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Regulation of the osteoblastic and chondrocytic differentiation of stem cells by the extracellular matrix and subsequent bone formation modes

Jing He [a], Bo Jiang [a], Yun Dai [a], Jianyuan Hao [c], Zongke Zhou [b], Zhili Tian [a], Fang Wu [a,*], Zhongwei Gu [a]

[a] National Engineering Research Center for Biomaterials, Sichuan University, Chengdu, China
[b] Orthopaedic Hospital, West China Hospital, Sichuan University, Chengdu, China
[c] University of Electronic and Electrical Science, Chengdu, China

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ABSTRACT

While various factors have been reported to direct stem cell differentiation lineage, little is known about how nature orchestrates the mesenchymal stem cell (MSC) differentiation and bone morphogenesis during skeleton development and bone regeneration. The present study reports that the matrix has a critical regulating effect on MSC differentiation and the subsequent bone formation modes. A simply combined hydroxyapatite (HA)-collagen matrix stimulates the MSC differentiation into the osteoblastic lineage and leads to a straightforward intramembranous bone formation mode, in contrast to the chondrocytic differentiation and endochondral mode observed on HA-synthetic hydrogel matrix. The accelerated MSC condensation and robust MSC-matrix and MSC–MSC interactions on collagen-based matrix might be the critical factors contributing to such events, likely through the orchestrated signal cascades and cellular events modulated by the extracellular matrix. The results demonstrate that matrix plays critical role in modulating the stem cell differentiation lineage and bone formation mode, which has been largely overlooked.

1. Introduction

During the skeletal development, bone is formed through two different processes: intramembranous ossification and endochondral ossification [1,2]. In the intramembranous mode the MSCs differentiate directly into the osteoblasts after condensation, whereas in endochondral bone formation mode the MSCs first differentiate into the chondrocytes followed by the invasion of blood vessels and the apoptosis of hypertrophic chondrocytes [3–5]. Critical transcription and growth factors that regulate the MSC condensation and differentiation into osteogenic and chondrogenic lineages have been identified, through the study of the embryonic bone development and skeletal diseases [6,7].

The MSC differentiation lineage is governed by its niche and various factors have been used to direct the MSC differentiation lineage, including bioactive molecules [8], growth factors and cytokines [9]. Different induction media, enriched with specified biological factors, have been widely used to direct the MSC differentiation into the desired lineage. Recent studies have suggested that material properties also have important influence on MSC differentiation lineage, including substrate elasticity [10], surface chemistry [11] and topography [12]. For instance, the hMSCs could be directed along neuronal, muscle, or bone lineages, by controlling the substrate elasticity [10]. The surface function groups also exert strong influence on the stem cell lineage specification, with charged phosphate groups inducting osteogenesis and hydrophobic t-butyl groups leading to adipogenesis [11]. Furthermore, it has been shown that nano-displaced topography significantly increases the osteospecific differentiation [12]. Nevertheless, little is known about how nature directs the MSC differentiation and bone formation during skeleton development and bone regeneration.

The interaction between the cell and matrix would influence strongly the biological responses of MSCs. The selection of the extracellular matrix (ECM) protein as the matrix would no doubt stimulate the MSC recruitment, attachment, condensation, proliferation and differentiation, resulting in cell-matrix and cell–cell interactions that may ultimately affect the final fate of MSCs.

* Corresponding author. Tel.: +86 28 8541 2923; fax: +86 28 8541 0246.
E-mail addresses: fangwu0808@yahoo.com, fwu@scu.edu.cn (F. Wu).

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To determine whether there is matrix dependence and how matrix modulates the MSC fate, we selected two different matrices: HA-collagen, HA-synthetic hydrogel. Type I collagen and a synthetic thermo responsive PLGA-PEG-PLGA hydrogel with a sol–gel transition temperature of 30 °C (SI 1), loaded with growth factors (BMP-2 and VEGF), were impregnated into a highly porous HA coating, respectively. The effect of the selected matrix on the osteoblastic and chondrocytic differentiation of MSC and subsequent bone formation mode have been studied through analyzing MSC responses in vitro and ectopic bone formation in-vivo.

