

# Influence of VEGF/BMP-2 on the proliferation and osteogenetic differentiation of rat bone mesenchymal stem cells on PLGA/gelatin composite scaffold

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**Abstract.** – **OBJECTIVE:** To investigate the influence of VEGF/BMP-2 on the proliferation and osteogenic differentiation of rat bone mesenchymal stem cells (BMSCs) on PLGA/gelatin composite scaffold.

**MATERIALS AND METHODS:** Randomly-oriented nanofibers with different ratios of Poly Lactic-co-Glycolic Acid (PLGA)/gelatin were produced through electrospinning. The mixture of nanofibers and BMSCs was pipetted onto the surface of the scaffolds, and BMSCs/PLGA/gelatin composite was obtained. The surface morphology, chemical structure, hydrophilicity and mechanical property of PLGA/gelatin nanofibers were revealed by scanning electron microscope. In vitro release kinetics of bone morphogenetic protein (BMP-2) and vascular endothelial growth factor (VEGF) were studied using ELISA kits. The cell adhesion, growth and proliferation of BMSCs on scaffolds were observed by scanning electron microscopy. The CCK-8 assay was used to evaluate the effects of VEGF/BMP-2 slow release system on the proliferation of BMSCs on scaffolds. RT-PCR was used to examine the activities of alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX-2), and osteocalcin (OCN).

**RESULTS:** In each group of cells in the *in-vitro* experiment, through electron microscope scanning, fiber scaffolds were interconnected three-dimensional reticular structure, BMSCs firmly attached to the fiber surface and internal stent, cells experienced a long spindle, polygon change, and branch-like protrusions on the cell surface were connected. Under the electron microscope, cell proliferation curve and osteogenesis markers (ALP, RUNX-2, OCN) expression in the dual factor group on cell adhesion, prolifer-

ation and differentiation were much better than those of blank control group and single factor groups.

**CONCLUSIONS:** In the successfully constructed gelatin/PLGA nanofiber scaffold, VEGF and BMP-2 can be sequentially released, during which VEGF and BMP-2 can promote the adhesion, proliferation, and differentiation of BMSCs.

*Key Words:*

BMSCs, Bone tissue engineering, PLGA/gelatin nanofiber stents, BMP-2 growth factor, VEGF growth factor.

## Introduction

At present, defect and loss of bone tissues caused by some diseases such as bone trauma and bone tumor have been frequently seen in clinical practice. Especially for elderly patients with a defect of bone tissues, simple repair and reconstruction using their own bone tissues could hardly repair the affected bone tissues<sup>1</sup>. Considering such a demand for clinical repair of bone tissues, many studies have focused on the development of substitute materials of bone tissues. In recent years, with the continuous development of tissue engineering, its effect on the treatment of diseases of bone tissue defect has also attracted the wide attention of scholars in China and other countries.

Currently, studies on the bone engineering mainly focus on the construction patterns of scaffold material, seed cells, and growth factors, in which good scaffold material and growth factors are essential for cell adhesion, proliferation, and differentiation<sup>2,3</sup>. However, the deficiencies of growth factors, such as poor stability and short half-time, have limited clinical application; but when the growth factor is loaded on a carrier or a sustained release system, not only can the biological activity of growth factor be protected, but also the release will be delayed. In this way, it can continuously promote the cell growth, repair, and regeneration of tissues. Thus, the application of materials that can be used as the carrier to control the release of growth factors has become one of the orientations in current studies<sup>4</sup>. At present, porous scaffold, prepared by the biological material, polylactic-co-glycolic acid (PLGA, a copolymer of polylactic acid and polyglycolic acid), has been frequently applied in biomedical engineering and pharmaceutical engineering for its excellent biological compatibility, nontoxicity, sacculation and film-forming capabilities<sup>5</sup>. Furthermore, via biological methods, growth factors can be loaded onto the PLGA nanofiber scaffold with the advantages like simplicity and the ability to sustain the activity of growth factors; thus, it is a better method to prepare the sustained release system<sup>6</sup>. In this study, biological methods were applied to load the vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) onto the gelatin/PLGA nanofiber scaffold to prepare the composite scaffold with the sustained release function of VEGF/BMP-2; in addition, to search for the ideal material for preparation of composite scaffold for bone tissue engineering through *in-vitro* experiment to provide the experimental basis for the treatment of bone tissue defect, we investigated the effect of the composite material on adhesion and proliferation bone mesenchymal stem cells (BMSCs) after the osteogenic induction of BMSCs.

## Materials and Methods

### Materials and Apparatus

#### *Animals*

SD rats (about 30 g) were provided by Experiment Animal Center of Harbin Medical University. The animal experiment was conducted under the guidance of *Regulation of Use and*

*Management of Experiment Animals of Harbin Medical University*, and researchers in this study should possess the relevant qualification certificate. During the operation, all procedures were in accordance with the requirement of Ethics.

#### *Main Reagents and Apparatus*

Gelatin, trifluoroethanol, and PLGA were purchased from Aladdin Biological Reagent Co., Ltd. (Denver, CO, USA); phosphate buffer (PBS) and low-sugar Dulbecco's Modified Eagle Medium (DMEM) medium were purchased from Hyclone (Logan, VT, USA); trypsin, fetal bovine serum (FBS), penicillin-streptomycin, ALP kit, dexamethasone sodium phosphate injection and alizarin red staining kit of L-ascorbic acid were purchased from Beyotime Biotech Co., Ltd. (Hangzhou, Zhejiang, China); DMSO was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China); DMSO and oil red O were purchased from Sigma-Aldrich (St. Louis, MO, USA); 0.25% glutaraldehyde and RIPA lysis buffer were purchased from Gibco (Grand Island, NY, USA), and ELISA kit from Peprotech (Rocky Hill, NJ, USA). BCD-182WE -25°C refrigerator was purchased from SANYO (Tokyo, Japan), and -80°C refrigerator from Jouan (Saint-Herblain, France); CO<sub>2</sub> incubator was purchased from Heraus (Hangzhou, Zhejiang, China); liquid nitrogen container was purchased from Thermo Forma (Boston, MA, USA); centrifugal machine was purchased from Heraus (Hangzhou, Zhejiang, China); optical microscope was purchased from Olympus (Tokyo, Japan); microplate reader was purchased from Reder (Tokyo, Japan); T25 culture bottle was purchased from Sigma-Aldrich (St. Louis, MO, USA); imaging system of microscope was purchased from Olympus (Tokyo, Japan); inverted microscope was purchased from Reder (Tokyo, Japan); electronic scale was purchased from Sartorius (Beijing, China).

### Methods

#### *Preparation and Features of Gelatin/PLGA Electrospun Nanofiber Scaffolds and Combination with BMP-2/VEGF*

The experimental gelatin solution (11%wt) was prepared by dissolving gelatin into trifluoroethanol and placed at room temperature for 24h. The same method was applied in the preparation of PLGA spinning solution. Before use, the spinning solutions were well mixed using magnetic stirrers.

