In Vitro Fabrication and Biocompatibility Assay of a Biomimetic Osteoblastic Niche

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Abstract

A novel HAp-CS/Gel biomimetic osteoblastic niche was fabricated by freeze-drying, and its mechanical strength and biocompatibility were characterized. HAp-CS/Gel scaffolds in various ratios of 100:5, 100:10, and 100:20 (CS/Gel to HAp) were prepared by freeze-drying prior to chemical cross-linking followed by infrared spectrum analysis, EDS, FITR, SEM, fluorescence microscopy, MTT, and ALP experiments. Results from the infrared spectrum analysis showed that HAp doping remained the surface morphology and the architecture of scaffold with interconnected pores in the size range of 135 to 150 μm . The HAp doping ratio of 100:20 was found to be optimal based on its high porosity of 90%, better water uptake folds of 19.1. In addition, EDS and FITR analyses demonstrated that HAp were uniformly distributed on the surface of a scaffold with aggregates and particles, which has sufficient roughness for cell attachment and proliferation of osteoblasts. Under SEM and fluorescent microscopy, osteoblasts seeded onto the scaffold showed evenly distributed viable cells, which is believed to form a biomimetic niche. In the present study, we further demonstrate that osteoblasts can maintain their function and grow well on the scaffold through MTT and ALP tests. Thus, the scaffold has favorable physical properties and biocompatibility to support the proliferation and differentiation of osteoblasts and further to support the constructs of biomimetic osteoblastic niche.

Keywords Osteoblasts · Biomimetic niche · Hematopoietic stem cell expansion · Dynamic environment · Cell function maintain

Introduction

Niche, as the bone marrow microenvironment of hematopoietic stem cells, was first proposed by Scofield in 1978 [1]. It contains the bone marrow mesenchymal stem and progenitor cells, osteolineage cells, sinusoidal endothelium, perivascular stromal cells, adipocytes, unmyelinated Schwann cells, immune cells (mainly regulating T cells), and many other components, including a variety of adhesion factors, growth factors, and chemokines [2]. Scofield believed that hematopoietic stem cells and bone marrow had a close contact, and the contact between cells and cells could guarantee the HSCs unlimited expansion capabilities and inhibit the maturation of HSCs. Secondly, the niche also provided a physical and functional environment for extrinsic factors controlling the fate of HSCs [3]. Generally speaking, as long as the stem cells can be closely attached to the niches, they will not be aging and still show the

characteristics and activity of stem cells. However, once the stem cells leave the niche (such as into the spleen), they will be aging and gradually lose the ability of re-parasitism. It was not widely recognized until 2008 that the niche, as the local microenvironment, plays a major role on the regulation of stem cell fate [4]. Recent advances have improved our understanding of the niches that maintain hematopoietic stem cells (HSCs). These advances include new markers for HSCs and niche cells, systematic analyses of the expression patterns of niche factors, genetic tools for functionally identifying niche cells *in vivo*, and improved imaging techniques. Together, they have shown that endothelial cells and mesenchymal stromal cells secrete factors that promote HSC maintenance in these niches, but other cell types also directly or indirectly regulate HSC niches [5, 6].

The niche has the two following functions: (1) regulating the balance of cell proliferation and silencing; (2) regulating the balance of cell self-renewal and differentiation [7]. Osteoblastic niche is located in the long bone cancellous bone region. The interface between bone and bone marrow is the inner lining of the bone. The inner layer of the inner lining of the bone is attached to a large number of osteoblasts and osteoclasts [8], and a large number of hematopoietic stem/progenitor cells are distributed in the vicinity of the lining of the bone. From the structure and distribution, the two kinds of cells have a close relationship. In fact, a large number of studies have indicated that osteoblasts are regulatory components of hematopoietic stem cells and also play a key role in survival, proliferation, and the maintenance of the hematopoietic stem cells [9–13]. Usually, its regulating function is mainly reflected in the following two aspects: (1) secreting related factors (receptors, cytokines, growth factors, etc.) to regulate the survival, regeneration, and maturation of HSC [14, 15]; (2) co-reconstructing the niche environment with HSC and other niche cells [16]. Although the expansion of hematopoietic stem cells has been widely studied, there is no reliable *in vitro* culture system to ensure the effective amplification of HSCs so far. Usually, HSCs will quickly differentiate, and then lose the stemness, which cannot meet the clinical needs [17, 18]. As we all know, the body's natural system can make HSCs keep the maintenance and self-renewal in the whole process of life, which forms a sharp contrast with the *in vitro* relatively low efficiency of the amplification system. Therefore, it is a promising method to construct the natural growth conditions.

