Frequent occurrence of non-malignant genetic alterations in clinical grade mesenchymal stromal cells expanded for cell therapy protocols

Human bone marrow mesenchymal stromal cells (BM-MSC) represent one of the most investigated "advanced therapeutic medicinal products".[1] Recent safety concerns have focused attention on the possible malignant transformation due to mutations acquired during their large-scale *in vitro* expansion.[2] Indeed, spontaneous oncogenic transformation has been described for murine MSC[3] although not for human cells,[4,5] with the exception of a few studies,[6] which were subsequently retracted when it was realized that this was due to cross-contamination by a tumor cell line.[7,8] One single report has described the *in vitro* outgrowth of a transformed subpopulation from a normal BM sample.[9] Furthermore, genetic aberrations of MSC have been very occasionally observed after long-term cultures[4,10,11] but interpreted to be related to senescence.[5]

In order to investigate the frequency of cytogenetic alterations in a broad "collection" of clinical-grade BM-MSC products, we performed cytogenetic analysis of 92 preparations expanded under Good Manufacturing Practice conditions.[12] More precisely, 67 expansions were performed from 33 healthy donors, 4 β–thalassemia patients and 21 multiple sclerosis patients (Table 1). MSC were expanded from BM washouts or aspirates using human platelet lysate as previously described.[12,13] Metaphases were prepared according to standard procedures[12] and analyzed by QFQ-banding. At least 20 metaphases per sample were analyzed. Karyotype was described according to the International System for Human Cytogenetic Nomenclature. Furthermore, p53 gene mutations were analyzed by deep sequencing of exons 5 to 11.

Chromosomal abnormalities were detected in 17 of 86 expansions (19.8%). In all cases, the genetic lesions were spontaneous abnormalities.[2] In 14 cases they were non-clonal: in 8 they involved one metaphase (MSC46, MSC70, MSC74, MSC79, MSC82, MSC87, MSC121, MSC126); in 5 two different chromosome abnormalities in two metaphases (MSC52, MSC55, MSC66 MSC100, MSC116). Only in one case were three different alterations in three metaphases found (MSC80). "Clonal chromosome changes"[2] were detected in 3 cases: in MSC114 monosomy of chromosome X was found in three metaphases, while in MSC119 and MSC122 inversion of chromosome 1 and a translocation involving chromosomes 9 and 4, respectively, were found in two metaphases. We also examined the results of multiple expansions from the same donors. Chromosomal anomalies were observed for 7 out of 13 donors (ns. 18, 20, 23, 32, 37, 50, 63), but these lesions were not recurrent and present only in some of the expansions performed. This suggests that chromosome aberrations do not associate with specific donors. In 6 cases, cytogenetic evaluation could not be performed on the final fresh P2 products due to lack of metaphases (Table 1) but in 5 of these the analyses could be repeated using a frozen P2 aliquot and in 4 cases karyotypes were normal. Similarly, when spontaneous and non-clonal abnormalities were detected, the karyotype analysis was repeated using a frozen P2 aliquot and was found normal in 7 of 12 cases (MSC46, MSC52, MSC55, MSC66, MSC79, MSC80, MSC87). Interestingly, for MSC116 two new anomalies appeared in 2 metaphases while for MSC100 trisomy of chromosome 5 recurred. The same aneuploidy, identified by Tarte *et al.*,[5] appears to be deleterious for cell survival. In support of this, when MSC displaying this aneuploidy were injected into immunocompromised mice, the authors reported no tumor formation after eight weeks as well as no hTERT expression or p53/p21 mutations in these cells.[5] The karyotyping test was repeated also in the 3 cases showing clonal lesions. For MSC122, the second karyotype resulted normal. For MSC119, the analysis confirmed the presence of the previous lesion in two metaphases. MSC114 required more extensive investigation. At the first analysis, 3 of 24 metaphases showed monosomy of X chromosome, 2 of 24 showed deletion of chromosome X and translocation involving chromosomes 15 and 17, and one showed translocation between chromosome 1 and chromosome 16 (Table 1 and Figure 1A). The second analysis confirmed the presence of two of the previous lesions (46,X,⸮del(X),⸮t(15;17)) in five metaphases and indicated the appearance of new lesions (47,XX,+⸮8,del(9)) in two additional metaphases. Due to the multiple genetic abnormalities of MSC114, these cells were further analyzed for transformation *in vitro*. To first exclude the possibility that the abnormal metaphases were derived from contaminating cells,[7] Short Tandem Repeat amplification was performed, and this resulted in a pattern corresponding to a single individual (Figure 1B). We then investigated the capacity of MSC114 P2 cells for anchorage independent growth[14] and found that MSC114, unlike control PDE-02 cell line, were unable to form colonies in methylcellulose (Figure 1C). Furthermore, *in vitro* long-term culture showed that MSC114 underwent complete growth arrest after 82 days and 35 PDs (Figure 1D), without any observable evidence of transformation (Figure 1E). Cytogenetic analysis, performed at P6, resulted in the same chromosome alterations detected at P2, with an additional chromosome 5 trisomy in one out of 19 metaphases (mos(45,X)[3]/46,X,⸮del(X),⸮t(15;17)[1]/46,XX,t(1;16)[7]/47,XX,+5[1]/46,XX[7]).

