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In vitro gene and chromosome characterization of expanded bone marrow mesenchymal stem cells for musculo-skeletal applications

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Abstract. – OBJECTIVE: A number of studies have shown the role of expanded Bone Marrow-derived Mesenchymal Stem Cells in the repair and regeneration of musculo-skeletal tissues. The current European regulations define *in vitro* expanded cells for clinical purposes as substantially manipulated and include them in the class of Advanced-Therapy Medicinal Products to be manufactured in compliance with current Good Manufacturing Practice. Among the characteristics that such cells should display, genomic stability has recently become a major safety concern. The aim of this study is to perform a chromosomal and genetic characterization of Bone Marrow-derived Mesenchymal Stem Cells expanded in compliance with Good Manufacturing Practice for a potential clinical use in orthopaedics.

MATERIALS AND METHODS: Mesenchymal Stem Cells, isolated from bone marrow, were expanded for six weeks in compliance with current Good Manufacturing Practice. DNA profiling analyses were applied to test cross-contamination absence. Genomic stability was evaluated by means of karyotyping, sequencing of TP53, p21/CDKN1A and MDM2 genes and the expression analysis of c-MYC and H-RAS oncogenes, p21/CDKN1A, TP53, p16/CDKN2A, RB1 and p27/CDKN1B tumor suppressor genes and hTERT gene.

RESULTS: The DNA profiling analysis showed a unique genetic profile for each Mesenchymal Stem Cell culture, indicating the absence of cross-contamination. Karyotyping evidentiated some chromosomal abnormalities within the 10% limit set by the Cell Products Working Party review, except for one patient. In all cases, the molecular biology analyses did not revealed DNA point mutations, acquisition or changes in gene expression. hTERT levels were undetectable.

CONCLUSIONS: Cultured Mesenchymal Stem Cells do not seem to be prone to malignant transformation. In fact, although some chromosomal aberrations were found, molecular biology analyses demonstrated that the expansion phase did not induce the acquisition of *de novo* genetic changes.

Key Words:
Mesenchymal stem cells, Karyotyping, DNA profiling, Direct sequencing, Oncogenes, Tumor suppressor genes, Musculo-skeletal applications.

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Abbreviations

ATMPs = Advanced-Therapy Medicinal Products; bp: base pair; cDNA = complementary DNA; BSE/TSE = Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy; DMEM = Dulbecco's Modified Eagle Medium; EDTA = Ethylenediaminetetraacetic acid; EMA = European Medicines Agency; FBS = Fetal Bovine Serum; GAPDH = Glyceraldehyde 3-Phosphate Dehydrogenase; GTG = G banding by Trypsin and Giemsa; GMP = Good Manufacturing Practice; hTERT: Human Telomerase Reverse Transcriptase; ISCN = International System for Human Cytogenetic Nomenclature; ISCT = International Society for Cellular Therapy; MSCs = Mesenchymal Stem Cells; mtDNA = mithochondrial DNA; PCR = Polymerase Chain Reaction; qPCR = quantitative Real-time RT-PCR; RMP = Random Match Probability; rRCS = revised Reference Sequence; RT-PCR = Reverse Transcriptase-PCR; SIGU = Italian Society of Human Genetics; STR = Short Tandem Repeat; SNP = Single Nucleotide Polymorphism.

Introduction

Several studies have shown the role of bone marrow-derived Mesenchymal Stem Cells (MSCs) in the repair of musculo-skeletal tissues[1-8]. Bone large segment regeneration after tissue lost, due to trauma, tumor resection or skeletal deformities, non-unions fractures and avascular necrosis may require a large amount of cells to achieve better results. Since MSCs display low density in bone marrow, an *in vitro* expansion phase is needed in order to obtain a suitable cell number[9-12].

The current European regulation defines such *in vitro* expanded cells as substantially manipulated and include them in the class of Advanced-Therapy Medicinal Products (ATMPs)[13,14]. Consequently, it becomes mandatory to produce these cell-based medicines in proper Facilities (authorized by each Member State) and in accordance with the current Good Manufacturing Practice (GMP)[15,16]. These rules, that have been already encoded for conventional drugs, allow for the manufacture of medicinal products in a standardized and controlled way minimizing, at the same time, contamination risks[17]. The GMP-Facilities consist in environments (cleanrooms) where specific parameters such as air filtration, ventilation, temperature, relative humidity, differential pressure, number of air particles and bacterial colony forming units are standardized and continuously monitored[15].

