Asymmetric Aneuploidy in Mesenchymal Stromal Cells Detected by In Situ Karyotyping and Fluorescence In Situ Hybridization: Suggestions for Reference Values for Stem Cells

Seon Young Kim,¹ Kyongok Im,² Si Nae Park,² Jiseok Kwon,² Jung-Ah Kim,¹ Qute Choi,¹ Sang Mee Hwang,^{1,3} Sung-Hee Han,⁴ Sunghoon Kwon,⁵ Il-Hoan Oh,⁶ and Dong Soon Lee^{1,2}

Cytogenetic testing is important to ensure patient safety before therapeutic application of mesenchymal stromal cells (MSCs). However, the standardized methods and criteria for the screening of chromosomal abnormalities of MSCs have not yet been determined. We investigated the frequency of cytogenetic aberrations in MSCs using G-banding and fluorescence in situ hybridization (FISH) and suggest reference values for aneuploidy in MSCs. Cytogenetic analysis was performed on 103 consecutive cultures from 68 MSCs (25 adipose-origin, 20 bone marrow-origin, 18 cord blood-origin, and 5 neural stem cells; 8 from adipose tissue of patients with breast cancer and 60 from healthy donors). We compared the MSC aneuploidy patterns with those of hematological malignancies and benign hematological diseases. Interphase FISH showed variable aneuploid clone proportions (1%–20%) in 68 MSCs. The aneuploidy patterns were asymmetric, and aneuploidy of chromosomes 16, 17, 18, and X occurred most frequently. Clones with polysomy were significantly more abundant than those with monosomy. The cutoff value of maximum polysomy rates (upper 95th percentile value) was 13.0%. By G-banding, 5 of the 61 MSCs presented clonal chromosomal aberrations. Aneuploidy was asymmetric in the malignant hematological diseases, while it was symmetric in the benign hematological diseases. We suggest an aneuploidy cutoff value of 13%, and FISH for aneuploidy of chromosomes 16, 17, 18, and X would be informative to evaluate the genetic stability of MSCs. Although it is unclear whether the aneuploid clones might represent the senescent cell population or transformed cells, more attention should be focused on the safety of MSCs, and G-banding combined with FISH should be performed.

Introduction

MESENCHYMAL STROMAL CELLS (MSCs) have attracted great interest for their potential use in cell therapy and tissue engineering. An expanding number of clinical trials has been conducted to examine the potential therapeutic applications of MSCs. However, the clinical use of MSCs is still controversial, due to concerns about their safety [1–3]. The most important concern is the tumorigenesis potential of the MSCs [4–7]. Chromosomal aberration is one of the hallmarks of human cancer, and therefore, it is important to evaluate the chromosomal stability and variability of MSCs before they are used in clinical applications [8]. Several studies have reported

chromosomal aberrations in cultured MSCs. The European Medicine Agency determined that the cytogenetic abnormalities of MSCs should be assessed [3,9].

There is a wide range of techniques that are used to assess the cytogenetic status, including conventional karyotyping, spectral karyotyping, fluorescence in situ hybridization (FISH), array comparative genomic hybridization (CGH), and microsatellite genotyping. From a regulatory point of view, the types of techniques that should be used to assess MSCs and the cutoff values to ensure the safety of MSCs deserve further discussion. Each technology has its advantages and pitfalls, including different sensitivities and costs. The conventional karyotyping method is the most basic and fundamental technique used to evaluate whole chromosomes. However, it is the least sensitive

¹Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea.

²Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea.

³Department of Laboratory Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea.

⁴Division of Molecular Genetics, Department of Laboratory Medicine, Seoul Medical Science Institute, Seoul Clinical Laboratories, Seoul, Republic of Korea.

⁵Department of Electrical and Computer Engineering, Inter-University Semiconductor Research Center, Center for Nanoparticle Research Institute for Basic Science, Seoul National University, Seoul, Republic of Korea.

⁶Catholic High-Performance Cell Therapy Center, Research Center for Stem Cell Therapeutics Evaluation, The Catholic University of Korea, Seoul, Republic of Korea.

method and can only be used to test metaphase nuclei. However, most of the nuclei are in interphase; therefore, important information can be missed if only the karyotyping method is used. Meanwhile, FISH is another universally used cytogenetic technique that can detect structural abnormalities and aneuploidies. Using the FISH technique, it is possible to investigate hundreds of interphase nuclei. Other studies using alternative techniques, such as array-CGH or spectral karyotyping, have shown that these techniques can provide very useful information about the chromosomal abnormalities of MSCs. However, array-CGH is not a sensitive method and requires 20%–30% of the cells to be abnormal [10,11].

It is universally accepted that cytogenetic testing is essential before the MSCs are used in clinical trials to ensure patient safety; however, because there is little information about the cytogenetic characteristics of MSCs, except for some sporadic reports, we do not know the appropriate methods and criteria to assess their safety. The safest option may be to perform all available tests and exclude MSCs with even a few ambiguous abnormalities when using tests with the greatest sensitivity. In reality, the number of MSCs available for preclinical testing is generally low, and preclinical screening for safety cannot be too extensive. Unreasonably strict regulations for MSCs may hinder the clinical application of MSCs and the application of powerful therapeutic tools for the treatment of intractable diseases in the future. Furthermore, several previous studies showed that human MSCs usually do not transform during *ex vivo* expansion, even with aneuploidy that can appear during culture but is not related to the transformation *per se* [9,12,13]. However, we still do not know much about the possibility of transformation based on experience from more than 15 years of clinical trials on MSCs. Moreover, we do not know much about the risk of MSCs with cytogenetic abnormalities. Therefore, from a regulatory point of view, we need to establish screening guidelines for cytogenetic abnormalities of MSCs, which require a deeper investigation into the possible risk of transformation.

In this study, we selected two of the most widely used techniques for cytogenetic testing, conventional karyotyping and interphase FISH. These two cytogenetic tests were performed based on previous efforts to optimize MSC screening. For conventional karyotyping, we used the established *in situ* karyotyping technique, which is the standard method for amniotic fluid analysis [14]. For interphase FISH, previous studies reported that the most prevalent abnormalities found in MSCs are aneuploidies, and we used FISH to enumerate the whole chromosomes. We performed these cytogenetic tests on a wide variety of MSCs that were generated from different origins and cultured in different conditions, and we tried to establish criteria to distinguish the normal MSCs from the abnormal MSCs. Clonal abnormalities are defined by the International System for Human Cytogenetic Nomenclature (ISCN) criteria, such as at least two cells with the same chromosome gain or structural rearrangement or three or more cells with the same missing chromosome [15]. However, we still do not know whether the same criteria can be applied to the MSCs examined using *in situ* karyotyping. Additionally, although aneuploidy has been frequently reported in MSCs, cutoff values of aneuploidy have not been established for the interphase FISH method, which can generate higher levels of background tetraploidy signals in highly proliferating cells. To assist in our determination, we also evaluated the aneuploidy patterns of malignant and non-malignant bone marrow hematopoietic cells, as determined by

FISH, in patients with hematological malignancies and in patients without malignancies.

The aims of this study were to use *in situ* karyotyping and FISH techniques to detect chromosomal abnormalities and aneuploidy in primary MSCs, to determine the most effective method to screen MSCs for medical use and to determine the criteria for the selection of safe MSCs.

Materials and Methods

Isolation and culture of human MSCs

A total of 103 cultures of primary MSCs established from 68 donors in 5 different laboratories was analyzed in this study. The isolation and culture of the MSCs were conducted according to each laboratory's protocol; however, these processes were generally conducted according to standard protocols. The MSC types, according to the origins from the 68 donors, included 25 cultures of adipose-origin MSCs (ADSCs), 20 cultures of bone marrow-origin MSCs (BMSCs), 18 cultures of umbilical cord-origin MSCs (UCSCs), and 5 cultures of neural stem cells (NSCs). Eight MSC cultures were isolated from abdominal adipose tissue of patients with breast cancer during plastic surgery, and the remaining MSCs were isolated from healthy donors. There were four MSC cultures that underwent genetic manipulation, including transduction with the *Ngn1* (in two BMSCs) and *hTERT* (in two NSCs) genes. The MSCs were transferred to a central laboratory for cytogenetic analysis. The MSCs were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine and were cultured at 37°C with 5% CO₂. The MSCs were analyzed at different passage numbers, ranging from passage 1 (p1) to p13, with a median passage number of 4. The passage numbers of the UCSCs (median, 5; range 3–13) and NSCs (median, 9; range 4–11) were higher than those of the ADSCs (median 3; range, 1–7) and BMSCs (median, 4; 2–11). For 11 ADSCs, *in situ* karyotyping and FISH analysis were performed both before and after storage at –80°C for several months to 1 year. This study was in compliance with Declaration of Helsinki and all patients gave written informed consent. This study (sample collection, stem cell culture, and testing) was approved by the Institutional Review Board (IRB) of Seoul National University College of Medicine (IRB no. C-1008-012-326 and no. H-1103-129-357); IRB of the Catholic University of Korea St. Mary's Hospital (IRB no. KC11TNSI0186); and IRB of Asan Medical Center (IRB no. 2012-0283).

