Layered biomimetic nanocomposites replicate bone surface in three-dimensional cell cultures

Yichun Wang, Edward Jan, Meghan Cuddihy, Joong Hwan Bang and Nicholas Kotov

Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan, USA; Biointerfaces Institute, University of Michigan, Ann Arbor, Michigan, USA; Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan, USA; Department of Material Science and Engineering, University of Michigan, Ann Arbor, Michigan, USA

ABSTRACT

Replication of bone marrow niches is essential for the evaluation of the toxicity and efficacy of multiple drug candidates and understanding of cell development. Recreation of the complex biological environment of bone marrow in a suitable format for such studies can be accomplished using bone-mimetic constructs based on three-dimensional (3D) nanocomposite-coated cell scaffolds with inverted colloidal crystal geometry. Layer-by-layer (LBL) assembled nanocomposites combine the mechanical properties and surface topography of bone with the transparency of 3D cell matrices. Such rare combination of properties allows for discrimination and control of complex biological processes such as differentiation of bone marrow stromal cells. LBL biomimetic nanocomposites open the pathway toward better understanding of the interactions between stromal cells and the extracellular matrix in the bone marrow microenvironment, in order to achieve the accurate engineering of the bone marrow niches.

1. Introduction

A heterogeneous population of stromal cells in bone marrow provides the structural and physiological support for hematopoietic cells [1]. The stromal cells of bone marrow play critical roles in presenting membrane-bound ligands, secreting chemokines and potentially other intercellular signaling processes. Numerous studies have proven the need of bone marrow stromal cells (BMSCs) for the maintenance of hematopoietic cells in long-term culture for tissue engineering in vitro [2,3] that could be used for personalized cellular transplants and immune therapeutics [4]. However, it is unclear whether or how BMSCs transform their phenotypes and differentiate in engineered bone marrow niches. BMSCs have the potential to replicate as undifferentiated cells or differentiate to various lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma [5]. Like many stem cells, BMSCs differentiate into distinct lineages depending
on which cues are present in the cell culture environment such as growth factors [6], cell adhesion [7], cell shape [8], mechanical forces [9], and substrate rigidity [10]. Although differentiation fates for hematopoietic stem cells and other stem cells are partially known, the fate of BMSCs co-cultured with them as well as the factors that control it remains enigmatic [4,11,12]. Exploration of the interactions between surrounding stromal cells, BMSCs, and the extracellular matrix (ECM) is necessary for better understanding of bone tissue engineering.

Traditional in vitro models of the bone marrow niche are dependent on hematopoietic cells co-cultured with BMSCs on 2D surfaces. However, they lack the dimensionality and ECM components of the bone marrow niche. Three-dimensional (3D) matrixes can possess sufficiently large surface area for cell attachment, high porosity for cell migration and transport of nutrients, substantial transparency for inspection of constructs with optical techniques, and variability in scaffold structure to control cell-to-cell contacts. As such, these are particularly suitable for replication of bone marrow niches with addition of surrounding stromal cells [13]. Various types of 3D models of the bone marrow niche were developed [14–17]. Yet these models lack systematic approaches due to limited controllability and reproducibility of microscale structures and surface properties. Whereas the surface properties of the scaffolds that determine cell adhesion and other substrate–cell interactions represent some of the key factors for bone surface, such as surface stiffness and surface charges. Nanocomposites provide a convenient toolbox for engineering of biomaterials with a variety of functions [18]. While many hybrid organic-inorganic materials with nanoscale structure have been developed for drug delivery, tissue regeneration, antibacterial coatings and other applications [19], 3D cell cultures in scaffolds usually rely on traditional polymers. The biomimetic properties of layered nanocomposites combined with toughness and transparency in a single 3D scaffold system opens unique opportunities to engineer and replicate bone marrow niches in vitro [13,20].

