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Isolation and Phenotypical Characterization of Mesenchymal Stem Cells from Human Fetal Thymus

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Stem cells from human fetal thymus ectomesenchyma capable of forming colonies during *in vitro* culturing were isolated and characterized. Selection of culturing conditions showed that the growth and phenotypical characteristics of these cultures depended on seeding density and presence of basic fibroblast growth factor in the medium. After nonspecific inhibition of proliferation clonogenic cultures of thymic mesenchymal stem cells differentiated into myoblasts, formed characteristic myotubes, and expressed specific myogenesis markers. Colonies of thymic mesenchymal stem cells differentiated into chondrogenic, osteogenic, and adipogenic lines under conditions described for bone marrow mesenchymal stem cells. Cytofluorometric analysis of surface epitopes of thymic mesenchymal stem cells showed that the majority of cells expressed mesenchymal markers Thy-1, CD44, and CD105. Testing for CD34, CD38, CD45, and HLA-DR were negative in all cases. The main cell population (70-95%) did not express MHCl antigens during long-term culturing.

Key Words: *mesenchymal stem cells; fetal thymus; immunophenotype; differentiation*

The thymus is a central organ of immunogenesis responsible for differentiation of bone marrow stem cells migrating into this organ to T lymphocytes. Morphological peculiarities of the thymus are early appearance (during embryogenesis), mature status in the newborns, and further development during early infancy.

The inductive effect of mesenchymal cells is obligatory for proliferation and maturation of thymic epithelium during the formation of the organ [3]. The significance of this relationship was experimentally proven in 1960 by R. Auerbach, who investigated the development of mouse thymus [2]. Active proliferation of epithelial cells was observed in thymic explants (12.5-day gestation), but not when the mesenchymal component of the developing thymus was removed. Recombination of the thymic epithelial component with the mesenchyma from different sources (lungs, submaxillary glands, or fetal rudiments of mouse limbs) showed that the origin of the mesenchyma and its presence are the key factors in the process of embryo development. Hence, the presence of the mesenchyma from the appropriate source and at certain developmental stage is an obligatory condition for the proliferation of pre-committed epithelial cells towards thymus development.

During embryogenesis, appreciable portion of the mesenchyma filling pharyngeal pouches and forming the substance of the pharyngeal region wall is formed from the neural crest cells, which migrate ventrolaterally and proliferate. Some clusters give rise to components of the nervous system (*e.g.* spinal ganglia). Other groups of cells form the ectomesenchyma. These cells are morphologically similar to mesenchymal cells developing from the mesoderma, but they are characterized by specific functional features. The ectomesenchymal cells interact with the epithelium by forming contacts or through secreted signal molecules, thereby stimulating proliferation, maturation, and differentiation processes. Under conditions of epithelial stroma microenvironment ensuring thymocytes pro-

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liferation and T-cell maturation, fetal thymus becomes competent for recruiting lymphoid precursor cells from the blood [4].

Epithelial and lymphoid components of the thymus attracted special interest of many scientists for a long time, but thymic ectomesenchyma cells were virtually never studied *in vitro.*

MATERIALS AND METHODS

Isolation of mesenchymal cells from the thymus. Human thymuses were obtained from stillborn fetuses after spontaneous abortions on gestation weeks 12-22 (n=8).

The thymuses were separated from the adjacent connective tissue, fragmented, and disaggregated in 0.2% dyspase solution (Invitrogen Corp.). The cell suspension was filtered through a stainless steel sieve and centrifuged. Cell precipitate was suspended in DMEM (Invitrogen Corp.) with 15% FCS selected for optimal culture growth at low density (HyClone, Fetalclone I, Lot No. AND18477) and 2 mM L-glutamine. The cells were disseminated in 90 mm plastic Petri dishes (Nunclon) in a concentration of 10^4 mononuclear cells/ml and incubated at 37°C and 5% CO_2. After 24 h nonadhesive cells were removed and adhesive cells were washed with Hanks' medium and incubated until confluence in a growth medium supplemented with fibroblast growth factor (10 ng/ml, Sigma) and heparin (8 U/ml).