In order to investigate the characteristics and fate of single MSC114 cells, 33 clones were generated by limiting dilution. Only 2 of these were able to grow for 20 PDs before senescence, and karyotypic analysis of these showed absence of metaphases, confirming the lack of a proliferative advantage of cells with abnormal karyotypes (*data not shown*). Altogether, these data suggest lack of transformation of MSC114, despite multiple and clonal chromosome alterations. As batch MSC114 could not be released for safety reasons, patient n. 63 underwent two further BM collections and MSC expansions. As shown in Table 1, the karyotypes of these expanded products, MSC120 and MSC123, were normal suggesting that the abnormalities observed in MSC114 were non-recurrent and donor independent.[2] Finally, as p53 expression has been involved in MSC transformation,[6] we analyzed the p53 DNA sequences of both MSC114 and MSC120 derived from the same patient. A median of 575 sequences (range 115-1051) and 690 sequences (range 77-1058) were performed, respectively, and the only genomic variation detected was a base substitution (rs1625895A/G) in exon 5 representing the most common single nucleotide polymorphism variant present in the Caucasian population.

In conclusion, conventional karyotype analysis performed as quality control test on 92 clinical-grade BM-MSC preparations, to our knowledge the largest collection reported so far, showed the presence of spontaneous, non-clonal and non-recurrent mutations in 14 of 86 cases (16.3%). A previous study by Ben-David *et al.*,[15] based on

Table 1. Karyotype analysis of clinical-grade human BM-MSC.