In situ karyotyping

The *in situ* conventional karyotyping technique, which uses cells grown on coverslips for G-banding, is the standard protocol for the chromosomal analysis of amniotic fluids [16,17]. Because this technique involves minimal cell manipulation and requires fewer cells, we used the *in situ* karyotyping technique to analyze the MSCs. In this study, we performed *in situ* karyotyping according to previously established procedures [14]. Approximately 1.5×10^5 MSCs were grown on 22 × 22 mm cover glass (Paul Marienfeld, Lauda-Königshofen, Germany) using the Mesenchymal Stem Cell Basal Medium (American Type Culture Collection, Manassas, VA) supplemented with Mesenchymal Stem Cell Growth Kit-Low serum (American Type Culture Collection). Once the cells reached 50%–70%

confluence, the cells were harvested for chromosome analysis. Colcemid solution was added at a final concentration of 0.1 $\mu\text{g}/\text{mL}$ (KaryoMAX Colcemid Solution; Invitrogen, Grand Island, NY), and the cells were incubated in a CO_2 incubator at 37°C for 40–50 min. The media was removed, and 2 mL of pre-warmed 0.075 M potassium chloride was added. The resulting mixtures were incubated in a CO_2 incubator at 37°C for 30 min. Then, 200 μL of Carnoy's fixative solution [3:1 (v/v) methanol/glacial acetic acid] was added to the edge of the cover glass at room temperature for 2 min. The solutions were removed, and 3 mL of Carnoy's solution was added and incubated at room temperature for 20 min. This step was repeated twice. The cover glass was mounted onto the glass slide with the cells facing downward. The slide was placed in a 56°C oven for 16 h and stained using Leishman's protocol [18]. All of the observed karyotypes were recorded, and clonal abnormalities according to the ISCN criteria were also recorded [19].

Interphase FISH analysis for MSCs

The numerical abnormalities were evaluated using oligo-FISH (OF4-0127-0100, OF4-0128-0100, OF4-0129-0100, OF4-0052-0100, OF4-0130-0100, BP4-0131-0100; Cellay, Cambridge, MA) in the interphase nuclei of MSCs according to the manufacturer's instruction. The oligo-FISH probe sets included five oligonucleotide probe cocktails (probes targeting chromosomes 3, 7, 12, and 16; chromosomes 2, 13/21, 18, and 20; chromosomes 6, 8, 9q12, and 11; chromosomes X, Yq12, 15, and 17; and 1q12, 4, 10, and 14/22) and one bacterial artificial chromosome (BAC) probe cocktail (probes targeting chromosomes 5, 19, 21, and 22). Briefly, the FISH slides with MSCs fixed in Carnoy's solution were denatured for 10 min at room temperature. The slides were dehydrated in cold ethanol. For the oligonucleotide probes, the probe cocktail was dropped onto the slide, and the slide was warmed at 37°C for 5–10 min and then immersed in saline sodium citrate (SSC, 0.03 M sodium citrate, 0.3 M NaCl, pH 7.0) for 5 min. The slide was then placed in a wash solution containing SSC and 0.1% sodium dodecyl sulfate, and 4', 6-diamidino-2-phenylindole (DAPI) was added. For BAC FISH, the probe was denatured for 10 min at 72°C in a water bath and was then placed at 37°C for a minimum of 30 min. The slides were immersed in denaturation solution at 37°C for a minimum of 30 min, followed by incubation at 72°C for 2 min. The slides were dehydrated in an ethanol series (85% and 100%, 1 min each), and the FISH probe mixture was then dropped onto the slides. The slides were stored at 37°C overnight for hybridization and placed in SSC for 5 min, followed by incubation in a wash solution containing 0.4% SSC and 0.3% nonylphenol polyethylene glycol (NP-40) at 72°C for 2 min, and finally in a second washing solution containing SSC and 0.1% NP-40 for 2 min. The slides were placed in SSC at room temperature, and DAPI was added. The fluorescent signals were analyzed using a fluorescence microscope (Zeiss, Göttingen, Germany). Interphase FISH signals were evaluated in at least 200 cells.

Interphase FISH data of patients with hematological malignancies and benign diseases

To compare the levels and patterns of aneuploidy in malignant and nonmalignant cells, we collected FISH results

from 259 patients with various hematological malignancies and from 22 patients with no malignant cells in the bone marrow. The hematological malignancies included acute lymphoblastic leukemia ($n=82$), acute myeloid leukemia ($n=56$), myelodysplastic syndrome (MDS; $n=22$), multiple myeloma (MM; $n=73$), and malignant lymphoma ($n=26$). Among the 22 patients with no malignant cells, 7 patients had nonmalignant diseases, including aplastic anemia ($n=2$), hemolytic anemia ($n=2$), immune thrombocytopenia ($n=1$), and normal amniotic fluid ($n=1$). The remaining 12 patients were in remission after chemotherapy for previously diagnosed acute leukemia. We selected patients using the laboratory informatics system based on the following criteria: patients who were tested using more than one FISH probe targeting different chromosomes, and patients who presented significant aneuploidy signals exceeding the cutoff value of the specific FISH probe (generally more than 2.5%–3%).

Statistical analysis

The data were compared using the Mann–Whitney U test and Kruskal–Wallis analysis for the continuous variables and using the chi-squared test and Fisher's exact test for the categorical variables. The percent coefficient of variation (% CV) was calculated as the ratio of the standard deviation (SD) to the mean. The reference intervals of the FISH aneuploidy levels were calculated using a robust method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [20]. The robust method was developed to determine the reference intervals when a limited number of samples are available. We also calculated the upper 95th percentile values after excluding the outliers (exceeding mean + 3.5 SD range) and the percentages of samples exceeding the mean + 2 SD and mean + 3 SD. The correlations are expressed as Pearson's coefficients. Statistical analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL). P values < 0.05 were considered statistically significant.

Results

Enumeration of the chromosomes using FISH

We evaluated the aneuploidy rates of 68 MSCs and a total of 103 cultures, including different passage numbers, using five sets of oligo-FISH probes targeting all of the chromosomes. The aneuploidy rates for the individual chromosomes and the average aneuploidy rate for the earliest passages of the 68 MSCs are displayed in Table 1. In the 68 MSCs, the maximum value of the chromosomal aneuploidy rates ranged from 1% to 20%, with a mean of 6.2% ($\pm 4.3\%$). The aneuploidy rates differed among the chromosomes. The mean aneuploidy rate for each chromosome ranged between 2.0% and 3.1%, with a mean of 2.5% ($\pm 2.0\%$). The tetrasomy level (mean, 1.9%) was significantly higher than the monosomy (mean, 0.3%; $P < 0.001$) and trisomy rates (0.3%; $P < 0.001$). For chromosomes 10, 16, 17, 18, and X, the mean aneuploidy rates of the 68 MSCs were $\geq 3\%$. The maximum aneuploidy level for each chromosome ranged between 8.0% and 20.0%. For chromosomes 1, 2, 17, 19, 20, and 22, aneuploidy rates $> 15\%$ were observed in the MSCs. According to pairwise multiple