In this work, we studied the behaviors of BMSCs in regard to the changes of substrate properties in an artificial bone marrow matrix in vitro based on a 3D scaffold with inverted colloidal crystal (ICC) geometry [21,22]. The ICC structure is geometrically similar to the 3D morphology of supporting bone marrow tissue in a trabecular bone. This is important for creating microenvironmental niches that maintain stem cell survival and promote maturation [23]. In addition, the structure of ICC scaffolds offers other benefits for bone tissue engineering such as a high degree of structural control, complete interconnectivity of cavities, and mechanical robustness [24]. To understand the effect of cell adhesion and substrate property changes on the BMSC supportive layer, scaffolds were coated with layers of transparent bone–mimetic nanocomposites. These coatings were made taking advantage of the layer-by-layer assembly (LBL aka LbL, or electrostatic self-assembly) [25–28] employing clay nanoplatelets (NPs) and polydiallyldimethylammonium bromide (PDDA) [29]. While many nanocomposites can offer toughness and stiffness typical of bone [30], layered nanocomposites made by LBL replicate the structure of hard mineralized tissues more accurately than other methods [31] and allow transparent coatings on highly curved surfaces [32]. The transparency of such nanocomposites is also essential for microscopy evaluation and other optical techniques relevant for the utilization of the scaffolds as an in vivo drug discovery platform [33]. The results demonstrated that the 3D-layered nanocomposites are capable of supporting BMSC adhesion, while their phenotypes expressed differently during the cell culture compared to in vitro 2D cell culture. These findings provided insights into replicating bone marrow niches based on 3D biomimetic nanocomposites.

2. Materials and methods
2.1. Preparation of colloidal crystals

Uniformly sized (120 μm) polystyrene beads suspension was added to water in a glass vial (Duke Scientific Corporation, 3 × 10^4 particles per milliliter). Two drops of the solution were dropped into the pipette every 20 min, so the beads slowly settled onto the bottom of the glass vial and assembled into a colloidal crystal (CC). After the desired volume of microspheres was added to reach the thickness of 0.5–1 mm, the beads and vial were left under sonication for an hour. After completely dried at 60°C overnight, the CCs were annealed at 120°C for 4 h. The final dimensions of freestanding CCs were 6.5 mm in diameter and 0.5–1 mm in thickness.

2.2. Preparation of ICC scaffolds

The CCs were removed from vials and placed into a 0.1 g/mL solution of 85:15 PLGA (Lactel Absorbable Polymers, Pelham, AL) and methylene chloride. The CCs were infiltrated by the PLGA solution by layer-by-layer assembly (LBL aka LbL, or electrostatic self-assembly) [25–28] employing clay nanoplatelets (NPs) and polydiallyldimethylammonium bromide (PDDA) [29]. While many nanocomposites can...
2.3. Layer-by-layer assembly on ICC scaffolds

ICC hydrogel scaffold surfaces were coated with the sequential deposition of positively charged 0.5 wt.\% poly (diallyl dimethylammonium chloride) (PDDA, Sigma, MW = 200,000 Da) solution, and a negatively charged 0.5 wt.\% clay platelet (average 1 nm thick and 70–150 nm in diameter, Southern Clay Products) dispersion for 30 min each. Each adsorption step was followed by washing in deionized water for 30 min, and all processes were performed under a gentle flow generated by a stirrer. Cyclic repetition of such process was carried out seven times to obtain seven bilayers of nanocomposites and the process ended with a clay NP layer.

2.4. Bone marrow stromal cell culture

Human BMSCs HS-5 (CRL-11882, ATCC) were cultured in Dulbecco’s Modified Eagle Media (DMEM) with 4 mM l-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin. Once cell growth reached approximately 80% confluence, they were detached from the culture flask using a 0.25% (v/v) Trypsin-EDTA solution and 80,000 cells were seeded on top of each UV-sterilized scaffold.

2.5. Live/dead assay analysis

Cell viability was observed using a Live/Dead Viability Kit (Invitrogen). 20 μL of 2 mM EthD-1 stock solution was added to 10 mL of sterile, tissue culture-grade PBS solution (PH = 7.4) to give an approximately 4 μM EthD-1 solution. The reagents were combined by transferring 5 μL of 4 mM calcein AM stock solution to the 10 mL EthD-1 solution. The resulting approximately 2 μM calcein AM and 4 μM EthD-1 working solution is then added directly to cell culture. The stained cells were