Gelatin solution and PLGA solution were mixed in a volume ratio of 7:3, 5:5, 3:7 and 1:9 to prepare the gelatin/PLGA electrospinning solutions in different concentrations, from which we took 10 mL gelatin/PLGA electrostatic spinning solution for following procedures. For electrospinning, 5 mL of the composite solution was placed in a 5 mL syringe for injection at a rate of 10  $\mu\text{m/s}$  at 10 kV, in which the distance between loading rollers was 10 to 15 cm and the needle was perpendicular to the receiving roller. Nanofiber scaffolds that were prepared by electrospinning in gelatin/PLGA spinning solutions in different concentrations were dried at room temperature in vacuum. After metal spraying, the material of scaffolds was scanned using a scanning electron microscope (SEM) to observe the morphology. After the gelatin/PLGA mixture was well mixed, the growth factors were added and stirred with a magnetic stir for 30 min followed by electrospinning. In each group, 10 mL electrospinning solution was injected into a syringe which was later placed into a micro-injection pump, and solution was injected at a rate of 10  $\mu\text{m/s}$  at 10 kV, in which the distance between loading rollers was 10 to 15 cm. Nanofiber scaffolds that were prepared by electrospinning were dried at room temperature in vacuum for 24h.

Gelatin and PLGA were dissolved in the tri-fluoroethanol to prepare 11%wt gelatin electrospinning solution and 11%wt PLGA electrospinning solution after mixing at room temperature. Based on the previous experiment results, the mixture of gelatin and PLGA in a volume ratio of 1:9 was taken as the standard electrospinning solution. Then, BMP-2 or VEGF were added into the well-mixed electrospinning solution to prepare the standard spinning solution of BMP-2 (2.5  $\mu\text{g/mL}$ ) and standard spinning solution of VEGF (2.5  $\mu\text{g/mL}$ ).

#### ***Isolation, Culture and Identification of BMSCs in Rats***

SD rats were executed and disinfected. In an aseptic environment, bilateral femurs and tibias were removed and rinsed using PBS 3 times. 5 mL syringe was used to rinse the marrow cavity repeatedly, and the bulk tissue fragments were filtrated. Rinsing solutions were collected using 15 mL centrifuge tubes for centrifugation at 2500 rpm/min for 30 min. The solution in middle layer of centrifuge tube was taken for centrifugation at 2000 rpm/min for 10 min, and the supernatant was discarded. DMEM culture medium

(10% FBS + 0.1% penicillin and streptomycin) was added into the solution for re-suspension of cells. The cell re-suspension was added into a 25  $\text{cm}^2$  culture bottle which was then placed in an incubator (37°C and 5%  $\text{CO}_2$ ) for 3 days of culture. Thereafter, cell adhesion was observed under the inverted microscope, and the medium was exchanged every two days; meanwhile, morphological changes of cells were observed. After 80% to 90% of cells were fused, a subculture of cells was carried out, in which 1 mL PBS was added to rinse cells two times. 2 to 3 mL 0.25% trypsin was added for digestion. When cells began to float or changes were observed in cell morphology, digestion was terminated. The cells at the bottom of the bottle were blown and beat. Then, cell solution was centrifuged for 5 min at 2000 rpm/min. After centrifugation, sediment was added into DMEM medium (containing 10% FBS + 0.1% penicillin and streptomycin), and then delivered into the corresponding culture bottles for culture followed by subculture in the ratio of 1:2. After 3 passages of subculture, cells were cryopreserved. After digestion and centrifugation, the density of cells in the 3<sup>rd</sup> generation was adjusted to  $1 \times 10^6$  mL. Using the flow cytometer, expressions of cell-surface antigens (CD29, CD44, CD90, CD34 and CD45) were detected, and the results were analyzed.

#### ***Observing the Distribution and Morphology of Rat BMSCs on the Materials Via SEM***

After 1 day of cell culture, the culture medium was removed and the cells were rinsed twice using PBS. Cells in each experiment group were fixed at 4°C for 24h using 2.5% glutaraldehyde solution and sequentially placed into 50%, 75%, 95% and 100% ethanol solutions for gradient dehydration (15 min in each solution of ethanol). Then, cells were soaked in the isoamyl acetate for 30 min. After being dried at a critical point of  $\text{CO}_2$  and metal spraying for cells in experiment groups, cells were placed under the SEM for observation.

#### ***Osteogenic Induction of Rat BMSCs***

Preparation of osteogenic induction medium: 5 mL FBS and 0.5 mL penicillin-streptomycin solution were added into 45 mL DMEM culture medium to prepare the complete medium. In the complete medium, 50  $\mu\text{M}$  ascorbic acid, 10 mM sodium  $\beta$ -glycerophosphate, 0.1  $\mu\text{M}$  dexamethasone, and 50 mg/L vitamin C were added to

prepare the osteogenic induction medium. Cells in the 3<sup>rd</sup> generation were collected and the density of cell solution was adjusted to  $5 \times 10^3/\text{cm}^2$ . Then, the solution was added into 2 mL osteogenic induction solution, and the experiment in each group was repeated 5 times. Morphological changes were observed and recorded every day. BMSCs on the 21<sup>st</sup> day of osteogenic induction were collected with the cell culture medium being removed, and were rinsed using PBS for 5 min. Then, BMSCs were fixed in 4% paraformaldehyde at room temperature for 15 min followed by washing using distilled water for 5 min. 40 mM alizarin red staining solution was added into cells (1 mL/well) for reaction at 37°C. After 30 min, free alizarin red was fully removed and the BMSCs were washed using distilled water for 5 min. Thereafter, cells were dried, sealed and placed under the inverted microscope to take a photograph and record.

#### ***Detecting the Proliferation of Rat BMSCs on the Nanofiber Scaffold Via CCK8***

The CCK-8 method was applied to detect the proliferation of rat BMSCs on the nanofiber scaffold. Tissue engineering scaffolds in each group that had been disinfected under an ultraviolet lamp were placed at the bottom of 24-well plate. Rat BMSCs of the 3<sup>rd</sup> generation were inoculated onto the scaffolds in each group in  $5 \times 10^3/\text{cm}^2$ , and in each group, there were 4 subgroups of experiment sample. 100  $\mu\text{L}$  high-glucose DMEM medium was added into each experiment well and the plate was placed into an incubator for incubation (37°C, 5%  $\text{CO}_2$ ). On the 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days of culture, 10  $\mu\text{L}$  CCK-8 solution was added into each well, and the plate was then placed into the cell incubator for 2 hours of incubation. Thereafter, 100  $\mu\text{L}$  culture solution was taken from each well and then transferred into a new 96-well plate.  $\text{OD}_{450}$  values in different groups were assayed using microplate reader. Cell growth curve was drawn for evaluating cell growth.

#### ***Detecting the Expressions of Osteogenic Differentiation Genes (RUNX-2, ALP, and OCN)***

Expressions of RUNX-2, ALP, and OCN were detected using RT-PCR. Those cells in different groups that had been processed were washed 3 times in pre-cooled PBS on ice. 1 mL Trizol RNA extraction was added into each well, and the extraction of total RNA of the cell was performed in accordance with the procedures in the instruction

of kit. The extracted total RNA was then used to detect the A260/A280 ratio via ultraviolet spectrophotometry, and after 3 times of detection, the concentration of RNA was calculated. In accordance with the instruction of reverse transcription kit, reverse transcription of extracted RNA was carried out to prepare the cDNA for PCR amplification. Primers used in PCR were synthesized by Invitrogen (Shanghai, China) as per the following sequences: GAPDH: upstream primer: 5'-ATGG-GGAAGGTGAAGGTCG-3', downstream primer: 5'-GGGTTCATTGATGGCAACAATATC-3'; Runx-2: upstream primer: 5'-TCTTCCCAAAG-CCAGAGCG-3', downstream primer: 5'-TG-CCATTCGAGGTGGTTCG-3'; ALP: upstream primer: 5'-CGTCTCCATGGTGGATTATG-3', downstream primer: 5'-CCCAGGCACAGTG-GTCAAG-3'; OCN upstream primer: 5'-AC-CCAGAATTACCTGATCC-3', downstream primer: 5'-AGACCACCCAGCACAAC-3'. Reaction conditions were set as follows: initial denaturation at 95°C for 3 min, 94°C for 30 s, 48°C for 30 s, 72°C for 1 min for a total of 35 cycles of amplification; extension at 72°C for 10 min. 1.5% agarose gel electrophoresis was carried out for PCR products, and grey scale scanning was performed in the images obtained from the gel imaging scanning system for 3 times.