Here are two essential factors to be considered for the construction of biomimetic niche: (1) three-dimensional porous cancellous bone-like scaffold structure provides space and carrier for other factors of niches; (2) the factors in the niche, especially the stromal cells, were fully introduced into the 3D scaffolds. The scaffold material has an important influence on the growth behavior of cell adhesion and proliferation [19, 20]. Ideally, the material chosen should provide sufficient three-dimensional growth space for the seed cells, while having good mechanical properties to allow it to quickly return, guide, and protect the developing tissue during exercise. In addition, biodegradability and degradation rate can be used as tunable parameters for evaluation. Among them, commonly used scaffold materials can be generally divided into natural polymer materials, synthetic polymer materials, and composite materials [21–23]. Chitosan, as a kind of natural biological material, has good biocompatibility and degradability [24–26]. Not only that, chitosan plays a supporting role in the process of osteoblast proliferation and differentiation [27]. In general, the chitosan solution in a suitable concentration can be of excellent pore structure after freeze-drying [28–30]. However, chitosan also has its shortcomings, lack of elasticity, flexibility, etc. Therefore, in order to strengthen the properties of chitosan in bone tissue engineering applications, it is often necessary to use it in combination with other materials [31, 32]. Gelatin is a biopolymer produced by the hydrolysis

of collagen with good biocompatibility, non-toxicity, and absorbable properties. Therefore, it is widely used in food-, pharmaceutical-, imaging-, and biomedical-related fields [33]. Hydroxyapatite has good biocompatibility, osteoconductivity, and osteoinductivity as the main mineral components of the extracellular matrix of bone [34]. In addition, the interaction between the nano-particles and the organic polymer material can improve the mechanical properties and biological properties of the stent [35, 36]. The nano-hydroxyapatite/chitosan hydrogel membrane after the addition of nano-hydroxyapatite greatly increased the crystallinity of the scaffold, demonstrating the excellent biocompatibility of the composite scaffold by human osteosarcoma cells [37].

Therefore, in this paper, the chitosan will be used as a substrate, combined with gelatin and hydroxyapatite, to prepare the composite scaffolds with excellent pore structure and bone conduction function. And bone cells will be inoculated to construct a bionic osteoblastic niche, so that an appropriate microenvironment could be provided for in vitro culture of hematopoietic stem cells.

Materials and Methods

Experimental Materials

Chitosan (degree of deacetylation > 85%, Amresco, USA); gelatin, lysozyme, calcein, propidium iodide (Sigma, USA); carbodiimide, N-hydroxy-succinamide, morpholine ethane sulfonate (Shanghai Maclean Biochemical Technology Co., Ltd.); glacial acetic acid (Tianjin Damao Chemical Reagent Factory); disodium hydrogen phosphate (Tianjin Komiou Chemical Reagent Co., Ltd.); hydroxyapatite (Shanghai Hualan Chemical Technology Co., Ltd.); fetal bovine serum, trypsin (GIBCO, USA); sodium edetate (SOLARIBIO, USA); HE dye solution, alkaline phosphatase dye solution, ALP staining kit (Biyuntian Biological Reagent Co., Ltd.); ALP assay kit (Nanjing Jianli Biological Reagent Co., Ltd.); MTT assay kit (Kaki Biotechnology Co., Ltd.); Hoechst 33258 (Shanghai Xiangsheng Biotechnology Co., Ltd.).

Freeze dryer (Shanghai Yuming Instrument Co., Ltd., FD-1A-50); scanning electron microscope (US FEI Company, QUANTA 450); collecting thermostatic heating magnetic stirrer (Gongyi City, China Instrument Co., Ltd., DF-101S); contact angle tester (DATAPHYSICS, OCAH200); Fourier transform infrared spectrometer (Thermo Fisher Nicolet 6700); inverted (phase difference) microscope (OLYMPUS, IX70); centrifuge (Herm, Z323); ultra-clean workbench (Shanghai Huilong Instrument Electronics Co., Ltd., CA-920); CO₂ incubator (Kendro Laboratory Products, HERA cell); ultrapure water machine (Millipore Corporation, Milli-Q); electronic balance (Shanghai Precision Scientific Instrument Co., Ltd., FA2004); porous culture plate (Saiye Biotechnology Co., Ltd.); microplate reader (TECAN, Sunrise).

Isolation and Culture of Osteoblast

Osteoblasts were extracted from the skull of neonatal rats (< 24 h) by enzymatic digestion. The newborn rats were taken from the SPK Animal Experimental Center of Dalian Medical University. The experiment has been reviewed by the Biotechnology and Medical Ethics Review Committee of the Dalian University of Technology. The newborn rats were sacrificed and sterilized in 75% alcohol for 30 min. The skull of the newborn rats was obtained by

surgery and soaked in PBS, and the skull was washed to remove the connective tissue such as the periosteum and blood vessels. The cleaned skull was cut into small pieces of 1 mm × 1 mm and transferred to a centrifuge tube. Trypsin was added and resuspended in a 37 °C water bath for 30 min to digest the fibrous tissue; then type I collagen was added and shaken in a 37 °C water bath for 90 min to separate the cells. The cell pellet was resuspended in complete medium, inoculated into a culture flask, and placed in a 37 °C, 5% CO₂ incubator. The presence of hybrid cells in the cultures is diminished by a complete medium change 3 days after plating the cells. Osteoblasts were isolated and purified by changing the medium, and passaged after the cells were grown to 80–90% confluence.