Figure 1. 3D-layered nanocomposite fabrication of the ICC scaffold. (a) SEM images of highly ordered colloidal crystals with the beads in size of 120 μm as the template. (b) Hydrogel precursor solution is later infiltrated into the colloidal crystals and polymerized. ICC scaffold geometry is created by dissolving beads from the hydrogel matrix. All pores are open to the outer cell culture media which facilitates cell motility and media exchange in dynamic culture conditions. (c) Scheme of PDDA, clay NPs and LBL surface coating procedures 1–4. (d) Seven bilayers of fluorescent labeled PDDA (green) and clay platelets on an ICC hydrogel scaffold soaked in (PBS). Scale bars (a) and (b): 250 μm.
visualized under fluorescence microscope (Zeiss Axiovert 100 M inverted microscope) at 20×.

2.6. Immunostaining of BMSCs in three potential lineages

Cultured HS-5 cells grown in scaffolds after 7 and 21 days were fixed with 4% freshly prepared paraformaldehyde for 20 min at room temperature. For specific protein detection such as nuclear proteins, the cells were permeated with 0.3% Triton X-100 (Sigma) for 5 min at room temperature followed by aspirating the fixative. Cells were washed three times for 5 min each with PBS after membrane permeation. Non-specific binding was blocked with PBS containing 1% rabbit serum in PBS for 2 h at room temperature. The cells cultured for 7 d were then incubated with primary antibodies (Santa Cruz): the chondrocyte-specific marker Sox-9, collagen type II (COL2A) and osteocyte-specific marker alkaline phosphatase (ALP), and runt-related transcription factor 2 (RUNX2) in 10% rabbit or mouse serum overnight at 4°C. Next, the cells were washed with PBS three times for 5 min each. The cells were then incubated with secondary antibodies anti-rabbit-FITC (Santa Cruz; 1:500) in 0.1% rabbit or mouse serum at 37°C for 30 min. After three 5 min washes with PBS, the cells were exposed to 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) solution for 1 min. As a result, the cell nuclei are stained blue. After washing the cells twice for 5 min each with PBS, confocal imaging was performed and analyzed.

Figure 2. HS-5 cells cultured on non-LBL scaffolds (a)–(c) and ICC scaffolds with seven bilayers of PDDA/clay (d)–(f). Confocal images were taken on days 3, 9, and 14 where cells were stained with a Live/Dead Viability/Cytotoxicity assay. (g) Cells per scaffold were counted on days 3, 9 and 14. Cell density at seeding is 80,000 cells/scaffold for all groups. Scale bar: 200 μm.
Following a 21-day culture period, adipogenic markers peroxisome proliferator-activated receptor (PPARγ2) and acyl-CoA synthetase (ACSL5) were examined by the same method above.

2.7. Scanning electron microscopy of BMSCs grown in ICC scaffolds

To investigate the attachment, spreading and differentiated morphologies of BMSC layer on scaffolds, samples were prepared for scanning electron microscopy after 3, 9, and 14 days of growth as follows. Firstly, the medium in these samples was replaced with 2.5% glutaraldehyde in Eagle’s Minimum Essential Medium (EMEM) without serum and fixed for 30 min at room temperature. The samples were then washed with PBS (pH 7.2) three times for 15 min each. The samples were dehydrated through an increasing series of ethanol washes from 50% to 60%, 70%, 80%, 95%, and finally 100%. The specimens were mounted on aluminum holders and sputter-coated with a conducting layer of gold platinum for 90 s. The samples were examined by scanning electron microscopy (SEM), using a voltage of 5 kV.

