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Original Research

Three-dimensional stress *In vitro* promotes the proliferation and differentiation of periodontal ligament stem cells implanted by bioactive glass

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Abstract: To analyze the biological and mechanical microenvironment on the directional differentiation of periodontal ligament stem cells (PDLSCs) *In vitro*. PDLSCs were cultured in three-dimensional stress system *In vitro* for 1, 2 and 3 weeks. Methods like immunohistochemistry and flow cytometry were adopted and the proliferation and differentiation situation of PDLSCs were determined. Bioactive glass (BAG) of 0%, 10%, 20%, 30% and 40% was implanted into PDLSCs with or without three-dimensional stress for 3 weeks, respectively. The proliferation and differentiation situation of PDLSCs were determined. The mRNA levels of Alkaline phosphatase (ALP), Type I Collagen (COL I), Type II Collagen (COL II), Bone sialoprotein (BSP), Osteocalcin (OCN) and Osteopontin (OPN) were determined by semi-quantitative RT-PCR. 30% BAG and three-dimensional stress for 3 weeks promoted the proliferation and differentiation of PDLSCs mostly. PDLSCs induced by BAG and 3D force and the control all expressed the mRNA of ALP, COL Ⅰ and COL Ⅱ. The BAG and three-dimensional stress induced PDLSCs also expressed the mRNA of BSP, OCN and OPN. BAG and three-dimensional stress indicated microenvironment *In vitro* can promote the proliferation and differentiation of PDLSCs.

Key words: Three-dimensional stress, PDLSCs, Bioactive glass, Differentiation.

Introduction

Periodontal disease is a chronic inflammatory condition characterized by destruction of the periodontal tissues and resulting in loss of connective tissue attachment, loss of alveolar bone, and the formation of pathological pockets around the diseased teeth (1). It has been found to be a potential risk factor for coronary heart disease (2). Drug treatment and periodontal surgery are traditional ways, which can kill pathogenic microorganisms, slow or stop the disease progression. Its disadvantage is not to recover and reconstruct the original parodontium and the structure of alveolar bone (3). In order to restore the height and volume of original alveolar bone, some synthetic or natural material, autologous and allogeneic bone graft have been applied to the clinical treatment of periodontal defects (4). But these materials lack the structure of natural alveolar bone and periodontal ligament, and allograft often cause immune rejection which results in the long time maintain is not stable (5).

The difficulties for treating periodontal disease is the regeneration of tissue and reconstruction of damaged alveolar bone and parodontium (6). Periodontal tissue engineering is a new and exciting technique which has the potential to create tissues and organs de novo (7). It brings new hope for the regeneration and repair of periodontal tissue. The reconstruction of periodontal ligament is a method using the principle and method of bioscience and engineering to exploit and study the substitutes for periodontal tissue (8). Tissue engineering technology is divided into four research dimensions: periodontal seed cells, support material, cell growth factor and reconstruction of micro environment in the body mechanics *In vitro* (9). Seed cells and microenvironment play a critical and decisive role in the formation and regeneration of periodontal tissue. In recent years, the research on stem cells opens up broad prospects for the regeneration of periodontal tissue (10). Cells and microenvironment influence and interact with each other, and collectively regulate the regeneration and repair of tissue. Periodontal ligament stem cells have strong amplification and differentiation ability. How to simulate microenvironment in biological tissues and organs, realize the reconstruction of tissues *In vitro* is the focus of the present study.

In this study, we simulated the period of mastication physiology and stimulated periodontal ligament stem cell in three-dimensional cultivation condition. The proliferation and differentiation were then determined and inspected.

Materials and Methods

Acquisition and identification of seed cells

Tissue engineering utilizing periodontal ligament stem cells (PDLSCs) has recently been proposed for the development of new periodontal regenerative therapies. PDLSCs were gently separated from orthodontic tooth extraction patients (age ranged from 12 to 15 years old) of oral and maxillofacial surgery clinics in Nanjing stomatological hospital. The selected teeth had no tooth decay, periapical periodontitis and periodontitis. Teeth were transmitted by DMEM after separated and PBS combining with antibiotic was used to wash fang repeatedly in laminar flow cabinet (Gelman Sciences, Australia) to remove blood. Two thirds of the

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periodontal membrane in fang was scraped by scalpel and moved into centrifuge tube after cutting into pieces. Collagenase (Sangon Co., Ltd. China) of 3mL (0.1%) was added to digest for 50-60min and 1% collagenase was added sequentially to digest for 25 min. When most cells dispersed into single cell suspension, 10% FBS was added to end digestion. Moderate culture fluid was added after centrifugation at 1000 rpm for 10 min and the cultures were maintained at 37°C in a 5% CO^2 humidified chamber. The culture fluid was changed every two days and when cells are filled with the bottom of the bottle, subculture was performed. Primary culture fluid was discarded and washed by PBS and trypsin was added. When most of the cells have been retracted at inverted microscope and a small number of cells suspended in the culture medium, equal amount of culture fluid containing serum was added and centrifugation was conducted at 1000 rpm for 10 min.

