# GENETICS

# Study of Genetic Stability of Human Bone Marrow Multipotent Mesenchymal Stromal Cells

**V. A. Nikitina, E. Yu. Osipova*, L. D. Katosova, S. A. Rumyantsev*, E. V. Skorobogatova*, T. V. Shamanskaya*, and N. P. Bochkov**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 150, No. 11, pp. 559-563, November, 2010
Original article submitted January 21, 2009

Immunophenotype, proliferation rate, and genetic stability parameters of bone marrow multipotent mesenchymal stromal cells were studied. Despite the reduction of proliferative activity by passages 11-12, the cells retained the characteristic immunophenotype. The incidence of spontaneous aneuploidy for autosomes 6, 8, 11 and sex chromosomes was evaluated. Two cultures of mesenchymal stromal cells carrying aneuploid cell clones were detected: with chromosome 8 trisomy and X chromosome monosomy. The results indicate the possibility of genetic transformation and selection of mesenchymal stromal cells with abnormal karyotype during *in vitro* culturing.

**Key Words:** *multipotent mesenchymal stromal cells; karyotype; aneuploidy; FISH*

Human bone marrow mesenchymal stromal cells (MSC) are characterized by great potentialities for clinical use as an independent or accessory cell therapy. These cells were used with good results in clinical trials in the treatment of osteogenesis imperfecta [4], metachromatic leukodystrophy [6,8], Hurler syndrome [6], and other diseases. The immunomodulating characteristics of MSC suggest their use as accessory cell therapy for alleviation of the graft-versus-host reaction [7]. In addition, they can serve as an extra source of cells in cases with insufficient levels of hemopoietic stem cells in the transplant or its incomplete engrafting. The interest to clinical use of MSC is explained also by their availability: they are easily isolated from the bone marrow or other tissues. Culturing and transplantation of the cells to the patient involve no problems, either technological or ethic. On the other hand, the safety of MSC culture injection as regards the probable genetic and oncogenic transformation of the cells has not been proven.

Genetic stability of MSC cultures intended for transplantation was intensively studied. The results are contradictory. Some authors showed that bone marrow MSC remain genetically stable throughout long culturing [2] even after reaching and surpassing the Hayflick limit [13]. Other authors indicate the possibility of spontaneous transformation of MSC isolated from the bone marrow and fatty tissue [9,10,14], which is proven by induction and formation of tumors from these cells in animals *in vivo* [10,12].

We studied the dynamics, immunophenotype, karyotype, and incidence of aneuploidy in MSC cultures during different periods of culturing.

## MATERIALS AND METHODS

**Isolation of MSC from the bone marrow and culturing.** The cells were isolated from healthy donor

Medical Genetic Research Center, the Russian Academy of Medical Sciences; *Federal Center of Pediatric Hematology, Oncology, and Immunology, the Russian Ministry of Health and Social Development, Moscow, Russia. ***Address for correspondence:*** vanikitina@mail.ru. V. A. Nikitina

0007-4888/11/1505-0627 

bone marrow, collected for allogenic transplantation to hematological patients, treated at Federal Center of Pediatric Hematology, Oncology, and Immunology.

Mononuclear cells isolated from the bone marrow were inoculated in ventilated culture flasks in DMEM with low glucose content with 20% FCS. Nonadherent cells were removed during medium replacement after 1-3 days. The cells were harvested from the plastic with Trypsin-EDTA after 14 days and reinoculated every 7 days until the end of culturing. Culturing was carried out at 37°C at absolute humidity and 5% $CO_2$. Starting from passage 3, MSC cultures were monomorphic and consisted of spindle-shaped cells (Fig. 1).

**Evaluation of proliferation rate and immunophenotype.** The dynamics of cell population growth was evaluated by cell increment multiplicity (number of cells obtained after current passage/number of cells inoculated at the previous passage). Surface markers were evaluated using a panel of antibodies to CD14, CD19, CD34, CD45, CD73, CD90, CD105, and HLA-DR. The percentage of cells expressing a certain antigen in MSC culture was evaluated.

