Study on Osteogenic and Endothelial Differentiation Potential of Human Dermal Fibroblasts

Taijun Chen¹, Yingying Wang¹, Wenzhen Peng², Bo Feng¹, Wei Zhi¹, Jie Weng¹, Jianxin Wang^{1*}

¹Key Laboratory of Advanced Technologies of Materials, School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu, People's Republic of China

²Department of Biochemistry and Molecular Biology, College of Basic and Forensic Medicine, Sichuan University, Chengdu, People's Republic of China

Received: October 21, 2016; Accepted: November 08, 2016; Published: December 07, 2016

***Corresponding author:** Jianxin Wang, School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu 610031, People's Republic of China, Tel: +86-28-87634023; E-mail: jwang@swjtu.edu.cn

Abstract

Tissue engineering has been proved one of the most effective ways for repairing bone defects. However, the insufficiency of seed cell source is a major obstacle. Human Dermal Fibroblasts (hDFs) are the most widely distributed in the body and very simple to culture or to obtain when compared with mesenchymal stem cells, and have become one of the most important cell sources. However, there is no report on using hDFs as an autologous seed cell source. This study aimed to examine whether hDFs would possess the capacity to differentiate into osteogenic and endothelial cell lineages. Low Intensity Pulsed Ultrasound (LIPUS) was applied to stimulate osteogenic and endothelial differentiations of hDFs. The results showed that hDFs were successfully differentiated into both osteogenic and endothelial cell lineages, which have been confirmed by checking ALP activity and calcium nodules as well as expression levels of CD80, RUNX2, OCN, CD31, AGTR1 and KDR. The application of LIPUS and RGD modification indicated much higher expression levels of these markers. These results revealed that hDFs possess the capacity to differentiate into both osteogenic and endothelial cell lineages and might be an alternative autologous seed cell source for bone tissue engineering.

Keywords: Bone tissue engineering; Human dermal fibroblasts; Osteogenic and endothelial differentiation; Oxidized sodium alginate/ N-succinyl chitosan hydrogel; RGD; LIPUS;

Introduction

The repair of bone defect has been being a hot spot for a few centuries. The repair of a large bone defect is still a difficult problem that has not been solved yet due to the lack of self-healing ability for large area of bone defect [1]. Autograft and allograft of bone tissue are considered to be the gold standard for the treatment of bone loss and defects caused by a traumatic injury. Although these approaches are very successful, there still exist some shortcomings including the difficulty in shaping the graft to fill the defects, the requirements for numerous procedures and lengthy recovery time, insufficient material supply, donor site morbidity and contour irregularities [2]. The recent development in the technology of bone tissue engineering provides an alternative approach to the existing treatment strategies for the

repair and regeneration of bone defects, especially the critical-sized bone defects resulting from trauma, surgical resection and congenital deformity corrections [3]. The construction of tissue engineering bone has been proved one of the most effective ways for repairing bone defects, especially the large bone defects [4].

The seed cell is one of three key factors for bone tissue engineering. The ideal seed cells should be those that have the following characteristics such as abundant source, easy accessibility, less injury to body and strong proliferation ability in vitro culture. However, the insufficiency of seed cell source is a major obstacle to the clinical application of bone tissue engineering [5-6]. Since the late 90s, stem cells, such as human embryonic stem cells, induced pluripotent stem cells and Mesenchymal Stem Cells (MSCs), have been considered as a potential source of seed cells for regenerative medicine and tissue engineering. Among these stem cells, MSCs show some advantages over the others, such as possible clinical application as autologous cells and low immunogenicity [7]. However, the extremely low proportion of MSCs in human body leads to a complicated process and in turn a great deal of time and expense required to isolate and to expand them in vitro, thus restricting the clinical application of autologous MSCs. As such, the clinical limitation of autologous MSCs has made researchers to have to look for an alternative autologous cell source for future tissue engineering [8-9].

Human Dermal Fibroblasts (hDFs) are the most widely distributed in the body and they are very simple to culture or to obtain when compared with MSCs. They are regarded as the greatest number of "seed cell banks" in the human body [10]. Human Dermal Fibroblasts (hDFs), derived from the embryonic mesoderm stromal cells, have become one of the most important sort of cells in tissue repair. In recent years, there has been increased interest in dermal fibroblasts in the field of differentiation research, although, in the past, dermal fibroblasts were only regarded as fully differentiated cell terminal during the development of human body, did not have the potential of multi-directional differentiation, could only be seed cells of

dermal tissue [11]. Lin et al found that fibroblasts in new bone trabeculae and partial granulation tissue can express BMP to make them to be able to differentiate into bone cells and that they can also participate in the process of the bone repair [12]. Junker et al found that fibroblasts possess multilineage potential towards fat-, cartilage- and bone-like cells [13]. Yadav et al used buffalo fetal fibroblasts to have successfully differentiated them into adipogenic and osteogenic cells [14]. Yin et al found that terminal differentiated hDFs are capable of transdifferentiating into chondrogenic lineage under stimulation with CDMP1 in vitro [15]. Hee et al found that the treatment of dermal fibroblasts with vitamin D3 induced the expression of the osteoblast-specific markers, alkaline phosphatase and osteocalcin [16]. Bi et al found that multipotent dermal fibroblasts could differentiate into islet-like cells under no genetic manipulation [17]. However, to our best knowledge, there has not been any report on the endothelial differentiation of hDFs while the osteogenic differentiation of hDFs and the related regulation mechanism are not clear. In this study, in vitro osteogenic and endothelial differentiation of hDFs would be conducted to examine whether human dermal fibroblasts would possess the capacity to differentiate into both osteogenic and endothelial cell lineages. The results will provide direct evidence for whether or not hDFs can be used in bone tissue engineering as

Materials and Methods

Materials

Sodium alginate (SA, Mw: 612 kDa) and chitosan (CS, Mw: 209 kDa) were purchased from Maichao Chemical Reagent (Shanghai, China) and Aokang Biological Technology Co. Ltd (Shandong, China), respectively. All other chemicals and solvents were purchased from Kelong Chemical Reagent Factory (Chengdu, China) without further purification.