In the document issued by the European Medicines Agency (EMA), the "Reflection paper on stem cell-based medicinal products", the characteristics (specifications) that such MCS-based products should display are set out in detail: absence of contamination, viability, growth kinetics, identity maintenance, purity, potency and genomic stability[18]. The latter has recently become a major safety concern, especially in orthopaedic field, where most cell therapy treatments are not life-saving. In fact, pre-clinical data evaluating the *in vitro* and/or *in vivo* transformation/oncogenic potential are nowadays mandatory before starting any trial in humans[9-12].

In the literature there are various studies investigating MSC genomic stability, but results are conflicting[19-24]. In particular, the effects of possible abnormalities remain to be elucidated.

Recently, the Cell Products Working party (CPWP) organized an expert meeting mainly focused on tumorigenicity of MSCs whenever used as therapeutic medicinal products[25]. Besides emphasizing the importance of GMP cultures, the CPWP suggested that karyotyping analysis would be sufficient as a release test with the exclusion of two identical abnormal metaphases on a total of 20 analyzed (10%), in comparison with patient karyotype status. Such limit has been defined as a clonal chromosome change by the International System for Human Cytogenetic Nomenclature[25].

Within this complex context, the present study investigates the chromosomal and genetic stability of bone marrow MSCs expanded in compliance with GMP in order to evaluate their potential clinical use in orthopaedics.

Materials and Methods

Donor Screening and Informed Consent

Bone marrow was harvested, under general anesthesia, from the iliac crest of six patients (4 females and 2 males; median age: 35.6 years; range: 22-39 years, hereafter identified as Patient 1-6) undergoing regenerative orthopaedic treatment employing autologous bone marrow stem cells transplantation within a study approved by the Ethical Committee of Rizzoli Orthopaedic Institute, Bologna, Italy.

For this research, 6-8 remaining milliliters of heparinized bone marrow from each donor were used, after obtaining written informed consent.

Peripheral blood samples (3-4 ml) were also harvested from each patient and utilized both to evaluate the presence of transmissible pathologies (Acquired Immune Deficiency Syndrome, Hepatitis B and C and syphilis) and as controls for karyotype and molecular biology analyses. An additional written consensus for the utilize of genetic data was obtained from each patient, as requested by current laws[26].

Reagents

All cell culture reagents were suitable for cell therapy applications: as specifically requested by current legislation, Trypsin-Ethylenediaminetetraacetic acid (EDTA) and Fetal Bovine Serum (FBS) were certified to be free from porcine and bovine mycoplasma and viral contaminations, respectively. In addition, FBS origin had to be from a country with a negligible risk of Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy (BSE/TSE)[27].

Bone Marrow MSC Isolation and Culture

Cultures were developed in a public, authorized, GMP compliant facility located inside Rizzoli Orthopaedic Institute. After passing donor screening controls, bone marrow aliquots were diluted 1:4 with Dulbecco's Modified Eagle Medium (DMEM) low glucose (1 g/L), supplemented with 10% FBS and L-Glutamine 4 mM (complete medium). Diluted samples were then seeded in vented cell culture flasks (BD Falcon™, San Jose, California USA) and incubated at 37°C, 5% CO_2, 95% RH. Cells were let adhere for 2-3 days. The non-adherent cells were then removed by medium replacing and cultures were carried on in monolayer for six weeks. During the whole expansion phase, medium was changed weekly; at 70% confluence, MSCs were trypsinized and used partly for gene expression analysis and partly passaged at low density. DNA profiling, gene sequencing, karyotyping and Human Telomerase Reverse Transcriptase (hTERT) expression analyses were performed at the end of the six weeks expansion period. Gene expression analysis were carried on at each passage until the end of the expansion phase.

DNA Profiling

For each patient, a peripheral blood sample collected during surgery and a MSC pellet (80.000 cells) of the corresponding culture harvested at the end of the expansion phase were utilized. Nuclear and mitochondrial DNAs were extracted by *QIAmp Mini Kit* (Qiagen, Hilder, Germany) following Manufacturer's instructions and visualized by electrophoresis on 1% agarose gel containing ethidium bromide.