3. Results and discussion

ICC scaffolds were used to design a 3D bone marrow analog, which are hydrogel matrices with uniformly shaped spherical cavities arranged in a hexagonal array where each cavity is connected to 12 adjacent cavities following the manufacturing protocol [23,34]. Throughout this study polystyrene beads with a diameter of 120 μm were used, producing the uniform cavities of the same size connected by 10–20 μm channels (Figure 1 (a) and (b)). The yielded cavity size of such scaffolds is larger than 100 μm and ideal for bone growth [24] because it provides efficient contacts between adhering and dispersing cells, and allows for natural cell migration through the channels between the cavities. ICCs scaffolds as prepared are 6.5 mm in diameter, 0.5–1 mm thickness, and have pores of 120 μm in diameter. The ICC scaffolds used here are made of biodegradable poly(lactic-co-glycolic acid) (PLGA), which were demonstrated to be biocompatible with...
human cell cultures [24]. To provide adequate adhesion of BMSCs, hydrogel matrixes were coated by clay NPs and PDDA multilayer by LBL (Figure 1(c)). Each bilayer contains one layer of clay NPs and PDDA as shown by steps 1–4 in the scheme (Figure 1(c)). The LBL coating was started with a PDDA layer and finished with a clay NP layer for a total of seven bilayers to support stromal cell adhesion [35]. Visualization of the PDDA/clay NP multilayers on the ICC surface was carried out by confocal microscopy using FITC as a fluorescent label added to PDDA during deposition (Figure 1(d)) [36]. The compressive modulus of the resulting 3D nanocomposites was $179.4 \pm 4.89 \text{kPa}$ compared to $146.67 \pm 3.23 \text{kPa}$ of the non-LBL 3D scaffold. Such nanocomposites consisting of hybrid organic–inorganic layers are compatible with the hydrogel mechanically while providing a high Young’s

Figure 4. Immunofluorescence staining of lineage-specific markers of HS-5 Cells on LBL ICC scaffold with 7 bi-layers of PDDA/Clay NP coating: markers of chondrocytes (Sox-9 and COL2A) (a), markers of osteocytes (ALP and RUNX2) (b) and markers of adipocytes (PPARγ2 and ACSL5) (c). Quantitative analysis of expression level is shown in (d). Scale bar: 200 μm.
modulus necessary for successful stromal cell adhesion [22].

Human BMSC line HS-5 (ATCC, Manassas, VA), was used to investigate cellular responses of the support stromal cells to surface properties of bone marrow scaffold in vitro. This cell line was stabilized and generally used as support cells for hematopoietic stem cells [2], which provides growth factors and signaling communication to stem cells to mimic bone marrow stromal tissue function. HS-5 cells were seeded in the scaffolds and maintained in culture media. Live/Dead Viability/Cytotoxicity staining (Life Technologies, Carlsbad, CA) was used to monitor the viability of BMSCs in the scaffolds with (LBL) or

Figure 5. Confocal z-stack images of HS-5 cells aggregates in pores of the LBL ICC scaffold cultured with HS-5 cells were taken every 8 μm in depth. Cells were stained with (a) COL2A and (b) RUNX2, respectively. RUNX2 and Sox-9 staining shows that osteocyte marker positive cells mostly attached on the surface of scaffolds and chondrocyte marker positive cells mostly formed plaque in the center (c). Quantitative analysis of the counterstaining images for RUNX-2 and Sox-9 (d). Scale bar: 100 μm (a) and (b).
without (non-LBL) surface modification on days 3, 9, and 14 with confocal microscopy. In the confocal images, green cells are alive, while red cells are dead. Cells in the scaffold both with and without LBL coating appeared green while there were no cells stained red. The result indicated the cells maintained good viabilities in both types of scaffolds (Figure 2(a)–(f)). In addition, the confocal images also show that the cells better adhesion on the LBL coated ICC scaffolds and most of the cells formed thick plaques on the porous surface (Figure 2(e) and (f)). In contrast, the cells in non-LBL scaffolds tend to aggregate in the center of the pores and form small spherical clusters. This trend became more obvious after 9 days of culture (Figure 2(b) and (c)). The nanocomposites with surface clay NPs enhance BMSCs adhesion on the substrate, thereby changing the shape and morphology of cells.

The density of cells was evaluated from scaffolds with and without LBL after imaging on days 3, 9 and 14. As a result, without LBL the cells proliferated faster and numbered 1,340,000 ± 50,000 cells/scaffold compared to 725,000 ± 25,000 cells/scaffold in the LBL group from the original seeding (Figure 2(g)). There are two possible causes to result in this difference of cell proliferation. One is that the better adhesion of cells on the surface in LBL scaffold prevented agitation from detaching the cells from the substrates in identical experimental conditions. The other is that BMSCs in LBL scaffolds are transforming into differentiated cells which proliferate more slowly, explaining the reduction of self-renewal cells and increase of differentiated cells seen in the 3D culture. To exclude the first proposed potential cause, the LBL scaffolds were checked after trypsin treatment and extraction of cells and showed only a few leftover cells attached. Thus, the latter case is considered to be the primary cause of density difference between the LBL and non-LBL scaffolds. Moreover, the morphology change of the plaque shape of BMSC clusters here in the LBL scaffolds are also potentially related to the differentiation of cells which slows proliferation.