2. Materials and methods

2.1. Materials preparation

2.1.1. Preparation of the porous HA coatings

Ti–6Al–4V disks with 14 mm diameter and 1.5 mm thickness were used as the substrates. The substrate surfaces were grit-blasted with alumina powder (380 μm particle size, 0.4–0.7 MPa grit-blast pressure) and were ultrasonically cleaned with acetone and ethanol prior to plasma spraying. Porous HA coatings were deposited onto the Ti–6Al–4V substrates with Metco MN air plasma spraying system (Metco Ltd. USA) and an AR2000 thermal spraying robot, using the liquid HA precursor with a solid content of 12.5% as the feedstock. Liquid precursor was transported by a peristaltic pump and was directly injected into the plasma plume through an atomizing nozzle. Nitrogen and hydrogen were used as the plasma forming gases. The thickness of the HA coatings were approximately 80 μm. More detailed information of the LPPS process and the spraying parameters can be found elsewhere [13–15].

2.1.2. Preparation of the HA-collagen matrix and the HA-synthetic hydrogel matrix

Type I collagen was extracted from bovine skin, dissolved in acetic acid (pH 5.0) at a concentration of 7.0 mg/ml. Then the collagen solution (in the presence and absence of growth factors) was dropped on the HA coating surface using a previous described method [13]. Briefly, a total of 400 μl collagen solution was dropped on each HA coating surface at two separate times, followed by air drying in a laminar flow cabinet. HA-collagen matrices (in the presence and absence of growth factors) were prepared and the collagen would spontaneously self-assemble into a fibrous network under biological conditions.

The Poly(D, L-lactide-co-glycolide)-Poly(ethylene glycol)-Poly(D, L-lactide-co-glycolide) (PLGA-PEG-PLGA) triblock thermogelling copolymer was synthesized by ring-opening polymerization of D, L-lactide (LA) and glycolide (GA) with a LA/GA molar feed ratio of 4: 1 in the presence of poly(ethylene glycol) (PEG) that has a number molecular weight of 1540. More detailed information of the synthesis and characterization of the synthetic hydrogel can be found in Supplementary information 1(SI1). The HA-synthetic hydrogel matrices were prepared in the same way as the preparation method for HA-collagen matrix. Briefly, each HA coating sample was added by 400 μl PLGA-PEG-PLGA hydrogel solution, alone or with additional incorporation of rhBMP-2 and rhBMP + VEGF, respectively.

2.1.3. Incorporation of BMP and VEGF into the HA-collagen and HA-synthetic hydrogel matrices

Selected growth factors were added into the type I collagen (pH 5.0) and PLGA-PEG-PLGA hydrogel prior to being applied on the HA coatings. RhBMP-2 (rhBMP-2, BioVision, BioVision Inc., USA) and VEGF ($VEGF_{165}$ Peprotech, Peprotech Inc., USA) were diluted in ultrapure water with concentrations of 1 mg/ml and 100 μg/ml, respectively. The rhBMP-2 and BMP + VEGF were uniformly mixed into the type I collagen solution at 4 °C with a concentration of 12.5 μg rhBMP-2 and 12.5 μg rhBMP-2 + 25 ng VEGF in 1 ml collagen solution, respectively. The BMP and BMP + VEGF were mixed with PLGA-PEG-PLGA hydrogel at 4 °C with the same concentrations, respectively.

The five kinds of coatings prepared were referred as HA-collagen, HA-collagen/BMP, HA-collagen/BMP + VEGF (HA-collagen group samples), HA-synthetic hydrogel/BMP, and HA-synthetic hydrogel/BMP + VEGF coatings (HA-synthetic hydrogel group samples), respectively. These samples were used to analyze the effect of matrix on MSC differentiation into the osteoblastic and chondrocytic lineages, as well as the MSC attachment, proliferation, and condensation.

To further analyze the selected markers for BMP mediated pathways, a follow-up experiment has been conducted by using the following samples: HA, HA-collagen, HA-collagen/BMP, HA-synthetic hydrogel, HA-synthetic hydrogel/BMP (rhBMP-2, Rebone, Shanghai Rebone Biomaterials Co. Ltd, China).