Nuclear DNA

1-4 ng of DNA template were utilized for fluorescent multiplex Polymerase Chain Reaction (PCR) with the AmpFlSTR Identifiler Amplification Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) to amplify 15 tetranucleotide Short Tandem Repeat (STR) loci (molecular size: 107-358 base pair, bp): D8S1179, D21S11, D7S820, CSF1PO, D3S1358, THO1, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA. Amelogenin locus for sex determination was also amplified and used as internal control. Dye-labeled PCR fragments analysis was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and alleles were genotyped by GeneMapper ID V3.2 software.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) analysis was performed in a reaction volume of 25 µl containing: 10 ng of genomic DNA, 1X PCR buffer, 1,5 µM $MgCl_2$, 200 mM of each dNTP, 1,5 U AmpliTaq DNA Polymerase (Applied Biosystems) and 0,2 mM of each primer (L15997-H16401 for HV1 control region and L00029-H00408 for HV2 control region). The amplification was carried out for 35 cycles: 3 min at 94°C, 1 min at 94°C, 30 sec at 56°C and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR products were run on a 2% agarose gel and stained with ethidium bromide. Amplicons were purified by ExoSAP-IT™ reagent (USB Corporation, Cleveland, OH, USA), according to Manufacturer's protocol. Sequencing was performed using BigDye Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems) and products were purified by ethanolic precipitation. Sequences were analyzed by capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and sequences were aligned and compared with the revised Reference Sequence (rRCS), using the Sequence Navigator computer program (Applied Biosystems, Sequence Navigator version 1.0.1).

Statistical Analysis

The calculation of Random Match Probability (RMP) based on genotype frequencies was estimated using the Italian database[28,29]. $p < 0.05$ was considered statistically significant.

Karyotyping

For each patient, karyotyping was performed both on a peripheral blood sample collected during surgery (control) and his relative MSC culture obtained at the end of the expansion phase.

Standard techniques were used for the preparation of metaphase spreads[30]. At least, 20 metaphases per samples were studied using G banding by trypsin and Giemsa (GTG) at a resolution of 400 bands for haploid set. Each case was analysed by two different operators in order to reduce slide subjective scanning impact. Cytogenetic investigations were performed in accordance with the International Guidelines of the Association of Cytogenetic Technologists (USA) and of the Italian Society of Human Genetics (SIGU). Karyotypes were formulated following the International System for Human Cytogenetic Nomenclature (ISCN, 2009) indications.

Oncogenes and Tumor Suppressor Genes Analysis

TP53, p21/CDKN1A and MDM2 Sequencing. A peripheral blood sample collected during surgery and a MSC pellet (500.000 cells) of the corresponding culture harvested at the end of the expansion phase were analyzed.

Genomic DNA from whole blood was extracted with the Beckman Biomek NX automated liquid handling workstation using Agencourt® Genfind™ v2 kit (Beckman Coulter Company, Miami, FL, USA), according to the protocols specified by Manufacturer.

MSCs were washed in PBS and DNA was extracted using the Nucleospin Blood kit (Macherey-Nagel, Düren, Germany).

DNA quality and quantity were checked with a NanoQuant Infinite M200 instrument (Tecan Group Ltd, Männedorf, Switzerland) before analyses. The 11 exons of TP53 gene, the 3 exons of p21/CDKN1A, along with exon–intron junctions, and Single Nucleotide Polymorphism (SNP)309 (rs2279744) in MDM2 gene were PCR-amplified. Gene-specific primers were designed using the software BLAST Primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/). Amplification products were purified using ExoSap-IT (USB Corp., Cleveland, OH, USA) and sequenced in both forward and reverse direction with the BigDye Terminator chemistry version 3.1 (Applied Biosystems). Sequencing products purification was performed with BigDye X-Terminator kit and samples were analyzed with ABI prism 3100 automated DNA sequencer (Applied Biosystems). Wild-type reference sequences for TP53, p21/CDKN1A and MDM2 were obtained from GenBank accession numbers NM_000546.4, NM_000389 and NM_002392.3, respectively. Mutation numbering was based on complementary DNA (cDNA), such that + 1 corresponds to the A of the ATG initiation codon, in the reference sequence.