TABLE 1. ANEUPLOIDY LEVELS IN 68 TYPES OF MSCs FOR EACH CHROMOSOME

Chromosome	Monosomy (%)		Trisomy (%)		Tetrasomy (%)		Total polysomy (%)		Total aneuploidy (%)	
	Mean $\pm$ SD	Maximum	Mean $\pm$ SD	Maximum	Mean $\pm$ SD	Maximum	Mean $\pm$ SD	Maximum	Mean $\pm$ SD	Maximum
Aneuploidy % of a chromosome with maximum value	1.6 $\pm$ 1.7	7.1	1.9 $\pm$ 1.9	11.5	4.8 $\pm$ 3.4	18.0	5.6 $\pm$ 4.0	19.0	6.2 $\pm$ 4.3	20.0
1	0.3 $\pm$ 0.6	2.0	0.2 $\pm$ 0.4	1.5	1.5 $\pm$ 2.0	12.0	1.8 $\pm$ 2.4	15.0	2.1 $\pm$ 2.4	16.0 ^a
2	0.1 $\pm$ 0.4	2.0	0.4 $\pm$ 0.8	3.3	2.1 $\pm$ 2.4	11.1	2.5 $\pm$ 3.0	15.2	2.7 $\pm$ 3.0	15.2
3	0.2 $\pm$ 0.4	2.0	0.5 $\pm$ 1.0	4.0	1.9 $\pm$ 2.0	9.0	2.5 $\pm$ 2.4	9.5	2.7 $\pm$ 2.4	10.5
4	0.2 $\pm$ 0.3	1.5	0.2 $\pm$ 0.5	2.0	1.8 $\pm$ 1.7	8.0	2.0 $\pm$ 2.0	8.0	2.2 $\pm$ 2.1	8.0
5	0.2 $\pm$ 0.5	2.0	0.2 $\pm$ 0.5	3.0	1.9 $\pm$ 2.5	11.0	2.1 $\pm$ 2.7	12.0	2.3 $\pm$ 2.9	12.0
6	0.3 $\pm$ 0.9	6.5	0.2 $\pm$ 0.5	2.0	1.8 $\pm$ 1.9	9.0	2.1 $\pm$ 2.1	9.0	2.3 $\pm$ 2.3	11.0
7	0.2 $\pm$ 0.5	2.0	0.3 $\pm$ 1.4	11.5	1.6 $\pm$ 1.7	7.0	1.9 $\pm$ 2.2	12.0	2.1 $\pm$ 2.4	12.5 ^a
8	0.3 $\pm$ 0.7	4.0	0.3 $\pm$ 0.6	3.0	1.4 $\pm$ 1.8	8.0	1.8 $\pm$ 2.1	10.0	2.0 $\pm$ 2.4	12.5 ^a
9	0.2 $\pm$ 0.5	2.0	0.2 $\pm$ 0.5	2.0	2.0 $\pm$ 1.9	7.1	2.3 $\pm$ 2.1	9.0	2.5 $\pm$ 2.2	9.0
10	0.4 $\pm$ 0.8	4.0	0.5 $\pm$ 0.8	4.0	2.1 $\pm$ 2.4	10.0	2.6 $\pm$ 2.9	11.0	3.1 $\pm$ 3.2	13.0
11	0.3 $\pm$ 0.5	2.6	0.3 $\pm$ 0.7	4.0	1.4 $\pm$ 1.4	6.0	1.8 $\pm$ 1.8	7.5	2.1 $\pm$ 2.0	8.5 ^a
12	0.4 $\pm$ 0.7	2.5	0.3 $\pm$ 0.5	2.0	1.9 $\pm$ 1.8	9.0	2.3 $\pm$ 1.9	9.0	2.6 $\pm$ 2.2	9.0
13	0.2 $\pm$ 0.5	2.0	0.3 $\pm$ 0.9	7.0	1.5 $\pm$ 1.6	8.0	1.8 $\pm$ 1.9	8.0	2.0 $\pm$ 2.1	8.0 ^a
14	0.1 $\pm$ 0.3	1.0	0.2 $\pm$ 0.5	2.5	1.9 $\pm$ 1.6	8.0	2.1 $\pm$ 1.8	8.0	2.2 $\pm$ 1.8	8.0
15	0.4 $\pm$ 0.9	5.5	0.2 $\pm$ 0.5	3.0	1.7 $\pm$ 1.8	7.0	2.0 $\pm$ 2.0	8.0	2.4 $\pm$ 2.5	12.0
16	0.4 $\pm$ 1.0	7.1	0.6 $\pm$ 1.1	5.0	2.1 $\pm$ 2.1	9.5	2.8 $\pm$ 2.7	11.0	3.1 $\pm$ 3.1	14.1 ^b
17	0.3 $\pm$ 0.5	2.0	0.6 $\pm$ 0.7	3.0	2.1 $\pm$ 2.9	17.0	2.7 $\pm$ 3.3	19.0	3.0 $\pm$ 3.4	20.0 ^b
18	0.4 $\pm$ 0.7	2.5	0.5 $\pm$ 0.7	3.0	2.0 $\pm$ 1.9	10.0	2.6 $\pm$ 2.4	10.0	3.0 $\pm$ 2.6	11.0 ^b
19	0.2 $\pm$ 0.5	2.5	0.1 $\pm$ 0.3	2.0	2.3 $\pm$ 3.1	18.0	2.5 $\pm$ 3.4	18.0	2.7 $\pm$ 3.4	18.0
20	0.2 $\pm$ 0.4	2.0	0.3 $\pm$ 0.7	4.5	2.2 $\pm$ 3.1	17.0	2.6 $\pm$ 3.4	18.0	2.8 $\pm$ 3.5	19.0
21	0.2 $\pm$ 0.4	1.5	0.3 $\pm$ 0.9	6.5	2.0 $\pm$ 2.1	10.0	2.4 $\pm$ 2.6	13.0	2.5 $\pm$ 2.7	13.0
22	0.2 $\pm$ 0.4	2.0	0.4 $\pm$ 1.0	7.0	2.1 $\pm$ 2.2	12.0	2.6 $\pm$ 3.0	16.0	2.7 $\pm$ 3.2	18.0
X	0.4 $\pm$ 1.2	7.0	0.4 $\pm$ 0.8	3.5	2.3 $\pm$ 2.4	9.3	2.8 $\pm$ 2.9	10.0	3.1 $\pm$ 3.2	13.0 ^b
Mean	0.3 $\pm$ 0.3	1.0	0.3 $\pm$ 0.4	1.8	1.9 $\pm$ 1.6	6.8	2.3 $\pm$ 1.9	8.8	2.5 $\pm$ 2.0	9.8
aneuploidy % in all chromosomes										

^aChromosomes with significantly lower aneuploidy rates in pairwise comparisons.

^bChromosomes with significantly higher aneuploidy rates in pairwise comparisons. MSC, mesenchymal stromal cell; SD, standard deviation.

comparisons, chromosomes 16, 17, 18, and X presented significantly higher aneuploidy rates than chromosomes 1, 7, 8, 11, and 13.

The reference ranges were determined using various cutoffs. The cutoffs for the maximum and mean aneuploidy rates, as determined by the upper 95th percentile values, were 13.0% and 5.9%, respectively (Table 2). The cutoffs determined by the mean + 2 SD and robust method were similar (12.7% and 5.8%; 12.7% and 5.7%, respectively), whereas the cutoffs determined using the mean + 3 SD or the nonparametric method covered a broader range (16.3% and 7.6%; 16.0% and 7.3%, respectively).

The proportion of cells in which the polysomy levels exceeded the various cutoff values was investigated in a total of 103 MSC cultures (Table 3). There were six MSC cultures (5.8%) that had at least one chromosome with an aneuploidy level exceeding $\geq 13\%$. There were 24 MSC cultures (23.3%) that had at least one chromosome with an aneuploidy level exceeding the upper 95th percentile values for each chromosome. There were 28 (27.2%), 22 (21.4%), and 12 (11.7%) MSC cultures that had at least one chromosome with an aneuploidy level $\geq 6\%$, $\geq 7\%$, and $\geq 10\%$, respectively. More than 13% of the polysomy cases were observed in six MSCs for chromosomes 1, 2, 12, 17, 19, 20, 22, and X (Table 3).

We compared the aneuploidy levels according to the origin of the MSCs. When the mean polysomy levels across all

chromosomes were compared, the ADSCs exhibited the highest polysomy rate, with a mean polysomy rate of 3.33%, and the NSCs exhibited the lowest polysomy rate (Table 4). When polysomy levels between 8 ADSCs obtained from abdominal fat tissue from breast cancer patients and 17 other ADSCs were compared, there were no significant differences in the mean polysomy percentages (4.04% vs. 2.99%, $P=0.130$) or the maximum polysomy rate (8.31% vs. 4.04%, $P=0.726$). In the four MSCs in which genetic manipulation was performed, no significantly high aneuploidy was observed.

When the specific percentages of aneuploidies for each chromosome were investigated, the MSCs exhibited a few chromosomes with high aneuploidy levels (asymmetric aneuploidy pattern) rather than generally high levels across all chromosomes (symmetric aneuploidy pattern). These asymmetric aneuploidy patterns of 24 MSCs, which had at least one chromosome with an aneuploidy level exceeding the upper 95th percentile, are described in Figure 1.

In situ karyotyping results of MSCs

In situ karyotyping analysis was performed for 61 cultures. The detailed karyotype results are summarized in Table 5. There were five MSCs that contained clonal chromosomal abnormalities, as defined by the ISCN criteria. Two numerical abnormalities were observed (trisomy 10 in an ADSC and

TABLE 2. REFERENCE RANGES OF THE POLYSOMY LEVELS IN 68 TYPES OF MSCs
DETERMINED USING THE VARIOUS CUTOFFS

Chromosome	n ^a	Upper 95th percentile ^a	Mean + 2 SD ^a	Mean + 3 SD ^a	Robust method		Nonparametric method ^a	
					Cutoff	(90% CI)	Cutoff	(90% CI)
Aneuploidy % of a chromosome with maximum value	68	13.0	12.7	16.3	12.7	(10.9–14.5)	16.0	(16.0–19.6)
1	67	6.0	5.3	7.1	6.1	(4.3–8.0)	6.5	(6.5–7.1)
2	68	9.5	8.6	11.6	8.1	(6.4–9.9)	11.4	(7.7–14.3)
3	68	8.0	7.3	9.6	7.1	(5.8–8.3)	9.1	(8.8–11.7)
4	68	6.0	6.0	8.0	5.9	(5.1–7.0)	8.0	(8.0–10.1)
5	67	8.0	6.9	9.4	7.0	(6.7–9.2)	9.6	(8.2–11.6)
6	68	6.0	6.2	8.3	6.1	(5.0–7.3)	9.0	(9.0–12.0)
7	67	5.0	5.4	7.2	6.1	(4.7–7.5)	7.0	(7.0–9.0)
8	68	6.0	6.0	8.1	5.7	(4.6–6.9)	8.9	(7.8–11.8)
9	68	6.5	6.6	8.7	6.3	(5.3–7.3)	7.6	(6.1–8.9)
10	45	10.0	8.3	11.2	8.1	(6.7–10.1)	10.9	(10.7–12.3)
11	68	5.9	5.4	7.2	5.0	(4.1–5.9)	7.1	(6.8–8.6)
12	68	6.0	6.1	8.1	5.8	(4.7–6.6)	7.6	(6.1–9.1)
13	68	6.0	5.6	7.5	5.5	(4.7–6.7)	8.0	(8.0–10.7)
14	68	5.0	5.6	7.4	5.5	(4.8–6.5)	6.9	(5.8–8.8)
15	68	6.5	6.0	8.0	5.9	(5.1–6.9)	7.3	(6.6–8.4)
16	68	8.0	8.1	10.7	7.7	(6.4–8.8)	9.9	(8.8–11.8)
17	67	7.5	7.7	10.3	8.7	(6.4–11.6)	10.4	(4.8–14.2)
18	67	8.0	7.3	9.7	6.7	(5.4–7.8)	9.3	(8.6–11.2)
19	66	7.3	6.7	9.0	8.3	(5.8–10.8)	8.7	(7.3–10.7)
20	67	8.0	8.1	10.9	8.7	(6.7–11.0)	12.0	(12.0–16.0)
21	68	7.0	7.5	10.1	6.9	(5.3–8.2)	10.8	(8.7–14.6)
22	68	6.5	8.6	11.6	8.4	(6.5–11.1)	16.0	(16.0–25.5)
X	68	9.0	8.5	11.4	8.3	(6.9–9.8)	9.5	(9.0–10.6)
Mean aneuploidy % in all chromosomes	68	5.9	5.8	7.6	5.7	(4.8–6.8)	7.3	(6.7–8.7)

^aExcluding outliers (out of the range of the mean $\pm$ 3.5 SD).