Bioactive glass (BAG) has found extensive application as an orthopedic and dental graft material and most recently also as a tissue engineering scaffold (11). To explore the influence of BAG on periodontosis, 0% to 40% BAG was implanted, and the growth, proliferation and differentiation of periodontal ligament stem cells were determined with and without outside three-dimensional stress. The force was performed to simulate the internal stress of alveolar bone in chewing environment in vivo to guide and promote the differentiation of human periodontal ligament stem cells to periodontium. We used the teeth "chewing" power with a cycle of 1HZ, 3 times per day and 30 min at per time.

Monoclonal proliferation

We cloned and separated PDLSCs using the method of limited dilution method description as Seo and Liu HW (12,13). The first generation of periodontal ligament cell suspension was diluted to a density of 10 / mL. Each hole of the 96 holes culture plate was added 0.15mL cell suspension to make every 0.l ml cell suspension contained 1 cell in each hole. The cells wall was examined under an inverted microscope after adhering on walls when maintaining at 37°C in a 5% CO^2 humidified chamber. Holes containing single cell were selected and added 0.lmL culture medium. When cells increased to occupy one-third or one-second of the hole bottom area, they were transferred to 24, 12 and 6 holes culture plate for expand training using digestion method and gradually expanded to 10^7 Seven Order of magnitude quantity level.

Immunohistochemistry

Cell histologic sections were deparaffinized, rehydrated, and blocked with methanolic 3% hydrogen peroxide. The slides were immersed in 10 mm citrate buffer, pH 6.0 and heated in a 1200W microwave oven at the highest power setting. Evaporated liquid was replenished at 5 minutes, then the slides were heated at high power for an additional 5 minutes. The slides were left in the buffer for an additional 10 minutes before being removed. The antibody used was a mouse monoclonal antibody raised against full-length recombinant Cdx2 protein (AM392—5 m; BioGenex, San Ramon, CA). Immunostaining was performed by hand using the prediluted antibody solution and a 30 minute incubation. After washing, antibody binding was visualized using the avidin-biotin-peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlington, VT), followed by incubation with 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin.

Analysis on STRO-1 and CD-146 by flow cytometry

To study the influence of expression changes of BAG and external force on the progression of cell cycle in PDLSCs, flow cytometry was used. The PDLSCs at logarithmic phase were selected and plated in a 96-well plate at a density of 2×10^3 cells/well in supplemented RPMI 1640 and incubated for 16 h before the cells were subjected to treatment in triplicate wells. After treatment, the cells were washed twice in phosphate-buffered saline (PBS) (2.68 mM KCl, 1.47M KH2PO4, 8 mMNa2KPO4, 136.75 mM NaCl) and counted. Fifty to one hundred thousand cells were selected and centrifuged 5 min at 1000r/min. Annexin V-FITC mixed liquor of 195 μL was added to resuspend cytotrophoblast cells and 5 μL was added to mix. Centrifugation at 1000r/min for 5 min was performed after cultivation 10 min. Sample was obtained after discarding supernatant and 10μL propidium iodide (PI) was added. Afterwards, the sample was stilled in dark for 30 min. Finally, the apoptosis was detected using flow cytometry (FCM) on the Moflo (Dako Cytomation, Glostrup, Denmark).