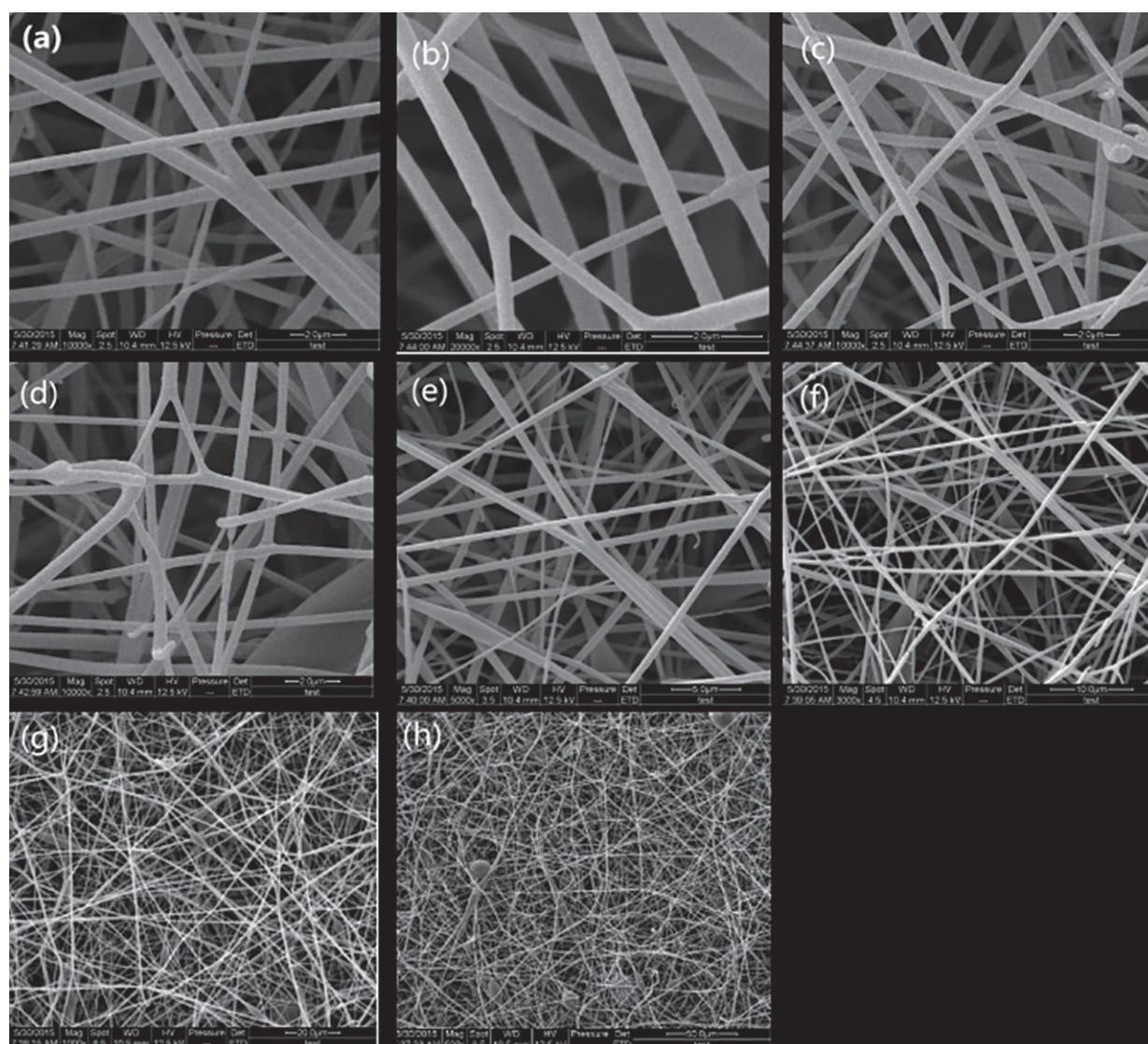
#### ***Statistical Analysis***

All values were presented by mean  $\pm$  standard deviation. In statistical analysis, SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was applied for single factor ANOVA.  $p < 0.05$  suggested that the difference had a statistical significance. Tukey's HSD (honestly significant difference) test was used in conjunction with an ANOVA to find means that were significantly different from each other.

## **Results**

#### ***Features of Gelatin/PLGA Nanofiber Scaffold Material***

Under the SEM, gelatin/PLGA nanofiber scaffold prepared via electrostatic spinning possessed smooth and bead-free surface in the uniform thickness. The result indicated that the mixture of gelatin and PLGA is capable of electrostatic spinning with a relatively high performance of spinning (Figure 1), nanofiber scaffolds in three-dimensional structures could be successfully prepared in different groups. Nanofibers, under 10000 $\times$  amplification, were randomly ori-



**Figure 1.** The surface morphology of electrospinning fiber under different ratio of gelatin/PLGA. (a) 1:9; (b) 3:7; (c) 5:5; (d) 7:3; (e) 1:9 5000 $\times$ ; (f) 1:9 3000 $\times$ ; (g) 1:9 1000 $\times$ ; (h) 1:9 500 $\times$ .

ented and in uniform diameter. In gelatin/PLGA (1:9) group and gelatin/PLGA (3:7), the smoothness and uniformity of nanofiber were better; in gelatin/PLGA (5:5) group and gelatin/PLGA (7:3) group, curves and crosslinking were observed in some nanofibers, and the viscosity between

nanofibers was more severe than that in gelatin/PLGA (1:9) group and gelatin/PLGA (3:7) group; in gelatin/PLGA (3:7) group, extremely thickened nanofibers were observed.

As shown in Table I, we could find that the diameter of the fiber in each group was about

**Table I.** Fiber diameter and distribution of electrospun gelatin/PLGA nanofiber with different ratios.

Radio of gelatin/PLGA	Average diameter (nm)	Distribution (nm)
1:9	231 $\pm$ 150	130-1105
3:7	374 $\pm$ 240	145-1235
5:5	450 $\pm$ 312	120-1576
7:3	649 $\pm$ 425	225-1789

100 nm, and the fibrous scaffolds met the requirements of nanofiber scaffold. Also, with a gradual increase in gelatin/PLGA ratio, the diameter was also increased, and so did the changes in the distribution of diameter of nanofibers. In gelatin/PLGA (1:9) group, the diameter of nanofiber was the smallest, and changes in the distribution of fiber diameter were also less obvious.

