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Expression of RUNX2 and Osterix in Rat Mesenchymal Stem Cells during Culturing in Osteogenic-Conditioned Medium

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We studied the expression of transcription factors RUNX2 and Osterix after addition of a concentrate of osteogenic-conditioned medium to the culture medium for osteogenic differentiation of mesenchymal stem cells (MSC). The obtained concentrate of osteogenic-conditioned medium containing a complex of bioactive substances with a molecular weight >10 kDa provided MSC differentiation into osteoblasts, which was confirmed by high level of expression of transcription factors RUNX2 and Osterix in comparison with the negative control. The highest expression of transcription factor Osterix was revealed on day 14 of MSC culturing in the presence of osteogenic supplement StemPro (positive control) and the studied concentrate of osteogenic-conditioned medium.

Key Words: *mesenchymal stem cells; osteoinduction; differentiation; osteogenic-conditioned medium*

Mesenchymal stem cells (MSC) hold great promise for cell therapy, tissue engineering, and regenerative medicine. One of the modern tasks of regenerative medicine is creation of bone grafts based on populations of living cells and paracrine factors [15]. Tissue engineering *in situ*, a new strategy for repairing bone defects [6], requires both biomimetic cell carriers and osteoinductors for *in situ* technologies. Isolation and use of paracrine factors are general approaches in enhancing the regeneration process based on MSC therapy [11]. The most widely used paracrine factors exhibiting osteoinductive properties are bone morphogenetic protein-1 (BMP), fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), stromal cell factor 1 (SDF-1), *etc*. [14,16].

In parallel, the possibility of using osteogenic-conditioned media for induction of osteoblastic differentiation of MSC is studied. It was shown that osteogenic-conditioned medium from human periosteum-derived cells (hPDC) stimulated the expression of receptor activator of NF-κB ligand (RANKL) and promoted osteoclastogenesis [9]. Interesting results were demonstrated by a group of researchers demonstrating a decrease in the level of some cytokines, chemokines, and growth factors not exhibiting direct osteoinductive activity in the conditioned medium obtained after culturing of adipose tissue-derived stem cells (ASC) and stem cells from human exfoliated deciduous teeth (SHED) with osteoinductive supplement [8]. We believe that a concentrate osteogenic-conditioned medium obtained from MSC after exposure to generally accepted combination of substances with osteoinductive activity can be used as osteoinductive supplement [13]. To verify this hypothesis, it was necessary to study the expression of transcription factors RUNX2 and Osterix, attesting to osteogenic differentiation of MSC. It is known that RUNX2 is required for direct differentiation of MSC towards osteoblasts lineage cells and maintains cells in the immature stage (preostoblasts), while accumulation of Osterix promotes maturation of preosteoblasts and their differentiation into osteoblasts [5,7].

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Our aim was to study the expression of transcription factors RUNX2 and Osterix upon addition of osteogenic-conditioned medium concentrate to the culture medium to differentiate MSC towards the osteogenic direction.

MATERIALS AND METHODS

The experiments were carried out on culture of MSC obtained from Wistar rats (vivarium of Research Institute of Pharmacology of Living Systems, Belgorod National Research University). All manipulations were carried out in accordance with GOST ISO 10993-2:2006 and Directive 86/609/EEC On the Protection of Animals used for Scientific Purposes.

MSC were isolated from red bone marrow using commonly accepted method [2] and were seeded in 25-cm^2 flasks; the cells were expanded in complete culture medium (CCM) based on DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (HyClone) in a CO_2 incubator MCO-5AC (Panasonic) at 37°C, 100% humidity, and 5% CO_2. After obtaining the required number of cells for *in vitro* experiments, the supernatant was removed from the flask, 5 ml 0.05% trypsin solution with EDTA (Capricorn Scientific) was added to remove and subculture the cells. At this stage, rat MSC (2×10^6 cells per ml) were phenotyped (detection of positive expression of CD90 (MA1-81572, Thermo Fisher Scientific) and CD29 (17-0291-80e, BD Bioscience) and negative expression of CD45 (12-0461-80 Invitrogen), CD31 (25-0310-80 BD Bioscience)) by flow cytometry using BD FACSCanto II and Software FACS Diva 8) [3] using facilities of industrial partner Innovation Center Biruch-NT.

