



## Revista Brasileira de Hematologia e Hemoterapia Brazilian Journal of Hematology and Hemotherapy

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### Scientific comment

## Genetic evaluation of mesenchymal stem cells



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The use of mesenchymal stem cells/mesenchymal stromal cells (MSCs) in regenerative medicine is increasing throughout the world. These cells can easily be isolated from different tissues and expanded *in vitro* to produce a sufficient number of cells that do not have infusion-related toxicity in humans during cell therapy. Moreover, they have the ability of multilineage differentiation and secrete ‘therapeutic’ factors that possess trophic, immunomodulatory, and anti-inflammatory activities and are unable to form teratomas.<sup>1</sup> Today, due to these characteristics, there are 385 MSC-based clinical trials registered at ClinicalTrials.gov, a service provided by the U.S. National Institutes of Health ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

However, there are substantial ambiguities and uncertainties in the literature concerning the genetic stability of these cells during *in vitro* expansion, and before implantation in the patient.<sup>2,3</sup> The reported *ex vivo* spontaneous malignant transformation of MSCs is limited to only two scientific articles,<sup>4,5</sup> with other authors reporting no tumor formation after *in vitro* manipulation, but stating that culture expansion induces recurrent genomic alterations, mainly aneuploidies, which rapidly accumulate in cultures. Thus, it is important to monitor the integrity of MSCs before their clinical use.<sup>2,6–10</sup> Other researchers did not find genetic alterations and say that if they do happen they will disappear during culture propagation and will not have any growth advantage *in vitro* and thus will not be a problem in clinical application, thereby concluding that MSCs have low risk of tumorigenicity.<sup>3,11–16</sup>

One of the articles published this year in the *Revista Brasileira de Hematologia e Hemoterapia* contributes to this discussion.<sup>17</sup> The article entitled “Genetic evaluation of mesenchymal stem cells through G-band karyotyping in a Cell

Technology Center” shows the G-band cytogenetic methodology performed at the Pontifícia Universidade Católica do Paraná (PUCPR) and the results of the first three years of genetic evaluation of MSC derived from the bone marrow of 21 patients. The main adaptations made by Borgonovo et al.<sup>17</sup> to achieve G-banded chromosomes from MSCs were: adjusting the time of colcemid action (from 2 to 6 h), using additional washes with higher concentrations of acetic acid in the fixative solution (2 methanol: 1 acetic acid), and alterations in the trypsin concentration (at least 0.002 g/mL) and time of exposure (from 5 to 20 s). On analyzing the cells before and after cell expansion at least until the second passage (P2), the authors concluded that the cells are chromosomally stable as no clonal alterations were found. However, they reported the presence of some signs of chromosomal instability (chromatid gaps and breaks, and tetraploidy) suggesting that “long term cultivation could provide an intermediate step for tumorigenesis”.<sup>17</sup>

This is an important Brazilian initiative that must be taken into account in all Cell Technology Centers; this kind of article leads us to think about some specific issues about the genetic evaluation of MSCs and the need for discussion, harmonization and regulatory points. By analyzing the articles concerning the genetic stability of MSCs, it highlights the existence of several variables such as cell origin, use of different protocols to isolate and expand MSCs, time of expansion and techniques used to analyze the stability. There are a lot of techniques that can be used to assess genetic stability of MSCs, including conventional karyotyping, spectral karyotyping (SKY), fluorescent *in situ* hybridization (FISH), genome-wide array comparative genomic

DOI of original article: <http://dx.doi.org/10.1016/j.bjhh.2014.03.006>.

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<http://dx.doi.org/10.1016/j.bjhh.2014.05.014>

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hybridization (aCGH), cytokinesis-block micronucleus assay (CBMN), microsatellite genotyping, single nucleotide polymorphism detection, RNA sequencing and detecting chromosomal aberrations based on gene expression patterns of these cells.<sup>4-19</sup> Each one of these methodologies has its degree of accuracy, sensitivity and limitations. These variations suggest an inexistence of regulation. The European Regulatory Authorities proposed that conventional karyotypes should be analyzed to ensure the safety of MSC production, and that aCGH or FISH are only necessary when recurrent abnormalities are found.<sup>20</sup>

In Brazil, the National Agency for Sanitary surveillance (ANVISA) published a resolution in 2011 that says that "Genetic control should be performed in stem cells when they are cultured and expanded *in vitro* or genetically modified". However, there is no specification of the method to be used and several questions arise such as: How many metaphases should be analyzed? What is the quality of the chromosomes that should be considered? What kind of alteration should be considered 'dangerous', not random nor related to methodological problems? Should the criteria determined in the International System of Cytogenetic Nomenclature (ISCN) related to abnormalities commonly seen in neoplasias be the same for MSCs? Are cytogenetic alterations randomly acquired after the *in vitro* expansion of MSCs or are specific chromosomes more prone to instability in MSCs? The human karyotype analysis requires extensive personnel training. Currently, in Brazil most cytogenetic tests are conducted within hospitals, and accreditations and validation that a testing laboratory should obtain to ensure the validity of the cytogenetic results do not exist. The scientific and clinical community must discuss and try to determine these aspects, as MSCs are already being used in regenerative medicine and, sometimes, sold as therapeutic products.

