Myogenesis in Hemopoietic Tissue Mesenchymal Stem Cell Culture

S. N. Gornostaeva*, A. A. Rzhaninova*, and D. V. Gol'dstein*,**

Translated from *Kletochnye Tekhnologii v Biologii i Medicine*, No. 2, pp. 63-69, April, 2006
Original article submitted March 6, 2006

The myogenic differentiation capacity of prenatal mesenchymal stem cells from the main sites of hemopoiesis (bone marrow, thymus, liver, and spleen) was studied. Myogenesis was observed in all studied cell cultures except splenic mesenchymal stem cells. Differentiating cells from the thymus, bone marrow, and liver were positively stained for skeletal muscle markers (myogenin and MyoD). Autonomously contracting structures positively stained for cardiotroponin I and slow muscle myosin, were detected in the same cultures. Our experiments revealed differences in differentiation of mesenchymal stem cells from hemopoietic organs depending on the source of cells.

Key Words: *mesenchymal stem cells; myogenesis; differentiation*

Mesenchymal stem cells (MSC) were for the first time derived from adult donor bone marrow, due to their capacity to adhere to plastic [7]. Recent studies showed that multipotent stromal cells differentiating into many cell lines can be isolated from various tissues [3,4,6]. These cells are precursors of stromal elements maintaining hemopoiesis *in vivo* and *ex vivo*; they produce matrix components, cytokines, and growth factors involved in migration, proliferation, and differentiation of hemopoietic cells.

Proliferation and differentiation of hemopoietic stem/progenitor cells during human ontogeny is realized in histologically different microenvironment. In adult human bone marrow MSC are closely related to hemopoietic cells. The liver, spleen, thymus, and bone marrow are well-known sites of active hemopoiesis during certain periods of prenatal development.

The data on *in vitro* myogenic differentiation of MSC are scanty and contradictory. Differentiation of MSC into cardiomyoblasts induced by cytotoxic agents (5-azacitidine), co-culturing, and addition of cardiomyogenic differentiation media was demonstrated [8,9,13]. No spontaneous differentiation was described. *In vitro* predifferentiation of undifferentiated stem cells into cardiomyogenic cells was attained by stem cell co-culturing with cardiomyocytes and END-2 cells or by using myogenic agents, for example, 5-azacitidine and other compounds [11]. However, the safety of this predifferentiation is not proven, and we do not know whether it will have a negative impact on stem cell capacity to migration, division, or alternative differentiation into other cell types. The capacity of MSC to differentiate into muscle cells was not persuasively demonstrated.

The study of prenatal MSC behavior in culture and specific features of their differentiation will help to understand the ontogenesis of these cells and presumably open new potentialities for cell and gene therapy.

MATERIALS AND METHODS

Isolation of mesenchymal stem cells from the bone marrow, thymus, liver, and spleen. Bone marrow MSC cultures were derived from adult donors (n=6),

*Institute of Stem Cell and Cell Technologies; **ReMeTeks, Moscow.
Address for correspondence: sgen@mail.ru. S. N. Gornostaeva

0007-4888/06/1414-0493

prenatal bone marrow (n=9), and thymus (n=8) as described previously [1,2].

The spleens (n=9) and livers (n=9) were separated from adjacent connective tissue and washed in Hanks' solution (PanEco) with gentamicin (50 μg/ml). The organs were mechanically crushed with scissors and disaggregated in 0.2% dispase solution (Invitrogen Corp.) for 20 min at 37°C. The resultant suspension was centrifuged for 5 min at 800 rpm (4°C), and the supernatant was discarded. Cold DMSI medium with 1% fetal calf serum was added to the precipitate. Cell suspension was filtered through a stainless steel sieve and centrifuged. Cell precipitate was suspended in DMSI (Invitrogen Corp.) with 10% FCS, selected for optimal growth of low-density cultures (HyClone, Fetal clone I, Lot No. AND18477), 2 mM L-glutamine, and 10 μg/ml gentamicin. Cell suspension was inoculated in 90-mm plastic Petri dishes (Nunclon) in a concentration of 10^4 mononuclear cells/ml and incubated at 37°C in a CO_2 incubator at 5% CO_2. After 24 h nonadherent cells were removed, adherent cells were washed twice in Hanks' solution and incubated until confluence in the growth medium containing additio-