Osteoblasts at passage 3 were inoculated onto the coverslips in culture dishes with a density of 2×10^4 cells/cm². After 3 days of culture, the cells grew to 80% and were fused. Dead/live staining of osteoblasts was measured. The cells were washed twice with PBS, and then calcein, Hoechst, and PI were added; the cells were incubated at 37 °C for 30 min and observed under an inverted phase difference microscope. After fixing the cells with 4% paraformaldehyde at room temperature for 10 min, they were washed twice with PBS.

Biological Detection of Osteoblasts

Osteoblasts at passage 3 were inoculated onto the coverslips in culture dishes with a density of 2×10^4 cells/cm². After 2–3 days of culture, HE staining was performed to observe the cell morphology. After 1 week of culture, ALP staining was performed and calcein was added; after 3 weeks of culture, alizarin red staining was performed; after 4 weeks of culture, Von Kossa staining was performed to investigate the early differentiation and cell viability of the cells.

Calcein Staining The medium was discarded in the sample wells at room temperature. Washing with PBS was done three times, and 20 µL of calcein staining solution was added. The stained samples were incubated at 37 °C for 30 min and washed with PBS three times, and the cells were observed under fluorescence microscopy.

HE Staining The cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature and washed twice with distilled water. The cells were added HE staining solution for 8 min, rinsed with tap water and 95% ethanol, stained with eosin for 1 min, washed twice with 70% ethanol, and observed its morphology of under a microscope.

ALP Staining The medium was discarded in the well, rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min, rinsed with distilled water several times, added NBT/BCIP dye solution, and covered the sample in the well. Then, it was incubated in the incubator in the dark for 30 min, washed several times with distilled water, and observed the staining under a microscope.

Von Kossa Staining The medium in the wells was discarded, washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, rinsed with distilled water, incubated with 5% sodium thiosulfate for 30 min, and then washed three times with distilled water. The sample was covered with 1% sodium thiosulfate, rinsed with distilled water after 30 min of UV irradiation, incubated with 5% sodium thiosulfate for 2 min, neutralized with 1% neutral red for 10 min, washed with distilled water, and observed under a microscope.

Alizarin Red Staining After discarding the medium, the cells were washed three times with PBS, fixed with a 95% alcohol solution for 15 min, rinsed with distilled water, and stained with 0.1% alizarin red for 30 min. After washing with distilled water, the cells were observed under a microscope.

Preparation and Detection of HAp-CS/Gel Loaded Scaffolds

HAp-CS/Gel solution in a different proportion of 100:5, 100:10, and 100:20 (CS/Gel to HAp) was injected into 24 role plates, followed by pre-freezing at -20°C and freeze drying at -50°C for 36 h. After freeze-drying, EDC/NHS/MES was added for cross-linking for 6 h. To remove the residual acetic acid, all scaffolds were washed in Na_2HPO_4 solution for 2 h, followed by rinsing with 40% (v/v) ethanol for four times (30 min/time). At the same time, a non-HAp scaffold was prepared as a blank control.

Surface Morphology HAp-CS/Gel Composite Scaffolds

The surface morphology of the prepared scaffolds were examined using the tungsten scanning electron microscope (SEM). The specimens were cut to 2-mm discs, mounted on aluminum sample stubs, and sputter-coated with platinum prior to examination in the SEM at a voltage of 2000 kV.

Semiquantitative Analysis of Pore Wall Elements of Composite Scaffolds

For the composite scaffold with the highest HAp ratio of (100:20), three feature points are selected on the pore wall of the stent when the environment is scanned, meantime, combined with EDS, finally analyze the element composition of white aggregates on the pore walls. One thing to note is that cross-link does not change the distribution of HAp scaffold after cross-link was chosen to perform EDS.

Infrared Spectrum Analysis of Composite Scaffolds

Cross-linked and non-cross-linked composite scaffolds were ground to powder. Incorporation of HAp and chemical cross-linking was analyzed using infrared spectrum analysis.