To verify the morphology change of BMSCs clusters in LBL scaffolds, SEM images were taken after culturing for 9 days. A dense plaque of cells was observed in the LBL scaffold (Figure 3(c) and (d)) whereas in the non-LBL scaffold the cells were washed away during sample preparation (Figure 3(a) and (b)). Noticeably, the LBL scaffolds still maintained the uniform porous structure compared to the deformed structure of the non-LBL scaffold during the dehydration process since the LBL coating enhanced the stiffness of the 3D matrix [13]. Furthermore, nanoscale porosity was observed on the surface of the scaffolds (Figure 3(b) and (c)) similar to the nanofibrous architecture of cartilage [37].

As stated previously, BMSCs have the potential to differentiate into multiple lineages of cells in bone tissue such as adipocytes, chondrocytes and osteocytes arisen from the supporting complex structure [5]. To understand how the LBL coating on the supporting scaffold influences phenotype changes, protein markers of three lineages were studied by immunofluorescent staining. The chondrocyte-specific marker Sox-9, collagen type II (COL2A) (Figure 4(a)) [38,39], osteocyte-specific marker alkaline phosphatase (ALP), and runt-related transcription factor 2 (RUNX2) [40–42] were examined on day 7 of cell culture (Figure 4(b)). Afterward, following a 21-day culture period, adipogenic markers peroxisome proliferator-activated receptor (PPARγ2) [43] and acyl-CoA synthetase (ACSL5) [44] were observed at the end of the culture and show very limited expressions (Figure 4(c)). The quantitative analysis by image processing (ImageJ) based on confocal images with immunofluorescence staining were shown in Figure 4(d). The gene expressions of all markers were compared with the original BMSCs in the 2D culture dish as a control in order to study the transition of phenotypes from the native state to their state in 3D nanocomposites.

As a control in 2D culture before seeding the ICC scaffolds, the expression of all markers for all three lineages on days 7 and 21 are significantly lower than in the LBL 3D, confirming that gene expression has changed with the transition of cells during culturing in LBL ICC scaffolds. Sox-9 expressed in 81.2 ± 5.2% of cultured cells, which is a sign of activation in all pre-chondrogenic mesenchymal condensations. 53.2 ± 3.8% of cells express COL2A, indicating that this phenotype of cells is more active in the chondrogenic state [38]. Osteogenic markers, RUNX2 and ALP are expressed in 27.3 ± 4.7% and 2.89 ± 1.4% of the population respectively. At the early stage of phenotype change, RUNX2 determines the osteoblast lineage from bone marrow multipotent mesenchymal cells, and further induces ALP activity at later stages. Therefore, RUNX2 shows dominant expression compared to ALP on day 7. However, osteogenic marker levels are still much lower than chondrogenic marker levels and shows insufficient features for osteogenesis towards mature osteocytes or osteoblasts. Following a 21-day culturing period, there was a significant upregulation of the adipocyte gene ACSL5, in BMSCs compared with the 2D cultured cells (Figure 4(d)), while there is no mature adipocyte marker PPARγ2 expressed in BMSCs cultured on the LBL ICC scaffold. This result shows no sufficient adipogenesis in the 3D culture.
In addition, the expression of chondrogenic and osteogenic markers were found to be related to the cells’ location of cells in the porous structure of the LBL scaffold. It was noticed that the BMSCs stained with chondrogenic marker COL2A, were mainly present at the center of the cell plaque (Figure 5(a)), whereas osteogenic marker, RUNX2, was predominantly localized at the periphery of the cell plaque or on the clay surface of the pore (Figure 5(b)). To observe the gene expression of two lineages at the same time, we counterstained the cells with both RUNX-2 and Sox-9 (Figure 5(c)). Quantitative analysis of the counterstaining images was processed by ImageJ to examine the overlap of the two markers (Figure 5(d)). The result shows that most of RUNX-2 positive cells also expressed chondrocyte specific markers Sox-9. 21.34 ± 3.2% of cells express both markers compared to 81.2 ± 6.0% of chondrocyte-like cells, and these cells were located in the plaques rather than adhered to the surface of the pore substrate.