Gene Expression Analysis

MSC pellets (500.000 cells) harvested at each passage until the end of the expansion phase were utilized. hTERT expression analysis was performed at the end of the expansion phase.

Total RNA was isolated from MSCs using the Qiagen RNeasy mini kit (Qiagen), according to Manufacturer's instruction. RNA quality was determined by 1% agarose gel electrophoresis. RNA concentration and 260/280 ratio were checked with a NanoQuant Infinite M200 instrument (Tecan Group Ltd, Mannedorf, Switzerland). From each sample, 500 ng of RNA were used for cDNA synthesis in a 50 μl reaction mixture using TaqMan Reverse Transcription Reagent (Applied Biosystems).

Gene expression analysis by quantitative Real-Time RT-PCR (qPCR) was performed on cDNA samples of c-MYC and H-RAS oncogenes and p21/CDKN1A, TP53, p16/CDKN2A, RB1 and p27/CDKN1B tumor suppressor genes. Gene-specific primers were designed using the software BLAST or selected from the RTPrimerDB database (http://www.rtprimerdb.org/). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH)" was used as endogenous control. Primers sequence and products size are reported in Table I. The RT^2 SYBR Green qPCR Master Mix (Qiagen) was used with 4 μl of 1:10 cDNA dilution and with 800 nM primers in a 25 μl reaction volume. All PCR reactions were performed in triplicate by using a Rotor-Gene Q instrument (Qiagen) at the following cycling conditions: 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 61°C for 1 min. After amplification, a melting step was performed within the 70°C-90°C range, rising at 1°C each step, and melting temperature of PCR products were used to check amplicon specificity. A negative control without any cDNA template (no template control) was run with every assay.

mRNAs relative expression was determined using the $\Delta\Delta$Ct method, that enables relative gene expression analysis as described by Livak and Schmittgen[31].

Table I. Primers used for gene expression analysis by quantitative Real-Time RT-PCR.

Gene	ENTREZ gene ID*	Forward primer 5′-3′	Reverse primer 5′-3′	Product size (bp**)
p21/CDKN1A	1026	CCTCATCCCGTGTTCTCCTTT	GTACCACCCAGCGGACAAGT	97
TP53	7157	ACGACGGTGACACGCTTCCCT	CTCAGAGGGGGCTCGACGCT	100
p16/CDKN2A	1029	CTCACGGCGTCCCCTTGCCT	CCTCCGGTGCTGGCGGAAGA	93
p27/CDKN1B	1027	CCGACGCCGGCAAGGTTTGG	AGGCAAGCGGAGAGGGTGGC	99
RB1	5925	GCTTCCACCAGGCCCCCTACC	CCCTCCAGGAATCCGTAAGGGTGA	110
H-RAS	3265	TGCGTGAGATCCGGCAGCAC	AGCTTGTGCTGCGTCAGGAGAG	105
c-MYC	4609	TCAAGAGGCGAACACACAAC	GGCCTTTTCATTGTTTTCCA	110
GAPDH	2597	GCAAATTCCATGGCACCGT	GCCCCACTTGATTTTGGAGG	104

*ID, identification; **bp, base pair.

For ΔΔCt analysis, the MSC sample harvested at the earliest available passage was used as calibrator ($2^{-\Delta\Delta Ct} = 1$).

hTERT expression was analyzed by Reverse Transcription-PCR (RT-PCR), along with the endogenous control human GAPDH, as described by Rosland et al[19]. PCR products have been analyzed using 2% agarose gel electrophoresis. A telomerase-negative osteosarcoma cell line (U2OS)[32] and a Ewing's tumor cell line expressing TERT (SK-ES-1)[33] were utilized as controls.

Results

DNA Profiling

Typing of the 15 STRs and the amelogenin locus showed a genetic autosomal profile unique and identical for each MSC culture and the relative patient's peripheral blood sample, as reported in Table II. The random match probability was estimated in a range from 1×10^{-15} to 1×10^{-18}.

Sequence of mitochondrial DNA HV1 and HV2 control regions did not show *de novo* heteroplasmic nucleotide positions neither mutations com-

Table II. DNA profiling analysis on Mesenchymal Stem Cell samples after a six weeks culture period. Cells and peripheral blood related control samples displayed the same DNA profiling, for each patient. Amelogenin locus for sex determination was used as internal control.