Preparation of Composite Hydrogels

Oxidized sodium alginate, RGD-grafted Oxidized Sodium Alginate (RGD-OSA) and N-Succinyl Chitosan (NSC) were prepared according to the previously reported method [18]. Briefly, 1 g of sterilized NSC and 2 g of sterilized RGD-OSA (or OSA) were dissolved in 40 ml and 20 ml sterilized phosphate buffer solutions (PBS, PH = 7.4), respectively, to obtain the solutions of NSC and RGD-OSA (or OSA). Both solutions were then mixed under stirring vigorously for a short time. Subsequently, the mixed solution was coated on clean square glass slides (1cm × 1cm) using a spinning coater. The coated solution finally formed into a RGD-OSA/NSC (or OSA/NSC) gel on the glass slides within a few minutes at 37°C via the reaction of Schiff Base. All the hydrogels-coated glass slides for cell culture were immersed in 75% ethanol for 2h for further sterilization and followed by rinsing three times with sterilized PBS before cell culture.

Cell Culture for Endothelial and Osteogenic Differentiation of hDFs

Human dermal fibroblasts were purchased from KUNMING CELL BANK and plated onto a T25 culture flask and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The

adherent hDFs were cultured in a growth medium consisting of high glucose Dulbecco's Minimum Essential Medium (H-DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin/streptomycin antibiotics. The cell culture medium was replaced with fresh medium every 3 days. At the third passage, hDFs were harvested from the culture flask via treatment with 0.25% trypsin-ethylenediaminetetraacetic acid solution and then were re-suspended in complete medium for later seeding when reaching 80% confluence. The re-suspended cells were seeded on blank square glass slides, square glass slides with OSA/NSC coatings and square glass slides with RGD-OSA/NSC coatings in 24-well plates at a density of 0.2×10^5 cells per well, and then they were cultured with induced medium.

Endothelial induction was performed using low glucose DMEM (L-DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 50 ng/ml vascular endothelial growth factor (VEGF₁₆₅) and 1% (v/v) penicillin/streptomycin antibiotics while osteogenic induction was conducted by applying high glucose DMEM (H-DMEM) supplemented with 10% FBS, 10 ng/ml bone morphogenetic protein (BMP-2), 50 μM L-ascorbic acid, 10 mM β-glycerophosphate, 0.1 μM dexamethasone and 1% (v/v) penicillin/streptomycin antibiotics. The cell culture medium was renewed every 3 days. All the groups (Blank group, gel group and RGD group) were treated with or without Low Intensity Pulsed Ultrasound (LIPUS) at an intensity of 200 mW/cm², a duty cycle of 20% and a repetition rate of 1 MHz for 10 minutes every day [19]. Such used parameters were based on our previous positive results in study of bi-lineage differentiation of hMSCs.

Proliferation of Cells during Endothelial and Osteogenic Differentiation of hDFs

After 1, 3, 7, 10, 14, 17 and 21 days of culture, cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) [20], which allowed very convenient assays by utilizing the highly water-soluble tetrazolium salt WST-8. In the presence of electronic coupling agent 1-methoxy radical-5-methyl phenazine dihydralazine sulfate dimethyl (1-Methoxy PMS), WST-8 can be reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells. Briefly, 100 μl of CCK-8 solution (the solution of DMEM containing 10% CCK-8) was added to each well, and the cells were incubated at 37°C for 4 h. DMEM containing 10% CCK-8 was used as a control. The number of formazan is proportional to the number of living cells, the absorbance value can be measured by using enzyme-linked immunosorbent assay at the wavelength of 450 nm. The experiments were repeated at least three times.

Morphological Observation for Endothelial and Osteogenic Differentiation of hDFs

After 4 weeks of endothelial and osteogenic differentiation of hDFs, the induced cells were rinsed three times with PBS and fixed in 10% formaldehyde solution at room temperature for 30 minutes. The fixed cells were then incubated in 0.1 ml PBS containing 1% Acridine Orange (AO) at 37°C for 15 minutes and

rinsed three times with PBS to remove excess AO. Microscopy observation was performed using an Olympus fluorescence microscope equipped with a digital camera (Radiance 2100; Biorad Laboratories, Hercules, CA, USA).

At 28th day of endothelial and osteogenic differentiation of hDFs, similarly, the induced cells were rinsed three times with PBS and fixed in 10% formaldehyde solution at room temperature for 30 minutes. The fixed cells were then incubated in hematoxylin solution at 37° for 5 minutes and rinsed with water for 3s to remove excess hematoxylin, then incubated in 1% hydrochloric acid and ethanol for 3s and rinsed with water for 30s, then incubated in 0.5% eosin solution for 3 minutes and rinsed with distilled water for 2s. Microscopy observation was performed using an Olympus microscope equipped with a digital camera.

Alkaline Phosphatase and Alizarin Red Staining for Osteogenic Differentiation of hDFs

At days 3,7,10 and 14 of osteogenic induction, the quantitative and qualitative examination of osteogenic differentiation of hDFs was conducted by monitoring the activity of ALP, a early marker of osteogenesis, using an alkaline phosphatase kit (Jiancheng Bioengineering Institute, Nanjing, China) as well as staining with a BCIP/NBT alkaline phosphatase kit (RD Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Additionally, at days 14, 21 and 28 of osteogenic induction, mineralization during osteogenic differentiation was analyzed by alizarin red staining according to the previously reported method [21], cells were rinsed three times with PBS and then fixed in 10% formaldehyde at room temperature for 30 minutes. In the meantime, calcium content was measured quantitatively by using Image Pro-Plus as described by Takayama et al [22].

Immunofluorescence Staining for Endothelial Differentiation of hDFs

After endothelial induction of 28 days, the endothelial differentiation of hDFs was identified by immunofluorescence staining, as described by Wang et al [23]. In brief, the induced cells were rinsed three times with PBS and fixed in 10% formaldehyde at room temperature for 30 minutes. After that, these cells were incubated with primary antibody (mouse anti human CD31, 1:25, Beijing Biosynthesis Biotechnology Co. Ltd) at 4° overnight and then the non-specific bindings were blocked by washing three times with the blocking buffer of PBS containing 4% bovine serum albumin. After the removal of the blocking buffer, these cells were incubated again with secondary antibodies (Fluorescein Isothiocyanate (FITC)-conjugated goat-anti-rabbit immunoglobulin G, 1:100, Beijing Zhongshan Glodenbridge Biotechnology Co. Ltd) in the dark at room temperature for 1h and then rinsed three times with the blocking buffer. The nuclei were counterstained with (DAPI) for visualization. Microscopy observation was performed using an Olympus fluorescence microscope equipped with a digital camera and Image Pro-Plus. Fluorescent intensity was also used to quantitatively measure the expression of CD31 by using Image Pro-Plus.