Colony counting. The cells were seeded in 90-mm plastic Petri dishes (30 cell/cm^2). The cultures were incubated for 10 days, after which the medium was discarded, the dishes were washed in phosphate buffer (pH 7.0) and stained with 0.5% Crystal Violet (Reakhim) in 70% ethanol for 5 min at 20-22°C. The cells were washed twice in distilled water and the colonies were counted. Colonies <2 mm in diameter were not counted.

Immunocytochemical studies. Cells for analysis were cultured on polylysin-treated slides, fixed in 4% formaldehyde (20 min), washed, and stored in phosphate buffer (pH 7.4) until analysis. Monoclonal antibodies to myogenin (Novocastra Lab. Ltd.), collagen II, and aggrecane (Sigma) were used in the study. Antibodies were detected using Novostatin Super ABC Kit (Novocastra Lab. Ltd.) or FITC-conjugated goat antimurine antibodies (Biotrend).

For detection of mineralization foci during osteogenic differentiation, the cell monolayer was washed with phosphate buffer and incubated with 0.2% Alizarin red S (pH 5.5) for 2-5 min at ambient temperature. Unbound stain was removed by washout.

Immunophenotyping. The cultures were incubated in growth DMEM with low FCS content (2%) for 24 h before analysis. The cells were treated with trypsin, suspended in phosphate buffer (pH 7.4) to a concentration of 100,000 cell/ml, fixed in 1% methanol at 4°C for 10 min, and washed. Nonspecific binding was blocked by incubation with 1% BSA and 0.1% goat serum for 1 h at ambient temperature. The cells were then washed in 3 volumes of phosphate buffer and centrifuged; the precipitate was resuspended in 0.5% working solution of the first antibodies in 1% BSA with 0.1% goat serum. After 40-min incubation at 4°C the cells were washed in phosphate buffer (pH 7.4). Murine monoclonal antibodies (PharMingen and Chemicon) were used. Nonspecific murine (rabbit) IgG of the same companies served as negative control. The preparations were incubated with FITC- or phycoerythrin-labeled antispecies antibodies for 20 min, after which the cells were washed in phosphate buffer (pH 7.4), and 1 ml suspension was analyzed in a FACS Calibur flow cytofluorometer (BD Biosciences). The results were processed using MDI 2.8 software.

Functional characteristics. For evaluation of osteogenic differentiation [4,11], 10^3 cell/cm^2 were seeded in 24-well plates (Nunclon) and cultured in the standard growth medium for 5 days until the formation of 50-70% monolayer. The medium was then replaced by osteogenic one: 10^8 M dexamethasone, 0.2 M ascorbic acid, and 10 mM glycerophosphate (Sigma). The medium was replaced every 3 days, after which the cultures were washed in phosphate buffer, fixed in 70% ethanol for 1 h, and stained in 1 ml 40 mM Alizarin Red S (pH 4.1, Sigma) for 10 min.

For adipogenic differentiation [5,12], 50-70% confluent cultures were incubated in complete medium with 0.5 μM hydrocortisone and 60 μM indomethacin. The medium was replaced every 3-4 days. After 21 days adipocyte-like cells were noted in cultures; these cells differed by the presence of phase-contrast lipid inclusions in the cytoplasm.

For differentiation into chondroblasts, the cells were treated with trypsin and centrifuged in polypropylene tubes (200,000 cell per tube). Differentiating medium (500 ng/ml BMP-6, 10 ng/ml TGF-β, 10^{-7} M dexamethasone, 25 mM glucose, and 50 g/ml ascorbic acid) was added to the precipitate and the preparation was incubated for 21 days.

RESULTS

Isolation of thymic mesenchymal cell colonies. After enzymatic disaggregation of the thymus, primary suspension of thymic cells contains an admixture of several cell types, which can grow in adhesive culture. In order to separate cell populations after the zero passage, the monolayer was subcultured to a density of