**Fixation of MSC and cytogenetic preparation making.** Fixation of MSC for cytogenetic analysis was carried out on days 2-3 after the last passage. Colchicine in a concentration of 0.5 μg/ml was added to culture flasks, after 1.5 h the cells were harvested, incubated (8-10 min) in hypotonic solution, and fixed in methanol:glacial acetic acid (3:1) mixture. Cytogenetic preparations for karyotyping were prepared by G staining. At least 15 metaphases were analyzed for each MSC culture. Interphase FISH analysis was carried out using centromer-specific DNA probes for chromosomes X (DXZ1), Y (DYZ3), 6 (D6Z1), 8 (D8Z1), and 11 (D11Z1) (Abbott, Vysis). Denaturation, hybridization, and washout were carried routinely. The nuclei were contrasted with DAPI. A total of 500-1000 interphase nuclei were analyzed in each culture. Due to the use of the dual- and triple-color FISH analysis it was possible to evaluate the incidence of two or three chromosomes aneuploidies simultaneously.

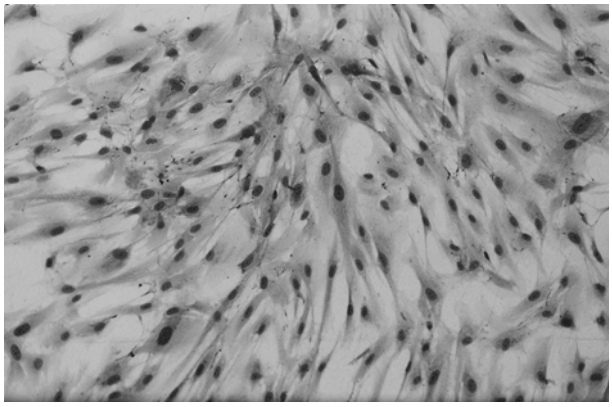

**Fig. 1.** Morphology of MSC culture. Hematoxylin and eosin staining, ×400.

The immunophenotype evaluation and cytogenetic study were carried out twice in the majority of MSC cultures: during passages 3-4 and 10-12.

## RESULTS

Proliferative activity of MSC at passages 3-4 (6-fold increment) was higher ($p$=0.01) than at passages 11-12 (2-fold increment).

High expression (>60%) of CD90, CD105, CD73 markers on bone marrow MSC was observed after 3-4 passages. Less than 5% stromal cells carried CD45, CD34, CD19, and CD45 markers (Table 1). With prolongation of MSC culturing to 10-12 passages, the percentage of cells expressing CD90 de-

**TABLE 1.** Changes in Surface Phenotype of Bone Marrow MSC during Long-Term Culturing *In Vitro*

| Surface marker | Antibody | Percentage of cells carrying the marker, median | | $p$ |
|---|---|---|---|---|
| | | passage 3-4 | passage 10-12 | |
| Monocyte marker | CD14 | 0.58 | 0.155 | ↓0.07 |
| B-cell marker | CD19 | 0.29 | 0.30 | 0.90 |
| Hemopoietic stem cell marker | CD34 | 0.46 | 0.64 | 0.67 |
| Leukocyte common antigen | CD45 | 2.75 | 1.30 | ↓0.049 |
| SH3 | CD73 | 97.50 | 98.50 | 0.34 |
| Thy-1 | CD90 | 67.29 | 45.27 | ↓0.05 |
| Endoglin, SH2 | CD105 | 69.6 | 57.71 | 0.44 |
| HLA-DR | Anti-HLA-DR | 1.24 | 0.36 | ↓0.07 |

**Note.** Arrows show significant differences between the percentage of cells at passages 3-4 and 10-12 (cell percentage decreased in all cases).

creased significantly, the admixture of hemopoietic cells and monocytes disappeared from cultures (Table 1). Hence, analysis of immunophenotype showed that all cell cultures during early and late passages could be referred to MSC in accordance with the requirements of International Society for Cellular Therapy [3,5].

In order to evaluate genetic stability parameters, comprehensive evaluation of aneuploidy incidence in interphase nuclei and of MSC karyotype were carried out. Cultures containing more than 10% cells with one chromosome abnormality were referred to clone-forming.

Karyotypes of 20 MSC cultures were analyzed. Eighteen cultures had normal karyotype (46,XY or 46,XX), which did not change during culturing (Fig. 2). Clones were detected in two cultures, which was confirmed by FISH analysis of interphase nuclei.

A clone with chromosome 8 trisomy was detected in one MSC culture (Fig. 3). The incidence of aneuploidy and karyotype of this culture evaluated three times. At passage 4, 24% analyzed cells contained an extra chromosome 8. At passage 6, the number of cells with chromosome 8 trisomy increased to 34%, but by passage 12 their relative content decreased to 16%. The conditions of cell culturing and reinoculation were similar, and hence, changes in the clone size can indicate different rate of division during culturing and more rapid proliferation and aging processes in aneuploid compared to diploid cells. In the other case a cell clone carrying one sex chromosome X was detected in MSC culture derived from normal female bone marrow. Twelve percent aneuploid cells were found at passage 4 and at passage 10 their content reached 91%.