Physical Properties of Composite Scaffolds

Water Absorption

The final casts of scaffolds were weighed as m_1 . After being soaked in distilled water for 10 days when the weight of wet scaffold did not change noticeably, the saturated absorbent scaffold was removed and the residual water on the surface of the scaffolds was absorbed with filter paper. The wet scaffolds were weighed as m_2 . The water absorption can be calculated through the following formation:

$$\phi = \frac{m_2 - m_1}{m_1} \times 100\%$$

Porosity HAp-CS/Gel at different proportions of HAp and CS/Gel was soaked in ethanol completely (V_1). The total volume of the scaffold and ethanol used for soaking was measured as V_2 . Therefore, the volume of “ $V_2 - V_1$ ” was considered as the total volume of the composite scaffold. Thereafter, the ethanol was extracted from the scaffold and the volume of ethanol was recorded as V_3 . The volume of ethanol ($V_1 - V_3$) was used as the volume of the pore volume of the scaffold. The macroscopic volume of the scaffold was then calculated as $V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$, and the porosity of the composite scaffold could be expressed as:

$$P = \frac{V_1 - V_3}{V_2 - V_3} \times 100\%$$

Water Contact Angle In order to investigate the effect of the incorporation of hydroxyapatite on the water contact angle, the contact angle on the top surface of the material was measured using a contact angle instrument.

In Vitro Construction of Biomimetic Osteoblastic Niche

HAp-CS/Gel scaffolds at a ratio of 100:20 (CS/Gel to HAp) were further analyzed for capability as biomimetic niches. The HAp-CS/Gel scaffold was sliced and placed in a 15-mL centrifuge tube, followed by sterilization. The osteoblasts (100 μ L) were evenly seeded on the scaffold at a cell density of 2×10^5 cells/mL and placed at a static condition of 37 °C, 5% CO₂ for 6 h incubation to construct the bionic osteoblastic niche.

Cell Growth on the Biomimetic Niches

The activity and distribution of cells in the bionic niches: post 3 days culturing, cell-seeded scaffold was examined to determine the survival status of cells using dead/live staining.

For observing cell distribution on the surface of the scaffold, three specimens were fixed by glutaraldehyde at 4 °C, following by dehydration in a series concentrations of ethanol (50%, 70%, 90%, and 100%). Post fixation, cell-seeded scaffolds were prepared as described above and scanned under a tungsten SEM at a voltage of 2000 kV.

Cell Proliferation in the Biomimetic Niches Cell proliferation rates of osteoblasts on the scaffold were measured by MTT assay at days 1, 3, 5, and 7, respectively. Cell growth in the orifice without the scaffold was used as the control group.

Preserve of Cell Function in the Biomimetic Niches The concentrations of ALP (alkaline phosphatase) in the scaffold for 7, 14, and 21 days were measured using an ALP quantitative assay kit, while cells growing without the scaffold were used as the control group.

Statistical Analysis

Data were presented as mean $\pm$ standard deviations. The one-way ANOVA was used to evaluate the inter-group comparison using the software Origin8.0 (OriginLab Corporation, USA). $P < 0.05$ was considered statistical difference.

Results and Discussions

Cell Morphology and Biological Detections of Osteoblasts

In this paper, enzymatic digestion was used to digest the bone fragments of newborn rats to extract osteoblasts. The cut bone pieces were inoculated into the culture flask. After 2 days of culture, some long spindle-shaped and fibroblast-shaped cells were found to climb out from the bone pieces, and some dead cells were not attached. When the cells were cultured for 5 days, the number of cells increased and maintained a variety of forms; there are triangles, long spindles, and so on. After the primary cells were trypsinized and passed to the first and second generations, the cells still showed good growth. With the increase of algebra, the morphology of the cells was more obvious and uniform, and most of them showed a long fusiform shape. However, there are still some fibroblasts (Fig. 1a).

Fig. 1 Culture and staining identification of SD rat osteoblasts. **a** Cell morphology of primary cultured and subcultured osteoblasts. Cell morphology of primary osteoblasts after 2 and 5 days in vitro culture (A1, A2); cell morphology of osteoblasts at passage 1 and 2 after 5 days of in vitro culture (A3, A4). **b** Detection of activity of osteoblasts in SD rats. Microscopic picture of SD rat osteoblast at passage 3 (B1); fluorescence microscopy picture of osteoblasts stained with calcein, Hoechst, and PI (B2, B3, B4). **c** Cell morphology of osteoblasts at passage 3 in vitro culture (C); fluorescence microscopy picture of osteoblasts stained with calcein (D), HE staining (E), ALP staining (F), Von Kossa staining (G), and Alizarin red staining (H), respectively

Part of the fibroblasts was still found in the first two generations of cells, and therefore, the cells of the second generation were purified by the differential adherence method. After purification and culture, it was found that most of the cells were long fusiform or triangular, full in shape, and had a strong three-dimensional sense, and the cells were well defined. After Hoechst staining, the nucleus emits bright blue fluorescence, and the nucleus is obviously round or oval; the cell cytoplasm is rich, and after being stained by Calcein, it emits green fluorescence. Therefore, it can be seen that the purity of the osteoblasts is improved to some extent after differential adherence purification, and the morphology of the cells is better and the distribution is more uniform, which is suitable as a seed cell for subsequent research (Fig. 1b).

