ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2019, Vol. 74, No. 4, pp. 235–239. © Allerton Press, Inc., 2019.
Russian Text © The Author(s), 2019, published in Vestnik Moskovskogo Universiteta, Seriya 16: Biologiya, 2019, Vol. 74, No. 4, pp. 294–300.

RESEARCH ARTICLE

Selection of the Optimal Protocol for Preparation of a Decellularized Extracellular Matrix of Human Adipose Tissue-Derived Mesenchymal Stromal Cells

D. K. Matveeva[a], E. R. Andreeva[a,*], and L. B. Buravkova[a,b]

[a] *Institute of Biomedical Problems, Russian Academy of Sciences, Moscow, 123007 Russia*
[b] *Ecological and Extreme Medicine, Moscow State University, Moscow, 119234 Russia*
**e-mail: andreeva1564@gmail.com*

Received June 20, 2019; revised September 16, 2019; accepted September 23, 2019

Abstract—Currently, biological scaffolds composed of extracellular matrix (ECM) are being actively examined for the needs of regenerative medicine. ECM substrates are prepared by decellularization and used to deliver cells to damaged tissue. Native scaffolds of ECM have an advantage over bioengineered ones because ECM retains natural biologic cues that provide efficient reparative cell functions. Mesenchymal stromal cells (MSCs) have a multipotent potential of differentiation and secrete a wide range of bioactive molecules. In this regard, MSCs are valuable intermediaries for tissue repair. The ECM as a critical component of the MSCs niche modulates their functional activity, including migration, proliferation, and differentiation, and supports their potential for self-renewal. In vitro investigations would be useful in elucidation of how biological scaffolds can affect the reparative functions of MSCs. There are several different protocols for decellularization. Since ECM of various cell types differs qualitatively and quantitatively, these protocols should be optimized for each specific case. In the present study we compared the effectiveness of approach to prepare decellularized ECM (dcECM) of adipose-derived MSC (adMSC): Triton X-100/NH_4OH solution in phosphate buffered solution or H_2O, and the possibility of using dcECM after spheroids were formed. ECM-derived substrates were analyzed with immunocytochemistry and scanning electron microscopy. During long-term culture, MSCs produced a well-developed ECM, which maintained a structure close to the native one after treatment with phosphate buffered solution of Triton X-100/NH_4OH. It was impossible to receive a uniform dcECM layer, when water solution of Triton X-100/NH_4OH was used. On the scanning electron microscopy images single fiber of ECM were revealed in this case. Fragments of ECM and cells after spheroids formation with RGD peptides were detected. Therefore, this method was not effective for obtaining dcECM of adMSCs.

Keywords: human mesenchymal stromal cells, extracellular matrix, decellularization, regenerative medicine, immunocytochemistry, scanning electron microscopy.

DOI: 10.3103/S0096392519040096

Mesenchymal stromal cells (MSCs) have become the subject of extensive research as a promising source of cells for regenerative medicine and cell therapy due to their high proliferative activity, multilineage differentiation potential, and production of a wide range of biologically active molecules [1]. In vivo, stem/stromal cells occupy specific niches formed by various cellular and noncellular components [2, 3]. The extracellular matrix (ECM) is an essential component of the MSC microenvironment. The ECM molecules are involved directly or indirectly in the regulation of physiological and reparative activity of cells. The physical properties of the ECM, including its rigidity, topography, porosity, and insolubility, provide physical cues to the cells related to adhesion, migration, proliferation, and polarity [4]. Interactions between MSCs and ECM are bidirectional. Cells in response to signals from the microenvironment remodel the ECM by means of specific remodeling enzymes [5].