DNA Loci	STR* polymorphims in MSC** samples					
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
D8S1179	13-14	12-12	15-16	12-13	12-13	13-15
D21S11	29-31.2	31.2-33.2	30-32.2	32-33.2	29-30	29-30.2
D7S820	10-11	10-12	8-11	7-12	10-12	10-12
CSF1PO	11-12	11-11	10-10	11-12	10-12	10-11
D3S1358	14-15	14-18	16-18	15-18	16-16	14-16
TH01	9.3-9.3	9-9	6-7	7-9.3	7-8	7-8
D13S317	9-11	8-12	11-12	12-14	9-11	9-12
D16S539	12-13	12-13	9-12	11-13	11-12	9-12
D2S1338	18-24	18-24	17-18	17-23	21-23	17-26
D19S433	13-15.2	13-15.2	12-14	13-15	14-15.2	14-15
vWA	18-19	17-19	16-19	16-17	16-18	18-18
TPOX	8-9	10-10	8-11	8-12	9-11	9-11
D18S51	16-16	16-17	16-17	12-15	14-17	13-14
D5S818	11-12	11-12	11-12	11-13	10-13	9-11
FGA	19-20	23-23	21-23	20-23	23-25	20-24
AMEL	XX	XY	XX	XX	XY	XX

*STR: Short Tandem Repeats; **MSC: Mesenchymal Stem Cell.

pared to blood reference samples sequences and revealed the variations indicated in Table III, with respect to Anderson reference Sequence[34].

Karyotyping

All peripheral blood samples showed a normal karyotype. Two MSC cultures showed a normal karyotype (patient 1 and 5). Some chromosomal non-clonal abnormalities were observed in three MSC cultures: cells from Patients 2 showed two different chromosome abnormalities in two metaphases: del (5)(p15.1) and del (12)(p12.3); in patient 4 MSCs one metaphase was found with a X chromosome monosomy; cultures from patient 6 showed one cell with an unbalanced rearrangement: der(5)t(5;?)(q35.1;?). Clonal chromosomal changes were found in patient 3 culture: 2 metaphases showed a reciprocal translocation: t(1;2)(p32;p16); 7 metaphases displayed a deletion: del(3)(q21).

Oncogenes and Tumor Suppressor Genes Analysis

TP53, p21/CDKN1A and MDM2 Sequencing. The analyses showed no point mutations for all the three genes, both in the MSC cultures and in the corresponding blood samples used as controls. For the TP53 gene some polymorphisms were detected in all samples and relative controls. In all patients the same two polymorphisms were found: Pol Etero IVS2(+38)c > g and Pol Omo IVS3(+41_+56) del in exon 3; Pol Etero c.215C > G and p.Pro 72Arg in exon 4. In exon 6 of patient 6 samples Pol Etero c.639A > G and p.Arg213Arg were detected. In exon 7 of patient 3 samples Pol Etero IVS7(+72) c > *t* and Pol Etero IVS7(+92) *t* > g were evidentiated.

No polymorphisms have been identified at the level of p21/CDKN1A gene.

SNP309 variant in the promoter of MDM2 gene was detected in three patients (rs2279744 homozygosis in patient 1 and 3, heterozygosis in patient 2).

Gene Expression Analysis

Mean expression mRNA values showed no differences among passages except for p21/CDKN1A which displayed a slight increase (Figure 1).

As shown in Figure 2, undetectable levels of endogenous hTERT were observed for all the MSC cultures analysed.

Discussion

In this study we developed MSC cultures in compliance with current GMP, mimicking a production process suitable for a clinical use in orthopaedic applications. The expansion phase has been carried on beyond the time hypothesized for a clinical administration (about three weeks) in order to evidentiate any increase in chromosomal aberration risk.

Table III. Mitochondrial DNA HV1 and HV2 hyper variable regions analyses on Mesenchymal Stem Cell samples after a six weeks culture period. *De novo* heteroplasmic nucleotide positions neither mutations were observed in comparison with each patient's peripheral blood reference samples sequence, with respect to Anderson reference.

mtDNA hyper variable regions	Nucleotde sequence variations in MSC* samples					
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
HV1	16184T 16357C	16093C 16224C 16311C	16111T 16187T 16223T 16290T 16319A 16362C	16224C 16311C	16093C/T 16126C 16292T 16294T 16296T 16304C	16192T 16256T 16270T 16325C 16362C 16399G
HV2	263G 309.1insC 315.1insC	73G 195C 263G 315.1insC	64T 73G 146C 153G 204C 235G 263G 309.1insC 315.1insC	73G 263G 315.1C	73G 263G 315.1C	73G 263G 315.1C

*MSC: Mesenchymal Stem Cell.