TABLE 3. PROPORTION OF CELLS FROM 103 CULTURES OF MSCs IN WHICH THE POLYSOMY LEVELS EXCEEDED THE VARIOUS CUTOFF VALUES

Chromosome	No. of MSCs exceeding cutoffs (%)						
	$\geq 6\%$ of polysomy	$\geq 7\%$ of polysomy	$\geq 10\%$ of polysomy	$\geq 13\%$ of polysomy	$\geq$ Upper 95th percentile	$\geq$ Mean + 3 SD	Cutoffs by Robust method
Total	28 (27.2)	22 (21.4)	12 (11.7)	6 (5.8)	24 (23.3)	16 (15.5)	25 (30.1)
1	5 (4.9)	2 (1.9)	2 (1.9)	2 (1.9)	5 (4.9)	2 (1.9)	5 (6.0)
2	8 (7.8)	7 (6.8)	2 (1.9)	2 (1.9)	4 (3.9)	2 (1.9)	5 (6.0)
3	6 (5.8)	6 (5.8)	1 (1.0)	1 (1.0)	3 (2.9)	1 (1.0)	6 (7.2)
4	5 (4.9)	4 (3.9)	1 (1.0)	1 (1.0)	5 (4.9)	2 (1.9)	6 (7.2)
5	8 (7.8)	6 (5.8)	2 (1.9)	0 (0)	3 (2.9)	2 (1.9)	6 (7.2)
6	4 (3.9)	3 (2.9)	1 (1.0)	1 (1.0)	4 (3.9)	3 (2.9)	4 (4.8)
7	6 (5.8)	4 (3.9)	2 (1.9)	1 (1.0)	10 (9.7)	4 (3.9)	6 (7.2)
8	4 (3.9)	3 (2.9)	1 (1.0)	1 (1.0)	4 (3.9)	3 (2.9)	6 (7.2)
9	7 (6.8)	4 (3.9)	1 (1.0)	1 (1.0)	6 (5.8)	3 (2.9)	7 (8.4)
10	6 (5.8)	5 (4.9)	2 (1.9)	1 (1.0)	2 (1.9)	1 (1.0)	4 (4.8)
11	6 (5.8)	4 (3.9)	1 (1.0)	1 (1.0)	8 (7.8)	4 (3.9)	10 (12.1)
12	5 (4.9)	4 (3.9)	2 (1.9)	2 (1.9)	5 (4.9)	3 (2.9)	9 (10.8)
13	5 (4.9)	4 (3.9)	1 (1.0)	1 (1.0)	5 (4.9)	4 (3.9)	9 (10.8)
14	6 (5.8)	4 (3.9)	3 (2.9)	1 (1.0)	9 (8.7)	4 (3.9)	7 (8.4)
15	5 (4.9)	2 (2.0)	1 (1.0)	1 (1.0)	3 (2.9)	1 (1.0)	6 (7.2)
16	9 (8.7)	8 (7.8)	2 (1.9)	1 (1.0)	4 (3.9)	2 (1.9)	5 (6.0)
17	10 (9.7)	8 (7.8)	3 (2.9)	3 (2.9)	7 (6.8)	3 (2.9)	4 (4.8)
18	9 (8.7)	6 (5.8)	1 (1.0)	1 (1.0)	4 (3.9)	2 (1.9)	9 (10.8)
19	9 (8.7)	8 (7.8)	2 (1.9)	2 (1.9)	7 (6.8)	3 (2.9)	4 (4.8)
20	9 (8.7)	8 (7.8)	5 (4.9)	2 (1.9)	5 (4.9)	4 (3.9)	5 (6.0)
21	8 (7.8)	7 (6.8)	2 (1.9)	1 (1.0)	7 (6.8)	2 (1.9)	8 (9.6)
22	7 (6.8)	4 (3.9)	3 (2.9)	3 (2.9)	5 (4.9)	3 (2.9)	3 (3.6)
X	14 (13.6)	13 (12.6)	4 (3.9)	2 (1.9)	6 (5.8)	2 (1.9)	8 (9.6)

The data are shown as a number (percentage).

TABLE 4. POLYSOMY LEVELS ACCORDING TO THE ORIGIN OF THE MSCs

<i>Polysomy % by FISH</i>	<i>ADSC (n=25)</i>	<i>BMSC (n=20)</i>	<i>UCSC (n=18)</i>	<i>NSC (n=5)</i>
Polysomy % of a chromosome with the maximum level				
Mean $\pm$ SD	8.02 $\pm$ 4.89	4.78 $\pm$ 2.22	4.36 $\pm$ 2.51	1.40 $\pm$ 1.47
<i>P</i> value		0.015	0.007	0.002
Mean polysomy % in all chromosomes				
Mean $\pm$ SD	3.33 $\pm$ 2.37	1.93 $\pm$ 1.15	1.67 $\pm$ 1.26	0.45 $\pm$ 0.56
<i>P</i> value		0.042	0.014	0.006
MSCs with polysomy clone ^a (%)				
<i>n</i> (%)	12/25 (48)	1/20 (5)	2/18 (11)	0/5 (0)
Odds ratios		0.06 (0.01–0.49)	0.14 (0.03–0.72)	<0.01 (<0.01–>99)
<i>P</i> value		0.009	0.019	0.974

^aNumber of MSCs having at least one chromosome with greater than upper 95th percentile levels of polysomy.

ADSC, adipose-origin MSCs; BMSC, bone marrow-origin MSCs; FISH, fluorescence in situ hybridization; NSC, neural stem cell; UCSC, umbilical cord-origin MSCs.

FIG. 1. Twenty-four mesenchymal stromal cells (MSCs) had at least one chromosome with an aneuploidy level that exceeded the upper 95th percentile value of each chromosome (which is presented in Table 2), as determined by fluorescence in situ hybridization (FISH). The detailed aneuploidy levels of each chromosome in these cells are presented as bar graphs (23 bars represent the percentages of monosomy, trisomy, tetrasomy, and pentasomy of chromosome 1–22 and chromosome X, sequentially from left to right). ADSC, adipose-origin MSCs; BMSC, bone marrow-origin MSCs; UCSC, umbilical cord-origin MSCs; p, passage. Color images available online at www.liebertpub.com/scd

monosomy 16 in a UCSC). The t(7;22)(p11.2;q13.3), t(7;22)(q11.21;q13.3), and t(15;17)(q22;q21) structural abnormalities were observed in three ADSCs, and der(7)dup(7)(q32q36)t(7;?)(q36;?) was observed in a UCSC. There were 12 MSCs in which the structural abnormalities were observed in only one metaphase. Most of the MSCs containing these nonclonal structural abnormalities, as defined by the ISCN criteria, were ADSCs ($n=11$). The observed structural abnormalities included deletions, such as del(3)(q12), del(6)(q12), del(10)(q22), and del(17)(q11.2), translocations, including t(X;2)(p11.4;p25), t(1;9)(p32;q34), t(1;10)(q21;q22), t(1;16)(q21;q12.1), t(7;22)(q11.22;q13.3), t(8;16)(p10;q10), and t(12;21)(q15;p10), and a marker chromosome. Fourteen MSCs exhibited metaphases with nonclonal chromosome losses or gains, which were interpreted as having a normal karyotype according to the ISCN criteria. The remaining 30 MSCs exhibited normal karyotypes. In summary, in situ karyotyping revealed a marker chromosome in 4.9% of the MSCs, deletions in 8.2%, and balanced translocations in 14.8%, when the abnormalities found in a single cell were taken account. When we applied the ISCN definition of a clone, 8.2% of the MSCs (5/61) showed chromosomal aberrations.

We successively cultured seven MSCs. For three MSCs with a normal karyotype or nonclonal structural abnormalities, no abnormalities were observed in the successive passages. For UCSC18, there was only one clone containing der(7)dup(7)(q32q36)t(7;?)(q36;?) at p8, which was not clonal according to the ISCN definition; however, the number of clones with the same abnormality increased with advanced passages. Additionally, for ADSC24, a structural abnormality appeared after several passages of cells with normal karyotypes. In contrast, in ADSC14-2, the clonal abnormality of t(7;22)(p11.2;q13.3) disappeared during successive passages. In ADSC10-2, in which only the numerical abnormality of trisomy 10 was observed, a structural abnormality of t(15;17)(q22;q21) emerged after additional passaging.

Comparison of in situ karyotyping and FISH results

When the in situ karyotyping and FISH results were compared, the MSCs with one or two nonclonal numerical abnormalities, according to in situ karyotyping, presented no significantly different aneuploidy levels than the MSCs with no abnormal karyotypes (Table 6). The MSCs with nonclonal structural abnormalities and clonal numerical and structural abnormalities tended to have slightly higher polyploidy levels; however, this trend was not statistically significant ($P=0.155$ and 0.809 , respectively). The specific aneuploidy levels of each chromosome were plotted and are shown in Figure 2. An MSC with the structural chromosomal abnormality t(7;22)(q11.21;q13.3) presented an asymmetric aneuploidy pattern by FISH and exhibited a higher polyploidy level for chromosome 7. However, in other MSCs, the cytogenetic abnormalities were not closely correlated with aneuploidy. Additionally, there were several MSCs with normal karyotypes and remarkably high aneuploidy levels.