Some components of ECM are used actively in laboratory and clinical practice, but the cellular microenvironment has still not been reconstituted completely [6]. Decellularized ECM (dcECM) of native tissues or cultured cells is gaining popularity in regenerative medicine as a source of biological scaffolds for tissue repair [7]. The main goal of decellularization is to remove the cellular materials and antigens, such as DNA, to minimize the risk of destructive immune responses. At the same time, integrity, composition, and bioactivity of the prepared dcECM must be preserved. Compared to bioengineered scaffolds, natural ECM obtained by decellularization is biocompatible and the tissue architecture and biochemical properties are preserved, which may be crucial for maintaining

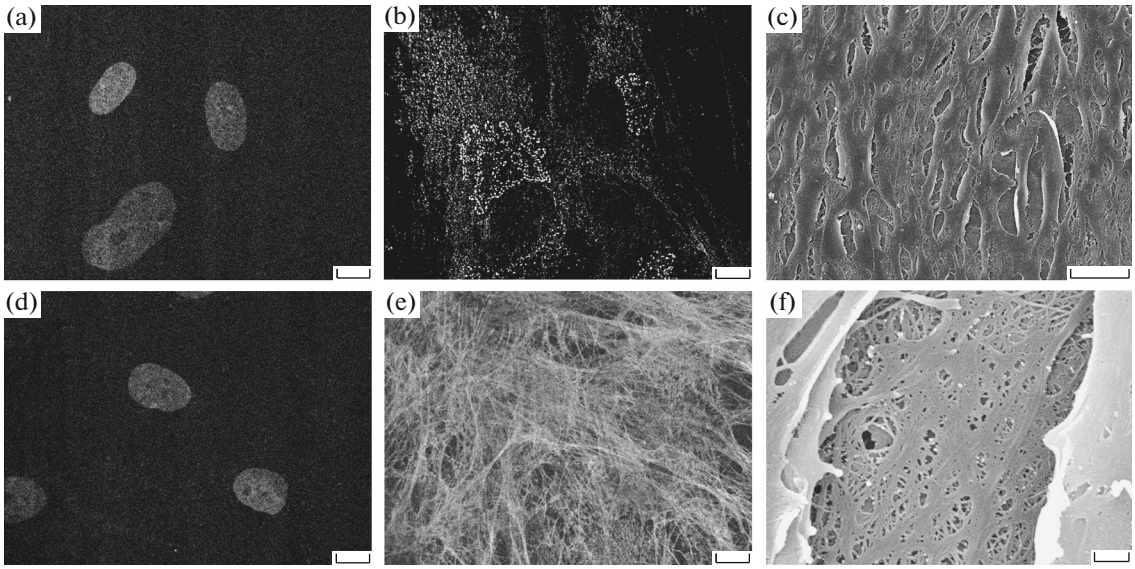

Fig. 1. Characterization of ECM produced by adMSCs over 14 days. (a, b) immuncytochemical detection of type I collagen; (d, e) immunocytochemical detection of fibronectin; (a, d) detection of nucleic acids using DAPI stain; (b, e) FITC-labeled antigens. Confocal microscopy LSM 780 Carl Zeiss. (c, f) Scanning electron microscopy (CamScanZ2). (c) Monolayer of adMSCs; (f) ECM in intercellular spaces. Bars (a, b, d, e) 20 μm, (c) 50 μm, (f) 1 μm.

cell viability and their participation in physiological and reparative remodeling [8].

The contribution of c cell types to the matrix's production and remodeling can be evaluated in vitro. MSCs, as key components of connective tissue, synthesize a sufficient amount of ECM with various fibrillar and soluble structural elements during cultivation [9]. The removal of cellular components by various agents preserves the matrix scaffold, which is usually dominated by fibrillar components, and the thickness and mechanical properties characteristic of the native tissue are retained [10].

There are various approaches for decellularization, and they use physical, chemical, and biological treatments: freeze–thaw cycles, protease and nuclease digestion, ultrasound, and detergent or acid extraction of cells [10].

The aim of this work was to select the optimal protocol for obtaining of dcECM of adMSCs and to characterize the morphology of the prepared dcECM.

