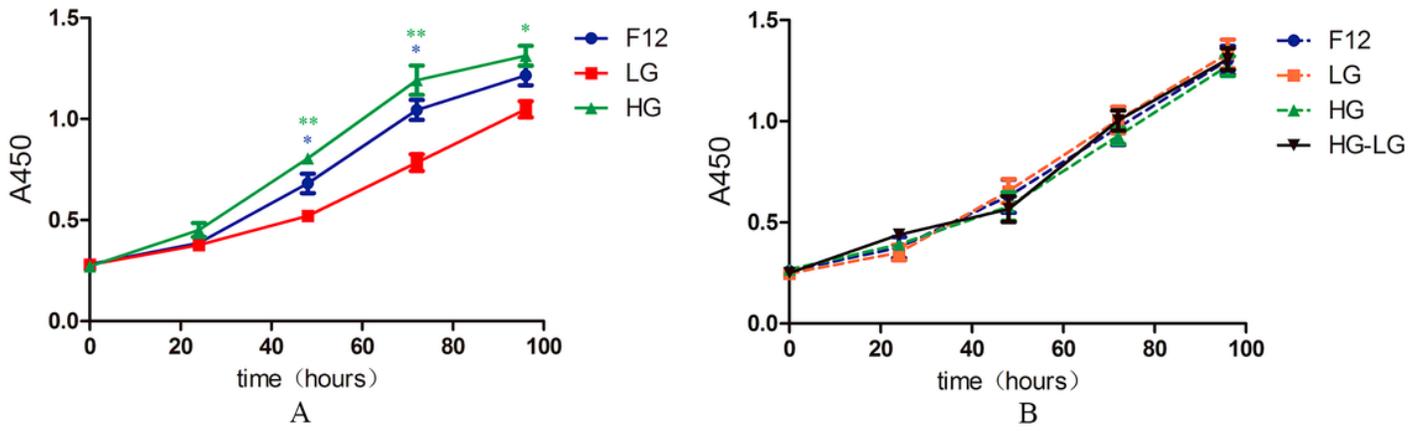
**Figure 5**

The multi-directional differentiation potential of the 3rd passage rBMSCs in 4 different culture groups A: The 3rd passage rBMSCs in 4 different culture groups all showed positive staining areas in Alizarin Red, Alison Blue and Oil Red O staining after induction. The osteogenic induction staining was black, the chondrogenic induction staining was blue, and the adipogenic induction staining was red.  $\times 100$ . B: Statistical analysis of multi-directional differentiation induced staining results.  $n=3$ .



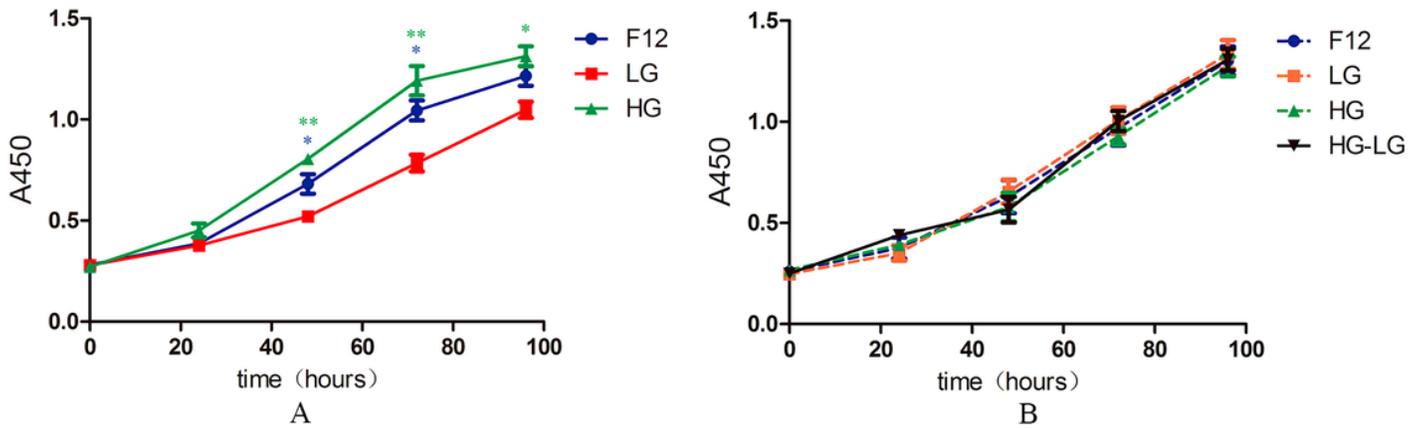
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**Figure 6**