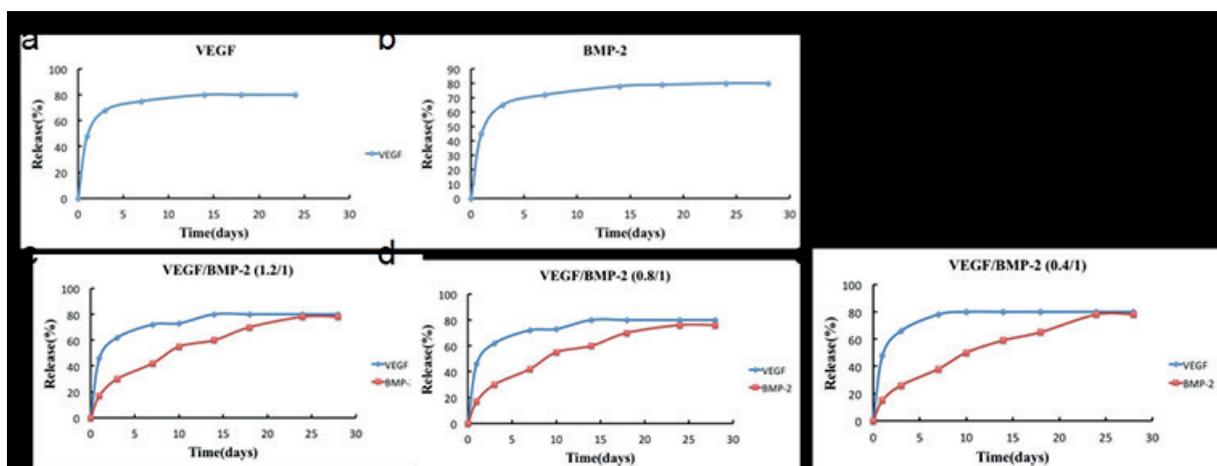
### Construction And In-Vitro Release of VEGF-BMP-2 Loaded Gelatin/PLGA Nanofiber Scaffolds

In each experiment group, no alterations were observed in the spinning efficiency and performance of nanofiber scaffolds following the addition of growth factor. This might be ascribed to the amount of growth factors that was less than the original spinning solution; thus, no alterations in the characteristics of nanofiber scaffolds were observed in each group when compared with those groups without addition of growth factors; moreover, compared to the single-layer spinning group, no significant changes were observed in spinning efficiency in newly constructed three-layer spinning structure. The release curve of growth factors in different groups was divided into 3 stages, i.e. stage of initial burst release, stage of slow-growth release and stage of persistent release. Burst release of growth factors in each group was observed on the 1<sup>st</sup> day. Subsequently, the stage of slow-growth release, followed by the slow but persistent release from the 7<sup>th</sup> to 14<sup>th</sup> days; the quantity of final release was around 80% (Figure 2).

### Morphological Observation and Identification of Rat BMSCs in Isolated Culture

In the primary culture of cells, the suspended cells were in round shape. Since the suspension contained various kinds of cells, such as BMSCs and blood cells, cells, other than BMSCs with the characteristic of adhesive growth, could be eliminated by exchanging the medium. In cell passages, other cells were gradually eliminated. On the 3<sup>rd</sup> day, long fusiform-shaped and polygon-shaped variations were observed in cells, and cell cluster appeared. During the cell growth, the quantity of cells adhering to the wall was increased, of which most cells were in spindle shape; cell cluster was fused, and monolayer growth and eddy-shaped cell alignment were observed. Passages were carried out when the 90% of the area in the bottom was covered by cells, and if BMSCs in the 5<sup>th</sup> generation were still in favorable morphology with stable proliferation rate, it suggested that BMSCs possess the capability to passage stably (Figure 3).

In rat BMSCs in the 3<sup>rd</sup> generation of isolated culture, expression of antigens (CD29, CD44, CD90, CD34, and CD45) was detected via flow cytometry. As shown in Figure 4, the expression rates of CD44, CD29, and CD90 were respectively  $93.55 \pm 0.27\%$ ,  $94.68 \pm 0.55\%$  and  $18.79 \pm 2.3\%$ ; the expression rates of CD45 and CD34 were respectively  $2.3 \pm 0.12\%$  and  $8.7 \pm 0.25\%$ . Based on the expression of antigens of cells, we could identify that the cells obtained through isolated culture were bone marrow stromal cells.



**Figure 2.** Release kits of VEGF/BMP-2 in different ratio of VEGF/PLGA. **A**, VEGF group; **B**, group of BMP-2; **C**, VEGF/BMP-2 1.2:1 group; **D**, VEGF/BMP-2 0.8: 1 group; **E**, VEGF/BMP-2 0.4: 1 group.



**Figure 3.** The morphology of separate culture BMSCs. **a**, Generation in primary culture on the third day; **b**, P3 subculture on the third day; **c**, The P5 subculture on the third day.

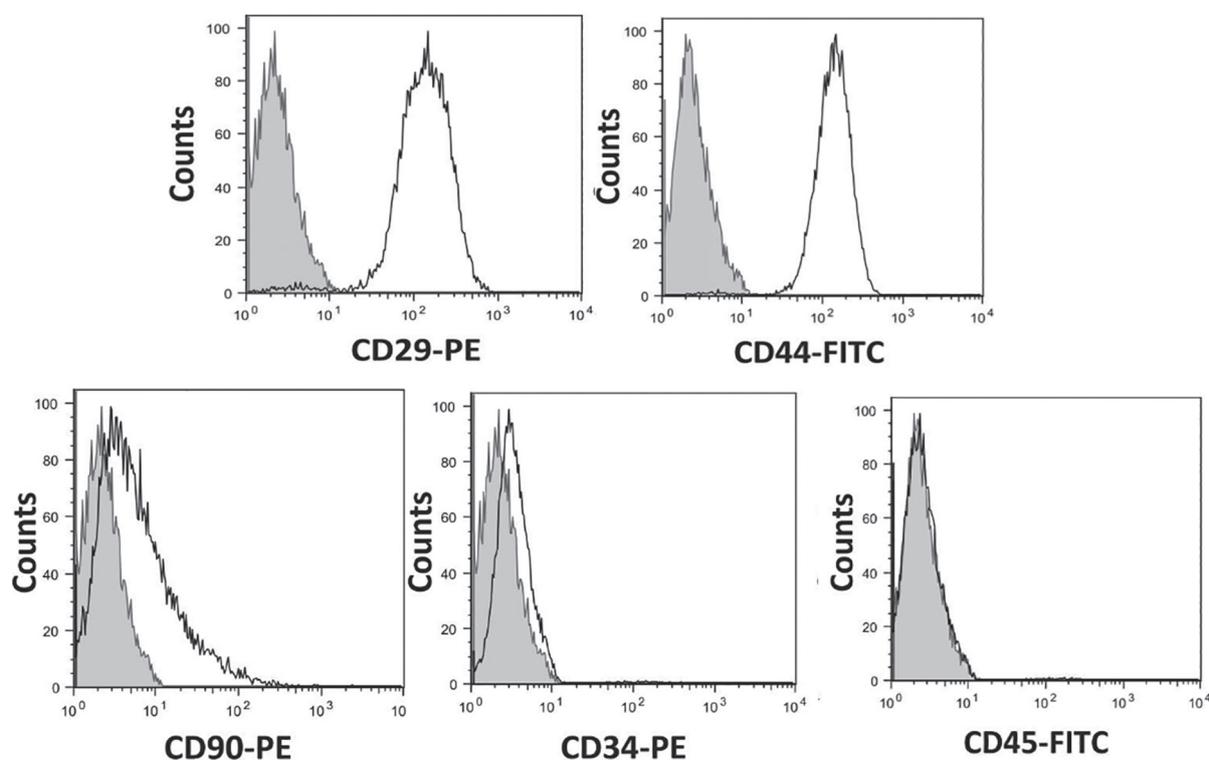
### ***Identification of Osteogenic Induction of rat BMSCs in Isolated Culture***