Here, we should consider that RUNX2 is not only a regulator for osteogenesis but also plays an important role in terminal chondrocyte differentiation [45]. Thus RUNX2 expression of BMSCs cultured on LBL scaffolds are representative of an advanced step of chondrogenesis adjacent to osteogenesis-positive cells on the surface of the substrate and the thick plaque of chondrogenesis-positive cells. Overall, the distribution of cells with two markers implied that surface modification of the substrates with LBL did have influence on cell phenotypes. This is potentially due to the change of mechanical properties of ICC scaffolds after LBL surface modification with a relatively high compressive modulus and mechanical strain. These features were proved to stimulate marrow-derived mesenchymal stem cells in an effort to induce chondrogenic and osteogenic phenotypes and to impede adipocyte formation [46]. Coated clay nanocomposites also created nanoscale roughness, increased charges on the surface, and a much stiffer film than hydrogel [31]. Increase of the Young’s modulus was shown to be the primary factor determining the adhesion of cells to materials [47]. These effects were verified to promote cell adhesion, and thus caused differentiation of the phenotypes of HS-5 cells attached to the substrate surface and in cell plaques. The transition to the osteocyte-like phenotype was stimulated by increasing negative charge on the surface, providing a comprehensive stiffness and compressive modulus for BMSCs growth.

4. Conclusion

BMSCs can change their phenotypes during culturing within the layered biomimetic nanocomposites based on 3D scaffold. Furthermore, one can vary the morphology of cultured BMSC aggregation by taking advantage of surface engineering using LBL. Thus, this study provides insights for 3D nanocomposites to replicate bone marrow niches that require co-culture of BMSCs.

Acknowledgements

The central part of this work was supported by the MURI project from the Department of Army [W911NF-10-1-0518] Reconfigurable Matter from Programmable Colloids; and for the National Science Foundation grant [DMR-9871177] for funding of the FEI Nova 200 analytical electron microscope used in this work. The authors also thank Harrison Zhou for assistance with writing the manuscript.

Notes on contributors

Yichun Wang is postdoc researcher in Department of Chemical Engineering at the University of Michigan. She has mainly published on three-dimensional cell cultures for tissue engineering and the evaluation of the toxicity and efficacy of nanomedicines.

Edward Jan is a senior product marketing manager at Paragon Genomics. He has mainly published on layer-by-layer assembled nanocomposites for neural interfaces and he has also worked on gene transfection and neuronal programming on electroconductive nanocomposite.

Meghan Cuddihy is a commercialization education project manager of Fast Forward Medical Innovation at University of Michigan Medical School. She has designed novel bio-degradable 3D structure and its production process for bone tissue engineering and developed ex vivo hematopoietic stem cell niche analog for pharmaceutical testing.

Joong Hwan Bahng is postdoc researcher at Nonlinear Photonics Laboratory at California Institute of Technology. He has mainly published the work on ‘hedgehog’ particles which exhibit long-term colloidal stability in both hydrophilic and hydrophobic media. He was also involved in the work of three-dimensional cell cultures.

Nicholas Kotov is the Joseph B. and Florence V. Cejka Professor of Chemical Engineering at the University of Michigan in Ann Arbor, MI. He received his M.S. (1987) and Ph.D. (1990) under the guidance of M. Kuzmin of the chemistry department at Moscow State University. He worked as a postdoctoral associate at Syracuse University in the group of J. Fendler. Prior to his current position, he was a professor at Oklahoma State University. His group at the University of Michigan is actively involved in research in the field of biomimetic nanostructures, self-organization of nanocolloids, ultrastrong nanocomposites, energy materials, chiral nanostructures, implantable biomedical devices.

ORCID

Yichun Wang http://orcid.org/0000-0002-4353-6660

References

1. Bianco P, Rimiruuci M, Gronthos S, et al. Bone marrow stromal stem cells: nature, biology,
and potential applications. Stem Cells. 2001;19: 180–192.