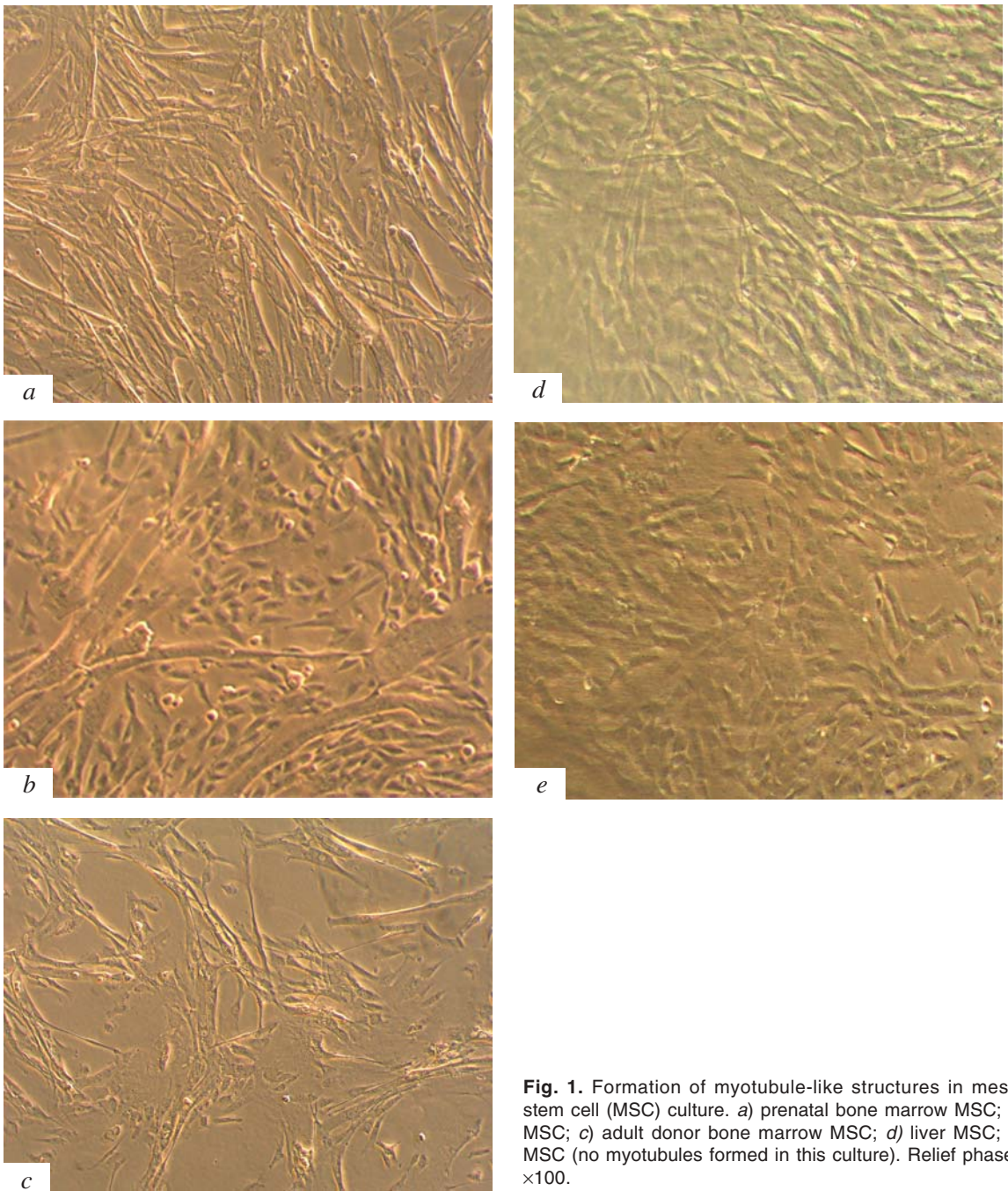

Fig. 1. Formation of myotubule-like structures in mesenchymal stem cell (MSC) culture. *a*) prenatal bone marrow MSC; *b*) thymus MSC; *c*) adult donor bone marrow MSC; *d)* liver MSC; *e*) splenic MSC (no myotubules formed in this culture). Relief phase contrast, ×100.

nally fibroblast growth factor (10 ng/ml; Sigma), heparin (8 U/ml), 1% insulin transferrin selenite (PanEco). The medium was replaced every 2-3 days depending on the culture status.