Multiple differentiation abilities of rat BMSCs obtained from culture were observed under the conditions of osteoblastic culture. After 3 days of culture under the osteogenic induction, enlargement was seen in a small fraction of cells; on the 7<sup>th</sup> day, cells were in loose arrangement, superposition was observed in cells and morphological changes were seen in most of the cells; on the 14<sup>th</sup> day, greater changes were observed in the morphology of all cells, and nodular mineralizer was found under the microscope (Figure 5); on the 21<sup>st</sup> day, after alizarin red staining, bulk complex

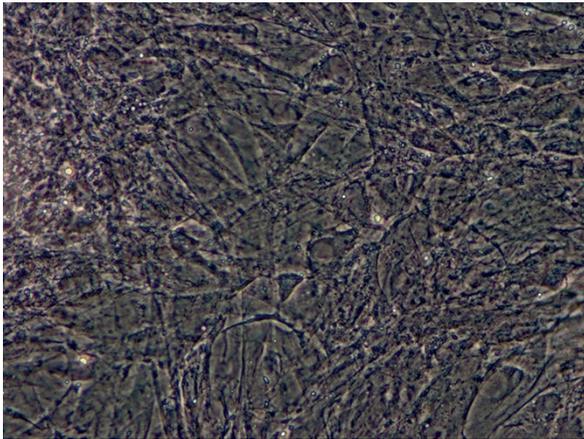
in deep red that was formed by the combination of alizarin red and Ca<sup>2+</sup> spread on the surface of osteoblasts, demonstrating that BMSCs obtained from osteoblastic culture were successfully differentiated into the osteoblasts (Figure 6).

### ***Adhesive Growth of BMSCs on Nanofiber Scaffolds***

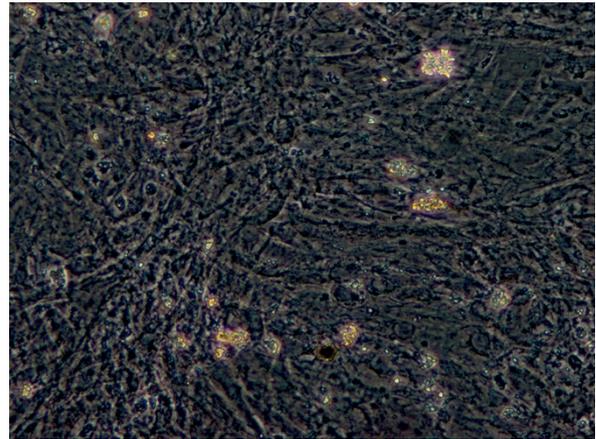
After rat BMSCs had been implanted onto the nanofiber scaffolds, we used the SEM to observe the growth of rat BMSCs on the nanofiber scaffolds in all experiment groups. In all experiment groups, there were cells in fusiform-shape adhering to the nanofiber scaffolds; it indicated that the



**Figure 4.** The surface antigen expression of BMSCs in rat.



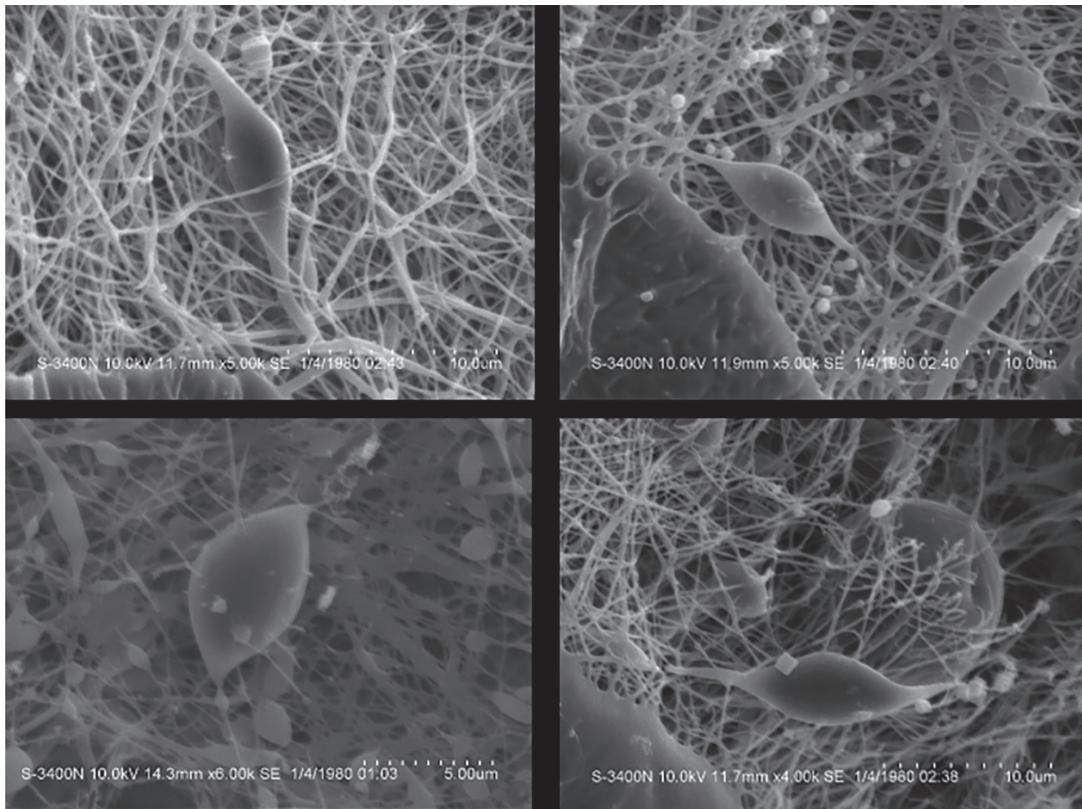
**Figure 5.** The microscopic observations of osteogenesis induced BMSCs in 14 days ( $\times 100$ ).