Flow Cytometry

Characterization of hDFs: To characterize the human dermal fibroblasts and to ensure whether or not they are pure human dermal fibroblasts, CD 90 (a fibroblast specific surface antigen) and CD 29 (a specific surface antigen marker of MSCs) were examined via flow cytometry. Briefly, human dermal fibroblasts were rinsed three times with PBS and then harvested with 0.25% trypsin-ethylenediaminetetraacetic acid solution. These cells were collected by centrifugation, and then the harvested cells were re-suspended in complete medium and mechanically dissociated to achieve a single-cell suspension. The single-cell suspension was divided into two parts and then the complete medium was discarded by centrifugation for both parts. One part was incubated with Fluorescein Isothiocyanate (FITC)-conjugated mouse anti human CD90 antibodies (Bio legend, USA) and another with Fluorescein Isothiocyanate (FITC)-conjugated mouse anti human CD29 antibodies (Bio legend, USA) at room temperature for 30 minutes. And then the incubated cells were washed with PBS, re-suspended in 0.5 ml PBS, and evaluated by using a FACS Aria instrument (BD Biosciences, USA). The cells that had adequate size and granularity were used for statistical analysis.

Identification for Endothelial Differentiation of hDFs: After endothelial induction of 28 days, the endothelial differentiation of hDFs was evaluated by flow cytometry. In brief, the induced cells were rinsed three times with PBS and then harvested with 0.25% trypsin-ethylenediaminetetraacetic acid solution. These cells were collected by centrifugation, and then the harvested cells were re-suspended in complete medium and mechanically dissociated to achieve a single-cell suspension. The complete medium was discarded by centrifugation and the induced cells were incubated with Fluorescein Isothiocyanate (FITC)-conjugated mouse anti human CD31 antibodies (Bio legend, USA) at room temperature for 30 minutes. Finally, the induced cells were washed with PBS, re-suspended in 0.5 ml PBS, and evaluated by using a FACS Aria instrument (BD Biosciences, USA). The cells that had adequate size and granularity were used for statistical analysis.

Identification for Osteogenic Differentiation of hDFs: After osteogenic induction of 28 days, the osteogenic differentiation of hDFs was examined by flow cytometry. In brief, the induced cells were rinsed three times with PBS and then harvested with 0.25% trypsin-ethylenediaminetetraacetic acid solution. These cells were collected by centrifugation, and then the harvested cells were re-suspended in complete medium and mechanically dissociated to achieve a single-cell suspension. The complete medium was discarded by centrifugation and the induced cells were incubated with PerCPcy5-conjugated mouse anti human CD80 antibodies (Bio legend, USA) at room temperature for 30 minutes. The induced cells were washed with PBS, re-suspended in 0.5 ml PBS, and evaluated by using a FACS Aria instrument (BD Biosciences, USA). The cells that had adequate size and granularity were used for statistical analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Endothelial differentiation of hDFs at 28th day, the induced cells were collected to quantify the express of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Angiotensin II Type1 Receptor (AGTR1) and Kinase Insert Domain Receptor (KDR). GAPDH was used as a reference gene. The details of primers were showed in Table 1.

Osteogenic differentiation of hDFs at 28th day, the induced were collected to quantify the expression of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN). GAPDH was used as a reference gene. The details of primers were showed in Table 2.

Total RNA was isolated and cDNA was synthesized as described as Chun et al [24]. The gene expression levels of AGTR1 and KDR were normalized according to the GAPDH gene reference

by ΔC_t method and presented as fold changes. Similarly, the gene expression levels of RUNX2 and OCN were normalized according to the GAPDH gene reference by ΔC_t method and presented as fold changes.

Statistical Analysis

All data were expressed as mean $\pm$ Standard Deviation (SD). Statistical significances of all data were determined using Student's t-test. The difference is considered significant if the p-value is less than 0.05. Indicated error bars correspond to the Standard Deviation (SD).

Results

Proliferation of Cells during Endothelial and Osteogenic Differentiation of hDFs

Cell proliferation was determined by the Optical Density (OD) values of the CCK-8 assay in our study. The OD values of the CCK-8 assay of all the groups exhibited a similar increasing tendency over time, as shown in Figure.1. It can be noted from Figure.1 that the OD values was low during first 3 days. The main reason for that might be that the hydrophilic surface of the hydrogel was not beneficial for the adsorption of proteins and was not conducive to the adhesion and growth of cells. It can be seen that the groups treated with LIPUS showed higher OD values compared with those treated without LIPUS, indicating a positive effect of LIPUS on enhancing the adhesion and growth of cells. A significant increase in cell proliferation was observed in all the groups from day 7 to day 17 for endothelial induction (Figure.1A) and from day 7 to day 14 for osteogenic induction (Figure.1B). The OD values of the groups treated with both LIPUS and RGD modification were higher than those of the groups with LIPUS only plus those of the groups with RGD modification only. It demonstrated that the synergistic effect of LIPUS and RGD on the enhancement of the adhesion and growth of cells. The significant synergistic effect of LIPUS and RGD on the enhancement of cell proliferation occurred at days 3, 10 and 14 both for endothelial and osteogenic induction.

Table 1: Details of primers for endothelial differentiation

Genes	Primer sequences	Annealing temp	Primer length
GAPDH	F:AAGCTCATTTCTGCTGATGACA; R:TCTTACTCCTTGGAGGCCATGT	54	86bp
AGTR1	F:GAATATTTGGAAACAGCTTGGT; R:CAAAGTCAGTAAAAAGCATAAG	54	119bp
KDR	F:AAAGAAGGAGCAACACACAG; R:CCACATTGAGATGGTGACCAAT	54	85bp

Table 2: Details of primers for osteogenic differentiation

Genes	Primer sequences	Annealing temp	Primer length
GAPDH	F:AAGCTCATTTCTGCTGATGACA; R:TCTTACTCCTTGGAGGCCATGT	54	86bp
RUNX2	F:CAAGAGTTTCACCTTGACCAT; R:GTCATCAAGCTTCTGTCTGTG	54	127bp
OCN	F:GTGCAGCCTTTGTGTCCAAG R:GTCAGCCAACCTCGTCACAGT	58	158bp

Figure 1: Cell proliferation by CCK assays on days 1, 3, 7, 10, 14, 17 and 21 during (A) endothelial induction and (B) osteogenic induction. Results were presented as the mean $\pm$ SD, and experiments were performed in triplicate.*Significantly different ($p < 0.05$, $n = 3$) to the blank group at the same time point; **significantly different ($p < 0.01$, $n = 3$) to the blank group at the same time point. (Herein, and later, the OSA/NSC groups, RGD-OSA/NSC groups, and blank control groups were denoted as gel, RGD and blank, respectively whilst these corresponding groups with LIPUS were named as L+G, L+R and L+B, respectively.)