The proliferation ability of the primary and the 3rd rBMSCs cultured in different media A: The blue, red and green colors in the figure represented the A450 curves of the primary rBMSCs in F12 group, LG group and HG group, respectively. During the culture process, the proliferation ability of rBMSCs in HG group was the strongest, while the proliferation ability of rBMSCs in LG group was the weakest. The green asterisk referred to HG versus LG, and the blue asterisk referred to F12 versus LG. B: The blue, red, green and black colors in the figure represented the A450 curves of the 3rd rBMSCs in F12 group, LG group, HG group and HG-LG group, respectively. n=3; \* P<0.05; \*\* P<0.01.



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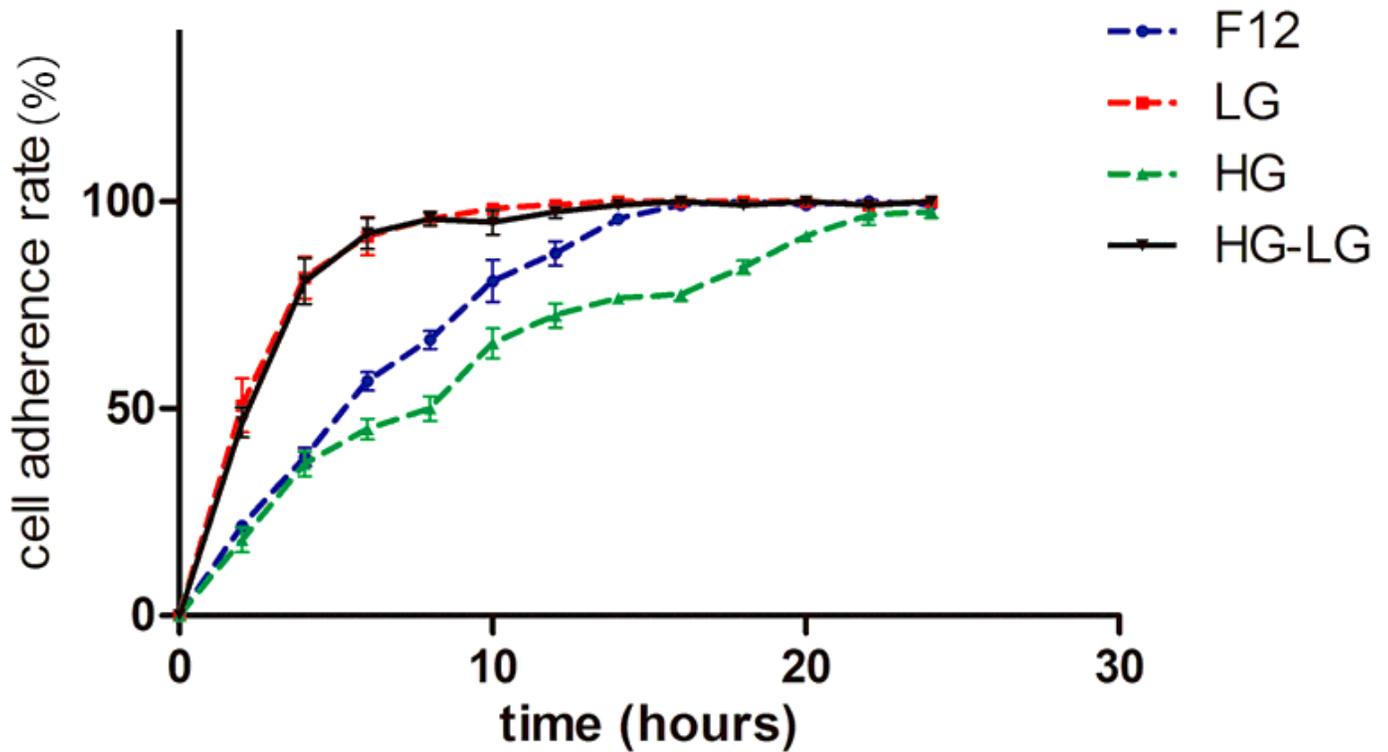
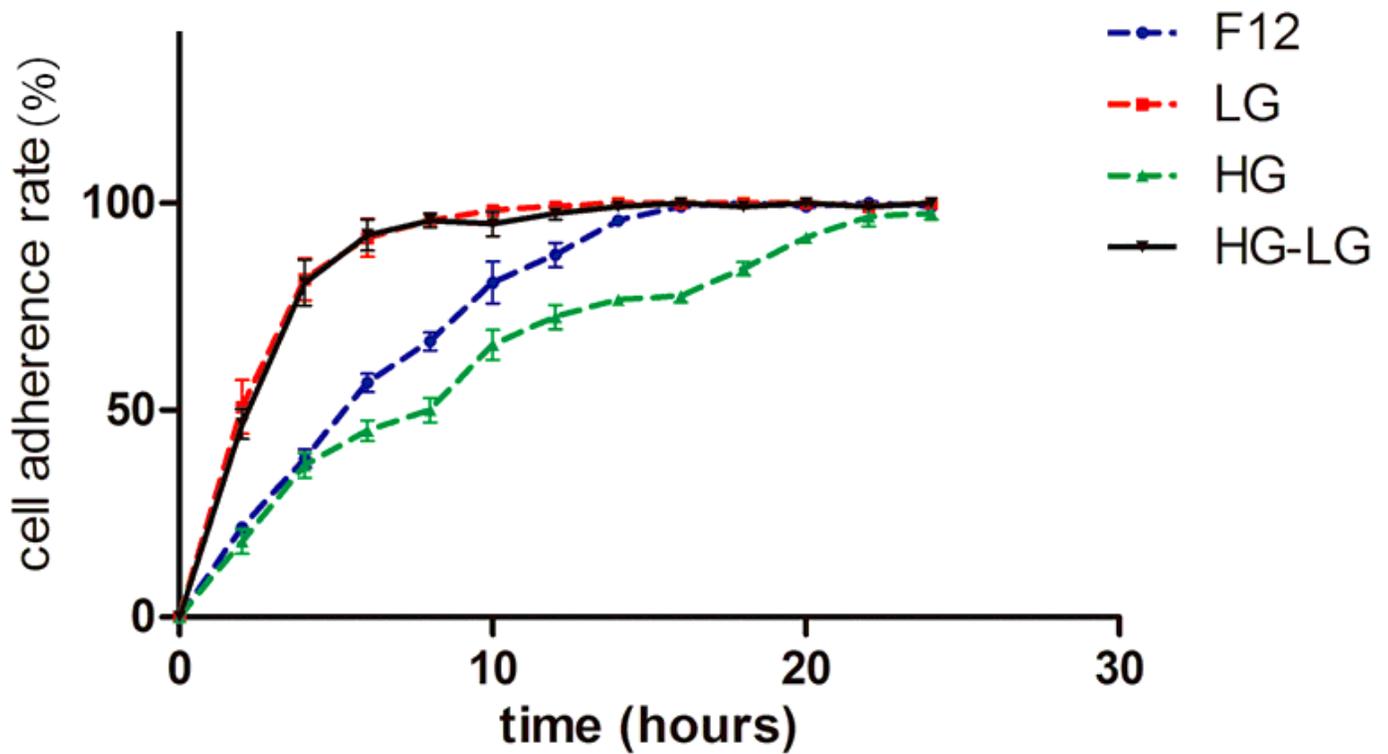