Immunocytochemical studies. Cells for analysis were cultured on polylysin-treated slides, fixed in 3.7% formaldehyde solution for 20 min, washed, and stored in phosphate buffer saline (pH 7.4). Monoclonal antibodies to MyoD, myogenin (Novocastra Lab. Ltd.), slow muscle myosin heavy chain (clone No. 0Q7.5.4D, Sigma-Aldrich), and troponin I (Abcam) were used. Antibodies were detected using Novostatin Super ABC Kit (Novocastra Lab. Ltd.) or FITC-conjugated goat anti-murine antibodies (Biotrend). Antibody staining was carried out by the Abcam protocol.

Directed cardiomyogenic differentiation of MSC by 5-azacitidine. Directed differentiation of bone marrow and splenic MSC into cardiomyoblasts was induced by adding demethylating agent 5-azacitidine. The cells were inoculated into 90-mm dishes (n=60) (10,000 cells/dish). 5-Azacitidine was added in doses of 1, 3, and 10 μl. In order to evaluate inhibition of proliferation, the cells were removed after 6 and 12 days of culturing with 5-azacitidine, stained with 0.2% Trypan Blue, and counted in a Goryaev chamber. In order to evaluate differentiation into cardiomyoblasts, after 8 weeks of culturing in cardiomyogenic medium the cells were fixed in 4% formaldehyde and stained using antibodies to slow muscle myosin and troponin I.

Phase contrast and fluorescent microscopy was carried out on an Olympus CK-40 microscope. Photographing and videoretyping were carried out using Olympus digital camera.

The data were statistically processed using methods of variation statistics (Student's t test) and Excel software.

RESULTS

Spontaneous myogenic differentiation. An obligatory condition for myogenic differentiation without addition of inductors is isolation of small cell colonies grown from a density of 6-30 cell/cm^2 in the presence the fibroblast main growth factor. Myo-