The concentrate of osteogenic-conditioned medium (cOCM) was obtained by culturing rat MSC in DMEM/F-12 growth medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (HyClone) at 5% CO_2, 37°C, in a humid atmosphere. After obtaining sufficient number of cells, the nutrient medium was removed, the cell monolayer was washed once with PBS. To stimulate osteogenic differentiation of MSC, CCM containing an osteoinductive supplement: dexamethasone (100 nM), ascorbic acid (50 μg/ml), and sodium β-glycerophosphate (10 mM) was used [12]. After a certain period of time, the osteoinductive medium was removed, the cell monolayer was washed with PBS, and the same amount of serum-free DMEM/F-12 growth medium without supplements was added. After culturing, a conditioned medium was collected and centrifuged at 3000 rpm for 10 min. The supernatant was passed through a 0.22-μ filter and concentrated by tangential flow ultrafiltration using a Vivaflow 200 system (Sartorius) that allows working with sample volumes from 0.1 liter to 1 liter in one step. For this, membrane modules with a cut-off threshold for molecules with a molecular weight of 10 kDa were used, because the main pool of MSC secretome proteins lies above this value [10]. Diafiltration of cOCM was also carried out using this system according to manufacturer's recommendations. Purified from salts cOCM was dried in a Rotavapor R-210 vacuum rotary evaporator (Buchi). Dried cOCM was collected from a round bottom reaction flask using a spatula into a microcentrifuge tube with observance of aseptic principles and stored in a freezer at -20°C.

The supplement StemPro Osteogenesis Differentiation kit (Life Technologies) to CCM was used as a positive control of osteoinductivity; CCM of standard composition without osteoinductive supplements was used as a negative control of osteoinductivity.

Prior to collection of osteogenic-conditioned medium, osteogenic differentiation of MSC was confirmed by alkaline phosphatase staining [1]. To this end, MSC were washed in PBS and fixed with 4% paraformaldehyde for 2 min. The fixed cells were washed with 1 ml wash PBS with 0.05% Tween-20 and incubated with BCIP/NBT substrate (Sigma-Aldrich) at room temperature for 10 min. After washing, alkaline phosphatase staining in MSC was detected under an Eclipse Ti-U microscope (Nikon) and its intensity (in arb. units) in comparison with the negative control MSC was evaluated using specialized Nikon EZ-C1 FreeViewer software (Nikon).

The obtained rat MSC culture was resuspended and seeded to 35-mm Petri dishes (SPL Lifesciences) in a volume of 200 μl (concentration 10^6 cells/ml). After 30-min incubation of MSC in a CO_2 incubator at 37°C, 100% humidity, and 5% CO_2, 2 ml of CCM with osteogenic-conditioned medium at a ratio of 1:50 was added and the cells were cultured in a CO_2 incubator at 37°C, 100% humidity, and 5% CO_2.

On days 7 and 14, the expression of transcription factors RUNX2 and Osterix was studied in rat MSC culture [4] by the immunofluorescence method. The following reagents were used to identify transcription factors RUNX2 and Osterix in MSC: primary antibodies to RUNX2 (ab76956; 1:50; Abcam) with Goat Anti-Mouse IgG H&L secondary antibodies (Alexa Fluor 488) (ab150113, 1:1000; Abcam) and primary antibodies to Osterix/Sp7 (ab22552, 1:500; Abcam) with Goat Anti-Rabbit IgG secondary antibodies H&L (Alexa Fluor 594) (ab150080, 1:1000; Abcam). All initial work with antibodies was carried out according to manufacturer's instructions. Prior to addition of antibodies, the culture medium was completely removed, 2 ml PBS was added, and shaken for 1 min. Then, PBS was completely removed, the cells were fixed with 10% formalin for 30 min, washed with PBS, and

permeabilized in 0.1% Triton X-100 in PBS for 15 min at room temperature. After permeabilization, the cells were washed twice in PBS and incubated with 1% solution BSA in PBS was for 30 min. Then, the cells were again washed twice with PBS and primary anti-RUNX2 or anti-Osterix antibodies in PBS (150 µl) were applied to the sample (directly to the bottom of a 35 mm Petri dish) and incubated for 1 h at room temperature. Then, the samples were washed twice with PBS and incubated with secondary antibodies in 150 µl PBS for 1 h at room temperature in the dark. After double washing with PBS, cell fluorescence was recorded using a Digital Eclipse C1 Plus confocal laser scanning microscope (Nikon). The fluorescence intensity of each cell and the number of cells in 10 fields of view were analysed. The proportion of RUNX2$^+$ and Osterix$^+$ cells was calculated by the formula: P (%)= B/A×100%, where P (%) is proportion of RUNX2$^+$ and Osterix$^+$ cells, A is the total number of cells per field of view, and B is the number of RUNX2$^+$ or Osterix$^+$ cells.

