Vital Dye Dil and Fluorescent Magnetic Microparticles Do Not Affect the Phenotype of Mesenchymal Stem Cells from Human Amnion and Their Differentiation Capacity

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We present a method of labeling of mesenchymal stem cells from human amnion with a fluorescent dye Dil and microspheres (Bangs Laboratories). The possibility of administration of loaded cell culture was verified and comparative analysis of the phenotype of mesenchymal stem cells by the expression of fibronectin, nestin, CD13, CD29, CD34, CD44, CD54, CD90, CD105, CD106, HLA-ABC, HLA-DR, and PCNA was carried out. The labeled cells retained osteogenic differentiation capacity. The results suggest that fluorescent dye Dil and microspheres from Bangs Laboratories can be used for monitoring of mesenchymal stem cells from human amnion in *in vivo* experiments.

Key Words: *mesenchymal stem cells; placenta; microparticles; phenotype; differentiation*

At present, many cell types rated among stem cells are planned for therapeutic administration [7]. Among these cells, a special place is occupied by mesenchymal stem cells (MSC) isolated from adult organism [9]. These multipotent cells can differentiate into various cell types including bone, adipose, cartilage, nervous, and muscular cells and stromal cells of the bone marrow [3,5], and exhibit unique immunomodulating properties: they suppress proliferation of lymphocytes *in vitro* [10] and promote engrafting of the skin transplant *in vivo* [4]. Postmorten and vital monitoring of cells with preliminary evaluation of the effect of labels on cell nativity, *i.e.* proliferative activity, phenotype, and differentiation, is an actual problem in *iv vivo* experiments, because maintenance of cells in the non-differentiated state is an obligatory condition for their therapeutic use.

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Here we evaluated the possibility of using fluorescent dye Dil and microspheres from Bangs Laboratories for detection of cell localization in *in vivo* experiments.

MATERIALS AND METHODS

We used DMEM-F12 growth medium, 100× solution of streptomycin, penicillin, and glutamine (Gibco), fetal calf serum (FCS, HyClone), trypsin, versen, Hanks solution, PBS (PanEko), Tween-20, DAPI (Sigma), mouse antibodies to human nestin, fibronektin, CD29, CD34, CD44, CD54, CD90, CD105, CD106, PCNA (proliferating cell nuclear antigen), HLA-ABC, and HLA-DR ("Chemicon"), and Envision+System-HRP kit for detection of protein expression by peroxidase activity (DakoCytomation).

For isolation of MSC from the terminal human placenta, a fragment of the amnion (~10 cm^2) adjacent to the umbilical cord was used. The material was washed with Hanks solution and chopped to

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2-3-mm fragments. The amnion was then treated with 0.1% collagenase 1 and collagenase 4 at 37°C for 2 h , washed with Earl medium, and transferred to a cultural flask. Placental cells were grown on a Dulbeco modified Earl medium and DMEM/F12 (1:1 mixture). The medium contained 10% PBS, 10 U/ml penicillin, 10 U/ml streptomycin, and 2 mM glutamine. The cells were grown at 37°C and 5% CO_2.

After loading with Dil dye and microparticles, the harvested cells were placed in 10 ml warm Hanks solution (37°C) and 10 µl Dil was added (final concentration of the dye was 1 µg/ml). The tube was incubated for 10 min at 37°C and 20 min at 4°C. Then, the cells were washed two times in the growth medium, transferred to flasks, and cultured until confluence. For loading of 4-5 mln cells with microparticles, at least 200 microparticles per cell were taken. Then, the particles were suspended in 5 ml PBS containing 0.1% sodium azide, incubated at 22°C for 20 min, and centrifuged at 600*g* for 10 min. The microspheres were suspended in 1 ml PBS containing 5% dimethylsolfoxide, incubated at 22°C for 20 min, and added to 10 ml cell suspension. The mixture was incubated at 22°C for 1 h (the suspension was agitated every 15 min). The cells were then transferred to a cultural flask.

For induction of osteogenic differentiation, the cells were seeded onto coverslips. After attaining 80% confluence, the growth medium was replaced with a differentiation medium DMEM/F12 containing dexamethasone (1 µM), ascorbic acid (0.05 mM), and calcium glycerophosphate (10 mM). The cells were cultured for 3 weeks, the medium was replaced every 3 days. Immunocytochemical staining was performed using Envision+System-HRP kit according to manufacturer's instruction.