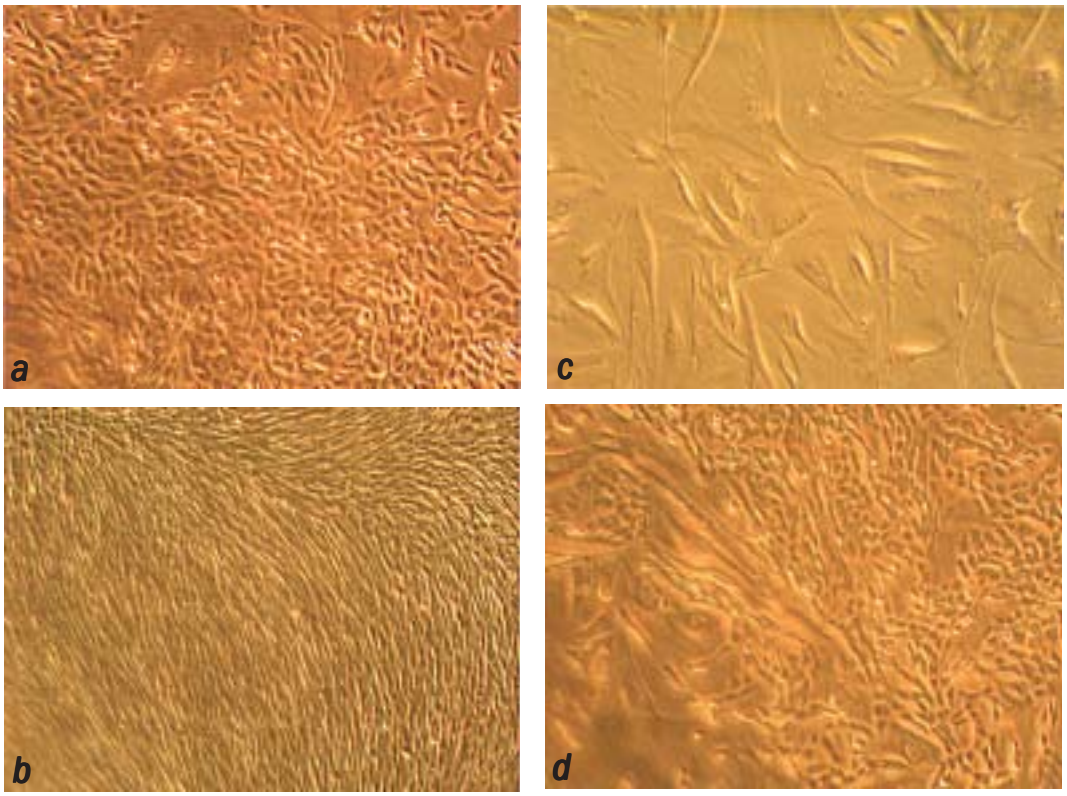

Fig. 1. Morphology of the main cell types present in thymic mesenchyma cultures during early passages. *a*) colonies of small polygonal cells; *b*) fibroblast-like colonies; *c*) foci of large stromal cell growth; *d*) mixed type colonies (relief phase contrast, ×100).

2000 cell/cm^2. As the culture grew, the plates with predominant growth of small mesenchymal cells (detected under a phase-contrast microscope) were selected. The selected cultures were seeded at clonal densities of 30 and 300 cell/cm^2. After 5-7-day incubation colony formation was observed; colonies larger than 2 mm in diameter formed after 14-15 days. The following cell types were detected in the culture at this stage of isolation: colonies of very small cells, fibroblast-like colonies, and foci of large stromal cell growth. Mixed colonies were sometimes seen (Fig. 1). Small-cell colonies were isolated and cloned repeatedly. The optimal clonal density was determined experimentally and was 30 cell/cm^2. This seeding density is sufficient for stable yield of colonies from different thymus samples; it allows isolation of individual (not aggregated) colonies >2 mm in diameter, as was described for bone marrow mesenchymal stem cells (MSC) [4]. The colonies were counted 15 days after seeding by staining with 0.5% Crystal Violet in 70% ethanol. The colonies appreciably varied in size, not only in cultures from different thymuses, but even within one sample (Fig. 2). Analysis of MSC cultures from 7 fetal thymuses showed the formation of 1-2 colonies per cm^2 at this stage of culturing (Table 1).