2. Roecklein B, Torok-Storb B. Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. Blood 1995;85:997–1005.

3. Mateos-Timoneda MA, Navarro M, Planell JA. Bioreponsive surfaces and stem cell niches. In: Taubert A, Mano JF, Rodriguez-Cabello JC, editors. Biomaterials surface science. 2013. p. 269–284. https://doi.org/10.1002/9783527694600.ch9.

4. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol. 2006;6:93–106.

5. Prokop DJ. Marrow stromal cells as stem cells for continual renewal of nonhematopoietic tissues and as potential vectors for gene therapy. J Cell Biochem. 1998;72:284–285.

6. Cals FLJ, Hellingman CA, Koevoet W, et al. Effects of transforming growth factor-β subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. J Tissue Eng Regen Med. 2012;6:68–76.

7. Belnoue E, Tougne C, Rochat A-F. Homing and adhesion patterns determine the cellular composition of the bone marrow plasma cell niche. J Immunol. 2012;188:1283–1291.

8. Gao L, McBeath R, Chen CS. Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. Stem Cells. 2010;28:564–572.

9. Mauney JR, Sjostrom S, Blumberg J, et al. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. Calcif Tissue Int. 2004;74:458–468.

10. Parekh SH, Chatterjee K, Lin-Gibbon S, et al. Modulus-driven differentiation of marrow stromal cells in 3D scaffolds that is independent of myosin-based cytoskeletal tension. Biomaterials. 2011;32: 2256–2264.

11. Civini S, Jin P, Ren J, et al. Leukemia cells induce changes in human bone marrow stromal cells. J Transl Med. 2013;11:298.

12. Cheung W-C, Van Ness B. The bone marrow stromal microenvironment influences myeloma therapeutic response in vitro. Leukemia. 2001;15: 264–271.

13. Cuddihy MJ, Wang Y, Machi C, et al. Replication of bone marrow differentiation niche: comparative evaluation of different three-dimensional matrices. Small. 2013;9:1008–1015.

14. Feng Q, Chai C, Jiang X-S, et al. Expansion of engraving human hematopoietic stem/progenitor cells in three-dimensional scaffolds with surface-immobilized fibronectin. J Biomed Mater Res. 2006; 78A:781–791.

15. Sasaki T, Takagi M, Soma T, et al. 3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics. Cytotransfer. 2002;4:285–291.

16. Cook MM, Putrega K, Osiecki M, et al. Micromarrows–three-dimensional coculture of hematopoietic stem cells and mesenchymal stromal cells. Tissue Eng Part C. Methods. 2012;18:319–328.

17. Leisten I, Kramann R, Ventura Ferreira MS, et al. 3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche. Biomaterials. 2012;33:1736–1747.

18. Furuya M, Shimono N, Okamoto M. Fabrication of biocomposites composed of natural rubber latex and bone tissue derived from MC3T3-E1 mouse preosteoblastic cells. Nanocomposites. 2017;7:76–83.

19. Rogalski JJ, Bastiaansen CWM, Peijs T. Rotary jet spinning review – a potential high yield future for polymer nanofibers. Nanocomposites. 2017;3: 97–121.

20. Lee J, Heckl D, Parekkadan B. Multiple genetically engineered humanized microenvironments in a single mouse. Biomater Res. 2016;20:19.

21. Kotov NA, Liu Y, Wang S, et al. Inverted colloidal crystals as three-dimensional cell scaffolds. Langmuir 2004;20(19):7887–7892. DOI:10.1021/la0499580.

22. Nichols JE, Cortiella J, Lee J, et al. In vitro analog of human bone marrow from 3D scaffolds with biomimetic inverted colloid crystal geometry. Biomaterials. 2009;30:1071–1079.

23. Zhang Y, Wang S, Eghtedari M, et al. Inverted-colloidal-crystal hydrogel matrices as three-dimensional cell scaffolds. Adv Funct Mater. 2005;15: 725–731.

24. Cuddihy MJ, Kotov NA. Poly(lactic-co-glycolic acid) bone scaffolds with inverted colloidal crystal geometry. Tissue Eng Part A. 2008;14:1639–1649.