RESULTS

The cells obtained from the placental amnion had fibroblast-like morphology and were positively stained with antibodies to HLA-ABC, CD10, CD13, CD29, CD31, CD44, CD49b, CD54, CD55, CD90, CD105, CD106, and CD166 proteins. During the early passages, they had spindle shape, especially if the seeding density was low (Fig. 1). MSC isolated from the bone marrow and umbilical cord had similar morphology [1,7]. Morphological changes observed during long-term culturing were most probably related to cell aging (cells became more flattened). In the present work, we used cells at the 5th passage: at the final stages, the preparations contained cells of both morphological types.

Long-chain dialkylcarbocyanines (*e.g.* Dil) are the most potent fluorescent dyes; this fact together with lipophilic structure of their molecule prompts

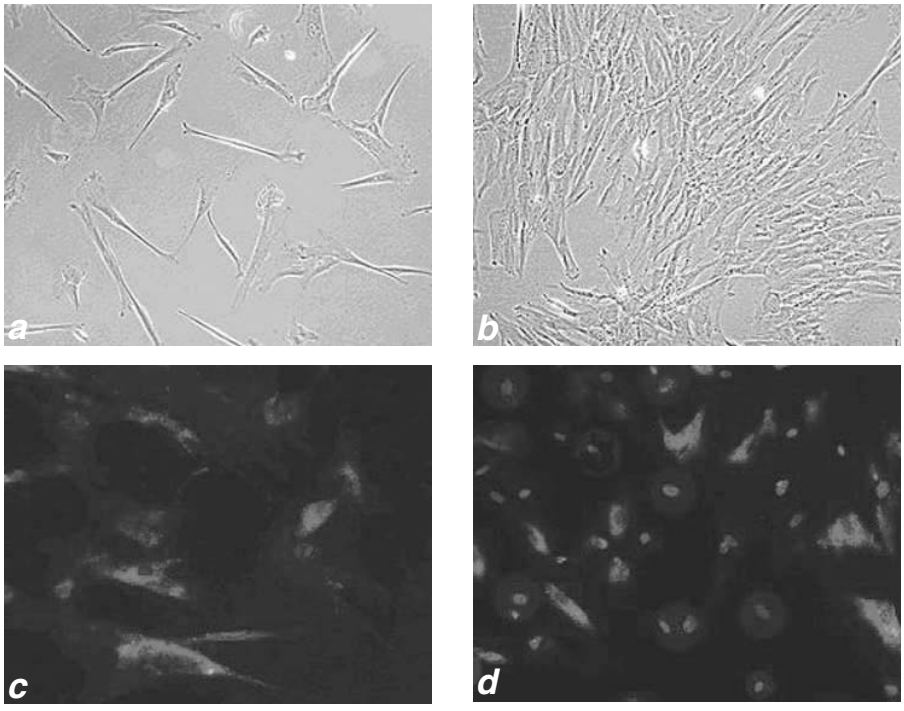

Fig. 1. Morphology and MSC from human amnion and distribution of fluorescent dye Dil. *a*) cells cultured with low seeding density; *b*) culture at 80% confluence; *c*) cells 1 day after addition of the dye; *d*) cells grown for 1 week after passage 1.