Figure 1c is a cell morphology diagram of osteoblasts at passage 3, which grows in an adherent manner and has a long spindle shape. Figure 1c (D) is a picture of calcein staining of osteoblasts. The cells are connected to form a network of spiral cells, indicating that the proliferation of osteoblasts *in vitro* is very good. Figure 1c (E) shows HE staining of osteoblasts for more intuitive observation of cell morphology. It can be seen that the osteoblasts' adherent growth is long fusiform and is spirally covered at the bottom of the well plate. By staining, the blue-purple nuclei and the red-colored cytoplasm can be clearly seen. The growth pattern is more obvious. Figure 1c (F–H) are ALP staining, Von Kossa staining, and alizarin red staining, respectively. The results of blue-violet precipitation, black staining, and red nodules appear in each staining result, indicating that the osteoblasts have strong proliferation and osteogenic ability, suitable for use as seed cells.

Morphology of HAp-CS/Gel Scaffold

HAp-Cs/Gel stent was successfully produced by freeze-drying with sufficient chemical cross-linking. The macroscopic structure of the scaffold appeared to be spongy with a fairly smooth surface as shown in Fig. 2a.

The SEM analysis demonstrated a similar porous morphology of chitosan scaffolds with the pore size measured in the range of 135–150 μm for blank chitosan scaffolds and HAp-incorporated chitosan scaffolds, suggesting that incorporation of HAp had no effect on the morphology or internal structure of CS/Gel scaffolds (Fig. 2b). Therefore, the physical structural support of the HAp-CS/Gel was believed to be comparable to blank CS/Gel scaffolds. In addition, the SEM analysis also revealed that with the increase of the HAp incorporation, white particles (undetermined HAp) were apparent on the surface of pores and evenly distributed (Fig. 2c).

Elements Analysis of Composite Scaffold Wall

Elements analysis was conducted on the chosen area (Fig. 2d) through SEM-EDS for the determination of HAp shown as white parties under SEM. The SEM-EDS analysis confirmed that three selected clusters contain calcium and phosphorus, in addition to the main elements carbon and oxygen, which can fully judge that the white area is incorporated with HAp, and it can further prove that HAp was successfully incorporated into the scaffold (Fig. 2e).

The existence of calcium and phosphorus was determined by EDS, and it can be further discovered that the ratio of calcium and phosphorus on the scaffold was close to the ratio of HAp. The amplitude of variation of the amount of calcium on three selected sites was minimal indicating HAp was evenly distributed on the surface of scaffolds.

Fig. 2 **a** The macrograph of HAp-CS/Gel scaffold. HAp-CS/Gel mixture with different HAp contents (A); the macrographs of the scaffold with HAp content of 100: 20 (B). **b** The micrograph of HAp-CS/Gel scaffold. The micrograph of the scaffold with HAp content of 100:0 (A); the micrograph of the scaffold with HAp content of 100:5 (B); the micrograph of the scaffold with HAp content of 100:10 (C); the micrograph of the scaffold with HAp content of 100:20 (D). **c** SEM images of HAp-Cs/Gel scaffold pore wall (scale bar 20 μ m). Pore wall of scaffold without HAp (A); pore wall of scaffold loading HAp with different content (B–D 100:0, 100:5, 100:10, and 100:2). **d** Energy spectrum of chosen sites in the pore wall of the scaffold (100:20). Specified sites onto pore wall (A); energy spectrum of the chosen sites (B–D, 1–3). **e** Atom content of Ca/P on the specified sites. **f** FITR analysis of the HAp-Cs/Gel scaffold (100:20)

Infrared Spectroscopic Analysis of HAp-CS/Gel Scaffold

The chemical bonds of HAp-CS/Gel scaffolds were characterized by the infrared spectroscopic analysis. Chitosan contains a large amount of hydroxyl and amidogen, while gelatin has a large amount of amino acid chains, among them are amidogen and carboxy group. Cross-linking reaction can be triggered by a cross-linking agent such as EDC, NHS, and MES, and post cross-linking, ester bonds and amide bonds are formed to enhance the combination. As shown in Fig. 2f, the absorption peaks of an amide bond (1342 cm^{-1}) and the stretching vibration band of C=O in amide bond (1685 cm^{-1} and 1776 cm^{-1}) were observed after cross-linking, indicating that cross-linking reaction sufficiently enhanced the combination of various composition. Such combination was believed to enhance the mechanical strength of the scaffold in the usage of bone tissue engineering.

Physical Properties of HAp-CS/Gel Support

In the present study, it was found that the capacity of water uptake was measured 20 times high based on the dry weight of the scaffold. This water uptake was increased with a higher proportion of HAp in the composite scaffolds, measuring at the tenth day for 100:0 and the

tenth day for 100:20 (Fig. 3b). This observation may be attributed to the water shielding effect of HAp which is able to increase mechanical strength in immersion. Therefore, the follow-up experiment will consider the use of HAp incorporation ratio of 100:20 composite scaffolds.