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted and isolated from cells using either the mirVana miRNA isolation kit (Ambion, Austin, TX) or the TRIzol method. Trizol of 1 mL was added and the solution was mixed homogeneously for 10 min. The mixture was then transferred into eppendorf tubes (EP, 1.5mL) with 200 μL chloroform. After 15 min shake, the EP tubes were centrifuged at 4°C for 15 min (12000 × g). The supernate was transferred into other EP tubes and mixed with isopyknic isopropanol for 15 s. The centrifugation (4°C, 10 min, 12000×g) was carried out again and the supernate was discarded. The precipitate was washed by 75 % ethonal twice and dissolved into 30 μL diethypyrocarbonate (DEPC) after dried to obtain RNA stock solution. After isolation, the concentration of RNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and the RAN solution was stored at -80 °C for further use. Genes were amplified by specific oligonucleotide primer, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. The detection and quantification contained the following steps: first, reverse transcription was performed at 55°Cfor 30 min, initial activation for 15 min at 95°C, next 40 cycles of denaturation were conducted at 94°C for 15 s, then annealing for 30 s at 55°C, extension for 30s at 72°C. The expression level was normalized using U6 small nuclear RNA by the $2-\Delta Ct$ method. The ΔCt values were normalized to GAPDH level. Primers sequence was as follows: Alkaline phosphatase (ALP), F, GGACAGGACACACACACACA; R, CAAACAGGAGAGCCACTTCA. Type I Collagen (COL I), F, ACGTCCTGGTGAAGTTGGTC; R, TCCAGCAATACCCTGAGGTC. Type IV Collagen (COLIV), F, GCCAGCAAGGTGTTACAGGATT; R,

AGAAGGACACTGTGGGTCATCTATT. Bone sialoprotein (BSP), F, GCATCGAAGAGTCAAAATAG; R, TTCTTCTCCATTGTCTTCTC. Osteocalcin (OCN), F, CAGACACCATGAGGACCATC; R, GGACTGAGGCTCTGTGAGGT. Osteopontin (OPN), F, TTGCTTTTGCCTCCTAGGCA; R, GTGAAAACTTCGGTTGCTGG. GAPH, F, AGGTCGGTGTGAACGGATTTG; R, TGTAGACCATGTAGTTGAGGTCA.

MTT assay

U266 and LP-1 cells in logarithmic growth phase were seeded in 96-well culture plates with 2.0 × 104/hole (200μL), and eight parallel holes were set. The cell viability was assessed on 24, 48, 72 and 96 h. MM cells were added 10mL MTT (5mg/ml PBS stock solution) and incubated for 4 hours, the liquid culture was aspirated off and 200μL dimethylsulfoxide (DMSO) was added. The absorbance was recorded at 570 nm. The migration index was defined as the number of migrated cells by cell counting in at least three random fields (magnification, ×200) per filter. Each experiment was repeated three separate times.

Osteogenic ability

The coloned PDLSCs cell suspension was inoculated on 6 well plate with 5×10^4 /mL and cultured with α-MEM containing 10% FBS for 24h. When cells extended to 60% and joined, mineralization induced liquid (l0mmol/L β- sodium glycerophosphate, 50 μg/mL vitamin C, 1×10-8 mol/L dexamethasone and α-MEM containing 10% FBS) was used for further culture. Cell multi layer growth was observed at microscope and culture was continued to 21 days when nodules appeared. The primary medium was discared and cells were washed with deionized water, fixed by 4% paraformaldehyde for 30 min and dyed with alizarin red.

Statistical analysis

Statistical analysis was performed by SPSS 16.0 statistical software. All data were expressed as means ± SD from at least three independent experiments. P values were determined using one-way ANOVA. Significance was defined as $P<0.05$.

Results

Identification on PDLSCs

Previous studies have demonstrated that human PDLSCs have a very strong clonality. In this study, we successfully separated and cloned 5 strains of PDLSCs used limiting dilution assay. Other cells were due to the discarded grew slowly and were aging gradually during clone transfer process. Amplified and purified PDLSCs grew in a manner of colony and the morphology of the most cells was long spindle. Results showed the cloning efficiency was about 60%. Immunocytochemical stain showed the expression of some Stro-1 cells was positive. The results of FCM showed 14.1% of PDLSCs cloned Stro-1 showing positive and 88.4% of PDLSCs cloned CD146 showing positive. Since Stro-1 and CD146 were both markers of early mesenchymal stem cells, it indicated PDLSCs which we obtained were mesenchymal stem cells. To validate the differentiation potential of PDLSCs, we made a bone induction of PDLSCs from multiple clones.