Observation of Cell Morphology during Endothelial and Osteogenic Differentiation of hDFs

It is generally accepted that hDFs are mainly fusiform and that the morphologies of cells have a close relationship with their differentiation and mechanical properties as some studies revealed that differentiation can result in the change of cell morphologies while they, in turn, affect differentiation [25]. Thus, the change of cell morphologies during the endothelial induction and osteogenic induction may be seemed as evidence for differentiation of hDFs.

As shown in *Figure.2A*, flow cytometry (FACS) was used to measure the expression levels of fibroblast specific surface marker CD 90 and MSCs specific surface marker CD 29 in hDFs. The used human dermal fibroblasts strongly indicated positive expression for CD 90 but negative for CD 29, hence, it can be safely concluded that the cells used for the endothelial and osteogenic differentiation were only human dermal fibroblasts which didn't contain any MSCs. This is the presupposition for the subsequent experiments and conclusions. After induction of 28 days, as shown in *Figure.2B*, it was observed that most cells presented totally different morphologies from those of human dermal fibroblasts, demonstrating that most of dermal human fibroblasts may have differentiated. The cells for the endothelial induction presented a typical flat polygonal appearance, like endothelial cells, while those for osteogenic induction exhibited spindle-shaped morphologies with relatively large nuclei similar to osteoblasts.

Alkaline Phosphatase and Alizarin Red Staining for Osteogenic Differentiation of hDFs

ALP activity is the most widely recognized biochemical marker for osteoblastic activity in the early stage. It is a characteristic protein that is used to identify osteoblast-phenotype and osteoblast differentiation [26]. *Figure.3A* shows the results of ALP staining of the induced cells at day 3, 7, 10 and 14. The expression of alkaline phosphatase was firstly observed at day 3, although its amount was low, which would increase with the increasing cultivation time and reached a peak by day 10 and then started to decrease [27]. The initial increase in ALP activity was a marker of the commitment towards osteoblastic lineage while the subsequent decrease should be attributed to the formation of

Figure 2: (A) Flow cytometry analysis of fibroblast specific surface marker CD 90 and MSCs specific surface marker CD 29 for human dermal fibroblasts; (B) Microscopic observation of cell morphology before and after osteogenic and endothelial differentiation of hDFs with acridine orange and HE staining.

Figure 3: (A) ALP staining assay after osteogenic induction of 3, 7, 10 and 14 days. (B) Images of mineralized nodule formation by staining with alizarin red S after osteogenic induction of 14, 21 and 28 days. Original magnification: 200×. (C) ALP activity assay after osteogenic induction of 3, 7, 10 and 14 days. (D) Quantitative analysis of calcium content after osteogenic induction of 14, 21 and 28 days. Results were presented as the mean±SD, and experiments were performed in triplicate.*Significantly different ($p < 0.05$, $n = 3$) to the blank group at the same time point; **significantly different ($p < 0.01$, $n = 3$) to the blank group at the same time point.

the advanced matrix mineralization and more mature phenotype [28]. A repeatable positive expression of alkaline phosphatase in the groups treated with RGD could be observed, although it was not very pronounced from a statistical point of view, compared with that in the groups treated with LIPUS, indicating that there existed a possible positive enhancement effect of RGD or LIPUS on the osteogenic differentiation of hDFs. Further work needs to be done to prove it. The similar results were also detected in the later measurement of ALP activity in *Figure.3C*. The use of LIPUS or RGD led to a much higher level of the ALP activity compared with that of the blank group, revealing that LIPUS or RGD did enhance the differentiation of cultured hDFs towards osteoblasts, as indicated by the increase in the ALP activity.

Alizarin Red S (AR-S) as a dye can bind selectively to calcium salts and hence is widely used for calcium mineral histochemistry and the assay of formation of new bone. This kind of histological staining is based on the capacity of alizarin red to specifically stain matrix containing calcium and its positive appearance is considered an expression of bone matrix deposition [29]. The ability of cells to form mineralized matrix is essential with regard to the development of materials for the formation of new bone. Normally, the measurement of ALP activity alone is not sufficient for determining bone formation. Hence, calcium nodulation is considered as another specific marker of extracellular mineralized deposit in the late stage [30]. As shown in *Figure.3B*, alizarin red positive nodular aggregates have already been observed in all the groups at day 14, indicating the formation of bone mineralization matrix at this time. At the same time point, the number and size of Alizarin red positive nodular aggregates in the groups treated with LIPUS or RGD modification were larger than those in the controls, demonstrating the enhancement effect of both LIPUS and RGD on calcium deposition and formation of bone matrix. The increased number and size of Alizarin red positive nodules

with the increasing induction time indicated that more and more calcium deposition had occurred.

The calcium content at different induction time points was measured by using Image Pro Plus to quantitatively assay the formation of bone matrix [31]. It can be seen from *Figure.3B*, the change of calcium content presented the same tendency over time as Alizarin red S staining as shown in *Figure.3D*. It can be seen that the use of LIPUS or RGD could induce a higher level of calcium deposition compared with those in the controls, further revealing that LIPUS and RGD could promote the differentiation of cultured hDFs towards osteoblasts, as indicated by the increase in the calcium content. In comparison, treatment with LIPUS led to a much higher level of calcium deposition and a much stronger enhancement effect on osteogenic induction than RGD modification. Moreover, there was a significant synergistic effect of LIPUS and RGD on the enhancement of the calcium nodules formation.

Immunofluorescence Staining for Endothelial Differentiation of hDFs

In our study, endothelial differentiation of hDFs on OSA/NSC and RGD-OSA/NSC hydrogels were conducted to evaluate their ability to induce vascularization. The immunofluorescence staining can provide a direct evidence for endothelial differentiation of the cells. The differentiated cells can be detected for the expression of endothelial cell markers CD31 that can be visualized by FITC-conjugated secondary antibody by immunofluorescence staining. We chose endothelial cell markers CD31 to assay the endothelial differentiation of hDFs and found that all the cells expressed specific endothelial markers of CD31 at day 28 and could be visualized by FITC-conjugated secondary antibody shown in *Figure.4A*, demonstrating that the hDFs had been successfully induced to differentiate into endothelial cells [32]. Additionally, fluorescent intensity has also been used to quantitatively assay the expression of CD31. The cells in the groups with LIPUS showed a much higher level of the positive expression of CD31 compared with those in the groups without LIPUS, revealing that LIPUS might be very useful for promoting the endothelial differentiation of hDFs. The highest expression level of CD31 was detected in the group treated with both LIPUS and RGD and it was much higher than the totality caused by LIPUS and RGD alone shown in *Figure.4B*, indicating the synergistic effect of LIPUS and RGD on the enhancement of the endothelial differentiation of hDFs.

