# Morphofunctional Status and Osteogenic Differentiation Potential of Human Mesenchymal Stromal Precursor Cells during *In Vitro* Modeling of Microgravity Effects

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We studied the effects of long-term (20-day) simulated microgravity (clinostatic exposure) and osteogenic differentiation stimuli on cultured mesenchymal stromal precursor cells isolated from human bone marrow. Clinostatic exposure significantly reduced proliferative activity of mesenchymal stem cells in comparison with the static and dynamic control, increased the number of large flat cells in the culture, and stimulated migration activity of cells. Phenotypic studies of surface antigens (CD90, CD54, CD106, CD105, CD34, CD45, class 1 HLA) during clinostatic exposure of mesenchymal stem cell cultures showed differences in their expression between experimental and control groups. Studies of osteogenesis of precursor cell showed that cell differentiation potential can be directed towards osteogenesis by a combination of clinostatic exposure and differentiation stimuli. The results confirm gravity sensitivity of human bone marrow precursor cells and open new vistas for understanding of the mechanisms of bone tissue loss in humans under conditions of space mission.

**Key Words:** *human mesenchymal stem cells; clinostatic exposure; proliferation; immunophenotypic characteristics; osteogenesis*

Space missions, hypokinesia and hypodynamia, aging, and some diseases impair homeostasis of the bone tissue, which leads to a decrease in its differentiation [1,2,10]. Several hypotheses were put forward on the causes of bone tissue loss in humans under conditions of space mission. A possible explanation is negative effect of microgravity on precursor cells leading to changes in their function and capacity to differentiate into components of the locomotor system, which can become a cause of impaired bone tissue remodeling under conditions of reduced locomotor load [1]. Mesenchymal stem cells (MSC) are a unique population of multipotent bone marrow stromal precursor cells capable of long proliferation and differentiation *in vivo* and *in vitro* at least into cells of mesenchymal origin, such as osteocytes, chondrocytes, adipocytes, and cells of hemopoiesis-maintaining stroma [9,13]. The MSC capacity to differentiate into tissue cells derivative from the mesenchyma underlies their regenerative and reparative potential. Some data indicate that MSC *in vitro* respond to reduced or increased mechanical stimulation by modification of their morphology, function, and differentiation potential [5, 11,12,15-18]. Culturing of human bone marrow MSC under conditions of a monoaxial cyclic mechanical stimulation for 14 days led to increased expression of type 1 collagen and osteonectin (re-

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gistered by increased immunofluorescence) and to decreased expression of CD90 [16]. Similar results were obtained for types 1 and 3 collagens and for fibronectin in another study [5]. Human MSC isolated from the fatty tissue during exposure to a pulsed liquid flow demonstrated a response similar to that characteristic of osteocytes, which manifested in increased production of NO and expression of *COX-2* gene [15]. Culturing of human bone marrow MSC for 7 days in a rotation bioreactor (NASA design) simulating the effects of microgravity led to a reduction of directed osteogenesis, because the cells did not express alkaline phosphatase, osteonectin, and collagen-1 mRNA, and *RANX2* early transcription factor essential for osteogenic differentiation of MSC [12,18]. Activation of *PPAR2* (early transcription factor of adipogenesis) expression was noted, despite cell culturing in the medium with osteogenic inductors; proliferative activity of cells did not decrease. It was shown that 7-day culturing of human MSC under conditions of randomized position of gravity vector of the culture in a 3D clinostat modulated the cell capacity to chondrogenic differentiation, because expression of mRNA for chondrogenesis markers aggrecan and collagen-2 decreased to an undetectable level [17]. This was paralleled by reduction in the counts of double-positive cells with CD44/CD29 and CD44/CD90 phenotypes and in their proliferative activity. Hence, these data confirm mechanosensitivity of human MSC, but these reports describe the effects of only short-term exposure, though it is known that, for example, osteogenic differentiation of MSC is a long-term process.

We studied the effect of long-term (20 days) clinostatic exposure on the proliferation, immunophenotype, and osteogenesis of MSC *in vitro*.