In contrast, HAp incorporation into gel scaffolds showed no significant effect on the porosity of the scaffolds. The porosity remained over 90% from HAp weight ratios in the range of 100:0 to 100:20 of HAp:CS/Gel. Thereafter, HAp and CS/Gel mass ratio of 100:20 composite scaffolds was chosen as the structural support for producing bionic niche.

The results from the surface contact angles indicated that the HAp incorporation affected the surface hydrophilicity of CS scaffolds: that the surface contact angle gradually reduced from 88.5° in the blank group to 45.8° when the HAp content increased from 0 to 20. This result suggested that the incorporation of HAp can be definitely applied for modifying the hydrophilicity of the hybrid scaffolds.

Cell Distribution and Activity in Biomimetic Niche

Calcein-AM staining, Hoechst staining, and PI staining confirmed the cells' viability in the scaffold with emitted green fluorescence signals. Few cells showed positive red fluorescence, which indicated that cells grow favorably in the scaffold. Moreover, blue fluorescence-stained cells were observed in the pores of scaffolds further demonstrating that cells attached and proliferated on the surface of the scaffolds desirably. The SEM analysis (Fig. 4) showed the characteristic cell morphology of osteoblasts which were attached on the surface of the scaffold desirably to stretch growth, and the trend of 3-dimensional growth was observed.

The Proliferation and Function Maintenance of Cells in Biomimetic Niche

During 1 week post seeding, the osteoblasts showed a desirable proliferation rate, although the proliferation rate was found slightly slower compared with those cells growing in a culture flask (Fig. 5a). The number of cells (OD value) was found gradually increasing approximately from 0.26 ± 0.01 at day 1 to 0.60 ± 0.02 at day 7 on scaffolds compared with 0.22 ± 0.01 at day

Fig. 3 Physical properties of HAp-CS/Gel scaffolds. **a** Surface contact angle of composite scaffold. Compressed scaffold (A); surface contact angles with HAp content increasing (B–E). **b** The water uptake capability and porosity of HAp-CS/Gel scaffold

Fig. 4 Evaluation of osteoblast activity, distribution, and infiltration in a biomimetic niche. Dead/live staining used to evaluate cell viability after 3 days of incubation. Cells were stained with calcein-AM, Hoechst 33342, and PI. **a** Calcein-AM staining. **b** Hoechst staining. **c** PI staining. **d** Morphology of scaffold under an inverted microscope. **e** Cell distribution in scaffold under SEM

1 and 0.46 ± 0.01 at day 7 in culture flasks. However, the proliferation advantage of cells in a T-flask has no significance when compared with cells on the scaffold, strongly indicating that the HAp-CS/Gel scaffolds provided a desirable environment for osteoblasts. In addition, the ALP content quantitatively detects the ability of osteogenic differentiation of osteoblasts. Figure 5b shows that the ALP content gradually increases with the osteoblast cultured from 7 to 21 days, indicating that the scaffold material contributes to osteogenic differentiation. The results showed that the ALP content of the cells cultured on the scaffold was slightly lower

Fig. 5 Expansion trend and maintenance of osteoblasts in a biomimetic niche. **a** Expansion trend of osteoblasts. **b** ALP content by osteoblasts.

than that of the cells cultured in the culture flask, but there was no significant difference. This result showed the potential of HAp-CS/Gel scaffolds to reconstruct biomimetic niche in vitro for supporting cell growth, e.g., hematopoietic stem cell.

Conclusion

The biomimetic osteoblastic niche was constructed in the presented study to provide a microenvironment for hematopoietic stem cells. We also showed that HAp can be successfully incorporated into the scaffold and evenly distributed which provided osteoblast with enough growth position for its proliferation and differentiation. Moreover, the combination of chemical bonds post HAp incorporation displaced inside the scaffold to increase the mechanical strength. The morphology and pore structure of Cs/Gel scaffold were not affected by the incorporation of HAp; the scaffold has desirable water absorbency, with the fact that HAp can block water around the scaffold and reducing the water absorbency. The high porosity of the scaffold can be achieved to 90 with better hydrophilia. This structure and advantages in physical properties will provide cells with a desirable physical environment.

Osteoblasts were favorably attached to the scaffold, maintaining desirable cell proliferation and differentiation trend, and no significant difference compared with traditional ways, with great potential to be utilized as this kind of cell/scaffold composite can be used as a biomimetic niche to support the growth of hematopoietic stem cells. In conclusion, the biomimetic osteoblastic niche constructed in this research process desirable biocompatibility, suitable to the proliferation and function-realization, thus setting a good foundation for the hematopoietic stem cells culture.

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Compliance with Ethical Standards

The experiment has been reviewed by the Biotechnology and Medical Ethics Review Committee of the Dalian University of Technology.