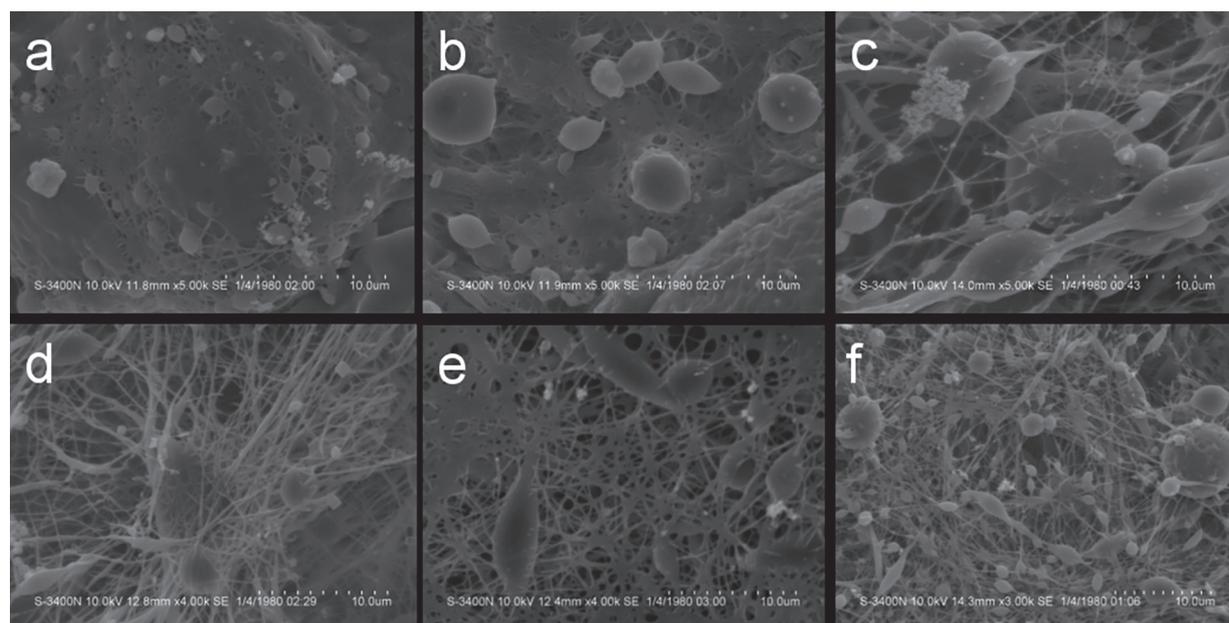
**Figure 6.** Alizarin red staining microscopic results of osteogenesis induced BMSCs in 21 days ( $\times 100$ ).

nanofiber scaffolds contributed to the adhesive growth of cells. In addition, cell entry could be observed inside the scaffolds, and there were cellular pseudopods around the scaffolds to assist the cells to adhere to nanofiber scaffold; meanwhile, surrounding the cells, there were white granular substances or filaments, including the matrix se-

creted by cells; this suggested that the porosity of scaffolds in this experiment was relatively large, which was beneficial to cell growth inside the scaffolds in addition to the growth on the surface, and could help the cells to fix on the scaffolds; thus, nanofiber scaffolds can promote the adhesive growth of cells (Figure 7). In Figure 8a (the



**Figure 7.** The 1-day electronic microscope observation of BMSCs cells cultured in PLGA/gelatin nanofiber scaffold loaded with BMP-2/VEGF.



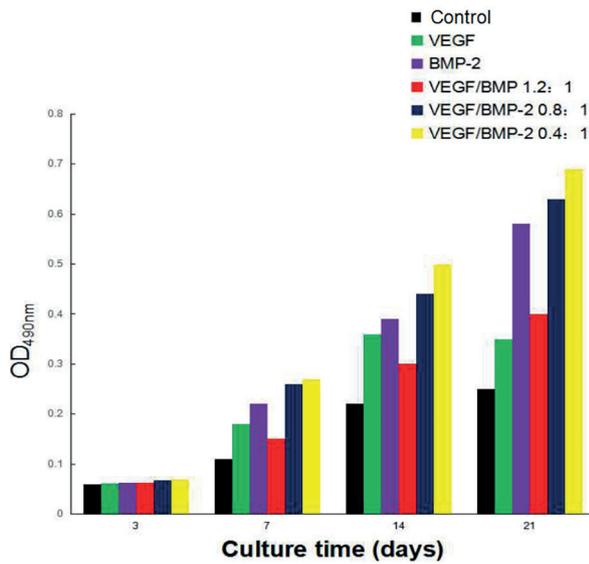
**Figure 8.** The 1-day electronic microscope observation of BMSCs cells cultured in PLGA/gelatin nano-fiber scaffold with different loading materials (5000 $\times$ ). **a**, negative control group; **b**, VEGF group; **c**, BMP-2 group; **d**: VEGF/BMP-2 1.2:1 group; **e**, VEGF/BMP-2 0.8: 1 group; **f**, VEGF/BMP-2 0.4: 1 group.

negative control group), under the SEM, cells on the scaffolds were in diffused distribution, and most of them on the scaffolds were the spherical cells that did not adhere to the scaffolds; under microscope, we found some tabular cells that did not fully adhere to the scaffolds, but no granular substance or filaments on the surface of cells, and most of cells were independent to each other. In Figure 8b (the VEGF group), compared with the cells in control group, the quantity of cells with tabular changes was increased; cell polarity could be identified; there was cell growth inside and on the surface of scaffolds, and the connections between the cells were increased. In Figure 8c (the BMP-2 group), fusiform changes could be seen in cell morphology; there were granular substances on the surface of cells, which were in more connections with the scaffolds than the filaments on the surface of cells. In Figure 8d (VEGF/BMP-2 1.2:1 group), fusiform changes were also observed in the cells, but the cell quantity and connections between cells were less than the BMP-2 group. In Figure 8e (VEGF/BMP-2 0.8:1 group) and Figure 8f (VEGF/BMP-2 0.4:1 group), cells were widely spread inside the scaffolds and on the surface of scaffolds, and most of the cells were in fusiform-shape; excretion of extracellular matrix was increased, the connections between cells were closer and more filament pseudopods

of cells were closely connected with the nanofiber scaffolds. In terms of adhesive growth of cell, the VEGF/BMP-2 0.4:1 group was the best, and the negative control group was the poorest. Thus, in this experiment, we found that PLGA/gelatin nanofiber scaffolds can promote adhesive growth of cells and accelerate the inward growth of cells in the scaffolds due to its special surface structure, while the PLGA/gelatin nanofiber scaffolds with the addition of BMP-2/VEGF can benefit the adhesion of rat BMSCs, and the excellent adhesive growth of cells can be attained in VEGF/BMP-2 0.4:1 group.

#### ***The effect of PLGA/Gelatin Nanofiber Scaffolds Containing Different Growth Factors on Cell Proliferation and Growth***

We applied the CCK-8 method to detect the effect of PLGA/gelatin nanofiber scaffolds containing different growth factors on cell proliferation. We assayed the OD values respectively on the 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days in culture to observe the proliferation of BMSCs in each experiment group. From Figure 9, we could find that there was a statistically significant difference in comparison of cell quantity between the negative control group and control group ( $*p < 0.05$ ), indicating that scaffolds can promote the cell proliferation; among all groups