MATERIALS AND METHODS

Cell culture. MSCs from the stromal-vascular fraction of human adipose tissue were cultured in α-MEM medium (Gibco, United Kingdom) with the addition of penicillin/streptomycin (100 u/mL and 100 mg/mL, respectively, PanEco, Russia) and fetal bovine serum (10%; HyClone, United States) in a CO_2-incubator (5% CO_2, 95% air; Sanyo, Japan). The cells were assessed for compliance with requirements for adMSCs: the ability of osteogenic and adipogenic differentiation, the expression of stromal cell markers CD73, CD90, CD105, and the lack of expression of the hematopoietic cell marker CD45 (data not shown). The seeding density was 3000–3500 cells/cm^2. The medium was replaced every 3–4 days. Cells of 3–5 passages were used in the experiments. To assess ECM production, adMSCs were seeded on culture plastic or cover glasses at high density (7500 cells/cm^2) and cultured for 14 days. To stimulate the production of the matrix components, 2-phospho-L-sodium ascorbate at a concentration of 50 μg/mL (Fluka, Germany) was added into the culture medium of cells in the premonolayer.

Decellularization methods. Cultured adMSCs were washed thrice with 20 mM phosphate buffered saline (PBS) (PanEco, Russia) to rid them of residual medium; dcECM substrates were prepared using the following approaches:

(1) Hypo-osmotic protocol: adMSCs were treated with hypoosmotic aqueous solution of 0.2% Triton X-100 (Ferac, Germany) for 5 min at room temperature and then 0.3% NH_4OH (Reakhim, Russia) at room temperature for 15 min [11].

(2) Iso-osmotic protocol: an iso-osmotic solution of 0.5% Triton X-100 in PBS (pH = 7.2) containing 20 mM NH_4OH at 37°C for 5 min was used [12]. Cell lysis in both cases was evaluated by phase contrast microscopy (Figs. 2a, 2e).

(3) RGD-protocol: dcECM scaffolds were prepared after spheroids were formed using a peptide containing the RGD-domain (Arg-Gly-Asp), i.e., cyclo-RGDfK (TPP) (RGD-peptide) (according to the protocol in [13]). After 14 days, the culture medium was