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Schofield, R. (1977). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, 4(1–2), 7–25.
2. He, N. N., Zhang, L., Cui, J., & Li, Z. J. (2014). Bone marrow vascular niche: home for hematopoietic stem cells. *Bone Marrow Research*, 2014, 128436.
3. Yu, V. W., & Scadden, D. T. (2016). Hematopoietic stem cell and its bone marrow niche. *Current Topics in Developmental Biology*, 118, 21–44.
4. Morrison, S. J., & Spradling, A. C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*, 132(4), 598–611.
5. Crane, G. M., Jeffery, E., & Morrison, S. J. (2017). Adult haematopoietic stem cell niches. *Nature Reviews. Immunology*, 17(9), 573–590.
6. Wang, A., & Zhong, H. (2018). Roles of the bone marrow niche in hematopoiesis, leukemogenesis, and chemotherapy resistance in acute myeloid leukemia. *Hematology*, 23(10), 729–739.

7. Müller, E., Ansorge, M., Werner, C., Pomer, T. (2014). Mimicking the hematopoietic stem cell niche by biomaterials. *Bio-inspired Materials for Biomedical Engineering*, 309–326.
8. Tabe, Y., & Konopleva, M. (2014). Advances in understanding the leukaemia microenvironment. *British Journal of Haematology*, 164(6), 767–778.
9. Taichman, R. S., Reilly, M. J., & Emerson, S. G. (1999). The hematopoietic microenvironment: osteoblasts and the hematopoietic microenvironment. *Hematology*, 4(5), 421–426.
10. Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringham, F. R., Milner, L. A., Kronenberg, H. M., & Scadden, D. T. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, 425(6960), 841–846.
11. Zhang, J. W., Niu, C., Ye, L., Huang, H. Y., He, X., Tong, W. G., Jason, R., Jeff, H., Teri, J., Jian, Q. F., Stephen, H., Leanne, M. W., Yuji, M., & Li, L. H. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, 425(6960), 836–841.
12. Bromberg, O., Frisch, B. J., Weber, J. M., Porter, R. L., Civitelli, R., & Calvi, L. M. (2012). Osteoblastic N-cadherin is not required for microenvironmental support and regulation of hematopoietic stem and progenitor cells. *Blood*, 120(2), 303–313.
13. Lu, T., Huang, Y., Wang, H., Ma, Y., & Guan, W. (2014). Multi-lineage potential research of bone marrow-derived stromal cells (BMSCs) from cattle. *Applied Biochemistry and Biotechnology*, 172(1), 21–35.
14. Gonzalez-Nieto, D., Li, L., Kohler, A., Ghiaur, G., Ishikawa, E., Sengupta, A., Madhu, M., Arnett, J. L., Santho, R. A., Dunn, S. K., Fishman, G. I., Gutstein, D. E., Civitelli, R., Barrio, L. C., Gunzer, M., & Cancelas, J. A. (2012). Connexin-43 in the osteogenic BM niche regulates its cellular composition and the bidirectional traffic of hematopoietic stem cells and progenitors. *Blood*, 119(22), 5144–5154.
15. Weber, J. M., & Calvi, L. M. (2010). Notch signalling and the bone marrow hematopoietic stem cell niche. *Bone*, 46(2), 281–285.
16. Reagan, M. R., & Rosen, C. J. (2016). Navigating the bone marrow niche: translational insights and cancer-driven dysfunction. *Nature Reviews Rheumatology*, 12(3), 154–168.
17. Walasek, M. A., van Os, R., & de Haan, G. (2012). Hematopoietic stem cell expansion: challenges and opportunities. *Annals of the New York Academy of Sciences*, 1266(1), 138–150.
18. Ewerth, D., Kreutmaier, S., Schmidts, A., Ihorst, G., Follo, M., Wider, D., Felthaus, J., Schüler, J., Duyster, J., Lena Illert, A., Engelhardt, M., & Wäsch, R. (2018). APC/CCdh1 regulates the balance between maintenance and differentiation of hematopoietic stem and progenitor cells. *Cellular and Molecular Life Sciences*, 76(2), 369–380 1–12.
19. Sarker, B., Hum, J., Nazhat, S. N., & Boccaccini, A. R. (2015). Combining collagen and bioactive glasses for bone tissue engineering: a review. *Advanced Healthcare Materials*, 4(2), 176–194.
20. Lantada, A. D., Romero, A. D. B., Moreno, S. V., Curras, D., Téllez, M., Schwenenwein, M., Jellinek, C., & Homa, J. (2016). *Tissue engineering scaffolds for bone repair: general aspects* (pp. 269–285). Cham: Springer.
21. Rezvani, Z., Venugopal, J. R., Urbanska, A. M., Mills, D. K., Ramakrishna, S., & Mozafari, M. (2016). A bird's eye view on the use of electrospun nanofibrous scaffolds for bone tissue engineering: current state of the art, emerging directions and future trends. *Nanomedicine*, 12(7), 2181–2200.
22. Siqueira, N. M., Paiva, B., Camassola, M., Rosenthal-Kim, E. Q., Garcia, K. C., dos Santos, F. P., & Soares, R. M. D. (2015). Gelatin and galactomannan-based scaffolds: characterization and potential for tissue engineering applications. *Carbohydrate Polymers*, 133, 8–18.
23. Khamhaengpol, A., & Siri, S. (2017). Composite electrospun scaffold derived from recombinant fibroin of weaver ant (*Oecophylla smaragdina*) as cell-substratum. *Applied Biochemistry and Biotechnology*, 183(1), 110–125.
24. Jacobsen, S., & Fritz, H. G. (1996). Filling of poly (lactic acid) with native starch. *Polymer Engineering & Science*, 36(22), 2799–2804.
25. Saravanan, S., Chawla, A., Vairamani, M., Sastry, T. P., Subramanian, K. S., & Selvamurugan, N. (2017). Scaffolds containing chitosan, gelatin and graphene oxide for bone tissue regeneration in vitro and in vivo. *International Journal of Biological Macromolecules*, 104(Pt B), 1975–1985.
26. Zhu, Y., Song, K., Jiang, S., Chen, J., Tang, L., Li, S., Fan, J., Wang, Y., Zhao, J., & Liu, T. (2017). Numerical simulation of mass transfer and three-dimensional fabrication of tissue-engineered cartilages based on chitosan/gelatin hybrid hydrogel scaffold in a rotating bioreactor. *Applied Biochemistry and Biotechnology*, 181(1), 250–266.
27. Senel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Australian Veterinary Journal*, 56(10), 1467–1480.
28. Morrison, S. J., & Scadden, D. T. (2014). The bone marrow niche for haematopoietic stem cells. *Nature*, 505(7483), 327–334.
29. LogithKumar, R., KeshavNarayan, A., Dhivya, S., Chawla, A., Saravanan, S., & Selvamurugan, N. (2016). A review of chitosan and its derivatives in bone tissue engineering. *Carbohydrate Polymers*, 151, 172–188.