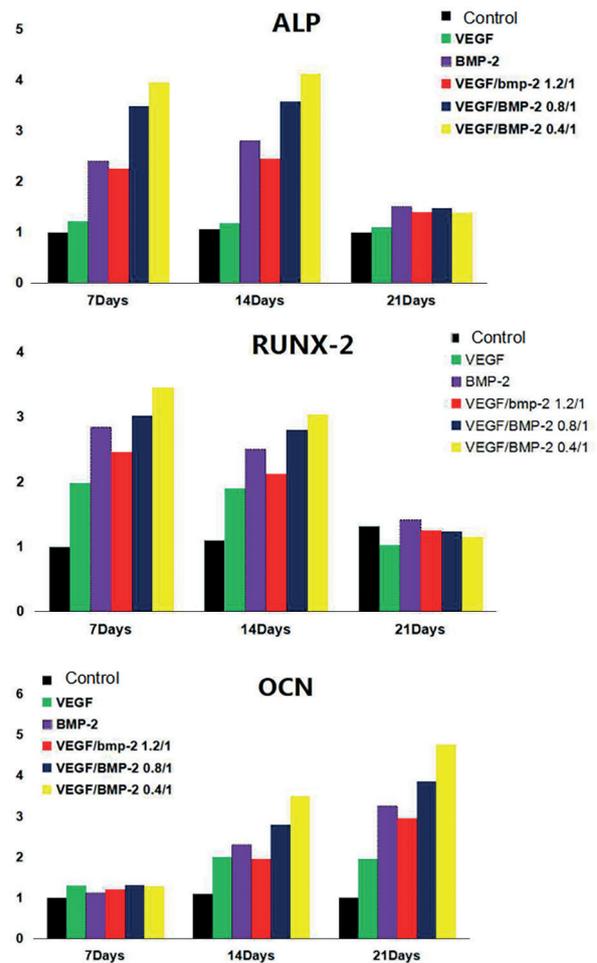


**Figure 9.** The OD values of PLGA/gelatin nanofiber stents loaded with different growth factors in different times.

with the load of growth factors, the quantity of cells was the lowest in the scaffolds only loading VEGF, yet higher than the negative control group; among the BMP-2 group and all experiment groups loading VEGF/BMP-2 in different ratios, there were no statistically significant differences in comparison of cell proliferation on the 3<sup>rd</sup> day of culture, but after 7 days of culture, the differences appeared and became more and more apparent with the extension of time. In VEGF/BMP-2 1.2 group, the OD values of cells on 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days were lower than the BMP-2 group. In VEGF/BMP-2 0.8 group and VEGF/BMP-2 0.4, the OD values of cells on 7<sup>th</sup>, 14<sup>th</sup>, and 21<sup>st</sup> days were significantly higher than the BMP-2 group ( $p < 0.05$ ), and the highest proliferation of cells was identified in the VEGF/BMP-2 0.4 group. This suggested that PLGA/gelatin nanofiber scaffolds, with excellent biocompatibility, can promote cell growth and proliferation. After 7 days of culture, the effect to promote cell proliferation in the groups of PLGA/gelatin nanofiber scaffolds loading VEGF/BMP-2 was superior to the groups of single growth factor. However, in all experiment groups, the PLGA/gelatin nanofiber scaffolds loading VEGF/BMP-2 in different ratios showed different effects on promoting the cell proliferation, in which VEGF/BMP-2 in the ratio of 0.4 was the most appropriate for cell proliferation.

### Gene Expressions of RUNX-2, ALP, and OCN

According to the gene expressions of RUNX-2, ALP and OCN at different time points in all experiment groups, we found that on the 7<sup>th</sup> day, i.e. the early stage of osteogenic differentiation, the gene expressions of RUNX-2 and ALP in BMP-2 group, VEGF group and VEGF/BMP-2 groups were significantly higher than the control group, in which the expression in VEGF group was significantly lower than other experiment groups. As for the expression of OCN, there was no statistically significant difference in comparison among groups. On the 14<sup>th</sup> day of osteogenic induction, among all VEGF/BMP-2 groups, the expressions of RUNX-2 and ALP in VEGF/BMP-2 0.8 group and VEGF/BMP-2 0.4 group were significantly higher than other groups, but the expressions of those two genes in VEGF/BMP-2



**Figure 10.** The ALP, RUNX-2 and OCN expression loaded with different growth factors in different times.

1.2 group were lower than the BMP-2 group. On the 21<sup>st</sup> day, a decreasing trend was identified in expressions of RUNX-2 and ALP in all groups. Persistent increase in expression of OCN was initially found on the 14<sup>th</sup> day, and the expressions in BMP-2 group, VEGF/BMP-2 0.8 group, and VEGF/BMP-2 0.4 group began to significantly increase, in which the OCN expression in BMP-2 group was the lowest, and in VEGF/BMP-2 0.4 group was the highest; the differences in gene expression among three groups had statistical significance ( $p < 0.05$ ). On the 21<sup>st</sup> day of osteogenic differentiation, a decreasing trend was identified in expressions of RUNX-2 and ALP in all groups.

## Discussion

In the world, there are a great number of patients requiring surgical treatment for the defect of bone tissue caused by various factors. However, reconstruction and functional restore of the affected bone tissue remain to be a challenge in clinical diagnosis and treatment. Generally, reconstruction via bone transplantation or other method is required in the treatment for defect of bone tissue. At present, the major methods employed in the bone repair include autogenous bone graft and bone allograft, in which the autogenous bone graft has been the most common method in clinical practice. Nevertheless, due to the limitations of materials, the autogenous bone graft is limited in clinical practice; while the application of bone allograft is bounded by disadvantages like immunological rejection and nosocomial infection. Thus, those methods are difficult to be generalized in clinical practice<sup>7</sup>. Construction of bone regeneration and repair via bone tissue engineering has become the major method for treatment of defect of bone tissue, in which the scaffold material is one of the key factors in constructing the bone via tissue engineering<sup>9</sup>. Currently, it has become a hotspot in research of bone tissue engineering to search for a kind of composite scaffold material that can induce the seed cells to exert the best biological function and possesses the sustained release system of growth factor.

Poly(lactic-co-glycolic acid) (PLGA), a kind of artificially synthesized high-molecular organic compounds, has been served as the most common scaffold material in tissue engineering research for its excellent biocompatibility and biodegradability. PLGA has currently been applied in the

domains of pharmaceuticals, medical engineering, and modern industries. Electrostatic spinning, frequently served as a method for preparation of nanofiber scaffolds<sup>9</sup>, is affected by various factors, such as the polymer's type, viscosity and surface tension, intensity of electric field, distance, temperature, and humidity. Through adjusting these factors, the characteristics of nanofiber scaffolds can be altered, such as the surface state of fiber, fiber diameter, and porosity<sup>10</sup>. A study<sup>11</sup> has shown that fibrous scaffolds in tissue engineering prepared by electrostatic spinning can effectively promote cell proliferation, which is mainly caused by the similarity between the structure of nanofiber scaffolds and the natural structure of the extracellular matrix. Thus, in this study, we chose gelatin/PLGA as the scaffold material, and construct the nanofiber scaffold via electrostatic spinning to observe the BMSCs' effects to promote cell proliferation and increase the osteogenic activity.

Cytokines are vital to proliferation, differentiation, and transformation of seed cells. VEGF plays an important role in the angiogenesis in repair and regeneration of bone defect<sup>12</sup>, while BMP-2 can exert vital functions in repair of a bone defect, especially in regulating the differentiation of stromal cells into the osteoblast and proliferation<sup>13</sup>. Three-dimensional porous scaffold prepared by biodegradation materials can realize the sequential release of factors promoting the angiogenesis and bone growth, which is of great significance for remodeling of bone tissues<sup>14</sup>.