TABLE 1. Numbers of Thymic MSC Colonies from 7 Fetal Thymuses

Sample	Gestation term, weeks	Passage No.	Number of colonies*
L31/TM	19-20	4	42
L26/TM	20	3	54
S76/T	12	4	59
L30/TM	21-22	3	67
L28/TM	22	3	103
L29/TM	22	3*	60
L27/TM	18-19	3	97

Note. *Mean number by the results for at least three 90-mm dishes is presented. Seeding density 30 cell/cm^2.

The largest colonies of small polygonal short-process cells were selected under an inverted microscope, multiplied, and used for further studies.

The presence of basic fibroblast growth factor (bFGF) in the growth medium is not obligatory for clonal culturing of the bone marrow MSC, though accelerates their growth [14]. In our experiments with MSC from fetal thymus two conditions were obligatory for effective isolation of multipotent cell colonies: culturing at low density and presence of bFGF in the growth medium.

The mitogenic effect of bFGF also manifested in culturing at high density (10^3-10^5 cell/cm^2, data not

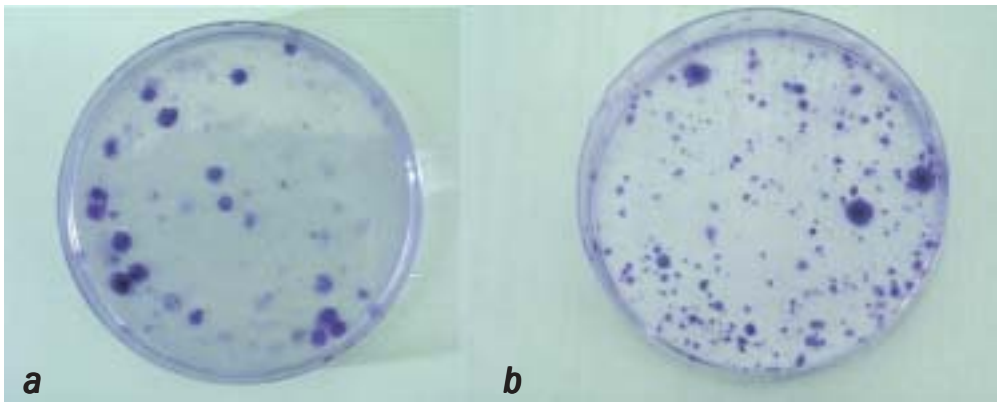

Fig. 2. Visualization of cell colonies from two thymuses of 18-20-weeks gestation by staining with 0.5% Crystal Violet. Mean diameter of colonies in dishes is 2.7 mm (*a*) and 4.5 mm (*b*).

presented), but the presence of this growth factor was not sufficient for retaining undifferentiated phenotype of the culture.

Cytofluorometric analysis of cell culture phenotype. The expression of surface epitopes was analyzed in MSC culture from 8 fetal thymuses of 12-22 weeks gestation. Since expression of surface antigens by fetal thymus mesenchymal cells was never studied before, we selected a group of markers most often used for the analysis of bone marrow MSC [4,8].

The main markers of hemopoietic cells (CD34, CD38, CD45) and HLA DR were expressed by less than 1% cells in thymic MSC clonal cultures (at the level of negative control, Fig. 3). The greatest cell populations were stained with antibodies to Thy-1 (CD90) (80-92%), CD44 (60-75%), and endoglin CD105 (about 50%). Cell populations expressing transferrin receptor CD71 were detected in all cultures, but the percentage of positive cells varied from 15 to 70. Our results indicate the absence of correlation between proliferative activity of cell cultures from fetal tissues and expression of transferrin receptor (our unpublished data). A small cell population (8-15%) stained with antibodies to ICAM1 (soluble intercellular adhesion molecule 1, CD54) was present in all analyzed cultures. However, we distinguished colonies with higher level of CD54 expression (>50%). It is known that ICAM1 is induced by a certain set of cytokines, such as IL-1, TNF-α, and IFF-γ. Presumably, activation of ICAM1 expression in these colonies was due to a specific cytokine profile characteristic of just few cell populations.

MHCl antigens (HLA-ABC) were present on the surface of 5-30% cells. The percentage of positive cells changed negligibly with passages, but in general remained low. Comparative studies showed that thymic MSC are characterized by the lowest expression of MHCl antigen in comparison with similarly isolated cultures from other fetal sources, including the bone marrow [1].