## MATERIALS AND METHODS

The cells were cultured in DMEM with 1 g/liter glucose (Biolot), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES buffer, 1 mM sodium pyruvate (all reagents from Gibco), and 10% FCS (HyClone) [11] in 25- and 75-cm$^2$ culture flasks (Nunc). The cells were washed from the medium in balanced Hanks' solution and harvested with 0.025% trypsin and 0.05% EDTA (Gibco). The medium was replaced every 2-3 days. Reinoculation in 1:3 proportion was carried out after the culture reached 90% confluence (every 7-10 days). Cells of passages 3-6 were used in experiments.

Microgravity was simulated by the method of clinostatic exposure consisting in the following: the gravity vector was randomized around the axis of cell culture horizontal rotation at a permanent velocity (2D clinostatic control) [3,11]. This experiment simulated the effect of cell culture gravity reduction. Mesenchymal stem cells cultured in a static horizontal position (static control) or on a shaker at 60 rpm (dynamic control for analysis of the impact of constant stirring of culture medium during clinostatic exposure) served as controls.

**TABLE 1.** Human MSC Phenotype during Different Passages after 20-Day Clinostatic Exposure and Culturing in Medium with Osteogenic Inductors

| Passage, group | | CD34 | CD45 | CD54 (ICAM-1) | CD106 (VCAM-1) | CD105 (endoglin) | CD90 (Thy-1) | Class 1 HLA |
|---|---|---|---|---|---|---|---|---|
| Passage 3 | SC | 0.73 | 3.04 | 1.7 | 88.3 | 98.6 | 85.7 | 99 |
| | $SC_{ost}$ | 0.09 | 4.07 | 35.9 | 98.1 | 99.9 | 96.6 | 99.5 |
| | CL | 0.75 | 0.43 | 17.9 | 84 | 99.3 | 90.7 | 99.4 |
| | $CL_{ost}$ | 0.43 | 11.6 | 66.2 | 99.6 | 99.9 | 98 | 99.5 |
| Passage 5 | SC | 0.34 | 1.08 | 2.3 | 60.6 | 96.3 | 89 | 87.6 |
| | $SC_{ost}$ | 0.17 | 2.5 | 3.3 | 72.4 | 95.1 | 91.1 | 80.0 |
| | CL | 0.42 | 3.02 | 2.4 | 63.5 | 90.3 | 85.2 | 76.3 |
| | $CL_{ost}$ | 0.27 | 0.96 | 2.6 | 46.9 | 93.6 | 82 | 74.3 |
| Passage 6 | SC | 0.20 | 4.4 | 11.3 | 83.4 | 96.9 | 77.3 | 80.9 |
| | $SC_{ost}$ | 0.47 | 0.20 | 13.4 | 95.1 | 99.6 | 87 | 94.4 |
| | CL | 0.11 | 1.6 | 2.5 | 84 | 99.3 | 77.1 | 76.9 |
| | $CL_{ost}$ | 0.02 | 0.38 | 2.4 | 74.4 | 97.7 | 75.8 | 70.0 |

**Note.** SC: static control; $SC_{ost}$: static control in osteogenic medium; CL: clinostatic exposure; $CL_{ost}$: clinostatic exposure in osteogenic medium.

Experimental MSC cultures were placed on a clinostat at rotation velocity of 6 rpm and cultured in a thermostat at 37°C. Three experimental series on different passages, each repeated several times, were carried out.

Dexamethasone ($10^{-8}$ M), 10 mM glycerol-2-phosphate, and 0.2 mM 2-phospho-L-ascorbic acid (Sigma) were added into growth DMEM for directed osteogenic differentiation [13]. Control cells were cultured in standard DMEM. The cells were inoculated in 24 $cm^2$ flasks (Nunc) at a density of $2.5\times10^3$ cell/$cm^2$ (in some cases $5\times10^3$ cell/$cm^2$) 24 h before the experiment. Before clinostatic exposure, the flasks were completely filled with appropriate medium (normal or osteogenic) and hermetically sealed with elastic film. Osteogenesis efficiency was evaluated after 20 days by the increment in alkaline phosphatase activity assessed by the naphthol method (86-C kit, Sigma) according to manufacturer's instruction and by mineralization of the extracellular matrix assessed by histochemical detection of Ca salts in cultures with 40 mM alizarine red S, pH 4.2 (Sigma).