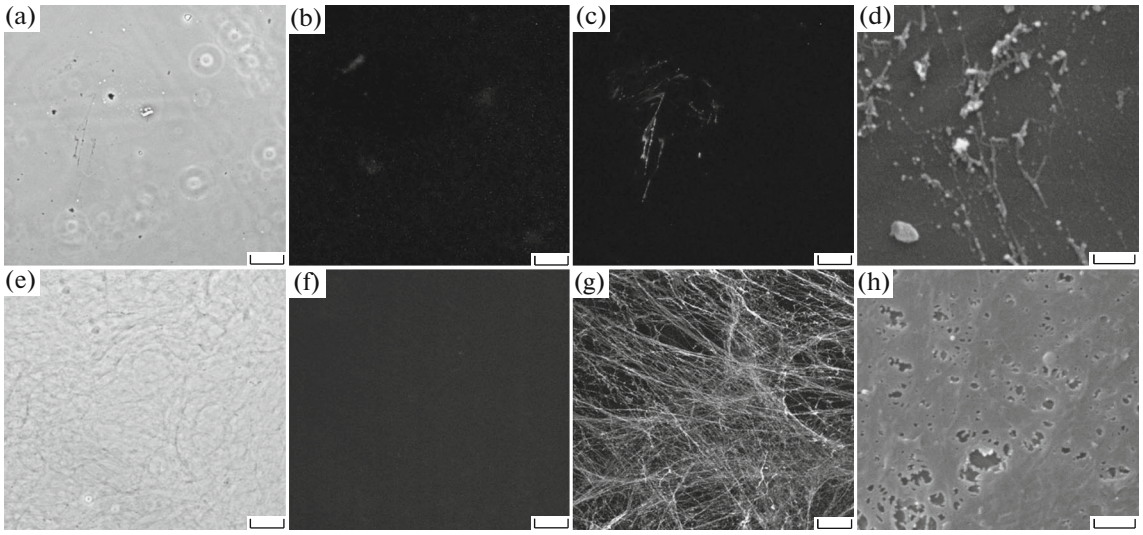

Fig. 2. Preparation of dcECM of adMSCs using Triton X-100 in combination with NH_4OH: (a–d) 0.2% Triton X-100 and 0.3% NH_4OH; (e–h) 0.5% Triton X-100 in PBS containing 20 мМ NH_4OH. (a, e) dcECM of adMSCs; (b, f) detection of DNA contamination with DAPI stain; (c, g) immunocytochemical detection of fibronectin in dcECM substrates (FITC); (a–c, e) Nikon Eclipse TiU (Nikon, Japan); (f, g) confocal microscopy LSM 780 Carl Zeiss; (d, h) visualization of dcECM with scanning electron microscopy (CamScanZ2). Scaling (a–c, e–g) 20 μm, (d, h) 1 μm.

replaced with a medium containing 80 mM of RGD peptide. After spheroids were formed (within 3–4 days), they were carefully removed with a pipette.

After decellularization, dcECM substrates were rinsed with PBS and used for analysis as described below.

Scanning electron microscopy (SEM). Cell or dcECM specimens were washed twice with PBS and fixed overnight with a mixture of 5% glutaraldehyde and 4% paraformaldehyde in a 1 : 1 ratio (both Merck, Germany) prepared with PBS. The specimens were dehydrated in increasing concentrations of ethanol (30, 40, 50, 60, 70, 80, 90, and 100%) followed by replacement of ethanol with acetone (Khimmed, Russia). The specimens were dried by a critical point drying technique, coated with gold-palladium and examined with the CamScanZ2a scanning electron microscope (Cambridge Instruments, United Kingdom). Microscopy was performed at the "General Faculty Laboratory of Electron Microscopy at Moscow State University" under the direction of G.N. Davidovich.

Immunocytochemistry. The major proteins of ECM were detected in cellular and decellularized specimens by indirect immunofluorescence staining. The cells were washed twice with PBS and fixed with 4% paraformaldehyde with 0.2% Triton X-100 at 37°C for 15 min (dcECM specimens were fixed without Triton X-100). After washing from the fixator in PBS, nonspecific binding of antibodies was blocked with 1% bovine serum albumin (BSA; Sigma, United States) for 15 min at room temperature. Next, the cells or dcECM were incubated with primary rabbit antibodies to type I collagen (5 μg/mL) and human fibronectin (20 μg/mL) (IMTEK, Russia). Instead of the primary antibodies, α-MEM with 10% FBS was used a negative control. For fluorescence, secondary antibodies were goat antirabbit labeled with fluorescein isothiocyanate (FITC) (10 μg/mL) (IMTEK, Russia). The first and second incubations were carried out for 30 min at room temperature in a humidified chamber. Between staining procedures, specimens were washed in a solution of PBS/BSA thrice for 5 min. The specimens were stained with Fluoroshield mounting medium containing DAPI nuclear dye (Sigma-Aldrich, United States). The specimens were analyzed using a Nikon Eclipse TiU fluorescent phase contrast microscope (Nikon, Japan) with a Nikon Intensilight C-HGFI lamp and an LSM 780 confocal microscope (Zeiss, Germany, in collaboration with Prof. S.V. Buravkov, MSU). Representative data from one of three independent experiments have been illustrated.

Image processing. SEM images were processed using ImageJ and Zeiss LSM Image Browser software. Brightness and contrast were adjusted on the images and fluorescence channels were merged.