Correlation between growth conditions and myogenic potential of the culture. Colonies of small cells grown from densely seeded (50-500 cell/cm^2) cultures differentiated into myoblasts without addition of inductive agents (*e.g.* 5-azocitidine). An obligatory condition for spontaneous myogenesis in colonies of this type is temporary inhibition of proliferation: the dishes with cultures were kept under conditions of

TABLE 2. Protocol of Experiments for Targeted Differentiation of Thymic MSC

Direction of differentiation	Culture type	Composition of differentiation medium	Period of observation, days
Chondrogenesis	Suspension	500 ng/ml BMP-6, 10 ng/ml TGF-β, 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid, ITS (1:100)	21
Osteogenesis	Monolayer	10^{-8} M dexamethasone, 0.2 mM ascorbic acid, 10 mM β-glycerophosphate	21
Adipogenesis	Monolayer	0.5 μM hydrocortisone, 50 μM indomethacin	21

CO_2 deficiency and lower (25°C) temperature for 30 min. The appearance of characteristic multinuclear promyotubes was observed in all studied cell cultures after 3-7 days (Fig. 4). Later myosymplasts increased in size and expression of myogenin (marker of late myogenic differentiation associated with cell fusion [10]) was detected in the cultures. No expression of myospecific proteins was detected in undifferentiated cells.

Myoid cells are a component of thymic stroma [9]. This population possesses characteristics of epi-