Statistical processing of the results was carried out using Statistica 6.0 software (StatSoft, Inc.) using Student's *t* test and presented as *M*±*SD*. The differences were significant at $p \leq 0.01$.

RESULTS

Flow cytometry showed that the studied cells expressed CD29 (98.8%), CD90 (98.8%), CD45 (1.7%), and CD31 (0.3%), which allowed classifying them as MSC (Fig. 1). Analysis of the expression of alkaline phosphatase in MSC in CCM with osteoinductive supplement (dexamethasone, ascorbic acid, and sodium β-glycerophosphate) confirmed osteogenic differentiation of cells: 150.0±5.5 arb. units (*vs* 2.0±1.5 arb. units in negative control; $p \leq 0.01$).

Immunofluorescence analysis showed that the proportion of RUNX2$^+$ MSC on day 7 of culturing both in the positive control and in the presence of cOCM was higher than in the negative control. On day 7, the proportion of RUNX2$^+$ MSC was 57.54% (83.60±18.99 cells) in the negative control and 100% in the positive control (123.90±19.12 cells); in the presence of cOCM, the proportion of RUNX2$^+$ MSC was also 100% (126.30±18.42 cells). The intensity of fluorescence of RUNX2$^+$ MSC in the positive control was 929.67±171.65 arb. units; in the presence of cOCM, this parameter was 496.10±114.94 arb. units, which exceeded more than 11 times the fluorescence intensity in the negative control (36.56±21.65 arb. units; $p \leq 0.01$). These findings confirm osteoinductive properties of cOCM.

On 14 day of MSC culturing, a decrease in the proportion of RUNX2$^+$ MSC to 51.69% (68.70±14.25 cells) was observed in cultures with cOCM, while in positive and negative controls, these indicators did not change (Figs. 2, 3). The proportion of RUNX2$^+$ MSC in the negative control was 54.70% (68.60±18.98 cells) and in the positive control 100% (127.30±19.76 cells). Interesting, that the intensity of RUNX2 fluorescence in all MSC decreased by day 14 of culturing to 696.33±170.33 arb. units in the positive control, to 302.77±122.92 arb. units in MSC cultured with cOCM, and to 33.90±17.21 arb. units in the negative control ($p \leq 0.01$).

Analysis of the expression of Osterix in MSC revealed no Osterix fluorescence in the negative control on days 7 and 14. Osterix fluorescence was also absent on day 7 in MSC cultured in the presence of cOCM. On day 7 the fluorescence of Osterix$^+$ MSC were detected in the positive control: 51.68% (64.70±17.06