Flow Cytometry

From the results of flow cytometry, it can be seen that all the groups were positive for the expression of endothelial cell-associated marker CD31 (*Figure.5A*), thus indicating the success in the endothelial differentiation. From *Figure.5A*, it can be observed that the fluorescence intensity peaks of all the experimental groups caused a certain degree of shift towards the high fluorescence intensity direction when compared with that of IgG isotype control, indicating the positive expression of endothelial cell surface marker CD31. As shown in *Figure.5C*, the mean fluorescence intensity of groups treated with LIPUS was

up-regulated, the ratios of the up-regulation of the blank, gel and RGD groups were 12%, 18% and 38%, respectively. The mean fluorescence intensity of RGD group was 1.11 times as much as that of the blank group; demonstrating that RGD was conducive to increasing the expression level of endothelial cell-associated marker CD31. The mean fluorescence intensity of RGD group treated with LIPUS was approximately 1.53 times as much as that of blank group, further revealing the synergistic effect ($0.53 > 0.12 + 0.11$) of LIPUS and RGD on the enhancement of the expression level of endothelial cell-associated markers CD31. Thus, the application of LIPUS and RGD modification can not only promote the endothelial differentiation of hDFs but also has a certain synergistic effect on it.

In addition, from the results of flow cytometry, it can be seen that all the groups were positive (>98%) for osteogenic cell-associated marker CD80 (*Figure.5B*), indicating the success in osteogenic differentiation. From *Figure.5B*, it can be observed that the fluorescence intensity peaks of all the experimental groups had an obvious degree of shift towards high fluorescence intensity direction when compared with that of IgG isotype control, indicating the positive expression of osteogenic cell surface marker CD80. As shown in *Figure.5D*, the application of LIPUS and RGD modification also led to the similar results in the osteogenic differentiation of hDFs. The mean fluorescence

Figure 4: (A) Images of immunofluorescence staining of cells after endothelial induction of 28 days. The differentiated cells were detected for the expression of (red) endothelial cell markers CD31 and visualized by FITC-conjugated secondary antibody. DAPI counter stained nuclei in blue. Original magnification: 200×. (B) Quantitative analysis of fluorescence intensity of CD31 was performed and values are expressed as IOD. Results were presented as the mean±SD, and experiments were performed in triplicate.*Significantly different ($p < 0.05$, $n = 3$) to the blank group at the same time point; **significantly different ($p < 0.01$, $n = 3$) to the blank group at the same time point.

Figure 5: Endothelial and osteogenic differentiation of human dermal fibroblasts. Flow cytometry analysis of CD31 (A) after endothelial induction of 28 days and CD80 (B) after osteogenic induction of 28 days. The corresponding mean fluorescence intensity of CD 31 (C) and CD80 (D) for (A) and (B).v

intensities of all the groups treated with LIPUS were higher than those groups treated without LIPUS, the increased ratios of the blank, gel and RGD groups were 10%, 14% and 19%, respectively. The mean fluorescence intensity of RGD group was 1.11 times as much as that of blank group; and the highest expression of CD80 was detected in the group with both LIPUS and RGD, it was approximately 1.31 times as much as that of blank group. Consequently, the application of LIPUS and RGD modification can promote both endothelial and osteogenic differentiations of hDFs.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

To confirm the endothelial differentiation of hDFs, a panel of endothelial related genes including Angiotension II Receptor Type1 (AGTR1) and Kinase Insert Domain Receptor (KDR) were measured by RT-PCR (Figure.6). The AGTR1 can be found in the heart, blood vessels and it seems to be re-expressed or up-regulated in vascular and wound healing. KDR, also known as Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2), plays an essential role in the regulation of angiogenesis, vascular development, vascular permeability, and embryonic hematopoiesis. It also promotes proliferation, survival, migration and differentiation of endothelial cells.

At 28th day after the endothelial differentiation of hDFs, it can be observed that all the groups were positive to express

these endothelial cell specific genes, indicating the success in the endothelial differentiation. As shown in Figure. 6A1 and A2, the results of all the experimental groups' minimum cycle number verified that the amplified products of AGTR1 and KDR had specificity. From the bar graph of Figure. 6C, it has been observed that the expression levels of both AGTR1 and KDR for all the groups treated with LIPUS have been elevated. The increased ratios of AGTR1 for the blank, gel and RGD groups were 30%, 70% and 130%, respectively, while those of KDR for the same groups were 30%, 70% and 220%, respectively; indicating that the application of LIPUS was conducive to increasing the expression levels of both AGTR1 and KDR. The expression levels of AGTR1 and KDR for RGD group were also increased and the increased ratios for them were 150% and 20%, respectively, when compared with the blank group, demonstrating that RGD modification was also beneficial for enhancing the expression levels of both AGTR1 and KDR. Furthermore, the expression levels of AGTR1 and KDR for RGD group treated with LIPUS were approximately 2.3 times and 3 times higher than that of blank group suggesting that the synergistic effect ($2.3 > 0.3 + 1.5$ for AGTR1 and $3 > 0.3 + 0.2$ for KDR) of LIPUS and RGD on the up-regulation of the expression level of both AGTR1 and KDR. The results of electrophoresis (Figure. 6B) showed that the electrophoresis bands of all the experimental groups were very clear and the variation trend of the electrophoresis brightness could match the results of the bar graph very well.

To identify the osteogenic differentiation of hDFs, the expression levels of osteogenesis-related genes including runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN) were measured by RT-PCR analysis (Figure.6). RUNX2, also known as Core Binding Factor alpha 1 (Cbfa 1), is an osteoblast specific transcription factor that regulates osteoblast differentiation and bone formation by initiating the expression of many bone specific genes. Osteocalcin (OCN), the most typical maker for late-stage osteogenesis, is already known to play an important role in the process of ossification for bone formation.