Donor N./ health status (gender, age)	MSC batch	Starting material	Karyotype on fresh P2 cells [Metaphases analyzed]	Karyotype on frozen P2 aliquot [Metaphases analyzed]
4/N (M,26)	MSC32	P1 vial	46,XY[21]	/
	MSC37	P1 vial	46,XY[12]	/
	MSC47	P1 vial	46,XY[20]	/
8/N (M,28)	MSC27	P1 vial	absence of metaphases	46,XY[21]
	MSC29	P1 vial	46,XY[15]	/
	MSC30	P1 vial	46,XY[7]	/
	MSC31	P1 vial	46,XY,[1]	/
	MSC33	P1 vial	absence of metaphases	46,XY[23]
	MSC34	P1 vial	46,XY[18]	46,XY[20]
	MSC35	P1 vial	46,XY[20]	/
	MSC48	P1 vial	46,XY[21]	/
9/N (M,48)	MSC26	P1 vial	46,XY[19]	/
	MSC67	P1 vial	46,XY[21]	/
	MSC68	P1 vial	46,XY[20]	/
	MSC72	P1 vial	46,XY[20]	/
	MSC77	P1 vial	46,XY[25]	/
13/N (M,47)	MSC57	P1 vial	46,XY[11]	46,XY[20]
16/N (M,33)	MSC28	BM	46,XY[15]	/
17/N (M,12)	MSC36	BMw	46,XY[20]	/
	MSC38	P1 vial	46,XY[21]	/
	MSC40	P1 vial	46,XY[20]	/
	MSC42	P1 vial	46,XY[24]	/
	MSC43	P1 vial	46,XY[20]	/
	MSC44	P1 vial	46,XY[22]	/
18/N (M,42)	MSC39	BMw	46,XY[21]	/
	MSC46	P1 vial	46,XY,del(13)[1]/46,XY[19]	46,XY[26]
19/N (M,16)	MSC41	BMw	46,XY[23]	/
	MSC49	P1 vial	absence of metaphases	46,XY[20]
	MSC50	P1 vial	absence of metaphases	46,XY[21]
20/N (M,20)	MSC45	BMw	46,XY[20]	/
	MSC51	P1 vial	46,XY[20]	/
	MSC52	P1 vial	46,XY,del(6)[1]/46,XY, tr(15)[1]/46,XY[21]	46,XY[20]
	MSC55	P1 vial	46,XY,t(1;9)[1]/46,XY[20]	
			46,XY,t(4;6)[1]/46,XY[18]	
21/N (M,18)	MSC53	BMw	46,XY[20]	/
	MSC60	P1 vial	46,XY[20]	/
	MSC65	P1 vial	46,XX[20]	/
22/N (M,37)	MSC54	BM	46,XY[20]	/
23/N (M,35)	MSC59	P1 vial	46,XY[22]	/
	MSC66	P1 vial	46,XY,del(3)[1]/ 46,XY,del(10)[1]/46,XY[21]	46,XY[20]
24/N (F,8)	MSC58	BMw	46,XX [1]	46,XX[22]
	MSC61	P1 vial	46,XX[20]	/
	MSC62	P1 vial	46,XX[20]	/
	MSC63	P1 vial	46,XX[23]	/
	MSC64	P1 vial	46,XX[22]	/
25/N (M,38)	MSC69	BMw	46,XY[20]	/
26/β-Thal (M,25)	MSC70	BM	46,XY,t(10;15)[1]/46,XY[22]	ND
27/N (M,16)	MSC71	BMw	46,XY[8]	/
28/N (M,34)	MSC73	BM	46,XY[20]	/
29/β-Thal (M,37)	MSC74	BM	46,XY,-8[1]/46,XY[20]	ND
30/β-Thal (F,11)	MSC75	BM	46,XX[20]	/