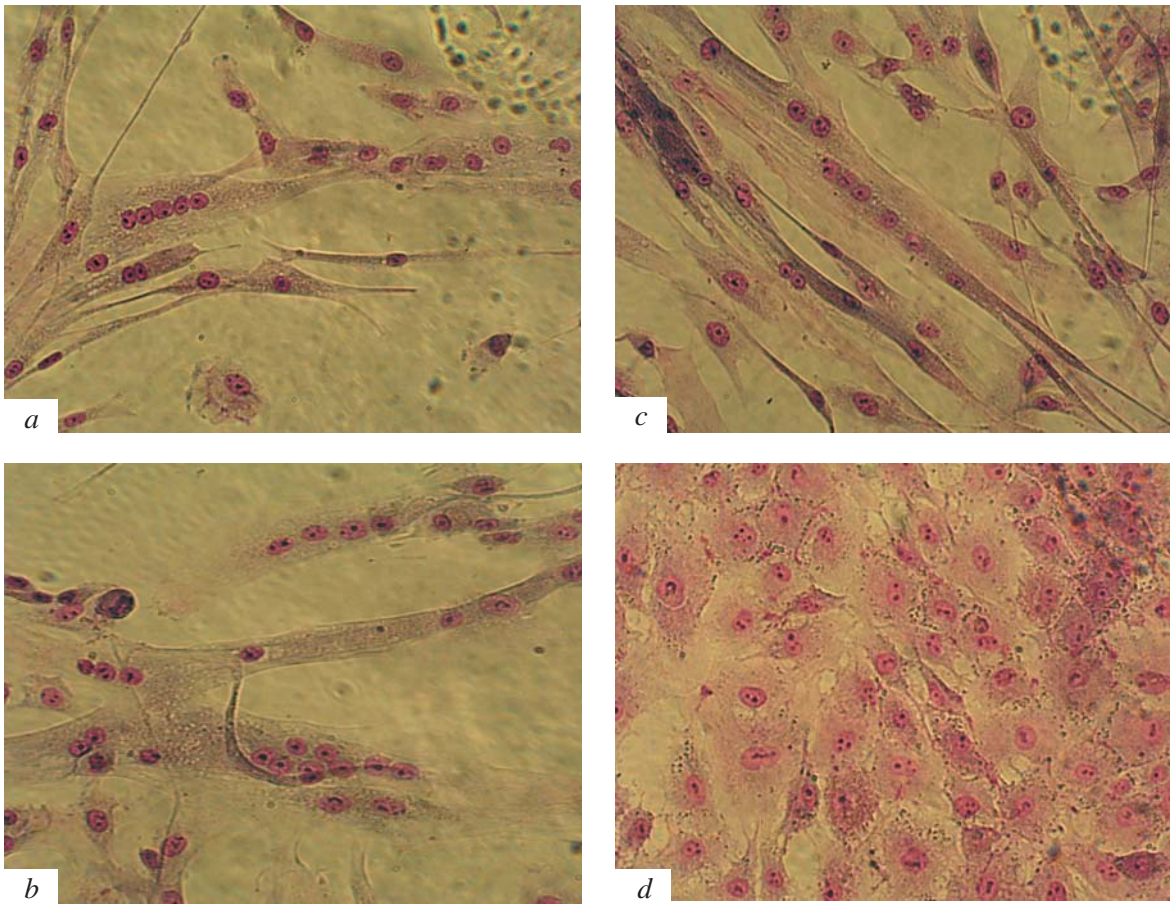

Fig. 2. Myogenesis in MSC culture. *a-c*) bone marrow, thymus, liver MSC; central location of the nuclei seen in the myotubules; *d*) no formation of myogenic structures in splenic MSC. Romanowskii staining, ×200.

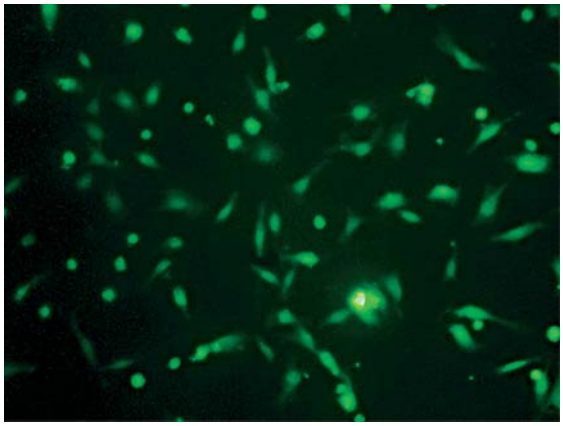

Fig. 3. Early myogenesis in MSC colonies. Staining for antibodies to MyoD, FITC-conjugated second antibodies.

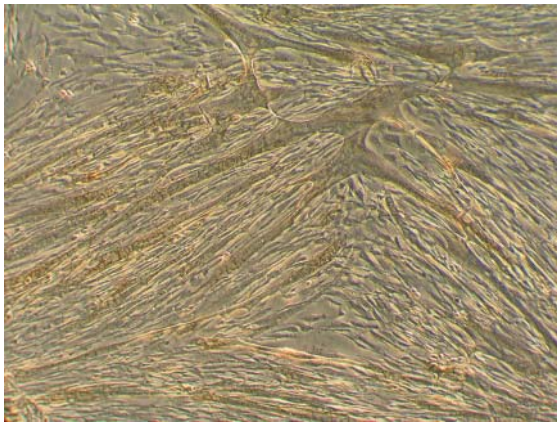

Fig. 4. Late myogenesis in MSC colonies. Staining for antibodies to myogenin. Peroxidase-conjugated second antibodies (Novocastra Super ABC-kit), ×100.

genesis in these colonies was stimulated as a result of time inhibition of proliferation: the dishes with cultures were kept for 30 min under conditions of CO_2 deficiency at lowered (25°C) temperature. Changes in cell morphology were detected under a phase contrast microscope only in cultures of bone marrow, liver, and thymus MSC. The cells stretched in one direction, fused, and after 7-8 days formed muscle tubules containing 3-10 nuclei per cell (Fig. 1). Romanowskii staining showed central position of the nuclei characteristic of early stage of the myosymplast formation (Fig. 2). After myogenic differentiation cell proliferation was decelerated, but did not stop. The myotubules disintegrated during reinoculation and formed again on the next day. The emergence of characteristic multinuclear myotubules was observed in all studied MSC cultures originating from the thymus, bone marrow, and liver, but not in splenic MSC (Fig. 1, *d*). Presumably, spontaneous myogenic differentiation de-