Comparison of in situ karyotyping and FISH results between MSC cultures before and after storage

For 11 types of ADSCs, in situ karyotyping and FISH analysis were performed before and after storage at -80°C

(Fig. 3). Seven ADSCs exhibited novel nonclonal structural abnormalities in a single cell. One ADSC presented with a newly appeared trisomy 10, and the clonal structural abnormality t(7;22)(p11.2;q11.3) occurred in one of the re-cultured ADSCs. The aneuploidy rates, as measured by oligo-FISH, did not display any significant patterns that corresponded to the appearance of nonclonal or clonal abnormalities. One re-cultured ADSC (ADSC17, as shown in Fig. 3) presented with high aneuploidy rates and an asymmetric pattern of aneuploidy (5.0%–36.6%). Re-cultured ADSC17 cells did not proliferate, and no mitotic cells were observed; therefore, in situ karyotyping was impossible. These results imply that highly abnormal and asymmetric aneuploidy patterns might be observed during the senescence of MSCs.

Asymmetric distribution of aneuploidy among the different chromosomes in MSCs and patients with hematological malignancies

For comparison of the aneuploidy patterns of MSCs, the patterns of aneuploidy were investigated in 259 patients with hematological malignancies compared to 22 patients with nonmalignant cells (Table 7 and Fig. 4). The patients with various hematological malignancies exhibited heterogeneous and asymmetric patterns of aneuploidies for the different chromosomes (Fig. 4A). However, the patients with no malignant cells displayed symmetric patterns of ploidy changes across all of the investigated chromosomes; in other words, the characteristic numerical chromosomal change in nonmalignant proliferative cells was tetraploidy (Fig. 4B). The mean aneuploidy percentages in the patients with nonmalignant cells were lower than the aneuploidy rates in acute leukemia patients, but they were not significantly different from the MDS and MM patients. However, the SDs of the aneuploidy rates among the chromosomes were significantly higher for all categories of hematological malignancies than in patients with nonmalignant cells (Table 7). When the aneuploidy patterns of MSCs were considered, MSCs with polysomy exhibited a significantly greater SD of aneuploidy rates among different chromosomes than that of MSCs without polysomy (2.6% vs. 1.1%, $P<0.001$), which also presented the asymmetric pattern of aneuploidy found in MSCs.

Discussion

Abnormalities in chromosome content are hallmarks of cancer; it is thought that genomic instability could result from tetraploidy, and this instability may play an important role in cancer, according to Boveri [21]. Boveri's hypothesis states that a tetraploid intermediate is a common precursor to aneuploidy, and subsequent chromosomal evolution results in aneuploid chromosomal complements. In esophageal carcinoma, tetraploid cells, which are defined as $>6\%$ of the cells, are predictors that are associated with a 12-fold increased risk of progression to cancer [22]. Meanwhile, polyploidy often occurs in specific tissues as part of terminal differentiation in humans [23]. For example, approximately half of human hepatocytes are polyploid, and aneuploidy affects 30%–90% of hepatocytes in humans [24,25]. Because spontaneous liver cancer is rare, hepatic chromosomal instability per se is not considered to be associated with

TABLE 5. IN SITU KARYOTYPING RESULTS OF 61 MSCs

<i>Description</i>	<i>n</i>	<i>Passages</i>	<i>Karyotype (ISCN)</i>	<i>All observed clones</i>
Normal karyotypes				
Total	30 (49%)	3 (2–12)		Normal
ADSCs	20	3 (2–12)		Normal
BMSCs	4	2 (2–2)		Normal
UCSCs	5	5 (3–8)		Normal
NSCs	1	9		Normal
ISCN Normal karyotypes with nonclonal losses or gains of chromosomes				
Total	14 (23%)	5 (2–13)		
ADSCs	3	3 (3–3)		46,XX[16]/92,XXXXX[1]
ADSC12-1		3	46,XX[17]	46,XX,14pstk + [20]/92,XXXXX[1]
ADSC13-1		3	46,XX,14pstk + [21]	46,XX[17]/92,XXXXX[1]
ADSC18-1		3	46,XX[18]	
BMSCs	7	5 (2–6)		46,XY[16]/43,X,-Y,-3,-4[1]/45,XY,-1[1]/45,XY,-21[2]/43,X,-Y,-8,-17[1]
BMSC03		5	46,XY[21]	
BMSC05		6	46,XY[8]	46,XY[7]/42,XY,-9,-16,-18,-19[1]
BMSC06		5	46,XY[9]	46,XY[7]/43,XY,-1,-19,-20[1]/43,XY,-8,-10,-15[1]
BMSC07		6	46,XY,22pstk + [24]	46,XY,22pstk + [16]/43,XY,-6,-10,-22[1]/45,XY,-9,22pstk + [1]/45,XY,-7,22pstk + [1]/45,XY,-2,22pstk + [1]/45,XY,-16,22pstk + [1]/44,XY,-6,-17,22pstk + [1]/45,XY,-22,22pstk + [1]/45,XY,-1,22pstk + [1]
BMSC11		2	46,XY[20]	46,XY[19]/44,XY,-16,-21[1]
BMSC12		2	46,XY[20]	46,XY[17]/42,XY,-3,-7,-19,-22[1]/44,XY,-17,-18[1]/45,XY,-9[1]
BMSC14		2	46,XY[6]	46,XY[5]/44,XY,-7,-14[1]
UCSCs	4	9 (4–13)		
UCSC07		13	46,XY[3]	46,XY[2]/44,XY,-1,-2[1]
UCSC08		5	46,XY[10]	46,XY[8]/47,XXYY[1]/45,XY,-15[1]
UCSC09		13	46,XY[16]	46,XY[12]/45,X,-Y,-13[1]/44,X,-Y,-8[1]/44,XY,-13,-20[1]
UCSC10		4	46,XY,9qh + [5]	46,XY,9qh + [3]/44,XY,9qh + , -21, -22[1]/43,X,-Y,9qh + , -18, -21[1]
ISCN normal karyotypes with nonclonal structural abnormalities				
Total	12 (20%)	4 (2–7)		
ADSCs	10	4 (3–7)		
ADSC08-2		4	46,XX[20]	46,XX,del(6)(q12)[1]/46,XX[19]
ADSC09-1		3	46,XX[20]	46,XX,-18,+mar[1]/46,XX,t(1;10)(q21;q22)[1]/46,XX[18]
ADSC09-2 ^a		5	46,XX[20]	47,XX,+2[1]/46,XX,inv(9)(p22q13)[1]/46,XX,t(12;21)(q15;p10)[1]/46,XX[17]
		6	46,XX[11]	45,X[1]/46,XX[10]
		7	45,XX[1]	46,XX[1]
ADSC11-2		4	46,XX,inv(9)(p11q13)[20]	46,XX,t(8;16)(p10;q10),inv(9)(p11q13)[1]/46,XX,inv(9)(p11q13)[19]
ADSC13-2		3	46,XX,14pstk + [20]	46,XX,14pstk + ,del(17)(q11.2)[1]/46,XX,14pstk + [19]
ADSC15-2		4	46,XX[20]	46,X,t(X;2)(p11.4;p25)[1]/46,XX[19]
ADSC16-2		4	46,XX[20]	47,XX,+18[1]/46,XX,t(1;16)(q21;q12.1)[1]/46,XX,t(4;5)(q33;q31)[1]/46,XX[17]

(continued)

TABLE 5. (CONTINUED)

<i>Description</i>	<i>n</i>	<i>Passages</i>	<i>Karyotype (ISCN)</i>	<i>All observed clones</i>
ADSC18-2		4	46,XX[20]	92,XXXX[1]/46,XX,del(3)(q12)[1]/46,XX[18]
ADSC22		7	46,XX[20]	46,XX,del(10)(q22)[1]/46,XX[19]
ADSC23 ^a		6	46,XX[23]	47,XX,+2[1]/46,XX,t(1;9)(p32;q34)[1]/46,XX[21]
		7	46,XX[20]	47,XX,+10[1]/46,XX,dup(10)(q22q24)[1]/46,XX[18]
BMSC10	1	8	46,XX[20]	46,XX[20] (p9-p18: normal karyotype)
		2	46,XY[20]	46,XY[16]/43,XY,-6,-14,-19[1]/44,XY,-20,-22[1]/45,XY,-9[1]/46,XY,-10,+mar[1]
UCSC15	1	5	46,XY[20]	46,XY,der(p10;q10),+7,t(7;22)(q11.22;q13.3)[1]/46,XY[19]
ISCN abnormal karyotypes				
Total	5 (8%)	5 (2-8)		
ADSCs	3	4 (1-5)		
ADSC10-2 ^a		5	47,XX,+10[3]/46,XX[17]	47,XX,+10[3]/47,XX,+12[1]/46,XX[16]
		6	46,XX[19]	46,XX[19]
		7	47,XX,+10,21psk + [3]/46,XX,t(15;17)(q22;q21),21psk + [3]/47,XX,+2,21psk + [1]/46,XX,21psk + [12]	
ADSC14-2 ^a		4	t(15;17)(q22;q21),21psk + [3]/46,XX,21psk + [13]	
		4	46,XX,t(7;22)(p11.2;q13.3),21psk + [2]/47,XX,21psk + , + mar[1]/46,XX,21psk + [17]	
		5	21psk + [2]/46,XX,21psk + [18]	
		6	46,XX,21psk + [20]	47,XX,+2,21psk + [1]/46,XX,21psk + [19]
			46,XX,21psk + [20]	47,XX,+10,21psk + [1]/46,XX,t(X;3)(p13;p22.3),21psk + [1]/46,XX,21psk + [18]
		7	46,XX,21psk + [20]	46,XX,21psk + [20]
		8	46,XX,21psk + [19]	46,XX,add(21)(q22.3),21psk + [1]/46,XX,21psk + [18]
		9	46,XX,21psk + [10]	46,XX,t(7;12)(q21.1;q24.33),21psk + [1]/46,XX,21psk + [9]
ADSC24 ^a		2	46,XX[20]	46,XX[20]
		3	46,XX[20]	46,XX[20]
		4	46,XX[13]	46,XX[13]
		5	48,XX,+7,t(7;22)(q11.21;q13.3),+9[4]/46,XX[21]	48,XX,+7,t(7;22)(q11.21;q13.3),+9[4]/46,XX[21]
UCSCs	2	7 (5-8)		
UCSC06		5	45,XY,-16[3]/46,XY[8]	46,XY[8]/45,XY,-16[1]/45,XY,-3,+del(6)(q21),der(14)t(7;14)(q32;q22),-22[1]/44,XY,-16,-21[1]/88,XXYY,-2,-9,-16,-16[1]/44,XY,-9,-9[1]/42,XY,-3,-18,-20,-20[1]
		8	46,XY[15]	46,XY,der(7)dup(7)(q32q36)t(7;?)(q36;?)[1]/46,XY[14]
		9	46,XY[15]	46,XY[15]
		10	46,XY[13]	46,XY[13]
		11	46,XY,der(7)dup(7)(q32q36)t(7;?)(q36;?)[3]/46,XY[20]	46,XY,der(7)dup(7)(q32q36)t(7;?)(q36;?)[3]/46,XY[20]
UCSC18 ^a		12	46,XY,der(7)dup(7)(q32q36)t(7;?)(q36;?)[7]/46,XY[13]	46,XY,der(7)dup(7)(q32q36)t(7;?)(q36;?)[7]/46,XY[13]