Proliferative activity of cells in experimental and control cultures was evaluated during the first 3 days of the experiment by counting the cells every 24 h in 8-9 fixed visual fields chosen at random under an Axiovert 25 (Zeiss) microscope with an AxioCam HRm digital videocamera and Sigma Scan Pro 5 image analyzer software (Sigma).

Immunophenotypic characterization of MSC was carried out using an Epics XL cytofluorometer (Beckman Coulter) and mouse monoclonal antibodies to surface antigens CD54-FITC, CD106, CD90-FITC, CD105, HLA ABC-FITC, CD34-FITC, and CD45-PE (Immunotech Coulter). Fluorescein isothiocyanate-conjugated $IgG_1$ (Sigma) served as the second antibodies; an appropriate isotypical (negative) control was used. A total of $5\times10^3$ events were analyzed for each sample.

The significance of differences was evaluated using Microsoft Excel software and nonparametric Mann—Whitney test.

## RESULTS

Clinostatic exposure significantly inhibited proliferative activity of precursor cells cultured in common medium and in medium with osteogenic differentiation inductors in comparison with the static and dynamic controls (Fig. 1, *a*, *b*; $p<0.01$). The MSC population in control cultures increased 2-fold every 24 after the 24-h lag phase; the growth of small fibroblast-like rapidly dividing cells, described as RS cells (rapidly self-renewing or recycling stem cells) [6] was activated. Control cultures reached the confluent state on days 5-6, while during clinostatic exposure the number of cells in separate visual fields negligibly decreased because of their separation from the substrate and/or migration and redistribution on the underlayer during the first 24 h; the cells were visually larger and flat. The MSC cultures exposed to clinostatic treatment did not reach complete confluence over the entire observation period (1 week). Proliferative activity of MSC was 2.5-6.5 times lower in comparison with the control (depending on the passage). The reduction of proliferation rate in clinostatic exposure was the greatest for MSC of passages 5-6 and the least in passage 3 cells.

These data are in line with our previous findings [11], but disagree with other data indicating that proliferative activity of human MSC *in vitro* exposed to microgravity did not change or in-

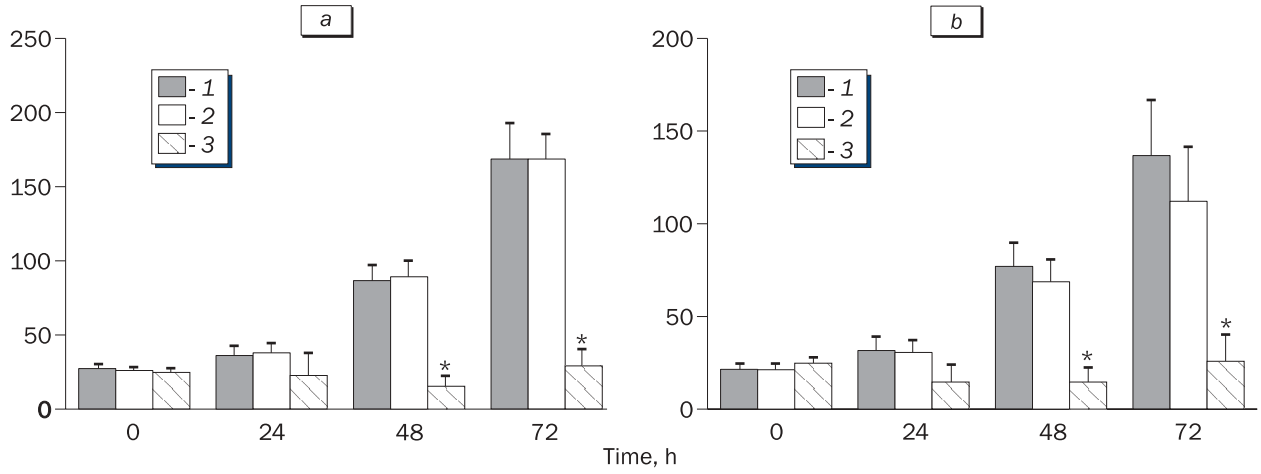


**Fig. 1.** Proliferative activity of passage 6 MSC during clinostatic exposure in normal (*a*) and osteogenic (*b*) medium. Ordinate: number of forming cells. *1*) static control; *2*) dynamic control; *3*) clinostatic exposure. *$p<0.01$ compared to static and dynamic control.

creased [17,18]. The differences can be explained by the use of different simulation systems and methods for evaluation of proliferative activity, as well as by different functional status of MSC (donor, passage, culturing conditions).