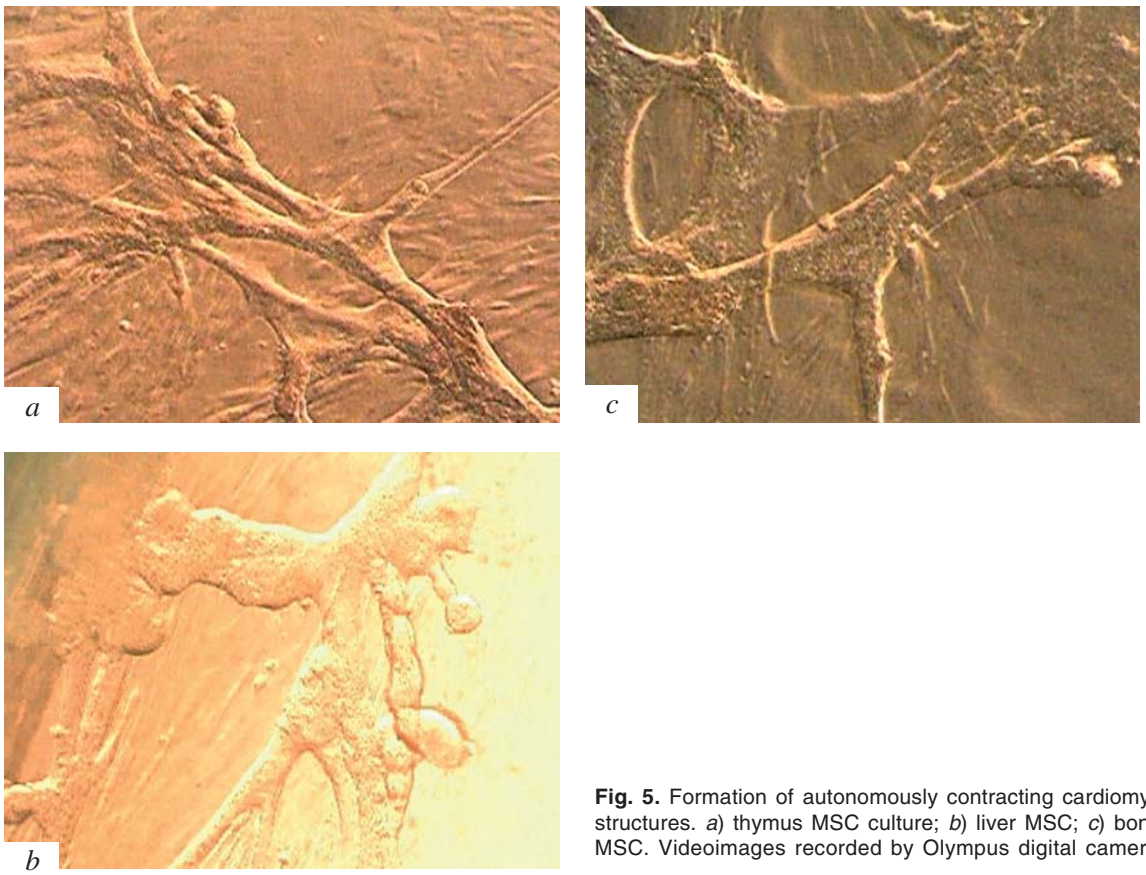

Fig. 5. Formation of autonomously contracting cardiomyocyte-like structures. *a*) thymus MSC culture; *b*) liver MSC; *c*) bone marrow MSC. Videoimages recorded by Olympus digital camera.

pends on fetal origin of the cells. In order to verify this hypothesis, MSC from the bone marrow of 6 adult donors were cloned; myogenic differentiation was observed in all samples (Fig. 1, *d*).

Myogenesis is regulated by MRF transcription factor family, including MyoD, Myf5, myogenin, and MRF4. MyoD and Myf5 are involved in the formation of the skeletal muscles during embryonal development, while myogenin is essential for terminal differentiation [12].

We selected MyoD for evaluating early myogenesis and myogenin as a late marker. Three-five days after nonspecific inhibition of proliferation the bone marrow, thymus, and liver MSC cultures were positively stained with antibodies to MyoD (Fig. 3). Expression of myogenin was detected in these cultures after the formation of myotubules (after 7-8 days) (Fig. 4).

The formation of autonomously contracting cardiomyocyte-like structures was observed about 1 month after the beginning of myogenesis (Fig. 5). Differentiated cells contracted repeatedly and autonomously without specific stimulation. Cultures in which these structures formed were positively stained for slow muscle myosin heavy chain and troponin I (Fig. 6). The number of cardiomyocyte-like structures per dish varied from 2 to 8. The structures formed virtually simultaneously, but started contracting at different time. The contractions were mainly asynchronous and with different amplitudes, but some structures contracted synchronously and rhythmically. Presumably, this was due to the formation of a pacemaker region. Contractions of cardiomyocyte-like structures were observed 48-72 h.