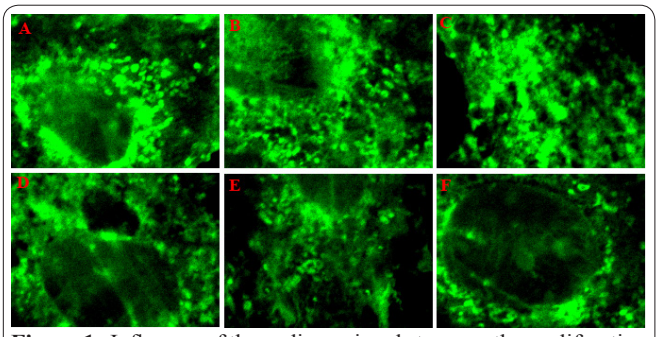

Figure 1. Influence of three-dimensional stress on the proliferation and differentiation of periodontal ligament stem cells (PDLSCs) *In vitro*. A, C and E: PDLSCs were treated with three-dimensional stress for 1, 2 and 3 weeks, respectively. B, D and F: PDLSCs were treated with medium for 1, 2 and 3 weeks, respectively.

Influence of BAG and microenvironment on the proliferation and differentiation of PDLSCs

To explore the influence of microenvironment on the proliferation and differentiation of PDLSCs, cloned PDLSCs were cultured *In vitro* with three-dimensional stress for 1, 2 and 3 weeks. Results showed the proliferation and differentiation capacity increased with time increase (Figure 1). The apoptosis of PDLSCs was also determined and results were shown in Figure 2. As shown, the number of living cells in force group increased with time increase and decreased in no force group. At 3 weeks, the number of PDLSCs in force group was 3 times the number of PDLSCs in no force group. As for death cells, the number in force group decreased evidently only in the first week and the number of death cells in force group and no force group had no evident difference. Considering all those results, 3 weeks was selected for the further study.

To study the influence of BAG on the proliferation and differentiation of PDLSCs, 0%, 10%, 20%, 30% and 40% BAG was implanted into periodontal ligament stem cells with or without three-dimensional stress for

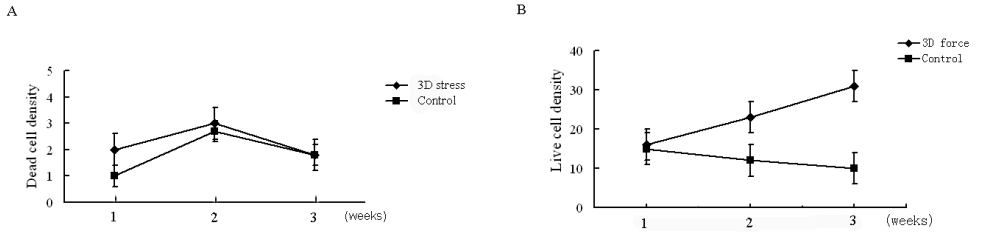

Figure 2. Influence of three-dimensional stress on the proliferation of PDLSCs by flow cytometr. A: Dead cell density of PDLSCs after treating with three-dimensional stress for 1, 2 and 3 weeks, respectively. B: Live cell density of PDLSCs after treating with three-dimensional stress for 1, 2 and 3 weeks, respectively.

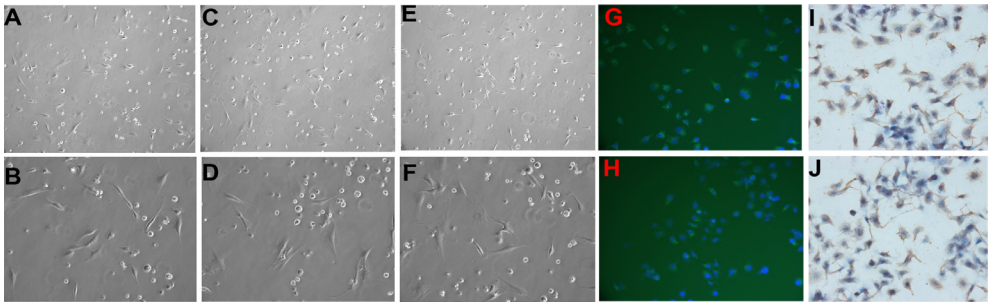

Figure 3. Influence of bioactive glass (BAG) on the proliferation and differentiation of periodontal ligament stem cells (PDLSCs) *In vitro*. A, C, E, G and I: PDLSCs were treated with 0%, 10%, 20%, 30% and 40% BAG and three-dimensional stress for 3 weeks, respectively. B, D, F, H and J: PDLSCs were treated three-dimensional stress for 3 weeks, respectively.

3 weeks. Results showed the proliferation and differentiation capacity increased with the increase of BAG concentration (Figure 3).