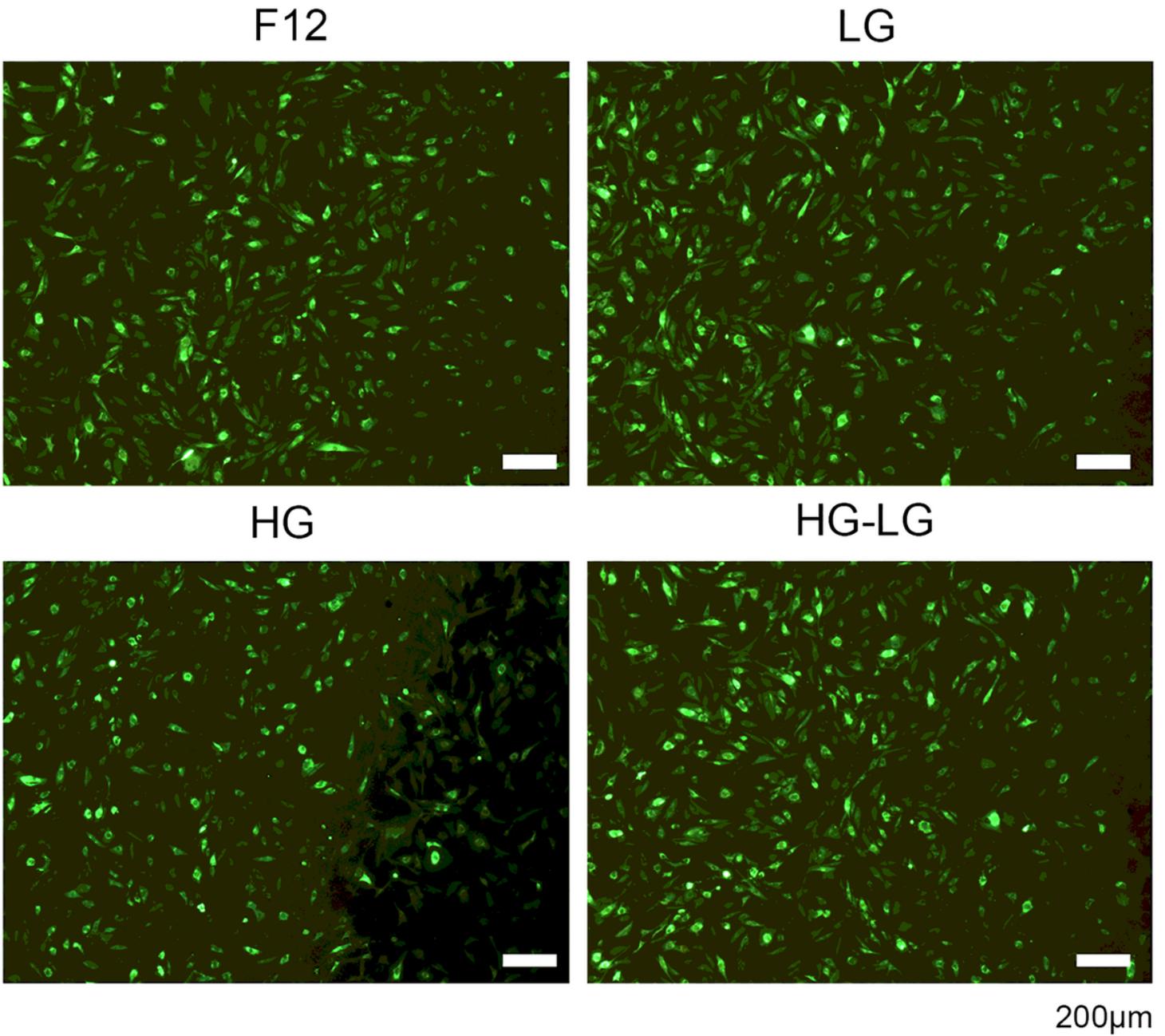
Figure 7

The adhesion ability of the 3rd passage rBMSCs in 4 different culture groups The blue, red, green and black colors in the figure represented the cell adherence rate curves of the 3rd passage rBMSCs in F12 group, LG group, HG group and HG-LG group, respectively. The 3rd passage rBMSCs in LG group and HG-LG group had the strongest adhesion ability, followed by DMEM/F12 group, and the adhesion ability of the 3rd passage rBMSCs in HG group was the weakest. n=3.