For evaluation of MSC differentiation into cardiomyocytes we used 5-azacitidine, co-culturing with cardiomyocytes, or addition of cardiomyogenic differentiation media [8,9,13]. Contractions of myotubules formed by adult donor bone marrow MSC differentiated under the effect of 5-azacitidine were described previously [10]. Spontaneous differentiation of MSC was characterized by the formation of clone-like structures resembled those forming during cardiomyogenic differentiation of fetal stem cells.

Spontaneous differentiation of fetal stem cells can be initiated by their high density [5]. However, MSC culturing after high density inoculation (more than $4\text{-}5\times10^3$ cell/cm^2) without isolation of colonies did not result in the formation of myotubules. Presumably, this was due to impossibility of selecting cell populations with myogenic potential at such inoculation density.

Directed myogenic differentiation. For evaluation of the capacity to directed myogenic differentiation, two cultures with different characteristics in a previous experiment were selected: bone marrow MSC exhibited capacity to spontaneous myogenic differentiation, splenic MSC showed no capacity of this kind. Non-cloned cultures without signs of spontaneous differentiation after standard 1:10 passages (corresponding to 4×10^3 cell/cm^2) were taken into the experiment. 5-Azacitidine (cytosine analog) served as differentiation agent. This agent is characterized by demethylating activity and, according to published data, is indicated for directed differentiation of MSC into cardiomyocytes [10]. The cells were inoculated in 90-mm Petri dishes (n=40), 5-azacitidine was added in concentrations of 1, 3, and 10 μM.

In order to evaluate the effect of 5-azacitidine on proliferative activity, the cells were removed after 6 and 12 days of incubation and the mean number of cells from 5 samples per point was evaluated. 5-Azacitidine inhibited MSC proliferation in cultures of both types in a dose-dependent manner (Figs. 7, 8). Proliferative activities of bone marrow and splenic cells treated with 1 μM 5-azacitidine

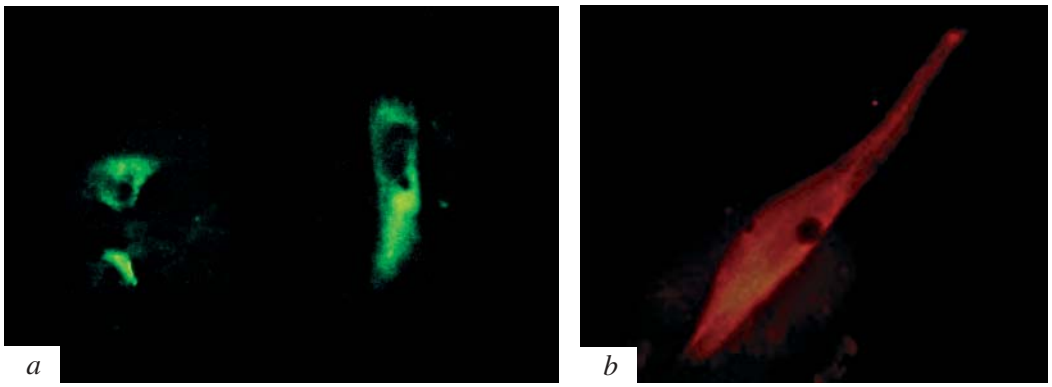

Fig. 6. Immunofluorescent staining of cultures with spontaneously contracting structures. *a*) contractions to cardiotroponin (FITC-conjugated second antibodies); *b*) contractions to slow muscle myosin heavy chain (phycoerythrin-conjugated second antibodies), ×200.