^aMSCs in which in situ karyotyping analysis was performed for multiple passages. ISCN, International System for Human Cytogenetic Nomenclature.

TABLE 6. POLYSOMY LEVELS IN 61 CULTURES OF MSCs ACCORDING TO THE CHROMOSOMAL ABNORMALITIES DETECTED USING IN SITU KARYOTYPING

Polysomy % by FISH	Normal karyotype (n=30)	ISCN normal karyotypes with nonclonal losses or gains of chromosomes (n=14)	ISCN normal karyotypes with nonclonal structural abnormalities (n=12)	ISCN abnormal karyotypes (n=5)
Polysomy % of a chromosome with the maximum value				
Mean $\pm$ SD	5.65 $\pm$ 3.95	6.00 $\pm$ 4.22	7.72 $\pm$ 4.71	7.60 $\pm$ 2.97
P value		0.791	0.145	0.329
Mean of polysomy % in all chromosomes				
Mean $\pm$ SD	2.39 $\pm$ 2.01	2.24 $\pm$ 1.61	3.32 $\pm$ 2.01	2.61 $\pm$ 1.47
P value		0.813	0.155	0.809
MSCs with polysomy clone ^a (%)				
n (%)	6/30 (20)	5/14 (36)	6/12 (50)	3/5 (60)
Odds ratios		2.2 (0.5–9.1)	4.0 (0.9–16.9)	6.0 (0.8–44.4)
P value		0.268	0.060	0.079

^aNumber of MSCs having at least one chromosome with greater than upper 95th percentile levels of polysomy.

malignancy [25]. Considering these normal cells, it may be difficult to conclude that aneuploidy per se is a definite indicator of malignancy. However, we do not understand the mechanism by which hepatocytes maintain normal function in the face of extreme chromosomal instability. With this lack of sufficient knowledge of physiologic polyploidization and aneuploidy and considering that it is well known that tetraploidization can initiate chromosomal instability and promote cell transformation, it would be more reasonable to consider chromosomal abnormalities as risk indicators for malignancy.

Indeed, many international and national regulations have determined that checking the MSCs for genomic instability is essential prior to infusion therapy. In Europe, the Cell

Products Working Party (CPWP) and the Committee for Advanced Therapies (CAT) have recommended that stem cells undergo a quality check for tumorigenicity and genomic instability [3]. However, technical protocols have not been standardized, and no guidelines have been established. Karyotyping using conventional G-banding, FISH, spectral karyotyping, array-CGH, cDNA array, and next-generation sequencing are currently being utilized [3,26].

Many reports on the chromosomal analysis of stem cells have utilized the G-banding method. The conventional G-banding method can detect changes in metaphase cells, which comprise <0.1% of the cells tested. Instead, most of the cells are in interphase; therefore, the abnormalities of the interphase cells cannot be detected using the G-banding

FIG. 2. The distributions of the polysomies in the 68 MSCs are presented as box plots of chromosomes 1–22 and chromosome X. The box plot includes the median (horizontal line), mean (diamonds), and interquartile range, and the whiskers indicate the minimum and maximum data values unless outliers are present, in which case the whiskers extend to a maximum of 1.5 times the interquartile range. The polysomy levels of the five MSCs with abnormal karyotypes, as determined using in situ karyotyping, are indicated as lines with different colors. Color images available online at www.liebertpub.com/scd

FIG. 3. Comparison of polysomy levels determined by interphase FISH between the cultures of cells before (green lines) and after (red lines) storage at -80°C for several months to 1 year. The results of in situ karyotyping are presented in the legend of each plot. Color images available online at www.liebertpub.com/scd

TABLE 7. DISTRIBUTION OF ANEUPLOIDY LEVELS AMONG THE DIFFERENT CHROMOSOMES IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES, PATIENTS WITH NO MALIGNANT CELLS IN THE BONE MARROW, AND MSCs

Samples	n	Monosomy (%) ^a		Trisomy (%) ^a		Tetrasomy (%) ^a		Total polysomy (%)		Total aneuploidy (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Patient samples											
Nonmalignancy	22	0 (0–2.5)	0 (0–2.2)	0 (0–2.3)	0 (0–1.1)	5.1 (1.8–30.5)	0.7 (0–2.3)	5.2 (2.2–30.5)	0.6 (0–2.1)	6.0 (2.2–30.5)	0.6 (0–2.7)
Hematological malignancy	259	0 (0–14.3)	0 ^b (0–33.9)	2.6 ^b (0–52.6)	3.9 ^b (0–54.8)	7.0 (0–77.8)	6.0 ^b (0–62.2)	13.4 ^b (0.7–91.0)	10.1 ^b (0–62.2)	14.7 ^b (1.2–91.3)	11.6 ^b (0–62.2)
ALL	82	0 (0–3.8)	0 (0–7.4)	5.8 ^b (0–52.6)	10.4 ^b (0–53.0)	12.9 (0.1–66.3)	24.5 ^b (0–62.2)	21.3 ^b (2.7–91.0)	31.4 ^b (0–62.2)	21.3 ^b (2.7–91.3)	31.4 ^b (0–62.2)
AML	56	0.1 (0–14.3)	0.2 ^b (0–33.9)	4.1 ^b (0–38.8)	7.4 ^b (0–46.6)	6.8 (0.1–51.7)	4.9 ^b (0.2–42.5)	12.4 (1.7–81.1)	14.5 ^b (0.5–46.8)	17.0 ^b (1.7–81.1)	14.3 ^b (0.7–46.8)
MDS	22	0.3 ^c (0–14.2)	0.5 ^b (0–31.2)	4.3 (0–28.8)	8.0 ^b (0–49.9)	4.7 (0.2–27.5)	1.9 (0.2–41.2)	11.6 (0.7–43.1)	8.3 ^b (0.4–50.2)	12.8 (2.6–43.1)	15.0 ^b (0.5–48.9)
MM and lymphomas	99	0 (0–10.7)	0 (0–18.8)	0.8 (0–39.8)	1.4 (0–54.8)	5.7 (0–77.8)	5.2 ^b (0–47.5)	8.3 (1.0–78.6)	5.9 ^b (0.4–58.3)	9.3 (1.2–78.6)	5.9 ^b (0.3–58.3)
Cultured MSCs											
Total MSCs	103	0.1 (0–1.0)	0.2 (0–1.5)	0.2 (0–1.8)	0.4 (0–2.4)	1.7 (0–23.5)	1.1 (0–9.4)	2.0 (0–24.0)	1.3 (0–9.5)	2.0 (0–24.0)	1.4 (0.3–9.5)
MSCs without polysomy ^c	79	0.1 (0–0.9)	0.2 (0–1.5)	0.1 (0–1.1)	0.3 (0–1.2)	1.5 (0–3.3)	1.0 (0–2.0)	1.8 (0–3.7)	1.1 (0–2.2)	1.8 (0–4.0)	1.1 (0.3–2.8)
MSCs with polysomy ^c	24	0.2 (0–1.0)	0.3 (0–1.3)	0.5 ^b (0–1.8)	0.8 ^b (0.2–2.4)	3.9 ^b (1.0–23.5)	2.2 ^b (0.8–9.4)	4.6 ^b (1.7–24.0)	2.5 ^b (1.4–9.5)	4.8 ^b (1.9–24.0)	2.6 ^b (1.4–9.5)

^aThe means and SDs of the tetrasomy, trisomy, and monosomy percentages of each chromosome were calculated for each patient. The distribution of the means and SDs of the aneuploidy levels among the chromosomes are presented as the median and range (in parentheses).

^b $p < 0.05$.

^cMSCs having at least one chromosome with greater than upper 95th percentile levels of polysomy.