Three-dimensional stress promoted osteogenic differentiation

To explore the influence of three-dimensional stress on osteogenic differentiation, the activity of alkaline phosphatase (ALP) was determined. ALP is present in human serum in the form of several isoenzymes and is expressed during osteoblastic differentiation. It plays an important role in the formation of mineralized tissues. The higher the ALP activity is, the higher the degree of cell differentiation and the ability to secrete matrix. In our results, the activity of ALP increased evidently after PDLSCs were induced by three-dimensional stress. The activity of ALP increased with BAG concentration increased from 0% to 30% and decreased with continue increase from 30% to 40% (Figure 4). It may be due to the long time culture *In vitro* resulting in the mineralization of PDLSCs. As for the control group, the activity of ALP increased gradually with BAG concentration increased from 0% to 30% and also decreased with the further increase of BAG concentration, which may be related with spontaneous differentiation of some PDLSCs.

Changes of genotype

The mRNA levels of ALP, COL I, COL II, BSP, OCN and OPN were determined by semi-quantitative RT-PCR. Results showed PDLSCs induced by BAG and three-dimensional stress and the control all expressed the mRNA of ALP, COL I and COL II. The BAG and three-dimensional stress induced PDLSCs also expressed the mRNA of BSP, OCN and OPN (Figure 5).

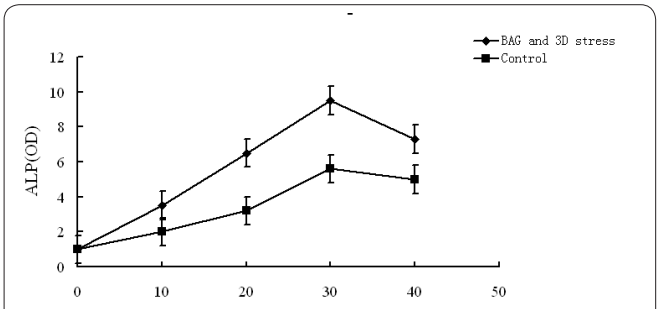

Figure 4. The influence of BAG and three-dimensional stress on ALP activity. BAG and 3D stress: The activity of ALP in PDLSCs after treating with 0%, 10%, 20%, 30% and 40% BAG and three-dimensional stress for 3 weeks, respectively. Control: PDLSCs were treated three-dimensional stress for 3 weeks.

Generally speaking, the mRNA of BSP, OCN and OPN often expressed in or cementoblast and osteoblast cells. OCN and OPN often express during osteoblastic differentiation.

Discussion

Periodontosis is an idiopathic degeneration of the periodontium which results in migration and loss of teeth (14). In the oral cavity, if alveolar bone loss the stimulation of physiological chewing function, it will atrophy gradually and periodontal tissue are diseased (15). Conversely, chew exercise can maintain the stability of alveolar bone and the healthy periodontal tissue (16). Further study showed external mechanical load affected the mechanics microenvironment in periodontal tissue, the formation of itself tissue, growth

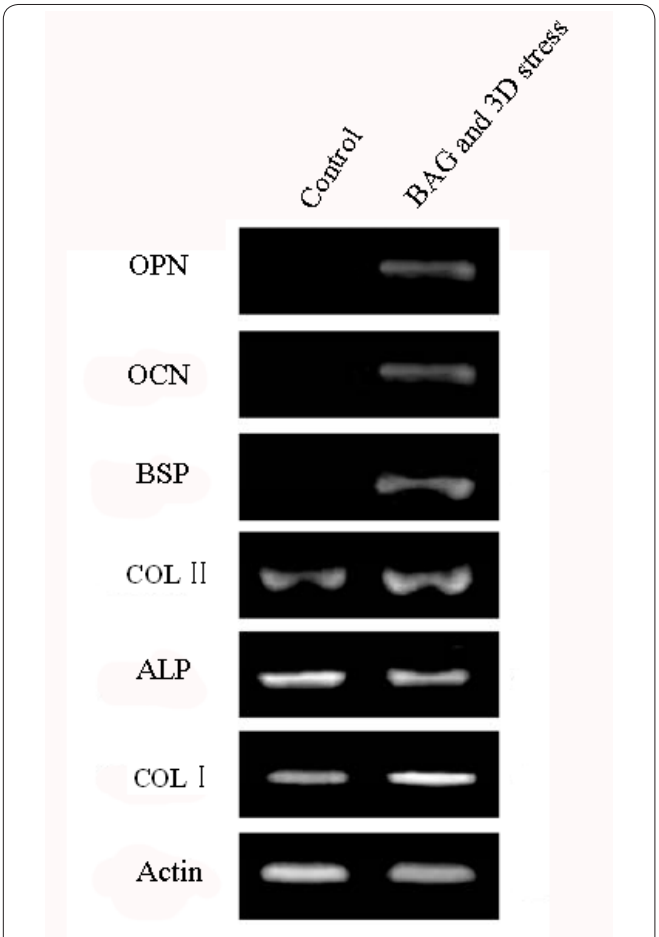

Figure 5. The mRNA levels of Alkaline phosphatase (ALP), Type I Collagen (COL I), Type II Collagen (COL II), Bone sialoprotein (BSP), Osteocalcin (OCN) and Osteopontin (OPN) determined by semi-quantitative RT-PCR.