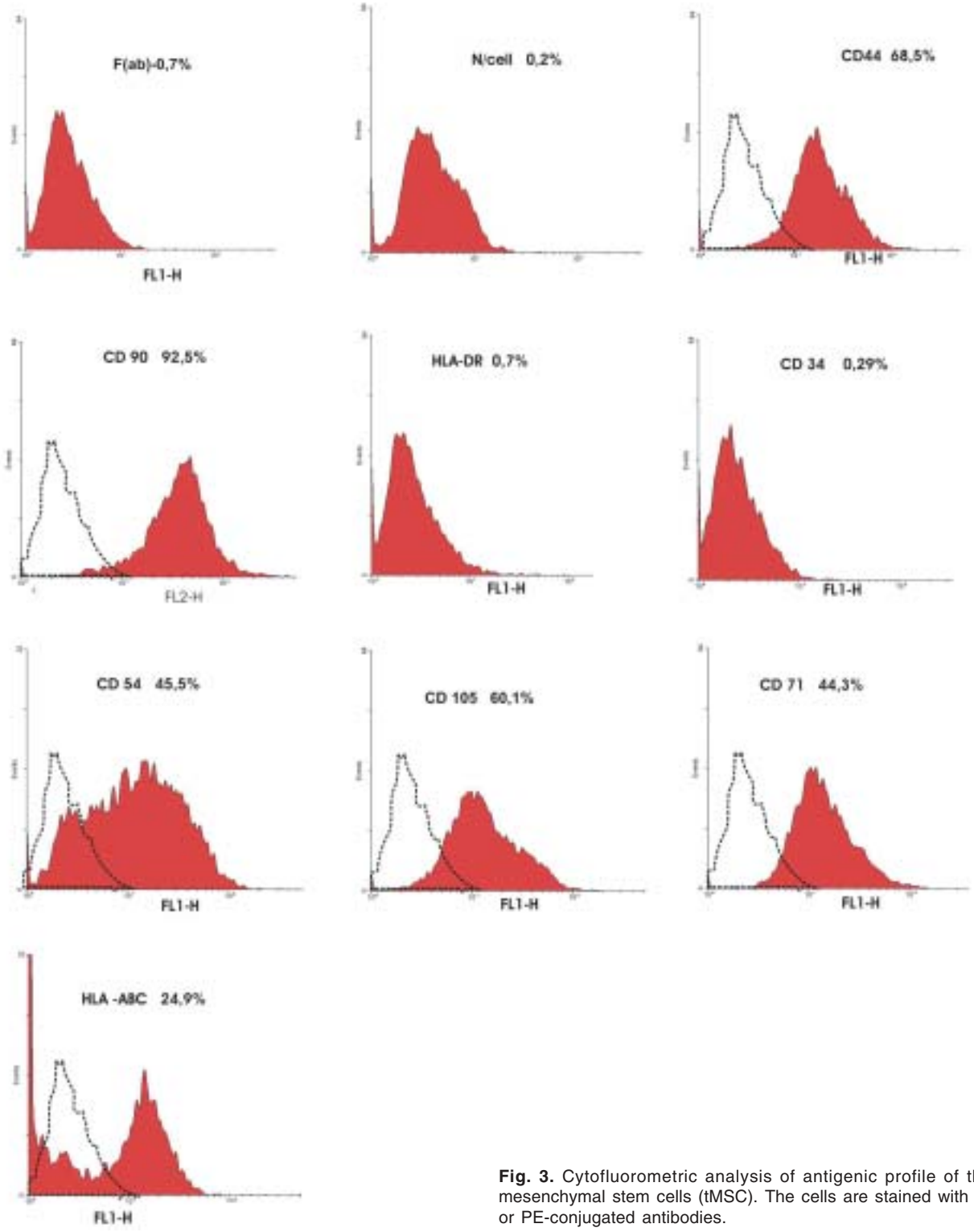

Fig. 3. Cytofluorometric analysis of antigenic profile of thymic mesenchymal stem cells (tMSC). The cells are stained with FITC- or PE-conjugated antibodies.

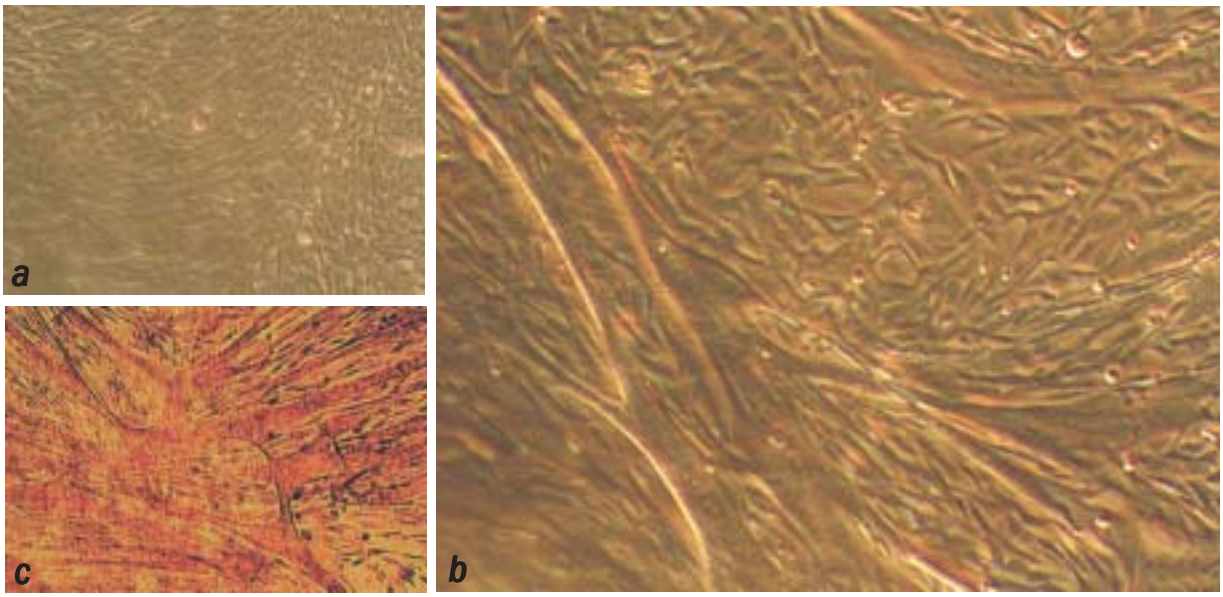

Fig. 4. Myogenesis in clonogenic tMSC cultures. *a*) before differentiation; *b*) formation of promyotubes (relief phase contrast, ×100); *c*) myogenin expression (immunoperoxidase staining, Novostatin Super ABC Kit, ×100).

thelial cells (positive markers — EMA, cytokeratins) and expresses myospecific proteins troponin T, desmin, and acetylcholine receptor [7,11]. Myoid cells are characterized by very stable immunophenotype, which can be detected both *in vivo* (e.g. in thymic tumor biopsy specimens [12,15]) and in immortalized MITC cells [9]. It seems that cell populations isolated by us are not myoid cells, because they are positive to mesenchyma markers (CD44, CD90, CD105, vimentine) and require additional induction of myogenesis.