RESULTS AND DISCUSSION

Immunocytochemical characterization of ECM proteins of adMSCs. Indirect immunocytochemical labelling was used to identify major collagen (collagen I) and noncollagen (fibronectin) proteins. Most of the structures stained positively using antibodies to collagen type I were localized intracellularly (Fig. 1a, b). Type I collagen is known to be secreted out of cells as procollagen molecules, and the final assembly of collagen fibrils occurs in the extracellular space [14, 15]; hence,

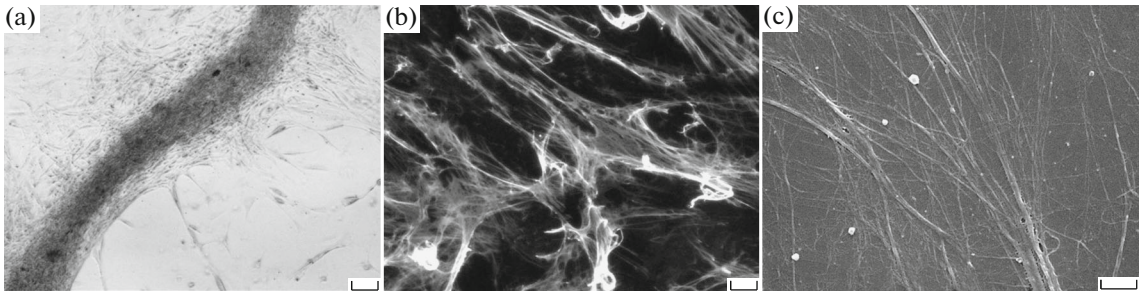

Fig. 3. Preparation of dcECM of adMSCs using RGD-peptide. (a) formation of spheroids, 96 h of incubation with the peptide; (b) immunocytochemical detection of fibronectin after removal of spheroids (FITC); (a, b) Nikon Eclipse TiU (Nikon, Japan); (c) visualization of dcECM with scanning electron microscopy (CamScanZ2). Scaling (a) 40 μm, (b) 20 μm, (c) 3 μm.

it is concluded that the intracellular staining revealed in this study corresponds to procollagen I molecules. Fluorescence microscopy showed that fibronectin is an abundant component in the extracellular space and forms a network of fine interconnected fibrils (Fig. 1d, e).

The ultrastructure of ECM of cultured adMSCs was investigated using SEM (Fig. 1c). ECM occupied the space under the cells and between the cells; individual fibrils were detected on the cell surface (Fig. 1f). ECM molecules typically formed a dense, membrane-like structure without clear separation into individual fibrils; regions with an interconnected network of ECM fibrils were present.

Preparation of dcECM scaffolds of adMSCs using the Triton X-100 as a detergent. Two modifications of the method using the Triton X-100 in combination with NH_4OH (hypo-osmotic aqueous solution and iso-osmotic solution in PBS) were used to obtain dcECM of adMSCs.

The protocol with hypo-osmotic solution (aqueous solution of 0.2% Triton X-100 and 0.3% NH_4OH) included two steps [11]. First, an aqueous solution of 0.2% Triton X-100 was added to a monolayer of adMSCs, and cell membrane lysis was evaluated by microscopy. The cell nuclei were visible. Subsequent incubation with NH_4OH resulted in the removal of the protein and nuclear components. However, immunocytochemistry (Fig. 2b) and SEM (Fig. 2c) of dcECM showed that ECM did not retain its structure; there were single fibrils and remnants of cell membranes. These results were reproduced in three independent experiments and, hence, the hypo-osmotic protocol was not subsequently used.

Uniform dcECM substrates were obtained using iso-osmotic solution of 0.5% Triton X-100 in PBS with 20 mM NH_4OH at 37°C. The ECM layer was clearly visible even in phase-contrast microscopy (Fig. 2e). Immunocytochemical assay revealed a dense network of fibronectin without DNA contamination, as evidenced by lack of DAPI-stained nuclear material (Fig. 2f). Similar results were obtained using mouse embryonic fibroblasts in the work of Harris et al. [16]. Collagen molecules were not detected in the composition of dcECM that was correlated to the data for cell specimens, when collagen type I was detected immunocytochemically inside of cells in the form of procollagen. SEM-analysis confirmed that the dcECM layer was uniform. The ECM after decellularization differed slightly from the picture observed for native specimens, but the structural features described for the intact cell monolayer were preserved (Fig. 2h).