30. Mesgar, A. S., Mohammadi, Z., & Khosrovan, S. (2018). Improvement of mechanical properties and in vitro bioactivity of freeze-dried gelatin/chitosan scaffolds by functionalized carbon nanotubes. *International Journal of Polymeric Materials and Polymeric Biomaterials.*, 67(5), 267–276.
31. Saravanan, S., Vimalraj, S., Vairamani, M., & Selvamurugan, N. (2015). Role of mesoporous wollastonite (calcium silicate) in mesenchymal stem cell proliferation and osteoblast differentiation: a cellular and molecular study. *Journal of Biomedical Nanotechnology.*, 11(7), 1124–1138.
32. Sainitya, R., Sriram, M., Kalyanaraman, V., Dhivya, S., Saravanan, S., Vairamani, M., Sastry, T. P., & Selvamurugan, N. (2015). Scaffolds containing chitosan/carboxymethyl cellulose/mesoporous wollastonite for bone tissue engineering. *International Journal of Biological Macromolecules.*, 80, 481–488.
33. Schönwälder, S. M. S., Bally, F., Heinke, L., Azucena, C., Bulut, Ö. D., Heißler, S., Kirschhöfer, F., Gebauer, T. P., Neffe, A. T., Lendlein, A., Brenner-Weiß, G., Lahann, J., Welle, A., Overhage, J., & Wöll, C. (2014). Interaction of human plasma proteins with thin gelatin-based hydrogel films: a QCM-D and ToF-SIMS study. *Biomacromolecules.*, 15(7), 2398–2406.
34. Gong, T., Xie, J., Liao, J. F., Zhang, T., Lin, S. Y., & Lin, Y. F. (2015). Nanomaterials and bone regeneration. *Bone Research.*, 3(3), 15029.
35. Webster, T. J., Ergun, C., Doremus, R. H., Siegel, R. W., & Bizios, R. (2015). Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *Journal of Biomedical Materials Research.*, 51(3), 475–483.
36. Elsayy, M. A., Kim, K. H., Park, J. W., & Deep, A. (2017). Hydrolytic degradation of polylactic acid (PLA) and its composites. *Renewable & Sustainable Energy Reviews.*, 79, 1346–1352.
37. Madhumathi, K., Shalumon, K. T., Rani, V. V. D., Tamura, H., Furuike, T., Selvamurugan, N., Nair, S. V., & Jayakumar, R. (2009). Wet chemical synthesis of chitosan hydrogel–hydroxyapatite composite membranes for tissue engineering applications. *International Journal of Biological Macromolecules.*, 45(1), 12–15.

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