2.2. MSC harvesting and culture

MSCs were harvested from 1-week-old New Zealand rabbits. In brief, MSCs were obtained from the femora and tibias by either aspirating or flushing out with a 16-gauge needle and a 10-ml syringe. After that, the bone marrow cells were plated in cell culture flasks (Falcon, BD, USA) containing 20 ml of α-MEM (HaLi Corporation, Chengdu, China) containing 20% fetal bovine serum (FBS, HyClone, Thermo Fisher Scientific Inc., USA) and 1% antibiotics (Gibco, Invitrogen Corporation, USA), and cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Non-adherent cells were removed by changing the culture medium after 3 days and the adherent cells were collected for further expanding. The medium was then renewed every 2 days, and all experiments were performed within passage 3.

2.3. Cell proliferation and morphology

Suspension of MSCs with number 2×10^4 per milliliter was seeded on the coating surfaces and cultured 3, 5, 7 and 14 days, respectively. Cell counting kit-8 (CCK-8) assay was selected and optical densities (OD) values at 450 nm were measured. The proliferation of MSCs was determined by the OD value. SEM and confocal microscopy were used to analyze the cell condensation on the coatings surface, after 5 days.

2.4. Enzyme-linked immunosorbent assay

The runt-related transcription factor-2 (Runx2), alkaline phosphatase (ALP) activity, osteopontin (OPN) and osteocalcin (OCN), Sry related HMG box 9 (Sox9), collagen type II (Col II), collagen type X (ColX), Osterix, distal-less homeobox5 (Dlx5), intercellular adhesion molecule 1 (ICAM-1) and N-Cadherin were measured after 3, 5, 7, 14 days, using the quantitative enzyme-linked immunosorbent assay (ELISA). All the experiments were conducted strictly according to the instructions of the ELISA kits (BlueGene, Bluegene Ltd., Shanghai, China). The expressions were determined by the absorbance measurements performed with a spectrophotometer (MK3, Thermo Electron Ltd., USA) at 450 nm, by comparing the measured OD values to the standard curve plotted using a set of standard samples.

2.5. Animal study

Three adult New Zealand rabbits with weight 2.5–3 kg were used in the experiment. The animals were anaesthetized with sodium pentobarbital (1 mg/kg). Then the dorsal regions of each rabbits were shaved and disinfected before the skin was incised. Titanium cylinders (dimensions $\Phi 4 \times 10$ mm) coated with HA coatings loaded with collagen/BMP and synthetic hydrogel/BMP (rhBMP-2, Peprotech, Peprotech Inc., USA, 142 μg for each sample) were used as the implant materials ($n = 3$ for each material) and were inserted into the muscular tissues of the animals. The animals were sacrificed under general anesthesia after 4 weeks of implantation. Then the implants with their surrounding tissue were retrieved and prepared for histological examination. The retrieved samples were fixed with 10% buffered formaldehyde for 3 days at 4 °C and flushed with flowing water for 24 h. Then the fixed samples were dehydrated with a graded ethanol series and embedded in polymethyl methacrylate. Afterwards, samples were cut with about 100 μm thicknesses. The prepared sections were stained with toluidine blue. Finally, the histological observations were conducted by a histopathologist with an optical microscopy fitted with a digital camera. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation (Huaxi Laboratory Animal Center, Sichuan University), regarding the care and use of animals in the experimental procedures.

2.6. Statistical analysis

All data were presented as means values ± standard deviation for $n = 3$. Differences between the various groups were analyzed using the one-way ANOVA test with Bonferoni's method. All tests were performed by SPSS and the level of significance was set at $p < 0.05$.

3. Results

3.1. MSC differentiation

3.1.1. Osteoblastic differentiation

The Runx2, ALP, OPN and OCN secretions (Fig. 1) were measured to analyze the osteogenic differentiation of MSCs. The HA-collagen group samples drove the MSCs to differentiate into the osteoblastic lineage, as evidenced by the significant up-regulation of ALP, OPN, OCN expressions quantified by Elisa (Fig. 1b–d). Especially, the ALP activities of the MSCs on the HA-collagen group samples increased significantly with cell culture time, exhibiting a strong upward trend, while the ALP activities of the HA-synthetic hydrogel group samples kept at stabilized levels with lower values (Fig. 1b). The ALP secretion levels in the HA-collagen/BMP sample were 7.7 and 8.2 fold of those of the HA-synthetic hydrogel/BMP sample at 7 and 14 days, respectively. For all the times, the OPN and OCN secretions