In this study, gelatin/PLGA nanofiber scaffolds were successfully established via electrostatic spinning with BMP-2 and VEGF being loaded, and sequential release of BMP-2 and VEGF was achieved through three-dimensional structure of nanofiber scaffold, through which we found that after the release of VEGF and BMP-2, adhesion, proliferation, and osteoblastic differentiation of BMSCs could be significantly promoted. Via SEM, we found that after the growth factors were added, cells could be better adhered to the nanofiber scaffolds; especially on the nanofiber scaffolds loaded with VEGF and BMP-2, cells were widely distributed inside the scaffolds and the surface of scaffolds, connections between cells became closer, and cells could excrete the extracellular granular substances. These phenomena, together with the VEGF and BMP-2 loaded on the nanofiber scaffolds, can act on the cells, resulting in a more rapid adhesion, proliferation, and differentiation

of BMSCs<sup>14</sup>. This suggested that VEGF and BMP-2 loaded on the scaffolds can be slowly released and possess the biological activity to promote the adhesion in an early stage. In detecting cell proliferation in different groups via CCK-8, we found that on the 7<sup>th</sup> day, the rate of cell proliferation in VEGF/BMP-2 group was significantly higher than that in the single factor group. We believed that this might be correlated with the addition of VEGF in the VEGF/BMP-2 group: After the addition of VEGF, VEGF can interact with BMP-2 to promote cell proliferation in an early stage. Boulet et al<sup>15</sup> found that VEGF can upregulate the expression of BMP-2, and accelerate the proliferation rate of BMSCs. The study of Cui et al<sup>16</sup> showed that VEGF and BMP-2 can interact with each other via cell signaling pathway to promote the proliferation of BMSCs and the differentiation of BMSCs into osteoblasts, but the pattern of interaction between VEGF and BMSCs is associated with the amount of these two growth factors<sup>16</sup>. The addition of VEGF could significantly accelerate cell proliferation in an early stage, but less evident effect was found in the simple application of VEGF. Furthermore, scaffolds could better promote the activity of RUNX-2, ALP and OCN of BMSCs in *in-vitro* trials, indicating that VEGF/BMP-2 can remarkably promote the osteoblastic differentiation of BMSCs on gelatin/PLGA scaffolds.

## Conclusions

In this study, two different growth factors, VEGF and BMP-2, were creatively loaded on the gelatin/PLGA nanofiber tissue engineering stent system, and the sequential release of these two factors was achieved in a nanofiber stent. Compared with previous studies on the bone tissue engineering, this invention is characterized by many advantages, such as simplicity and feasibility in operation. Moreover, not only does this invention sufficiently exploit the biological features of gelatin/PLGA nanofiber tissue, but also it gives full scope to the effects of VEGF and BMP-2. Thus, it can fully exert its effect on promoting the adhesion and proliferation of BMSCs, and effectively facilitate the osteoblast differentiation of BMSCs in the long term. Furthermore, the sequential release of different growth factors can be accurately regulated, or adjusted according to the state of treatment, thereby realizing the indi-

vidual treatment under different circumstances. Meanwhile, this also provides us with the new orientation for future studies, in which the synergistic effect of multiple different growth factors on a stent will be more frequently utilized in the clinical practice and research of bone tissue engineering. However, the application of VEGF/BMP-2 loaded gelatin/PLGA nanofiber scaffolds is still limited in *in vivo* or *in-vitro* studies, and its osteogenic capability and the in-depth mechanism to promote the angiogenesis remain unclear. Thus, to apply the bone tissue engineering into the clinical treatment of bone defect, we need to carry out more studies in the future.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

## References

- 1) PANTALONE A, ANTONUCCI I, GUELFU M, PANTALONE P, USUELLI FG, STUPPIA L, SALINI V. Amniotic fluid stem cells: an ideal resource for therapeutic application in bone tissue engineering. *Eur Rev Med Pharmacol Sci* 2016; 20: 2884-2890.
- 2) FERNANDEZ-YAGUE MA, ABBAH SA, McNAMARA L, ZEUGOLIS DI, PANDIT A, BIGGS MJ. Biomimetic approaches in bone tissue engineering: integrating biological and physicommechanical strategies. *Adv Drug Deliv Rev* 2015; 84: 1-29.
- 3) XU J, LU H, MIAO ZN, WU WJ, JIANG YZ, GE F, FANG WF, ZHU AH, CHEN G, ZHOU JH, LU YZ, TANG ZF, WANG Y. Immunoregulatory effect of neuronal-like cells in inducing differentiation of bone marrow mesenchymal stem cells. *Eur Rev Med Pharmacol Sci* 2016; 20: 5041-5048.
- 4) FAROKHI M, MOTTAGHITALAB F, SHOKRGOZAR MA, OU KL, MAO C, HOSSEINKHANI H. Importance of dual delivery systems for bone tissue engineering. *J Control Release* 2016; 225: 152-169.
- 5) ORTEGA-OLLER I, PADIAL-MOLINA M, GALINDO-MORENO P, O'VALLE F, JÓDAR-REYES AB, PEULA-GARCÍA JM. Bone regeneration from PLGA micro-nanoparticles. *Biomed Res Int* 2015; 2015: 415289.
- 6) ZHU XH, WANG CH, TONG YW. In vitro characterization of hepatocyte growth factor release from PHBV/PLGA microsphere scaffold. *J Biomed Mater Res A* 2009; 89: 411-423.
- 7) CHANCHAREONSOOK N, JUNKER R, JONGPAIBOONKIT L, JANSEN JA. Tissue-engineered mandibular bone reconstruction for continuity defects: a systematic approach to the literature. *Tissue Eng Part B Rev* 2014; 20: 147-162.
- 8) JAKOB F, EBERT R, IGNATIUS A, MATSUSHITA T, WATANABE Y, GROLL J, WALLEES H. Bone tissue engineering in osteoporosis. *Maturitas* 2013; 75: 118-124.

- 9) HAMORI M, NAGANO K, KAKIMOTO S, NARUHASHI K, KIRIYAMA A, NISHIMURA A, SHIBATA N. Preparation and pharmaceutical evaluation of acetaminophen nano-fiber tablets: application of a solvent-based electrospinning method for tableting. *Biomed Pharmacother* 2016; 78: 14-22.
- 10) HOLMES B, ZHU W, LI J, LEE JD, ZHANG LG. Development of novel three-dimensional printed scaffolds for osteochondral regeneration. *Tissue Eng Part A* 2015; 21:403-415.
- 11) LI D, WU T, HE N, WANG J, CHEN W, HE L, HUANG C, EI-HAMSHARY HA, AL-DEYAB SS, KE Q, MO X. Three-dimensional polycaprolactone scaffold via needleless electrospinning promotes cell proliferation and infiltration. *Colloids Surf B Biointerfaces* 2014; 121: 432-443.
- 12) HU K, OLSEN BR. The roles of vascular endothelial growth factor in bone repair and regeneration. *Bone* 2016; 91: 30-38.
- 13) SCHWARTING T, SCHENK D, FRINK M, BENÖLKEN M, STEINDOR F, OSWALD M, RUCHHOLTZ S, LECHLER P. Stimulation with bone morphogenetic protein-2 (BMP-2) enhances bone-tendon integration in vitro. *Connect Tissue Res* 2016; 57: 99-112.
- 14) KANZLER JM, GINTY PJ, WHITE L, CLARKE NM, HOWDLE SM, SHAKESHEFF KM, OREFFO RO. The effect of the delivery of vascular endothelial growth factor and bone morphogenic protein-2 to osteoprogenitor cell populations on bone formation. *Biomaterials* 2010; 31: 1242-1250.
- 15) DECKERS MM, VAN BEZOOIJEN RL, VAN DER HORST G, HOOGENDAM J, VAN DER BENT C, PAPAPOULOS SE, LÖWIK CW. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* 2002; 143: 1545-1553.
- 16) HU Z, PEEL SA, HO SK, SÁNDOR GK, CLOKIE CM. Role of bovine bone morphogenetic proteins in bone matrix protein and osteoblast-related gene expression during rat bone marrow stromal cell differentiation. *J Craniofac Surg* 2005; 16: 1006-1014.