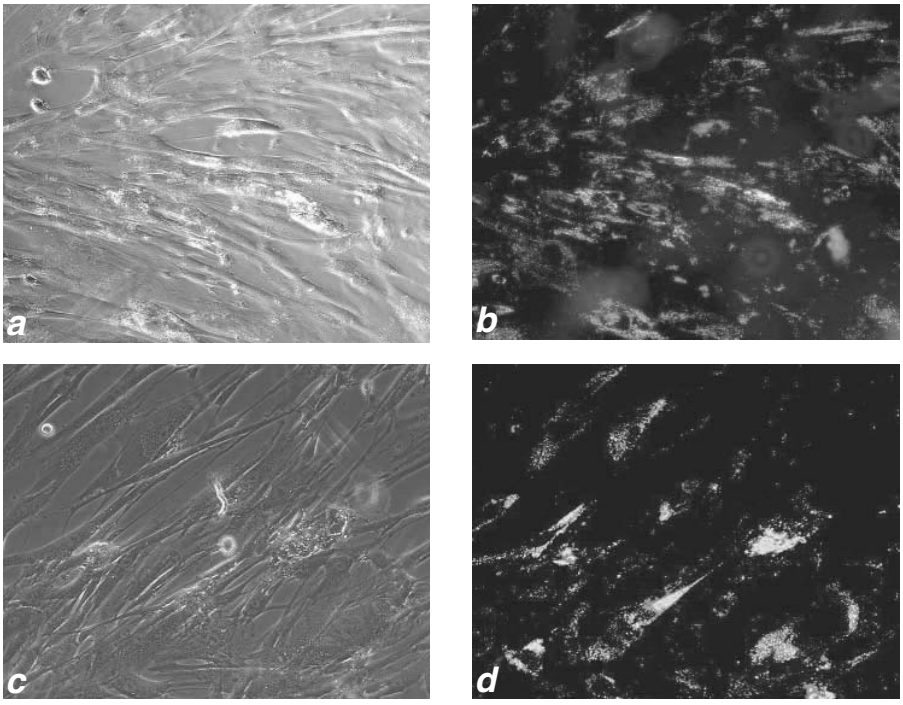

Fig. 2. Capture and retention of microparticles FC02F (0.21 μ; *a*, *b*) and FC03F (0.51 μ; *c*, *d*). *a*, *c*) light microscopy; *b*, *d*) fluorescent microscopy.

using these agents for long-term marking of viable cell structures [11]. All cells were effectively loaded and retained this dye (Fig. 1). During further culturing, Dil after cell division was distributed between the daughter cells; therefore, intensively dividing cells lost bright fluorescence (Fig. 1). Nevertheless, if the cell was highly loaded with Dil, the content of the dye in daughter cells remained sufficient for visualization by fluorescent microscopy. However, the decrease in fluorescence intensity due to cell division should be taken into account during postmortem examination of animals.

In the present study, we used different types of microspheres manufactured by Bangs Laboratories Company. Two types of microspheres were tested: 1) FC02F, FC03F are fluorescent microparticles with a diameter from 0.21 to 0.99 μ; 2) ME02F, ME03F, and MC05F are magnetic microspheres with a diameter from 0.90 to 1.630 μ carrying a fluorescent label. Both types of particles have the same fluorescent dye Dragon Green, an analog of fluorescein-5-isothiocyanate and a shell made of a polymer with carboxyl groups. There are published data that these types of particles are captured by cells in the form of endosomes and are concentrated in the perinuclear space [8].

Since the surface functional groups were similar in all microspheres, the efficiency of incorporation and retention depended only on particle size (Fig. 2, 3). The particles with a diameter of 0.21

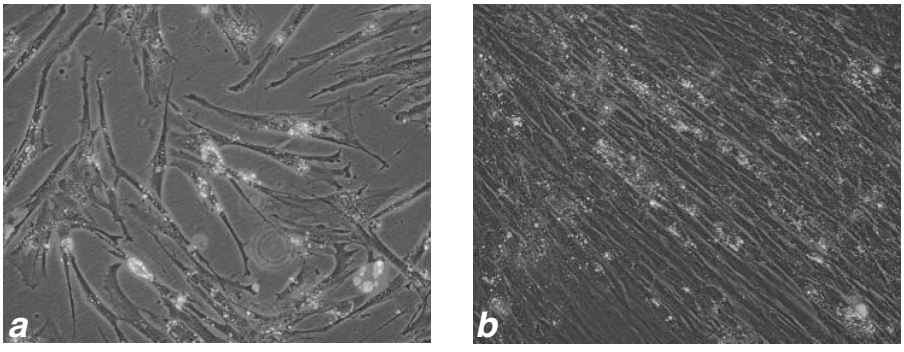

Fig. 3. Distribution of microparticles FC03F (0.51 μ) in 1 passage cells 1 day (*a*) and 1 week (*b*) after subculturing.