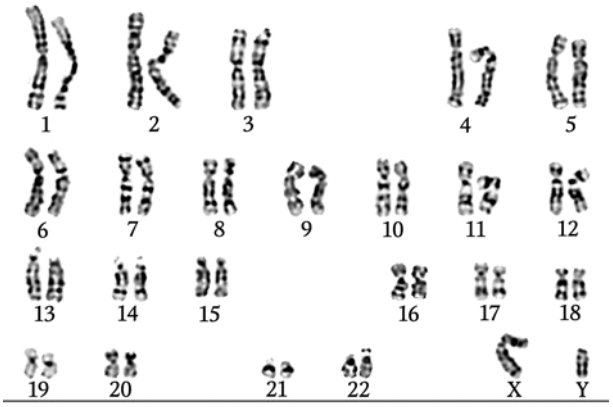


**Fig. 2.** Karyotype of MSC from a healthy donor (46,XY).

Hence, the clones were found at the early passages and were retained until late stages of culturing. The clones could originate from abnormal cells of

**TABLE 2.** Incidence of Autosome 6, 8, 11 Aneuploidy in MSC during Early (3-4) Passages

| MSC culture No. | Chromosome 6 | | Chromosome 8 | | Chromosome 11 | |
|---|---|---|---|---|---|---|
| | incidence | | incidence | | incidence | |
| | monosomy, % | trisomy, % | monosomy, % | trisomy, % | monosomy, % | trisomy, % |
| 1 | 0.51 | 0.58 | 0.10 | 0.00 | 1.15 | 0.29 |
| 2 | 0.59 | 0.39 | 1.69 | 0.00 | 0.59 | 0.00 |
| 3 | 0.50 | 0.00 | 0.46 | 0.09 | 0.20 | 0.00 |
| 4 | 1.25 | 0.29 | 0.29 | 0.10 | 1.64 | 0.00 |
| 5 | 0.93 | 0.00 | 0.78 | 0.19 | 3.23 | 0.38 |
| 6 | 1.64 | 0.48 | 0.31 | 0.31 | 1.21 | 0.22 |
| 7 | 0.49 | 0.10 | 0.87 | 0.48 | 0.00 | 0.39 |
| 8 | 0.59 | 0.10 | 0.59 | 0.00 | 0.59 | 0.29 |
| 9 | 0.44 | 0.55 | 0.59 | 0.10 | 1.30 | 0.95 |
| 10 | 0.68 | 0.20 | 0.89 | 0.00 | 1.61 | 0.85 |
| 11 | 0.95 | 0.19 | 1.37 | 0.20 | 2.56 | 0.09 |
| 12 | 0.97 | 1.46 | 1.24 | 0.10 | 0.19 | 0.00 |
| 13 | 0.96 | 1.73 | 0.97 | 0.49 | 0.90 | 0.10 |
| 14 | 2.36 | 0.59 | 1.15 | 0.96 | 1.65 | 0.24 |
| *M*±*m* | 0.92±0.14 | 0.48±0.14 | 0.81±0.12 | 0.22±0.07 | 1.20±0.24 | 0.27±0.08 |

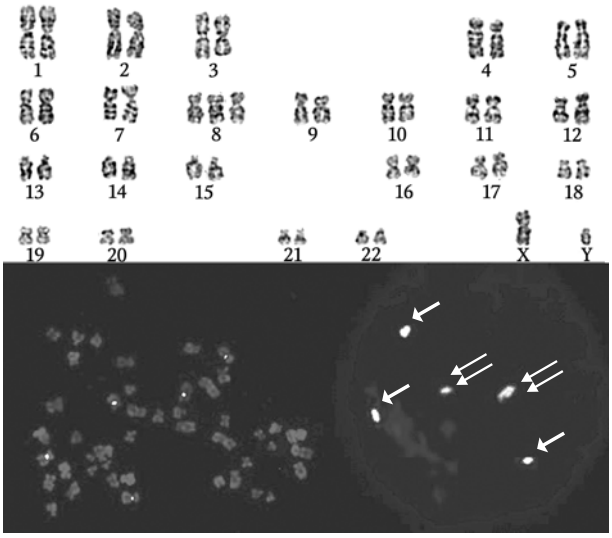


**Fig. 3.** Chromosome 8 trisomy in bone marrow MSC: karyotyping (*a*) and FISH analysis (*b*). Arrows: chromosome 8 replicas; double arrows: chromosome 6 replicas.

donor bone marrow, for which no apoptosis mechanism had been triggered *in vivo*, or from aberrant cells emerging *in vitro* during isolation from the bone marrow and during early stages of MSC culturing.