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma.

technique. Furthermore, if cells become quiescent under stressful culture conditions, chromosomal abnormalities cannot be detected by G-banding. Previous studies have demonstrated that the aneuploidy condition during interphase may be more representative of the general status of the cell population [27,28]. Additionally, the advantages of FISH include a 10-fold increase in sensitivity compared with G-banding and a short turnaround time. In the present study, we evaluated the metaphase cells using the G-banding method and the interphase cells using FISH for each chromosome, which allowed us to examine the entire cell population.

Previous studies reported that stem cells exhibited aneuploidy, which may be the result of a random process or a tendency of certain types of stem cells to develop abnormalities in specific chromosomes. In a previous study, four primary MSCs derived from adipose tissue were analyzed using FISH probes for chromosomes 8, 11, and 17, and these cells exhibited a substantially high percentage of aneuploidy, ~15%–24%, even during the early passages of p2 to p5 [29]. In another study investigating induced pluripotent stem cells (iPSCs), the average aneuploidy rate was 2.1% for iPSCs and 4.2% for embryonic stem cells (ESCs) [30]. In another study, gains of chromosomes 12, 17, 1, and X were reported for iPSCs. In a study using nine MSCs derived from bone marrow, a conventional cytogenetic analysis revealed random aneuploidies. In particular, one MSC presented with clonal trisomy 16 in p6 and p8 [31].

In our study, we investigated the aneuploidy of all of the chromosomes. The aneuploidy rates detected by FISH ranged from 1% to 20% among the 68 MSCs. Polysomies, particularly tetrasomies, were more common than monosomies. Because there are no consensus cutoff values to determine whether certain FISH signals are abnormal or within normal background levels for MSCs, we applied various methods to determine a reference range of aneuploidy levels for FISH analysis of MSCs. Using the Robust method and the upper 95th percentile value, the cutoffs for maximum polysomy was ~13%. When these cutoffs were applied to our whole set of MSCs, ~6% of the cells were determined to be abnormal. Interestingly, in individual MSCs, we observed asymmetric patterns of aneuploidy among the chromosomes, which was similar to the pattern of aneuploidy found in malignant cells. The tetrasomy rates for chromosomes 16, 17, 18, and X were significantly higher in MSCs. These observations indicate that some chromosomal gains or losses are most likely random, while others are selected for, as they give the clone a competitive advantage over the other stem cells. Because we investigated the chromosomal changes of hematological malignancies as an illustrative disease due to large preexisting FISH data, we could only compare the patterns of aneuploidy between malignant and nonmalignant cells. Further investigation into the chromosomal abnormalities found in MSC-derived tumors may be helpful to gain a deeper understanding for specific chromosomal abnormalities found in cultured MSCs.

To analyze a sufficient number of metaphase cells, a relatively large amount of cells is required. Therefore, we established an in situ karyotyping method for MSCs that can be used to analyze MSCs in a manner similar to amniotic embryonic cells. Conventional G-banding includes several manipulations, and as the nuclear membranes of the cells rupture, chromosomes can be artificially gained or lost. To

rule out random artificial chromosomal abnormalities, a clone is defined by the loss of chromosomes in ≥ 3 cells, by a gain of chromosomes in ≥ 2 cells or by the presence of a structural anomaly in ≥ 2 cells by ISCN criteria. In situ culture and karyotyping does not include cell manipulation; therefore, whether the same definition of a clone can be applied is questionable. During prenatal diagnosis, chromosomal abnormalities detected using in situ culture of amniocytes are interpreted using a different strategy [32]. A single abnormal cell observed by amniocyte in situ culture results in additional workups, and the laboratory usually will not report a single cell observation if the analysis of additional cells fails to confirm the abnormality [32]. In our study, in situ karyotyping revealed a marker chromosome in 3.3% of the cultures, deletions in 8.2%, and balanced translocations in 14.8% when abnormalities found in a single cell were considered. When we applied the ISCN definition of a clone, 8.2% of the MSCs (5/61) displayed chromosomal aberrations. Interestingly, in this study, the structural abnormalities frequently involved 7q and were found in 2/4 (50%) of MSCs with structural chromosome abnormalities. Deletions of 7q are common chromosomal abnormalities in myeloid neoplasms and are associated with poor prognosis [33,34]. Meanwhile, to our knowledge, 7q abnormalities have not been significantly noted in stem cells. Further investigation of MSCs with 7q abnormalities may uncover novel molecular mechanisms that induce chromosome instability in stem cells. We passaged seven MSCs, including three MSCs with non-clonal single metaphase abnormalities at initial karyotyping. Two MSCs showed the disappearance of a single cell abnormality, resulting in a reversion to a normal karyotype. However, in an MSC, one cell with der(7)dup(7)(q32q36)t(7;?)(q36;?) expanded into clones with abnormalities following continued passage. In addition, when we compared the FISH and in situ karyotyping results, we found no significant correlation between these data, which indicate the need for investigating both interphase and metaphase cells by FISH and karyotyping.

In fact, the potential role of MSCs as the origin of several tumors is still being debated in many studies [7]. Studies using murine MSCs have shown that transformed murine MSCs formed sarcomas in vivo [4,5]. Data concerning human MSCs revealed no consistent evidence of spontaneous tumor formation, which is contrary to the animal model [35,36]. There are sporadic reports of bone marrow transplant recipients who developed sarcoma [37]. However, some reports of spontaneous transformation events from adult stem cells to tumors may be the result of cross-contamination artifacts [38]. In previous studies, no or only rare clonal cytogenetic abnormalities increased during culture over time, and the injection of MSCs into immunodeficient mice did not produce tumors [39–41]. Additionally, previous studies showed that human MSCs do not transform during ex vivo expansion, and aneuploidy is not related to transformation but instead is related to senescence [12,13].

In our study, some MSCs contributed to expanding clones with clonal chromosomal abnormalities. In addition, there was an MSC with high aneuploidy levels and loss of mitotic activity after cryopreservation. Considering that previous studies reported an increase in aneuploidy levels in ESCs after cryopreservation [42,43], we speculated that this MSC with high aneuploidy levels was damaged during storage or

freezing and thawing and was senescent without mitotic activity when thawed for re-culture. The chromosomal abnormalities of this MSC can only be detected by interphase FISH analysis, and thus karyotyping was impossible. Both transformation and senescence of MSCs would be reasons to prohibit the clinical use of MSCs. The risk of cross-contamination of tumor cells might be another reason for a strict quality check prior to MSC treatment. Until we have a more clear understanding of MSCs, a high level of caution should be used before MSC therapy in patients. We should establish fast and efficient screening methods for MSCs. We suggest that cytogenetic studies using in situ karyotyping and FISH may be promising options. Because automated high-throughput FISH analysis is possible, FISH is a potential screening method for stem cells. It would be practical to investigate the most frequent chromosomal changes using a panel test. Based on our data, we suggest screening chromosomes 16, 17, 18, and X to ensure the quality of the MSCs. We believe that a warning should be given when a population of cells with aneuploidy above a certain level is used. Because FISH cannot detect structural chromosomal abnormalities, both G-banding and FISH panel screening should be performed to check the safety of the MSCs.

In this study, we included MSCs with various origins, in different passages, and from donors with heterogeneous ages and health conditions because we wanted to suggest general guidelines that can be universally applied to any MSC for which the clinical application is intended and because increasing the number of samples can reduce the statistical uncertainty. Previous studies indicated that MSC conditions, including culture conditions (such as hypoxia), passages, and donor status (such as age), influence the aneuploidy levels of MSCs [1,8,27]. Therefore, our results should be analyzed cautiously when applied to an individual MSC. The abnormal screening results should be confirmed by further workup, and possible conditions causing the abnormalities should be investigated.

In conclusion, we established the following guidelines to check the safety of MSCs: (i) When single cell abnormalities by G-banding are observed, we recommend a further workup. Serial passage of the cells and analysis by two blinded investigators are needed to exclude random artifacts. Alternatively, animal experiments to examine tumorigenesis are another option. (ii) When aneuploidy is observed in the cells by G-banding, we recommend that chromosome enumeration by FISH should be performed for the corresponding chromosome. (iii) General screening for chromosomes 16, 17, 18, and X and karyotyping would be helpful in detecting the abnormal MSCs.

Acknowledgments

This study was supported by (1) a grant (10172KFDA993) from the Korean Food and Drug Administration (2) a grant from the Korean Healthcare Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (A120216) and (3) the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (grant number 20120009555).

Author Disclosure Statement

All authors declare that there are no conflicts of interest.