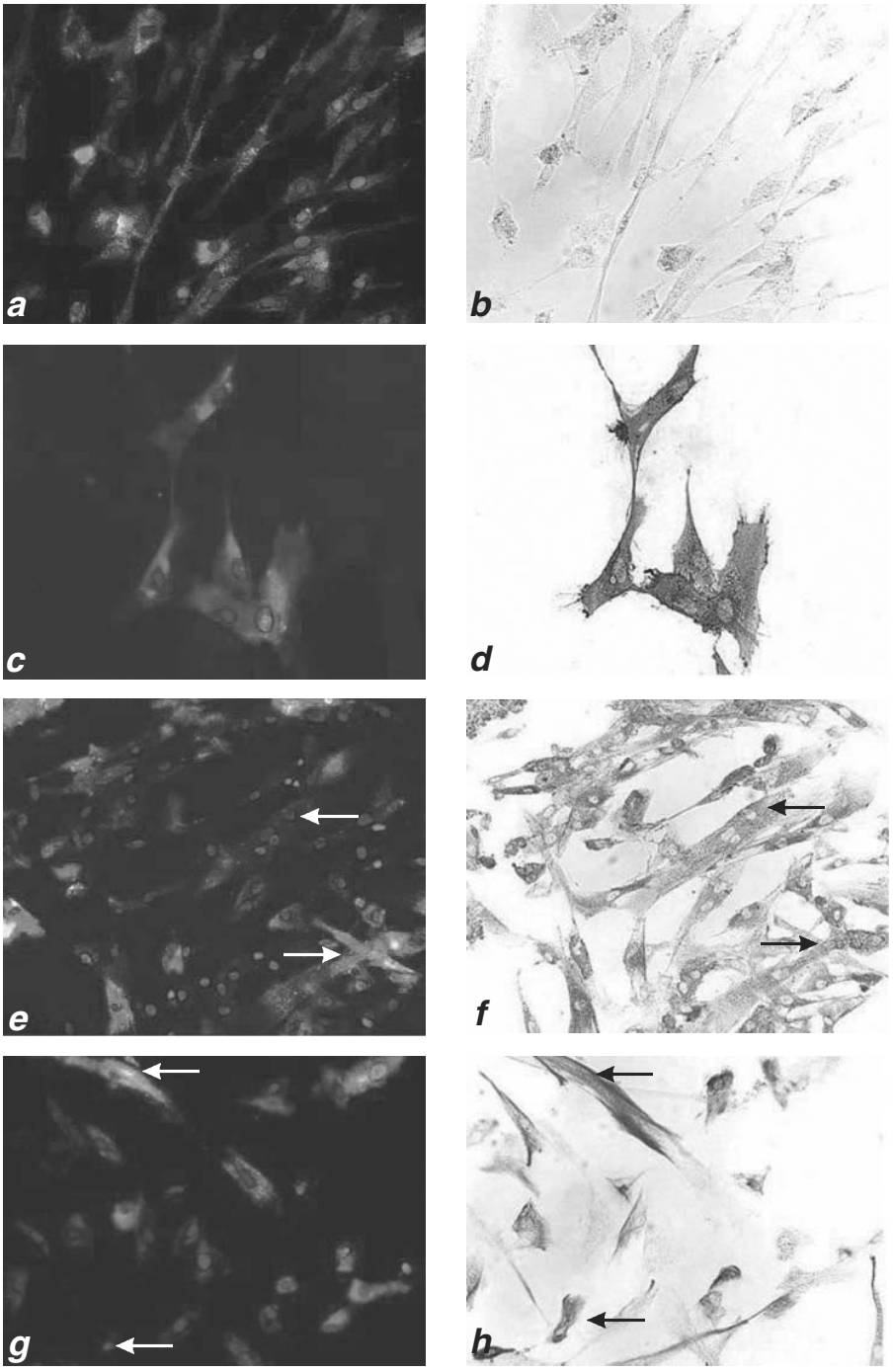

Fig. 4. Expression of HLA-DR (*a*, *b*), HLA-ABC (*c-f*) and nestin (*g*, *h*) in cells loaded with fluorescent dye DiI. *a*, *c*, *e*, *g*) DiI fluorescence; *b*, *d*, *f*, *h*) coloration reflecting the level of the expression of the test protein. *a-d*, *g*, *h*) cells seeded after loading with the dye; *e*, *f*) passage 1 cells grown to 70% confluence.

and 0.51 μ were captured best of all. The efficiency of incorporation decreased with increasing the particle diameter. The incorporated particles were well retained and were not lost during passaging. Thus, the use of DiI and microspheres did not complicate cell culturing and allowed evaluation of these agents on cell phenotype and differentiation potential.

Since DiI and fluorophore of particles are characterized by a wide fluorescence emission band, the most convenient method of evaluation of pro-