Intercellular adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM-1), a regulatory element for TGF-β family receptors (endoglin), Thy-1 (an immunoglobulin superfamily member, according to some data essential for the formation of hemopoietic cell colonies), and class 1 HLA participating in immune reactions are present on the MSC membrane [4,7,8]. In our study the cells expressed differentiation clusters (CD) CD90 (Thy-1), CD105 (endoglin), CD54 (ICAM-1), CD106 (VCAM-1), and class 1 HLA and did not express CD34 (hemopoietic stem cell marker). The expression of CD45 panleukocytic antigen was below 5%, which attested to high purity of MSC population (absence of hemopoietic cells.

Immunophenotypic analysis of cultures showed that osteogenic induction selectively (not for all passages) changed the expression of surface antigens characteristic of MSC during clinostatic exposure (Table 1). In passage 3 MSC, the expression of CD54 adhesion molecule significantly increased during clinostatic exposure and expression of CD45 panleukocytic marker significantly increased in osteogenic medium during clinostatic exposure. After 20 days of culturing, different CD patterns were detected in passages 5-6 control cultures growing in normal and osteogenic growth media, while the corresponding cultures exposed to clinostatic control had similar phenotype. The expression of class 1 HLA, CD90, CD54, and CD106 somewhat decreased (by 10-25%) after clinostatic exposure of passage 6 MSC in osteogenic medium in comparison with the corresponding static control. A similar regularity was characteristic of passage 5 cells, but only for CD106 and CD90.

Hence, changed expression of precursor cell differentiation clusters can indicate modification of the function of MSC cultured in osteogenic medium during clinostatic exposure. It is known that osteoclasts are involved in normal remodeling of bone tissue at the expense of its resorption; osteoclast precursors descend from hemopoietic stem cells of the granulocyte/macrophage origin; the growth, maturing, and activity of osteoclasts are regulated by cytokines and receptor molecules released by osteoblasts and, presumably, by osteogenic precursor cells [10,14]. Reduced expression of class 1 HLA, Thy-1, and VCAM-1 in the culture during clinostatic exposure can indicate changed immunological status of osteogenic precursor cells, the possibility of "recognition" of MSC and their more committed "descendants" by immune cells, as well as their interactions with hemopoietic progenitors. Increased expression of CD45 panleuko-