and development. Biochemical power can influence the phenotype and activity of cells and balance the function of anabolism constructive metabolism and absorption (17). When periodontal ligament stem cells were stimulated by specific periodic mechanics, they would be induced and migrated proliferated and differentiated directly. Periodontal ligament stem cells have strong amplification and differentiation ability. How to simulate microenvironment in biological tissues and organs, and realize the organization reengineering *In vitro* and application is the focus of the present research. In traditional two-dimensional culture and stress application *In vitro*, cells often occur the dedifferentiation and lose normal morphology and function.

In this study, we simulated the period (frequency) of physiological chew and stimulated periodontal ligament stem cells cultured in three-dimensional cultivation condition. The proliferation and differentiation of PDLSCs were then determined. Results showed the proliferation and differentiation capacity of PDLSCs increased with the increase of strength time and 3 weeks were selected for further experiment. Bioactive glasses (BAG) enhances bone formation and bond directly to bone, and has emerged as promising substrates for bone tissue engineering applications (18). BAG are reported to be able to stimulate more bone regeneration than other bioactive ceramics but they lag behind other bioactive ceramics in terms of commercial success (19). It can not only have combined with bone tissues, but also connected with soft tissue. Since different proportion of BAG has different influence on bone growth, we used 0%, 10%, 20%, 30% and 40% BAG to implant PDLSCs with and without stress, respectively. Results showed the proliferation and differentiation capacity increased with the increase of BAG concentration.

The mRNA levels of Alkaline phosphatase (ALP), Type I Collagen (COL I), Type II Collagen (COL II), Bone sialoprotein (BSP), Osteocalcin (OCN) and Osteopontin (OPN) were determined by semi-quantitative RT-PCR. Results showed PDLSCs induced by BAG and three-dimensional stress and the control all expressed the mRNA of ALP, COL I and COL II. The BAG and three-dimensional stress induced PDLSCs also expressed the mRNA of BSP, OCN and OPN. ALP is a widely distributed non-specific phosphomonoesterase that functions through formation of a covalent phosphoseryl intermediate (E–P) (20). It constitutes a system of multiple molecular forms of enzymes in which heterogeneity is partly due to genetic factors and partly to posttranslational modifications (21). It is a marker of osteoblast differentiation (22). COL I, the most abundant protein component of the extracellular matrix of skin, tendon and bone, is a heterotrimer consisting of two α1(I) and one α2(I) chains encoded by the COL1A1 and COL1A2 genes, respectively (23). The pro-alpha chains of type I collagen contain an uninterrupted helical region consisting of 338 repeats of the Gly-Xaa-Yaa triplet (24). BSP is an anionic phosphoprotein in the extracellular matrix of mineralized tissues, and a promoter of biomineralization and osteoblast development (25). It is highly expressed in early bone deposition and may play a part in primary bone mineralization (26). BSP is also found at high levels in mineralized tissues (27). OCN is a bone-specific extracellular matrix protein expressed by mature osteoblasts during late stages of differentiation (28). OCN originates from osteoblastic synthesis and is deposited into bone or released into circulation, where it correlates with histological measures of bone formation (29). OPN, an extracellular matrix (ECM) cytokine, is highly expressed by macrophages and plays a key role in the pathology of several chronic inflammatory diseases including atherosclerosis and the foreign body reaction (30). OPN is the main non-collagenous proteinof cementum and plays an important role in the formation and regeneration of cementum (31). Moreover, research showed OPN can accelerate the repair and growth of alveolar bone evidently (32). The increase of their expression after inducing by BAG and three-dimensional stress indicated microenvironment *In vitro* can promote the proliferation and differentiation of PDLSCs

In conclusion, we may construct parodontium and alveolar bone *In vitro* to substitute the clinical grown parodontium and alveolar bone by analyzing biomechanics and mechanical microenvironment.

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