31/β-Thal (F,6)	MSC76	BM	46,XX[20]	/
32/N (M,47)	MSC78	BMw	46,XY[20]	/
	MSC80	P1 vial	46,XY, t(6;11)[1]/46,XY, t(6;7;9;11)[1]/ 46,XY,t(2;7)[1]/ 46,XY[17]	46,XY[20]
33/N (na)	MSC79	BMw	46,XY,t(12;17)[1]/46,XY[20]	46,XY[20]
34/N (F,27)	MSC81	BMw	46,XX[22]	/
35/N (M,61)	MSC82	BMw	46,XY,t(5;13)[1],46,XY[19]	absence of metaphases
37/N (M,32)	MSC87	P1 vial	46,XY,t(6;13)[1]/46,XY[19]	46,XY[20]
	MSC94	P1 vial	46,XY[22]	/
	MSC95	P1 vial	46,XY[20]	/
38/N (F,51)	MSC85	BMw	absence of metaphases	absence of metaphases
39/N (F,17)	MSC86	BMw	46,XX[20]	/
40/N (F,35)	MSC88	BMw	46,XX[12]	/
41/N (M,47)	MSC91	BM	46,XY[22]	/
42/N (M,30)	MSC90	BM	46,XY[20]	/
43/N (M,31)	MSC92	BMw	46,XY[20]	/
44/N (M,34)	MSC93	BMw	46,XY[23]	/
46/N (M,52)	MSC96	BMw	46,XY[7]	/
47/N (M,45)	MSC97	BMw	46,XY[20]	/
48/MS (F,27)	MSC98	BM	46,XX[20]	/
50/MS (F,49)	MSC100	BM	46,XX,del(7)[1]/47,XX,+5[1]/ 46,XX[19]	47,XX,+5[1]/ 46,XX[19]
	MSC106	BM	46,XX[20]	/
57/MS (M,49)	MSC105	BM	46,XY[20]	/
58/MS (M,28)	MSC108	BM	46,XY[20]	/
59/MS (F,44)	MSC112	BM	46,XX[20]	/
60/MS (M,28)	MSC110	BM	46,XY[20]	/
61/N (M,21)	MSC111	BMw	absence of metaphases	ND
62/MS (F,40)	MSC113	BM	46,XX[20]	/
63/MS (F,38)	MSC114	BM	mos(45,X)[3]/46,X,?del(X), ?t(15;17)[2]/46,XX,t(1;16) [1]/46,XX[18]	46,X,?del(X), ?t(15;17) [5]/47,XX,+?8, del(9)[2]/ 46,XX[13]
	MSC120	BM	46,XX[20]	/
	MSC123	BM	46,XX[6]	/
64/N (M,29)	MSC115	BMw	46,XY [20]	/
65/N (M,31)	MSC116	BMw	46,XY,t(1;11)[1]/46,XY, del(5)[1]/46,XY[20]	46,XY,del(6) [1]/46,XY, inv(6)[1]/46, XY[19]
66/MS (M,42)	MSC117	BM	46,XY[20]	/
67/MS (M,37)	MSC118	BM	46,XY[20]	/
68/MS (M,34)	MSC119	BM	46,XY,inv(1)[2]/46,XY[18]	46,XY,inv(1) [2]/46,XY[18]
69/MS (M,47)	MSC121	BM	45,Y,-X,t(4;11),-7,-1,+mar1, +mar2[1]/46,XY[19]	ND
70/MS (F,38)	MSC122	BM	46,XX,?t(9;4)[2]/46,XX[20]	46,XX[20]
71/MS (M,30)	MSC124	BM	46,XY[20]	/
72/MS (F,21)	MSC125	BM	46,XX[20]	/
73/MS (M,31)	MSC126	BM	46,XY,t(9;15)[1]/46,XY[19]	ND
74/MS (F,37)	MSC127	BM	46,XX[21]	/
75/MS (F,31)	MSC128	BM	46,XX[23]	/