The incidence of aneuploidy by autosomes 6, 8, 11 and sex chromosomes in MSC are presented in Tables 2, 3, and 4. A total of 60,000 interphase nuclei were analyzed at different passages.

The incidence of chromosomes 6, 8, and 11 monosomy and trisomy did not differ ($p>0.05$) and was 1 and 0.3%, respectively (Table 2). Monosomic nuclei predominated over trisomic ones. These data are in line with our previous data on MSC isolated from the adipose tissue [1]. The incidence of the studied autosome aneuploidy did not change over the course of MSC culturing ($p>0.05$; Table 3). However, comparison of aneuploidy levels in chromosomes 8 and 11 at late passages showed that the incidence of chromosome 11 monosomy was higher than the incidence of chromosome 8 monosomy (Table 3). Since the levels of trisomy by these chromosomes did not differ, we cannot speak about different contribution of individual chromosomes to the formation of chromosome aberrations. This fact most likely indicates the need in accumulation of more representative scope of data on the incidence of aneuploidy by different chromosomes. The incidence of aneuploidy by other chromosomes did not differ.

Spontaneous incidence of chromosome Y loss in MSC derived from male donors was higher than X chromosome loss ($p<0.05$; Table 4). According to our observations, X chromosome nullisomy is an extremely rare phenomenon and can be related to detec-

**TABLE 3.** Incidence of Autosome 6, 8, 11 Aneuploidy in MSC during Late (10-12) Passages

| MSC culture No. | Chromosome 6 | | Chromosome 8 | | Chromosome 11 | |
|---|---|---|---|---|---|---|
| | incidence | | incidence | | incidence | |
| | monosomy, % | trisomy, % | monosomy, % | trisomy, % | monosomy, % | trisomy, % |
| 1 | 0.28 | 1.23 | 0.39 | 0.20 | 1.26 | 0.00 |
| 2 | 0.10 | 0.20 | 0.45 | 0.00 | 0.68 | 0.29 |
| 3 | 0.44 | 0.11 | 0.39 | 0.10 | 0.56 | 0.23 |
| 4 | 1.00 | 0.00 | 0.67 | 0.29 | 0.68 | 0.58 |
| 5 | 1.55 | 0.48 | 0.49 | 0.39 | 0.77 | 0.00 |
| 6 | 0.00 | 0.10 | 0.40 | 0.00 | 1.51 | 0.34 |
| 7 | 0.87 | 0.77 | 1.16 | 0.29 | 1.55 | 0.29 |
| 8 | 0.59 | 0.10 | 0.30 | 0.10 | 3.62 | 0.12 |
| 9 | 0.59 | 0.00 | 1.26 | 0.48 | 2.41 | 0.00 |
| 10 | 2.79 | 0.48 | 1.17 | 0.00 | 3.68 | 0.36 |
| 11 | 1.05 | 0.48 | 1.17 | 0.00 | 0.67 | 0.57 |
| 12 | 1.72 | 0.48 | 1.44 | 0.10 | 1.34 | 0.48 |
| *M*±*m* | 0.92±0.23 | 0.37±0.10 | 0.77±0.12* | 0.16±0.05 | 1.56±0.32* | 0.27±0.06 |

**Note.** Here and in Table 4:*$p<0.05$.

**TABLE 4.** Mean Incidence of Sex Chromosome Aneuploidy in MSC

| Cells | Early passages | | Late passages | |
|---|---|---|---|---|
| | nullisomy | disomy | nullisomy | disomy |
| Male | | | | |
| X chromosome | 0.13±0.05* | 1.36±0.19 | 0.01±0.01 | 2.00±0.43 |
| Y chromosome | 0.56±0.15* | 0.97±0.18 | 0.32±0.17 | 1.68±0.40 |
| Female | | | | |
| X chromosome | 1.30±0.31 | 0.21±0.10 | 0.68±0.29 | 0.19±0.03 |

tion of solitary events of the only X chromosome loss or because of hybridization artifact. The incidence of chromosomes X and Y disomies did not change after long culturing and did not differ (Table 4). Analysis of X chromosome aneuploidy in MSC cultures derived from women showed higher incidence of monosomy *vs.* trisomy, similarly as for autosomes ($p<0.05$). Comparison of the incidence of sex chromosome aneuploidy at the early and late stages revealed no differences. The incidence of autosome and sex chromosome aneuploidies did not differ and presumably characterized spontaneous level of aneuploidy in MSC.