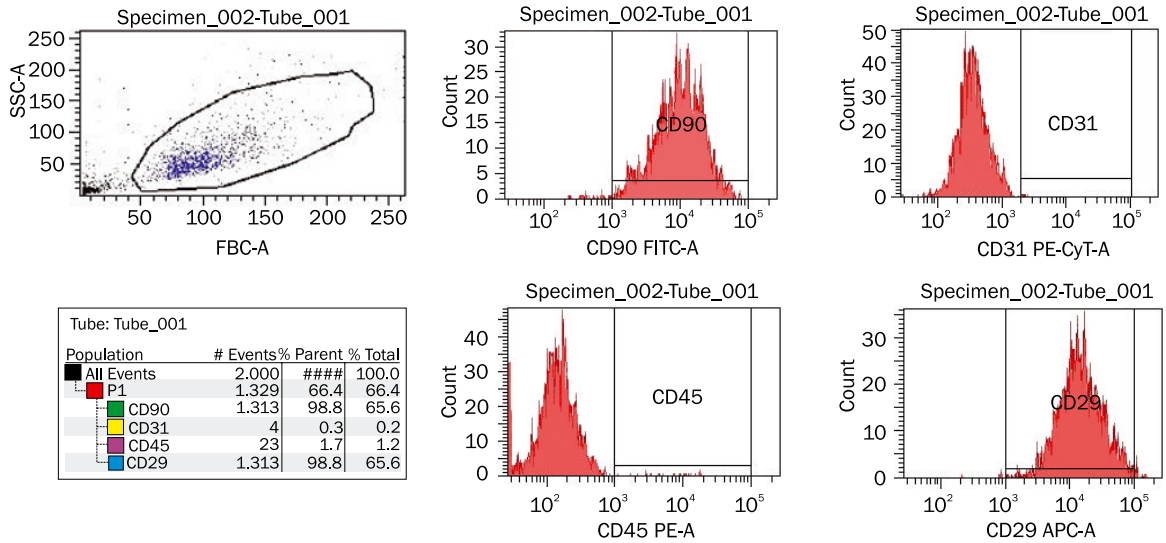

Fig. 1. Expression of CD90, CD31, CD45, and CD29 on the surface of MSC isolated from rat red bone marrow.