References

1. Ferreira RJ, AC Irioda, RC Cunha, JC Francisco, LC Guarita-Souza, GV Srikanth, S Nityanand, R Rosati, JC Chachques and KA de Carvalho. (2012). Controversies about the chromosomal stability of cultivated mesenchymal stem cells: their clinical use is it safe? *Curr Stem Cell Res* 7:356–363.
2. Wang Y, ZB Han, YP Song and ZC Han. (2012). Safety of mesenchymal stem cells for clinical application. *Stem Cells Int* 2012:652034.
3. Barkholt L, E Flory, V Jekerle, S Lucas-Samuel, P Ahnert, L Bisset, D Buscher, W Fibbe, A Foussat, et al. (2013). Risk of tumorigenicity in mesenchymal stromal cell-based therapies—bridging scientific observations and regulatory viewpoints. *Cytotherapy* 15:753–759.
4. Tolar J, AJ Nauta, MJ Osborn, A Panoskaltsis Mortari, RT McElmurry, S Bell, L Xia, N Zhou, M Riddle, et al. (2007). Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 25:371–379.
5. Mohseny AB, K Szuhai, S Romeo, EP Buddingh, I Briaire-de Bruijn, D de Jong, M van Pel, AM Cleton-Jansen and PC Hogendoorn. (2009). Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. *J Pathol* 219:294–305.
6. Jeong JO, JW Han, JM Kim, HJ Cho, C Park, N Lee, DW Kim and YS Yoon. (2011). Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circ Res* 108:1340–1347.
7. Mohseny AB and PC Hogendoorn. (2011). Concise review: mesenchymal tumors: when stem cells go mad. *Stem Cells* 29:397–403.
8. Ueyama H, T Horibe, S Hinotsu, T Tanaka, T Inoue, H Urushihara, A Kitagawa and K Kawakami. (2012). Chromosomal variability of human mesenchymal stem cells cultured under hypoxic conditions. *Cell Mol Med J* 16:72–82.
9. Sensebe L. (2013). Beyond genetic stability of mesenchymal stromal cells. *Cytotherapy* 15:1307–1308.
10. Miller DT, MP Adam, S Aradhya, LG Biasecker, AR Brothman, NP Carter, DM Church, JA Crolla, EE Eichler, et al. (2010). Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. *Am J Hum Genet* 86:749–764.
11. Scott SA, N Cohen, T Brandt, G Toruner, RJ Desnick and L Edelmann. (2010). Detection of low-level mosaicism and placental mosaicism by oligonucleotide array comparative genomic hybridization. *Genet Med* 12:85–92.
12. Bernardo ME, N Zaffaroni, F Novara, AM Cometa, MA Avanzini, A Moretta, D Montagna, R Maccario, R Villa, et al. (2007). Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 67:9142–9149.
13. Tarte K, J Gaillard, JJ Lataillade, L Fouillard, M Becker, H Mossafa, A Tchirkov, H Rouard, C Henry, et al. (2010). Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 115:1549–1553.
14. Hwang SM, C-J See, J Choi, SY Kim, Q Choi, JA Kim, J Kwon, SN Park, K Im, I-H Oh and DS Lee. (2013). The application of an in situ karyotyping technique for mesenchymal stromal cells: a validation and comparison study with classical G-banding. *Exp Mol Med* 45:e68.
15. International Standing Committee on Human Cytogenetic Nomenclature, LG Shaffer, J McGowan-Jordan and M Schmid. (2013). *ISCN 2013: An International System for Human Cytogenetic Nomenclature (2013)*. Karger, Basel, Unionville, CT.
16. Schmid W. (1975). A technique for in situ karyotyping of primary amniotic fluid cell cultures. *Humangenetik* 30:325–330.
17. Miron PM. (2012). Preparation, culture, and analysis of amniotic fluid samples. *Curr Protoc Hum Genet* 8.
18. Barch MJ, T Knutsen, JL Spurbeck and AoG Technologists. (1997). *The AGT Cytogenetics Laboratory Manual*. Lippincott-Raven Publishers, Philadelphia, PA.
19. International Standing Committee on Human Cytogenetic Nomenclature., LG Shaffer, ML Slovak and LJ Campbell. (2009). *ISCN 2009: An International System for Human Cytogenetic Nomenclature (2009)*. Karger, Basel, Unionville, CT.
20. Horowitz GL, S Altaie, JC Boyd, F Ceriotti, U Garg, P Horn, A Pesce, HE Sine and J Zakowski. (2008). *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory: Approved Guideline, 3rd edn*. Clinical and Laboratory Standards Institute, Wayne, PA.
21. Satzinger H. (2008). Theodor and Marcella Boveri: chromosomes and cytoplasm in heredity and development. *Nat Rev Genet* 9:231–238.
22. Rabinovitch PS, G Longton, PL Blount, DS Levine and BJ Reid. (2001). Predictors of progression in Barrett's esophagus III: baseline flow cytometric variables. *Am J Gastroenterol* 96:3071–3083.
23. Storchova Z and C Kuffer. (2008). The consequences of tetraploidy and aneuploidy. *J Cell Sci* 121:3859–3866.
24. Duncan AW, AE Hanlon Newell, W Bi, MJ Finegold, SB Olson, AL Beaudet and M Grompe. (2012). Aneuploidy as a mechanism for stress-induced liver adaptation. *J Clin Invest* 122:3307–3315.
25. Duncan AW. (2013). Aneuploidy, polyploidy and ploidy reversal in the liver. *Semin Cell Dev Biol* 24:347–356.
26. Goldring CE, PA Duffy, N Benvenisty, PW Andrews, U Ben-David, R Eakins, N French, NA Hanley, L Kelly, et al. (2011). Assessing the safety of stem cell therapeutics. *Cell Stem Cell* 8:618–628.
27. Mukherjee AB, J Alejandro, S Payne and S Thomas. (1996). Age-related aneuploidy analysis of human blood cells in vivo by fluorescence in situ hybridization (FISH). *Mech Ageing Dev* 90:145–156.
28. Mukherjee AB and C Costello. (1998). Aneuploidy analysis in fibroblasts of human premature aging syndromes by FISH during in vitro cellular aging. *Mech Ageing Dev* 103:209–222.
29. Estrada JC, C Albo, A Benguria, A Dopazo, P Lopez-Romero, L Carrera-Quintanar, E Roche, EP Clemente, JA Enriquez, A Bernad and E Samper. (2012). Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis. *Cell Death Differ* 19:743–755.
30. Dekel-Naftali M, A Aviram-Goldring, T Litmanovitch, J Shamash, H Reznik-Wolf, I Laevsky, M Amit, J Itskovitz-Eldor, Y Yung, et al. (2012). Screening of human pluripotent stem cells using CGH and FISH reveals low-grade mosaic aneuploidy and a recurrent amplification of chromosome 1q. *Eur J Hum Genet* 20:1248–1255.
31. Binato R, T de Souza Fernandez, C Lazzarotto-Silva, B Du Rocher, A Mencalha, L Pizzatti, LF Bouzas and E Abdelhay. (2012). Stability of human mesenchymal stem cells during

- in vitro culture: considerations for cell therapy. *Cell Prolif* 46:10–22.
32. Mir P, L Rodrigo, E Mateu, V Peinado, M Milan, A Mercader, P Buendia, A Delgado, A Pellicer, J Remohi and C Rubio. (2010). Improving FISH diagnosis for preimplantation genetic aneuploidy screening. *Hum Reprod* 25:1812–1817.
 33. Jerez A, Y Sugimoto, H Makishima, A Verma, AM Janowska, B Przychodzen, V Visconte, RV Tiu, CL O'Keefe, et al. (2012). Loss of heterozygosity in 7q myeloid disorders: clinical associations and genomic pathogenesis. *Blood* 119:6109–6117.
 34. Hosono N, H Makishima, A Jerez, K Yoshida, B Przychodzen, S McMahon, Y Shiraishi, K Chiba, H Tanaka, et al. (2014). Recurrent genetic defects on chromosome 7q in myeloid neoplasms. *Leukemia* 28:1348–1351.
 35. MacIsaac ZM, H Shang, H Agrawal, N Yang, A Parker and AJ Katz. (2012). Long-term in-vivo tumorigenic assessment of human culture-expanded adipose stromal/stem cells. *Exp Cell Res* 318:416–423.
 36. Aguilar S, E Nye, J Chan, M Loebinger, B Spencer-Dene, N Fisk, G Stamp, D Bonnet and SM Janes. (2007). Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. *Stem Cells Int* 25:1586–1594.
 37. Berger M, M Muraro, F Fagioli and S Ferrari. (2008). Osteosarcoma derived from donor stem cells carrying the Norrie's disease gene. *N Engl J Med* 359:2502–2504.
 38. Torsvik A, GV Rosland, A Svendsen, A Molven, H Immervoll, E McCormack, PE Lonning, M Primon, E Sobala, et al. (2010). Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track: letter. *Cancer Res* 70:6393–6396.
 39. Jones M, M Varella-Garcia, M Skokan, S Bryce, J Schowinsky, R Peters, B Vang, M Brecheisen, T Startz, N Frank and B Nankervis. (2013). Genetic stability of bone marrow-derived human mesenchymal stromal cells in the Quantum System. *Cytotherapy* 15:1323–1339.
 40. Roemeling-van Rhijn M, A de Klein, H Douben, Q Pan, LJ van der Laan, JN Ijzermans, MG Betjes, CC Baan, W Weimar and MJ Hoogduijn. (2013). Culture expansion induces non-tumorigenic aneuploidy in adipose tissue-derived mesenchymal stromal cells. *Cytotherapy* 15:1352–1361.
 41. Roselli EA, S Lazzati, F Iseppon, M Manganini, L Marcato, MB Gariboldi, F Maggi, FR Grati and G Simoni. (2013). Fetal mesenchymal stromal cells from cryopreserved human chorionic villi: cytogenetic and molecular analysis of genome stability in long-term cultures. *Cytotherapy* 15:1340–1351.
 42. Iwarsson E, M Lundqvist, J Inzunza, L Ahrlund-Richter, P Sjoblom, O Lundkvist, N Simberg, M Nordenskjold and E Blennow. (1999). A high degree of aneuploidy in frozen-thawed human preimplantation embryos. *Hum Genet* 104:376–382.
 43. Salumets A, N Horelli-Kuitunen, AM Suikkari, A Metspalu and T Tuuri. (2004). Elevated incidence of chromosomally chaotic embryos among frozen-thawed preimplantation embryos. *Eur J Obstet Gynecol Reprod Biol* 114:59–63.

Address correspondence to:

Prof. Dong Soon Lee

Department of Laboratory Medicine

Seoul National University College of Medicine

101 Daehak-Ro

Jongno-Gu

Seoul 110-744

Republic of Korea

E-mail: soonlee@snu.ac.kr

Received for publication March 18, 2014

Accepted after revision July 11, 2014

Prepublished on Liebert Instant Online July 14, 2014