In the classical protocol, after the use of lysing agents, dcECM scaffolds were additionally treated with DNase I to remove DNA contamination. However, some authors did not use this enzyme to prepare pure samples appropriate for subsequent recellularization [17–19]; hence, this step appears to be optional.

Preparation of dcECM scaffolds using RGD-peptide. In addition to the most commonly used decellularization protocols, we tested a method used to prepare multicellular spheroids. To do this, the adMSC monolayer was treated with RGD-peptide. The amino acid sequence of RGD (Arg-Gly-Asp) is found in domains of ECM proteins, such as collagen type I and fibronectin. This sequence is a ligand for integrin binding during cell adhesion [20]. Peptides dissolved in the medium are competitors for cell integrins, which contributes to the cell detachment from the substrate and the formation of multicellular spheroids [21, 22]. We assumed that dcECM will remain on the plasticware after removal of the formed spheroids.

We failed to obtain fully formed cell spheroids; the cells were detached as a whole layer (Fig. 3a). This was apparently happened as spheroid formation typically occurs over quite short cultivation time from a high initial density of cells that give rise to a monolayer very quickly (within a few days). Our protocol involved 14-day cultivation of adMSCs using ascorbate to enhance ECM production, resulting in the cells being "embedded" into the matrix. It is assumed that the hard attachment of adMSCs to the matrix prevented spheroid formation. In addition, this apparently contributed to the fact that no pure layer of dcECM remained after cell removal (Fig. 2g). A SEM image shows areas of cells that are attached to the sub-

strate with long processes. In cell-free spaces, single fine fibrils of the matrix and fragments of the fibronectin network were identified (Fig. 2h).

Thus, ECM proteins of adMSCs have been detected by immunocytochemistry and the ultrastructure of ECM in the intercellular space in the monolayer has been revealed. The most effective way to prepare dcECM of adMSCs is the use of iso-osmotic Triton X-100 in combination with NH_4OH, which allows obtaining a uniform layer of dcECM without contaminations. The data hold promise for further investigation of ECM influence on the functional activity of adMSCs and application of dcECM as a source of biocompatible coatings for scaffold fabrication.