Hence, MSC karyotype and aneuploidy level of the majority of MSC cultures in our study did not change even after long-term culturing. In some cultures, clones with abnormal karyotype were detected at the early passages. The immunophenotype of these cultures did not change, while the capacity to clone formation, intrinsic for MSC, led to formation of clones.

It is known that chromosome aberrations characterize genetic instability of the culture, which can lead to malignant transformation. However, some authors think that cultures with karyotypical changes are not hazardous for transplantation. Clones with various chromosome aberrations most likely differ by their potential hazards. A cell clone with one sex chromosome X can be considered as safe as regards its oncogenic hazard. After transplantation these cells will most likely restore the capacity to hemopoiesis or will compensate for the enzyme deficiency. As for clone with chromosome 8 trisomy, it can potentially promote the development of tumor. Chromosome 8 trisomy is one of the most incident aberrations in malignant myeloid diseases; it is detected as the only chromosome abnormality in 8-15% patients [15]. Cells with chromosome 8 trisomy are resistant to chemotherapy [11] and the lifespan of patients with chromosome 8 trisomy is shorter than of patients with the normal karyotype [15]. The biological role of chromosome 8 trisomy in the pathogenesis of tumors can be related to greater number of copies and more intense expression of C-*MYC* oncogene located on chromosome 8 long arm.

At present, evaluation of genetic stability is not included in the protocol of obligatory pretransplantation procedures. Further studies in this direction will lead to creation of methodological approaches to rapid comprehensive evaluation of genetic stability of transplanted cells, for detection and characterization of aberrant clones with potential oncogenic characteristics before their clinical application.

The study was supported by the Russian Foundation for Basic Research (grant No. 09-04-00948-a).

## REFERENCES

1. N. P. Bochkov, V. A. Nikitina, O. A. Buyanovskaya, *et al.*, *Byull. Eksp. Biol. Med.*, **146**, No. 9, 320-323 (2008).
2. M. E. Bernardo, N. Zaffaroni, F. Novara, *et al.*, *Cancer Res.*, **67**, No. 19, 9142-9149 (2007).
3. M. Dominici, K. Le Blanc, I. Mueller, *et al.*, *Cytotherapy*, **8**, No. 4, 315-317 (2006).
4. E. M. Horwitz, P. L. Gordon, W. Koo, *et al.*, *Proc. Natl. Acad. Sci. USA*, **99**, No. 13, 8932-8937 (2002).
5. E. M. Horwitz and W. R. Prather, *Isr. Med. Assoc. J.*, **11**, No. 4, 209-211 (2009).
6. O. N. Koç, J. Day, and M. Nieder, *Bone Marrow Transplant.*, **30**, No. 4, 215-222 (2002).
7. K. Le Blanc, F. Frassoni, L. Ball, *et al.*, *Lancet*, **371**, 1579-1586 (2008).
8. N. Meuleman, G. Vanhaelen, T. Tondreau, *et al.*, *Haematologica*, **93**, No. 1, e11-e13 (2008).
9. L. A. Meza-Zepeda, A. Noer, J. A. Dahl, *et al.*, *J. Cell. Mol. Med.*, **12**, No. 2, 553-563 (2008).
10. D. Rubio, S. Garcia, M. F. Paz, *et al.*, *PLoS One*, **3**, No. 1, e1398 (2008).
11. M. Schaich, R. F. Schlenk, H. K. Al-Ali, *et al.*, *Haematologica*, **92**, No. 6, 763-770 (2007).
12. N. Serakinci, P. Guldberg, J. S. Burns, *et al.*, *Oncogene*, **23**, No. 29, 5095-5098 (2004).
13. T. Soukup, J. Mokry, J. Karbanova, *et al.*, *Acta Medica*, **49**, No. 1, 27-33 (2006).
14. Y. Wang, D. L. Huso, J. Harrington, *et al.*, *Cytotherapy*, **7**, No. 6, 509-519 (2005).
15. S. R. Wolman, H. Gundacker, F. R. Appelbaum, *et al.*, *Blood*, **100**, No. 1, 29-35 (2002).