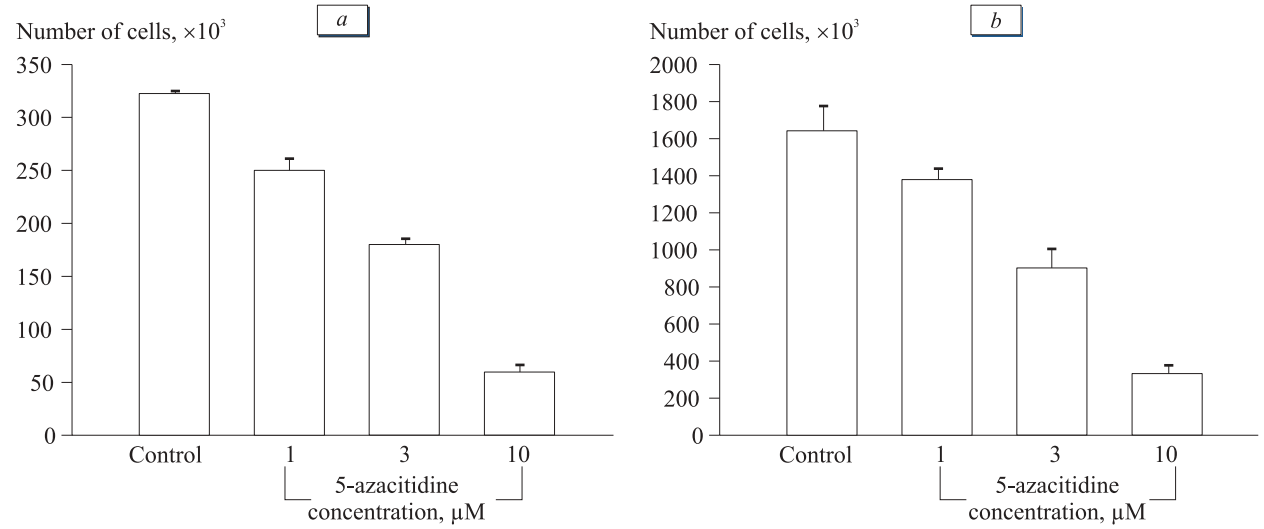

Fig. 7. Relationship between 5-azacitidine concentration and splenic MSC proliferation after 6 (*a*) and 12 (*b*) days of incubation. $p<0.01$.

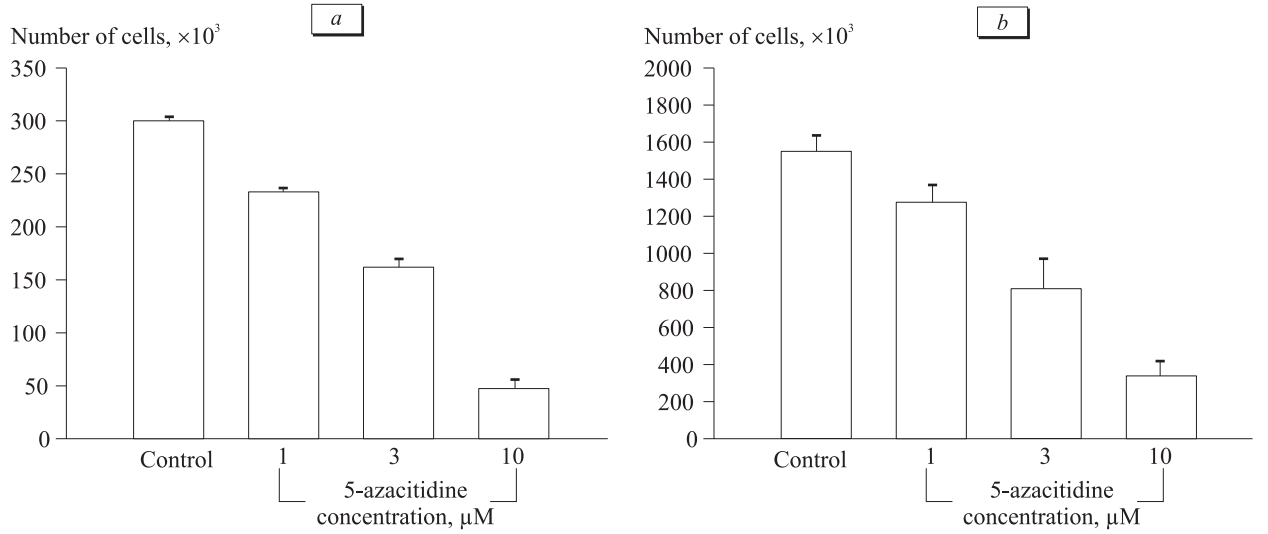

Fig. 8. Relationship between 5-azacitidine concentration and bone marrow MSC proliferation after 6- (*a*) and 12- (*b*) day incubation. $p<0.01$.