FUNDING

The study was supported by Program of the Presidium of the Russian Academy of Sciences #19.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Parekkadan, B. and Milwid, J.M., Mesenchymal stem cells as therapeutics, *Annu. Rev. Biomed. Eng.,* 2010, vol. 12, pp. 87–117.
2. Schraufstatter, I.U., Discipio, R.G., and Khaldoyanidi, S., Mesenchymal stem cells and their microenvironment, *Front. Biosci.,* 2011, vol. 16, pp. 2271–2288.
3. Gattazzo, F., Urciuolo, A., and Bonaldo, P., Extracellular matrix: A dynamic microenvironment for stem cell niche, *Biochim. Biophys. Acta,* 2014, vol. 1840, no. 8, pp. 2506–2519.
4. Lu, H., Hoshiba, T., Kawazoe, N., and Chen, G., Autologous extracellular matrix scaffolds for tissue engineering, *Biomaterials,* 2011, vol. 32, no. 10, pp. 2489–2499.
5. Hynes, R.O., The extracellular matrix: Not just pretty fibrils, *Science,* 2010, vol. 326, no. 5957, pp. 1216–1219.
6. Joddar, B., Hoshiba, T., Chen, G., and Ito, Y., Stem cell culture using cell-derived substrates, *Biomater. Sci.,* 2014, vol. 2, no. 11, pp. 1595–1603.
7. Hoshiba, T., Lu, H., Kawazoe, N., and Chen, G., Decellularized matrices for tissue engineering, *Expert Opin. Biol. Ther.,* 2010, vol. 10, no. 12, pp. 1717–1728.
8. Nöth, U., Rackwitz, L., Steinert, A.F., and Tuan, R.S., Cell delivery therapeutics for musculoskeletal regeneration, *Adv. Drug. Deliv. Rev.,* 2010, vol. 62, nos 7–8, pp. 765–783.
9. Bornstein, P., Duksin, D., Balian, G., and Davidson, J.M., Organization of extracellular proteins on the connective tissue cell surface: relevance to cell-matrix interactions in vitro and in vivo, *Ann. N. Y. Acad. Sci.,* 1978, vol. 312, pp. 93–105.
10. Crapo, P.M., Gilbert, T.W., and Badylak, S.F., An overview of tissue and whole organ decellularization processes, *Biomaterials,* 2011, vol. 32, no. 12, pp. 3233–3243.
11. Harvey, A., Yen, T., and Aizman, I., Proteomic analysis of the extracellular matrix produced by mesenchymal stromal cells: Implications for cell therapy mechanism, *PLoS One,* 2013, vol. 8, no. 11.
12. Lin, H., Yang, G., Tan, J., and Tuan, R.S., Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential, *Biomaterials,* 2012, vol. 33, no. 18, pp. 4480–4489.
13. Akasov, R., Gileva, A., Zaytseva-Zotova, D., Burov, S., and Guedon, E., 3D in vitro co-culture models based on normal cells and tumor spheroids formed by cyclic RGD-peptide induced cell self-assembly, *Bioteshnol. Lett.,* 2016, vol. 39, no. 1, pp. 45–53.
14. Myllyharju, J. and Kivirikko, K.I., Collagens, modifying enzymes and their mutations in humans, flies and worms, *Trends Genet.,* 2004, vol. 20, no. 1, pp. 33–43.
15. Myllyharju, J., Prolyl-4-hydroxylases, the key enzymes of collagen biosynthesis, *Matrix Biol.,* 2003, vol. 22, no. 1, pp. 15–24.
16. Harris, G.M., Raitman, I., and Schwarzbauer, J.E., Cell-derived decellularized extracellular matrices, *Methods Cell Biol.,* 2018, vol. 2, no. 143, pp. 97–114.
17. He, F., Chen, X., and Pei, M., Reconstruction of an in vitro tissue-specific microenvironment to rejuvenate synovium-derived stem cells for cartilage tissue engineering, *Tissue Eng., Part A,* 2009, vol. 15, no. 12, pp. 3809–3821.
18. Sun, Y., Li, W., Lu, Z., Chen, R., Ling, J., Ran, Q., Jilka, R.L., and Chen, X.D., Rescuing replication and osteogenesis of aged mesenchymal stem cells by exposure to a young extracellular matrix, *FASEB J.,* 2011, vol. 25, no. 5, pp. 1474–1485.
19. Prewitz, M., Seib, F.P., von Bonin, M., Stißel, A., and Niehage, C., and Werner, C., Tightly anchored tissue-mimetic matrices as instructive stem cell microenvironments, *Nat. Methods,* 2013, vol. 10, no. 8, pp. 788–794.
20. Ruoslahti, E., RGD and other recognition sequences for integrins, *Annu. Rev. Cell Dev. Biol.,* 1996, vol. 12, pp. 697–715.
21. Zhang, L., Hum, M., Wang, M., Li, Y., Chen, H., Chu, C., and Jiang, H., Evaluation of modifying collagen matrix with RGD peptide through periodate oxidation, *J. Biomed. Mater. Res. A,* 2005, vol. 73, no. 4, pp. 468–475.
22. Lam, J. and Segura, T., The modulation of MSC integrin expression by RGD presentation, *Biomaterials,* 2013, vol. 34, no. 16, pp. 3938–3947.

Translated by M. Novikova