Ben-David et al.<sup>7</sup> presented a comprehensive analysis of chromosomal aberrations in 144 samples of MSCs and found that common aberrations in MSC cultures resembled characteristic aberrations of mesenchymal tumors, such as monosomy 13 (with downregulation of the retinoblastoma tumor suppressor gene - RB1) that was recurrent in independent studies of MSC cultures and is a common monosomy in bone and soft tissue tumors.<sup>21</sup> The associations can imply that specific aberrations at least confer growth advantage in a cell lineage-specific manner, both for stem cells *in vitro* and tumors *in vivo*, or may indicate the beginning of a spontaneous transformation in culture.<sup>2</sup> On the other hand, Tarte et al.<sup>12</sup> identified, by conventional cytogenetic analysis, donor-dependent chromosomal abnormalities in five out of 20 MSC cultures isolated from bone marrow, including trisomies of chromosomes 5, 8 and 20, that disappeared in almost all cultures at P2. Trisomies of chromosomes 5, 7 and 9 were identified in two out of seven MSC samples analyzed by Redaelli et al.<sup>22</sup> Both teams of researchers concluded that negative selection of aneuploid clones might occur in later passages, indicating a general chromosomal stability of MSCs. However, what calls attention is that the authors did not discuss the fact that trisomies of chromosomes 5, 7 and 20 are frequently found in myeloid and lymphoid malignancies and both originate from the leukemogenesis process that takes place in the bone marrow.<sup>21</sup>

Another important point to consider in stem cell therapy is the genetic stability of these cells after cryopreservation. Cryopreservation is necessary when the use of these cells will not occur immediately after their isolation, and so banks of MSCs are being created all around the world, but the research on chromosome instability is not proportional to this increase. Almost 2300 studies were performed with cryopreservation related to stem cells but the majority of them look at the maintenance of stem cell properties (<http://www.ncbi.nlm.nih.gov/pmc/?term=cryopreservation+stem+cell> by 03/13/2014). Only five articles were found addressing the question of MSC cytogenetic instability (<http://www.ncbi.nlm.nih.gov/pubmed/?term=mesenchymal+stem+cell+cytogenetic+cryopreservation> by 03/13/2014). Our group wrote one of these articles,<sup>23</sup> and similar to the results obtained by Borgonovo et al.,<sup>17</sup> we found non-clonal chromosomal alterations in MSCs derived from the umbilical cord vein after cryopreservation. We believe it is important to describe them because they are correlated to overall genetic instability. Of the other articles, only one described a non-clonal chromosomal alteration,<sup>24</sup> but there are a lot of differences between all these papers, making it difficult to directly compare the results. The variables involved are the origins of the MSCs, the method of cryopreservation, time of conservation and number and quality of metaphases analyzed. In addition, there was no description of the criteria used to determine chromosomal alterations. The methods of cryobanking of human stem cells are not fully defined, which is particularly critical for the umbilical cord cryopreservation process where the cells will be stored for an indeterminate time span; at least G-band chromosome analysis should be performed for each sample after thawing and before implanting the cells into patients.

In conclusion, we agree with Borgonovo et al.<sup>17</sup> and confirm conventional karyotyping as an useful and valuable tool to analyze chromosomal stability during *in vitro* expansion of MSCs. Complementary molecular cytogenetic techniques such as FISH and aCGH could be used to help to detect low mosaicism and to increase the sensitivity of genetic stability analysis. It is important to establish the biosafety profile of MSCs prepared for use in regenerative medicine, in particular it is necessary to identify and define culture conditions during *in vitro* MSC expansion, in order to avoid the occurrence of chromosomal abnormalities.

The importance and applicability of MSCs is undeniable; however their biological characteristics must be well studied and the risk-benefit ratio linked to their clinical use must be well defined. Rules must be established to standardize the genetic stability analysis and guarantee better biosafety with the use of these cells.

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## Conflicts of interest

The authors declare no conflicts of interest.

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