was inhibited negligibly, though on day 6 decreased to 233.0±3.6 (bone marrow) and 250±10 (spleen) in comparison with the control (300±5; $p<0.001$ and 322±2.5; $p<0.005$, respectively); on day 12 it reached 1275.0±90.1 in the bone marrow (1550.0± 86.6 in control, $p<0.001$) and to 1379±56.5 in the spleen (1642±131 in control, $p<0.001$). Treatment with 3 μM 5-azacitidine led to more pronounced inhibition of bone marrow MSC growth: 162.0±7.2 on day 6 (300±5 in control, $p<0.001$) and 808.3± 162.6 on day 12 (1550.0±86.6 in control, $p<0.001$); splenic MSC proliferation reached 180±5 on day 6 (322.5±2.5 in control, $p<0.001$) and 901.6±102.5 on day 12 (1642.0±131.1 in control, $p<0.001$). Proliferative activity decreased more than 5-fold after treatment with 10 μM 5-azacitidine: on day 6 this parameter for bone marrow MSC was 47.3±8.7 (*vs.* 300±5 in control, $p<0.001$), on day 12 339.3±80.5 (1550.0±86.6 in control, $p<0.001$); for splenic MSC 59.6±5.9 (322.5±2.5 in control, $p<0.001$) on day 6 and 332.0±43.2 (1642.0±131.1 in control, $p<0.001$) on day 12.

Cardiomyogenic differentiation was evaluated after 8 weeks. After induction splenic MSC became hypertrophic and their growth was inhibited; numerous adipocyte-like cells appeared. No signs of myogenic differentiation were detected in splenic MSC culture.

Solitary adipocyte-like cells were detected in bone marrow MSC culture, but induction with 5-azacitidine led to myogenic differentiation of these cells. The most pronounced effect (20-30% cells)

was noted after addition of 10 μM 5-azacitidine. The majority of cells were mononuclear; there were many binuclear cells and several were multinuclear (with 3-10 nuclei). The use of 1 μM 5-azacitidine brought about a negligible effect; just solitary binuclear cells appeared. A more pronounced result was observed after addition of 3 μM 5-azacitidine: about 10% differentiated cells.

The mechanism of 5-azacitidine effect on cardiomyogenic differentiation remains unclear. Presumably, it is related to modification of gene expression caused by demethylation [5]. In general, induction by 5-azacitidine led to less pronounced myogenic differentiation in comparison with spontaneous differentiation of cloned cultures. Directed differentiation required more time (4 weeks, instead of 7 days), while the percent of differentiated cells was lower (20-30% *vs.* 40-50% during spontaneous differentiation). Cloning and selection of MSC provide cardiomyogenic differentiation without 5-azacitidine treatment.

Hence, we attained spontaneous cardiomyogenic differentiation of MSC *in vitro*. MSC from hemopoiesis and immunogenesis organs possess different myogenic potential in culture: MSC from the thymus, bone marrow, and liver differentiated into skeletal myoblasts and cardiomyocytes, while splenic MSC did not differentiate spontaneously or after induction.

REFERENCES

1. A. A. Rzhaninova, S. N. Gornostaeva, D. V. Gol'dstein, *et al.*, *Byull. Nauch. Tsentra im. Bakuleva*, **5**, No. 5, 314 (2004).
2. A. A. Rzhaninova, S. N. Gornostaeva, and D. V. Gol'dstein, *Kletoch. Tekhnol. Biol. Med.*, No. 1, 34-41 (2005).
3. H. A. Awad, D. L. Butler, G. P. Boivin, *et al.*, *Tissue Eng.*, **5**, 267-277 (1999).
4. S. P. Bruder, A. A. Kurth, M. Shea, *et al.*, *J. Orthop. Res.*, **16**, 155-162 (1998).
5. S. C. Choi, J. Yoon, W. J. Shim, *et al.*, *Exp. Mol. Med.*, **36**, No. 6, 515-523 (2004).
6. J. E. Dennis, A. Merriam, A. Awadallah, *et al.*, *J. Bone Miner. Res.*, **14**, 700-709 (1999).
7. A. J. Friedenstein, N. N. Kulagina, *et al.*, *Exp. Hematology*, **2**, 83-92 (1974).
8. K. Fukuda and J. Fujita, *Kidney Int.*, **68**, No. 5, 1940-1943 (2005).
9. Y. Iijima, T. Nagai, M. Mizukami, *et al.*, *FASEB J.*, **17**, No. 10, 1361-1363 (2003).
10. S. Makino, K. Fukuda, S. Miyoshi, *et al.*, *J. Clin. Invest.*, **103**, No. 5, 697-705 (1999).
11. C. Mummery, D. Ward-van Oostwaard, P. Doevendans, *et al.*, *Circulation*, **107**, No. 21, 2733-2740 (2003).
12. L. A. Sabourin and M. A. Rudnicki, *Clin. Genet.*, **57**, No. 1, 16-25 (2000).
13. W. S. Shim, S. Jiang, P. Wong, *et al.*, *Biochem. Biophys. Res. Commun.*, **324**, No. 2, 481-488 (2004).