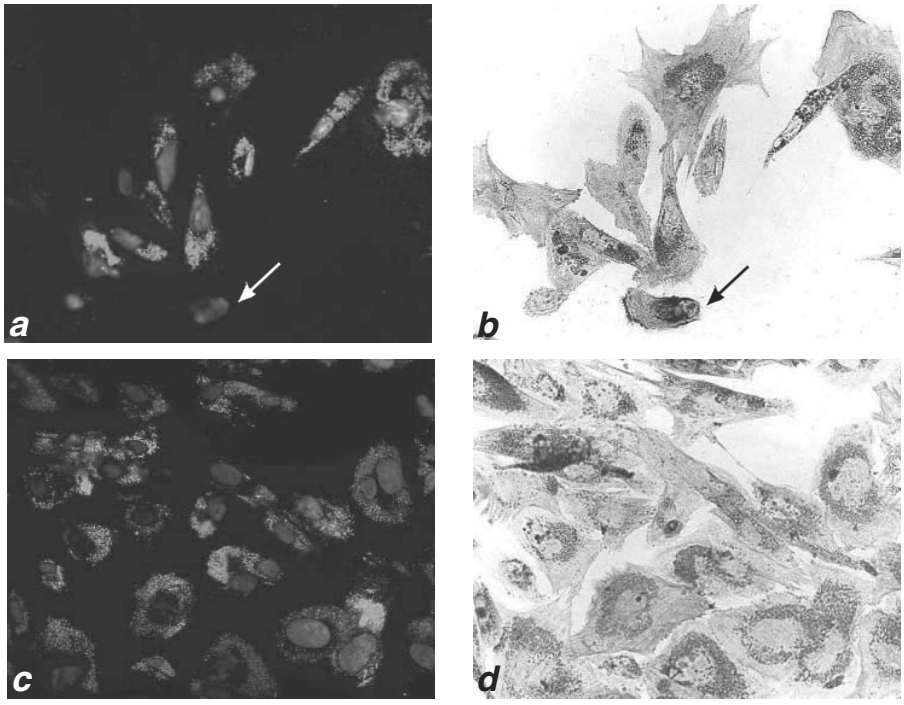

Fig. 5. Expression of CD90 (*a*, *b*) and CD54 (*c*, *d*) in cells containing ME02F microparticles (diameter 0.96 μ). *a*, *c*) light microscopy; *b*, *d*) fluorescent microscopy.

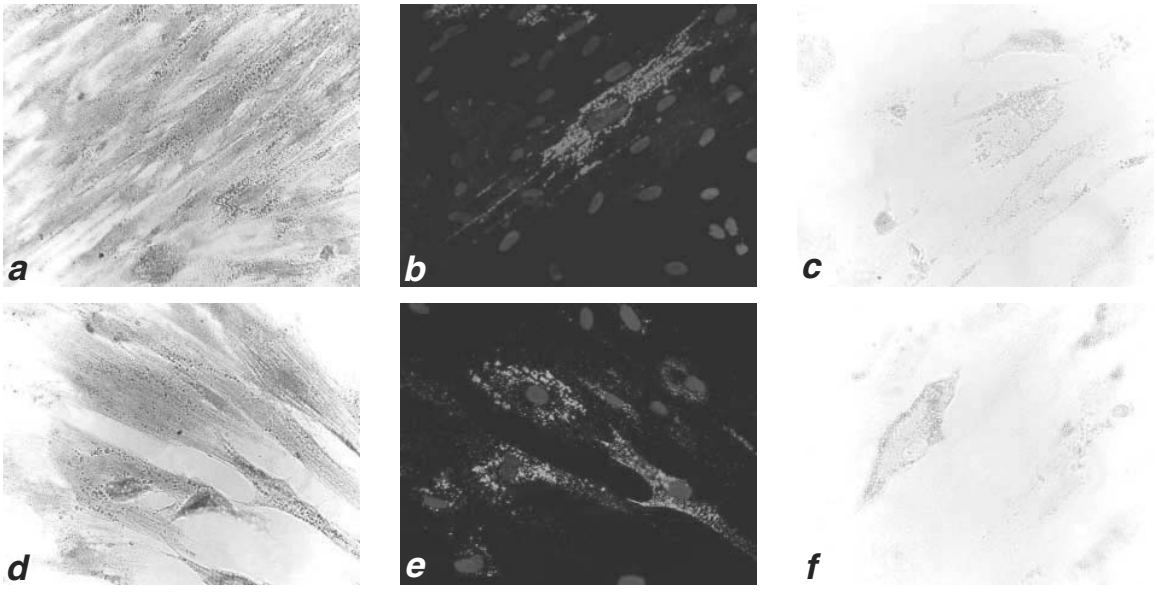

Fig. 6. Osteogenic differentiation (by the expression of osteonectin) of cells labeled with DiI (*a-c*) and FC03F microparticles (0.51 μ; *d-f*). *a*, *d*) peroxidase staining; *b*, *e*) fluorescence; *c*, *f*) control (cells grown without differentiation medium).

tein expression is color staining with peroxidase, the method used for the analysis of cell phenotype and osteogenic differentiation.