continued in the next column

N: normal donor; M: male; na: not available; P1 vial: cells frozen at P1 and later thawed; BM: bone marrow; BMw: BM washout; F: female; β-thal: β-thalassemia donor; MS: multiple sclerosis donor; ND: not done.

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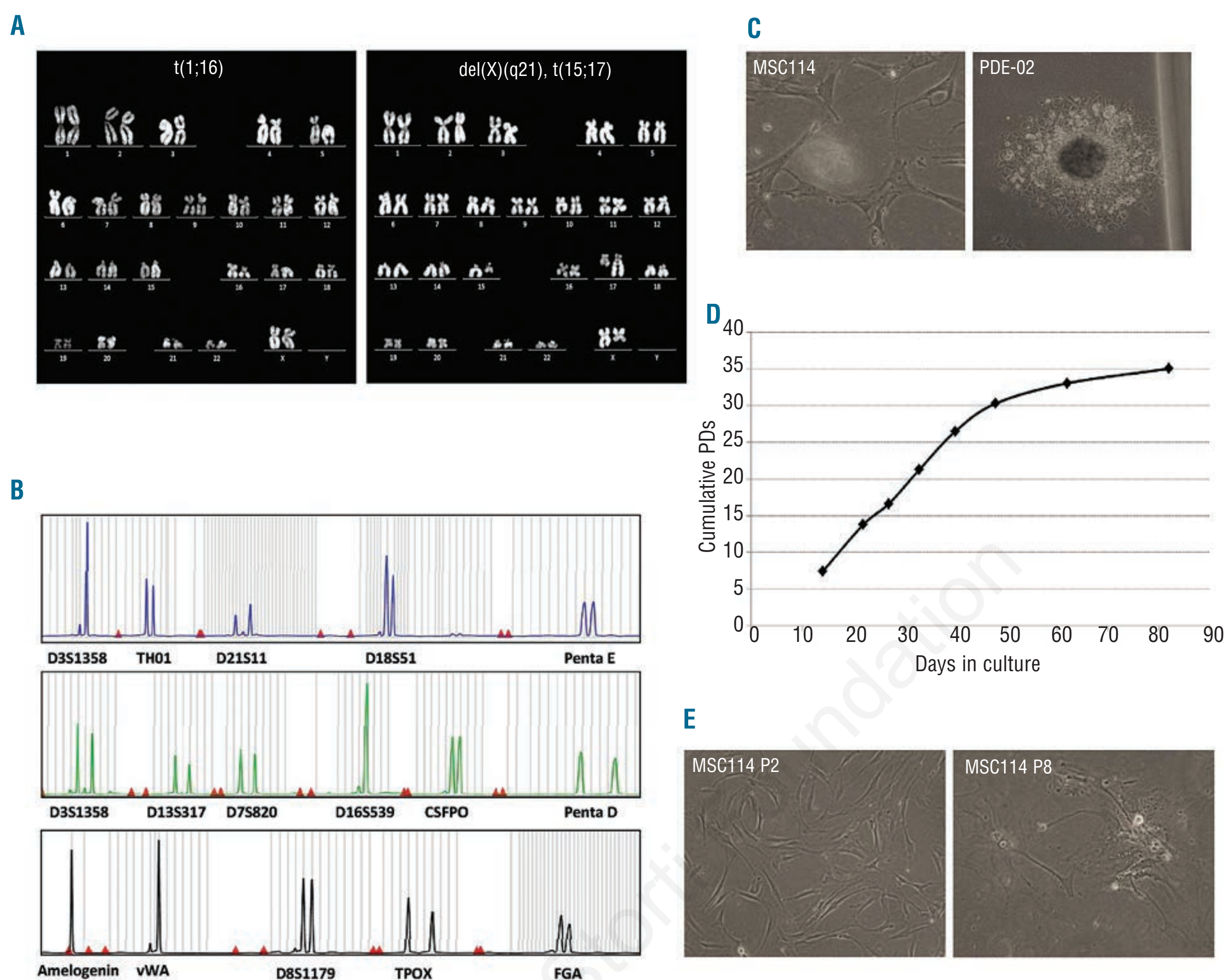

Figure 1. Characterization of MSC114. (A) Examples of the types of chromosomal aberrations found in MSC114. Karyogram was performed using the Ikaros imaging system (MetaSystems, Germany). (B) Short Tandem Repeats (STR) analysis of MSC114 DNA. Detection and genotyping of PCR products were carried out on the Abi Prism 3130 Genetic Analyser (Life Technology, Foster City, CA, USA) with a sensitivity of 5%. The electropherogram shows the presence of a maximum of two alleles (peaks) for each of the 15 genetic loci analyzed. This testifies the presence of DNA deriving from a single individual. The Amelogenin locus presents only the peak derived from the X chromosome demonstrating the female origin of the sample. (C) Anchorage-independent growth in methylcellulose for MSC114 P2 and human sarcoma cell line PDE-02 (original magnification X200). Images were acquired by an inverted microscope (Axiovert 25, Zeiss, Oberkochen, Germany). (D) Long–term growth kinetics for MSC114. Cell numbers were determined at the end of every passage and cumulative population doublings (PDs) were calculated in relation to the cells number plated. (E) Morphology comparison between MSC114 P2 and MSC114 P8 (original magnification X100). Images were acquired by an inverted microscope (Axiovert 25, Zeiss, Oberkochen, Germany).

DNA microarray analysis, reported 4% aberrations over 135 MSC samples. In only 3 of 86 cases (3.5%), was evidence of clonal mutations obtained, but these were not associated with a malignant transformation and transformed phenotype *in vitro*. Nevertheless, for safety reasons and in the light of the Cell Product Working Party (CPWP) review,[2] the lack of clonal chromosome aberrations or the presence of non-clonal chromosome anomalies on 10% or less of metaphases were set as release criteria before MSC distribution for exploitation in clinical trials.

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