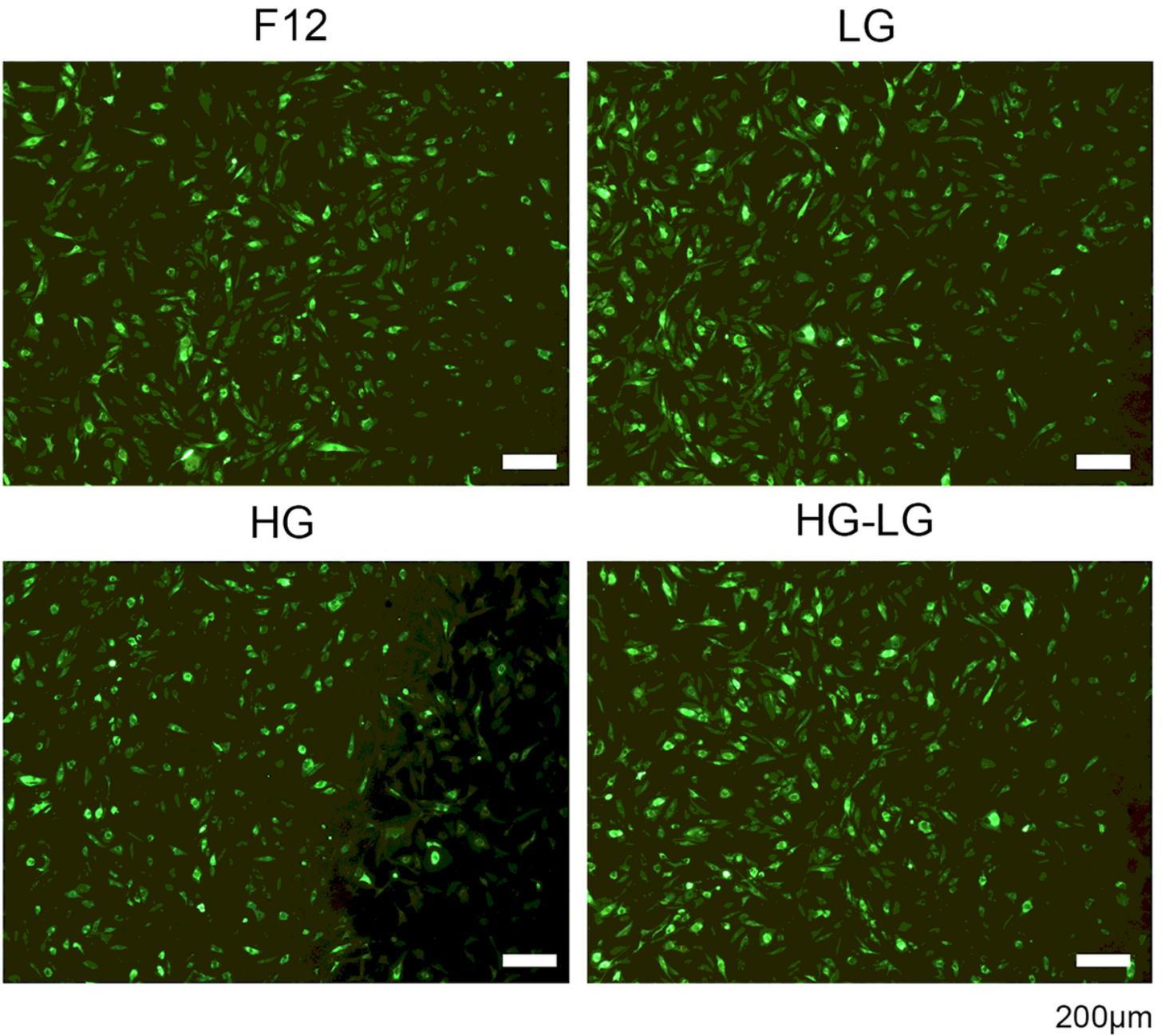
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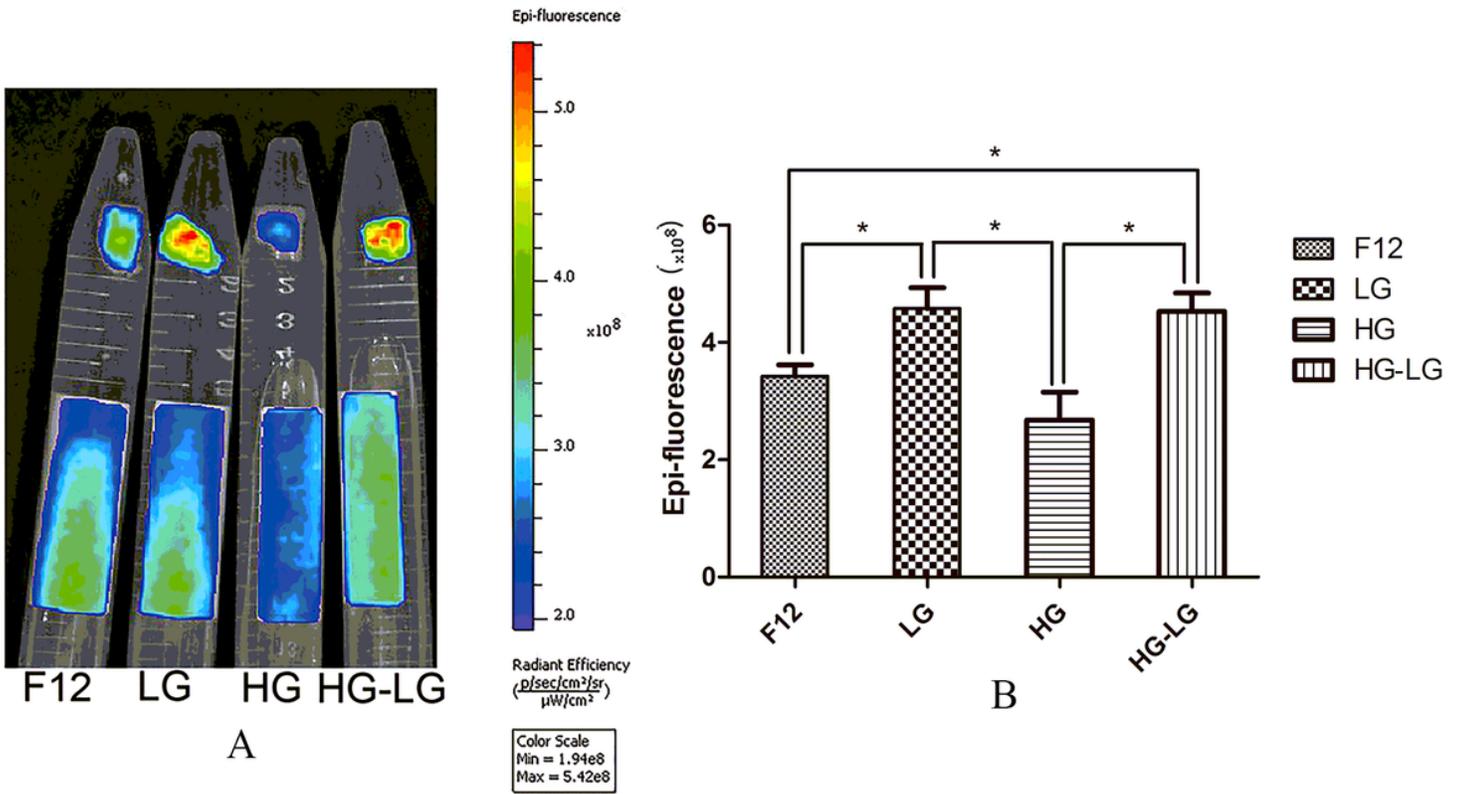
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The expression of GFP fluorescence of the 3rd passage rBMSCs in 4 different culture groups The 3rd passage rBMSCs in the 4 groups were highly expressed GFP fluorescence after staining.  $\times 60$ ;  $n=3$ .