25. Decher G, Eickle M, Schmitt J, et al. Layer-by-layer assembled multicomposite films. Curr Opin Colloid Interf Sci. 1998;3:32–39.

26. Hammond PT. Building biomedical materials layer-by-layer. Mater Today. 2012;15:196–206.

27. Jiang C, Tsukruk VV. Freestanding nanostructures via layer-by-layer assembly. Adv Mater. 2006;18: 829–840.

28. Kotov NA. Ordered layered assemblies of nanoparticles. MRS Bull. 2001;26:992–997.

29. Tang Z, Wang Y, Podaślado P, et al. Biomedical applications of layer-by-layer assembly: from biomimetics to tissue engineering. Adv Mater. 2006;18: 3203–3224.

30. Greenfeld I, Wagner HD. Nanocomposite toughness, strength and stiffness: role of filler geometry. Nanocomposites. 2015;1:3–17.

31. Tang Z, Kotov NA, Magonov S, et al. Nanostructured artificial nacre. Nat Mater. 2003;2: 413–418.

32. Crisp MT, Cotov NA. Preparation of nanoparticle coatings on surfaces of complex geometry. Nano Lett. 2003(2):173–177. DOI:10.1021/nl025896.

33. Ablade-Cela S, Ho S, Rodriguez-González B, et al. Loading of exponentially grown LBL films with silver nanoparticles and their application to generalized SERS detection. Angew Chem Int Ed Engl. 2009;48:5326–5329.

34. Lee J, Shanbhag S, Kotov NA. Inverted colloidal crystals as three-dimensional microenvironments for cellular co-cultures. J Mater Chem. 2006;16: 3558.

35. Podaślado P, Qin M, Cuddihy M, et al. Highly duc- tile multilayered films by layer-by-layer assembly of oppositely charged polyurethanes for biomedical applications. Langmuir. 2009;25:14093–14099.

36. Lee J, Cuddihy MJ, Cater GM, et al. Engineering liver tissue spheroids with inverted colloidal crystal scaffolds. Biomaterials. 2009;30:4687–4694.
37. Xu L, Zhao X, Xu C, et al. Water-rich biomimetic composites with abiotic self-organizing nanofiber network. Adv. Mater. 2018;30:1703343.
38. Foster NC, Henstock JR, Reinwald Y, et al. Dynamic 3D culture: Models of chondrogenesis and endochondral ossification. Birth Defect Res C. 2015;105:19–33.
39. Lefebvre V, de Crombrugghe B. Toward understanding SOX9 function in chondrocyte differentiation. Matrix Biol. 1998;16:529–540.
40. Shui C, Spelsberg TC, Riggs BL, et al. Changes in Runx2/Cbfa1 expression and activity during osteoblastic differentiation of human bone marrow stromal cells. J Bone Miner Res. 2003;18:213–221.
41. Fujita T, Azuma Y, Fukuyama R, et al. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J Cell Biol. 2004;166:85–95.
42. Tsai M-T, Li W-J, Tuan RS, et al. Modulation of osteogenesis in human mesenchymal stem cells by specific pulsed electromagnetic field stimulation. J Orthop Res. 2009;27:1169–1174.
43. Ren D, Collingwood TN, Rebar EJ, et al. PPARgamma knockdown by engineered transcription factors: exogenous PPARgamma 2 but not PPARgamma 1 reactivates adipogenesis. Genes Dev. 2002;16:27–32.
44. Hall JA, Ribich S, Christofiolete MA, et al. Absence of thyroid hormone activation during development underlies a permanent defect in adaptive thermogenesis. Endocrinology. 2010;151:4573–4582.
45. Enomoto H, Enomoto-Iwamoto M, Iwamoto M, et al. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem. 2000;275:8695–8702.
46. Li R, et al. Mechanical strain regulates osteogenic and adipogenic differentiation of bone marrow mesenchymal stem cells. Biomed Res Int. 2015;2015:873251.
47. Thompson MT, Berg MC, Tobias IS, et al. Tuning compliance of nanoscale polyelectrolyte multilayers to modulate cell adhesion. Biomaterials. 2005;26:6836–6845.