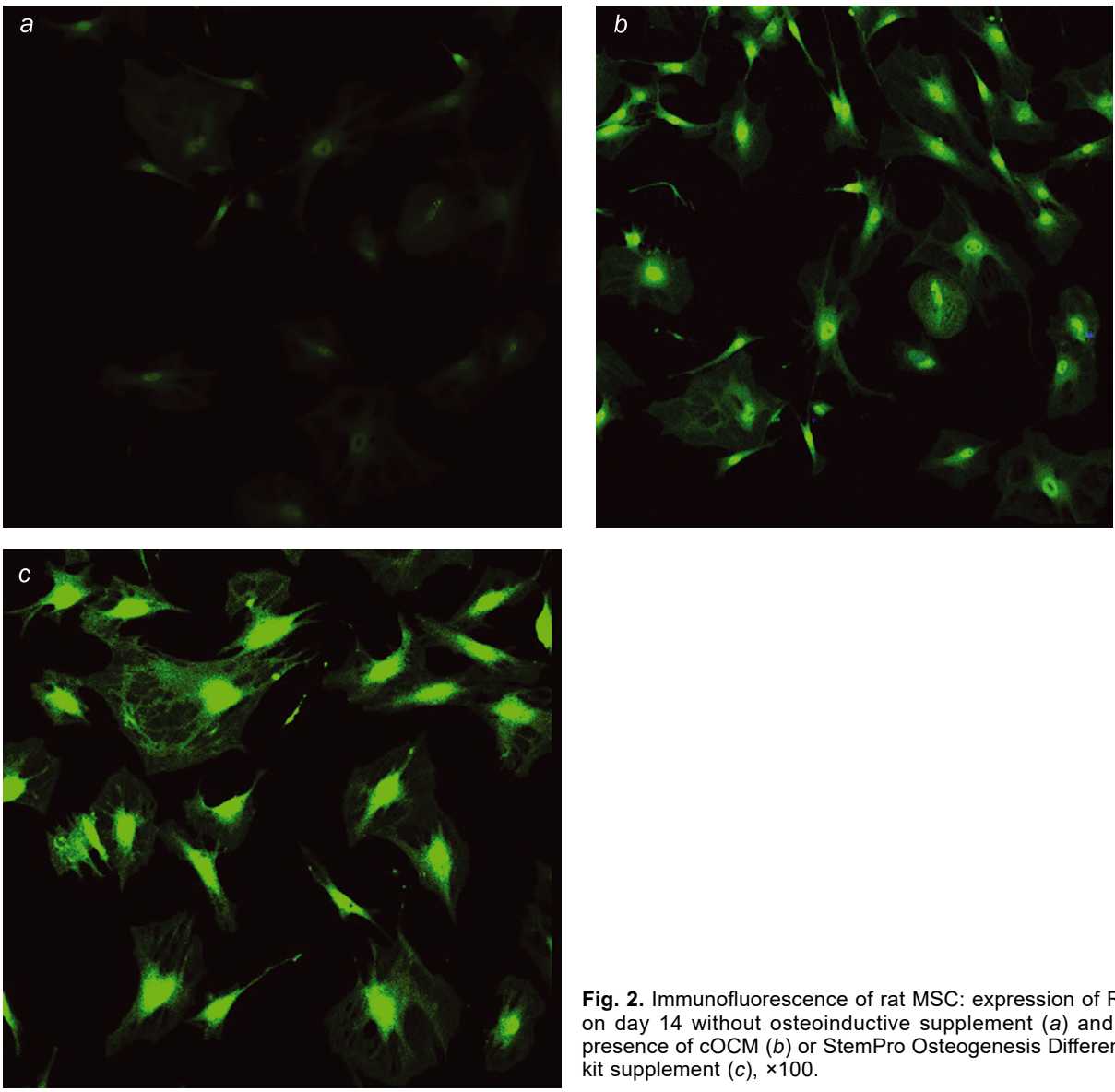

Fig. 2. Immunofluorescence of rat MSC: expression of RUNX2 on day 14 without osteoinductive supplement (*a*) and in the presence of cOCM (*b*) or StemPro Osteogenesis Differentiation kit supplement (*c*), ×100.

cells); fluorescence intensity 139.33±67.28 arb. units. On day 14, Osterix$^+$ MSC were detected both in the positive control and in cultures incubated with cOCM. In the positive control, the proportion of fluorescent cells was 100% (124.80±19.35 cells), while in the presence of cOCM, the proportion of fluorescent cells was 43.91% (62.60±15.09 cells). The Osterix fluorescence intensity in MSC was maximum in the positive control (456.10±162.98 arb. units) and lower in MSC cultured with cOCM (319.67±166.33 arb. units).

The expression of RUNX2 transcription factor at the level of background values in the negative control MSC (without osteoinductive factors) could be explained by the fact that MSC were passaged only several times and retained their multipotency. In the course of MSC differentiation towards osteogenic lineage cells, the expression of transcription factor RUNX2 decreased and expression of transcription factor Osterix increased. The highest expression of Osterix transcription factor was observed on day 14 of culturing both in the positive control with osteogenic StemPro supplement and in the presence of cultured the studied cOCM.

Thus, the concentrate of osteogenic-conditioned medium containing a complex of bioactive substances with a molecular weight >10 kDa stimulates differentiation of MSC into osteoblasts, which is confirmed by high expression of transcription factors RUNX2 and Osterix in comparison with the negative control. These findings indicate the possibility of obtaining of cOCM with a complex of paracrine factors of directed activity.

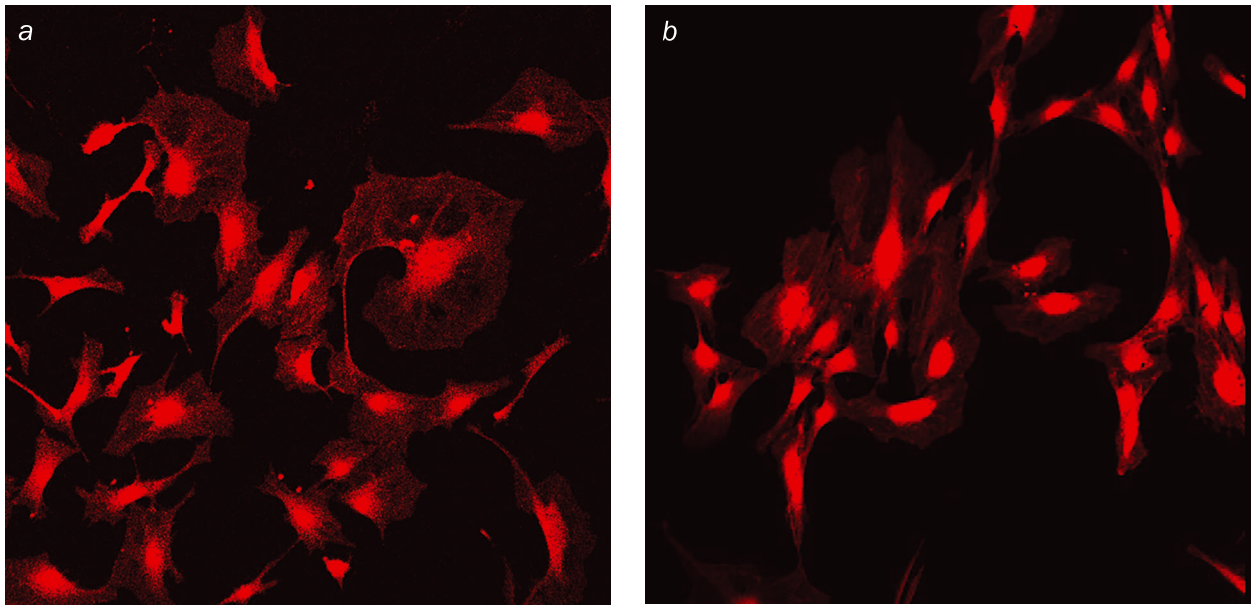

Fig. 2. Immunofluorescence of rat MSC: expression of Osterix on day 14 in the presence of cOCM (*b*) and StemPro Osteogenesis Differentiation kit supplement (*c*), ×100.