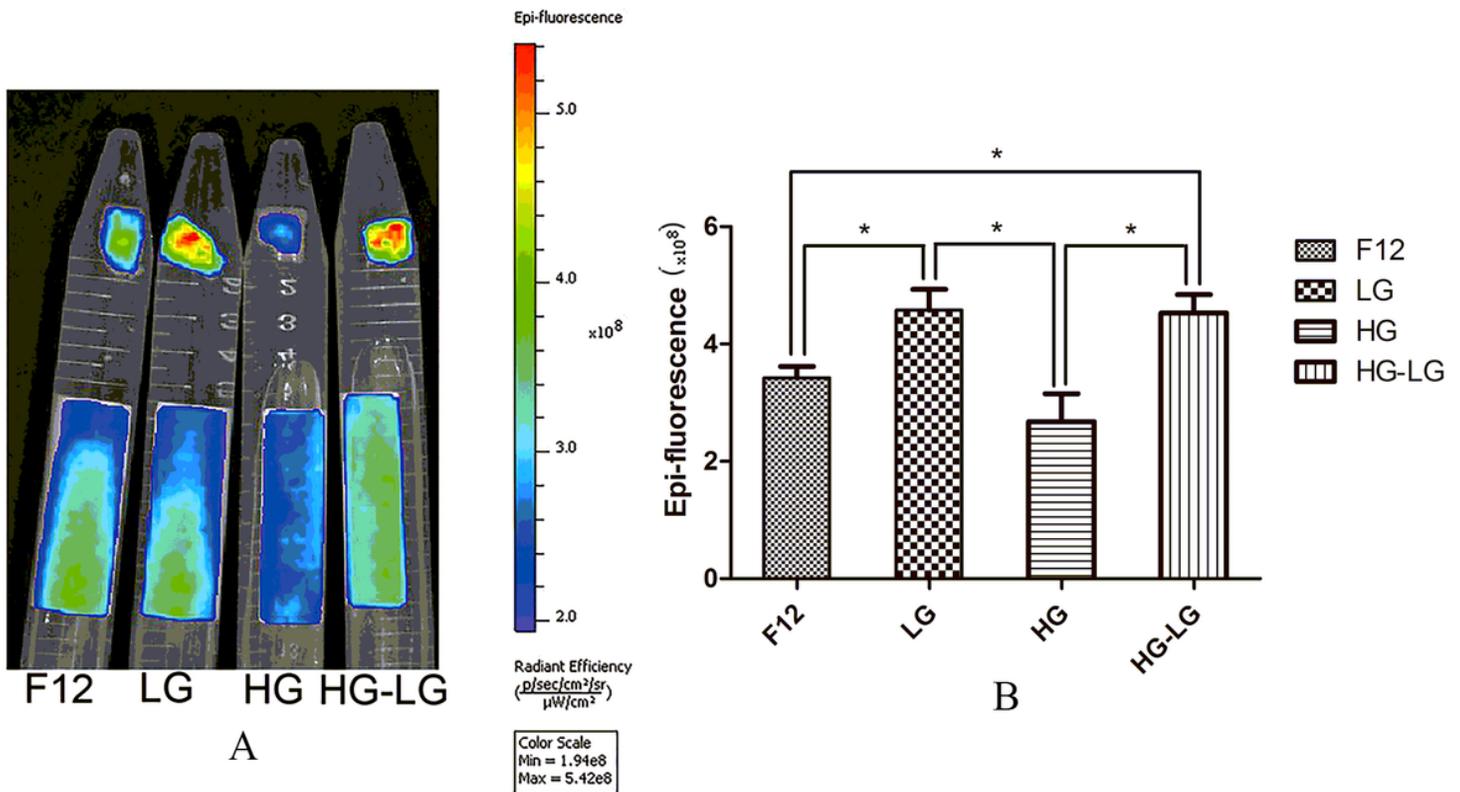
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**Figure 9**

The vitality of the 3rd passage rBMSCs in TEB in 4 different culture groups A: The GFP fluorescence expression of TEB in 4 different culture groups. B: The results of statistical analysis of GFP fluorescence intensity of TEB in 4 different culture groups. The fluorescence intensity of TEB of LG group and HG-LG group was higher than that of F12 group and HG group.  $n=3$ ; \*  $P < 0.05$ .



**Figure 9**

The vitality of the 3rd passage rBMSCs in TEB in 4 different culture groups A: The GFP fluorescence expression of TEB in 4 different culture groups. B: The results of statistical analysis of GFP fluorescence intensity of TEB in 4 different culture groups. The fluorescence intensity of TEB of LG group and HG-LG group was higher than that of F12 group and HG group. n=3; \* P<0.05.

## Supplementary Files

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