At 28th day after the osteogenic differentiation of hDFs, it has been observed that all the groups were positive to express these osteogenesis-related genes, indicating the success in osteogenic differentiation. As shown in Figure. 6A3 and A4, the specificity of amplified products of RUNX2 and OCN was verified by the minimum cycle number of all the experimental groups. From the histogram of Figure 6C, it can be seen that the expression levels of RUNX2 and OCN for all the groups treated with LIPUS have been improved. The increased ratios of RUNX2 for the blank, gel and RGD groups were 100%, 110% and 140% while the increased ratios for OCN of blank, gel and RGD groups were 50%, 60% and 80%, respectively, indicating that LIPUS contributed to promoting the expression levels of RUNX2 and OCN. The expression levels of RUNX2 and OCN in RGD group were also elevated when compared with the blank group, the raised ratios for RUNX2 and OCN were 20% and 60%, respectively, also demonstrating that RGD was beneficial for up-regulating the expression levels of RUNX2 and OCN. Additionally, the expression levels of RUNX2 and OCN for RGD group treated with LIPUS were approximately 3 times and 1.6 times higher than that of blank group, respectively, suggesting the synergistic effect ($3 > 1 + 0.2$ for RUNX2 and $1.6 > 0.5 + 0.6$ for OCN) of LIPUS and RGD on the up-regulation of the expression levels of RUNX2 and OCN. Moreover, the electrophoresis bands of all the experimental groups (Figure.6B) were very clear and

the variation trend of the electrophoresis brightness was in good agreement with the results of the histogram.

In brief, the application of LIPUS and RGD modification not only can promote the endothelial and osteogenic differentiations of hDFs but also has a certain synergistic effect on them.

Discussion

The Effect of RGD Modification on the Proliferation and Differentiation of hDFs

Owing to good biocompatibility, biodegradation and appropriate mechanical properties, hydrogels have good prospects in biomedical applications. However, hydrophilic surfaces of hydrogels are not beneficial for the adsorption of protein and not conducive to the adhesion and growth of cells [33]. As a result, these will allow the biomedical applications of hydrogels to be limited. Therefore, the functionalization and modification of hydrogels are becoming very important in tissue engineering. In this study, the modification of hydrogels by grafting cell adhesive molecules RGD has been proved to be a promising approach to overcome these issues. It has been seen that the RGD-OSA/NSC samples presented a higher absorbance value than the OSA/NSC ones. This result demonstrated that the RGD modification of hydrogels could improve the adhesion, growth and proliferation of hDFs to a certain extent indeed. Moreira et al.'s study showed that the seeded cell population in the 3-D dense collagen scaffolds can affect the degree of osteoblastic cell proliferation, differentiation and some aspects of matrix remodeling activity [34]. In this present study, the groups treated with RGD modification indicated a higher expression levels of numerous markers and genes associated with endothelial and osteogenic differentiation, suggesting that RGD can promote the differentiation of hDFs. This promoted effect of RGD modification on the differentiation of hDFs might be attributed to the much higher cell density caused by RGD modification. These results

Figure 6: Endothelial and osteogenic differentiation of human dermal fibroblasts. RT-PCR analysis was performed for AGTR1 (A1); KDR (A2) after endothelial induction of 28 days and for OCN (A3); RUNX2 (A4) after osteogenic induction of 28 days. The relative gene expression levels were normalized by GAPDH and shown in (C) and (D). *Significantly different ($p < 0.05$, $n = 3$); **significantly different ($p < 0.01$, $n = 3$).

are similar to those that we have reported on previously in the differentiation of hMSCs [19].

The Effect of LIPUS with 1 MHz and 200 mW/cm² on the Proliferation and Differentiation of hDFs

Takayama et al. reported that transient LIPUS stimulation (1.5 MHz, 30mW/cm², 20 minutes everyday) did not affect the rate of cell proliferation [22]. Parvizi et al. reported that pulsed ultrasound stimulates (1 kHz) had no effect on cell proliferation [35]. However, Bohari et al found that LIPUS alone showed a positive effect on human dermal fibroblasts proliferation and collagen deposition even without growth factor supplements [36]. We speculate that the bio-effects of cells caused by LIPUS depends on appropriate ultrasound dosage and ultrasound stimulates have no effect on cell proliferation under too high or too low intensity and frequency. In this present work, it can be seen that all the experimental groups treated with LIPUS exhibited a higher OD values compared with those groups treated without LIPUS. Our present and previous results showed that 1 MHz and 200 mW/cm² might be a suitable frequency and intensity that was beneficial for the proliferation of most kinds of cells [19]. Warden et al. found that LIPUS could stimulate the expression of the immediate early response genes c-fos and COX-2, and elevate mRNA levels for the bone matrix proteins ALP and OCN, suggesting that LIPUS has a direct effect on bone formation [37]. Mostafa et al found that LIPUS treatment enhanced osteogenic differentiation potential of human gingival fibroblasts as determined by significant up-regulation of specific ALP activity and osteopontin (OPN) expression [38]. The present study also showed that all the groups treated with LIPUS showed higher expression levels of ALP activity, calcium nodules, osteogenic cell associated surface antigen marker CD80 and osteogenesis related genes RUNX2 and OCN when compared with those groups treated without LIPUS. However, up to date there has not been any report on the endothelial differentiation of hDFs. In this study, all the groups treated with LIPUS showed a higher expression level of endothelial cell associated surface antigen marker CD31, higher expression levels of endothelial related genes AGTR1 and KDR when compared with those groups treated without LIPUS. Hence, the application of LIPUS can promote not only the proliferation but also endothelial and osteogenic differentiation of hDFs.

Synergistic Effect of LIPUS and RGD on the Proliferation and Differentiation of hDFs

In our study, the proliferation, endothelial and osteogenic differentiations of hDFs were all significantly enhanced by the simultaneous application of LIPUS and RGD modification. To our best knowledge, there has been no any report on them yet. We speculate that the synergistic effect on the enhancement of proliferation is achieved by RGD promoting cell adhesion to create a higher cell density and LIPUS stimulating hDFs to release ECMs, which further enhance the adhesion of hDFs. Similarly, for the synergistic effect on the enhancement of hDFs differentiation, the application of RGD can promote cell proliferation to achieve a higher cell density, thus enabling LIPUS to stimulate more hDFs to release growth factors to form a microenvironment with high

concentrations of growth factors where differentiation of hDFs can be successfully induced. More importantly, in our previous study on the differentiation of hMSCs, we have reported on some similar results and proposed a signaling pathway-based mechanism on a positive synergistic effect of LIPUS and RGD on differentiation of hMSCs [19]. During this process some same receptors might simultaneously respond to a few “chemical ligands”, for example, integrins respond to not only RGD but also LIPUS. We hence inferred that LIPUS can stimulate hMSCs by activating some special mechanosensors while these sensors can trigger signaling pathways to activate transcription factors to bind with the appropriate cis elements so as to modulate the expression of the gene and then the protein, the changes of which in turn modulate the function of the hMSCs and finally result in the differentiation of hMSCs [39]. Moreover, LIPUS can increase the permeability of the cell membrane and accelerate signal transmission and enhance the role of cytokines and RGD, promoting the differentiation of hMSCs [40]. Hence, a positive synergistic effect of LIPUS and RGD can be expected. For the differentiation of hDFs, we speculate that the same mechanism and signaling pathways might be responsible for the differentiation of hDFs, hence, it might also be true for the positive synergistic effect of LIPUS and RGD on differentiation of hDFs in the same way.