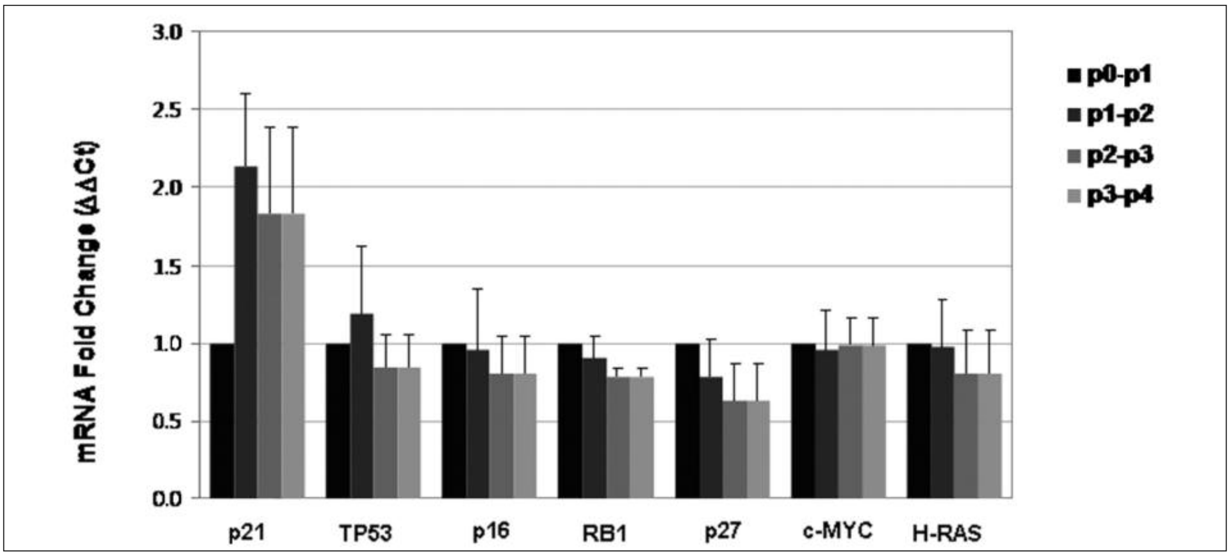

Figure 1. Mean expression levels of tumor associated genes in Mesenchymal Stem Cell samples at different culture passages. Results are expressed as fold changes relative to the earliest culture passage ($2^{-\Delta\Delta Ct} = 1$). Each analysis has been performed in triplicate. Values are the mean ± SD.

A potential application of such expanded cells is bone reconstruction after large tissue loss[1,2,4,6,8]: the MSC-based medicines developed could be delivered to the patients directly in the lesion site as a suspension or implanted in association with a scaffold/biomaterial.

We found that cell features such as viability, morphology, immuno-phenotype, proliferation rate (population doublings) and differentiation ability were in line with the literature[9-12,35] and normative requirements[18,36] (data not shown). We have particularly focused on genomic stability which is still a matter of debate[24,37].

The data obtained from karyotype analysis showed in the cultures some chromosomal abnormalities such as chromosome X monosomy that has been already described in the literature[38]. In particular, Verp evidentiated that cultured fibroblastic lines with X monosomy displayed a growth disadvantage when compared to normal ones[39]. Recent articles highlighted the role of specific chromosomal abnormalities as an *in vivo* selective growth disadvantage[40,41]. On the other side, it cannot be ignored that chromosomal abnormalities have been associated with pathologies development or neoplastic transformation[42]. For instance, chromosome 7 trisomy has been described in cultured synovial and cartilage cells from patients with osteoarthritis and rheumatoid arthritis[43]. The chromosomal abnormalities that we found in the MSC cultures were within the 10% limit set by the Cell Products Working Party (CPWP) review to allow implantation, except for one patient[25]. However, the scarcity of the starting bone marrow (see Materials and Methods) did not allow to perform karyotyping also at the time of the supposed clinical release (three weeks) and to generate multiple cultures from each patient sample in order to both confirm the data and to evidentiate the possible presence of recurrent abnormalities, as stated by the CPWP review. If confirmed, safety reason altogether with the CPWP recommendation, impose not to use in the clinics the cultures carrying abnormalities that exceed the posed limit[37].