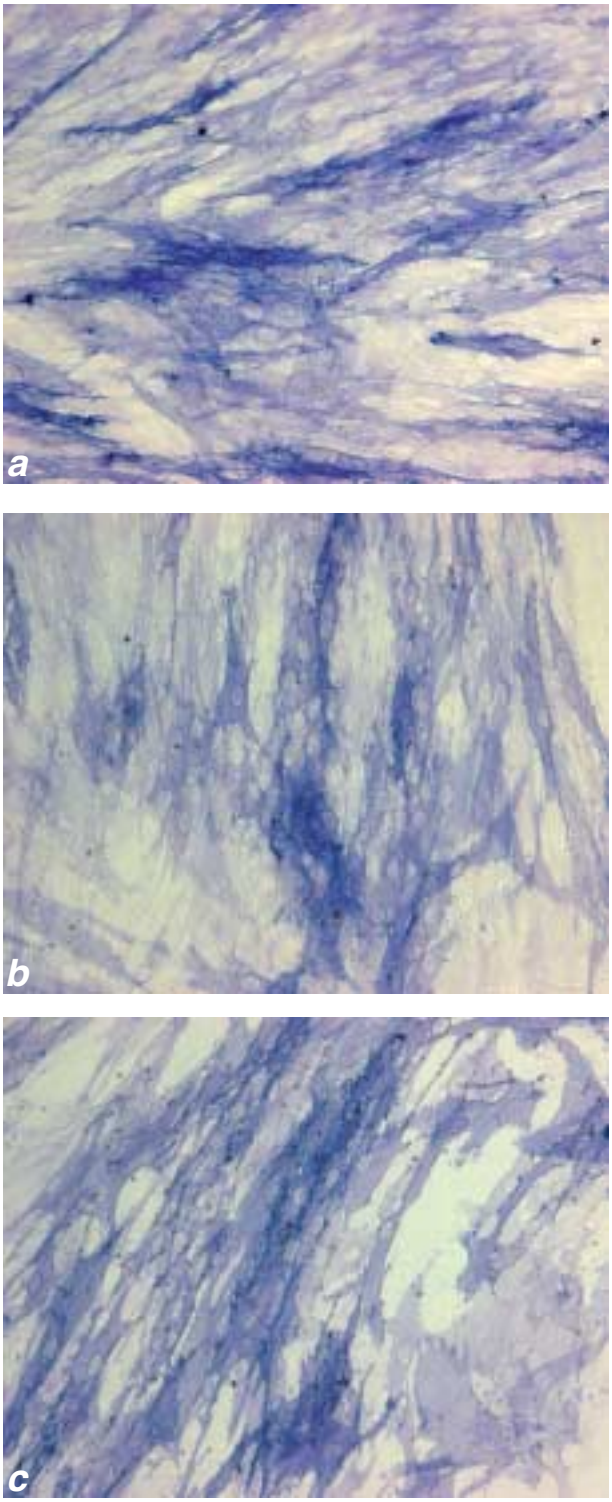


**Fig. 2.** Alkaline phosphatase activity: induced osteogenic differentiation of MSC (20 days of culturing; objective ×20). *a*) static control; *b*) shaker; *c*) clinostatic exposure.

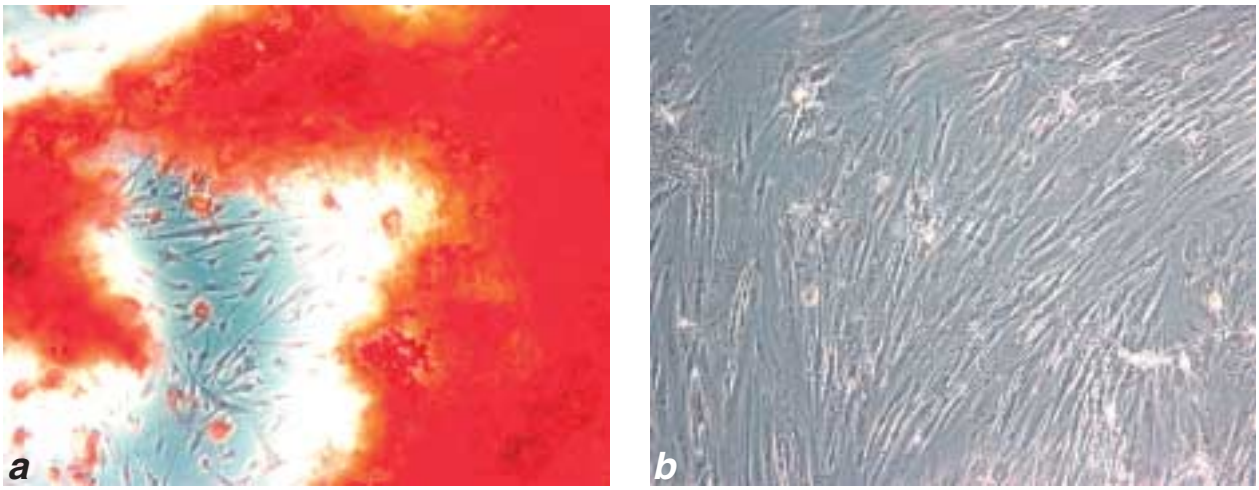


**Fig. 3.** Calcium mineralization of extracellular matrix, detected by alizarine red staining in passage 6 cells (objective ×10). *a*) static control; *b*) clinostatic exposure.