For the evaluation of the resistance of cell phenotype to loading with DiI and microparticles we used various surface and intracellular markers, some of these markers constitute the standard of mesenchymal nature of cells [7]. Expression of class I and class II major histocompatibility complex proteins HLA-ABC and HLA-DR, as well as CD13, CD29, CD44, CD54, CD90, CD105, CD106, hemopoietic marker CD34, and intracellular proteins fibronectin,

nestin, and PCNA was evaluated. This spectrum of proteins reflects mesenchymal nature of cells, while expression of nestin and PCNA reflects retention of non-differentiated status and proliferative activity, respectively. Both, short-term (1 day after loading) and delayed (1 week after passage 1) effects were studied.

Expression of HLA-ABC and HLA-DR proteins remained unchanged after loading with Dil (Fig. 4). Expression of HLA-DR was not detected (there were no staining), while expression of HLA-ABC was determined in cells 1 day after staining and was retained over 1 week after passage 1. The level of expression of this protein did not depend on the content of Dil in cells. In some cells, the content of Dil was close to the background value because of high proliferative activity, while other cells exhibited bright fluorescence. However, expression of HLA-ABC was pronounced in both cases. Immunocytochemical reaction with peroxidase conjugate served as the negative control: in this case, no staining was detected (similarly as in the absence of expression of HLA-DR and CD34 proteins. Cells not loaded with Dil and stained with the antibodies served as the positive control.

The cells expressed adhesion molecules CD29, CD44, CD54, CD106, (carrying important functions including regulation of cell differentiation and proliferation), CD13 (membrane glycoprotein exhibiting enzyme activity), CD105 (growth factor receptor), CD90, fibronectin, and PCNA. Some cells do not express nestin or expression of this marker was low. This can lead to erroneous conclusion that loading with Dil inhibited expression of nestin. However, we observed cells with high content of Dil and intensive expression of nestin, therefore the effect of the dye cannot be regarded as negative (Fig. 4). Thus, in none cases loading with fluorescent dye Dil affected the expression of vitally important proteins and nativity of cells.

Similar approach was used for evaluation of the effect of microspheres on cell phenotype. Despite the degree of cell loading and their proliferative activity depend on the size of the label, the expression of all markers in cells carrying different microparticles did not change compared to the control (cells loaded with fluorescent microspheres ME02F). Particles with a diameter of 0.96 μ are well discernible under a light microscope (Fig. 5).

Expression of markers does not depend on the content of microspheres in the cell preparation (Fig. 5). After cell division, cells with and without fluorescence are seen in the same field of view. This can be due to unequal labeling of cells during loading and the absence of microparticles in daughter cells. The label can be distributed unequally between the daughter cells. Thus, as in experiments with Dil, microspheres do not affect the expression of surface and intracellular markers reflecting functional status of the cell, its proliferative potential and non-differentiated status. However, the most valuable test for non-differentiated status of the cells is *in vitro* differentiation.

The properties of native cells should be carefully extrapolated for cells carrying foreign inclusions. Thus, the maintenance of differentiation capacity depends on cell characteristics, labeling technique, and the type of particles used [2]. That is why, verification of the differentiation potencies is an obligatory step in experiments with new types of labels. In our experiments, the capacity of cells loaded with the test agents for osteogenic differentiation was verified by the expression of osteonectin, a marker of osteogenic differentiation [7]. Osteonectin, a glycoprotein of the bone and dentin, is characterized by high affinity for type 1 collagen and hydroxyapatite; it contains Ca-binding domains and maintains the concentration of calcium and phosphorus in the presence of collagen. Similarly to bone sialoprotein, osteonectin is an important component of the extracellular matrix of the bone tissue. It is hypothesized that this protein participates in the cell-matrix interaction.

When cells enter the osteogenic differentiation, the number of cells carrying Dil or microspheres decreases due to asymmetrical distribution during division or higher proliferative activity of unlabeled cells, but the expression of osteonectin in both types of cells is similar (Fig. 6).

Thus, we showed that two types of cell labeling do not affect the phenotype MSC isolated from the amnion of human placenta and their differentiation potencies. Hence, Dil can be used as a tool for postmortem, and microspheres from Bangs Laboratories for vital study of the distribution of these MSC in laboratory animals. Our results give hope that investigators will get adequate picture of the therapeutic effect of MSC irrespective of whether these cells are labeled or not with the test agents.

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