Promyotubes did not form in cultures of large stromal cells grown at a density of 10^3 cell/cm^2 and higher without bFGF.

Study of multipotency (functional activity) of isolated cultures. The functional activity of thymus MSC cultures was evaluated by their capacity to purposeful differentiation. Differentiation of fetal thymus MSC into chondrogenic, osteogenic, and adipogenic lines was evaluated in inductive media under experimental conditions described previously for bone marrow MSC [5,12] (Table 2).

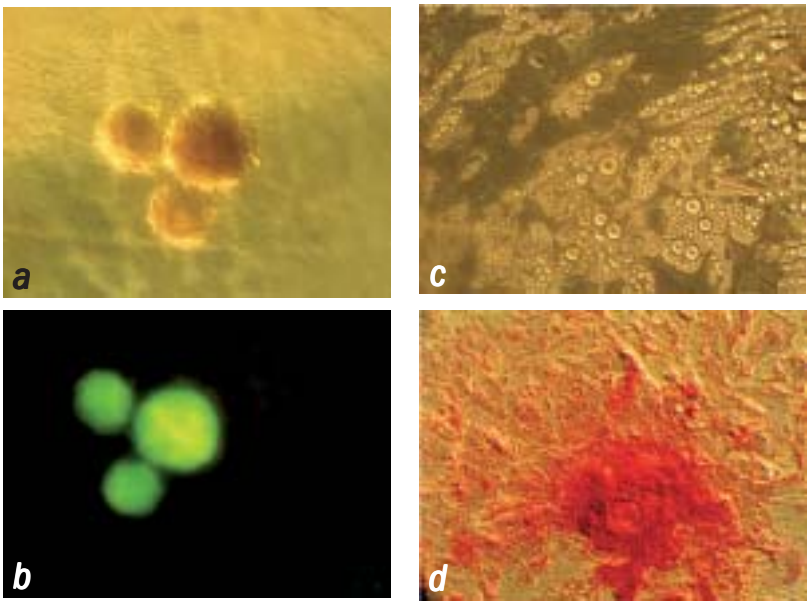

Fig. 5. Targeted differentiation in clonogenic tMSC cultures. *a*, *b*) chondrogenesis (spheroids express aggrecane, immunofluorescence, ×200); *c*) adipogenesis (relief phase contrast, ×200); *d*) osteogenesis (mineralization focus stained by Alizarin Red S, ×200).

For chondrodifferentiation the cells were incubated under nonadhesive conditions (in polypropylene tubes). After 2 weeks of culturing the formation of clone-like spheroids consisting of live cells not incorporating trypan blue was observed. On day 21 the cultures were fixed with 0.4% formaldehyde and stained with antibodies to specific chondrogenesis markers (type 2 collagen and aggrecane) (Fig. 5).

Long-term incubation in osteogenic medium induced changes in the culture morphology: the cells grew hypertrophic and did not proliferate. After fixation of the monolayer in 0.4% formaldehyde mineralization foci were detected by staining with Alizarin Red ethanol solution (pH 4, Fig. 5). Stained areas were seen at sites of the most dense cell growth and were rare: no more than 6-8 foci per 90-mm dish.

The formation of phase-contrast lipid inclusions in the cytoplasm was observed not only after incubation in adipogenic medium, but also spontaneously, in case of violation of the culturing protocol (Fig. 5). Differentiation of solitary adipocyte-like elements was observed in cultures enriched with large stromal cells.

Hence, the thymus from fetuses of the 2nd trimester of gestation is a source of MSC. Selection of the optimal culturing conditions allowed isolation of cell populations forming colonies of multipotent cells with high level of plasticity in culture. The profile of surface epitope expression, low antigenic activity of thymic MSC, and high technology of culture isolation recommend this type of cells for the use in cell replacement therapy.

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