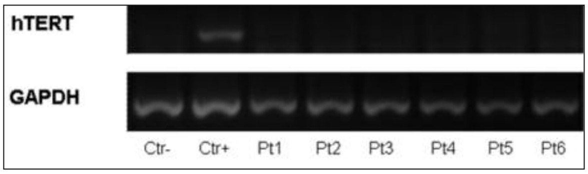

Figure 2. Agarose gel electrophoresis of human Telomerase Reverse Transcriptase RT-PCR products in Mesenchymal Stem cells samples after a six weeks culture period. Ctr-: U2OS used as negative control; Ctr+: SK-ES-1 used as positive control; Pt1-6: Patients.

In literature there are various reports on MSC chromosomal stability, but results are conflicting[19-24]. Some authors have demonstrated that these cells display stable karyotypes even after long-term expansion[22,44]. Other researches have reported a number of chromosomal abnormalities that gave rise to neoplastic transformation[23,45].

However, these last have subsequently retracted their own data since the transformed cells found were coming from tumor lines simultaneously cultured in the same laboratory[46]. In general, GMP compliance protects cells from cross-contamination risks (i.e. disposable materials and cultures production by campaign followed by appropriate and validated cleaning procedures). However, in order to give evidence of cross-contamination absence in our cultures we decided to apply the DNA profiling technique, a method already proved to be effective for this aim[47]. The DNA profiling analysis showed a unique genetic profile for each MSC culture, indicating no mix-up.

Recently, some papers analyzed a set of karyotypes from GMP grade MSCs, highlighting the presence of abnormalities in some cells. Evidence of clonal mutations was obtained, but not associated with *in vitro* malignant transformation. The studies generally included a parallel analysis of a panel of well-known genes involved in tumorigenesis or cell cycle control that showed stability[24,37].

The molecular biology analyses that we performed on a set of oncogenes and tumor suppressor genes evidentiated that our MSC cultures have not acquired DNA point mutations or significant changes in gene expression levels that may indicate a tendency to neoplastic transformation[22,24,48]. In particular, all TP53 polymorphisms were detected in both MSC samples and relative blood controls, thus without *de novo* mutations acquired in culture. Moreover, these polymorphisms are described in the IARC DataBase (DataBase IARCTP53; http://www.p53.iarc.fr/PolymorphismView.asp) as non-pathological variations.

MDM2 is the primary regulator of TP53. SNP309 is a functional single-nucleotide T to G polymorphism located in the promoter of the MDM2 gene (rs2279744)[49]. Bond et al. demonstrated that the GG genotype of SNP309 enhanced the affinity of the transcription factor Sp1 to the MDM2 promoter in cell lines, and consequently enhanced the expression of MDM2 RNA and protein, resulting in an attenuation of the p53 pathway[50]. However, the possible association between SNP309 and cancer risk remains contradictory[51]. In our study, SNP309 variant was detected in three patients, both in the MSC cultures and in the corresponding blood samples controls, further confirming that we did not detect new sequence variations at the end of the expansion phase. No polymorphisms have been identified at the level of p21/CDKN1A gene.

The gene expression analysis showed, in general, no differences among passages. The slight increase detected in p21/CDKN1A mRNAs has been already described in MSC senescent cultures and is therefore indicative of a controlled and not disorganized proliferation[52]. Moreover, the undetectable endogenous hTERT levels observed in all the MSC cultures are not related with a tumorigenic pattern, in agreement with previous Literature[19,22]. In fact, the hTERT gene, plays a key role in cellular senescence and is normally repressed in somatic cells, thereby leading to progressive telomere shortening[53].

Conclusions

Our results suggest MSC GMP cultures genetic stability, in line with literature data[54], but the karyotype analyses are not conclusive, due to the starting small bone marrow samples. Therefore, more in depth studies are needed with an increased number of patients, number of samples per patient, bone sample volume appropriateness to test cell tumorigenic potential with additional *ex vivo* models.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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