Conclusion

This in vitro study was successfully designed to evaluate the capacity of hDFs to differentiate into both osteogenic and endothelial cell lineages and in turn to see whether or not they can be used as an alternative for seed cells in tissue engineering. The results showed that the endothelial and osteogenic differentiations of hDFs could be successfully induced, which have been confirmed by checking ALP activity and calcium nodules as well as expression levels of CD80, RUNX2, OCN, CD31, AGTR1 and KDR. The results revealed that the application of LIPUS and RGD modification improved the proliferation, endothelial and osteogenic differentiations of hDFs to different degree, especially when they were simultaneously applied. From the obtained result data, it could be concluded that hDFs possess the capacity to differentiate into both osteogenic and endothelial cell lineages and might be an alternative autologous cell source for future bone tissue engineering.

Acknowledgements

This work was supported by the National Key R&D Program of China (13th Five-Year Plan, 2016YFC1102001), the National Natural Science Foundation of China (No. 51072167 and No. 31370966).seed cells.

References

1. Reichert JC, Woodruff MA, Friis T, Quent VMC, Gronthos S, Duda GN, et al. Ovine bone- and marrow-derived progenitor cells and their potential for scaffold-based bone tissue engineering applications in vitro, and in vivo. *J Tissue Eng. Regen. Med.* 2010;4(7):565–576. doi: 10.1002/term.276.
2. Zhou J, Hong L, Fang TL, Li XL, Dai WD, Uemura T, et al. The repair of large segmental bone defects in the rabbit with vascularized tissue engineered bone. *Biomaterials.* 2010;31:1171–1179. doi: 10.1016/j.biomaterials.2009.10.043.

3. Morishita T, Honoki K, Ohgushi H, Kotobuki N, Matsushima A, Takakura Y. Tissue Engineering Approach to the Treatment of Bone Tumors: Three Cases of Cultured Bone Grafts Derived From Patients' Mesenchymal Stem Cells. *Artif Organs*. 2006;30(2):115-118. DOI:10.1111/j.1525-1594.2006.00190.x.
4. Jiang J, Fan CY, Zeng BF. Experimental Construction of BMP2 and VEGF Gene Modified Tissue Engineering Bone in Vitro. *Int J Mol Sci*. 2011;12(3):1744-1755. doi: 10.3390/ijms12031744
5. Ghodsizad A, Ruhparwar A, Bordel V, Mirsaidighazi E, Klein HM, Koerner MM, et al. Clinical Application of Adult Stem Cells for Therapy for Cardiac Disease, *Cardiovasc. Ther.* 2013;31(6):323-334. doi: 10.1111/1755-5922.12032.
6. Kagami H, Agata H, Tojo A, Inoue M, Asahina I, Yamashita N, et al. Bone marrow stromal cells (bone marrow-derived multipotent mesenchymal stromal cells) for bone tissue engineering: basic science to clinical translation, *Int J Biochem. Cell Biol.* 2011;43:286-289.
7. Marin-Bañasco C, García MS, Guerrero IH, Sánchez RM, Estivill-Torrús G, Fernández LL, et al. Mesenchymal properties of SJL mice-stem cells and their efficacy as autologous therapy in a relapsing-remitting multiple sclerosis model, *Stem Cell Res. Ther.* 2014;5(6):134. doi: 10.1186/scrt524.
8. Lorenz K, Sicker M, Schmelzer E, Rupf T, Salvetter J, Schulz-Siegmund M, et al. Multilineage differentiation potential of human dermal skin-derived fibroblasts. *Exp Dermatol*. 2008;17(11):925-932. doi: 10.1111/j.1600-0625.2008.00724.x.
9. Yamanaka S. Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. *Cell Proliferation*. 2008;41:51-56. doi: 10.1111/j.1365-2184.2008.00493.x.
10. Han X, Han J, Ding F, Cao S, Lim SS, Dai Y, et al. Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. *Cell Res*. 2011;21(10):1509-1512. doi: 10.1038/cr.2011.125.
11. Yamanaka S, Takahashi K, Okita K, Nakagawa M. Induction of pluripotent stem cells from fibroblast cultures. *Nat. Protoc*. 2007;2(12):3081-3089. DOI:10.1038/nprot.2007.418
12. Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. *J Cell Biochem*. 2011;112(12):3491-3501. doi: 10.1002/jcb.23287.
13. Junker JP, Sommar P, Skog M, Johnson H, Kratz G. Adipogenic, Chondrogenic and Osteogenic Differentiation of Clonally Derived Human Dermal Fibroblasts. *Cells Tissues Organs*. 2010;191(2):105-118. doi: 10.1159/000232157.
14. Yadav PS, Mann A, Singh J, Kumar D, Sharma RK, Singh I. Buffalo (*Bubalus bubalis*) Fetal Skin Derived Fibroblast Cells Exhibit Characteristics of Stem Cells. *Agric Res*. 2012;1(2):175-182. doi:10.1007/s40003-012-0013-y.
15. Yin S, Cen L, Wang C, Zhao G, Sun J, Liu W, et al. Chondrogenic transdifferentiation of human dermal fibroblasts stimulated with cartilage-derived morphogenetic protein 1. *Tissue Eng Part A*. 2010;16(5):1633-1643. doi: 10.1089/ten.TEA.2009.0570.
16. Hee CK, Nicoll SB. Endogenous bone morphogenetic proteins mediate 1 α , 25-dihydroxyvitamin D3 -induced expression of osteoblast differentiation markers in human dermal fibroblasts. *J Orthop Res*. 2009;27:162-168.
17. Bi D, Chen FG, Zhang WJ, Zhou GD, Cui L, Liu W, et al. Differentiation of human multipotent dermal fibroblasts into islet-like cell clusters. *BMC Cell Biol*. 2010;11:46. DOI: 10.1186/1471-2121-11-46.
18. Liu X, Peng WZ, Wang YY, Zhu MH, Sun T, Peng Q, et al. Synthesis of an RGD-grafted oxidized sodium alginate-N-succinyl chitosan hydrogel and an in vitro study of endothelial and osteogenic differentiation. *J Mater Chem B*. 2013;1:4484-4492. DOI: 10.1039/C3TB20552E.
19. Wang YY, Peng WZ, Liu X, Zhu MH, Sun T, Peng Q, et al. Study of bi-lineage differentiation of hMSCs in oxidized sodium alginate/N-succinyl chitosan hydrogels and synergistic effects of RGD modification and low-intensity pulsed ultrasound. *Acta Biomater*. 2014;10(6):2518-2528. doi: 10.1016/j.actbio.2013.12.052.
20. Seo J, Lee H, Jeon J, Jang Y, Kim R, Char K, et al. Tunable layer-by-layer polyelectrolyte platforms for comparative cell assays. *Biomacromolecules*. 2009;10(8):2254-2260. DOI: 10.1021/bm900439r.
21. Chen DC, Chen LY, Ling QD, Wu MH, Wang CT, Kumare SS, et al. Purification of human adipose-derived stem cells from fat tissues using PLGA/silk screen hybrid membranes. *Biomaterials*. 2014;35(14):4278-4287. doi: 10.1016/j.biomaterials.2014.02.004.
22. Takayama T, Suzuki N, Ikeda K, Shimada T, Suzuki A, Maeno M, et al. Low-intensity pulsed ultrasound stimulates osteogenic differentiation in ROS 17/2.8 cells. *Life Sci*. 2007;80(10):965-971. DOI: 10.1016/j.lfs.2006.11.037.
23. Wang MK, Su YP, Sun HQ, Wang T, Yan GH, Ran XZ, et al. Induced endothelial differentiation of cells from a murine embryonic mesenchymal cell line C3H/10T1/2 by angiogenic factors in vitro. *Differentiation*. 2010;79(1):21-30. doi: 10.1016/j.diff.2009.08.002.
24. Chun CJ, Lim HJ, Hong KY, Park KH, Song SC. The use of injectable, thermosensitive poly (organophosphazene)-RGD conjugates for the enhancement of mesenchymal stem cell osteogenic differentiation. *Biomaterials*. 2009; 30(31):6295-6308. doi: 10.1016/j.biomaterials.2009.08.011.
25. Kim BS, Kim JS, Chung YS, Sin YW, Ryu KH, Lee J, et al. Growth and osteogenic differentiation of alveolar human bone marrow-derived mesenchymal stem cells on chitosan/hydroxyapatite composite fabric. *J. Biomed Mater Res. Part A*. 2013;101(6):1550-1558. doi: 10.1002/jbma.34456.
26. Kim DY, Jung MS, Park YG, Yuan HD, Quan HY, Chung SH. Ginsenoside Rh2(S) induces the differentiation and mineralization of osteoblastic MC3T3-E1 cells through activation of PKD and p38 MAPK pathways. *BMB Rep*. 2011;44(10):659-664. doi: 10.5483/BMBRep.2011.44.10.659.
27. Magnusson P, Larsson L, Magnusson M, Davie MW, Sharp CA. Isoforms of Bone Alkaline Phosphatase: Characterization and Origin in Human Trabecular and Cortical Bone. *J Bone Miner Res*. 1999;14(11):1926-1933. DOI:10.1359/jbmr.1999.14.11.1926.
28. Dimitrievska S, Bureau MN, Antoniou J, Mwale F, Petit A, Lima RS, et al. Titania-hydroxyapatite nanocomposite coatings support human mesenchymal stem cells osteogenic differentiation. *J Biomed Mater Res. Part A*. 2011; 98(4):576-588. doi: 10.1002/jbma.32964.
29. Wang D, Haile A, Jones LC. Dexamethasone-induced lipolysis increases the adverse effect of adipocytes on osteoblasts using cells derived from human mesenchymal stem cells. *Bone*. 2013;53(2):520-530. doi: 10.1016/j.bone.2013.01.009.
30. Reichert JC, Quent VMC, Burke LJ, Stansfield SH, Clements JA, Huttmacher DW. Mineralized human primary osteoblast matrices as a model system to analyse interactions of prostate cancer cells with the bone micro environment. *Biomaterials*. 2010;31(31):7928-7936. doi: 10.1016/j.biomaterials.2010.06.055.

31. Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin and osteocalcin expression in developing rat bone visualized by in situ hybridization. *J Bone Miner Res.* 1998;5(8):831-842. DOI:10.1002/jbmr.5650050806.
32. Bailey AS, Jiang S, Afentoulis M, Baumann CI, Schroeder DA, Olson SB, et al. Transplanted adult hematopoietic stems cells differentiate into functional endothelial cells. *Blood.* 2004;103(1):13-19. DOI:10.1182/blood-2003-05-1684.
33. Parhi P, Golas A, Vogler EA. Role of Proteins and Water in the Initial Attachment of Mammalian Cells to Biomedical Surfaces: A Review. *J Adhes Sci Technol.* 2010;24(5):853-888. doi.org/10.1163/016942409X12598231567907.
34. Moreira SM, Andrade FK, Domingues L, Gama M. Development of a strategy to functionalize a dextrin-based hydrogel for animal cell cultures using a starch-binding module fused to RGD sequence, *BMC Biotechnol.* 2008;8:78. doi: 10.1186/1472-6750-8-78.
35. Parvizi J, Wu CC, Lewallen DG, Greenleaf JF, Bolander ME. Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression. *J Orthop Res.* 1999;17(4):488-494. DOI: 10.1002/jor.1100170405.
36. Bohari SP, Grover LM, Hukins DW. Pulsed low-intensity ultrasound increases proliferation and extracellular matrix production by human dermal fibroblasts in three-dimensional culture. *J Tissue Eng.* 2015;6:1-9. doi:10.1177/2041731415615777.
37. Warden SJ, Favaloro JM, Bennell KL, McMeeken JM, Ng KW, Zajac JD, et al. Low-Intensity Pulsed Ultrasound Stimulates a Bone-Forming Response in UMR-106 Cells. *Biochem Biophys Res Commun.* 2001;286(3):443-450. DOI: 10.1006/bbrc.2001.5412.
38. Mostafa NZ, Uluda H, Dederich DN, Doschak MR, T.H. El-Bialy TH. Anabolic effects of low intensity pulsed ultrasound on gingival fibroblasts *Arch. Oral Biol.* 2009;54(8):743-748. doi: 10.1016/j.archoralbio.2009.04.012.
39. Chien S. Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. *Am J Physiol Heart Circ. Physiol.* 2007;292(3):1209-1224. DOI:10.1152/ajpheart.01047.2006.
40. Dinno MA, Dyson M, Young SR, Mortimer AJ, Hart J, Crum LA. The significance of membrane changes in the safe and effective use of therapeutic and diagnostic ultrasound. *Phys Med Biol.* 1989;34(11):1543-1552.