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REFERENCES

1. Bao X, Li Z, Liu H, Feng K, Yin F, Li H, Qin J. Stimulation of chondrocytes and chondroinduced mesenchymal stem cells by osteoinduced mesenchymal stem cells under a fluid flow stimulus on an integrated microfluidic device. Mol. Med. Rep. 2018;17(2):2277-2288.
2. Donzelli E, Salvadè A, Mimo P, Viganò M, Morrone M, Papagna R, Carini F, Zaopo A, Miloso M, Baldoni M, Tredici G. Mesenchymal stem cells cultured on a collagen scaffold: In vitro osteogenic differentiation. Arch. Oral. Biol. 2007;52(1):64-73.
3. Fitzpatrick EB, Dehart MJ, Brown TA, Salgar SK. Mesenchymal stem cell therapy to promote limb transplant functional recovery. Microsurgery. 2016;37(3):222-234.
4. He G, Chen J, Huang D. miR-877-3p promotes TGF-β1-induced osteoblast differentiation of MC3T3-E1 cells by targeting Smad7. Exp. Ther. Med. 2019;18(1):312-319.
5. Kanatani N, Fujita T, Fukuyama R, Liu W, Yoshida CA, Moriishi T, Yamana K, Miyazaki T, Toyosawa S, Komori T. Cbf beta regulates Runx2 function isoform-dependently in postnatal bone development. Dev. Biol. 2006;296(1):48-61.
6. Khaled EG, Saleh M, Hindocha S, Griffin M, Khan WS. Tissue engineering for bone production- stem cells, gene therapy and scaffolds. Open Orthop. J. 2011;5(Suppl. 2):289-295.
7. Komori T. Regulation of osteoblast differentiation by transcription factors. J. Cell. Biochem. 2006;99(5):1233-1239.
8. Mussano F, Genova T, Petrillo S, Roato I, Ferracini R, Munaron L. Osteogenic differentiation modulates the cytokine, chemokine, and growth factor profile of ASCs and SHED. Int. J. Med. Sci. 2018;19(5). pii: E1454. doi: 10.3390/ijms19051454
9. Park HC, Son YB, Lee SL, Rho GJ, Kang YH, Park BW, Byun SH, Hwang SC, Cho IA, Cho YC, Sung IY, Woo DK, Byun JH. Effects of osteogenic-conditioned medium from human periosteum-derived cells on osteoclast differentiation. Int. J. Med. Sci. 2017;14(13):1389-1401.
10. Park YJ, Koh J, Gauna AE, Chen S, Cha S. Identification of regulatory factors for mesenchymal stem cell-derived salivary epithelial cells in a co-culture system. PLoS One. 2014;9(11). e112158. doi: 10.1371/journal.pone.0112158
11. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-147.
12. Pittenger MF, Mbalaviele G. Black M, Mosca JD, Marshak DR. Mesenchymal stem cells. Human cell culture. Vol. V. Primary Mesenchymal Cells. Koller MR, Palsson BO. eds. Kluwer Academic Publishers. 2001. P. 189-207.
13. Scuteri A, Donzelli E, Foudah D, Caldara C, Redondo J, D'Amico G, Tredici G, Miloso M. Mesengenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells. Int. J. Stem Cells. 2014;7(2):127-134.
14. Sobacchi C, Palagano E, Villa A, Menale C. Soluble factors on stage to direct mesenchymal stem cells fate. Front. Bioeng. Biotechnol. 2017;5. ID 32. doi: 10.3389/fbioe.2017.00032
15. Vanden Berg-Foels WS. In situ tissue regeneration: chemoattractants for endogenous stem cell recruitment. Tissue Eng. Part B. Rev. 2014;20(1):28-39.
16. Wang R, Liu W, Du M, Yang C, Li X, Yang P. The differential effect of basic fibroblast growth factor and stromal cell-derived factor-1 pretreatment on bone morrow mesenchymal stem cells osteogenic differentiation potency. Mol. Med. Rep. 2018;17(3):3715-3721.