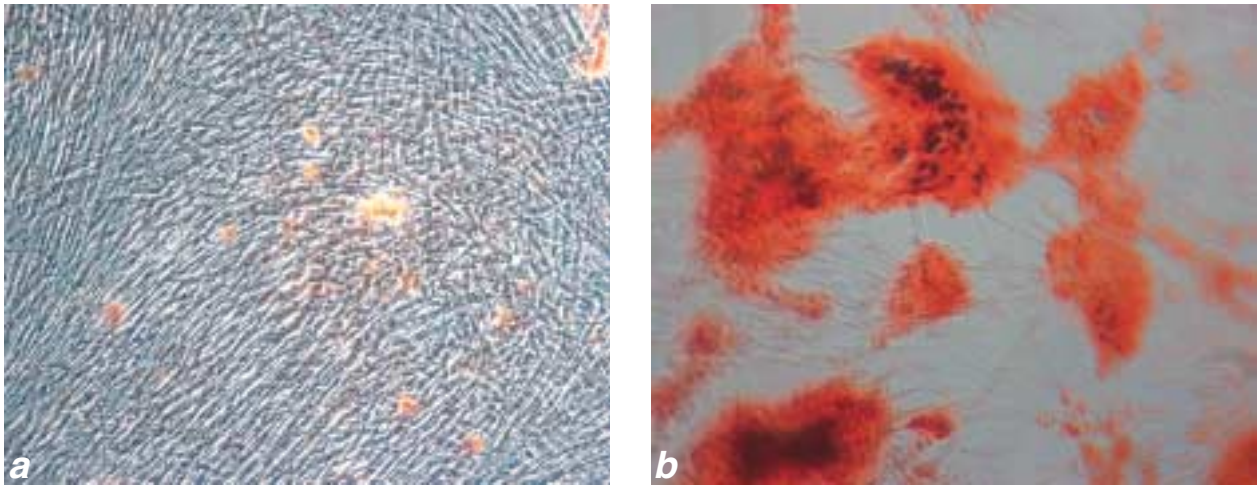


**Fig. 4.** Calcium mineralization of extracellular matrix, detected by alizarine red staining in passage 3 cells (objective ×10). *a*) static control; *b*) clinostatic exposure.

cytic antigen can indirectly attest to activation of the hemopoietic cell pool (usually eliminated from the culture towards later passages) during clinostatic exposure of cells in osteogenic medium. It is assumed that the adhesion interactions between osteoblasts and osteoclasts are mediated primarily by VCAM-1 [14]. Hence, decreased expression of this molecule by osteogenic precursor cells can modify commitment or activity of osteoclast precursors. It is known that ICAM-1 and VCAM-1 are also involved in the regulation of cell adhesion, proliferation, and migration [4], and hence, changed expression of these adhesion molecules can be responsible for detected separation of the cells from the matrix, high migration activity, and reduced rate of MSC proliferation in clinostatic exposure. Increased migration of cells during clinostatic exposure was previously shown on another model (cultured human endothelium, also expressing ICAM-1 and VCAM-1) [3].

Alkaline phosphatase activity is the earliest marker of osteogenic commitment of MSC; its expression significantly decreases under conditions of zero gravity and simulated microgravity [1, 18]. In our study its expression virtually did not change during clinostatic exposure, at least after 20-day culturing (Fig. 2).

Calcium mineralization of the extracellular matrix (a later indicator of MSC osteogenesis and a sign of osteoblast functional activity [2,13]) was evaluated by alizarine red S staining. Induced formation of mineralized matrix was inhibited in passage 6 cells during clinostatic exposure (Fig. 3), because no $Ca^{2+}$-positive staining or so-called "bone nodules" were detected in the culture after 20-day growth. Histochemical and morphological studies of passage 3 cell cultures detected initial stages of calcification in MSC exposed to clinostatic treatment, but virtually no calcification was detected in

the control. "Bone nodules" were detected in experimental and control cultures (Fig. 4).

Studies of stromal precursor cell osteogenesis demonstrated changes in the MSC differentiation potential after clinostatic exposure. Similarly as with the phenotypic characteristics of MSC exposed to clinostatic treatment, cells of different passages differently responded to the 20-day exposure. It seems that precursor cells of later passages are most sensitive to clinostatic effects, because they react to changes in the gravity not only by modification of their morphology and function, but also by modification of their commitment in the osteogenic direction.

Hence, simulation of microgravity exposure of cultured human bone marrow MSC showed that clinostatic exposure inhibited proliferative activity of cells and modulated their adhesive and migration characteristics. The detected functional changes in MSC culture, including modified expression of some cellular antigens and precursor cell differentiation potential, indicate gravity sensitivity of these cells. These results are important for further studies of the stem cell commitment and differentiation directions and for the development of notions on the regeneration and reparation processes under conditions of microgravity.

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